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POULTRY REOVIRUS INFECTION AND DEVELOPMENT OF ITS SPECIFIC PREVENTION IN HENS

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Summary. The article analyzes the epizootic situation on poultry farms in Ukraine to reovirus infection among different bird species (chickens, broilers, ostriches, geese, perching ducks), molecular-biological properties of isolates based on the selected reovirus strain with the aim to create the inactivated emulsion vaccine against AVRI (tenosynovitis) chickens.

Epizootological, serological and virological studies have demonstrated the circulation of the causative agent of AVRI among poultry in 7 oblasts of Ukraine. Serological studies carried out among different bird species for observation period 2006–2014. It was found four field isolates of reovirus and studied their biological properties. Technological parameters of manufacture of inactivated emulsion vaccine against AVRI chickens of local strain Br-06 were developed and substantiated as well as studied formation characteristics, dynamics, level and duration of specific immunity in birds vaccinated in the laboratory and found high against epizootic effectiveness in production.

It is proved the opportunity of using inactivated emulsion-vaccine against AVRI of strain Br-06 to immunize parental poultry flock. It is also established its use as import-substitute biological products for specific prevention of parental poultry flock. State vaccine does not surrender in immunogen ratio to the imported vaccine of 'Merial' firm (France).

Keywords: chicken reovirus infection (tenosynovitis), epizootiology, reovirus biological properties and specific prevention

Introduction. Scientists paid great attention to the study of chickens viral arthritis (tenosynovitis), the pathogen which belongs to the family Reoviridae in 80–90th of the last century. The infection has been registered for the first time among broiler chickens in the United States and England in 1954. Today it is observed among poultry in many countries in the world. The most susceptible are the young growth of the first three weeks of life (turkeys, ducklings, goslings, ostriches and chicks) and adult bird meat and egg breeds and crosses (Aliev, 2002; Jones, 2013; Parkhomenko, Ihnatov, and Zemlianska, 2006; Herman, I. V., Nevolko and Khamko, 2007).

Reovirus persistence in healthy bird organisms may be the cause of latent infections, lesions of the lymphatic system and immune suppression.

The disease significantly influences on economic profitable of poultry farming due to the birds death (from 5 to 20%), delayed growth and development (to 40%) and deficiency of poultry products (Malkinson, Perk and Weisman, 1981; Jones, 2000; Dzhavadov, 2008; Trefilov and Pruglo, 2003; Nalyvaiko, 2013; Nikolaenko, 2015).

In recent time, Ukraine imports a large number of different species and breeds of poultry from countries with different epizootic situation and so, it is not excluded the importation of new pathogens of infectious diseases, which include reovirus infection. The messages about the circulation and dissemination of the pathogen on the territory of Ukraine for the last 10 years remain limited.

Countries with developed poultry farming use alive and inactivated virus-vaccines to the control and prevent of this infection.

Today in Ukrainian market has registered a significant quantity of vaccine different companies against infectious diseases of poultry. Vaccine against Newcastle disease, chicken infectious bronchitis, Borsalino infectious disease and adenovirus infection plays the most important role (Hassan et al., 1993; Borisov et al., 2005; Trefilov, 2000; Bezrukava et al., 2005).

Vaccines of avireovirus infection (ARVI) of state production on Ukrainian market do not exist. Therefore, vaccination remains one of the most effective measures to provide stable prosperity concerning the disease and prevention of the occurrence this infection.

Therefore, the aim of our work was to study biological properties of isolated pathogens and development on their base state inactivated emulsion-vaccine against chicken reovirus tenosynovitis.

Materials and methods. The experimental part of the work was carried out in 2006–2014 at the Institute

of Poultry of NAAS and on poultry farms in the Kharkiv, Ivano-Frankivsk, Donetsk, Dnipropetrovsk, Luhansk, Odesa and Cherkasy oblasts of Ukraine. Industrial reovirus strains of (Br-06, isolated from a clinically sick chickens on farms in Kharkiv oblast) and epizootic (Str-07, isolated from sick ostriches on farms in Kharkiv oblast; G-07 — from sick geese on poultry farming in Ivano-Frankivsk oblast; K-14 — from sick ducks of the private farming in Odessa oblast) were used at investigation.

Culture of cells. Primary tripsinized culture of cells fibroblast, free of pathogen flora (SPF) of chicken embryos, aimed for reproduction and virus titration, were prepared of skin and muscle tissue of 10 days SPF embryos (Syurin et al., 1998; German, 2007). In addition, were used vaccinated cell cultures: Vero (was received from State Scientific-Control Institute of Biotechnology and Strains of Microorganisms, c Kyiv), LC, KC, BGM, BHK-21 (was received from Federal State Institution 'All-Russian Scientific Research Institute of Animal Protection' (FSI 'RSRIAP'), Vladimir, RF).

Adjuvant. It was used oil adjuvant 'Montanide ISA 70 VG' production 'Seppic' company (France).

Serological methods. Sampling, sample preparation and study of blood serum was carried out from recuperated and vaccinated against poultry reovirus infection. Antibody titers were determined in the indirect haemagglutination reaction (IHR) by erythrocyte diagnostic (National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv) and by enzyme-linked immunosorbent assay (ELISA) using the 'Kit for detection of antibodies to the pathogen poultry reovirus infection by enzyme immunoassay method' (FSI 'RSRIAP', Vladimir, RF) and the test-system 'Avian Reovirus Antibody Test Kit' ('BioChek', the Netherlands).

Indication of pathogen in the pathological material was carried out by PCR (polymerase chain reaction) with inverse transcription. *Identification* of the isolated viral agents were carried out by using PH with positive to strain 1133 blood serum (FSI 'RSRIAP', Vladimir, RF).

Virological investigations were carried out according to standard methods on 9–10 daily chicken and 11-days-old duck embryos. The infectious activity of the isolated strains was determined by titration and calculated for Reed-Muench method (Syurin et al., 1998).

Biological properties of field isolates reovirus were studied on chicken embryos, chicken embryo fibroblasts (CEF), duck embryo fibroblasts and revaccined cell cultures Vero, LC, KC, BGM, BHK-21 in comparison with the reference strain 1733.

Study of virulent properties strain Br-06 were carried out in 2 experiments, before the experimental procedure the live weight of day-old chicks was, on average, 41.0–46.5 g. In the first experiment 3 day-old intact chickens were infected with field strain Br-06 and control pathogenic strain 1733 reovirus in soft tissue of paws in volume of 0.2 cm^3 per os — 0.5 cm^3. In the second experiment the first chicken group was infected with a field strain Br-06 in the soft tissue of paws in a volume of 0.2 $см^3$, the second — per os — 0.5 $см^3$, the third in soft tissue of paws and per os in the same volume. Control chicken group received saline in the appropriate volumes in both experiments.

Virus inactivation was carried out by using ethylenimine production of the Institute of Chemical Physic named after M. M. Semenova of the Russian Academy of Sciences (Moscow, RF) in three final concentrations of (0.05, 0.1 and 0.15%) at two temperature conditions (25±0.5 °C i 37±0.5 °C) in period 12, 24, 30, and 42 hours. Control of inactivation completeness was carried out by conducting four consecutive 'blind' passages on cell culture of chicken embryos fibroblasts (CEF).

The lack of contamination by bacterial and fungal microflora was determined according to DSTU 4483:2005 and DSTU 4517:2006

Antigenic activity and immunogenicity of vaccine was tested at the repair young growth hens at 90–120 days age, which were once immunized intramuscularly at dose 0.5 cm^3, on harmlessness 1.5 cm^3. Antigenic activity of research series was studied in comparison with inactivated against infectious Borsalino disease vaccine and viral bird arthritis 'Galimun 201' of strain 1133 ('Merial', France).

Test of inactivated emulsion vaccine. Commission interdepartmental check '*Inactivated emulsion vaccine* against reovirus infection of chickens (AVRI) of strain Br-06' was carried out at the Institute of Poultry of National Academy of Agrarian Sciences in quality factor, with this aim the repair young growth of hens at 90–120 days age were immunized, that was obtained on free-problem infectious diseases farms. Antigenic activity and immunogenicity was tested monthly using ELISA for 360 days. Industrial testing was carried out on two poultry farms in Kharkiv and one farm in Donetsk oblasts.

Results. The data about infection of reovirus diseases among poultry according to the reports of Kharkiv branch of the State Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Inspection and the State Veterinary and Phytosanitary Service of Ukraine to nowadays are not available. On this point during 2006–2014 epizootology monitoring of reovirus infection was carried out on 11 poultry farms

of different ownership forms in 7 oblasts of Ukraine (Kharkiv, Donetsk, Luhansk, Dnipropetrovsk, Ivano–Frankivsk, Cherkasy, Odesa), where the poultry were not vaccinated against reovirus infections. It was studied 820 samples of blood serum from clinically sick birds of different species. Among broiler chickens by serological studies was found 79.3 % positively reacting to reovirus infection with antibody titers from 1:8 to 1:128 (IHR) and 1:400–1:800 (ELISA). On poultry farms of Dnipropetrovsk and Luhansk oblasts in blood serum of investigated poultry positive titers of antibodies to reovirus is not detected.

Sick birds had clinical signs of disease that were characterized by feed refusal, retarded growth and development, edema and erosion of cartilage joint limbs, arthritis, lameness, diarrhoea. On post mortal were noted hemorrhages under skin and chest muscles, spleen involution, flabby liver with ocher-green colored insulas, kidney marbling and hemorrhagic enteritis.

Economic losses from the disease at the period of study among birds of different age groups, species, breeds and crosses ranged from 2 to 36 %. Poultry was imported from abroad to the farms, where the research was conducted.

Molecular genetic studies. By polymerase chain reaction (PCR), with inverse transcription in postmortem material from diseased broiler-chickens and ostriches was found the plot of RNA genome poultry reovirus and sequenced of segment S3 order with the length of 985 b.p., carried out its comparative analysis with sequences of the strains presented in GenBank. It is defined that the selected isolates belong to the group vaccine strain 1133 of Reoviridae family.

Virological tests. For 3 serial passages in chicken embryos from pathological specimens (synovial fluid) selected from clinically sick birds, secured 4 isolates (Br-06, Str-07, G-07 and K-14), 3 of which led to the death of 10 daily chicken and duck embryos with specific reovirus infection modifications: Br-06 and K-14 in 4 days, Str-07 — on the 9th day of the 2nd serial. Tissue swelling and torso hyperemia, injection of the blood vessels in the chorioallantoic membrane, edema and hemorrhage at occiput and increase liver blood circulation with parts of necrosis, the blood filling of the kidneys, brain deformation with hemorrhages were observed from dead embryos. Consequently, it is established that isolates isolated by PH identification of isolates, secured from broiler-chickens, ostriches, geese and ducks belong to reovirus.

Biological properties of field isolates Br-06, Str-07 and K-14. According to the results carried out on chicken embryos, (CEF), duck embryos fibroblasts and revaccinated cell cultures Vero, LC, KC, BGM, BHK-21 studies, the highest infectious titers were determined in Br-06 and 1733 strain on the chicken embryos fibroblasts and 7.55 and 6.5 lg TCD_{50}/cm^3, accordingly. Other isolates were characterized by lower infectious titers, therefore, they have not been used in further researches.

While determining of level accumulation strain Br-06 reovirus chickens in stationary cell cultures was secured probable difference between the infectious titers on different cultivation systems. The greatest activity was observed when it was cultured on the chicken embryos fibroblasts (CEF) (from 6.5±0.14 to 7.5±0.07 lg TCD_{50}/cm^3) ($p \leq 0.01$) compared to cell cultures NT and LEK (from 5.4±0.18 to 4.1±0.19 lg TCD_{50}/cm^3) ($p \leq 0{,}001$). It is established that the sowing concentration of 600–800 ths cells/cm^3 with the dose of infection of 0.1 lg TCD_{50}/cell provided optimal accumulation of viral biomass in the titers from 6.00±0.14 to 7.37±0.11 lg TCD_{50}/cm^3 for 3 serials.

Study of virulent properties of the strain Br-06 on chickens. It is determined that the incubation period of infection with Br-06 strain is 9 days and strain 1733 — 3 days, with specific reovirus infection modifications at necropsy. Causative agent was taken from the dead birds.

In 20 days after infection with the reaction of indirect hemagglutination specific to reovirus antibodies in diagnostic titers (3.0–3.6 $\log_2$) with their further increase to 40th day (3.9–4.5 $\log_2$) were secured. In chicken control group, that were treated with saline, specific to reovirus antibodies were absent throughout the whole study period.

During the growth period (from 7th to 42nd days) average chicken live body weight in experimental groups, ranges from 156.9±6.88 to 232.8±43.86 g, and the control group was within the normal range (2,400 g). At the end of the study, the difference in weight between broiler chickens of control and test groups probable ($p \leq 0{,}001$) was 2,117.2 g.

Thus, confirmed etiological role of field strain Br-06 reovirus for chicks and found that the incubation period of the disease depends on the strain that poultry has been infected.

Developing procedures for the inactivation of Br-06 and 1733 strain reovirus and determining the ratio of adjuvant to inactivated antigen. The research result was found that ethylenimine at final concentration of 0.1% within 24 h at temperature of 37.5 °C completely inactivates the infectivity of strains of Br-06 and 1733 reovirus, while this retaining antigenic features. Poultry injection by inactivated ethylenimine antigen determines the formation of specific to reovirus antibodies in the titers from 1:5909±812 to 1:8304±327 in ELISA.

It is determined that combination of inactivated antigen with adjuvant 'Montanide ISA 70 VG'

in ratio 30:70 allows to obtain the stable emulsion for the manufacture of inactivated experimental samples vaccines against AVRI as well as from Br-06 and as 1733 strains.

Making inactivated emulsion vaccine against reovirus infection using state Br-06 strain reovirus. It was made 6 lab series *inactivated emulsion vaccine* against AVRI in total number of 2,400 doses. After checking experimental vaccine samples of Br-06 strain (C-1) and the control strain 1733 (C-3) as to the inactivation completeness, emulsion stability, sterility it was immunized reparing young chickens at the age of 90 days. Induction of specific antibodies to reovirus and duration of humoral immunity in vaccinated by two samples of the vaccine birds were checked by ELISA monthly over 390 days. It is established that the prepared of Br-06 and 1733 strains samples of inactivated emulsion vaccine against of ARVI provided persistent protective antibody levels of to the causative agent of the disease in vaccinated chickens. Birds immunized by experimental vaccine of strain 1733 (C-3), saved protective antibody titers within 360 days at 1:1,200 level, but in 390 days they were down to 1:881. Whereas the chickens vaccinated by the experimental sample made of strain Br-06 (C-1), had protective antibody titers higher and remained them during 390 days of the productive period (observation period) at 1:1,207 level. The control was unvaccinated poultry with the antibody titer in ELISA not higher than 1:400 (Fig. 1).

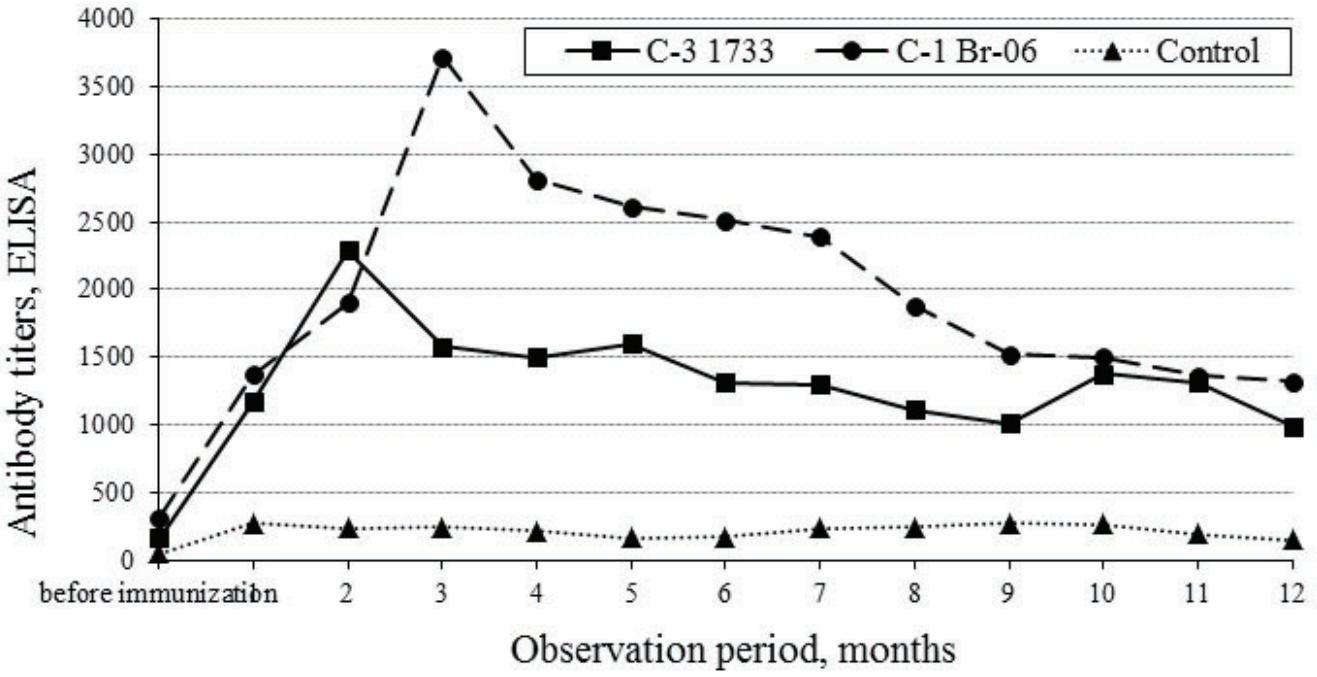

Figure 1. Dynamics of humoral immunity level of chicken after immunization by experimental samples of inactivated emulsion vaccine made of Br-06 (C-1) and 1733 (C-3) strains

The repair young growth hens at the age of 110 days were immunized by research samples of inactivated monovalent vaccine of strains Br-06 (C-2) and 1733 (C-1) reovirus to study immunity tension The dynamics of specific antibodies level to poultry vaccination was studied by ELISA, in 30 days after immunization and monthly during 330 days. Antibodies to reovirus were not found at experimental poultry before vaccination. Control infection of immunized chickens (experimental-infected and control-uninfected) was performed intramuscularly in a volume of 3.0 cm^3 by control pathogenic strain 1733 with infectious titer 6.6 lg TCD_{50}/cm^3. The birds were observed for 390 days. Vaccinated chickens before infection had antibodies titer to reovirus 1:3085, in 30 days after infection, it was reduced to 1:1,651, and in 60 days was increased to the previous level (1:3,221). However, the infected birds were decreased in egg production from 48.6 to 42.8%, that was rebounded to 56.6% in 30 days after infection.

Thus, use of the experimental sample of developed 'Emulsion vaccine inactivated against of chicken reovirus infection (AVRI) of strain Br-06' in 100%

of cases prevented the disease development after infection by the virulent control 1733 strain.

Comparative assessment of immunity tension in vaccinated by inactivated vaccines against AVRI of 1733 and Br-06 strains reovirus and 1133 of the company 'Merial (France) poultry. It is defined that protective antibody titers in ELISA for chickens vaccinated by three biological products were almost identical and remained their properties for 11 months of the productive period (observation period) at enough high protection level. But it should be noted that the average titer of antibodies in the case of the use of vaccines, made of Br-06 antigen, was higher (1:1,885±188) compared with these indicators according to vaccines of 1733 strains (1:1,324±164) and 1133 (1:1,435±222).

Therefore, developed by us sample of state inactivated emulsion-vaccine against reovirus infection made of state strain Br-06 did not surrender to inactivate vaccine of 'Merial' company (France) in immunologic. Antibody titers in chicken blood serum, vaccinated by state vaccine samples were higher in 1.2 times.

Detection of expire date of laboratory series vaccine against AVRI. Expire date of inactivated emulsion vaccine against *AVRI* was determined on three samples made of strains 1733 (C-2, C-3) and Br-06 (C-1), which had been stored respectively for 7 and 12 months at a temperature 4–8 °C. It is defined that the experimental vaccine samples after the deadline has remained the original antigenic activity to reovirus. In 30 days after vaccination, the antibody titers in ELISA were, in average, for C-1 — 1:2,873, C-2 — 1:1,880 and C-3 — 1:1,282. So, the obtained results indicate that the experimental vaccine samples of state vaccine were characterized by high immunogenicity. However, the sample made from antigen strain Br-06 (C-1), induced the formation of specific antibodies in 1.5–2.2 times at the highest titres.

Study of chick transovarial immunity formation made of vaccinated hens. With the aim of study transovarial immunity formation of hens parental flock, immunized with inactivated vaccine against AVRI of strain Br-06, it was received the chicks for 6 months while the productive period. In 30 days after hen vaccination the antibody titers in ELISA were 1:2,081±263, and in 180 days — 1:2,520±440. Antibody titers in decedents blood serum obtained of these poultry, were examined in the first day and daily for 21 days. On the basis of received results it was defined that the average protective titer of maternal antibodies in ELISA of young growth chickens at 14 days age was 1:424±7,22, that is confirmed by their control infection by pathogenic 1733 strain reovirus at 7 days age. Specific to reovirus antibodies were detected on the 21st day, however, their level did not reach the protective titers (1:21–1:170 ELISA). So, the young growth chickens of experimental groups, obtained from vaccinated progenitors, despite of low antibody titers, remained resistant to infection by pathogenic 1733 strain (clinical signs of reovirus infection were not observed). Infected in 1 day age chickens of the control groups were sensitive to the disease in 100% of cases.

Chickens, obtained of immnized against reovirus infection parental herd, were resistant to infection within 14 days despite of the low protect antibody titers ELISA, that were in the range of 1:400–1:450 (Fig. 2)

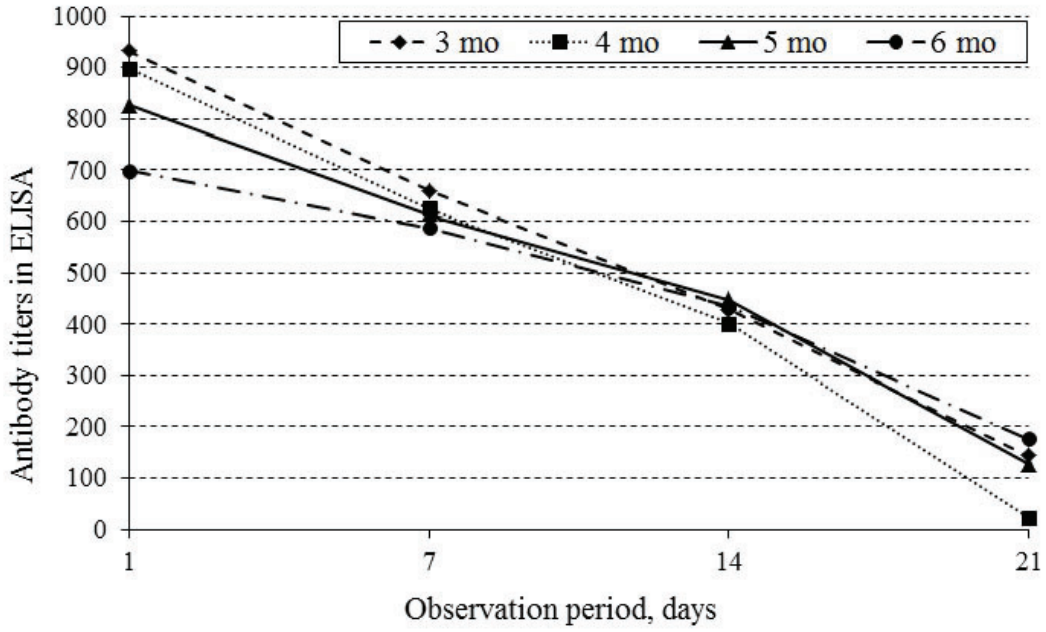

Figure 2. Transovarial dynamics of chicks immunity obtained from vaccinated against AVRI hens

Thus, the results indicate that the experimental sample of 'Inactivated emulsion vaccine against chicken reovirus infection (AVRI) of Br-06 strain' provided the tension immunity in 100% vaccinated birds for 6 months (observation period).

Commission used test 'Inactivated emulsion vaccine against chickens reovirus infection (AVRI) of strain Br-06'. Testing of series experimental vaccine was performed in the following parameters: appearance, color, presence of impurities, emulsion stability, bacterial and fungal contamination, viscosity, pH, immunogenicity, harmlessness.

It is defined, that experimental range inactivated emulsion vaccine, in 30 days after immunization of rearing chickens provided induction of antibodies to reovirus in protective titers that were persisted for 360 days (observation period). Harmlessness of the vaccine was tested within 30 days (observation period) on chickens, which were injected by triple dose of a biological product (1.5 cm^3). Cases of illness or death were not noted among the vaccinated chickens, as well as the remnants of emulsion and inflammation while autopsy at injection site. Conducted commission test of antigenic activity and immunogenicity showed that using of prototypes state 'Inactivated emulsion-vaccine against chicken reovirus infection (AVRI) of strain Br-06' provided 100 % immunity tension of vaccinated birds with antibody titers ELISA from 1:1,453±301.01 to 1:2,333±561.05 for 10–12 months of productive period (observation period).

Testing of native developed 'Inactivated emulsion vaccine against chicken reovirus infection (AVRI) of strain Br-06' in production conditions. Experimental samples inactivated emulsion vaccine against AVRI made of strain Br-06 of reovirus, passed the industrial test on two poultry farms in Kharkiv and one poultry farm in Donetsk oblasts at the repair young growth hens at the age of 90–120 days. The vaccine was injected intramuscularly once at 0.5 cm^3 dose. The tension immunity against AVRI in vaccinated chickens was controlled by ELISA method. before vaccination Experimental poultry did not have antibodies to reovirus before vaccination.

According to immune monitoring test among chickens on two poultry farms of Kharkiv oblast were found the antibody titers from 1:2,081±264 to 1:4,087±568 and from 1:4,916±452 to 1:7,539±599, respectively. On poultry farm in Donetsk oblast it is from 1:1,056±167 to 1:2,193±127. Results showed tension immunity to AVRI in vaccinated chickens was 100% for 10–12 months of the productive period (observation period).

Conclusions. Epizootological, serological and virological studies defined the pathogen circulation and spread of poultry reovirus infections with the fluctuation of sickness from 2 to 36% on 11 poultry farms of different ownership forms in 7 oblasts of Ukraine. Biological properties of four isolates, observed from broiler chickens, ostriches, geese and, firstly in Ukraine, from musk ducks were studied. Strains that are isolated from clinically ill poultry (Br-06, Str-07, G-07, K-14), were identified with PH and defined their relationship with the standard strain 1133 reovirus.

State 'Inactivated emulsion-vaccine against chicken reovirus infection (AVRI) of strain Br-06' is antigen active, immunogenic, harmless and provides 100% tension of immunity to causative disease agent. The vaccine could be used as import-substitute of biological product for the specific prevention of parental chicken flock, and does not surrender in immunogenic ratio to the activated vaccine of company 'Merial' (France).

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