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Isolation and study of biological properties of *Bordetella bronchiseptica*-specific bacteriophages

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SUMMARY

The paper presents results of the studies on isolation of bacteriophages active against *Bordetella bronchiseptica*. Three new bacteriophages were recovered from clinical samples from animals with respiratory signs:*vB_BbrS_2/200220.7.2*, *vB_BbrS_4/200220.7.1*, *vB_BbrM_5/200220.7.2*. The bacteriophage isolation method and basic biological properties thereof have been specified in detail. The lytic activity of isolated bacteriophages was determined by the agar layer method varied from $(2.3 \pm 1.4) \times 10^8$ to $(9.0 \pm 0.2) \times 10^8$ PFU/mL, and the lytic spectrum ranged from 61.5 to 76.9%. The bacteriophage titer stability was shown during 8-month storage of phage lysate with no preservative added. The morphology of bacteriophage plaques was tested in various nutrient media and analyzed based on two parameters: size and transparency. Dissociation of plaques into clear colonies, turbid colonies, and clear colonies with turbid halos was observed in the media. Plaques also were varied in size from 0.6 ± 0.2 to 2.6 ± 0.1 mm. Great thermal stability was noted during exposure of bacteriophages to high temperatures ranging from +40 to +95 °C with 5 °C increment. The specificity study showed that the isolated bacteriophages lyse closely-related bacteria. The electron microscopy of each bacteriophage revealed such parameters as the average diameter of the head and the average length of the tail. In accordance with the international classification of viruses by morphological characteristics the *vB_BbrS_2/200220.7.2* and *vB_BbrS_4/200220.7.1* phages have been assigned to the family *Siphoviridae*, *vB_BbrM_5/200220.7.2* bacteriophage has been assigned to the family *Myoviridae*. The obtained results of *in vitro* studies have shown that the isolated bacteriophages can be promising for phage therapy of *Bordetella bronchiseptica*-induced diseases in veterinary medicine.

Keywords: bacteriophages, *Bordetella bronchiseptica*, phage therapy, phage prophylaxis

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Выделение и изучение биологических свойств бактериофагов, специфичных к *Bordetella bronchiseptica*

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РЕЗЮМЕ

Представлены результаты собственных исследований по выделению бактериофагов, активных в отношении *Bordetella bronchiseptica*. Из клинического материала от животных с признаками респираторных заболеваний выделено три новых бактериофага: *vB_BbrS_2/200220.7.2*, *vB_BbrS_4/200220.7.1*, *vB_BbrM_5/200220.7.2*. Подробно описана методика выделения бактериофагов и их основные биологические свойства. Литическая активность выделенных бактериофагов, определяемая методом агаровых слоев, варьировала от $(2,3 \pm 1,4) \times 10^8$ до $(9,0 \pm 0,2) \times 10^8$ БОЕ/мл, а спектр литического действия составил от 61,5 до 76,9%. Показана стабильность титра бактериофагов при хранении фаголизата в течение 8 месяцев без добавления консерванта. Морфологию негативных колоний бактериофагов изучали на различных питательных средах и анализировали по двум признакам: размер и прозрачность. На средах наблюдалась диссоциация негативных колоний на прозрачные, мутные, и прозрачные с мутными ореолами. Бляшки также

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различались по размеру: от 0,6 ± 0,2 до 2,6 ± 0,1 мм. Отмечены высокие показатели температурной устойчивости при воздействии на бактериофаги высокой температуры от +40 до +95 °С с шаговым интервалом 5 °С. Изучение специфичности показало, что выделенные бактериофаги лизируют близкородственные бактерии. В ходе электронно-микроскопических исследований для каждого бактериофага были определены такие параметры, как среднее значение диаметра головки и среднее значение длины хвоста. В соответствии с международной номенклатурой вирусов по морфологическим параметрам фаги *vB_BbrS_2/200220.7.2* и *vB_BbrS_4/200220.7.1* отнесены к семейству *Siphoviridae*, бактериофаг *vB_BbrM_5/200220.7.2* – к семейству *Myoviridae*. Полученные результаты исследований *in vitro* показали, что выделенные бактериофаги могут быть перспективными для применения в ветеринарной медицине в фаготерапии заболеваний, вызванных бактерией *Bordetella bronchiseptica*.

Ключевые слова: бактериофаги, *Bordetella bronchiseptica*, фаготерапия, фагопрофилактика

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INTRODUCTION

Bordetella bronchiseptica is an aerobic gram-negative motile bacterium. It causes respiratory diseases in most domestic animals (pigs, rabbits, cats, dogs) and wild animals of all ages, however, young individuals under one year of age and animals with chronic diseases are at risk of infection most of all [1–3]. The bacteria multiply in the epithelial cilia of the respiratory tract; infection is airborne, the incubation period lasts from 5 to 20 days [2, 4].

Respiratory infection (bordetellosis) caused by *B. bronchiseptica* develops quite quickly, causing infectious tracheobronchitis in dogs, commonly known as "kennel cough", pneumonia, rhinitis in cats and rabbits [3, 5, 6]. In some cases, the disease can actively progress to bronchopneumonia and result in death [1, 7, 8]. *B. bronchiseptica* is the cause of atrophic rhinitis and bronchopneumonia in pigs. Atrophy of the nasal septum and nasal conch can occur [3, 4, 9, 10]. It has been reported cases of human infections with *B. bronchiseptica* bacteria [2, 3, 5, 6, 11–14]. The disease mainly affects children, people with chronic diseases and weakened immunity. People get infected by direct contacts with sick animals, the possibility of cross-infection between humans is not excluded [5, 6, 11–14].

To date, the main means of bordetellosis therapy in pets and livestock are antibiotics, but their effectiveness is reduced due to the spread of *B. bronchiseptica* antimicrobial resistant strains [15, 16]. This entails an active increase in morbidity among animals, a reduced productivity of farm animals, and, therefore, economic losses in animal production. There is a direct threat to human health, since there is a risk of infection with antimicrobial-resistant strains of *B. bronchiseptica* from carrier animals.

The development of new tools and methods of prevention and treatment of bordetellosis is an urgent task in veterinary medicine. One of the promising and safe solutions to this issue is the use of products based on bacteriophages. The prospects for the use of phages active against *B. bronchiseptica* are conditioned by their safety, and the lack of a negative impact on the normal flora of the body [4, 17].

Bacteriophages can be used not only as an alternative to antibiotics, but also in combination with all types of traditional antimicrobial therapy [17]. In a study conducted by G. Y. Park et al. [10], it was found that bacteriophages have therapeutic potential against respiratory diseases caused by *B. bronchiseptica* and can participate in the suppression of bacterial inflammation. However, the mechanism of this process remains under-studied.

The purpose of this study was to isolate bacteriophages active against *B. bronchiseptica*, to study their biological properties for further evaluation of potential practical application in veterinary medicine.

MATERIALS AND METHODS

Strains of *B. bronchiseptica* bacteria were isolated from clinical material from rabbits, dogs and cats (mucosal swabs, faecal samples, water from drinkers). Samples were submitted from animal shelters, veterinary clinics and veterinary institutes of Moscow and the Moscow Region. The *B. bronchiseptica* pathogenic isolates were selected based on the results of identification of microorganisms by microscopic, biochemical and mass spectrometric methods to create a working collection of strains of RPC "MikroMir". The strains were certified and deposited into the collection of microorganisms of RPC "MikroMir". All bacterial strains under study were preliminarily tested for the absence of profages in the culture by S. Luria and D. Darnell method [18], as well as using induction by ultraviolet radiation [19].

B. bronchiseptica bacteria were cultured at a temperature of (37 ± 0.5) °C for 24 hours on BHI agar (HiMedia Laboratories Pvt. Limited, India) with 5% sterile defibrinated sheep blood added.

The isolation of bacteriophages and the study of their biological properties were carried out by methods proposed by M. Adams [20] and D. M. Goldfarb [21]. Bacteriophages were isolated from samples of biomaterial of animals from which strains of *B. bronchiseptica* had been previously isolated. The samples were resuspended in 20 mL of isotonic saline solution. Large particles and bacteria were removed from the resulting suspension by low-speed centrifugation (5,000 rpm, 20 min) using Avanti J-E centrifuge (Beckman Coulter, Inc., USA) [22]. Pathological materials of the liquid phase were centrifuged without preliminary resuspending at the same parameters.

The supernatant was separated from the precipitate and centrifuged on an Optima L-90K ultracentrifuge (Beckman Coulter, Inc., USA) at a high speed (27,000 rpm, for 120 min). The precipitate was resuspended in 0.05 M Tris-HCl buffer (pH 7.0–7.2) and filtered through membrane filters (pore sizes 1.2; 0.45; 0.22 microns) of Sartorius, Germany. The presence of phages in the filtrate was detected by the Gratia method [20, 21]. The detection of various types of plaques on the bacterial lawn of the test culture suggested the presence of several types of phages in the tested material [22]. Pure bacteriophage lines were obtained from morphologically homogeneous plaques. For this purpose, 0.1 mL of an 18-hour culture of the test strain was inoculated into flasks with 20 mL of BHI broth and incubated in a growth chamber (Binder, Germany) at 37 °C. A fragment of an agar plate with a single phage plaque was introduced into the log-phase culture. A flask without a plaque fragment served as a control. The contents of the flasks were cultured in a growth chamber (Binder, Germany) at 37 °C for 24 hours, after this, clearing was observed in the flasks with the test strain, and pronounced turbidity of the medium was observed in the control flask. Then the contents of the flasks were centrifuged for 20 min at 5,000 rpm on an "Avanti J-E" centrifuge (Beckman Coulter, Inc., USA) [22]. The collected supernatant was successively filtered through different membranes (pore size 1.2; 0.45; 0.22 microns). The obtained phagolysate was tested again by the agar layer method [20, 21]. The procedure was repeated until homogeneous plaques were obtained.

To obtain a sufficient amount of phagolysate with a consistently high titer, bacteriophages were cultured according to the following method: 0.3 mL of a phage-sensitive culture of *B. bronchiseptica* was added to flasks with 50 mL of BHI broth. The suspension was incubated in an orbital shaker-incubator (BioSan ES-20/60, Latvia) at 140 rpm at 37 °C for 2.5 hours. Then 3.0 mL of a pure bacteriophage line was introduced into the flasks and cultured at 37 °C for 18–24 hours at 140 rpm. After this time, the contents of the flasks were centrifuged at a low speed for 20 min at 5,000 rpm, then the phage particles were re-precipitated at 27,000 rpm for 120 min on an "Optima L-90K" centrifuge (Beckman Coulter, Inc., USA). The precipitate was resuspended in 0.05 M Tris-HCl buffer (pH 7.0–7.2) and filtered through membrane filters (pore sizes 1.2; 0.45; 0.22 microns). To determine the titer, the phagolysate was titrated according to generally accepted methods on dense nutrient media (Gratia method) [20, 21], after which the filtrate was placed in sterile test tubes for storage at 4 °C.

The lytic activity of the isolated phages was determined by the Gratia method [20, 21].

The spectrum of lytic activity was studied on 13 test cultures of *B. bronchiseptica* by spot testing [22].

The bacteriophage plaque morphology was studied on various dense nutrient media: 1.5% BHI-agar (HiMedia Laboratories Pvt. Limited, India) with the addition of 5% sterile defibrinated sheep blood, 1.5% BHI-agar (HiMedia Laboratories Pvt. Limited, India), 1.5% FPH agar (FBSI SSC PMB, Russia), 1.5% Bordetelagar (FBSI SSC PMB, Russia) [23]. 1.0 mL of dilutions of the titrated bacteriophage and 0.1 mL of bacterial suspension were added to tubes with 0.8 or 0.4% BHI-agar (HiMedia Laboratories Pvt. Limited, India), then were inoculated on pre-prepared plates with solid agar (1.5%). The morphology of plaques was studied after incubation for 18–20 hours at 37 °C [22].

Electron microscopy of the obtained bacteriophages, counterstained with 1% uranyl acetate, was performed on a transmission electron microscope JEM-1011 (JEOL, Japan). The figures were taken using a side-mounted camera Erlangshen ES500W (Gatan, USA). The bacteriophage parameters were measured using the ImageJ program.

The specificity of the phage was studied by spot testing of phagolysate on the lawn of closely related bacteria: *Bordetella parapertussis* C-95, *Bordetella parapertussis* C-94, *Bordetella pertussis* C-99. The strains were obtained from the RPC "MikroMir" collection of microorganisms.

To study the thermal stability of isolated bacteriophages, phagolysates were heated in a dry block heater "Termit" ("DNA Technology" LLC, Russia) with a temperature increase from 40 to 95 °C. A sample was taken from the test tube every 20 min, while increasing the temperature by 5 °C [24]. The control tubes were not heated. The activity of the tested phages was determined by the Gratia method [20, 21].

The titer stability of the phage in the sealed tube was studied using the Gratia method and stored at 4–8 °C without the addition of a preservative for 8 months [20, 21].

RESULTS AND DISCUSSION

Three new virulent bacteriophages were isolated from samples of animal biomaterial (Fig. 1), which were designated as: *vB_BbrS_2/200220.7.2*; *vB_BbrS_4/200220.7.1*; *vB_BbrM_5/200220.7.2*.

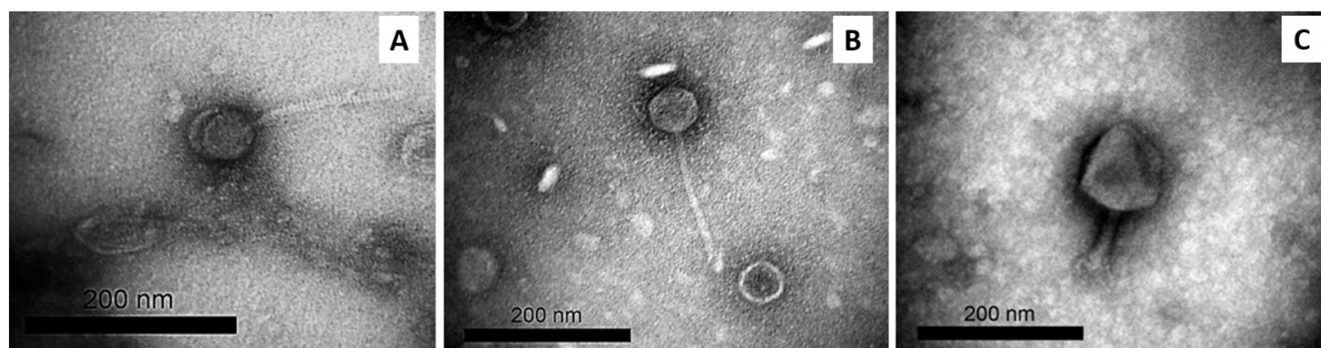

Fig. 1. Electron micrographs of bacteriophages active against B. bronchiceptica: A – vB_BbrS_2/200220.7.2; B – vB_BbrS_4/200220.7.1; C – vB_BbrM_5/200220.7.2 (1% uranyl acetate solution was used as a counterstain, 250,000× magnification)

Table 1
Morphology of bacteriophage plaques

Nutrient medium	Plaque morphology		
	vB_BbrS_2/200220.7.2	*vB_BbrS_4/200220.7.1*	*vB_BbrM_5/200220.7.2*
1.5% BHI-agar with blood	– clear, Ø 1.8 ± 0.3 mm; – turbid, Ø 0.9 ± 0.1 mm; – clear with turbid halo, Ø 2.3 ± 0.3 mm	– clear, Ø 1.2 ± 0.3 mm; – turbid, Ø 1.0 ± 0.1 mm; – clear with turbid halo, Ø 2.4 ± 0.1 mm	– clear, Ø 0.6 ± 0.2 mm; – turbid, Ø 1.9 ± 0.1 mm
1.5% BHI-agar	– turbid, Ø 1.1 ± 0.1 mm	– turbid, Ø 1.0 ± 0.1 mm	– turbid, Ø 1.0 ± 0.1 mm
1.5% FPH-agar	– turbid, Ø 1.0 ± 0.1 mm	– turbid, Ø 1.0 ± 0.1 mm	– turbid, Ø 1.0 ± 0.1 mm
1.5% Bordetelagar	– clear, Ø 2.2 ± 0.3 mm; – turbid, Ø 0.9 ± 0.1 mm; – clear with turbid halo, Ø 2.4 ± 0.2 mm	– clear, Ø 1.6 ± 0.2 mm; – turbid, Ø 1.0 ± 0.1 mm; – clear with turbid halo, Ø 2.6 ± 0.1 mm	– clear, Ø 1.5 ± 0.2 mm; – turbid, Ø 1.0 ± 0.1 mm; – clear with turbid halo, Ø 2.1 ± 0.1 mm

The plaque morphology was studied on various nutrient media and analyzed by two parameters: size and transparency. The manifestation of these signs depended on the composition of the nutrient medium and the concentration of agar in the upper layer. Table 1 presents the results of the analysis of the morphology of plaques obtained in *B. bronchiseptica* strains. Dissociation of plaques into clear, turbid and clear with turbid halos was observed on various media. Plaques also varied in size, the smallest ones had a diameter of 0.6 ± 0.2 mm, and the largest – 2.6 ± 0.1 mm. It is worth noting that this plaque morphology was observed when 0.8% agar was used in the upper layer. At the same time, when using 0.4% agar in the upper layer, mainly large (2.5 ± 0.1 mm) clear plaques were formed on BHI- and FPH-agar nutrient media, i.e. agar dilution promotes the formation of more clear plaques and an increase in their size. This means, the composition of the nutrient medium has a significant effect on the morphology of plaques, which corresponds to the conclusions made by N. Ramesh et al. [23]. In addition, in M. Adams' publication [20] it was also noted that the plaque count will not give the absolute number of phage particles

Table 2
Lytic spectrum of isolated bacteriophages active against *B. bronchiseptica*

No.	Strains	Bacteriophages		
		vB_BbrS_2/200220.7.2	*vB_BbrS_4/200220.7.1*	*vB_BbrM_5/200220.7.2*
1	*B. bronchiseptica* 200220.7.1	++++	++++	++++
2	*B. bronchiseptica* 200220.7.2	++++	++++	++++
3	*B. bronchiseptica* 200220.6.1	–	+	–
4	*B. bronchiseptica* 1	+	–	++
5	*B. bronchiseptica* 2	++++	++++	–
6	*B. bronchiseptica* 3	++	++	++
7	*B. bronchiseptica* 4	–	+	–
8	*B. bronchiseptica* 200220.4.4	+++	–	–
9	*B. bronchiseptica* C-93	–	+	–
10	*B. bronchiseptica* C-97	+	+++	+
11	*B. bronchiseptica* C-98	+	+	+
12	*B. bronchiseptica* 43	–	+	+
13	*B. bronchiseptica* 44	–	–	++

"–": no plaque; "+": a plaque with multiple secondary bacterial colonies; "++": a plaque with few secondary bacterial colonies; "+++": a plaque with sporadic secondary bacterial colonies; "++++": clear plaques without any secondary colonies of bacteria grown [25].

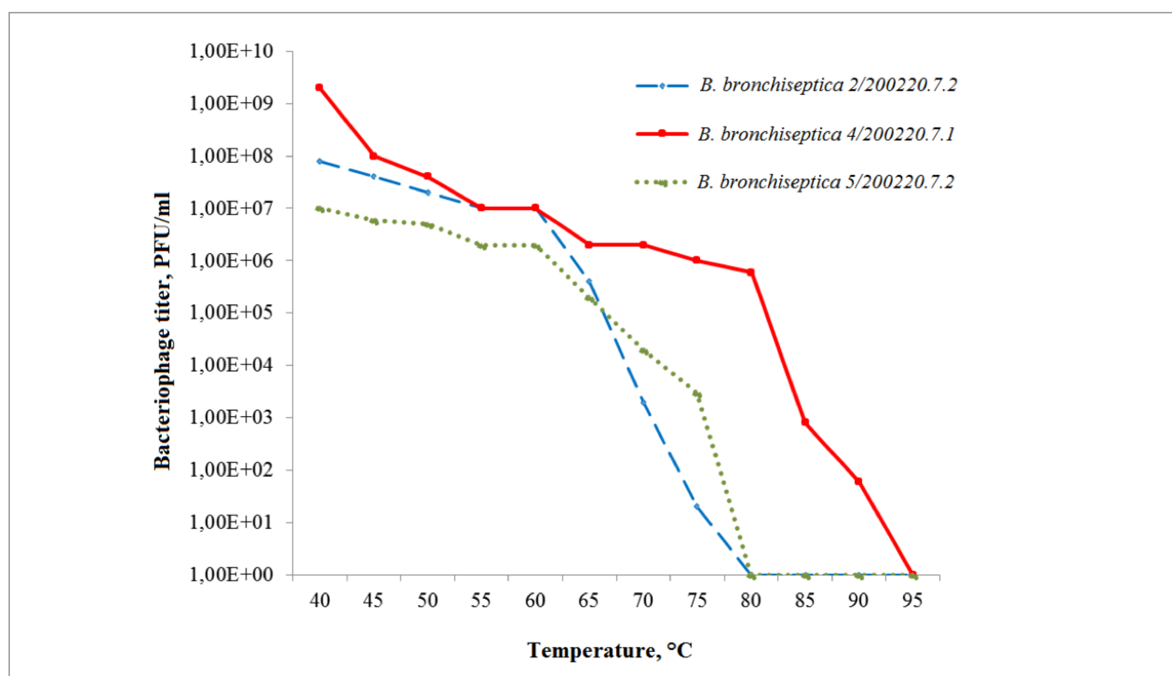

Fig. 2. Heat resistance of isolated bacteriophages

Table 3
Temporal variations in lytic activity of bacteriophages active against *B. bronchiseptica*

Bacteriophage	Titer before storage, PFU/mL	Titer after 8 months of storage, PFU/mL
vB_BbrS_2/200220.7.2	$(2.3 \pm 1.4) \times 10^8$	$(3.1 \pm 0.4) \times 10^8$
vB_BbrS_4/200220.7.1	$(9.0 \pm 0.2) \times 10^8$	$(8.6 \pm 0.2) \times 10^8$
vB_BbrM_5/200220.7.2	$(6.1 \pm 1.2) \times 10^8$	$(4.1 \pm 1.1) \times 10^8$

present in the inoculum; this number depends on the nutrient medium and the strain of sensitive bacteria.

Titers of *vB_BbrS_2/200220.7.2*; *vB_BbrM_5/200220.7.2* bacteriophages were determined on a test strain of *B. bronchiseptica* 200220.7.2 and were $(2.3 \pm 1.4) \times 10^8$; $(6.1 \pm 1.2) \times 10^8$ PFU/mL, respectively. The bacteriophage *vB_BbrS_4/200220.7.1* had the highest titer – $(9.0 \pm 0.2) \times 10^8$ PFU/mL. Its titer was determined on a test strain of *B. bronchiseptica* 200220.7.1.

The results of tests of lytic activity spectrum of isolated bacteriophages are presented in Table 2. The tests were performed in triplicate. Tests demonstrated that the isolated bacteriophages had different spectrum of lytic action. The maximum spectrum of lytic action was shown by phage *vB_BbrS_4/200220.7.1*, which lysed 10 out of 13 bacterial strains (76.9%). Bacteriophages *vB_BbrS_2/200220.7.2* and *vB_BbrM_5/200220.7.2* 61.5% of *B. bronchiseptica* strains were lysed.

For each isolated bacteriophage, parameters such as the average diameter of the head (from vertex to vertex) and the average length of the tail were determined. Virions of the phage *vB_BbrS_2/200220.7.2* consist of 49 ± 2.67 nm icosahedral head and a flexible non-contractile tail, 171 ± 2.26 nm long. Virions of the bacteriophage *vB_BbrS_4/200220.7.1* have morphology, which is similar to the *VB_BbrS_2/200220.7.2* phage: 61 ± 2.88 nm icosahedral head and a flexible non-contractile tail, 158 ± 6.17 nm long. Virions of the phage *vB_BbrM_5/200220.7.2* consist of 108 ± 3.49 nm icosahedral head and a straight contractile tail, 69 ± 5.37 nm long. In accordance with the international classification of viruses by morphological parameters phages *vB_BbrS_2/200220.7.2* and *vB_BbrS_4/200220.7.1* are assigned to the family *Siphoviridae*, bacteriophage *vB_BbrM_5/200220.7.2* to the family *Myoviridae*.

The specificity test showed that the isolated bacteriophages lyse not only the strains of *B. bronchiseptica*, but also active against the strains of *Bordetella parapertussis* C-95 and *Bordetella parapertussis* C-94.

The testing of bacteriophage thermal stability demonstrated that heating of phages for 20 min at more than 40 °C results in a decrease in their lytic activity (Fig. 2). Bacteriophages *vB_BbrS_2/200220.7.2* and *vB_BbrM_5/200220.7.2* are completely inactivated at 80 °C, bacteriophage *vB_BbrS_4/200220.7.1* is inactivated at 95 °C. The tests were performed in triplicate.

To use the isolated phages in production, it is necessary to study the change in their lytic activity over time [26, 27]. Bacteriophage filtrates were stored in vials at 4–8 °C without any preservative added. Lytic activity was determined after 8 months. It was found that during this period of time, the titer of bacteriophages did not decrease. The results are presented in Table 3.

CONCLUSION

Thus, bacteriophages *vB_BbrS_2/200220.7.2*, *vB_BbrS_4/200220.7.1*, *vB_BbrM_5/200220.7.2*, according to the results of *in vitro* studies, are promising for further scientific research in the context of phage therapy of diseases caused by the bacterium *B. bronchiseptica*. The use of drugs based on virulent bacteriophages in veterinary medicine will safely and effectively eliminate the infection without affecting the normal flora, minimize the risk of transmission of *B. bronchiseptica* antimicrobial-resistant strains to humans, improve the livestock productivity and economic performance in the animal production.

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