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PROPERTIES OF EXPERIMENTAL SAMPLES OF VACCINE AGAINST AVIAN INFECTIOUS CORYZA

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Abstract

Avian infectious coryza is caused by the bacteria *Avibacterium paragallinarum* and occurs throughout the world in countries with a well-developed poultry industry, causing significant economic losses to the poultry industry. The specific prevention is the main link in combating infectious rhinitis of chicken. Vaccination of birds provides expressed immunity due to generation of anti-hemagglutinating antibodies. The presented research study is the first to report on immunobiological properties of two formulations of a developed experimental vaccine against avian infectious coryza which contains the formaldehyde-inactivated antigen of a new *A. paragallinarum* strain No. 5111 (serogroup B). The aim of the work was to evaluate the safety, antigenic and protective properties of the absorbed and emulsion-based formulations of a vaccine against chicken infectious rhinitis based on the *A. paragallinarum* strain No. 5111. A whole-cell antigen of the *A. paragalli*narum strain No. 5111 (serotype B-1) inactivated with formaldehyde was used to produce experimental samples of the vaccine for trials. A dose for immunization (0,5 cm^3) contained 10^9 inactivated microbial cells and 3.75 mg of aluminum hydroxide for the absorbed vaccine formulation or oil adjuvant Montanide ISA 70 VG («SEPPIC», France, 70 % wgt) for the emulsion-based formulation. The immunobiological properties of the vaccine were tested on 125 Haysex brown chickens (*Gallus gallus* L.) of 1.5-2.0 months of age which were seronegative to *A. paragallinarum*. The safety of the vaccine samples was tested by injecting chickens in a 2-fold dose (1.0 cm^3). Each sample was injected subcutaneously in the middle third of the neck and intramuscularly in the chest using 5 chickens per each formulation. The clinical status of the birds was observed daily for 42 days. At the end of the experiment, the chickens were slaughtered and the incision of the injection site was visually examined. Three groups of chickens (25 birds each, 75 chickens in total) were assigned to determine protective properties of the vaccine. The birds of group I were immunized with the absorbed formulation of the vaccine, the chickens of group II were injected with the emulsion-based formulation. The birds were injected subcutaneously into the middle third of the neck at a dose of 0.5 cm^3 twice with a 20-day interval. Unvaccinated chickens of group III were used as a control. In 15 days after revaccination, the chickens of groups I, II, and III were infected with a 1-day broth culture of the *A. paragallinarum* strain No. 5111 with a concentration of 5 units according to the optical standard of bacterial suspension turbidity. The clinical status of the chickens was observed during 7 days after infection. The post-mortem examination was performed with a bacteriological analysis of the contents of the nasal sinuses during the experiment and at the end of the experiment. The vaccine antigenicity and the duration of immunity were determined on 30 birds (three groups of 10 birds each). The chickens of group I were immunized with the absorbed vaccine sample, group II — with an emulsion-based sample, and unvaccinated birds of group III served as a control. The vaccine antigenicity was assessed based on humoral antibody level using the hemagglutination inhibition test (HI test). The mild to moderate tissue lesions were observed at the injection site without an obvious inflammatory reaction. For the absorbed formulation, slight subcutaneous swellings and hyperemia were observed in some chickens at the injection site. For the emulsion-based formulation, the formation of connective tissue granules with the vaccine residues without necrotic lesions and an obvious inflammatory reaction of the surrounding tissues occurred at the injection site in all birds. No significant differences in the condition of chicks from vaccinated groups were

observed ($p > 0.05$), but there was a significant difference between the birds of the test and control groups ($p < 0.05$). The level of protection of chickens after double immunization with the adsorbed vaccine and the emulsion-based vaccine was 92 % and 88 %, respectively. Twenty days after the first vaccination with absorbed and emulsion-based formulations, the average antibody titers were below the threshold level ($p > 0.05$). Increased antibody titers in chicken sera were observed only at day 15 post the second immunization. At day 60 post vaccination, the antibody levels in the chicken sera reached their maximum, i.e., 7.5 ± 0.8 $\log_2$ in poultry immunized with the adsorbed vaccine and 8.9 ± 0.7 $\log_2$ in birds immunized with the emulsion-based vaccine ($p > 0.05$). In chickens vaccinated with the vaccine containing aluminum hydroxide gel, a decrease in the antibody titer to 5.5 ± 0.7 $\log_2$ was observed at day 240 while in birds immunized with the emulsion-based vaccine the titer remained at the level of 8.7 ± 0.8 $\log_2$ ($p > 0.05$). No specific antibodies to the causative agent of infectious coryza were detected in chickens of the control group during the entire observation period, including the diseased and convalescence period. Thus, our findings show that the adsorbed and emulsion-based experimental formulations of the developed vaccine against avian infectious coryza are safe and demonstrate high antigenicity and immunogenicity after double administration.

Keywords: avian infectious coryza, *Avibacterium paragallinarum,* antigen, adjuvant, candidate vaccine

Bacterial diseases at industrial poultry farms pose an urgent problem for veterinary medicine. Infectious rhinitis (hemophilia) of chickens caused by *Avibacterium paragallinarum* has recently become a widespread respiratory pathology. The clinical sighs of this acute enzootic infectious disease are catarrhal inflammation of the mucous membranes of the upper respiratory tract and edema in the subcutaneous tissue of the facial part of the head [1-5].

Infectious rhinitis is typical for all countries with developed poultry farming, including the Russian Federation [4-6]. The disease causes serious economic damage to the industry due to growth retardation, loss of egg production (up to 40 %), and costly preventive and recreational measures.

Antigenic diversity of the pathogen is important for the epizootology. The currently recognized nine serovars of *A. paragallinarum* are A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, and C-2 which are organized into three serogroups termed A, B, and C. Different serovars and serogroups of the pathogen dominate in different countries [1, 7-9]. In natural illness, birds, as a rule, develop weak and short-term immunity, therefor, recurrences of the disease are possible [6, 10].

Vaccination is the main measures in combating chicken infectious rhinitis as it ensures the development of strong immunity due to generation of anti-hemagglutinating antibodies. A close relationship was revealed between the protective immunity and the titer of specific antibodies in the hemagglutination inhibition (HI) assay [7, 11, 12]. Vaccination allows significant reduction of antibiotic use which, in turn, helps to prevent the emergence of antibiotic resistance in microorganisms and decreases the residual amounts of antibiotics in poultry products [12-13].

Until the mid-1990s, most commercial vaccines against chicken infectious rhinitis were produced from strains of serogroups A and C, which negatively affected their effectiveness, especially in regions with active circulation of the pathogen of serogroup B. However, over the past 20 years, express methods for the *A. paragallinarum* serotyping have been developed and introduced into laboratory practice, allowing accurate detection of antigenic profiles of the pathogen. Most modern vaccines are positioned as universal, since they contain a set of *A. paragallinarum* strains of serogroups A, B, and C. Individual serotypes within serogroups A and C ensure cross-protection, whereas effectiveness of vaccination against the pathogen of serogroup B directly depends on the antigenic correspondence of the vaccine strain and the epizootic strain circulating in a particular geographic region.

Currently, in Russia, single trivalent vaccine against avian infectious coryza of has been developed and approved the use of which is limited due to its

high reactogenicity. For the prevention of the disease, foreign vaccines are mostly used, which creates the dependence on imported drugs.

Diagnostic studies of pathological material carried out at FGBI ARRIAH identified 12 isolates of *A. paragallinarum* from regions of Russia [2]. Serotyping revealed their belonging to the B-1 serovar. In 2014, two strains were isolated from sick chickens during an outbreak of the disease at large poultry farms which used a commercial trivalent emulsion vaccine against avian infectious coryza. This fact casts doubt on the effectiveness of the vaccine used in the territory of the Russian Federation. In addition, outbreaks of *A. paragallinarum* serotype B-1 have also increased in recent years in Europe and Asia, despite the use of commercial vaccines. The weak cross-protection between strains of serotype B-1 has not yet been explained. Since the studied strains of serotype B-1 provide only partial cross-protection, it is likely that an effective vaccine can be made only from the antigen of a strain isolated in a specific geographic region where this serotype is endemic [7, 10, 14]. The wide distribution of the pathogen of infectious rhinitis of chickens of serotype B-1 in Russia indicates the advisability of using the strain to develop a domestic vaccine [1, 15].

Effective vaccination is largely determined not only by the antigen properties, amount, and the administration routs but also by the appropriate adjuvants [16-18]. A stimulant of nonspecific immunity must primarily be safe [3, 19, 20].

Cultured *A. paragallinarum* No. 5111 of B-1 serovar endemic to the Russian Federation can reach high cell density while maintaining stable hemagglutinating activity and high virulence. Vaccine against avian infectious coryza based this strain in combination with various adjuvants is of great importance for veterinary medicine.

This work it the first to present characterization of the harmlessness, antigenic properties and protective effect of candidate vaccines based on the antigen of a new domestic strain of *A. paragallinarum* No. 5111 of serogroup B.

The work aimed to assess the immunobiological properties of absorbed and emulsion formulations of a candidate vaccine against avian infectious coryza.

Materials and methods. The whole cell antigen of *Avibacterium paragallinarum* No. 5111 (serotype B-1) was inactivated with formaldehyde. The concentration of bacteria in each inoculated dose (0.5 cm^3) was 10^9 microbial cells according to the optical turbidity standard. The absorbed formulation contained 3.75 mg of aluminum hydroxide per dose. The emulsion formulation contained 70 % (w/w) oil adjuvant Montanide ISA 70 VG (SEPPIC, France).

The immunobiological properties of the vaccine were evaluated in 2019 on 125 1.5-2.0-month-old Hisex brown chickens (*Gallus gallus* L.) which were delivered from a poultry farm free from infectious diseases and seronegative to *A. paragallinarum*.

The harmlessness of the formulations was determined by injecting chickens in a 2-fold dose (1.0 cm^3). Each formulation was injected subcutaneously into the middle third of the neck from the dorsal side and intramuscularly into the chest (5 animals per formulation). The examination of clinical state of the birds and the injection site were performed daily for 42 days. At the end of the experiment, the poultry were slaughtered and the tissue condition at the injection site was visually assessed on the incision. The degree of tissue damage after intramuscular injection was assessed according to the Stone's criteria [21]: weak lesions (blanching of the tissues surrounding the injection site with no signs of inflammation and residues of the encapsulated vaccine), moderate lesions (hyperemia and edema

of surrounding tissues ranging in size from 1.0 to 2.0 cm in diameter with the presence of residues of an encapsulated or diffusely distributed vaccine), severe lesions (pronounced tissue inflammation with the formation of granulomas with a diameter of 3.0 to 4.0 cm, the contents of a liquid consistency flow out on a cut or have a cheese-like appearance).

The protective properties of the formulations were assessed on 75 chickens (three groups of 25 animals each). Group I was immunized with the absorbed formulation, group II with the emulsion formulation, group III of unvaccinated chickens was used as a control. The birds were twice injected (20 days apart, subcutaneously into the middle third of the neck from the dorsal side) with a dose of 0.5 m^3. Fifteen days after the revaccination, the chickens were infected with a 1-day broth culture of *A. paragallinarum* No. 5111 (5 U optical turbidity standard). The microbial cell suspension (0.2 cm^3) was administered intranasally. For 7 days since infection, the clinical state of the chickens was monitored daily. The protective properties of the formulations were assessed according to the method proposed by Soriano et al. [22] to determine the *A. paragallinarum* virulence, with the exception that the significant difference between the average score for the experimental and control groups testified to the immunogenicity of the formulation.

The degree of the developed clinical signs in infected birds was scored as follows: 0 points mean no symptoms; 1 point means slight nasal discharge, slight swelling of the nasal sinus region, or both; 2 points mean moderate nasal discharge, moderate swelling of the nasal sinus region, or both; 3 points mean abundant nasal discharge, pronounced swelling of the nasal sinus region, or both; 4 points mean abundant nasal discharge and pronounced swelling of the nasal sinus region, wheezing. When symptoms of disease were detected, the scores for individual animals were summed up and divided by the total number of infected chickens in the group. During the experiment and at its end, a pathoanatomical autopsy was carried out with a bacteriological analysis of the contents of the nasal sinuses from dead and slaughtered chickens. Birds that had no clinical signs, no pathoanatomical changes, and a negative bacteriological test were considered protected from the disease.

The antigenic properties of the vaccine and the duration of immunity were determined using 30 birds (three groups of 10 birds each). Group I was immunized with the adsorbed formulation, group II with the emulsion formulation, group III of unvaccinated birds served as a control. The scheme of immunization and the method of administration of the vaccine were used the same as in studying the protective properties. The antigenic activity of the candidate vaccine formulations was assessed by the titers of humoral antibodies in the hemagglutination inhibition (HI) test [23, 24]. Blood samples were taken from the axillary vein before immunization, 20 days after the first vaccination and 15, 40, 100, 160, and 220 days after revaccination. In HI test, the antibody titers ≥ 4.0 $\log_2$ were considered positives, ≤ 2.0 $\log_2$ as negatives.

The data were processed to determine the arithmetic mean values (M) and standard errors of the mean ($\pm$SEM). The significance of differences was assessed by the Student's t-test. The difference between the values was considered statistically significant at $p < 0.05$.

Results. The first step was to determine the safety of the candidate vaccine formulations. One day after the intramuscular injection of the emulsion formulation, in three chickens, a slight swelling occurred at the injection site which disappeared on its own within 3-5 days.

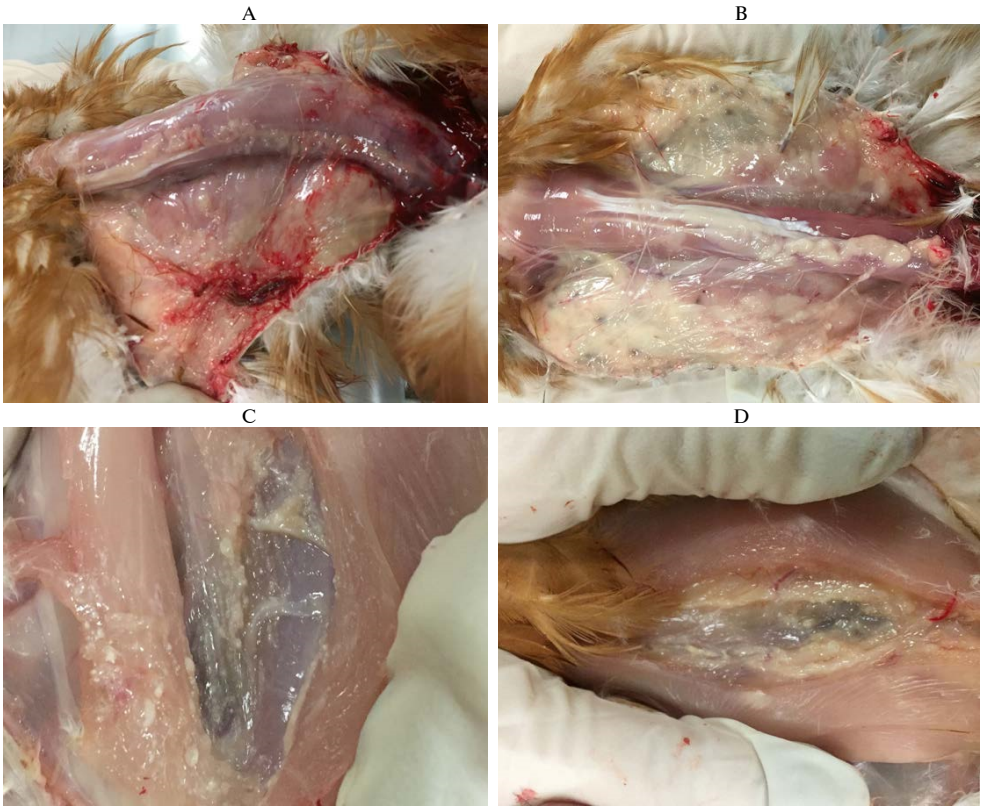

Fig. 1. Hisex brown chickens (*Gallus gallus* L.) injected with an avian infectious coryza candidate vaccine based on *Avibacterium paragallinarum* No. 5111 (serotype B-1) antigen: A — swelling and hyperemia at the site of subcutaneous injection of the absorbed vaccine formulation, B — connective tissue granuloma with vaccine residues at the site of subcutaneous injection of the emulsion vaccine formulation, C — no muscle tissue lesions at the site of intramuscular injection of the he absorbed vaccine formulation, D — encapsulated emulsion residues in the tissues at the site of intramuscular injection of the absorbed vaccine formulation (lab tests).

At the end of the experiment, autopsy of the slaughtered birds revealed tissue lesions form mild to moderate severity without a pronounced inflammation. At the site of subcutaneous injection of the absorbed formulation, slight edema and hyperemia of the subcutaneous tissue was observed in some chickens, whereas for the emulsion formulation, in all birds, there were connective tissue granulomas with vaccine residues though without necrotic lesions and a pronounced inflammation of the surrounding tissues (Fig. 1).

Intramuscular injection of the absorbed vaccine formulation led to a slight tissue damage with no pronounced signs of inflammation and residues of the encapsulated vaccine. With a similar administration of the emulsion vaccine formulation, there were a moderate tissue inflammation, granulomas up to 2.0 cm in diameter, and vaccine residues which looked like small encapsulated droplets located along the muscle fibers. No necrosis and hemorrhages were seen in the surrounding tissues. In totality, these data indicated the safety of both vaccine formulations when administered subcutaneously.

To assess protective properties of the candidate vaccine formulations, the control infection of chickens with 1-day broth culture of *A. paragallinarum* No. 5111 was carried out. One to two days after infection n most birds from the control group and in 3-4 days in some birds of the test groups, the same types of clinical signs (rhinitis, sinusitis, and conjunctivitis) developed. At the initial

stage of the disease, birds showed transparent discharge from the nasal passages and slight one- or two-sided swelling in the nasal sinuses. Subsequently, in sick chickens of the control group, nasal discharge became cloudy and acquired a viscous consistency. The inflammatory exudate often blocked the nasal passages and the birds began to breathe through the mouth. Further development of the disease in chickens from the control group showed pronounced swelling of the nasal sinuses and conjunctival sacs. Most sick birds were depressed with partial or complete refusal of food and water. In some chickens, when the infection developed in the deep parts of the respiratory tract, breathing was accompanied by wheezing. In immunized birds, there were only a slight watery discharge from the nasal passages and subtle one- or two-sided swelling of the nasal sinuses. The average duration of illness in vaccinated chickens was 3-4 days, while in birds from the control group it was 5-7 days (Table).

Protective properties of formulations of a candidate vaccine against avian infectious coryza in Hisex brown chickens (*Gallus gallus* L.) infected with 1-day broth culture of *Avibacterium paragallinarum* No. 5111 (laboratory studies)

Formulation	Birds				Disease severity, the sum of scores per group (M±SEM)	Efficacy, %
	total number	sick	died	in which the pathogen was detected		
Absorbed	25	3	0	3	0.12±0.03	92
Emulsion	25	4	0	4	0.16±0.04	88
Control (no vaccination)	25	23	7	25	3.00±1.00	

When comparing the development of control infection in birds immunized with different vaccine formulations, no significant differences were observed ($p > 0.05$), however, a significant difference occurred between the experimental and control groups ($p < 0.05$). In group I immunized with the absorbed formulation, the protective efficacy was 92 %. The total score characterizing the severity of the disease was 25 times lower compared to the control birds. In group II immunized with the emulsion formulation, the effectiveness was 88 %. In the control birds, clinical signs were observed in 23 chickens, of which 7 birds died.

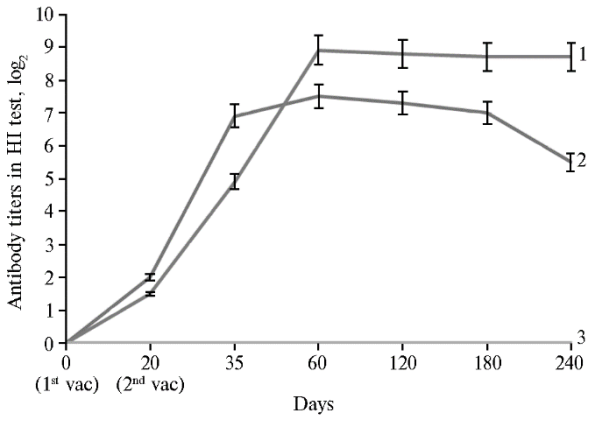

Fig. 2. The titer of humoral antibodies in the hemagglutination inhibition (HI) **test and the duration of immunity in Hisex brown chickens (*Gallus gallus* L.) immunized with two formulations of a candidate vaccine against avian infectious coryza based on the *Avibacterium paragallinarum* № 5111 antigen (B-1 serovar):** 1 — emulsion formulation, 2 — absorbed formulation, 3 — control (no vaccination); 1st and 2nd vaccinations (M±SEM, laboratory tests).

Pathological changes in chickens were observed mainly in the upper parts of the respiratory tract. In the immunized chickens with clinical signs of the disease during life, a small amount of serous exudate was seen in the nasal sinuses. In most of the birds of the control group, the subcutaneous tissue in the facial part of the head had a pronounced edema and a gelatinous consistency. The nasal passages and sinuses in all chickens were filled with fibrinous or fibrinous-purulent exudate. In the conjunctival sacs, serous-purulent exudate with fibrin films was often seen. When distal part of the respiratory tract was affected, fibrinous pneumonia and aerosacculitis usually developed. The

causative agent of the disease was isolated from the inflammatory exudate form the nasal sinuses. The original *A. paragallinarum* No. 5111 strain was isolated from 3 birds of group I, 4 chickens of group II, and from all control birds.

Analysis of the dynamics of the formation of specific antibodies in the blood sera of birds after double immunization testified to the high antigenic activity of both vaccine formulations (Fig. 2).

Twenty days after the first vaccination, in birds vaccinated with both formulations, the average antibody titers were below the threshold value ($p > 0.05$). A significant increase in the blood antibody levels occurred only 15 days after the second immunization. Sixty days after the immunization, the antibody titers in groups I and II were the highest, up to 7.5 ± 0.5 and 8.9 ± 0.2 $\log_2$ ($p < 0.05$), respectively. In 240 days, in chickens immunized with the absorbed formulation, the antibody titers decreased to 5.5 ± 0.6 $\log_2$, while in birds immunized with an emulsion formulation, the titer was 8.7 ± 0.8 log2 ($p < 0.05$). During the entire period, no specific antibodies to *A. paragallinarum* were detected in the control group.

According to the literature, emulsion vaccines are more immunogenic than absorbed vaccines, but when they are used, there is a likelihood of developing local inflammatory reactions and even abscesses. Absorbed vaccines, as a rule, have less reactogenicity for animals; however, when administered subcutaneously, they sometimes cause the formation of connective tissue granulomas [21, 25, 26].

It is known that the effectiveness of inactivated vaccines directly depends on the amount of bacterial antigen per dose. An excessive amount of antigen can suppress the body's immune system up to the development of immunological tolerance and cause undesirable reactions at the injection site. In turn, at an insufficient dose, the antigen does not induce immunological processes in the body [10, 13]. Our experiments showed that the tested vaccine formulations are safe for birds when injected subcutaneously. Evaluating the protective properties confirmed a pronounced immunogenic activity of the formulations. An effective inactivated vaccine must provide protection for at least 80 % of the vaccinated population. In our experiments, the protective activity reached 92 % for the absorbed formulation of the candidate vaccine and 88 % for the emulsion formulation. Similar results were obtained by another scientists. For example, Blackall and colleagues conducted trials to assess the safety and efficacy of inactivated vaccines containing mineral oil and aluminum hydroxide gel as adjuvants. Both types of the drug were injected subcutaneously into the middle third of the neck from the dorsal side. After 3 weeks, the birds were subjected to experimental infection. The emulsion preparation provided 80 % protection, a vaccine based on an aluminum hydroxide gel provided 94 % protection [10, 26]. Our results are also consistent with the data of other authors, who assert that, after 2-fold use, absorbed and emulsion formulations have high antigenic activity against avian infectious coryza [27].

Thus, the tested formulations of a candidate vaccine against avian infectious coryza based on the *Avibacterium paragallinarum* strain No. 5111 (serotype B-1) antigen are harmless when administered subcutaneously to Hisex brown chickens at a 2-fold dose. At the site of injection of the absorbed formulation, some chickens developed slight edema and hyperemia of the subcutaneous tissue. When the emulsion formulation was injected, all birds developed connective tissue granulomas with the presence of vaccine residues

without a pronounced inflammatory reaction of the surrounding tissues. The efficacy of the absorbed formulation was 92 %, of the emulsion formulation 88 %. Both formulations induced production of antihemagglutinating antibodies. The antibody titers were the highest 60 days after vaccination. In birds vaccinated with the absorbed and the emulsion formulations, the antibody titer averaged 7.5±0.8 and 8.9±0.7 $\log_2$, respectively ($p > 0.05$). For early protection of birds from avian infectious coryza, the absorbed vaccine formulation is preferable while the oil adjuvant formulation ensures a more intense and lasting immunity.

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