

SHORT COMMUNICATION

Characterization of lytic activity of *Phage SAvB14* on *Staphylococcus aureus* variant *bovis*

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ABSTRACT

Objective: The objective of this study was to investigate the intensity of phage infection caused by *Phage SAvB14*, which was isolated from dairy farms, depending on the initial number of *Staphylococcus aureus* cells in the medium.

Material and methods: To evaluate the impact of the viable bacteria *S. aureus* var. *bovis* on the intensity of phage infection caused by *Phage SAvB14*, 1 mg of phagolysate (phage titer 10⁵ CFU/ml) was introduced in 9 ml of nutrient broth with an appropriate amount of daily culture of *S. aureus* var. *bovis* under study. The number of viable staphylococci was determined by total viable count/ml.

Results: In this experiment, we found that the intensity of phages lytic activity was dependent on the number of sensitive bacterial cells in the volume of the culture medium. Effective phage therapy requires a high concentration of phages in the medium (inflammation foci) for rapid contact of the virus with bacteria.

Conclusion: When developing a phage drug to treat subclinical mastitis, it is necessary to increase the phage titer in the drug or its dosage compared to the clinical form, as there is a lower probability of phage contact with a susceptible microbial cell. Besides, at a high concentration of bacteria, there is a gradual decrease in nutrients in the medium, resulting in phages going back to the condition of lysogeny.

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Introduction

Cow mastitis is an inflammation of breast caused by pathogenic microflora [1–3]. This disease is one of the largest production problems in the dairy industry, not only in Ukraine but worldwide [3,4]. Among mastitis pathogens, *Staphylococcus aureus* is mostly attracted by the low level of effectiveness of antibiotic therapy and remains in the herd in the form of undetected subclinical infections. Studies report that the effectiveness of therapy for intramammary infections caused by *S. aureus* in the lactation period is up to 35%, and in the treatment of staphylococcal mastitis in the dry period is up to 80%

[5,6]. Overall, the resistance rates of *S. aureus* to antimicrobial agents vary widely by region. Also, multi-resistant strains are allocated from raw milk, especially methicillin-resistant *S. aureus* and vancomycin-resistant *S. aureus*, which pose a risk to humans [7]. Therefore, there is an urgent need to seek and develop new therapeutic mastitis treatments targeting this pathogen. Treatment of bacterial infections by phages is one of the alternative methods [8–10]. Studies indicate that only lytic phages should be used for effective phage therapy [11]. However, some lytic phages have also been reported to be known as lysis inhibition [12]. Chronically

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infected phage cells produce offspring that slowly depart from the cell or are transmitted to daughter cells without lysis. However, neither the phage genome integration into the host genome nor bacterial lysis occurs. This phenomenon is considered as a chronic phage infection or “carrier state.” This strategy is thought to help the phages to persist in the host when they lack nutrients to support the growth of germs or high levels of virions in the extracellular environment [13,14]. However, these phenomena are insufficiently disclosed and need a more detailed study.

Therefore, because of the above-mentioned issues, when developing cost-effective methods and treatments for mastitis of cows using phages, it is necessary to conduct a thorough research on the selection of specific, environmentally active and environmentally resistant phages.

The purpose of work was to determine the intensity of phage infection caused by *Phage SAvB14*, which was isolated on dairy farms, depending on the initial number of *S. aureus* cells in the medium.

Materials and Methods

To achieve this purpose were investigated the cultures of *S. aureus* and the specific *Phage SAvB14*, which we isolated from the secret of the mammary gland of cows with mastitis and investigated by the State Scientific Control Institute of Strains and Microorganisms. In the experiments, the strains of *S. aureus var. bovis* were used and were lysed by the *Phage SAvB14* using the Otto method [15].

To evaluate the impact of viable bacteria *S. aureus var. bovis* on the intensity of phage infection caused by the *Phage SAvB14*, 1 ml of phagolysate was injected with a phage titer of 10^4 PFU/ml in 9 ml of nutrient broth with a number of 5.0×10^2 , 1.0×10^3 , 1.0×10^4 and 1.0×10^5 daily culture of tested microorganisms. The number of viable staphylococci was determined by sowing them in the BD Baird-Parker Agar medium (HiMedia, India) according to

standard procedures. The studies were carried out in three replicates.

Statistical processing of results was carried out by variational statistics methods using the program Statistica 6.0 (StatSoft Inc., Tulsa, OK). Nonparametric research methods were used (Wilcoxon, Mann-Whitney criteria). The difference between the compared values was considered significant at $p < 0.05$.

Results

Virulent phages in contact with sensitive bacterial cells inhibit their metabolism, which leads to complete lysis of microorganisms. Our previous studies found that the isolated staphylococcal *Phage SAvB14* from cows with mastitis showed high lytic activity against *S. aureus var. bovis* cultures.

Table 1 shows the results of the study of the effect of *Phage SAvB14* at a concentration of 10^4 PFU/ml on strains of susceptible bacteria *S. aureus var. bovis* within 2 h.

The data of Table 1 indicate the dependence of the intensity of phage infection on the initial *S. aureus* content of the medium. After 2 h of phage interaction with microbial cells, the slowest spread of phage infection occurred in medium with an initial *S. aureus* content of $(5.0 \pm 0.1) \times 10^2$ CFU/ml. During this period, the number of staphylococci decreased by 1.1 times. Increasing the initial number of *S. aureus* in the medium to $(1.0 \pm 0.07) \times 10^3$ CFU/ml increased the incidence of phage infection, as the number of microbial cells decreased by 1.23 times after 2 h ($p < 0.05$). A similar pattern with respect to the spread of phage infection was observed in the greater initial number of *S. aureus* in the medium ($10^4 - 10^5$ CFU/ml). With this initial content of microbial cells, the effect of phage within 2 h led to a decrease in the number of staphylococci, an average of 1.3 times ($p < 0.05$). This indicated that bacterial lysis took place due to the rapid spread of phage infection among staphylococci.

In a nutrient BD Baird-Parker Agar medium with an initial number of *S. aureus* $1.0 \pm 0.002 \times 10^5$ CFU/ml,

Table 1. Influence of *Phage SAvB14* on *S. aureus var. bovis* within 2, 4, 24, and 48 h.

The initial number of <i>S. aureus var. bovis</i> , CFU/ml	Phage titer, PFU/ml	Number of <i>S. aureus var. bovis</i> after the influence of phage, CFU/ml			
		2 h	4 h	24 h	48 h
$5.0 \pm 0.1 \times 10^2$	10^4	$4.5 \pm 0.1 \times 10^2$	$1.8 \pm 0.07 \times 10^2$	0	0
$1.0 \pm 0.07 \times 10^3$	10^4	$8.1 \pm 0.1 \times 10^2$ *	$3.2 \pm 0.1 \times 10^2$	0	0
$1.0 \pm 0.008 \times 10^4$	10^4	$7.6 \pm 0.1 \times 10^3$ *	$2.3 \pm 0.1 \times 10^{3\Delta\Delta}$	0	0
$1.0 \pm 0.002 \times 10^5$	10^4	$7.5 \pm 0.1 \times 10^{4*\Delta}$	$2.2 \pm 0.05 \times 10^{4\Delta\Delta}$	$7.8 \pm 0.3 \times 10^2$	$7.1 \pm 0.2 \times 10^2$

CFU = colony-forming unit; PFU = plaque-forming unit.

* $p < 0.05$ in comparison with initial number; Δ – compared to a number of *S. aureus* $(4.5 \pm 0.1) \times 10^2$ CFU; $\Delta\Delta$ – compared to initial number of *S. aureus* $4.5 \pm 0.1 \times 10^2$ CFU/ml and $1.0 \pm 0.07 \times 10^3$ CFU/ml.

1.6 times ($p < 0.05$) faster killing of microbial cells was observed, compared to a medium with $5.0 \pm 0.1 \times 10^2$ CFU/ml and 1.5 times ($p < 0.05$) faster compared to $1.0 \pm 0.07 \times 10^3$ CFU/ml. With an initial *S. aureus* content in the nutrient broth of $1.0 \pm 0.008 \times 10^4$ CFU/ml, the intensity of phage infection was also 1.5 and 1.4 times faster ($p < 0.05$) than in the broth with the number of staphylococci. $5.0 \pm 0.1 \times 10^2$ and $1.0 \pm 0.07 \times 10^3$ CFU/ml, respectively. This indicates that the greater number of microbial cells in the medium, the faster the contact between the virus and the bacterium, which leads to the spread of phage infection.

At the same time, during 24 and 48 h of phage exposure to microbial cells in variants of experiments with an initial number of *S. aureus* up to $1.0 \pm 0.008 \times 10^4$ CFU/ml, complete bacterial lysis occurred, and they were not isolated from the nutrient medium. At the initial number of staphylococci in the broth $1.0 \pm 0.002 \times 10^5$ CFU/ml after 48 h of action, the phage of complete lysis of microbial cells was not observed. The number of *S. aureus* decreased by several orders of magnitude of $7.1 \pm 0.2 \times 10^2$ CFU/ml.

Discussion

Phages are natural viruses that use bacterial cells for growth, as soon as the phage overcomes the host's immunity (i.e., Clustered Regularly Interspaced Short Palindromic Repeats; CRISPR defenses), the cell may undergo lytic or lysogenic cycle [16,17]. The ability of phages to influence bacteria depends on the possible adsorption of phages into the host cell. It is believed that the more phage sensitive bacteria present in the medium, the greater the likelihood that free phage will be adsorbed [18–20].

In this experiment, we detect a virulent phage infection of a sensitive bacterial culture that results in the inhibition of *Staphylococcus* cell metabolism and almost complete bacteriolysis. However, the intensity of the lytic activity of phages depended on the number of sensitive bacterial cells in the volume of culture medium. When introduced into the nutrient broth $5.0 \pm 0.1 \times 10^2$ CFU/ml of microbial cells of staphylococci, it was found that after 2 h of phage–bacterial interaction, the number of viable cells decreased by 1.1 times compared with the original number. At the same time, due to the effect of phage on bacteria with an initial *Staphylococcus* count of $1.0 \pm 0.008 \times 10^4$ and $1.0 \pm 0.002 \times 10^5$ CFU/ml of medium, the lytic activity was more effective as the bacterial count decreased by an average of 1.3 times ($P < 0.05$). A similar pattern was found after 4 h of phage infection for staphylococci. Similar results were

obtained by other scientists who noted that the optimal ratio of phage and bacteria (virus-to-bacterium ratio or VBR) is a crucial factor in the fight against bacterial pathogens. Studies showed that the ratio of phage and bacteria of 1:100 caused a slight decrease in viable *P. aeruginosa* cells.

In contrast, a ratio of 1:1000 was 10 times more effective within 24 h of treatment [21–23]. Other scientists studied the interaction of phages with 72 h biofilm of *E. coli*. In the short period (30 min), significantly more biofilm disturbance was observed in the ratio of phage and bacteria of 1:100 compared to the ratio of 1:10 [24,25]. Therefore, effective phage therapy requires a high concentration of phages in the medium (inflammation foci) for rapid contact of the virus with bacteria. Even if phages cause the infection of one pathogenic cell, the closest susceptible microbial cell can be far enough from the infected one. Therefore, the spread of the phage epidemic in the inflammation site requires the achievement of significant infection of microbial cells by phage.

Our previous studies on examining bacterial pathogens in various forms of mastitis have found that in subclinical mastitis, the number of *S. aureus* was, on average three times lower than in acute form [26]. Therefore, the approach to the use of phage drugs for the treatment of subclinical mastitis should be different from the clinical one. When developing a phage drug for the treatment of subclinical mastitis, it is necessary to increase the phage titer in the drug or its dosage compared to the clinical form, since there is a lower likelihood of phage contact with a sensitive microbial cell.

Studies have reported [27–30] that the coexistence of bacteria and phages may be unstable due to nutrient deficiencies for bacteria, low intensity of phage infection, and the like. The results of studies found that after 24 h of *Staphylococcus* interaction with phage in the variant with a large initial number of microbial cells (10^5 CFU/ml) did not occur complete lysis of staphylococci, and their number was $7.8 \pm 0.3 \times 10^2$ CFU/ml. This suggests that with a high bacterial concentration, there is a gradual decrease in nutrients in the medium, causing the phages to become lysogenic. Lysogenesis is a parasitic symbiosis where phages delay cell lysis, integrate into the bacterial genome, and are in a weak active state [30]. According to studies [31–33], lysogenesis is more common when the multiplicity of infection is large and nutrient deficiency occurs.

Therefore, summarizing the results of the study, the prospect of effective use of specific Staphylococcal Phage SA_vB14, selected by us, in the mastitis of cows caused by *S. aureus* var. *bovis* can be noted. However, when selecting the dose and concentration of phage, it is necessary to take

into account the specific features of phage–bacterial interaction to ensure effective treatment.

Conclusion

The lytic activity of phages has been found to be dependent on the number of susceptible bacterial cells in the volume of the medium. With an initial amount of *S. aureus* $5.0 \pm 0.1 \times 10^2$ CFU/ml of the medium, after 2 h of phage–bacterial interaction, the viable cell counts decreased by 1.1 times. At the same time, due to the effect of phage on staphylococci with an initial amount of 10^4 and 10^5 CFU/ml of the medium, the bacterial content decreased by an average of 1.3 times. It has been found that after 4 h of action of bacteriophage in an environment with an initial amount of *S. aureus* $1.0 \pm 0.002 \times 10^5$ CFU/ml, there has been observed 1.6 times faster death of microbial cells compared to the medium with an amount of $5.0 \pm 0.1 \times 10^2$ CFU/ml and 1.5 times faster than $1.0 \pm 0.07 \times 10^3$ CFU/ml. This indicates that the larger the number of microbial cells in the environment, the faster the contact between the virus and the bacterium. It has been established that during 24 and 48 h of phage exposure to microbial cells in variants with an initial amount of *S. aureus* up to 1.0×10^4 CFU/ml, complete bacterial lysis occurs. At the initial *Staphylococcus* count in the environment of $1.0 \pm 0.002 \times 10^5$ CFU/cm³, complete lysis of microbial cells has been not observed. The number of *S. aureus* decreased by several orders of magnitude and amounted to $7.1 \pm 0.2 \times 10^2$ CFU/ml. This suggests that with a high bacterial concentration, there is a gradual decrease in nutrients in the medium, causing the phages to become lysogenic.

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contribution

Y. Horiuk, M. Kukhtyn, V. Horiuk developed the experiment, analyzed data, and wrote the manuscript. A. Tsvihun and S. Kernychnyi helped in manuscript writing, setting, and data analysis.

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