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Isolation and characterization of *Bordetella bronchiseptica* bacteriophages using prophage induction and enrichment methods

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Bordetella bronchiseptica is an important bacterial pathogen of respiratory infections in companion animals and a promising target for bacteriophage application. However, the efficiency of isolating specific bacteriophages from clinical material remains low, and the factors associated with successful phage isolation have not been sufficiently studied. The aim of this study was to optimize methods for the isolation of *B. bronchiseptica* bacteriophages and to determine the factors associated with the efficiency of their isolation from clinical material. A total of 437 dogs and cats with and without symptoms of respiratory diseases were examined. The presence of *B. bronchiseptica* was determined using polymerase chain reaction and bacteriological methods. PCR was used as a confirmatory method for the presence of the pathogen, whereas bacteriological isolation of the culture was evaluated as an indicator of the presence of a viable bacterial host required for bacteriophage replication. Phage isolation was performed using the direct isolation method, as well as with the application of prophage induction, preliminary enrichment, and chloroform treatment. According to the PCR results, *B. bronchiseptica* DNA was detected in 124 of 437 animals (28.4%), whereas viable cultures of *B. bronchiseptica* were isolated only in 53 cases (12.1%). The highest frequency of bacteriological isolation of the pathogen was established in animals with an acute course of respiratory infection (31.7%), whereas significantly lower frequencies were observed in animals with a chronic course of respiratory disease and in animals without clinical signs of respiratory pathology (6.7% and 1.4%, respectively). When using the direct isolation method, bacteriophages were detected only in 3 of 124 PCR-positive samples (2.4%). In contrast, the use of a combined protocol including prophage induction, preliminary enrichment, and chloroform treatment made it possible to increase the frequency of bacteriophage isolation to 8.9% (11 of 124 samples), which statistically exceeded the efficiency of the classical direct isolation method (OR = 3.9; 95% CI: 1.0–15.0; P = 0.048). In bacteriologically positive samples, bacteriophages were isolated significantly more frequently than in samples positive only according to PCR results (OR = 7.1; 95% CI: 1.5–34.0). Of the 11 isolated bacteriophages, 10 were obtained from animals with an acute course of respiratory infection. The obtained results indicate that the key factors for the effective isolation of *B. bronchiseptica* bacteriophages are the presence of a viable bacterial host and the acute phase of the infectious process. To increase the frequency of bacteriophage isolation, it is advisable to collect clinical material from animals with an acute course of bordetellosis and to use a combined protocol including prophage induction, preliminary enrichment, and chloroform treatment.

Keywords: *Bordetella bronchiseptica*; isolate; bacteriophage; clinical status; dogs; cats; bacteriophage isolation; lytic activity; bacteriophage induction; phage enrichment.

Introduction

Respiratory infections are among the most common groups of diseases in dogs and cats and represent an important problem of modern veterinary medicine (Dear, 2020; Rybolt et al., 2022). Among the bacterial pathogens of respiratory diseases, *Bordetella bronchiseptica*, a Gram-negative bacterium capable of causing lesions of the upper and lower respiratory tract in various animal species, occupies an important place (Belcher et al., 2021; Kameyama et al., 2022). Infection caused by this microorganism is an important component of the canine infectious respiratory disease complex (CIRDC) and may cause both acute and chronic forms of pathology (Miguelena Chamorro et al., 2023; Nicholson & Shore, 2024; Hadzevych et al., 2026). In addition, recent studies indicate an increasing prevalence of antibiotic-resistant strains of microorganisms, which complicates the treatment of animals and increases the relevance of searching for alternative methods for controlling the diseases they cause (Paliy et al., 2023; Atencia et al., 2025; Tang et al., 2026).

The problem of treating infectious diseases, including those associated with *B. bronchiseptica*, is further complicated by the ability of bacteria to form biofilms (Kolchyk et al., 2022; Mugni et al., 2025), which reduces the effectiveness of traditional antibacterial therapy. Particular attention should be paid to the development of increased resistance of clinical isolates of microorganisms to disinfectants (Paliy, 2018; Walczak et al., 2025). In this regard, interest in alternative approaches to the control of bacterial infections has increased significantly in recent years, with particular attention being paid to bacteriophages and phage therapy (Strathdee et al., 2023; Faruk et al., 2025). In addition, modern genomic studies indicate the gradual accumulation of antibiotic resistance genes among *Bordetella* spp., which emphasizes the relevance of searching for new antimicrobial strategies (Ma et al., 2026; Tang et al., 2026).

The spread of *B. bronchiseptica* among animal populations largely depends on housing conditions, population density, and the presence of concomitant infectious agents. In particular, a direct influence of climatic conditions on the microbial and plant world has been noted (Bogach et al., 2020; Chakraborty et al., 2024). A particularly high level of pathogen circulation is observed in kennels, shelters, and other places with group housing of animals (Lavan & Knesl, 2015). According to modern studies, *B. bronchiseptica* is capable of long-term persistence in the respiratory tract of animals and may act as a primary or opportunistic pathogen, thereby complicating the course of respiratory diseases (Zhang et al., 2021; Yi et al., 2024; Jang et al., 2025). In addition, some studies have shown that the interaction of

B. bronchiseptica with bacteriophages may affect bacterial virulence and the characteristics of their persistence in the host organism (Chen et al., 2020; Park et al., 2020).

An important factor in the pathogenicity of *B. bronchiseptica* is the ability of the bacterium to express a wide range of virulence factors, including adhesins, dermonecrotic toxin, adenylate cyclase toxin, and components of the type III secretion system. These factors ensure effective colonization of the mucous membranes of the respiratory tract, suppression of the local immune response, and long-term persistence of bacteria in the respiratory tract of animals (Badhai & Das, 2023; Belhart et al., 2023). In addition, the ability of *B. bronchiseptica* to form biofilms may further increase its resistance to antimicrobial agents and complicate the elimination of the pathogen from the host organism (Miguelena Chamorro et al., 2023).

It is known that in the acute phase of respiratory infection caused by *B. bronchiseptica*, the bacterial load in the respiratory tract of animals is maximal, which creates favorable conditions for active bacteriophage replication. In contrast, during the chronic phase of the disease, the number of viable bacterial populations may decrease as a result of the host immune response or previous use of antibacterial drugs. Modern studies on bacteriophage ecology emphasize that the density and physiological state of the bacterial agent are among the key factors determining the efficiency of replication and accumulation of phage particles in the environment (Dion et al., 2020; Venturini et al., 2022; Costa et al., 2024).

Phage therapy is being actively studied as an alternative or adjunctive approach to the treatment of infections in medicine and veterinary medicine (Ferriol-González & Domingo-Calap, 2021; Strathdee et al., 2023; Uchechukwu & Shonekan, 2024). Studies of *B. bronchiseptica* bacteriophages have shown that certain phages may exhibit pronounced lytic activity and can potentially be used for the control of infections caused by this pathogen (Szymczak et al., 2020; Hosseindoust et al., 2023; Huang et al., 2025). In addition, modern studies demonstrate the potential of using both natural and genetically modified bacteriophages (Łobocka et al., 2021; Faruk et al., 2025).

Particular interest in modern studies is focused on temperate bacteriophages and prophages integrated into the bacterial genome. It has been shown that prophages can influence the virulence, adaptation, and biological properties of bacterial cells, and their activation often occurs in response to stress factors or bacterial DNA damage (Chen et al., 2021; Cornuault, 2024). In this regard, methods of prophage induction and preliminary enrichment of phage suspensions are often used for the effective isolation of bacteriophages from clinical material (Dion et al., 2020; Cornuault & Moineau, 2021).

At the same time, the isolation of bacteriophages from natural or clinical sources is often a difficult task, since their concentration in the investigated material may be low, and a significant proportion of phages remain in the prophage state within the genome of bacterial cells. In addition, the efficiency of phage isolation may depend on the physiological state of the bacterial hosts, cultivation conditions, and the laboratory methods used (Dion et al., 2020; Venturini et al., 2022; Jo et al., 2023). Some studies have also shown that *B. bronchiseptica* bacteriophages may be characterized by different types of interaction with bacterial cells, including the ability to integrate into the bacterial genome or to interact with host organism cells, which may potentially affect the efficiency of phage therapy and the results of phage isolation from clinical material (Chen et al., 2019; Bichet et al., 2021). Modern studies emphasize that optimization of methods for bacteriophage isolation and accumulation is an important prerequisite for the further development of phage therapy and the creation of phage preparations (Nikolich & Filippov, 2020; Huang et al., 2022).

In this regard, studies aimed at the detection of *B. bronchiseptica* in clinical material and the optimization of methods for the isolation of its bacteriophages are of important scientific and practical significance. Despite the increasing number of studies devoted to bacteriophages, data regarding *B. bronchiseptica* phages remain limited. In particular, the factors influencing the efficiency of bacteriophage isolation from clinical material, as well as the relationship between the clinical status of animals, bacteriological confirmation of the pathogen, and the probability of phage isolation, remain insufficiently studied. In addition, the biological properties of *B. bronchiseptica* phage isolates and the possibilities of their practical application in veterinary medicine require further investigation. In this regard, the aim of the present study was to optimize methods for the isolation of *B. bronchiseptica* bacteriophages and to determine the factors associated with the efficiency of their isolation from clinical material.

Materials and methods

All manipulations involving animals during the collection of clinical material were carried out in accordance with bioethical principles and in compliance with the Law of Ukraine "On Protection of Animals from Cruel Treatment" and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986). Collection of clinical material and clinical examination of animals were performed in accordance with generally accepted veterinary practices with the consent of the owners. All procedures were non-invasive and were not associated with injury to the animals.

The study was conducted in 2025 at the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine". A total of 437 animals were examined. Depending on the clinical condition, the animals were divided into three groups: animals with an acute course of respiratory infection, animals with a chronic course of respiratory disease, and animals without clinical signs of respiratory system lesions. Clinical material was collected from the mucous membrane of the upper respiratory tract of animals using sterile swabs. The samples were placed into transport medium and delivered to the laboratory for further investigation.

The presence of *B. bronchiseptica* in clinical material was determined using polymerase chain reaction (PCR) and bacteriological methods. DNA extraction and PCR detection of *B. bronchiseptica* were performed according to existing protocols (Dong et al., 2022; Tabatabaei & Rohani, 2022). Bacteriological examination was carried out using selective nutrient media followed by incubation at 37.0 ± 0.5 °C for 24–48 h. Identification of the isolated cultures was performed according to their morphological, cultural, and biochemical properties (Zhang et al., 2021).

Clinical isolates of *B. bronchiseptica* (BB-1, BB-2, BB-3, BB-4, BB-5) obtained during the bacteriological examination of clinical material from animals were used in the study. These isolates were used to determine the spectrum of lytic activity and the stability of the isolated bacteriophages.

Bacteriophages were isolated from clinical material in which the presence of *B. bronchiseptica* had been confirmed by PCR. Primary isolation of bacteriophages was performed using the direct isolation (direct plating) method (Dion et al., 2020; Costa et al., 2024). The presence of bacteriophages was determined by the formation of lysis zones on the bacterial lawn of a sensitive *B. bronchiseptica* culture.

For prophage induction, ultraviolet irradiation of the bacterial culture and treatment with mitomycin C were used (Chen et al., 2021; Guerrero-Bustamante & Hatfull, 2024). Bacterial suspensions were exposed to ultraviolet irradiation (254 nm) at a distance of 20 cm for 1 min, followed by incubation at 37.0 ± 0.5 °C for 18 h. Mitomycin C was added to bacterial cultures to a final concentration of 0.6 μg/mL, followed by incubation at 37.0 ± 0.5 °C for 24 h. For preliminary enrichment, clinical material was mixed with a broth culture of *B. bronchiseptica* in the logarithmic growth phase (approximately 10^8 CFU/mL) at a ratio of 1:1 and incubated at 37.0 ± 0.5 °C for 18 h. To increase the efficiency of bacteriophage isolation, a method of preliminary enrichment of the investigated material using a sensitive *B. bronchiseptica* culture was applied. For the release of bacteriophages and destruction of bacterial cells, the samples were treated with chloroform at a final concentration of 5.0% (v/v) for 15 min. The morphological properties of bacteriophages were evaluated according to the characteristics of plaques formed on the bacterial lawn of a sensitive *B. bronchiseptica* culture, in particular by their diameter, shape, and degree of transparency (Dion et al., 2020; Costa et al., 2024). The spectrum of lytic activity of bacteriophages was determined by the spot-test method using a panel of clinical *B. bronchiseptica*

isolates. A drop of phage suspension was applied to the surface of the bacterial lawn followed by incubation at 37.0 ± 0.5 °C and evaluation of lysis zone formation. The sensitivity of bacterial isolates to bacteriophages was determined (Dion et al., 2020; Guerrero-Bustamante & Hatfull, 2024).

The stability of bacteriophages to temperature and changes in medium acidity was investigated by incubating phage suspensions under different conditions followed by determination of their lytic activity against a *B. bronchiseptica* culture (Dion et al., 2020; Costa et al., 2024). The effects of different temperatures (4, 25, 37, 50, 60 °C), as well as pH values (3, 5, 7, 9, 10), were studied. After incubation of the phage suspensions for one hour, the residual lytic activity of the bacteriophages was determined.

Statistical analysis of the results was performed using Fisher's exact test and McNemar's test. To evaluate the relationship between the bacteriological isolation of *B. bronchiseptica* and the probability of bacteriophage isolation, the odds ratio (OR) was calculated. Differences were considered statistically significant at $P < 0.05$.

Results

The prevalence of *B. bronchiseptica* among animals (n = 437) with different clinical status was evaluated in order to determine the conditions associated with successful isolation of bacteriophages of this pathogen. The study included animals with clinical signs of respiratory system lesions, as well as clinically healthy animals kept in the same kennels. According to PCR results, *B. bronchiseptica* DNA was detected in 124 of 437 examined animals (28.4%), whereas viable cultures of the pathogen were isolated in 53 animals (12.1%) by bacteriological examination (Table 1). No cases of bacteriological isolation of *B. bronchiseptica* with a negative PCR result were detected.

Table 1
Detection of *B. bronchiseptica* by PCR
and bacteriological methods in examined animals

Method	Positive samples, n	Positive rate, %
PCR	124	28.4*
Bacteriological culture method	53	12.1

Note: * difference between methods was statistically significant (McNemar test, $P < 0.05$).

For further analysis of factors that may influence the isolation of *B. bronchiseptica* bacteriophages, the examined animals were divided into three groups according to clinical status: animals with an acute course of respiratory infection, animals with a chronic course of the disease, and clinically healthy animals without signs of respiratory system lesions. Such grouping made it possible to evaluate the possible relationship between the clinical course of infection and the frequency of bacteriological isolation of *B. bronchiseptica*.

The highest frequency of bacteriological isolation of *B. bronchiseptica* was established in animals with an acute course of respiratory infection (31.7%), whereas significantly lower frequencies were observed in animals with a chronic course and in animals without clinical signs of respiratory pathology – 6.7% (6/89) and 1.4% (3/209), respectively (Table 2).

The obtained results indicated an association between the acute course of respiratory infection and a higher frequency of bacteriological isolation of *B. bronchiseptica*. To evaluate the possibility of bacteriophage isolation, 124 clinical material samples in which the presence of *B. bronchiseptica* DNA had been confirmed by PCR were investigated. This made it possible to evaluate the efficiency of bacteriophage isolation both in samples with bacteriologically confirmed presence of the pathogen and in PCR-positive samples without bacteriological isolation of the culture (Table 3).

According to the results of direct isolation, *B. bronchiseptica* bacteriophages were detected only in 3 of 124 investigated PCR-positive clinical samples, which amounted to 2.4%. In 121 samples (97.6%), no signs of lytic activity were detected. Thus, the direct isolation method was characterized by low efficiency of *B. bronchiseptica* bacteriophage isolation from clinical material.

Table 2
Detection of *B. bronchiseptica*
by bacteriological culture depending on clinical status of animals

Clinical status	Animals, n	*B. bronchiseptica* isolated, n	Detection rate, %
Acute respiratory infection	139	44	31.7
Chronic respiratory disease	89	6	6.7**
Animals without clinical signs of respiratory disease	209	3	1.4***

Note: statistical significance was assessed using the χ^2 test; ** $P < 0.01$ vs. acute respiratory infection group; *** $P < 0.001$ vs. acute respiratory infection group.

Table 3
Isolation of *B. bronchiseptica* bacteriophages
by direct isolation method

Parameter	Number, n	Percentage
Clinical samples examined	124	100.0
Bacteriophages isolated	3	2.4
No bacteriophages detected	121	97.6

Given the low efficiency of direct isolation, optimization of methods for the isolation of *B. bronchiseptica* bacteriophages was carried out. For this purpose, prophage induction followed by enrichment of the investigated material and chloroform treatment of samples were applied. For prophage induction, two approaches were used: ultraviolet irradiation (UV) and treatment with mitomycin C. The use of two inducers made it possible to evaluate differences in the efficiency of prophage induction in *B. bronchiseptica*. As a result of UV induction, bacteriophages were isolated in 5 of 124 PCR-positive samples (4.0%), whereas after treatment with mitomycin C they were isolated in 6 samples (4.8%, Table 4). No statistically significant difference between the efficiency of the applied induction methods was established (Fisher's exact test, $P > 0.05$).

Table 4
Isolation of *B. bronchiseptica* bacteriophages
using different prophage induction methods

Induction method	Samples examined, n	Bacteriophages isolated, n	Detection rate, %
UV irradiation	124	5	4.0
Mitomycin C	124	6	4.8

Note: no statistically significant difference between induction methods was detected (Fisher's exact test, $P > 0.05$).

The frequency of bacteriophage isolation was evaluated at different stages of optimization of the isolation methods among 124 PCR-positive samples (Table 5). The use of prophage induction alone was not associated with a statistically significant increase in the frequency of bacteriophage isolation compared with the direct isolation method (4.0% vs. 2.4%; OR = 1.7; 95% CI: 0.4–7.1; P = 0.47). The use of the enrichment step was accompanied by a tendency toward an increase in the frequency of bacteriophage isolation to 6.5% (OR = 2.8; 95% CI: 0.7–10.7; P = 0.22), however, no statistically significant differences compared with the direct isolation method were established. At the same time, the additional use of chloroform treatment was associated with a statistically significant increase in the frequency of bacteriophage isolation to 8.9% (OR = 3.9; 95% CI: 1.0–15.0; P = 0.048).

To evaluate the relationship between bacteriological isolation of *B. bronchiseptica* and the frequency of isolation of the corresponding bacteriophages, a statistical analysis of the obtained results was performed (Table 6). The frequency of bacteriophage isolation was statistically significantly higher in samples in which *B. bronchiseptica* had been isolated by the bacteriological method compared with samples positive only according to PCR results. In total, 11 *B. bronchiseptica* bacteriophages were isolated during the study. Of these, 9 were obtained from samples in which the pathogen had been confirmed by both PCR and bacteriological methods, whereas 2 bacteriophages were isolated from samples positive exclusively by PCR. A statistically significant association was established between the bacteriological isolation of *B. bronchiseptica* and successful isolation of the corresponding bacteriophages (OR = 7.1; 95% CI: 1.5–34.0; P = 0.018).

The highest frequency of isolation of *B. bronchiseptica* bacteriophages was established in animals with an acute course of respiratory infection (Table 7).

Table 5
Efficiency of different methods for isolation of *B. bronchiseptica* bacteriophages

Method	Samples examined (n)	Bacteriophages isolated (n)	Isolation rate (%)	OR	95% CI	P-value
Direct isolation	124	3	2.4	reference	–	–
Prophage induction	124	5	4.0	1.7	0.4–7.1	0.47
Prophage induction + enrichment	124	8	6.5	2.8	0.7–10.7	0.22
Prophage induction + enrichment + chloroform treatment	124	11	8.9	3.9	1.0–15.0	0.048

Note: statistical analysis was performed using Fisher's exact test; Odds ratios (OR) were calculated relative to the direct isolation method.

Table 6
Association between bacteriological isolation of *B. bronchiseptica* and frequency of bacteriophage isolation

Sample category	Bacteriophages isolated, n	No bacteriophages isolated, n	Total
Culture-positive samples	9	44	53
PCR-positive / culture-negative samples	2	69	71

Note: association was assessed using Fisher's exact test; OR = 7.1; 95% CI: 1.5–34.0; P = 0.018.

Table 7
Frequency of bacteriophage isolation depending on clinical status of animals

Clinical status	Bacteriophages isolated, n	No bacteriophages isolated, n	Bacteriologically positive samples, n
Acute respiratory infection	10	34	44
Chronic respiratory disease	1	5	6
Animals without clinical signs of respiratory disease	0	3	3

Note: analysis of bacteriophage isolation frequency according to clinical status was performed for all phage-positive samples regardless of bacteriological culture results; association between clinical status and bacteriophage isolation was assessed using Fisher's exact test.

For further analysis of the biological properties of the phages, primary screening was performed according to plaque morphology and the level of lytic activity. Plaque morphology was evaluated on solid nutrient agar after incubation with a *B. bronchiseptica* culture. For most of the investigated bacteriophages, the source of isolation was animals with an acute course of respiratory infection. In particular, isolates BBPh-1, BBPh-2, BBPh-3, BBPh-4, BBPh-6, BBPh-7, BBPh-8, BBPh-9, BBPh-10, and BBPh-11 were obtained from animals with acute respiratory infection. Only isolate BBPh-5 was isolated from an animal with a chronic course of respiratory disease. All investigated bacteriophages formed round plaques of different sizes and degrees of transparency. The mean plaque diameter, depending on the isolate, ranged from 0.6 ± 0.2 to 2.2 ± 0.4 mm (Table 8). The largest transparent plaques were formed by isolates BBPh-1, BBPh-2, and BBPh-3, which indicated higher lytic activity of these bacteriophages compared with the other investigated isolates.

For further analysis of the spectrum of lytic activity, five bacteriophage isolates (BBPh-1 – BBPh-5), which differed in plaque morphology and level of lytic activity according to the results of primary screening, were selected. The spectrum of lytic activity was determined against a panel of *B. bronchiseptica* isolates using the spot-test method (Table 9).

The investigated bacteriophages differed in the spectrum and intensity of lytic activity against *B. bronchiseptica* isolates. Phages BBPh-1 and BBPh-2 were characterized by the broadest spectrum of lytic activity and provided pronounced lysis of most of the investigated bacterial isolates. Isolate BBPh-3 also demonstrated high lytic activity; however, its spectrum of activity was somewhat narrower. Bacteriophages BBPh-4 and BBPh-5 were characterized by lower lysis intensity and a limited spectrum of activity.

Table 8
Plaque morphology and lytic activity of *B. bronchiseptica* bacteriophages

Phage isolate	Plaque diameter, mm	Plaque morphology	Lytic activity
BBPh-1	2.2 ± 0.4	Clear	High
BBPh-2	2.0 ± 0.3	Clear	High
BBPh-3	1.8 ± 0.4	Clear	High
BBPh-4	1.4 ± 0.3	Semi-clear	Moderate
BBPh-5	0.9 ± 0.2	Turbid	Low
BBPh-6	1.1 ± 0.3	Turbid	Low
BBPh-7	0.8 ± 0.2	Turbid	Low
BBPh-8	0.6 ± 0.2	Turbid	Low
BBPh-9	1.2 ± 0.3	Semi-clear	Moderate
BBPh-10	0.7 ± 0.2	Turbid	Low
BBPh-11	1.0 ± 0.3	Semi-clear	Moderate

Note: plaque diameter values are presented as mean ± standard deviation based on 10 independent measurements.

Table 9
Lytic activity of *B. bronchiseptica* bacteriophages against different bacterial isolates

B. bronchiseptica isolate	BBPh-1	BBPh-2	BBPh-3	BBPh-4	BBPh-5
BB-1	++++	++	+++	++	+
BB-2	++++	+++	+++	+	–
BB-3	+++	++++	++	++	+
BB-4	++++	++++	+++	++	++
BB-5	+++	++	+++	+	–

Note: "++++" – very strong lysis; "+++" – strong lysis; "++" – moderate lysis; "+" – weak lytic activity; "–" – no visible lysis.

The stability of bacteriophages to the effects of temperature was investigated by incubating phage suspensions under different temperature conditions followed by determination of lytic activity against a *B. bronchiseptica* culture (Table 10). Bacteriophages BBPh-1, BBPh-2, and BBPh-3 retained high lytic activity within the temperature range from 4 °C to 37 °C. At 50 °C, a partial decrease in lytic activity was observed for most isolates, whereas incubation at 60 °C resulted in complete loss of lytic activity of all investigated bacteriophages.

Table 10
Effect of temperature on the lytic activity of *B. bronchiseptica* bacteriophages

Temperature, °C	BBPh-1	BBPh-2	BBPh-3	BBPh-4	BBPh-5
4	++++	++++	++++	+++	++
25	++++	++++	++++	+++	++
37	++++	++++	++++	+++	++
50	++	±	+	±	–
60	–	–	–	–	–

Note: "++++" – complete preservation of lytic activity; "+++" – high residual activity; "++" – moderate reduction of activity; "+" – weak residual activity; "±" – partial preservation of activity; "–" – complete loss of lytic activity.

The effect of medium pH on the lytic activity of bacteriophages was evaluated by incubating phage suspensions in media with different pH values followed by determination of lytic activity against a *B. bronchiseptica* culture (Table 11).

The investigated bacteriophages retained the highest lytic activity at neutral pH values (pH 7). Under acidic (pH 3) and alkaline conditions (pH 10), all isolates completely lost lytic activity. At moderately

acidic (pH 5) and moderately alkaline pH values (pH 9), a partial decrease in lytic activity was observed, the degree of which depended on the phage isolate. Bacteriophages BBPh-1 and BBPh-2 were found to be the most stable, retaining moderate lytic activity over a wider range of pH values compared with the other isolates.

To evaluate the reproductive properties of bacteriophages, the dynamics of their replication in a *B. bronchiseptica* culture were investigated. Bacteriophage growth curves were obtained by periodic determination of phage particle titers after infection of the bacterial culture (Fig. 1).

Table 11
Effect of pH on lytic activity of *B. bronchiseptica* bacteriophages

pH	BBPh-1	BBPh-2	BBPh-3	BBPh-4	BBPh-5
3	–	–	–	–	–
5	++	++	+	±	–
7	++++	++++	++++	+++	++
9	++	++	±	+	±
10	–	–	–	–	–

Note: "++++" – complete preservation of lytic activity; "+++" – high residual activity; "++" – moderate reduction of activity; "+" – weak residual activity; "±" – partial preservation of activity; "–" – complete loss of lytic activity.

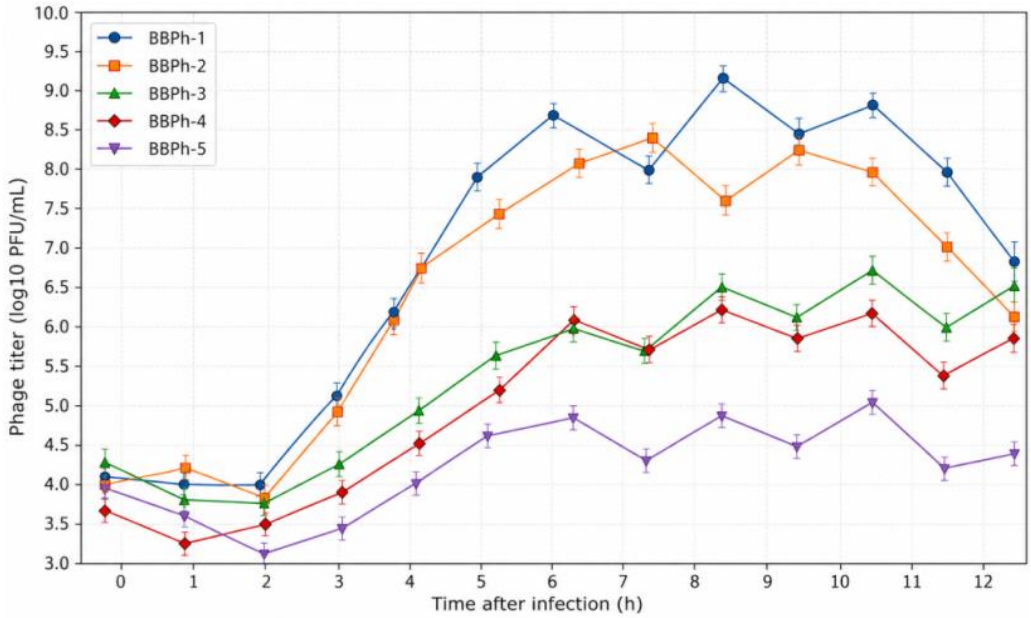

Fig. 1. Growth kinetics of *B. bronchiseptica* bacteriophages: results are presented as mean ± SD based on three independent replicates

Analysis of the growth curves showed a gradual increase in bacteriophage titer after infection of the *B. bronchiseptica* culture. The most intensive increase in the concentration of phage particles was observed during the first 4–6 h of cultivation. Subsequently, the bacteriophage titer remained relatively stable without significant fluctuations.

The highest levels of phage particle accumulation were characteristic of isolates BBPh-1 and BBPh-2, whereas BBPh-4 and BBPh-5 were characterized by lower levels of replication. The obtained results confirmed differences between the investigated bacteriophages in terms of lytic activity and reproductive properties.

Discussion

Diseases of companion animals caused by *B. bronchiseptica* are registered worldwide (Kadhim et al., 2025). The spread of this disease determines the relevance of searching for effective means for its prevention and control. The obtained results showed that the efficiency of isolation of *B. bronchiseptica* bacteriophages largely depends on the presence of a viable bacterial culture, the clinical status of animals, and the isolation methods used. During the study, a significant difference was established between the frequency of detection of *B. bronchiseptica* by PCR and the results of bacteriological examination. According to PCR results, pathogen DNA was detected in 28.4% of examined animals, whereas viable bacterial cultures were isolated only in 12.1% of cases.

Similar differences between molecular and culture-based methods for the diagnosis of *B. bronchiseptica* have also been described in other studies and are explained by the high analytical sensitivity of PCR, which makes it possible to detect even small amounts of bacterial DNA in clinical material (Tabatabaei & Rohani, 2022; Atencia et al., 2025). In addition, PCR is capable of detecting DNA of nonviable bacteria or microorganisms with reduced metabolic activity, which may lead to higher detection rates compared with culture-based methods.

It was established that *B. bronchiseptica* bacteriophages were isolated significantly more frequently from samples in which the pathogen had been confirmed by the bacteriological method. Of the 53 culture-positive samples, bacteriophages were isolated in 9 (17.0%) cases, whereas among 71 samples positive only according to PCR results, phages were isolated in only 2 (2.8%) cases. A statistically significant association was found between bacteriological isolation of *B. bronchiseptica* and successful isolation of bacteriophages (OR = 7.1; 95% CI: 1.5–34.0; P = 0.018). These findings indicate that the presence of a viable bacterial culture is one of the key factors for successful bacteriophage isolation, since phage particles are capable of replication only in metabolically active bacterial cells. Similar patterns have been described in studies devoted to the ecology and biology of bacteriophages, where it has been shown that the density and physiological state of the bacterial population directly influence the efficiency of phage replication and their accumulation in the environment (Dion et al., 2020; Venturini et al., 2022).

Analysis of the distribution of *B. bronchiseptica* depending on the clinical status of animals showed that the highest frequency of bacteriological isolation of the pathogen was observed in animals with an acute course of respiratory infection. In particular, culture confirmation of *B. bronchiseptica* was obtained in 44 animals with an acute form of the disease, whereas among animals with a chronic course of respiratory pathology the pathogen was isolated only in 6 cases, and among clinically healthy animals in 3 cases. In addition, *B. bronchiseptica* bacteriophages were most frequently isolated from samples obtained from animals with acute respiratory infection: 10 of the 11 isolated phages were obtained from animals of this group. The obtained results indicate that the acute course of infection may be accompanied by more active replication of the pathogen and a higher density of the bacterial population, which creates favorable conditions for the accumulation of bacteriophages. Similar patterns are consistent

with modern concepts of the pathogenesis of infection caused by *B. bronchiseptica* and the characteristics of interaction between bacteriophages and bacterial populations during the period of active infectious process (Dion et al., 2020; Miguelena Chamorro et al., 2023; Nicholson & Shore, 2024).

According to literature data, during the acute phase of *B. bronchiseptica* infection, the pathogen actively colonizes the mucous membrane of the respiratory tract, intensively replicates, and expresses a wide spectrum of virulence factors, including adhesins, dermonecrotic toxin, and components of the type III secretion system, which ensure effective colonization of the respiratory tract and modulate the immune response of the macroorganism (Badhai & Das, 2023; Belhart et al., 2023; Miguelena Chamorro et al., 2023). It is believed that precisely during the acute course of infection the bacterial load in the respiratory tract reaches its maximum, which increases the probability of bacteriological isolation of a viable pathogen culture and creates favorable conditions for the replication and accumulation of specific bacteriophages.

In animals with a chronic course of respiratory pathology, the frequency of bacteriological isolation of *B. bronchiseptica* was significantly lower compared with the acute form of infection: the pathogen culture was isolated only in 6 cases, whereas bacteriophages were isolated in only one sample. The decrease in the frequency of detection of viable cultures during the chronic phase of the infectious process may be associated both with partial elimination of bacteria by the host immune system and with previous use of antibacterial drugs. In addition, according to modern studies, chronic respiratory diseases in dogs and cats are often polyetiological in nature and may be accompanied by changes in the composition of the respiratory microbiota, the addition of secondary bacterial microflora or viral agents, which complicates the bacteriological diagnosis of *B. bronchiseptica* (Nicholson & Shore, 2024; Yi et al., 2024). A decrease in the number of viable bacterial populations in such animals probably also limits the possibility of effective replication of specific bacteriophages.

In the study, the efficiency of different approaches to the isolation of *B. bronchiseptica* bacteriophages from clinical material was evaluated. When using the classical direct isolation method, bacteriophages were detected in only 3 of 124 investigated samples (2.4%). The low efficiency of direct isolation is probably associated with the low concentration of phage particles in clinical material. Similar results have also been described in other studies, which indicate that effective isolation of bacteriophages from clinical or natural material often requires preliminary enrichment of phage suspensions (Stewart et al., 1998; Dion et al., 2020; Olszewska et al., 2025).

In addition, some bacteriophages may exist in the form of prophages integrated into the bacterial genome and may not enter the lytic cycle without the action of external inducing factors (Chen et al., 2021; Cornuault, 2024; Guerrero-Bustamante & Hatfull, 2024). This feature of the bacteriophage life cycle may also significantly limit the efficiency of their direct isolation from clinical material.

To increase the efficiency of bacteriophage isolation, a combined approach was used in the study, which included prophage induction, preliminary enrichment of clinical material, and chloroform treatment of samples. Prophage induction was performed using two methods – ultraviolet irradiation and treatment with mitomycin C. The use of UV irradiation and mitomycin C is based on their ability to damage bacterial DNA and activate the SOS response of bacterial cells, which leads to prophage induction and the transition of phages into the lytic cycle (Dion et al., 2020; Chen et al., 2021; Cornuault & Moineau, 2021). Similar approaches are widely used in studies of temperate bacteriophages to increase the efficiency of their isolation and to investigate bacterial prophage systems.

In our study, after induction by ultraviolet irradiation, bacteriophages were isolated in 5 of 124 PCR-positive samples (4.0%), whereas after treatment with mitomycin C they were isolated in 6 samples (4.8%). Despite the slightly higher frequency of phage isolation when using mitomycin C, no statistically significant difference between the induction methods was established. The absence of a statistically significant difference between the methods is probably associated with the limited number of isolated phage isolates, which reduces the statistical power of the comparison.

Each of the applied methods of prophage induction had its own advantages and limitations. Ultraviolet irradiation is a technically simple method that does not require the use of additional chemical reagents and can be easily implemented in laboratory practice. At the same time, the efficiency of UV induction may significantly depend on irradiation intensity, duration of exposure, and the physiological state of the bacterial culture, which may reduce the reproducibility of the results. In contrast, mitomycin C is considered a more specific inducer of the bacterial SOS response and often provides more stable activation of prophages; however, its use requires additional reagents and compliance with biosafety measures (Chen et al., 2021; Cornuault & Moineau, 2021; Cui et al., 2024). The obtained results indicate that both methods can be effectively used for the induction of temperate *B. bronchiseptica* bacteriophages under laboratory conditions. In our study, the frequency of bacteriophage isolation after the use of mitomycin C was slightly higher compared with ultraviolet irradiation (4.8% vs. 4.0%), although no statistically significant difference between the methods was established.

The combined protocol, which included prophage induction, preliminary enrichment of the material, and chloroform treatment of samples, proved to be the most effective in our study. Using this approach, *B. bronchiseptica* bacteriophages were isolated in 11 of 124 investigated samples (8.9%), which was statistically significantly higher than the efficiency of the classical direct isolation method. The obtained results indicate that the combination of several stages of material preparation substantially increases the probability of successful bacteriophage isolation from clinical samples. The enrichment step provides preliminary replication of phage particles in a sensitive bacterial culture and promotes an increase in their concentration, whereas chloroform treatment leads to destruction of bacterial cells and release of intracellular phages. Similar combined approaches are widely used in modern bacteriophage studies and are considered one of the most effective ways to increase the frequency of phage isolation from natural and clinical sources (Dion et al., 2020; Costa et al., 2024; Olszewska et al., 2025).

At the same time, the enrichment method has certain limitations that should be taken into account when interpreting the obtained results. In particular, during repeated passage of bacteriophages on laboratory bacterial strains, selective accumulation of individual phage variants may occur, which can potentially lead to underestimation of the actual diversity of the phage population in a natural or clinical sample (Dion et al., 2020; Costa et al., 2024). In addition, the enrichment step may promote preferential selection of phages with a higher replication rate and more pronounced lytic activity, which may also influence further analysis of the biological properties and structure of the phage population.

Analysis of the distribution of bacteriophages depending on the clinical status of animals also confirmed the identified pattern. The highest frequency of isolation of *B. bronchiseptica* bacteriophages was established in animals with an acute course of respiratory infection – 10 of 11 isolated phages were obtained specifically from animals of this group. In contrast, in animals with a chronic course of respiratory pathology, bacteriophages were isolated in only one case, whereas no phages were detected among clinically healthy animals. The obtained results indicate a tendency toward more frequent isolation of bacteriophages during the acute phase of infection, although interpretation of these data should be carried out taking into account the limited number of observations in the comparison groups. This is probably associated with a higher bacterial load during the acute period of the disease, which creates favorable conditions for the replication and accumulation of phage particles. Similar patterns have also been described in studies of bacteriophage ecology, where it has been shown that the density of the bacterial host population is one of the key factors determining the efficiency of phage replication and the abundance of phages in the environment (Dion et al., 2020; Costa et al., 2024; Strathdee et al., 2023).

At the same time, the conducted study had certain limitations that should be taken into account when interpreting the obtained results.

The total number of isolated *B. bronchiseptica* bacteriophages was relatively small – 11 isolates, which may have limited the statistical evaluation of the efficiency of individual prophage induction methods and reduced the statistical power of comparisons between the experimental groups. In addition, only selected factors influencing the efficiency of bacteriophage isolation were analyzed in the study, whereas other parameters, including the composition of the nutrient medium, the physiological state of the bacterial culture, the characteristics of phage–host interactions, and cultivation conditions, may also significantly affect the replication and accumulation of phage particles (Venturini et al., 2022; Cui et al., 2024; Huang et al., 2025). Nevertheless, the obtained results made it possible to determine the most effective approaches for the isolation of *B. bronchiseptica* bacteriophages from clinical material and to characterize the main biological properties of the isolated phage isolates.

The obtained results indicate that the efficiency of isolation of *B. bronchiseptica* bacteriophages largely depends on the clinical status of animals, the presence of a viable bacterial culture, and the isolation methods used. The most effective approach in our study was the combined protocol, which included prophage induction, preliminary enrichment of the material, and chloroform treatment. Similar combined approaches are considered a promising way to increase the efficiency of bacteriophage isolation from clinical and natural sources (Dion et al., 2020; Costa et al., 2024; Olszewska et al., 2025). The obtained results provide a basis for further investigation of the biological properties of *B. bronchiseptica* bacteriophages and evaluation of the prospects for their practical application in veterinary medicine.

The *B. bronchiseptica* bacteriophages obtained in the study were characterized by differences in morphological and biological properties. The mean plaque diameter varied from 0.6 ± 0.2 to 2.2 ± 0.4 mm, whereas the intensity of lytic activity ranged from low to high depending on the phage isolate. The most pronounced lytic activity and the largest transparent plaques were formed by isolates BBPh-1, BBPh-2, and BBPh-3. The established differences between the phage isolates indicate the heterogeneity of the *B. bronchiseptica* bacteriophage population. Such diversity of phage populations is typical for natural ecosystems and reflects the complex processes of coevolution between bacteria and their viruses (Dion et al., 2020; Costa et al., 2024; Cui et al., 2024). Similar results have also been described in studies devoted to *B. bronchiseptica* bacteriophages, where differences in plaque size, spectrum of lytic activity, and efficiency of bacterial culture lysis were demonstrated (Szymczak et al., 2020; Petrovic Fabijan et al., 2021).

Analysis of the spectrum of lytic activity showed that individual *B. bronchiseptica* bacteriophages were characterized by different abilities to infect the investigated bacterial isolates. In particular, phages BBPh-1 and BBPh-2 demonstrated high or moderate lytic activity against most tested *B. bronchiseptica* isolates, whereas BBPh-4 and BBPh-5 were characterized by a narrower spectrum of lytic activity. Similar patterns have been described in studies devoted to *B. bronchiseptica* bacteriophages, where it has been shown that certain phages are capable of infecting several bacterial strains and may be considered promising agents for phage therapy (Szymczak et al., 2020; Huang et al., 2025; Li et al., 2026). The presence of phages with a relatively broad spectrum of lytic activity is of important practical significance for their potential use in the control of infections caused by *B. bronchiseptica* in veterinary medicine (Loponte et al., 2021; Ferriol-González & Domingo-Calap, 2021).

The study of the stability of *B. bronchiseptica* bacteriophages showed that most phage isolates retained high lytic activity within the temperature range from 4 to 37 °C. At 50 °C, a partial decrease in lytic activity of individual isolates was observed, whereas at 60 °C all investigated bacteriophages completely lost the ability to lyse the bacterial culture. Analysis of the effect of pH showed that the phages were most stable at pH values of 5–9, whereas in strongly acidic (pH 3) and alkaline (pH 10) environments their lytic activity was completely lost. The obtained results are consistent with data from other studies demonstrating the sensitivity of most bacteriophages to extreme physicochemical conditions (Dion et al., 2020; Venturini et al., 2022; Cui et al., 2024). The stability of phages within physiological temperatures and moderate pH values is considered an important prerequisite for their potential use in veterinary medicine and for the development of phage preparations (Strathdee et al., 2023; Olawade et al., 2024).

At the same time, the obtained results should be interpreted taking into account certain limitations of the study. The total number of isolated *B. bronchiseptica* bacteriophages was relatively small, which does not allow definitive conclusions to be drawn regarding the complete diversity of phage populations in the investigated animal groups. Further studies using a larger number of bacterial isolates and bacteriophages, as well as a more detailed analysis of their biological and genetic properties, are required to confirm the identified patterns (Dion et al., 2020; Venturini et al., 2022; Costa et al., 2024).

Thus, the results of the conducted study made it possible to determine the conditions that promote effective isolation of *B. bronchiseptica* bacteriophages from clinical material. The highest frequency of bacteriophage isolation was observed in animals with an acute course of respiratory infection and bacteriologically confirmed presence of the pathogen. In addition, the use of a combined protocol, which included prophage induction, preliminary enrichment of the material, and chloroform treatment, made it possible to increase the efficiency of bacteriophage isolation compared with the classical direct isolation method. The obtained results indicate that the combination of bacteriological confirmation of *B. bronchiseptica* with the use of combined methods of induction and enrichment is a promising approach for effective bacteriophage isolation. Similar approaches are considered in modern studies as an important basis for further investigation of the biological properties of bacteriophages and the development of phage preparations for the prevention and treatment of bacterial infections in veterinary medicine (Venturini et al., 2022; Strathdee et al., 2023; Olawade et al., 2024).

Conclusion

The classical method of direct isolation of *B. bronchiseptica* bacteriophages from clinical material is characterized by low efficiency (2.4%). The use of a combined approach including prophage induction, enrichment of the investigated material, and chloroform treatment of samples made it possible to increase the frequency of phage isolation to 8.9%, which was statistically significantly higher than the efficiency of the classical method.

The probability of isolation of *B. bronchiseptica* bacteriophages significantly increased in the presence of a viable bacterial culture. In bacteriologically positive samples, phages were isolated in 9 of 53 cases, whereas among samples positive only according to PCR results, they were isolated in 2 of 71 cases (OR = 7.1; 95% CI: 1.5–34.0; P = 0.018).

The highest frequency of bacteriophage isolation was established in animals with an acute course of respiratory infection, from which 10 of 11 phage isolates were obtained. This indicates the advisability of collecting clinical material specifically during the acute phase of the disease in order to increase the efficiency of phage isolation. The isolated *B. bronchiseptica* bacteriophages were characterized by variable plaque morphology, as well as different spectra and intensities of lytic activity. Most phage isolates retained stability within the temperature range of 4–37 °C and at pH values of 5–9, which highlights their potential for further investigation and practical application in veterinary medicine.

The authors declare no conflict of interest.

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