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Address: build. 16/1, office 36, pr. Polesskii, Moscow, 125367 Russia
Tel: + 7 (916) 027-09-12
E-mail: felami@mail.ru, elein-k@yandex.ru **Internet:** http://www.agrobiology.ru

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POULTRY BEHAVIOR REACTIONS AND WELFARE
(review)

L.I. SULIMOVA, K.V. ZHUCHAEV, M.L. KOCHNEVA

Novosibirsk State Agrarian University, 160, ul. Dobrolubova, Novosibirsk, 630039 Russia, e-mail zhuchaev-kv@mail.ru (✉ corresponding author), Sulimova88@yandex.ru, mlkochneva@rambler.ru
ORCID:
Sulimova L.I. orcid.org/0000-0002-6612-3770
Kochneva M.L. orcid.org/0000-0002-5214-2401
Zhuchaev K.V. orcid.org/0000-0002-5748-1525
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Abstract

In connection with the need to improve competitiveness with foreign poultry farming, the problem of ensuring the well-being of poultry and producing high-quality products is an urgent issue (Welfare Quality® Assessment for poultry, 2009; I.J.H. Duncan, 1981; J.A. Mench, 1992). Special attention on the way to improving the welfare of animals is given to the compliance of technology with the biological characteristics of animals (D.A. Orlov et al., 2016). The level of well-being of poultry is affected by many factors: illness, stress, nutrition, conditions of housing (D.C. Jr Lay et al., 2011). A bird within certain limits is able to adapt to various environmental conditions (M. Brantsæter et al., 2018). Inability to adapt is expressed in changes in physiological status, as well as behavioral disorders that can harm both animals and maintenance personnel. Stress sensitivity is manifested in the behavior of chickens, which serves as the best indicator of well-being. Strong manifestations of fear, such as panic or abrupt escape attempts, not only increase energy costs, but can also cause damage or even death when the birds start moving through obstacles and hurt each other (S. Waiblinger et al., 2006). Fear, like an unwanted emotional state, reduces the overall activity of animals. Regular negative stimuli inhibit social interactions between animals (J.A. Mench, 2004; B. Forkman et al., 2007). Human is one of the main sources of stress for animals, the fear of human affects their well-being and productivity (T. Kutzer et al., 2015; M.A. Sutherland et al., 2012; F. Barone et al., 2018). Relationships between humans and animals can include visual, tactile, olfactory, and group perception (S. Waiblinger et al., 2006). A hen is sensitive to visual contact with a person, but some neutral interaction, such as moving a person's hands to the side of a cage or approaching a bird, even for short periods, can decrease stress (J.A. Mench, 2004). Measuring the response of animals to humans leads to conclusions about how they perceive all people or a particular person. It depends on the type of animal and the housing system, on the nature of its interactions with a person (positive, neutral or negative), on the quality of care for animals and poultry (S. Waiblinger et al., 2006). Understanding behavior is an important aspect of the concept of animal welfare (V.N. Tikhonov et al., 2008). From the point of view of ensuring the welfare of animals, it is significant to have the possibility of living their natural life through the manifestation of natural behavior and the presence of elements in the environment that bring it closer to the natural environment (Animal Welfare Issues Compendium; D. Fraser, 2008). Poultry have retained a significant part of the behavioral needs of the wild forms (M.S. Dawkins, 1988). The main needs for the behavior of poultry are nesting, food and drinking behavior, the provision of physical and comfort activity and social interactions (I.J.H. Duncan, 1998; T. Shimmura et al., 2018). The restriction of natural behavior leads to a deterioration in the well-being of the bird. Environmental factors, such as high light intensity and crowding, also contribute to the high likelihood of behavioral disturbances (M.C. Appleby et al., 2004). Animals that are kept in captivity may exhibit behavioral disorders, including "stereotypes," such as repetitive fixed cycles performed for no apparent purpose, aggressive behavior, pecking eggs (G.J. Mason, 1991; M.C. Appleby et al., 2004; I.J.H. Duncan, 1998). Hens contained in traditional cage batteries (for 4-5 heads) are less prone to problems with aggressive behavior due to the smaller number of birds in the group (H. Lukanov et al., 2013). At the same time, in floor systems, the size of the group can exceed 1000 heads, which expands the possibilities of the exploratory behavior of poultry, but increases the risk of peck and cannibalism (D.C. Jr Lay et al., 2011). The behavioral preferences of animals are the basis for designing technologies that ensure the animal welfare (M.S. Dawkins, 1988).

Keywords: poultry welfare, behavior, behavioral infractions, behavioral needs, stress

According to experts, Russia is able to become a major supplier of poultry products to the world market, but for this it is necessary to produce products that meet the requirements of foreign importers. Unfortunately, at present, this potential is poorly realized: in 2017, 165 thousand tons of poultry meat and 435 million eggs were exported. The programs provide for a significant increase in the export of poultry products [1], therefore, the production of high-quality products and ensuring the well-being of poultry become urgent.

Well-being assessment is actively used in foreign enterprises in biologizing technologies, in increasing the productive longevity of animals, and observing ethical standards. The concept of providing welfare, based on a synthesis of the psychoemotional and physical characteristics of animals [2-4], can be also implemented at Russia.

The Brambell Committee which published its first report in 1965 proposed the "five freedoms" as a measure of animal welfare: freedom from hunger and thirst; freedom from discomfort; freedom from pain, damage and disease; freedom in the expression of natural behavior; freedom from fear and suffering [5].

The purpose of this review is to analyze the disturbances in the poultry behavior resulted from adversities, as well as the consideration of behavioral preferences as conditions for ensuring well-being.

There are three types of standards for protecting the welfare of poultry. The firs is the basic/model standards promoted by producer associations and restaurant chains in the United States that require approximately 440 cm^2 of floor space and free access to water and feed. The second one is the improved standards implemented in the European so-called enriched cages with 750 cm^2 area, nests, perches and litter. The third one is the alternative, used in certain production systems (for example, free-range and organic, requiring the same conditions as improved standards, as well as access to open space and natural daylight) [6].

Particular attention is paid to the conformity of the technologies to the biological features of animals [7]. Thus, a detailed comparison of the health and productivity of laying hens in different types of cages revealed frequent cases of feather cover damage and problems with limbs. Most birds had significant foot damage in cages with a sloping metal floor. Severe neck injuries of chickens were noted during feeding from a feeder set too high for comfortable access [8]. Injuries to the foot pads were found in 13% of laying hens of Lohmann cross at the age of 32 weeks, contained in specially equipped ("furnished") small-sized cages [9]. In such situations, it is enough to make some changes in the design of the cages: install solid partitions between the sections, which reduces feather loss due to wear and tear, use plastic coated floors with a slight slope and abrasive strips against claw ingrowth, reinstall the feeders [6].

Obviously, many factors affect the welfare of animals and birds: diseases, skeleton and musculoskeletal system health, parasites and parasitic infestations, stress, nutrition, and living conditions [10]. An unsatisfactory state of health, regardless of its causes, indicates a violation of the well-being of animals. Poor housing conditions lead to the aggravation of existing, including hereditary, problems [11]. Broiler health potential is undermined by genetic selection in order to accelerate growth and increase meat yield. They have an excessively rapid increase in muscle mass as compared to growth of the skeleton and internal organs, the capacity of the lungs and heart necessary to provide muscle functions is reduced. Broiler chickens suffer from limb deformity and lameness, and abnormalities in walking are found in 90% of individuals [12]. The frequency of limb problems is related to the growth rate: lameness was detected in 85% of broiler chickens of

fast-growing crosses. The poultry of "slow" crosses turned out to be more prosperous on this basis (up to 27% of lame individuals) [13].

One of the most important factors of well-being is the absence of stress. The most frequently used nomenclature defines environmental stimuli leading to an imbalance of homeostasis as stressors, and the corresponding protective reactions of the animal as stress reactions, with the brain playing a central role in binding stressors to reactions. Responses include changes in behavior in the immune system, activation of the neuroendocrine system (hypothalamic-pituitary-adrenal axis) and the autonomic nervous system [14]. Catecholamines, adrenaline and norepinephrine are involved in many metabolic processes, regulate emotions and provide motivation for action. Many authors point to a positive correlation between corticosterone level and social stress [15, 16]. It was shown that the blood level of corticosterone in meat ducks depends on the stocking density, being significantly higher in groups with a high stocking density ($p < 0.05$) compared to the control (91.42 and 28.71 ng/ml, respectively). In groups with low (3 birds/m^2) and medium (4 birds/m^2) stocking density, the concentration of corticosterone was similar (61.33 and 62.96 ng/ml). The corticosterone concentration in the group with a high stocking density (6 birds/m^2) exceeded the indicator values in the control group and in the groups with low and medium density [17]. An almost 3-fold decrease in corticosterone release in chickens is reported in response to stress when using a stress-protective antioxidant (55.5 versus 148.14 nmol / L) (16).

Under stress, changes occur in the blood morphology [18]. So, at rest, the ratio of heterophiles and lymphocytes was 0.29 in stress-sensitive chickens, and 0.23 in stress-resistant chickens. Under the influence of a stimulant (turpentine test), the ratio of heterophiles and lymphocytes in sensitive and stress-resistant chickens increased to 0.64 and 0.36, respectively [19].

Stress sensitivity is manifested in a peculiar behavior of chickens [19, 20], which is the best indicator of well-being. Strong manifestations of fear, such as panic or sudden attempts to escape, not only provoke expend of more energy, but can also lead to damage and even death [21]. Fear is considered an undesirable emotional state. When the level of fear is low, the activity of animals increases, when it is high, activity decreases. Regular stressors inhibit social interactions between animals. The conflict between negative emotional state and positive motivation can lead to behavioral disorders [22, 23].

Fear of personnel affects the well-being and productivity of animals, since a person becomes one of the main sources of stress. Studies on cattle and pigs showed that animals with a pronounced reaction of human fear are characterized by reduced productivity (24-26). A high degree of animal fear of human affects the productivity of laying hens [27]. Thus, the variability of the peak productivity index of a laying per day varied from 53 to 61% under the influence of this factor [28]. It is worth noting that the chicken fear of personnel depends on the cross. For example, it is more pronounced in Dekalb White cross chickens than in ISA Brown [29].

There are several types of contact between people and animals: visual (non-mobile) presence; movements between animals without tactile contact (with the possible use of voice contact); physical contact; feeding (promotion); invasive, fear-provoking [21]. The poultry is sensitive to visual contact with personnel, but some interactions, such as moving a person's hands to the side of the cage or short touches of the bird, can reduce its timidity [22].

According to the assessment of corticosterone concentration, regular processing by personnel does not lead to the formation of a habit of a certain type of processing, especially if it is catching with loading birds into transport [30]. In Leghorn chickens, when sampling blood through a catheter without contact, the

amount of plasma corticosterone was significantly lower than in the birds of the control groups, which was fixed manually (3.67±0.316 and 4.63±0.303 ng/ml, p = 0, 0422, $n = 19$), and the ratio of heterophiles and lymphocytes was significantly higher ($p < 0.001$) [31].

To prevent a bird from perceiving a person as a predator, it is important for personnel to move slowly and carefully around the poultry house [22]. J. Rushen et al. [32] identified several aspects in the personnel–animal interaction that can be used to improve well-being: positive contact with humans, especially when animals are young and more sensitive to various procedures; human understanding of what kind of behavior an animal can scare; creating conditions to reduce the number of rough treatments; avoidance of aversive techniques in handling animals, for example, the use of electrical stimulants [22].

Shy poultry avoid people. This behavior is observed in herds where farm workers, busy with their tasks, move too quickly. Fright (for example, due to a strong and sudden sound) causes broiler crowding, sometimes leading to asphyxiation. In shy chickens, a decrease in live weight gain is observed. Voice signals can indicate an emotional state (positive or negative) [11].

Estimation of animal reactions allows us to draw conclusions about how they perceive all people or a specific person. The reactions of animals reflect a mixture of different emotions. Of paramount importance, most likely, is fear. The degree of its manifestation depends on the type of animal and the keeping system, the nature of interactions with humans (positive, neutral, or negative), and the quality of care [21]. Personnel training is crucial in improving behavior towards animals, reduces problems associated with shyness. An increase in the number of low-paid and uneducated labor in livestock breeding in some countries represents a significant obstacle to good governance, since small companies are not profitable by investing in vocational training and staff incentives [22).

The birds within certain limits can adapt to various environmental conditions. Inability to adapt is expressed in violation of behavior. Behavioral problems can be associated with keeping technology (fewer problems are noted in "furnished", specially equipped cages) and microclimate disorders ($p = 0.001$) [33]. Animals that are kept in captivity exhibit stereotypes — repeated invariant patterns of behavior that are executed without a visible goal [34]. Stereotypies serve as a means of adaptation to captivity through the release of endorphins. Nevertheless, if the animals exhibit stereotypical behavior over time, the degree of well-being decreases [35). Environmental factors, such as high light intensity and crowding, contribute to the biting of eggs by chickens. Another example is the feather bite which was observed in 80% of chickens at one of the farms at the age of 14 weeks [36]. Bitings were observed in young chickens in 13 of 24 herds (54%) using the organic production system. If the youngster was prone to pecking the pen, then this behavior was observed in her further in 90% of cases, which confirms the influence of individual characteristics on the manifestation of the trait [37].

Some studies show that feather damaging is the so-called biased behavior related to feeding behavior or to taking dust baths [38]. The hypothalamus is considered to be a potentially important part of the brain for the study and control of behavior associated with pecking [38, 39]. There is no complete understanding of the fundamental biological mechanisms of feather pecking, but they can be due to the synthesis of serotonin and/or dopamine, associated with the content of aromatic amino acids tryptophan, phenylalanine and tyrosine in blood plasma. For example, in the laying hen line with low mortality, a large number of aggressive pecks was associated with an increased tyrosine content ($n = 78$, $r = 0.643$, $p < 0.001$) and a low tryptophan/(phenylalanine + tyrosine) ratio ($n = 78$, $r = –0.541$, $p < 0.001$). In highly productive laying hens, the correlations

for tyrosine (n = 73, r = –0.308, p = 0.005) and the ratios of tryptophan/(phenylalanine + tyrosine) (n = 73, r = 0.314, p = 0.004) were reversed [40].

Often, pecking-related problems are controlled via debeaking (beak trimming), but in some countries it is prohibited [41]. It should be understood that pecking problems are multifactorial [42]. The age of the bird, feather color, feeding regime, the ability to exhibit natural behavior (search and getting feed) should be regarded, and individuals initiating pecking should be removed. That is, the pecking-related problems can be prevented via effective flock management without beak trimming [41, 42].

Regulation of the light regime is a key factor determining the frequency and severity of pecking and cannibalism in chickens [43]. Not only lighting, but also its intensity plays an important role in the regulation of behavior [44, 45]. For example, low-intensity red light decreases the frequency of pecking and damage to plumage, timidity, and cannibalism-related mortality in chickens [46]. The risk of pecking increases with more intense lighting under commercial automation systems for poultry farming, so a 5-10 lux lighting intensity is advisable to maintain [47]. It was found that feather cleaning, pecking and aggressive behavior of 3-week-old turkey poults significantly increased at 50 lux against 5 and 25 lux [48]. Blue light resulted in higher frequency of feather pecking in Lohmann Brown Classic laying hens (65.7% for blue light vs. 45.2% for fluorescent light, 52.9% for incandescent light, and 53.7% for daylight lamps) [49]. Besides, the frequency of feather pecking depended on an increase in the intensity of blue light (18.7% at 50 lux vs. 11.7% at 5 lux) [49[.

Aggressive behavior of poultry is less common in small herds, which is associated with the identification and memorization of relatives [19, 20]. That is why, according to a number of authors, poultry kept in traditional battery cages 4-5 birds each is at a lower risk of aggressive behavior due to the smaller number of individuals in the group [50]. The group size in cage-free poultry can exceed 1000 birds, which stimulates exploratory behavior, but increases the risk of pecking and cannibalism [10]. Food deprivation also leads to higher aggression. This is the most common procedure to induce artificial molting [51]. Triggers of forced molting are determined by the functional state of the pituitary gland. Its activity through the hypothalamus is regulated by the nervous system, which, in turn, adapts the body to stressors that induce molting [52]. A direct correlation was found in chickens between the period of food deprivation and the frequency of aggressive contacts [53]. Most of the chickens kept in cages, especially the light hybrid lines, become more aggressive before egg laying [54]. Serotonin has been shown to be the primary regulator of aggression [55]. An increase in the level of aggression is associated with an increase in dopamine concentration [56].

Understanding behavior is an important aspect in the concept of poultry well-being. Behavior as an adaptive trait is associated with the reproductive traits and characterizes animal fitness and adaptive ability [57]. From the point of view of ensuring animal well-being, experts especially emphasize the possibility of living a natural life through the manifestation of natural behavior and presence in the animal environment of elements which bring it closer to natural conditions [51, 58]. Therefore, the behavioral needs of birds are of great importance.

It is the behavioral preferences of animals that should be the basis for the design of technologies that ensure well-being. A domesticated poultry is prone to exhibit behaviors similar to the behavior of a wild birds, which serves as the basis for survival [35]. When considering the concept of “behavioral needs”, it is important how often various patterns of behavioral reactions are observed in a bird, what exactly causes the manifestation of natural instincts and how strongly their

occurrence is motivated [54]. The main behavioral needs include nesting, perching, fodder getting, dust baths, comfortable behavior, physical activity, exploratory behavior, feeding and drinking behavior, personal care (preening, cleaning), social interactions [54, 59, 60]. Obviously, in industrial poultry farming, development of territories, sexual behavior, gestation and incubation, reaction to predators should be excluded from the basic needs.

Exploring is useful for animals for several reasons: it motivates to gain knowledge about the environment, gives freedom of choice and certain skills [59, 61]. In laying hens kept in cages the exploring time was 326 minutes for 16 hours for a group size of 7 birds and stocking density of 430 cm^2 per hen. Less exploring activity (227 minutes for 16 hours) was noted for a group size of 2-3 birds and landing densities of 333 and 455 cm^2 per hen [61]. Significant differences were found between the manifestation of exploring behavior in poultry when kept on the floor (20.1%) and in cages (14.7%) [62]. It is believed that the exploring reaction to a new object is associated with human fear and characterizes the well-being of the bird [2].

Brooding and incubating behavior is characterized by morphological, behavioral, and physiological changes [63, 64]. This is accompanied by an increase in concentration of prolactin [65] which helps suppress the secretion of luteinizing hormone during incubation, possibly acting on the hypothalamus and the anterior pituitary gland [66]. In the experiments, laying turkey hens showed a gradual decrease in egg laying during the time they were receiving ovine prolactin (oPrl) [64]. Research by S. Crisystomo et al. [65] confirms the hypothesis of a regressive effect of a high prolactin content on steroidogenesis, which subsequently leads to ovarian regression. This can be controlled, for example, by passive immunization of poultry with rabbit serum containing antibodies against recombinant turkey prolactin. It has been shown that even a single injection of antiserum to prolactin to Bentham chickens during manifestation of brooding instinct leads to an increase in the content of luteinizing hormone and disrupts this behavior [67, 68].

It should be noted that in hybrid lines there is no behavior associated with brooding which does not arise either from environmental influences or during hormonal stimulation; presumably, it is due to the lack of reaction of the corresponding brain regions due to selection [54, 69]. Thus, frequency of brooding in Bentham and White Leghorn hens varied significantly (78.6 and 0%, respectively). Frequency of incubation behavior of crosses from direct and reverse crossing of Leghorn and Bentham breeds did not coincide with the maternal breed (61.6 and 56.8%, respectively). The incubation behavior during backcrossing crossbred males Leghorn × Bentham with Leghorn females was only 5.8%, which was significantly less ($p < 0.001$) than expected (39.3%). It was revealed that the incubation behavior is not controlled by a major gene (or genes) on Z chromosome. It has been suggested that there are two dominant autosomal genes that equally affect the expression of behavior [70].

Sexual behavior is largely driven by external stimuli [71-73]. Before egg laying, hens sometimes demonstrate squatting down or sitting, which is associated with an increase in the content of sex hormones [73]. Dominant males can interrupt copulation attempts by subordinates (up to 78% of cases) [74].

In red partridge males, the duration of a voice call as an external stimulus positively correlates with the size of the crest ($F_{1.7} = 19.88$, $p = 0.003$) [75]. Pedigree Bentham males manifest sexual behavior at 8-12 weeks of age [76]. Camphor stimulates sexual behavior of Japanese quails when added to the feed at 0.5 g/kg ($p < 0.05$) [77].

For laying hens during the laying period, exercising is important [78], associated with free movement in space. It was found that about 24% of chickens

kept in cages at the end of the productivity period suffer from bone fractures. Greater bone strength is observed in birds, which are kept on a deep litter with perches [79, 80]. The need for long exercises or their compensation with short walks (as in extensive containment systems) has not yet been proved in intensive poultry industry [36, 54]. However, a higher ($p < 0.01$) concentration of blood corticosterone was detected in birds kept on a litter without motions than in free range poultry. Less feed conversion ($p < 0.05$), egg laying time ($p < 0.001$), frequency of feather cleaning ($p < 0.05$), dust baths ($p < 0.001$) and mating ($p < 0.01$) were observed indoor compared to birds under free-range system. At the same time, the bird, when kept indoor, consumed more water ($p < 0.001$), rested more ($p < 0.01$) and was more aggressive ($p < 0.05$) [81].

The movement of birds in nature is accompanied by getting feed, which is prevented in cages. Feeding behavior [82] accompanied by sewing beak and claws, free walking [83]. It is worth noting that broiler pullets have less pronounced feeding behavior (by 57%) in the evening than in the morning [84]. It is noteworthy that females more often and actively manifest feeding behavior ($F_{1.16} = 63.3$, $p < 0.001$), regardless of breed [85]. Red junglefowl spends about 34% of time budget actively scratching and foraging, and 60% of the time is spend for ground pecking [86-88]. Equipping cages with a special material for scratching stimulate scraping in birds [89]. In some species held in captivity (for example, in mink) stereotypes associated with food consumption are mostly observed before feeding. In birds, the number of stereotypes increases after eating. The lack of space may prevent the manifestation of the motor form of stereotype before feeding [90]. Feeding behavior is affected by a stocking density. Thus, at stocking density of 2000 birds/ha, the time spent for searching feed decreased compared to that at 10,000 and 20,000 birds/ha [87]. In a cage for 20 birds, feed getting took 98.4% of the time, and at 40 and 60 birds per cage this time decreased to 96.37 and 94.45%, respectively [89].

Preening behavior [91] is necessary as a response to external influences on the feather, as well as a substitution upon a slight degree of frustration or conflicts between individuals. Its frequency is affected by stocking density and microclimate conditions [92]. Thus, the Preening frequency ($F_{2.16} = 8.19$, $p < 0.05$) decreased in small commercial cages (70×30×55 cm) [93]. In medium-sized cages (160×75×70 cm) with a 7.14 birds/m^2 stocking density, the frequency of preening increased up to 21 repetitions per hour ($p < 0.05$) compared to small cages (120×50×45 cm) with a stocking density of 10 birds/m^2 and 3.6 repetitions per hour [94]. In turkeys, at low ambient temperatures (–18 °C), the frequency of feather cover cleaning decreased to 5.1% [95].

Nesting [88] is defined as the basic behavioral need of laying hens. Nesting behavior is a characteristic of a sequence of behavioral reactions associated with nest-site selection, nest construction, and egg laying. In the wild, 90 minutes before laying, the hens go away to a secluded place, carefully dig a small depression in the ground and build a nest. Similar behavior is observed when keeping hens on floor. Hens are initially motivated to gain access to the nesting place during the laying period [96, 97]. Other factors influencing nesting behavior are the ability to use perch [10] and the design of nests. In large cages with open nests hens were more active during 1 hour of observation than hens in nests closed with a plastic curtain (56.15±6.79 vs. 28.79±2.85 nesting acts per hour; $p = 0.0003$) In small cells, the frequency of aggressive interactions was higher in open nests than in closed nests (66.00 ± 15.97 vs. 9.65±2.10 acts per hour; $p < 0.0001$) [98]. The number of eggs laid both in cages with open and closed nests was small (0.8-1.5%) [99]. It was also established that hens in small cages laid more eggs on red smooth floors than on yellow wire mesh floors (55.6±2.3 vs. 43.4±2.3%, $p = 0.0012$). No

similar differences were found for large cages (50.7±3.4 vs. 48.4±3.4%; $p = 0.89$) [99]. Suppression of the nesting instinct upon cage keeping system often leads to severe frustration [21].

Birds is anatomically adapted to perching and roosting [100], that is, their limbs are evolutionally adapted to climbing trees. The use of perches plays an important role in maintaining healthy bones and avoiding interactions with more aggressive relatives. In the wild, birds spend night time and rest time on trees [101-103]. As a rule, hens do not give preference to either round or hexagonal perches ($p = 0.59$-0.98). With age, birds use perch more often ($p < 0.01$), regardless of its shape. In total, hens spend about 10% daytime on perches. More than 75% of hens are perching nightly [104)]. Chickens of the first week of life prefer a flat surface and realize more behavioral needs on the ground (52%) compared to a 5-9-week-old birds ($p < 0.0001$). Aboveground surface (15-69 cm height), chickens begin to use from 2-week age and realize 45% of their behavioral needs there [105].

Normally, poultry should not be thirsty. More than a century ago, the behavior was described of chicks that consumed water only from droplets on plant leaves. In industrial poultry farms, chickens begin to drink from a fixed water surface and then adapt to a variety of water supply technologies [36]. Broilers are known to spend 16% of their time for drinking behavior [82]. This type of behavioral activity is described as pecking at the water bowls, which increases by 49% in the evening compared to the morning time. This is not associated with water consumption and is regarded as a manifestation of stereotype. The frequency of water bowl pecking is significantly reducing during 10 to 16-17 weeks of age (0.28±0.04 vs. 0.05±0.02 acts per chicken for 15 minutes, $p < 0.0001$) [84]. The stocking density affects the water consumption frequency which is higher at moderate (4 birds/m^2) than at low stocking density (3 birds/m^2) [17].

Dustbathing [88] helps maintain skin and feather health. It has been experimentally proven that it balances the amount of feather lipids. Such behavior is controlled by both the nervous system and external factors. For example, in cages equipped with perches, nests and a special bath, birds took dust baths once in the afternoon for 5 minutes. In traditional cages, the dust baths were brief and fragmented (3 times for 10 s) [106). Optimal lighting, temperature, and a dry, loose substrate contribute to this behavior [89, 107]. The frequency of dustbathing was higher at a temperature of 22 °C ($p < 0.01$) than at 10 °C [108].

Dustbathing helps to maintain feather cover in many bird species, including broiler chickens. Thanks to them, chickens get rid of contaminants, including litter particles. Failure to use dust baths may indicate problems with beddings or flooring (e.g. humidity or excessive stiffness) [11]. The frequency of such behavior is also affected by age. According to G. Vasdal et al. [109], dustbathing was observed in broilers aged 16 days ($p = 0.009$), but was not recorded at the age of 30 days.

Engaging in comfort behavior (other maintenance behavior) [54, 110] is important to maintain good condition and feather cover. It includes sipping, flapping wings, swinging the body. Cage keeping has a negative effect on manifestation of this behavior because of too small space [111, 112]. When kept in cages, chickens are more likely to stand or to sit ($p < 0.05$), while in free-range systems they demonstrate running and jumping at certain hours [113]. In using systems with peat-rich, alfalfa-rich and raised platforms, 16- and 30-day-old broilers flapped their wings ($p = 0.016$) and shocked their bodies ($p = 0.002$) more often than in systems without enrichment [109].

The comfort of the birds during sleep and rest (sleeping behavior) is equally important [93]. The bird's natural position for sleep is perched, but birds

can quite easily adapt to other conditions. All housing systems for laying hens provide for a substantial period without lighting for rest. Broiler housing systems have long light periods. Currently, regimes of Intermittent lighting or a gradual increase in lighting are being introduced to deal with the problem of rapid growth in poultry [36]. The frequency of sleep ($p < 0.01$) increases significantly in industrial small cages (0.70×0.30×0.55 m) than in medium-sized (1.00×0.33×0.55 m) and large cages (1.30×0.36×0.55 m) [93].

Response to predators [54] arises as a result of external factors in the presence of a stimulus. In the poultry keeping system, there is no key stimulus, i.e. association with a predator, although under certain conditions a person is perceived as such [21, 36, 79]. In experiments using a prey bird model, the number of hens with normal behavior decreases ($p < 0.001$) and does not remain unchanged during the day under the predator model exposure ($p = 0.12$). In addition, the influence of time (before/after exposure of the predator model) and the number of days of exposure on anxious ($p < 0.001$) and panic behavior ($p < 0.001$) was noted, i.e., the bird gets used to the attacks of the model predator [114].

Social interactions [115, 116] are necessary for any keeping systems [21, 36, 79]. An increase in aggression against partners may indicate a decrease in the well-being of the aggressor [53]. It has been shown that the manifestation of aggression while reducing the critical distance between individuals is affected by the general activity of the bird [117] and changes in the environment. So, after the removal of perches, social interactions in cages increased by 19.3% [115]. It is known that red junglefowl hens exhibit synchronous social behavior, which is manifested, among other things, in the pecking of the feathery coat of relatives ($p = 0.058$) [85]. An unfavorable microclimate, air pollution, and ammonia concentration increase aggression in the flock and lead to a decrease in well-being [81].

Thus, animal behavior characterizes its adaptiveness and may be the best indicator of welfare. The degree of well-being of poultry is influenced by many factors: diseases, stress, nutrition, and living conditions. Inability to adapt is expressed in changes in physiological status, as well as in behavioral disorders that can harm both animals and maintenance personnel. From the point of view of ensuring animal well-being, they especially emphasize the ability to exhibit natural behavior and the presence of elements in an environment that brings it closer to the natural environment. Domestic hens have retained a significant part of the behavioral needs of wild relatives (the main are nesting, feeding and drinking behavior, motor activity, comfort behavior, and social interactions). The restriction of natural behavior leads to a deterioration in poultry welfare. The consequence of this may be a violation of homeostasis, leading to a decrease in stress resistance and an increase in risks to poultry health. Productivity of modern poultry breeds and crosses is so high that under industrial technologies, when birds fail to realize behavioral needs, any deviations from the standards of keeping and feeding lead to significant losses. Effective production performance of commercial poultry requires a balance between financial interests and bird welfare.

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ASSISTED REPRODUCTIVE TECHNOLOGIES: THE HISTORY AND ROLE IN THE DEVELOPMENT OF GENETIC TECHNOLOGIES IN CATTLE

(review)

N.A. ZINOVIEVA[1], S.V. POZYABIN[2], R.Yu. CHINAROV[1]

[1]*Ernst Federal Science Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail n_zinovieva@mail.ru (✉ corresponding author), roman_chinarov@mail.ru;
[2]*Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology*, 23, ul. Akademika K.I. Skryabina, Moscow, 109472 Russia, e-mail jippo77@mail.ru
ORCID:
Zinovieva N.A. orcid.org/0000-0003-4017-6863
Chinarov R.Yu. orcid.org/0000-0001-6511-5341
Pozyabin S.V. orcid.org/0000-0003-1296-2840
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The development of "active" transgenesis technologies has allowed targeted modifications (gene editing, GE) in the genome of farm animals belonging to different species with relatively high efficiency (reviewed by S.Y. Yum et al., 2018; A.L. Van Eenennaam, 2019; N.A. Zinovieva et al., 2019). However, effective improvement of livestock production systems based on GE technologies requires the development of an integrated approach that combines biotechnology, population genetics, quantitative genomics, and assisted reproductive technologies (ARTs) (C. Rexroad et al., 2019). The development of ART, including germ plasma collection for gene editing in animals, the effective production of GE-offspring, and their possible earlier multiplication are an integral requirement for the successful development and implementation of GE technologies in cattle breeding (A.L. Van Eenennaam, 2019). This review provides a retrospective analysis of the development of ART, including artificial insemination (R.H. Foote, 2002; R.G. Saacke, 2012; P. Lonergan, 2018), embryo transfer (K.J. Betteridge, 2003; R. J. Mapletoft, 2013), *in vitro* production of embryos (IVP) (L. Ferrй et al., 2019), oocyte retrieval in living animals (Ovum-Pick-Up) (R. Boni, 2012; M. Qi et al., 2013), and somatic cell nuclear transfer (C.L. Keefer, 2015; K.R. Bondioli, 2018; A.V. Lopukhov et al., 2019). We describe the state of the research and discuss the areas requiring further improvement in ART for the development of genetic technologies in cattle breeding, including gene editing. This review shows that for more than 100 years, significant progress has been made in the development of ART for cattle, many of which are now actively used in practical animal breeding (C. Smith, 1988; L. Ferrй et al., 2019) and became the basis for the development of effective programs for genetic improvement of livestock, including genomic selection (P.M. VanRaden et al., 2009). Current research priorities are focused on ensuring further progress in cattle breeding through the integration of GE technologies into livestock breeding programs (C. Rexroad et al., 2019, A.L. Van Eenennaam, 2019). ARTs are expected to play a crucial role in this ambitious task.

Keywords: cattle, assisted reproductive technologies, gene editing

Advancements in genetic technologies, including genome editing (GE), for application in agriculture and, in particular, animal breeding is one of the important goals of science and technologies around the world [1, 2]. Genomic

selection has become one of the most significant scientific advances in the last decade and has been implemented in practical animal breeding [1, 3]. One of the most promising scientific breakthroughs that can be achieved in the next decade is associated with the "ability perform routine gene editing of agriculturally important organisms" [4, cited according 1]. The development of "active" transgenesis technologies, such as CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9), has allowed the incorporation of targeted modifications (gene editing, GE) in the genome of farm animals of different species with relatively high efficiency [reviewed by 5-7]. However, effective improvement of livestock production systems based on GE technologies requires the development of integrated approaches involving biotechnology, population genetics, quantitative genomics, and advanced reproductive technologies [1]. The development of assisted reproductive technologies (ARTs), including collection of germ plasma for GE from animals with the desired genetic characteristics (for example, having a high breeding value due to economically important traits) and the effective production of GE-offspring and their possible earlier multiplication are an integral requirement for the successful development and implementation of GE technologies in cattle breeding [6].

This review provides a retrospective analysis of the development of ART, describes the state of the art research, and discusses the areas for further improvement in ART to enable the development of genetic technologies in cattle breeding, including GE.

In its early stages, the main goal of ART was to increase male fertility. The first such technology, which was applied in livestock species, was artificial insemination (AI). Several reviews have described in detail the history of AI in cattle [8-10]. In the present paper, we briefly focus on the milestones involved in the development of this technology.

The development of AI in mammals began in 1780, when the Italian physiologist (professor of natural history) Lazzaro Spallanzani (1729-1799) performed AI in a female dog for the first time, resulting in offspring [11, cited according 12]. In 1799, John Hunter (1728-1793) successfully employed this method in humans, which resulted in the birth of a healthy child [13, cited according 12]. The first application of AI which can be received from one male in mammals to increase the number of offspring dates back to the late 19th century. In 1885, the breeder of Basset hounds sir Everett Millais (1856-1897) divided a single ejaculate into three parts and inseminated three female dogs, all of which successfully gave birth to offspring [14, cited according 12]. At about the same time, a French veterinarian demonstrated the effectiveness of AI for improving fertility in horses as well [15, cited according 16]. Further development in AI technology was achieved thanks to the works of a reproductive biologist from Cambridge, Walter Heape (1855-1929), who performed experiments on dogs, rabbits, and horses [12]. The practical bases of the AI for domestic animals were laid in 1899 by the Russian scientist Ilya Ivanovich Ivanov, who proposed the application of this method for multiplying the offspring from the best males to accelerate the improvement of breed qualities and productivity of animals [17, 18]. The ideas of I.I. Ivanov were further developed by V.K. Milovanov, who implemented large-scale AI projects in cattle and designed the first artificial vagina, similar to what is used nowadays [19]. By 1938, approximately 1.2 million cows were artificially inseminated in the USSR annually.

The role of AI in improving the genetic characteristics of domestic

animals was further increased by the proof that animal spermatozoa remain viable after freezing (cryoconservation) and storage at low temperatures. In 1942, the ability of the spermatozoa of higher vertebrates to maintain fertilizing capacity after freezing and thawing was demonstrated for the first time on fowl; fertilized eggs were obtained after insemination of chickens with sperm stored at –79 °C for one hour, but all the embryos died within 10-15 h after fertilisation [20]. Soviet scientists Milovanov V.K. (Doctor of biological Sciences) and Sokolovskaya I.I. (Doctor of biological Sciences Smirnov I.V) developed the method for cryoconservation of the semen of domestic animals which then served as a foundation for a wide range of practical applications of AI technology. In 1947, Sokolovskaya I.I. published this work in the journal "Proceedings of the Soviet Academy of Agricultural Science", in which, for the first time, she showed the ability of mammalian spermatozoa to maintain their viability after freezing/thawing and to produce viable offspring. Sixty-nine healthy offspring were born after insemination of rabbits with semen subjected to freezing in carbon dioxide vapor and subsequent thawing [21]. This was a scientific breakthrough that served as the basis for research on other farm animal species. The next major milestone was the use of glycerol as a cryoprotectant in the process of freezing and preserving the semen at low temperatures. Although most studies have attributed the cryoprotective role of glycerol to Christopher Polge [22], in 1937, Soviet scientists Bernstein A.D. and Petropavlovsky V.V. used glycerol to freeze the bull, ram, stallion, boar, and rabbit spermatozoa at –21 °C [23]. However, their work was published in Russian, and therefore, was not widely recognized. Using glycerol as a cryoprotectant after insemination with frozen-thawed semen, viable offspring were produced in chickens [24] and cattle [25] in 1951, and in pigs [26] and horses [27] in 1957. AI technology has been widely used in cattle breeding since the late 50s and early 60s, becoming the basis for the development of large-scale breeding [28, 29]. An additional technical advantage of AI technology is the development of a method for sorting spermatozoa carrying X-and Y-chromosomes [30, 31, 32], which is based on the measurement of DNA content in mammalian spermatozoa using flow cytometry [33]. Subsequently, this method was improved and numerous practical applications were identified [31]. Today, in countries with developed cattle breeding, AI is used in 100% of the dairy cattle population. The genetic potential of the best breeding bulls is replicated from several hundred thousand to more than a million offspring (http://www.holsteinusa.com, cited according 34), significantly increasing the degree of genetic gain.

The main milestones in the development of AI technology are summarized in Figure 1. AI technology has become the basis for the development of other ARTs, such as embryo transfer, in vitro embryo production, cloning, transgenesis, and GE.

Another important aspect is multi-replication of the genetic potential of highly productive cows. Relatively late puberty (12-13 months and older), single birth, and a relatively long period of pregnancy when using traditional AI technology ensures that the first offspring is produced in cows two years and older, and in the subsequent period (under optimal conditions), an average of one calf per year. The solution to this problem is highly relevant for the implementation of programs for the conservation of rare (small and gene-pool breeds) and unique (genetically modified animals) genetic resources. In this respect, the goal of ART development is to ensure that more offspring from a single female are produced as early as possible.

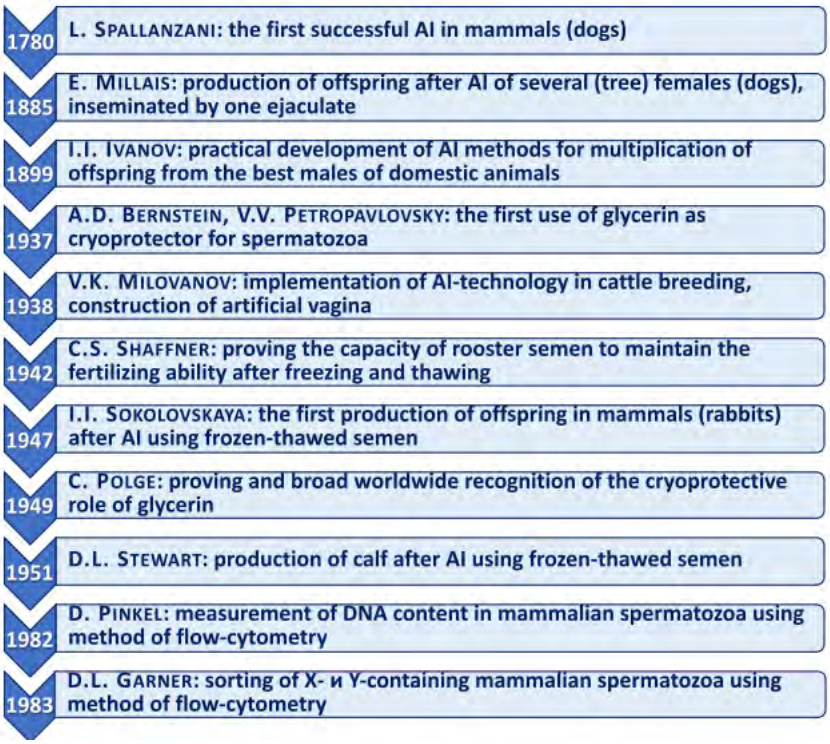

Fig. 1. The main stages of development of artificial insemination (AI) technology. L. Spallanzani [11], W. Heape [12], E. Millais [14], I.I. Ivanov [17], A.D. Bernstein, V.V. Petropavlovsky [23], V.K. Milovanov [19], C.S. Shaffner [20], I.I. Sokolovskaya [21], Polge C. et al. [22], Stewart D. et al. [25], Pinkel D. et al. [33], Garner D.E. et al. [30].

The first such technology was embryo transfer (ET), which includes the induction of superovulation in donor cows through hormonal treatment, artificial insemination, followed by embryo washing (on days 6-7 after AI), and embryo transfer to recipient cows [review 35, 36]. The first ET calf after surgical transplantation of a 5-day-old embryo obtained at a slaughterhouse was born in 1951 in the United States [37]. At the early stages, embryo recovery and transplantation were performed surgically, which limited the practical use of ET. In 1976, non-surgical embryo recovery was performed for the first time [38], and in the early 80s, non-surgical embryo transfer was performed in cows [39], which allowed these operations to be performed on the farm. The main goal of early ET programs was to distribute desirable phenotypes in herds. In 1988, scientists at the University of Guelph proposed the concept of multiple ovulation and embryo transfer (MOET) [40] to increase the genetic potential of herds. It was shown that the establishment of nucleus herds and "juvenile MOET" in the offspring of heifers can almost double the degree of genetic progress compared to traditional schemes of progeny testing evaluation. According to the International Embryo Transfer Society (IETS, http://www.iets.org/comm_data.asp) from 1997 to 2005 there was a progressive increase in the number of MOET embryos, i.e., approximately 450 thousand to almost 800 thousand per year, after which from 2005 to 2013 the production of embryos was stabilized at 700-800 thousand embryos per year. From 2014-2016, embryo production decreased to about 610-660 thousand, mainly due to an increase in the number of embryos obtained in vitro [cited according 41].

The main disadvantage of ET technology is the need for hormonal treatment: (1) it is known that not all donors respond equally well to hormonal stimulation; (2) the effectiveness of superovulation with each subsequent hormonal

treatment decreases (as a rule, the effective response to hormonal stimulation in cows is observed during 2-4 consecutive treatments); (3) a break of 2-3 months between hormonal treatments is required, which increases the cost of maintaining the donor cows. In addition, it is impossible to obtain embryos in cases of oviduct pathology [42, 43].

The next scientific breakthrough in the development of ART was in vitro embryo production (IVP) [review 41]. Classical IVP technology involves obtaining oocytes from the ovaries of cows by follicle aspiration, followed by in vitro maturation (IVM), in vitro fertilisation (IVF), and in vitro development of embryos (IVD) to stages suitable for transplantation or freezing (usually, late morula and blastocyst). The first calves resulting from in vitro fertilisation of the ovulated oocytes maturated in vivo were born in 1981 [44]. The first calves produced exclusively by IVP, including IVM, IVF, and IVD, were reported in the late 80s [45]. Initially, IVP embryos were produced using oocytes recovered from the ovaries of cows after slaughter (post-mortem), which limited the use of this technology for the genetic improvement of cattle.

Integration of IVP technology into programs for the genetic improvement of livestock began with the development of Ovum-Pick-Up (OPU), a method for the non-invasive recovery of oocytes [reviews 46, 47] from antral follicles in live animals [48-50]. In vivo retrieval of cow oocytes was first performed by Canadian scientists using endoscopy through the right paralumbar fossa [51]. In 1987, transcutaneous aspiration of cow follicles under ultrasound control was proposed in Denmark [52]. The next step was the development of a method for the retrieval of cow oocytes by ultrasound-assisted transvaginal aspiration of follicles, in 1988 by Dutch scientists [53]. This method has superseded all the methods mentioned above and is currently the standard method for obtaining of cow oocytes. Unlike MOET, OPU does not interfere with the normal reproduction and production cycle of the donor (no long-term negative effects on the fertility of donor cows were observed, even after OPU was performed twice a week for more than a year [54, 55]). Any female between three months and six months of pregnancy and shortly after calving (2-3 weeks) can become a suitable donor [47]. Currently, OPU is a practical alternative to the traditional MOET strategy [48, 49] and is increasingly used in commercial programs around the world [50, 56]. Although there are significant differences between individual donors, the joint use of OPU/IVP can result in the production of more than 50 calves per donor cow per year. Therefore, Kruip et al. [57] performed OPU twice a week for five months and obtained an average of 340 oocytes and 54 suitable embryos from one cow. In 2016, the number of IVP cow embryos produced in the world was more than 600 thousand and for the first time exceeded the production of MOET embryos (IETS, cited according 41). Considering the important role of OPU in the development of highly advanced genetic technologies, such as embryonic breeding [58] and genome editing [review 7], we focused on the research areas for improving OPU technology in more detail.

To increase the efficiency of OPU technology, studies have been conducted to identify factors that affect the number and quality of the obtained oocytes. The original OPU technology does not include hormonal stimulation, which limits the number of oocytes received. In this regard, various schemes of hormonal stimulation of donor cows using gonadotropins of the placenta (for example, pregnant mare serum gonadotropin, PMSG) and pituitary origin (for example, follicle-stimulating hormone, FSH) are used to obtain more oocytes per session [48, 59, 60]. We performed a study on the effect of hormonal stimulation using FSH on OPU in Simmental heifers. FSH treatment resulted in an average 3.2-fold increase

(from 4.5 to 14.6) in the number of ultrasound-visible follicles (3 mm or more in diameter), as well as the number of recovered cumulus oocyte complexes (COCs) per session (from 2.4 to 7.7). At the same time, we did not find any differences in the quality of COCs obtained with or without hormonal stimulation [61].

Along with the advantages, using hormonal stimulation for OPU has some unresolved key issues. The prolonged use of exogenous hormones can disrupt the endocrine system of the donors, which can lead to infertility. Responses of donor to hormonal stimulation are different: when using FSH, the number of oocytes obtained per session varied in different animals from 0 to 26 [62]. Even the same donor may show different reactions in different sessions, which leads to unstable results. In this regard, it is ideal to use hormones for a short period, leaving time for regulation and rehabilitation of the endocrine system [47).

Other factors that affect the effectiveness of OPU are time regimen (frequency of OPU sessions), technical and technological parameters of OPU, individual characteristics of donors (breed, age, reproductive phase, and individual response), availability of necessary nutrients in the diet [63], climatic conditions [64, 65], and operator experience [review 47].

The classic OPU procedure (without hormonal stimulation), in most cases, involves performing a puncture twice a week (2/w). The choice in favor of the 2/w regimen is due to an increase in the frequency of follicular waves, a delay in the estrous cycle, follicle maturation, and ovulation. Animals subjected to OPU in the 2/w regimen come to a so-called paraphysiological state in which follicular waves are independent of the estrous cycle [57]. When using this regimen, the dominant follicle does not develop, since all visible follicles are aspirated during the OPU process. When OPU is performed once a week (1/w) and less often, in most cases, the dominant follicle develops, which leads to regression and degeneration of subordinate follicles.

Comparative analysis of OPU in 1/w and 2/w regimens did not reveal differences in the number of aspirated follicles, recovered oocytes, and blastocysts obtained on the 7th day of cultivation, per cow per session. However, on a weekly basis, all three parameters were significantly higher for the 2/w regimen compared to 1/w [66-68). We studied the effect of two different time regimens on the OPU in Simmental heifers in terms of the quantity and quality of oocytes obtained [69]. On average, 4.4 oocytes were received from each donor per session using both regimens. We found a significant 1.2-fold increase ($p < 0.05$) in the rate of OPU oocytes of good quality characterized by normal morphology when performing the 2/w regimen (65.7±4.0% of the total number of recovered oocytes) compared to the 1/w regimen (53.6±3.0%). Considering the values of the rate of oocyte maturation (74.0%), the rate of cleavage of fertilized oocytes (on average 63.5%), the rate of development of embryos to the blastocyst stage (on average 16.7%), and the increase in the rate of good-quality oocytes compared to OPU sessions once a week, the 2/w regimen produced 2.5 times more embryos in the blastocyst stage from one donor for a certain period of time [69].

The influence of age and various physiological conditions on the effectiveness of OPU has been established. Rizos et al. [70] showed higher OPU performance in Holstein heifers than in cows: the total number of recovered oocytes was 4.7 vs. 2.8, respectively, including the number of oocytes of the 1st-2nd degree - 3.0 vs. 1.8. Significant differences in the cleavage rate of fertilized oocytes and blastocyst yield between heifers and cows were not observed [70].

The influence of the physiological state on the ability of OPU-oocytes to further develop has been established. In an experiment on Japanese Black cattle, it was shown that the cleavage rate of fertilized oocytes and their development to

the blastocyst stage, as well as survival after freezing, was higher for embryos obtained from oocytes recovered from pregnant cows than for embryos from non-pregnant cows [71].

Significant differences in the efficiency of OPU between the *Bos Taurus* and *Bos indicus* breeds (zebu cattle) have been revealed. Significantly more oocytes were obtained from *Bos indicus* donors [72, 73], mainly due to the larger population of follicles in the ovary. Between 18 and 25 oocytes were obtained from donors of Nelore zebu cattle bred in Brazil without the use of exogenous hormones or synchronisation protocols [74, 75]. A comparative study of the Holstein breed (*Bos Taurus*) and the Gir breed (*Bos indicus*) was performed by Pontes J.H.F. et al. [50]. The number of viable oocytes recovered per one OPU session in donor cows of the Holstein breed, the Gir breed, the 1/2 Holstein * 1/2 Gir and 1/4 Holstein * 3/4 Gir crosses was 8.0±2.7, 12.1±3.9, 24.3±4.7, and 16.8±5.0, respectively. The rate of IVP embryos obtained after insemination by sexed semen did not differ significantly between the groups and was 36-40% [50]. There were no noticeable differences in OPU efficiency between different *Bos taurus* cattle breeds. To predict the number of antral follicles in the ovaries of *Bos taurus* and *Bos indicus* cows and, consequently, the efficiency of OPU, measurement of the concentration of anti-muller hormone in blood plasma, which is produced by follicle cells during their maturation, can be used [76].

The sensitivity of ultrasound devices, the type (sector or linear), frequency of the probe used [57, 77], vacuum characteristics [78, 79, 80], the diameter and length of the slope of the needle [57, 81, 82], scrolling the needle inside the follicle during aspiration [80, 83], and removal of the dominant follicle are technical factors that affect the efficiency of OPU [66, 84].

Another possibility of obtaining oocytes from live cows is the laparoscopic collection of oocytes (L-OPU), which was first used in cattle in 1992 [85]. L-OPU has a number of advantages over the classical OPU procedure, including the choice of follicles for aspiration, the possibility of aspiration of follicles with a smaller diameter (2 mm or more), direct observation of the reproductive organs and ovary, visual control of the aspiration procedure, and reduced risk of ovarian damage. Comparative studies have shown that using the classical OPU technology, more oocytes of good quality were obtained and, as a result, a higher yield of embryos at the morula/blastocyst stage was achieved compared to L-OPU [86, 87]. L-OPU technology is used for obtaining oocytes from prepubertal females (aged 2 months and older), on which the use of classical OPU technology is impossible. Using L-OPU on heifers aged 2-6 months, 4.6 [85], 21.4 [88], and 42.6 oocytes [89] were obtained in one session. The use of prepubertal females with high breeding value as oocyte donors, selected based on the results of genomic evaluation, can reduce the generation interval and, as a result, increase the genetic progress [90]. However, for the practical application of this technology, it is necessary to improve the protocols for in vitro production of embryos using juvenile oocytes.

Reichenbach et al. [91] proposed a modification of the L-OPU method, in which access to the ovaries of cows is achieved through the vaginal fornix of cows. The procedure can be performed on animals under epidural anesthesia in less than 15 minutes, does not require surgery, and can be performed in the field.

Another scientific breakthrough in the development of ART as a basis for the development of advanced genetic technologies in cattle was the successful somatic cell nuclear transfer (SCNT). SCNT is a method in which the somatic cell nucleus is transferred to an enucleated oocyte to produce a new individual that is genetically identical to the somatic cell donor [reviews 92-94]. The

production of calves by somatic cloning was first reported in 1993 [95]. Inner cell mass (ICM) cells obtained from blastocysts and cultured from six to 100 days before use were used for cloning. In 1998, the first calves obtained by SCNT using differentiated somatic cells (foetal fibroblasts) were reported [96].

Thus, over a century-old history, various technologies have been developed and implemented into practice in cattle breeding (Table 1), which became the basis for the development of effective technologies for the genetic improvement of cattle, including genomic selection [3, 97].

1. Milestones in the development of assisted reproductive technologies that became the basis for the development of genetic technologies in cattle breeding

Events	Year
I.I. Ivanov: production of offspring after artificial insemination of cows	1899
D. STEWART: birth of calf after insemination by frozen-thawed semen	1951
E.L. WILLETT: birth of first ET calf after surgical transfer of 5-day embryos	1951
R.P. ELSDEN: non-surgical recovery of cows' embryos	1976
R.F. ROWE: non-surgical transfer of cows' embryos	1980
B.G. BRACKETT: birth of calf after *in vitro* fertilization of the oocyte, matured *in vivo*	1981
K. GOTO: birth of calf, produced exclusively from IVP embryo, including IVM, IVF and IVD	1988
C. SMITH: MOET to improve the genetic potential of herds	1988
M.C. PIETERSE: recovery of oocytes from lived animals using ultrasound-guided transvaginal follicular aspiration (Ovum-Pick-Up)	1988
M. SIMS, N.L. FIRST: first birth of calves after SCNT using ICM-cells, cultured *in vitro*, as donor cells	1993
X. VIGNON: first birth of calves after SCNT using differentiated somatic cells as donor cells	1998

N o t e. Ivanov I.I. [17], Stewart D. et al. [25], Willett E.L. et al. [37], Elsden R.P. et al. [38], Rowe R.F. et al. [39], Brackett B.G. et al. [44], Goto K. et al. [45], Smith C. et al. [40], Pieterse M.C. et al. [53 , Sims M., First N.L. [95], Vignon X. et al. [96]; ET — embryo transfer, IVP — in vitro production, IVM — in vitro maturation, IVF — in vitro fertilization, IVD — in vitro development, MOET — multiple ovulation and embryo transfer, SCNT — somatic cell nucleus transfer, ICM — inner cell mass.

The improvement of ART, including IVP and ET technologies, and their introduction into routine laboratory practice, initiated attempts to introduce genetic changes in early embryos of farm animals. At the initial stage of development of transgenic technologies, microinjection of a DNA solution of gene constructs into the pronucleus of zygotes was used for these purposes [reviews 5, 98, 99]. The efficiency of this method for generating transgenic mammals was initially demonstrated in mice [100]. Transgenic farm animals were first reported in 1985 by two laboratories in the United States [101] and Germany [102]. The first transgenic calves carrying the human lactoferrin gene under the control of the bovine alpha-S1-casein promoter were produced in 1991 [103]. In subsequent years, various genetic modifications in farm animals of different species, including cattle, were performed using the microinjection method [review 104]. The main disadvantage of microinjection is its very high labor intensity and low efficiency: (1) to produce a single transgenic calf, more than 1000 zygotes must be injected [105]; (2) only about 70% of transgenic founder animals are able to transmit the transgene to their offspring; and (3) of the obtained transgenic lines, only 50% have an expression level sufficient for subsequent practical use [106]. Due to the high material costs of producing transgenic animals by microinjection, the main goals of genetic modification of domestic animals were shifted from agricultural use to biomedical use, in which higher revenues from implementation are expected [106]. In the mid-90s of the XX century, the method of microinjection into the pronucleus of zygotes was almost completely replaced by the method of SCNT using genetically transformed cells (Fig. 2).

The advantages of SCNT in comparison with the method of microinjection are the ability to select donor cells of a certain sex and the in vitro preselection of cells that carry the specified genetic changes. As a result, 100% of

the produced offspring will have the desired sex and carry the necessary genetic modifications. Another advantage of SCNT in the subsequent application of the technology for agricultural purposes is the possibility of obtaining donor cells from highly productive animals as well as highly reliable prediction of the breeding value of future offspring using genomic estimation [6]. The disadvantages of this method include reduced viability of embryos obtained by SCNT, which is revealed by 60% higher embryonic mortality between 35 and 60 days of pregnancy compared to IVP embryos [108]. With the production of the first transgenic calf in 1998 carrying the reporter genes beta-galactosidase and neomycin [109], the SCNT method has been the dominant method for producing transgenic cattle for more than 15 years.

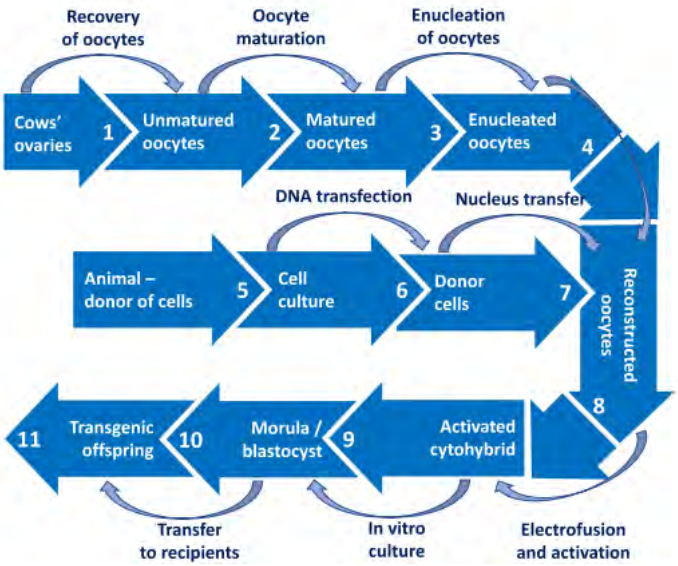

Fig. 2. Scheme for producing genetically modified animals using technology of somatic cell nuclear transfer (SCNT).

Fig. 3. Development of methods for genetic modification of cattle.
N o t e. Krimpenfort P. et al. [103], Cibelli J.B. et al. [109], Chan A.W.S. et al. [110), Kuroiwa Y. et al. [111], Hofmann F. et al. [112], Richt J.A. et al. [113], Yu S. et al. [114], Liu X. et al. [115], Proudfoot C. et al. [116], Wu H. et al. [117], Yum S.Y. et al. [118], Gao Y. et al. [119].

In combination with various ART, several different methods for the production of transgenic animals have been developed for over more than 30 years,

which have been successfully used to generate genetic GE cattle (Fig. 3). However, the use of transgenic technologies in cattle breeding has been limited until recently due to the relatively high cost of producing transgenic cattle, as well as owing to the lack of a reliable method that can ensure the introduction of specific genetic changes in the target genome regions with high efficiency [5, 6].

Further progress in the field of genetic engineering of domestic animals is associated with the development of technologies for GE, which enables the generation of targeted (site-specific) modifications of the genome [120]. Technologies involving DNA transposons [118] and site-specific nucleases, including ZNF "zinc finger" nucleases [114, 115], TALEN-transcription activator-like effector nuclease [116, 117], and CRISPR/Cas9-based systems are used as tools for GE in cattle [120]. Due to the relatively simple creation of gene constructs, the latter are becoming increasingly popular for GE in farm animals [review 7].

Two main methods are used to introduce targeted genetic changes in the germ lines of farm animals by GE, i.e., nucleus transfer of somatic cells (usually, embryonic fibroblasts) previously modified in vitro (see Fig. 2), and microinjection of the RNA form of gene constructs into the zygote. We discussed the advantages of the SCNT method above; however, SCNT is still not a routine procedure in many laboratories [121]. The microinjection method is relatively easy to perform. In contrast to the classical method of microinjection into the pronucleus of zygotes [101, 102], gene constructs are directly into the cytoplasm of zygotes. Although only a portion of animals derived from injected embryos carry the expected genetic changes, the microinjection method has been successfully implemented to create GE cattle [7]. In combination with the OPU/IVP technology—which allows a large number of zygotes to be obtained from parents with high breeding value—the method of microinjection can become the standard for use in programs for genetic improvement of cattle through GE.

Thus, for more than 100 years, significant progress has been made in the development of ART in cattle, and many of these techniques are now actively used in practical animal husbandry and have become the basis for the development of effective programs for genetic improvement of livestock, including genomic selection. Current research priorities are focused on ensuring further progress in cattle breeding by integrating GE technology into livestock breeding programs. ART will play a crucial role in this ambitious task.

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IDENTIFICATION OF "SELECTION SIGNATURES" IN PIGS AND WILD BOARS

(review)

L.V. GETMANTSEVA[1], A.A. TRASPOV[1], N.F. BAKOEV[1], Yu.A. PRYTKOV[1], S.Yu. BAKOEV[1], L.V. PETRIKEEVA[1, 2], O.V. KOSTYUNINA[1]

[1]*Ernst Federal Science Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail ilonaluba@mail.ru (✉ corresponding author), nkvdeshnik@mail.ru, nekruz82@mail.ru, prytkov_y@mail.ru, siroj1@yandex.ru;
[2]*Timiryazev Russian State Agrarian University—Moscow Agrarian Academy*, 49, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail ulreeka@gmail.com
ORCID:
Getmantseva L.V. orcid.org/0000-0003-1868-3148
Traspov A.A. orcid.org/0000-0003-1618-2577
Bakoev N.F. orcid.org/0000-0001-5495-8191
Prytkov Yu.A. orcid.org/0000-0003-0843-1297
Bakoev S.Yu. orcid.org/0000-0002-0324-3580
Petrikeeva L.V. orcid.org/0000-0001-9663-7978
Kostyunina O.V. orcid.org/0000-0001-8206-3221

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Abstract

The pig is one of the few species that has living wild ancestors, which provides a unique opportunity to track the evolutionary history of mammals and determine the "selection signatures" caused by both domestication and natural selection (K. Chen et al., 2007). Animal selection leads to changes in certain regions in the genome associated with economically significant traits, adaptation to climate and stress conditions, immune response and resistance to diseases, and as a result of its pressure, traces are formed in the genome of animals (S.R. Keller et al., 2008), known as selection signatures (M. Kreitman, 2000). Identification of selection prints attracts special attention of evolutionary geneticists, since it can serve as a source of information, ranging from basic knowledge about evolutionary processes to functional information about genes/genomic regions (C. Schlцtterer, 2003; C. Horscroft et al.). The purpose of this review is to summarize approaches used to identify "selection signatures", as well as to analyze the detected traces of selection in domestic pigs and wild boar. The development of modern methods of full-scale research has significantly expanded the arsenal of tools that allow searching for regions subjected to selection pressure at a fundamentally new level. Analysis of data obtained using full-genome resequencing (C.J. Rubin et al., 2012; X. Li et al., 2017), full-genome genotyping on biochips of different densities (M. Huang et al., 2020; M. Mucoz et al., 2019), RADseq (Y. Li et al., 2017), RNA-seq (M. Li et al., 2017; Y. Yang et al., 2018), GBS (Y. Ma et al., 2018; K. Wang et al., 2018) is used to search for selection prints in *Sus scrofa*. Methods are based on scanning areas of homozygosity, as well as evaluating differences in the frequency of alleles or haplotypes between populations or generations within a population. The most commonly used statistical methods for identifying selection prints are extended haplotypic homozygosity (EHH) (P.C. Sabeti et al., 2002), integrated haplotype estimation (iHS) (B.F. Voight et al., 2006), runs of homozygous segments (ROH) (J. Gibson et al., 2006), Fst-statistics (R.C. Lewontin, J. Krakauer, 1973), haplotype-based analysis (hapFLK) (M.I. Fariello et al., 2013), composite selection signal method (CSS) (I.A. Randhawa et al., 2014), and a combination of these methods. Breeding models in pig breeds differ depending on their evolution and breeding history, so studying the "selection signatures" of a large number of different breeds will help to better understand the genetic variations underlying the traits of interest. Based on these methods, large-scale fingerprint scans of diversifying selection have been successfully applied to domestic pigs. In most cases, the research was aimed at studying the evolutionary and selection mechanisms of the genome of Chinese pigs (X. Li et al., 2017; M. Chen et al., 2018). The research found genomic regions that contribute to adaptation to various climatic conditions (R.J. Cesconeto et al., 2017), as well as candidate genes associated with growth, development (K. Wang et al., 2018), reproductive traits (Z. Zhang et al., 2018) and certain aspects of the immune response (S. Yang et al., 2014). Full-genomic research of domestic resources (A. Traspov et al., 2016) showed that pig populations bred on the territory of the Russian Federation, including local ones, are a cultural achievement of domestic animal science and have their own unique structure, even though they

originated with the participation of imported breeds. This may be due to several factors, including differences in origin, long periods of genetic isolation, and differences in climate and food resources. However, domestic breeding resources remain poorly studied at the moment. Thus, the proposed approaches designed to identify "selection signatures" in pigs bred on the territory of the Russian Federation and wild boar can be used to search and analyze the detected traces of selection in domestic pigs and wild boar of domestic origin.

Keywords: pigs, selection prints, domestication, genome-wide genotyping, haplotype, homozygosity

The pig, which belongs to the mammalian order *Artiodactyla*, is one of the first domesticated animals that is important in agriculture as a source of nutritious protein. The pig is also of interest as a biomedical model with high anatomical and immunological similarity to humans [1]. It is one of the few species that have living wild ancestors, which provides a unique opportunity to track the evolutionary history of mammals and determine the "selection signatures" caused by both domestication and natural selection [2].

Natural selection is the process by which populations can adapt, survive, and reproduce in their environment. Individuals with traits that improve viability and reproductive qualities can pass these on to their descendants, which over time affects the increase in representation of useful traits in the population [3]. Individuals with a favorable allele have increased adaptability to environmental conditions and better chance for reproduction compared to individuals lacking this allele [4].

The occurrence of selection creates deviations from the expectations of the neutral theory in models of molecular variability [5]. Each form of both natural and artificial selection causes specific changes in the dependent loci and associated neutral loci. Animal selection leads to changes in certain areas of the genome that affect economically significant traits as well as characteristics related to climate and stress adaptation, immune response, and disease resistance. Therefore, as a result of selection pressure, there are traces formed in the genome of animals [6], which are known as "selection prints" ("signature of selection") that can be used to identify loci subjected to selection pressure [7]. The search for selection signatures has attracted special attention from evolutionary geneticists, who can obtain both basic knowledge about evolutionary processes and functional information about genes/genomic regions [8, 9]. In addition, it provides an opportunity to better understand the history of populations and genetic mechanisms that affect the phenotypic differentiation of wild and domestic animals [10]. Determining the genes that are under selection pressure will make it possible to detect causal mutations in regions previously identified through quantitative trait loci (QTL) mapping experiments, and genes associated with environmental traits (such as adaptation) that are difficult to find experimentally [5]. Such studies will be useful when searching for genes or gene networks that play an important role in the formation of the same phenotypic traits but vary among breeds. They can also reveal the genes involved in the domestication process [8, 11].

Domestic pigs (*Sus scrofa*) have significant differences in morphological, behavioral, and environmental characteristics [12]. The use of this species in a wide variety of production systems around the world has led to a huge variety of breeds, each of which is adapted to specific conditions [5]. The search for signature of selection in pigs will help to show the genetic determination of quantitative traits and the mechanisms of adaptive reactivity of the species.

The purpose of this review is to describe the approaches used to identify signature of selection, as well as to analyze the detected selection traces in domestic pigs as compared to wild boar.

With the development of modern genome-wide research methods, the variety of tools that allow searching for regions that are subjected to selection pressure has significantly expanded. Full-genome resequencing to identify "selection prints" in *S. scrofa* [13, 14] was performed using biochips of different densities: Affymetrix Axiom Pig1.4M (Affymetrix™, Thermo Fisher Scientific Inc., USA) [15], GeneSeek® Geno-mic Profiler (GGP) 70 K HD Porcine chip (Illumina, Inc., USA) [16-18], Illumina Porcine SNP60 BeadChip (Illumina, Inc., USA) [19-21], and also restriction-site associated DNA sequencing (RADSeq) [22], RNA sequencing (RNA-seq) [23, 24], and genotyping by sequencing (GBS) [25, 26].

Recognizing the molecular traces left by different types of selection is the main task in identifying the parts of the genome that are being selected. In this case, the neutral theory serves as the basis for statistical tests designed to detect selection traces. However, in natural populations, some assumptions of the neutral theory can be violated (for example, as a result of population growth, migration, and "bottleneck"), which leads to signals that mimic selection traces [5]. The interaction between different types of selection and between selection and demographic factors can shift the traces of such processes left in the genome [5, 7, 27]. In this regard, it is worth noting that when detecting traces of breeding in farm animals, a significant number of false positive results are expected due to genetic drift and the founder effect, which are especially important in the formation and development of breeds [28]. It is also important to distinguish positive selection prints from those formed as a result of neutral evolution, and to select appropriate statistical tests and software for screening them to determine the genomic regions involved in adaptation processes [29].

There are several approaches for detecting selection signatures. As a rule, they are based on the search for homozygous regions and the assessment of differences in the frequency of alleles or haplotypes between populations or generations within the population [30]. Several methods were developed for the statistical processing of the data obtained.

Extended haplotype homozygosity (EHH) [31-33] is the maximum value for a small number of different haplotypes with unequal frequency distribution. This test reveals selection traces by comparing the base (main) haplotype, which is characterized by high frequency and extended homozygosity, with other haplotypes at the selected locus.

The integrated haplotype score (iHS) [34] was proposed for genomic scale work based on information obtained using high-density single nucleotide polymorphism (SNP) chips. The iHS value shows how unusual the haplotypes around the SNP are compared to the genome.

Runs of homozygosity (ROH) [10] were first introduced by Gibson et al. [35] as adjacent homozygous segments in the genome that are present in an individual due to transmission of identical haplotypes from parents to offspring. The frequency of ROH varies widely within and between chromosomes; along with hot spots ("islands"), ROH is met with cold spots ("deserts") [36]. The reasons for this are of great interest, since the distribution of ROH across chromosomes is not random [37, 38]. The number of ROHs and their size distribution are important determinants of long-standing and recent events in the population. Long sections of ROH appear more often in regions with low recombination located in the middle of the chromosome, and the smallest ROH with higher density is distributed in the direction of telomeric regions [37]. Accordingly, the presence of long sections of ROH indicates a recent common ancestor. Conversely, shorter ROH regions indicate greater temporal distance from the common ancestor. ROH identification and characterization provide insight into how population structure and

demographic events evolved over time. In addition, ROH has recently been increasingly used in the search for genome regions associated with selection pressure. It is assumed that most of the genome is in the selection process, while all functional sites in the genome are under the pressure of selection [39] or adaptive evolution [40]. One strength of ROH analysis is that long homozygous segments can be reliably identified even with relatively low marker densities [10].

Haplotype-based analysis (hapFLK) [41-43], unlike most existing statistical data processing methods, accounts for the hierarchical structure of the selected populations. Using computer modeling, Fariello et al. [44] showed that using information about haplotypes and the hierarchical structure of populations significantly increases the power of detecting selected loci, and combining them in hapFLK statistics provides even greater efficiency. It has also been demonstrated that the hapFLK method produces reliable results in bottleneck and migration conditions, and in many other cases exceeds the existing approaches.

The composite selection signals (CSS) method [45-47] combines three different approaches: determining the fixation index (F_{ST}) of population differentiation (allows the detection of selection traces from genetic polymorphism data by paired comparison of two modern populations); evaluating changes in the frequency distribution of derived allele frequencies (ΔDAF) or changes in the direction of the selected allele frequency (ΔSAF); and statistics of extended haplotype homozygosity (EHH), depending on the frequency of the allele and the strength of the linkage disequilibrium (LD) with neighboring loci.

The FST, first defined by Wright [48, 49], is a measure that uses differences in allele frequencies to determine genetic differentiation between populations or generations [50]. Akey et al. [51] proposed using loci in the tails of the empirical FST distribution as potential selection targets.

Tests based on LD [25] are used to detect selection traces in pigs. Usually, several breeds are compared, and the genetic basis of various characteristics of the breed, such as productivity [52-54], morphology [55], and adaptation to local conditions such as climate [56, 57], are put at the forefront. Selection signatures associated with growth traits, reproductive qualities, coat color, or ear shape were found, and several genes that significantly influence these traits were identified [18, 13]. However, the selection models of pig breeds differ depending on their evolution and demographic history, therefore, studying the selection signatures of a large number of breeds will help to better understand the genetic mechanisms that cause the manifestation of interesting traits.

Attempts were made to analyze the mechanisms underlying the phenotypic differentiation of pigs caused by selection pressure [58-60]. Gurgul et al. [61] found selection traces at the whole genome level in three native populations of pigs (Puławska, Złotnicka White and Złotnicka Spotted) and the Polish Landrace. To identify the selection prints in the analyzed breeds, they applied the method based on FST and aimed at detecting selection diversification among breeds, as well as relative extended haplotype homozygosity (REHH) statistics [31, 62], allowing the detection of permanent selection in the breed. It was shown that both FST and REHH statistics are useful for detecting selection prints [63] and largely complement each other. The REHH test can detect selection prints within breeds with high efficiency and is more accurate in the case of ongoing selection, whereas the F_{ST} is useful for detecting selection prints among breeds represented mainly by loci that are differentially fixed in different breeds [64].

Li et al. [65] found selection prints in native Chinese pigs by comparing variations using the non-equilibrium coupling method (LD) [66]. Yang et al. [19] performed genome-wide scanning of selection prints in Chinese local and commercial breeds using High-FST and identified 81 candidate genes with a high

degree of confidence of positive selection. In addition, the gene network analysis results showed that genes of traits subjected to positive selection were mainly involved in the development of tissues and organs, and in the immune response [17]. Rubin et al. [13] used pig genome sequencing (S.scrofa10.2) [67] and re-sequencing of the entire genome of domestic pigs and boars to identify loci that were subjected to selection pressure during and after domestication. This study was based on the search for genetic variants with noticeable differences in the frequency of alleles between populations of pigs and wild boar. The strongest selection fingerprint (ZHp = -5.82) was present on the 1st chromosome of *S. scrofa* (SSC1) for the locus that includes the *NR6A1* (nuclear receptor 6 A1) gene. Also significant was the region on SSC4 (ZHp = -5.77), which includes *PLAG1* (pleomorphic adenoma gene 1), and the region on SSC8 (ZHp = -5.29), which covers the entire coding region of the ligand-dependent corepressor-like nuclear receptor (*LCORL*). The *NR6A1* gene is associated with the number of vertebrae in pigs (boar usually has 19, and pigs have up to 21) [68]. The melanocortin receptor 1 (*MC1R*) gene was identified as an artificial selection gene associated with coat color in domestic pigs in China [69].

Genetic adaptation to different climatic conditions has formed distinct thermoregulatory mechanisms for high and low temperatures, which are mainly manifested in pigs of local breeds. To identify genomic loci that contribute to adaptation to various climatic conditions, Ai et al. [57] studied Chinese pigs from the southern and northern regions. A total of 774 regions located on autosomes and the X chromosome were identified. Ontology analysis identified genes that are involved in biological processes that contribute to maintaining thermoregulation during heat or cold. These processes are associated with hair development, differentiation of neurons in the thalamus, kidney development, energy exchange, and blood circulation. For example, genes involved in hair cell differentiation (*ATOH1*, *JAG1*, and *RAC1*) and maturation of hair follicles (*BARX2* and *TBC1D8*) were isolated. This is consistent with the fact that southern Chinese pigs have sparse, short hair, which facilitates heat loss, whereas northern Chinese pigs usually have long, thick hair that forms a dense coat. The genes involved in the differentiation of neurons in the thalamus (*DLX1*, *DLX2*, *RAC1*, *ROBO1* and *SALL1*) are described, and explain the important role of the nervous system in acclimatization. The *BMP4*, *BMP7*, *MYC*, *SALL1*, *SPRY1*, and *KLHL3* genes are responsible for processes that affect kidney development, which may be associated with a tendency to increase kidney mass at low ambient temperatures and decrease it at high [70]. Genes related to blood circulation are also known, and are involved in the development of arteries (*BMP4*, *CITED2*, and *JAG1*), and the embryonic heart tube (*CITED2*, *INVS*, *RYR2*, *SUFU*, and *TBC1D8*). In the process of temperature adaptation, biological mechanisms that ensure blood flow play an important role, since heat stress can lead to an increase in the number of platelets and blood viscosity, which in turn increases the risk of cerebral and coronary thrombosis [71]. In southern Chinese pigs, a missense-mutation in the *VPS13A* gene was found to reduce the risk of thrombosis by modulating the number of platelets and blood viscosity.

The search for selection signatures related to climate conditions was performed in Brazilian pigs by Cesconeto et al. [72]. As a result of their work, genomic regions were identified that contribute to the adaptation of pigs to various environmental conditions: temperature, precipitation, and solar radiation.

Traditional breeding programs for the most common commercial pig breeds (Large White, Yorkshire, Landrace, Duroc, and Pietren) focus mainly on growth rates and feed conversion. Thus, Wang et al. [25] found in the Landrace

and Yorkshire breeds, 540 potential regions (50 kb) controlling these traits, which contained 111 genes.

Most candidate genes were associated with growth, development, and other aspects of the immune response (*COL11A1*, *GHR*, *IGF1R*, *IGF2R*, *IFNG*, and *MTOR*), and only a few were associated with meat quality (*ACACA* and *MECR*). Chinese pigs are characterized by slow growth rates, the ability to quickly accumulate fat deposits, high meat quality indicators, and earlier puberty. A study of selection prints performed on Large White pigs [65], Chinese breeds, and South American native pigs [73, 74] identified four genomic regions on the 7th, 9th, 13th, and 14th chromosomes. For Chinese pigs, a significant region localized on SSC14 included the genes *MORC2*, *SMTN*, *INPP5J*, *PLA2G3*, and *RNF185* associated with the content of linoleic acid [75, 76], one of the polyunsaturated fatty acids, the content of which is characterized by a high positive correlation with the flavor of pork meat [77, 78]. This region was also associated with the early sexual maturity characteristic of Chinese pigs [79]. In the study of Li et al. [65] selection signatures were also identified for Large White pigs, located on the 7th and 9th chromosomes. One interesting gene found in these regions is *ADAMTSL3* (SSC7], which is a candidate gene that determines the length of the trunk [80]. In the works previously conducted by Wilkinson et al. [20] and Li et al. [81], this gene was also recognized as a significant selection signature for European commercial breeds of pigs.

Zhang et al. [11] studied the genetic basis of phenotypic differences between Chinese and Western pig breeds. Numerous genes (*IGF1R*, *IL1R1*, *IL1RL1*, *DUSP10*, *RAC3*, *SWAP70*, *SNORA50*, *OR1F1*) related to growth, immunity, smell, reproduction, and meat quality were identified as differentiated candidate genes that could be associated with phenotypic differences in Western and Chinese pigs. Evaluation of FST signals allowed us to identify differentiated features in Chinese pig breeds. There were 75 genes near strong FST signals. The most significant SNPs were located near the *JPH3* (SSC6) gene associated with skatole content [82] and meat quality indicators [83]. On the 4th chromosome, the signal was detected in the *ZFPM2* gene, for which Zhao et al. [84] established a connection with pig scrotal hernias. On the 15th chromosome, a signal was detected near the *CNTNAP5* gene, previously noted by Rohrer et al. [85] as a candidate gene for the number of vertebrae in pigs. Expression mapping helped to identify the genetic basis of phenotypic features of Western pigs (growth, feed intake, meat yield, fat thickness) and Chinese pigs (good adaptation, immunity, high quality of meat, and reproductive qualities).

Wang et al. [25] showed that pigs of the Taihu, Meishan, Fengjing, Shawutou, Erhualian, Jiaxing Black, and Mizhu breeds were subjected to less intensive selection in contrast to Western commercial breeds. Different regions of their genomes were subjected to selective pressure. For Western pigs, more pressure was directed to the areas that cause signs of fattening and meat production (growth indicators, fat thickness, precocity, and muscle depth). The selection signatures showed a significant number of genes involved in lipid metabolism and reproduction, which is expected, given the breeding programs of commercial breeds of pigs aimed at increasing fattening productivity and reproductive function. In the Taihu pig genomes, most regions were associated with reproduction and relatively high disease resistance.

The unique feature of the Laiwu pig breed, associated with a high content of intramuscular fat, led to their use as model animals in determining the selection prints for fat deposition in muscles and identifying genes associated with the formation of intramuscular fat. In the work of Chen et al. [86], the search for genomic regions in pigs of Laiwu (n = 50) and Yorkshire (n = 52) breeds was

performed using three methods. The length of the genomic regions identified by at least one method was 465 Mb. On SSC8, one region (2.75-3.00 Mb) was identified in all three ways. From the results of at least two methods, 175 candidate regions were identified and unevenly distributed; most are located on SSC8 (86 regions) and none on SSC12. A total of 438 genes were identified in these regions, including *NPY1R*, *NPY5R*, *PIK3R1*, and *JAKMIP1* associated with feed intake and fat deposition, *ESR1* and *PTHLH* associated with reproductive functions, and *CXCL2*, *CXCL8*, and *TLR2* associated with immune responses. In addition, approximately 25% of the signals were registered in intergenic regions, which indicates an important function of non-coding sequences in the selection process. An additional study of the functions of annotated genes showed that the most significant genes involved in the regulation of metabolic processes, cell proliferation, feeding behavior, immunity, pathways for transmitting signals of the epidermal growth factor receptor, and neuropeptide. It should be noted that in other studies, some of these functional genes have also been observed as being under selection pressure or related to energy balance.

Zhu et al. [87] identified 14 genomic regions associated with selection during domestication in Chinese pigs (16 boars were selected from 7 locations in China and 54 individuals from 9 Chinese native breeds: Bamaxiang, Erhualian, Hetao, Jinhua, Luchuan, Wuzhishan, Neijiang, Bamei, Baoshan). Products of genes localized in these regions are functionally involved in the metabolic processes that ensure growth and development, reproduction, smell, behavior, and activity of the nervous system of animals. The most interesting genes are *TBX19* (involved in metabolic changes and development of Chinese domestic pigs) and *AHR* (associated with sow reproduction).

Based on the analysis of functional enrichment of the regions of the "selection signatures" [88], 449 protein-coding genes were identified in Rongchang pigs. Among them, 10 genes (*CNTN4*, *DLL3*, *GHSR*, *LHX5*, *MAP1B*, *MBP*, *METRN*, *NUMBL*, *TNFRSF12A*, and *REST*) were identified in supporting brain development, neuron function, and behavior. These results support the view that mutations associated with reduced fear and aggression towards humans are at the heart of domestication [89]. In addition, four genes (*CYP2A6*, *GMPS*, *UPB1*, and *UPP2*) were identified that are responsible for the metabolism of narcotic drugs, which is probably due to the constant exposure of domestic pigs to high doses of chemicals.

Studies of local pig breeds carried out in several countries have shown [90] that many of them retain unique features and differ from the main commercial breeds, such as the Large White and Landrace breeds. This may be due to differences in the origin of populations, long periods of genetic isolation, and differences in climate and available food between Western Russia, Belarus, Ukraine, and Western Europe [90].

Therefore, the analysis of the literature data demonstrated a clear interest in the in-depth study of the genetics of the *Sus scrofa* species. Various genomic and statistical methods based on the search for homozygosity regions and the estimation of differences in the frequency of alleles or haplotypes between populations have been tested. Most often, extended haplotypic homozygosity, integrated assessment of haplotypes, identification of extended homozygous segments, FST-statistics, haplotype-based analysis, the method of composite selection signals and their combination are used for the analysis of selection prints in pigs and boars. Studying the selection prints of many different breeds will help to better understand the genetic variations underlying the traits of interest. However, most of the work is carried out on Chinese pigs, while Russian breeding resources remain

poorly studied. Therefore, the use of the described approaches for identification of selection prints in pigs, including those bred in the territory of the Russian Federation, and wild boar is a necessary condition for the effective development of pig breeding.

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GENOMIC VARIABILITY ASSESS FOR BREEDING TRAITS IN HOLSTEINIZATED RUSSIAN BLACK-AND-WHITE CATTLE USING GWAS ANALYSIS AND ROH PATTERNS

A.A. SERMYAGIN[1], O.A. BYKOVA[2], O.G. LORETTS[2], O.V. KOSTYUNINA[1], N.A. ZINOVIEVA[1]

[1]*Ernst Federal Science Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk, Moscow Province, 142132 Russia, e-mail alex_sermyagin85@mail.ru (✉ corresponding author), kostolan@mail.ru, n_zinovieva@mail.ru;
[2]*Ural State Agricultural University,* 42, ul. Karl Liebknecht, Ekaterinburg, Sverdlovsk Province, 620075 Russia, e-mail olbyk75@mail.ru, rector.urgau@yandex.ru
ORCID:
Sermyagin A.A. orcid.org/0000-0002-1799-6014
Bykova O.A. orcid.org/0000-0002-0753-1539
Loretts O.G. orcid.org/0000-0002-9945-5691
Kostyunina O.V. orcid.org/0000-0001-8206-3221
Zinovieva N.A. orcid.org/0000-0003-4017-6863
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Abstract

For using the pedigree information recorded in dairy herds rational, the creation of reference groups with a high reliability the estimated breeding value of animals is required. This is a necessary requirement not only for the genetic assessment procedure, but also for the introduction of genomic selection methods. However, without high-quality phenotyping cannot be this achieved. Therefore, it seems relevant to conduct model studies using the highly productive herd of Holsteinizated Russian Black-and-White cattle in the Urals as an example to study the genomic variability of phenotypic traits in animals' different generations using data from genome-wide analysis (GWAS) and runs of homozygosity (ROH), as well as to replenish the Russian reference population. The novelty of the work is to assess the dynamics of genomic inbreeding variability (F_{ROH}) in animal generations ("dam of mother"—"mother"-"daughter") and its comparison with direct genomic value (DGV), as well as the search for new mechanisms to confirm the scientific hypothesis of using a limited experimental dataset in GWAS. The object of this research was 76 cows and heifers of Holsteinizated Russian Black-and-White breed, as well as 9 Holstein sires genotyped for 139 thousand SNPs by the Bovine GGP HD platform (Illumina/Neogen, USA). To calculate the DGVs of studied animals the Russian reference bulls and cows' group that include 591 individuals was used. GWAS and ROH analyzes were performed based on 110448 SNPs. The reliability (p-values) of genome-wide associations with direct cows' phenotypes ranged from 2.31×10^{-5} to 1.08×10^{-7}. Quantitative traits loci on autosomes BTA1, BTA2, BTA5, BTA7, BTA8, BTA10, BTA11, BTA12, BTA14, BTA16, BTA20, BTA21, and BTA26 were found. For milk yield a region on BTA14 (1.44-1.59 Mb) with the genes *ZNF16*, *ARHGAP39*, *ZNF7* associated with an increased fat milk yield was detected. For the number of inseminations found SNPs included into the genes (*ARHGAP31*) or located close to the genes (*SERPINA5*) and associated with the growth intensity to a mature state, as well as ovarian function in animals. The characteristic of ROHs depending on the length of their fragments in the genome is given. Conservative homozygous regions on BTA12, BTA14, BTA26, and BTA29 and the most significant genes entering them were identified, which are potentially associated with selection pressure in the studied population mainly by milk production traits, reproduction, and udder type measurement parameters. The value of F_{ROH} significantly ($p < 0.05$-0.001) increased in the offspring—parent generations: by +0.012 or 1.2 % for mothers, and +0.029 for daughters. The highest values of $F_{ROH} = 0.135$ were noted for bulls that were signed as fathers of cows (generation of "mother") and heifers (generation of "daughter"). Each subsequent generation of individuals showed an average increase in DGV for milk yield by +94.2 kg, fat milk yield by +4.4 kg and protein milk yield by +3.0 kg, reflecting clear the strategy to improve milk production traits in the herd for obtaining new cattle genotypes. Thus, the possibilities for assessing the variability of direct cattle phenotypes, as a model for studying

genomic variability in a single herd, based on the search for associations and loci in the genome under selection pressure are shown.

Keywords: cattle, GWAS, ROH, reference population, genomic inbreeding, genomic evaluation, milk production, fertility

Over the past century, methods for improving animal populations have undergone a number of changes: from selection of individuals by their ancestors and phenotype, to the offspring's quality evaluation, and further on by the totality of genetic markers. The key to the successful development of genomic selection approaches is to obtain accurate phenotypic information on economic and biological traits. Therefore, the efficiency of further measures of improving the genetic potential in dairy cattle breeding depends both on the quality of pedigree records and on the chosen strategy for animal breeding in the population. Datasets accumulated in cattle herds are increasing annually. The genomic passport is added to the pedigree background and phenotype of the animal, which allows you to clarify the origin of the individual and its genetic value [1, 2].

Studies on the genomic architecture of animal productivity traits are actively being conducted both in Russia and worldwide. The characteristic of variability of quantitative and qualitative parameters can be measured in the components that are genetic, phenotypic, and adjusted for a number of regression factors. For example, previous genome-wide associative studies (GWAS) on the holsteinized Black Pied livestock population of Moscow Oblast and Leningrad Oblast showed the possibility of using estimated breeding values (EBV) in searching for single nucleotide polymorphisms (SNPs) associated with QTL. Significant mutations were found in the genes DGAT1 ($p = 6.8\times10^{-22}$), PLEC ($p = 6.9\times10^{-20}$) and GRINA ($p = 4.2\times10^{-10}$) and they are associated with the fat content in milk [3]. GWAS among different populations of dairy cattle in the USA, Germany, Holland, Australia and China revealed a pool of common genes responsible for indicators of milk productivity of cows and the amount of milkfat in particular: DGAT1, SCD1, GHR, EPS8, GPAT4, casein cluster genes (Hapmap24184-BTC-070077) [4-7]. It is worth noting that the pleiotropic effect of a gene was observed in the Holstein cattle population of Chinese origin according to DGAT1, in terms of the content and yield of milk protein and milk yield [8].

Simultaneously, there are studies on using absolute phenotypic values or "direct" phenotypes in GWAS. Highly reliable associations with the main genes were obtained for meta-analysis of complex of milk productivity traits, fertility and body type of dairy cattle [9], and for evaluation of feed efficiency in beef cattle [10], which indicates genes' determination of phenotypic variability. Similar studies are conducted on Large White, Landrace, and Duroc pigs, showing moderate convergence in the search for quantitative trait loci (QTL) in animals belonging to different populations [11, 12, 13]. It is worth noting that the main factor of GWAS in this case is the accuracy of phenotyping of characters and strict consideration of environmental factors for each single experiment. The practice of using data from long-term observations is more typical for research herds in Europe, North America and Australia with the aim of obtaining "pure" animal phenotypes not just for standard productivity traits, but also for indicators requiring laborious accounting (methane emission, residual feed intake, fat and amino acid composition of milk and meat, etc.) [14].

Another powerful tool of genomic analysis is the assessment of animal individual autozygosity or extended homozygous nucleotide fragments — ROH (Runs of Homozygosity) patterns. Using an example of analysis of demographic events in populations of Pinzgauer, Brown Swiss and Tyrolean cattle breeds, thresholds are determined by ROH length categories, based on the probability of inheritance of genome segments from a common ancestor for 50, 25, 6 and 3

generations ago (respectively, with ROH >1, >2 , >8 and >16 Mb) [15]. Fixation of ROH with a low frequency of recombination of loci in animal generations may indicate formation of gene clusters under evolutionary pressure – primarily, positive selection for productivity traits, as well as adaptation to changing environmental conditions [16, 17]. Nevertheless, ROH patterns allow us to evaluate the level of genomic inbreeding (F_{ROH}) both in a population (herd) and for a single animal more efficiently, relative to the pedigree information. Thus, the average variability of the F_{ROH} values between four herds of Russian Black Pied cattle of the Leningrad Oblast was 5.5-8.0% for the 46-85 selected animals [18].

The abovementioned GWAS and ROH results are more relevant to the study of mechanisms of variability of the genetic architecture of quantitative and qualitative traits in animals. The practical direction of using genomic data is focused on developing methods for early selection of the best individual genotypes, i.e. the genomic selection. This approach is a routine procedure worldwide, but in Russia it is at the stage of development and implementation. The first results show that the values of the genomic forecast should be no lower than +900 kg of milk, +31 kg of milkfat and +23 kg of protein for the young bulls of Russian origin that are being evaluated in order to use them in the reproduction of herds [19].

The scientific novelty of this work, built as an experiment, is the integration of genomic analysis and forecasting methods for animals of three generations that are simultaneously in a highly productive herd, with the aim of developing a model for their selection by a set of indicators – the level of genomic inbreeding and genomic breeding value in the early stages. Approaches for testing the hypothesis about the possibility of using direct phenotypic data on cattle productivity in searching for associations with individual nucleotide mutations and determining loci in the genome under evolutionary pressure are considered. For the first time on the basis of the Russian reference group of stud bulls and cows, it has been shown that its expansion can be achieved by including a population of holsteinized Russian Black Pied cattle from Ural.

The objective of our comprehensive research was to search for genome-wide associations, as well as loci that are associated with the intensity of selection in the population of holsteinized Russian Black Pied cattle from Ural for characterizing the genomic variability of trait of milk production and fertility of animals belonging to different offspring generations.

The research aims were: i) to conduct genotyping of 138 thousand SNPs of stud bulls of the Holstein breed and Holsteinized Russian Black Pied cows and heifers in the generations of mother's mother—mother—daughter, owned by PJSC "Kamenskoye", Sverdlovsk Province. In accordance with the biochip design, determine the carriage of valuable alleles of genes associated with the qualitative and quantitative composition of milk, as well as recessive mutations that cause loss of reproductive ability; ii) to characterize GWAS with direct indicators of productivity and reproductive qualities of cows in order to search for loci of quantitative traits in the genome of Ural cattle; and iii) to evaluate the level of genomic inbreeding in generations for the following groups: mother's mothers—mothers—daughters and bull-fathers of cows and heifers, in order to clarify the information in the analysis of the selection results of parental pairs, as well as the search for regions in the genome (ROH) that are subjected to evolutionary pressure. To calculate DGV for traits of milk production and to study the dynamics of its change in different generations of descendants.

Materials and methods. The Bovine GGP 150K biochip (Illumina/Neogen, USA) with overlap of 138974 SNP was used for the animal genotyping procedure. Based on the results of quality control of reading genomic information and conducting pedigree counting, genotypes with a presence of at least 110,448 SNPs for

85 animals were selected, including 9 stud bulls of Holstein breed. (OJSC "Uralplemcentr"), Holsteinized Russian Black Pied cows and heifers, which are descendants of these bulls, in the amount of 76 animals (PJSC "Kamenskoye" in Sverdlovsk Province). The average proportion of Holstein genes in the sample of Russian Black Pied breed was 97.7% with fluctuations from 75% to 99%. In order to study the dynamics of accumulation of homozygosity and the genomic breeding value in generations, experimental groups of animals were formed and individuals were selected in three generations of the ancestors and their descendants: mothers' mothers (25 cows, 2012-2014 years of birth), mothers (30 cows, 2015-2017 years of birth) and daughters (21 heifers, 2018-2019 years of birth). The milk productivity in the mothers' mothers and mothers groups was, on average, 7666 and 8461 kg of milk per 305 days long lactation, with 3.95% and 3.99% of mass fraction of fat, 3.20% and 3.21% of mass fraction of protein, 302.0 and 338.1 kg of milkfat, as well as 245.0 and 274.4 kg of milk protein.

The editing of biochip data for the construction of adapted extension files (.ped, .map, .fam, .bed, .bim), as well as the calculation of genome-wide associations, was performed in the Plink 1.9 program [20]. The distribution diagrams of GWAS and ROH on the chromosomes were constructed using the "qqman" and "ggplot2" packages in the "R" programming and visualization language. The averaged indices of "direct phenotypes" of 44 cows for 1-5 complete lactations were used in the GWAS. Based on the biochip design (https://genomics.neogen.com/pdf/slicks/ggp_bovine150k.pdf), selection-significant point mutations included in genes and having polymorphic variants of genotypes were pre-selected: *DGAT1* (diacylglycerol O-acyltransferase 1); *LEP* (leptin); *kCSN_A(CE)* (kappa-casein (A, C, E alleles); *kCSN_AB* (kappa-casein (A, B alleles); *HH1, HH3* (fertility haplotypes); *GHR* (growth hormone gene receptor); *CSN2_I* (beta-casein (allelic variant I); *ABCG2* (ATP-binding cassette of the G subfamily); *BLG* (beta-lactoglobulin); *YellowFat* (accountable for changes in the carotene content in fat cells).

The ROH patterns were studied using the cgaTOH program [21] in a combined selection of cows (grandmothers, mothers), heifers (daughters) and bulls-fathers for calculating the genomic inbreeding coefficient in each group. The conditions were accepted according to the Ferenčaković method [15], which provided for the differentiation of ROH into groups, according to their length in connection with the moment of the occurrence of a demographic event: [1; 2], (2; 4], (4; 8], (8; 16] and > 16 Mb. The following results were visualized and the common ROH segments that were found with the highest frequency in at least 30-40% of animals were determined (due to the small experimental selection sample, the threshold was reduced to 30%).

Summary of the identified associations between SNPs and phenotypes of animals, as well as the localization of ROH patterns and genes included, was performed by the cattle genome assembly Bos_taurus_UMD_3.1.1 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000003055.6, access date 01.30.2020). The "CattleQTLdb" database [22] was used for searching the QTL and regions under evolutionary pressure on chromosomes that are interfaced with the functional characteristics of animals.

The calculation of genomic estimates of breeding value (DGV) based on the sum of SNP markers for a selected population of the three generations brood stock and bulls according to traits of milk productivity was carried out on the basis of the Russian reference group of animals in the amount of 591 animals [19], according to the GBLUP algorithm proposed by VanRaden [23].

Results. At the first stage of studies on a selected population of the herd, the frequencies of genes associated with milk composition and animal fertility, the

level of polymorphism, and their genetic equilibrium in the Holstein cattle population in Ural were determined (Table 1).

1. Estimation of occurrence frequency of breeding-valuable genetic markers in the studied cattle population of Holsteinized Russian Black Pied breed (n = 85, PJSC "Kamenskoye", Sverdlovsk Province, 2019)

Gene	Frequency	Genotypes			Allele frequency		χ^2	*Ca*
		11	12	22	1	2		
DGAT1	O	0.389±0.035	0.474±0.036	0.137±0.025	0.626	0.374	0.014	0.532
	E	0.392	0.468	0.140				
LEP	O	0.200±0.021	0.547±0.026	0.253±0.022	0.474	0.526	1.816	0.501
	E	0.224	0.499	0.277				
kCasein_A(CE)	O	0.792±0.017	0.208±0.017	0	0.896	0.104	3.894	0.813
	E	0.803	0.187	0.010				
kCasein_AB	O	0.531±0.021	0.396±0.020	0.073±0.011	0.729	0.271	0.734	0.605
	E	0.532	0.395	0.073				
HH3	O	0.990±0.004	0.010±0.004	0	0.995	0.005	0.008	0.989
	E	0.990	0.010	0				
HH1	O	0.979±0.006	0.021±0.006	0	0.010	0.990	0.032	0.979
	E	0.979	0.021	0				
GHR	O	0.646±0.035	0.333±0.034	0.021±0.010	0.813	0.187	0.849	0.695
	E	0.660	0.305	0.035				
CSN2_I	O	0.990±0.005	0.010±0.005	0	0.995	0.005	0.005	0.989
	E	0.990	0.010	0				
ABCG2	O	0.292±0.033	0.510±0.036	0.198±0.029	0.547	0.453	0.085	0.504
	E	0.299	0.496	0.205				
BLG	O	0.095±0.012	0.432±0.021	0.474±0.021	0.311	0.698	0.018	0.572
	E	0.096	0.428	0.475				
YellowFat	O	0.990±0.004	0.010±0.004	0	0.005	0.995	0.011	0.989
	E	0.990	0.010	0				

N o t e. Coding options for genotypes and allele frequencies are given here. *HH1* and *HH3* are indicated according to the chip design. Ca is Robertson homozygosity coefficient, O and E are observed and expected frequencies.

It was found that polymorphic variants of genotypes were found for all detected point mutations in the genes, with a variation in the level of homozygosity (according to Robertson) from 0.501 to 0.989. A slight deviation from genetic equilibrium (at a threshold up to $\chi^2 = 3.840$, $p < 0.05$, when equilibrium is not disturbed) was found for the kappa-casein gene of the allelic variant *A(CE)* ($\chi 2 = 3.894$). Carriage of recessive mutations in the heterozygous state with occurrence frequencies of 1.0% and 2.1% was established for the *HH1* and *HH3* fertility haplotypes, respectively.

The desired genotypes (including heterozygotes) for milk protein genes, the casein kappa form, were 20.8% (AE genotype of the kCasein_A gene(CE), 7.3% (BB genotype of the kCasein_AB gene), and only 1.0% for the beta form (A1I genotype of the CSN2_I gene) , respectively, which is generally specific for the Holstein cattle population, which was designed for production of significant volumes of whole milk, in comparison with the products of its processing (higher yield of cheese, cottage cheese, butter). The *DGAT1* and *ABCG2* genes, associated with the percentage yield of fat and the amount of milk protein, obtained a moderate frequency distribution of the desired genotypes for the alternative allele 22 (AA) — 13.7% and 19.8%, respectively, which indicates hereditarily predetermined aspects of selection according to the quantitative composition of milk components (the frequencies of the desired alleles for each of the genes were 0.374 and 0.453, respectively). The beta-lactoglobulin (BLG) gene is significant in controlling the allergenic properties of cow milk as a part of human nutrition; the occurrence frequency of the most desirable genotype 11 (AA), which was 9.5% or 31.1% for the allele 1 in the studied population, which indicates the potential for animal selection when creating herds that are specific for this gene in production of milk with low allergenic properties.

Along with indicators of milk productivity, the animal growth and development and the ability of intense fattening (bulls) are of no small importance. The

obtained data show that for livestock genotypes of the leptin gene and growth hormone gene receptor, the desired allelic variants were 20.0% (LEP_TT) and 64.6% (GHR_AA), respectively.

In order to confirm the hypothesis about the genomic dependence of the "direct" phenotypes (indicators of own productivity) on the traits of milk productivity and fertility of cows, the GWAS was performed. The division into generations of grandmothers, mothers and daughters was carried out in order to take into account the influence of the population structure with a limited set of observations, and also to identify segments in the genome that have a common identity by origin. The increase in average productivity for 1-5 lactations per generation amounted to +795 kg of milk, + 0.04% of mass fraction of fat, +0.01 of mass fraction of protein, +36.1 kg of milkfat, +29.4 kg of milk protein. Multiple analysis of variance in order to determine the influence of paratypical factors on the variability of characters showed that the year of calving, the farm and the bull-father did not have substantial significance, except for the mass fraction of protein index ($p < 0.05$ to $p < 0.001$). In this regard, it was decided to use "direct" cow phenotypes for GWAS without adjusting for differences in environmental factors.

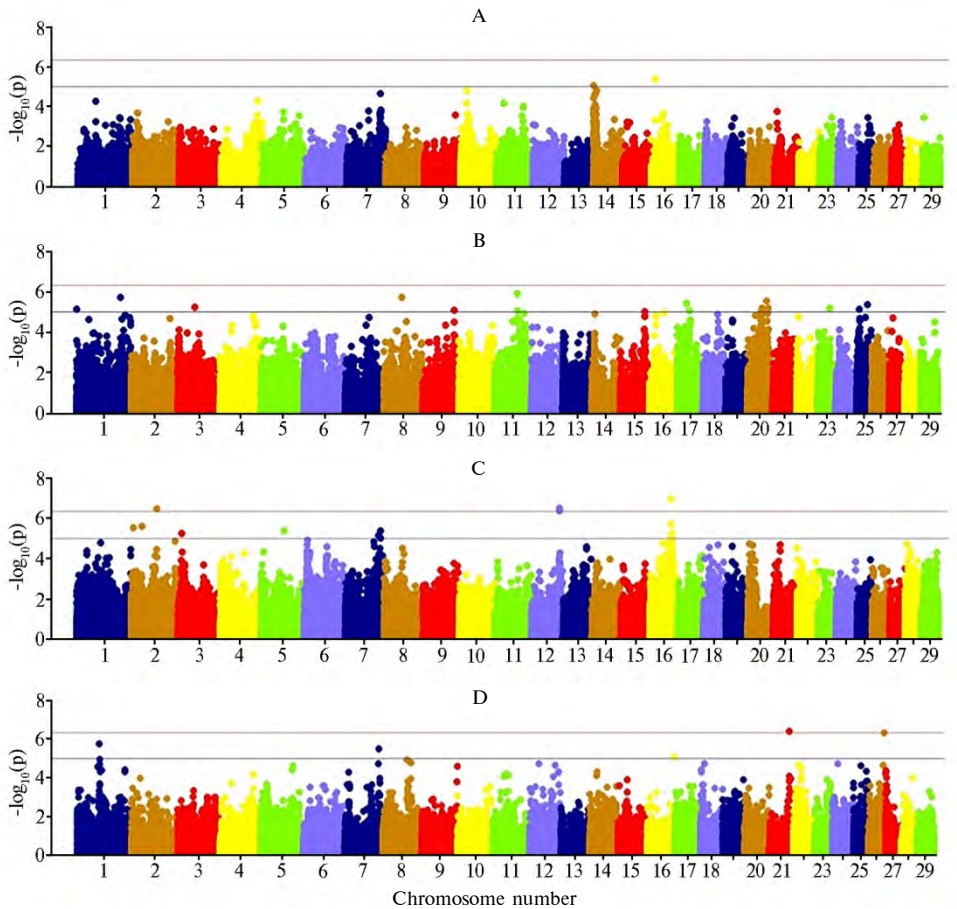

Fig. 1. Distribution of single nucleotide mutations in chromosomes of the studied cow group of Holsteinized Russian Black Pied cattle in connection with the significance level for indicators of own productivity on average per 305 days long lactation (the upper horizontal line is the significance threshold for genome-wide associations, $-\log_{10}(p) = 4{,}5\times10^{-7}$; the lower horizontal line is the significance threshold for suggestive associations $-\log_{10}(p) = 1{,}0\times10^{-5}$): A — milk yield per one lactation, B — mass fraction of fat, C — mass fraction of protein, D — conception rate ($n = 44$, PJSC "Kamenskoye", Sverdlovsk Province, 2019).

Figure 1 presents the results of the analysis of genome-wide associations with traits of milk productivity and fertility of the cows of the studied selected population. A significant correlation was established between the genotype by SNP markers and the animal's own productivity for: milk yield for 305 days long lactation on cattle chromosomes (BTA) 7, 10, 14, 16; MJ on BTA1, 8, 11, 20; CSBM on BTA2, 5, 7, 12, 16; conception rate on BTA1, 7, 8, 21, 26.

2. Significant single nucleotide mutations, associated with phenotypic traits of cows of the studied group of Holsteinized Russian Black Pied cattle (PJSC "Kamenskoye", Sverdlovsk Province, 2019)

SNP	BTA	Position, bp	p-value	Closest gene	Distance to the gene, bp
Average milk yield per 305 days long lactation, kg					
BovineHD1600003292	16	12318853	4.18×10^{-6}	*CDC73*	+325765
BovineHD1400000152	14	1439476	8.54×10^{-6}	*ZNF16*	Inside
BovineHD1400000187	14	1585385	8.54×10^{-6}	*ARHGAP39*	Inside
BTA-34956-no-rs	14	1514056	8.54×10^{-6}	*ZNF7*	+1864
BovineHD1000006525	10	19972763	1.58×10^{-5}	*HCN4*	+7449
BovineHD1400002920	14	10319796	1.58×10^{-5}	*EFR3A*	–37385
				KCNQ3	–234570
ARS-BFGL-NGS-42106	7	97772240	2.23×10^{-5}	*ELL2*	–78731
BovineHD0700028539	7	97833516	2.23×10^{-5}	*PCSK1*	+322158
BovineHD4100010843	14	7332355	2.31×10^{-5}	*KHDRBS3*	+99747
Average mass fraction of fat, %					
BovineHD1100019146	11	67734294	1.21×10^{-6}	*GFPT1*	Inside
Hapmap59899-ss46527105	11	67678534	1.21×10^{-6}	*ANTXR1*	–88003
ARS-BFGL-NGS-36975	1	128799966	1.83×10^{-6}	*ZBTB38*	–263595
BovineHD0100036417	1	128823979	1.83×10^{-6}	*SPSB4*	+6929
BovineHD0800015859	8	52869025	1.83×10^{-6}	*RFK*	–65127
BovineHD0800015899	8	52984674	1.83×10^{-6}	*PRUNE2*	Inside
BTB-00347944	8	53031744	1.83×10^{-6}	*PRUNE2*	Inside
ARS-BFGL-NGS-38258	20	56721394	2.79×10^{-6}	*MYO10*	-149181
Average mass fraction of protein, %					
ARS-BFGL-NGS-45195	16	63612072	1.08×10^{-7}	*STX6*	Inside
BovineHD4100009755	12	81991589	3.23×10^{-7}	*ITGBL1*	Inside
BTA-87771-no-rs	12	82056537	3.23×10^{-7}	*ITGBL1*	Inside
BovineHD0200021465	2	74934453	3.31×10^{-7}	*IGDCC3*	+455733
BovineHD1200023614	12	82094359	4.37×10^{-7}	*ITGBL1*	Inside
ARS-BFGL-NGS-102876	16	63521833	2.00×10^{-6}	*XPR1*	-19726
BovineHD0200009386	2	31672054	2.52×10^{-6}	*COBLL1*	Inside
BovineHD0200002141	2	7215096	2.95×10^{-6}	*COL3A1*	+102191
Hapmap53461-rs29027660	7	102855103	4.37×10^{-6}	*AP3S1*	142023
BovineHD0500018676	5	66809794	4.38×10^{-6}	*IGF1*	–206095
				PAH	+140707
Average conception rate, number					
BovineHD2100017363	21	59895529	4.13×10^{-7}	*SERPINA5*	–99716
ARS-BFGL-NGS-119213	26	47375257	4.87×10^{-7}	*DOCK1*	–79054
BovineHD0100018320	1	64773642	1.79×10^{-6}	*ARHGAP31*	Inside
BovineHD0700029413	7	100618564	3.17×10^{-6}	*CHD1*	–51472
BovineHD1600023747	16	81193491	8.92×10^{-6}	*KIF14*	–1809
				DDX59	Inside
BovineHD1600023756	16	81215628	8.92×10^{-6}	*CAMSAP2*	+62155
BovineHD0100018663	1	66151741	1.14×10^{-5}	*STXBP5L*	+61871
ARS-BFGL-NGS-108666	8	71352779	1.18×10^{-5}	*LOXL2*	Inside
BovineHD0800021457	8	71309172	1.18×10^{-5}	*LOXL2*	Inside
BovineHD0800024578	8	82536035	1.70×10^{-5}	*FBP2*	–97218

Note. For SNP, single nucleotide substitutions are presented in order of decreasing of association significance level (p-value); BTA means cattle chromosome; "+" marks the distance from the corresponding SNP to the gene, "–" marks the distance against this direction.

Based on the publicly available "CattleQTLdb" database, quantitative trait loci in the livestock genome are determined, which include genes and SNPs that are reliably associated with selection indicators in the studied Ural livestock population (Table 2). Ehe following significant polymorphisms were detected in the genes *ZNF16, ARHGAP39, EFR3A, KCNQ3, KHDRBS3*, for milk productivity, which were associated with milk yield, milk fat and protein yield, and corresponded to loci found in similar works by foreign authors [24]. The *HCN4* and

PCSK1 genes were characterized by a conjugation with growth and development parameters, as well as behavioral responses, which suggests their possible impact on milk productivity [25, 26].

Locus at position 1.44-1.59 represents the milk yield trait on BTA14, which included the three most significant genes, and at a distance of 210 kb there is the *DGAT1* gene, which plays an important role in the conversion of diacylglycerides and CoA enzymes of fatty acids into triglycerides, which are essential for synthesis of fats. Associations with a mass fraction of fat had a moderate level of significance $p = 2{,}79\times10^{-6}$ to $1{,}21\times10^{-6}$, while the *ANTXR1*, *ZBTB38*, *PRUNE2*, *MYO10* genes showed correlation with QTL, annotated with increased traits of temperament and residual feed intake, as well as growth parameters, body length and chest circumference [27-30].

More convincing results of GWAS were obtained about the mass fraction of protein, $p = 4{,}38\times10^{-6}$ to $1{,}08\times10^{-7}$. The *COL3A1, IGF1*, and *PAH* genes showed an ambiguous relationship with QTL in previous similar studies in terms of meat marbling, milk yield, percentage and yield of milk protein, live weight, conception rate, and a number of parameters for assessing the quality of the udder and legs [31-33].

At the same time, conception rate had QTL due to the functional relationship of genes with the reproductive qualities of animals responsible for: ovarian regulation and follicular atresia — *SERPINA5* ($p = 4{,}13\times10^{-7}$, including nearby variants of the super family *SERPINA1, 10, 3-2, 11*) [34]; live weight at 18 months of age (period of successful insemination) — *ARHGAP31* ($p = 1{,}79\times10^{-6}$) [35]; early preimplantation development of embryo — *CHD1* ($p = 3{,}17\times10^{-6}$) [36]; fertility of bull-daughters — *STXBP5L* ($1{,}14\times10^{-5}$) [24]. At the same time, polymorphisms in the genes were found to be correlated between the number of inseminations and the yield of milk fat — *LOXL2* ($1{,}18\times10^{-5}$) [24], the growth parameters of dairy cattle — *FBP2* ($1{,}70\times10^{-5}$) [37] and those without a link, for example, with resistance to a number of parasitic diseases with a 22.1 kb cluster on BTA16 — *KIF14, DDX59* and *CAMSAP2* ($p = 8{,}92\times10^{-6}$) [38].

The single nucleotide substitutions detected by GWAS were largely consistent with previously annotated genes and QTL for milk productivity and cattle fertility traits by a number of researchers. However, some of the SNPs only indirectly confirmed their conjugation with “direct” animal phenotypes, which indicates their pleiotropic effect, or a different nature of the combinational variability of the quantitative indicators taken into account (phenotyping accuracy, gene drift, selection intensity or lack of it).

The present-day expansion of the Holstein cattle population in one or another region of its breeding faces a number of difficulties: avoidance of close degrees of kinship, control of homozygosity in the population and lethal recessive genetic mutations, reproduction of their own breeding resources (stud bulls, rearing flocks) with given parameters of breeding values. Achieving high performance of cows is possible due to creation of an animal genotype that steadily transfers valuable alleles and/or haploblocks from generation to generation, as well as having a moderate level of homozygosity.

The pattern search algorithm is designed to scan sections of successively arranged homozygous single nucleotide substitutions; the principle is in continuous checking along the entire length of the chromosome. ROH was determined provided that 15 or more consecutive homozygous SNPs were present in the studied genome at a density of at least 1 SNP for every 100 kb, with gaps between them of no more than 1000 kb. A number of conditions were implemented as well:

starting from the ROH group with a length of (8; 16] Mb, 2 missing SNPs per segment were allowed, and then, at a level >16 Mb, 4 missing nucleotide substitutions and 1 heterozygous allele were allowed. Table 3 shows the statistics of the SNP number, included in the ROH of different lengths depending on the implemented allowances.

3. Statistical parameters of the number of single nucleotide polymorphisms (SNP) **for homozygosity patterns** (ROH) **of different lengths in the studied group of Holsteinized Russian Black Pied cattle** (n = 85, PJSC "Kamenskoye", Sverdlovsk Province, 2019)

Statistics	ROH length by groups, Mb				
	[1;2]	(2;4]	(4;8]	(8;16]	> 16
SNP number	58.6±0.4	122.0±1.1	233.8±2.0	449.8±5.0	904.2±27.0
Standard deviation	22.3	44.4	67.8	110.2	268.3
Minimum	15	15	27	253	617
Maximum	289	476	668	1050	2214
Average ROH length, Mb	1.40±0.01	2.82±0.01	5.49±0.03	10.77±0.10	22.01±0.65
Total ROH length, Mb	48.3±1.2	59.0±2.0	71.0±2.6	63.8±3.4	42.7±3.9

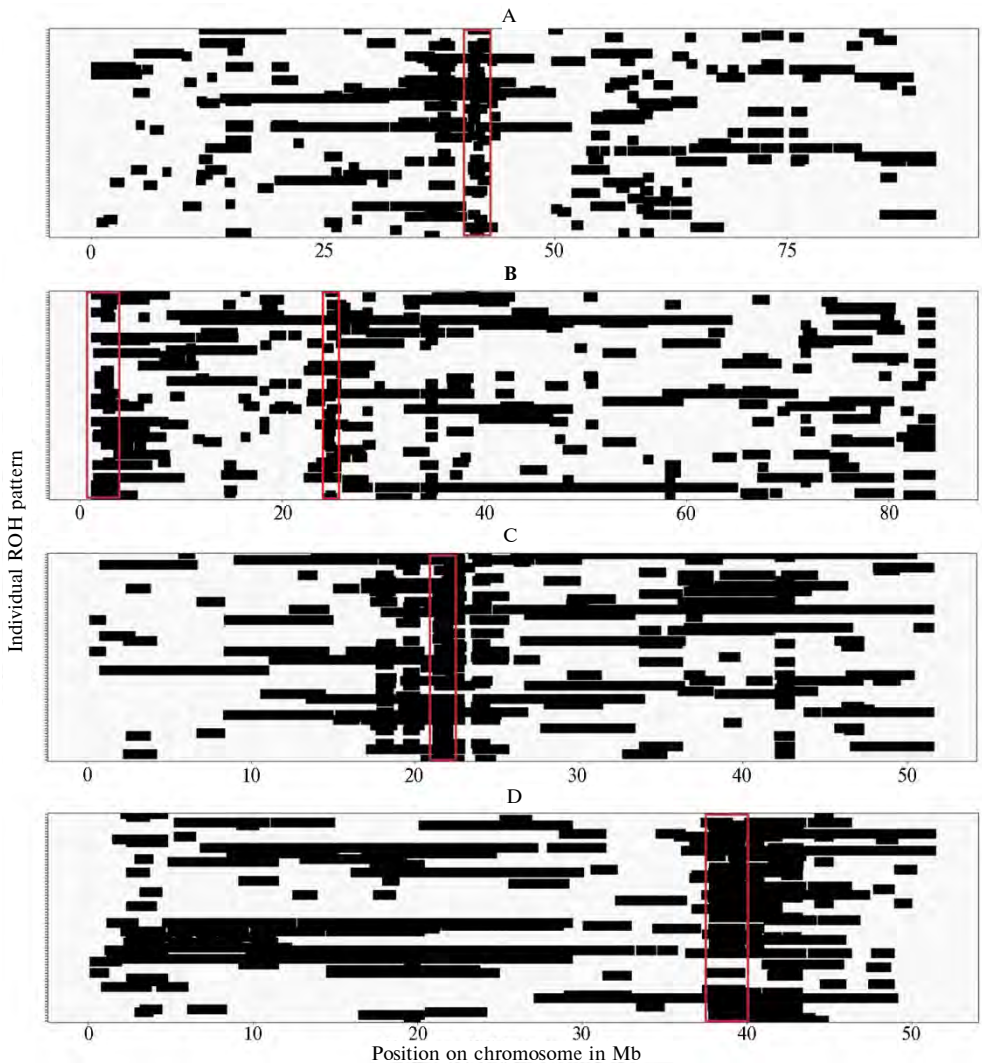

Fig. 2. Identification of ROH patterns for the breeding-valuable loci (indicated by the rectangular frame) **on the bovine chromosomes** (BTA) **12** (A)**, 14** (B)**, 26** (C)**, and 29** (D) of Holsteinized Russian Black Pied cattle (each row represents one animal; n = 76, females of different generations, PJSC "Kamenskoye", Sverdlovsk Province, 2019).

With an increase of the patterns' length, the number of SNPs entering the segment almost proportionally increased, reaching a maximum of 2214 markers. The average ROH length with increasing pattern size from 1-2 Mb to > 16 Mb increased from 1.40 to 22.01 Mb. While the total ROH length (the sum of all segments on average per animal by gradations) did not change significantly, and even tended to decrease from 48.3 Mb to 42.7 Mb.

A search for causal mutations in genes using data visualization (Fig. 2) and an analysis of the ROH patterns occurrence frequency in the studied Ural dairy cattle population (Table 4) made it possible to establish a number of conservative regions in the genome (clusters with protein-encoding genes included) which, we assume, are under the evolutionary pressure.

4. Genomic regions and the most significant protein-coding genes that are under evolutionary pressure and that are identified by ROH (extended homozygous fragments) in the studied group of Holsteinized Russian Black Pied cattle (n = 76, PJSC "Kamenskoye", Sverdlovsk Province, 2019)

BTA	ROH average detected position, Mb		Ratio of animals having ROH in the region, %	The number of genes	Most significant genes
	beginning±σ	end±σ			
12	35.258±1.967	39.747±3.407	44	25	*IL17D, PARP4*
14	1.580±0.454	5.102±2.424	35	66	*ARHGAP39*, *LRRC14*, *PPP1R16A*, *FOXH1*, *CYHR1*, *TONSL*, *CPSF1*, *ADCK5*, *SLC52A2*, *TMEM249, SCRT1*, *DGAT1*, *BOP1*, *MROH1*, *MAF1*, *OPLAH*, *SMPD5*, *SPATC1*, *PLEC*, *GRINA*, *MAPK15*, *EPPK1*, *SCRIB*, *EEF1D*, *RHPN1*, *GPIHBP1*, *GML*, *ADGRB1*, *TSNARE1*, *SLC45A4*, *DENND3*, *PTK2*, *AGO2*, *TRAPPC9*, *KCNK9*
14	24.412±1.828	26.621±2.346	59	18	*XKR4*, *PLAG1*, *CHCHD7*, *SDR16C5-SDR16C6*, *FAM110B*, *SDCBP*, *NSMAF*, *TOX*
26	22.142±1.102	23.877±1.878	73	48	*PKD2L1*, *SCD*, *BTRC*, *ELOVL3*, *GBF1*, *SUFU*, *CNNM2*
29	37.953±1.177	41.982±2.334	62	117	*PAG1-PAG20*, *FADS1-FADS3*, *INCENP*, *SLC3A2*

N o t e. BTA is bovine chromosome.

Due to limitations of the selected experimental population sample, the beginning and end of each of the detected ROH regions is shown as the average value in Mb with a deviation in sigma in order to maximize the accuracy of variation in the homozygous segment. Having analyzed the distribution of ROH across 29 chromosomes in three generations of animals, 5 regions in the genome were identified with a maximum occurrence in at least 30% of individuals on BTA12 (35.3-39.7 Mb), 14 (1.6-5.1 Mb; 24.4-26.6 Mb), 26 (22.1-23.9 Mb) and 29 (38.0-42.0 Mb). The SNP annotation was carried out in order to search for the most significant genes and the QTLs formed by them. It was found that from 35% to 73% of individuals from the total sample had a similar arrangement of ROH patterns with detectable positions.

Annotation of genes that are forming clusters of increased selection pressure was carried out after the visual search and analysis of the ROH occurrence frequency. Among the 25 genes on BTA12, only 2 were classified as the most significant – *IL17D* (interleukin 17D) [39], PARP4 (Poly(ADP-ribose)-polymerase, member of the family 11) [40]. These genes are involved in the immune processes of mammary glands, as well as adipogenesis. In general, on the basis of the functional abstract of the "Cattle QTL Database", it can be summarized that the QTLs are concentrated in this region of the chromosome 12, which are associated with the milkfat and protein yield, total number of somatic cells, duration of a productive life, lactation persistence, live weight, calving difficulties, stillbirths, conception rate, quality of the udder and limbs.

Two regions formed by ROH were identified for BTA14, which are quite

informative in terms of breeding dairy cattle. The first one included 66 genes, of which the most studied ones were the DGAT1 (diacylglycerol acyltransferase 1), the PLEC (pectin) and the GRINA (glutamine receptor), and they are involved in synthesis of lipid components — milkfat and several fatty acids [3]. QTLs were more associated with milk yield per lactation, fat, casein, phosphorus, calcium, milk fatty acids, somatic cells, mastitis, ketosis, resistance to tuberculosis and heat stress in animals. The second region on the chromosome contained 18 genes that were part of the QTL that were responsible for the growth and development parameters, live weight, average daily gain, qualitative and quantitative post-mortem characteristics of carcasses, participation in the synthesis of insulin-like growth factor 1. One of the pre-determining genes in this case is the PLAG1 (gene of pleiomorphic adenoma 1), which is important for growth and reproduction of animals, and also acts as a transcription factor in regulating the expression of insulin-like growth factors [41].

Only 18 genes were detected on BTA26, and the *SCD* (stearoyl-CoA desaturase 1), *PKD2L1, BTRC, ELOVL3, GBF1, SUFU, CNNM2* are being direct precursors for the synthesis of milk fatty acids (linolenic, stearic, myristic and palmitoleic, mono-unsaturated and other acids) [42]. Also, chromosome 26 shows the QTL series for milk yield, content of fat, protein, lactose, linoleic, lauric, myristic and conjugated linoleic fatty acids, milk casein, and qualitative characteristics of the cattle udder.

The discovered genes on BTA29, which are part of the homozygous locus, suggest a predefined effect on animal fertility: a group of glycoproteins associated with pregnancy — the *PAG1 ... PAG20*, as well as the *INCENP*, *SLC3A2* genes, which showed a connection to sperm motility and general fertility index using the QTL database [43, 44]. In turn, a cluster of fatty acid desaturase genes (*FADS1 ... 3*), which is a key enzyme in the metabolism of fatty acids of cow milk, was also identified in the ROH pattern [45]. In general, this QTL region also caused variability of traits, i.e. the interval between calving and heat (estrous), conception rate, semen quality, fertility of bull-daughters and content of protein, casein and fat in milk.

The use of homozygosity patterns to search for the selection “fingerprints” in the animal genome is not limited to this. Using the sum value of the total length of ROH per individual divided by the total length of the autosomal genome (2516398 Mb, in this particular research), it is possible to evaluate the individual autozygosity of the animal. It is assumed that by the number and sum of extended ROH segments, we can judge the level of the so-called genomic inbreeding, which should show higher accuracy compared to the pedigree background.

In this regard, ROH-based inbreeding estimates were calculated depending on their extent in animal generations, the grandmothers, mothers, daughters, including the control group of fathers (Table 5). This approach provided a more accurate assessment of the accumulation of homozygosity in a population by sequential selection of parental pairs of animals. The most accurate assessment of genomic inbreeding was given by the $F_{ROH} > 1$ Mb gradation, which shows the growth in generations, for grandmothers/mothers from 8.6% to 9.8%, or 1.2% ($p < 0.05$), and for mothers/daughters from 9.8% to 12.7%, or 2.9% ($p < 0.001$). $F_{ROH} > 1$ Mb of bull-fathers reached a value of 13.5%. There is an opinion that use of ROH > 1 Mb leads to an overestimation of the genomic inbreeding rate due to difficulties in identifying common ancestors in the pedigree background and because of high recombination frequency; therefore, some authors consider it appropriate to determine F_{ROH} by segments over 4 Mb [18]. Thus, the population of Holsteinized Russian Black Pied cattle, using the example of the highly productive herd of Ural, shows the accumulation of general homozygosity and, as a

result, the formation of a consolidated group of animals according to the genetic characteristics inherent in the Holstein breed. With an increase of ROH patterns size to more than 16 Mb, the observed value of inbreeding in animal generations was lower and amounted to 1.4%/1.4%/2.6% ($p < 0.001$). This suggests that a significant share in the homozygosity assessment is given by ROH with a relatively small extent, which is a reflection of demographic events at the breed level as a whole. The use of a limited number of stud bulls of the current livestock population (genealogical complexes — breeding lines and branches), selected to obtain a high level of milk productivity, has led to an increase in the overall level of homozygosity. Further increase of genomic inbreeding over 15.0% should be controlled in order to timely reduce the possible negative effect on reproductive traits of animals.

5. Comparative estimates of the genomic inbreeding level (F_{ROH}) **of the total length of homozygous fragments** (ROH_{total}, Mb) **in relation to the ROH length, the generation of daughters-mothers-grandmothers and the value of the direct genomic breeding value** (DGV) **in the studied group of Holsteinized Russian Black Pied cattle** ($n = 85$, $M \pm$SEM, PJSC "Kamenskoye", Sverdlovsk Province, 2019)

Parameter	Animal generations (year of birth)			
	mother's mother, $n = 25$ (2012-2014)	mother, $n = 30$ (2015-2017)	daughter, $n = 21$ (2018-2019)	cow's father, $n = 9$ (2008-2015)
Level of genomic inbreeding in the studied population sample (ROH)				
$F_{ROH} > 1$	0.086±0.004	0.098±0.004*	0.127±0.005***	0.135±0.009
$F_{ROH} > 2$	0.068±0.004	0.080±0.004*	0.105±0.005***	0.114±0.009
$F_{ROH} > 4$	0.051±0.004	0.061±0.003*	0.083±0.005***	0.092±0.009
$F_{ROH} > 8$	0.028±0.002	0.034±0.003	0.053±0.004***	0.059±0.009
$F_{ROH} > 16$	0.014±0.002	0.014±0.002	0.026±0.003***	0.032±0.005
$ROH_{total} > 1$	215.3±10.1	247.3±9.3*	319.9±12.7***	338.7±23.1
Genomic breeding value based on milk productivity (DGV)				
Milk yield, kg	448.7±37.8	645.5±39.7**	677.1±57.1	730.3±113.0
Mass fraction of fat, %	0.0005±0.0108	–0.0024±0.0101	0.0049±0.0133	–0.0295±0.0126
Milkfat, kg	18.3±1.4	24.9±1.6**	27.1±2.1	27.3±15
Mass fraction of protein, %	–0.0017±0.0032	–0.0144±0.0042*	–0.0059±0.0041	–0.0165±0.0056
Milk protein, kg	9.6±1.3	13.7±1.2*	15.5±1.9	16.7±3.1

*, **, *** Differences in sequential generations (mother—mother's mother or daughter—mother) are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

A preliminary analysis of the phenotypic traits of cows with their genomic inbreeding indicated a positive correlation between them: for milk yield $r = 0.464$, for mass fraction of fat $r = 0.121$, for milkfat content $r = 0.463$, for milk protein content $r = 0.389$, for conception rate $r = 0.168$, with the exception of mass fraction of protein $r = –0{,}400$. So, with an increase in the overall level of homozygosity (ROH), a simultaneous improvement in the parameters of milk yield, yield of milk fat and protein was observed, with a moderate decrease in fertility rates.

Based on the Russian reference group of dairy cattle, the DGV forecast was calculated for three generations of animals, including bull-fathers (see Table 5). It was shown that mothers significantly exceeded the generation of grandmothers for genomic breeding value in milk yield by +157 kg of milk, +6.6 kg of milkfat and +4.1 kg of milk protein, ceding by -0.003% to mass fraction of fat (unreliable) and by -0.013% to mass fraction of protein. The daughters' generation had positive growth dynamics of DGV, compared to the mothers by +32 kg of milk, +2.2 kg of fat, +1.8 kg of protein, +0.007% fat and +0.008% protein. The bull-fathers of the studied offspring showed the highest level of genomic breeding value, but like their daughters, they did not have significant superiority over the last generation of animals in the experimental sample. This may indicate an exit to the breeding

plateau in the herd through saturation of the offspring genotype with a combination of valuable alleles for traits of milk productivity obtained as a result of selection of the parental pairs of animals. Further improvement in the genetic value of individuals is possible through the use of new generations of bulls with higher DGV values, or with high accuracy and level of breeding value in the offspring quality.

Hence, it was shown that the control of monogenic genetic mutations (defects) and selection-determined DNA markers can provide efficient selection of animals at the herd level (or population as a whole) and they can be used in the selection of parental pairs for producing offspring with desired parameters of breeding qualities. Analysis of genome-wide associations with traits of milk productivity and fertility in cattle made it possible to establish causal mutations that are localized close to the genes or within the genes, of which *ZNF16*, *ARHGAP39*, *EFR3A*, *KCNQ3*, *KHDRBS3*, *IGF1*, *SERPINA5*, *ARHGAP31*, *CHD1* and *STXBP5L* are the most significant ones. Value of significant associations for point mutations was within $p < 2{,}3\times10^{-5}$ to $1{,}1\times10^{-7}$. The identified genetic polymorphisms are consistent with studies conducted in Russia on the example of Holsteinized Russian Black Pied cattle, as well as with the data on the Holstein breed in North America and several European countries. According to the ROH analysis, the regions in the genome that are subject to the highest evolutionary pressure on chromosomes 12, 14, 26, and 29, were identified, which showed the presence of QTLs that are associated with traits of milk productivity, animal fertility, fatty acid composition of cow milk, cattle body type, growth and development parameters. Experimental data were obtained on the basis of homozygosity patterns in order to clarify the level of livestock inbreeding, as an example for use in animal selection programs at the level of individual herds and the population as a whole. Genomic inbreeding showed positive dynamics of population increase, while at the same time there was an increase in the forecast value of their genomic breeding value. This shows that the complex of genomic analysis tools makes it possible to be more efficient about planning of obtaining animals with desired breeding value that would meet the economic needs of dairy cattle breeding. The replacement of the reference group of dairy cattle in Russia through the integration of regional animal populations, primarily the genotyped cows and the stud bulls, provides prospects for expanded use of prognosis of genomic breeding value for reproduction of both bull and pedigree livestock of Holsteinized Russian Black Pied and Holstein cattle.

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A SEARCH FOR GENOMIC REGIONS CARRYING THE LETHAL GENETIC VARIANTS IN THE DUROC PIGS

O.V. KOSTYUNINA[1], A.S. ABDELMANOVA[1], E.U. MARTYNOVA[2], N.A. ZINOVIEVA[1]

[1]*Ernst Federal Science Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail kostolan@mail.ru (✉ corresponding author), preevetic@mail.ru, n_zinovieva@mail.ru;
[2]*Center of Life Sciences, Skolkovo Institute of Science and Technology*, 3, ul. Nobelya, Moscow, 143026 Russia, e-mail elenamartynovaster@gmail.com

ORCID:
Kostyunina O.V. orcid.org/0000-0001-8206-3221
Abdelmanova A.S. orcid.org/0000-0003-4752-0727
Martynova E.U. orcid.org/0000-0003-0802-0236
Zinovieva N.A. orcid.org/0000-0003-4017-6863

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Abstract

The necessity to address the problem of reducing embryonic losses, which in pigs are estimated at the level up to 30%, is not in doubt. LoF (Loss of Function) mutations, which in the homozygous state can lead to the termination of synthesis or synthesis of non-functional proteins, are considered as one of the genetic factors that cause embryonic mortality. While in cattle, an intensive search for LoF mutations is carried out, in pigs, studies of such mutations are still performed on a smaller scale. Whole-genome analysis using medium- and high-density SNP chips which are uniformly covering the entire genome gives researchers new methodology to identify positional candidates for lethal recessive variants. One approach is to assess the level of linkage disequilibrium (LD) of SNP alleles. In this work, we applied the LD analysis of alleles in SNP loci to detect genome areas with presumptively lethal recessive variants in Duroc pigs (*Sus scrofa*) and for the first time revealed in the genes a series of single nucleotide polymorphisms that significantly affect various physiological processes. Studies were carried out with 715 Duroc boars bred in JSC Top Gen (Voronezh region) in 2017-2019. Whole-genome genotyping was carried out using Porcine GGP HD DNA chips (Neogene/Illumina Inc., USA) containing about 70 thousand SNP. After the quality control, 42981 polymorphic SNP were selected for analysis. Search of reference sequences (rs) and clarification of their localization was carried out using the Ensembl database (http://www.ensembl.org). Functional gene annotations were performed using the GeneCards database (http://www.genecards.org/). Analysis of the maintenance of genetic equilibrium showed the presence of 990 SNPs with the absence of one of the homozygous genotypes (2.30% of the total number of polymorphic SNPs), which were distributed among all pig chromosomes, including 205 SNPs, which were in the linkage disequilibrium (0.48%). Chromosomes SSC9 (0.8 %), SSC5 (0.77 %), SSC7 (0.68%) and SSC2 (0.68%) were characterized by the highest ratio of SNPs in linkage disequilibrium, while chromosomes SSC13 (0.28%), SSC4 (0.29%) and SSC10 (0.30%) were the lowest. For 52 SNPs, of which 25 SNPs were localized within genes, differences in observed and expected heterozygosity frequencies were statistically significant ($p < 0.01$). Among SNPs located in intergenic regions, two SNPs (rs81350198 and rs81337222) are associated with important phenotypes from earlier GWAS studies. For 12 of the 25 identified positional candidate genes (*OR4C45*, *EPHB4*, *EML4*, *SLC4A1AP*, *ZFAT*, *CELSR2*, *NEGR1*, *LRRC32*, *MYOCD*, *HUNK*, *RPH3A*, and *DOCK1*), we obtained the information on their role in various processes in organisms of mammals, including nervous system development, angiogenesis, cardiogenesis, cell differentiation, apoptosis and many others. The integration of DNA markers associated with lethal phenotypes into breeding programs, in addition to DNA markers identified by GWAS studies, will significantly improve the efficiency of marker and genomic breeding programs in pigs.

Keywords: pigs, linkage disequilibrium, lethal variants, LoF, loss of function, single nucleotide polymorphisms

Embryonic losses have significant negative impact on the efficiency and profitability of animal husbandry. In pig breeding, embryonic losses make up to 30% [1]. The so-called LoF (loss of function) mutations, which in the homozygous state lead to premature termination of protein synthesis or to the synthesis of non-functional proteins, are considered among the genetic factors associated with embryonic mortality [2]. The most active search for LoF mutations is carried out in various cattle breeds using the appropriate diagnostic test systems [3]. In pig breeding, studies of LoF mutations are less extensive. To identify mutations leading to visible phenotypic changes, an approach based on the analysis of pedigrees is often used [4]. However, it is not suitable for the identification of LoF mutations, since their phenotypic effects consist in the reduction of multiple pregnancies, which may be due to several other reasons.

The development of methods which enable to analyze whole-genome data has opened up new possibilities for the search for genetic factors associated with embryonic mortality. Sufficient levels of linkage disequilibrium (LD) between the marker allele (alleles) and the lethal variant (variants) are required to successfully use DNA markers for identification of lethal recessive variants which cause prenatal mortality [5]. Two alleles at different loci are in linkage disequilibrium, if the frequency of the haplotype which contains both of them is significantly different from the frequency expected in the case of random allele segregation. Haplotype-based approach is used to more accurately identify rare and atypical variants which are generally not included within the single nucleotide polymorphism (SNP) panels used for genotyping. Phasing the genotype data allows more clearly defining haplotype heterogeneity and makes it possible to draw conclusions about the haplotypes of non-genotyped ancestors and animals that were genotyped using lower density panels. This approach was used to identify the lethal recessive haplotype associated with stillborn piglets' number [6].

Derks et al. [7] searched for lethal alleles segregating in the Landrace (n = 28,085) and Duroc (n = 11,255) pig populations using a medium density SNP chip (Illumina, Inc., USA). Using the overlapping sliding window method, the authors have identified a single strong candidate haplotype (DU1) carrying a lethal recessive allele in the Duroc pig population and four candidate haplotypes in the Landrace breed (LA1-4). No homozygotes were detected for the DU1, LA1, and LA3 haplotypes, while their expected number was 26, 126, and 16, respectively. For the LA2 and LA4 haplotypes, genetic equilibrium disturbance was also observed, which may indicate incomplete LD between the haplotypes and lethal recessive mutations. The association between all five haplotypes and a significant decrease in the total number of born piglets (total number born, TNB) and the number of live born piglets (number born alive, NBA) has been shown. At the same time, no significant increase in the number of stillborn or mummified piglets was observed, which indicates that homozygous carriers die at the early stages of gestation [7].

In the presented work, we have for the first time performed a search for genomic regions which may be associated with lethal recessive defects in Duroc pigs using the analysis of the degree of linkage disequilibrium, and revealed a number of highly significant single nucleotide polymorphisms localized within genes and playing an important role in various physiological processes.

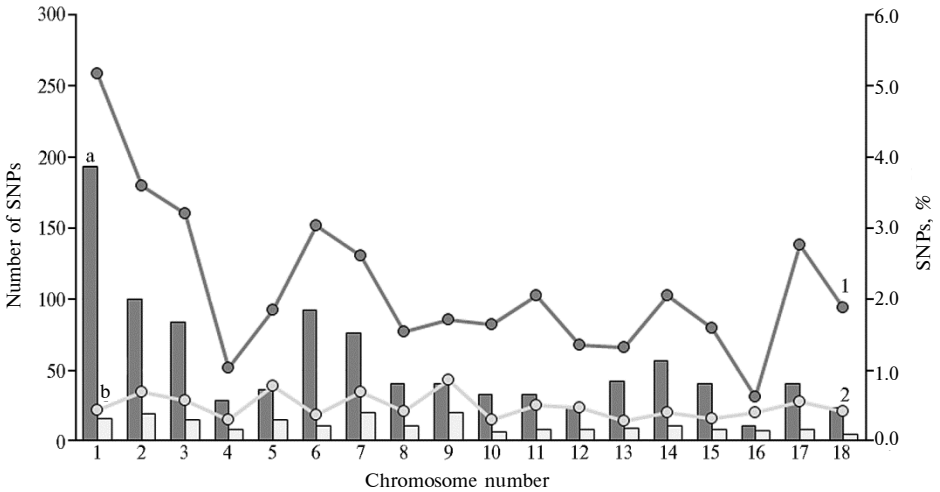
Our goal was to search for the regions bearing presumptively lethal recessive variants in Duroc pigs (*Sus scrofa*) based on the analysis of the degree of linkage disequilibrium between the alleles at SNP loci.

Materials and methods. The study was carried out on 715 boars of the Duroc breed (JSC Breeding-Hybrid Center, Voronezh Region, 2017-2019).

Genomic DNA was extracted from tissue samples (ear plucks) using the DNA Extran-2 kit (Syntol, Russia). DNA quality and concentration were determined using the NanoDrop8000 spectrophotometer (Thermo Fisher Scientific, United States) and the Qubit 2.0 fluorimeter (Invitrogen/Life Technologies, United States), respectively.

Whole-genome genotyping was carried out using the Porcine GGP BeadChips (Neogene/Illumina, Inc., United States) containing about 70 thousand SNPs. 42,981 polymorphic SNPs were selected for the analysis using the Plink 1.9 software (http://zzz.bwh.harvard.edu/plink/) according to genotyping quality (higher that 90%), minor allele frequency (not higher than 0.5%), and linkage disequilibrium (at 50 kb intervals) [8]. The search for reference sequences (reference sequence, rs) and adjustment of their localization was carried out in the Ensembl database (http://www.ensembl.org, accessed date: August 2019). Functional gene annotation was performed using the GeneCards database (http://www.genecards.org, accessed: August 2019).

Results. Genetic equilibrium analysis revealed the presence of 990 SNPs with the absence of one of the homozygous genotypes, among which 205 SNPs were in linkage disequilibrium. These SNPs constituted 2.30 and 0.48% of the total number of polymorphic SNPs, respectively (Fig.).



Chromosomal distribution of SNPs (single nucleotide polymorphisms) for which the absence of one of the homozygous variants and linkage disequilibrium were observed in the Duroc boars (*Sus scrofa*): a — number of SNPs for which one of the homozygous genotypes is missing, b — number of SNPs in linkage disequilibrium (bar graph); 1 — portion of SNPs for which one of the homozygous genotypes is missing in the total number of polymorphic SNPs on the chromosome, 2 — ratio of SNPs that are in linkage disequilibrium in the total number of polymorphic SNPs on the chromosome (line graph) (n = 715, JSC Breeding-Hybrid Center, Voronezh Province, 2017-2019).

The largest number of SNPs for which the absence of one of the homozygous genotypes was observed was detected on chromosome 1 (*Sus scrofa* chromosome 1, SSC1) — 193 SNPs, and the largest number of SNPs in linkage disequilibrium, on the chromosomes SSC7 and SSC9 (20 SNPs), while the smallest number, on SSC16 (11 SNPs) and SSC18 (5 SNPs), respectively. The largest ratio of SNPs in linkage disequilibrium was demonstrated for the chromosomes SSC9 (0.85%), SSC5 (0.77%), SSC7 (0.68%), and SSC2 (0.68%), the smallest ratio, for SSC13 (0.28%), SSC4 (0.29%), and SSC10 (0.30%).

The most significant polymorphisms for which linkage disequilibrium was observed ($p \leq 0.01$) are presented in Table 1.

1. Most significant single nucleotide polymorphisms in linkage disequilibrium detected in the Duroc boars (*Sus scrofa*) (n = 715, JSC Breeding-Hybrid Center, Voronezh Province, 2017-2019)

SSC	RS	Position on chromosome (assembly v.10.2)	A1A1	A1A2	A2A2	H_o	H_e	p
1	rs81350198	226 188 042	0	163	547	0.2296	0.2032	5.1×10^{-5}
1	rs80795638	277 854 630	0	227	440	0.3403	0.2824	1.7×10^{-10}
1	rs81002425	296 461 073	0	700	9	0.9873	0.4999	5.4×10^{-195}
1	rs334911415	312 050 746	0	662	45	0.9364	0.4980	1.2×10^{-152}
1	rs342062641	312 083 991	0	645	63	0.9110	0.4960	1.2×10^{-137}
2	rs81362641	12 082 068	0	618	92	0.8704	0.4916	1.7×10^{-117}
2	rs81255095	14 056 188	0	345	309	0.5275	0.3884	5.2×10^{-28}
2	rs319913462	14 759 005	0	522	126	0.8056	0.4811	4.0×10^{-84}
2	rs323641934	15 384 171	0	537	172	0.7574	0.4706	1.1×10^{-76}
2	rs343381067	15 551 096	0	665	45	0.9366	0.4980	2.0×10^{-153}
2	rs80911461	160 111 983	0	186	506	0.2688	0.2327	7.8×10^{-7}
3	rs344115015	8 107 291	0	139	570	0.1961	0.1768	9.7×10^{-4}
3	rs323044318	104 257 589	0	120	590	0.1690	0.1547	6.4×10^{-3}
3	rs81375606	116 445 052	0	124	586	0.1746	0.1594	4.1×10^{-3}
3	rs81375903	118 615 471	0	142	568	0.2000	0.1800	6.0×10^{-4}
3	rs80828678	132 719 179	0	348	305	0.5329	0.3909	1.9×10^{-28}
3	rs327044542	141265 677	0	170	540	0.2394	0.2108	1.8×10^{-5}
4	rs331053365	4 353 353	0	156	554	0.2197	0.1956	1.4×10^{-4}
4	rs323787335	7 185 799	0	591	114	0.8383	0.4869	1.8×10^{-103}
4	rs343205058	109 273 333	0	604	106	0.8507	0.4889	5.1×10^{-109}
4	rs80949619	121 334 654	0	123	587	0.1732	0.1582	6.7×10^{-3}
5	rs340620949	1 453 085	0	163	546	0.2299	0.2035	5.1×10^{-5}
5	rs81323749	18 046 364	0	184	526	0.2592	0.2256	2.4×10^{-6}
5	rs80875559	99 245 749	0	119	591	0.1676	0.1536	6.5×10^{-3}
6	rs81337222	13 141	0	709	1	0.9986	0.5000	3.3×10^{-210}
6	rs81476539	67 022 132	0	237	471	0.3347	0.2787	6.2×10^{-11}
6	rs337799081	130 798 722	0	130	580	0.1831	0.1663	2.5×10^{-3}
7	rs319008071	27 213 610	0	237	451	0.3445	0.2851	3.0×10^{-11}
7	rs80944793	129 041 116	0	120	590	0.1690	0.1547	6.4×10^{-3}
7	rs331172717	131 889 604	0	614	79	0.8860	0.4935	4.5×10^{-122}
8	rs81399201	31 445 286	0	134	576	0.1887	0.1709	1.6×10^{-3}
8	rs322099448	78 592 741	0	580	127	0.8204	0.4839	5.5×10^{-97}
9	rs343201786	11 913 668	0	137	573	0.1930	0.1743	9.8×10^{-4}
9	rs346413844	12 946 073	0	638	3	0.9953	0.5000	2.7×10^{-185}
9	rs81337172	15 049 063	0	125	585	0.1761	0.1606	4.0×10^{-3}
10	rs81305281	76 905 575	0	520	189	0.7334	0.4645	4.7×10^{-70}
11	rs80816476	939 424	0	131	579	0.1845	0.1675	2.5×10^{-3}
11	rs325221950	21 018 670	0	145	561	0.2054	0.1843	3.7×10^{-4}
11	rs329067201	21 371 677	0	148	562	0.2085	0.1867	3.7×10^{-4}
12	rs81478101	26 712 700	0	697	13	0.9817	0.4998	2.4×10^{-189}
12	rs81436301	50 907 615	0	342	368	0.4817	0.3657	1.9×10^{-24}
12	rs81228589	59 880 454	0	137	573	0.1930	0.1743	9.8×10^{-4}
13	rs329645817	5 325 304	0	320	390	0.4507	0.3491	5.3×10^{-21}
13	rs322958990	205 932 444	0	276	404	0.4059	0.3235	7.0×10^{-16}
13	rs328137225	218 478 357	0	135	575	0.1901	0.1721	1.6×10^{-3}
14	rs80958173	41 518 669	0	125	585	0.1761	0.1606	4.0×10^{-3}
14	rs80862470	95 881 035	0	624	86	0.8789	0.4927	1.6×10^{-121}
14	rs80993446	148 199 735	0	128	582	0.1803	0.1640	4.3×10^{-3}
15	rs337254355	2 154 617	0	138	570	0.1949	0.1759	9.6×10^{-4}
16	rs334615079	81 560 836	0	381	321	0.5427	0.3955	5.0×10^{-32}
17	rs80988530	36 703 557	0	587	68	0.8962	0.4946	4.3×10^{-120}
17	rs345268841	66 549 094	0	258	450	0.3644	0.2980	5.09×10^{-13}

N o t e. SSC — chromosome number, RS — reference sequence, A1 — allele 1, A2 — allele 2, H_o — observed heterozygosity, H_e — expected heterozygosity, and p — level of statistical significance.

A total of 52 significant polymorphisms were identified, for which the differences in the observed and expected heterozygosity were statistically significant. These SNPs were distributed between 17 chromosomes (except for SSC18), including 5 SNPs on SSC1, 6 on SSC2, 6 on SSC3, 4 on SSC4, 3 on SSC5, 3 on SSC6, 3 on SSC7, 2 on SSC8, 3 on SSC9, 1 on SSC10, 3 on SSC11, 3 on SSC12, 3 on SSC13, 3 on SSC14, 1 on SSC15, 1 on SSC16, and 2 on SSC17. Two SNPs (rs81350198 on SSC1 and rs81337222 on SSC6) were identified

as the DNA markers associated with economically-important phenotypes based on the results of the previous GWAS (genome-wide association study) analysis [9, 10]. The rs81350198 polymorphism is associated with the taste of meat in non-castrated boars due to the accumulation of skatol and androstenone during puberty [9]. For the rs81337222 polymorphism, a moderate association was found ($p = 2.4\times10^{-5}$) with the development of umbilical hernia [10]. Although no quantitative trait loci that are associated with umbilical hernia have been identified in the immediate vicinity of rs81337222, and the identified genes for which the association with this phenotypic trait was confirmed have not been localized, its presumable QTL may be located in the upstream region of SSC6 (6:3 814 021-3 870 534) identified by the CNV (copy number variation) analysis [11]. Therefore, at the start of SSC6 there are regulatory regions that require additional research.

2. Single nucleotide polymorphisms localized within the genes and presumptively associated with lethal recessive variants in Duroc pigs (*Sus scrofa*) ($n = 715$, JSC Breeding-Hybrid Center, Voronezh Province, 2017-2019)

RS (p)	Gene name (Ensembl)	Candidate gene	Mutation type
rs343381067 (2.0×10^{-153})	ENSSSCG00000031436	*OR4C45*	Substitution within intron
rs344115015 (9.7×10^{-4})	ENSSSCG00000007675	*EPHB4*	Substitution within intron
rs323044318 (6.4×10^{-3})	ENSSSCG00000008467	*EML4*	Substitution within intron
rs81375606 (4.1×10^{-3})	ENSSSCG00000008533	–	Substitution within intron
rs81375903 (6.0×10^{-4})	ENSSSCG00000008549	*SLC4A1AP*	Substitution at the 3′-end of the gene
rs327044542 (1.8×10^{-5})	ENSSSCG00000049737	–	Substitution within intron
rs323787335 (1.8×10^{-103})	ENSSSCG00000030947	*ZFAT*	Mutation in 3′-UTR
rs343205058 (5.1×10^{-109})	ENSSSCG00000006694	–	Substitution within intron
rs80949619 (6.7×10^{-3})	ENSSSCG00000034360	*CELSR2*	Substitution within intron
rs340620949 (5.1×10^{-5})	ENSSSCG00000024474	–	Substitution at the 3′-end of the gene
rs81323749 (2.4×10^{-6})	ENSSSCG00000024474	–	Substitution at the 3′-end of the gene
rs81476539 (6.2×10^{-11})	ENSSSCG00000003444	–	Substitution at the 3′-end of the gene
rs337799081 (2.5×10^{-3})	ENSSSCG00000025085	*NEGR1*	Substitution within intron
rs319008071 (3.0×10^{-11})	ENSSSCG00000001395	–	Synonymous mutation
rs343201786 (9.8×10^{-4})	ENSSSCG00000014869	*LRRC32*	Mutation in 3′-UTR
rs325221950 (3.7×10^{-4})	ENSSSCG00000045677	–	Substitution at the 3′-end of the gene
rs81436301 (1.9×10^{-24})	ENSSSCG00000017853	–	Substitution at the 3′-end of the gene
rs81228589 (9.8×10^{-4})	ENSSSCG00000031988	*MYOCD*	Substitution within intron
rs322958990 (7.0×10^{-16})	ENSSSCG00000029392	*HUNK*	Substitution within intron
rs80958173 (4.0×10^{-3})	ENSSSCG00000009883	*RPH3A*	Substitution within intron
rs80862470 (1.6×10^{-121})	ENSSSCG00000043778	–	Substitution within intron
rs80993446 (4.3×10^{-3})	ENSSSCG00000035045	*DOCK1*	Substitution within intron
rs337254355 (9.6×10^{-4})	ENSSSCG00000044919	–	Substitution within intron
rs80988530 (4.3×10^{-120})	ENSSSCG00000007155	*C20orf194*	Substitution within intron
rs345268841 (5.09×10^{-13})	ENSSSCG00000007525	–	Missense mutation

N o t e. *OR4C45* — olfactory receptor, family 4, subfamily C, member 45; *EPHB4* — EPH receptor B4; *EML4* — protein associated with excessively expressed proliferation; *SLC4A1AP* — kanadaptin; *ZFAT* — zinc finger protein; *CELSR2* — Cadherin EGF LAG Seven-Pass G-Type Receptor 2; *NEGR1* — Neuronal Growth Regulator 1; *LRRC32* — Leucine-Rich Repeat-Containing protein 32; *MYOCD* — myocardin; *HUNK* — Hormonally Up-Regulated Neu-Associated Kinase; *RPH3A* — rabphilin 3A; *DOCK1* — Dedicator of Cytokinesis. RS — reference sequence, p — level of statistical significance, UTR — untranslated region of the gene. The type of mutation is given as per Borisevich et al. [12]. Dashes indicate the absence of data.

The analysis of the genomic regions in which we found the detected SNPs showed that 25 SNPs were located within genes. By the type of localization, most SNPs were mutations in introns (a total of 15 mutations). Nucleotide substitutions were also found in the 3′-terminal sequences of genes (6 mutations) and in the 3′-untranslated region (2 mutations), along with one missense mutation and one synonymous mutation (Table 2).

For 12 out of the 25 identified positional candidate genes, the data are available on their role in various processes in the mammalian organism. For example, the *OR4C45* gene encodes an olfactory receptor (OR) protein, which is important for maintaining intestinal homeostasis. OR is expressed in the enterochromaffin cells of the mucous membrane. Odorant ligands through OR present in enterochromaffin cells cause serotonin release, which controls motility and intestinal secretion and is involved in the pathological conditions such as vomiting and diarrhea [13]. The role for the OR ligand in the regulation of epithelial permeability and secretion of electrogenic anions in human colon has been reported [14]. *EPHB4* plays a special role in various biological processes, such as neuronal development, bone homeostasis, and angiogenesis [15]. Genetically modified mouse embryos homozygous for the *EphB4taulacZ* allele had cardiovascular defects and were characterized by embryonic mortality with very high penetrance. In such embryos, growth retardation, lack of blood flow, and cardiac development arrest were observed [16]. EML4 is a poorly characterized microtubule-associated protein. It is assumed that its natural function is to stabilize microtubules in the axons and dendrites of neuronal cells. Chimeric EML4-ALK causes the development of lung cancer in humans [17].

Kanadaptin (SLC4A1AP) is a nuclear protein with unknown function that is widely expressed in mammalian tissues. The ubiquitous distribution of kanadaptin in mammals suggests that it should play an important physiological role [18]. ZFAT is involved in the development and peripheral homeostasis of T cells. There is evidence that a deletion in the *Zfat* gene in mice leads to embryonic death and disrupts primitive hematopoiesis in the blood islands of the yolk sac [19, 20]. In pigs, ZFAT is associated with susceptibility to enterotoxin infection caused by *Escherichia coli* [21]. CELSR2 is expressed in all brain areas and regulates the maintenance and growth of dendrites. Mice homozygous for the *CELSR2* mutation develop hydrocephaly due to a decrease in the number, size, and orientation of ependymal cilia [22]. NEGR1 is involved in the regulation of neurite proliferation in the developing brain [23]. As a result of the search for QTL associated with obesity in humans and pigs, three most probable genes have been identified, including *NEGR1*, which is responsible for genetic predisposition to the common obesity types, in particular, for the thickness of subcutaneous fat [24]. LRRC32 functions as a receptor for latent transforming growth factor molecules; it has been detected in regulatory T cells [25]. Its important role in immune regulation has been noted. GWAS studies revealed an association between rs11236909 located approximately 58 kb upstream from the *LRRC32* gene and certain parameters of human sperm motility [26].

MYOCD contributes to heart development and differentiation of cardiomyocytes. It was noted that mutant mice with the knockout of the *MYOCD* gene developed dilated cardiomyopathy, which was accompanied by the disturbance of the structural organization of cardiomyocytes and severe depression of systolic function [27]. The functions of the *HUNK* gene are still not clear. Probably, it is involved in the transfer of phosphorus-containing groups and possesses transferase and protein tyrosine kinase activities. RPH3A plays an important role in the adhesion of neutrophils to endothelial cells during inflammatory reactions [28]. DOCK1 regulates phagocytosis, fusion of myoblasts and cell migration, is involved in embryonic development. The detected underdevelopment of all skeletal muscle tissues in *Dock1*- knockout embryos allowed to identify DOCK1 as an important regulator of the fusion stage in the myogenesis in mammals [29]. No information on the roles of the *ENSSSCG00000024474* gene has been found in open databases; however, GWAS of DNA methylation in lard, lean, and miniature pig breeds, identified it as a differentially methylated region [30]. The *C20orf194 gene* (194th

open reading frame on the 20th chromosome) encodes an uncharacterized protein with the C-terminal coiled coil. The gene is located on the 20p13 chromosome in the 1.8 Mb region associated with the spinocerebellar ataxia phenotype in humans. The work of Ponsuksili et al. [31] concerned with the description of the regions associated with behavioural reactions in Landrace pigs revealed the presence of the rs80988530 SNP, which we detected in the present work in the region with high degree of linkage disequilibrium, within this gene.

To summarize, the genome-wide study which was conducted using the GGP Porcine HD beadchips allowed us to identify the regions mutations in which may cause lethal effects. The most significant single-nucleotide polymorphisms which are in linkage disequilibrium in the boars of the Duroc breed are localized in the following genes: *OR4C45* (olfactory receptor, family 4, subfamily C, member 45); *EPHB4* (EPH receptor B4), *EML4* (protein associated with excessively expressed proliferation), *SLC4A1AP* (kanadaptin), *ZFAT* (zinc finger protein), *CELSR2* (Cadherin EGF LAG Seven-Pass G-Type Receptor 2), *NEGR1* (Neuronal Growth Regulator 1), *LRRC32* (Leucine-Rich Repeat-Containing 32), *MYOCD* (myocardin), *HUNK* (Hormonally Up-Regulated Neu-Associated Kinase), *RPH3A* (rabphilin 3A), and *DOCK1* (Dedicator of Cytokinesis). For almost each identified candidate gene, an important role in various processes, including the development of the nervous system, angiogenesis, cardiogenesis, cell differentiation, apoptosis, etc., has been demonstrated by now. In humans, many of these genes are associated with various organ and tissue disorders, hence, their participation in the occurrence of lethal effects in pigs cannot be ruled out. Understanding the processes which take place during the growth and development of embryos and using this knowledge in the analysis of the actual zootechnical data will expand the arsenal of tools which would allow to propose approaches for the genetic improvement of breeding products in proper time. The integration of DNA markers associated with lethal phenotypes into breeding programs along with the DNA markers identified based on the results of GWAS studies, will significantly increase the efficiency of marker and genomic selection programs in pig breeding.

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THE GENOMIC ARCHITECTURE OF THE RUSSIAN POPULATION OF SAANEN GOATS IN COMPARISON WITH WORLDWIDE SAANEN GENE POOL FROM FIVE COUNTRIES

T.E. DENISKOVA[1], A.V. DOTSEV[1], M.S. FORNARA[1], A.A. SERMYAGIN[1], H. REYER[2], K. WIMMERS[2], G. BREM[1, 3], N.A. ZINOVIEVA[1]

[1]*Ernst Federal Science Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail horarka@yandex.ru (✉ corresponding author), asnd@mail.ru, margaretfornara@gmail.com, alex_sermyagin85@mail.ru, n_zinovieva@mail.ru;
[2]*Institute of Genome Biology, Leibniz Institute for Farm Animal Biology (FBN),* Mecklenburg-Vorpommern, 18196 Dummerstorf, Germany, e-mail reyer@fbn-dummerstorf.de, wimmers@fbn-dummerstorf.de;
[3]*Institut für Tierzucht und Genetik, University of Veterinary Medicine (VMU),* Veterinärplatz, A-1210, Vienna, Austria, e-mail gottfried.brem@agrobiogen.de
ORCID:
Deniskova T.E. orcid.org/0000-0002-5809-1262
Dotsev A.V. orcid.org/0000-0003-3418-2511
Fornara M.S. orcid.org/0000-0002-8844-177X
Sermyagin A.A. orcid.org/0000-0002-1799-601
Reyer H. orcid.org/0000-0001-6470-0434
Wimmers K. orcid.org/0000-0002-9523-6790
Brem G. orcid.org/0000-0002-7522-0708
Zinovieva N.A. orcid.org/0000-0003-4017-6863

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Abstract

The Saanen goat breed is valued for its high milk productivity and good adaptive qualities, which contributed to its worldwide distribution outside Switzerland. In Russia, the Saanen is a popular breed that had been officially recommended for breeding and had a pedigree status. Breeding in local environments as well as regional specifics of the used breeding strategies can lead to a significant change in the allele pool of breeds, and therefore, it is relevant to conduct genomic studies of national populations of world breeds to establish their current genetic status. Here, for the first time we presented the results of whole-genome analysis of the Russian population of goats of the Saanen breed in comparative aspect with the original (Switzerland) and the world gene pool of the Saanen breed, represented by four countries. The aim of our work was to assess genetic diversity and to study population structure of the Saanen goats of Russian selection in comparison with representatives of this breed from five different countries (Switzerland, Italy, France, Argentina and Tanzania) whose whole-genome SNP-profiles were obtained from the database of the AdaptMap project. The studies were conducted on 21 goats of the Saanen breed (RUS), bred in one of the Russian breeding farms, in 2019-2020. DNA was extracted from the selected ear fragments using DNA Extran-2 kits (Syntol CJSC, Russia). Genotyping was performed using a GoatSNP50 BeadChip DNA chip (Illumina, Inc., USA) containing 53347 SNPs and providing coverage of the average interval between SNPs in 40 kb. To assess the genetic diversity and to perform comparative analysis of the Russian goat population with the representatives goats of this breed from five different countries, we used SNP-profiles of the Saanen goats bred in Switzerland (SWI, n = 38), Italy (ITA, n = 22), France (FRA, n = 55), Argentina (ARG, n = 11) and Tanzania (TNZ, n = 8), which were downloaded from the publicly available digital data repository Dryad and generated in within the AdaptMap project. The Swiss population of the Saanen breed was assumed as a sample of the original gene pool. Bioinformatic processing and visualization of whole-genome genotyping data was performed in the PLINK 1.90, Admixture 1.3, SplitsTree 4.14.5 software, in R packages "diveRsity" and "pophelper". The observed heterozygosity varied from 0.381 in SWI to 0.423 in FRA and was high in RUS (Ho = 0.418). In SWI, ITA, FRA populations the values of the inbreeding coefficient were close to zero level; RUS, ARG, and TNZ showed heterozygote deficiencies, which were 1.5%, 8.9, and 6.0%, respectively. Allelic richness was maximal in ARG, RUS, and FRA (Ar ≥ 1.979) and minimal in SWI (Ar = 1.934). The Principal component analysis and the phylogenetic tree showed a clear differentiation between the national and original populations of the Saanen breed. Analysis of population structure demonstrated

the presence of the genetic component of the SWI cluster in goats from the RUS group. RUS had the smallest genetic distances with FRA (F_{ST} = 0.02; R_{ST} = 0.189) and ITA (F_{ST} = 0.023; R_{ST} = 0.215); and RUS was highly differentiated from TNZ (F_{ST} = 0.054; R_{ST} = 0.311) and SWI (F_{ST} = 0.06; R_{ST} = 0.276). Thus, different selection strategies resulted in genetic differences between the national goat populations of the Saanen breed. However, genomic components of the original gene pool are still present in the Russian goat population of the Saanen breed.

Keywords: Saanen breed, domestic goats, SNP markers, DNA chips, genetic diversity, AdaptMap

More than 50 dairy goat breeds have been created over the history of domestic goat breeding worldwide [1], among which the Saanen breed is the most popular. The breed got its name from the Swiss Zaanental Valley, where it was bred using the method of long-term folk selection in the middle of the 19th century [2, 3]. Among goats, the Saanen breed is an analogue of the Holstein cattle breed, characterized by high milk productivity (from 300 to 2000 kg for 150-300 days of lactation) [3]. Goats of this breed are easily acclimatized to different conditions of keeping and feeding without significant loss of milk production. They were imported from Switzerland to most countries in Europe [3, 4], North [5] and South America [6], Australia [7], Asia [8], and Africa [9], where the use of their valuable gene pool significantly transformed the national dairy industry.

According to official data [10], pedigree dairy goat breeding in Russia is represented by four foreign breeds: Alpine (since 2015), Saanen (since 1993), Murciano Granadina (since 2019), and Nubian (since 2018). Nevertheless, according to data for 2018, the Saanen breed is the most numerous [11]. The breeding stock of goats of the Saanen breed is concentrated in three stud farms, six breeding reproducers, and one gene pool enterprise and amounts to 12.3 thousand heads. The average milk yield in herds varies from 822 kg per 305 days of lactation in the breeding reproducer KH Rus-1 LLC (Stavropol Province, Budyonnovsky District, Pokoynoye village) to 961 kg per 305 days of lactation in the gene pool enterprise LLC “Berezka” (Kursk Province, Kursk District, Petrovskoye village) [4].

Along with the use of pure-bred Saanen goats for dairy production, their crossings with local goat populations are promising [1, 12]. Therefore, goat sires of the Saanen breed are a high-quality improving material that allows for short term conversion of mongrel low-productive groups of goats (with a milk yield of 200-250 kg for 305 days of lactation) into reliable milk producers with a milk yield of up to 663 kg for 305 days of lactation [1, 12].

Genomic studies of domestic goats were not as common as those of other livestock species [13]. In 2013, three large scientific groups conducting projects to search for single nucleotide polymorphisms (SNPs) joined into the International Goat Genome Consortium (IGGC) to create a DNA chip [14]. As a result of the SNP selection, 53347 SNPs were included in the final set in the Goat SNP50 BeadChip chip (Illumina, Inc., USA).

The development of this DNA chip has led to increased interest in studies of the genome organization of domestic goats. For example, L. Nicoloso et al. [15] investigated the genetic diversity of 14 Italian goat breeds based on genome-wide data. Mdladla et al. [16] demonstrated the applied value of a whole-genome SNP analysis in their studies of indigenous goat breeds in South Africa. Using Goat SNP50 BeadChip, Rahmatalla et al. [17] identified the genes associated with the growth and development of bones and the formation of the immune system in Sudanese goats. Brito et al. [18] studied genetic diversity in more than 1000 goats from nine popular commercial breeds.

Numerous scientific works resulted in the generation of SNP profiles of

goat breeds from different countries around the world (3171 goats from 117 populations), which were collected in the AdaptMap project database [19, 20] and were used to identify runs of homozygosity [20], establish historical goat migration routes [21], and search for loci under pressure of selection [22]. In addition, the creation of publicly accessible databases of SNP profiles allows for the study of breeds imported to various countries to assess their discrepancy or similarity with the original gene pool.

Here, we present the first reported genome-wide analysis of the Russian population of Saanen goats, for which a comparative assessment was performed with the original (Switzerland) and the world gene pool of the Saanen breed, which was represented by four countries.

Our aim was to evaluate the genetic diversity and establish the structure of the Russian population of the Saanen breed in comparison to the gene pool of goats of this breed from five different countries (Switzerland, France, Italy, Argentina, and Tanzania), whose genome-wide SNP profiles were obtained from the AdaptMap project database.

Material and methods. The studies were performed in 2019-2020 on goats (*Capra hircus*) of the Saanen breed (n = 21, RUS) bred at one of the breeding reproducers in the Russian Federation. DNA was isolated from selected fragments of the auricle using DNA Extran-2 kits (Syntol CJSC, Russia). Genotyping was performed using a GoatSNP50 BeadChip DNA chip (Illumina, Inc., United States) containing 53347 SNPs and providing an average 40 kb spacing between SNPs [14]. Reading of DNA chips was performed on an iScan Reader System (Illumina, Inc., USA), and the raw data were loaded into GenomeStudio 2.0 (Illumina, Inc., USA) to call genotypes.

For parameters characterizing reading quality (GenCall, GC) and clustering of SNP markers (GenTrain, GT), cutoffs were set to 0.5 [23]. In PLINK 1.90 [24], we selected SNP markers with a minor allele frequency (MAF) more than 5% that did not deviate from Hardy-Weinberg equilibrium at $p < 10^{-6}$, were in linkage equilibrium, and were located only on autosomes.

To assess the genetic diversity and perform comparative analysis of the Russian goat population with the global gene pool, we used SNP profiles of Saanen goats bred in Switzerland (SWI, n = 38), Italy (ITA, n = 22), France (FRA, n = 55), Argentina (ARG, n = 11), and Tanzania (TNZ, n = 8), which were obtained from the Dryad publicly available digital data repository [25] and generated within the AdaptMap project [20, 21]. The Swiss population of the Saanen breed was chosen as the original gene pool.

Observed heterozygosity (H_o), unbiased expected heterozygosity (uH_e), allelic richness (Ar), inbreeding coefficient F_{is} (with a confidence interval of 95%), pairwise F_{ST} [26], and R_{ST} (Reynolds distance) values [27] were calculated in the R package "diveRsity" [28].

Principal component analysis (PCA) was performed in PLINK 1.90 with subsequent plotting in the R package "ggplot2" [29]. A NeighborNet graph based on the matrix of pairwise F_{ST} values was visualized in SplitsTree 4 software [30].

The population structure and genetic homogeneity of the Russian and other national groups of Saanen goats were established in Admixture 1.3 software [31] with a graphical representation using the R package «pophelper» [32]. The most probable number of ancestral clusters (K) was determined by calculating values of the cross-validation error (CV error) for K from 1 to 7 in Admixture 1.3 software.

Bioinformation processing and data visualization were performed using the R Project for Statistical Computing software environment [33].

Results. An analysis of genetic diversity (Table 1) showed that the observed heterozygosity ranged from 0.381 in SWI to 0.423 in FRA. The H_o value in RUS (0.418) was ranked second after the maximum. In three populations (SWI, ITA, FRA), the inbreeding coefficient was insignificant, which could indicate a state close to genetic equilibrium in these groups. In the RUS group, a small deficit of heterozygotes was noted (1.5%). The ARG and TNZ groups were characterized by more significant heterozygote deficiencies of 8.9% and 6.0%, respectively.

1. Characterization of the genetic diversity of the Russian and five national populations of goats (*Capra hircus*) of the Saanen breed, evaluated using the GoatSNP50 BeadChip DNA chip (2019-2020)

Group	*n*	H_o	uH_e	uF_{is}	Ar
RUS	21	0.418	0.424	0.015 (0.013; 0.017)	1.979
SWI	38	0.381	0.380	–0.002 (–0.003; –0.001)	1.934
ITA	22	0.417	0.418	0.002 (0; 0.004)	1.975
FRA	55	0.423	0.422	–0.002 (–0.003; –0.001)	1.979
ARG	11	0.386	0.426	0.089 (0.086; 0.092)	1.980
TNZ	8	0.388	0.414	0.06 (0.057; 0.063)	1.970

Note. *n* — sample number; H_o — observed heterozygosity, uH_e — unbiased expected heterozygosity, uF_{is} — inbreeding coefficient, Ar — rarified allelic richness. In parentheses, magnitude of the F_{is} variability is given at a confidence interval of 95%. The arithmetic mean error for the parameters H_o, uH_e and Ar is ±0,001. Groups of goats of the Saanen breed: RUS — Russian; SWI — Swiss; ITA — Italian; FRA — French; ARG — Argentinean; TNZ — Tanzanian.

The highest allelic richness was found in the ARG (Ar = 1.980), RUS (Ar = 1.979), and FRA (Ar = 1.979) populations. For the SWI group, it was minimal (Ar = 1.934).

Principal component analysis (Fig. 1) showed that the first principal component, responsible for 7.76% of genetic variation, separated the SWI group from the remaining five populations, including RUS. The second principal component, corresponding to 4.28% of genetic variability, separated SWI, ITA, and partially FRA from RUS (some of which were located almost on the axis), ARG, and TNZ (located in the most distant sector of the PCA-plot) populations.

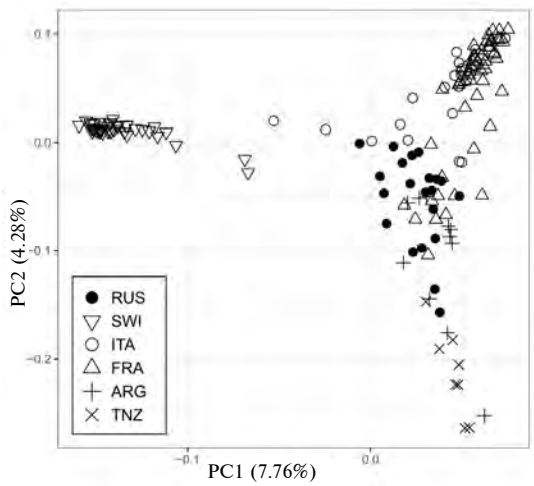

Fig 1. Results of principal component analysis (PCA) for Russian and five national goat populations (*Capra hircus*) of the Saanen breed based on SNP profiles obtained using the GoatSNP50 BeadChip DNA chip (2019-2020)**.** Groups of goats of the Saanen breed: RUS — Russian; SWI — Swiss; ITA — Italian; FRA — French; ARG — Argentinean; TNZ — Tanzanian.

There were two distinguished clusters in the structure of the genetic network, demonstrating the relationships between the Russian and five national populations of goats of the Saanen breed (Fig. 2). The first included a long isolated SWI branch, an ITA + FRA subcluster adjacent to it, and a more distant short RUS branch (at the junction of clusters). The second cluster was formed by separate branches of ARG and TNZ.

F_{ST} and R_{ST} values were calculated to achieve a better understanding of genetic differentiation between the studied goat populations. Their values (Table 2) were maximal between the SWI and TNZ groups (F_{ST} = 0.109; R_{ST} = 0.374), as

well as between SWI and ARG (F_{ST} = 0.078; R_{ST} = 0.321). Minimal differentiation was observed between ITA and FRA (F_{ST} = 0.008; R_{ST} = 0.154).

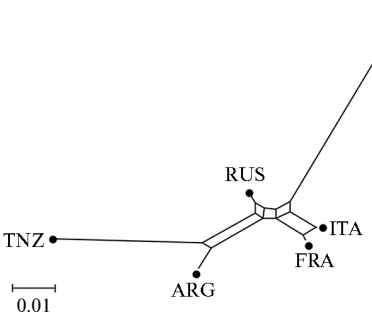

Fig. 2. NeighborNet graph based on pairwise F_{ST} values demonstrating relationships between the Russian and five national populations of goats of the Saanen breed based on SNP profiles obtained using the GoatSNP50 BeadChip DNA chip (2019-2020)**.** Groups of goats of the Saanen breed: RUS — Russian; SWI — Swiss; ITA — Italian; FRA — French; ARG — Argentinean; TNZ — Tanzanian.

By studying the genetic relationships of RUS with the populations from other countries, we observed minimal genetic distances between RUS and the FRA and ITA groups (F_{ST} = 0.02; R_{ST} = 0.189 and F_{ST} = 0.023; R_{ST} = 0.215), and the greatest differentiation was with the TNZ and SWI groups (F_{ST} = 0.054; R_{ST} = 0.311 and F_{ST} = 0.06; R_{ST} = 0.276, respectively).

In order to analyze the population structure of the studied groups of goats, we assessed the most probable number of clusters (Fig. 3, A). The lowest cross-validation error was identified at K = 3 and amounted to 0.64445. At K = 2, the SWI group formed its own isolated cluster (see Fig. 3, B). At K = 3, the ITA and FRA groups showed a similar population structure. Furthermore, the FRA population consisted of heterogeneous individuals, most of which were highly consolidated in their cluster, and the second part (about 30% of the animals) showed the presence of other genomic components. The TNZ group formed its own cluster. The RUS and ARG populations were characterized by the presence of all three identified genetic components, while RUS retained the largest share of the original element (SWI).

2. Genetic differentiation between the Russian and five national populations of goats (*Capra hircus*) of the Saanen breed, estimated using the F_{ST} and R_{ST} indicators based on genome-wide genotyping data using the GoatSNP50 BeadChip DNA chip (2019-2020)

Group	RUS	SWI	ITA	FRA	ARG	TNZ
RUS		0.276	0.215	0.189	0.254	0.311
SWI	0.060		0.275	0.276	0.321	0.374
ITA	0.023	0.059		0.154	0.263	0.327
FRA	0.020	0.065	0.008		0.248	0.316
ARG	0.029	0.078	0.035	0.035		0.318
TNZ	0.054	0.109	0.066	0.065	0.044	

N o t e. The pairwise F_{ST} values are presented below the diagonal, the Reynolds distances R_{ST} are above the diagonal. Groups of goats of the Saanen breed: RUS — Russian; SWI — Swiss; ITA — Italian; FRA — French; ARG — Argentinean; TNZ — Tanzanian.

Highly productive livestock breeds that are attractive for breeding are widespread outside of their original breeding area. In cattle breeding, such breeds include Holstein and Simmental; in pig breeding, this includes Landrace, Duroc, and Large White; in sheep breeding, this includes Rambouillet and Romney Marsh; and in goat breeding, the world leader is the Saanen breed. In exporting countries, the breeding process of imported genetic material begins. Thus, the assessment of the identity of national populations to the original gene pool is of practical importance because it helps to determine the general direction of selection and allows breeders to make possible adjustments to achieve the aimed goal [34]. Therefore, a comparative analysis of the SNP profiles of Simmental cattle of German-Austrian and Russian breeding revealed genomic regions with a high frequency of identical haplotypes, despite certain differences for breeding pur-

poses in the studied populations [34].

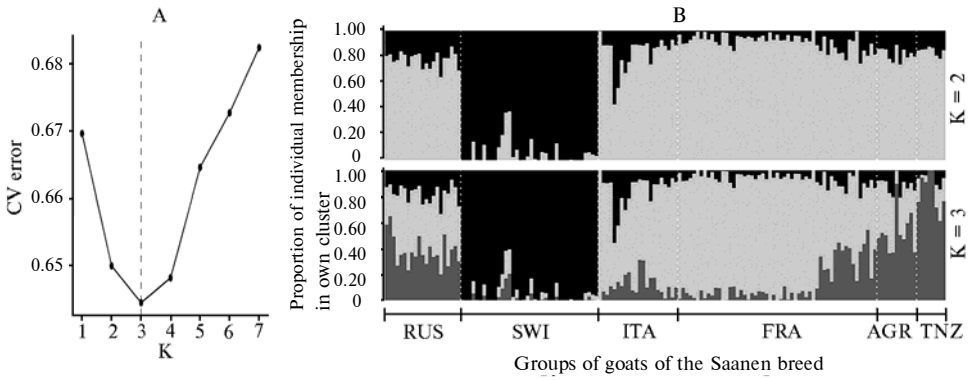

Fig. 3. Comparison of the population structure and analysis of the genetic homogeneity of the Russian and five national populations of goats (*Capra hircus*) of the Saanen breed using the GoatSNP50 BeadChip DNA chip (2019-2020): A — a graph showed cross-validation errors (CV error), calculated by testing the number of ancestral clusters (K) from 1 to 7 (the dashed line indicates the number of clusters for which the cross-validation error was the lowest); B — structure of the studied populations with the number of clusters equal to two and three. Groups of goats of the Saanen breed: RUS — Russian; SWI — Swiss; ITA — Italian; FRA — French; ARG — Argentinean; TNZ — Tanzanian.

The Saanen breed was involved in the development and testing of the GoatSNP50BeadChip DNA chip; therefore, the differentiations between the national populations of the Saanen breed would be completely determined by their genetic differences and would not depend on possible errors caused by the intraspecific biases in the polymorphic loci. For example, the Angora goat breed was not included in the initial list of breeds for the development of a DNA chip; thus, Lashmar et al. [35] preliminarily evaluated the informativeness of the GoatSNP50BeadChip for this breed.

Comparing the genetic diversity indicators calculated in our work for national populations of goats of the Saanen breed, high values were found in all groups, except for the original Swiss one. Burren et al. [36] investigated the genetic diversity in 10 local Swiss goat breeds, in which the observed heterozygosity ranged from 0.369 (Appenzell and Toggenburg) to 0.401 (Grisons striped and Peacock goat), and allelic richness varied from 1.531 (Stifelges) to 1.941 (Chamois colored). The observed heterozygosity was 0.385, 0.384, 0.338, 0.379, and 0.353 for the Alpine, La Mancha, Nubian, Saanen, and Toggenburg breeds, respectively, of the Canadian selection. In addition, in all the listed breeds, a minor deficiency of heterozygotes was recorded [18]. The Ho and Ar values in local Swiss goats and dairy breeds of Canadian selection corresponded to those evaluated for the original SWI group and were a bit inferior to those estimated for the RUS, ITA, FRA, ARG, and TNZ populations.

Like the Saanen, the Angora breed, originating from Turkey, is widespread in many countries of the world. C. Visser et al. [37] used the Goat SNP50 BeadChip to study the degree of geographical isolation and genetic variability in three populations of the Angora goat breed from South Africa, France, and Argentina. Among the national populations of the Angora breed, a difference in the expected and observed heterozygosity was recorded (H_e = 0.371-0.397; H_o = 0.365-0.414), which is comparable with the difference calculated in our research (H_e = 0.380-0.424; H_o = 0.381-0.423).

By summarizing the PCA results and the structure of the phylogenetic tree, we revealed a clear differentiation between the national (except for ITA and FRA) and the original populations of goats of the Saanen breed. Visser et al.

[37] also reported that the national populations of the Angora goats were very clearly separated from each other. The F_{ST} value between Angora populations was 0.120, while in our research the maximum F_{ST} value was 0.109 between TNZ and SWI. This might indicate that the populations of the Saanen breed did not move as far away from the original Swiss group as in the case of the Angora goats.

Interestingly, in accordance with the F_{ST} values, the greatest differentiation was found between RUS and SWI, while according to R_{ST} values, the RUS group was most isolated from TNZ, which was confirmed by the position of the corresponding groups on the PCA plot. According to Laval et al. [38], the calculation of Reynolds distances is the best method for estimating the divergence between closely related groups, which is consistent with our data. In addition, analysis of the population structure provided evidence of the conservation of the genomic components of SWI in goats of the RUS group.

Thus, the whole-genome study of the Russian population of goats of the Saanen breed showed that this group exceeds the original Swiss population by the values of genetic and allelic diversity indices and corresponds to those estimated in the French and Italian groups. The results of our work confirmed that various breeding strategies had led to genetic differences between national populations of the Saanen breed, including the Russian one. Nevertheless, the Russian population of the Saanen breed retains the genomic components inherent in the original gene pool, which, although it creates the necessary variability for selection, it also leaves the possibility of returning to the original type of the Swiss breeding.

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in vitro DEVELOPMENT OF CLONED EMBRYO IN CATTLE IN RELATION WITH FUSION AND ACTIVATION PARAMETERS

G.N. SINGINA, A.V. LOPUKHOV, E.N. SHEDOVA

Ernst Federal Science Center for Animal Husbandry, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail g_singina@mail.ru (✉ corresponding author), shedvek@yandex.ru
ORCID:
Singina G.N. orcid.org/0000-0003-0198-9757
Shedova E.N. orcid.org/0000-0002-9642-2384
Lopukhov A.V. orcid.org/0000-0002-1284-1486
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Abstract

Embryo production through somatic cloning technology has the perspectives for application in reproductive biotechnologies in cattle in order to multiply the most productive and unique genotypes in livestock breeding and create new genotypes using genome editing. Success of somatic cloning depends on the ability of donor somatic cell nucleus (karioplast) to be reprogrammed to totipotent state. Relevant transformations of donor nucleus are mediated by oocyte cytoplasmic factors (cytoplasts) and start from the moment of their association (fusion). Effects of oocyte cytoplasm are direct and depend on many factors. The objective of the present study was to evaluate the cloning efficiency in terms of time of oocyte cytoplasm exposure to donor nucleus before activation, the time of oocyte maturation before their activation in the fused complexes (cytohybrids), and repeated electrofusion of the cytoplast and karyoplast. The effects of these factors on formation of cloned embryos and development to blastocyst stage were studied. Isolated oocyte-cumulus complexes (OCCs) were in vitro matured in TC-199 medium supplemented with 10 % fetal bovine serum, 10 μg/ml of FSH and 10 μg/ml of LH. After 20-24 h of maturation, OCCs were treated with a 0.1% hyaluronidase, then cumulus cells were mechanically removed and the oocytes with the first polar body were selected. Long-time conserved fetal fibroblasts were in vitro cultured up to monolayer and maintained in contact inhibition during 2 days. Then, cell suspension was prepared for transferring into enucleated oocyte. Somatic cell was transferred to perivitelline space of the oocyte, and two consecutive rectangular 20 μs pulses at constant current with a voltage of 35 V were performed (once or twice if there were no signs of cell-oocyte fusion). The obtained cytohybrids were activated with the ionomycin 1 or 2 hours after fusion (recipient oocytes were matured either 23-25 hours or 26-28 hours). Activated cytohybrides were then cultured up to blastocyst stage. Oocyte cleavage rate were similar in all experimental groups (60.7 to 70.4 %). Blastocyst development rate did not differ between the groups where single or double fusions were performed (29.4±4.4 and 22.8±3.5 %, respectively). Blastocyst rate was 17.4±2.6% at 1-hour interval between fusion and activation. Two-hour interval increased blastocyst rate to 31.1±3.8% ($p < 0.05$). In the case of early activation (23-25 hours of maturation), 29.4±4.8% of fused complexes developed to the blastocyst stage. With an increase of oocyte maturation time to 26-28 hours, blastocyst rate decreased to 14.6±2.2% ($p < 0.05$). Therefore, cloning efficiency depends on the interval between cytohybrid fusion and activation, and the age of MII oocytes at the time of activation of the fused complexes; 2 hours and 23-25 hours, respectively, were the optimal parameters. In addition, the repeated electrofusion of the enucleated oocytes and somatic cells did not affect cytohybrid quality, and, therefore, this procedure can be used for somatic embryo cloning in cattle.

Keywords: cattle, somatic cell nuclear transfer, fusion, activation, embryo development

Modern reproductive cell technologies, in particular generation of Bovine cloned embryos, have broad prospects in multiplication of the most productive

and unique genotypes in pedigree animal husbandry [1-4], as well as in creation of new genotypes by genomic editing methods [5-9]. However, efficiency of high-quality embryo production through somatic cloning remains low, including in cattle, while abortion, perinatal mortality, and birth of low viable offspring are high, which inhibits practical application of this technology [10-12].

In somatic cloning, a female reproductive cell (oocyte), instead of its own chromosomal material, contains the injected nucleus of a somatic cell derived from an animal selected for genetic copying. In this, the epigenetic pattern of differentiated somatic cells is erased, and the embryonic epigenetic characteristics and gene expression patterns are restored to a totipotent embryonic state. The resulting cloned embryos with totipotent status are again able to differentiate into various types of somatic cells. This process involves various molecular and epigenetic modifications, on which the cloning efficiency ultimately depends, and is called nuclear reprogramming [13]. It is believed that changes of somatic cell nucleus (karyoplast) are mediated by the cytoplasm of oocyte (cytoplast) and start upon the karyoplast—cytoplast fusion [14]. The effects of cytoplasmic environment on nuclear reprogramming depend on many factors and, therefore, can be altered purposefully [15].

Available data on improving efficiency of cloning embryos are methodologically contradictory and require additional elaboration to select the optimal parameters for manipulations. So, there is no consensus on the time of donor nucleus exposure to oocyte cytoplasm. Whereas in some works, the development of cloned embryos in cattle requires a long exposure before activation [16], in others it is reported that excessive exposure is unfavorable [17]. It was shown that the percentage of development of cloned blastocysts decreases as the period between fusion and activation increases from 1 to 5 hours [18]. A number of authors regard the 2-2.5-hour interval optimal [19, 20].

Cytoplasmic maturation of the oocyte which integrates donor nucleus is no less important for generation of viable cloned embryos. A mature oocyte in metaphase II is regarded the most suitable recipient cell [21]. However, aging of mature mammalian oocytes, which negatively affects their quality and competence to further embryonic development, occurs in the absence of activating stimuli [22, 23]. Prolonged culture of a mature oocyte due to its late activation during donor nucleus transfer is probably also accompanied by a complex of intracellular processes called “oocyte aging” [22, 24]. However, this aspect of oocyte quality deterioration, unfortunately, has not yet been regarded when improving in vitro culture systems, the optimal age of mature oocytes for cloning has also not yet been determined [25].

Commonly, to generate cloned cattle embryos, oocyte in metaphase II (MII) stage of meiotic division is fully enucleated, and somatic donor cell is injected into perivitelline space of the cytoplast [21]. Upon completion of enucleation, the cytoplast and karyoplast are fused in a pulsed electric field with the breakdown of their membranes at the contact point. The in vitro development of cloned embryos directly depends on how efficiently the recipient and donor cells fuse and whether they remain viable during such aggressive manipulations. [26]. The reconstructed oocytes failed to fuse are again subjected to electrofusion to increase the yield of cytohybrids [27]. This may have an adverse effect on a cloned embryo development that is not yet elucidated.

This paper reports optimized parameters of cattle embryo cloning protocol

(i.e. the time during which the donor nucleus should be exposed to the cytoplasm of reconstructed oocyte prior to activation, and the timing of the oocyte maturation until activation in the cytohybrid). Also, for the first time, we showed no negative effects of a re-fusion commonly used to increase the yield of cytoplast-karyoplast complexes on in vitro development of cloned cattle embryos.

Our objective was to assess effects of fusion-activation interval, the age of MII oocytes at activation, and re-fusion on the efficiency of cloning in cattle, i.e. on embryo formation and development to blastocyst stage.

Materials and methods. In all experiments, except for specially indicated, reagents from Sigma-Aldrich (USA) were used. Oocytes and embryos were cultured at 38.5 °C, 90% humidity and a 5% CO_2 atmosphere; outside the incubator, all manipulations were carried out at 37 °C.

Preparation of donor cells. Cattle fetal fibroblasts were donor cells. Uteri of cows on day 55 of pregnancy was delivered to the laboratory, the uterine horn containing the fetus was treated with 70% ethanol, the extracted fetus was released from head, limbs, and internal organs. The resulting fetal tissue was washed repeatedly in phosphate-buffered saline (PBS) with antibiotics and an antimycotic (100 IU/ml penicillin, 100 μg/ml streptomycin, 100 ng/ml amphotericin), mechanically fragmented and treated with 0.25% trypsin solution for 30 min at 37 °C. Trypsin was neutralized with an equivalent volume of DMEM manipulation medium (Gibco, USA, Cat. No. 31966021) containing 5% fetal bovine serum (FBS) and gentamicin (50 μg/ml) (DMEM-M). Cell suspension was filtered through a sieve with a pore of 100 μm diameter and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed, the cell pellet was resuspended in DMEM-M medium, centrifuged again, after cultured to form a monolayer on 100-mm-diameter Petri dishes with DMEM growth medium (Gibco, USA, Cat. No. 31966021), supplemented with 15 % FBS, 1% non-essential amino acids (Gibco, USA) and 50 μg/ml gentamicin (DMEM-P).

To get enough fibroblasts, the primary cell culture was propagated by passaging the formed monolayer (1:4). Petri dishes, after the growth medium replacement with trypsin/EDTA solution (Gibco, USA), were incubated at 37 °C, the cell suspension was transferred to centrifuge tubes with DMEM-M to neutralize trypsin and centrifuged at 1500 rpm for 5 minutes The supernatant was removed, the pellet was resuspended and re-cultured on 100-mm-diameter Petri dishes with DMEM-P, as described above. After the second passage, the cells were frozen in DMEM (Gibco, USA, Cat. No. 31966021) with 40% FBS and 10% dimethyl sulfoxide (DMSO) and stored in 1 ml cryovials (Corning, USA) at 196 °C until use (for 8 years).

Seven days before somatic cloning, the frozen fibroblasts were thawed in cryovials in a water bath at 37 °C, centrifuged at 1500 rpm in tubes with 10 ml of DMEM-M, cultured in DMEM-P until complete monolayer formation, and allowed for 2-day contact inhibition to synchronize the cell cycle. The cells were suspended in TC199 medium containing 10% FBS and 50 μg/ml gentamicin (TC199-M) at most 30 minutes before transfer to an enucleated oocyte.

Preparation of recipient oocytes. Cows' ovaries delivered at 30-35 °C within 3-5 hours were released from surrounding tissues and washed many times in sterile saline with antibiotics (100 IU/ml penicillin and 50 μg/ml streptomycin). Cumulus-oocyte complexes (COC) were isolated from follicles, washed 3 times in TC199-M medium with heparin (10 μg/ml), and morphologically examined. Round shaped oocytes with a homogeneous cytoplasm, uniformly wide

zone pellucida and multilayered compact cumulus were selected and cultured for 19-23 hours, 20-30 OCCs per 500 μl of TC-199 medium containing 10% PBS, 1 mM sodium pyruvate, 50 μg/ml gentamicin, 10 μg/ml follicle-stimulating hormone (FSH) and 10 μg/ml luteinizing hormone (LH).

Reconstruction of mature oocytes. Matured oocytes were released freed from cumulus cells in a 0.1% hyaluronidase solution in TC199-M medium) for 1 min at 37 °C, followed by disaggregation of the complexes by pipetting. Only oocytes with a first polar body (FPB) were selected for cloning.

Fifteen to twenty oocytes were microsurgically manipulated at once in 20 μl drops of TC199-M medium which were put on a Petri dish bottom pre-covered with light mineral oil. The procedure was performed with an inverted microscope Diafort (Nikon Corporation, Japan) equipped with a Narishige micromanipulation system (Japan). During the reconstruction, the oocytes were focused with a holding pipette in the field of view of the microscope in a position that allows clear visualization of a first polar body (PB1) in the perivitelline space in the position to 1 or 5 hours of the conditional dial. A biopsy micropipette (13-15 μm internal diameter) was brought close to oocyte membrane, the zone pellucida was punctured at the site of PB1 localization, chromosomes were removed blindly by aspiration of the PB1 and 10-20% of adjacent cytoplasm. A somatic cell was introduced into the perivitelline space of a fixed oocyte with the micropipette, previously used for PB1 biopsy, through an opening formed during enucleation.

To obtain a cloned cytohybrid, the enucleated oocyte and somatic cell were electro-fused with an Eppendorf multiporator (Great Britain). The oocyte/somatic cell complexes were placed in a microchamber pre-filled with buffer (270 mM mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$), with a 0.2 mm distance between the electrodes and were first exposed to an alternating current electric field (5 V, 5 s) to drive cell complexes apart towards the electrodes, then two consecutive rectangular pulses of direct current (35 V, 20 μs) were applied. Treated cell complexes were short-cultured in 50 μl TC199-M drops covered with light mineral oil. After 1 h incubation, morphologically normal cloned cytohybrids formed from oocyte/somatic cell complexes were selected. The complexes with no signs of the oocyte and somatic cell fusion were repeatedly subjected to electrofusion procedure as described above.

Activation and post-activation culture of cloned cytohybrids. The cytohybrids were activated 1 or 2 hours after fusion (i.e. 23-25 or 26-28 hours from the start of maturation of recipient oocytes) by incubation for 5 min in a 5 mM ionomycin-containing Tyrode solution [28], followed by culture of reconstructed oocytes in CR1aa medium [29] with 2 mM 6-dimethylaminopurine and 10 μg ml cyclohexedine. After 4 hours, putative zygotes were transferred to CR1aa medium and cultured for 4 days, after which the developing embryos were transferred into the same medium with 5% FBS. On day 2 after activation of cytohybrids, morphologically of the cleaved zygotes were evaluated; on day 7, the number of embryos developed to the blastocyst stage was determined. Evaluation was performed with a SMZ stereo microscope (Nikon, Japan, magnification ×40).

One-way analysis of variance was performed with a SigmaStat software (Systat Software, Inc., USA). The data are presented as mean values (*M*) and standard errors of the mean (±SEM). The Tukey's test ($p \leq 0.05$) was used to assess the significance of differences between the compared means.

Results. Figure 1 illustrates the stages of preparation of fetal fibroblasts and cattle oocytes for the cloning procedure (a — a monolayer of cultured donor

cells, b — a suspension of fetal fibroblasts, c — selection of oocytes with the first polar body).

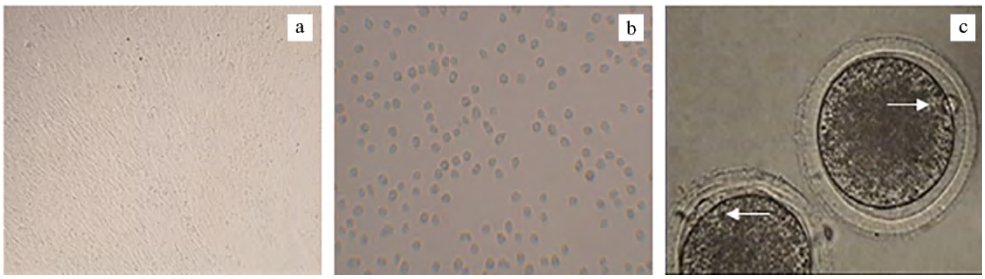

Fig. 1. Cattle fetal fibroblasts and oocytes during somatic cell nuclear transfer (SCNT) procedure: a — culture of fetal fibroblasts after 2 days of contact inhibition; b — fetal fibroblasts suspension (magnification ×200); c — oocytes at metaphase II stage (white arrow indicates the first polar body, magnification ×400) (an Eclipse Ti-U microscope, Nikon, Japan).

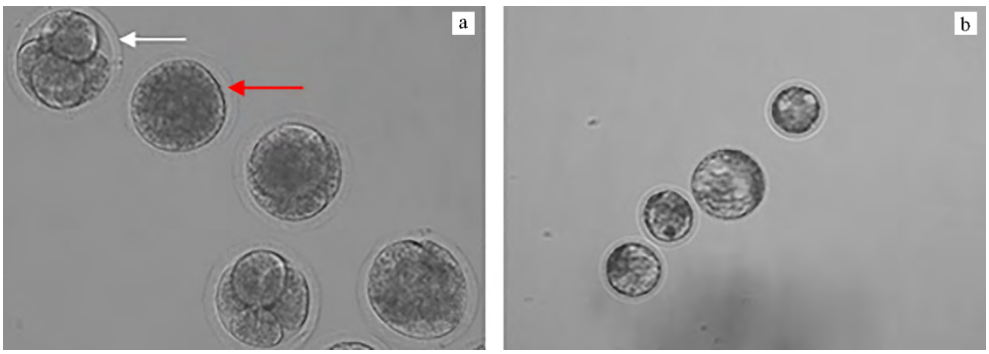

Fig. 2. Cloned cattle embryos derived from fused enucleated MII oocytes and somatic cells (fetal fibroblasts) after activation: a — fused (red arrow) and cleaved (white arrow) cytohybrids (magnification ×200); b — cytogybrids developed to the blastocyst stage (magnification ×100) (an Eclipse Ti-U microscope, Nikon, Japan).

The time of exposure of the oocyte cytoplasm to the donor nucleus before activation is known to be critical for the development of cloned embryos [18, 20]. Reprogramming processes that are necessary for a cell to return to the totipotency [21] are initiated under the influence of cytoplasmic factors in nucleus of somatic cell from the moment of its integration into the cytoplast via fusion.

The influence of the interval between fusion and activation (1 or 2 hours) on somatic nucleus reprogramming efficiency was assessed by the ability of activated cytohybrids (n = 142) to enter the first cleavage division (Fig. 2, a) and to reach the blastocyst stage (see Fig. 2, b). The cleavage rate of activated oocytes on day 2 did not differ for 1- and 2-hour pre-activation exposure of donor nucleus to oocyte cytoplasm (66.6±4.9 and 70.0±5.8%, respectively). However, 1- and 2-hour intervals resulted in different blastocyst yields, i.e. 17.4±2.6% for 1.0 hour vs. a significant increase to 31.1±3.8% ($p < 0.05$) for 2 hours (Fig. 3).

These data are partially consistent with the results of other researchers [19, 20, 30] and suggest that the 2-hour interval between karioplast-cytoplats fusion and activation of resultant cytohybrids provides donor nuclei with enough exposure to the MII oocyte cytoplasm to initiate reprogramming events, while the 1-hour interval reduces blastocyst-stage embryo production. K.I. Aston et al. [20] also reported a positive effect of 2-hour exposure on donor nuclei, but at the same time, they found similar effect for 1-hour exposure. Perhaps such differences are due to peculiarities of somatic cells used for cloning. In our experiments, we used long-stored fetal fibroblasts.

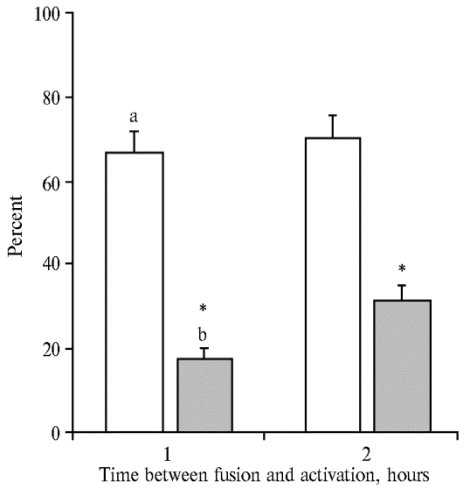

Fig. 3. In vitro development of Bovine cloned embryos as influenced by intervals between karyoplast-cytoplast fusion and activation: a — cleavage rate, b — blastocyst rate. Standard errors of the mean (±SEM) are indicated for $n = 6$ (independent experiments). An asterisk (*) indicates statistically significant differences between the compared groups at $p < 0.05$.

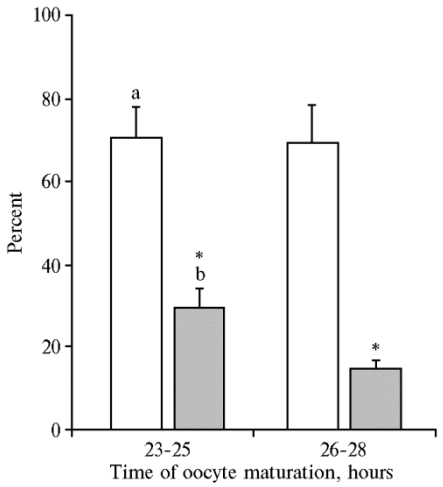

Fig. 4. In vitro development of cytohybrids to the blastocyst stage as influenced by the age of MII oocyte at activation: a — cleavage rate, b — blastocyst rate. Standard errors of the mean (±SEM) are indicated for $n = 9$ (independent experiments). An asterisk (*) indicates statistically significant differences between the compared groups at $p < 0.05$.

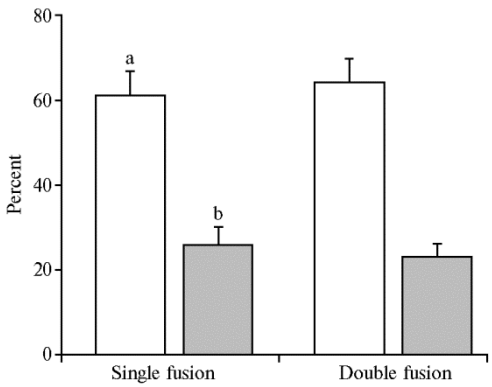

Fig. 5. In vitro development of cloned embryos to the blastocyst stage as influenced by re-fusion of enucleated oocyte and somatic cell: a — cleavage rate, b — blastocyst rate. Standard errors of the mean (±SEM) are indicated for $n = 9$.

Cytoplasmic maturation of the original germ cells is essential to generate cloned embryos capable of normal development [21]. MII oocytes are convenient recipient cells for cloning, since in their cytoplasm, due to specific changes, there are factors ensuring formation of embryonic competence in cloned cytohybrids [21, 24]. However, aging processes are shown to negatively affect quality of mature oocytes not subjected to activation [22, 23]. As capability of MII oocytes to acquire competence to activation in cytohybrids is age-dependent, the age of MII oocytes can also critically affect the development of cloned embryos [24, 25].

We compared developmental competence in cytohybrids activated in 23-25 hours ($n = 104$) and 26-28 hours ($n = 78$) after the maturation of recipient oocytes begins (Fig. 4). The number of cleaved oocytes did not differ between the variants and ranged from 69.4 to 70.4%. Upon early activation, cytohybrids developed to the blastocyst stage constituted 29.4±4.8%. Note, this parameter significantly decreased to 14.6±2.2% ($p < 0.05$) with an increase in the age of oocytes to 26-28 hours. Our data indicate the adverse effect of prolonged culture of mature oocytes on the development of cloned cattle embryos. Also, the age of MII oocytes subjected to activation in hybrid complexes should not exceed 26 hours. Other researchers also indicate the advantage of earlier activation to produce viable offspring [25, 31].

We also investigated the effects of re-fusion on in vitro embryonic development in complexes with no signs of combining an enucleated oocyte and a somatic cell 1 hour after the first fusion (Fig. 5). The electrofusion parameters were the same as in the first procedure. Analysis of in vitro development of the resultant embryos did not reveal adverse effects of the repeated manipulation on the percent of cleaved cytohybrids. Also, there was no decrease in the competence of cytohybrids to form blastocysts neither upon single nor double fusion (29.4±4.4 and 22.8±3.5%, respectively).

Practically, repeated electrofusion is used in somatic cloning to obtain a larger number of cell complexes with signs of combining an enucleated oocyte and a donor cell [27], and also as a method for activating cytohybrids [31]. At the same time, given possible negative consequences of repeated electrofusion, as a rule, more gentle electric pulse modes are used. In our study, these parameters were similar in both manipulations, however, we did not observe any deterioration in in vitro development of the embryos till blastocyst stage.

Thus, we have confirmed that the efficiency of producing cloned cattle embryos of preimplantation stages depends on the interval from fusion to activation, and also on the age of MII oocytes in activated cytohybrids. As per the in vitro cloning protocol we suggest, 2 hours and 23-25 hours, respectively, are the optimal. It is also obvious that the repeated electrofusion of an enucleated oocyte and a somatic cell does not adversely affect the quality of the resulting cytohybrid, and therefore can be used for obtaining cloned embryos in cattle.

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GENETIC MODIFICATION OF ROOSTERS' GERM CELLS USING VARIOUS METHODOLOGICAL APPROACHES

A.N. VETOKH, L.A. VOLKOVA, B.S. IOLCHIEV, E.K. TOMGOROVA, N.A. VOLKOVA

Ernst Federal Science Center for Animal Husbandry, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail anastezuya@mail.ru (✉ corresponding author), ludavolkova@inbox.ru, baylar1@mail.ru, tomgorova@rambler.ru, natavolkova@inbox.ru

ORCID:
Vetokh A.N. orcid.org/0000-0002-2865-5960
Volkova L.A. orcid.org/0000-0002-9407-3686
Iolchiev B.S. orcid.org/0000-0001-5386-726
Tomgorova E.K. orcid.org/0000-0001-5398-8815
Volkova N.A. orcid.org/0000-0001-7191-3550

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Abstract

Gonad germ cells of male farm birds are considered as promising target cells for introducing recombinant DNA for the purpose of targeted genetic modification of their genome. Germ cell precursors (primordial germ cells and spermatogonia cells) are of most interest for modifications. The transplantation of these transformed cells and their successful colonization in the gonads of recipient individuals would allow a population of transformed mature germ cells — sperm — to be obtained, which can be used for female insemination in order to obtain transgenic offspring. The results of studies on the genetic modification of rooster germ cells by transfection of primordial germ cells in embryos and spermatogenous cells in the testes were presented in this work. The novelty of the research consists in the development and optimization of individual stages of local transformation in roosters' spermatogenic cells in vivo to obtain a genetically modified sperm. Our aim was to evaluate the effectiveness of the genetic transformation in roosters' germ cells using various methodological approaches. The study was carried out with poultry (*Gallus gallus domesticus*) of the Russian White breed. Primordial germ cells were isolated from 6-day-old embryos. The resulting culture of PGCs was transformed by electroporation using the Neon system (Thermo Fisher Scientific, USA). For transfection, the ZsGreen1-N1 plasmid (Addgene, USA) with the *ZsGreen* gene under the CMV promoter was used. Transformed cells in the amount of 400, 700 and 1000 were introduced into the dorsal aorta of 2.5-day-old embryos. The embryos of the control group were injected with DMEM growth medium in the dorsal aorta. To transform spermatogenic cells in vivo, a viral preparation was used, which was injected directly into the testes of roosters by multiple injection. The introduction of the viral drugs was carried out once at the age of 3 or 4 months and twice at the age of 3 and 4 months. The viral preparation at a concentration of 1×10^7 CFU/ml was introduced at the rate of 0.5 ml per testis. The lentiviral vector contained the *ZsGreen* reporter gene under the CMV promoter. Histological sections of the testes from experimental males were obtained and analyzed to assess the efficiency of colonization and development of donor primordial germ cells (PGCs) in the gonads of recipients, as well as to evaluate the effectiveness of spermatogenic cell transformation in vivo. As a control, we used histological sections of the testes from non-transgenic roosters, selected on the basis of analogues (age, breed). The fertilizing ability of the sperm from experimental roosters and the proportion of embryos with *ZsGreen* gene expression were evaluated. The transformation efficiency of target cells was determined by expression of the *ZsGreen* reporter gene using a Nikon Ni-U microscope (Nikon, Japan). The chicken embryonic cell culture obtained in the first stage of the experiment consisted of the several types of cells. The proportion of PGCs did not exceed 3%. The percentage of PGCs in the cell suspension increased to 81% after separating the different types of embryonic cells by adhesion. The PGCs culture transformation efficiency was 12 %. The presence of fluorescent spermatogenic cells in the testes seminiferous tubules was established both with the introduction of transformed donor PGCs and with a lentiviral vector. With the introduction of donor PGCs at a concentration of 400, 700 and 1000 cells per embryo, the percentage of chickens with transformed germ cells was 16%, 23% and 26%, respectively. With the twofold introduction of the viral drug into the testes at the age of 3 and 4 months, the highest transformation efficiency of spermatogenic testicular cells in vivo was established, which amounted to 10%.

With a single injection of the viral drug, this indicator was 2 times lower. The possibility of using the obtained individuals with transformed germ cells to obtain transgenic offspring is shown. The efficiency of obtaining transgenic embryos is 6-10%.

Keywords: roosters, embryos, primordial germ cells, spermatogenic cells, lentiviral vector, transgenesis

Male germ cells can be used for targeted delivery of recombinant DNA to generate genetically modified individuals [1, 2]. The success of this largely depend on the improvement of artificial insemination method, which greatly enhance potential of using genetically modified semen to generate population of individuals with desirable traits. The genetic transformation of male germ cells makes it possible to purposefully affect specific target cells, reducing the risks of transgenic mosaics unable to produce transgenic offspring. Primordial germ cells (PGCs, the germ cell precursors) [3, 4], spermatogonia (undifferentiated male germ cells) [5, 6] and sperm (mature male germ cells) [7] can be used as targets in genetic transformation of male germ cells of farm birds.

PGCs and spermatogonia are the most effective in producing transgenic and chimeric individuals, since these cells, during differentiation, can form a significant population of transformed mature germ cells [8, 9]. An in vitro culture of poultry germ and spermatogenic cells allows the number of techniques to be involved in delivering recombinant DNA into target cells with the use of liposomal transfection [10], electroporation [11, 12], transposon-mediated manipulations [13), cationic polymers [14], lentiviral (15) and retroviral vectors [16, 17].

PGCs and spermatogonia donor cells, after isolation and transformation, should be transplanted into the gonads of recipients to subsequently generate offspring with the acquired trait. Colonization of donor germ cells during their transplantation into the gonads of male recipients have been initially shown in laboratory animals [18, 19]. There are reports on efficiency of donor germ cell transplantation in various farm animals, i.e. in pigs [20, 21], sheep [22], goats [23], and bulls [24]. For poultry, works have been done on roosters [25, 26] and quails [27].

The novelty of this research is the development and optimization of particular stages of local transformation of rooster spermatogenic cells by transfecting PGCs of embryos and testicular spermatogenic cells during early differentiation.

Our goal was to evaluate the effectiveness of various techniques in genetic transformation of rooster's germ cells.

Materials and methods. Primordial germ cells were isolated from 6-day-old embryos of Russian White chicken (*Gallus gallus domesticus*). The embryos were subjected to mechanical dissection/dissociation and enzymatic dissociation with a 0.05% trypsin solution. The resulting cell suspension was cultured in Petri dishes in DMEM growth medium (Dulbecco's Modified Eagle Medium) (Thermo Fisher Scientific, USA) with high glucose concentration (4.5 g/l), bovine fetal serum (10%), glutamine (2 mM), 2-mercaptoethanol (10^{-6} mM) and gentamicin (50 μg/ml).

PGCs transformation was carried out by electroporation (a Neon system, Thermo Fisher Scientific, USA). ZsGreen1-N1 plasmid (Addgene, USA) with *ZsGreen* gene under the control of a CMV promoter was used for transfection. Transformed cells (400, 700, and 1000 cells for test groups I, II, and III, respectively) were injected into dorsal aorta of 2.5-day-old embryos, which were incubated until hatching (RCOM Maru 190 Deluxe MAX, Rcom, South Korea). The chicks, when aged 1.5-2-month, were slaughtered, and the reproductive organs were sampled to assess the effectiveness of germ cell transformation. In the control, DMEM growth medium was injected into the dorsal aorta of embryos.

For in vivo transformation of spermatogenic cells, a lentiviral vector preparation (1×10^7 CFU/ml) was injected directly into the testes of roosters (0.5 ml per testis), once at the age of 3 months (group I), once at the age of 4 months (group II), and twice at the age of 3 months and 4 months (group III). The lentiviral vector contained *ZsGreen* reporter gene under the control of a CMV promoter. Upon reaching maturity, testicular tissues were sampled, and histological sections were prepared (a Shandon Cryotome E cryostat, Thermo Fisher Scientific, USA). Non-transgenic roosters of the same age and breed were control.

The roosters with donor germ cells (group I with transforrmed PGCs introduced into embryos) and their own transformed germ cells (group II with the viral vector injected into the testes) were used to inseminate the females. The fertilizing ability of the semen and the proportion of embryos with *ZsGreen* gene expression were evaluated to assess the transgenic offspring production.

Target cell transformation efficiency was determined by expression of the *ZsGreen* reporter gene. Analysis of cytological and histological preparations was carried out using a Nikon Ni-U microscope with NIS-Elements imaging software (Nikon, Japan).

Statistical analysis was performed with Microsoft Excel 2016 software (*t*-test). The tables show arithmetic means (*M*) and mean errors (±SEM). Differences were deemed statistically significant at $p < 0.01$

Results. The obtained chicken embryonic cell culture consisted of several types of cells, mainly fibroblasts, with PGCs portion not exceed 3%. Separation of different types of embryonic cells by adhesion allowed us to increase the PGCs percentage in cell suspension up to 81% (Fig. 1, A, B). This cell culture was used for transformation with recombinant DNA. The efficiency of PGCs transformation was 12%. This culture was used as donor cells for injection into embryos (see Fig. 1, C, D).

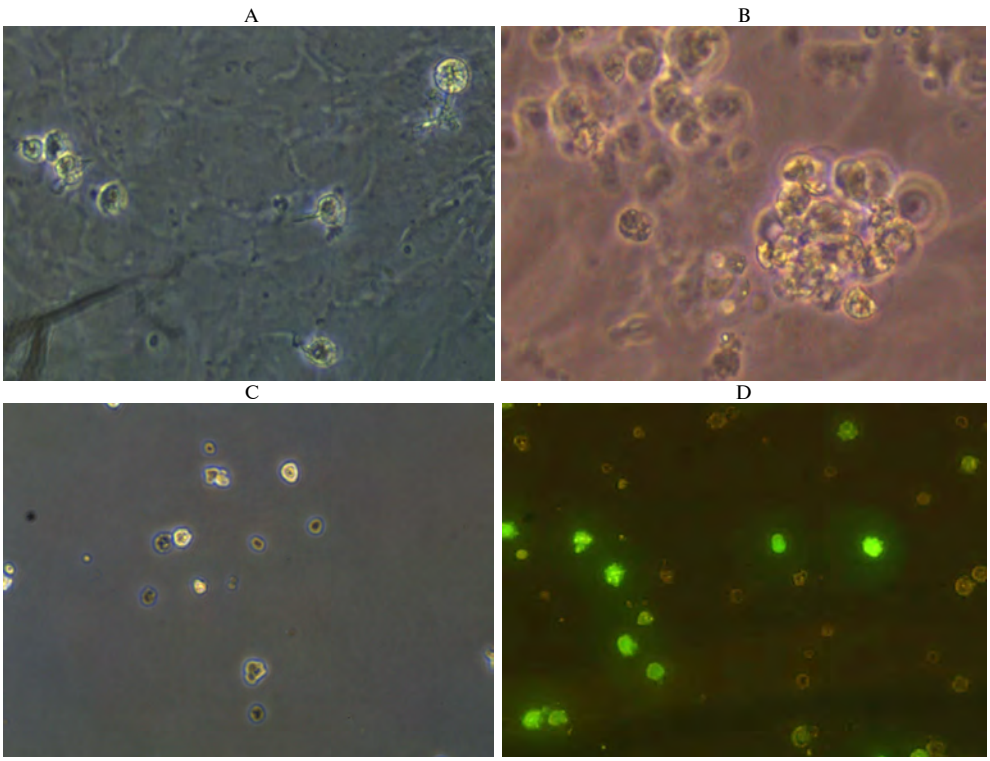

Fig. 1. Culture of primordial germ cells of Russian White chickens: A — day 2 of culture, B — day 7

of culture (colonies of primordial germ cells — PGCs), C — native suspension of PGCs before transformation, G — PGCs suspension after transformation (transformed cells fluoresce). Light microscopy, phase contrast (A-C); fluorescence microscopy (D) (magnification ×400, Nikon Ni-U microscope, Nikon, Japan).

A culture of transformed donor PGCs in various concentrations was introduced into the dorsal aorta of 2.5-day recipient embryos (152 embryos in total). The number of the introduced donor cells did not significantly affect development of the embryos. The proportion of developed embryos varied between test groups from 78 to 85%, 83% value in the control group. In the test groups, a stop in the embryo development occurred mainly on days 6 to 10 and at the end of incubation, while in the control group at the end of incubation. Increased embryonic mortality in the test groups during early embryogenesis may be associated with the genetic engineering manipulations.

Colonization efficacy and the development of donor PGCs in recipient chickens were investigated in birds aged 1.5-2 months. Fluorescence microscopy reveled green-fluorescent spermatogenic cells in the seminiferous tubules of testicle (Fig. 2, A). The number of chickens with transformed germ cells varied depending on the number of administered donor cells, being 16% for group I (400 PGCs), 23% for group II (700 PGCs), and 26% for group III (1000 PGCs) (Table 1).

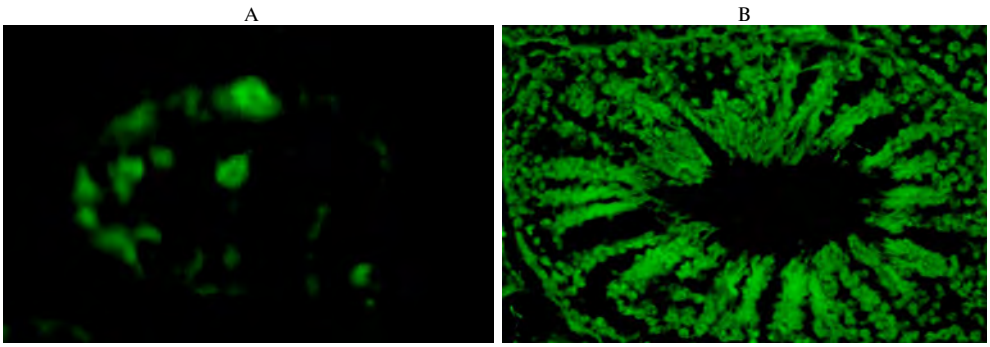

Fig. 2. Expression of transferred *ZsGreen* gene in gonad cells of Russian White roosters of different age: A — 1.5 month of age (after injection of 400 transformed primordial germ cells to a recipient embryo)**, B — 9 month of age** (in vivo viral-vector mediated testis gene transfer). Fluorescent spermatogenic cells at different stages of differentiation are visible inside the seminiferous tubules of the testes (A, B), including spermatozoa in the lumen of the transformed tubule (B). Fluorescence microscopy (A — magnification ×400, B — magnification ×160, Nikon Ni-U microscope, Nikon, Japan).

1. Colonization efficacy of transformed donor primordial germ cells (PGCs) **in recipient Russian White chicken embryos**

Indicator	Control	Group I	Group II	Group III
Embryos per group, *n*	30	50	52	50
Donor cells, *n*		400	700	1000
Developed embryos, *n* (%)	25 (83)	40 (80)	44 (85)	39 (78)
Hatched chickens:				
in total, *n* (%)	18 (60)	31 (62)	33 (63)	29 (58)
with genetically modified germ cells, *n*		8	12	13
Efficacy of transgenesis, %		16	23	26

N o t e. For a description of the groups, see the Materials and methods section. Efficacy of transgenesis is the ratio of the individuals with transformed germ cells to the total number of injected embryos.

Along with transplantation of transformed donor PGCs, a technique was developed for the in vivo transformation of spermatogenic cells in rooster's testes to generate genetically modify sperm. The efficiency of transformation of spermatogenic cells depended on the scheme of the lentiviral vector administration

(see Fig. 2, B, Table 2). In the roosters upon one injection of the vector at 3-month age (group I), the percentage of seminiferous tubules with transformed spermatogenic cells varied from 3 to 8% and averaged 5.8±0.4%. The efficiency of single administration of the lentiviral vector to roosters aged 4 months (group II) was less effective than in group I and did not exceed 4.0±0.6% on average. A 2-fold administration of the viral preparation (group III) contributed to a reliable 1.7-fold and 2.4-fold ($p < 0.01$) increase in the efficiency of in vivo transformation as compared to group I and group II, respectively.

2. Efficiency of in vivo viral-vector mediated gene transfer to testicular spermatogenic cells of Russian White roosters (*M*±SEM)

Indicator	Group I	Group II	Group III
Roosters per group, *n*	5	5	5
Age of lentiviral vector administration	3 months	4 months	3 and 4 months
Seminiferous tubules per rooster, *n*	201±1	252±13	257±24
Seminiferous tubules with transformed spermatogenic cells, *n*	12±1	10±1	24±2[a, c; b, c]
Efficacy of transgenesis, %	5.8±0.4	4.0±0.6	9.6±0.8[a, c; b, c]

N o t e. For a description of the groups, see the Materials and methods section. Efficacy of transgenesis is the ratio of the number of seminiferous tubules with transformed spermatogenic cells to total number of seminiferous tubules в.

[a, c] Differences between group I and group III are statistically significant at $p < 0.01$.

[b, c] Differences between group II and group III are statistically significant at $p < 0.01$.

The percentage of live sperm cells in the semen of transgenic roosters was slightly lower compared to non-transgenic analogues at less than 2% differences (Table 3). In the test groups, the number of morphologically abnormal spermatozoa was 3% higher as compared to the control. Defects of sperm flagella were the most frequent in both control and test roosters.

Sperm fertilizing ability of transgenic roosters was 5-6% lower compared to the control. The expression of reporter *ZsGreen* gene was detected in 10% of embryos in group I and 6% of embryos in group II.

3. Sperm quality and fertilizing ability in non-transgenic and transgenic Russian White roosters produced by transplanting genetically transformed donor primordial germ cells and in vivo viral-vector mediated gene transfer of spermatogenic cells (*M*±SEM)

Indicator	Control	Group I	Group II
Sperm motility, %	84±1	82±1	82±2
Proportion of live spermatozoa, %	88±1	87±2	86±2
Proportion of sperm with abnormal morphology, %	7±1	9±1	10±1
Fertilizing ability:			
incubated eggs, *n*.	50	50	50
developed embryos on day 6 of incubation, *n* (%)	43 (86)	41 (80)	42 (81)
Embryos with *ZsGreen* gene expression, *n* (%)		5 (10)	3 (6)

N o t e. For a description of the groups, see the Materials and methods section.

Various techniques have now been suggested for transformation of PGCs and spermatogenic cells. As per J. Macdonald et al. [13], an efficiency of chicken PGCs transfection with piggyBac and Tol2 transposons was 5.4 and 25.5%, respectively. Sawicka et al. [29] and Tyack et al. [10] effectively transfected chicken PGCs using transposon vectors in combination with lipofection. Naito et al. [28] derived cell culture from chicken PGCs and transformed it by nucleofection with an efficiency of 10%. The cultured GFP-positive PGCs were transferred into the bloodstream of recipient embryos. Test mating revealed one chimeric chicken, which produced one donor-derived offspring (with detected reporter GFP) of 270 examined. These data are consistent with 12% efficiency of chicken PGCs transformation we obtained in our research by electroporation.

Min et al. [30] and Li et al. [14] used direct injection of SofastTM cationic polymer in combination with a genetic construct into the testis parenchyma for in

vivo transformation of rooster spermatogenic cells. Min et al. [30] conducted research on the production of chickens resistant to avian influenza virus. The transformation efficiency of spermatogenic cells was 72.2%. The transgene was found in 10% of sperms and in blood of 7.8% of the resulting F_1 progeny. In the experiments of B. Li et al. [14] the efficiency of spermatogenic cell transformation did not exceed 12-19%.

We used the lentiviral vector carrying *ZsGreen* reporter gene for in vivo transfection of roosters' testicular spermatogenic cells. As a result, GFP gene expression was detected in 10% of the spermatogenic cells and in 6% of embryos after the sperm of the transgenic roosters was used for insemination.

In conclusion, our findings confirm that male primordial germ cells (PGCs) and spermatogonia are good targets for recombinant DNA transfer. Resultant male recipients with genetically modified germ cells in the gonads can generate transgenic offspring with certain traits. In our research, when 1000 donor PGCs after genetic transformation were transferred to a recipient embryo, the transgenesis efficiency was the highest: 26% of chickens with GFP expression in gonads vs. 16% and 23% for 400 and 700 PGCs per embryo, respectively. A 2-fold administration of the lentiviral vector to males at the age of 3 and 4 months provided 10% in vivo modification of testicular spermatogenic cells which was 2 times higher compared to a single injection of the lentiviral vector. Six to ten percent of offspring from the roosters with genetically transformed germ cells were transgenic.

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INTESTINAL MICROFLORA AND EXPRESSION OF IMMUNITY-RELATED GENES IN HENS AS INFLUENCED BY PREBIOTIC AND PROBIOTIC FEED ADDITIVES

I.I. KOCHISH[1], O.V. MYASNIKOVA[1], V.V. MARTYNOV[2], V.I. SMOLENSKY[1]

[1]*Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology*, 23, ul. Akademika K.I. Skryabina, Moscow, 109472 Russia, e-mail prorector@mgavm.ru, omyasnikova71@gmail.com, Smolensky-vgnki@mail.ru (✉ corresponding author);
[2]*Moscow Region State University*, 24, ul. Very Voloshinoi, Mytishchi, Moscow Province, 141014 Russia, e-mail martynov.vik@gmail.com
ORCID:
Kochish I.I. orcid.org/0000-0001-8892-9858
Martynov V.V. orcid.org/0000-0003-1784-3429
Myasnikova O.V. orcid.org/0000-0002-9869-0876
Smolensky V.I. orcid.org/0000-0002-2124-0017
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Abstract

It is known that probiotic and prebiotic feed additives improve the function of the intestines and lead to normal the processes of digestion of food for animals. Colonization of the gastrointestinal tract with beneficial microflora helps to reduce the negative impact of pathogenic or opportunistic pathogenic microflora, maintain optimal acidity of the gut, prevent dysbiosis, and stimulate local and general immune factors. However, the biological mechanisms for the implementation of such properties of these drugs are still not fully understood. We evaluated the effect of two Russian products, the multifunctional feed additive Profort® (Biotrof LLC, Russia), combining the qualities of an enzyme and probiotic, and the prebiotic feed additive Vetelact (SVC, Agrovetzashchita, Russia) on the quantitative and qualitative composition of the intestinal microbiota in egg layers to compare the effect of these feed additives on the intestinal microbiota with the expression of the β-defensin 9 (AvBD9), interleukin 8 (IL8), gallinacin-10 (Gal-10) and proenkephalin (PENK) genes that are associated with immune systems. Lohmann white LSL hens with an egg laying intensity of at least 95 % at the age of 25 weeks were used in the experiment (the conditions of vivarium, 2019). The hens were assigned to three groups (20 birds each). Feeding the birds was carried out with mixed feed, the feed specification were calculated according manual from Lohmann Tierzucht. Birds of the control group received only mixed feed. Birds of the experimental groups were also fed with biological additives for 28 days. The egg production was recorded daily, the egg laying intensity, egg weight and body weight were recorded weekly. After the termination of the experiment, the composition of the microbiota of the blind processes of the intestine was determined using NGS sequencing and the expression levels of the β-defensin 9 (*AvBD9*), interleukin 8 (*IL8*), gallinacin-10 (*Gal-10*), and proenkephalin (*PENK*) genes were assessed. It is known that β-defensin 9 and gallinacin-10 belong to the family of endogenous peptides, which are an important element of the innate immunity system and a link between innate (non-specific) and acquired (adaptive, specific) immunity, proenkephalin is one of six opioid peptides that regulate signaling between cells and affect many biological processes in vertebrates, including development, growth and reproduction, and interleukin 8 is one of the main pro-inflammatory chemokines formed by macrophages, epithelial and endothelial cells which also plays an important role in the innate immune system. It was established that the hens receiving probiotic had the highest egg productivity (3.33 % higher than the control, $p < 0.05$), while their final body weight was minimal. Feeding a prebiotic led to a 0.24-0.45 % ($p > 0.05$) decrease in egg production with the body weight 0.9 % ($p > 0.05$) higher compared to the control. Feeding the prebiotic contributed to an increase in the total number of microorganisms in the intestinal contents to 7.625±0.74 lg CFU/g (the microbial number in the control was 7.598±1.01 lg CFU/g), while the feeding with probiotic reduced the number of microorganisms to 7.565±0.56 lg CFU/g ($p > 0.05$). Both feed additives contributed to an increase in the number of bifidobacteria and cellulolytic bacteria in the intestine and reduced the total amount of pathogenic and undesirable microflora by 25-50 % vs. control. A decrease in the proportion

of pathogenic and undesirable microorganisms in the composition of microbiota naturally reduced the body's need for non-specific defense factors and pro-inflammatory cytokines. In the birds receiving feed additives, the expression of the β-defensin 9 gene was 3.3-5.0 times lower, and the interleukin 8 (*IL8*) gene expression level was reduced by 8-36 % compared to the control. Along with a decrease in the expression of β-defensin 9 and interleukin-8 genes, a 1.48-1.55-fold increase in the expression of the gallinacin-10 gene and 1.11-1.91-fold increase in proenkephalin were established, which is probably associated with strengthening the protective functions of the body. The selective effect of probiotic and prebiotic on the reproduction of various types of bacteria in the intestine, confirmed by the negative expression of genes associated with immunity, justifies the promise of using the studied products to increase the resistance of poultry and normalize functions of the immune system without compromising of poultry performance.

Keywords: commercial poultry, probiotic, prebiotic, intestinal microbiota, immune factors, β-defensin 9, interleukin 8, gallinacin-10, proenkephalin, genes expression

Modern immunologists appreciate application of bioactive feed additives favorable for normal intestinal microflora and stimulating body defenses as a better approach in counteracting infectious processes [1, 2].

The intestines of farm animals and poultry play an important role not only in the assimilation of food nutrients, but also in maintaining the body's immune defense [3, 4]. The barrier function of the intestinal microvilli of the cylindrical epithelium cannot fully protect the body from the invasion of pathogenic bacteria and viruses if the intestines are not colonized by a number of beneficial microorganisms. It has been proven that normal microflora stimulates the development of some cecum tissues in mammals [5]. The gut microbiota of birds performs numerous functions to maintain homeostasis and resistance. It takes part in the normal functioning of the cardiovascular, endocrine, hematopoietic, nervous and other systems. The intestinal microflora synthesizes amino acids, enzymes, antibiotics, vitamins, and other metabolites valuable for the macroorganism [2, 6]. Microbiota also plays a significant role in maintaining the body's defense system [7, 8].

The intestines are one of the main sites of invasion and habitat of pathogenic microorganisms. Therefore, the intestinal functionalities provide for a special mechanism which is extremely important for the fight against the pathogenic microflora [7] with two groups of factors involved. The first group includes physical barriers and special conditions of the internal environment [8], e.g. mucous layer and the protective properties of mucin, preventing penetration and attachment of microorganisms to the intestine villi, acidic pH in the small intestine, normal oxygen levels in the intestinal environment which prevent proliferation of anaerobes, etc. [9, 10]. The second group comprises immune factors, e.g. antimicrobial peptides (defensins), neuropeptides and interleukins, which regulate synthesis of mucin and intestinal immunoglobulins.

Mucins are glycosylated proteins with a molecular weight of up to 20 kDa, which play a key role in preventing pathogen penetration through the intestinal mucosa [11, 12]. There is a distinct relationship between intestinal microflora and the amount of mucin [13].

Antimicrobial peptides are the key components of innate immune system in animals. These peptides are capable of disrupting integrity of the membranes of microorganisms [14]. α-Defensins are characteristic only for mammals, while birds possess only β-defensins [15]. Four types of defensins called gallinacins have been identified in chickens. Gallinacins are specific towards *Campylobacter* sp., *Salmonella* sp., *Clostridia* sp., *Escherichia coli*, can suppress their growth, change the morphology and ultimately, cell lysis and death [16]. Gallinacins are expressed in the small intestine, liver, gall sac and spleen of chickens. Other recently studied chicken defensins possess tissue specificity, e.g. AvBD1, AvBD7 and AvBD9 are

expressed in goiter, AvBD8, AvBD10 and AvBD13 in the intestine, AvBD1 and AvBD7 in spleen [14, 17, 18].

Canadian researchers [19] showed that a probiotic containing *Lactobacillus acidophilus*, *L. casei*, *Streptococcus faecium*, *Saccharomyces cerevisiae* and organic acids positively influenced intestinal morphology in chickens (the duodenum of test birds was longer than in control). Expression of immunity-related genes gave conflicting results. Drinking probiotics for 7 or 14 days leads to an increase in the expression of AvBD3, IL6, IL10 genes, while in interleukin 12 (IL12) and γ-interferon (INF-γ) genes it was lower compared to control.

Proenkephalin, like other neuropeptides, not only affects regulation of the inflammatory process, but also coordinates cell-cell signaling and reduces the activity of cellular alkaline phosphatase [20-22]. The main function of proinflammatory interleukins (proinflammatory cytokines) is attracting additional leukocytes from the blood to the pathological focus to increase the resistance of the epithelial cells to the infection [14, 23].

Most recent studies on bioactive feed additives in poultry farming have evaluated the effects of these drugs on productivity, intestinal health, and gene expression in broiler chickens as the most convenient model. Laying hens, the production cycle of which exceeds 80 weeks, remained outside the scope of such studies, though the search for drugs that will preserve intestinal health and immunity in laying hens remains urgent.

This work is the first study in Russia showing effects of dietary pre- and probiotic supplementation not only on poultry gut microbiota via stimulation of beneficial microflora, but also on expression of immune-related genes.

This study aimed to assess the effect of prebiotic and probiotic preparations on productive performance, gut microbiota patterns, and the expression of immunity-associated genes in laying hens.

Materials and methods. For experiments (vivarium of Scriabin MVA, 2019), Lohmann white LSL cross female chickens aged 18 weeks were placed in individual cages to determine the rate of lay. Hens with an egg laying rate of at least 95 % at the age of 25 weeks were assigned into 3 groups of 20 bird each.

The control birds (C) were fed standard compound feed (basic ration, BR) in accordance with the recommendations for the cross, including wheat, sunflower and soybean meal, grass meal, sunflower oil, vitamins and mineral supplements. Feed additives were used for 28 days as per the manufacturers' instructions. Chickens of group I (test) received a lactulose (50%)-based prebiotic Vetelact (NEC Agrovetzashchita SP, Russia) on at 0.1 ml/kg body weight. Chickens of group II (test) received probiotic Profort® (Biotrof LLC, Russia) containing live cells of *Bacillus megaterium* B-4801 and *Enterococcus faecium* 1-35 (at least 7.0 lg CFU/g, 500 g/t feed).

Laid eggs were counted daily, the egg laying rate was calculated weekly. Egg weight and poultry body weight were determined weekly by individual weighing (electronic scales ME-R 326AFU, Mercury Equipment, China).

At the end of the experiment, 5 individuals from each group were euthanized. Cecal tissue fragments and cecal contents were sampled to assess expression of β-defensin 9, gallinacin-10, interleukin 8, proenkephalin genes and to profile microbiota composition.

Microbial DNA was extracted using QIAamp Power Fecal DNA Kit (Qiagen, USA) as per the manufacturer's protocol. DNA was quantified with a Qubit 3.0 fluorimeter (Thermo Fisher Scientific, Inc., USA).

Total microbial counts per unit volume were determined by quantitative PCR (qPCR, a Light Cycler® 96 System thermocycler, Roche, Switzerland) with

Maxima SYBR Green/ROX qPCR Master Mix (dye SYBR Green fluorescent detection, Termo Fisher Scientific, Inc., USA). The primers used were Eub338 5′-ACTCCTACGGGAGGCAGCAG-3′, Eub518 5′-ATTACCGCGGCTGCTGG-3′ (Eurogen, Russia). To quantify microbial community composition, extracted DNA was processed using 16S Metagenomik Kit and Ion 520 & Ion 530 ™ Kit-OT2 kit (Thermo Fisher Scientific, Inc., USA) as per the manufacturer's instructions and loaded on the chip for NGS sequencing (an Ion GeneStudio™ S5 System, Thermo Fisher Scientific, Inc., USA). In total, 2 million reads with 300-400 bp read length were obtained (211,000 reads on average per sample). DNA sequencing data analysis was performed with Ion Reporter network software (https://ionreporter.thermofisher.com/ir/).

In addition to a simple comparison of the microbiome profiles, the number of taxa weighted by relative abundance per the Shannon and Simpson diversity indexes was determined [24].

To assess gene expression, total RNA was extracted from cecal tissue fragments. RNA purity was estimated by classical agarose gel electrophoresis method (Mini-SubCell GT camera, Bio-Rad, USA). cDNA was synthesized from RNA template via reverse transcription (iScript kit for cDNA synthesis, Qiagen, USA). Analysis of gene expression, allowing detection of its activation upon a particular effect, was performed by real-time PCR (a LightCycler® 96 System thermocycler, Roche, Switzerland) with 2× Quanti Nova SYBR GREEN PCR kit (Qiagen, Austria). The reference genes were the "housekeeping" genes *TBP* (TATA-binding protein) and *ACTBL2L* (β-actin) (since the primer annealing temperature was different for the studied genes, two "housekeeping" genes corresponding to primers were taken). the cycle threshold (Ct) value was determined for each reference and analyzed genes. The data were processed by the Livak and Shmitgen method [25] with calculation of mean values of threshold cycles in the group and ΔCt (difference between threshold cycle values for the desired gene and the "housekeeping" gene), then of differences between ΔCt for test and control groups $\Delta\Delta Ct = \Delta Ct2 - \Delta Ct1$. Relative gene expression was calculated as the threshold cycle value normalized by control ($2^{-\Delta\Delta Ct}$) [25].

Mathematical and statistical analysis was carried out using standard methods of correlation and analysis of variance (Excel 2007 software). The means (*M*) and standard error of the mean (±SEM) were calculated. The results were checked for the significance of differences according to Student's *t*-test (https://gallery.shinyapps.io/dist_calc/). Differences were deemed statistically significant at $p < 0.05$. Each cDNA sample was examined in real-time PCR in triplicate. Assessment of biological diversity and processing of microbiota data was performed using Qiime 2.0 bioinformatics platform (https://qiime2.org/).

Results. According to the manufacturer's information, the Profort® probiotic feed additive is able to normalize microflora and increase the safety and productivity of poultry. The probiotic bacteria of Profort® synthesize lactic acid and vitamin B_{12} which stimulates regeneration of intestinal epithelium, participates in synthesis of nucleic acids and accelerates restoration of antioxidants in the body [26]. Studies on broilers showed that the growth rate in chickens fed with dietary Profort® was 6.9% higher than in the control group, while feed conversion was 3% better [27]. Prebiotic preparation Vetelact contains lactulose, the disaccharide of galactose and fructose, which is cleaved in colon into low molecular weight organic acids. These acids stimulate growth of beneficial bifidobacteria and lactobacilli, inhibit potentially pathogenic clostridia and Escherichia, stimulate intestinal motility, improve absorption of phosphorus and calcium salts, and promote excretion of ammonium ions [28]. The use of Vetelact increased broiler safety by 2.85% and body weight at the end of growing by 2.38-3.52% while

reducing the cost of feed per bird by 3.3-3.6% and per 1 kg weight gain by 5.8-7.1% [29].

In our tests, the body weight of chickens, egg production and egg weight did not differ significantly between all groups (Table 1).

1. Productivity of Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, $M \pm$SEM, vivarium of Skryabin MVA, 2019)

Indicator	C (control)	Group I (prebiotic)	Group II (probiotic)
At the begining of the experiment (175 days of age)			
Live weight, g	1434.4±20.76	1451.1±26.27	1417.5±22.44
Egg weight, g	56.19±0.82	57.24±1.18	55.13±0.67
Egg production, pcs.	28.39±2.04	27.69±1.98	28.18±2.11
Egg-laying rate, %	95.23±0.82	95.26±0.83	95.36±0.82
At the end of the experiment (203 days of age)			
Live weight, g	1468.1±16.02	1482.13±29.31	1467.53±18.22
Egg weight, g	58.19±0.53	58.05±1.31	58.91±0.83
Egg production, pcs.	21.47±0.25	21.38±0.34	22.24±0.30
Egg-laying rate, %	93.35±1.09	92.93±1.48	96.68±1.32*

* Difference from control are statistically significant at $p < 0.05$.

Table 1 shows that feeding chickens of group II with Profort® led to 1.23% increase in egg weight ($p > 0.05$) and 3.3% increase in egg-laying rate ($p < 0.05$) compared to the control. Since birds of this group expended more energy or egg production, at the end of the experiment, they were 0.03% inferior to the control in average body weight ($p > 0.05$). Prebiotic Vetelact did not have a significant effect on productivity and even slightly decreased it (by 0.24-0.45%, $p > 0.05$) as compared to control. Moreover, the weight of chickens in the test slightly exceeded that in the control (by 0.9%, $p > 0.05$).

However, the prebiotic slightly increased the total number of microorganisms in the intestinal contents (7.625±0.74 log CFU/g) while the probiotic, on the contrary, reduced this indicator value to 7.565±0.56 log CFU g (in control 7.598±1.01 log CFU/g). However, the revealed differences between the groups were unreliable, being just a trend.

2. Microbial profiles (%) of cecal contents in Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, $M \pm$SEM, vivarium of Skryabin MVA, 2019)

Taxon	C (control)	Group I (prebiotic)		Group II (probiotic)	
		total	Δ to control, %	total	Δ to control, %
Phylum *Actinobacteria* including:	0.08±0.03	0.12±0.10	+50.00	0.18±0.15	+125.00
order *Bifidobacteriales*	0.08±0.02	0.10±0.10	+20.00	0.07±0.05	–12.50
Phylum *Bacteroidetes*	32.00±2.20	27.10±1.80	–15.40	37.4±2.74	+16.80
Phylum *Firmicutes* including	52.40±2.40	55.00±2.70	+4.92	47.2±4.04*	–9.90
family *Lactobacillaceae*	32.20±3.80	36.90±4.50	+14.70	23.1±3.8*	–28.30
family *Clostridiaceae*	13.30±5.30	16.10±2.30	+20.60	21.4±2.1*	+60.40
family *Ruminococcaceae*	5.86±0.95	4.78±0.60	–18.40	7.08±0.92	+20.80
genus *Selenomonadales*	0.12±0.02	0.14±0.03	+16.70	0.21±0.15	+75.00
Phylum *Proteobacteria* including	15.20±2.31	17.70±1.06	+16.50	14.7±1.66	+39.10
family. *Enterobacteriacea*	0.75±0.17	0.35±0.09	–53.30	0.51±0.26	–32.00
Phylum *Synergistetes*	0.03±0.01	0.05±0.01	+66.60	0.03±0.01	0
Phylum *Tenericutes* including	0.06±0.03	0.04±0.02	–33.30	0.09±0.05	+50.00
family *Mycoplasmataceae*	0.04±0.03	0.00±0.00	–91.70	0.01±0.01	–75.00
Phylum *Spirochaetes*	0.01±0.00	0.00±0.00	–66.60	0.02±0.02	+100
Pathogens and undesirable microorganisms in total	0.88±0.10	0.44±0.07	–50.00	0.66±0.20	–25.00
Uncultured	0.23±0.20	0.02±0.01	–91.30	0.32±0.26	–3.10

* Difference from control are statistically significant at $p < 0.05$.

A comparison of microbial profiles of cecal bacterial community in the test and control groups (Table 2) revealed six main phyla, *Actinobacteria*,

Bacteroidetes, *Firmicutes*, *Proteobacteria*, *Synergistetes* and *Tenericutes*. The exception was bacteria of the phylum *Spirochaetes* absent in birds of the test group I. The bacteria of this phylum belong mainly to pathogenic and undesirable microflora, and in healthy individuals (control and test groups in the experiment) its content is allowed in minimal quantities. The genus *Lactobacillus* is important for intestinal microbiota, as it provides nutrients to the host and protects against opportunistic microflora, and bacteria of the *Bifidobacteriales* order can synthesize vitamins to supply the host body [6].

The abundance of *Actinobacteria* phylum increased by 50.00% in birds from group I fed with the prebiotic preparation (including 20.00% growth in counts of order *Bifidobacteriales*), the level of bacteria from family *Lactobacillaceae* increased by 14.70%, while the number of cellulolytic bacteria of family *Ruminococcaceae*, as well as pathogenic and undesirable microflora decreased by 18.40 and 50.00%, respectively. In birds from group II fed with a probiotic the number of bacteria of the phylum *Bacteroidetes* decreased by 12.50%, of *Lactobacillaceae* family by 28.30%, while the number of cellulolytic bacteria increased by 20.80%, and the abundance of pathogenic and undesirable microflora decreased by 25.00% (see Table 2). Publications on age-related changes in microbiota in laying hens have reported a decrease in the abundance of cellulosolytic bacteria with age and an increase in abundance of phylum *Bacteroidetes* and lactobacilli [30]. In experiments on chickens [6], feeding a phytobiotic with *Macleaya cordata* plant extract of led to an increase in the number of lactobacilli, a decrease in the abundance of pathogenic microflora, and a decrease in the expression of cytokine and immunoglobulin genes (IL-4, IFN-γ).

Thus, in our study, the prebiotic and probiotic had a multidirectional effect on the abundance of bifidobacteria and cellulolytic bacteria, but equally affected the decrease in the number of pathogenic and undesirable microorganisms.

Analysis of α-diversity of the chicken cecal microbiota using the Shannon index (3.27±0.10 in control, 3.16±0.10 in group I, and 3.40±0.04 in group II) and the Simpson index (0.84±0.02; 0.81±0.03 and 0.86±0.001, respectively) showed that the differences between the groups are not statistically significant ($p > 0.05$), which allows us to conclude only about a trend.

β-Defensins and gallinacins of birds play a vital role in innate antibacterial immunity [31, 32]. Defensins, being cationic peptides, are active against bacteria, fungi, enveloped and non-enveloped viruses. Immune cells use defensins to kill bacteria absorbed in phagocytosis [2].

Immune-related genes and primers that we used to study their expression in birds as influenced by dietary probiotic and prebiotic additives are shown in Table 3.

3. Primers used to assess the immune-related gene expression in Lohmann White cross chickens (vivarium of Skryabin MVA, 2019)

Gene, protein	Primer
«Housekeeping» genes:	
ACTBL2L (β-actin)	F: 5′-ATTGTCCACCGCAAATGCTTC-3′
	R: 5′-AAATAAAGCCATGCCAATCTCGTC -3′
TBP (TATA-binding protein)	F: 5′-GAACATCATGGATCAGAACAACA-3′
	R: 5′-ATAGGGATTCCGGGAGTCAT-3′
AvBD9 (defensin 9)	F: 5′-AACACCGTCAGGCATCTTCACA-3′
	R: 5′-CGTCTTCTTGGCTGTAAGCTGGA-3′
Gal-10 (gallinacin-10)	F: 5′-GCTCTTCGCTGTTCTCCTCT-3′
	R: 5′-CCCAGAGATGGTGAAGGTG-3′
PENK (proenkephalin)	F: 5′-GCTGGATGAGAACCATCTGC-3′
	R: 5′-AGCCTCCGTACCTCTTAGCC-3′
IL8 (interleukin 8)	F: 5′-GGAAGAGAGGTGTGCTTGGA-3′
	R: 5′-TAACATGAGGCACCGATGTG-3′

In birds fed with probiotic and prebiotic drugs, the expression of *AvBD9* gene reduced significantly (5.0 and 3.3 times, respectively) (Table 4). The expression of β-defensins in the intestine is induced by pro-inflammatory cytokines [26], as well as by microorganisms (for example, in humans, by *Escherichia coli*, *Helicobacter pylori* or *Pseudomonas aeruginosa*) [33]. In our tests, a decrease in the number of pathogenic microorganisms seemed to reduce the need for pro-inflammatory cytokines, which was reflected in the level of β-defensin 9 synthesis. This pattern was also characteristic of gene *IL8* expression but to a lesser extent (Table 5).

4. β-Defensin 9 gene *AvBD9* expression in cecal tissues of Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, M±SEM, vivarium of Skryabin MVA, 2019)

Group	Ct *TBP*	Ct *AvBD9*	ΔCt	ΔΔCt	Values nirmalized by control ($2^{-\Delta\Delta Ct}$)
C (control)	25.91±0.77	29.34±0.84	3.44	0	1
I (prebiotic)	24.85±0.43	30.60±0.65	5.75	2.31	0.20*
II (probiotic)	22.92±0.47	28.14±0.92	5.22	1.78	0.29*

Note. ΔCt = Ct *AvBD9* – Ct *TBP*; ΔΔCt = ΔCt test – ΔCt contol; *TBP* (TATA-binding protein) — a «housekeeping» gene.
* Difference from control are statistically significant at $p < 0.05$.

5. Interleukin 8 gene *IL8* expression in cecal tissues of Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, M±SEM, vivarium of Skryabin MVA, 2019)

Group	Ct *ACTBL2L*	Ct *IL8*	ΔCt	ΔΔCt	Values nirmalized by control ($2^{-\Delta\Delta Ct}$)
C (control)	16.99±0.58	23.19±0.41	7.08	0	1
I (prebiotic)	15.79±0.55	22.99±0.72	7.20	0.13	0.92
II (probiotic)	14.33±0.36	22.04±0.21	7.71	0.63	0.64*

Note. ΔCt = Ct *IL8* – Ct *ACTBL2L*; ΔΔCt = ΔCt test – ΔCt contol; *ACTBL2L* (β-actin) — a «housekeeping» gene.
* Difference from control are statistically significant at $p < 0.05$.

Haghighi et al. [34] showed that the expression of IL6, IL10, IL12 interleukin genes in broiler chickens increased upon *Salmonella typhimurium* infection, however, when feeding the probiotic, the expression did not differ from that in the uninfected control. The probiotic also affected the expression of the interferon gene (*INF*). Upon infection in the birds receiving probiotic, the expression was lower than in infected individuals not fed with probiotic [34]. In the report of Ateya et al. [35], feeding the experimental broiler chickens with probiotic, synbiotic and acidifier upon *Escherichia coli* infection led to a decrease in the expression of a number of proinflammatory factors (Il6, Il8, AvBD2, AvBD9), while the anti-inflammatory cytokine IL10 gene (*IL10*) showed a sharp increased expression when compared to uninfected control [35].

We found a positive correlation ($r = 0.442$, $p < 0.05$) between the number of bacteria from *Firmicutes* phylum and the *AvBD9* gene expression. A similar trend for *IL8* gene expression was reveled for the polynomial equation; the detected correlation turned out to be very low and negative ($r = –0.006$). In the work of Oakley and Kogut [36], the level of cytokine expression, as a rule, negatively correlated with the relative abundance of various members of the *Firmicutes* group and positively correlated with an abundance of proteobacteria. Correlations between the microbiome structure and the specific transcription of cytokine mRNA indicate the importance of gut microbiome for poultry health and productivity and can be a successful tool for identifying bacterial taxa with certain immunomodulating properties. In our studies, when feeding the prebiotic, the number of phylum *Firmicutes* microorganisms and proteobacteria increased by 5 and 16%, respectively ($p > 0.05$), while IL8 cytokine expression remained practically unchanged ($p > 0.05$). For the probiotic, we found a lower abundance of the same

microorganisms, by 10% ($p < 0.05$) and 3% ($p > 0.05$), respectively, with a decrease in IL8 gene expression by 36% ($p < 0.05$).

Under the influence of stimuli causing stress, and in response to factors enhancing stress response (corticotropin-releasing factor, cytokines, catecholamines, etc.), immunocytes begin to secrete opioids. These peptides activate peripheral opioid receptors and cause a feeling of analgesia, suppressing excessive excitation of sensory neurons and facilitating the secretion of neuropeptides. Opioid peptides, including proenkephalin, enkephalins, endorphins, are currently being studied more and more intensively [20-22]. In studies of scientists from South Korea, there was a significant variation in the expression of a number of genes, including the proenkephalin gene, in connection with processes of egg formation [37]. Table 6 shows the results of our analysis of the expression of the proenkephalin gene in chickens. It can be seen that, unlike cytokines, the expression of the proenkephalin gene (*PENK*) under the influence of the probiotic increased 1.11 times ($p > 0.05$), under the influence of the prebiotic 1.91 times ($p < 0.05$).

6. Proenkefalin gene *PENK* expression in cecal tissues of Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, $M \pm$SEM, vivarium of Skryabin MVA, 2019)

Group	Ct *TBP*	Ct *PENK*	ΔCt	ΔΔCt	Values nirmalized by control ($2^{-\Delta\Delta Ct}$)
C (control)	25.91±0.77	22.63±0.83	–3.28	0	1
I (prebiotic)	24.85±0.43	20.64±0.45	–4.21	–0.93	1.91*
II (probiotic)	22.92±0.47	19.49±0.92	–3.43	–0.15	1.11

Note. ΔCt = Ct *PENK* – Ct *TBP*; ΔΔCt = ΔCt test – ΔCt contol. *TBP* (TATA-binding protein) — a «housekeeping» gene.
* Difference from control are statistically significant at $p < 0.05$.

The expression of the gallinacin-10 (*Gal-10*) gene also increased (Table 7), that is, despite the fact that this protein is quite close to β-defensin 9, the body response was the opposite. Note that other researchers also noted conflicting results for the expression of β-defensin 9 and β-defensin 3 in broilers [19, 35].

7. Gallinacin-10 gene *Gal-10* expression in cecal tissues of Lohmann White cross chickens fed with prebiotic Vetelact and probiotic Profort® (for each group $n = 20$, $M \pm$SEM, vivarium of Skryabin MVA, 2019)

Group	Ct *ACTBL2L*	Ct *Gal-10*	ΔCt	ΔΔCt	Values nirmalized by control ($2^{-\Delta\Delta Ct}$)
C (control)	16.99±0.58	23.19±0.41	5.36	0	1
I (prebiotic)	15.79±0.55	22.99±0.72	4.79	–0.56	1.48
II (probiotic)	14.33±0.36	22.04±0.21	6.75	–0.63	1.55

Note. Ct = Ct *Gal-10* – Ct *ACTBL2L*; ΔΔCt = ΔCt test – ΔCt contol; *ACTBL2L* (β-actin) — a «housekeeping» gene.

Our studies indicate (see Table 7) that the expression of gallinacin-10 gene when feeding prebiotic and probiotic increased 1.48 times ($p > 0.05$) and 1.55 times ($p < 0.05$), respectively. In total, 14 genes of defensins and gallinacins with various antimicrobial activity are revealed in chickens [38]. In broilers infected by *Salmonella enterica*, the expression of the gallinacin-10, gallinacin-11, gallinacin-13, and gallinacin-14 genes was suppressed, while the expression of defensins 1, 2, 7, 8, and 9 remained unchanged. The differential expression of defensins and gallinacins indicates the features of the participation of these genes in the immune response and a different response not only to pathogens, but also to food factors [39].

Normal microbiota which provides resistance to colonization and intestinal health, is a key condition for the proper development of the intestinal tract and the complete maturation of the immune system of the mucous membrane [40]. Our study is the first attempt to understand the interactions between commensal microbiota and the expression of regulatory cytokines in the cecum of

laying hens based on the identification of specific taxa the abundance of which significantly correlates with the expression of cytokine genes.

Thus, our experiments on healthy laying hens have confirmed the fact that dietary probiotic and prebiotic supplements positively affects the intestinal microflora with a minimal effect of these additives on productivity. Both tested feed additives contributed to an increase in the abundance of bifidobacteria and cellulolytic bacteria in the intestine and reduced the total number of pathogenic and undesirable microflora by 25-50%. The studied bioactive additives had a multidirectional effect on the functional activity of immune-associated genes (*AvBD9*, *IL8*, *PENK*, and *Gal*-10) with a general tendency to stabilize the state of the body and readiness to suppress the inflammatory process. The revealed trend of a significant increase in the number of vital bacteria and a similar decrease in pathogenic microflora in the intestine shows the promise of probiotic and prebiotic application to optimize immune functions, which will ultimately improve the health and productivity of poultry.

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

D.D. SMIRNOV, A.V. KAPUSTIN, P.N. SHASTIN, M.I. GULYUKIN, A.I. LAISHEVTCEV

Federal Scientific Centre Skryabin and Kovalenko All-Russian Research Institute of Experimental Veterinary RAS, 24/1, Ryazanskii Prosp., Moscow, 109428 Russia, e-mail ddsmirnov2010@mail.ru, kapustin_andrei@mail.ru, shastin.pasha@yandex.ru, admin@viev.ru, a-laishevtsev@bk.ru (✉ corresponding author)
ORCID:
Smirnov D.D. orcid.org/0000-0003-23-44-3755
Kapustin A.V. orcid.org/0000-0003-0136-2487
Shastin P.N. orcid.org/0000-0001-7360-927X
Gulyukin M.I. orcid.org/0000-0002-7489-6175
Laishevtcev A.I., orcid.org/0000-0002-5050-2274
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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 1.7×10^7-9.4×10^8 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 (1.5×10^9, 1.5×10^8, 1.5×10^7, and 1.5×10^6 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 (1.0×10^9, 1.5×10^9 and 2.0×10^9 cells) adsorbed on three adjuvants — GOA, PEG-6000, and Acrypol®971P as follows: 1 — 1.0×10^9 cells. + GOA; 2 — 1.5×10^9 cells + GOA; 3 — 2.0×10^9 cells + GOA; 4 — 1.0×10^9 cells + PEG-6000; 5 — 1.5×10^9 + PEG-6000; 6 — 2.0×10^9 cells + PEG-6000; 7 — 1.0×10^9 cells + Acrypol®971P; 8 — 1.5×10^9 cells + Acry-pol®971P; 9 — 2.0×10^9 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 — 8.1×10^7, No. 425 — 1.1×10^8, No. 426 — 7.8×107, No. 837 — 2.9×107, No. 838 — 2.6×10^8, No. 839 — 1.8×10^8, No. 1096 — 8.0×10^7, No. 1481 — 2.1×10^8, No. 1517 — 1.7×10^7, No. 1647 — 2.3×10^8, No. 1865 — 9.4×10^8 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 1.7×10^7-9.4×10^8 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 1.5×10^9 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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EPIZOOTIC SITUATION AND MODELING OF POTENTIAL NOSOAREALS OF PESTE DES PETITS RUMINANTS, SHEEP AND GOAT POX AND RIFT VALLEY FEVER UP TO 2030

V.A. ZHURAVLYOVA, A.V. LUNITSIN, A.V. KNEIZE, A.G. GUZALOVA, V.M. BALYSHEV

Federal Research Center for Virology and Microbiology, 1, ul. Akademika Bakuleva, pos. Vol'ginskii, Petushinskii Region, Vladimir Province, 601125 Russia, e-mail 33Milita@rambler.ru (✉ corresponding author), lunicyn@mail.ru, knize65@mail.ru, guz-anna@yandex.ru, balyshevvm@rambler.ru
ORCID:
Zhuravlyova V.A. orcid.org/0000-0003-0768-3484
Lunitsin A.V. orcid.org/0000-0002-5043-446X
Kneize A.V. orcid.org/0000-0003-4526-1354
Guzalova A.G. orcid.org/0000-0002-8248-9684
Balyshev V.M. orcid.org/0000-0001-8224-9333
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Abstract

The current global epizootic situation is characterized by a pronounced increase in the tension for a number of special danger viral infections of livestock including sheep and goat diseases. The above diseases include peste des petits ruminants (PPR), sheep and goat pox (SGP) and Rift Valley fever (RVF), the probability of their entering the Russian Federation being rather high. In this report we have pioneered determination of PPR, SGP and/or RVF potential nosoareas varying in the above infections emergence danger levels, both in the Russian Federation and the neighboring countries, based on the monitoring of the global epizootic situation using mathematical extrapolation of regressive models. Also, the natural ecological factor was shown to have the most serious impact on the intensity of an epizootic process. Our work was aimed at evaluation of the spatial-dynamic features and regularities of the global spreading of peste des petits ruminants, sheep & goat pox, and Rift Valley fever, as well as evaluation of the risks of these infections emerging and spread in the Russian Federation and the neighboring countries in the period of 2020 to 2030. We used the statistical data of Food and Agriculture Organization (FAO) and Office International des Epizooties (OIE) reflecting the global epizootic situation for PPR, SGP and RVF in 1984 to 2018, the data on the economic status of sheep and goat husbandry worldwide, and also some information on a range of special danger animal infections from the Federal Service for Veterinary and Phytosanitary Surveillance, and The Veterinary Center of Russia. The epizootological method of the research applied here included calculation of indices of the intensity of an epizootic situation, namely the stationarity index and the incidence index. Also, statistical verification of a relation of the epizootic situation intensity with some natural and socio-economic factors was performed, and the informational impact indicator (III) was calculated. To model and predict the dynamics and the structures of PPR, SGP and/or RVF nosoareas, the calculation of regression & information models was used. The probability of a disease emergence was calculated through spatially dynamic modeling of its incidence in the nosoarea-involved countries in 1984-2018, taking into account the factors of the natural and/or socio-economic background on the stationarity index values within the global nosoarea. According to the summarized data, the largest numbers of PPR- or RVF-affected countries were registered in the African continent in 1984 to 2018. A few more countries affected with SGP were found in Asia. Nevertheless, the numbers of PPR (more than 38 thousand) and SGP (more than 39 thousand) outbreaks recorded in Asia significantly exceeded the respective values as observed in African countries. The autocorrelation analyses revealed 13- to 15-year cyclicity for PPR, 12- to 13-year or 21- to 22-year one for SGP, and 25- to 27-year or 8- to 10-year for RVF infections. In Russia, as many as three potential nozoreas for PPR and/or SGP and two potential nozoareas for RVF were identified which varied in the quantitative indicators of their incidence. Also, natural environment and climatic factors were found to have the greatest influence on the intensity of an epizootic situation. In the period up to 2030, the emergence and spread of SGP and/or PPR is possible throughout the territory of the Russian Federation, the highest probability being

predicted in the North Caucasus Federal District (the Republic of Dagestan, the Republic of Ingushetia, Kabardino-Balkaria, the Republic of North Ossetia, Chechnya or Stavropol Territory) and the South Federal District (Krasnodar Territory, the Republic of Adygeya, and the Republic of Crimea). Furthermore, there is a low likelihood of RVF introduction and emergence in the above regions. Among the neighboring countries, Tajikistan, Kyrgyzstan, Kazakhstan, Uzbekistan, Afghanistan, Turkmenistan, Armenia, Georgia, Azerbaijan, Turkey, Iran, Mongolia and China pose the greatest danger for PPR and/or SGP while countries of the African continent, Arabian Peninsula, and the southern region of Asia for RVF. The data obtained indicate the requirement for carrying out a comprehensive monitoring of the epizootic situation for PPR, SGP and RVF worldwide combined with the development of forecasts for these infections and the implementation of a set of preventive antiepizootic measures to ensure sanitary and epizootic welfare of animal husbandry in the Russian Federation.

Keywords: stationarity index, incidence index of outbreaks, Rift Valley fever, epizootic monitoring, intensity of epizootic situation, sheep and goat pox, potential nosoarea, epizootological prediction, spatial-dynamic model, peste des petits ruminants

According to FAO (Food and Agriculture Organization, FAO) and OIE (Office International des Epizooties, OIE) information, in recent years the epizootic situation for some transboundary special danger viral diseases of small ruminants like peste des petits ruminants (PPR), sheep and goat pox (SGP), Rift Valley fever (RVF) in the world remains difficult [1, 2]. The causative agents of these special danger infections can be used for bioterrorism. While PPR and SGP affect mainly sheep, goats and wild artiodactyls, which can be carriers of the infections, Rift Valley fever belongs to zooanthroponoses. Not only the mentioned animals but also cattle, horses, camels, antelopes, monkeys, some other mammals, as well as humans are susceptible to RVF [3, 4; FAO/OIE/WHO. Animal health yearbook 1985-1995 FAO, Rome, 1986-1996] which poses a special threat.

Over the past decade, a marked tendency towards international spread and an increase in the number of epizootic outbreaks has begun to appear on these diseases, which causes significant economic damage to the affected countries, both directly due to the increase in the numbers of diseased animals and their death rates and in connection with the necessary quarantine and other restrictive measures [5].

The causative agents of SGP, PPR and RVF are not identical in their taxonomic affiliations and biological characteristics, their high contagiousness for small ruminants being the common feature. The mortality rates in the primary foci of infection can reach 50-90% [10]. Along with the high virulence of the pathogens, this is explained by the method of sheep and goat breeding when large groups of animals are located in limited area.

The peste des petits ruminants has been known since 1942, and is currently not registered in the Russian Federation. The modern nosoareal of the disease covers the countries of Africa and Eurasia, of which the states bordering the Russian Federation or having close economic ties with it, in particular China [6, 7], Mongolia [8], Kazakhstan [7], Georgia [8], Tajikistan [7], Turkey [7, 9], and Iran [7] are the most dangerous for Russia. China is recognized as a country endemic for PPR, with the infection foci having been determined in Manchuria bordering the Far Eastern Federal District [6, 7]. In total, as many as 57 countries of Africa and Eurasia were recognized as the disease-affected from 1985 to 2014 [8].

In 1984 to 2014, sheep and goat pox was registered in 34 countries of the African continent and in 42 countries of Eurasia. In the second half of the 20th century, SGP outbreaks among small ruminants in ex-USSR countries occurred mainly in the republics of Central Asia, Transcaucasia, Kazakhstan and Kyrgyzstan [10, 11]. In the Russian Federation, the epizootic situation for sheep and goat pox aggravated in 1994-1998, when the disease was detected in 12 regions of the country. In 2010-2015, SGP was detected in Primorsky [12-14] and Trans-Baikal [12-14] regions, in the Amur [12-14] and Chita [13] regions, in Dagestan [12, 13, 15] and

Kalmykia [12, 14, 16].

Rift Valley fever is a vector-borne infection, the virus vectors being mosquitoes of the genera *Culex*, *Aedes* and *Erenmopodites* spread throughout Russia, which poses a threat of the pathogen entering this country [3, 13]. At the end of the 20th century, RVF was most common in southeastern Africa [17-20]. In 2006, RVF caused major epizootics in Kenya, Tanzania, and Somalia, where the number of diseased animals (i.e., small ruminants and cattle) exceeded 36 thousand, and more than 4 thousand animals died [21]. In Asia, RVF was first described in Saudi Arabia in 2000 [22-24], then in Turkey in 2010 [13] and in China in 2016 [25, 26]. New outbreaks of RVF in Yemen and Saudi Arabia, as well as the case of the disease introduction to the Iberian Peninsula, indicate its spread beyond the African continent and the possibility of its spreading to countries of Asia and/or Europe [13, 27, 28].

To substantiate and work out antiepizootic action plans against PPR, SGP or RVF, constant monitoring of the global epizootic situation for the above infections is required which comprises identification of possible pathways of the pathogens invasion and spread, as well as considering probable livestock population losses.

In the presented work, with the use of regression models for mathematical extrapolation of global epizootic monitoring data, we first determined potential nosoareas for PPR, SGP and RVF in the Russian Federation and neighboring countries during the next decade, which differ in the disease emergence risk levels. Furthermore, some environmental factors were found have the most serious effect upon epizootic tension.

Our goal was to assess the spatial and dynamic characteristics and patterns of the global spread of peste des petits ruminants, sheep and goat pox and Rift Valley fever and to assess the risk of these infections emergence and spread both in the Russian Federation and its neighboring countries in 2020-2030.

Materials and methods. We used the OIE and FAO statistics on the world epizootic situation for PPR, SGP and RVF for 1984-2018, the data on the economic status of the world sheep and goat breeding, as well as the information obtained from the Federal Service for Veterinary and Phytosanitary Surveillance and the Center for Veterinary for special danger animal diseases [12, 13, 16, 29]. The epizootological research method included calculation of the indicators of an epizootic situation: the stationarity index (SI) as the ratio of the number of years during which the disease was recorded in the country/region to the number of years of observation, and the incidence index (II) as the ratio of the number of susceptible livestock in the country to the number of new epizootic outbreaks within 12 months [30-32].

The estimates of the influence of various systems of factors (namely, natural and socio -economic) on the epizootic situation (through the of stationarity and incidence indexes) for the infections was calculated according to the formula of the informational impact indicator (III):

$$\sqrt{\frac{\sum H(A) - \frac{\sum(n_k \cdot \sum H(A/b_k))}{n}}{\sum H(A)}},$$

where $\sum H(A)$ is total entropy of the complex, and $\frac{\sum(n_k \cdot \sum H(A/b_k))}{n}$ is entropy of random diversity.

For the III calculation, tables of communication channels were used reflecting the calculations and relationships of specific values of risk factors with certain values of indicators characterizing epizootic situation.

The statistical significance characterizing the relationship of epizootic tension with natural and socio-economic factors was evaluated in accordance with the methods adopted [30]. Calculation of regression and information models [30] was used to simulate and predict the dynamics of PPR, SGP, RVF and the structure of their nosoareas.

The probability of a disease incidence was calculated through spatial-dynamic modeling of the frequency of its occurrence in nozoarea countries in 1984-2018 with regard to the influence of natural and socio-economic factors on the stationarity index within the global nozoarea. In constructing diagrams, a method of aligning empirical series was used, in particular, a regression analysis and periodic functions [33].

Results. The current nozoarea of PPR covers 38 African countries, 23 Asian countries, Georgia and Bulgaria [8, 12, 34]. According to the OIE information, the number of PPR outbreaks in the world over a 30-year period has exceeded 54 thousand, of which over 15.8 thousand took place in Africa, over 38.2 thousand in Asia, and in Georgia and Bulgaria were 10 outbreaks (namely in 2016 and 2018, 3 and 7 foci of the disease, respectively).

In the Russian Federation, PPR cases have not been registered. Statistically significant rises of SGP incidence in the world occurred in 1985-1989, 1996-1997, and 2012-2013. In 2016-2017, as many as 116 PPR outbreaks were identified in Turkey, and 295 and 998 in Afghanistan and Iran, respectively. Currently, Mongolia and China are endemic for PPR. The infection foci have been determined in Manchuria and in other China regions bordering the Far Eastern Federal District of Russia, Kazakhstan and Mongolia. In Kazakhstan, PPR outbreaks were diagnosed in 2003, 2005, 2006. In Mongolia, PPR was first reported in August 2016, and in 2017 several foci of the disease were detected among saigas (the number of infected and dead animals was more than 5,000). Thus, saigas can become an intermediate link when the infection enters susceptible livestock population (i.e., sheep and goats) in the unaffected regions.

In 1984-2018, SGP were recorded in 77 countries (34 countries of Africa, 38 countries of Asia and 5 countries of Europe with more than 20,960, 39,131 and 509 outbreaks detected, respectively). The incidence of SGP among sheep and goats markedly increased in 1989-1993, 1999-2005 and 2010-2012. The countries of Africa (Algeria, Nigeria, Cameroon, Libya, Ethiopia, Mali, Mauritania, Morocco, Niger, Senegal, Tanzania, Uganda) and Asia (China, Pakistan, India, Iran, Qatar, Kuwait, Tajikistan, Turkey, Israel, Kazakhstan, Mongolia, Kyrgyzstan, Kazakhstan, Vietnam) are permanently affected with SGP. In Europe, sheep pox has been registered in Greece, Russia and Bulgaria. In Central and South America (Costa Rica, Bolivia), SGP outbreaks were registered in 1990. In the ex-USSR, the disease outbreaks occurred mainly in the republics of Central Asia, Transcaucasia, Kazakhstan, and Kyrgyzstan in the second half of the 20th century [10, 11]. In 2000-2002, SGP outbreaks characterized by high morbidity and mortality levels occurred in Angora goats in the Republic of Tajikistan on the border with Afghanistan.

In the Russian Federation, the SGP outbreaks of 1994-1998, when the disease was first registered in Dagestan (5 affected points) and then in the Stavropol Territory and 10 more regions, caused significant economic damage to sheep breeding. In 2008, there was a pox outbreak among goats in the Khabarovsk Territory, in 2010-2015 sheep and goat pox was registered in four Russian regions bordering China, as well as in Dagestan and Kalmykia, in 2016, pox was diagnosed in sheep in seven districts of the Yaroslavl region, and in 2018 in the Moscow and Tula regions, and the Republic of Kalmykia [13, 16]

In 1984-2015, RVF was registered in 30 countries on the African continent, in 4 countries of Asia, and in one country in Europe [13, 35]. In total, more than 1.6 thousand outbreaks of the disease occurred during this period including 1.2 thousand in Africa (the highest numbers of 0.7 and 0.16 thousand having been observed in South Africa and Kenya, respectively), more than 200 in Asia (in Yemen, Saudi Arabia, Turkey and China), and one in Europe (in Portugal). The disease spread mainly among sheep, goats and cattle and to a lesser extent among other artiodactyls. The epizootic situation for RVF was characterized by a pronounced increase in tension in 1984-2018 with periods of 8-10 and 25-27 years. Within the 10-year cycles, the highest RVF incidence values were seen in 1988, 1998, 2006, and 2016. The seasonality of the increase in the number of RVF outbreaks in the tropical and subtropical zones of Africa and Asia was associated with the heat and moisture regime of the nosoareal. The disease incidence rates were higher in the countries of eastern, southern and western parts of Africa (SI of 0.4-0.6 and higher), the highest II levels being characteristic of the countries of the southeast and west of the African continent, and the Arabian Peninsula.

As per the data summarized in Table 1, the largest amounts of PPR- and/or RVF-affected countries fell on the African continent. A few more SGP-affected countries were found in Asia. At the same time, the numbers of outbreaks of PPR (more than 38 thousand) and SGP (more than 39 thousand) in Asia significantly exceeded the corresponding figure for African countries.

1. World incidence of peste des petits ruminants (PPR), sheep and goat pox (SGP) and Rift Valley fever (RVF) in 1984-2018

Continent/subcontinent	Number of countries			Number of outbreaks		
	PPR	SGP	RVF	PPR	SGP	RVF
Africa	38	34	30	15824	20960	1200
Eurasia:						
total	25	43	5	38281	39640	< 200
in Asia	23	38	4	38271	39131	< 200
in Europe	2	5	1	10	509	1
Total in the world	63	77	35	54105	60600	< 1400

An autocorrelation analysis of the data characterizing the dynamics of the epizootic situation revealed a 13-15-year disease cycle for PPR, 12-13-year and 21-22-year cycles for SGP, and 25-27-year and 8-10-year cycles for LDR.

To perform a multidimensional informational cartographic analysis of the structure of PPR, SGP and RVF nosoareas, we used nosogeographic maps that reflect the spatial distribution of the SI and II values in the affected countries for the period of 1984-2018 [30-32]. The natural background maps reflected the spatial distribution of geographical types of landscapes and the small ruminant population zoogeographic distribution. The socio-economic maps reflected regionalization of agricultural production worldwide, in particular, the economic growth indices for sheep and goat husbandry and the provision of veterinary services.

2. Information analysis of the worldwide structure of nosoareas for peste des petits ruminants (PPR), sheep and goat pox (SGP) and Rift Valley fever (RVF) based on epizootic development intensity and impact factors (1984-2018)

Factors	Stationarity index			Incidence index		
	PPR	SGP	RVF	PPR	SGP	RVF
Environmental	0.603	0.580	0.416	0.600	0.420	0.317
Socio-economic	0.514	0.450	0.320	0.516	0.530	0.387
Complex of environmental and socio-economic factors	0.730	0.650	0.610	0.700	0.650	0.490
Statistical significance, α-value	0.05	0.05	0.05	0.05	0.05	0.05

The III values for environmental (natural) and socio-economic factors (as per the indicators of epizootic intensity) for PPR, SGP and RVF ranged from 0.317

to 0.730 (Table 2). The greatest impact on the epizootic situation for these diseases was, as a rule, characteristic of environmental factors (SI from 0.416 to 0.603, II from 0.317 to 0.600), the values for the socio-economic complex were slightly lower (SI and AI from 0.320 to 0.516) (the example of the III calculation for PPR in Excel see the website http: //www.agrobiology.ru). The distribution of data by gradations of epizootic intensity was close to lognormal indicating the possibility of using regression analysis for epizootic forecasting.

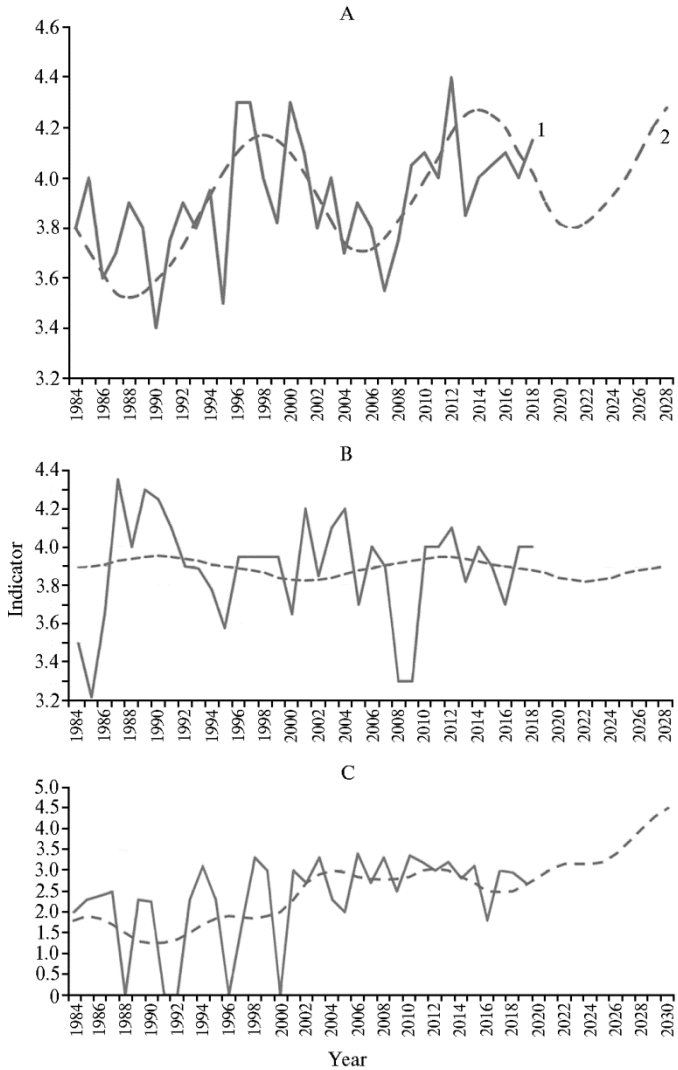

Fig. 1. Actual incidence index (1) **and incidence index model up to 2030** (2) **for peste des petits ruminants** (A)**, sheep and goat pox** (B) **and Rift Valley fever** (C) **outbreaks in potentially affected regions worldwide.**

Math extrapolation of the regression models allowed us to make a forecast for the period until 2030 for PPR, SGP and RVF epizootic situation in potentially affected regions worldwide (Fig. 1). In 2019-2023, the downward trend for PPR and SGP showed a decrease in the epizootic tension, and the upward trend for RVF predicted its increase due to cyclical fluctuations in the epizootic situation.

A potential nosoarea within Africa and Eurasia, including the territory of the Russian Federation, is differentiated into 5 regions depending on the probability (P) of the disease emergence: P ranging from 0 to 0.2 corresponds to a minor risk

level, from 0.2 to 0.4 to low, from 0.4 to 0.6 to medium, from 0.6 to 0.8 to significant, and from 0.8 to 1.0 to high risk of the infection entry.

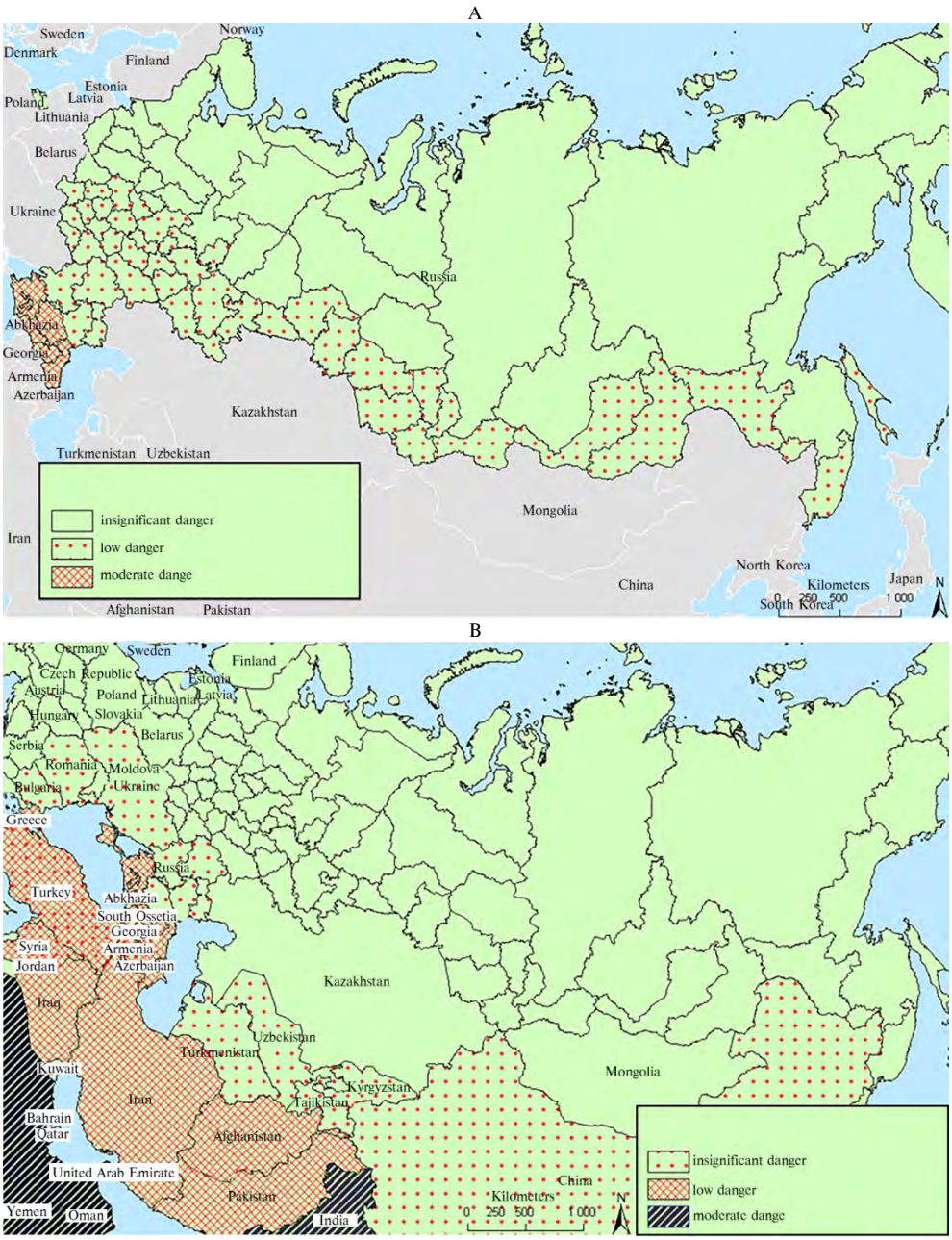

Fig. 2. The level of danger of emergence and spread of special danger viral diseases of sheep and goats in the Russian Federation given the epizootic situation in the surrounding territories (2020-2030): A — peste des petits ruminants, B — Rift Valley fever. Detailed data on the subjects of the Russian Federation are presented in tables (see the website http://www.agrobiology.ru).

In Russia, zones in 2020-2030 with an average, low, and negligible risk levels for the above infections' emergence are distinguished. Zones with an average risk level (with an outbreak probability of 0.4 to 0.6) include the Republic of Dagestan, the Republic of Ingushetia, the Kabardino-Balkarian Republic, the Republic of North Ossetia, the Chechen Republic, the Stavropol Territory (North Caucasus Federal District), the Krasnodar Territory, the Republic of Adygea, the

Republic of Kalmykiya, the Republic of Crimea (Southern Federal District), as well as areas bordering Mongolia and China. Regions with a low and negligible risk levels for SGP and PPR (with a probability of less than 0.4) are the subjects of the Central, the Volga, the Siberian, the Far Eastern and the North-Western federal districts (Fig. 2, A). At the same time, a significant risk of SGP and/or PPR outbreaks is predicted for the countries of South-West Asia and Southern Europe

A potential RVF nosoarea worldwide is differentiated into the following zones of the disease probable emergence: high risk level (P from 0.6 to 1.0; agroecosystems of the African continent, Arabian Peninsula, and southern region of Asia), moderate risk (from 0.4 up to 0.6; countries of the northern region of Africa and southwestern Asia covering Afghanistan, Iraq, Iran and Pakistan), low risk level (from 0.2 to 0.4; countries of southern Europe, Transcaucasia and Central Asia), an negligible risk level (from 0 to 0.2; agroecosystems of the temperate climatic zone of Europe in which RVF has not been registered earlier). For the Russian Federation, the spatial prognosis of the potential RVF nosoarea for 2020-2030 indicates the possibility of the disease emergence in two zones. The first zone with a probability of the diseases incidence below the average value includes the territories of the North Caucasus and Southern Federal Districts (the Krasnodar Territory, the Republic of Adygea, the Republic of Dagestan and the Crimea). The second zone with a low probability of RVF emergence includes some subjects of the Southern Federal District (Astrakhan, Rostov and Volgograd regions, the Republic of Kalmykia and the Stavropol Territory) (Fig. 2, B).

The most important task of epizootological forecasting is to determine the risk of an infectious disease emergence associated with the interaction of a pathogen with a population of susceptible animals in specific environmental and socio-economic conditions to ground taking effective anti-epizootic measures [35, 36]. In the present work, the study was carried out in accordance with the "Methodological recommendations for epizootological monitoring of exotic highly dangerous and/or exotic animal diseases" developed with our participation [30]. We confirmed the effectiveness and reliability of this method earlier when monitoring and predicting other infections like nodular dermatitis of cattle [37], Newcastle disease [38], African swine fever (ASF) [39].

It should be noted that a significant number of publications has been devoted to the development of epizootics and methods for their prediction. For example, the results of studies using the cluster analysis method show that the intensity of ASF outbreaks spread in a certain area is associated with the denseness indices of pig-breeding complexes and/or private farms, the road network density, and anthropogenic activities [40, 41]. In the epizootological forecasting of special danger diseases of humans and animals, the basic methods of statistical analysis, analytical epidemiology and system modeling with the Pausson model are used [42]. Also, some modern geographic information systems based on computer technologies for automated processing, storage and analysis of epidemiological information with its visualization on maps have been developed in recent years [43-45].

Thus, a long-term (2020–2030) prognosis of the spread of peste des petits ruminants (PPR), sheep and goat pox (SGP) and Rift Valley fever (RVF) among sheep and goats indicates a trend towards an aggravation of the world's epizootic situation for these diseases. Of the countries with which Russia is bordering and/or has close trade and economic ties, Tajikistan, Kyrgyzstan, Kazakhstan, Uzbekistan, Afghanistan, Turkmenistan, Armenia, Georgia, Azerbaijan, Turkey, Iran, Mongolia and China pose the greatest danger for PPR and SGP, and the countries of the African continent, the Arabian Peninsula and the south of Asia for RVF.

In the period until 2030, the emergence and spread of PPR and SGP is possible throughout the territory of the Russian Federation, with the North Caucasus and the Southern Federal Districts exhibiting the highest predicted probability of the above infections emergence. Also, there is a low probability of RVF entry in these regions. Our data indicate the requirement of systemic monitoring and forecasting of the epizootic situation for PPR, SGP and RVF and carrying out antiepizootic and preventing campaigns to ensure sanitary and epizootic safety.

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STUDY ON ANTIGENIC RELATIONSHIPS AND BIOLOGICAL PROPERTIES OF SWINE INFLUENZA A/H1N1 VIRUS STRAINS ISOLATED IN NORTHERN KAZAKHSTAN IN 2018

N.T. SAKTAGANOV, N.G. KLIVLEYEVA, N.S. ONGARBAYEVA, T.I. GLEBOVA, G.V. LUKMANOVA, A.M. BAIMUKHAMETOVA

Research and Production Center for Microbiology and Virology, A26T6C0 Republic of Kazakhstan, Almaty, st. Bogenbai Batyr, 105, e-mail biochem_vir@mail.ru, nsaktaganov1984@mail.ru (✉ corresponding author), i_nailya@list.ru, nuray.syrlybay@gmail.com, taty1962@mail.ru, gal_l@bk.ru, a_baimukhametova@mail.ru
ORCID:
Saktaganov N.T. orcid.org/0000-0001-6526-956X
Klivleyeva N.G. orcid.org/0000-0002-4094-137X
Ongarbayeva N.S. orcid.org/0000-0001-9022-331X
Glebova T.I. orcid.org/0000-0002-1139-9868
Lukmanova G.V. orcid.org/0000-0002-9809-6674
Baimukhametova A.M. orcid.org/0000-0002-8959-126X
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Abstract

Swine influenza is a highly contagious acute disease characterized by pronounced fever, general weakness, and disorders of the respiratory system. Swine influenza virus can cause disease in humans and, on the contrary, swine may be infected by human influenza virus. In the pig's organism, simultaneously infected with viruses of different origin, genetic reassortment takes place with the risk of occurrence of new dangerous highly pathogenic strains. The study of influenza viruses circulating in the pig population therefore plays an important role in preventing the development of dangerous outbreaks of the disease and planning preventive measures. In this work we studied the characteristics of the newly isolated strains of swine influenza virus which are epizootically relevant in the specified region at the present time. Our purpose was to identify the biological and antigenic characteristics of strains of swine influenza A/H1N1virus, circulating in the North Kazakhstan oblast of the Republic of Kazakhstan in 2018. Influenza A/H1N1 virus strains were studied including those isolated from pigs in pig farms of the North Kazakhstan oblast, the A/swine/Petropavlovsk/01/18, A/swine/Petropavlovsk/02/18, and A/swine/Petropavlovsk/03/18, and also the reference strains A/swine/Iowa/15/30, A/swine/USA/1976/31, and A/California/04/09 pdm. The strains were cloned in 10-day-old developing chicken embryo systems. The antigenic properties of surface glycoproteins of the strains were examined by cross-reactivity hemagglutination inhibition assay with rabbit immune sera. Infectivity was determined in chicken embryos (CE) and MDCK cell culture. The adsorption properties were studied on formalinized chicken red blood cells under constant stirring at 4°C for 18 hours. Elution from red blood cells was determined after 30, 60, 120, 180, and 240 min in buffered saline at 37°C. The heat sensitivity of hemagglutinin was assessed by the ability to agglutinate red blood cells after heating at 56 °C for 5, 10, 15, 30, and 60 minutes. The hemagglutinating activity of strains was assayed using 0.75% suspensions of chicken, guinea pig, ram, horse erythrocytes, and blood group I(0) erythrocytes of human. The susceptibility of isolates to nonspecific inhibitors was determined in the hemagglutination inhibition assay with native and heated (30 min at 62 °C and 10 min at 100 °C) blood sera of guinea pig, chicken, and rabbit. The susceptibility of virus strains to different concentrations of antiviral drugs was evaluated by the level of reproductive suppression of lg 100 EID_{50}/0.2 ml of virus in CE. The antigenic relationship of the examined variations of influenza A/H1N1 virus between each other and with the reference strains A/swine/USA/1976/31 and A/swine/Iowa/15/30 was revealed as well as their difference from the strain A/California/04/09 pdm. The studied strains in high titers agglutinated all types of red blood cells taken in the experiment. The infectious activity of swine influenza virus strains ranged within 6.5-7.9 lg EID_{50}/0.2 ml in chicken embryos, and 3.5-4.3 lg TCD_{50}/0.2 ml in MDCK cell culture. After heating at 56 °C, all strains agglutinated chicken erythrocytes in high titers ($\log_2 = 6.3\pm0.6$-9.6 ± 0.8) and were characterized as thermostable. The isolated strains possessed good adsorption ability against chicken erythrocytes (90-100 %) and eluted from them after 30-60 min incubation at 37 °C. The strains revealed inhibitory resistance with native nonspecific sera and were suppressed by inhibitors of the heated sers only. The studied strains proved to be susceptible to Tamiflu and Remantadine (the inhibitory concentrations were 5.6-6.6 and 3.7-12.7 μg/ml, respectively). Viruses exhibited resistance to the drugs Arbidol and Ingavirin. Thus, the study revealed similarity of isolated and reference A/H1N1 strains in ther-

mostability of the hemagglutinin, adsorption rate and susceptibility to antiviral drugs, as well as differences in infectious activity and the rate of elution from chicken red blood cells.

Keywords: swine influenza virus, A/H1N1, strain, isolate, antigen, hemagglutinin, infectivity, thermostability, resistance, drug susceptibility

Influenza A viruses are unique infectious agents in humans, as well as in other mammals and birds [1, 2]. Interspecific transmission of influenza A/H1N1 viruses in humans and animals is important for studying the evolution, ecology and epidemiology of the pathogen. Theoretically, influenza A virus transmission is possible between birds of the near-water complex and other marine inhabitants, birds and pigs, seals and humans, pigs and humans [3, 4].

In pigs infected by influenza virus clinical signs often do not appear, and the mortality rate does not exceed 1-4%. In temperate climates, animals can be infected by influenza virus year-round, but in cold weather the probability of infection increases [5)].

Swine flu virus has been found in many countries with developed livestock farming, including Kazakhstan [6-9]. Currently, three subtypes of influenza A virus have been identified in pig populations: H1N1, H3N2, H1N2. Antigenically different bird H1N1 variation has been isolated in pigs since 1979. The most common is A/H1N1, antibodies to which were detected in pigs in all countries of the world [10]. Strains of the influenza virus with the antigenic formula A/H3N2, which became the result of interspecific transmission of the virus from humans to pigs, were first discovered in 1970. The A/H1N2 strains, resulting from reassortment of swine, human, and bird flu viruses, were isolated from pigs in 1994 and now continue to circulate [11].

Pigs can be infected with human and bird flu viruses. Under simultaneous infection with these two viruses, genetic material is exchanged between strains of various origins; as a result, newly emerged virions acquire the ability to be transmitted from person to person with the likelihood of pandemic [12].

Monitoring of the virus spread, understanding etiology of the disease, and a comprehensive description of the infectious agent are important to prevent possible epizootics. Determination of biological and antigenic features of circulating viruses allows us to identify the main parameters of variability, phylogenetic relationships of strains that were previously discovered and constantly appearing in different countries and regions. Information on epidemiologically relevant strains makes it possible to understand the origins of current and future epidemics and to find the most accurate means and methods for preventing influenza, as well as a treatment strategy

In this work, giving characterization of biological properties of influenza A/H1N1 strains isolated in a pig population in Northern Kazakhstan, we first established a higher degree of similarity of these strains with the classic A/H1N1 swine influenza virus (A/Swine/USA/1976/31 and A/Swine/Iowa/15/30) than with related to A/California/4/09 pdm strains which are currently circulating in the human population. In addition, the sensitivity of isolated strains to anti-influenza drugs of the adamantane series (Rimantadine) and neuraminidase inhibitors (Tamiflu®), as well as resistance to Arbidol® and Ingavirin®, were found.

Our goal was to identify the biological and antigenic features of strains of swine influenza virus A/H1N1, circulated in the North Kazakhstan region of the Republic of Kazakhstan in 2018.

Materials and methods. Influenza A/H1N1 virus isolates from samples collected in 2018 in pig farms in the North Kazakhstan region (A/pig/Petropavlovsk/01/18, A/pig/Petropavlovsk/02/18 and A/pig/Petropavlovsk/03/18), as well as reference

strains A/Swine/Iowa/15/30, A/Swine/USA/1976/31 and A/California/04/09 pdm (collection of the Laboratory of Virus Biochemistry, Research and Production Center for Microbiology and Virology) were investigated. Viruses were cultivated in 10-day-old developing chicken embryos (CE) with inoculation of viral material into the chorioallantoic cavity. The collected allantoic fluid was centrifuged at 24,000 rpm for 180 min at + 4 °C. Further purification and concentration of viruses was carried out in a sucrose density gradient (a Beckman centrifuge, Beckman Coulter, USA; Ti 45 rotor, 37,000 rpm, 90 min, + 4 °C) [13].

Antigenic properties of viral glycoproteins were studied by a cross-reactivity in hemagglutination inhibition assay (HIA) as per the recommendations of the World Health Organization [14] with rabbit immune sera [15]. Specific hyperimmune rabbit sera were obtained by 3-fold immunization of chinchilla rabbits weighing 2.5-3 kg. The concentrated virus was injected subcutaneously, 150 μg per animal with a 21-day interval. The specific activity of the obtained hyperimmune rabbit serum was determined in HIA with a set of antigens for the diagnosis of influenza viruses (LLC PDPP, St. Petersburg, Russia) with antigenic formulas A/H1N1, A/H3N2 and type B.

Virus infectivity of the isolated strains was determined on 10-day CE and in an MDCK cell culture (continuous line of Madin-Darby canine kidney epithelial cells, ATCC Catalog of cell lines & hybridomas, 7th edn. Rockville, MD, 1992) according to L. Reed and H. Muench [16] by the assessment of embryonic infectious dose and tissue cytopathogenic dose and expressed in lg EID_{50}/0.2 ml and lg TCD_{50}/0.2 ml, respectively.

The adsorption properties were investigated on 50% formalized chicken erythrocytes for 18 h at 4 °C with constant stirring. Formalized chicken erythrocytes were obtained by treating pre-washed chicken erythrocytes with a 40% formaldehyde solution (1:1). After erythrocyte contact with formalin for 6 days with periodic resuspension, formalin was removed by repeated washing with sterile saline followed by centrifugation at 3000 rpm (Rotanta 460, Hettich, Germany). Elution from red blood cells at 37 °C was evaluated in buffered saline after 30, 60, 120, 180, and 240 min. The heat sensitivity of hemagglutinin (HA) was assessed by the ability of viruses to agglutinate red blood cells after heating at 56 °C for 5, 10, 15, 30, and 60 min [17]. Viral hemagglutinating activity was assayed with 0.75% suspensions of erythrocytes of chicken, guinea pig, ram, horse and human with I(0) blood group in hemagglutination tests (HT) [18]. Red blood cells were washed thrice in a sterile buffered physiological solution by sedimentation for 10 min at 1500 rpm (CM-6M, Elmi, Latvia). A 0.75% suspension was prepared from the precipitate of washed red blood cells.

Sensitivity of the isolates to nonspecific inhibitors was assayed in HIA with guinea pig, chicken and rabbit sera (native and warmed 30 min at 62 °C and 10 min at 100 °C).

The sensitivity of the isolates to different concentrations of antiviral drugs was evaluated by suppression of viral reproduction in CE (100 EID_{50}/0.2 ml). Remantadine (100 mg/capsule, JSC Olainfarm, Latvia), Tamiflu® (75 mg/capsule, Cenexi SAS, France, packed by F. Hoffmann-La Roche AG, Switzerland), Arbidol® (100 mg/capsule, umifenovir hydrochloride monohydrate expressed as umifenovir hydrochloride, Pharmstandard-Leksredstva OJSC, Russia); Ingavirin® (90 mg/capsule, imidazolyl ethanamide pentandioic acid — vitaglutam, Valenta Pharmaceuticals OJSC, Russia). Drugs were dissolved in a phosphate-buffered solution (50 mg/ml), the resultant solutions were used as initial ones. The dose that suppresses 2 times the titer of the virus in HT as compared

to control was deemed an inhibitory concentration (IC_{50}) [19].

The results were statistically processed using Microsoft Office Excel 2010 software. For all series of results, the geometric mean inverse binary logarithms of hemagglutination titers (geometric mean titer, GMT) were found and their standard deviations (±SD) were calculated.

Results. To study the antigenic relationships of the isolated strains, viral preparations obtained in CE after purification were concentrated in a 2.0-2.7 ml phosphate-buffer solution. The protein content in the samples was 0.336-0.683 mg/ml, the hemagglutinating activity was 256000-512000 HAU/ml. When rabbits were immunized with purified virus-containing suspensions (150 μg protein per animal), hyperimmune polyclonal sera were obtained. For further work, the serum was diluted (1:10) and warmed up according to the standard procedure at 56 °C for 30 min to inactivate the heat-labile proteins of the complement system. The titers of specific antibodies of the obtained sera in HIA with homologous strains were 1:320 and 1:5120

Immune rabbit sera to Kazakhstan strains of swine influenza virus in titers 1:160 inhibited the hemagglutinating activity of influenza virus A/H1N1. Serum did not interact with heterologous A/H3N2 and type B viruses.

1. Cross-reaction of inhibition of hemagglutination (HIA**) of the reference strains of swine influenza virus A/H1N1 and isolates form pig farms of Northern Kazakhstan** (2018)

Isolate, strain	Antiserum to viruses					
	1	2	3	4	5	6
A/swine/Petropavlovsk/01/18	320	160	2560	1280	320	40
A/swine/Petropavlovsk/02/18	160	320	2560	1280	160	40
A/swine/Petropavlovsk/03/18	160	160	2560	1280	160	40
A/Swine/USA/1976/31	80	160	1280	640	640	20
A/Swine/Iowa/15/30	320	80	2560	640	640	40
A/California/04/09 pdm	20	40	20	20	40	160

N o t e. Inverse titers of anti-hemagglutinins are presented. 1 — A/pig/Petropavlovsk/01/18, 2 — A/pig/Petropavlovsk/02/18, 3 — A/pig/Petropavlovsk/03/18, 4 — A/pig/Petropavlovsk/, 5 — A/Swine/Iowa/15/30, 6 — A/California/04/09 pdm.

The isolated strains A/swine/Petropavlovsk/01/18, A/swine/Petropavlovsk/02/18, and A/swine/Petropavlovsk/03/18 interacted with immune sera to the reference viruses A/Swine/USA/1976/31 (H1N1) and A/Swine/Iowa/15/30 (H1N1) in high titers (Table 1), while with the antiserum to the drift variation A/California/04/09 (H1N1) pdm they reacted in lower titers. According to the antigenic structure of HA, the studied strains did not differ significantly from each other. We revealed the antigenic relationship of influenza A(H1N1) virus isolates from pigs among the isolates themselves and with reference strains A/H1N1 (A/Swine/USA/1976/31 and A/Swine/Iowa /15/30), and also their difference from strain A/California/04/09 pdm.

The infectious activity of the isolates varied within 6.5-7.9 lg EID_{50}/0.2 ml on CE, and within 3.5-4.3 lg TCD_{50}/0.2 ml on MDCK cell culture. Thence, the isolates were slightly inferior to the reference strains A/Swine/Iowa/15/30 and A/Swine/USA/1976/31 with 8.7 and 8.0 lg EID_{50}/0.2 ml and 5.2 and 5.1 lg TCD_{50}/0.2 ml, respectively (Table 2). Strain A/swine/Petropavlovsk/01/18 in its infectious activity was close to the reference strain A/California/04/09 pdm.

As to heat sensitivity of HA, the tested isolates of the swine influenza virus, similar to reference strains, were assigned to thermostable, since they retained the ability to agglutinate chicken erythrocytes in high titers (log2 from 6.3±0.6 to 9.6±0.8) after heating at 56 °C for 60 min (see Table 2). It was established that all the studied strains can well adsorb chicken erythrocytes (90-

100%); their elution occurred within 30-60 min of incubation at 37 °C.

2. Biological properties of swine influenza virus A/H1N1 isolates from pig farms of Northern Kazakhstan (2018)

Isolate, strain	IA		HT		Ad	El
	1	2	3	4		
A/swine/Petropavlovsk/01/18	6.5	3.5	9.7±0.2	9.6±0.8	90	1.0
A/swine/Petropavlovsk/02/18	7.8	4.3	9.6±0.4	9.6±0.8	100	1.0
A/swine/Petropavlovsk/03/18	7.9	4.3	9.7±0.6	9.3±0.9	100	0.5
A/Swine/USA/1976/31	8.8	5.2	9.7±0.6	6.3±0.6	100	1.0
A/Swine/Iowa/15/30	8.0	5.2	8.7±0.6	6.6±0.6	90	0.5
A/California/04/09 pdm	6.0	3.8	8.7±0.6	6.6±0.6	100	1.0

N o t e. IA — infectious activity: 1 — for chicken embryo, lg EID_{50}/0.2 ml, 2 — for MDCK cells, lg TCD_{50}/0.2 ml; HT — hemagglutinin thermal stability (GMT±SD): 3 — intact viral preparation, 4 — viral preparation heated at 56 °C for 60 min; Ad — adsorption on chicken erythrocytes, %; El — time of elution from chicken erythrocytes at 37 °C, hours.

The isolates, as well as the reference strains A/Swine/Iowa/15/30 and A/Swine/USA/1976/31, activated all types of red blood cells in high titers (Table 3).

3. Hemagglutinating activity of swine influenza virus A/H1N1 reference strains and isolates from pig farms of Northern Kazakhstan (GMT±SD, 2018)

Isolate, strain	Chicken	Guinea pig	Sheep	Horse	Human I(0)
A/swine/Petropavlovsk/01/18	9.5±0.4	10.1±0.2	10.0±0.0	9.6±0.4	10.4±0.2
A/swine/Petropavlovsk/02/18	9.6±0.3	10.5±0.4	10.0±0.0	9.7±0.2	10.4±0.2
A/swine/Petropavlovsk/03/18	9.5±0.4	10.5±0.4	10.0±0.0	9.5±0.4	10.3±0.0
A/Swine/USA/1976/31	9.7±0.6	12.0±0.0	9.5±1.4	8.7±0.3	9.6±1.5
A/Swine/Iowa/15/30	8.7±0.6	11.0±0.0	8.2±1.6	9.7±0.4	9.3±1.2
A/California/04/09 pdm	8.7±0.6	9.3±0.6	6.6±0.6	1.0±0.0	8.0±0.0

N o t e. Geometrical mean binary logarithm for reverse hemagglutinin titers (GMT) are presented.

In all tested isolates, hemagglutinating activity was not suppressed by nonspecific tested native sera (Table 4). However, heating of the sera contributed to an increase in their inhibitory activity. Inhibitor titers increased in sera heated for 30 min at 62 °C, and even more when boiling for 10 min at 100 °C.

4. Sensitivity of swine influenza virus A/H1N1 isolates from pig farms of Northern Kazakhstan (2018) **to different nonspecific inhibitors**

Isolate, strain	Serum								
	guinea pig			chicken			rabbit		
	1	2	3	1	2	3	1	2	3
A/swine/Petropavlovsk/01/18	< 20	20	160	< 20	40	80	< 20	20	20
A/swine/Petropavlovsk/02/18	< 20	40	80	< 20	80	80	< 20	80	160
A/swine/Petropavlovsk/03/18	< 20	40	80	< 20	160	160	< 20	80	160
A/Swine/USA/1976/31	< 20	40	80	< 20	< 20	40	< 20	80	160
A/Swine/Iowa/15/30	< 20	80	80	< 20	< 20	40	< 20	40	160
A/California/04/09 pdm	< 20	40	80	< 20	< 20	40	< 20	80	80

N o t e. 1 — intact serum, 2 — serum heated for 30 min at 62 °C, 3 — serum boiled for 10 min at 100 °C. Inverse titers of nonspecific inhibitors are presented.

A preliminary study on CE did not reveal the embryotoxic effect of the used antiviral drugs in all the doses studied (20, 21).

5. Sensitivity of swine influenza virus A/H1N1 isolates from pig farms of Northern Kazakhstan (2018) **to different to antiviral drugs** (GMT±SD)

Isolate, strain	Inhibiting concentration, μg/ml			
	Tamiflu®	Remantadine	Arbidol®	Ingavirin®
A/swine/Petropavlovsk/01/18	6.6±0.1	6.9±0.0	No inhibition	No inhibition
A/swine/Petropavlovsk/02/18	5.6±2.0	12.7±0.1	No inhibition	No inhibition
A/swine/Petropavlovsk/03/18	5.7±1.7	3.7±0.2	No inhibition	No inhibition
A/Swine/USA/1976/31	6.5±0.1	6.7±0.2	No inhibition	No inhibition
A/Swine/Iowa/15/30	6.6±0.6	7.0±0.1	No inhibition	No inhibition
A/California/04/09 pdm	3.5±0.0	No inhibition	No inhibition	No inhibition

N o t e. The concentration causing a 2-fold decrease in virus reproduction in chicken embryos is indicated.

The studied isolates of A/H1N1 virus and the reference strains were sensitive to Tamiflu® and Remantadine (Table 5). The inhibitory concentration was 5.6-6.6 and 3.7-12.7 μg/ml, respectively. Also, the isolates, as well as the reference strains, turned out to be resistant to Arbidol® and Ingavirin® which did not inhibit viral reproduction even in high concentrations (50 μg/ml).

To summarize, it should be noted that a comparison of the swine influenza viruses isolated in the Republic of Kazakhstan in 2018 and in 2010-2016 [9, 22] revealed similarities in the main biological characteristics that allows us to assign these isolates into a single rather homogeneous group. However, the isolates show slight antigenic heterogeneity that indicates a prolonged circulation of antigenically homogeneous swine flu virus strains among pigs. There is also a greater similarity of the isolates with the reference strains of swine influenza virus (A/Swine/USA/1976/31 and A/Swine/Iowa/15/30) than with A/H1N1 strains which have been circulating in the human population since 2009 and are genetically related to A/California/04/09 pdm. The susceptibility to avian and human influenza A viruses, detected in pigs, allows us to refer them as intermediate hosts which provide a reassortment between the genes of influenza viruses of various origins. This can lead to the emergence of new antigenic variations of the influenza virus with epidemic potential [23]. The degree of pathogenicity and epidemic activity is not the same for different influenza viruses and depends both on their molecular biology and ecological features. Comparison of cross-reactivity of influenza viruses by HIA discloses the nature of their serological relations which may reflect small antigenic differences in hemagglutinin between similar strains and/or indicate differences between groups of strains [24, 25].

Thus, the isolates of swine influenza virus of 2018 from pigs in Northern Kazakhstan are antigenically similar to each other, to the strains that circulated earlier in the pig population in the Republic of Kazakhstan, and to classical standards of swine flu virus, but different from strains circulating in the human population. The isolates of 2018 are similar to each other and to reference strains in thermostability of hemagglutinin and the rate of adsorption on chicken erythrocytes, but they differ in the rate of elution from red blood cells, sensitivity to antiviral drugs, and also in infectious activity. Two strains, the A/swine/Petropavlovsk/02/18 and A/swine/Petropavlovsk/03/18, are similar to the reference strains A/Swine/Iowa/15/30 and A/Swine/USA/1976/31 in infectivity, while strain A/swine/Petropavlovsk/01/18 shows similarities with the reference strain A/California 04/09 pdm. The tested isolates are sensitive to antiviral drugs Tamiflu® and Remantadine and resistant to Arbidol® and Ingavirin®. Data on the antigenic and biological properties of animal influenza viruses reveal the patterns of their circulation and the development of infection, which is necessary to predict an epidemic situation and an appropriate strategy and tactics for preventive and anti-epidemic measures. Identification of emerging variations of the influenza viruses in populations of susceptible animal species, especially pigs, is extremely important, therefore, we plan to continue monitoring studies.

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A PROBIOTIC BASED ON THE *Escherichia coli* ŽP STRAIN. I. EFFICIENCY ASSESSMENT OF THE CONJUGATIVE TRANSFER OF THE COLICIN E7 ACTIVITY GENE INTO AVIAN PATHOGENIC *E. coli* STRAINS in vitro AND in vivo

M.V. KUZNETSOVA[1], I.L. MASLENNIKOVA[1], J.S. GIZATULLINA[1], D. ŽGUR BERTOK[2], M. STARČIČ ERJAVEC[2]

[1]*Institute of Ecology and Genetics of Microorganisms, Perm Federal Research Center of Ural Branch RAS,* 13, ul. Goleva, Perm, 614081 Russia, e-mail info@iegm.ru, mar@iegm.ru (✉ corresponding author), i.maslennikova1974@gmail.com, gizatullina.julia@yandex.ru;

[2]*Department of Biology, Biotechnical Faculty, University of Ljubljana,* Jamnikarjeva 101, 1000 Ljubljana, Slovenia, e-mail biologija@bf.uni-lj.si, darja.zgur@bf.uni-lj.si, marjanca.starcic.erjavec@bf.uni-lj.si

ORCID:
Kuznetsova M.V. orcid.org/0000-0003-2448-4823
Maslennikova I.L. orcid.org/0000-0002-2776-8023
Gizatullina J.S. orcid.org/0000-0001-9625-1151
Žgur Bertok D. orcid.org/0000-0003-4166-552X
Starčič Erjavec M. orcid.org/0000-0003-0200-573X

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Abstract

The protection of farm animals against infectious diseases is a priority in veterinary medicine. The wide spread of pathogenic and opportunistic bacteria resistant to antibiotics on poultry farms requires the development of modern methods to maintain the health of birds in industrial production. A promising direction to improve measures to prevent and limit the spread of pathogens resistant to antimicrobial agents is the use of targeted bacterial drugs — probiotics. Veterinary probiotics based on genetically modified microorganisms can be used for the treatment and prophylaxis of infectious diseases in farm animals. We demonstrated the antagonistic effect of the genetically modified *Escherichia coli* ŽP strain against agents of avian colibacillosis, the avian pathogenic *Escherichia coli* (APEC) in both in vitro and in vivo models. The *colE7* gene (colicin E7 synthesis gene) carried on a conjugative plasmid was efficiently transferred by conjugation in conditions of planktonic and biofilm growth in vitro. The *E. coli* ŽP strain was shown to actively colonize the intestine of rats and quails and to contribute to beneficial effects of the microbiota. Our aim was to evaluate the competitive ability of the *E. coli* ŽP strain as well as the efficiency of killing APEC based on the conjugative transfer of the *colE7* gene, encoding a DNasae, in vitro and in vivo. We used the ColE7-mediated kill—anti-kill system based on the Nissle 1917 probiotic strain, the genetically modified *E. coli* ŽP strain (killer donor) carrying the *colE7* gene on the conjugative plasmid pOX38a, as well as the *immE7* gene (colicin E7 immunity gene) on the chromosome. As control, the *E. coli* N4i strain without *colE7* gene on the conjugative plasmid pOX38 (control donor) was used. Six APEC strains with resistance to ampicillin isolated from organs of broilers with colibacillosis were used as recipients in performed conjugation assays. The phylogenetic group of used APEC strains was detected with quadruplex PCR. The conjugation transfer was conducted in Luria-Bertani (LB) medium in immunological polystyrene 96-walls flat bottom plates in plankton and in biofilm culture. The experiments with rats (Wistar line) and Manchurian quail (*Coturnix coturnix*) were conducted in the vivarium of the Wagner Perm State Medical University. The competitive ability of *E. coli* ŽP strain was confirmed in co-cultivation assays with APEC strains, including bacteriocin producers, in various models. Conjugative *colE7* gene transfer to APEC was detected in vitro in plankton and in biofilm: in experiments with the control donor strain *E. coli* N4i the conjugation frequency varied from 10^{-6} to10^{-2}. In vivo, it was shown that *E. coli* ŽP strain was able to effectively colonize the rat (line Wistar) and Manchurian quail (*Coturnix coturnix*) intestine and persist there at least for a month. Introduced *E. coli* ŽP cells increased the total amount

of commensal *Escherichia* in the intestine of the animals and reduced the growth of the pathogenic microbes without affecting the lactic acid bacteria. The transfer ability of the conjugative plasmid pOX38 in the intestinal tract of both animal species was demonstrated. The conjugation in the intestinal tract occurred with a high frequency, on average 10^{-2}. No transconjugants were detected in both in vitro and in vivo experiments with the *E. coli* ŽP strain harboring the conjugative plasmid pOX38a; the recipient cells that received the *colE7* gene via the conjugative transfer expressed it and were lysed due to the colicin DNase activity. In groups in which the strain ŽP was applied, the number of APEC recipients also decreased. The obtained results indicated that the *E. coli* ŽP strain was able to colonize the intestine of animals and had antibacterial activity against enteropathogens due to the conjugative mechanism of colicin gene transfer. As the ŽP strain was also effective on APEC cells that were resistant and tolerant to bacteriocins, it has the potential to become the basis for a highly effective new generation of probiotics.

Keywords: colicins, ColE7, conjugative transfer, kill—anti-kill system, antibiotic alternative, probiotics, avian pathogenic *Escherichia coli* (APEC), animal models

Poultry farming is one of the fastest growing segments of agriculture [1, 2]. To increase the efficiency of poultry production, various feed additives, such as synthetic hormones and antibacterial drugs, are widely used [3]. Long-term use of antibiotics led to the appearance of gram-positive and gram-negative microorganisms resistant to these substances, which become the main cause of death of young birds in poultry farms [4, 5]. In addition, infected poultry are a potential reservoir of pathogens of acute intestinal infections for humans [6]. In 2014, the World Health Organization (WHO) adopted a strategy to limit the use of antibacterial drugs for the prevention of infectious diseases in farm animals [7], and in 2017, Russia joined this program [8].

Probiotics, the bacterial targeted drugs that play a leading role in replacing antibiotics, are promising in improving measures to prevent and limit the spread of pathogens resistant to antimicrobial agents [9, 10]. Probiotics are live cells of microorganisms of a single species or several species which are used as feed additives and act as a growth stimulant, and also beneficially affect the host physiological parameters and microbiota [11, 12]. In the first hours of life, the animal intestines are artificially colonized with bacterial strains and/or biocomplexes that provide an antagonistic effect against pathogenic or conditionally pathogenic microorganisms due to the competitive displacement by bacteria producing bacteriocins, the antibacterial substances which inhibit closely related microbial taxa. There are many reports on the positive results of using probiotics to prevent and treat infections in birds [1, 13-15]. Artificially constructed strains with multiple production of bacteriocins attract special attention [16-19]. For example, to restore intestinal competitiveness of probiotic strain *Escherichia coli* M17 which lost its antagonistic properties it was proposed to use recombinant plasmids for production of colicin E1 [20] or microcin C51 [21]. However, even these drugs may not be effective enough as bacteriocin-resistant bacteria appear [22] which possess modified surface receptors and translocation systems. Colicin delivery via horizontal *col*-gene transfer using conjugative plasmids may be an alternative approach [23] that allows us to target bacteriocin resistant/tolerant bacterial strains.

The antimicrobial bacterial kill—anti-kill system was tested with the reference (*E. coli* K-12 TG1) and uropathogenic (*E. coli* DL82) strains. Real-time PCR, a bioluminescent method, and flow cytometry confirmed the conjugative transfer of plasmid pOX38a carrying *colE7* to the recipient cell where transcription of *colE7* gene begins immediately and the synthetized bacteriocin kills the recipient [24].

This paper is the first report on the antagonistic effect of the genetically

modified *E. coli* ŽP strain against APEC (avian pathogenic *Escherichia coli*, the causative agents of escherichiosis in birds) in in vitro and in vivo experimental models. It was found that *in vitro* conjugative transfer of the colE7 gene to APEC cells is effective both under planktonic growth and biofilm development. In vivo, the strain *E. coli* ŽP was able to actively colonize the intestines of rats and Manchurian quail and persist there, contributing to the normalization of animal microbiota. Our results confirmed that the conjugative plasmid transfer from the used donor to APEC strains occured in the intestinal tract with a high frequency, and resulted in colicin acting also on cells that were resistant and tolerant to bacteriocins. This suggests the possibility of creating a highly effective probiotic drug of a new generation based on conjugative transfer of colicin synthesis genes.

The aim of our study was to evaluate the efficiency of APEC (avian pathogenic *Escherichia coli*) killing upon conjugative transfer of colicin E7 gene and the competitiveness of *Escherichia coli* ŽP strain in vitro and in vivo.

Materials and methods. ColE7-mediated kill anti-kill system based on a genetically modified Nissle 1917 probiotic strain, the *E. coli* ŽP pOX38a Gm^rCm^r strain (killer donor, KD) that carries the colicin E7 synthesis gene (*colE7*) with DNase activity on the conjugative plasmid and the colicin E7 immunity (*immE7*) gene on the chromosome, and the *E. coli* N4i pOX38 Gm^rCm^r strain (control donor, D) without *colE7* on the plasmid [25]. Recipients (R) were ampicillin-resistant *E. coli* strains ($n = 6$) isolated from the internal organs of infected broiler chickens (APEC). The strains had an individual genetic profile according to rep-PCR typing with ERIC 1R/ERIC 2 primers [26]. The phylogenetic affiliation of the isolates was determined by the multiplex polymerase chain reaction (quadruplex PCR) [27]. The primers used in this work were synthesized in Syntol LLC (Syntol, Russia). Amplification was run on a DNA Engine Dyad Thermal Cycler (Bio-Rad, USA). The bands were visualized using the Gel-Doc XR gel documentation system (Bio-Rad, USA).

The strains were deposited into the Ex culture collection at the Department of Biology, Biotechnical Faculty, University of Ljubljana (Univerza v Ljubljani, Slovenia).

APEC cultures were screened for sensitivity to bacteriocins using the BZB collection of indicator strains producing bacteriocins and microcins (University of Ljubljana) using the agar overlay method [22]. *E. coli* DH5α was used as the control (bacteriocin-sensitive) strain. The bacteriocin production by APEC strains and the sensitivity of *E. coli* ŽP to bacteriocins of the studied strains were assessed by a similar method [22].

Conjugative transfer was performed in Luria-Bertani (LB) medium (Amresco, USA) for 6 and 24 hours in plankton culture and biofilm in polystyrene flat-bottomed immunological 96-well plates (Lenpolymer, Russia).

The conjugation mixture contained 100-fold diluted night cultures (standardized to 2.0 according to the McFarland turbidity standard) of the donor and recipient in a 1:4 ratio. Pre-washed (0.89% NaCl) biofilms were destroyed by ultrasound (37 Hz, Elmasonic 30S, Elma Schmidbauer GmbH, Germany) in 100 μl of saline for 1 min (5 cycles). The colony forming units (CFU) were calculated from the counts on selective media (on LB medium supplemented with 50 μg/ml chloramphenicol and 50 μg/ml ampicillin for transconjugants, on LB medium with 50 μg/ml ampicillin for recipient cells, and on LB medium with 40 μg/ml gentamicin for donor cells). Conjugation transfer frequency (Y)

was estimated as the transconjugant cells (T) CFU to recipient cells (R) CFU ratio [28].

Biofilm biomass was determined as described by Merritt et al. [29] on a Benchmark Plus microplate reader (Bio-Rad, USA) at $\lambda = 570$ nm.

Bacterial growth in co-culture was assessed in a polystyrene flat-bottomed immunological 96-well plate on rich (LB) and minimal (M9) media at 37 °C from 1 to 3 days. Plating was performed from decimal dilutions of the bacterial suspension on the corresponding selective agars with antibiotics.

Tests on Wistar rats and Manchurian quail (*Coturnix coturnix*) were performed in a vivarium (Wagner Perm State Medical University). Stocking density, feeder space and watering space, temperature, humidity, lightness were within the recommended limits (VNITIP). Care for rats and poultry was carried out in accordance with GOST 34088-2017 [30].

In the first in vivo conjugative transfer experiment, 30-day-old male rats of 175.25±10.31 g weight were assigned into three groups, 10 animals per group. The rats were kept for 21 days in plastic cages (5 animals per cage) in a ventilated room at 21-23 °C and natural lighting, with access to food and water ad libitum. Live strains of the control donor (group I) and killer donor (group II) were added to water at 10^8 cells per rat for 7 days. Intestinal colonization was determined on day 3 and day 6 by plating feces on selective media. From day 8 to day 21, in both test groups, live culture of APEC strain resistant to ampicillin (the recipient) were added to water at 10^8 bacteria per rat. After 6 hours, water was changed and donor strains were added. The control group did not receive any of the *E. coli* strains. The presence of ampicillin-resistant recipients, control/killer donors, and transconjugants in the intestine was determined on days 10, 14, and 21.

In the second in vivo experiment, Manchurian quails of 114.0±7.50 g weight were assigned into three groups, 5 individuals each, and kept for 1 week under conditions similar to those in the first experiment. Live cells of the control donor (group I) and killer donor (group II) were administered via water (10^8 bacteria per quail) for 3 days, with daily control of intestinal colonization by plating feces on selective media. From day 2 to day 6, live cells of the ampicillin-resistant APEC strain (10^8 bacteria per quail) were administered in both experimental groups, after 6 h water was changed and donor strains were applied. The control group did not receive any *E. coli* strains. The presence in the intestine of ampicillin-resistant recipients and transconjugants was determined on days 2, 3, and 6.

In both experiments, the number of viable cells was calculated per 1 g feces, the conjugation frequency was determined similarly to the in vitro experiment.

Statistical analysis was performed using Microsoft Office Excel and STATISTICA 10 software (StatSoft, Inc., USA). The results are presented as arithmetic mean (M) and its error (±SEM). The significance of differences between the average values was assessed by Student's t-test at $p < 0.05$ and the relationship between the quantitative values by the linear Pearson correlation coefficient (r_p).

Results. The results of quadruplex PCR revealed that the APEC strains used in this study belonged to the phylogenetic groups B1, B2, and E. Colicinogenesis was detected in four cultures, one of them turned out to be insensitive to bacteriocin ColE7 (Fig. 1, Table 1). All APECs were resistant to ten or more bacteriocins, including those with pore-forming, DNase, rRNAse, and tRNAse action.

1. Characterization of *Escherichia coli* strains from the internal organs of infected broiler chickens (avian pathogenic *E. coli*, APEC)

APEC strain	Phylogroup	Bacteriocines	Resistance to colicins	Resistance to microcins
RB1	B1	No	A, B, D, E1, E3, E5, E7, Ia, Ib, K, N, M, S4	B17, C7, V
RB2	E	No	A, B, D, E1, E5, E7, Ia, Ib, K, N, M, S4	C7, V
RB3	E	Yes	A, B, D, E1, E2, E3, Ia, Ib, K, N, M, S4	B17, C7, V
RB4	E	Yes	A, B, D, E1, E, E5, E7, E8J, Ia, Ib, K, N, M	B17, C
RB5	B1	Yes	A, B, D, E1, E5, E6, Ia, Ib, K, N, M, S4	B17, C7, V
RB6	B2	Yes	A, B, D, Ia, Ib, N, M, S4	B17, C7, V

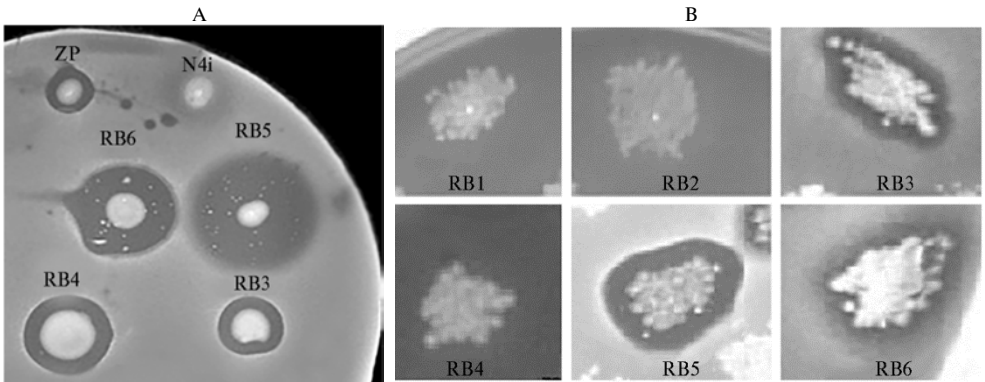

Fig. 1. Production of bacteriocins (A) **and sensitivity to bacteriocins** (B) **in APEC (avian pathogenic *Escherichia coli*) strains:** A — *E. coli* ŽP, N4i, APEC RB1-RB6 strains; after growing test strains, the indicator strain *E. coli* DH5a was grown on top of them, B — strain ŽP with different APEC strains (RB1-RB6) in the top layer (the agar overlay method).

In experiments with the control donor strain *E. coli* N4i, the frequency of conjugative transfer in 6 hours varied within 10^{-6}-10^{-2} and turned out to be higher in the biofilm than in plankton growth ($1.73\times10^{-2}\pm2.24\times10^{-2}$ vs. $2.27\times10^{-5}\pm2.40\times10^{-5}$, respectively), and after 24 hours it was comparable in both models ($4.45\times10^{-4}\pm5.46\times10^{-4}$ vs. $6.25\times10^{-3}\pm8.63\times10^{-3}$) (Table 2). A biofilm model was necessary because in vivo, including in the intestine, microorganisms exist mainly as part of attached communities. Upon a 24-hour exposure, the correlation between plasmid transfer in plankton and biofilm was $r_p = 0.905$ ($p = 0.05$).

In all variants of conjugation of the killer donor *E. coli* ŽP with recipient strains, transconjugants were not detected. That is, recipient cells that received *colE7* gene via conjugative transfer expressed it and were lysed due to the colicin DNase activity.

After 24 hours we also determined biofilms biomass in mixed cultures. For different strains, the OD_{570} value ranged from 0.100 to 0.187, in average 0.144 ± 0.007. An inverse moderate relationship was revealed between the biofilm biomass and the frequency of conjugation ($r_p = -0.630$, $p = 0.05$).

It should be noted that after 24 hour incubation of conjugation mixture the average counts of control and killer donors were $2.22\times10^7\pm2.03\times10^7$ and $1.02\times10^8\pm7.69\times10^7$ CFU/ml in plankton, and $7.38\times10^6\pm2.33\times10^6$ and $9.89\times10^6\pm1.13\times10^7$ CFU/ml in biofilms, respectively, which may indicate a high

2. The number of donor, recipient, transconjugant cells (CFU/ml) **and the conjugation frequency in plankton growth and in biofilms upon conjugative transfer of plasmid pOX38 to APEC** (avian pathogenic *Escherichia coli*) **strains in vitro** ($M \pm$SEM)

APEC strain	Group I, *E. coli* N4i (control donor)				Group II, *E. coli* ŽP (killer donor)			
	D	R	T	Y	KD	R	T	Y
				Plankton growth				
RB1	$1.04\times10^{7}\pm1.13\times10^{6}$	$1.70\times10^{8}\pm2.50\times10^{7}$	$2.10\times10^{5}\pm7.25\times10^{4}$	$1.33\times10^{-3}\pm6.22\times10^{-4}$	$3.14\times10^{7}\pm2.13\times10^{6}$	$9.13\times10^{7}\pm3.75\times10^{6}$	0.00	0.00
RB2	$1.76\times10^{6}\pm6.63\times10^{5}$	$1.16\times10^{8}\pm1.13\times10^{7}$	$2.62\times10^{6}\pm2.21\times10^{6}$	$2.46\times10^{-2}\pm2.13\times10^{-2}$	$8.43\times10^{7}\pm5.08\times10^{7}$	$3.86\times10^{7}\pm3.63\times10^{6}$	0.00	0.00
RB3	$5.71\times10^{7}\pm2.79\times10^{7}$	$5.50\times10^{7}\pm2.25\times10^{7}$	$2.16\times10^{5}\pm2.01\times10^{5}$	$2.92\times10^{-3}\pm2.46\times10^{-3}$	$1.70\times10^{8}\pm3.40\times10^{7}$	$6.80\times10^{7}\pm1.45\times10^{7}$	0.00	0.00
RB4	$1.74\times10^{6}\pm5.63\times10^{5}$	$3.65\times10^{8}\pm1.56\times10^{7}$	$1.84\times10^{5}\pm1.66\times10^{5}$	$5.03\times10^{-4}\pm4.55\times10^{-4}$	$2.03\times10^{7}\pm1.45\times10^{7}$	$1.59\times10^{8}\pm3.63\times10^{7}$	0.00	0.00
RB5	$2.36\times10^{7}\pm7.63\times10^{6}$	$2.89\times10^{8}\pm4.88\times10^{7}$	$1.13\times10^{4}\pm1.25\times10^{3}$	$3.94\times10^{-5}\pm2.31\times10^{-6}$	$6.83\times10^{7}\pm4.75\times10^{7}$	$1.88\times10^{8}\pm3.25\times10^{7}$	0.00	0.00
RB6	$3.88\times\pm1.13\times10^{7}$	$2.79\times10^{7}\pm1.46\times10^{7}$	$2.80\times10^{5}\pm2.20\times10^{5}$	$8.13\times10^{-3}\pm3.63\times10^{-3}$	$2.35\times10^{8}\pm1.84\times10^{8}$	$3.58\times10^{7}\pm2.34\times10^{7}$	0.00	0.00
				Biofilm				
RB1	$1.15\times10^{7}\pm6.15\times10^{6}$	$1.15\times10^{7}\pm1.04\times10^{6}$	$5.13\times10^{2}\pm3.63\times10^{2}$	$4.89\times10^{-5}\pm2.06\times10^{-5}$	$7.94\times10^{6}\pm1.29\times10^{6}$	$3.04\times10^{7}\pm2.67\times10^{7}$	0.00	0.00
RB2	$4.07\times10^{6}\pm3.81\times10^{6}$	$7.76\times10^{6}\pm3.56\times10^{6}$	$7.38\times10^{3}\pm6.88\times10^{3}$	$1.60\times10^{-3}\pm3.42\times10^{-4}$	$3.43\times10^{7}\pm2.22\times10^{7}$	$3.03\times10^{7}\pm2.62\times10^{7}$	0.00	0.00
RB3	$5.32\times10^{6}\pm3.93\times10^{6}$	$4.78\times10^{6}\pm8.75\times10^{5}$	$1.13\times10^{3}\pm7.75\times10^{2}$	$2.13\times10^{-4}\pm1.23\times10^{-4}$	$4.70\times10^{6}\pm2.08\times10^{6}$	$5.25\times10^{6}\pm2.00\times10^{6}$	0.00	0.00
RB4	$8.16\times10^{6}\pm3.44\times10^{6}$	$3.96\times10^{7}\pm7.56\times10^{6}$	$2.49\times10^{4}\pm1.91\times10^{4}$	$5.58\times10^{-4}\pm3.76\times10^{-4}$	$1.48\times10^{6}\pm3.25\times10^{5}$	$2.83\times10^{7}\pm2.50\times10^{7}$	0.00	0.00
RB5	$7.43\times10^{6}\pm3.07\times10^{6}$	$2.02\times10^{7}\pm1.73\times10^{7}$	$2.50\times10^{1}\pm2.50\times10^{1}$	$8.47\times10^{-6}\pm8.47\times10^{-6}$	$1.55\times10^{6}\pm3.00\times10^{5}$	$2.96\times10^{6}\pm1.16\times10^{6}$	0.00	0.00
RB6	$7.82\times10^{6}\pm3.20\times10^{6}$	$2.01\times10^{7}\pm1.75\times10^{7}$	$1.10\times10^{3}\pm1.00\times10^{2}$	$2.40\times10^{-4}\pm2.13\times10^{-4}$	$9.38\times10^{6}\pm3.63\times10^{6}$	$7.74\times10^{6}\pm5.26\times10^{6}$	0.00	0.00

Note. D — control donor. KD — killer donor, R — recipient, T — transconjugant, Y — frequency of conjugation transfer.

competitiveness of donors in polymicrobial communities. This assumption was verified in experiments with the co-culture of the killer donor and APEC strains.

Growth of cultures of the killer donor *E. coli* ŽP and three APEC strains, RB2 (not producing bacteriocins and insensitive to ColE7), RB3 (producing bacteriocins and sensitive to ColE7) and RB4 (producing bacteriocins and insensitive to ColE7) on rich (LB) and minimal (M9) media are shown in Figure 2.

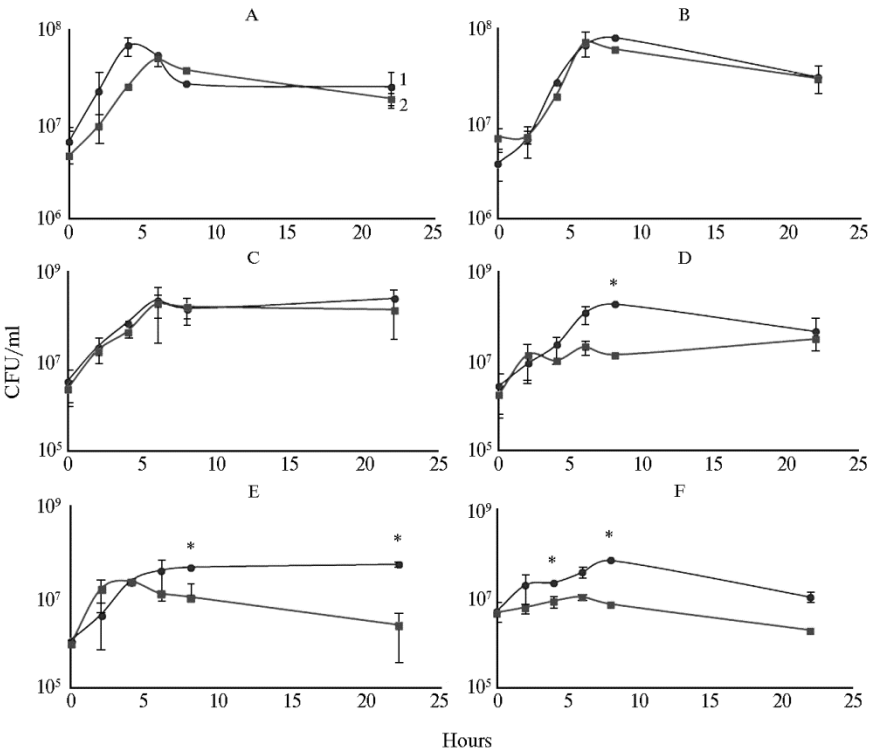

Fig. 2. Growth of APEC (avian pathogenic *Escherichia coli*) **strains in co-cultures with *E. coli* ŽP in rich (LB) medium** (left) **and minimal** (M9) **medium** (right)**:** 1 — recipient (APEC), 2 — killer donor (ŽP); A, B — strain RB2, C, D — strain RB3, E, F — strain RB4. Asterisks (*) indicate statistically significant differences ($p \leq 0.05$) between the growth of the recipient and the killer donor in the corresponding periods.

The dynamics of competition between *E. coli* ŽP and APEC strains in a rich medium (see Fig. 2, A, B, D) differed significantly. In the absence of antagonism between the strains (RB2 recipient), immediately after inoculation, the number of cells of both cultures increased, and a slight change in the ratio of bacteria of competing strains was most likely due to the advantage in the growth rate of the natural strain RB2. After 5 hours of co-culture, the amount of *E. coli* ŽP cells was stable. There was a tendency toward a decrease in the abundance of *E. coli* RB2 bacteria due to their lysis resulting from conjugative plasmid transfer, which was quite effective at a frequency of $2.46\times10^{-2}\pm3.02\times10^{-2}$. In co-culture of *E. coli* ŽP and RB4, the number of *E. coli* ŽP cells towards the stationary phase, on the contrary, decreased. Apparently, at this stage the antagonistic activity of the APEC strain was manifested due to the synthesis of bacteriocin, which is induced by an increase in the culture density, starting from the end of the logarithmic growth. Considering that the bacteria *E. coli* RB4 are insensitive to ColE7 and the conjugation rate was low, the cell ratio after 24 hours in this pair is explainable. Interestingly, in a co-culture of *E. coli* ŽP and RB3, in which RB3 also produces bacteriocins but is sensitive to ColE7, antagonistic activity

3. The number of donor, recipient, transconjugant cells (CFU/ml) **and the conjugation frequency in intestines of Wistar rats and Manchurian quails upon conjugative transfer of plasmid pOX38 to APEC** (avian pathogenic *Escherichia coli*) **strains in vivo** ($M \pm$SEM)

Days	Group I, *E. coli* N4i (control donor)				Group II, *E. coli* ŽP (killer donor)			
	D	R	T	Y	KD	R	T	Y
				Rats				
3	$3.50\times10^{4}\pm2.15\times10^{3}$	nd	nd	nd	$1.50\times10^{4}\pm1.25\times10^{3}$	nd	nd	nd
6	$7.21\times10^{5}\pm5.29\times10^{4}$	nd	nd	nd	$2.34\times10^{5}\pm1.74\times10^{5}$ [a]	nd	nd	nd
10	$2.96\times10^{6}\pm2.01\times10^{6}$ [a]	$3.54\times10^{3}\pm1.23\times10^{3}$	$2.15\times10^{2}\pm1.75\times10^{2}$	$6.07\times10^{-2}\pm5.58\times10^{-2}$	$5.97\times10^{5}\pm2.58\times10^{5}$ [a]	$2.19\times10^{3}\pm1.25\times10^{3}$	0.00	0.00
14	$1.26\times10^{7}\pm8.64\times10^{6}$ [a]	$5.21\times10^{5}\pm2.13\times10^{4}$	$4.11\times10^{4}\pm4.00\times10^{3}$	$7.87\times10^{-2}\pm8.32\times10^{-3}$	$5.64\times10^{7}\pm8.96\times10^{6}$ [a]	$4.22\times10^{4}\pm2.36\times10^{4}$	0.00	0.00
21	$7.25\times10^{7}\pm2.35\times10^{7}$ [a]	$8.69\times10^{6}\pm5.55\times10^{6}$ [a]	$6.15\times10^{4}\pm1.02\times10^{3}$	$7.08\times10^{-3}\pm9.64\times10^{-4}$	$2.23\times10^{7}\pm1.00\times10^{7}$ [a]	$5.91\times10^{4}\pm4.98\times10^{3}$ [b]	0.00	0.00
				Manchurian quails				
1	$8.00\times10^{3}\pm5.21\times10^{3}$	nd	nd	nd	$5.04\times10^{4}\pm1.22\times10^{4}$	nd	nd	nd
2	$2.70\times10^{6}\pm2.01\times10^{5}$ [a]	$5.00\times10^{5}\pm4.55\times10^{5}$	$5.00\times10^{3}\pm1.12\times10^{3}$	$1.00\times10^{-2}\pm1.12\times10^{-1}$	$8.12\times10^{5}\pm2.22\times10^{5}$	$4.68\times10^{4}\pm2.25\times10^{4}$	0.00	0.00
3	$4.45\times10^{6}\pm3.05\times10^{6}$ [a]	$3.05\times10^{6}\pm2.41\times10^{5}$	$1.05\times10^{4}\pm1.11\times10^{4}$	$3.44\times10^{-3}\pm2.46\times10^{-3}$	$1.74\times10^{6}\pm1.96\times10^{6}$ [a]	$7.39\times10^{5}\pm5.57\times10^{5}$	0.00	0.00
6	$6.42\times10^{7}\pm6.16\times10^{7}$ [a]	$9.12\times10^{6}\pm7.15\times10^{6}$ [a]	$4.18\times10^{5}\pm4.00\times10^{5}$	$4.58\times10^{-2}\pm2.12\times10^{-1}$	$2.57\times10^{7}\pm3.01\times10^{7}$ [a]	$8.29\times10^{5}\pm5.55\times10^{5}$ [b]	0.00	0.00

N o t e. D — control donor. KD — killer donor, R — recipient, T — transconjugant, Y — frequency of conjugation transfer, nd — not detected.

[a] Statistically significant differences ($p \le 0.05$): D: value compared with the day 3 value for rats and day 1 for quails; R: value compared with day 10 value for rats and day 2 value for quails; KD: data compared with day 3 value for rats and day 1 value for quails).

[b] Statistically significant differences ($p \le 0.05$) between the group with the control donor and the group with the killer donor.

between the strains did not appear.

On M9 medium (see Fig. 2, B, D, E), the ratio between *E. coli* ŽP and RB2 did not change, while APECs producing colicins (RB3 and RB4) suppressed the growth of the killer donor at the end of the logarithmic—the beginning of the stationary phase of growth. However, the absence of significant differences between associates at the end of culture, apparently, can be associated with ineffective conjugation of *E. coli* ŽP and low colicin synthesis by recipients (RB3 and RB4) when grown on a poor culture medium.

In our preliminary studies of in vivo conjugative plasmid transfer, *E. coli* bacteria resistant to ampicillin, chloramphenicol, and gentamicin were not found in the intestines of rats and quails. In group I of rats, on day 3 the number of viable cells of the control donor and recipient averaged $3.50\times10^4\pm2.15\times10^3$ and $3.54\times10^3\pm1.23\times10^3$ CFU/g, respectively (Table 3). *E. coli* Amp^rCm^r transconjugants were detected on day 3 after the administration of the recipient, on average, their number was $2.15\times10^2\pm1.75\times10^2$ CFU/g, the frequency of conjugation was $6.07\times10^{-2}\pm 5.58\times10^{-2}$. In this group, an increase in the cell counts of both the donor and the recipient was observed throughout the experiment. In group II, the number of viable cells of the killer donor and the recipient on day 3 was $1.50\times10^4\pm1.25\times10^3$ and $2.19\times10^3\pm1.25\times10^3$ CFU/g, respectively. Further, the abundance of *E. coli* ŽP increased to 10^7 CFU/g, while the number of recipient cells after the first administration increased insignificantly. Transconjugants in this group were not found in any assayed time point. In the group of animals that did not receive donor strains, *E. coli* resistant to ampicillin, chloramphenicol and gentamicin were not detected in any assayed time point.

Given the results on colonization of rat intestines with donor strains in a preliminary experiment, in the model with Manchurian quail, the frequency of conjugative transfer in vivo was followed for 6 days. Just 2 days after the administration of the bacterial suspension, the number of cells of the donor and killer strains of *E. coli* in the poultry intestine was $2.70\times10^6\pm2.01\times10^5$ and $8.12\times10^5\pm2.22\times10^5$ CFU/g, respectively. The frequency of conjugative transfer in group I (control donor) remained at 10^{-2}-10^{-3} level for 6 days of observation. Similar to the experiment with the rat model, transconjugants in group II (killer donor) were not detected. In the control, *E. coli* bacteria resistant to ampicillin, chloramphenicol, and gentamicin were also not detected.

Development of new methods and means of specific prophylaxis and/or therapy of bacterial infections in farm animals is actively carried out in Russia and abroad [31]. Maintaining effective symbiosis between the poultry organism and the intestinal microbiota is a necessary component of a successful feed strategy and the livestock safety and wellness. Highly effective probiotic preparations are being developed to control causative agents of escherichiosis, salmonellosis, campylobacteriosis and other intestinal infections in poultry. Evidence is provided that bacteriocins can replace antibiotics in animal husbandry.

The search for microbial producers of bacteriocins that can be used as probiotics, is constantly conducted by researchers. Torshizi et al. [32] screened lactobacteria isolated from chicken intestines and found two isolates (*Lactobacillus fermentum* and *Lactobacillus rhamnosus*) capable of inhibiting *Escherichia* growth in vitro. Ogunbanwo et al. [33] studied the potential therapeutic efficacy of the bacteriocinogenic strain *Lactobacillus plantarum* in experimental *E. coli* infection of broiler chickens. The strain *E. coli* S5/98 producing microcin B 5/98 with a wide range of antagonistic activity against *Escherichia*, *Salmonella*, *Klebsiella*

bacteria, which was isolated from feces of adult pigs, is already used to produce liquid and dry form of Microcycol probiotic [34]. Tests on broiler chickens showed that Microcycol can effectively control the intestinal microbiota balance, increase nonspecific resistance, preservation and productivity of poultry, and also improve meat quality [35]. Colicins, a class of bacteriocins produced by *E. coli* and acting against closely related taxa, were studied as a possible alternative to antibiotics. Colicin E1 inhibited growth of enteropathogenic *E. coli* strains (ETEC) [36], and, when added to feed, reduced the frequency and the severity of experimental diarrhea caused by ETEC [37]. These results show that the use of bacteria producing bacteriocins or pure bacteriocins in animal rearing can positively affect the safety of livestock and poultry products which are the main source of diarrhea strains of *E. coli* causing escherichiosis and toxicoinfections in humans.

It is believed that biological products for medical and veterinary use based on recombinant microorganisms with proven safety can be targeted and effectively used for the treatment and prevention of various diseases [16]. To date, the use of combined probiotic preparations combining several cultures with different properties or artificially constructed strains with multiple production of bacteriocins is approved. Members of the natural gastrointestinal microbiota, such as lactobacteria (*Lactobacillus* and *Bifidobacterium*) and *Escherichia*, e.g. *E. coli* Nissle 1917 are the source for developing novel probiotics [38]. Being commensal microorganisms, they usually have a therapeutic effect on their own and are excellent for use in engineering synthetic biology [39, 40]. pColap and pPAL3 vectors, *E. coli* M17 and natural producer *E. coli* S5/98 were used to create recombinant *E. coli* M17 strains pPAL4 which produces microcin B and colicin E1 and pPAL5, synthesizing microcin but resistant to colicin E1 [35]. Romacol, a probiotic based on a genetically engineered strain *E. coli* M17 (p74), which produces C51 microcin, has been proposed [21].

E. coli ŽP is promising as the basis for a probiotic preparation due to the possibility to affect enterobacteria which are resistant to antibiotics and bacteriocins and circulate in poultry and livestock farms. Incorporation of *E. coli* ŽP into gut microbiota will increase the total level of commensal *Escherichia* in the intestines of animals, while inhibiting pathogenic representatives of this species without a noticeable effect on lactobacteria and bifidobacteria. The effectiveness of the strain is determined by an alternative system of colicin delivery, which ensures its high competitiveness in various ecological niches where bacteriocin producers can also be present.

Thus, in co-cultures, the interaction between *Escherichia coli* ŽP and APEC (avian pathogenic *E. coli*) strains is determined not only by the growth rate and sensitivity to bacteriocins, but also by conjugative plasmid transfer. The combination of these factors will determine the dynamics of competitiveness of a new probiotic strain *E. coli* ŽP and heterogeneous APEC in gastrointestinal tract of birds. Our experiments confirmed that *E. coli* ŽP strain is able to effectively colonize the intestines of rats and Manchurian quail and persist for a long time. Conjugative transfer of the plasmid from the control donor occurs in intestinal tract with a rather high frequency, while in the group with the killer donor transconjugants are absent. The reduced number of recipient cells in the second group also proves the effectiveness of the studied strain against pathogenic forms of *E. coli*. The use of conjugative mechanism in creating probiotic agent for poultry farming is in line with global trends. More research allows us to fully reveal the biotechnological potential of the genetically modified strain *E. coli* ŽP, including its effect on zootechnical indicators of birds. In particular, it seems important to study its effectiveness in treatment and

prevention of escherichiosis in animals and to evaluate the therapeutic and anti-epidemic potential.

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PECULIARITIES OF DIAGNOSTICS AND PATHOMORPHOLOGY OF EIMERIIDOSES IN THE MINK FARMS OF THE NORTHWESTERN REGION OF THE RUSSIAN FEDERATION

Yu.E. KUZNETSOV[1], L.M. BELOVA[1], N.A. GAVRILOVA[1], M.E. MKRTCHYAN[1], K.V. SIDORENKO[1], A.B. MUROMTSEV[2]

[1]*Saint-Petersburg State Veterinary Academy*, 5, ul. Chernigovskaya, St. Petersburg, 196084 Russia, e-mail Fish2017@yandex.ru (✉ corresponding author), larissabelova2010@yandex.ru, nadezhda.gavrilova65@mail.ru, laulilitik@yandex.ru, capricorn26.12.94@yandex.ru;
[2]*Kaliningrad State Technical University,* 1, Sovetskii prosp., Kaliningrad Province, Kaliningrad, 236022 Russia, e-mail muromtsev.a@mail.ru
ORCID:
Kuznetsov Yu.E. orcid.org/0000-0001-9095-7049
Belova L.M. orcid.org/0000-0003-4473-1940
Mkrtchyan M.E. orcid.org/0000-0002-0615-5703
Gavrilova N.A. orcid.org/0000-0001-5651-5976
Sidorenko K.V. orcid.org/0000-0001-8987-9426
Muromtsev A.B. orcid.org/0000-0002-2456-7023
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Abstract

Parasitic diseases are widespread in fur-bearing animals, especially in minks. Coccidioses occupy a special place among invasive diseases, as they often occur without any symptoms and in some cases are not timely diagnosed. Despite the mild clinical manifestation of invasion, it causes serious damage to animal health and significant economic damage to fur-bearing animal farms. The pathogenic effect of eimeriids on the body of fur-bearing animals consists of mechanical, toxic and inoculative effects. As a result, accumulations of mucus are found in the intestinal contents, sometimes with bloody patches. Subacute catarrhal hemorrhagic enteritis occurs, which is manifested by areas of hyperemia and edema of the mucous membrane of the small intestine, desquamation of the epithelium and is accompanied by a violation of the structure of the villi. In the presented work, in the fur bearing animal farms of the Northwestern region of the Russian Federation, the parasitic fauna, prevalence rates (PR) and invasion intensity (II) of minks were studied for the first time, the species composition of eimeriids was clarified by the molecular-genetic method, the clinical and biochemical composition of blood, as well as pathomorphological changes in intestines in animals with eimeriidosis were studied. *Isospora eversmanni* was discovered in the Kaliningrad region for the first time and we managed to discover ill mink puppies from 13 days of age. Even with low II in adult minks, pathomorphological changes in the small intestine were observed. With high II, all layers of the intestinal mucosa were affected and marked diffuse, subacute lymphoplasmacytic enteritis was noted. In ill mink, changes in the composition of the blood were revealed. The objective of our work was to develop an integrated approach to the diagnosis of mink eimeriidosis, including the study of the species composition of parasitic protozoa, the assessment of PR and II, the determination of the clinical and biochemical blood parameters of healthy and eimeriids infected animals, and the establishment of pathomorphological changes typical for eimeriosis and isosporosis that occur in chronic and asymptomatic form. In total, from 2013 to 2019, 6118 minks (*Mustela vison*, *M. lutreola* Linnaeus, 1761, *Neovison vison* Schreber, 1777) were studied in six fur-bearing animal farms of the North-Western region of the Russian Federation using the coprological method. Of these, 294 minks were studied intravitally (clinical study of animals, morphological and biochemical blood tests) and postmortem (autopsia of minks after euthanasia, histological and immunohistochemical tests). At the same time, parasitic fauna was studied in six animal farms of the North-West region of the Russian Federation, 2687 of the examined minks were infected, the prevalence rates (PR) were 43.92 %. It was found that two species of eimeria parasitize in minks, *Eimeria vison* and *E. furonis* and two isospores, *Isospora laidlawi* and *I. eversmanni*. The latter species was discovered by us in the Kaliningrad region of the Russian Federation for the

first time. A deep sequencing of the V4 region of the 18S rDNA gene and bioinformatics analysis were performed, which made it possible to determine OTUs (operational taxonomic units) and establish coccidia's taxonomic affiliation. Thus we were able to confirm the results of light microscopy and determine the taxonomic affiliation of the isolated oocysts. As a result of the analysis, it was found that the sequence of the *E. vison* DNA fragment of 383 bp is most similar (99.48 %) to the sequence of another species (*E. ictide*) found in the GenBank. Data on high morphological and genetic similarities raise the question of the taxonomic affiliation of these two species and require additional detailed study. Most often, eimeriidoses of minks proceeded in the form of mono infections (37.20 %), mixed infections with two parasites were 6.15 %, mixed infections with three protozoans made 0.57 % of cases. The peak of PR in young and adult minks occurred in the summer in the Northwestern region of the Russian Federation. In animals aged 1.5-6 months, eimeriosis and isosporosis proceeded mainly in acute and subacute forms, in minks older than 6 months — in subacute, chronic and latent. The content of hemoglobin and red blood cells in the blood of ill mink with eimeriidosis was significantly lower than in healthy minks, while the number of leukocytes, on the contrary, increased. Eosinophilia, segmented neutrophilia were also observed in ill animals, the number of basophils increased by 2 times, the content of stab neutrophils increased by 1.6 times. Proteinemia was observed in ill animals, the total bilirubin and creatinine content increased by 33.83 and 31.90 %, respectively, and the amount of urea decreased by 21.19 %. A histological examination of material from various parts of the intestine from animals infected with eimeriids revealed that at a low intensity of invasion (II) (in adult minks), although the disease was not clinically manifested in this group of animals, nevertheless, pathological changes in the histological level have already been recorded in small areas and were noted mainly only in the epithelial plate of the intestinal mucosa. With high II, damage to all layers of the intestinal mucosa was observed. Pronounced diffuse, subacute lymphoplasmacytic enteritis was discovered. The pathological processes caused by the parasitism of eimeriid in minks are often similar to those for various infectious diseases, such as the carnivorous plague virus, Aleutian mink disease and coronavirus born disease. To exclude the possibility of diagnostic errors, the material was sent to the laboratory for immunohistochemical studies (IHC), as a result of which antigens of the carnivorous plague virus, coronavirus and Aleutian mink disease were not detected. Nucleic acids of viruses were not detected in all studied samples; the result of IHC was negative in all samples.

Keywords: mink, eimeria, isospora, protozoa, pathogenesis, pathomorphology, histology, immunohistochemistry

Diseases caused by coccidian parasites are among the main reasons for growth slowdown and death of young minks, and deterioration of fur quality in adults [1-4]. *Eimeria* and *Isospora* parasites invade the epithelial cells of the intestinal mucosa and cause catarrhal hemorrhagic enteritis with hyperemia and edema of the small intestine mucosa, desquamation of the epithelium, and impaired villi structure [5]. Because of the pathogenic effect of the parasite, the intestinal mucosa is covered with viscous transparent mucus, thickens, and areas with point hemorrhages appear on it [6, 7]. Infestations may be chronic and asymptomatic, which complicates their timely diagnosis.

Coccidia significantly affect the morphobiological and immunological properties of mucus, which is important for the microbiota of vertebrates [8, 9]. Intestinal protozoa stimulate increased mucus production through the immune response of type 2 T-helpers (Th2), in which interleukins (IL)-13 and (IL)-22 (the cytokines involved in the regulation of inflammatory bowel reactions) control proliferation and goblet cell hyperplasia [10] during the immune response of a host organism trying to get rid of the parasite [6, 7].

Structural and chemical changes in mucin (glycoprotein, which forms the basis of mucus) especially often occur in case of simultaneous invasion of several parasitic protozoa species [6, 11]. Similar processes can occur in carnivores during infectious diseases [4]. Pathomorphological changes in the intestines of minks are mainly described in acute eimeridosis, while the chronic form of pathology is less studied. [12-15].

In the presented work, in the fur farms of the Northwestern region of the Russian Federation, the parasitic fauna, invasion extensity (IE) and invasion intensity (II) in minks were investigated for the first time, the species of eimeriids was specified by molecular methods, the blood clinical and biochemical analyses

were conducted, and pathomorphological changes in the intestine in animals with eimeriidosis were described. For the first time, *Isospora eversmanni* was discovered in the Kaliningrad region, and sick mink puppies were identified from 13 days of age. Pathomorphological changes in the small intestine in adult minks were observed even under low II value. With high II value, all layers of the intestinal mucosa were affected, and marked diffuse subacute lymphoplasmacytic enteritis was noted. Sick minks had changes in blood parameters.

The goal of our work was to suggest an integrated approach to the diagnosis of mink eimeriidosis, including detection of parasitic protozoa species, the assessment of the extensivity and intensity of invasion, the determination of the clinical and biochemical blood parameters of healthy and sick animals, and the pathomorphological changes upon chronic and asymptomatic eimeriosis and isosporosis.

Material and methods. From 2013 to 2019, 6118 invaded by eimeriids and intact minks (*Mustela vison*, *M. lutreola* Linnaeus, 1761, *Neovison vison* Schreber, 1777) were examined in six fur farms of the North-West region of the Russian Federation (Leningrad and Kaliningrad regions). In addition to intravital methods used (clinical observation, blood morphological and biochemical tests, coprological flotation method), pathoanatomic, histological and immunohistochemical studies were performed after euthanasia [16, 17].

For coprological examination, 10-20 g of feces per animal were hermetically packed in plastic bags and delivered to the laboratory at +4 °C. The Darling method using a universal flotation diagnostic fluid was applied [18]. The specimens were viewed with a Mikroton-200M light microscope (Petrolaser LLC, Russia) and a Primo Star microscope (Carl Zeiss, Germany) at 10×10, 10×20 and 10×40 magnification using the OMOM LOMO Micrometer nozzle (JSC LOMO, Russia). Image registration was carried out with a microscope camera and a smartphone camera Mi MIX 2 (Xiaomi, China). Invasion intensity (II) was assessed by counting eimeriid oocysts in 1 g of feces (a VIGIS counting chamber, VIGIS, USSR).

For DNA extraction, oocysts (10 specimens per sample) after a morphometry were frozen 3 times in liquid nitrogen (–196 °C) to destroy the walls and release sporocysts. Extraction and purification of genomic DNA from sporocysts was done as described [19, 20]. The DNA concentration in samples was measured (an SS2107 spectrophotometer, MEDIORA OY, Finland), and the obtained DNA preparations were stored at +4 or –20 °C.

Genotyping of each sample was carried out for two loci, the nuclear 18S rDNA (SSUrDNA) and subunit I of mitochondrial cytochrome oxidase (mt COI). Sequences of nu 18SSUrDNA and mtDNA Cytochrome Oxidase Subunit I (mt COI) were amplified by polymerase chain reaction (PCR) with the following primers: CYC1FE — 5′-TACCCAATGAAAACAGTTT-3′, CYC4RB — 5′-CGTCTTCAAACCCCCTACTG-3′ [21], Cocci 18S 595F 5′-CCGCGGTAATTCCAGCTCCAAT-3′, Cocci 18S 847R 5′-GCTGMAGTATTCAGGGCGACAA-3′, Lank 18S 224F 5′-TCATAGTAACCGAACGGATC-3′ [22], Api SSU 2733R 5′-CGGAATTAACCAGACAAATC-3′ [21-23]. Real-time PCR amplification was performed for all samples on Veriti® Thermal Cycler (Life Technologies, Inc., USA) in a 25 μl reaction mixture containing ~ 100 ng of genomic DNA, 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM deoxyribonucleotide triphosphates (dNTP), 400 nM of each primer and 1 unit of Invitrogen Platinum TaqDNA polymerase (Thermo Fisher Scientific, Canada). For sequencing, PCR fragments were obtained using a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, Singapore). Amplification mode: 3 min at 95 °C; 30 s at 94 °C, 30 s at 56-62 °C, 30-

75 s at 72 °C (35 cycles); 7 min at 72 °C (final elongation). PCR amplification products were separated electrophoretically in a 2% agarose gel, stained with ethidium bromide, visualized (a WUV-M10 ultraviolet transilluminator, DAIHAN Scientific, South Korea) and separated in agarose gel with fluorescence detection. DNA fragments were analyzed using a CEQ 8000 automatic sequencer (Beckman Coulter, USA) according to the manufacturer's recommendations. The error of the CEQ 8000 was no more than 5%. DNA was also extracted from 10 formalin-fixed and paraffin-embedded tissue samples (5-6 μm) (FFPE) using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) to detect genomic material of the carnivore plague virus, coronavirus and Aleutian mink disease virus by PCR according to the prescribed research protocol (TLVet Path International Consultants — Animal Eye Consultants of Iowa, USA).

Geneious database software (https://www.geneious.com/) was used for bioinformatics analysis, for taxonomic annotation of eimeriids, the BLAST search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and nucleotide sequences published in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) were used.

The blood total protein, glucose, total bilirubin, urea, uric acid, creatinine, cholesterol, triglycerides, the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), acid phosphatase (AcP), α-amylase, creatine kinases (CK), lactate dehydrogenases (LDH) were quantified using a Sapphire-400 biochemistry analyzer (Tokyo Boeki Medisys Inc., Japan).

Died (n = 6) and forcedly euthanized (n = 20) animals were investigated by the Scriabin's method of incomplete helminthological autopsy. Mucosal scrapings of the affected areas of gastrointestinal tract membrane were collected, and the smears were prepared and stained according to Romanovsky-Giemsa method. A total of 20 intestines of minks spontaneously infected by eimeriids were examined. Samples from the central part of pathological foci (no more than 1 cm^3) and the area bordering the unaffected tissues were collected no later than 1 h after the death of animals.

Specimens were fixed for 7 days in a 10% buffer formalin solution (pH 6.8-7.0), the amount of which was 10-20 times the volume of the test sample. After washing in running water, 3-4 mm thick fragments cut out from fixed samples through the entire thickness of the tissue were dehydrated in alcohols of increasing concentration (50, 75, 90, 100, and 100%) and paraffin-embedded. An automatic carousel-type machine for histological processing STP-120 (MICROM International GmbH, Germany) was used for dehydration, a AP 280 station (MICROM International GmbH, Germany) was used for paraffin embedding. Slices 5-7 μm thick were made on a rotational microtome HM 320 E (MICROM International GmbH, Germany) with STS section transfer system (MICROM International GmbH, Germany). The slices were transferred to glass microscope slides and allowed to dry overnight. The deparaffining and staining with hematoxylin and eosin were carried out (an HMS 70 linear tissue staining machine, MICROM International GmbH, Germany).

To exclude infectious diseases, immunohistochemical (IHC) analysis was performed. For laboratory diagnostics, the REVEAL Biotin-Free Polyvalent DAB antigen (AG) detection system (Spring Bio Science, USA) was used as per the manufacturer's recommendations. IHC staining was carried out manually; to avoid unwanted evaporation of the liquid and to prevent slides from drying out, we used a special stand with a lid. The glass slides on the stand were covered with a Hydrogen Peroxide Block solution (Cell Marque Corporation, USA) to block endogenous peroxidase and allowed for 10 min at 18-25 °C, then the glasses were washed

3 times in a phosphate-buffered solution (FBI) [25]. To unmask AG, the slides were placed in cuvettes with citrate buffer (pH 6.0), which was heated in a water bath to 95 °C for at least 30-40 min, then cooled (the buffer was not poured out) and washed twice with distilled water. To block non-specific binding, a Protein Block DPB-125 solution (Spring Bio Science, USA) (pH 7.6) was applied to glass with tissue samples for 10 min at 18-25 °C. After removing Protein Block DPB-125 solution the sections were not washed. The slides were incubated with a set of primary antibodies (AT) against the carnivorous plague virus, coronavirus and Aleutian mink disease (Abcam, USA) at 18-25 °C for 25-30 min. The sections were covered with a solution of a conjugate of secondary antibodies with horseradish peroxidase and incubated for 15 min at 18-25 °C. The slides were washed 3 times with buffer solution and stained with a chromogen solution (0.020 cm^3 per 1 cm^3 DAB Substrate), which was applied to a tissue section. The slides were dark-incubated at 18-25 °C for 7-10 min, then washed 3 times with phosphate-buffered saline, stained with Mayer hematoxylin for 3-5 min at 18-25 °C. The dye was removed, and the slides were placed in distilled water for 3-5 min. Prior to coverslips were placed over the slides, tissue samples were dehydrated in 65% ethyl alcohol for 1-2 min, in 80% ethyl alcohol for 1-2 min, and 95% ethyl alcohol for 1-2 min, then placed in xylene for 1 min and air-dried for 10-15 min in a fume hood [24]. All tests were done in triplicate to detect AG of the carnivore plague virus, coronavirus and Aleutian mink disease virus. The results of IH tests were examined under a Primo Star microscope (Carl Zeiss, Germany; the magnification ×100 or ×400). IHC staining in negative control preparations ("tissue control" and "reaction control") was not allowed. The results of the IH tests were evaluated as follows: "–" — no AG of the virus was detected; "+" — single viral AG-positive focus; "++" — several viral AG-positive foci; "+++" — multiple viral AG-positive foci [24, 25].

Statistical processing was performed with Microsoft Excel 2013 and Primer of Biostatistics 4.03 for Windows. Variation statistics method was applied. A simple comparison of averages with the two-sided Student's *t*-test in Tippett's modification was used. Mean values (*M*) and standard error of mean (±SEM) were calculated for clinical and biochemical blood parameters. A p-value = 0.05 was deemed statistically significant; values were ranked by three levels of statistically significant differences: $p \leq 0.05$; $p \leq 0.01$; $p \leq 0.001$.

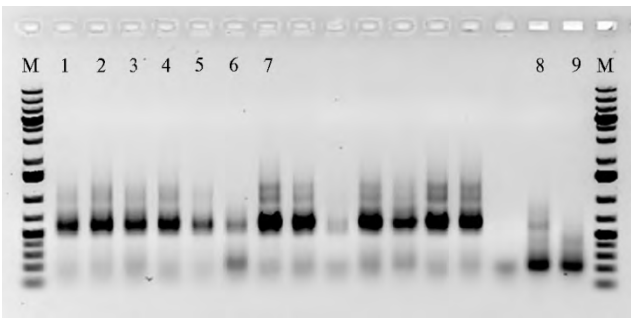

Fig. 1. An example of analysis of 18S rDNA libraries by gel electrophoresis when genotyping of eimeriids isolated form minks in fur farms of the Kaliningrad region in 2018. Samples (different DNA amounts): 1-3 — Nor1 (1, 2, 3 μl), 4, 5 — Nor5 (1, 0.5 мкл), 6, 7 — Nor5PC (1, 0.5 μl); 8, 9 — Ctrl_NegControl (in duplicates); M — molecular weight marker (GeneRuler 1 kb Plus DNA Ladder, Thermo Fisher, USA) (2 % agarose gel).

Results. In a survey of fur farms in the North-Western region of Russia we have established high rate of eimeriidosis. The causative agents were detected in 2687 animals out of 6118 tested, that is, the invasion extensity (IE) was 43.92% (Table 1).

Sequence analysis of 18S rDNA gene was performed in order to establish species affiliation of the isolated causative agents of mink eimeriidosis (Fig. 1).

The deep sequencing of the 18S rDNA gene V4 region and bioinformatics analysis made it possible to determine OTUs (operational taxonomic units) and establish their affiliation. The predominant species of parasitic protozoa were *Eimeria vison*, *E. furonis*, *Isospora laidlawi*, and *I. eversmanni*. In oocysts defined as *E. vison* a 383 bp fragment of 18S rDNA had the greatest (99.48%) similarity with the sequences of *E. ictide*. This suggests that more detailed molecular genetic studies of eimeriids are required. Moreover, high morphological and genetic similarities of *E. vison* and *E. ictidea* raise the question of possible synonymization of these two species.

The results of genotyping eimeriids by mt COI gene sequencing were similar to those for nuclear 18S rDNA.

1. Extensity of invasion (IE) **by eimeriids in minks form fur farms of the North-West region of the Russian Federation** (n = 6118, 2013-2019)

Eimeria and *Isospora* species	Number of invaded animals	IE, %
Eimeria vison	869	14.20
E. furonis	48	0.78
Eimeria invasion (in total)	917	14.99
Isospora laidlawi	1356	22.16
I. eversmanni	3	0.05
Isospora invasion (in total)	1359	22.21
Monoinvasions (in total)	2276	37.20
E. vison + *E. furonis*	34	0.56
E. vison + *I. laidlawi*	294	4.81
E. vison + *I. eversmanni*	2	0.03
E. furonis + *I. laidlawi*	34	0.56
E. furonis + *I. eversmanni*	1	0.02
I. laidlawi + *I. eversmanni*	11	0.18
Invasion with two parasites (in total)	376	6.15
E. vison + *I. laidlawi* + *I. eversmanni*	4	0.07
E. vison + *E. furonis* + *I. laidlawi*	31	0.51
Invasion with three parasites (in total)	35	0.57
Total	2687	43.92

In all farms, monoinvasions by eimeriids prevailed (37.2%), the rate of mixtinvasions with two parasites was 6.15%, mixtinvasions with three protozoa occurred in 0.57% of cases. The most common species in mono-invasions was *I. laidlawi* (see Table 1). IE for this parasite was 22.16%. It should be noted that rare species *I. eversmanni* were identified in one farm in the Kaliningrad region. IE for this parasite was insignificant (up to 0.05%). In this farm *I. eversmanni* was discovered for the first time and only in animals imported from the Stavropol Territory. Earlier, *I. eversmanni* was found in minks in Kazakhstan and Belarus in 1956 and 2006, respectively [26]. Among eimeria, the species *E. vison* prevailed (14.2%), while *E. furonis* was rare (0.78%). In all affected animals, IE was 50.5% for *I. laidlawi*, 32.3% for *E. vison*, and the association of these two protozoa was in third place (10.9%).

For all seasons during the observation years, with the exception of winter periods, IE in young minks was higher than in adults. E.g., in the winter 2016 in the Leningrad region the infection rate of eimeriids was 12.7% for young minks (n = 150) and 15.8% for adults (n = 120). The peak of invasion in both young and adult animals (50.7 and 43.3%, respectively) occurred in the summer. In spring and autumn, IE of young animals remained approximately the same, 38.0 and 36.0%, while in adults it was 30.8 and 24.2%. II also varied from season to season. Protozoa excretion from the body are known to occur with a certain periodicity, depending on the type of parasite, the internal conditions and external factors [2, 4, 12]. We found out that in adult animals from the Leningrad region, II value was the lower, the higher the IE. In the summer decade in the Leningrad region, II in females decreased to 2-58 oocysts, in males to 1-10 oocysts per sample. In autumn and winter, the number of oocysts per sample increased in females up to 480, in males to 240. In spring, II value decreased in females to 1-180, in males to 1-12 oocysts per sample. In young minks, the II reached up to 280 oocysts per sample.

In lab tests we managed for the first time to identify sick mink puppies, starting from 13 days of age (Leningrad region, 2016). In this group (n = 6), single eimeria oocysts were found, IE was 12%. A study of the monthly dynamics of invasion with eimeriids in puppies showed the peak to occur at 2 months of age (June-July), during this period the number of infected young animals reached 30 out of 50 examined, IE was 60%. We associate this increase in invasion in young animals with stress caused by weaning of puppies from nursing females on days 42-43 after birth. Having reached a peak, IE began to decline, reaching 56% in August and 50% in September. In winter, the number of sick animals decreased to a minimum, IE in February was only 6%, that is, 10 times less than in July. Therefore, by the age of 7-9 months, young minks infected with protozoa develop cell-mediated and humoral immunity the intensity of which depends on the type of pathogen and the physiological state of the animals. In 10- and 11-month-olds, a sharp increase in invasion was observed (on average up to 46-56% of the number of young minks examined), which we associate with a change in the of feeding, keeping conditions, grouping and assignment of females to males. However, as noted above, in the autumn-winter period, the II value increases in both young animals and adults.

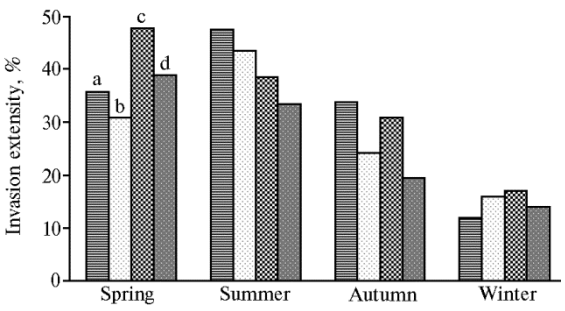

Fig. 2. Seasonal dynamics of eimeriid invasion of young (a, c) **and adult** (b, d) **minks in fur farms of the Leningrad** (a, b, 2016) **and Kaliningrad** (c, d, 2018**) regions.**

As per the publications [4, 26], young animals are more susceptible to eimeriidosis, so we compared the dynamics of the the disease that we identified in the Leningrad region with that in the Kaliningrad region, where the mink rutting occurs earlier.

The invasion peak in young minks and adults in the fur farms of the Kaliningrad region (2018) was in spring (May). IE value during this period was 47.7% in puppies, and 38.9% in animals of the main stock (Fig. 2). In the summer months, IE decreased in both groups to 38.5 and 33.3%, respectively. There was a relationship between animal IE and water supply. In farms with auto drinkers and unlimited access to water, IE was higher than in a farm where animals were supplied with water manually. We admit possible coincidence, however, these facts should be further examined. In autumn and winter, IE in animals from the Kaliningrad region decreased, as in the Leningrad region. However, in winter, in the Leningrad region the IE of minks was higher among adults (15.8%) than that of young animals (12.7%), while in the Kaliningrad region IE of young animals (16.9%) turned out to be higher than in adults (13.9%). A similar trend we observed throughout the year (see Fig. 2).

In both regions, a predominantly acute and subacute of eimeriosis and isosporosis was characteristic of minks from 1.5 to 6 months of age, in animals older than 6 months, a subacute, chronic and latent forms occurred. Animals with chronic form had a decreased activity and appetite, dull fur and diarrhea with an admixture of blood and mucus. The urge to defecate in sick minks was 2-4 times more often than in healthy minks. The body temperature in sick and healthy animals averaged 37.7±0.24 and 38.6±0.15 °C, respectively. Muscle tremor and photophobia were observed in eight sick individuals.

2. Blood parameters of healthy minks and minks infected with eimeriids (fur farms of the North-West region of the Russian Federation, 2017)

Indicator	Healthy minks (n = 12)		Sick minks (n = 40)		Test	
	M±SEM	Cv, %	M±SEM	Cv, %	$t_{cr.}$	p-value
Hemoglobin, g/l	170.00±5.50	10.7	147.00±4.80	20.7	3.151	0.0028
Red blood cells, $\times 10^{12}$/l	8.90±0.60	22.4	6.40±0.40	39.5	3.467	0.0011
Platelets, $\times 10^{9}$/l	447.80±15.10	11.2	417.00±19.60	29.7	1.245	0.219
White blood cells, $\times 10^{9}$/l	5.40±0.40	24.6	7.80±0.30	24.3	4.800	1.47×10^{-5}
Basophils, %	0.30±0.42	464.3	0.60±0.36	379.5	0.542	0.5900
Eosinophils, %	1.80±0.28	51.6	7.50±0.42	35.4	11.292	2.30×10^{-15}
Young neutrophils, %	0±0		0.36±0.40	702.7	0.900	0.3724
Band neutrophils, %	4.61±1.00	71.9	7.36±1.20	103.1	1.761	0.084
Segmented neutrophils, %	48.20±4.50	31.0	63.80±2.80	27.8	2.943	0.005
Lymphocytes, ×1012/l	42.99±3.90	30.1	17.44±4.60	166.8	4.237	9.73×10^{-5}
Monocytes, %	2.1±0.16	25.3	2.94±0.90	193.6	0.919	0.363
MID cells, $\times 10^{9}$/l	3.20±0.60	62.2	3.40±0.20	37.2	0.316	0.753
Total protein, g/l	74.46±3.42	15.2	64.70±2.14	20.9	2.419	0.019
Total bilirubin, гmol/l	7.08±0.32	15.0	10.70±0.90	53.2	3.790	0.0004
Glucose, mmol/l	9.10±0.51	18.6	5.63±0.84	94.4	3.531	0.001
Urea, mmol/l	6.18±1.13	60.6	4.87±0.36	46.8	1.105	0.275
Creatinine, μmol/l	47.80±1.87	13.0	70.20±2.41	21.7	7.343	1.74×10^{-9}
Uric Acid, μmol/l	48.10±2.54	17.5	54.60±1.62	18.8	2.158	0.036
Total lipids, mmol/l	6.71±0.06	3.0	7.14±0.13	11.5	3.003	0.0042
Cholesterol, mmol/l	6.53±0.58	29.5	8.70±1.40	101.8	1.432	0.1584
Triglycerides, mmol/l	1.01±0.05	16.4	1.34±0.07	33.0	3.836	0.0004

N o t e. The Student's t-test was used to compare the independent samples and assess the statistical significance of differences between the average values for each indicator. For groups, df = 50. At a significance level of p = 0.05, the value of Student's t-test $t_{cr.}$ = 2.009, with $t_{fact.} < t_{cr.}$ the difference in means is not statistically significant. The calculated p-value allow us to assess the differences in indicators in sick minks from those in healthy minks.

An analysis of hematological and biochemical parameters in clinically healthy and sick minks showed that the coefficient of variation for most values did not exceed 33% (Table 2), therefore, the analyzed set is homogeneous, and the data have small dispersion, which indicates a slight deviation of the observed values from the average values. The cellular components of blood in clinically healthy and sick minks differed (see Table 2). The hemoglobin content in sick animals was lower than in healthy ones, 147±4.8 vs. 170± 5.5 g/l, respectively ($p = 0.0028 < 0.05$), while the number of red blood cells was $(6.4\pm0.4)\times10^{12}$/l and (8.9 $0.6)\times10^{12}$/l ($p = 0.0011 < 0.05$). The counts of leukocytes were significantly greater in infected minks compared to healthy minks, $(7.8\pm0.3)\times10^{9}$/l vs. (5.4±0.4) Ч10^{9}/l ($p = 1.47\times10^{-5} < 0.05$). Sick animals showed a slight decrease in platelet count.

The leukogram (see Table 2) showed that most blood parameters in both healthy and sick animals remained within the reference values, with some exceptions. In infected minks, eosinophilia occurred, and the number of basophils was 2 times more. Young neutrophils appeared in the blood of infected minks, the number of lymphocytes decreased sharply, segmented neutrophilia was observed ($p = 0.005 < 0.05$), but this indicator remained within the reference values. The counts of stab neutrophils also increased 1.6 times.

Proteinemia was observed in sick animals. The total protein level was 13.1% less than in healthy ones ($p = 0.019 < 0.05$). Total bilirubin and creatinine in infected minks increased by 33.83 and 31.9% ($p = 0.0004 < 0.05$ and $p = 1.74\times10^{9} < 0.05$, respectively), the urea concentration was 21.19% lower ($p = 0.036 < 0.05$).

Fat metabolism indicators (total lipids, cholesterol, triglycerides) which should be in focus upon invasion with eimeriids since the liver and intestinal mucosa are involved in the biosynthesis of these components were 6.0, 25.0, and 24.6% higher in sick minks than in healthy minks (see Table 2).

Postmortem pathomorphological examination of died and euthanized sick minks revealed a small amount of light-yellow liquid in the abdominal cavity.

Hemorrhagic inflammation was observed along the entire length of intestines. In duodenum, jejunum, cecum there were mucosal folds with spot and band-shaped hemorrhages (Fig. 3). Within the lumen of the small intestine there were gas and contents with streaks of blood and mucus. Meronts and merozoites of eimeriids were found in Romanovsky/Giemsa-stained smears of scrapings from the intestinal mucosa.

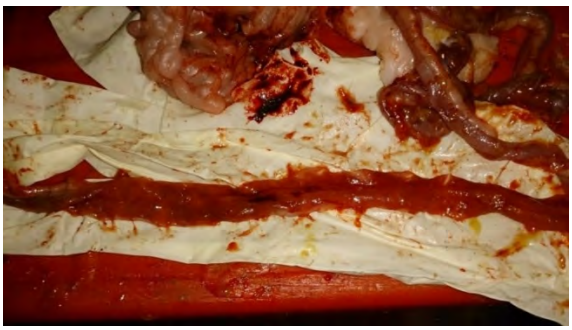

Fig. 3. Hemorrhagic inflammation of small intestine in a mink infected with eimeriids (a fur farm in the Leningrad region, 2016).

Despite the absence or weakly expressed clinical sings of eimeriidosis due to the low intensity of invasion in adults, the histological examination of the intestinal wall epithelium of sick minks revealed pathological processes. With a low degree of infection, the main pathomorphological changes were observed only in the epithelial plate of the intestinal mucosa. With high II, all the layers of the mucous membrane were involved in pathological process manifested by diffuse enteritis with a pronounced lymphoplasmacytic profile. Over the entire wall of the intestine, the vessels were moderately filled with blood, however, a well-defined vasculature were found in the small intestine (Fig. 4). In the own plate of the mucous membrane a polymorphic cell infiltration was revealed, which reached the muscle plate of the intestinal mucosa. At the same time, small-focal concentration of cell groups in the submucosa of the intestinal wall was noted.

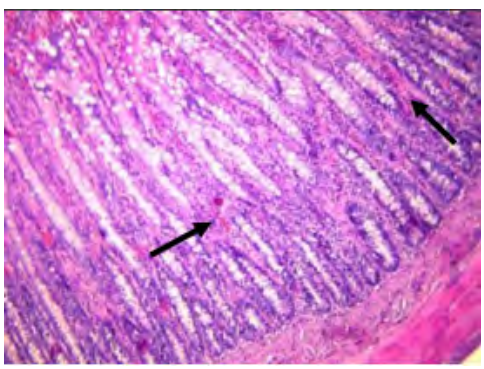

Fig. 4. The small intestine of a mink infected with eimeriids (see cell infiltration and vessels moderately filled with blood). The arrows indicate the areas of pronounced blood-filling of vessels in the own plate of the mucous membrane of the small intestine wall (staining with hematoxylin and eosin; microscope Mikroton-200M, LLC Petrolazer, Russia; magnification ×400) (a fur farm in the Kaliningrad region, 2018).

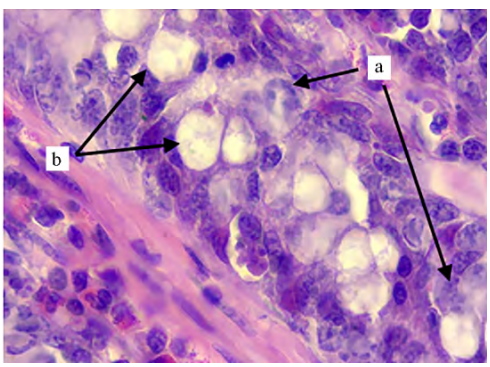

Fig. 5. Mitotic activity of enterocytes (a large number of cells in the late prophase of mitosis) (a) **and proliferation of goblet cells** (b) **(increase in their number and size) in the epithelial plate of small intestine mucous membrane in a mink infected with eimeriids** (staining with hematoxylin and eosin; microscope Mikroton-200M, LLC Petrolazer, Russia; magnification ×400) (a fur farm in the Kaliningrad region, 2018).

In the epithelial plate throughout the intestine with varying degrees of severity, the mitotic activity of enterocytes and the proliferation of goblet cells increased (Fig. 5). In part of the epithelial cells, necrotic processes took place, which was confirmed by the presence of picnotic, karyorectic and karyolytic nuclei. In the epithelium of the small intestine, there were uncharacteristic oval,

sometimes roundish formations with a diameter of 12-25 μm. They contain eimeria merozoites, which were stained with the alkaline dyes (hematoxylin) in blue-violet color. Using ultrastructural studies, multiple endozoites were found inside the meronts (Fig. 6).

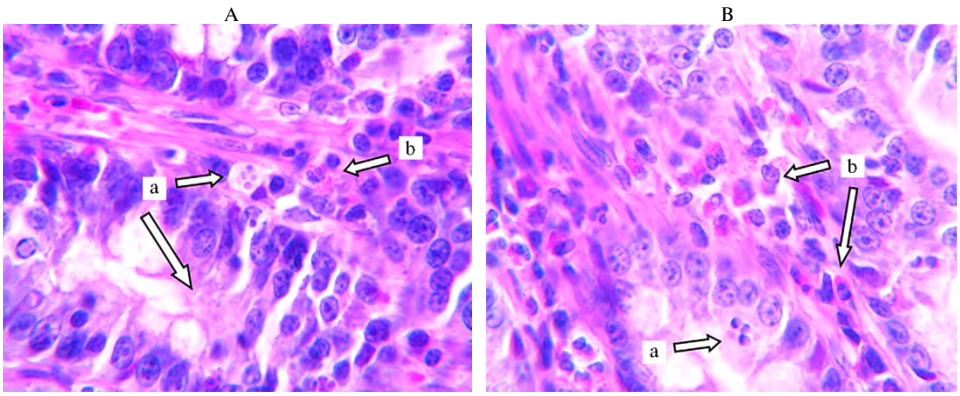

Рис. 6. Parasitophorous vacuole of coccidia merohogony (A) **and eosinophil** congestion **in the lesion caused by coccidia** (B) **in the small intestine of a mink infected with eimeriids:** a — meronts containing eimeria endozoites, b — eosinophils (staining with hematoxylin and eosin, microscope Primo Star, Carl Zeiss, Germany; magnification ×400) (a fur farm in the Kaliningrad region, 2018).

In the mucous membrane of the small intestine, necrotic changes in the cells and desquamation of the single-layer limbic epithelium were revealed, which spread into the crypt mucosal plate. A large number of cells of desquamated epithelium were found in the lumen of the intestine. The development of multicellular meronts (Fig. 7) and the further release of merozoites led to the destruction of surface epithelial cells and atrophy of the small intestine villi. Small sections of the small intestine were found in which the epithelial plate was completely absent. In the own plate, foci of infiltration by lymphocytes, plasmacytes, neutrophils, and eosinophils were present (Fig. 8). Particularly large aggregates of eosinophils surrounded the foci of localization of the endogenous stages of the eimeria.

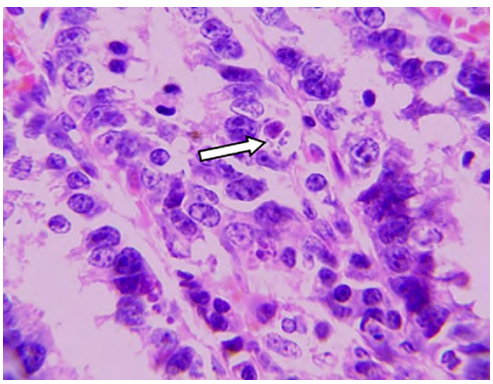

Fig. 7. Meront of coccidia containing coccidia endozoytes (marked by an arrow) **in the destroyed villi of the small intestine of a mink infected with eimeriids** (staining with hematoxylin and eosin; microscope Mikroton-200M, LLC Petrolazer, Russia; magnification ×400) (a fur farm in the Kaliningrad region, 2018).

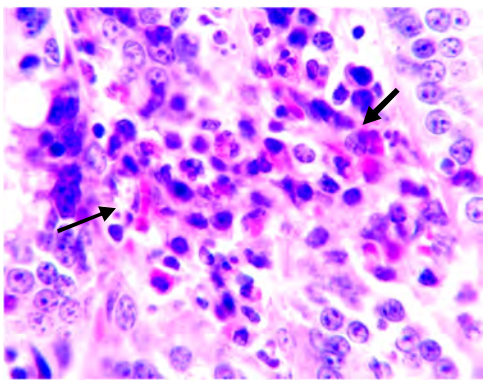

Fig. 8. Eosinophilia in the mucous membrane of the small intestine of a mink infected with eimeriids. The arrows indicate the eosinophil aggregates in the own plate of the intestinal mucosa (staining with hematoxylin and eosin; microscope Primo Star, Carl Zeiss, Germany; magnification ×400) (a fur farm in the Kaliningrad region, 2018).

The endogenous stages of coccidia in minks caused mild eosinophilic (see Fig. 6, Fig. 7) and lymphoplasmacytic enteritis, and in some cases were accompanied by necrosis of crypts. A small number of eosinophils, rare neutrophils,

plasmacytes, lymphocytes and multinucleated cells (see Fig. 8), indicating epithelial syncytium, were present in the own plate of the small intestine villi. Within the middle layer of the mucosa and intestinal crypts, individual necrotic epithelial cells were scattered. Intestinal crypts were occasionally replaced by residues of necrotic cells and a small number of degenerative neutrophils. At the same time, a moderately increased number of mitoses was observed in the remaining undamaged enterocytes of crypt cells. Goblet cell hyperplasia and severe lymphoid clusters were also observed.

Pathogens of plague of carnivores, Aleutian mink disease and coronavirus cause in minks pathomorphological processes similar to those caused by eimeriids. To exclude diagnostic errors, histological preparations of the small intestine (20 histosections) were stained with hematoxylin and eosin and immunohistochemically studied. Antigens of the plague virus, coronavirus and Aleutian mink disease virus were not detected in the studied histological preparations. Nucleic acids of these viruses were also not detected in any of the studied samples; the result of IHC in all samples turned out to be negative (–AG). Nevertheless, the eosinophilic component, together with detected parasitophorous vacuole, confirmed the presence of endoparasites (eimeriids) in the samples.

Our data on the taxonomic affiliation of eimeriids isolated from minks are consistent with the results of other researchers. In 2008-2017, *E. furonis* и *Isospora* (= *Cystoisospora*) *laydlaw* found in mink feces and during postmortem autopsies were studied in Canada [23]. The parasites were characterized using complete mitochondrial genome sequences and 18S rDNA nuclear sequences of *E. ictidea* and *E. furonis*; for comparison, *I.* (= *C.*) *laydlawi* was used [23]. DNA extraction from formalin-fixed paraffinized tissues and sequencing of PCR amplicons made it possible to identify coccidia in the studied samples with high reliability [23]. From 1990 to 2006, V.A. Gerasimchik studied the parasitic fauna of fur animals in the Republic of Belarus, and in 17.45% of animals in farms with different fur production technologies, he identified 4 species of eimeriids: two species of *E. vison* and *E. furonis* and two isospores, the *I. laidlawi* and *I. eversmanni* [26]. However, unlike our data, *E. vison* prevailed (57.03%) in all 24 examined animal farms, which may be due to different production conditions and diet. In the Republic of Karelia, according to V.S. Anikanova [1], only two eimeria species, *E. vison* and *E. furonis*, and one species of isospores, *I. laidlawi*, were identified. The latter species was more common than the rest [1], which is confirmed by findings on the fur parasitic fauna of animals in the Leningrad region, bordering Karelia. In the Republic of Kazakhstan, *E. vison* also prevailed among minks [2, 26]. Eumeria, in particular *E. vison*, were more common in the southern regions, and *I. laidlawi* remained the dominant species in the northern.

As per records in fur farms, breeding minks enter the Russian Federation mostly from Denmark, though also from other states. A number of researchers [13, 23, 27, 28] state that clinical eimeriosis is rarely found in Dutch and Danish mink farms, although coccidiidoses in association with other non-pathogenic or low pathogenic microorganisms are known to cause death of young mink [12, 23].

Analysis of previous studies [2, 3] and our data indicate that the species composition of emeriids is associated with animal age but does not depend on mink color and farm location. Gerasimchik also notes that the species composition of eimeria and isospores is independent of gender and color of minks [26].

It is known that parasitic infestations often occur in association with viral and bacterial infections, while complex relationships develop between animals and the entire complex of microparasitocenosis. In minks seropositive for Aleutian

disease (viral plasmacytosis), a wider variety of protozoan fauna and higher II have been established [26]. Perhaps this is due to a decrease in animal immunity, and the seropositivity of minks can play an important role in the investigation of their parasitic fauna, which is the opinion of many researchers [1, 13, 22].

It is known that the biochemical and physiological properties of coccidia parasitizing in different animal species are similar [1]; therefore, it seems interesting to us to compare the pathomorphology of eimeriidosis that we identified in minks with the course of coccidiosis infestations in other fur animals, in particular rabbits.

Thus, during 2013 to 2019, the eimeriids *Isospora laidlawi* with the invasion extensity of 22.16% were the most common in the surveyed fur farms of the North-West region of the Russian Federation. The species *Eimeria vison* (14.2%) prevailed among eimeria, and *E. furonis* was rare (0.78%). In one of the farms in the Kaliningrad region, *I. eversmanni* was found for the first time. Eimeriidosis of minks mostly occurred as a monoinvasions (37.2%), the rates of mixed invasions by two and three parasites were 6.15% and 0.57%, respectively. The peak of invasion extensity (IE) in the North-West region of the Russian Federation in young and adult minks occurred in the summer. Single oocysts of eimeriids in puppies were found from the 13-day age (IE = 12%). In minks aged 1.5-6 months, eimeriosis and isosporosis proceeded mainly in acute and subacute form, in animals older than 6 months the forms were subacute, chronic and latent. The blood level of hemoglobin and erythrocytes in the minks with eimeriidosis was significantly lower than in healthy minks, and the number of leukocytes, on the contrary, increased. Eosinophilia and segmented neutrophilia were also observed in sick animals, the number of basophils was 2 times higher, the counts of stab neutrophils were 1.6-fold. Proteinemia was characteristic of sick animals, the total bilirubin and creatinine levels increased by 33.83 and 31.9%, respectively, and the urea concentration decreased by 21.19%. Immunohistochemical studies did not detected antigens of the plague virus of carnivores, coronavirus and Aleutian mink disease virus. Mild eosinophilic and lymphoplasmacytic enteritis was detected, accompanied by rare crypt necrosis due to lesion caused by endogenous stages of coccidia. Coccidia infection as monoinvasion or parasite associations is accompanied by a violation of the integrity of the intestinal mucosa. At a high invasion rate, both the intrinsic and muscular plates and the submucosal base are involved in polymorphic cell infiltration. In this case, a syndrome of increased intestinal permeability arises, which is accompanied by a violation of the function of the gastrointestinal tract.

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THERAPEUTIC EFFICIENCY OF SORBENTS MODIFIED BY HYDROXIC ACIDS DURING ANIMAL EXPERIMENTAL POISONING WITH IVERMECTIN

V.I. DOROZHKIN[1], Yu.N. FEDOROV[2], L.K. GERUNOVA[3], L.G. P'YANOVA[4], T.V. GERUNOV[3], M.S. DELYAGINA[4], A.A. TARASENKO[3]

[1] *All-Russian Research Institute of Sanitary, Hygiene and Ecology,* 5, Zvenigorodskoe sh., Moscow, 123022 Russia, e-mail tox.dor@mail.ru (✉ corresponding author);
[2]*All-Russian Research and Technological Institute of Biological Industry,* 17, pos. Biokombinata, Shchelkovskii Region, Moscow Province, 141142 Russia, e-mail fun181@mail.ru;
[3]*Stolypin Omsk State Agricultural University,* 1, Institutskaya pl., Omsk, 644008 Russia, e-mail lk.gerunova@omgau.org, vsed@mail.ru, tarasenko_pharm@mail.ru;
[4]*Center of New Chemical Technologies of the Federal Research Center Boreskov Institute of Catalysis, Siberian Branch RAS,* 54, ul. Neftezavodskaya, Omsk, 644040 Russia, e-mail medugli@ihcp.ru
ORCID:
Dorozhkin V.I. orcid.org/0000-0003-1188-4449
Fedorov Yu.N. orcid.org/0000-0001-7268-3734
Gerunova L.K. orcid.org/0000-0003-0835-9352
P'yanova L.G. orcid.org/0000-0002-6207-0878
Gerunov T.V. orcid.org/0000-0002-5594-2666
Delyagina M.S. orcid.org/0000-0003-2846-8628
Tarasenko A.A. orcid.org/0000-0001-7314-9998

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Abstract

The development of modified sorbents combining the adsorption properties of the initial porous material and the biological activity of the modifiers is a current issue of sorption therapy. The purpose of the study was to develop a method for modifying carbon sorbents with hydroxyacids and to conduct a comparative assessment of their effectiveness upon poisoning animals with ivermectin. A granular carbon enterosorbent with a specific surface area of 300-400 m^2/g was the matrix for the preparation of biospecific sorbents, glycolic acid (50 wt.%, manufactured by Merk Schuchardt OHG, Germany) and lactic acid (80 wt.%, produced by MOSREACTIVE, Russia) and a copolymer thereof were used as modifiers. To study the morphology and surface topography of the obtained samples, scanning electron microscopy (JSM 6460LV, JEOL Ltd, Japan) was used. The therapeutic efficacy of the modified sorbents was measured in 5 groups (10 animals each) of outbred adult white rats weighing 180-200 g. During 5 days animals of different groups received one of the modified sorbents in doses of 0.3 g/kg upon acute intoxication with Baymec® (1 % solution of ivermectin, Bayer Animal Health GmbH, Germany; manufactured by Federal Centre for Animal Health, Russia). The controls were intact and intoxicated animals. At the end of the experiment, blood was taken from all animals for biochemical studies, as well as histological examination of organs and tissues was performed post mortem. Electron microscopic examination showed that upon modification, the size and shape of the granules of the initial sorbent are preserved, but surface topography and morphology change. The results of in vivo experiments showed varying degrees of efficiency of enterosorption in rats when modified sorbents were applied to the animals intoxicated with ivermectin-containing Baymec® preparation. Creatinine varied from 40.86±0.66 μmol/l ($p < 0.0001$) for the sorbent with lactic acid to 82.32±2.74 μmol/l ($p = 0.4698$) for the sorbent with glycolic acid; in intoxicated animals, the creatinine level reached 87.00±5.52 μmol/l, $p < 0.0001$). Intoxication was accompanied by an increase in the total protein content in blood of rats (73.08±0.96 g/l, $p = 0.0001$), mainly due to globulins. At the same time, the concentration of urea decreased (2.44±0.05 mmol/l, $p = 0.0001$) but the content of total bilirubin increased (1.80±0.07 μmol/l, $p = 0.0379$), while the level of creatinine increased 2.3 times compared to intact animals. Post-mortem analysis indicated the polytropic nature of the drug. In animals, acute congestive hyperemia of the internal organs and the brain was found. Under the epicardium, petechial hemorrhages were noticeable. There were also marked the heart dilatation, granular dystrophy of cardiomyocytes, protein-fatty degeneration of the liver, emphysematous changes in the lungs, congestive hyperemia and hemorrhages in the kidneys. In the gastrointestinal tract signs of acute catarrh were prevailing. The most distinct corrective effect of enterosorption was noted when using a sorbent

modified with lactic acid oligomer. The research results confirm the prospects for the development of bifunctional sorbents as well as expand the possibilities of sorption therapy.

Keywords: ivermectin, Baymec®, modified enterosorbents, hydroxyacids, lactic acid, glycolic acid, clinical pathology, blood biochemistry, histopathology, intoxication, sorbent therapy

Sorption therapy is a promising area of detoxification measures. A distinctive feature of biospecific sorbents is a combination of the adsorption properties of the initial porous material and the biological activity of the modifiers. Chemical modification of the surface of sorbents can increase the selectivity of sorption. Known methods of chemical modification include immobilization of enzymes, polyenzyme complexes, cell organelles [1-4], incorporation of heteroatoms (Si, N), functional groups (−NH2, −COOH, −C = O, phosphonic acid residues) or carriers of functional groups followed by treatment of the modified sorbents with various reagents and protein solutions. Currently, interest in polymer compounds is growing [5-9]. Oligo- and polymers of hydroxy acids (glycolic and lactic) can be used as modifiers of carbon sorbents. They are approved for use in medicine, since they do not have pronounced toxicity, are compatible with body tissues and biodegradable [10-14]. An important advantage of immobilizing acids on a carbon matrix is the preservation of the antimicrobial properties of immobilized compounds and the high adsorption capacity of the carbon material [15-19]. Upon exogenous poisoning, a change in the pH in the gastrointestinal tract during acid desorption is of great importance, since this significantly affects the rate of absorption and elimination of toxicants.

In this work, the feasibility of a new carbon sorbent modified with lactic acid for enterosorption upon acute poisoning with ivermectin has been proved for the first time using a rat model (outbred white rats). Our findings prove the advantages of the proposed modifier, as compared to glycolic acid and a copolymer of these hydroxy acids.

Our subjective was to develop a method for modifying the carbon sorbents with hydroxy acids and to compare their therapeutic efficacy upon ivermectin poisoning.

Materials and methods. Novel carbon sorbents were synthetized at the Institute of Hydrocarbon Processing SB RAS (Omsk) via modification of granular carbon enterosorbent (300-400 m^2/g) glycolic acid (GA, 50 wt.%, Merk Schuchardt OHG, Germany), lactic acid (MK , 80 wt.%, MOSREACTIVE, Russia), and their copolymer according to an original methodology.

The morphology and surface topography of the obtained sorbents were investigated by scanning electron microscopy (JSM 6460LV, JEOL Ltd, Japan) with vacuum coating of specimens with a gold film 10-15 nm thick (voltage 15-20 kV, current 10-30 mA to ensure contrasting). Five to ten granules of sorbents with different modifiers were examined.

Therapeutic efficacy of the modified sorbents was evaluated using 50 outbred mature white rats with a body weight of 180-200 g. Animals were analogues in age, gender and body weight. For simulation of acute poisoning with ivermectin the Baymec® preparation (Federal Center for Animal Health, Vladimir) containing 1% ivermectin was applied. Prior to the experiment, the rats were clinically supervised during a 2-week quarantine in order to exclude infectious diseases. Throughout the entire period, the animals were fed a full-grain cereal mixture according to common laboratory standards.

The rats were assigned into five groups of 10 animals each. In the control (group I), intact rats were not subjected to any manipulations. The rest animals were injected subcutaneously with Baymec® at a dose 10 times higher than the therapeutic dose. In group II, no sorbents were administered, rats of group III, IV and

V received sorbents modified with glycolic acid, lactic acid, and with a copolymer of glycolic and lactic acids, respectively. All enterosorbents were administered as bread boluses (0.3 g/kg body weight) 2 times a day for 5 days, starting from 1 day after the date of experimental poisoning.

The rats were weighed before and after the experiment. At the end of the experiment, blood was sampled from all animals for biochemical analysis, and then they were euthanized. Fragments of internal organs, mesenteric lymph nodes and the brain were collected for histological examination.

Pathological material was fixed in 4% neutral formaldehyde and Carnoy's solution, and paraffin-embedded to make sections (Rotational Microtome Labo-Cut 4055, Slee, Germany). To investigate histomorphological features, the specimens were stained with Hansen hematoxylin and eosin, and also as per Van Gieson [20]. Glycogen and neutral glycosaminoglycans (neutral GAGs) were detected by Schiff (PAS) reaction according to Shabadash method [21]. Specimens were studied using light microscopy (Altami Bio, Altami Russia, zoom Ч300, Ч600).

The principles set forth in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, March 1986) and the Declaration of Helsinki (Helsinki, June 1964) were observed.

Statistical processing was done using Statistica 6.0 software (StatSoft, Inc., USA). The data were checked using the Shapiro-Wilk normality *W*-test, after which the Student *t*-test was used for independent samples. Differences were deemed statistically significant at $p < 0.05$. The results were presented as arithmetic mean and standard error of the mean ($M \pm SEM$).

Results. The matrix for the preparation of biospecific sorbents was a granular carbon enterosorbent (CE) with a mesoporous structure, high chemical purity, biocompatibility, and specific surface area of 300-400 m^2/g. The modifiers should be safe, soluble, having reactive groups, capable of fixing on the sorbent surface and biodegradable [22]. Glycolic and lactic acids fully possess these properties [23] and, therefore, were used as surface modifiers of the carbon sorbent.

A two-step matrix modification with a glycolic acid oligomer includes i) the carbon sorbent impregnation with an aqueous solution of glycolic acid (50 wt%) for 8 hours in air at 20-25 °C (sorbent:modifier as 1:2), and ii) heat treatment in a sand bath at 105±5 °C and 195±5 °C for 1 h followed by heating at 225±5 °C for 5 h. The CE-GA modified carbon sorbent contains 12-15 wt% GA oligomer, has a specific surface area of 180-210 m^2/g, and provides a low pH of saline solution (pH 4.3-4.6) after a 1-day contact with a specimen.

Modification of the carbon sorbent with lactic acid oligomer also was a two-step procedure which includes i) impregnation with an aqueous solution of lactic acid (50 wt%) for 24 h in air at 20-25 °C (sorbent:modifier as 1:2), and ii) heat treatment in a sand bath at 130±5 °C for 2 h and at 150±5 °C for 4 h, than at 170±5 °C for 18 h in a tube furnace with a desiccant in an argon stream. The CE-LA modified carbon sorbent contains 25-30 wt% LA oligomer, has a specific surface area of 30-60 m^2/g, and provides a low pH of saline solution (2.3-2.6) after a 1-day contact with a specimen.

CE modification with GA-LA copolymer was a tree-step and includes i) the sorbent impregnation with aqueous solution of hydroxy acids (GA:LA 70:30 wt%) for 24 h at room temperature (sorbent:hydroxy acids as 1:2), ii) drying in a sand bath for 1 h at 103-107 °C, and iii) a 14-h heat treatment at 160-170 °C in a sand bath with a molded NaA-U zeolite (Ishimbay Specialized Chemical Catalyst Plant LLC, Republic of Bashkortostan) as a desiccant, followed by 7-h

heating in tube furnace with zeolite at 170-180 °C in an argon stream.

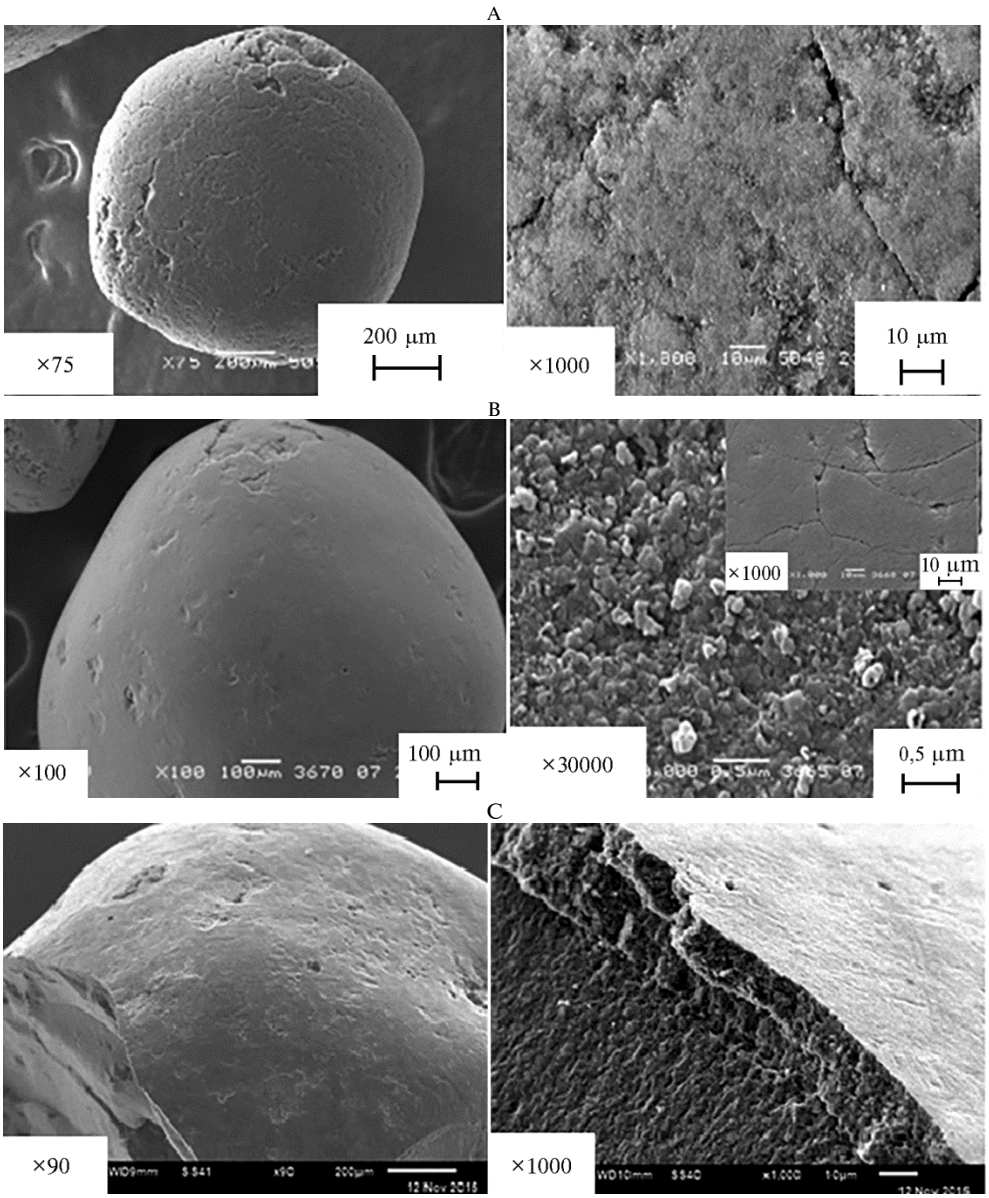

Fig. 1. Granules (left) **and chips** (right) **of sorbents:** A — carbon sorbent, B — carbon sorbent modified with glycolic acid, C — carbon sorbent sample modified with lactic acid (scanning electron microscopy with gold coating, JSM 6460LV, JEOL Ltd, Japan).

Several papers describe methods for generation of polyglycolic, polylactic acids and their copolymer by heating with compounds based on antimony, zinc, tin, etc., as catalysts [24, 25]. All these methods of the synthesis of polymers and copolymers of hydroxy acids are not safe when producing materials for medical and veterinary purposes, since they involve the use of organic solvents and heavy metal-based catalysts that are toxic to the human and animals. Our methods for producing modified sorbents does not use such chemical compounds.

Electron microscopy showed that upon modification, the size and shape of the granules of the initial sorbent are retained. However, the relief and surface morphology of the modified samples noticeably change. In contrast to the initial

sorbent (Fig. 1, A), small polymer particles of various shapes smaller than 1 μm in size are observed on the carbon surface of the samples modified with the glycolic acid oligomer (see Fig. 1, B). They are distributed locally, mainly in cracks, pores, and other surface defects. The LA modifier formed an uneven polymer film screening the carbon surface (see Fig. 1, B).

In vivo tests showed varying efficiency of modified sorbents in enterosorption upon acute intoxication of rats with the ivermectin-containing Baymek® drug. Clinical symptoms of acute intoxication developed in rats within 2 hours after poisoning simulation. Tousled fur and hypersalivation indicated worsening of the rats. Over time, the response to sound and tactile stimuli intensified. Periods of anxiety gave way to oppression, a decrease in motor activity. These signs are due to the mechanism of action of the drug. The injected ivermectin interacts with glutamate GABA-ergic receptors of the Cl^- channels and receptors of glycine-activated channels, which leads to hyperpolarization of postsynaptic membranes and impaired interneuron transmission of impulses [26-28].

A day after the start of the experiment, intoxicated animals remained sedentary, with dull hair and, slight hypersalivation and reduced appetite. Rats of test groups (III-V) receiving sorbents began to show interest in food faster and performed grooming. In 48 h after the start of the experiment and until its end, there were no significant differences in the behavior and clinical signs in animals upon enterosorption.

The behavior of intact animals (control) remained unchanged throughout the observation period. Comparing the data of control weighings did not reveal statistically significant differences at the beginning and at the end of the experiment.

The blood tests (Table) showed that rat intoxication by Baymec® led to an 11.9% ($p = 0.0001$) increase in the total blood protein in and a significant increase in the globulin index (by 17.86%, $p = 0{,}0002$). The urea concentration decreased (by 29.89%, $p = 0.0001$), while the total bilirubin level increased (by 26.76%, $p = 0.0379$), indicating the hepatotoxic effect of Baymec® [29]. However, an increase in creatinine concentration was more significant (a 2.3-fold). These changes indicate a decrease in protein catabolism and impaired renal filtration. At the same time, the activity of alanine aminotransferase in intoxicated rats decreased. The LA-enterosorbent contributed to a 7.5% ($p = 0.0014$) increase in the protein concentration compared to this indicator in the rats intoxicated without correction. The sorbents modified with hydroxy acids affected the total blood protein content in rats ambiguously.

The sorbent modified with LA-GA, on the contrary, reduced the blood protein concentration by 17.8% compared to rats intoxicated with Baymec®. Moreover, the first of the sorbents offered (with lactic acid) equally increased the concentration of albumin and globulins, the second one (with glycolic acid) predominantly decreased the globulin fractions, but did not decrease the creatinine concentration as much as the LA-sorbent. The GA-sorbent did not cause statistically significant changes in creatinine level compared to that in the intoxicated animals which were not treated.

All sorbents declined the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). However, for making clinical decisions, it is of fundamental importance that the ALT activity in intoxicated rats was lower than in intact rats, although the increase in blood alanine aminotransferase levels more often indicates hepatotoxic effects [30, 31].

Blood biochemical parameters of outbred white rats intoxicated with Baymec® when corrected with modified carbon sorbents (M±SEM, lab tests)

Total protein, g/l	Albumins, g/l	Globulins, g/l	Urea, mmol/l	TB, rmol/l	Creatinine, rmol/l	Glucose, mmol/l	AP, U/l	AST, U/l	ALT, U/l	Ca, mmol/l	P, mmol/l	Fe, rmol/l	Ca, mmol/l
Control (n = 10)													
65.3±0.36	27.58±0.69	37.72±0.86	3.48±0.13	1.42±0.13	38.22±2.03	5.60±0.28	433.22±46.07	148.88±2.49	79.64±5.23	3.08±0.03	2.82±0.11	40.56±2.88	8.64±0.37
Rats intoxicated with Baymec® without correction													
73.08±0.96	28.62±0.44	44.46±0.52	2.44±0.05	1.80±0.07	87.00±5.52	5.52±0.10	394.56±6.26	155.76±1.70	58.62±0.16	2.84±0.02	2.40±0.20	37.34±0.55	8.82±0.12
$p_к$ = 0.0001	$p_к$ = 0.2424	$p_к$ = 0.0002	$p_к$ = 0.0001	$p_к$ = 0.0379	$p_к$ < 0.0001	$p_к$ = 0.7979	$p_к$ = 0.4298	$p_к$ = 0.0745	$p_к$ = 0.0039	$p_к$ = 0.0007	$p_к$ = 0.1094	$p_к$ = 0.3046	$p_к$ = 0.6576
Rats intoxicated with Baymec®:													
upon correction with sorbent modified with glycolic acids (n = 10)													
73.32±0.94	29.42±0.47	43.90±0.88	3.74±0.10	2.04±0.22	82.32±2.74	7.58±0.37	339.88±23.01	122.84±0.83	49.30±2.52	2.94±0.05	2.28±0.10	52.86±0.86	9.98±0.42
$p_б$ = 0.8635	$p_б$ = 0.2504	$p_б$ = 0.6023	$p_б$ < 0.0001	$p_б$ = 0.3344	$p_б$ = 0.4698	$p_б$ = 0.0007	$p_б$ = 0.0510	$p_б$ < 0.0001	$p_б$ = 0.0062	$p_б$ = 0.1151	$p_б$ = 0.6143	$p_б$ < 0.0001	$p_б$ = 0.0315
upon correction with sorbent modified with lactic acid (n = 10)													
78.56±0.63	31.20±0.46	47.36±0.88	4.16±0.09	0.89±0.01	40.86±0.66	8.06±0.10	194.88±2.89	120.38±0.76	53.02±1.20	3.16±0.05	2.12±0.03	54.22±0.43	9.06±0.16
$p_б$ = 0.0014	$p_б$ = 0.0039	$p_б$ = 0.0228	$p_б$ < 0.0001	$p_б$ < 0.0001	$p_б$ < 0.0001	$p_б$ < 0.0001	$p_б$ < 0.0001	$p_б$ < 0.0001	$p_б$ = 0.0017	$p_б$ = 0.0005	$p_б$ = 0.2109	$p_б$ < 0.0001	$p_б$ = 0.2805
upon correction with sorbent modified with copolymer of glycolic and lactic acids (n = 10)													
60.10±0.38	26.48±0.43	33.62±0.32	3.76±0.12	0.81±0.01	66.82±2.81	2.80±0.23	466.80±46.51	66.02±17.41	57.60±4.73	2.76±0.04	3.08±0.23	37.68±1.13	10.62±0.46
$p_б$ < 0.0001	$p_б$ = 0.0085	$p_б$ < 0.0001	$p_б$ < 0.0001	$p_б$ < 0.0001	$p_б$ = 0.0116	$p_б$ < 0.0001	$p_б$ = 0.1624	$p_б$ = 0.0008	$p_б$ = 0.8350	$p_б$ = 0.1265	$p_б$ = 0.0599	$p_б$ = 0.7941	$p_б$ = 0.0058

Note. The t-test is applied for independent samples. $p_к$ — significance level compared to the control group, p_b — significance level compared to the group intoxicated with Baymec®; TB — total bilirubin, AP — alkaline phosphatasea, AST — aspartate aminotransferase, ALT — alanine aminotransferase.

LA-sorbent caused a maximum increase in the blood iron (by 45.2% at $p < 0.0001$) and calcium (by 11.26% at $p = 0.0005$) levels in rats but did not significantly affect the phosphorus concentration. The bilirubin concentration was the lowest while the glucose concentration was the highest.

Analysis of post-mortem changes in rats subjected to Baymec® acute intoxication indicated the polytropic nature of the drug action, noted by other authors in their study of adverse systemic effects of ivermectin [32].

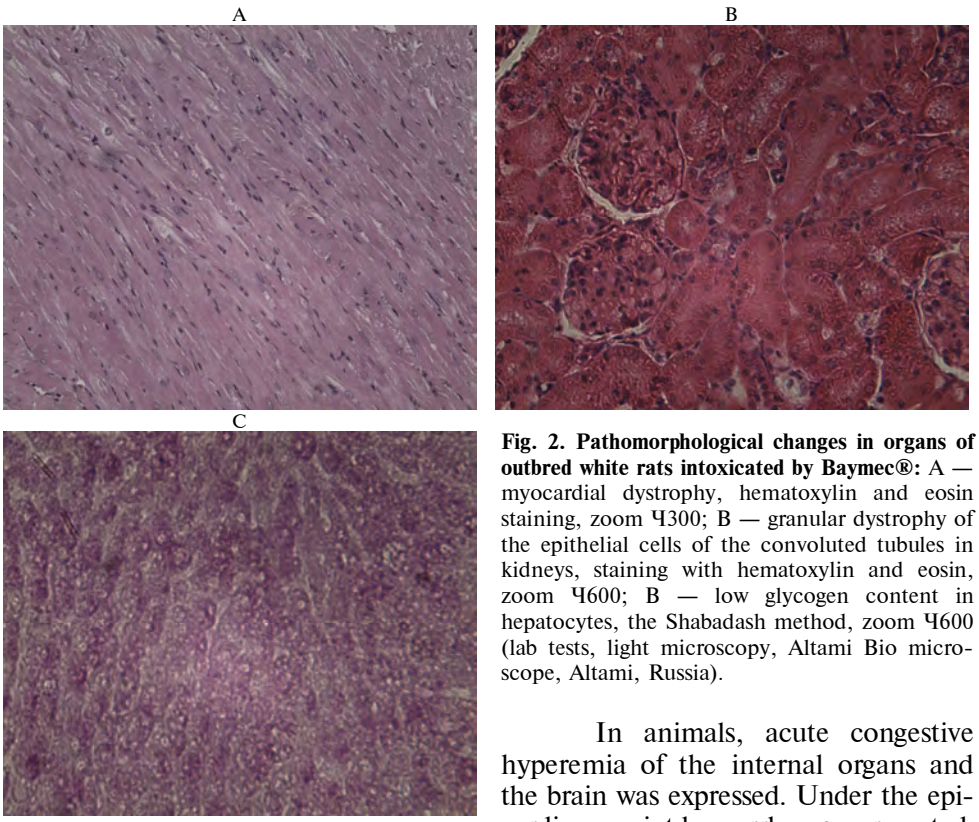

Fig. 2. Pathomorphological changes in organs of outbred white rats intoxicated by Baymec®: A — myocardial dystrophy, hematoxylin and eosin staining, zoom Ч300; B — granular dystrophy of the epithelial cells of the convoluted tubules in kidneys, staining with hematoxylin and eosin, zoom Ч600; B — low glycogen content in hepatocytes, the Shabadash method, zoom Ч600 (lab tests, light microscopy, Altami Bio microscope, Altami, Russia).

In animals, acute congestive hyperemia of the internal organs and the brain was expressed. Under the epicardium, point hemorrhages were noted. The heart was enlarged, its cavities were filled with blood. Myocardium had a grayish tint. Histological examination showed pronounced swelling of muscle fibers with a loss of transverse striation of cardiomyocytes (Fig. 2, A). Glycogen content in cardiomyocytes decreased.

The kidneys had a red-brown color with light gray patches. Their capsule was easily removable. Microscopic examination revealed hyperemia of the kidneys, and hemorrhages in the medulla. The gaps of the convoluted tubules were narrowed and contained an oxyphilic colored mass. The cytoplasm of the proximal nephron epithelial cells had a granular structure, part of the nuclei in the epithelial cells were in a state of pycnosis or lysis (see Fig. 2, B). The liver was unevenly colored. Microscopy showed a sharp decrease in the glycogen content in hepatocytes (see Fig. 2, B), pronounced blood-filled vessels with expanded intralobular sinusoidal capillaries, hepatocytes were in a state of protein-fatty degeneration. The nuclei of many hepatocytes were pyknotic or lysed (Fig. 3, A).

The lungs of the rats were unevenly colored, with patches of red and pale pink. A histological examination revealed focal hemorrhages, overflow of blood vessels with the blood. In areas of emphysema, stretching of the alveoli, thinning and rupture of their walls were observed. In some animals, infiltrates, mainly consisting of lymphoid cells, were noted around the bronchi. The surface of the

bronchial epithelium was covered with a significant amount of mucus.

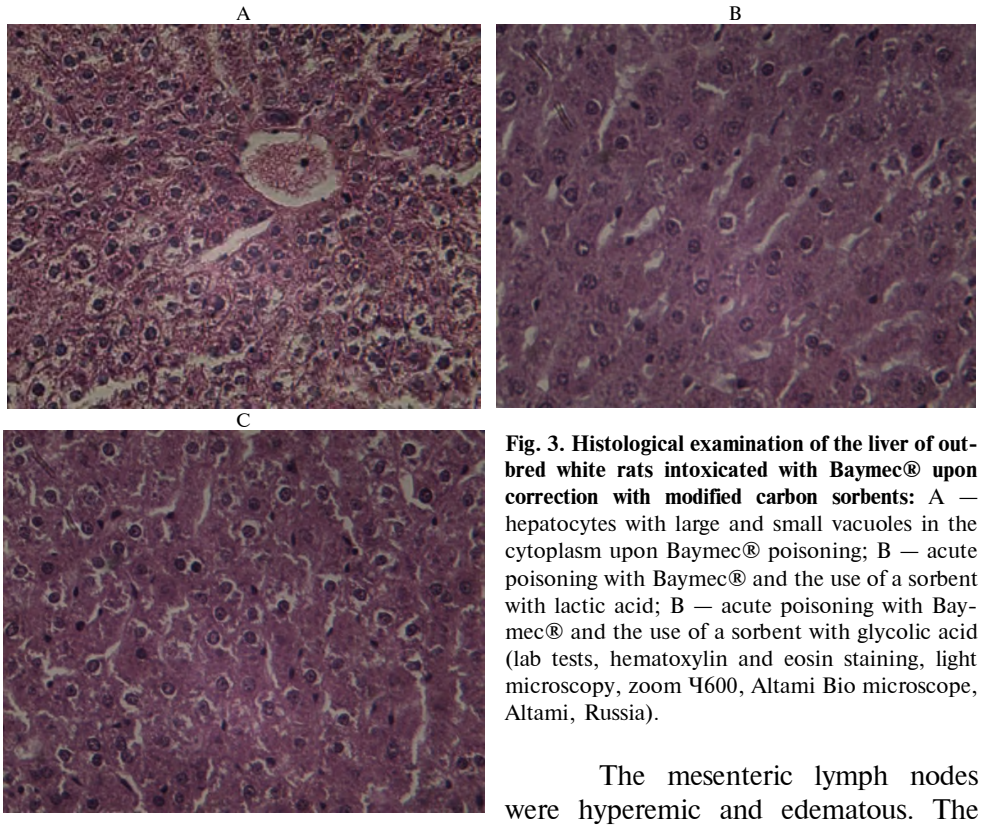

Fig. 3. Histological examination of the liver of outbred white rats intoxicated with Baymec® upon correction with modified carbon sorbents: A — hepatocytes with large and small vacuoles in the cytoplasm upon Baymec® poisoning; B — acute poisoning with Baymec® and the use of a sorbent with lactic acid; B — acute poisoning with Baymec® and the use of a sorbent with glycolic acid (lab tests, hematoxylin and eosin staining, light microscopy, zoom Ч600, Altami Bio microscope, Altami, Russia).

The mesenteric lymph nodes were hyperemic and edematous. The mucous membrane of the stomach and intestines was swollen, reddened, with hemorrhages. On the surface of the mucous membrane, mucus is detected containing a significant amount of desquamated epithelial cells.

Application of GA-, LA- and GA-LA-modified enterosorbents decreased the intensity of pathological processes in animals. A comparative pathomorphological study of rats subjected to Baymec® intoxication and pharmacocorrection allowed us to conclude that the LA-modified sorbent has a more pronounced detoxifying effect. Histological examination of the liver when using this sorbent showed only signs of granular dystrophy (see Fig. 3, B). Enterosorbents with glycolic acid and a hydroxy acid copolymer did not completely eliminate necrobiotic changes in hepatocytes (see Fig. 3, C), as well as signs of congestive hyperemia of organs, which explained the revealed changes in the metabolic profile of animals.

The noted differences in the action of sorbents modified with hydroxy acids are probably due to a lower pH of the LA-sorbent. Ivermectin undergoes biotransformation in the liver with the participation of cytochrome P450, mainly its isoenzyme CYP3A4, the oxidative activity of which increases significantly in an acidic environment [33].

Thus, for the first time, methods have been developed for modifying the surface of a carbon sorbent with glycolic and lactic acid oligomers, as well as their copolymer. The technology is based on sorbent impregnation with aqueous solutions of hydroxy acids or their mixture and prolonged heat treatment (polycondensation) without the use of toxic catalysts and organic solvents. This made it possible to obtain bifunctional sorbents combining the adsorption properties of the matrix and the biological activity of the modifiers. Our findings confirm the

therapeutic efficacy of sorbents modified with hydroxy acids for the intoxication of animals with ivermectin containing Baymec® drug. The sorbent modified with lactic acid expresses the most pronounced detoxifying effect. With its use, the concentration of blood globulins maximally increases, the total bilirubin is halved, and mineral metabolism is activated. Histological investigation revealed only slight dystrophic changes in parenchymal organs after enterosorption. The use of the modified sorbents significantly increases the effectiveness of detoxification and provides new possibilities for sorption therapy.

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INTESTINAL MICROBIOTA AND BROILER PERFORMANCE UPON ADMINISTRATION OF PHYTASE TO INCREASE PHOSPHORUS DIGESTIBILITY AND NUTRIENT UTILIZATION FROM FEED

T.N. LENKOVA[1], I.A. EGOROV[1], T.A. EGOROVA[1], V.A. MANUKYAN[1], V.G. VERTIPRAKHOV[1], A.A. GROZINA[1], I.N. NIKONOV[1], V.A. PHILIPPOVA[2], E.A. YILDIRIM[2], L.A. ILINA[2], A.V. DUBROVIN[2], G.Yu. LAPTEV[2]

[1]*Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS,* 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail olga@vnitip.ru, dissovet@vnitip.ru (✉ corresponding author) , manukyan@vnitip.ru, eta164@mail.ru, ilnikonov@yandex.ru, vertiprakhov63@mail.ru, alena_fisinina@mail.ru;
[2]*JSC «Biotrof+»,* 19 korp. 1, Zagrebskii bulv., St. Petersburg, 192284 Russia, e-mail ilina@biotrof.ru, deniz@biotrof.ru, laptev@biotrof.ru
ORCID:
Lenkova T.N. orcid.org/0000-0001-8026-3983
Egorov I.A. orcid.org/0000-0001-9122-9553
Egorova T.A. orcid.org/0000-0002-5102-2248
Manukyan V.A. orcid.org/0000-0003-4564-4427
Vertiprakhov V.G. orcid.org/0000-0002-3240-7636
Grozina A.A. orcid.org/0000-0002-3088-0454
Nikonov I.N. orcid.org/0000-0001-9495-0178
Philippova V.A. orcid.org/0000-0001-8789-9837
Yildirim E.A. orcid.org/0000-0002-5846-5105
Ilina L.A. orcid.org/0000-0003-2490-6942
Dubrovin A.V. orcid.org/0000-0001-8424-4114
Laptev G.Yu. orcid.org/0000-0002-8795-6659

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Abstract

Phosphorus is an essential element in the nutrition of humans, animals, and plants. Due to the short growth period (34-42 days of age), fast growth of live bodyweight and skeleton the problem of mineral nutrition and balance (including calcium and phosphorus) is especially urgent for broiler chicks (*Gallus gallus* L.). The predominant form of phosphorus in vegetable feed ingredients is phytate which is an antinutritive factor for poultry and cannot be digested. As a result the supplementation of diets for poultry with phytases, enzymes degrading the indigestible phytate complexes, has gradually become a common practice worldwide. The recent data of Russian (O.V. Trufanov, 2011; E.V. Anchikov, 2012) and foreign authors (S.W. Kim et al., 2018; C.L. Walk et al., 2019; O.O. Babatunde et al., 2019) evidenced that the supplementation of diets with reduced phosphorus content with different doses of phytase improves daily weight gains, phosphorus content in blood serum, tibial strength, and tibial contents of minerals. The efficiency of supplementation of diets for broilers (cross Cobb 500) with reduced by 0.1 % phosphorus content with two innovative new-generation phytase preparations (Feedbest-P and Berzyme-P, produced by Sibbiopharm Co., Russia) was studied; the data of pioneer research of the effects of different phytase preparations on the cecal microbial community are also presented. The trials were performed in the Center for Genetics & Selection Zagorskoye EPH (Moscow Province) in 2018. In the first trial the diets for experimental treatments were supplemented with phytase preparation 1 with activity 10,000 FTU/g (20, 40, and 60 ppm); in the second trial phytase preparation 2 was used with activity 50,000 FTU/g (6, 12, and 30 ppm). The supplementation of diets with preparation 1 increased live bodyweight in broilers at 37 days of age by 2.7; 3.0 and 3.7 % (respective to aforementioned doses) in compare to non-supplemented control treatment; feed conversion ratio (FCR) in these treatments was better by 2.9; 4.0 and 4.6 %, respectively. The respective improvements with different doses of preparation 2 were 1.3; 3.1 and 2.0 % and 1.9; 5.6 and 3.7 %. Positive effects on the digestibility of dietary nutrients, deposition of calcium and phosphorus, mineral contents in the skeleton were found with both phytase preparations. The investigation of cecal microbiota using T-RFLP (terminal restriction fragment length polymorphism) method revealed the significant increase in the pool of cellulolytic bacterial species in the phytase-fed broilers; the increases in the species of *Eubacteriaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae,* and

Bacteroidetes were also found. The most of the identified bacterial species in cecal population were ascribed to the phylums *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Fusobacteria*.

Keywords: *Gallus gallus*, phytase, compound feeds, broiler chicks, productive performance, cecal microbiota

Enzyme preparations have a high potential for practical use in feed production. Recently, up to 90 % of poultry feeds are enriched with phytases, the enzymes that break down indigestible phytate containing complexes. The use of phytases allows better absorption of plant food P by poultry, reduces the level of inorganic phosphates in diets and the excretion of phosphorus in droppings [1-3]. Young birds, in particular broiler chickens, are especially in dire need of phosphorus. Selection for growth rate has caused the development of chicken skeleton to lag behind the formation of muscle tissue which often results in leg abnormalities of non-infectious etiology [4].

The market of commercial bacterial and fungal phytases for animal husbandry is quite diverse and saturated. The activity of phytase preparation determines its dose in feeds, thermostability, costs and effectiveness of practical use [5]. Phytase-enriched premixes or direct enrichment of animal feed in the farm can be applied which requires due equipment to mix feeds, premixes and supplements.

Dietary phytase can affect the physical and chemical properties of the chyme in the gastrointestinal tract, especially pH, which leads to shifts in the profiles and activity of the gut microbiota. Phytases show highest activity at acidic pH of 3 to 6, i.e., phytases can release phosphorus already in crop and gizzard [6]. From 0 to 12,000 FTU/kg live weight of broilers, upon a decrease in the amount of calcium and phosphorus, contributed to a significant increase in the average daily weight gains in chick, blood phosphorus, and tibia strength and ash content [7]. However, phytase can adversely impact the absorption of lysine, cysteine, aspartic acid, glycine, methionine, tryptophan, and serine [8]. Regardless of poultry age, diet and products the birds are farmed for, phytase increases the availability and amount of phytic phosphorus in the jejunum contents. Phytase acts more effective in 14-22-day old chickens when bone tissue formation and growth intensify [9]. The size of mineral component particles also affects the effectiveness of dietary phytase [10]. Upon a combined use of phytase with xylanase or β-glucanase, broiler chickens showed a decrease in feed intake and *Escherichia coli* colonization of the intestines [11] without detectable changes in the digestive system.

The microbiocenosis of poultry intestines is a fairly rich and complex community of symbiotic microorganisms, comprising bacteria, archaea, micromycetes, protozoa, and viruses [12-15]. Bacteria are the main members of chicken intestinal microbiome [16]. The 16S rRNA gene sequencing technique revealed 13 bacterial phyla in the intestinal microbial ecosystem of chickens, with *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* dominating (> 90%). Of more than 900 equivalents of operational taxonomic units (OTUs) found in the intestines of chickens 117 OTUs belong to known bacterial genera. The effect of dietary phytases on the microbial ecosystem of the gastrointestinal tract is extremely poor studied.

In this work, the alterations of cecum microflora in broiler chickens when phytase of different activity were added to the feed were first studied by the T-RFLP (terminal restriction fragment length polymorphism) method which has a number of advantages compared to methods of classical bacteriology. It was shown that the Russian biopreparations Feedbest-R and Berzyme-R provide competitive advantage of the chicken gut normoflora, while the counts of conditionally pathogenic and pathogenic members of the microbial community decrease. Feedbest-R and Berzyme-R increase P utilization from feeds with low digestible phosphorus, improve protein and fat digestibility, as well as utilization of nitrogen, amino acids,

calcium, phosphorus and trace elements (iron, manganese, copper, zinc).

Our subjective was to investigate effects of two Russian phytase containing preparations on broilers' productive performance, bone mineralization, nutrient digestibility and use, and cecum microbiome composition.

Material and methods. Experiment 1 and experiment 2 were performed in vivarium (Zagorsk EPH Selection and Genetic Center, Sergiev Posad, Moscow Region, 2018) on birds from 1 day to 37 days of age, and from 1 day to 36 days of age, respectively. For each experiment, Cobb 500 broilers (*Gallus gallus* L.) were assigned into four groups of analogues in live weight (control group I, test groups II-IV, 35 birds per group, 280 birds in total). Chickens were kept in AviMax cell batteries (Big Dutchman, Germany) as per stated technological parameters [17]. For the first 5 days, chickens of all groups received the same starter compound feed. The feeds corresponded to the age norms for the cross (from day 6 to day 21 and from day 22 until the end of the experiment). In the control groups, the nutrients content the feeds were equal, in the experimental groups, the amount of digestible phosphorus was reduced by 0.1%. The enzyme was mixed with feed in a stepwise fashion. In experiment 1, the Feedbest-R (LLC PO Sibbiofarm, Berdsk, Russia) doses were 20, 40 and 60 g/t feed for groups II, group III and group IV, respectively. In experiment 2, Berzyme-R (PO Sibbiofarm LLC, Berdsk, Russia) doses were 6, 12 and 30 g/t feed for groups II-IV, respectively. The activity of Feedbest-R is standard (10,000 FTU/g), while of Berzyme-R it is 5-fold (50,000 FTU/g).

The investigated parameters were mortality, live weight at 1, 7, 14, 21 and 36-37 days of age (from day 21, females and males were weighed separately), feed conversion and average daily weight gain. At the age of 28-36 days, physiological balance experiments were carried out, in which the protein, fat, fiber digestibility and the balance of nitrogen, calcium, and phosphorus were determined. For this, 3 birds (analogues in live weight) from each group were kept in a cage to determine the amount of consumed feed and the amount and chemical composition of poultry manure. At the end of the experiments, the chemical composition of the tibia, pectoral and foot muscles were determined. After slaughter, anatomical cutting of carcasses was carried out and the slaughter yield of the gutted carcass was determined [18].

Cecal content was sampled for microbial analysis after slaughter of chickens at 36 days of age (three replicates from each group) strictly aseptically [18] and immediately frozen.

Microbial composition of the cecal content was assayed by T-RFLP (term-minimal restriction fragment length polymorphism) method. Total DNA was extracted with DNA Purification Kit (Fermentas, Inc., Lithuania) as per the manufacturer's recommendations. PCR (Verity DNA Amplifier, Life Technologies, Inc., USA) was performed with eubacterial primers 63F 5′-CAGGCCTAACACATGCAAGTC-3′ labeled at the 5′-end (D4 WellRED fluorophore, Sigma-Aldrich, Inc., USA) and 1492R 5′-TACGGHTACCTTGTTACGACTT-3′ to amplify the 16S pRNA gene fragment (positions from 63 to 1492, numbering indicated for *Esherichia coli* 16S pRNA gene) in the following mode: 3 min at 95 °C (1 cycle); 30 s at 95 °C; 40 s at 55 °C; 60 s at 72 °C (35 cycles); 5 min at 72 °C. Labeled amplicons of 16S pRNA gene DNA were purified by a standard method [19]. Concentration of purified fragments was determined (a Qubit 2.0 fluorimeter, Invitrogen, Germany) according to the manufacturer's recommendation. HaeIII, HhaI, and MspI (Fermentas, Lithuania) were used for PCR amplicon restriction endonuclease analysis (the amplicon amount of 30-50 ng). Cleaved DNA fragments were sequenced (CEQ 8000, Beck-man Coulter, USA). Fragment Sorter

program (available from http://www.oardc.ohiostate.edu/trflpfragsort/index.php) were used for phylogenetic affiliation.

Mathematical and statistical processing was carried out by standard methods of analysis of variance [20] using Microsoft Excel 2010 software. We used parametric (Student's *t*-test) and nonparametric (Wilcoxon-Mann-Whitney method) statistical methods. Mean values (*M*) and standard errors of the mean (±SEM) were calculated. Biological diversity was evaluated using the Shannon and Simpson indices in the Past program (http://folk.uio.no/ohammer/past/). The causal relationship between cecal microflora composition and phosphorus utilization was assessed using Pearson correlation coefficients, which makes it possible to establish direct relationships between the variables by their absolute values [20]. Correlation indicators were analyzed for microorganisms comprising over 1% of the total microbial counts in the community.

Results. Feedbest-R and Berzyme-R supplements increased the absorption of phytic phosphorus, the digestibility and use of other feed nutrients. In experiment 1, the Feedbest-R increased the use of phosphorus by 4.2% in group II, by 6.6% in group III and by 7.1% in group IV compared to control (Fig. 1, A). In test groups II, III and IV, the feed dry matter digestibility was 1.2; 2.8 and 3.0 % higher, respectively, and fat digestibility was 3.0; 3.7 and 3.9% higher, as compared to control.

Phytates bind positively charged metal ions, the macro- and microelements (calcium, zinc, iron, manganese, magnesium ions), as well as proteins, amino acids, and starch, reducing their bioavailability [9]. In the experiment 1, the phytase contributed to a better use of calcium in groups II, III and IV, by 1.8; 3.0 and 3.5%, respectively. The phytase has improved the digestibility of animal protein upon a sufficiently low its amount in the diet. Protein digestibility in groups II, III and IV was higher than in the control by 1.2; 1.4 and 1.5%. The test broilers also utilized feed nitrogen better (2.2-2.8%) than the control birds. The digestibility of essential amino acids lysine and methionine was 3.0-3.5 and 3.1-4.0% higher, respectively.

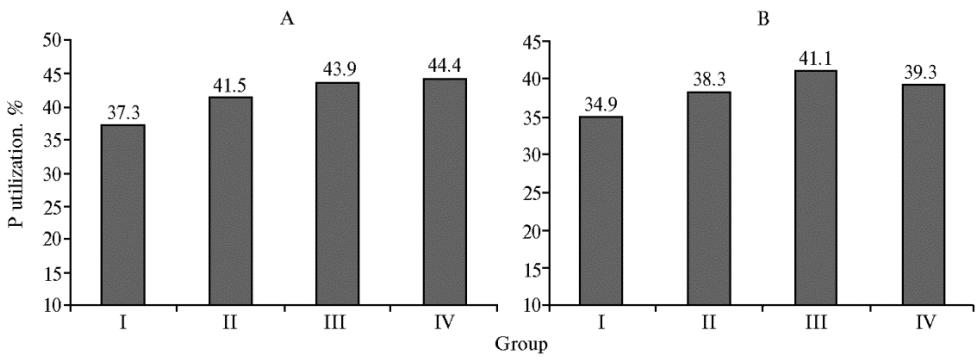

Fig. 1. Phosphorus utilized by Cobb 500 broilers (*Gallus gallus* L.) upon different dosage of dietary phytase preparations Feedbest-R (A) **and Berzyme-R** (B)**.** For feed composition in groups, see the Material and methods section ($n = 35$ per group; vivarium of the Zagorsk EPH Breeding and Genetics Center, Sergiev Posad, Moscow Region, 2018).

As a result of improved phosphorus and calcium utilization under the influence of phytase, the accumulation of these trace elements in tibias showed a tendency to rise. In addition, birds of all experimental groups showed a higher concentration of iron (by 2.54-2.91 mg%), manganese (by 0.05-0.13 mg%), copper (by 0.06-0.013 mg%), and zinc (0.45-1.16 mg%) in the bones.

Due to higher digestibility and utilization of feed nutrients, the live weight of 37-day-old broilers was 2.7; 3.0 and 3.7% higher in groups II, III, and IV compared to control (group I), being 1.6; 2.1 and 2.5% higher in females and 3.6; 3.9 and 4.8% higher in males. Feed conversion in these groups was 2.9; 4.0 and 4.6% better than in control.

The Berzyme-R-enriched feeds (experiment 2) also positively affected the physiological state of the birds (see Fig. 1, B). In test groups II, III and IV, as compared to control, the use of phosphorus was 3.4; 6.2 and 4.4% higher, the use of calcium was 1.5; 3.2 and 2.3% higher, respectively, the digestibility of feed dry matter increased by 0.8-2.6%, of fat by 1.1-3.2%. The feed protein digestibility and the nitrogen utilization in the test groups were 0.9-2.9 and 1.3-2.8% higher, respectively.

Significant differences in the crude ash content in the tibias were not observed among broilers from different groups. There was a slight tendency towards an increase in calcium and phosphorus accumulation in the skeleton of poultry from the test groups. As in experiment 1, the iron, manganese, copper, and zinc levels were higher in tibia of broilers fed phytase than in the control. The body weight of 36-day-old broilers increased in test groups II, III and IV by 1.3; 3.1 and 2.0%, respectively, compared to the control. Moreover, the body weight in the groups turned out to be 1.14; 3.0 and 2.9% higher in the females and by 1.5; 3.3 and 1.3% higher in males.

The key commercial criterion of broilers is the slaughter yield of meat of gutted carcasses. According to the results of both experiments, this indicator was higher in the experimental groups. Chemical analysis of broiler pectoral and foot muscles showed the absence of significant differences between the experimental and control groups in terms of moisture, crude protein, fat, ash.

T-RFLP analysis revealed 78±3.9 to 108±5.4 bacterial phylotypes in the cecal microflora of chickens in experiment 2 (Table). A more pronounced taxonomic diversity and complexity of the microbial communities was noted in the control group not fed the phytase additive, as well as in the group III fed Berzyme-R at a dose of 12 g/t feed. This indicates the heterogeneity of the compositions of the microbiocenosis, the accumulation of entropy and some disorganization in these groups as compared to test groups II and IV.

Biodiversity of microorganisms in the cecum of Cobb 500 broilers (*Gallus gallus* L.) depending on the dosage of a dietary phytase preparation Berzyme-R (n = 35 per group; M±SEM, Vivarium of Breeding and genetic center Zagorsk EPH, Sergiev Posad, Moscow province, 2018)

Indicator	Group I	Group II	Group III	Group IV
Number of phylitypes	101.0±4.60	107.0±6.20	78.0±3.90	108.0±5.40
Community dominance index	0.04±0.002	0.33±0.018	0.04±0.002	0.31±0.014
Shannon index	3.57±0.180	1.46±0.062	3.67±0.150	1.64±0.050
Simpson index	0.96±0.038	0.67±0.020	0.96±0.041	0.69±0.047
Margalef index	21.2±1.50	11.4±0.49	16.7±0.72	13.6±0.59

N o t e. For description of the groups see section Material and methods.

A significant proportion of detected microorganisms could not be attributed to any existing taxon (Fig. 2). The unidentified bacteria ranged from 4.7±0.3 to 12.3±0.52%, depending on the group. The number of such microorganisms was the largest in group IV. Other researchers have also earlier identified a high proportion of uncultured bacteria in the composition of the gut microbiota of chickens [21].

The composition of the identified gut microorganisms as a whole was similar in all groups of broilers (see Fig. 2). In most samples, the bacteria were

assigned to the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria*. Bacteria from *Firmicutes* phylum were dominant in all cases. These data partially agree with the results obtained previously [22-24]. The 16S rRNA gene sequencing showed that the members of phyla *Firmicutes* and *Proteobacteria* dominate in gut microbial communities of broilers, reaching over 90% of the analyzed sequences [25].

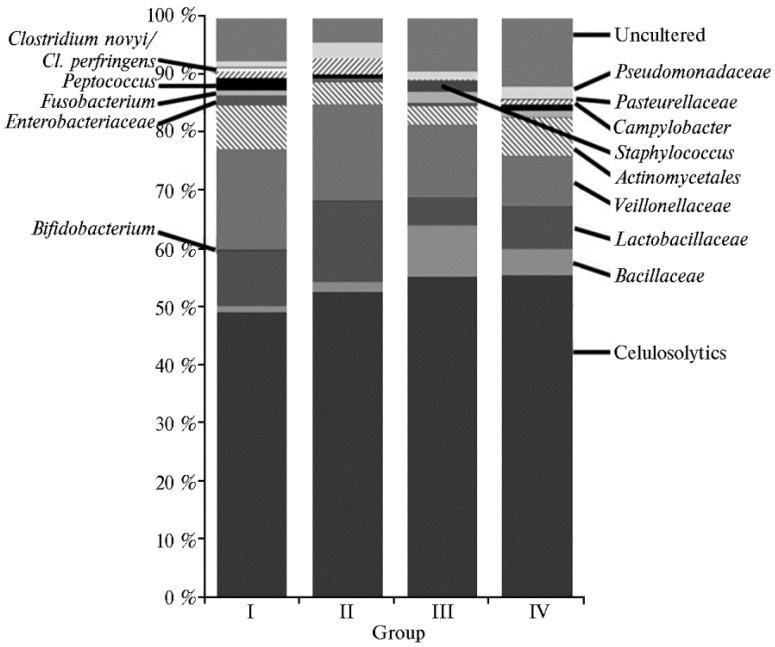

Fig. 2. Profiles of Cobb 500 broiler (*Gallus gallus* L.) cecal microbiocenosis depending on the dosage of dietary phytase preparations (the profiles are based on the T-RFLP analysis data)**.** For description of the groups see section Material and methods.

Interestingly, the addition of phytase promoted an increase in a pool of cellulolytic bacteria of the families *Eubacteriaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae* and phylum *Bacteroidetes*. These microorganisms play an important role in bird's digestion, since they produce a number of digestive enzymes, including cellulases, which allows the macroorganism to effectively use the energy of feeds rich in fiber. We revealed a 1.2-2.3-fold decrease in the abundance of order *Actinomycetales* by 1.2-2.3 times and a 2.8-14.4-fold decrease in *Enterobacteriaceae* family in broilers receiving a concentrated phytase preparation as compared to the control group. The *Enterobacteriaceae* family comprises pathogenic species of the genera *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter* etc., therefore, a decrease in the number of this bacterial group may indicate a correction of dysbiotic disorders in the intestines of broilers.

The *Lactobacillaceae* family was the most abundant (13.8±0.62%) in chickens of group II. The main metabolite of lactobacilli of family *Lactobacillaceae* is lactate which reduces the pH of the chime and thus suppresses pathogenic forms. Also, the smallest portion of the genus *Fusobacterium* members, among which pathogens of inflammatory diseases are often found, was characteristic of the chickens in group II. In addition, the number of bacteria *Clostridium novyi/Cl. perfringens*, representatives of the genus *Staphylococcus* and the family *Pasteurellaceae* were below the level of reliable determination by T-RFLP method. Nevertheless, these microorganisms were detected in the intestines of chickens, i.e. staphylococci in group III (1.88%) and group IV (0.19%), clostridia in the control

(0.73%), pasteurella in the control (0.09 %) and group IV (0.23%). Importantly, such pathogens as *Staphylococcus aureus* and *Pasteurella multocida* are often found among bacteria of the genus *Staphylococcus* and the family *Pasteurellaceae*. *Clostridium novyi* and *Cl. perfringens* are often associated with gastroenteritis and lameness in chickens [26, 27].

The Pearson correlation coefficients confirmed the relation between the abundance of functionally significant representatives of the cecal microbial community and the dosage of dietary phytase. An increase in the assimilation of phosphorus had a reliable direct correlation with an increase in the number of cellulolytic bacteria ($r = 0.98$ at $p \leq 0.05$), in particular, with more abundant members of the phylum *Bacteroidetes* ($r = 0.99$ at $p \leq 0.001$) and the family *Lachnospiraceae* ($r = 0.84$ at $p \leq 0.05$). Also, the higher P availability was associated with lower counts of bacteria from *Veillonellaceae* ($r = –0.84$ at $p \leq 0.05$) and *Enterobacteriaceae* ($r = –0.92$ at $p \leq 0.01$) families.

The modulating effect of phytase on the cecal microflora can be associated with an increase in the availability of phosphorus, a change in pH, as well as other physicochemical parameters in the lumen of the gastrointestinal tract. Despite the fact that information about changes in gut microbial communities of chickens under the influence of phytase added to feed is extremely limited, there are papers indicating that the use of this enzyme affects the microbiome structure. A. Ptak et al. [25] showed an increase in the number of *Lactobacillus* sp. and *Enterococcus* sp. when chickens received feed supplemented with phytase.

The researchers indicate the unequal efficacy of different phytase preparations [28-31]. Summarizing the results of our experiment, we can state that both Russian phytase preparations (Feedbest-R and Berzyme-R), when added to broiler feed, allows for lower concentration of inorganic phosphorus in the diet, while increasing bird productivity. The phytases we tested can increase the average daily in live weight gain of broilers and improve feed conversion similar to that reported by other researchers [5, 32]. As per P.H. Selle et al. [29], the use of dietary phytase significantly increased the calcium (32.2%) and phosphorus (28.0%) utilization in the ileum of broilers that received wheat-type feed. In our experiments, exogenous phytases contributed to a more efficient utilization of feed nutrients, as well as calcium and phosphorus. This is explained by the cleavage of phytates [32], which not only serve as a reservoir of phosphorus, but also bind a significant part of microelements, proteins, carbohydrates, amino acids, turning them into complex insoluble conglomerates. Phytases improve protein digestibility and the use of feed amino acids thus reducing endogenous loss of amino acids [33]. Better skeleton mineralization in chickens under the influence of phytases noted in our experiments was also reported earlier [34-36].

Thus, phytase activity of Feedbest-R and Berzyme-R preparations is high, which allows a 0.1% decrease in the digestible phosphorus in feed for broilers. Moreover, Feedbest-R and Berzyme-R improve the digestibility of protein, fat, and the use of nitrogen and amino acids of the feed. The digestibility of calcium and phosphorus increases by 3.4-7.1% depending on the dosage of the additives, and trace elements (iron, manganese, copper, zinc), as well as protein and amino acids are better used. Application of phytase-containing supplements increases bone mineralization. A concentrated phytase-based preparation fed to broilers changed the qualitative and quantitative composition of the cecal microbiome. The normal flora mainly possessed a competitive advantage, whereas the number of conditionally pathogenic and pathogenic bacteria decreased

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