AGRICULTURAL BIOLOGY,
2020, V. 55, № 2, pp. 328-342
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

*UDC 636.5:619:579.62:615.371*

doi: 10.15389/agrobiology.2020.2.328eng
doi: 10.15389/agrobiology.2020.2.328rus

# DEVELOPMENT OF A VACCINE AGAINST ENTEROCOCCOSIS FOR FARM BIRDS AND ASSESSMENT OF ITS SPECIFIC EFFECTIVENESS

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The authors declare no conflict of interests
*Received January 28, 2020*

Abstract

Enterococcosis in poultry is a disease which affects the organs of locomotor system and is accompanied by lameness, ataxia, spondylitis, necrosis of the femoral head and bacterial chondronecrosis. The main pathogen of this disease on the territory of the Russian Federation is bacteria of the species *Enterococcus cecorum* (EC). The disease can occur among young herds for replacement (mainly cockerels) at the age from 1 to 7 weeks; commodity broiler aged 3-5 weeks; and parent stock during peak production. In this work we represent for the first time the results of the development and tests of the domestic means of specific prevention from enterococcosis in poultry. The experimental series of vaccine, tested in industrial environment on poultry, was produced on the basis of the selection of production-control enterococcus species and the measurement of the optimal immunizing dose and adjuvant. The proposed medicine possesses areactogenicity and high specific effectiveness when used for poultry of different age groups. The aim of the work is the development of means of specific prevention from enterococcosis of poultry and evaluation of its effectiveness. The study of the epizootic situation on enterococcosis in poultry on the territory of the Russian Federation in 2017-2018 showed that 11 poultry enterprises in the Belgorod, Vladimir, Yaroslavl, Kaluga, Chelyabinsk, Tver and Penza regions, as well as in the Republics of Mari El and Udmurtia, were enterococcosis positive. All in all, 647 samples of parenchymal organs and tissues obtained from birds of Cobb 500 cross with typical clinical morphological manifestation of enterococcosis were examined. Strains *E. cecorum* Nos. 414, 425, 426, 837, 838, 839, 1096, 1481, 1517, 1647, 1865 were selected during the complex bacteriological diagnosis of breeding material. It was determined that 72.73 % of enterococci are resistant to ampicillin and penicillin, 45.45 % to vancomycin, 27.27 % to levofloxacin, linezolid, tetracycline, 18.18% to norfloxacin, rifampicin, chloramphenicol and ciprofloxacin, and 9.09% to doxycycline. The largest number of species are sensitive to gentamicin and levofloxacin (72.73 %); doxycycline, linezolid, rifampicin, chloramphenicol (54.55 %), respectively. All the studied strains led to the death of 100 % of laboratory mice within 24-96 hours after intraperitoneal infection. The $LD_{50}$ of enterococcal cultures was in the range of $1.7\times10^7$-$9.4\times10^8$ microbial cells. When determining the antigenic properties of EC species in the agglutination reaction, it was confirmed that they are all homologous to each other, i.e. belong to the same serotype. Evaluation of the level of antibodies in doubly immunized white mice with vaccines from strains No. 414 and No. 1517 showed that they have the highest antigenicity, inducing immunity in the titer of 1:26.66±9.23, while the antigenicity of other strains was 1:21.33±9.23 and less. Based on this result, strains No. 414 and No. 1517 were subsequently used for control and production. Evaluation of the immunogenic activity of the experimental medicine on white mice showed that the vaccine ensures the safety of 90 % of infected animals, while mortality among the mice of the control group was 100 %. To ensure high efficiency of the developed means, 1.5 billion microbe cells EC are needed, and the optimal amount of a single dose is 0.2 $cm^3$. Formalin (0.3%) was used as an inactivant and polyethylene glycol 6000 (PEG-6000) as an adjuvant at the rate of 10 % v/v. Phosphate-buffered saline (PBS) was used as diluent, the pH level was set to 7.2 with a 20 % sodium hydroxide solution. The vaccine provoked the formation of immunity 12-14 days after a double intramuscular injection, which lasts at least 4 months. Clinical trials on chickens of Cobb 500 cross proved the safety and the high specific effectiveness of the vaccine for poultry. Double vaccination of replacement herds in poultry led to a 4.6 % increase in uniformity and a 0.13 % decrease in total

waste. The analysis of production indicators of vaccinated laying hens showed a 1.81 % decrease in total mortality and a 1.7 % increase in egg productivity of. After the first vaccination of the parent livestock, the average antibody level in the bird was 1:5.60±2.00 ($n$ = 25), and 14 days after the second vaccination, the titer reaches 1:43.52±15.67, which exceeds the value of the protective level of antibodies (1:26.66±9.23). The results obtained allow us to talk about the possibility of further implementation of the medicine developed on the basis on *Enterococcus cecorum* strains in practical use.

Keywords: *Enterococcus cecorum*, EC, osteomyelitis, femoral head necrosis, enterococcosis, clinical signs, preventive measures, vaccination

*Enterococcus cecorum* (EC) is the prevailing pathogen of enterococcosis in birds in the Russian Federation. The disease that it causes is a damage to musculoskeletal system. The causative agent is widespread not only in Russia, but also in many European countries [1-3]. For a long time, EC was regarded as commensal, but such estimate of the pathogenic potential was incorrect. The signals about the role of EC in infections were first done by medical microbiologists who isolated EC from people with infectious pathologies of the respiratory organs, oral cavity, bile and urinary tract, and vagina [4, 5]. In addition, numerous cases of enteritis, peritonitis, septicemia, local and mass abscesses caused by EC were reported (6). Epidemiologists noted an increase in the incidence of infections of the circulatory system, including those in children, which were provoked by multiresistant pathogen isolates [7]. It has been proven that *E. cecorum* plays an important role in various infectious pathologies in humans [8, 9].

For a long time, veterinary specialists also referred EC to bacterial agents of no etiological significance. However, the role of EC in the infectious pathology of birds is currently established [10]. The causative agent is found in the natural gut microflora of chickens, mainly in cecum [11]. Besides chickens, EC is found in pathologies of the gastrointestinal tract in many birds and mammals, i.e. turkeys, ducks, pigs, calves, horses, cats, dogs [12-15].

The reasons for increased enterococcosis incidence in meat poultry farms are currently not completely clear, nevertheless, several predisposing factors can be distinguished. Firstly, these are a decrease in the immune status and natural resistance of birds as a result of primary infections, the intestinal dysbiosis caused by an undue diet or a shift in the diet, the use of antibiotics, and other internal changes in the body. Secondly, the environment, in particular factors influencing zoohygienic parameters, affect enterococcosis incidence [16, 17]. Some experts believe that an increase in the occurrence of enterococcosis in the world should be associated with a decrease in the use of antibiotics (e.g. the EU ban of January 2014 on the use of lincomycin and spectinomycin in the first days of bird life) which were applied to prevent EC-caused pathologies of limbs [18].

Operational factors provoking EC-caused pathogenesis is especially significant. These are the use of dirty eggs for incubation, improper sanitation and disinfection of the hatching eggs and poultry farm buildings, infection of chickens in an incubator, rearing chickens from a dirty and clean hatching egg in one premise, improper antibiotic dosages for replacement stock and when broilers are placed, poor laboratory control of cocci pathogens during poultry house and equipment sanitation.

Under favorable conditions and the influence of predisposing factors, EC can move from the digestive system to various organs and tissues of the susceptible organism, leading to sepsis, osteoarthritis, and osteomyelitis [19, 20]. The disease occurs in young birds of replacement stocks (mainly males) at the age of 1-7 weeks, in commercial broilers aged 3-5 weeks and in parent stocks during the peak of productivity. It is accompanied by lameness and ataxia [21]. Virulent EC isolates upon colonization of thoracic spine can cause spondylitis resulting in lameness and paresis of the extremities. If this is followed by necrosis of the femoral

head and bacterial chondronecrosis, mortality reaches 5-15% [22].

Pathomorphological examination of EC-infected birds reveals degenerative changes in the mobile segment of the thoracic spine [3]. Mechanical action on the abdominal part of the thoracic vertebrae leads to microtrauma with subsequent inflammation and hemorrhage. The accumulating exudate hardens, deforming and compressing the spinal cord [23].

Antibiotic therapy under enterococcosis in poultry is ineffective because of microbial antibiotic resistance, specific bioavailability of antibacterial drugs and high tropism of the pathogen. Besides, antibiotics cannot always be used in industrial poultry farming since the producer must avoid antibiotic contamination in poultry products [23].

In the present work, the investigation of pathogenic, antigenic and immunogenic properties of *Enterococcus cecorum* isolates allows us to develop the first domestic vaccine against enterococcosis. Farm testing in an industrial poultry enterprise has proved its harmless to poultry of various ages and protective effectiveness.

The purpose of the work is the development and evaluation of the effectiveness of specific vaccine against poultry enterococcosis.

*Materials and methods.* Poultry farms of several Russian regions (Belgorod, Vladimir, Yaroslavl, Kaluga, Chelyabinsk, Tver, Penza regions, Mari El Republic, Udmurtia Republic) were surveyed in 2017-2018 for the poultry enterococcosis epizootic situation. A total of 647 samples from 11 poultry enterprises were examined (92 lungs, 102 hearts, 43 spleens, 159 livers, 57 intestines, 118 affected femoral joints, 76 fragments of affected spines). Samples of parenchymal organs and tissues were collected from birds of the Cobb 500 cross of various age and physiological groups (broilers, replacement and parent stocks, hens and chickens) with clinical and morphological signs typical for enterococcosis.

Examination of organs and tissues of birds and laboratory animal was performed in accordance with generally accepted recommendations (24). Autopsy of corpses, died and euthanized animals was carried out with complete evisceration.

Lab diagnostics and isolation of *E. cecorum* strains were performed by routine bacteriological methods. For the initial isolation, modified esculin agar, broth with bromocresol purple, tryptone soy broth (HiMedia Laboratories Pvt Ltd, India) and Colombian agar (Oxoid Ltd, Great Britain) were used. Defibrinated blood added to culture media was obtained from a donor ram according to GOST 31746-2012 [25].

Species of bacteria were identified using MALDI-ToF time-of-flight mass spectrometry method (a Maldi Biotyper equipment, Bruker Daltonics, Inc., USA) according to MR 4.2.0089-14 [26]. The sensitivity of microorganism cultures to various antibiotics was determined as per MUK 4.2.1890-04 [27].

Preclinical trials were carried out at the experimental station of Vyshnevolotsky branch of Federal Research Center Kovalenko All-Russian Research Institute of Experimental Veterinary Medicine RAS (Lysiy Island, Tver Province).

The pathogenicity of the isolates was determined on 16-18 g outbred white mice (without gender separation) ($n$ = 3 per strain), which were injected intraperitoneally with a 1-day culture (0.5 cm$^3$ containing 1.5 billion microbial cells, mc.). Virulence was assessed by intraperitoneal inoculation of white mice ($n$ = 5 per group) using 10-fold dilutions of the bacterial suspension of each strain ($1.5\times10^9$, $1.5\times10^8$, $1.5\times10^7$, and $1.5\times10^6$ microbial cells in 0.5 cm$^3$). The period of observation of white mice was 10 days or until death. To confirm the causes of death, organs were removed for bacteriological investigation (Koch triad). The culture was recognized as pathogenic in the case of death of all individuals in the infected

group, followed by isolation of the infecting culture.

As per steps of the investigation (identification and serotyping of EC field isolates with an assessment of the antigenicity and immunogenicity; identification of an immunizing dose of a vaccine and adjuvant, preclinical trials; clinical trials under farm conditions), several series and variants of the vaccine were developed with different concentration of bacterial cells and adjuvant. EC strains were run in deep culture (a BIOSTAT-A fermenter, Sartorius AG, Germany) on tryptone-soy broth for 16-18 hours at 37 °C and a pH of 7.2-7.8. During cell growth, a 20% alkali solution and 40% glucose were added to maintain pH value and glucose concentration. Bacterial antigens were inactivated by formalin (0.3% of the volume of the broth culture) for 3 days at 21±1 °C. The bacteria were concentrated by centrifugation (an MPW-380R, MPW Med. Instruments, Poland) for 1 hours at 3,000 rpm and relative centrifugal field (RCF) of 1861. The following adjuvants were used: 15% aluminum hydroxide (GOA, FKP Armavir Biofactory, Russia), 10% polyethylene glycol (PEG-6000, LLC Norkem, Russia), 10% Acrypol®971P (Corel Pharma Chem, India); sodium merthiolate (1:10000 v/v) was a preservative; the hydrogen ions concentration was regulated with alkali solution to a pH value of 7.2-7.6.

EC inactivation was tested by the absence of viable cells in the concentrates used as antigens and in the formulated preparations, as well as by harmlessness of the inactivated bacteria in the bioassay on white mice ($n$ = 5 for each antigen) when administered subcutaneously at a dose 2 times higher than recommended. Sterility of the vaccines was controlled in accordance with GOST 28085-2013 [28].

EC strain serotyping, as well as the assessment of antibodies to the pathogen in vaccinated animals and birds, was carried out in tube agglutination test (agglutination reaction, AR). Monovaccines (3 billion cells per $cm^3$ with GOA as adjuvant) were made from each culture of enterococcus and used for immunization of Soviet chinchilla rabbits with 2.5-3 kg body weight ($n$ = 3 per each variant of the vaccine). The drugs were administered 2 times intramuscularly at a dose of 0.5 $cm^3$ with an interval of 14 days. Blood sera for AR test were sampled before each vaccination and 14 days after the re-vaccination.

In serotyping, each strain was examined in AR test with each serum obtained from previously vaccinated rabbits. Controls were the test culture mixed with a drop of physiological saline (pH 7.2) and with a drop of 1:10 diluted normal rabbit serum to exclude self-agglutination. RA test was positive when microbial cells formed grains or flakes of various sizes, with complete or partial transparency of the liquid and no agglutination in the control.

Immunogenicity of the vaccines was evaluated in two groups of 16-18 g white mice ($n$ = 10 per in each). Ten animals were placed in a polycarbonate container with steel wire bar lids and free access to food and water ad libitum. In the vivarium, standard microclimate conditions were maintained (30-70% relative air humidity, 22-24 °C, illumination of 110 lux at a 1 m distance from the floor). The animals were fed with granulated extruded rodent feed as per GOST R 50258-92 [29]. Drinking water corresponded to SanPiN 2.1.4.1074-01 [30]. Sterile sawdust was used as litter. Before the experiment, the mice were kept in a 14-day quarantine in accordance with SP 2.2.1.3218-14 [31].

Test group was vaccinated 2 times (with a 14-das interval) with 0.5 $cm^3$ of a monovaccine from EC strain No. 414, for which antibody production in the maximum titer was confirmed in AR with rabbit sera. The control group of mice was not vaccinated. Fourteen days after re-vaccination, mice of both groups were infected with pathogenic EC strain No. 1517 at 5 $LD_{50}$ in 0.5 $cm^3$. The period of animal observation was 14 days. The vaccine was recognized as immunogenic if the safety of the animals of the experimental group after infection was at least 80%

with a 80-100% mortality in the control.

The immunizing dose and adjuvant were selected in experiments on Cobb 500 of 10-day-old chickens assigned into groups (5 birds per each) and kept in cages (a cage per group). The microclimate conditions in the vivarium were as follows: 30-70% relative humidity, 27-28 °C, illumination of 70 lux at a 1 m distance from the floor). PK-5 compound feed (Russia) was used according to GOST 18221-2018 [32], drinking water corresponded to SanPiN 2.1.4.1074-01 [30]. Food and water were given ad libitum. Nine groups were tested. Birds were vaccinated with monovacines containing different amounts of antigen ($1.0\times10^9$, $1.5\times10^9$ and $2.0\times10^9$ cells) adsorbed on three adjuvants — GOA, PEG-6000, and Acrypol®971P as follows: 1 — $1.0\times10^9$ cells. + GOA; 2 — $1.5\times10^9$ cells + GOA; 3 — $2.0\times10^9$ cells + GOA; 4 — $1.0\times10^9$ cells + PEG-6000; 5 — $1.5\times10^9$ + PEG-6000; 6 — $2.0\times10^9$ cells + PEG-6000; 7 — $1.0\times10^9$ cells + Acrypol®971P; 8 — $1.5\times10^9$ cells + Acry-pol®971P; 9 — $2.0\times10^9$ cells + Ac-rypol®971P. For ease of use, the single dose volume for poultry was 0.2 $cm^3$. The estimates were based the severity of local and systemic adverse reactions in birds, as well as the antibodies titers.

Clinical trials of the drug were carried out at the poultry enterprise LLC Rovensky broiler (Belgorod Province, Rovensky District) in 2018. Pedigree Cobb 500 chickens were vaccinated at the age of 12 and 26 days (7019 test birds, 7020 control birds), breeding replacement stocks at the age of 121 and 135 days (9030 test birds, 8821 control birds). Birds were assigned into test and control groups as analogues. The drug was injected into pectoral muscle. Chickens and young birds were kept on the sawdust floor. Chickens were raised at 26.7 ° C and 60% humidity, and young stocks were reared at 20 °C and 50-60% humidity. Illumination for chickens was 80-100 lux in the brooding area and 10-20 lux in the poultry house, for replacement young stock 10-20 lux. An 8-hour daylight was applied to all groups and ages. Birds were fed with granulated feed PK-3 with feeding spaces of 5 cm per chicken and 15 cm per young bird. Watering was ad libitum with nipple drinkers. the watering space of 8-12 individuals per nipple. The stocking density for cockerels was 3-4 per $m^2$, for young hens 4-7 per $m^2$. The trials met the requirements of the Federal Law on the Circulation of Medicines No. FZ-61 and included drug assessments after the first and second administration in the recommended dose. The criteria were as follows: absence of side effects, reduction in mortality and forced culling, percentage of total losses, uniformity and egg productivity of the birds. Birdwatching upon vaccination lasted 28 days (14 days after the first vaccination and 14 days after the second vaccination) with clinical observation (examination of the injection site, thermometry) and postmortem autopsy at the end of the observation period in order to fix possible changes in the site of the drug injection. The effectiveness of the drug was evaluated by production indicators after the 140-day old young birds were transferred to parent stock. In order to ensure safety and minimize culling of the control chickens, antibacterial drugs based on amoxiclav and tetracycline were used; in the test group of birds, antibacterial drugs against infections of the musculoskeletal system were not used during the entire rearing. The effectiveness of vaccination of birds from the replacement stock was evaluated at the time of reaching the peak egg production on day 239 of life.

Under farm conditions, antigenic activity of the vaccine (serum titers of antibodies to EC in RA) was assayed in 25 birds from the replacement stock before the first vaccination, before the second vaccination, and 14 days after the second vaccination.

Statistical processing was carried out BioStat 2009 software (AnalystSoft, Inc., USA) and Microsoft Excel. The virulence of bacterial cultures ($LD_{50}$) was determined by probit analysis, arithmetic mean (*M*) and standard error of the

mean (±SEM) were calculated. The Student's *t*-test at $p = 0.05$ was used to assess the statistical significance of the differences.

*Results*. A survey of the epizootic situation for enterococcosis of birds showed spread of the infection in Belgorod, Vladimir, Yaroslavl, Kaluga, Chelyabinsk, Tver and Penza regions, as well as in the Republics of Mari El and Udmurtia. *Enterococcus cecorum* were detected with different incidence in all parenchymal organs and tissues from poultry contained in 11 poultry enterprises in these regions. In individuals with clinical and morphological signs of infection, frequency of EC isolation from lungs was 4.34%, from heart 24.51%, from spleen 27.9%, from liver 44.65%, from intestines 15, 79%, from joints 81.36%, and from spine 76.31%.

Analysis of the obtained epizootic data suggests a widespread occurrence of enterococcosis in poultry in several regions of the Russian Federation, that is, the infection can be recognized as epizootic. This is probably due to the fact that the progenitor and parent stock of birds in Russia are supplied by one group of enterprises, and the main way of spreading the disease is vertical. It is worth noting that EC bacteria were isolated from chickens and adults of different crosses, but the typical clinical and morphological sings of infection was manifested exclusively in the Cobb 500 cross.

Lab diagnostic revealed 11 EC strains: Nos. 414, 425, 426 (Belgorod region), No. 837 (Vladimir region), No. 838 (Yaroslavl region), No. 839 (Kaluga region), No. 1096 (Chelyabinsk region), No. 1481 (Republic of Mari-El), No. 1517 (Tver region), No. 1647 (Republic of Udmurtia), No. 1865 (Penza region). All enterococcal isolates had typical morphological, tinctorial, cultural and biochemical properties.

**1. Sensitivity of *Enterococcus cecorum* field isolates from nine regions of the Russian Federation to various antimicrobial agents**

| Antobiotics, concentration | Standard, mm | | | Growth inhibition zones in strains, mm | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S | I | R | 414 | 425 | 426 | 837 | 838 | 839 | 1096 | 1481 | 1517 | 1647 | 1865 |
| Ampicillin, 10 μg | ≥17 | – | ≤16 | 13 | 17 | 14 | 11 | 19 | 15 | 14 | 9 | 18 | 16 | 13 |
| Vancomycin, 30 μg | ≥17 | 15-16 | ≤14 | 16 | 14 | 17 | 22 | 13 | 11 | 14 | 19 | 15 | 12 | 24 |
| Gentamicin, 120 μg | ≥10 | 7-9 | ≤6 | 12 | 15 | 8 | 14 | 16 | 9 | 18 | 11 | 8 | 15 | 12 |
| Doxycycline, 30 μg | ≥16 | 13-15 | ≤12 | 17 | 14 | 20 | 18 | 14 | 11 | 18 | 15 | 13 | 19 | 22 |
| Levofloxacin, 5 μg | ≥17 | 14-16 | ≤3 | 19 | 22 | 17 | 18 | 13 | 18 | 11 | 24 | 19 | 13 | 18 |
| Linezolid, 30 μg | ≥23 | 21-22 | ≤20 | 26 | 28 | 23 | 26 | 18 | 22 | 17 | 27 | 19 | 26 | 22 |
| Norfloxacin, 10 μg | ≥17 | 13-16 | ≤12 | 16 | 9 | 17 | 14 | 15 | 18 | 13 | 21 | 16 | 10 | 19 |
| Penicillin, 10 units. | ≥15 | – | ≤14 | 13 | 8 | 11 | 15 | 17 | 14 | 9 | 18 | 13 | 8 | 10 |
| Rifampicin, 5 μg | ≥20 | 17-19 | ≤16 | 15 | 18 | 22 | 17 | 20 | 13 | 24 | 23 | 19 | 24 | 28 |
| Tetracycline, 30 μg | ≥19 | 15-18 | ≤14 | 13 | 24 | 25 | 17 | 20 | 14 | 15 | 28 | 13 | 19 | 17 |
| Phosphomycin, 200 rg | ≥16 | 13-15 | ≤12 | 19 | 22 | 18 | 14 | 20 | 16 | 18 | 18 | 24 | 20 | 19 |
| Chloramphenicol, 30 μg | ≥18 | 13-17 | ≤12 | 14 | 21 | 19 | 18 | 15 | 8 | 15 | 21 | 11 | 18 | 20 |
| Ciprofloxacin, 5 μg | ≥21 | 16-20 | ≤15 | 18 | 27 | 14 | 19 | 25 | 23 | 18 | 20 | 15 | 19 | 17 |
| Erythromycin, 15 μg | ≥23 | 14-22 | ≤ 3 | 17 | 14 | 20 | 26 | 18 | 14 | 22 | 29 | 25 | 21 | 14 |

N o t e. S — sensitive group, I — intermediate group, R — resistant group, «–» — not indicated.

In commercial poultry, the only way to combat EC infection still remains antibiotic therapy. To predict the effectiveness of antibiotics in treating birds with clinical and morphological manifestations of this infection, we determined the sensitivity of epizootic EC isolates to various antimicrobial agents (Table 1).

All EC strains had different antibacterial profiles. Most isolates (72.73%) were resistant to ampicillin and penicillin, 45.45% isolates were resistant to vancomycin, 27.27% to levofloxacin, linezolid, tetracycline, 18.18% to norfloxacin, rifampicin, chloramphenicol and ciprofloxin, and 9.09% to doxycycline. Gentamicin and levophloxacin showed efficacy against 72.73% of enterococci isolates, doxycycline, linezolid, rifampicin, chloramphenicol against 54.55%, tetracycline against 45.45%, vancomycin and norfloxacin against 36.36%, ampicillin, penicillin, ciprofloxacin, erythromycin against 27.27%. The proportion of EC strains sensitive to

phosphomycin was 90.91%.

Despite the high or moderate sensitivity of EC cultures to some antibiotics in the lab tests, therapeutic efficacy in poultry farms could be significantly lower. This is primarily due to the high tropism of the pathogen, which is able to penetrate the joints and spinal canal, as well as to the bioavailability of the antibiotics themselves, which do not always reach the site of infection.

The pathogenicity assessment of enterococci showed that all the studied strains caused the death of 100% of laboratory mice within 24-96 hours after intraperitoneal infection. EC cultures were isolated from the liver and spleen, as well as from blood collected directly from the hearts of dead animals. The virulence ($LD_{50}$) of the studied EC strains was as follows: No. 414 — $8.1\times10^7$, No. 425 — $1.1\times10^8$, No. 426 — 7.8×107, No. 837 — 2.9×107, No. 838 — $2.6\times10^8$, No. 839 — $1.8\times10^8$, No. 1096 — $8.0\times10^7$, No. 1481 — $2.1\times10^8$, No. 1517 — $1.7\times10^7$, No. 1647 — $2.3\times10^8$, No. 1865 — $9.4\times10^8$ microbial cells. The pathogenicity of the EC isolates for laboratory animals explains the mass morbidity in poultry at the enterprises where the samples were collected. In lab diagnostics, it should be borne in mind that commensal EC isolates (from pigs, calves, geese, ducks, turkeys, chickens) that are not involved in the development of any diseases are also widespread in animal husbandry [33]. Therefore, a bioassay is important in confirming the final diagnosis.

As per serological characteristics of the pathogen in AR, EC cultures showed high antigenic activity (from "++" to "++++") with all sera, therefore, all strains belonged to the same serotype. It was not possible to trace circulation of other EC serotypes in the Russian Federation in this work, despite the fact that at least two serotypes of *E. cecorum* are known [33]. Isolation of the second EC serotype is possible by expanding the area of epizootic monitoring

These data allowed us to use one strain of *E. cecorum* in the design of the target product, as, due to identical antigenic properties of pathogen isolates circulating throughout the country, such vaccine will induce pronounced cross-immunity.

Assay of antigenic activity of the enterococcal isolates in rabbits revealed that after 2-fold vaccination, the antibody titer ranged within 1:8-1:32, with agglutination estimates "+++" and more. The highest average antibody titers was in rabbits vaccinated with strains No. 414 and No. 1517 (1: 26.66±9.23); vaccination with strains No. 426, No. 1096 and No. 1865 generated an average antibody titer of 1:21.33±9.23, with strains No. 425, No. 838, No. 839 and No. 1647 1:13.33±4.61, and with strains No. 837 and No. 1481 1:10.66±4.61

It was not possible to reveal a statistically significant difference between antibody titers in rabbits after vaccination with various drug variants. This can be explained by the fact that all used EC strains had similar antigenicity. It is also impossible to exclude the option that with an increase in the sample of animals or when conducting an identical study on a naturally susceptible birds, statistical reliability will be significant.

Since the highest titer of antibodies appeared upon vaccination with preparations based on EC strains No. 414 and No. 1517, further experiments were carried out with these cultures. Strain No. 414 was used as a production strain, since it had more stable growth properties, and strain No. 1517 was used as a control in tests for immunogenic activity of the developed agent. The bacterial concentration the EC No. 414 strain achieved in culture was 2-3 billion cells per $cm^3$ more compared to other strains under identical conditions. In addition, this strain (in contrast to EC No. 837, No. 1096 and No. 1865) did not spontaneously form a dense precipitate in culture. The experiment on white mice showed 90% survival rate in the experimental group after infection (one mouse died 6 days after challenge), while 100% control mice died for 96 hours These results allowed

*Enterococcus cecorum* strains No. 414 and No. 1517 to be used as control and producer cultures for production of specific prophylaxis mean against poultry enterococcosis, and also indicated that the titer of antibodies 1:26 was able to protect laboratory animals from infection and death.

Different immunizing doses and adjuvants did not cause any systemic and local side effects. Hence the final choice was due to the titer of the resulting antibodies (Table 2).

**2. Antibody titer in Cobb 500 chickens vaccinated with *Enterococcus cecorum* No. 414 as depended on bacterial cell concentration and adjuvants** ($M$±SEM, vivarium)

| Microbial cells in 0.2 cm$^3$ | Adjuvant | | |
|---|---|---|---|
| | aluminum hydroxide | PEG-6000 | Acrypol®971P |
| 1.0×10$^9$ | 1:25.60±8.76 (group 1) | 1:38.40±14.31 (group 4) | 1:25.60±8.76 (group 7) |
| 1.5×10$^9$ | 1:28.80±7.15 (group 2) | 1:44.80±17.52 (group 5) | 1:25.60±8.76 (group 8) |
| 2.0×10$^9$ | 1:35.20±17.52 (group 3) | 1:44.80±17.52 (group 6) | 1:28.80±7.15 (group 9) |

The maximum antibody titer was in birds vaccinated 2 times using PEG-6000 as an adjuvant (1: 44.80±17.52). It is important to note that it was not possible to fix statistically significant differences in the antibody titers between test groups according to the Student's $t$-test. This confirms the same ability of the studied strains of pathogenic enterococci to induce antibodies. Despite the absence of a statistically significant difference, the concentration of protective antigen 1.5×10$^9$ cell per 0.2 cm$^3$ should be deemed optimal, since a further increase in concentration did not lead to an increase in antibody level. The choice of PEG-6000 as an adjuvant for further testing was also due to its technological simplicity in comparison with GOA and Acrypol®971P.

Clinical study after the first and second vaccinations of chickens testified to the harmlessness of the drug. The chickens in the test and control groups did not differ in mobility, water and feed intake. Neither systemic no local reactions were observed during drug administration.

At 140-day age, the number of died birds in the test group was 1.31% less than in the control group (Table 3). There was a 1.00% increase in culling in the test group compared to the control group, so the resultant total loses in the test group was 0.13% lower than in the control. Additionally, a 4.6% increase in uniformity was noted the test group.

**3. Performance of 140-day old Cobb 500 chickens vaccinated 2-fold (on days 12 and 26 of age) with *Enterococcus cecorum* strain No. 414-based experimental vaccine** (clinical trials, Rovensky Broiler LLC, Belgorod Province, Rovensky District, 2018)

| Group | $n$ | Died, $n$/% | Culled, $n$/% | Total losses, $n$/% | Uniformity, % |
|---|---|---|---|---|---|
| Test | 7019 | 199/2.84 | 144/2.05 | 343/4.89 | 93.7 |
| Control | 7020 | 291/4.15 | 74/1.05 | 365/5.02 | 89.1 |
| Deviation from control | +1 | –92/–1.31 | +70/+1.0 | –22/–0.13 | +4.6 |

**4. Performance of 239-day old Cobb 500 chickens from the replacement stock vaccinated 2-fold (on days 121 and 135 of age) with *Enterococcus cecorum* strain No. 414-based experimental vaccine** (clinical trials, Rovensky Broiler LLC, Belgorod Province, Rovensky District, 2018)

| Group | $n$ | Died, $n$/% | Culled, $n$/% | Total losses, $n$/% | Egg productivity, % |
|---|---|---|---|---|---|
| Test | 9030 | 182/2.01 | 281/3.11 | 463/5.12 | 82.9 |
| Control | 8821 | 239/2.70 | 373/4.22 | 612/6.93 | 81.2 |
| Deviation from control | –209 | –57/–0.69 | –92/–1.11 | –149/–1.81 | +1.7 |

N o t e. Egg productivity: gross egg production × 100/poultry population.

Testing on chickens of the replacement stock repairing young animals with 2-time administration of the vaccine showed its harmlessness. There were no systemic and local reactions, feed and water consumption, poultry mobility did not

differ in both groups. On average, the death rate in the test group was 0.69% lower than in the control group (Table 4). The number of culled birds in the test group was 1.11% less than in the control, and the total losses was 1.81% lower. The average productivity of vaccinated birds was 1.7% higher than in the control group.

Estimates of the antigenic activity indicate that the first vaccination of the replacement stock generates an average antibody titer 1:5.60±2.00 ($n$ = 25), and 14 days after the second vaccination, it increased to 1:43.52±15.67 (p = 0.05) exceeding the protective level of antibodies.

Infectious disease of commercial poultry caused by *Enterococcus cecorum* is widespread throughout the world and provokes massive pathologies of the musculoskeletal system [2, 3, 11]. In turn, this leads to an increased death rate, culling and resultant decrease in production indicators [34]. The causative agent of the disease possesses a high tropism to joints and spinal column, from where it is isolated using routine bacteriological methods [10, 35, 36]. Experts also emphasize the possibility of isolating EC not only from the affected joints and spine, but also from the intestines of 7-10-day old chickens [11, 37]. Besides EC localizations indicated earlier, in this work, we have established the possibility to detect the microorganism in the heart, spleen, liver and lungs. Thence, the microbiological investigation helps not only to assess the severity of the infectious process, but also to determine the risks of musculoskeletal pathologies prior to their actual manifestation. The pathogen excretion from the lungs of birds have not been previously described, and alimentary and contact ways were considered the main in EC transmission [37]. However, our data suggest the possibility of aerogenic transmission of the infection. High tropism of the pathogen should be associated with its virulent properties, which we have confirmed in lab tests [35]. The intraperitoneal introduction of the EC culture to outbred white mice provoked their death within 24-96 hours. These findings confirm the possibility of using mice model to finalize diagnosis. The model can be an alternative to that of chicken embryos as described by A. Jung et al. [38]. These researchers propose to determine *Enterococcus cecorum* culture pathogenicity on 11-day-old chicken embryos. For this, the test strain ($10^2$ cells) is introduced into the allantoic cavity. In case of pathogenicity the death of embryos should occur within 5-7 days. However, the use of white mice to estimate pathogenic and virulent properties of EC, in our opinion, has its advantages. First, the manipulation is simple, and death in mice occurs faster than in chicken embryos. Secondly, the use of mice will allow more accurate estimates of $LD_{50}$ of strains, which, in turn, is necessary to control immunogenicity of the tested products. In addition, the control of immunogenic activity of the drug can also be carried out on mice, and not on chicken embryos.

Serotyping of the obtained strains of enterococci confirmed their homology, which, in our opinion, can be associated with a single source of origin of the progenitor and parent livestock of the bird. This hypothesis can be confirmed or disproved by genotyping cultures.

Due to the wide spread of enterococcosis in various regions of the country, the pathogenicity and high tropism of its pathogen [35], as well as the development and spread of antibiotic resistance [39, 40], developing a vaccine is the most promising way to combate the disease. L.B. Borst et al. [33] also proved the possibility of effective control of enterococcosis through the use of inactivated vaccines in broiler stock. At the same time, they note that the antigenic composition of EC can comprise several heterologous serotypes that do not show cross antigenic activity, which is why the effectiveness of a vaccine preparation from one serotype turned out to be low [33]. Other studies established the existence of at least seven groups of EC genotypes forming two serological groups [38, 41], for the control of which inactivated polyvalent vaccines from two *Enterococcus cecorum* strains

have been proposed [33]. This combination showed high protective efficacy against all 7 genotypes. This agent and the vaccine we have developed and tested differ in the strain and adjuvant used. Foreign researchers used an oil adjuvant, while we used PEG-6000, but despite this, the results can be considered comparable.

Thus, the musculoskeletal infection caused by *Enterococcus cecorum* (EC) is widespread in poultry farms in various regions of the Russian Federation, which indicates an epizootic of enterococcosis. Bacteriological studies of biomaterial from birds with typical clinical and morphological manifestations of infection indicate a high tropism of the pathogen with penetration into various parenchymatous organs and tissues. Over the survey the number EC isolates from the affected joints and fragments of the spine were maximum (81.36 and 76.31%, respectively). Most pathogen isolates (72.73%) are resistance to ampicillin and penicillin, while the highest antibacterial effectiveness is characteristic of phosphomycin (90.91%), gentamicin (72.73%) and levofloxacin (72.73%). The antigenic homology of all studied enterococci cultures (i.e. their belonging to one serotype) and high pathogenicity for laboratory animals upon intraperitoneal administration ($LD_{50}$ of $1.7\times10^7$-$9.4\times10^8$ cells) have been were established. Double-immunization of rabbits with monovaccines based on different enterococcus isolated provides antibody titer of 1:8-1:32. Moreover, according to testing the immunogenic activity of the developed product on white mice, the protective titer of antibodies against the EC was on average 1:26. The immunizing dose of $1.5\times10^9$ bacterial cells in combination with PEG-6000 ensure the maximum antibody titer in a poultry. Clinical trials on Cobb 500 chickens and young broilers from replacement stock confirmed the harmlessness and high efficiency of the suggested bioproduct. Application of our experimental vaccine in farm trials decreased losses of chickens and young stock by 0.13 and 1.81%, respectively, improved uniformity by 4.6% and increased egg productivity by 1.7%. The results of clinical trials indicate a high protective efficacy of the drug against enterococcal infection in poultry.

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