Part 1. Veterinary medicine

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STUDY OF THE PATHOGENIC PROPERTIES OF *AVIBACTERIUM PARAGALLINARUM* CULTURES ISOLATED IN 2019–2020

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Summary. Both viruses and bacteria, including *Avibacterium paragallinarum*, formerly known as *Haemophilus paragallinarum*, can be etiological agents of respiratory infections of birds. Cultural-morphological and molecular-biological studies established that three isolates selected during 2019–2020 from pathological material (swabs from the subocular sinuses) of 42–180 days old birds (No. 1 — SS 6/20, serotype A; No. 2 — SS 7/20, serotype B; No. 3 — SS 8/20, serotype C) belonged to the species *A. paragallinarum* and they formed a heterogeneous group. During the study of the virulence of isolates on birds, it was found that it varies: *A. paragallinarum* SS 6/20 is virulent (the average value of the sum of points is from 0.5 to 0.7); *A. paragallinarum* SS 7/20 is low virulent (the average value of the sum of points is from 0.2 to 0.3); *A. paragallinarum* SS 8/20 is virulent (the average value of the sum of points is from 0.8 to 0.9). Also, isolates were heterogeneous in terms of pathogenicity. The pathogen *A. paragallinarum*, SS 7/20 had the lowest pathogenicity, while when infected with *A. paragallinarum* isolates, SS 6/20 and *A. paragallinarum*, SS 8/20, the morbidity of birds was 80–100%

Keywords: avian infectious rhinitis, avian infectious coryza, virulence

Introduction. Infectious rhinitis (poultry hemophilosis) is an acute enzootic highly infectious disease of the upper respiratory tract of poultry, primarily chickens, characterized by catarrhal inflammation of the mucous membranes of the nasal cavity, conjunctiva and sinuses, as well as subcutaneous swelling of the head and in rare cases — pneumonia.

This disease spreads horizontally. The source of infectious rhinitis is a sick and recovered bird, in the body of which bacteria can persist for 6–12 months (Baydevlyatov et al., 1980; Calnek, 2003; Korniienko et al., 2012; Orlov, 1971; Orlov and Prokof'eva, 1962). Information on the carrier of *A. paragallinarum* by wild birds has been published, which makes it a potential reservoir of infection (Calnek, 2003).

Birds of all ages are susceptible to infectious rhinitis, but especially chickens over 4 days old. It is believed that infectious rhinitis is not a systemic disease and does not cause high mortality in susceptible birds, but during acute outbreaks, flock losses can reach 10%. Economic losses are also manifested in the reduction of egg laying in hens by up to 40%, especially at the peak of productivity, and growth retardation in young birds (Korniienko et al., 2012).

In young birds, the disease, as a rule, begins with nonspecific clinical signs, such as depression, growth retardation, drowsiness ('sleeping bird' syndrome). Older chickens have sinusitis, hemorrhagic conjunctivitis, serous and/or serous-fibrinous rhinitis. As the disease progresses, a syndrome a syndrome occurs, which is often seen in avian metapneumovirus infection, better known as SHS (or 'swollen head syndrome'); it causes blindness. Infectious rhinitis is also associated with such pathological changes as fibrinous lesions of the submandibular area, one- or two-sided aerosacculitis, septic lesions of the liver and kidneys, less often, as already mentioned, with pneumonia.

According to antigenic properties, strains of *A. paragallinarum* are classified following two main schemes proposed by Page (1962) and Kume et al. (1983), Morales-Erasto et al. (2014). The scheme of Blackall et al. (1990), in which all strains of the causative agent of infectious rhinitis are divided into three serotypes: A, B, and C, is the most widespread. These serotypes do not have cross-protection during poultry immunization.

The work is aimed to study the cultural and morphological characteristics of epizootically relevant strains (isolate No. 1 — SS 6/20, serotype A; isolate No. 2 — SS 7/20, serotype B; isolate No. 3 — SS 8/20, serotype C) *A. paragallinarum*, which were isolated in recent years (2019–2020), as well as to determine their pathogenicity and virulence in susceptible birds with further use of the knowledge gained for the construction of an inactivated vaccine and as control strains to determine the immunogenicity of a prophylactic biological preparation.

Materials and methods. In order to definitively identify *A. paragallinarum* isolates (SS 6/20, serotype A;

SS 7/20, serotype B; SS 8/20, serotype C), which were isolated in 2019–2020 from pathological material, additionally a number of laboratory studies of cultural and biochemical properties were carried out. We also took into account the ability of cultures to grow on nutrient media without blood serum under the increased content of carbon dioxide in the atmosphere, to produce various metabolites and enzymes, to break down carbohydrates.

Biochemical properties were determined in *A. paragallinarum* cultures grown on a dense nutrient medium after 20–24 hours of incubation at 37 °C by culturing in the nutrient medium 'Broth base with phenol red M 054', produced by 'Himedia'. Oxidase activity was determined using a commercial kit following the instructions for use. The absence of catalase production was checked using a 3% hydrogen peroxide solution.

The next stage of laboratory research of selected isolates was to determine their pathogenicity for birds.

In a series of experiments, a 20-hour broth culture of the three *A. paragallinarum* isolates was used, in which the number of viable cells was determined by plating serial dilutions on a dense nutrient medium — Columbia serum agar with the addition of NAD. 8-week-old broiler chickens of the cross 'KOBB-500' were used as test objects. Each experimental group consisted of five birds, the experiment was carried out in two repetitions.

The experiment on virulence (clinical manifestation of infection) we used the methodology proposed by Soriano et al. (2004). The presence and degree of the upper respiratory tract damage in the infected poultry was assessed following to the scoring system:

0 — absence of clinical signs;

1 — scanty discharge of exudate from the nasal passages and/or slight swelling of the infraorbital sinuses;

2 — moderate discharge of exudate from the nasal passages and/or moderate swelling of the area of the infraorbital sinuses;

3 — profuse nasal discharge of exudate and/or severe swelling of the infraorbital sinuses;

4 — profuse nasal discharge of exudate and pronounced swelling of the infraorbital sinuses, wheezing.

Clinical signs were recorded daily in each bird separately. 5 days after infection, the sum of points for each group was determined, which was divided by the total number of infected birds.

Criteria for assessing virulence:

≤ 0.5 — low virulent;

0.5–0.9 — pathogenic;

≥ 1.0 — highly virulent.

21 days after experimental infection, all chickens were subjected to forced slaughter. After the post-mortem examination of poultry, bacteriological analysis of the infraorbital sinuses' contents was carried out to re-isolate the causative agent for the confirmation of the disease specificity.

The pathogenicity of the isolates was established by determining the minimum infectious dose that would ensure the disease of 80–100% of the birds in the experiment (Anjaneya et al., 2013).

Results. According to the previously obtained results from the study of cultural and morphological properties and PCR of three isolates of chicken hemophilosis (No. 1 — SS 6/20, serotype A; No. 2 — SS 7/20, serotype B; No. 3 — SS 8/20, serotype C) it was established that all cultures belong to the species *A. paragallinarum*.

The characteristics of the biochemical activity of these isolates are presented in Table 1, where we can see that they are also a homogeneous group in terms of cultural properties and enzymatic activity.

Table 1 — Cultural properties of *A. paragallinarum* isolates

Isolate	Galactose	Glucose	Mannitol	Sorbitol	Saccharose	Trehalose	Catalase	Oxidase	Indole	H_2S	Nitrate recovery	Hemolysis on blood agar	NAD dependence	Blood serum requirement
SS 6/20, serotype A	–	+	+	+	+	–	–	–	–	–	+	–	+	+
SS 7/20, serotype B	–	+	+	+	+	–	–	–	–	–	+	–	+	+
SS 8/20, serotype C	–	+	+	+	+	–	–	–	–	–	+	–	+	+

The characteristic properties of the bacteria were: the ability to reduce nitrates to nitrites, the absence of indole, hydrogen sulfide, catalase and oxidase production.

The saccharolytic activity of the selected isolates also did not differ. Thus, all isolates fermented glucose and sucrose, but not lactose, trehalose and galactose.

It is well known that there are various types of pathogenicity factors of infectious diseases agents, including hemophilosis of birds, from the presence of receptors for attachment to cells, properties regarding colonization (distribution throughout the body) and invasion (penetration into cells), negative impact on the body's immune response (binding antibodies with pathogen proteins) and reproduction of toxins. Therefore, to characterize different degrees of pathogenicity and its quantitative expression, the term virulence (degree of pathogenicity) is used.

In our study, the assessment of *A. paragallinarum* (SS 6/20, serotype A; SS 7/20, serotype B; SS 8/20, serotype C) virulence was performed following the

method of Soriano et al. (2004). Poultry was infected intranasally with a 20-hour daily broth culture of isolates in a dose of 0.5 cm^3, with a content of at least 10^8 CFU of the pathogen. The results of these studies on broiler chickens are presented in Table 2.

Table 2 — Virulence of *A. paragallinarum* isolates for 'KOBB-500' 8-week-old broiler chickens

Isolate	Time after infection, days						Average value
	1	2	3	4	5	6	
	Sum of points by groups						
SS 6/20, serotype A (I experiment)	0.5	0.6	0.8	0.8	0.6	0.4	0.5
SS 6/20, serotype A (II experiment)	0.6	0.8	1.0	0.8	0.5	0.4	0.7
SS 7/20, serotype B (I experiment)	0	0	0.2	0.4	0.4	0.4	0.2
SS 7/20, serotype B (II experiment)	0	0.2	0.4	0.5	0.5	0.4	0.3
SS 8/20, serotype C (I experiment)	0.6	0.8	0.8	1.0	0.8	0.8	0.8
SS 8/20, serotype C (II experiment)	0.6	0.8	1.0	1.0	1.0	0.8	0.9

As can be seen from the results of laboratory studies, the virulence of *A. paragallinarum* isolates is different and the average value of the sum of points is: SS 6/20, serotype A — from 0.5 to 0.7; SS 7/20, serotype B — from 0.2 to 0.3; SS 8/20, serotype C — from 0.8 to 0.9.

The results of experimental infection of poultry are presented in Table 3. Different infecting doses of isolates were used to infect poultry. The doses depended on the concentration of viable cells and varied from 4.78 ± 0.13 to $6.25 \pm 0.32 \times 10^8$ CFU.

The first clinical signs of the disease in poultry were observed 24–48 hours after infection. Symptoms were manifested in the form of liquid discharge from the nostrils and slight one- or two-sided swelling of the infraorbital sinuses.

Later, unilateral or bilateral catarrhal conjunctivitis developed in some birds, sometimes fibrin appeared in the exudate, which led to swelling of the eyelids and narrowing of the eye slit. When the infection was localized in the deeper parts of the respiratory tract, breathing was accompanied by wheezing in some chickens. In our studies, the period of clinical manifestation of the disease was 3–6 days.

During the analysis of the obtained results, it was established that isolates of the causative agent of chicken infectious rhinitis are heterogeneous in terms of pathogenicity. The pathogen *A. paragallinarum*, SS 7/20, serotype B had the lowest pathogenicity, at the same time in the case of infection with isolates *A. paragallinarum*, SS 6/20, serotype A and *A. paragallinarum*, SS 8/20, serotype C, the morbidity of poultry was 80–100%. 21 days after experimental infection, the pathogen was re-isolated from the contents of the infraorbital sinuses in most birds, regardless of the presence and severity of clinical signs during the disease period.

Table 3 — Study of the pathogenic properties of *A. paragallinarum* isolates for 8-week-old broiler chickens, cross KOB-500

Strain name	Infectious dose, $\times 10^8$ CFU	Number of birds		Morbidity, %	Reisolation of the pathogen, %
		infected	with clinical manifestation of infection		
SS 6/20, serotype A (experiment I)	4.78±0.13	5	4	80	60
SS 6/20, serotype A (experiment II)	5.13±0.16	5	4	80	80
SS 7/20, serotype B (experiment I)	5.60±0.19	5	2	40	60
SS 7/20, serotype B (experiment II)	5.87±0.23	5	3	60	80
SS 8/20, serotype C (experiment I)	6.10±0.25	5	4	80	80
SS 8/20, serotype C (experiment II)	6.25±0.32	5	5	100	100

Discussion. Among the infectious diseases of poultry, chicken hemophilus, the causative agents of which are bacteria *Avibacteruim paragallinarum*, formerly known as *Haemophilus paragallinarum* (Calnek, 2003; Blackall and Soriano-Vargas, 2020; Blackall et al., 2005), is one of the main problems for commercial poultry farming worldwide (Blackall et al., 2005; Blackall et al., 1997; Kelser, 1997; Blackall and Yamamoto, 1998; Poernomo et al., 2000).

According to preliminary cultural and morphological studies and PCR, it has been established that three isolates (No. 1 — SS 6/20, serotype A; No. 2 — SS 7/20, serotype B; No. 3 — SS 8/20, serotype C) isolated during 2019–2020 from the pathological material of birds aged 42–180 days with clinical signs of the disease (rhinitis, fibrinous inflammation of the mucous membranes of the sinuses), belong to the species *A. paragallinarum* and they form a heterogeneous group. Thus, the cultures were capsule-forming short rods or coccobacilli that required V-growth factor.

In addition, all *A. paragallinarum* isolates were NAD-dependent, although in the special literature there are reports on the NAD-independent isolates of the pathogen (Mouahid et al, 1992). The dependence of the cultivation of isolates on the presence of blood serum in the nutrient medium turned out to be absolute, which is

consistent with the results of research by other authors (Calnek, 2003; Deshmukh, 2015). Growth of *A. paragallinarum* on serum-free medium could not be obtained, even when it contained the optimal amount of V-factor. In addition, a number of foreign researchers claim that the cultivation of the causative agent of infectious rhinitis of chickens is possible only in the presence of an increased content of carbon dioxide (8–10%) in the atmosphere (Calnek, 2003; Deshmukh, 2015). However, in our experiments, the isolates did not show such a dependence. The morphology and size of the colonies obtained under the conditions of a normal atmosphere did not differ in any way from cultures with an increased content of carbon dioxide. The variability of signs was observed in the ratio of mannitol and mannose, as evidenced by the results of other authors (Blackall et al., 2005; Deshmukh, 2015).

In addition, according to literature data, it is known that apathogenic species of hemophilic bacteria *A. avium, A. volantium* do not require blood serum, produce catalase, ferment trehalose and galactose (Patil et al., 2017).

The virulence of the isolates was determined by intranasal administration of bacterial suspension of the pathogen to susceptible broiler chickens. There are many reports on these studies in the literature (Matsumoto and Yamamoto, 1975; Rimler et al., 1977; Bragg, 2002a; Bragg, 2002b).

Thus, in our studies, the virulence of experimental *A. paragallinarum* isolates varied. A high average score was noted using isolates SS 8/20 (serotype C) — from 0.8 to 0.9 (virulent) and SS 6/20 (serotype A) — from 0.5 to 0.7 (virulent). In turn, isolate SS 7/20 (serotype B) had the lowest value — from 0.2 to 0.3 (low virulence).

It should also be noted that clinical signs of the disease in the infected poultry already began to be noted on the first or second day (discharge from the nostrils, slight swelling of the infraorbital sinuses). In the future, the clinical manifestations of the disease progressed, which led to swelling of the eyelids and narrowing of the eye slit. In addition, in some chickens, breathing was accompanied by wheezing.

In general, the investigated *A. paragallinarum* isolates were not homogeneous in terms of pathogenicity. Thus, isolate SS 8/20 (serotype C) demonstrated high pathogenicity in relation to chickens, as well as high rates of re-isolation (80–100%). According to the literature, such a high pathogenicity of the SS 8/20 isolate may be related to its high reproductive capacity (Bragg, 2002b). In turn, SS 6/20 and SS 7/20 isolates (80% and 40–60%) were somewhat inferior to SS 8/20 in terms of pathogenicity; in terms of reproducibility, they were also lower than SS 8/20, but the same between themselves (60–80%).

Conclusions. 1. Based on the results of cultural, morphological and biochemical properties, three isolates of avian hemophilosis selected from clinically sick birds (SS 6/20, serotype A; SS 7/20, serotype B; SS 8/20, serotype C) were assigned to the species *A. paragallinarum*. Cultures are gram-negative capsule-forming short rods or coccobacilli, which require the presence of V-factor and blood serum during cultivation; they reduce nitrates to nitrites, ferment glucose and sucrose, but not lactose, trehalose and galactose; the absence of indole, hydrogen sulfide, catalase, and oxidase production is noted.

2. It has been established that the virulence of *A. paragallinarum* isolates is different and is: *A. paragallinarum* SS 6/20, serotype A is virulent (the average value of the sum of points is from 0.5 to 0.7); *A. paragallinarum* SS 7/20, serotype B is low virulent (the average value of the sum of points is from 0.2 to 0.3); *A. paragallinarum* SS 8/20, serotype C is virulent (the average value of the sum of points is from 0.8 to 0.9).

3. *A. paragallinarum* isolates are heterogeneous in pathogenicity. The pathogen *A. paragallinarum*, SS 7/20, serotype B had the lowest pathogenicity, while in the case of infection with *A. paragallinarum* isolates, SS 6/20, serotype A and *A. paragallinarum,* SS 8/20, serotype C, the morbidity of birds was 80–100%. The development of infection was proven by reisolation of the pathogen 21 days after experimental infection from the contents of the infraorbital sinuses, regardless of the presence and severity of clinical signs.

4. Taking into account the pathogenic characteristics and the degree of virulence, these isolates are available for obtaining a highly specific and active antigen, production of experimental series of inactivated vaccines and conducting immunogenicity control.

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