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BACTERIOLOGICAL STUDIES OF PROBE SWABS WITH NASOPHARYNGEAL SECRETIONS FROM CANINES DIAGNOSED WITH BORDETELLOSIS

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Summary. At the present work was developed the greatest effectiveness of bacteriological investigations and detection of clinical isolates of *Bordetella bronchiseptica* from nasopharyngeal secretions was observed when the casein-charcoal agar (CCA) was frozen with 5% blood and cephalexin. Thus, during the first trial and sampling of animals, 12 (66.67%) positive results were recovered, and with repeated sampling and sampling of animals after 24 h and 48 h, 15 (83.33%) positive results were recovered from the total number of animals with bordetheliosis based on the results of PCR. The addition of cephalexin in the form of a selective component in nutrient media allows increasing the effectiveness and speed of bacteriological investigations by 2–4 doses per month, suppressing the growth of third-party nasopharyngeal microflora. This is a simple vision of pure culture. Repeated sampling and sampling of nasopharyngeal secretions from sick animals at a short interval of 24 h and 48 h allows us to obtain 16.66% more positive results from clinical isolates of *B. bronchiseptica* using the bacteriological method of investigation

Keywords: *Bordetella bronchiseptica*, tracheobronchitis, canine infectious respiratory disease complex

Introduction. Bordetellosis is a highly contagious infectious disease of farm and small domestic animals widespread in many countries. It is characterized by general malaise, dry, painful cough, conjunctivitis, rhinitis, tracheobronchitis, vomiting, progressive weight loss, and animal death (Rampelotto et al., 2016; Prüller et al., 2015). Bordetellosis is caused by *Bordetella bronchiseptica* (Stępniewska et al., 2014; Carbonetti et al., 2016; MHU, 2005).

In the natural environment, *B. bronchiseptica* most commonly infects dogs, causing kennel cough, and cats and pigs, causing atrophic rhinitis. Animals of all ages are susceptible to the disease, but the highest incidence is in dogs and cats under one year and pigs aged under 4 months. In dogs, *B. bronchiseptica* is a major cause of canine infectious respiratory disease complex (CIRDC). The severity of the disease is associated with the presence of *B. bronchiseptica* pathogenicity factors: adhesins (filamentous hemagglutinin, pertactin, and fimbriae) and toxins (dermanecrotic, adenylate cyclase hemolysin and lipopolysaccharide) (Carbonetti et al., 2016; MHU, 2005).

B. bronchiseptica is a causative agent of chronic and often asymptomatic respiratory infections in animals, making diagnosis, prevention, and treatment of the disease very difficult (Rampelotto et al., 2016).

According to the scientific literature, researchers and specialists in diagnostic laboratories are currently focusing on developing and applying modern molecular genetic methods to detect *B. bronchiseptica* for diagnosing bordetellosis in animals. It is important to note that the polymerase chain reaction (PCR) has a sensitivity of several bacteria in a sample and a specificity of about 100%. In cases of negative bacteriological tests, PCR is positive in 71% of cases (MHU, 2005; Miguelena Chamorro et al., 2023; Goodnow, 1980; Mattoo et al., 2001; Goto et al., 2023).

Some authors have noted that the bacteriological method of laboratory diagnosis of bordetellosis is practically retrospective in nature, with the final result obtained at least a week after the start of the study. The diagnostic efficiency of the test in clinical practice usually does not exceed 10.0–38.0% (Prüller et al., 2015; Fastrès et al., 2020). The duration and low efficiency of the study are due to contamination of the test material with other microorganisms, imperfect formulation of nutrient media, and unsatisfactory selection of selective components (Stępniewska et al., 2014; MHU, 2005; Coutinho et al., 2009). Some authors recommend adding penicillin to the culture medium to suppress the unwanted growth of extraneous nasopharyngeal microflora. However, some of the associated microflora remains due to penicillin resistance and interferes with bacteriological studies (Prüller et al., 2015). Methicillin and cefsulodin (12 mg/l) exhibit superior inhibitory effects on nasopharyngeal microflora compared to penicillin, and cephalexin demonstrates superiority over methicillin (Chambers et al., 2019; Rodriguez and Berliner, 2023; Stępniewska et al., 2014; MHU, 2007). Additionally, cephalexin-rich casein-charcoal agar (CCA) can serve as an effective selective medium (Carbonetti et al., 2016).

Bacteriologic studies for diagnostic purposes are recommended to be performed early in the course of the disease (but not later than week 3), because later the inoculability of the pathogen decreases sharply. It is necessary to take samples for research twice, repeatedly at short intervals every day or every other day, and for culturing the material it is necessary to use Bordet–Gengou medium or casein-charcoal agar with the addition of blood and penicillin or cephalexin as selective components (MHU, 2005).

Our work **aimed** to determine the efficiency of isolating clinical isolates of *B. bronchiseptica* by

bacteriologic examination of nasopharyngeal secretion swabs from dogs with bordetellosis. To determine the effect of the frequency of sampling of nasopharyngeal secretions during repeated examinations of sick animals at short intervals, twice daily or every other day. Also, to determine whether Bordet–Gengou medium or casein-charcoal agar with blood and penicillin or cephalexin as the selective component is best for primary isolating of clinical isolates of *B. bronchiseptica* by bacteriologic examination of nasopharyngeal secretions from dogs with bordetellosis.

Materials and methods. The process of isolating clinical isolates of *B. bronchiseptica* from nasopharyngeal secretions consisted of three steps:

The first stage is the collection and inoculation of biological material (nasopharyngeal secretion) on nutrient media. Sampling of nasopharyngeal secretions for *Bordetella* isolating was performed using a sterile disposable probe-tampon from the mucous membrane of the tonsillar and peripheral areas. For this purpose, the probe-tampon was applied to the posterior pharyngeal wall with a spatula after firm fixation of the studied animals. After touching the mucous membrane of the tonsillar and peripheral pharyngeal areas, the probe tampon was carefully removed from the mouth without touching the tongue and cheeks, and the tampon was rotated around its axis, The nasopharyngeal secretion was rubbed in the center of the Petri dish in a circular motion with a Z-shaped stroke and in the periphery in the form of 4–5 areas with CCA (casein-charcoal agar with 5% blood) and Bordet–Gengou medium (potato-glycerol agar with 20% blood). In addition, the biological material was simultaneously inoculated on other nutrient media (MPA, MPB, blood agar, Endo, McConkey, Sabouraud, etc.) to isolate and identify other types of microorganisms-associates. This is due to the fact that different types of microorganisms can be found in biological material and it is necessary for diagnostics to grow as many as possible (Carbonetti et al., 2016). Inoculation was performed in such a way as to obtain separate isolated colonies (MHU, 2005). The cultures were placed in a thermostat at a temperature of 35.5 ± 0.5°C for 48 h, and to detect Candida fungi, the cultures were placed in a thermostat at a temperature of 28.0 ± 0.5°C for 5 days. The growth of microorganisms on the media was evaluated daily. The morphology of the colonies was examined under an MBS-9 microscope.

In parallel, they were inoculated on CCA Petri dishes with 5% blood and Bordet–Gengou with 20% blood, spread with a thin layer 1.5–2.0 mm to evaluate the hemolytic properties of clinical isolates of *B. bronchiseptica*. And to find out the effect of selective components on the inhibition of foreign microflora during the isolating of clinical isolates of *B. bronchiseptica*, samples of nasopharyngeal secretions were also inoculated on Bordet–Gengou medium and CCA with blood and penicillin (50 IU/100 ml) and cephalexin (4 mg/100 ml) by adding antibiotics to the media, as recommended in the guidelines for the microbiological diagnosis of pertussis and parapertussis (MHU, 2005). Sampling of nasopharyngeal secretions for isolating of *Bordetella* was performed in 24 1.5–3-month-old dogs (n = 24) with bordetellosis (according to anamnesis, clinical picture and detection of *B. bronchiseptica* genetic material by PCR). At the time of biological sampling, the dogs had a specific characteristic dry painful cough, rhinitis, conjunctivitis and signs of tracheobronchitis. No antibacterial drugs were used to treat the animals at the time of biological sampling. Samples from sick animals were taken at an early stage of the disease (but not later than week 3) because, as scientists point out (MHU, 2005), susceptibility to pathogens decreases sharply at a later stage. In order to obtain objective results for the isolating of clinical isolates of *B. bronchiseptica*, 36 samples of nasopharyngeal secretions from the mucous membrane of the tonsillar and peripheral regions were collected from each dog using a separate sterile disposable probe-tampon for each sample (Fig. 1).

Figure 1. Sterile disposable probe swabs for sampling nasopharyngeal secretions from the mucous membrane of the tonsillar and around the pharyngeal areas of animals for isolating of bordetella.

Samples of nasopharyngeal secretions from the same sick dogs were collected three times in a row during repeated examinations of the animals at short intervals of 24 h. The selected samples were inoculated on CCA with 5% blood, on CCA with 5% blood and penicillin, on CCA with 5% blood and cephalexin, on Bordet–Gengou with 20% blood, on Bordet–Gengou with 20% blood and penicillin, on Bordet–Gengou with 20% blood and cephalexin. The inoculation of the selected biological material was performed in such a way that two samples from each experimental dog were necessarily inoculated on two Petri dishes with each type of medium, including media with the addition of selective components. Media and specific nutrients were used from HiMedia Laboratories Pvt. Limited, India and Pharmaktiv LLC, Ukraine.

The second step is to obtain a pure culture of a clinical isolate of B. bronchiseptica. The cultures were examined on nutrient media. The appearance of the

colonies (size, color, shape), certain rapid tests and light microscopy after appropriate staining with aniline dyes determined the possible type of microorganisms and their significance in a particular case (Stępniewska et al., 2014; Carbonetti et al., 2016; MHU, 2005). Selected colonies were inoculated onto nutrient media to accumulate a pure culture of microorganisms. In the case of growth of colonies of only one species, the identification and determination of antibiotic sensitivity was performed without the accumulation stage of pure culture.

The third step is the identification of microorganisms. Depending on the type of microorganism, its identification (determination of biochemical and antigenic properties) was performed using a fixed kit of substrates, diagnostic selective media, and sera. The substrates are carbohydrates, amino acids, polyhydric alcohols, and other complex compounds. The results were used to determine the genus and species of the microorganism (Stępniewska et al., 2014; Carbonetti et al., 2016; MHU, 2005). To study the biochemical properties of microorganisms, we used selective and diagnostic-selective media and specific components manufactured by HiMedia Laboratories Pvt. Limited, India and Pharmaktiv LLC, Ukraine. Hiss medium with sugars and polyhydric alcohols, acetate agar, nitrate agar for determining the reduction of nitrates to nitrites, gelatinase medium, Pizou medium, Simmons citrate agar, etc. were used.

To determine the catalytic activity of clinical isolates of *B. bronchiseptica*, a microbial mass was looped onto a slide, and a drop of 3% hydrogen peroxide solution was immediately added to it. The release of oxygen bubbles indicated the presence of catalase in the microbes.

Antibiotic susceptibility was determined by the disc diffusion method by studying the zones of inhibition of microbial growth around a paper disc impregnated with an antibiotic. The determination of sensitivity to antibacterial drugs was performed according to the guidelines 'Determination of the sensitivity of microorganisms to antibacterial drugs' approved by the Ministry of Health of Ukraine (MHU, 2007). Depending on the zones of growth retardation, resistant, moderately resistant, and sensitive strains of microorganisms to a particular antibiotic were determined. The pathogenicity of the isolated clinical isolates was determined by bioassay on white mice (Goodnow, 1980; Tizolova et al., 2014).

Results. According to the results of bacteriologic studies of nasopharyngeal secretion samples, clinical isolates of *B. bronchiseptica* were isolated from 15 dogs (n = 15) using CCA with 5% blood and cephalexin, which is 83.33% of the total number of animals with bordetellosis by PCR.

The isolated clinical isolates of *B. bronchiseptica* had morphological and biochemical characteristics typical of the species. They looked like small gram-negative ovoid rods 1–1.2 μm long and 0.3–0.5 μm wide. They were motile by peritrichial flagella and had no spores or capsules. They were weakly stained with aniline dyes, more intensely at the poles.

Biochemically, the isolated clinical *B. bronchiseptica* were inactive. They did not degrade proteins and carbohydrates, but secreted catalase (Fig. 2). They were oxidase-positive, urease-positive, and nitrate-positive without gas formation. They did not produce hydrogen sulfide on iron trisugar agar. *B. bronchiseptica* are resistant aerobes which are sensitive to growth conditions, so we used Bordet–Gengou medium with 20% blood and CCA with 5% blood for primary isolating. After primary isolating, clinical isolates of *B. bronchiseptica* were well cultured on blood agar, MPA, MPB, McConkey, Endo, and SS agar.

B. bronchiseptica on CCA with 5% blood and Bordet–Gengou with 20% blood grew as small, smooth, shiny, round, convex, almost transparent colonies. The growth of *B. bronchiseptica* colonies was observed after 18–24 h of cultivation at a temperature of 35.5 ± 0.5°C. Their diameter ranged from 1.0 mm to 1.5 mm. After 48 h of cultivation, the diameter of *B. bronchiseptica* colonies was 1.0–2.0 mm. The growth of bronchoseptic microbes on CCA medium with 5% blood and Bordet–Gengou with 20% blood was not accompanied by a change in their color. A small zone of β-hemolysis was observed around the colonies on Petri dishes with CCA with 5% blood and Bordet–Gengou with 20% blood spread in a thin layer 1.5–2.0 mm to evaluate the hemolytic properties of clinical isolates of *B. bronchiseptica*.

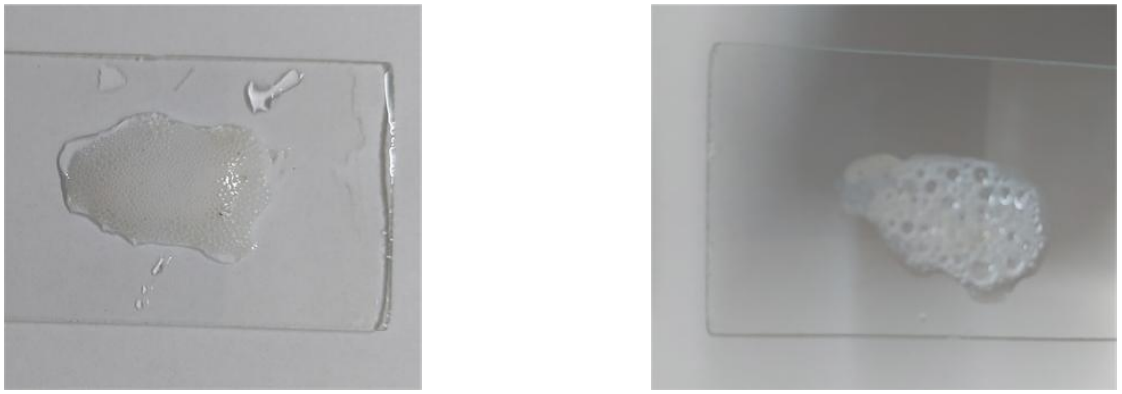

Figure 2. Release of oxygen bubbles during the determination of the catalytic activity of clinical isolates of *B. bronchiseptica*.

In blood broth and MPB, the isolated clinical isolates of *B. bronchiseptica* formed uniform turbidity, light sediment, and a wall ring. On semi-liquid media (0.7% MPA), growth was observed over the entire surface of the medium. On blood agar, colonies were surrounded by small zones of β-hemolysis.

As shown in Table 1, the highest efficiency of bacteriologic studies and isolating of clinical isolates of *B. bronchiseptica* from nasopharyngeal secretions was observed when using CCA with 5% blood and cephalexin. Thus, 12 (66.67%) positive results were obtained during the initial examination and sampling of animals, and 15 (83.33%) positive results were obtained during the repeated examination and sampling of animals after 24 h and 48 h.

The lowest result for the isolating of clinical isolates of *B. bronchiseptica* was obtained using Bordet–Gengou medium with 20% blood without the addition of selective components. During the initial examination and sampling of sick dogs, 5 (22.78%) positive results were obtained, which is 43.89% less compared to the isolating of clinical isolates of *B. bronchiseptica* on CCA with 5% blood and cephalexin. The use of Bordet–Gengou medium with 20% blood with the addition of cephalexin allowed to obtain 10 (55.56%) positive results during the re-examination and sampling of animals after 48 h, which is 27.77% less compared to the result of isolating clinical isolates of *B. bronchiseptica* on CCA with 5% blood and cephalexin.

Table 1 — Results of bacteriological studies of probe swabs with nasopharyngeal secretions obtained from dogs with bordetellosis

Parameters		Type of medium used for primary isolating clinical isolates of *B. bronchiseptica*					
		CCA with 5% blood	**CCA with 5% blood and penicillin**	**CCA with 5% blood and cephalexin**	**Bordet–Gengou with 20% blood**	**Bordet–Gengou with 20% blood and penicillin**	**Bordet–Gengou with 20% blood and cephalexin**
Results of isolating clinical isolates of *B. bronchiseptica* during the first examination and sampling of animals							
The number of animals with bordetellosis (according to PCR results) from which nasopharyngeal secretions were collected for bacteriological studies		18	18	18	18	18	18
The number of positive samples	n	7	10	12	5	7	6
	%	38.89	55.56	66.67	22.78	38.89	33.33
Results of isolating clinical isolates of *B. bronchiseptica* during the repeated examination and sampling of animals after 24 hours							
The number of animals with bordetellosis (according to PCR results) from which nasopharyngeal secretions were collected for bacteriological studies		18	18	18	18	18	18
The number of positive samples	n	10	11	15	7	8	9
	%	55.56	61.11	83.33	38.89	44.44	50.00
Results of isolating clinical isolates of *B. bronchiseptica* during the repeated examination and sampling of animals after 48 hours							
The number of animals with bordetellosis (according to PCR results) from which nasopharyngeal secretions were collected for bacteriological studies		18	18	18	18	18	18
The number of positive samples	n	10	11	15	7	8	10
	%	55.56	61.11	83.33	38.89	44.44	55.56

The use of penicillin as a selective component allowed only a slight increase in the results of isolating clinical isolates of *B. bronchiseptica* due to the inhibition of extraneous nasopharyngeal microflora. Thus, when re-examining and sampling from sick dogs after 24 h and 48 h using CCA with 5% blood and penicillin, 11 (61.11%) positive results were obtained, and using Bordet–Gengou with 20% blood and penicillin, 8 (44.44%) positive results were obtained, which is 22.22% and 38.89% less than the result of isolating of clinical isolates of *B. bronchiseptica* on CCA with 5% blood and cephalexin. The results of Table 1 indicate that repeated examination and sampling of nasopharyngeal secretions from sick animals at short intervals of 24 h and 48 h allows more positive results in isolating clinical isolates of *B. bronchiseptica* by bacteriological examination.

Thus, using CCA with 5% blood and cephalexin, 12 (66.67%) positive results were obtained at the initial examination and sampling of nasopharyngeal secretions from animals, and 15 (83.33%) positive results were obtained at the repeated examination and sampling of animals after 24 h and 48 h. That is, repeated examination and sampling of nasopharyngeal secretions from animals after 24 h and 48 h allowed us to obtain 16.66% more positive results of isolating clinical isolates of *B. bronchiseptica*. In our opinion, it is the most expedient to repeat the examination and sampling of nasopharyngeal secretions from animals after 48 h, when the preliminary result of the first inoculation of biological material has already been obtained and it is possible not to repeat samples with a positive result.

As shown in Table 2, the duration of isolating and identification of clinical isolates of *B. bronchiseptica* was due to contamination of the test material with concomitant foreign nasopharyngeal microflora. The studies were delayed by 2–4 days due to additional inoculations for isolating pure culture.

Thus, the duration of isolation and identification of clinical isolates of *B. bronchiseptica* on CCA medium with 5% blood and cephalexin was 5–7 days. When using CCA or Bordet–Gengou medium without selective components, clinical isolates of *B. bronchiseptica* were isolated and identified within 6–9 days. The study results indicate that it is necessary to use a selective component of cephalexin in the culture media to inhibit the growth of extraneous nasopharyngeal microflora.

In our opinion, as pointed out by Kennedy et al. (2024) and Dong et al. (2022), to reduce the time for isolation and identification of clinical isolates of *B. bronchiseptica* in clinical practice, it is necessary to improve the formulation of nutrient media using selective-elective components, as well as to optimize the use of selective diagnostic media. This will significantly reduce the time for isolation and identification of clinical *B. bronchiseptica* isolates.

It should be emphasized that the duration and efficiency of bacteriological examination of nasopharyngeal secretion samples for isolating clinical isolates of *B. bronchiseptica* are greatly affected by untimely and incomplete examination of animals, violation of rules for collection and transport of biomaterial, insufficient number of examinations, low qualification of diagnosticians, as well as use of antibiotics before the analysis.

Table 2 — Duration of isolation and identification of clinical isolates of B. bronchiseptica on different media

Parameters	Type of medium used for primary isolating clinical isolates of *B. bronchiseptica*					
	CCA with 5% blood	CCA with 5% blood and penicillin	CCA with 5% blood and cephalexin	Bordet–Gengou with 20% blood	Bordet–Gengou with 20% blood and penicillin	Bordet–Gengou with 20% blood and cephalexin
Number of days required to obtain primary growth of microorganisms on the medium	1–2	1–2	1–2	1–2	1–2	1–2
Number of days required to obtain a pure culture of a clinical isolate of *B. bronchiseptica*	2–4	2–4	1–2	2–4	2–4	1–2
Number of days required to identify a clinical isolate of *B. bronchiseptica* by biochemical properties	3	3	3	3	3	3
Duration of isolation and identification of clinical isolates of *B. bronchiseptica*, days	6–9	6–9	5–7	6–9	6–9	6–9

These factors require further study and careful analysis to avoid false-positive and false-negative bacteriologic test results in the future. It is also important to optimize and standardize the conditions and steps of the methods for isolating and identification of clinical *B. bronchiseptica* isolates by bacteriological testing. First of all, it is necessary to analyze the currently available nutrient media for isolating *B. bronchiseptica* and the selective components used to suppress the associated nasopharyngeal microflora.

Conclusions. 1. The highest efficiency of bacteriologic examinations and isolating of clinical isolates of *B. bronchiseptica* from nasopharyngeal secretions was observed when using CCA with 5% blood and cephalexin. Thus, during the initial examination and sampling of animals, 12 (66.67%) positive results were obtained, and during the repeated examination and sampling of animals after 24 h and 48 h, 15 (83.33%) positive results were obtained from the total number of animals with bordetellosis by PCR.

2. Repeated examination and sampling of nasopharyngeal secretions from sick animals with a short interval of 24 h and 48 h allowed us to obtain 16.66% more positive results of isolating clinical isolates of *B. bronchiseptica* by bacteriological examination.

3. The use of cephalexin as a selective component in culture media can increase the efficiency and reduce the duration of bacteriological studies by 2–4 days by inhibiting the growth of extraneous nasopharyngeal microflora and significantly simplify the isolating of pure culture.

Prospects for further research. The disadvantage of the bacteriological method of laboratory diagnosis of bordetellosis is the complexity and duration of the research. The final result can be obtained at least 5–9 days after the start of the test. However, the advantage is that it is available and traditional for most domestic laboratories and specialists and remains the 'gold standard' for bordetellosis laboratory diagnosis in our country. The results of the study indicate that to reduce the time for isolating and identification of clinical isolates of *B. bronchiseptica* in clinical practice, it is necessary to improve the formulation of nutrient media using selective-elective components, as well as to optimize the use of selective diagnostic media. This will significantly reduce the time for isolation and identification of clinical *B. bronchiseptica* isolates.

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