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Characteristics of bacteriophages of the *Staphylococcus aureus* variant *bovis*

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Abstract: Bacteriophages may be an alternative method of treatment for antibiotic-resistant bacteria, including mastitis in cows. Our study describes the initial isolation and bacteriological activity of bacteriophages, circulating on dairy farms, the against *S. aureus* var. *bovis*. Samples of cow's milk secretions with signs of mastitis and sewage water were used as the study material. The isolation and production of pure bacteriophage lines were performed according to the double agar method. The method of studying a single cycle of phage reproduction was used to determine the duration of the latency period. Determination of the spectrum of the lytic activity of bacteriophages against the clinical isolates of the microorganisms was carried out by the drop method. As a result of the research, four phages, specific for *S. aureus* var. *bovis* were isolated: *Phage SAvB07*, *Phage SAvB08*, *Phage SAvB12* and *Phage SAvB14*. The negative colonies of the isolated phages were 1–2 mm in size, rounded with clear edges, with varying degrees of transparency. The latency period of *Phage SAvB14* was 35 min, with the number of active virions increasing by 8 orders. In the study on growth curves of other bacteriophages, taken in the experiment, the latency period was more than 35 min, and their titre increased by only two orders. *Phage SAvB07*, *Phage SAvB08* and *Phage SAvB12* were able to lyse the bacterial strains of *S. aureus* var. *bovis* in 25–45.6% of the cases (low lytic activity), whereas *Phage SAvB14* lysed 94.1% of *S. aureus* strains were isolated from the cows. Studies have shown that among the bacteriophages we have studied, *Phage SAvB14* with a short latency period has the best lytic action on the culture *S. aureus* var. *bovis*. The resulting bacteriophage strain can be used to create a bacteriophage-based drug for the treatment of mastitis in cows.

Keywords: phages; *S. aureus*; mastitis; latency period; lytic activity

Interest in the use of phages as antimicrobials is increasing not only from the side of human medicine (Leskinen et al. 2017), but also veterinary medicine (O'Flaherty et al. 2005). Phages are an effective microbiological tool capable of fighting specific bacterial strains that cause infectious diseases (Kazmierczak et al. 2014; Iwano et al. 2018). The antibacterial effect of bacteriophage agents is

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when the phage genome enters the bacterium with the subsequent lysis of the infected cell through the phage reproduction. After lysis, the bacteriophages that are released into the external environment reinfect healthy bacterial cells, until they are completely destroyed in the inflammation site (Han et al. 2013). That is why bacteriophages play an important role in the evolution of bacteria and the realisation of their pathogenic potential (Ganaie et al. 2018). In addition, phages contribute to the formation of genetic diversity in the bacteria (Synnott et al. 2009). Bacteriophages can carry out horizontal genetic exchange between strains of germs by lysogenic conversion or transduction. Therefore, the use of non-virulent (moderate) bacteriophages for the treatment and prevention of infections is absolutely unacceptable (Young 1992; Nelson et al. 2001; Lu et al. 2003). Therefore, the main criterion for effective phagotherapy is the use of highly virulent bacteriophages. In this connection, it is important to search for lytic phages concerning their therapeutic use.

Lytic bacteriophages are attracting the attention of researchers as a possible means of combatting antibiotic-resistant bacteria, among which *S. aureus* is one of the most dangerous (Kwiatek et al. 2012; Ganaie et al. 2018; Horiuk et al. 2019). Some researchers describe the isolation and characterisation of bacteriophages that are specifically active against *S. aureus*, which are causative agents of mastitis in cows on dairy farms (O'Flaherty et al. 2005; Synnott et al. 2009; Han et al. 2013; Li and Zhang 2014; Xia and Wolz 2014; Zhang et al. 2017; Hamza et al. 2016; Uchiyama et al. 2017; Azam and Tanji 2019). However, there is no information on bacteriophages circulating directly on dairy farms in Ukraine as they can be used to create drugs effective in treating mastitis caused by *Staphylococcus aureus*.

The purpose of study has been to conduct a microbiological characterisation of staphylococcal phages, isolated on Ukrainian dairy farms, to create drugs for the treatment of mastitis in cows.

MATERIAL AND METHODS

Samples of cows' secretions with signs of mastitis and sewage water were used as the study material. A working set of microorganisms from field strains isolated on dairy farms in the western region of Ukraine have been created as hosts for the cultivation of phages active against *S. aureus* var. *bovis*.

The isolation and production of pure bacteriophage lines was performed according to the method developed by Oliveira et al. (2009). To determine the morphology of the negative colonies, the phages were diluted in 10^8–10^9 on Petri dishes by the method of agar layers. The crops have been cultured at 37 °C. The study of the morphology of the negative colonies was performed after 6, 10, 16, 24 hours. The size of the negative colonies, their shape, the degree of the transparency, the nature of the edges of the colonies were noted.

To determine the duration of the latency period, the method of studying a single cycle of the phage reproduction was used (Kutter and Sulakvelidze 2004). The determination of the spectrum of the lytic activity of the bacteriophages against the clinical isolates of the microorganisms was carried out by the drop method. For this purpose, 3–4 drops of an 18–24-hour broth culture of thetested microorganisms was applied to the surface of the BD Baird-Parker Agar medium (HiMedia, Mumbai, India) in the Petri dishes. The optical density of the inoculum was 0.5 units according to McFarland (control using a densitometer), which corresponds to 1.5×10^8 microbial cells/ml. Then the cultures were evenly distributed on the surface of the medium with a sterile spatula. The dishes with the seeded media were dried in a thermostat for 15–20 minutes. After that, the test bacteriophage was applied to the surface of the medium with a light touch of droplets, with an amount of at least 10^8 PFU/ml, tilting the dish so that the droplets were glassy and then incubated at 37 °C. The results were evaluated after 18–24 hours. As a control, a sterile nutrient broth was applied to the surface of the dense culture medium. The studies were performed in three replicates. As the experimental cultures, 101 strains of *Staphylococcus aureus* bacteria of different biotypes were used.

RESULTS

In the first stage of the study, we isolated bacteriophages on Ukrainian dairy farms and gave them characteristics (Table 1). When assessing the shape of the colonies of the selected phages and characterising their margins, no difference was found – all the phages of the colonies had a round form and

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Table 1. Characteristics of the negative colonies of the isolated phages

Studied phages	Diameter of negative colonies, mm	Shape of negative colonies	Degree of transparency	Nature of edges of colonies
Phage SAvB07	1.0 ± 0.1	round	half-transparent	even
Phage SAvB08	1.5 ± 0.1	round	half-transparent	even
Phage SAvB12	2.0 ± 0.1	round	half-transparent	even
Phage SAvB14	1.5 ± 0.1	round	transparent	even

equal edges. In terms of transparency, the isolated phages formed half-transparent zones, except for *Phage SAvB14*, in which the zone was transparent. The diameter of the phage colonies ranged from 1.0 mm to 2.0 mm, the smallest colonies in *Phage SAvB07* were 1.0 ± 0.1 mm, and the largest diameter in *Phage SAvB12* was 2.0 ± 0.1 mm. The phage that formed the transparent colony (*SAvB14*) had a diameter of 1.5 ± 0.1 mm.

Figure 1 shows the growth curves of the isolated bacteriophages. It can be seen that in phages *SAvB07* and *SAvB08* (Figure 1A and 1B), the latent period, during which the microbial cell destruction occurred, was 35–40 minutes. During this time period, the number of active phages increased by an average of two orders to 6 log PFU/ml. In *Phage SAvB12* (Figure 1C) the latent time period increased 1.7 times ($P < 0.05$) compared to phages *SAvB07* and *SAvB07* and amounted to 60 minutes. The number of virions also increased by two orders. *Phage SAvB14* also had a short latency period of 35 min, but it released a large amount of virions, which was 12 PFU/ml, log. This gives reason to believe that there is an infection and destruction of a large number of staphylococcal cells. Comparing the growth curves of the isolated phages, the bacteriophages *SAvB07*, *SAvB08*, and *SAvB12* can be attributed to a moderate lysogenic phage, because there was the lysis of the individual microbial cells of the staphylococci and release of a small number of virions. At the same time, *Phage SAvB14* had a short latency period and resulted in the release of virions 6 orders more than the phages *SAvB07*, *SAvB08*, and *SAvB12*.

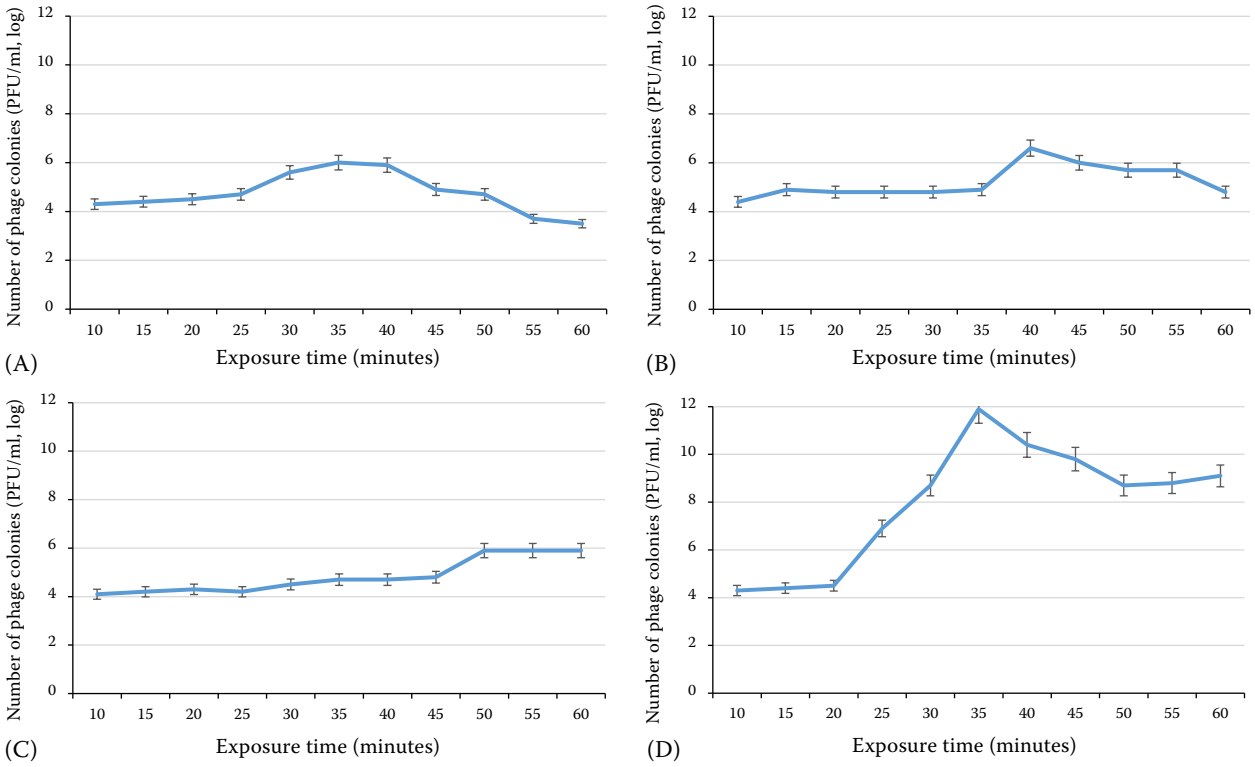

Figure 1. Growth curve of bacteriophages: (A) *Phage SAvB07*; (B) *Phage SAvB08*; (C) *Phage SAvB12*; (D) *Phage SAvB14*. Each data point is the mean value ± standard deviation from three independent experiments

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Table 2. Spectrum of the lytic action of the isolated bacteriophages

Studies of bacteriophage strains	Number of lysed cultures			
	S. aureus var. *bovis* (*n* = 68)		*S. aureus* var. *hominis* (*n* = 33)	
	n	%	*n*	%
Phage SAvB07	17	25.0	0	0
Phage SAvB08	21	30.8	0	0
Phage SAvB12	31	45.6	0	0
Phage SAvB14	64	94.1	1	3.1

Table 2 shows the results of the studies of the lytic action of the isolated phages to the staphylococci of different biotypes.

The phages were found to exhibit different lytic activity toward the culture *S. aureus* var. *bovis.* The staphylococcus cells were lysed the most under the action of *Phage SAvB14* – 94.1% of the cultures, the other three isolated phages (*SAvB07, SAvB08* and *SAvB12*) lysed the *Staphylococcus aureus* biotype from 25.0% to 4.5% of the cases. The cells of *S. aureus* var. *hominis* were practically not lysed with the phages isolated from the cows with mastitis, only 3.1% of cultures were sensitive to *Phage SAvB14.*

DISCUSSION

The size and shape of the negative colonies is an important characteristic of a phage (Lu et al. 2003; McCallin et al. 2018). A negative colony morphology can be used as a taxonomic feature during the primary phage classification because it is extremely specific for a particular strain and sometimes for a group of related bacteriophages (Kwiatek et al. 2012; Xia and Wolz 2014). Therefore, in the first stage of study, we carried out the isolation of bacteriophages by sowing the test material by the method of a two-layer agar. To obtain a pure phageline, five to seven passages were carried out from the isolated negative colonies. As a result, the negative colonies of the phages, which had a size of 1–2 mm, round in shape with even edges, were obtained. This type is characteristic of bacteriophages of *Staphylococcus aureus*. However, the resulting phage plaques were of varying degrees of transparency.

The results of the spot test and the analysis of the plaque formation reflect two different mechanisms underlying the phage bacteriolysis (Nelson et al. 2001). The formation of a lytic stain is a combination of the lysis, associated with the cycle of the phage (lytic) replication, and the lysis, caused by the direct binding of the phage to the bacterium (Young 1992). The second mechanism involves the occurrence of the lysis simply as a result of the attachment of the phages to the surface of a bacterial cell with the subsequent suppression of any multiplicative process of the virus propagation. Therefore, the transparency of the phage plaques directly depends on the ability of the phage to lyse the bacterial culture (Young 1992; Nelson et al. 2001). Thus, translucent colonies usually form moderate bacteriophages, since most bacterial cells in the middle of the colony remain in a state of lysogeny. Similar results have been obtained by scientists in the study of phages on dairy farms. Thus, phage *ΦSA039* lysed all the isolated staphylococcus cultures on the dairy farms, but the colonies were half-transparent (Synnott et al. 2009), which is evidence of a poor lytic activity.

Strains of bacteriophages with a short latency period and with a large number of virions after the bacterial cell destruction are considered ideal for the creation of therapeutic agents (O'Flaherty et al. 2005; McCallin et al. 2018). The results of the study revealed that the latency period of *Phage SAvB14* averaged 35 minutes. The number of active virions increased by 8 orders compared to the original number. Whereas, in the study on the growth curves of other bacteriophages, taken in the experiment, the latent period was 35–60 min, and an increase in the bacteriophage titre occurred by 2 orders. In the study (Kwiatek et al. 2012), virulent bacteriophages (MSA6) were isolated from cows with mastitis. MSA6 had a short latency period (15 min) and a relatively small burst size (23 PFU/cell). The obtained phages have been rec-

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ommended for the production of an agent on the basis of a phage cocktail for the treatment of diseases in cows.

There are only a few studies concerning the clinical use of bacteriophages for the treatment of mastitis in cows (Poutrel and Lerondelle 1980; Gill et al. 2006), and the effectiveness of such treatments is quite low. This may be due to the different mechanisms of the phage-bacterial interaction and the influence of external factors that require more detailed studies.

Therefore, the next step is to determine the host range of the isolated bacteriophages and their lytic activity against them. For this purpose, we studied the effect of the selected phages on cultures of *Staphylococcus aureus* of different biotypes. Thus, all the isolated phages have been, to some extent, lysed with *Staphylococcus aureus* strains isolated from cows. *Phage SAvB07, Phage SAvB08* and *Phage SAvB12* lysed the bacterial strains in 25–45.6% of the cases, i.e., they showed poor lytic activity. *Phage SAvB14* alone lysed 94.1% of the *Staphylococcus aureus* strains isolated from the cows. A study on the lytic activity of bacteriophages that can be used in mastitis therapy was performed by Ganaie et al. (2018). Their study describes the isolation and characterisation of two lytic phages SAJK-IND and MSP specific for *Staphylococcus aureus*. In this case, SAJK-IND showed 100% lytic activity against several strains of *Staphylococcus aureus* isolated from cows with mastitis, whereas MSP only showed 40% lytic activity.

We investigated strains of *S. aureus* isolated from humans. This made it possible to confirm the specificity of hosts, since only *Phage SAvB14* inhibited the growth of one culture *S. aureus* var. *hominis*. Similar results have been obtained by researchers (O'Flaherty et al. 2005) in the study of the hosts of phages CS1 and DW2, isolated from cows with signs of mastitis. These phages have been lysed with bacterial strains isolated from cattle. However, both phages did not form noticeable colonies when cultured on strains isolated from humans in Irish hospitals.

Thus, the studies indicate that among the bacteriophages that we have studied, *Phage SAvB14* with a short latency period shows the best lytic activity on the culture *S. aureus* var. *bovis*. The resulting bacteriophage strain can be used to create a bacteriophage-based drug for the treatment of mastitis in cows.

Conflict of interest

The authors declare no conflict of interest.

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