Immunological evaluation of inactivated Newcastle disease vaccine depending on adjuvant composition

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Newcastle disease is a global problem that is being recorded in most countries and also a serious obstacle to exchange of genetic material of poultry in various countries of the world. Control of the Newcastle disease comprises correct injection of efficacious vaccines so as to decrease or eliminate the clinical disease. Our goal was to perform comparative studies of the vaccines against Newcastle disease of water in oil type, the adjuvant being mineral oil mixed with emulsifiers (Span-80 and Tween-80) and ready-to-use adjuvant system (Montanide ISA 70), and study the impact of composition of adjuvant constituent on physical-chemical and immunogenic properties of inactivated vaccines. To reproduce virus-containing material and carried out titration of the viruses, we used chicken embryos free of pathogenic microflora. Aqueous phase for the preparation of emulsion-based vaccines of water in oil type consisted of antigen to Newcastle disease of La-Sota strain, manufactured by Biotestlab Ltd, and phosphate-saline buffer. To evaluate the effectiveness of the vaccine and induce immune response, we used 1-day old pathogen-free chickens, which were obtained from chicken embryos free of pathogenic microflora. As the positive control in the experiment, we used commercial vaccine. One-day chickens were divided into 3 groups (I, II, III) comprising 12 individuals each and one group (IV) consisting of 8 individuals as the control group with individual numeration. Chickens in groups I, II and III were divided into two subgroups (n = 8 and n = 4) to determine immunogenic efficiency and safety of the vaccine. Immunization was carried out through single subcutaneous injections in the region of the neck. To study immunogenic efficiency, the chickens were immunized with the dose of 0.1 mL (1 dose), and 0.2 mL (2 doses) to determine safety. After the immunization of 1-day old pathogen-free chickens with 0.1 mL dose, the obtained level of antibodies in the serum of vaccinated chickens on days 14, 21, 28, 35 and 42 after the vaccination indicated the ability of provoking the immune response to Newcastle disease at high level and safety of the vaccination for chickens. All the recipes of the examined series of the vaccines and the commercial vaccine produced appropriate level of viscosity according to the criterion equaling ≤ 200 mm^2/s at P <0.05, promoting fluidity of the vaccine and providing easier passage through the needle during the application. Both of the studied vaccines may be used in poultry farming for prophylaxis of Newcastle disease among chickens.

Keywords: poultry; Newcastle disease; inactivated vaccine; adjuvant; postvaccinal immune response; reaction of hemagglutination.

Introduction

Newcastle disease (ND) is an international problem recorded in most countries around the world and also a serious obstacle to exchange of genetic material in poultry in different countries of the world. Economic losses imposed by Newcastle disease comprise mass morbidity and 90–100% mortality of poultry, 20–60% decrease in productivity of vaccinated poultry and also significant costs for carrying out measures to liquidate and prevent it (Ashraf et al., 2014; Ganar et al., 2014). The commonest and most harmful virulent disease of poultry which affects the poultry farming, caused by infections of virulent viruses of *Avulavirus* genus and avian avulavirus 1 types, is broadly known as Newcastle Disease (NDV) and shortened as avian paramyxovirus 1 (APMV 1) (Afonso et al., 2016; Amarasinghe et al., 2018). The disease is extremely contagious and causes high morbidity and mortality to unvaccinated and badly vaccinated chickens, and also decrease in egg productivity, especially in developing countries, when no adequate strategy of combating it is implemented (Miller et al., 2013; Absalón et al., 2019; Ogali et al., 2020). Infectious Diseases 2020 Electronic Resource reported that after two decades free of Newcastle Disease, Belgium faced epizootopy of orthoavularis of avian type 1 in

2018. In Belgium, 20 cases were diagnosed, including 15 – in flocks, 2 – in flocks of specialized poultry and 3 – in flocks of poultry for retail. The disease has spread from Belgium throughout the BeNeLux region. According to the data of the World Organization for Animal Health, numerous outbreaks of Newcastle disease were recorded in 2020 in regions of Russia and individual cases in the territory of Romania, Bulgaria, Israel, Macedonia.

Because no treatment of ND exists, removal of infected individuals following rigid biological safety and aggressive vaccination protocols is the most suitable way of fighting the outbreaks (Dimitrov et al., 2017; Absalón et al., 2019).

Because Newcastle disease (ND) remains a constant threat to producers of poultry-products due to low level of antibodies, the aim of ND control is adequate injection of effective vaccines and decreasing or eliminating the clinical disease (Chen et al., 2021). Live and inactivated vaccines have been broadly used since the 1950s, recombinant and antigenically selected vaccines were recently adopted in some countries (Sedeik et al., 2019). However, there were reports about unsuccessful vaccinations with live attenuated vaccines due to problems in required storage and transport (Ruan et al., 2020). The disadvantage of inactivated anti-ND

vaccines is that they require a withdrawal period before the vaccinated birds can be processed for consumption by humans and each vaccine needs to be subcutaneously or intramuscularly injected individually. However, inflammatory reactions often occur in the places where injection has been made. Also, unfavourable local reactions after injecting emulsion-based vaccines, such as granulomes, are the main factor that should be taken into account when using liquid paraffin as adjuvant (Fukanoki et al., 2001). Nonetheless, birds vaccinated with inactivated vaccines usually have a high level of humoral antibodies compared with birds vaccinated with live anti-ND vaccines (Miller et al., 2013; Schijns et al., 2014). Furthermore, birds were observed to have no specific side effects after immunization with anti-ND vaccines. In all the experimental birds, mild conjunctivitis was observed, which was gone in 2 days (Miller et al., 2009).

Despite decades of studies and development of an optimum vaccine against ND and presence of sufficient amount of commercial vaccines, improvement of the recipe would be still relevant. Improvement of the vaccine consists of developing antigen variations (Sedeik et al., 2019) and various emulsion adjuvants. Also, to prevent safety-related problems, one should pay special attention to the quality of selected surfactants and natural oils which are being implemented (Arous et al., 2013). A study was made of serologic reaction of chickens, which varied 10–100 times after vaccination with different types of adjuvants or emulsifiers of ordinary homogenization (Brugh et al., 1983). Emulsion adjuvant accounts for a large part of the formulation of efficient inactivated vaccine for birds. Safe water-in-oil adjuvants induce humoral immune response, production and potentiation of antibodies, increase duration of antibodies' response, reduce frequency of vaccination and the amount of necessary antigen in each dose of vaccine, and therefore are broadly used in the compound of inactivated vaccines (Jafari et al., 2017; Yuan et al., 2020). Search of new adjuvants that would increase the cellular response or decrease the dose of injected vaccine and number of necessary vaccinations, or antigenic load in vaccine, may help enlarge the cross protection against various virulent strains or serotypes (Arous et al., 2013; Gharajalar et al., 2020). The assortment of registered vaccines against Newcastle disease in Ukraine is represented by 6 leading foreign producers and one domestic one, developed by Biotestlab Ltd, which were developed in the form of emulsion and contain various emulsion adjuvants.

Studies often focus on implementing effective ready-to-use adjuvants based on specific mineral oils such as Montanide ISA 71 VG or ISA 70, or application of well-known available oils, such as palm oil, with addition of antioxidants (Wanasawaeng et al., 2009; Arous et al., 2013).An additional substance Montanide is a mineral oil-based adjuvant that was developed for the production of water emulsions in mineral oil, consists of enriched light mineral oil that can stimulate both humoral and cellular immune responses, with extremely refined emulsifier obtained from mannitol and purified oleic acid of plant origin. This adjuvant has been developed specifically to stimulate the cell-mediated immunity, and has been earlier observed to induce strong humoral and cellular immune responses in various models of poultry diseases (Muzyka, 2013; Ali et al., 2017; Hn, 2018; Ismail et al., 2018).

The objective of the study was immunological evaluation of the effect of the composition of adjuvant component and immunogenity of inactivated vaccine against Newcastle disease in poultry.

Materials and methods

This study was performed according to the principles described in the guidelines of maintenance and use of experimental animals, according to the ethic norms written in Order No. 3447-IV of 21.02.2006 "On Protection of animals against abuse".

To prepare the experimental series of vaccine, we used local vaccine Newcastle disease virus strain La-Sota, obtained from the strain bank Biotestlab Ltd and replicated using the method of inoculation into the chorioallantoic membrane of 9–11 days old pathogen-free chicken embryos produced by VALO BioMedia, Germany. Correspondence of the strain was confirmed using the method of real time polymerase chain reaction. Hemagglutinin activity of Newcastle disease virus La-Sota strain was determined in hemagglutination reaction (OIE Terrestrial Manual,

2018) by preparing sequential two-fold dilutions of the material in 96-well plates with following addition of 1% suspension of erythrocytes from rooster. To prepare the series of vaccines, we used oil adjuvants: 1) mixture of mineral oil (Parafluid GmbH, Germany) combined with emulsifiers Span-80 (Shenzhen Ruiqi Import&Export Co., Ltd, China) and Tween-80 (Shenzhen Ruiqi Import & Export Co., Ltd, China); 2) Montanide ISA 70 (Seppic, France).

Specific free pathogen (SPF) chicken embryos manufactured by VALO BioMedia, Germany were used for replication of virulent material and titration of the viruses. To assess the efficiency of the vaccine and induction of the immune response, we used 1 day-old SPF chickens, obtained using SPF chicken embryos. All experimental studies of the poultry were conducted at the basis of BIOTESTLAB Ltd vivarium. The vivarium was equipped according to the sanitary hygienic norms (19–24 °C temperature, moisture no higher than 50%, in daylight regime "day–light"). During the experiment, chickens of all the studied groups were kept in SPF boxes for isolated maintenance (Qingdao, China). Maintenance, feeding and watering were the same for all the poultry groups.

Throughout the monitoring of the immunogenic efficiency and safety, the chickens were kept on a balanced fodder diet and had free access to food and water. All the procedures with the animals were performed following the International Rules and Norms of Bioethics.

Emulsion-based vaccines of water-in-oil type were prepared with the concentration of virus antigene equivalent to 109.0 ELD_{50} (average lethal dose for embryo) per 0.1 mL of vaccine (1 dose) (Reed et al., 1938). The aqueous phase for preparation of emulsion-based vaccines of water-in-oil type consisted of an inactivated antigen of Newcastle disease, La-Sota strain, manufactured by Biotestlab Ltd and phosphate-saline buffer (hereinafter – PSB). The antigen was accumulated in SPF chicken embryos aged 10 days, the incubating regime being 72–96 h in 37–38 °C temperature and 40–70% moisture. Inactivation of the antigen was carried out by introducing phormaldehide in the dose of 0.05% of the final concentration to the amount of antibody, which was then kept for 24 h in 20–25 °C temperature, being regularly stirred (Hongzhuan et al., 2020). Completeness of inactivation of the virus was performed using the methods according to E.P.9.3.01/2017:0870 Newcastle disease vaccine (inactivated). 2.5.1. The residual live virus was made by infecting 10 chicken embryos aged 9–10 days and three subsequent "blind" passages using a mixture of allantoic fluid, taken in equal amounts from each embryo of the previous passage, for the purpose of infecting. Collected allantoic fluid was examined in accelerated qualitative reaction of hemagglutination using 5% suspension of erythrocytes from rooster.

The oil phase for preparation of series I–WO PST of the emulsion-based vaccine consists of a mixture of mineral oil with surfactants Tween-80 (manufactured by Shenzhen Ruiqi Import & Export Co., Ltd) and Span-80 (manufactured by Shenzhen Ruiqi Import & Export Co., Ltd). For II–WO MISA series of the vaccine, we used the ready-to-use adjuvant system Montanide ISA 70 for emulsification.

The vaccines were prepared by homogenizing the emulsion with laboratory homogenizer IKA ULTRA–TURRAX T manufactured by IKA® – Werke GmbH & Co. KG, Germany. The aqueous phase was added to the oil phase in small portions, while constantly stirring the oil phase using magnate (anchor) stirrer for 10–15 min until the formation of pre-emulsion. After mixing the oil and water phases, the mixture was emulsified again.

Oil adjuvants were mixed with aqueous phase in the proportion that would provide the formation of stable emulsion of reverse type water in oil. Recipes of the series of emulsion-based vaccines are provided in Table 1.

Table 1
Recipe of experimental series of emulsion-based inactivated vaccines of overall volume measuring 250 mL

Studied materials	Aqueous phase, mL	Oil phase, mL
Series I–WO PST	97.5	152.5
Series II–WO MISA	75.0	175.0
Commercial vaccine	commercial inactivated vaccine	
Control (PSB)	250.0	–

Note: series I–WO PST – vaccine prepared using mineral oil mixed with Tween-80 and Span-80 as adjuvant; series II–WO MISA – vaccine prepared using Montanide ISA 70 as adjuvant; "–" – not used.

The prepared experimental series of inactivated vaccine against Newcastle disease corresponded to the quality control according to the following parameters: sterility, safety, kinetic viscosity, stability of emulsion, immunogenic efficiency, residual amount of formaldehyde.

The greater is fluidity, the thicker (more viscous) is the fluid, which makes the passage of the emulsion through the needle harder and may be a factor for the duration of vaccination of a large population of poultry. Kinematic viscosity was determined using capillary viscosimeter VPJ-1 (manufactured by Ecros, Russia). The temperature of the tested liquid was 20 °C. Time for the liquid to pass from one mark of the viscosimeter to another was measured using a stop watch with accuracy up to 0.2 s. The obtained data were considered favourable if two subsequent measurements were different by no higher than 1%. Time of leaking of the liquid was determined as mean value of no less than three measurements. Kinematic viscosity was calculated using the formula $v = kt$ and expressed in square millimeters per second (mm^2/s). Kinematic viscosity of the vaccine should be $\leq 200\ mm^2/s$.

Stability of vaccine's emulsion was tested using the centrifugation method with employment of laboratory centrifuge Centrisart G-16C (manufactured by Sartorius, Germany). The flacon with the vaccine was thoroughly shaken, and then we added 8.0 mL of the vaccine to each centrifuge test tubes and centrifuged at 3,000 rpm for 20 min. The height of the column of transparent fraction was measured in mm. The emulsion was considered stable if the test tubes were observed to have no development of transparent fraction in the upper part or if the height of the column of the transparent fraction, which could develop in the upper part, did not exceed 10% of the volume, and the divided fractions easily formed homogenous emulsion after shaking.

Safety was determined using the method developed according to the European Pharmacopeia 9.3, 04/2013:50206 Evaluation of safety of veterinary vaccines and immunosera. Safety of 1 administration of an overdose. The vaccine in the volume of 0.2 mL (2 doses) was injected to the chickens aged one day with the SPF status into the region of the neck. The monitoring of chickens lasted for 14 days. The vaccine was considered safe if all the chickens stayed healthy for 14 days of the monitoring and had no clinical manifestations that are characteristic of Newcastle disease. The tissues' reaction in the place of the vaccine injection was determined by dissection on the 14th day after the vaccination. We evaluated the condition of the neck muscles in the region of the injection and determined the tissues' reaction according to the following gradation: moderate level (pale muscles in the region of injection and absence of vaccine residuals), intermediate level (the surface muscles were pale to red and a small amount of the vaccine was present as drops), severe level (inflammation of the surface and deep muscles and residuals of the vaccine in the regions of injections, observed during autopsy) (Wanasawaeng et al., 2009).

Pathogen-free chickens, obtained from SPF embryos, were kept in boxes for isolative maintenance (Qingdao, China). Single-day SPF chickens were divided into three groups (I, II, III) each comprising 12 individuals (n = 12) and one group (IV) containing 8 individuals as the control group with individual numeration. Chickens in groups I, II and III were divided into two subgroups (n = 8 and n = 4) to determine immunogenic efficiency (a) and safety (b) of the vaccine. Immunization was carried with single subcutaneous injection in the region of the neck. To study the immunogenic efficacy, the chickens were immunized with the dose of 0.1 mL (1 dose), to study safety – in the dose of 0.2 mL (2 doses). Groups Ia, Ib were vaccinated with series I–WO PST. Groups IIa, IIb received series II–WO MISA. Groups IIIa, IIIb were vaccinated with the commercial vaccine. Group IV – the control – was vaccinated with PSB. Immunization matrix of poultry is presented in Table 2.

Table 2

Matrix of immunization of poultry with experimental series of vaccine for the purpose of determining the immunogenic efficiency of the vaccine

Group of studied animals	Number of chickens in group	Examined parameter	Vaccination at 1 day of age	Study method
Ia	8	immunogenic efficiency	subcutaneously in the region of the neck (series I–WO PST in the dose of 0.1 mL (1 dose)	blood draw
Ib	4	safety	subcutaneously in the region of the neck (series I–WO PST in the dose of 0.2 mL (2 doses)	dissection of chickens
IIa	8	immunogenic efficiency	subcutaneously in the region of the neck (series II–WO MISA in the dose of 0.1 mL (1 dose)	blood draw
IIb	4	safety	subcutaneously in the region of the neck (series II–WO MISA in the dose of 0.2 mL (2 doses)	dissection of chickens
IIIa	8	immunogenic efficiency	subcutaneously in the region of the neck in the dose of 0.1 mL (1 dose)	blood draw
IIIb	4	safety	subcutaneously in the region of the neck (commercial vaccine in the dose of 0.2 mL (2 doses)	dissection of chickens
IV	8	control of the study of immunogenic efficiency	subcutaneously in the region of the neck (sterile PSB)	blood draw

Note: see Table 1.

Blood of chickens was drawn every 7 days starting from the 14th day after the vaccination, in particular days 14, 21, 28, 35 and 42. Blood samples were taken from the ulnar wing in the amount of 2.0–2.5 mL using a sterile 5.0 mL syringe, transferred to test tubes and centrifuged at 1,500 rpm for 10–15 min. We separated the serum for serologic studies.

To evaluate the immunogenic efficiency of the vaccine, we studied blood serum of chickens in the reaction of hemagglutination inhibition (HI) according to the generally accepted methods, recommended by the OIE (OIE Terrestrial Manual, 2018) and method of enzyme-linked immuno sorbent assay, ELISA. The studies using the hemagglutination inhibition reaction are based on the fact that antibodies form an antigen-antibody complex when they meet the virus and neutilize hemagglutinins of the virus. Titer of antibodies to Newcastle disease was studied using 96-well plates, testing the samples against their homologic antigens of the vaccines (Rahman et al., 2017).

Of the studied blood serums of chicken, we prepared subsequent two-fold dilutions with additions of PBS. Then, equal amounts of operating dilution in concentration of 4 HA of ND virus were added to each well. The plate was incubated at the temperature of 37 ℃ for 30 min to achieve interaction between the examined serum and viruses. After the exposure, an equal amount of 1% suspension of rooster`s erythrocytes was added to each well and the plates were incubated in 20 ℃ temperature for 20–30 min. Erythrocytes were the indicator of presence of the virus in the mixture. Agglutination of erythrocytes indicated the presence of the virus in the mixture, while its absence indicated the absence of the virus because the antibodies had completely neutralized the hemagglutination activity of the virus. HI titer is the greatest dilution of the serum which causes complete inhibition of 4 HA antigen. Agglutination was assessed by tilting the plates. The result was considered positive after sedimentation of erythrocytes at the bottom of the plate's wells. Significance of the result was assessed by comparing it to the control serum (negative control) that contained no antibodies to virus hemagglutinin of Newcastle disease. Only the wells in which erythrocytes leaked at the same rate as the control were considered as those that demonstrated the inhibition (OIE Terrestrial Manual, 2018). Titer was recorded as log_2 of greatest dilution of serum, at which the hemagglutination inhibition (absence of hemagglutination) was observed, i.e. presence of homogenous layer of erythrocytes as sediments at the bottom of the wells of the plate. Titer of antibodies to ND 1:16 (4 log_2) and more was considered protective. ELISA is based on the main immunological concept of antibody that binds to its specific antigen, the-

reby allowing an identification of the amount of antibodies in the sample. ELISA has been developed on the general immunologic concept of specific interaction between antibody and antigen which allows assessment of the level of antibodies in sample of blood serum (Gan et al., 2013). Blood serum of chickens drawn 14, 21, 28, 35 and 42 days after the immunization was examined using a commercial ELISA test kit (IDEXX Newcastle Disease Virus Antibody Test Kit, USA) to determine specific antibodies to the Newcastle disease virus of chickens. The kit contained 96-well plates, the wells coated with antigen of Newcastle disease virus. The serum samples were prepared in 1:5,000 dilution and incubated in 96-well plates according to the manufacturer`s recommendations concerning the ELISA. After introduction of ND antibodies-holding serum to the wells, immune reaction occurred, forming a specific antigen-antibody complex, which strongly stood on the surface of the well. After the wells had been rinsed to remove non-specific components, the conjugant was introduced (Goat anti-chicken: HRPO), binding specifically to the antigen-antibody complex. The wells were rinsed to remove the residuals of the conjugant, and then we added TMB substrate for the enzymatic ELISA phase that occurred having changed the colour in the wells, the intensity being directly proportionate to the amount of antibodies in the sample. Colour intensity was measured using the reader (microplate spectrophotometer) at the wavelength of 650 nm. The kit was used for both qualitative detection of antibodies to Newcastle disease virus and calculation of the quantitative level of antibodies (titers) in the examined serum.

Presence or absence of antibodies to Newcastle disease was determined according to the value that is estimated as the ratio of optical density (OD) of the examined sample to optical density of the mean value of the positive control. As positive control, we used the kit`s standardized sample of blood serum of chickens with a certain level of antibodies to Newcastle disease virus.

Relative level of antibodies in the examined samples was determined according to S/P parameter that was calculated as ratio of difference of optical density parameters of the sample and mean value of optical density of the negative control to the mean values of positive and negative control.

In evaluating the results, if the value of the ratio of the serum sample to the positive control was ≤ 0.2, such sample of serum was considered negative, if the value was higher than 0.2, the sample was considered positive, indicating presence of antibodies to Newcastle disease.

Titer of antibodies was calculated according to the formula:

$$Log_{10}\text{Titer} = 1.09(log_{10} S/P) + 3.36$$

The result of the analysis was considered actual if the difference between the mean value of optical density for the positive control and mean optical density for the negative control was more than 0.075, and the average value of negative controls was no more than 0.150. Titer of antibodies to Newcastle disease at the level of 397 and higher was considered protective.

We calculated the variation coefficient (% CV) – statistical measure of dispersal of the data around some mean value. Variation coefficient was calculated as ratio of mean square deviation to mean value, indicating the level of variability of some selection of the data to their mean value. The lower was CV % parameter, the more homogenous were considered the results.

The data were analyzed in ANOVA software. The results were statistically analyzed with calculation of mean arithmetic value (x), standard error (SE) and standard deviation (SD) and using regression and correlation analyses, and the difference was considered significant at P < 0.05 (taking into account Bonferroni correction).

Results

All chicken embryos that were injected formaldehyde-inactivated virus survived more than 120 h, and no viruses were found by the express test for HA (hemagglutination reaction), indicating complete inactivation of Newcastle disease virus.

The results of determining the kinematic viscosity in II–WO MISA series were at the level of 40–42 mm²/s, which is lower compared with the results of I–WO PST series – 80–88 mm²/s. All the recipes of the examined series of the vaccines provided an acceptable level of viscosity according to the adopted criterion equaling ≤ 200 mm²/s at P < 0.05, which pro-

moted fluidity of the vaccine and provided easy passage through the needle. The prepared emulsion-based vaccines of water in oil type of series I–WO PST, series II–WO MISA and the commercial vaccine exhibited no division of oil and water phase during the maintenance for more than 4 weeks in 4 °C. In 37 °C, sample of II–WO MISA series and commercial vaccine displayed stability for more than 4 weeks and sample of I–WO PST series demonstrated separation of oil and water phases by layers on the 4th week of monitoring (Table 3), which, however, was easily restored in homogenous emulsion after intense shaking.

Table 3
Results of determining kinetic viscosity and stability of series of emulsion vaccines (x ± SD)

Studied materials	Monitoring period	Time, s	Kinematic viscosity, ≤ 200 mm²/s	Stability, weeks	
				4ºC	37ºC
Series I–WO PST	starting point 0	247.0 ± 2.3	80 ± 0.6		
	10 months	251.2 ± 0.6	81 ± 0.6	>4	<4
	16 months	271.4 ± 1.2	88 ± 0.6		
Series II–WO MISA	starting point 0	123.0 ± 0.2	40 ± 0		
	10 months	126.6 ± 0.5	41 ± 0	>4	>4
	16 months	130.7 ± 0.7	42 ± 0.6		
Commercial vaccine	16 months	163.0 ± 2.2	53 ± 0.6	>4	>4

Note: the tests were made in 20 °C, 45% moisture; the presented values are values ± standard deviations, difference was considered significant at P < 0.05.

The results of safety tests of the experimental samples of the vaccine were analyzed by assessing the vitality of chickens at dissection 14 days after the vaccination. All chickens (group Ib, group IIb and group IIIb – 12 chickens total) remained healthy for 14 days of monitoring and had no clinical manifestations that are characteristic of Newcastle disease. The results of the autopsy of the chickens of groups Ib, IIb and IIIb are provided in Table 4.

Table 4
Reaction of the tissues of chickens 14 days following the introduction of the examined samples of emulsion-based vaccines

Group of examined animals	Examined materials	Level of the tissue reaction		
		moderate	intermediate	heavy
Ib	series I–WO PST	6/8	2/8	0/8
IIb	series II–WO MISA	7/8	1/8	0/8
IIIb	commercial vaccine	6/8	2/8	0/8

Note: the results are presented as a/b, where a is number of chickens with identified level of damage to the tissue, b is number of chickens in group.

Fourteen days after the vaccination, titers of antibodies of groups Ia and IIa, determined using hemagglutination inhibition reaction, equaled 1:52 and 1:108 respectively, while the values of chickens that received the commercial vaccine accounted for 1:15. Twenty one days after the vaccination, the quantitative parameters of titers of antibodies increased as follows: group Ia – 1:272, group IIa – 1:336, group IIIa – 1:28. The results of titer of antibody 28 days after the vaccination revealed the quantity of antibodies in blood serum of experimental groups Ia and IIa at the levels of 1:400 and 1:512 respectively, titer of antibodies of chickens of group IIIa equaled 1:64. Thirty five days following the vaccination, the quantitative parameters of antibodies in poultry groups Ia and IIa equaled 1:368 and 1:496 respectively, the parameters for the commercial vaccine accounted for 1:80. The parameters of blood serum of the control groups of chickens (group IV) were within 1:0–1:2 throughout the study.

In all the studied samples of serum, 16 chickens (67% of vaccinated chickens) were observed to have protective level of the immunity, being at the level of 6 log₂–7 log₂ (groups Ia and IIa) on the 14th day after the vaccination; the formation of antibodies at the protective level measuring 4–5 log₂ was observed in 8 chickens of group IIIa (33% of vaccinated chickens) on the 21st day after the vaccination. Antibodies of all chickens of group IV (100% of non-vaccinated chickens) were at the maternal level. Table 5 presents the determined mean level of antibodies in the reaction of hemagglutination inhibition in blood serum of examined groups Ia, IIa, IIIa and IV after the vaccination. Figure 1 shows the dynamics of the development of the immunity in chickens after the vaccination with the examined series of vaccines according to the results

of determining the mean level of antibodies in the reaction of hemagglutination inhibition. Comparative analysis of the selections was carried out within each group in ANOVA software.

Table 5
Quantitative values of mean level of antibodies in blood serum of chickens after vaccination, determined in hemagglutination inhibition (x ± SE, n = 8)

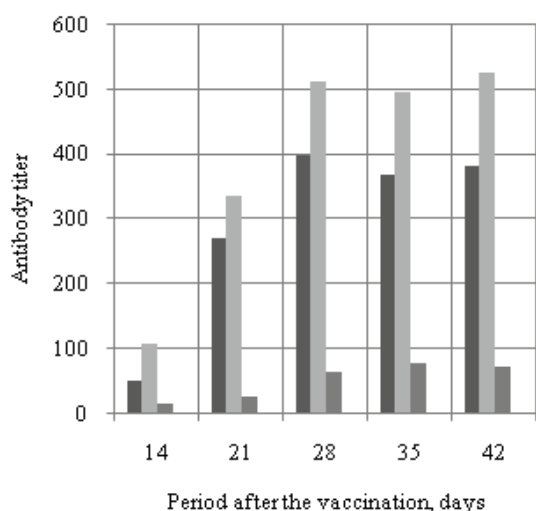| Groups of chickens	Period after the vaccination, days				
	14	21	28	35	42
Ia	52 ± 6	272 ± 56	400 ± 101	368 ± 56	384 ± 97
IIa	108 ± 25	336 ± 54	512 ± 119	496 ± 124	528 ± 120
IIIa	15 ± 3	28 ± 3	64 ± 11	80 ± 11	72 ± 8
IV	0 ± 0	2 ± 0	2 ± 0	0 ± 0	1 ± 0

Fig. 1. Dynamics of development of immunity in chickens after vaccination with the studied series of vaccines according to the results of determining mean level of antibodies in the reaction of hemagglutination inhibition (HI)

Parameters of average titer of antibodies in blood serum of chickens of groups Ia and IIa, determined using the method of immunoenzymatic analysis 14 days after the vaccination, equaled 281 and 487 respectively, and 286 in group IIIa. Twenty one days following the vaccination, the parameters achieved the levels of 1,455, 4,094 and 1,330 in groups Ia, IIa and IIIa respectively. Twenty eight days after the vaccination, mean titers of antibodies of chickens from groups Ia, IIa and IIIa were 4,676, 10,139 and 3,064 respectively. Thirty five days after the vaccination, the parameters in blood serum were at the levels of 9,154 and 14,024 in chickens of groups Ia and IIa respectively, and 5,938 in group IIIa. Parameters of blood serum of the control group of chickens (group IV) was within 1–77 throughout the study.

According to the results of the study, antibodies in all the serum samples of all 100% of vaccinated chickens were at the protective level on the 21st day after the vaccination, though the level of the immunity in group IIa was 2.5 times higher compared with the chickens of groups Ia and IIIa. The highest level of antibodies in the examined chickens was observed on the 35th day following the vaccination.

Table 6 provides the results of determining mean level of antibodies using the method of immunoenzymatic analysis in blood serum in examined groups Ia, IIa, IIIa and IV after the vaccination.

Using the parameters of immunogenity determined using ELISA method 35 days after the vaccination with the studied series of vaccines, we carried out integrated assessment of the degree of immunity development in the context of the parameters of the level of antibodies according to titer groups and inferred variation coefficient (% CV) for each studied group of

chickens. The determined variation coefficient was 24.4% in chickens of group Ia (series I–WO PST), 37.6% in group IIa (series II–WO MISA), 19% in group IIIa (commercial vaccine).

Table 6
Results of quantitative determining of mean level of antibodies in blood serum of chickens after the vaccination using immunoenzymatic method (x ± SE, n = 8)

Groups of chickens	Period after the vaccination, days				
	14	21	28	35	42
Ia	281 ± 70	1 455 ± 381	4 676 ± 657	9 154 ± 789	7 436 ± 693
IIa	487 ± 179	4 094 ± 1 635	10 139 ± 1 964	14 024 ± 1 865	10 101 ± 1 300
IIIa	286 ± 57	1 330 ± 200	3 064 ± 207	5 938 ± 401	4 924 ± 483
IV	49 ± 12	8 ± 3	1 ± 0	4 ± 2	77 ± 14

Figure 2 demonstrates the dynamics of development of the immunity in chickens after vaccination with the examined series of the vaccine according to the determined mean levels of antibodies using the immunoenzymatic analysis (ELISA). Comparative analysis of the selections was performed within each group in ANOVA software.
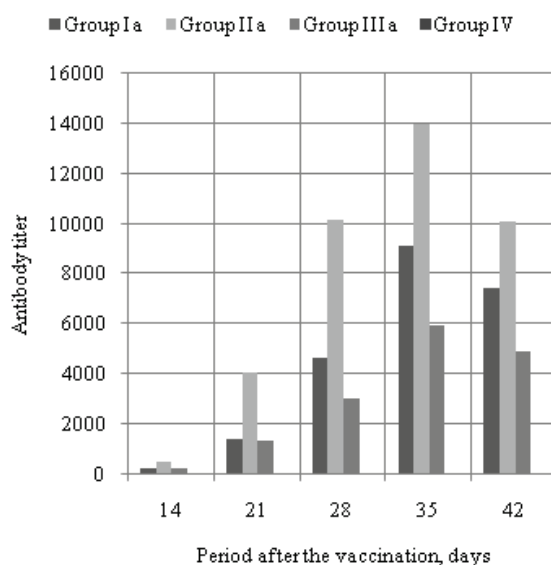

Fig. 2. Dynamics of the development of immunity in chickens after the vaccination using vaccine series according to the determined mean levels of mean level of antibodies using immunoenzymatic analysis (ELISA)

Discussion

Vaccination using La-Sota vaccine strain has successfully protected the birds against the mortality caused by Newcastle disease and decreased expressiveness of the clinical signs, pathological changes and cloacal isolation of the virus (Abd-Ellatieff et al., 2021). Lentogenic La-Sota strain for the vaccination of newborn single day chickens provided protection of broiler chickens throughout the life cycle (Cvetić et al., 2021).

Inactivated vaccines that are used to protect poultry require selecting oil adjuvants in order to induce the sufficient level of immune response. Safety of adjuvant in emulsion of water-in-oil formulation is important for the development of receipt of effective inactivated vaccine against Newcastle disease (Arous et al., 2013; Aljumaili et al., 2020). Adjuvants enhance immunity to vaccines and experimental antigens through various mechanisms. Advantages of oil adjuvants which one may expect include increase in the immune response and duration of the protection provided by the vaccine (Coffman et al., 2010). Furthermore, alterations in quantitative and qualitative characteristics of adjuvants in the content of the vaccine may be achieved by reducing the injection dose of the vaccine,

number of necessary injections and antigen load in the vaccine. Emulsions of water-in-oil type which contain liquid paraffin as adjuvant are used for injecting the food animals, and change of such an adjuvant is advisable because of deleterious side effects on animals and risk of residuals of liquid paraffin in food derived from those animals (Heegaard et al., 2011). Montanide ISA 70, which is an oil-based ready-to-use adjuvant, is to a high degree used in the production of oil emulsions for vaccines against poultry diseases. Using ready-to-use Montanide ISA system as adjuvant reduces the number of technological stages during the preparation of emulsion, thereby making a significant difference in the overall duration of vaccine preparation as compared with using multi-component adjuvants that require additional time and costs to prepare emulsions (Cahyani et al., 2020). Despite the fact that inactivated vaccines containing Montanide ISA 70 as oil adjuvant demonstrate significant efficacy and safety profile compared with other analogues, there is a need for development and selection of other, cheaper oil adjuvants.

In this research, we compared the studies of the vaccines against Newcastle disease of water in oil type with mineral oil mixed with emulsifiers (Span-80 and Tween-80) as adjuvants and ready-to-use adjuvant system (Montanide ISA 70) and studied the impact of adjuvant component on physical-chemical and immunogenic properties of the vaccines. We used oil adjuvant in the compound of the vaccine against Newcastle disease to increase the humoral response. Novel Montanide ISA 70 adjuvant is expected to improve the efficiency of vaccines for poultry, because the adjuvant system consists of enriched light mineral oil that is able to stimulate both humoral and cellular immune responses; it has been developed specifically for the purpose of stimulation of cellular immunity. Also, Montanide ISA 70 has been found to provoke strong humoral and cellular immune responses in subunit vaccines for chickens (Jang et al., 2011). The anti-Newcastle disease vaccine series that we prepared were sterile, had no alien contaminations and were safe for vaccination of chickens and caused no manifestation of the disease after the injection into the chickens' bodies according to the recommendations of the OIE (OIE Terrestrial Manual, 2018).

We determined that kinematic viscosity of series II–WO MISA was two-fold lower compared with the sample of series I–WO PST and 1.5 times lower compared with the commercial vaccine, which would make the application of the vaccine much easier in the process of immunization of poultry. Nonetheless, all examined receipts of vaccine series produced acceptable level of viscosity according to the criterion established as ≤ 200 mm^2/s at $P < 0.05$, which promoted fluidity of the vaccine and provided easy passage through the needle during the injection.

All the vaccines were observed stable in 4 ºC for more than 4 weeks in all the studied samples of emulsifying vaccines, including the commercial vaccine. In 37 ºC, the duration of the stability of emulsifying vaccine of series I–WO PST was determined as less than 4 weeks, there was observed layer separation of oil and aqueous phases on the time point of 4 weeks of the observation, but this may easily reduce into homogenous emulsion after the intensive shaking. At the same time, other samples of the vaccines exerted stability throughout more than 4 weeks maintenance period.

To study the safety of vaccines, we carried out the monitoring of chickens vaccinated with different vaccines for 14 days following the vaccination. After the experiment, we evaluated the histological damages to the tissues in the injection region at autopsy. The tissues in the region of the injections of vaccines (neck muscles) were treated and evaluated by medical examiners. The results of studying the level of tissue damages revealed that no serious damage was caused to any of the chickens. Also, we determined efficiency and safety of mineral oil in the mixture with emulsifiers Span–80 and Tween–80 as adjuvant in the compound of inactivated vaccine against Newcastle disease, which leads to no greater harm compared with Montanide ISA 70 as adjuvant, which is a standard for other oil adjuvants.

To compare the protective efficiency of these vaccines, we obtained the responses of antibodies of birds vaccinated with various vaccines and the level of immune response in the vaccinated and control groups of birds was determined according to mean numbers of antibodies to Newcastle disease using the (ELISA) method and reaction of hemagglutination inhibition (HI). Newcastle disease virus is known as avian paramyxovirus that

causes economically harmful and contagious avian disease. NDV contains three proteins associated with nucleocapsid proteins (NP, P and L), two glycoproteins (hemagglutinin-neuraminidase (HN) and hybrid protein (F)) and protein M (Fukanoki et al., 2001). Protein HN of NDV has both hemagglutinative and neuraminidase activities and plays a certain role in the disease, causing virus to bind to the receptor of the host cell. Test for hemagglutination inhibition (HI) is used in ND serotology most commonly, its usefulness in diagnostics depends on the immune status of the poultry vaccine that is being tested, as well as the dominating diseases (OIE Terrestrial Manual, 2018). Using HI reaction, one can solve diagnostic tasks such as detecting and identifying the titer of antibodies in the blood serum using a known virus, and also identify unknown virus by studying it with various known serums (antibodies).

The literature reports that vaccination-caused stress increased the level of adrenocorticotropic hormone and cortisol in blood serum, affected the growth parameters (mean daily increment, mean daily food consumption and coefficient of fodder conversion (Li et al., 2020). After immunizing one-day old SPF chickens with 0.1 mL dose, the level of antibodies in their blood serum was much higher on days 14, 21, 28, 35 and 42 following the vaccination in the group injected with adjuvant vaccine of series II–WO MISA, compared with the other groups, though both of the experimental vaccines of I–WO PST and II–WO MISA series induced the immunity up to the protective level. The highest parameters of the immune protection were seen 35 days after the vaccination. The levels of antibodies after the vaccination with the commercial vaccine were found to be much lower than in cases of injecting the experimental series of emulsifying vaccines throughout the study. In the conducted study of immunogenic efficiency, all the vaccinated groups of chickens exhibited increase in titers of antibodies compared with non-vaccinated chickens, which allowed determining the dynamics of the immunity development. The applied adjuvants that were included in the tested series of the vaccines exerted immunogenic properties, leading to protective immunity. The data obtained in our study are valuable for testing the potency of inactivated vaccines, because the European Pharmacopoeia does not indicate this antigen, which should be used in HI test. Serologic test for hemagglutination inhibition (HI) is simple to use, but is difficult to standardize among laboratory operators and in inter-laboratory control. Test for hemagglutination inhibition is considered the standard serologic test for detecting antibodies to Newcastle disease virus (Hassan et al., 2020). Nowadays, kits for diagnosing NDV using ELISA are broadly available because of a number of obvious advantages, including high sensitivity, specificity and recreation of the results, possibility of using minimum amounts of studied samples of biological fluids, availability and stability of the reagents, simplicity and fast reaction, instrumental account of the results and automatization of almost all ELISA stages (Tabidi et al., 2004).

The study of the dynamics included comparing the reaction ability of PFS chickens' blood serums to NDV using two methods of serological control of the vaccine efficiency, such as ELISA and HI. One-day old chickens were vaccinated with the experimental series of NDV vaccine of water in oil type. Titers of antibodies measuring up to the protective level were first seen already 14 days after the vaccination, identified using ELISA (S/P > 0.2 in 3/24 of birds; 12.5%) and HI reaction technique (titer > 1:16 in 87.5%). Titer of HI equaling 1:16 was considered minimum indicator of protection against the virulent virus, while titer of HI measuring 1:108 indicated good immune protection. In both serological surveys, the highest antibody titers were found after vaccination with II–WO MISA, which provided a significant level compared with the control group. The results of the study of serums following the vaccination with commercial vaccine revealed the lowest levels of development of antibodies compared with blood serums of pathogen-free chickens vaccinated with experimental series I–WO PST and II–WO MISA. On the 35th day of the experiment (35 days following the vaccination), all birds had significant levels of antibodies according to the results of serological studies using ELISA technique (group Ia – 9,154; group IIa – 14,024; group IIIa – 5,938) and HI reaction (group Ia – 368; group IIa – 496; group IIIa – 80) and had developed the protective immunity against NDV. The variation coefficient was calculated as 24.4% in chickens of group Ia, 37.6% in group IIa, and 19% in group IIIa. Despite the highest parameters of mean

titer of antibodies determined using ELISA in chickens vaccinated with the experimental series II–WO MISA, the most homogenous result according to the variation coefficient (% CV) was considered that of vaccination with series I–WO PST – 24.4 %.

Both methods of serological control of the level of immune protection were determined to be sensitive and able to detect the protective antigens to NDV in blood serums of SPF chickens. Also, we determined high positive correlation between the results of ELISA and HI reaction, indicating appropriateness of both methods for evaluating post-vaccination immunity. As a conclusion, by comparing both methods, we determined that HI reaction is more sensitive than ELISA and detects the post-vaccine antibodies to a higher degree in the earlier period following the vaccination. On the other hand, ELISA was observed to be a faster method of controlling the efficiency of the vaccination and more convenient in analysis of the results during the monitoring of dynamics of post-vaccine immunity, though more expensive than the HI test that was used to detect antibodies to NDV.

Conclusion

We prepared an experimental series of vaccines against NDV of water in oil type using mineral oil mixed with adjuvants (Span-80 and Tween-80) and ready-to-use adjuvant system (Montanide ISA 70) as emulsifiers and performed comparative studies of the effects of the adjuvant component on physical-chemical and immunogenic properties. We determined that during the use of 0.1 to 0.5 mL doses that are usually used for the standard vaccines against ND, injection with 0.1 mL of vaccine with Montanide ISA 70 adjuvant and mineral oil mixed with emulsifiers (Span-80 and Tween-80) were sufficient to provide complete protection of chickens and cause protective immune response on the model of Newcastle disease. The results we obtained demonstrate that both variants and mineral oil mixed with emulsifiers and Montanide ISA 70 are effective adjuvants that should be included in receipes of poultry vaccines, which would allow reduction of the vaccine dose for the protection of chickens to 0.1. On the other hand, Montanide ISA 70 adjuvant is an expensive, rare and imported raw material in Ukraine. Furthermore, its homogenization requires high-tech equipment. Both studied vaccines are safe for vaccination of chickens and both of them in the dose of 0.1 mL promoted induction of a sufficient level of antibodies to Newcastle disease in chickens from the 14th to 42nd day (monitoring period).

Both vaccines may be used in poultry to prevent ND in chickens.

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