

IMPROVEMENT OF THE METHOD FOR THE BACTERIOLOGICAL ISOLATION OF *BORDETELLA BRONCHISEPTICA* FROM NASOPHARYNGEAL SWABS OF DOGS

Hadzevych D. V., Paliy A. P., Hadzevych O. V., Kovalenko L. V., Yurko P. S.

National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine, e-mail: d5195681@gmail.com

Summary. This study aimed to develop a selective medium for the preliminary enrichment of clinical material and to improve the efficiency of the bacteriological isolation of *Bordetella bronchiseptica* from nasopharyngeal swabs collected from dogs. The study was conducted from 2023 to 2025. The study material consisted of 63 nasopharyngeal swabs from dogs with clinical signs of respiratory disease, of which 21 had an acute course and 42 had a chronic course. To choose selective components, the reference strain *B. bronchiseptica* UNCSM-110 and 14 clinical isolates of the pathogen were used. The study used bacteriological, biochemical, spectrophotometric, and molecular genetic methods. It was found that the most promising selective components were cephalixin (40 mg/L), gentian violet (0.0025 g/L), and basic fuchsin (0.005 g/L), which effectively suppressed the accompanying nasopharyngeal microflora while allowing *B. bronchiseptica* to grow. Based on these, three variants of selective media were developed for the preliminary enrichment of clinical material. *B. bronchiseptica* DNA was detected by PCR in 47 of 63 samples (74.6%). In direct bacteriological culture, the pathogen was isolated in 8 cases (12.7%), whereas after selective enrichment, it was isolated in 14 cases (22.2%), a statistically significant increase compared with direct culture ($p < 0.05$). Maximum efficiency of bacterial isolation was observed after 6 hours of selective enrichment. The isolation rate of *B. bronchiseptica* following selective enrichment in dogs with acute respiratory disease was 42.9%, and in those with chronic respiratory disease, it was 11.9% (OR = 5.55; 95% CI 1.56–19.73). The use of the developed selective enrichment method reduces the negative impact of concomitant microflora and increases the efficiency of bacteriological isolation of viable *B. bronchiseptica* cultures from clinical samples obtained from dogs

Keywords: bordetellosis, selective medium, selective enrichment, Regan–Lowe agar

Introduction. In modern dog breeding, infectious respiratory diseases remain one of the most common reasons why pet owners seek care at veterinary clinics. Of particular importance among these is the canine infectious respiratory disease complex (CIRDC), which is characterized by a polyetiological nature and widespread prevalence in populations of domestic and shelter animals (Day et al., 2020; Ouyang et al., 2024; Reagan and Sykes, 2020). One of the main bacterial pathogens of CIRDC is *Bordetella bronchiseptica* — a gram-negative coccobacillus capable of colonizing the mucous membranes of the upper and lower respiratory tracts of dogs and causing acute and chronic respiratory diseases (Jang et al., 2025; Miguelena Chamorro et al., 2023; Yondo et al., 2023).

According to the results of epizootiological studies, *B. bronchiseptica* is regularly detected in both animals with clinical signs of respiratory disease and clinically healthy dogs. This indicates its important role in the circulation of CIRDC pathogens and the development of latent carriage (Lavan and Knesl, 2015; Okonkowski et al., 2021; Tabatabaei and Rohani, 2022). The detection rate of *B. bronchiseptica* can vary significantly depending on animal housing conditions, age, the presence of concomitant infections, and the diagnostic laboratory methods used (Atencia et al., 2025; Marshall et al., 2026; Snedden et al., 2025). Additionally, numerous studies confirm *B. bronchiseptica*'s importance as a key bacterial agent in developing respiratory tract co-infections in dogs. These co-infections significantly complicate the disease's progression and reduce the effectiveness of

therapeutic measures (Jang et al., 2025; Michael et al., 2021; Yondo et al., 2023).

Important factors in the pathogenicity of *B. bronchiseptica* include its ability to adhere to the surface of respiratory tract epithelial cells, the production of a dermonecrotic toxin, the formation of biofilms, and a complex system regulating the expression of virulence factors via BvgAS (Jang et al., 2024; Miguelena Chamorro et al., 2023; Wang et al., 2020). The high genetic diversity of pathogen isolates and the variability in the expression of pathogenicity factors can influence both the clinical course of the infection and the results of laboratory diagnosis (Hadzevych et al., 2026; Nicholson et al., 2025; Nicholson and Shore, 2024).

Despite significant progress in the development of molecular biological diagnostic methods, the bacteriological isolation of *B. bronchiseptica* remains an important step in laboratory testing, since only the isolation of a viable culture of the pathogen allows for its subsequent phenotypic and genotypic characterization, determination of antimicrobial susceptibility, investigation of pathogenicity factors, and utilization of isolates in scientific and biotechnological research (Atencia et al., 2025; Dong et al., 2022; Kaul et al., 2025). At the same time, the efficiency of bacteriological isolation of *B. bronchiseptica* from clinical specimens often remains insufficient, particularly when examining nasopharyngeal swabs from dogs with chronic forms of respiratory disease (Marshall et al., 2026; Reagan and Sykes, 2020; Snedden et al., 2025).

One of the main reasons for the low effectiveness of bacteriological testing is the complex microbial composition of dogs' upper respiratory tracts. Under normal conditions and during pathological processes, the mucous membranes of the nasopharynx are colonized by numerous representatives of gram-positive and gram-negative microflora, including staphylococci, streptococci, enterococci, *Pasteurella*, enterobacteria, and other opportunistic microorganisms (Korkmaz et al., 2022; Mizgala-Izworska et al., 2023; Vientós-Plotts, Ericsson and Reiner, 2023). When clinical material is inoculated onto culture media, the associated microflora often grows more rapidly than *B. bronchiseptica*, which complicates the isolation of single colonies and reduces the likelihood of obtaining a pure culture of the pathogen (Meyer, Maier and Hein, 2023; Nielsen et al., 2023; Vientós-Plotts, Ericsson and Reiner, 2023).

Additional challenges stem from the fact that *B. bronchiseptica* may be present in clinical specimens in small quantities, particularly in cases of chronic disease or following antibiotic therapy (Canonne et al., 2021; Korkmaz et al., 2022; Ouyang et al., 2024). Furthermore, in recent years, there have been increasing reports of the spread of *B. bronchiseptica* isolates with various antimicrobial resistance profiles and the ability to form biofilms, which may also affect the effectiveness of bacteriological isolation of the pathogen (Mavrides et al., 2022; Nicholson and Shore, 2024; Yi et al., 2024). According to some studies, biofilm formation provides additional protection for bacterial cells against adverse environmental factors and contributes to the pathogen's long-term persistence in the animal's body (Miguelena Chamorro et al., 2023; Yi et al., 2024; Zhang et al., 2021).

To improve the efficiency of *Bordetella* spp. isolation, selective culture media are traditionally used; among these, the best-known is the Regan–Lowe medium, which was proposed for the isolation of *Bordetella* as early as the late 20th century (Regan and Lowe, 1977). However, even the use of selective media does not always ensure effective suppression of the accompanying microflora in clinical specimens, especially in cases of high sample contamination or the presence of microorganisms with increased resistance to selective components (Korkmaz et al., 2022; Mavrides et al., 2022; Teale and Borriello, 2021). In this regard, a key area of focus in modern diagnostic microbiology is the development of new approaches to the selective enrichment of clinical material before bacteriological testing (Srednik et al., 2025; Teale and Borriello, 2021).

Despite the significant number of studies devoted to the diagnosis of *Bordetella* infection, most current research focuses primarily on the use of molecular biological methods for detecting the pathogen, while the issue of improving the efficiency of bacteriological isolation of *B. bronchiseptica* from clinical specimens remains understudied (Dong et al., 2022; Kaul et al., 2025; Snedden et al., 2025). This problem is particularly relevant when examining material from animals with a chronic course of the disease, low levels of bacterial

colonization, or significant contamination by associated microflora (Korkmaz et al., 2022; Marshall et al., 2026; Reagan and Sykes, 2020).

The use of traditional selective media only partially addresses the problem of suppressing the accompanying microflora, since their effectiveness depends largely on the composition of the sample, the quantitative ratio of microorganisms, and the sensitivity of the contaminating microflora to the selective components (Mavrides et al., 2022; Regan and Lowe, 1977; Teale and Borriello, 2021). Furthermore, the lack of standardized media for preliminary selective enrichment of clinical material in the bacteriological diagnosis of bordetellosis limits the ability to isolate viable *B. bronchiseptica* cultures and reduces the diagnostic value of the bacteriological method (Atencia et al., 2025; Srednik et al., 2025; Tabatabaei and Rohani, 2022).

In this regard, it is important to identify new selective components and their optimal concentrations capable of effectively suppressing the associated microflora while preserving the viability and growth activity of *B. bronchiseptica* (Korkmaz et al., 2022; Meyer, Maier and Hein, 2023; Srednik et al., 2025). Of particular interest is the development of selective media for the preliminary enrichment of clinical specimens, which can increase the likelihood of isolating the pathogen in cases of low concentration or significant microbial contamination (Nicholson and Shore, 2024; Teale and Borriello, 2021; Yi et al., 2024).

Thus, improving methods for the bacteriological isolation of *B. bronchiseptica* remains a pressing challenge in veterinary microbiology and is of great importance for enhancing the effectiveness of laboratory diagnosis of bordetellosis in dogs.

This study aimed to develop a selective medium for the preliminary enrichment of clinical specimens during the bacteriological isolation of *B. bronchiseptica* and to evaluate its effectiveness in the analysis of nasopharyngeal swabs from dogs with respiratory diseases.

Materials and methods. The study was conducted from 2023 to 2025 at the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine) and included 63 dogs with clinical signs of respiratory tract disease and coughing. Twenty-one animals were diagnosed with an acute course of the disease, and 42 with a chronic course. For laboratory testing, nasopharyngeal swabs were collected from each animal and used for polymerase chain reaction (PCR), direct bacterial culture on nutrient media, and bacterial examination following selective enrichment.

All manipulations with experimental animals were carried out in accordance with the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), and under Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006) and basic

bioethical principles (Simmonds, 2017). Under the current procedure, the research program was reviewed and approved by the Bioethics Committee of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine).

To evaluate the selective properties of the components of the medium under development, the following were used: the control test strain *B. bronchiseptica* UNCSM-110 (National Center for Microorganism Strains, State Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise, Kyiv), the virulent *B. bronchiseptica* strain BorA1 from the microorganism collection of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv), and 14 clinical isolates of *B. bronchiseptica* obtained from dogs with respiratory pathology during the bacteriological examination of 63 nasopharyngeal swabs. The isolates were identified based on a combination of morphological, cultural, and biochemical characteristics typical of *B. bronchiseptica*, in accordance with the methodological approaches described in the works (Atencia et al., 2025; Dong et al., 2022; Kaul et al., 2025). Detection of *B. bronchiseptica* DNA was performed by polymerase chain reaction (PCR) using the primers and amplification conditions described previously (Atencia et al., 2025; Kaul et al., 2025).

To evaluate the selective effects of individual components of the culture medium, we determined the growth rates of *B. bronchiseptica* and museum strains of microorganisms — typical representatives of the normal nasopharyngeal microflora of dogs. The test cultures were incubated in a liquid culture medium with the addition of selective components at various concentrations. Similar cultures grown under the same conditions without the addition of selective components served as controls.

The growth rate of microorganisms was assessed spectrophotometrically by determining the optical density of the culture medium at a wavelength of 600 nm (OD₆₀₀). The obtained values were used to calculate the growth inhibition index using the following formula:

$$I = \frac{K - D}{K} \times 100$$

where I — growth inhibition index (%), K — culture growth rate in the control medium, D — culture growth rate in the test medium.

The inhibition index characterizes the degree to which microorganisms are suppressed by the tested component. Higher values of the index correspond to more pronounced growth inhibition.

Statistical analysis of the results was performed using biostatistical methods. For quantitative variables, the arithmetic mean (M) and standard deviation (SD) were calculated. Fisher's exact test was used to compare the frequency of *B. bronchiseptica* isolation among the study groups. The strength of the association between the clinical course of the disease and the probability of

bacteriological isolation of the pathogen was assessed using the odds ratio (OR) with a 95% confidence interval (95% CI). Differences were considered statistically significant at $p < 0.05$.

Results. One of the main challenges in the bacteriological isolation of *B. bronchiseptica* from clinical specimens collected from dogs with respiratory disease was the presence of rapidly growing, abundant concomitant nasopharyngeal microflora. During direct plating, this microflora rapidly colonized the culture medium surface, formed a continuous growth layer, and significantly complicated the isolation of pure cultures of *B. bronchiseptica* (Fig. 1).



Figure 1. Overgrowth of concomitant nasopharyngeal microflora, which complicates the bacteriological isolation of *B. bronchiseptica* during direct culture of a nasopharyngeal swab from a dog.

Particularly pronounced inhibition of *B. bronchiseptica* growth was observed in samples obtained from dogs with chronic respiratory disease, which were characterized by significant diversity and intense growth of associated bacterial microflora.

Due to the high density of the associated nasopharyngeal microflora, selective components were identified that are capable of inhibiting the growth of foreign microorganisms while maintaining sufficient growth of *B. bronchiseptica*. To this end, the effect of antibacterial agents, dyes, and individual components of the culture medium at various concentrations on the growth of museum strains of microorganisms — typical

representatives of the associated microflora of the upper respiratory tract of dogs — was evaluated. The results of the study are presented in Tables 1–3. To quantitatively assess the effect of the studied components on microbial growth, the growth inhibition index was used, calculated based on the results of spectrophotometric determination of the optical density of the culture medium at a wavelength of 600 nm (OD600).

Table 1 — Effect of antibacterial and antifungal selective components at various concentrations on the growth of *B. bronchiseptica* and museum strains of microorganisms — typical representatives of the associated nasopharyngeal microflora of dogs (growth inhibition index, %)

Microorganism	Cephalexin			Polymyxin B			Amphotericin B		
	10 mg/L	20 mg/L	40 mg/L	500 IU/L	1,000 IU/L	2,500 IU/L	2 mg/L	5 mg/L	10 mg/L
<i>B. bronchiseptica</i> BorA1	16.8 ± 2.4	34.7 ± 3.1	57.9 ± 4.0	28.5 ± 3.0	54.1 ± 4.3	79.2 ± 3.7	3.4 ± 1.1	5.7 ± 1.3	7.6 ± 1.5
<i>Staphylococcus aureus</i> No. 3Γ	43.2 ± 4.3	71.5 ± 4.8	94.1 ± 2.0	97.8 ± 0.9	96.1 ± 0.5	91.4 ± 0.4	2.8 ± 0.9	3.7 ± 1.0	5.4 ± 1.2
<i>Staphylococcus epidermidis</i> No. 72	39.6 ± 3.9	67.8 ± 4.4	92.7 ± 2.3	96.9 ± 1.2	94.8 ± 0.6	92.2 ± 0.5	3.1 ± 1.0	4.2 ± 1.2	5.8 ± 1.3
<i>Streptococcus pyogenes</i> No. 4K	61.4 ± 4.5	86.3 ± 3.0	98.2 ± 0.8	99.3 ± 0.5	95.6 ± 0.3	94.7 ± 0.2	22.7 ± 2.8	27.9 ± 3.1	33.5 ± 3.6
<i>Escherichia coli</i> No. 2A	46.8 ± 4.9	69.7 ± 5.1	89.4 ± 3.0	17.3 ± 2.5	39.5 ± 3.7	64.8 ± 4.3	2.4 ± 0.8	3.1 ± 0.9	4.7 ± 1.1
<i>Proteus vulgaris</i> No. 22	57.5 ± 5.1	63.8 ± 4.7	82.6 ± 4.1	35.7 ± 3.9	40.8 ± 4.2	74.6 ± 4.8	3.6 ± 1.1	4.8 ± 1.3	6.1 ± 1.4
<i>Proteus mirabilis</i> No. M11	48.3 ± 4.4	73.6 ± 4.6	88.7 ± 3.4	19.8 ± 3.0	45.9 ± 4.5	69.8 ± 4.7	2.6 ± 0.9	3.8 ± 1.1	5.0 ± 1.2
<i>Klebsiella pneumoniae</i> No. 17	34.7 ± 3.7	41.5 ± 4.2	61.8 ± 4.7	14.6 ± 2.6	36.2 ± 3.8	43.7 ± 4.4	2.2 ± 0.8	2.9 ± 0.9	3.9 ± 1.0
<i>Pseudomonas aeruginosa</i> No. P25	44.1 ± 4.6	68.2 ± 4.5	87.1 ± 3.7	11.8 ± 2.4	33.6 ± 3.5	59.7 ± 4.6	2.0 ± 0.7	3.2 ± 1.0	5.9 ± 1.5
<i>Enterococcus faecalis</i> No. 1K	34.8 ± 3.7	52.6 ± 4.1	71.4 ± 4.4	12.4 ± 2.3	18.6 ± 2.8	24.5 ± 3.1	3.0 ± 0.9	4.5 ± 1.2	5.5 ± 1.4
<i>Pasteurella multocida</i> No. 9K	13.2 ± 2.3	31.4 ± 3.5	54.7 ± 4.2	73.8 ± 4.4	78.6 ± 4.1	81.3 ± 3.8	3.3 ± 1.0	4.3 ± 1.1	6.2 ± 1.5
<i>Candida albicans</i> No. C37	1.8 ± 0.7	2.5 ± 0.8	3.1 ± 1.0	0.9 ± 0.3	1.1 ± 0.4	1.5 ± 0.5	61.8 ± 4.2	84.7 ± 3.1	92.4 ± 0.8

Note. The average growth inhibition index (%; M ± SD) from three independent replicates (n = 3) is presented. The inhibition index was calculated relative to a control culture grown under similar conditions without the addition of selective components. Higher values of the index indicated more pronounced inhibition of microbial growth by the tested component.

Table 2 — Effect of selective dyes at various concentrations on the growth of *B. bronchiseptica* and museum strains of microorganisms — typical representatives of the associated nasopharyngeal microflora of dogs (growth inhibition index, %)

Microorganism	Gentian violet			Basic fuchsin			Brilliant green		
	0.001 g/L	0.0025 g/L	0.005 g/L	0.001 g/L	0.005 g/L	0.01 g/L	0.0025 g/L	0.005 g/L	0.01 g/L
<i>B. bronchiseptica</i> BorA1	34.8 ± 3.4	58.1 ± 4.1	82.7 ± 3.6	29.7 ± 3.1	55.6 ± 4.0	79.8 ± 3.9	37.5 ± 3.8	61.2 ± 4.4	84.6 ± 3.7
<i>Staphylococcus aureus</i> No. 3Γ	83.4 ± 3.6	91.8 ± 2.5	88.2 ± 0.9	79.6 ± 3.8	87.4 ± 1.1	92.9 ± 0.6	90.8 ± 0.8	98.7 ± 0.5	99.2 ± 0.4
<i>Staphylococcus epidermidis</i> No. 72	81.7 ± 3.8	89.9 ± 2.9	94.8 ± 1.0	77.8 ± 3.9	93.9 ± 1.3	98.7 ± 0.7	87.4 ± 0.9	98.4 ± 0.6	99.1 ± 0.4
<i>Streptococcus pyogenes</i> No. 4K	87.9 ± 0.8	92.8 ± 0.5	90.3 ± 0.3	82.4 ± 3.5	87.8 ± 1.0	89.1 ± 0.5	88.2 ± 0.7	93.1 ± 0.4	95.5 ± 0.2
<i>Escherichia coli</i> No. 2A	11.7 ± 2.2	38.4 ± 3.9	44.2 ± 4.3	9.8 ± 2.0	36.7 ± 3.7	41.8 ± 4.0	56.9 ± 4.6	82.8 ± 3.5	93.6 ± 0.9

Table 2 — continuation

Microorganism	Gentian violet			Basic fuchsin			Brilliant green		
	0.001 g/L	0.0025 g/L	0.005 g/L	0.001 g/L	0.005 g/L	0.01 g/L	0.0025 g/L	0.005 g/L	0.01 g/L
<i>Proteus vulgaris</i> No. 22	13.4 ± 2.5	42.7 ± 4.1	67.8 ± 4.5	12.1 ± 2.3	39.4 ± 4.0	46.5 ± 4.3	59.8 ± 4.8	84.1 ± 3.3	92.1 ± 0.8
<i>Proteus mirabilis</i> No. M11	28.6 ± 3.0	56.4 ± 4.2	63.9 ± 4.4	10.4 ± 2.1	41.8 ± 4.1	48.3 ± 4.5	61.5 ± 4.6	86.2 ± 3.1	88.4 ± 0.7
<i>Klebsiella pneumoniae</i> No. 17	14.1 ± 2.4	36.5 ± 3.8	43.7 ± 4.2	11.3 ± 2.2	38.2 ± 3.9	45.6 ± 4.1	58.7 ± 4.4	83.6 ± 3.4	91.8 ± 0.8
<i>Pseudomonas aeruginosa</i> No. P25	12.8 ± 2.3	47.1 ± 4.0	58.4 ± 4.5	10.9 ± 2.2	44.6 ± 4.2	63.5 ± 4.7	34.5 ± 3.7	58.8 ± 4.4	81.3 ± 3.8
<i>Enterococcus faecalis</i> No. 1K	88.6 ± 2.8	91.2 ± 1.1	98.8 ± 0.5	84.1 ± 3.1	87.5 ± 0.9	91.0 ± 0.4	92.1 ± 0.7	90.0 ± 0.4	98.4 ± 0.3
<i>Pasteurella multocida</i> No. 9K	31.4 ± 3.2	57.8 ± 4.0	81.9 ± 3.8	27.6 ± 3.0	54.2 ± 4.1	78.5 ± 3.9	63.8 ± 4.5	86.4 ± 3.0	87.9 ± 0.7
<i>Candida albicans</i> No. C37	9.6 ± 1.8	41.5 ± 3.7	46.8 ± 4.2	12.7 ± 2.1	44.3 ± 3.9	68.7 ± 4.4	37.8 ± 3.6	62.4 ± 4.1	86.7 ± 3.2

Note. The average value of the growth inhibition index (%; $M \pm SD$) obtained in three independent replicates ($n = 3$) is presented. The inhibition index was calculated relative to a control culture grown under similar conditions without the addition of selective components. Higher values of the index indicated more pronounced inhibition of microbial growth by the selective dye under study.

Table 3 — Effect of certain components of the culture medium at various concentrations on the growth of *B. bronchiseptica* and museum strains of microorganisms — typical representatives of the associated nasopharyngeal microflora of dogs (growth inhibition index, %)

Microorganism	Potassium tellurite			Sodium sulfite			Urea			Barium chloride		
	0.001 g/L	0.0025 g/L	0.005 g/L	0.1 g/L	0.5 g/L	1.0 g/L	2 g/100mL	3 g/100mL	5 g/100mL	2 g/L	5 g/L	10 g/L
<i>B. bronchiseptica</i> BorA1	37.5 ± 3.8	62.4 ± 4.1	84.8 ± 3.5	11.7 ± 2.1	36.5 ± 3.7	58.9 ± 4.2	14.8 ± 2.4	34.2 ± 3.6	56.7 ± 4.1	31.4 ± 3.4	58.7 ± 4.2	83.5 ± 3.6
<i>Staphylococcus aureus</i> No. 3Г	84.2 ± 3.1	89.1 ± 1.0	97.8 ± 0.5	38.6 ± 3.8	67.4 ± 4.3	88.7 ± 2.9	41.8 ± 4.1	71.2 ± 4.5	89.5 ± 2.8	81.5 ± 3.2	87.6 ± 0.8	92.1 ± 0.4
<i>Staphylococcus epidermidis</i> No. 72	82.5 ± 3.4	96.8 ± 1.1	98.6 ± 0.6	34.5 ± 3.7	42.7 ± 4.0	64.8 ± 4.3	38.4 ± 3.9	69.5 ± 4.2	87.3 ± 3.0	79.6 ± 3.3	86.9 ± 0.9	98.8 ± 0.5
<i>Streptococcus pyogenes</i> No. 4K	98.2 ± 0.7	99.0 ± 0.4	99.5 ± 0.2	61.4 ± 4.4	68.2 ± 4.5	88.1 ± 3.1	58.5 ± 4.3	84.6 ± 3.2	98.1 ± 0.8	97.6 ± 0.9	99.1 ± 0.4	99.6 ± 0.2
<i>Escherichia coli</i> No. 2A	58.7 ± 4.4	82.5 ± 3.3	97.4 ± 0.8	39.8 ± 4.0	66.5 ± 4.4	86.7 ± 3.2	35.7 ± 3.8	63.1 ± 4.1	85.4 ± 3.4	82.1 ± 3.1	89.0 ± 2.8	97.3 ± 0.9
<i>Proteus vulgaris</i> No. 22	62.8 ± 4.5	86.1 ± 3.0	88.1 ± 0.7	56.7 ± 4.3	63.8 ± 4.4	82.9 ± 3.6	12.5 ± 2.2	28.6 ± 3.2	54.7 ± 4.0	85.4 ± 2.9	88.1 ± 0.7	89.2 ± 0.4
<i>Proteus mirabilis</i> No. M11	57.4 ± 4.2	81.7 ± 3.2	97.8 ± 0.8	62.5 ± 4.4	82.4 ± 3.5	90.0 ± 0.8	10.8 ± 2.1	24.4 ± 3.0	49.3 ± 3.8	73.6 ± 3.0	87.8 ± 0.8	89.1 ± 0.4
<i>Klebsiella pneumoniae</i> No. 17	56.8 ± 4.1	84.4 ± 3.4	91.1 ± 0.9	41.2 ± 4.1	69.3 ± 4.2	72.8 ± 4.1	39.4 ± 3.9	42.1 ± 4.0	65.5 ± 4.2	58.4 ± 4.0	84.8 ± 3.5	90.4 ± 0.6
<i>Pseudomonas aeruginosa</i> No. P25	82.1 ± 3.2	97.5 ± 0.9	98.9 ± 0.5	54.3 ± 4.2	63.5 ± 4.3	71.6 ± 4.1	42.5 ± 4.0	45.8 ± 4.2	67.3 ± 4.4	56.7 ± 4.1	61.8 ± 4.2	82.6 ± 3.7
<i>Enterococcus faecalis</i> No. 1K	84.8 ± 0.8	89.1 ± 0.4	92.6 ± 0.2	58.4 ± 4.2	86.7 ± 3.0	91.4 ± 0.7	57.5 ± 4.1	83.8 ± 3.1	93.5 ± 0.7	90.1 ± 0.8	92.3 ± 0.3	96.7 ± 0.2
<i>Pasteurella multocida</i> No. 9K	61.5 ± 4.4	84.1 ± 3.2	97.6 ± 0.8	37.5 ± 3.9	66.1 ± 4.4	87.2 ± 3.0	34.8 ± 3.7	61.7 ± 4.0	86.4 ± 3.2	81.2 ± 3.0	87.5 ± 0.8	89.1 ± 0.4
<i>Candida albicans</i> No. C37	12.4 ± 2.2	38.7 ± 3.8	62.8 ± 4.2	8.6 ± 1.7	11.5 ± 2.0	13.8 ± 2.3	18.5 ± 3.7	12.6 ± 2.2	9.4 ± 1.8	14.7 ± 2.3	37.5 ± 3.8	42.1 ± 4.0

Note. The average growth inhibition index (%; $M \pm SD$) from three independent replicates ($n = 3$) is presented. The inhibition index was calculated relative to a control culture grown under similar conditions without the test components in the culture medium. Higher values of the index indicated more pronounced inhibition of microbial growth.

An analysis of the results presented in Tables 1–3 indicated that cephalixin, gentian violet, and basic fuchsin are the most promising agents for inhibiting the growth of associated microflora during the bacteriological isolation of *B. bronchiseptica*. At the concentrations studied, these components inhibited the growth of most members of the associated microflora while maintaining sufficient growth of *B. bronchiseptica*. After examining the results, a method was created to improve the efficiency of isolating *B. bronchiseptica* from nasopharyngeal swabs of dogs. The method is based on preliminary selective enrichment of clinical material before bacteriological culture on Regan–Lowe agar.

The method involved the use of three variants of a selective medium. All medium variants contained the same basic nutrient base, which included peptone, sodium chloride, urea, and activated charcoal. The differences between the variants lay in the composition of the selective components. Variant I contained only cephalixin, whereas variants II and III additionally included gentian violet or basic fuchsin, respectively, to enhance the suppression of concomitant nasopharyngeal microflora. Concentrations of gentian violet (0.0025 g/L) and basic fuchsin (0.005 g/L) were selected as optimal because they provided marked suppression of most members of the associated nasopharyngeal microflora while maintaining sufficient growth of *B. bronchiseptica*.

In addition to evaluating each option individually, the effectiveness of using all selective enrichment options simultaneously was also assessed. To this end, clinical specimens were incubated in parallel in three separate test tubes containing different selective media, followed by bacteriological plating on Regan–Lowe agar. This approach was intended to increase the likelihood of bacteriological isolation of *B. bronchiseptica*, taking into account the possible variability in the susceptibility of individual clinical isolates to the selective components of the medium. Furthermore, we considered the potential diversity of the composition of the accompanying nasopharyngeal microflora in different clinical samples, which may vary in their susceptibility to various selective components.

A liquid culture medium consisting of 1.5% peptone and 0.5% sodium chloride, without the addition of carbohydrates, was used as the basis for selective enrichment. The absence of readily available energy sources was intended to limit the vigorous growth of the accompanying microflora in the clinical specimen. At the same time, for *B. bronchiseptica* — an obligate aerobic microorganism with a predominantly oxidative metabolism — the presence of carbohydrates in the medium is not critically necessary for short-term selective enrichment. Urea (5 g/L) was also added to the medium and used as an additional selective and differential component, taking into account the urease activity of *B. bronchiseptica*, as well as activated charcoal (10 g/L). Activated charcoal was used to adsorb toxic metabolites and reduce the negative impact of bacterial growth products on

B. bronchiseptica. Incubation was carried out under conditions of constant aeration by vigorously shaking the test tubes on a shaker, which ensured oxygen saturation of the culture medium and created favorable conditions for the growth of *B. bronchiseptica*. The composition of the developed selective medium variants is presented in Table 4.

Table 4 — Composition of selective media variants for the preliminary enrichment of clinical specimens during the bacteriological isolation of *B. bronchiseptica* from nasopharyngeal swabs collected from dogs

Component	Variant I	Variant II	Variant III
Peptone	15 g/L	15 g/L	15 g/L
Sodium chloride	5 g/L	5 g/L	5 g/L
Urea	5 g/L	5 g/L	5 g/L
Activated charcoal	10 g/L	10 g/L	10 g/L
Cephalixin	40 mg/L	40 mg/L	40 mg/L
Gentian violet	—	0.0025 g/L	—
Basic fuchsin	—	—	0.005 g/L

Note. The symbol ‘—’ indicates that the corresponding component is not present in the selective medium.

After developing the proposed method, we evaluated its effectiveness in a study of nasopharyngeal swabs from dogs with respiratory disease. The detection of *B. bronchiseptica* in nasopharyngeal swabs from dogs with respiratory disease was performed comprehensively, taking into account the clinical presentation of the disease, the results of polymerase chain reaction (PCR), direct bacteriological examination, and bacteriological examination following preliminary selective enrichment of clinical material using various versions of the developed culture media.

Table 5 presents the results of a comparative evaluation of the effectiveness of PCR, direct bacteriological cultivation, and bacteriological examination following selective enrichment in detecting *B. bronchiseptica*.

In addition to evaluating the effectiveness of various selective enrichment methods, the optimal time for plating clinical specimens on Regan–Lowe agar after the start of enrichment was determined. To this end, control plateings were performed after 2, 4, 6, 8, 12, and 24 hours of incubation. The results are presented in Table 6.

The highest rate of bacteriological isolation of *B. bronchiseptica* was observed after 6 hours of selective enrichment. Extending the duration of selective enrichment beyond 6 hours was accompanied by a decrease in the isolation rate of the pathogen, which was likely due to the active growth of concomitant microflora.

In addition to a comparative assessment of various methods for the bacteriological isolation of *B. bronchiseptica*, the influence of the clinical course of respiratory disease on the effectiveness of *B. bronchiseptica* isolation was analyzed. Table 7 presents the study results.

Table 5 — Results of *B. bronchiseptica* detection in nasopharyngeal swabs from dogs using various laboratory testing methods

Research method	Positive samples (n)	% of total (n = 63)
PCR for <i>B. bronchiseptica</i>	47	74,6
Direct bacterial cultivation on Regan–Lowe agar	8	12,7*
Selective enrichment (variant I) + cultivation on Regan–Lowe agar	9	14,3*
Selective enrichment (variant II) + cultivation on Regan–Lowe agar	11	17,5*
Selective enrichment (variant III) + cultivation on Regan–Lowe agar	10	15,9*
Simultaneous use of three selective enrichment variants + cultivation on Regan–Lowe agar	14	22,2*†

Notes: the efficiency of the selective enrichment step was monitored by plating the material onto solid Regan–Lowe agar medium after 2, 4, 6, 8, 12, and 24 hours of incubation. The results presented were obtained with a selective enrichment duration of 6 hours, which ensured maximum efficiency in the bacteriological isolation of *B. bronchiseptica* (Table 6); * — $p < 0.001$ compared with PCR results; † — $p < 0.05$ compared with direct bacteriological cultivation.

Table 6 — The effect of the duration of selective enrichment on the frequency of bacteriological isolation of *B. bronchiseptica*

Duration of selective enrichment	Positive isolates of <i>B. bronchiseptica</i> (n)	% of the total number of dogs (n = 63)
2 hours	1	1.6
4 hours	3	4.8
6 hours	14	22.2*
8 hours	9	14.3
12 hours	4	6.3
24 hours	1	1.6

Notes: plating on Regan–Lowe agar was performed 2, 4, 6, 8, 12, and 24 hours after starting selective enrichment of the clinical material; * — $p < 0.05$ compared with the results obtained after 2, 4, 8, 12, and 24 hours of selective enrichment.

Following selective enrichment, *B. bronchiseptica* was isolated from 42.9% of dogs with an acute course of respiratory disease and from 11.9% of animals with a chronic form of the disease. The rate of bacteriological isolation of the pathogen was statistically significantly higher in dogs with acute disease compared to those with chronic disease.

The proposed method allows for reducing the negative influence of concomitant nasopharyngeal microflora and increasing the likelihood of isolating viable *B. bronchiseptica* cultures, especially when examining samples from dogs with acute respiratory disease.

Discussion. One of the study's key stages involved selecting components that could effectively inhibit the growth of the associated nasopharyngeal microflora in dogs while preserving the growth activity of *B. bronchiseptica*. This approach is necessary because clinical material from the upper respiratory tract is characterized by a high level of microbial contamination and a significant diversity of microorganisms. When these microorganisms are directly inoculated, they often outgrow *B. bronchiseptica*

Table 7 — Effect of the clinical course of a respiratory disease on the efficiency of bacteriological isolation of *B. bronchiseptica*

Clinical course	Number of dogs	Direct cultivation n (%)	After selective enrichment n (%)
Acute	21	5 (23,8)	9 (42,9)
Chronic	42	3 (7,1)	5 (11,9)*
Total	63	8 (12,7)	14 (22,2)

Notes: * — $p = 0.008$ compared with the group of dogs with an acute course of respiratory disease after selective enrichment (Fisher's exact test). The odds of bacteriological isolation of *B. bronchiseptica* after selective enrichment in dogs with acute disease were 5.55 times higher compared with animals with chronic disease (OR = 5.55; 95% CI 1.56–19.73).

and complicate the isolation of pure colonies of the pathogen (Korkmaz et al., 2022; Mizgala-Izworska et al., 2023; Vientós-Plotts, Ericsson and Reinero, 2023).

Studies have shown that, among the antibacterial drugs examined, cephalexin was the most promising selective component. At a concentration of 20–40 mg/L, it provided marked inhibition of the growth of most representatives of the associated microflora, including staphylococci, streptococci, enterobacteria, and *Proteus*, while maintaining an acceptable growth rate of *B. bronchiseptica*. The results obtained are consistent with data on the use of selective antibacterial components in culture media for the isolation of *Bordetella* and other fastidious microorganisms from contaminated clinical specimens (Regan and Lowe, 1977; Srednik et al., 2025; Teale and Borriello, 2021).

An important finding of the study was the identification of the selective properties of gentian violet and basic fuchsin. At certain concentrations, both dyes provided additional inhibition of the growth of gram-positive microflora, which is one of the main competitors of *B. bronchiseptica* in bacteriological examinations of

nasopharyngeal swabs from dogs. At the same time, the use of gentian violet at a concentration of 0.0025 g/L and basic fuchsin at a concentration of 0.005 g/L did not result in critical inhibition of the growth of the pathogen under study, which allowed them to be considered as promising components of a selective medium. A similar approach to using dyes to enhance the selectivity of culture media is widely applied in diagnostic microbiology when isolating bacteria from material containing a significant amount of associated microflora (Meyer, Maier and Hein, 2023; Srednik et al., 2025; Teale and Borriello, 2021).

The results obtained indicate that the effectiveness of a selective medium is determined not by the maximum possible inhibition of contaminating microflora, but by achieving an optimal balance between selectivity and the ability to support the growth of the target microorganism. That is why the developed media included components that exerted a moderate effect on *B. bronchiseptica* while significantly limiting the growth of most contaminating microorganisms. This approach is consistent with modern principles for the design of selective culture media for the bacteriological diagnosis of infectious diseases (Regan and Lowe, 1977; Srednik et al., 2025; Teale and Borriello, 2021).

Based on the study results, several variants of a selective medium were developed for the preliminary enrichment of clinical material prior to bacteriological culture on Regan-Lowe agar. The use of a selective enrichment step was aimed not only at suppressing the accompanying microflora but also at creating conditions for the rapid accumulation of *B. bronchiseptica* cells to a concentration sufficient for subsequent culture isolation. This approach is particularly relevant when examining clinical samples in which the pathogen is present in small quantities or is affected by adverse environmental factors and antimicrobial agents (Atencia et al., 2025; Marshall et al., 2026; Ouyang et al., 2024).

The results demonstrated that the highest frequency of bacteriological isolation of *B. bronchiseptica* was achieved after 6 hours of selective enrichment. It is presumed that during this period, the most favorable conditions for the accumulation of pathogen cells were created while maintaining a sufficient level of medium selectivity. A subsequent increase in the incubation period was accompanied by a gradual decrease in the frequency of *B. bronchiseptica* isolation. We believe that this may be associated with the adaptation of individual members of the accompanying microflora to the selective conditions and their subsequent proliferation, which led to competitive inhibition of the growth of the target microorganism (Korkmaz et al., 2022; Mizgala-Izworska et al., 2023; Nielsen et al., 2023).

The data obtained are consistent with the general principles of selective enrichment of bacterial cultures. According to these principles, excessively prolonged incubation often leads to a loss of the benefits of the selective stage due to the accumulation of contaminating microflora and depletion of the culture medium (Regan

and Lowe, 1977; Srednik et al., 2025). As described in the research, similar patterns have been observed in the development of selective media for the isolation of other bacterial pathogens from contaminated clinical specimens. In these cases, maximum efficiency of bacteriological isolation was also achieved during the early stages of selective enrichment (Srednik et al., 2025; Teale and Borriello, 2021).

Thus, the results of the study indicate that the effectiveness of preliminary selective enrichment is determined not only by the composition of the medium but also by the duration of its use. The established optimal incubation time — 6 hours — ensures the best balance between the medium's selectivity and the accumulation of viable *B. bronchiseptica* cells, which is of great practical importance for improving the effectiveness of bacteriological diagnosis of bordetellosis.

A comparative evaluation of various laboratory diagnostic methods revealed significant differences between the results of PCR and bacteriological testing. *B. bronchiseptica* DNA was detected by PCR in 74.6% of the samples tested, whereas viable cultures of the pathogen were isolated by direct bacteriological culture in only 12.7% of cases. After applying the developed selective enrichment method, the rate of bacteriological isolation of *B. bronchiseptica* increased to 22.2%, indicating an improvement in the effectiveness of bacteriological diagnosis due to the preliminary accumulation of the pathogen and a reduction in the influence of concomitant microflora.

The results obtained are consistent with data from numerous studies in which PCR demonstrated a significantly higher detection rate for *B. bronchiseptica* compared to the bacteriological method (Atencia et al., 2025; Dong et al., 2022; Kaul et al., 2025). This is due to the fact that molecular biological methods allow for the detection of bacterial genetic material regardless of the bacteria's viability. In contrast, bacteriological testing detects only live cells capable of growing on culture media (Snedden et al., 2025; Tabatabaei and Rohani, 2022). The reliability of bacteriological testing is contingent on several factors, including the quantity of the pathogen in the clinical specimen, the conditions during transport, the presence of concomitant microflora, and prior use of antimicrobial agents (Canonne et al., 2021; Marshall et al., 2026; Reagan and Sykes, 2020).

At the same time, obtaining viable isolates of *B. bronchiseptica* is of great practical importance, since only the bacteriological method allows for further study of the pathogen's cultural and biological properties, determination of its antimicrobial susceptibility, investigation of pathogenicity factors, and conduct of epizootiological monitoring of the circulation of specific variants of the microorganism (Hadzevych et al., 2026; Jang et al., 2024; Nicholson and Shore, 2024). Given the increasing prevalence of antibiotic-resistant *B. bronchiseptica* isolates and the accumulation of data on the pathogen's genetic diversity, the importance of bacteriological

methods is not only not diminishing but is becoming even more relevant (Nicholson et al., 2025; Yi et al., 2024; Zhang et al., 2021).

It should be noted that the use of selective enrichment is not considered an alternative to PCR, but rather a tool for improving the effectiveness of bacteriological testing itself. The results obtained indicate that the use of the developed approach allows for an increase in the number of viable *B. bronchiseptica* cultures isolated compared to direct culturing of clinical material, which expands the possibilities for further microbiological, epizootiological, and biotechnological studies.

An important finding of this study was the establishment of a correlation between the frequency of bacteriological isolation of *B. bronchiseptica* and the clinical course of the respiratory disease. The highest frequency of pathogen isolation was observed in dogs with an acute course of the infectious process, whereas in animals with chronic respiratory diseases, bacteriological confirmation of *Bordetella* infection was recorded significantly less frequently. The results indicate that the effectiveness of bacterial isolation of *B. bronchiseptica* depends to a large extent on the stage of the infectious process and the intensity of bacterial shedding.

The data obtained are consistent with current understanding of the pathogenesis of bordetellosis in dogs. It has been established that in the early stages of the disease, *B. bronchiseptica* actively colonizes the mucous membranes of the upper respiratory tract, multiplies rapidly, and is shed into the environment, creating favorable conditions for its bacteriological detection (Jang et al., 2025; Michael et al., 2021; Ouyang et al., 2024). Subsequently, under the influence of the body's immune response, antimicrobial therapy, and competitive interactions with the commensal microbiota, the number of viable pathogen cells gradually decreases, which negatively affects the results of bacteriological testing (Marshall et al., 2026; Nicholson et al., 2025; Reagan and Sykes, 2020).

In addition, during the chronic phase of infection, the ability of *B. bronchiseptica* to form biofilms and persist for long periods on the mucous membranes of the respiratory tract may play an important role (Miguelena Chamorro et al., 2023; Nicholson and Shore, 2024; Yi et al., 2024). It is known that bacteria within biofilms

exhibit altered metabolic activity and may be less amenable to isolation using traditional bacteriological methods (Wang et al., 2020; Yi et al., 2024; Zhang et al., 2021). This partly explains the lower rate of bacteriological confirmation of bordetellosis in animals with chronic disease compared to acute cases.

The practical significance of these findings lies in the fact that the use of selective enrichment increases the likelihood of isolating viable *B. bronchiseptica* cultures even when the clinical material has a low bacterial load. This is particularly important for conducting epizootiological monitoring, controlling the circulation of the pathogen in dog populations, determining the antimicrobial susceptibility of isolates, and establishing collections of production and reference strains of *B. bronchiseptica* (Hadzevych et al., 2026; Mavrides et al., 2022; Nicholson et al., 2025).

Conclusions. 1. A method for the preliminary selective enrichment of clinical material was developed to improve the efficiency of the bacteriological isolation of *B. bronchiseptica* from nasopharyngeal swabs collected from dogs. Cephalixin, gentian violet, and basic fuchsin were found to be the most effective selective components.

2. In a study of 63 nasopharyngeal swabs from dogs with respiratory disease, *B. bronchiseptica* DNA was detected by PCR in 74.6% of the samples, whereas direct bacteriological culture isolated the pathogen in only 12.7% of cases. After applying selective enrichment, the rate of bacteriological isolation of *B. bronchiseptica* increased to 22.2%.

3. It was determined that the optimal duration of selective enrichment is six hours, at which the maximum rate of bacteriological isolation of *B. bronchiseptica* (22.2%) was achieved. Extending the incubation period beyond six hours was accompanied by a decrease in the efficiency of pathogen isolation.

4. The rate of bacteriological isolation of *B. bronchiseptica* after selective enrichment was statistically significantly higher in dogs with an acute course of respiratory disease (42.9%) compared with animals with a chronic form of the disease (11.9%; $p = 0.008$).

Declaration of competing interest. The authors declare that they have no conflict of interest.

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2026 © Hadzevych D. V.  0000-0002-5859-3609, Paliy A. P.  0000-0002-9193-3548,

Hadzevych O. V.  0000-0001-7115-200X, Kovalenko L. V.  0000-0003-1856-1298,

Yurko P. S.  0000-0003-4870-1570



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