Influence of staphylococcal Phage SAvB14 on biofilms, formed by *Staphylococcus aureus* variant bovis

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The use of bacteriophages for the treatment of chronic inflammatory processes has proved to be relevant, especially during isolation of antibiotic-resistant pathogens formed in biofilms. The article presents the results of research on the influence of Phage SAvB14 on young and mature biofilms formed by *Staphylococcus aureus* var. bovis. In the experiments we used cultures of *S. aureus* and a specific Phage SAvB14 isolated from the secretion of the mammary gland of cows suffering from chronic mastitis. In the study of the influence of bacteriophage on formed biofilms we determined the optical density of the dye solution that was washed from the biofilm photometrically on a spectrophotometer PE-5400UV (Eurosphim, Russia) and the number of staphylococcal cells in the biofilm after the action of the bacteriophage on 24-hour and 72-hour biofilms by a ten-fold dilution on beef-extract agar. It was determined that under the influence of the bacteriophage on young 24-hour biofilms of *S. aureus* var. bovis, the optical density of the dye solution from biofilm increased within 4 hours up to 10% and the number of microbial cells increased by 1.8 times. After 32 hours of bacteriophage action, the optical density of the dye solution decreased on average by 34% compared to the initial density and the number of *S. aureus* cells in the biofilm decreased by 30 times. This indicates that microbial cells of young biofilms are not subject to complete lysis during the action of even this specific bacteriophage. Degradation of 77.5% of biofilm under the influence of the bacteriophage was observed on mature 72-hour biofilm within 32 hours at 37 °C. At the same time, viable cells of *S. aureus* were not isolated from the biofilm. This indicates the high lytic activity of the bacteriophage against mature biofilm bacteria and the possibility of its use in chronic staphylococcal infections caused by *S. aureus* var. bovis. Thus, the obtained data indicate that when mature 72-hour biofilms are exposed to the researched bacteriophage, their degradation is more intense compared with the young 24-hour biofilms, and the amount of destroyed biofilm was on average 2 times higher. This suggests that the use of specific staphylococcal Phage SAvB14 isolated by us for the destruction of biofilm, formed by *S. aureus* var. bovis, is promising.

**Keywords:** phage activity; degradation of biofilm; staphylococci; 24- and 72-hour biofilms.

Current studies have revealed that biofilm is the “ecological variability”, as soon as the biofilm is formed, film is their “ecological variability”, as soon as the biofilm is formed, proliferation. Then, the bacteria in biofilm gradually lose their direct
phosphate buffer, 5 cm³ of sterile 0.5% sodium chloride solution was added and microbial biofilm was carefully washed off the walls and the bottom of the dish using a sterile tampon. From the dishes, 1.0 cm³ of fixated with 96% of ethyl alcohol during 10 minutes. Then they were stained shing three times with phosphate buffer, dried and the formed biofilms were added and incubated at 37 ºC for 24 and 72 hours. After incubation, the optical density of biofilm, the number of staphylococcal cells in the solution was measured spectrophotometrically at the wavelength of 570 nM (Stepanovic et al., 2000).

Many phages produce polysaccharide depolymerases and when phage infection develops, phage enzymes decompose capsular polysaccharides (CPSS), O-polysaccharide chains of lipopolysaccharide (LPS) molecules, or extracellular polysaccharides (EPs) that form the matrix of biofilm (Latka et al., 2017). This facilitates the penetration of phages into deeper layers of biofilm with subsequent lysis of target bacteria (Parraison et al., 2014). However, at present, researchers are not focusing enough attention on the possibility of using specific bacteriophages isolated from the infection center against a particular disease. This is especially true for such a disease as subclinical mastitis of cows, which causes significant economic losses due to the development of antibiotic resistance in pathogens and the necessity of rejecting milk. Therefore, the development of economically beneficial methods and preparations with the use of specific bacteriophages against the main pathogen S. aureus var. bovis is promising and relevant.

The objective of this work was determining the effect of Phage SAvB14 on young and mature biofilms formed by S. aureus var. bovis.

Materials and methods

We studied 8 cultures of S. aureus and the specific bacteriophage Phage SAvB14, which we isolated from the secretion of mammary glands of cows suffering from chronic mastitis and placed at the State Scientific Control Institute of Biotechnology and Strains of Microorganisms. In the experiments we used strains of S. aureus var. bovis, which were lysed by Phage SAvB14 according to the double-layer agar method (Wills et al., 2005).

Disposable plastic Petri dishes were used to determine the optical density of the formed biofilms. Into each dish, 5 cm² of beef-extract broth and 1 cm² of daily culture of S. aureus at the concentration of 10⁷ CFU/cm² were introduced and incubated at 37 °C for 24 and 72 hours. After incubation, the dishes were cleaned from the planktonic (unbraced) microorganisms by washing three times with phosphate buffer, dried and the formed biofilms were treated with 96% of ethyl alcohol during 10 minutes. Then they were stained with 0.1% solution of crystal violet for 10 minutes. In the Petri dishes, 3.0 cm² of 96% of ethyl alcohol were added; the Petri dishes were left for 20–30 minutes, occasionally being shaken. The optical density of alcohol solution was measured spectrophotometrically at the wavelength of 570 nM (Stepanovic et al., 2000).

During the study of the effect of bacteriophage on formed biofilms, the optical density of biofilm, the number of staphylococcal cells in the biofilm and the bacteriophage titre were determined. Determination of the number of staphylococci in biofilm after bacteriophage action was performed on young (24-hour) and mature (72-hour) biofilms grown in plastic Petri dishes. After 24- or 72-hour incubation of cultures, the dishes were washed three times with sterile phosphate buffer to remove the planktonic (unbraced) microorganisms and 5 cm² of Phage SAvB14 was introduced. During the 32 hours exposure for (every 4 hours), the phage was poured out, the dishes were washed three times with sterile phosphate buffer, 5 cm² of sterile 0.5% sodium chloride solution was added and microbial biofilm was carefully washed off the walls and the bottom of the dish using a sterile tampon. From the dishes, 1.0 cm³ of suspension was taken, a number of ten-fold dilutions were prepared, 1.0 cm³ of each dilution was inoculated in a Petri dish, MPA was poured and incubated at 37 °C for 24–48 hours to determine the number of staphylococci. Along with the study of the number of staphylococcal cells in the biofilm, every 4 hours rinses were extracted to determine the titre of the phage according to the double-layer agar method (Wills et al., 2005).

Statistical processing of the results was carried out according to methods of variation statistics using the program Statistica 9.0 (StatSoft Inc., USA). Non-parametric methods of research were used (Wilcoxon-Mann-Whitney test). The arithmetic mean (x) and the standard error (SE) were determined. The difference between the comparable values was considered significant at P < 0.05.

Results

Under the influence of bacteriophage on 24-hour biofilms of S. aureus var. bovis, we noted the 10.0 ± 0.3% (P < 0.05) growth of the optical density of the dye solution during 12-hour incubation compared with their initial density (Fig. 1).

Fig. 1. Influence of bacteriophage on the 24-hour biofilm S. aureus variant bovis: solid line – optical density of dye solution from biofilms without the influence of bacteriophage (control); dashed line – optical density of dye solution from biofilms under the influence of bacteriophage (experiment); data are shown as mean ± SD

During the following incubation hours, a gradual decrease in the optical density of dye from biofilms was observed compared to the initial density. On the 28th hour of contact of the bacteriophage with the biofilm formed by the strain of S. aureus var. bovis, the density of the dye decreased by 63.7 ± 2.4% (P < 0.05) compared to the biofilm that was not affected by the bacteriophage and by 34.5 ± 0.7% (P < 0.05) compared to the initial density. During the following effect of bacteriophage on the biofilm, the optical density of dye solution of the biofilm did not decrease below 0.69 ± 0.02 units. Therefore, under the influence of the bacteriophage, the young 24-hour biofilm was destroyed, but most of it (about 60%) during the period of 32 hours was not subjected to lysis.

Under the influence of the bacteriophage, the number of microbial cells in biofilm during 4 hours of interaction did not decrease, but even increased by 1.8 times (P < 0.05) (Table 1).

After 8 hours, their number decreased by 4.7 times (P < 0.05) compared to the initial content. Starting almost on the 8th hour of the bacteriophage’s contact with microbial cells an active process of staphylococcal lysis took place. At the 24th hour from the time of infection with bacterial cells, the number of staphylococci per 1 ml of extract from the biofilm was 1.9 ± 0.1 × 10⁷ CFU/mL. Subsequent incubation up to the 32nd hour did not significantly reduce staphylococcal content in the biofilm.

The number of bacteriophages in the biofilm practically did not change during 8 hours of contact with microbial cells, although a ten-
dency towards their growth was observed. By the 20th hour from the time of infection with staphylococci, the bacteriophage titre had increased by thirty times equaling $1.9 \pm 0.2 \times 10^9$ PFU/mL ($P < 0.05$). The increase in the number of bacteriophages in the biofilm coincided with the active lysis of staphylococci, since between the 20-28th hours their content was the lowest in the biofilm. After 32 hours of influence of the bacteriophage on the film-forming staphylococci, the titre of bacteriophages was $3.2 \pm 0.2 \times 10^5$ PFU/mL. That is, we noted the regularity of gradual reduction of bacteriophage titre in the biofilm, which is related to the significant staphylococcal lysis. Thus, under the impact of bacteriophages on young 24-hour biofilms of \textit{S. aureus} var. \textit{bovis}, the full lysis of microbial cells did not occur within 32 hours of contact with the virus.

The time period during which a slight increase ($4.6 \pm 0.2\%$) occurred in the optical density of the dye from the biofilm was 8 hours (Fig. 2). At the 12th hour of interaction of the bacteriophage with the microbial cell, a decrease by $22.0 \pm 1.3\%$ ($P < 0.05$) in the optical density of dye was observed, compared to the biofilm grown without the bacteriophage, and by $11.2 \pm 0.3\%$ compared to the initial density. During the next hours of the impact of the bacteriophage, destruction of 77.5 $\pm$ 1.4\% ($P < 0.05$) of the biofilm occurred and at the 28th hour the density of the washed dye equaled 0.44 $\pm$ 0.02 units. Under the influence of the bacteriophage on mature 72-hour biofilms, their degradation was more intense compared with the young 24-hour biofilms – the amount of destroyed biofilm was on average 2 times ($P < 0.05$) higher.

**Table 1**

<table>
<thead>
<tr>
<th>Time of bacteriophage influence, hours</th>
<th>Bacterial viable count, CFU/mL</th>
<th>Phage titre, PFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (start)</td>
<td>$1.5 \pm 0.1 \times 10^6$</td>
<td>$1.6 \pm 0.1 \times 10^6$</td>
</tr>
<tr>
<td>4</td>
<td>$2.7 \pm 0.2 \times 10^6$</td>
<td>$1.8 \pm 0.2 \times 10^6$</td>
</tr>
<tr>
<td>8</td>
<td>$3.2 \pm 0.3 \times 10^6*$</td>
<td>$1.9 \pm 0.2 \times 10^6$</td>
</tr>
<tr>
<td>12</td>
<td>$8.1 \pm 0.7 \times 10^6*$</td>
<td>$1.1 \pm 0.1 \times 10^6*$</td>
</tr>
<tr>
<td>16</td>
<td>$4.3 \pm 0.4 \times 10^6*$</td>
<td>$2.7 \pm 0.3 \times 10^6*$</td>
</tr>
<tr>
<td>20</td>
<td>$2.3 \pm 0.2 \times 10^6*$</td>
<td>$1.9 \pm 0.2 \times 10^6*$</td>
</tr>
<tr>
<td>24</td>
<td>$1.9 \pm 0.1 \times 10^6*$</td>
<td>$1.6 \pm 0.1 \times 10^6*$</td>
</tr>
<tr>
<td>28</td>
<td>$1.5 \pm 0.1 \times 10^6*$</td>
<td>$4.8 \pm 0.5 \times 10^6*$</td>
</tr>
<tr>
<td>32</td>
<td>$1.3 \pm 0.1 \times 10^6*$</td>
<td>$3.2 \pm 0.2 \times 10^6*$</td>
</tr>
</tbody>
</table>

Note: $\*$ – the biofilm was grown for 24 hours and then the bacteriophage was introduced; CFU – colony-forming unit; PFU – plaque-forming unit; $\*$ – $P < 0.05$; $\*$* – $P < 0.05$ in comparison with the initial number; $\*$* – $P < 0.05$ in comparison with the number of staphylococci.

During 4 hours of interaction of the bacteriophage and staphylococcal cells in biofilm, changes in the number of bacteria did not occur, and the number of phages increased by twenty times (Table 2).

**Table 2**

<table>
<thead>
<tr>
<th>Time of phage influence, hours</th>
<th>Bacterial viable count, CFU/mL</th>
<th>Phage titre, PFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (start)</td>
<td>$9.3 \pm 0.9 \times 10^6*$</td>
<td>$1.6 \pm 0.1 \times 10^6*$</td>
</tr>
<tr>
<td>4</td>
<td>$9.4 \pm 0.9 \times 10^6*$</td>
<td>$1.2 \pm 0.1 \times 10^6*$</td>
</tr>
<tr>
<td>8</td>
<td>$5.2 \pm 0.5 \times 10^6*$</td>
<td>$1.1 \pm 0.2 \times 10^6*$</td>
</tr>
<tr>
<td>12</td>
<td>$6.7 \pm 0.7 \times 10^6*$</td>
<td>$2.4 \pm 0.2 \times 10^6*$</td>
</tr>
<tr>
<td>16</td>
<td>$4.4 \pm 0.4 \times 10^6*$</td>
<td>$3.7 \pm 0.4 \times 10^6*$</td>
</tr>
<tr>
<td>20</td>
<td>$9.1 \pm 0.9 \times 10^6*$</td>
<td>$2.1 \pm 0.2 \times 10^6*$</td>
</tr>
<tr>
<td>24</td>
<td>$2.2 \pm 0.2 \times 10^6*$</td>
<td>$1.8 \pm 0.2 \times 10^6*$</td>
</tr>
<tr>
<td>28</td>
<td>$1.0 \pm 0.1 \times 10^6*$</td>
<td>$3.5 \pm 0.4 \times 10^6*$</td>
</tr>
<tr>
<td>32</td>
<td>$0$</td>
<td>$10^6*$</td>
</tr>
</tbody>
</table>

Note: $\*$ – the biofilm was grown for 72 hours and then the bacteriophage was introduced; CFU – colony-forming unit; PFU – plaque-forming unit; $\*$ – $P < 0.05$; $\*$* – $P < 0.05$ in comparison with the initial number; $\*$* – $P < 0.05$ in comparison with the number of staphylococci.

After 8 hours of contact of the virus and bacteria, the lysis process of microbial cells began and their number decreased by ten times ($P < 0.05$), and the number of bacteriophages increased to $1.1 \pm 0.2 \times 10^6$ PFU/mL ($P < 0.05$). After 12 hours of exposure to the bacteriophage, the process of fading of staphylococcal biofilm continued. At the same time, the bacteriophage content ceased to increase and their number was $2.4 \pm 0.2 \times 10^6$ PFU/mL. During the next hours of interaction of bacteriophages and staphylococcal biofilm in the biofilm, the lytic action of bacteriophages continued, and after 32 hours from the beginning of the phages’ contact with the biofilm no bacterial cells were isolated.

**Discussion**

Bacteriophages can harmfully interact with bacteria that form biofilms at different stages of its formation: “before” formation, “during” maturation and “after” maturation (Wills et al., 2005; Kelly et al., 2012). Their potential to interact with biofilm bacteria at any of these stages depends on the amount of bacterial targets and the susceptibility of microorganisms to phage adsorption (Donlan, 2009). The lysis of microbial cells in a biofilm exposed to bacteriophages is possible only if the exopolysaccharide matrix is destroyed. This occurs when phages produce specific depolymerase enzymes that destroy a significant part of the matrix and make film-forming bacteria vulnerable (Azeredo et al., 2008; Fischetti, 2008; Vorobyev et al., 2017).

The obtained data showed that the optical density of dye solution from the young biofilm (24-hour) of \textit{S. aureus} var. \textit{bovis} under the influence of reproduction of Phage SAvB14 bacteriophage decreased by 34.5 $\pm$ 0.7\% after 32 hours. This indicates that the matrix has constituents that are not lysed by enzymes of this bacteriophage. The variety of structural components of the biofilm matrix has been reported in a number of studies (Flemming & Wingender, 2010; Hyman et al., 2013; Vorobyev et al., 2017), which revealed 12.0\% content of exopolysaccharides and about 15.0\% nucleic acids in biofilms of \textit{S. aureus}. It is also reported that active lysis of biofilm by phage depolymerases requires a specific susceptibility of bacterial cells to phage receptors (Weissbaucher, 2004; Briandet et al., 2008; Hyman et al., 2013). We think that during the passive effect of bacteriophages on young 24-hour biofilms, pseudolizogenic infection of microbial cells occurs, as during 8 hours of interaction of the phage and staphylococcus, the biofilm was not destroyed, but even grew. This indicates that during this time, the bacteriophage increased the number of staphylococcal cells in biofilm, but their lysis does not occur as a result of the development of pseudolysogeny: form of interaction of phage and microbial cell, in which the nucleic acid of the virus is present in the cell in an unstable, inactive state. In such cells, due to the instability of their development, the phage does not have enough energy to initiate genetic expression for the lytic reaction (Abedon, 2009; Howard-Varona et al., 2017). Therefore, during this period, increase in biofilm occurs and,
Accordingly, increase in optical density of its dye solution. At the same time, the number of staphylococcal cells increased by 1.8 times within 4 hours of infection. In addition, exposure of the young biofilm to the bacteriophage for over 32 hours did not result in the total death of microbial cells, and the number of staphylococci was 1.3 ± 0.1 × 10^7 CFU/mL of rinsed extract. Therefore, the research has shown that mature 72-hour biofilm forms of staphylococci are more actively lysed under the influence of the bacteriophage than their young 24-hour biofilm forms.

Kasman et al. (2002) report that in order to inhibit fast-growing bacterial cells in vitro, a constant high concentration of bacteriophages equaling 10^7–10^8 PUFU/mL is needed. Probably, during an acute septic process, the use of phages will be less effective than during chronic sepsis, since the population of pathogenic bacteria would not completely be destroyed, but maintained at a certain high level. A number of studies (Kelly et al., 2012) have shown that the effective removal of bacterial biofilm forms using a mixture of phages in the concentration of 10^7 PUFU/mL, depended on the time of exposure, and at 37 ºC, the greatest destruction of microbial cells occurred after 72 hours. Other researchers (Alves et al., 2014) by using the combined staphylococcal bacteriophage for the degradation of biofilm formed by S. aureus completely removed it in 48 hours at 37 ºC. In laboratory conditions, staphylococcal bacteriophages such as ISP, Romulas and Rumus in the concentration of 10^7 PUFU/mL reduced biofilms by 37.8%, 34.4% and 60.4%, respectively, after 24 hours (Vandersteegen et al., 2013). Similar results were obtained in the studies (Gutieras et al., 2015) where a mixture of phages phiPLA-RODI and phiPLA-C1C was used against the formed biofilm of S. aureus. The phages reduced the number of microbial cells by 2 log in the biofilm 8 hours after treatment at 37 ºC incubation.

Subclinical mastitis of cows in 90–95% of cases occurs in chronic form and the main pathogen S. aureus forms mature biofilms that influence the effectiveness of antibiotic therapy (Horuk, 2019). In our studies, under the effect of Phage SAVB14 on 72-hour biofilms formed by S. aureus var. bovis, their degradation by 77.5 ± 1.4% occurred during 32 hours at 37 ºC. In this case, no viable microbial cells from the biofilm were isolated, and the bacteriophage titre was about 10^1 PUFU/mL. In this case, we can state that the phages penetrated and reached staphylococcal cells throughout the entire biofilm layer and the bacteria were susceptible to this phage. That is, a passive impact on biofilms by phages occurred, which lysis depended on the rate of absorption of the virus. Despite the fact that we obtained a rather effective bacteriophage action against the biofilm formed by S. aureus var. bovis in conditions in vitro, a number of authors (Hyman & Abedon, 2010) report that the in situ process of biofilm lysis depends on many factors associated with the physiological state of the host. However, some researchers (Abedon, 2011; Gutieras et al., 2015; Lopetuso et al., 2019) agree unanimously in situ that bacterial biofilms are more actively lysed under the influence of the bacteriophage than their young 24-hour biofilm forms.

Therefore, the results of laboratory studies indicate the prospect of efficient use of the specific staphylococcal bacteriophage Phage SAVB14, which we isolated for the destruction of biofilm formed by S. aureus var. bovis – from cows with mastitis.

Conclusion

Under the influence of staphylococcal bacteriophages on young 24-hour biofilms of S. aureus var. bovis we observed decrease in the optical density of dye solution by 34.5 ± 0.7% after 32 hours at 37 ºC, compared with the initial density. Also, decrease from 1.5 ± 0.3 × 10^7 to 1.3 ± 0.2 × 10^7 CFU/mL of the rinsed extract was observed for the number of S. aureus cells in biofilm after 32 hours of the experiment. This indicates that microbial cells of young biofilms are not susceptible to complete lysis at the impact of even a specific bacteriophage. At the same time, at the influence of the bacteriophage on mature 72-hour biofilms, 77.5 ± 1.4% degradation in the biofilms was observed after 32 hours at 37 ºC. At the same time, no viable cells of S. aureus were isolated from the biofilm. This indicates the high lytic activity of the bacteriophage towards mature biofilm bacteria and the possibility of its use in treating chronic staphylococcal infections caused by S. aureus var. bovis.

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