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CRYOBANKING OF SOMATIC CELLS IN CONSERVATION OF ANIMAL GENETIC RESOURCES: PROSPECTS AND SUCCESSES

(review)

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Abstract

Extinction of many species is irreversible and is a part of the natural evolution, but human activities have influenced this process, making it much faster comparing to speciation. According to FAO, approximately 20 % of the breeds of cattle, goats, pigs, horses and poultry in the world are currently at risk of disappearance, many have died in the past few years, as a result their genetic characteristics lost forever. The role of banks in the management of genetic resources and the conservation of endangered species is particularly noticeable in the last decade. Most cryobanks focus on the cryopreservation of gametes (primarily sperm) and embryos. Their main goal is to produce offspring using reproductive technologies, which include artificial insemination, in vitro fertilization and embryo transfer. The discovery of the phenomenon of reprogramming somatic cell nuclear allowed expanding the range of forms of biological material in programs for cryopreservation. Creating cryobanks of somatic cells as donors of nuclei for cloning considered an auxiliary instrument for the preservation and improvement of the gene pool of farm animals and poultry. To obtain viable cryopreserved cell lines very small amount of biopsy material, including that of dead animals, is sufficient, but such lines contain the complete genome and proteome. In contrast to germ cells, embryos and generative tissues, the cryopreserved somatic cells after repeated thawing are capable to regenerate, i.e. almost infinitely may serve as a source of biomaterial for use in assisted reproductive technologies and biological research, including retrospective reconstruction. Furthermore, due to the small size the somatic cells are more resistant to cryopreservation. This review also provides a brief description of the principles and history of cloning. The advantages of the use of different cell types as karyoplasts are discussed. In particular, almost all types of cells (e.g. embryonic cells, mammary cells, cumulus, granulosa, oviduct, liver, fibroblasts, white blood cells and embryonic stem cells) can be used for the production of cloned animals, but the cloning efficiency depends significantly on the type of cells. Aiming embryo development and birth of live offspring, the fetal fibroblasts as donors of nuclear material for cloning are most effective. Alternatively, the stem cells may be a source of the nuclei. Stem or progenitor cells (i.e., stem, determined to differentiate in specific type cells) are easier reprogrammed than terminally differentiated cells. Also when stem cells nuclei are used as karyoplasts the number of cloned embryos significantly increased. The advances in interspecific cloning as a strategy for restoration of rare and endangered species are discussed. Numerous examples show that somatic cells can be considered the most promising material for the recovery of animal genetic resources of different types. Particularly from 1997 to 2012 using the differentiated somatic cells domestic and wild animals of different species were obtained such as sheep, mice, cows, goats, pigs, guar, mouflon, domestic cat, rabbits, mule, horse, rat, wildcat, dog, banteng, ferret, wolves, buffalo, deer, mountain goat, camel, coyote. Cattle are still the leader in the production of cloned offspring with the efficacy 10 % on average, and in some cases up to 25 %, while for most other animals it does not exceed 1 %. Under controlled conditions in farms with good management, the productivity of clones should vary only within the remaining natural variability and mitochondrial genetic variability due to cloning technology.

Keywords: somatic cells cryobanks, cloning, biodiversity, animal genetic resources.

Extinction of many species is irreversible and is a part of the natural evolution. People activities, particularly uncontrolled hunting and fishing, destruction of environment, along with animal competition for food and terri-

tory influence this process making extinction much faster comparing to speciation (1). Changing demands of the market and the intensification of agriculture have also increased tendency to reduction of biodiversity of domestic animals. In agriculture small farms are gradually replaced by large commercial enterprises. Recent reproduction technologies, an unlimited transfer of genetic material, breeding programs implemented by national and international companies lead to dominance of certain livestock breeds (2). According to FAP reports, about 20 % of cattle, goats, pigs, horses and poultry breeds in the world are currently under threat of disappearance. Many breeds have become extinct during past few years with their genetic characteristics lost irreversibly (3). With the view to bio-, environmental and food safety it is essential to maintain biodiversity and alternative and potentially useful genes in the gene pool (4). In 2007 109 countries have approved Global Plan of Action for Animal Genetic Resources due to understanding this problem (5).

Obviously both wild and domestic animals should be best preserved in situ, i.e. in their natural habitat meaning nature or commercial farming with specific technology used for each breed. This is costly as the extensive infrastructure and management are required. Besides, in case of small population and necessity to keep up an adequate genetic diversity this approach could be insufficient (2). Banking of genetic recourses allows solving these problems additionally to in situ preservation (6-8).

The role of genetic resource banks, which provide collection, processing and storage of biomaterial, in the management and conservation of endangered species is particularly noticeable in the last decade (9). Under correct usage these recourses are enough to keep up current genetic diversity in the populations and allow their reproduction in the future using biotechnology (10). A core problem in creating such banks is to determine the quantity and type of preserved genetic material. Most cryobanks focus on cryopreservation of the gametes (primarily sperm) and the embryos, being targeted to offspring reproduction by means of assisted reproductive technologies, including artificial insemination, in vitro fertilization and embryo transfer (11). Somatic cell nuclei reprogramming allows expanding the range of biological material used in programs for cryopreservation. Cryobanking of somatic cells as the nuclei donors for cloning is recently considered the additional approach to preservation and improvement of agricultural animals and poultry gene pools (12).

Cultivation and freezing somatic cells allow to obtain the hundreds of millions of cells from an individual animal (and then preserve them for many years) that is similar in number to the culture of microorganisms. Specific complex media, which consist of amino acids, vitamins, sugars and blood serum containing sets of growth factors, are developed for effective somatic cell cultivation. The air CO_2 concentration and the temperature optimal for cultivation are specified. Developed techniques of stable line cultures allow cultivating cells for a dozen passages with no changes in karyotype and all traits of normal cells (13).

Unlike the gametes and embryos the somatic cells are smaller and, therefore, more resistant to cryopreservation. After decades of application and improvement the cryopreservation became a routine procedure for most cell types. It includes cell isolation from tissue, cultivation, obtaining primary cell culture, cell biomass accumulation, freezing and storing in liquid nitrogen (13).

In cryobanks the samples should be stored at the temperature below –146 °C, providing their high chemical and physical stability. Therefore, the stored biological material is valuable both for conservation of genetic resources ex situ and future research projects and investigations (14). Very small amount of biopsy material, including that of dead animals, is sufficient to obtain the

viable cryopreserved cell lines, but such lines contain the complete genome and proteome. In contrast to gametes, embryos and generative tissues, the cryopreserved somatic cells are capable to regenerate after repeated thawing, i.e. almost infinitely may serve as a source of biomaterial for use in assisted reproductive technologies and biological research and manipulations, including retrospective reconstructions (9, 15).

The activities of Frozen Ark, the international consortium (16), and its members, particularly the Can Diego Zoo (California, USA) (17), LaCONES (India) and Genome Resource Bank (CGRB) for Korean Wildlife (Seoul National University) (10), clear demonstrate the role of cryobanks in preservation of genetic resources of the domestic animals and wildlife. Creating somatic cell and tissue banks is a part of national programs of genetic resource conservation in Canada, Brazil, Chine, Germany, Poland, Spain, Turkey (10, 15, 18-23). Noteworthy results were achieved when the somatic cell banks and cloning technology were used to preserve the native Anatolian breeds of domestic animals (23).

Cloning means an excision of nucleus from mature oocyte and its replacement by the donor nucleus from somatic cell. The donor nucleus influenced by different ooplasmic factors is undergone the epigenetic reprogramming. As a result the differentiated donor nucleus becomes active and initiates embryo development instead of somatic cell division (24).

Cloning was first suggested in 1938 by H. Spemann who showed the plurypotency of cell nuclei until 16 cell stage in salamander embryo (25), but the experimental nuclei transfers in mammals were reported much later in the 1980s. As mammalian zygotes are small in size, it causes technical difficulties in manipulations. Nevertheless, first reports about mouse cloning were dated 1981. In 1986 S.M. Willadsen reported the first successful nuclear transfer in sheep (26). Cloned sheep was obtained by the microsurgical enucleation of oocytes at MII with further fusion to 8 and 16 cell blastomeres. After the success with early blastomeres there were attempts to use cultivated animal cells in cloning. In 1996 in Scotland University K.H.S. Campbell et al. (27) used donor nuclei from blastocyst inner cell mass. In these experiments two lambs, Megan and Morag, were born that became a crucial step towards obtaining clones using somatic cells of an adult animal.

The first cloned progeny via somatic cell nuclei transfer in mammals was obtained in the same university in 1997 (28). The birth of cloned sheep Dolly generated great scientific interest and contributed to further numerous studies in cloning other animal species by means of somatic cell nuclei transfer.

A number of wild and domestic animal species have been recently cloned using differentiated somatic cells (28-49) (Table).

First cloned progeny in different animal species

Year	Animal species	References
1997	Sheep Овцы	A.E. Schnieke et al. (Great Britain) (28)
1998	Mice	T. Wakayama c et al. (USA) (29)
1998	Cows	J.B. Cibelli et al. (New Zealand) (30)
1999	Goats	A. Baguisi et al. (Japan) (31)
2000	Pigs	I.A. Polejaeva et al. (Great Britain) (32)
2000	Guar	R.P. Lanza (USA) (33)
2001	Mouflon	P. Loi et al. (Italy) (34)
2002	Domestic cat	T. Shin et al. (USA) (35)
2002	Rabbits	P. Chesne (Chine) (36)
2003	Mule	G.L. Woods et al. (USA) (37)
2003	Horse	C. Galli et al.. (Italy) (38)
2003	Rat	Q. Zhou c соавт. (France, Chine) (39)
2004	Wild cat	M.C. Gomes c соавт. (USA) (40)

Table (continued)

2005	Dog	B.C. Lee с соавт. (Korea) (41)
2005	Banteng	M.J. Sansinena с соавт. (USA) (42)
2006	Ferret	Z. Li с соавт. (Chine, USA, France) (43)
2007	Wolf	M.K. Kim с соавт. (Korea) (44)
2007	Buffalo	D. Shi с соавт. (Chine) (45)
2007	Red deer	D.K. Berg с соавт. (New Zealand) (46)
2009	Mountain goat	J. Folch с соавт. (Spain) (47)
2010	Camel	N.A. Wani с соавт. (UAE) (48)
2012	Coyote	I. Hwang с соавт. (Korea) (49)

Almost any cell types can be used in animal cloning (12). There are reports on cells of embryos (50), mammary glands, cumulus, granulosa, oviduct, liver (29, 51-55), fibroblasts (56), leukocytes (57) and embryo stem cells (58) used as nuclei donors, but cloning efficacy essentially depends on the cell type. In view to successful embryonic development and the birth of viable animal the fetal fibroblasts are the most beneficial donors of nuclei under cloning. For these cells a low level of mutations and high proliferation are characteristic (12). However, sometimes there are no reasons and chance to obtain fetal biomaterial, when somatic cell banks are created, and then the tissues of adult animals could be the cell sources, mainly skin, muscles and cartilage. Fresh, stored at +4 °C for not more than 2 weeks or frozen biomaterial is suitable (59). A disadvantage of such cells is their lower capability to reprogramming and embryo development compared to fetal fibroblasts (60, 61).

The stem cells are recently considered the alternative source of nuclei for cloning. These cells are in each organ of adult animals providing structural and functional homeostasis. A proliferative ability and higher plasticity are the valuable traits of stem cells compared to differentiated somatic cells (62). Experiments on the mice neural stem cells showed an easier reprogramming of stem or progenitor cells comparing to differentiated cells, moreover, when stem cell nuclei were used as karyoplast the number of cloned embryos significantly increased (63). Nowadays, mesenchymal stem cells are considered the most attractive source of nuclei for cloning (64-66).

The adult animal cells significantly increase a potential of cloning technology in conservation of animal and poultry genetic resources and also in breeding. Cloning adult animals allows to replace the genetic selection by a phenotypic selection. Chromosomal gene combination under cloning remains unchanged thus not only the additive gene effects can be used. Under controlled conditions in farms with effective management the productivity of cloned animals should differ only within a residual natural variability and also due to mitochondrial genetic variability occurred because of used cloning technology. As a result, one generation in the herd is enough to reach the best productivity. In that connection the selection of animals with high lifetime productivity is particularly important (22).

Despite the recent impressive successes in cloning, its efficacy remains extremely low with the high level of embryo abnormalities and a decreased viability of the offspring (67, 68). Cattle still remains the leaders among cloned species with an average 10 % frequency of the offspring birth, being even 25 % in some cases, in contrary to most other animal species with not more than 1 % frequency as a rule. So, by creating somatic cell and tissue banks now we provide biomaterial for future applying when improved cloning technology will allow its effective use (69).

A search for universal cytoplast to be used in somatic cell nuclei transfer is an important point of cloning. These experiments became the most relevant in connection with programs of wildlife genetic resource conservation when obtain-

ing autogenic cytoplasts for obvious reasons is impossible or difficult. The oocytes used for interspecial cloning should meet special requirements. Technology of cytoplast processing should not be expensive or difficult, and cytoplast should be capable to reprogram the somatic cells of another species and support the embryo development of an interspecial cytohybrid.

In 1999 T. Dominko et al. (70) first demonstrated the capability of cattle oocyte cytoplasm to reprogram the nuclei of somatic cells of other animal species. After the transfer of nuclei from sheep, pig, monkey or rat skin cells to MII enucleated cattle oocyte the cytoplast and xenogenic karyoplast joined. In further experiments cattle oocytes were used as cytoplasts in transfer of somatic cell nuclei of pigs (71), koala (72), antelope, goat (73), horses (74), black bear (75), mountain antelope (76), hens (77), yak and dog (78), and buffalo (79). These cytoplasts are preferable because of their low cost (due to isolation from ovaries of slaughtered animals) and easy cultivation which allows 90 % maturation. Moreover, the technology of oocytes and embryos cultivation in this species is currently considered the most improved.

In 2000 the cloning of gaur, being on the edge of extinction, was first reported. In this experiment the nuclei of adult male gaur fibroblasts were injected into enucleated cow oocytes, then 44 in vitro cultivated embryos were transplanted to 32 cows and a calf was born alive (33).

Interspecial cloning of banteng (*Bos javanicus*) is good example of species revivalism. For recent 15-20 years it decreased in number by 85 %. In 2003 in USA the measures have been taken to preserve this rare species. Because of absence of autogenic oocytes the interspecial transformation was impossible, so the cow oocytes were used as recipients in transfer of the nuclei from adult banteng male and female skin fibroblasts. The biomaterial was received from the San Diego Zoo's Center for Reproduction of Endangered Species (CRES), where the tissues of endangered animals are stored. In the experiment two calves were born after transplantation of 30 balstocysts to surrogate mother cows (42).

Intriguing progress has been achieved on the home sheep and their wild relatives, especially in the European mouflon. Somatic cell nuclei of adult mouflon female found died in a pasture were injected into enucleated oocytes of domestic sheep, and the recipient sheep were transplanted with embryos, resulting in alive offspring birth. In 2001 (34) the reproductive cloning has been successfully applied to the vanishing species *Ovis musimom.* In the experiment the dead females were a source of genetic material and the efficacy was much higher than at Dolly cloning.

In Spain in 2009 a cloned cub of extinct subspecies *Capra pyrenaica pyrenaica* was born after the bucardo somatic cells were transferred into domestic sheep oocytes and the surrogate mothers of other subspecies or hybrids from crossing domestic and wild goats were transplanted with the embyos. Of 439 obtained embryos the 57 ones were implanted into surrogate uterus, and one bucardo female cub was born but died 7 minute after birth because of breathing problems (47).

A potential of cloning technology discussed hereinabove is based on its current achievements. Nevertheless, it is still unclear whether the cloning technology could be improved soon to optimize the expenses for using nuclei of adult animal somatic cells and the efficiency of the procedure at an acceptable level.

Thus, cryobanking of somatic cells as nuclei donors in cloning is regarded as an assisted technique for conservation and improving gene pools of agricultural animals and poultry. In contrast to gametes, embryos and generative tissues the cryopreserved somatic cells are capable to regenerate after repeated thawing, i.e. almost infinitely may serve as a source of biomaterial for use in

assisted reproductive technologies and biological research, including revivalism and retrospective reconstructions. Besides, the somatic cells are more resistant at cryoconservation due to smaller size. Almost any cells could be used for cloning, but the cell type is essential for efficiency of the procedure. The highest results of embryo development and offspring birth are reached when fetal fibroblasts are used as nuclei donors. Stem cells are an alternative nuclei source. The domestic and wild animals of different species such as sheep, mice, cows, goats, pigs, guar, mouflon, domestic cat, rabbits, mule, horse, rat, wildcat, dog, banteng, ferret, wolves, buffalo, deer, mountain goat, camel, coyote have been obtained by cloning. Cattle still remains the leader in cloning due to offspring output of 10 % and sometimes up to 25 %, while in other species the cloning efficiency is still not more than 1 %. If cloning are used, the best productivity can be reached in commercial herd during one generation, but practical expansion of cloning technology, particularly use of cryopreserved biological material, depends largely on whether there would be the acceptable relationship between the cost of implementation and economical impact.

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MAMMALS AND BIRDS AS AGRICULTURAL PESTS: A GLOBAL SITUATION

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Abstract

On the globe there are approximately 7 thousand species of pests injuring agricultural crops. Because of pests' activity from 33 % (D. Geinrikh et al., 2003) to 40 % (D. Pimentel et al., 1999) of world agricultural products are lost with annual financial losses estimated at $ 244 billion (D. Pimentel et al., 1997). In spite of the fact that the consequences of plant diseases and harmful insects for plant growing and forage production are discussed more often, economic loss caused by mammals and birds, is quite significant. It surpasses substantially losses put by nematodes, mites and molluscs, though far less than caused by harmful insects. As a whole it is to be noted that size of damage from mammals and birds as agricultural pests decreases gradually, remaining, however, still high. The reason of it consist both in application of modern scientific-design development (new technologies of harvesting, methods of pest control, etc.) and decreasing of populations size of wild animals due to reduction of their area and deterioration of the conditions of the habitat. As a preferred group of animals − agricultural pests, rodents were chosen. Their impact on condition of such branches, as production of grain, forage grasses and ensilage crops as well as vegetable growing, orcharding, and melon-growing is described. In California the annual economic loss from *Spermophilus beecheyi* at grain production is $ 8-12 million (S.A. Shwiff et al., 2009). Methods of struggle with rodents are presented. Other types of mammals, the most significant from the standpoint of their injuriousness are described. Information on their composition in a series of world regions is presented. For instance, they are deer, raccoons, and coyotes in the USA, and wild boar, bear and some ungulates in forest temperate zone. The figures of financial loss from them in some countries are given. Impact of plant-eating and omnivorous species of birds on grain-growing, gardening, vegetable growing, seed farming, and breeding of new cultures of plants is considered. In Australia more than 60 avian species are the pests of garden plantings, which cause the annual material loss of $ 300 million (Yu.M. Markin, 2008). The species of birds the most harmful on a global scale are listed, the figures of economic losses from them for a series of countries are presented.

Keywords: rodents, pests, economic loss, damage control measures, globe, agriculture, mammals, birds.

A global influence of nature on people activities as well as anthropogenic impact on environment are surveyed according to the program for the study of human interaction with the environment (1). Pests are a biotic factor which largely determines the effectiveness of providing mankind with food. The issue is very important because of the extent of the damage caused. In the world the losses from 33 % (2) to 40 % (3) and their annual estimation at $ 244 billion (4) were reported.

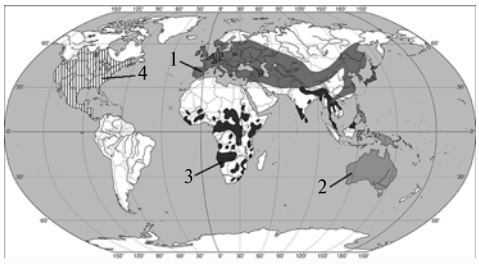

Fig. 1. Global distribution of some mammals and birds damaging agricultural crops: 1 — rooks, 2 — cockatoo, 3 — elephants, 4 — raccoons (http://commons.wikimedia.org; http://www.waza.org).

On the globe there are approximately 7 thousand species of pests damaging agricultural crops (4), the distribution of four of them is illustrated by Fig. 1. Most of them are insects (*Insecta*). A number of harmful pests are mites from *Arachnida* class related to insects. Some pests are described among *Mol-*

lusca: *Gastropoda* and *Vermes*: *Nematoda*).

In this article the role of mammals and birds as agricultural pests are discussed.

Rodents. *General characteristics.* This is the most damaging pests among mammals. Basically they are small animals ranged in weight from one or two dozen to a few hundred grams. Rodents are mainly herbivorous, but in the diet of many species there are also insects, larvae of invertebrates, small reptiles, etc. Rodents differ according to the plant foods they consume. Many species feed on the green parts of plants, which also serve as a source of water. Other rodents require a high-calorie food, and seeds are their preferable diet. In the absence of the necessary feed rodents easy enough change the energy source, for instant they can eat the bark of trees in winter (5).

Extremely high fecundity is a characteristic feature of rodents. The number of small rodents (mice, voles), giving birth to 3-12 cubs up to 5 times a year, can in some years grow 100 times or more (6).

In Russia the rodents damaging the field crops and trees are from *Sciuromorpha*, *Glirimorpha* and *Myomorpha* groups. Among *Sciuromorpha* some species such as *Pteromys volans* L., several species of marmots and ground squirrels, *Castor fiber* L. and *C. canadensis* are considered the potential pests. In *Glirimorpha* the *Eliomys quercinus* L., *Glis glis* L., etc., are potentially harmful. The harmfulness of these pests is mainly not high because of their low number.

With regard to pest species number and their harmfulness the *Myomorpha* group is the most important. *Spalax microphthalmus* Güld., *Cricetus cricetus* L., *Arvicola terrestris*, *Clethrionomys glareolus* Schr., *Microtus arvalis* Pall., *M. levis* and *M. socialis* are the pests from this group.

Rodents mainly damage grains, vegetables and fruits. Herbivorous forms additionally harm grazing livestock. The harmful effect of rodents is primarily due to their diet

The impact of rodents on crops. On grain crops the harm caused by rodents is quite diverse. Rodents can dig up the sown grain, devour seedlings, gnaw stems, cut and eat the ears, etc. The crop losses vary greatly depending on the pests. In the years favorable for their reproduction the consequences are disastrous. For example, in 1914 in Ukraine voles destroyed 80 % of all crops of rye and wheat. In Czechoslovakia in 1950 the damage of winter wheat, rye and barley crops was estimated at 22.3-68.7 %, 19.1-71.6 % and 8.5-15.0 %, respectively. However, even in the years when their number is not above an average score the damage they cause can be quite significant. Thus, in Rostov Province in 1977 the wheat yield was 2.8 or 1.8 t/ha in cases the defense measures against voles were or were not applied (5).

In Indonesia rodents are the main pests on rice crops decreasing the yield by 15 % and more. In Tanzania rodents consume 5 to 15 % of the corn crop (7). In the south-eastern Australia house mouse (*Mus domesticus*) in some years reduces the wheat yield by 3-4 % (8). The annual damage to grain production in California only by California ground squirrel (*Spermophilus beecheyi*) amounts from $ 8 to 12 million (9).

In XX century the losses were mostly related to knitting sheaves at harvesting. Nowadays, in the regions with simultaneous ear maturation the direct harvesting is applied excluding storage of sloping plants in the field and thus significantly reducing the damage caused by rodents. Nevertheless, in case of harvesting by separate combining, when plants are mowed and then dried to ripeness in rolls, the damage from rodents remains significant.

During the development of virgin lands, where the combination of virgin and cultivated territories creates the optimal living conditions for *Citellus*, the

damage caused by them was huge. For example, in 1957 in Alma-Ata region gophers completely destroyed 900 ha of winter wheat crops (5). The greatest damage was typical for a field edge of 50-100 m, where up to 60-80 animals per 1 ha were often caught. Nevertheless, their number was managed and the grain loss minimized.

So far as gophers can cause damage throughout the plant development cycle from seedling emergence to heading, their harmfulness is high. Another important trait is their voracity, as one animal is able to eat up to 22.5 kg of wheat for the season (10). Thus, during the development of virgin lands the losses could amount 1.8 t/ha. Currently, however, due to a sharp decline in the number of gophers they have no economic value. Some destruction of cereal crops is also caused by jerboa, damaging mainly the shoots of maize and wheat, and by hamsters.

Rodent-related damage to vegetable and melon crops. In general, such feeding behavior is less characteristic to rodents and these plants are extra components in their diet. However, the damage is rather clear. Wood mouse damages vegetables and melons on the distance up to 2 km from the woods. It gnaws the flesh and eats away seeds from watermelon, melon, cucumber, tomato. Field mouse damages the root-crops and zucchini.

All melons are damaged by gophers. Jerboa digs the sowings and eats the germinating seeds of watermelon, melon, cucumber and sunflower. Mole rats and *Ellobius*, constantly dwelling belowground, may mainly harm the root-crops. Hamsters and *Myospalax psilurus* Milne-Edwards commonly eat various root vegetables such as beets, turnips, carrots, etc. Field mouse, common hamster, Manchurian Zokor, water voles and great mole rat eat potato leading to yield loss. Vegetable and melon crops are also much harmed by *Nesokia indica* Gray (11).

In Rajasthan (India) the losses of vegetables from the rodents vary from 4.1 to 19.9 % depending on the crop and are of 8.7 % on average. The major pests are *Mus booduqa* Gray and *Gerbillus gleadowi*, at that the tomatoes and eggplants are most harmed (12).

Fig. 2. Loir eats the apple (Great Britain). The animal eats walnuts, chestnuts, berries, fruits such as pears, apples, grapes, cherries, plum, mulberry. In the Northern Caucasus during summer each loir consumes 400 pears. Steven Morris photos, September, 2010.

Impact on fruit crops. Rodents eat seeds and fruits, dug seedling from the soil, gnaw the bark and thin twigs. Feeding specialization among rodents differs. Loirs damage nut crops, as well as stone fruits, gnawing out pits and eating flesh of apple (Fig. 2), peaches and apricots, and also grape is often damaged. Forest dormouse eats and damages apples and pears. Voles gnaw the bark and roots, hares gnaw the bark and lower branches in winter, eat the young shoots, and mole rats and *Ellobius* gnaw roots of fruit and berry crops, causing their weakness.

The fruit yield losses from rodents can be rather significant. In Germany when the number of *Microtus arvalis* increased, the losses of apple trees production were estimated at € 25 million per year (13). In the East USA the *Microtus pennsylvanicus* and *M. pinetorum*, gnawing the bark, damage 5.6 % apple trees, being equal to annual losses from $ 40 million (14) to $ 50 million (15). In hazelnut plantations of Cuneo, Italy, the number of *Glis glis* ranges from 4.13 to 247 individuals per 1 ha, and in the areas of the highest concentration they consume up to 61 % of the hazelnut crop (16).

Impact on other crops. Their list is not limited to grain, vegetable and fruit crops. The *Meriones erythrourus* Gray significantly damage cotton plants, crunching boxes and husking seeds. Field and wood mouse, as well as gophers actively damage the sunflower crops. Thus, *Citellus pygmaeus* Pall. gnaw off the upper part of seedlings what makes a young plant to produce a lot of side shoots, and as a result such plants are removed at weeding.

Many rodents are the pests of sugarcane. They feed both on upper parts of the plant and on its roots. They also damage the roots when digging holes, causing plant lodging in the wind. In addition, microorganisms can get the injured stems, thus reducing the sugar content. In India the major pests of sugar cane are *Bandicota bengalensis*, *Tatera indica* and *Mus musculus.* They cause from 8.6 to 12.1 % losses in sugarcane with 31.5 % decrease in weight and 24.5 % decrease in sugar content (17).

Rodents as pests of pastures. In addition to a significant destruction of green mass, rodents alter the qualitative composition of grasses, eating the most valuable forage species. The *Rhombomys opimus* Licht. is a harmful pest of pastures in the Central Asia and Southern Kazakhstan. These animals eat prostrate summer-cypress, sedge, wheatgrass, astragalus, and thus are a serious competitor to livestock, especially sheep (5).

The following data can illustrate the rodent-related damage to livestock. Thus, *Meriones erythrourus* Gray in case its number is within an average limits can eat up to 20-30 % of valuable grasses. At gophers' population density of 30 individuals per 1 ha, the yielded green fodder dry weight may be annually reduced by more than 1125 kg/ha, or by 38 %. Vole population density of 250 individuals per 1 ha is capable of destroying up to 4 % of the annual alfalfa harvest. Marmots eat daily up to 0.45 kg of green fodder, and even more plants they trample when paving their paths. Gophers from North and Central America consume a quarter to a third of the crop of alfalfa (10). In Poland during the outbreaks of common vole *Microtus arvalis* these animals damage up to 45.8 % of the alfalfa (13). One pika when procuring food can collect plants in haystacks of 20 kg in weight (18).

Damage from rodents and their control. It is obvious that the global damage from pests like rodents is very significant. According to some estimates, they damage or destroy $^1/_5$ to $^1/_3$ of agricultural products (19), but these figures are obviously overestimated. The damage is basically rat- and mice-associated. In Asia rats eat annually the food enough to feed 200 million people (20). In 10 counties of the State of California (USA) the annual financial losses of crop from rodents and birds range from $ 168 to 504 million (9).

Technological, mechanical, chemical and biological approaches can be used to prevent losses from rodents.

Technological measures are mostly preventive and restrict animal spreading and reproduction. For instance, in case the yield is completely removed from fields, the rodents in winter are short of food that they need to stay alive and reproduce. Deep plowing which destroys holes and crop rotation also can serve as preventive technologies. Mechanical methods include the extermination of rodents with traps, snares, etc. Poisoned food baits, powdered preparations for dusting burrows and the gaseous toxic substances, mainly used indoors against rats, are the most popular chemical preparations applied. Biomethods include the use of natural enemies of rodents such as cats, dogs, кошек, hedgehogs, ferrets, mongooses and birds of prey, etc.

Other mammals. *General characteristics.* In most agricultural pests the harmfulness is related to plant consumption as a fodder. In mammals the harmfulness is mainly caused by simple moving through and under the plantings.

The *Talpa europaea*, when daggering their tunnels, destroy plant roots thus interfere plant nutrition that often results in plant death, also they eat the earthworms valuable for soil fertility. A significant harm is due to crop trampling by large mammals. In general, destroying agricultural plants by wild animals is rather common.

In different regions the list of large animals injuring crops changes significantly. In USA the 53 % of the interviewed farmers indicated *Odocoileus* spp. being the most harmful, while *Procyon lotor* and *Canis latrans* were considered the most problem species by 25 % and 24 % of respondents, respectively (21).

In South China (Yunnan) the major pests are elephants, monkeys, bears, gaurs, deer and wild pigs, at that 90 % losses are due to *Elephas maximus* (22).

In Africa many animal species cause losses in agriculture. Of them the elephants, baboons, monkeys, hippos, porcupines and bats are the most harmful, and the banana, cocoa, maize, rice and sorghum plantations are most damaged.

In each country the main pests are specific. In some territories of Uganda they are elephants *Loxodonta africana* which destroy up to 21 % of total yield (23), and in Entebbe suburb green monkeys *Cercopithecus aethiops* mostly harm the fruit plantations (24). In Zambia *Potamochoerus porcus*, *Loxodonta africana*, *Papio ursinus*, *Cercopithecus aethiops*) *Hippopotamus amphibius* and *Hystrix cristata* are the main harmful mammalians (25).

In the forest area of the temperate zone the problem species are wild boar, bear, and some ungulates. Thus, bears damage the oat fields that they visit as the grain begins to fill, but especially strong impact occurs at the phase of wax ripeness (26). In the remote areas of the Leningrad and Vologda provinces 2-3 bears got into the habit to visit oat crops sometimes completely suck and trample up to 10-15 ha during an autumn season (27). In France crop damage by wild boars and deer in 2007 was estimated at 22-23 million Euros (28).

Sometimes foxes and jackals damage the crops. Finding melon field, they regularly visit it until the end of the harvest, and eat only ripe melons. In some areas these animals consume up to 5-7 % of the crop (29).

In many parts of the globe the dangerous pests include hares, i.e. *Lepus europaeus*, *L. timidus*, *L. tolai* and *L. mandshuricus.* Hares eat sunflower, buckwheat, cereals, vegetables and melons, they also can chew the bark of 10-15 apple and pear trees during a night. The most serious problems are caused by hares in Argentina, Australia, and to a lesser extent in North America (30).

Economic damage from wild mammals. Evaluations of economic damage due to them are few, and those reported are not reliable as often based just on the words of the victims. In the United States in 1992 each of the surveyed farmers spent an average of 43.6 hours and 1002 dollar, trying to stop the damage from wildlife or prevent it (18). Throughout the country only in 1989 the wildlife-induced crop damage amounted $ 343 million. The main part of the losses ($ 247 million) occurred in field crops, $ 53 million lost resulted from the damaged vegetables, fruits and nuts, and another $ 16 million loss was related to other agricultural crops (31).

P.D. Curtis (22) estimates the annual crop losses from wild animals in USA at $ 500 million. The total losses, including livestock husbandry, exceeded $ 1 billion (32). In Xishuangbanna of Yunnan, China, in 1994 the losses from wild animals in agriculture reached 4 million yuans, being equal to about $ 482 thousand (22).

Indian elephant as a harmful species. These animals are the most harmful pests among mammals. About 20 % of the world's population presently lives in the elephants' area or near the places of their habitat. In most areas where the elephants are spread they greatly harm the crops, mainly rice, corn, bananas and

green peppers (22).

Just trampling can cause significant losses. In Salakpra of Thailand in 2006 there were registered 462 invasions of elephants to farmers' fields (33). Elephants also mark trees and bushes, and sometimes they knocked them to demonstrate or test the power or for emotional release.

Fig. 3. Canadian geese on a wheat field in Colorado (USA). They are not priority pests, but eat both young seedlings and grain (http://lib.colostate.edu/research/agnic/crops.html).

Very big losses, an average 1,121 billion Rs, or $ 11,928 million are due to elephants in Sri Lanka (34). The plantations are most attacked in the southern regions, where an average loss during vegetation is 12049 Rs per each farmer, or $ 128, that is about a third of earn. Besides, most farmers, up to 70 %, should spend significant money to protect their yield. Some of them cultivate less valuable crops, such as sweet potato and cassava, at the edges of fields to reduce the damage of valuable crops, and some use unfixed dates for seeding and harvesting in the view not to lose the whole yield at an overnight raid of elephants. Another way to protect crops is using plants rejected by elephants. In the areas of the most violent conflict the peasants simply leave the arable land because the tries to grow crops are obviously senseless (35).

Birds as pests. This problem is known for a very long time. The study of frescoes and paintings on ancient Greek and Egyptian vases shows that even then man suffered from an attack of birds on vineyards and orchards. The main damage from birds is due to their food-getting activity, which leads to losses of agricultural and raw materials at all stages of growth, primary processing, storage, transportation and industrial use. Herbivorous and omnivorous birds cause damage to grain (Fig. 3), fruit, vegetables, and seed production, and also to plant breeding (36).

The use of those or other plants is determined by taxonomic position and the ability to find suitable feed. Thus, the *Fringillidae* birds such as finches, siskins, goldfinches, crossbills, etc., and *Ploceidae*, namely sparrows, fanciscanus, etc., cause the extensive damage to agriculture in the southern regions of the European Russia, Ukraine, Moldova, Kazakhstan and in the Central Asia, eating seeds of sunflower, cannabis, millet, and wheat (10).

In Krasnodar Krai the rooks and other corvids, starting with the spring time, hatch out of the ground the just sown seeds of sunflower, soybeans, and later damage their seedlings by pulling young plants or biting their appeared cotyledons (37). In the state of North Dakota (USA) the sunflower and corn crop losses from birds were 1.2-3.0 % (38).

A threat from some species can be great. Thus, the population of *Quelea quelea* from Africa is the biggest. These birds form migrating colonies, each being up to 30 million individuals in number. They eat sorghum, wheat, barley, rice, sunflower and maize. A colony of 2 million individuals can consume 50 tons of grain per day, for which they are sometimes called feathered locust (39). In Scotland up to 25 % of oat and 20 % of barley are eaten by rooks. In USA an average grain losses from *Agelaius phoeniceus* is 16.2 % (40). Annual grain

losses of 60 ton from *Grus grus* L. are reported by Yu.M. Markin in the European Russia (41).

Fig. 4. Young starling pecks pear (New South Wales, Australia). Starlings are among the most harmful birds, they are most dangerous for gardening (eat grapes, cherry, plums, cherries, apricots, apples, etc.). Photo: B. Lukins. January 30, 2004.

Significant damage to vineyards in Southern Europe and North Africa, up to a third of the harvest, is caused by birds, mostly pink starlings and common starlings (42). Stone fruit trees such as cherries, plums, apricots, etc., are suffered from these representatives of the avifauna. In Uzbekistan the range of grape pests is much wider and includes Turkestan starlings, common myna, several species of sparrows, crows and magpies. According to A. Dzhabarov (43), from 7.0 до 23.2 % of grape yield is damaged in the southern-western Uzbekistan. Birds spoil the best berries, and the grapes lose the marketable condition. Woodpeckers, starlings, magpies, and fieldfare peck different kinds of berries, the damage to strawberry is particularly widespread. In central Russia pecking soft apples is typical.

In Ferrara province, the Northern Italia, pheasant is most economically important pest bird, damaging crops and vegetables. Starlings and sparrows cause significant damage to fruit and grapes. Magpies damage melons, watermelons and melons. Wild pigeons and ducks consume forage grasses (44).

Economic losses caused by birds on crops. Damage from birds is quite large. For example, in Australia the annual losses only in gardening are $ 300 billion (42). In this country there are more than 60 species of birds, damaging horticultural crops (Fig. 4). In Africa, the damage to crops only from *Quelea quelea* is $ 70 million per year (39), including $ 6.3 million in Sudan and about $ 1 million in Somalia (45). The annual cost of the vineyard invasions of common and pink starlings only in Tunisia amounts 8-10 million DM (42).

Over time the species composition of bird pests can vary, sometimes dramatically. For example, in Germany in the late 1970s the greatest damage was due to starlings (10 million DM), blackbird (2.4 million DM), wood pigeon (2 million DM), and rook (0.1 million DM). In the early 1990s wild wintering ducks and geese (42 million DM), wood pigeon (1 million DM) and corvids such as crows, jackdaws, rooks, magpies, etc. (0.5 million DM) became the most dangerous (46).

In conclusion it should be emphasized that the financial damage from mammals and birds is still very significant. For example, in the United States in 2001 it amounted to $ 619 million for field crops and $ 146 million for the fruit and nut crops (47).

Thus, despite the consequences of the diseases and invasion of insects for crop and forage production are commonly discussed, the damage from mammals and birds is much greater than, for example, the losses inflicted by nematodes, mites and slugs, although much less than those caused by insect pests. In general, the damage caused by mammals and birds as agricultural pests is gradually reduced, however, remaining still high. The reasons lie in the application of modern scientific and engineering developments such as new harvest technology, the measures for plant protection, etc., and also in reduction of wildlife populations due to the limitations of their natural habitat and deterioration of environmental conditions. Among the animals the rodents still are a priority group of

agricultural pests, but significant damage is caused by other species. Their sets vary depending on the specificity of regional conditions and cultivated crops.

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PROBLEM OF EGG PRODUCTIVITY IN HENS AND ITS EARLY PREDICTION

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Abstract

The generalized processes of natural maturation of oocyte with egg yolk in the ovary, production of egg white and eggshell in the oviduct, as well as ovulation and laying cycle are under the genotype control. Besides, they are affected by the environment and regulated by neurohumoral (neurohormonal) system. All these factors determine ultimately the egg productivity in poultry. Thus, it is limited by time, required for the oocyte maturation and yolk synthesis, and also by the decreasing reproductive potency of hens and egg quality because of aging and/or influence of technological factors. Whatever it was, the observed phenotypic variability due to selection pressure and the applied intensive technologies in poultry indicates the physiological limits of productivity (i.e. egg-laying rate, egg weight and quality) to become wider in hens with intensive metabolism. In the modern egg hybrid crosses a daily ovulation occurs synchronously with a mature egg formation and laying during long productive usage. The egg laying chickens can be forecasted in the postembryonic ontogenesis by the number of large follicles which are maturing in the ovary, if 85-90 % egg productivity is reached within 5-6 weeks after the laying begins. The steady cycle of egg laying with small interval (1-2 days) is also of importance. Thus, the rapid formation of 5-6 follicles in the ovary coinciding with weekly maturation of the yolk in ovogenesis determines the biological potency of reproductive function in high-yield laying hens. The use of these parameters for assessing egg chicks and their offspring gives an opportunity to predict the high-producers in the early ontogenesis. It will speed up breeding to improve modern egg crosses, create new ones, and also to optimize the intensive technologies in poultry.

Keywords: egg productivity, chicken genome, oocyte, ovary, ovogenesis, egg-laying, integrity, concept, prediction.

Since 1950s due to long and intensive breeding and hybridization the annual egg productivity in hen crosses increased almost 2 times and amounts 320-330 in number and 60-65 g in an average weight compared to 55-60 g. The weight of shell and white of the eggs mostly increases, while the yolk weight in selected lines of egg crosses is stabilized at 16-17 g, being up to 18 g on average in hybrids (1). Hence, egg productivity almost reached the biological limits.

Complicated processes of natural maturation of oocyte with the yolk in the ovaries, the egg white and shell formation in the oviduct, ovulation and oviposition are genetically based, neurohumorally regulated and depend on the environment. Therefore, egg production is limited by the time of an oocyte maturation and the yolk formation, on one side, and a reduced productivity and egg quality upon hen aging and(or) because of used poultry technologies, on the other side.

Study of genome structure and functions in the hens with high egg production are being intensified resulting in developed and improved technologies for poultry breeding and commercial use. Thus, early prediction of egg production is essential.

Genetic bases for egg productivity and breeding. Selection and crossing based on advances in population genetics are the main approaches in poultry breeding. The high yielding egg hen hybrids is due to strict selection for desired traits in the lines and under crossing the matching lines. For long

time the occurring genetic diversity was enough to keep up the variability of polygene traits and increase hen productivity. The DNA technologies based on genetic polymorphism and multiple molecular markers allowing early forecasting are currently used (1).

Hen haploid genome sequencing showed 24 000 genes and more than 1×10^9 bp in polymorphic regions reflecting genetic diversity. SNPs (single nucleotide polymorphism) are basic at marker assisted selection (2). This approach is most effective in case the valuable traits are sex-linked. Early screening young cocks from the lines and related groups reduces the time required for selection over generations.

In hen genome above 3 million SNPs are reported allowing to locate the markers for polygenic quantitative traits (3) and to identify genes of structural proteins, multiple regulatory proteins and the markers being used in genome mapping and poultry breeding. A development of the chips for more than 600 000 nucleotide sequences identified in the commercial lines has been reported (4). Complete hen genome sequencing should simplify detecting polymorphic DNA regions, marking specific nucleotide sequences, and mapping structural genes and loci involved in the production control. Particularly, the cytogenetic mapping, linkage groups and polymorphic markers were basic in the last reported version of the hen integrated chromosome map, including 1889 loci of which 450 loci formed 50 linkage groups (5).

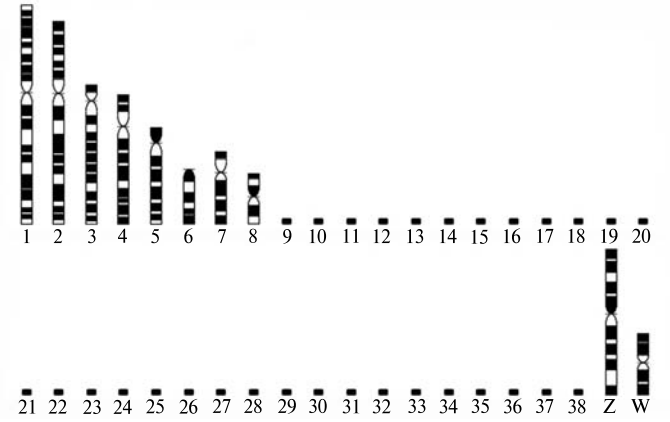

Fig. 1. The chicken (*Gallus gallus*) chromosome karyotype (a haploid set and two sex chromosomes) (38) (http://www.thearkdb.org).

Genomic QTLs screening revealed 29 loci on 5 of 8 described macro chromosomes, the chromosome 2, 3, 4, 5 and 8, and also on sex Z chromosome (Fig. 1). There were the loci contributing to body weight and egg size, feed conversion, etc. The 8 macro chromosomes and Z chromosome are 70 % of the chicken genome nucleotide sequences, and the rest 30 % sequences are the micro chromosomes. Gene activity and indirect evidence of an increased gene density in the micro chromosomes have also been shown (6).

Chicken Z or X chromosomes contain the genes affecting productivity. There are auto sex homozygotes different in color due to *K* and *k* alleles and in the rate of feathering due to *S* and *s* alleles, resulting in clear differences between the day-old chickens depending on their sex. Using dwarf gene (*dw*/*DW*) the maternal lines with reduced weight and increased egg productivity were developed and further involved into meat cross Hubbard ISA F15 breeding (7).

The alleles of restricted (*ro*) and normal (*RO*) ovulation located on Z chromosome are significant in view of egg productivity prediction. Sometimes the laying hens homozygous for *ro* are unable to ovulation of the oocyte with yolk because of a spot mutation and insufficient transport of lipoproteins from liver to follicles at yolk formation (8). Also the size of some bones in wings and legs as well as their mineral content are influenced by genes of restricted ovulation contributing the increase in weight compared to highly productive

poultry (9).

The Hy-Line International (USA) recently uses the genome marker assisted selection focused on the desired commercial traits and the early predicted productivity. The main valuable traits are the high lifelong productivity in hybrids during 80 weeks and more, tolerance to stresses and disease resistance (http://www.hyline.com).

Ovognesis in high-producing laying hens. Morphogenesis of ovaries and oviduct, development and maturation of oocyte, egg formation and time the hen reaches puberty and starts to produce eggs are related to ontogenetic periods. Due to breeding for past 60-70 years the age of the first oviposition in egg hens decreased 1.5 times from 6 months (26-27 weeks) to 4 months (17-18 weeks). However, bone strengths in wings and legs during post embyo development also lowered. The selection for strong bones together with limited feeding, and also the stabilization of 1.2-1.3 kg weight from the beginning to the end of hen commercial use are the countermeasures applied (10).

Some changes in the development of ovary function in hybrids occur at generative phase as observed in Loghmann Brown. The gonad rudiments are formed during 4 days, and from day 8 their differentiation into ovaries or testes begins. From day 11 the primordial germ cells turn into oogonia, and their number reaches several hundred thousands due to fast mitotic divisions. To day 19 most oogonia die, and just several differentiate into oocytes able to form follicles (11).

Until chick hatching the follicular stage prevails and results in formation of primary oocyte. In the ovary of 1 day hen there are 3500-4000 primary oocytes of 0.01-0.02 mm in diameter and 30-50 mg in total weight. The developing oocyte and the ovary interact via the follicle. Primary oocyte (with no yolk) contains the large nucleus with diploid chromosomal set tightly surrounded with vitelline membrane envelop (12, 13).

At vegetative phase the primary oocyte growth and development occur in the ovarian follicles until the mature oocyte ovulates into the oviduct. In turn, this phase is subdivided into several stages (Table 1). During 1-6 weeks the oocyte slightly increases in size due to cytoplasm accumulation, the latter being 0.5-1.5 mm in diameter, the germinal disc appears, and nucleus increases in size.

1. Phases of oogenesis in high-producing laying hens

Phase, stage	Object, organ	Time	Product
Generative phase (embryo period)	Embryo—one day old chick	8-21 days	Primordial germ cells — oogonia → oocytes
Vegetative phase (post embryo period)	Ovary	From 1 day to 17 weeks	Oocyte — primary oocyte → mature oocyte
Growth of oocyte	Ovary	From 1 day to 6 weeks	Oocyte with germinal disc and cytoplasm
Development of oocyte	Ovary	7-16 weeks	Oocyte with developing yolk as a single cell
Maturation of oocyte	Ovary	16-17 weeks	Mature oocyte with yolk
Ovulation	Ovary	About 30 minute	Gamete with haploid chromosomal set redy to fertilization
Laying period in hens	Ovary and oviducти	18-73 weeks and more	Primary oocytes at different staged in the course of rhythmic ovulation and oviposition)

The longest second phase of ovogenesis occurs from week 7 to week 16, when yolk mass increases due to blood transport of lipo- and glycoproteins, triglycerides, phospholipids, cholesterol and other biologically active substances from liver to the follicle and their accumulation.

Oocyte maturation occurs from week 16 to week 17. An accelerated yolk formation is subsequently completed in several large follicles within 1 week period. Fast yolk accumulation in the ovarian follicle takes 5 to 6 days, when pri-

mary oocyte turns into mature oocyte, or secondary oocyte, and ovulates into the oviduct. Breaking follicle envelope and ovulation itself take 15-30 minutes.

A mature oocyte (blastodisk) is located on yolk surface as a single cell surrounded by vitelline membrane. During diploid to haploid transformation the meiosis I is completed before ovulation, and meiosis II occurs after the ovulation in the oviduct resulting in a haploid gamete. Due to yolk it is provided with nutrients and the required bioactive substances. Each oocyte with yolk is subjected to long changes in hens from 1 day to 16-17 week age regardless of whether there was or was not the fertilization. After egg white and shell appear, the egg formation is completed.

Domestic hens are peculiar in their capability to produce eggs without fertilization. This is essential to determine the period of their productive use and biological limits of productivity both in cases the fertilized eggs used for incubation and unfertilized eggs used for food.

Current concept for egg formation. After ovulation, as the yolk is moving along the egg tube, the egg white and shell formation occur, resulting in egg laying. It takes about 22-24 hours, including 20 minutes in the infundibulum, 3 hours in the magnum to add albumen, 1 hour in the isthmus, and 18-19 hours in the utherus. Shell formation takes 17-18 hours, thus being second essential factor to restrict egg production in hens (14).

Earlier the maximal rate of transfer of nutrient from blood to follicle per its surface area was observed for 2.5 g oocytes (cited by 13). It could be considered the initial follicle in each set of fast maturating follicles. As the surface in each next follicle enlarges the rate of yolk formation increases because of increased follicle envelope.

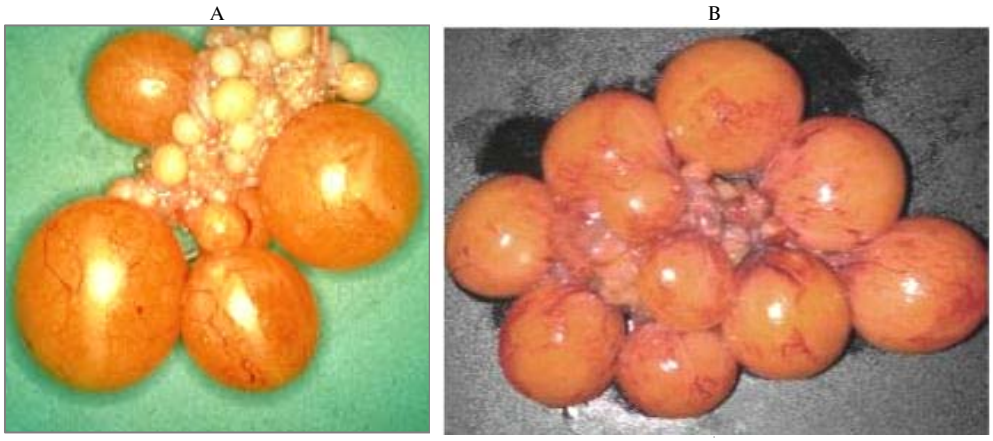

Fig. 2. Large follicle in ovaries of high-producing laying hens (A) **and hens with combination of meat and egg productivity (B) in White Plimutrok poultry (Cobb cross) (10).**

In high producing laying hens with more than 300 egg per year there is the first biggest follicle (Fig. 2, A), designated as the pre-ovulate one, with the rest ranged on their size and weight. In the photo the minimal number of follicles indicates the ovulation of the biggest follicle in the set occurred earlier. Yolk weight in hybrid hens is reported to be 18 g (15).

At relatively low egg production of 210-230 eggs, which are characteristic for the modern crossed with combination of meat and egg production and also for the maternal line of meat crosses, not less than 8-10 large follicles could occur simultaneously in the ovary. It leads to desynchronized ovulation and oviposition, especially during the second half of productive use, resulting in two yolk eggs of 75-80 g in weigh.

In ovary of White Plimutrok hens (see Fig. 2, B) none of three the largest follicles differed from each other in size, so each could be a pre-ovulate one. In such a case almost simultaneous ovulation of two follicles is possible leading to double yolk eggs and indicating desynchronized ovulation, laying and oviposition. Double yolk eggs also could be produced by young hens with still unstable cycle of egg laying. The eggs of changed shape or deviations in other morphometric parameters can indirectly indicate a desynchronized laying.

Laying cycle being individual for each hen is significant for high and sustainable productivity. The period of successively laid eggs defines the laying cycle. In high-producing hens there are long cycles of 50 to 80 days followed by 1-2 day interval. The frequency of repeated cycles with intervals determines the laying rhythm during time of productive use.

High producing hens are peculiar in ability to rapid accumulation of the nutrients and bioactive substances in 5-6 large successfully formed follicles at 6-7-day period of oocyte maturation. In the biggest one the yolk weight mostly increases 24 hours before the ovulation, with the follicle diameter of 2-35 mm in young hens and 40-42 mm in the adults. The follicle number in the ovary reflects the level of reproductive function and laying potency in poultry (16).

Egg white and shell formation is synchronized with the ovulation and laying cycle, determining time of egg formation under different follicle number in the ovary (Fig. 3). We suggest the coefficient of follicle growth as an indicator of its daily increase in weight. This coefficient is calculated from the difference in weigh gain (18.0-2.5 g) divided by the number of follicle in the ovary (see Fig. 3).

In case there are 5 follicle in the ovary the coefficient of growth is 3.10 thus being the highest, while for 6, 7, 8 and 9 follicles it is 2.58, 2.21, 1.94 and 1.92, respectively. So if in the ovary there are 5-6 follicles with maturing oocyte and yolk the growth coefficient is higher.

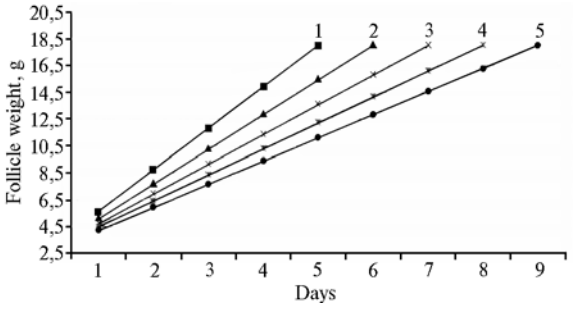

Fig. 3. Calculated time for the large follicle formation depending on their number in the ovary of laying hens: 1-5 — 5, 6, 7, 8 and 9 follicle, respectively.

Previously 7-9 and more large follicles per ovary considered appropriate and determining egg productivity in meat and egg producing breeds. However, it should be noted that the less number of successively maturing follicles is found, the faster ovulation occurs, resulting in higher egg productivity observed.

During ontogenesis the genetically determined, complicated and long processes of oocyte-yolk complex growth and maturation are mostly limiting for egg production. In high-producing poultry the biosynthetic activity and accumulation of yolk components in the large follicles are enough to provide their rapid formation and daily ovulation with corresponding rhythm of the oviposition. Laying 361 eggs for 364 days by a Leghorn hen probably is a biological limit of the egg productivity.

Forecasting egg production. Hybrid hens are accelerated in growing and start to lay eggs being 16-17 week-old. The earliness of maturity in a hen group is estimated at an average 5 % laying intensity, being determinative for further total egg number. Inherited laying rate at the beginning of oviposition is the factor mostly impacting egg production.

In laying hens the ovaries and oviduct, as well as other organs, have to function hard, especially at the beginning and on the top of oviposition. The nutrients and bioactive components of yolk, white and shell actively produced in liver are transferred to follicles, ovary and oviduct. The age of puberty also is an important factor. In high-producing egg hens the puberty is considered reached at 50 % laying rate achieved. In the crosses of the Hy-Line International (USA) this time has been decreased by almost 3 weeks over past 30 years (17). Accelerated growth and puberty are also peculiar to other egg crosses determining speedup of top productivity which is typical for hen hybrids (Table 2).

The weight from 1 day-old chicken until the end of laying period is the main controlled parameter indicating growth, development and physiological performance in hens. Breeding for increased egg production is effective due to weight optimization by limited feeding. In closed flocks the line breeding allows permanently lengthening the period of oviposition with keeping up the laying rate above 85 %.

2. The main parameters of egg productivity in Hy-Line International hen crosses (USA)

Parameter	1980	2002	2009
Age of 50 % productivity, days	161	145	143
Top laying rate, %	92	95	96
Survival, %	92	96	96-97
Total egg number per a hen	263	323	326
Weight of a 74 week-old hen, g	2470	2000	1980

During 2009-2010 international competition held in Czech Republic the Novogen Brown hens of Novogen S.A.A. (France) produced 351 eggs on average for 385 days at 91.2 % intensity (18). This level is almost the same as biologically limited (Table 3). Hereinabove we ranged these results with respect of total egg weight in kg as general commercial parameter of egg production.

3. Яичная продуктивость гибридных кур (55 нед, международные конкурсные испытания, Чехия, 2009-2010 годы)

Crosses	Laying rate per a hen		Average egg weight, g	Total egg weight, kg	Survival, %	Feed conversion ratio per 1 kg of total egg weight
	egg number	%				
Novogen Brown (Novogen S.A.A., France)	351,1	91,2	61,5	21,59	99	2,32
Hisex (Hendrix Poultry Breeder, Netherlands)	346,1	90,0	61,7	21,35	96	2,32
Lognmann (Loghmann Tierzucht GmbH, Germany)	338,9	88,0	62,5	21,18	99	2,34
isa Brown (ISA A Hendrix Genetics Company, Netherlands)	342,1	88,6	61,6	21,07	98	2,37
Hy-line (Hy-Line International, USA)	336,6	87,4	61,9	20,84	98	2,33
Super Nick (H&N International GmbH, Germany)	330,1	85,7	62,3	20,56	96	2,44
Tetra («Bábolna Tetra Kft., Hungary)	330,1	85,7	62,1	20,50	95	2,41
Bovans (ISA A Hendrix Genetics Company, Netherlands)	326,8	84,5	62,7	20,49	96	2,45

Thus, phenotypic changes due to selection and breeding show more wide physiological limits for egg productivity. High-producing layers are capable of producing a mature oocyte-yolk complex which ovulates with further egg white and shell formation and oviposition at daily cycle. The laying hens with high egg production can be forecasted in the postembryonic ontogenesis by the number of large follicles which are maturing in the ovary, if 85-90 % egg productivity is reached within 5-6 weeks after the laying begins. In case there are 5 to 6 large follicles in the ovary their maturation takes 6-7 days. Just a coincidence of these

parameters we consider the key determinative for the reproduction potency and early forecast of egg production in poultry.

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FORAGE CROPS SELECTION: PROGRESS AND CHALLENGES

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Abstract

In the Breeding Center of V.R. Williams All-Russian Fodder Research Institute more than 150 varieties of forage plants have been created for 42 years. Of these, the most widespread 85 varieties cultivated in Russia are not inferior to the best foreign varieties in productivity and exceed them in hardiness, the edaphic resistance to acidity and salinity, and phytocoenotic compatibility in mixtures. The *Trifolium pratense* L. varieties VIK 7, tetraploid VIK, Altyn, Topaz, Rannii 2, Trio, Mars, and the *T. repens* L. varieties Yubileynii, VIK 70 are characterized by a precisely expressed ecological individuality, symbiotic activity and different maturing time. The *Medicago* L. new varieties Vega 87, Lada, Nakhodka, Pastbishchnaya 88, Lugovaya 67, Selena, Soleustoichivaya, with the dry matter yield at 12-14 t/ha in the Central non-Chernozem zone, possess various phytocenotic, edaphic, symbiotic properties. More than 40 new varieties of *Bromopsis inermis* Leys., *Phleum pratense* L., *Festuca pratensis* Huds, *Dactylis glomerata* L., *Lolium perenne* L., *Poa pratensis* L., providing yield of 11-12 t/ha and above, are characterized by improved fodder quality and resistance to diseases. In recent years, the varieties of *Vicia sativa* L. and *V. villosa* Roth., high-resistant to environmental and phytocoenotic factors, are created for different use. The *V. sativa* L. varieties Lugovskaya 48, Uzunovskaya 91, Vera, and the *V. villosa* Roth. variety Lugovskaya express precocity and a reduced insistence to heat, especially during fruiting. For restoration of lost biodiversity and efficiency of the degraded pastures in arid areas of the Russian South, 18 new varieties of fodder xerohalophytes are created, particularly Barkhan, Dzhangar of *Kochia prostrata* (L.) Schrad., Salang of *Salsola orientalis* S.G. Gmell., Nogana, Alsu of *Camphorosma lessingii* (Litv.), Favorite, Tulkin, Bar of *Eurotia ceratoides* (L.) C.A. Mey., etc. These varieties are widely used for restoration of deserted lands. With reference to perennial bean and cereal grasses, more than 25 breeding technologies are patented in the Russian Federation. Adaptive systems for seed reproduction and the «know-how» for growing perennial and annual grasses are offered. With regard to the achieved results and recent data of fundamental biology a new breeding paradigm is substantiated, based on biogeocenotic principles.

Keywords: breeding, seed, fodder crops, breeding techniques, alfalfa and perennial grasses, arid forage plants.

December 2014 marks the 42nd anniversary of the founding the selection center as part of the W.R. Williams All-Russian Fodder Research Institute. Formed in the Soviet Union the complex for breeding and seed production of fodder crops currenly has fairly high scientific potential of 6 specialized and 12 complex breeding centers and more than 20 scientific breeding and seed production units in research institutions. The Breeding Center of the W.R. Williams All-Russian Fodder Research Institute is leading in the development of theory and practice of seed selection.

The main fundamental and applied aspects the researchers of the institute are working on are as follows: i) creating system of climatically and environmentally differentiated and economically specialized fodder crops complementary each other; ii) development and improvement of breeding and selection technologies; iii) original and commercial seed production; iiii) education; iiiii) coordinating national and international programs on fodder crops.

In the W.R. Williams All-Russian Fodder Research Institute there were created more than 150 varieties of clover, alfalfa, annual leguminous fodder crops, perennial cereal grasses, arid fodder plants of new generation, including 85 varieties for past 20 years. The progress in fodder grass production in most

Russia regions, particularly in the humid zone, is most due to clover breeding. The studies of biological, ecological and agronomic peculiarities of clover to which P.A. Lisitsyn, I.S. Travin, V.D. Shcherbachev, P.A. Sergrrv, the famous Russian scientists, greatly contributed, were initiated in 1930s. Thereat an extensive breeding based on mass selection was mainly used. Since the middle 1950s the research group of clover breeding, and then the Department, this group was transformed into in 1972, were headed by A.S. Novoselova.

Due to theoretical and methodological advance, 25 climatically and ecologically differentiated clover varieties such as VIK 7, Tetraploidnii VIK, Mars, Rannii 2, Trio, Altyn, Topas, Dobrynya, VIK 77, TOS 870, Orlik, VIK 84, Stodolich, Ratibor, Mariya, Pamyati Lisitsyna, Pamyati Burlaca (*Trifolium pratense*); Yubileinii, VIK 70, Lugovik (*Trifolium repens*); Pervenets, Mayak (*Trifolium hybridum*) were derived in this Deparment, recently headed by M.Yu. Novoselov. Chemical mutagenesis and experimental polyploidy were basically used. These varieties are effective in the northern Russian regions providing dry weight mass yield of 10-12 t per 1 ha. Ultra early ripening clover varieties contribute to expansion of sustainable seed production zone 300-350 km to the north and up to 70 km to the east with the seed yields of 306 centner per 1 ha, being enough to meet the demand on seeds in Russia and to sell abroad to Belarus and Germany (1-4). The Topaz variety obtained by edaphic selection is tolerant to soil acidification (pH 4.5-5.5) and produces the dry weight biomass and seeds at 10-11 t per 1 ha and 2-3 centner per 1 ha, respectively (1).

Our priority lies in scientific, methodological and management interrelations of the experts from 14 institutions taking into account varying soil and climatic conditions on the Russia territory. The monograph «Ecological breeding of clover» (Moscow, 2012) summarizes the results of these complex investigations. In 1999 the creation of the varieties of red clover basic for sustainable forage production and agriculture biologization in Chernozem zone of Russia was awarded the State Prize of the Russian Federation in Science and Technology.

Alfalfa is an important leguminous fodder plant in the world. Nevertheless, it was atypical in the Russian Nechernozem'e under relatively low temperatures, specific water regime and the acidic podzol soils. Due to theoretical experiments initiated in 1930s, in 1956 the Severnaya Gibridnaya variety capable to accumulate enough biomass but, unfortunately, failed to produce mature seeds has been originated by A.M. Konstantinova. Further a new generation of the alfalfa varieties was obtained, namely Vega 87, Lada, Pastbishnaya 88, Lugovaya 67, Selena, Soleustoichivaya, Sonata, Nadezhda, Nakhodka, Galiya, Agniya, which are specific with respect to their environmental, phytocenotic, edaphic traits and agrotechnologies. Vega variety with seed productivity at 2-4 centner per 1 ha was the first variety capable to reproduce successfully in the Central Nechenozem zone. Using phytocenotic selection the Pastbishnaya 88, Lugovaya 62, Nakhodka varieties were bred which in combination with cereal grasses guarantee the dry weight yield of fodder mass at 11-13 t/ha and up to 2.5 t/ha protein output. A phytocenotic longevity is the main peculiarity of these varieties, as after 4-5 year exploitation the ratio of leguminous component is not less than 35-45 % (5, 6). According to L.G. Ramenskii (9), there is a threshold for prosperity of the species when its number is enough competitive. With respect to the competitiveness the Pastbishnaya and Lugovaya varieties overcome the threshold for prosperity being sustainable botanical species in polycomponent agrophytocenosis. Alfalfa edaphic breeding also has been developed, and the obtained Selena plants can produce 10-12 t/ha dry mass on acidic soils at pH 4.5-5.6.

Symbiotic technologies are the promising strategy as it allows improving the nitric and phosphorus plant nutrition due to the plant-microbe interaction.

This approach based on integration of the genetic systems of plants and microorganisms is successfully being developed (7, 8), and the methods for isolation, selection and estimation of rhizobia strains have been patented. Symbiotic selection was used to design the integrated variety-and-microorganism effective systems such as the alfalfa Vega, Lugovaya 67, Pstbishchnaya varieties and 415b rhizobia strain, and Selena and Agniya varieties with 404b strain, and the clover varieties with local K-18 strain. These symbiotic systems allow to harvest 12-14 t dry matter and 2.0-2.5 t protein per 1 ha and accumulate biological nitrogen in soil of 150-200 kg/ha due to roots and crop residues. In the terms of ecology such systems are a biogeocenotic formation at a supraorganismal level denoted by botanist L.G. Rameskii in 1925 (9) and zoologist V.N. Beklemishev in 1951 (10) as consortia which are the primary units of self-organized and self-sustainable agroecosystems sufficient in nitrogen and partly in phosphorus nutrition.

Due to the developed theoretical bases and methodology the varieties of annual legumes with different climatic and environmental features and economic specialization have been originated. They are the spring vetch Lugovskaya 83, Lugovskaya 85, Lugovskaya 98, Vera, Lugovchaanka, Lugovskaya 24, Vakentina, Neposeda and the winter vetch Lugovskaya 2 (11). Many of them are early ripening and produce seeds at 3.0-4.0 t/ha over 65-88 day vegetation. Besides, they are less heat-loving, thus being successfully cultivated in regions with insufficient heat and excessive soil moisture. Yu.S. Tyuring has suggested creating a specialized forage vetch, and the Lugovskaya 98 variety is now widely used.

Perennial forage grasses and landscape are major components of the hay and pasture agro-ecosystems, so more than 20 varieties of perennial grasses were zoned. They are smooth brome-grass Fakelnii and Morshanets, timothy grass VIK 85, meadow fescue Krasnopoimskaya 92, tall fescue Lira, cocksfoot Morshanskaya 89, perennial ryegrass Tetraploidnii, VIK 66 and Duet, meadow grass Pobeda, Tambovets, fescue-ryegrass hybrid VIK 90 (Festulolium). They produce 11-12 t/ha dry matter, are more nutritionally valuable and more tolerant to diseases (12, 13).

Drought and salt tolerant fodder xerohalophytes are used on the arid Russia territories, i.e. kochia Barkhan, Dzhangar, Delta and Istok varieties, eurotia Favorite and Bar varieties, salsola Salang variety, Camphorosma lessingii Alsu and Nogana varieties, buckwheat Tsag variety, artemisia Sonet variety, and licorice Fortuna variety, which were obtained in cooperation with the researchers from A.N. Kostyakov Institute of Hydro Engineering and Land Reclamation (Moscow) and Kalmykia Research Institute of Agriculture (Elista). These fodder crops are widely used in degraded dry steppes and semi-steppes to increase productivity of the pastures (14-16).

Collecting gene pool together with application of biotechnology and investigations on plant immunity helpful to create tolerant initial material are being the basic approaches (17, 18). In 1930s the mobilization of fodder plant genetic recourses began. In more than 45 expeditions held in cooperation with the N.I. Vavilov Research Institute of Plant Industry (VIR, St. Petersburg), over 6 000 specimens of the wild grass seeds have been sampled in different regions. As a result, valuable alfalfa, clover, sweet clover, sainfoin, rump, foxtail, wheatgrass, wheatgrass forms, as well as endemics have been found, and this work is ongoing. Currently the gene pool collection includes 447 species and about 6500 specimens (16). For clover the polymorphic molecular markers are patented (19). Two clover genetic maps are built in cooperation with Japanese researchers.

Biotechnolgies were used to create promising forms for breeding (20-23) such as the clover tolerant to soil acidity, the transgenic clove and alfalfas regenerants, the breeding alfalfa forms tolerant to salination. Using extremely high

tolerance to salinity the alfalfa Soleustoichivaya variety was obtained which is successful on the secondary salinized soils in Nizhnee Povolzhie.

In view of disease-resistant fodder plant development the infectious agents were indicated with identification of those the most aggressive. The procedure for cultivation of these pathogens was suggested. Using field test the tolerance was estimated in more than 3500 plants. The disease resistant plants were derived by subjecting to high levels of disease pressure with further recurrent selection, hybridization and polycrossing. As a result the clover, smooth bromegrass and timothy grass resistant to diseases have been developed (24).

Generally, plant tolerance to stresses, the selection of disease-resistant forms, genetic transformation and regeneration, and finding microsymbionts of the selected plants were under consideration (25-38).

Much attention was paid to seed production of fodder crops such as fescue Krasnopoimskaya 92 and Kvarta varieties, hairy vetch, alfalaf Sonet and Tambovchanka varireties, red fescue Diana and Sigma varieties, festulolum VIK 90 and Izumrudnii varieties, meadow grass Tambovets variety.

The location of cultivation areas are revised for clover seed reproduction, for cultivation and commercial reproduction of alfalfa perennial ryegrass, timothy, meadow fescue, cocksfoot and smooth brome-grass, and 16 parameters are currently developed for seed quality assessment.

The leading position of the Center is due to wide research cooperation with other Russian researchers and the foreign scientists from Kazakhstan, Uzbekistan, USA, Israel, Belarus, and Mongolia. Thus, the mobilization of genetic resources and breeding fodder crops is particularly curried out together with Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (Germany). The breeders of the Fodder Research Institute were recognized the leaders in Russia in developing botanic, geographical, environmental and evolutionary bases for breeding fodder plants to manage a sustainable forage production.

The fundamental aspects of modern strategy for current fodder crop breeding and seed reproduction are based on mobilizing genetic resources of both cultivated and wild plants, using data banking systems, collecting genetic resource as the donors of desired traits, QTLs marking and genetic mapping in clover and other valuable fodder plants, designing gene constructions to develop transgenic plants resistant to fungal pathogens and environmental stresses. An induced recombinogenesis and mutagenesis, as well as interspecial and intraspecial interrelateions of the plants different in adaptive strategy are also helpful to design the parental forms by means of the improved phytocenotic selection. One more edaphic approach involves plant adaptation to acidic and salinized soils and toxic chlorine, manganese and sodium ions. As far as the plant-microbe interactions are related to fodder plant productivity, it necessitates the study of their efficacy during plant ontogenesis as influenced by the genetic traits and geographic origin of the partners. To substantiate the principles and approaches to preemptive breeding disease-resistant plants, it is essential to forecast changes of a microbial landscape. The study of reproductive peculiarities of developed or introduced fodder plants is helpful with the view of optimization of seed reproduction. Both global and regional survey of climatic changed and their impact allows to suggest the strategy for breeding climatically and edaphically specialized fodder crops.

Applied researches are focused on minimization of the global deficit in resources and the negative effects of modern agriculture. Thus, development of the complementary varieties with climatic and environmental specialization provides for a sustainable livestock forage production subject to the principles of ecological agriculture. Thus the clover, alfalfa and grassland legumes with different economic specialization, high green mass and seed yield, enhanced nitrogen

fixation and an increased resistance to most diseases and abiotic factors should be developed. Using biogeocenotic and improved breeding techniques the perennial cereal grasses with high forage value, disease-resistant, tolerant to abiotic stresses should be obtained to be used for mowing and grazing. Phytocenotic selection and induced recombinogenesis are applied to create spring and winter vetch for different economic purposes. An adaptive selection is suggested for cole fodder crops such as rape, colza, etc., with high yield, increased nutritive value and tolerant to biotic agents and edaphic factors. Arid fodder plants with specific edaphic and phytocenotic traits and more drought- and salt-tolerant should be developed for restoration of zonal biodiversity and fertility in soils after salination and in the degraded pastures. Environmental and phytocenotic approach is basic in agro ecological seed reproduction and more effective realization and maintenance of plant genetic potential. It is also helpful in improving specific agro technology for each variety in view of anticipated climatic changes and also at estimation of germination of seeds in fodder plant species and varieties.

Environmental and evolutionary principles are basic in both fundamental and applied studies. This approach at studying biological phenomena, particularly appearance and development of adaptive capability during phylo- and ontogenesis has been suggested by A.A. Zhuchenko in the monographs «Ecological genetics of cultivated plants: adaptation, recombinogenesis, agrobiocenosis» (Moscow, 1980) and «Ecological genetics of cultivated plants as a scientific discipline» (Moscow, 2010).

We suggested a breeding paradigm based on fundamental biogeocenotic principles of fodder crop breeding and seed production as a part of adaptive plant cultivation (39-41). According to the paradigm, the phothotrophic fodder plants trigger the flow of organic matter and energy in soil, resulting in formation of a biotic complex, the fodder agro biocenosis, being a system of functionally related components. In the system a phytocenotyc interaction between the plants, an edaphic interaction between plants and soil, and plant-microbe symbiotic and/or associative interaction are dominant. These interactions are the breeding potential resources which are essential for design the fodder crops as a biocenotic units possessing phytocenotic, edaphic, symbiotic and ecotypic specificity enough to form an adaptive self-organizing sustainable and high-yielding structures. A capability to self-optimization in these systems is functionally irreplaceable and economically valuable. Therefore, the biogeocenotic breeding of fodder crops can be considered as special scientific discipline.

Thus, more than 150 fodder crop varieties developed for past 42 years and widely cultivated in Russia are not inferior to the foreign varieties on productivity but more frost resistant, edaphic tolerant to soil acidity and salination and compatible in mixtures. More than 40 varieties of perennial grasses, specifically smooth brome-grass, timothy grass, meadow fescue, cocksfoot, perennial ryegrass, meadow grass, with increased productivity above 11-12 tons per hectare, higher disease resistance and improved nutritive value are developed. For restoration of biodiversity and soils in degraded pastures of the arid southern regions of Russia 18 varieties of fodder xenohalophytes are suggested such as *Kochia prostrata* (L.) Schrad. Barkhan and Dzhabgar varieties, *Salsola orientalis* S.G. Gmell. Salang variety, *Camphorosma lessingii* (Litv.) Nogana and Alsu-varieties, *Eurotia ceratoides* (L.) C.A. Mey. Favorit, Tulkin and Bar varieties, etc., wildly used for ecological restoration of desertified soils. The applied researches are influenced by recent state of agriculture, global deficit of natural resources and climate changes and based on fundamental research in genetics, gene engineering, biotechnology, botany, ecology, etc., elucidating expression of valuable traits and their inheritance in a sustainable developing population.

As a conclusion the paradigm is suggested based on the fundamental biogeocenotic approaches and principles of adaptive plant cultivation, particularly breeding and seed production technologies. The biogeocenotic breeding of fodder crops can be considered as special scientific discipline.

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GUT MICROBIOTA OF BROILER CHICKENS INFLUENCED BY PROBIOTICS AND ANTIBIOTICS AS REVEALED BY T-RFLP AND RT-PCR

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Abstract

In poultry, and birds in general, the gut is not only the first line of the defense from exogenous pathogens, but also the biggest immunity organ. The microflora inhabiting blind gut plays multifunctional role in the maintenance of homeostasis in macroorganism, being involved in different processes including fodder digestion. Probiotic, antibiotic, prebiotic, symbiotic preparations, enzymes, etc., affecting directly or ndirectly the gut microflora, can thus increase daily weigh growth and viability in chickens, and the slaughter yield. They also improve a digestibility of fodder nutrients, quality and sanitary condition of the birds. However, the influence of these agents on gut microflora in chickens is still not cleared up. Recent molecular approaches allow studying microbial biodiversity without traditional limitations we are faced with when applying microbiological techniques. By T-RFLP and RT-PCR, the composition and growth dynamics of gut microflora were examined in the Cobb 500 broiler chickens fed with probiotic Cellobacterin-T and antibiotic Stafak-110 as feed additives to the complete diet. By these methods, the probiotic and antibiotic additives were shown to contribute to the development of microbiota. In particular they increased the total number of bacteria, what is more, the normal gastrointestinal flora, i.e. cellulolytic bacteria, lactobacilli, bifidobacteria, bacilli, selenomonas species, increased, while the unwanted enterobateria and actinimycetes decreased. In case we used the probiotics in poultry diet when compared to antibiotics, in the studied parts of the intestinal of broiler chickens there were more cellulolytic bacteria, and in the blind gut the number of lactobacilli, bifidobacteria and bacilli was 10-100 times higher, while clostridia and transit bacteria number decreased. Numerous investigations affirmed the effectiveness of probiotics in poultry basing on zootechnical parameters, however, only molecular identification of the gut microbiota members by means of T-RFLP-PCR and RT-PCR analysis allows detecting and attributing their taxonomic groups influenced by a specific feed additive for further justification of its rational use.

Keywords: broiler chickens, digestive tract, duodenum, blind gut, microflora, pathogenic microflora, T-RFLP, RT-PCR.

An advanced genetic research, breeding technologies and feeding together with effective veterinary control provide for high poultry meat production in the world (1). Genetically determined potential for growth and effective feed conversion in modern poultry crosses makes it possible to reach 2.2-2.3 kg weight of 38-day old chicken at 1.45-1.50 kg fodder consumed per 1 kg weight gain (2). The weight increases 3-4 times during the chicken's first week of life, at that the gut is even more intensely growing. As the higher the productivity, the more stress sensitive poultry is, it often results in health deterioration, and therefore many difficulties are arising at breeding (3).

Numerous researches show the role of normal microflora involved in the control of cardiovascular, endocrine and nervous system functions, hematopoesis, immune reactivity, synthesis of antibiotic substances, proteins, enzymes, hormones, vitamins, etc., and also in preventing infection and unlimited patho-

gens' proliferation in the host's body (4-6). Influencing fodder digestibility and the digestive tract development in poultry, the microflora affects significantly on the nutrients absorption, nutritional demands, physiological status and growth (7).

Few days before and after hatching are crucial for chicks' development and survival because of metabolic and physiological changes occurred as the dry fodder consumption starts instead of yolk utilization. At that the gut are to develop intensely providing for the effective use of fodder nutrients. In case of improper gut microbiota formation during first days of life the chicks are dramatically influenced by sanitary parameters of feeds, water and farm conditions (8).

Gut is commonly known to be not only the first defense line against exogenous pathogen colonization of a host macroorganism cells and tissues, but the biggest immune organ in the body. Any changes in gut morphology influence nutrient absorption and activate secretion and excretion, leading to diarrhea and a decreased disease resistance and productivity in general (9). Compared to other parts of the poultry digestive tract the blind gut is the most populated by microorganisms with their total number reaching 10^{10}-10^{11} per 1 g content (10). In various ways, including feed digestion, the blind gut microflora diversely provides homeostasis of the macroorganism.

Colonization resistance is a most important feature of normal microflora whereby it performs the defensive role. Adverse conditions destroy the established microbial community, and change the adhesiveness and colonization resistance of the indigenous flora leading to disbiosis (disbacteriosis) (11). As a result the uncharacteristic microorgatisms colonize the gut and the bacterial strains different from their obligate forms can prevail. At disbacteriosis more than 50 % of *Escherichia coli* cells possess hemolytic activity and produce hyaluronidase indicating an increased virulence. Besides, a multidrug resistance is characteristic for bacteria isolated at disbacteriosis. Moreover, there are a decreased number of gut lacto- and bifidobacteria essential in performing different functions such as protection of the gut mucosa from penetration of pathogenic and opportunistic bacteria into the blood, production of antibiotic-like substances and organic acids, vitamin B group synthesis, and activation of immune response (12-14).

The diet for high-producing poultry is specially balanced to provide the highest growth rate over a short time. However, an increased level of nutrients could cause a misbalance in gut microbiota (15). Commonly used antibiotic feed additives, the acidifiers and other chemicals influence the gut microbiota. Besides, expansion and granulation of compound feeds also can inactivate many bacteria (16).

Bio-industry recently produces a variety of preparations, namely probiotics, antibiotics, prebiotics, symbiotics, enzymes, etc., which influence the poultry gut mictobiota directly or indirectly, resulting in a greater daily weight gain and chickens' survival, the higher weight at slaughter, and also in better digestibility of the feed nutrients and improved quality and sanitary condition of the birds. However, in fact the effect of these preparations on gut microflora in chickens is not completely clarified.

Antibiotics as feed additives are used to control animal health and achieve economic benefit, but as they lead to the occurrence of antibiotic resistant pathogenic bacteria, that in turn can affect people health, currently they are not fed to poultry and pigs in the European countries, particularly in Sweden since 1986 and in EU since 2006 (18). Because of improper antibiotic use the helpful gut flora decreased in number, moreover, each 2-3 years new antibiotic resistant strain are isolated. The development of each next antibiotic formula takes more time, but the effect is not obvious as often the intestinal motor disturbance occurs (19). Thus the replacement of antibiotics in feed is attractive

and stimulates the search for alternative ways.

Probiotic microorganisms are widely used in EU since 2006 due to forbiddance of feed antibiotics and some other antibiotic substances. Probiotic preparations usually contain living bacteria normally inhabiting the gastrointestinal tract, such as lactobacteria, bifidibacteria, spore forming microorganisms, yeasts and some fungi (20). Probiotics are used in poultry to treat and prevent the gastrointestinal infections, to stimulate a non-specific immunity, to correct the disbacteriotic symptoms caused by an abrupt change of a compound feed composition or violation of the diet, as well as stresses under the translocations, and also they are applied to replace antibiotics in the compound feeds (21).

Because of large selection of the manufactured compound feeds and feed additives for poultry farming, their effects on gastrointestinal bacteria should be reasonably compared by precise, accurate and low-costing attributing (22).

The data on composition and the role of gastrointestinal microbial community were mostly obtained using methods of classical microbiology. However, they are significantly limited and having disadvantages. Particularly counting bacteria is incorrect in case the colonies are derived from bacterial agglomeration but not an individual cell (23). Besides, gastrointestinal bacteria were shown to be mostly not routinely cultivated on growth media (they are called uncultivated bacteria) (24). Uncultivated state is noted to be a global problem topical in soil, plant microbiology, medicine and veterinary medicine. Cell wall-less bacteria are very special but widely spread phenomenon not based on the principles of classical microbiology. Because of bacterial transformation into uncultivated L-forms the pathogens are hard to detect, and false negative results usually occur (25).

Modern molecular methods allow studying diversity of microorganisms without limitations in cultivation. T-RFLP-PCR analysis based on bacterial DNA restriction fragment amplification is considered the most advanced (26) as a good tool to evaluate the total number and the relative species abundance, and to attribute taxonomically all bacteria of the microbial ecosystem. Molecular methods enable a large-scale and detailed comparison of development and changes in the microbial community (27).

In Russia a modified molecular technique is recently used to study the poultry gastrointestinal bacteria (28). The developed protocol enables their prompt and accurate identification and quantitation, early pathogen detection and estimation of the feed additives' effects on the gut microbiota.

Herein, we studied the effects of antibiotic and probiotic feed additives on gastrointestinal microbiota and its qualitative and quantitative changes in growing broiler chicks as detected by T-RFLP—RT-PCR analysis.

Technique. During the experiment held in 2013 the Cobb 500 cross broiler chickens were kept in the vivarium at battery cage system R-15 from a day of age until day 35. Day-old baby chicks were grouped upon the weight by 70 chicks per group and 35 chicks per cage. All chicks were fed compound feed on full ration basis according to the poultry breeding company's recommendations:

	0-5 days	6-15 days	15-36 days
Wheat, %	60,40	59,96	67,11
Soybean meal, %	23,03	19,43	11,05
Sunflower meak, %	6,00	7,20	7,31
Fish meal, %	4,00	2,50	0
Meat and bone meal, %	0	2,00	5,00
Soybean oil, %	3,11	5,33	6,00
Limestone, %	0,87	1,01	0,19
Mono calcium phosphate, %	1,00	0,70	0
Tricalcium phosphate, %	0	0,37	1,84
Vitamin and mineral premix, %	1,50	1,50	1,50

In the control group I chicks were fed balanced feed (the basic ration,

BR) with 4.0, 4.5 and 5.0 % animal protein at 0-5 days, 6-15 days and from 15 days of age, respectively. For feeding the chicks from the groups II and III the antibiotic Stafac 110 (Phibro Animal Health Corporation, USA) or probiotic Cellobacterin-T (Biotrof Ltd, Russia) at 180 g per ton and 1 kg per ton, respectively, were mixed with feed. In each group 6 chicks were arbitrarily slaughtered at days 1, 7, 14, 21 and 35 for sampling. For more reliable results we selected the chicks with filled crop, and a day old chicks were surveyed 24 hours after feeding. The contents of the duodenum and the blind gut were aseptically sampled into Eppendorf tubes and frozen at –20 °C until transportation at –18 °C.

T-RFLP and RT-PCR were performed as reported (29, 30) after the approbation (31).

To extract DNA for T-RFLP the Genomic DNA Purification Kit (Fermentas, Lithuania) was used. A 200 μg sample in 1.5 ml Eppendorf tube with 400 μl lysing reagent was vortexed on a CVP-2 (BioSan, Latvia) and incubated in a thermoshaker TS-100 (Biosan, Latvia) at 65 °C for 30 minutes. After the precipitation solution was added the mixture was vortexed on V-32 (BioSan, Larvia) and centrifuged at 14000 rpm for 10 minutes (Beckman Coulter centrifuge, USA). The precipitate was vortexed with 100 μl NaCl solution and 300 μl 96 % ethanol and centrifuged as hereinabove. The final precipitates were thermostaed at 45 °C until complete drying. After 100 μl sterile water addition the samples were vortexed. The obtained DNA preparations were stored at –20 °C. To control DNA purification, the preparations were separated electroforetically in 1 % agarose gel (Bio-Rad, USA) with TAE (Tris-acetate-EDTA) buffer (Fermentas, USA) at 50 V for 1 hour using Gene Ruler molecular marker (Fermentas, USA). The gels were viewed with a UV transilluminator (New England BioGroup, USA).

For T-RFLP-PCR we used 10 pM primers, 10 units of Taq DNA Polymerase, 10× Taq DNA Polymerase buffer, 2.5 mM $MgCl_2$, 2.5 mM dNTPs (Fermentas, USA), 10 ng template DNA. To amplify 16S-rRNA bacterial gene, the specific 5′-end fluorescently labeled oligos, the 63f, 1087r, were chosen. A negative control with no added DNA and a positive control containing successfully amplified DNA were used. PCR was carried out in a iCycler (Bio-Rad, USA) according to the following protocol: initial denaturation at 95 °C for 3 minutes; 34 cycles of denaturation, annealing and elongation at 95 ° for 30 seconds, 42-60 °C for 30-60 seconds, and 72 °C for 1 minute, respectively; and final elongation at 72 °C for 10 minutes. The amplicon patterns were analized electrophoretcally (Bio-Rad, USA) as described hereinabove.

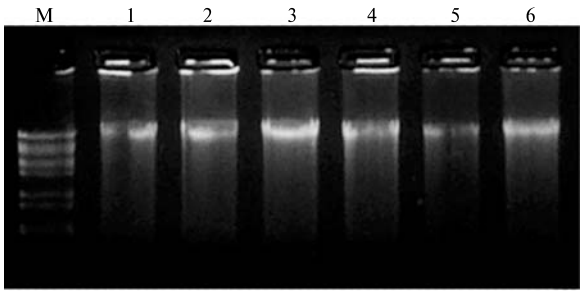

Fig. 1. Typical electrophoretic patterns of DNAs used in T-RFLP—RT-PCR analysis of poultry gut microbial community: M — molecular marker Gene Ruler (Fermentas, USA), 1-6 — DNA samples (1 % agarose gel, TAE buffer, 50 V, 1 hour).

DNA extraction from the gel slices was performed using Genomic DNA Purification Kit (Fermentas, Lithuania) according to the manufacturer's instructions. The obtained DNA precipitates were solubilized in 15 μl deionized water. For restriction a special kit («Fermentas», USA) was used with 1.5 μl restriction buffer and 10 units of Hae III, Hha I and Msp I endonucleases in the final volume brought to 15 μl, and heat termination at 72 °C for 20 minutes was applied.

Capillary electrophoresis was performed on a CEQ8000 DNA sequenser (Beckman Coulter, USA) with 0.2 μl of DNA Size Standard Kit-600 marker (Beckman Coulter, USA) added to each DNA probe to estimate fragment lengths. Based on a T-RFLP-PCR product fluorescence ranged from 10 to 220 thousand units and a control 600 bp fragment length, the data matrices were created in MS Excel and bacteria were phylogenetically attributed using FragSort program (http://mica.ibest.uidaho.edu). An accurate taxonomic attribution was obtained from the data for all three endonucleases, the Hae III, Hha I and Msp I, and a percentage of each bacterial group in microbial community was calculated.

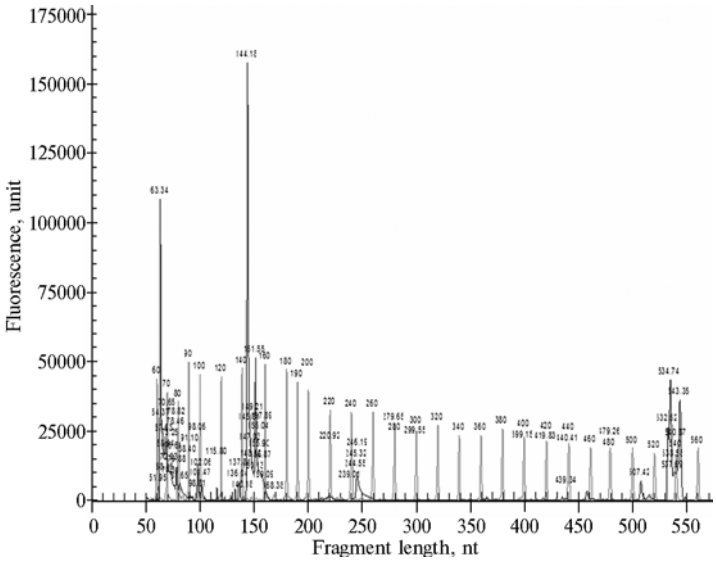

Fig. 2. A T-RFLP-PCR pattern for poultry gut bacterial DNA (gray peaks are the markers, and black peaks indicate DNA fragments in the sample).

In RT-PCR for a bacterial group quantitation the DNA extraction, fragmentation control and 16S-rRNA gene amplification were the same as described hereinabove (iCycler, Bio-Rad, USA), using DNA of a tested microorganism in a serial concentrations as four positive controls instead of a single one. A real-time calibration curves were developed to estimate the genome content in the probe. Results were processed mathematically using MS Excel.

Results. DNA gel electrophoresis indicated an admissible fragmentation (Fig. 1), and there were specific T-RFLP-PCR patterns obtained for DNA from gut bacteria (Fig. 2).

Stafac 110 possesses a bacteriostatic effect on most gram positive bacteria and some gram negative ones, being bactericidal at high concentrations. Its active substance, the virginiamycin, is not absorbed in the gastrointestinal tract and is not subjected to digestion, thus its concentration and antibiotic activity in the gut remain high for a long time. There is no virginiamycin accumulation in organs and tissues, thus it is excreted unchanged with feces (32). Probiotic Cellobacterin-T contains a microbial association of the animal rumen, being a multi active preparation (33). It contains cellulolytic bacteria and lactobacilli, being both enzymatic and prophylactic. Bacteria of this association produce xylanases, pectinase and β-glucanase, able to hydrolyze cellulose in feed, therefore the preparation is added to fodder with high cellulose content used at animal and poultry farming.

A total bacterial number were higher in case the antibiotic or probiotic preparation was used in the group II and group III compared to control (Table 1). More apparent differences were observed in 1-14 day-old chicks. Particularly in day-old chicks the total bacterial number in duodenum and blind gut was 100 and 10 times higher compared to control, respectively, that evidenced a more rapid microbial colonization important for gut development. At 7 and 14 days of age in the groups II and III the number of bacteria in the duodenum and the

blind guts was 10-fold as compared to that in control, moreover, in 14 day-old chicks in the blind guts it was up to 100 time higher compared to that in poultry fed antibiotics. It could be a result of a changed diet and increased content of animal protein. By day 21 the total bacterial number did not differ, and in 35-day-old chicks it was almost the same, except group II poultry demonstrated 10 times increased bacterial number in the duodenum.

1. Dynamics of total bacterial number (genomes/g) **in the gastrointestinal tract** (GIT) **content of Cobb 500 broiler chicks fed antibiotic and probiotic feed additives estimated by T-RFLP-RT-PCR analysis**

Group	GIT	1 day	7 days	14 days	21 days	35 days
I (control)	Duodenum	9.6×10^{7}	2.6×10^{8}	2.1×10^{8}	1.5×10^{9}	2.2×10^{9}
	Blind gut	7.0×10^{9}	1.6×10^{10}	2.2×10^{10}	2.8×10^{10}	8.3×10^{10}
II (antibiotic Stafac 110)	Duodenum	1.9×10^{10}	6.4×10^{9}	1.3×10^{9}	1.5×10^{9}	7.1×10^{9}
	Blind gut	2.5×10^{10}	1.4×10^{11}	2.4×10^{9}	3.1×10^{10}	6.3×10^{10}
III (Probiotic Cellobacterin-T)	Duodenum	4.0×10^{10}	1.1×10^{8}	1.5×10^{9}	2.3×10^{9}	7.0×10^{10}
	Blind gut	1.4×10^{10}	1.2×10^{11}	1.3×10^{11}	2.6×10^{10}	8.3×10^{10}

Among normal gut microbe community the following cellulolytic bacteria are essential: *Bacteroidacea*, *Lachnospiracea*, *Ruminococcacea*, *Thermoanaerobacteriacea*, *Clostridiacea* (34) because of absence of the birds' own enzymes for degradation of celluloses and other non-starch polysaccharides.

The highest percentage of cellulolytic bacteria was calculated for blind gut of broilers fed the antibiotic and probiotic additives (Table 2).

2. Dynamics of total number of normal microflora (genomes/g) **in the gastrointestinal tract** (GIT) **content of Cobb 500 broiler chicks fed antibiotic and probiotic feed additives estimated by T-RFLP-RT-PCR analysis**

Group	GIT	1 day	7 days	14 days	21 days	35 days
		Cellulolytic bacteria				
		Cellulolytics				
I (control)	Duodenum	1.9×10^{7}	1.6×10^{7}	1.1×10^{7}	2.4×10^{8}	5.8×10^{6}
	Blind gut	7.0×10^{5}	1.9×10^{9}	3.6×10^{9}	9.4×10^{9}	8.7×10^{9}
II (antibiotic Stafac 110)	Duodenum	1.1×10^{9}	6.8×10^{8}	$< 1.0\times10^{5}$	9.3×10^{7}	1.6×10^{8}
	Blind gut	5.2×10^{8}	3.1×10^{10}	6.2×10^{8}	8.0×10^{8}	1.6×10^{10}
III (Probiotic Cellobacterin-T)	Duodenum	1.7×10^{9}	1.4×10^{7}	3.6×10^{7}	3.3×10^{8}	8.3×10^{9}
	Blind gut	4.6×10^{8}	2.5×10^{10}	3.1×10^{10}	8.0×10^{9}	1.6×10^{10}
		Bacteroidaceae				
I (control)	Duodenum	7.3×10^{6}	1.6×10^{7}	7.0×10^{6}	8.0×10^{8}	9.8×10^{5}
	Blind gut	7.0×10^{5}	6.1×10^{8}	1.5×10^{8}	5.1×10^{8}	8.0×10^{6}
II (antibiotic Stafac 110)	Duodenum	4.2×10^{7}	1.4×10^{7}	$< 1.0\times10^{5}$	5.5×10^{7}	1.4×10^{7}
	Blind gut	8.8×10^{7}	1.4×10^{10}	4.0×10^{7}	5.0×10^{9}	3.6×10^{9}
III (Probiotic Cellobacterin-T)	Duodenum	1.1×10^{9}	1.2×10^{7}	1.9×10^{7}	2.3×10^{8}	3.6×10^{9}
	Blind gut	1.9×10^{8}	1.3×10^{10}	7.8×10^{9}	1.5×10^{9}	2.4×10^{9}
		Clostridiaceae				
I (control)	Duodenum	1.9×10^{6}	3.4×10^{6}	2.0×10^{4}	3.3×10^{7}	3.0×10^{6}
	Blind gut	4.5×10^{9}	4.3×10^{9}	6.7×10^{9}	2.8×10^{8}	1.9×10^{9}
II (antibiotic Stafac 110)	Duodenum	3.4×10^{7}	6.0×10^{5}	$< 1.0\times10^{5}$	$< 1.0\times10^{5}$	3.3×10^{7}
	Blind gut	7.5×10^{9}	5.8×10^{9}	2.4×10^{7}	2.0×10^{9}	4.0×10^{8}
III (Probiotic Cellobacterin-T)	Duodenum	1.9×10^{8}	4.2×10^{5}	1.4×10^{7}	2.1×10^{7}	5.0×10^{6}
	Blind gut	2.3×10^{9}	1.2×10^{10}	5.3×10^{9}	1.6×10^{8}	5.5×10^{6}
		Lactobacilli				
		Lactobacillaceae				
I (control)	Duodenum	5.8×10^{6}	1.7×10^{8}	1.6×10^{8}	9.1×10^{7}	1.3×10^{8}
	Blind gut	1.8×10^{7}	4.0×10^{9}	1.2×10^{9}	3.7×10^{9}	1.7×10^{9}
II (antibiotic Stafac 110)	Duodenum	1.5×10^{10}	2.0×10^{9}	1.2×10^{9}	8.4×10^{8}	1.4×10^{9}
	Blind gut	5.5×10^{8}	1.0×10^{10}	9.6×10^{7}	8.0×10^{8}	3.0×10^{8}
III (Probiotic Cellobacterin-T)	Duodenum	3.1×10^{10}	4.6×10^{7}	8.9×10^{8}	8.7×10^{8}	3.1×10^{9}
	Blind gut	3.7×10^{8}	2.7×10^{11}	4.4×10^{9}	4.0×10^{9}	1.7×10^{10}
		Bifidobacteriaceae				
I (control)	Duodenum	1.9×10^{6}	3.0×10^{6}	2.0×10^{4}	6.0×10^{7}	2.0×10^{4}
	Blind gut	7.0×10^{5}	2.0×10^{6}	2.4×10^{7}	9.0×10^{7}	8.0×10^{6}
II (antibiotic Stafac 110)	Duodenum	2.0×10^{6}	6.0×10^{5}	$< 1.0\times10^{5}$	1.5×10^{7}	4.3×10^{8}
	Blind gut	3.0×10^{6}	6.6×10^{8}	4.8×10^{6}	3.0×10^{6}	6.2×10^{8}
III (Probiotic Cellobacterin-T)	Duodenum	3.2×10^{8}	2.1×10^{6}	3.0×10^{7}	6.7×10^{7}	2.1×10^{9}
	Blind gut	1.0×10^{6}	1.8×10^{8}	1.0×10^{7}	1.4×10^{8}	6.8×10^{8}

Table 2 (continued)

		Helpful bacilli and selenomonas				
		Bacillaceae				
I (control)	Duodenum	1.6×10^{7}	1.3×10^{7}	7.5×10^{6}	5.0×10^{8}	2.0×10^{7}
	Blind gut	7.0×10^{5}	6.2×10^{8}	3.4×10^{8}	6.0×10^{8}	8.0×10^{6}
II (antibiotic Stafac 110)	Duodenum	1.9×10^{8}	4.6×10^{7}	1.0×10^{5}	1.2×10^{8}	3.3×10^{7}
	Blind gut	6.0×10^{8}	9.8×10^{9}	3.4×10^{7}	7.4×10^{8}	3.0×10^{9}
III (Probiotic Cellobacterin-T)	Duodenum	1.7×10^{9}	1.7×10^{7}	3.5×10^{7}	5.3×10^{8}	5.5×10^{8}
	Blind gut	6.5×10^{8}	8.4×10^{9}	1.0×10^{9}	1.2×10^{9}	1.1×10^{10}
		Veillonellaceae				
I (control)	Duodenum	1.5×10^{5}	2.0×10^{4}	2.0×10^{4}	1.0×10^{5}	2.0×10^{5}
	Blind gut	7.0×10^{5}	3.8×10^{8}	2.2×10^{9}	1.1×10^{9}	4.1×10^{8}
II (antibiotic Stafac 110)	Duodenum	9.0×10^{7}	5.1×10^{7}	1.0×10^{5}	1.0×10^{5}	1.9×10^{7}
	Blind gut	3.0×10^{6}	1.6×10^{10}	1.5×10^{8}	3.0×10^{9}	4.0×10^{8}
III (Probiotic Cellobacterin-T)	Duodenum	4.0×10^{6}	6.8×10^{5}	2.2×10^{6}	1.3×10^{7}	6.2×10^{8}
	Blind gut	1.0×10^{6}	5.5×10^{9}	3.5×10^{10}	4.7×10^{9}	6.8×10^{8}

In blind gut of control day-old chicks the celullolytic bacteria number was not more than 7×10^{5}, being in the group II and III almost 1000 times higher. In duodenum it was 100 times higher on average compared to control. In 7 day-old chicks in blind gut this parameter was about 10 times higher compared to control, and in duodenum of the chicks fed the antibiotic additive it was about 10 times higher if compared to other chicks. In 14 day-old chicks in both duodenum and blind gut cellulolytic bacteria were the most numerous when probiotic additive was used, and the smallest number was found in case the antibiotics was used. These indexes are very important as they reflect changes in the poultry diet. In 21 day-old chicks no significant differences in cellulolytics were found, except group II in which their number significantly decreased, probably due to their decreased number in 14 day-old poultry. At 35 days of age the cellulolytics were the most numerous in chicks fed the probiotic additive, and the smallest number was observed in the control chicks.

Special attention should be paid to *Bacteroidaceae*m which can also ferment starch feed components (35). During the experiment their number was the highest in poultry fed the probiotic additive and the smallest in control. Also clostridia are considered normal for bacterial community in poultry due to cellulose fermentation (36), but some pathogenic clostridia species can cause gastroenteritis and intoxication. The clostridia number varied significantly, nevertheless in blind gut of chicks fed probiotic preparation this index was generally lower.

Among lactobacilli *Lactobacillaceae* and *Bifidobacteriaceae* were identified (see Table 2).

Lactobacillaceae are helpful due to antimicrobial activity against pathogenic microorganisms (37). Their number was the highest in the gastrointestinal tract of broilers from group II and group III, and the smallest one in control poultry, being at that 10-100 times higher in the blind gut of the chicks from group III compared to group II.

Bifidobacteriaceae from poultry gut also possesses antimicrobial activity against pathogens (38). They were the most numerous in the group II and group III with the smallest number found in control. In the blind gut of broilers from the group III they were 10-100 times higher than in group II.

Also helpful *Bacillaceae* with antimicrobial activity and the *Veillonellaceae* able to degrade organic acids (39) were identified (see Table 2).

The largest bacilli populations were found in groups II and III compared to control, being at that 10-100 times higher in the blind gut of the chicks from group III compared to group II. The most *Veillonellaceae* were also observed in poultry fed the additives if compared to control chicks.

Enterobacteriaceae, including salmonellas, colibacteria, proteus, etc., are undesirable as often cause gastroenteritis (40). Their number was the lowest in

control and in broilers fed the probiotic additive, being however the highest in the blind gut (Table 3).

3. Dynamics of total numbers of enterobacteria and actimomycetes (genomes/g) **in the gastrointestinal tract** (GIT) **content of Cobb 500 broiler chicks fed antibiotic and probiotic feed additives estimated by T-RFLP-RT-PCR analysis**

Group	GIT	1 day	7 days	14 days	21 days	35 days
		Enterobacteriacea				
I (control)	Duodenum	2.7×10^{6}	3.5×10^{6}	9.2×10^{5}	1.1×10^{8}	4.2×10^{6}
	Blind gut	7.0×10^{5}	4.8×10^{8}	6.3×10^{8}	7.0×10^{9}	3.3×10^{9}
II (antibiotic Stafac 110)	Duodenum	6.1×10^{8}	7.7×10^{8}	7.5×10^{6}	8.7×10^{7}	5.5×10^{8}
	Blind gut	4.4×10^{8}	3.5×10^{9}	7.2×10^{8}	1.5×10^{9}	2.1×10^{9}
III (Probiotic Cellobacterin-T)	Duodenum	4.0×10^{6}	5.6×10^{6}	1.4×10^{7}	3.8×10^{7}	2.0×10^{7}
	Blind gut	1.6×10^{9}	4.6×10^{9}	1.1×10^{9}	2.0×10^{9}	5.2×10^{7}
		Actinomyces				
I (control)	Duodenum	3.5×10^{5}	2.0×10^{4}	2.0×10^{4}	1.4×10^{7}	1.1×10^{6}
	Blind gut	1.5×10^{7}	5.7×10^{8}	3.8×10^{9}	1.6×10^{9}	3.0×10^{10}
II (antibiotic Stafac 110)	Duodenum	5.1×10^{7}	1.8×10^{7}	1.0×10^{5}	1.0×10^{5}	7.1×10^{7}
	Blind gut	4.3×10^{9}	1.4×10^{10}	5.7×10^{8}	1.6×10^{9}	5.7×10^{9}
III (Probiotic Cellobacterin-T)	Duodenum	8.0×10^{8}	2.4×10^{5}	5.1×10^{6}	3.2×10^{7}	2.8×10^{7}
	Blind gut	1.5×10^{9}	1.1×10^{10}	8.6×10^{9}	2.0×10^{9}	6.9×10^{9}

Actinomyces are also undesirable in poultry gut as they cause actinomycosis (41). They were the least in number in the gastrointestinal tract of control broilers from day 1 to day 14, and in blind gut of the chicks fed the additives from day 14 to day 35.

Pathogenic microflora was not found in broiler chicks as the number of staphylococci, fusobacteria, pasteurella and campylobacter was not noticeable and did not differ significantly among the chicks' groups.

Besides, in all chicks the transit microorganisms were detected which occur in the feed and are not important for fermentation. In the blind gut of 1 day-old chicks fed the probiotic additive their number was much lower compared to gut of other broilers. It probably indicates earlier gut colonization by helpful microflora in case the probiotic additive fed. From day 7 to day 21 the number of transit microorganisms increased possibly due to BR change (Table 4).

4. Dynamics of total numbers of transit microflora (genomes/g) **in the gastrointestinal tract** (GIT) **content of Cobb 500 broiler chicks fed antibiotic and probiotic feed additives estimated by T-RFLP-RT-PCR analysis**

Group	GIT	1 day	7 days	14 days	21 days	35 days
I (control)	Duodenum	2.6×10^{5}	4.6×10^{6}	2.0×10^{4}	1.0×10^{5}	2.0×10^{5}
	Blind gut	2.5×10^{9}	1.7×10^{9}	2.0×10^{6}	2.8×10^{8}	2.6×10^{8}
II (antibiotic Stafac 110)	Duodenum	2.0×10^{6}	9.0×10^{7}	1.0×10^{5}	9.4×10^{6}	2.6×10^{8}
	Blind gut	7.5×10^{9}	1.1×10^{10}	2.0×10^{5}	8.4×10^{8}	2.3×10^{9}
III (Probiotic Cellobacterin-T)	Duodenum	7.2×10^{7}	2.2×10^{6}	3.0×10^{7}	4.2×10^{7}	1.9×10^{7}
	Blind gut	1.9×10^{8}	1.2×10^{10}	2.2×10^{9}	1.1×10^{9}	2.2×10^{7}

Thus, T-RFLP—RT-PCR analysis of gut microbial community in growing chicks showed that in case the antibiotic or probiotic feed additives are used the total bacterial number increases, mainly due to helpful cellulolytic bacteria, lactobacilli, bifidobacteria, bacilli, *Veillonellaceae*, while the percentage of undesirable enterobacteria and actinomyces decreases. In case of the probiotic additive, if compared to the antibiotic, the gastrointestinal microbial community was enriched with cellulolytic bacteria, and in the blind gut there was 10-100 times more lactobacteria, bifidobacteria, bacilli, while clostridia and transit microorganisms decrease in number. A numerous research based on the production parameters confirmed the benefit of probiotic additives in poultry. However the molecular identification of gut microflora such as T-RFLP—RT-PCR analysis provides reliable data on changes caused in different gut microbial groups by different preparations to make a decision on their proper use.

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DISTRIBUTION AND GENETIC VARIABILITY OF AVIAN HEPATITIS E VIRUS ON POULTRY FARMS IN ex-USSR

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Abstract

Russian commercial poultry industry is tightly tied to international trade. To date, avian hepatitis E virus (aHEV) has been isolated in Australia, US, Spain, Hungary, etc., being a serious threat to commercial poultry. In hens, aHEV affects liver and immune system, namely spleen and thymus, disrupting homeostasis and hematological parameters. In Russia, aHEV genetic identification was first reported in 2009, but the current epizootic situation in poultry remains to be unknown. In this paper we report the results of genetic analysis of 415 liver samples from hens and broiler chicks aged 4-56 weeks. The samples were obtained from 68 commercial farms in 36 regions of the Russian Federation and 4 commercial farms from Belarus, Kazakhstan and Ukraine during 2009 to 2012. A total of 14 isolates were detected and sequenced. Isolates of aHEV were detected in both clinically healthy and affected chickens. No agents causing similar clinical signs, i.e. leucosis virus, Marek's disease virus and adenovirus, were identified. In Russia, aHEV was isolated in Amurskaya, Vologodskaya, Ivanovskaya, Kaluzhskaya, Moskovskaya, Samarskaya, Saratovskaya provinces, and also in the Republic of Mari El. Ross 308 hens showed highest PCR-positive rates (8 of 14 isolates). According to RT-PCR analysis of capsid protein gene (ORF2), aHEV18198, aHEV19555, aHEV16211, aHEV18479 and aHEV18481 of 14 Russian isolates fall within European genotype 3. The aHEV16211, aHEV18479, aHEV18481 isolates are related to Chinese (China-09-G57), US (NY449, CA697A) and European (06-4582) isolates, and aHEV18198 and aHEV19555 are related to those from Australia and USA (Guelph01, CA518.3). Of 14 aHEV isolates studied, 7 samples formed a distinct genetic group. Two more isolates, aHEV18381 and aHEV18505, group outside the three genotypes described. Overall, the pool of HEV isolates identified in Russian chicken flocks consists of isolates belonging to European genotype 3 and isolates not yet assigned to a specific genotype. The analysis of genetic variability of aHEVs isolates from different countries strongly suggests that the current classification into genotypes by geographical origin should be revised.

Keywords: PCR, hepatitis E, hens.

To date the hepatitis E in poultry has been reported in Australia (1), the United States (2), Spain (3), Hungary (4), Russia (5), etc. Its causative agent, the aHEV (*Hepevirus: Hepeviridae*) is a non-enveloped virus with a single-stranded, positive-sense RNA genome (6) that is approximately 6600 bp in size and contains three open reading frames (ORFs) (7). 5′-end ORF1 encodes the nonstructural polypeptide with several domains, including a methyltransferase domain, a papain-like cystein protease domain, a helicase domain and a RNA-dependent RNA polymerase domain (8), and ORF2 and ORF3 encode the capsid protein and a posphoprotein, respectively (9-11).

aHEV attacks liver, as well as spleen and thymus involved in immune response, and constitutes a real threat to poultry commercial farming. Big liver and spleen disease (BLS) and hepatit-splenomegaly syndrome (HSS) are reported as manifestations of the infection. aHEV is shown to cause deep disturbance in homeostasis, especially in hematological indexes (12).

In Sverdlovsk Province the antibodies against aHEV were detected in 2004 in blood serum of 18.3 % hens indicating aHEV circulation in flocks (13). In Russia aHEV has been first identified genetically in 2009 (5), nevertheless, the prevalence and current epizootic situation are still poor studied.

In this paper a large-scale survey for aHEV and its genetic variability

based on the capsid protein gene sequencing are reported.

Technique. From 2009 to 2012 415 specimens of liver were sampled from Rodonit 3, Ross 308 and Hubbard ISA laying hens and broilers of different age in 68 poultry commercial farms from 36 regions of the Russian Federation and 4 commercial farms from Belarus, Kazakhstan and Ukraine.

Viral RNA was isolated using RIBO-sorb kit (Russia) composed of the lysing reagent, rinse and elution buffers, and silicate sorbent, according to manufacturer's recommendation. The RT-PCR primers and protocol described (14) were modified.

A 242 nt fragment of aHEV capsid protein gene was sequenced on a ABI Prism 3130 (Applied Biosystems, USA) with ClustalW sequence alignment algorithm (BioEdit v. 7.0.5.3). The Neighbor-Joining bootstrapped trees (n = 1000) were generated using MEGA software version v. 3.1 (available at http://megasoftware.net). The aHEV sequences used for alignment were retrieved from GenBank (the accession numbers FM872312 to FM872320, FM872322 to FM872330, FN557166 to FN557172, and AM943646), and also a swine HEV capsid protein gene sequence as an external control.

Results. Analysis of the samples. By RT-PCR (14) a total of 14 aHEV isolates were detected in poultry with big liver syndrome and in apparently healthy birds unchanged in egg production and viability (Table).

Characteristics of the avian hepatitis virus E isolates from poultry farms in ex-USSR (2009-2012)

Isolate	Country and the region of origin	Date	Clinical symptoms	Cross	Poultry age, days	GenBank accession number of a sequence
aHEV16050	Kazakhstan, no data	2009	Enlarged liver	Rodonit 3	Pooled sample	JQ814691
aHEV16211	Russia, Vologodskaya Province	2010	Enlarged liver and spleen, liver hemorrhage	Ross 308	230	JQ814690
aHEV16279	Russia, Kaluzhskaya Province	2011	Enlarged liver	Ross 308	237	JQ814692
aHEV18196	Russia, Ivanovskaya Province	2012	No symptoms	Ross 308	117, 350	JQ814688
aHEV18198	Russia, Saratovskaya Province	2012	No symptoms	Hubbard ISA	315	JQ814689
aHEV18381	Russia, Moscow Province	2012	No symptoms	no data	no data	–
aHEV18383	Russia, Amurskaya Province	2012	No symptoms	Hubbard ISA	30-36	–
aHEV18479	Russia, Vologodskaya Province	2012	Enlarged liver and spleen	Ross 308	193	–
aHEV18481	Russia, Vologodskaya Province	2012	Enlarged liver and spleen	Ross 308	250	–
aHEV18505	Russia, no data	2012	No symptoms	no data	н.д.	–
aHEV19551	Russia, Marii El Republic	2012	No symptoms	Ross 308	241	–
aHEV19553	Russia, Marii El Republic	2012	No symptoms	Ross 308	394	–
aHEV19555	Russia, Saratovskaya Province	2012	No symptoms	Hubbard ISA	306	–
aHEV20088	Russia, Saratovskaya Province	2012	No symptoms	Ross 308	224	–

C o m m e n t s. Dashes means no sequences accessed in GenBank.

Fig. 1. Prevalence of avian hepatitis virus E (aHEV) in Russian poultry farms: 1 — Amurskaya Province, 2 — Vologodskaya Province, 3 — Ivanovskaya Province, 4 — Kaluzhskaya Province, 5 — Marii El Republic, 6 — Moscow Province, 7 — Samarskaya Province, 8 — Saratovskaya Province; the regions with no aHEV isolates are dark grayed, and those not surveyed are light grayed (2009-2012).

The territories of the Russian Federation where aHEV has been isolated

are mapped hereinabove (Fig. 1).

Phylogenetic analysis. In total of 14 obtained aHEV isolates of different origin and location the capsid gene fragment was sequenced and subjected to phylogenetic analysis (Fig. 2).

According to publications, there are three different genotypes of aHEV, namely Australian (genotype 1), American (genotype 2) and European (genotype 3) (15, 16). Besides, a Hungarian isolate HU-16773 also shown on the dendrogam (see Fig. 2) is considered a potentially individual genotype.

The Russian isolates aHEV18198, aHEV19555, aHEV16211, aHEV18479 and aHEV18481 apparently belong to the European genotype 3 (see Fig. 2). At that three ones, the aHEV16211, aHEV18479 and aHEV18481, were genetically similar to the aHEV isolates of the genotype 3 from China (China-09-G57, etc.), the United States (NY449, CA697A) and Europe (06-4582, etc.), and aHEV18198 and aHEV19555 were similar to the Australian and Canadian isolates (05-6745-2, Guelph01). Thus the Russian isolates shared 84.0-97.6 % homology with the other representatives of the genotype 3.

Nevertheless, among 14 recovered Russian isolates the aHEV16050, aHEV19553, aHEV19551, aHEV18196, aHEV16279, aHEV20088 and aHEV18383 differed significantly from the other described strains and isolates of the virus and segregated into a distinct genetic lineage (see Fig. 2). They shared 89.6-99.2 % sequence homology with each other compared to 76.0-87.2 % nucleotide identity with the other aHEV strains and isolates.

The aHEV18505 and aHEV18381 Russian isolates did not segregate into any described genetic group on the dendrogram and shared 83.2 % similarity to each other while their sequence identity with the rest used strains and isolates ranged from 76.8 to 84.8 %.

Russian poultry industry is tightly tied to international trade, particularly incubator eggs and day-old chicks for broiler and laying hen flocks are being imported. Therefore, a wide prevalence of aHEV in Australia (1), USA (2), Spain (3), Hungary (4), Korea, Czech Republic, England, Ukraine, Poland, Israel (16) and China necessitates studying the epizootic situation in Russia. In the Russian commercial poultry flocks a hepato- and splenomegaly, hepatic subcapsular hematomas, a breakable consistence of the liver, bloody liquid in abdominal cavity, drop in egg production and increased mortality rates were earlier reported but only recently the eHEV was identified as an etiological agent (5).

Of note, the aHEV was detected both in sick poultry with affected livers and in clinically healthy birds (see Table), being in line with the other data reported (3, 14, 16, 17). So X.J. Meng et al. (17) found out the aHEV infection to be mostly subclinical. In our survey a majority of the recovered Russian isolates, exactly 9 of the 14 samples reported herein were detected in the individuals with no signs of illness. There are the evidences that clinical manifestations in birds may be influenced by a viral dose and strain specificity, by associated pathogens, and also by the poultry age, breed and vaccination (3, 7, 16). Thus, the suggestion was reasonable that the hens with liver pathology might be infected at high viral dose and influenced by some stress factors. However, no associated agents causing similar manifestations such as leucosis virus, Marek's diseases virus or adenovirus were detected (data not shown). All tested probes have been sampled from the hens over the age of 200 days. Importantly, a majority of aHEV isolates, exactly 8 of the 14, were detected in Ross 308 cross.

According to similarity of the nucleotide sequence of ORF2 capsid protein gene 7 of 14 Russian aHEV isolates segregated into a genetic group to which a lineage of another two isolates was close (see Fig. 2).

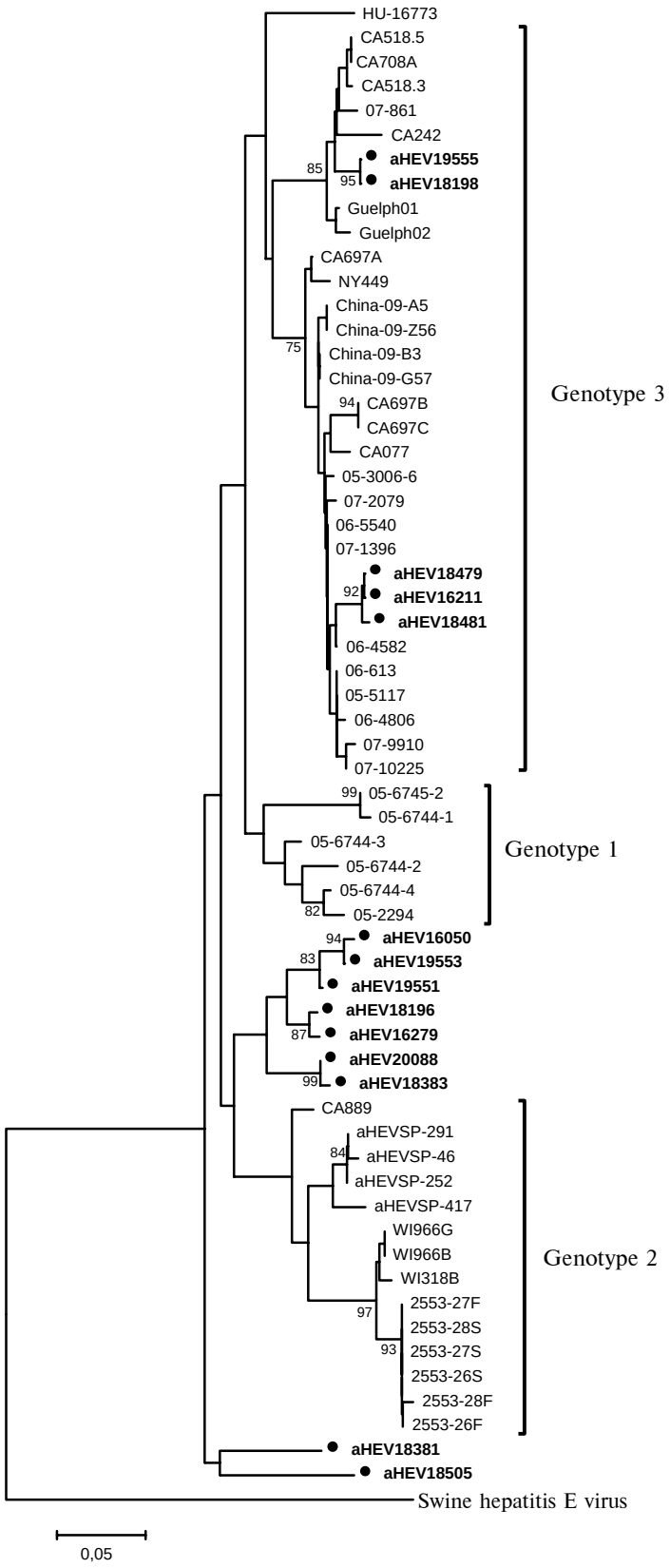

Fig. 2. Dendrogram constructed with the capside gene sequences of avian henatitis virus E (aHEV) of different origin (bootstrap values ≥ 75 %; the 14 aHEV isolates marked bold have been recovered on the poultry farms in the Russian Federation, Belarus, Kazakhstan and Ukraine from 2009 to 2012;

for details, refer to the text of the article).

Based on previous phylogenetic data three aHEV genotypes are suggested according to the geographic origin, i.e. the United States, Australia and Europe, (15, 16), and the Hungarian aHEV is considered a candidate genotype 4. Of note, the Russian isolates (see Fig. 1), too, can probably segregate into a specific genotype, however, the genome sequencing should be copleted for elucidation.

Z.F. Sun et al. (14) reported 76-100 % similarity among aHEV isolates from the US compared to their 48-54 % identity to swine and human HEV. I. Bilic et al. (15) found out the nucleotide sequence homology of aHEV capside gene fragment ranging from 84.0 to 100 % among the European isolates and from 85.1 to 99.4 % among the Australian isolates. The similarity extents of 80.6-85.7 %, 76.6-82.9 %, and 76.0-80.6 % were detected between European and Australian isolates, European and the US isolates, and Australian and the US isolates, respectively.

Three Russian isolates of European genotype 3, the aHEV16211, aHEV18479 and aHEV18481, are the most close to Australian 06-4582 isolate (see Fig. 2), and two isolates, the aHEV18198 and aHEV19555, are closer to Australian and the US isolates, the Guelph01 and CA518.3. The extent of sequence identity of these five Russian isolates to other members of the genotype 3 group reached 85.6-96.8 % (see Fig. 2), and other 9 isolates were not clustered with any currently known genotypes. Thus in Russia both aHEV isolates of European genotype 3 and those not yet assigned to a specific genotype are circulating. Our data evidence the previously reported high genetic heterogeneity of aHEV strains circulating worldwide (14, 15).

Variability of aHEV isolates from different countries strongly suggests the geographically based classification to be revised (15, 16). Despite the distance between Korea and Australia, the Korean aHEV isolates belong phylogenetically to Australian genotype group (18). The same is reported for aHEV isolates from Europe and China. To clarify possible reasons for genetic relationship between the geographically remote isolates more viral samples should be analyzed.

In Russia aHEV seroconversion was first reported in 2004 for Ekaterinburg region by Ibrakhim El-Morsi (13) based on 18.3 % seropositive hens in the flocks surveyed. Nevertheless, these results indirectly indicated aHEV circulation in flocks, and the estimation was incomprehensive. In other countries the aHEV seroprevalence indicates its wide distribution. (1, 19). Particularly, in Korea antibodies against aHEV were detected in 57 % hens (18). In the United States 71 % of 1276 blood serum samples obtained from 76 flocks were seropositive (7). The seroprevalence was higher in the hens over the age of 18 weeks rather than in young poultry (7). In out survey all hens with positive PCR-test were seropositive (data not shown). Moreover, specific antibodies were also identified in RT-PCR-negative blood serum samples. Therefore, aHEV seems to circulate in hen flocks very widely.

According to observations of P. Billam et al. (20), the aHEV natural infection can be first detected in hens aged about 12 weeks with the viremia occurred from 14 to 17 weeks of age. In the hens over 20 weeks of age the PCR-positive probes were low in number despite the high seroprevalence, probably due to viral clearance at sampling. In our survey aHEV was detected in 4 to 56 week-old hens (see Table).

Thus, using RT-PCR a total of 14 aHEV isolates, exactly 13 from Russia and 1 from Kazakhstan, together referred to as the Russian isolates, have been recovered. Majority of the isolates, exactly 9 of 14 reported, were detected in hens with no manifestations of liver and spleen pathology and no decrease in egg production and viability. Of 14 aHEV isolates studied, 7 samples formed a dis-

tinct genetic group. Two more isolates group outside the three genotypes described. The rest 5 isolates group with the European genotype 3 aHEVs. Overall, the pool of aHEV isolates identified in Russian chicken flocks consists of isolates belonging to European genotype 3 and isolates not yet assigned to a specific genotype. Our data are in line with the reported high genetic variability in aHEV strains circulating worldwide. The analysis of genetic variability of aHEVs isolates from different countries strongly suggests that the current classification into genotypes by geographical origin should be revised.

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DETECTION OF AKABANE VIRUS GENOME IN ORGANS AND BLOOD OF EXPERIMENTALLY INFECTED CAVIES

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Abstract

Akabane disease, a transmissible pathology of cattle, sheep and goats, is caused by *Simbu* serotype virus (*Bunyaviridae*) and results in significant economical losses due to abortions, unviable and abnormal calves, or dead embryos and calves born. The Akabane disease epizooties, characterized by geographic locations and the coincidence with definite seasons, are widely registered. For preparative accumulation of Akabane virus the 1-2 day mice are used as the most sensitive system for *Bunyaviridae* isolation. Earlier we reported the development of a test system for Akabane virus RNA indication by real time reverse transcription PCR. Its efficacy was approved using infected mice and cell cultures. Furthermore, it was of interest to estimate this test system with respect to more wide range of model animals to be involved in the study and reproduction of Akabane virus. In this investigation, healthy cavies (n = 20, the animal weight of 400 g) were infected with a concentrated viral culture (B8935 strain) of 7.0 lg $TCID_{50}/cm^3$. In blood the Akabane virus RNA was detected in four animals only 4 days after inoculation, but not shown in the rest probes, which were sampled from 2 to 6 days, and one cavy died a day after infection, probably due to nonspecific reaction as the Akabane virus genome was not detected in the post mortem tissue samples of all the organs of the animal tested. After 4 days the Akabane virus RNA was also indicated in brain, lung, kidney, hart and lymphatic gland. Thus, the developed test-system is effective for Akabane disease diagnosis, and the experimentally infected cavies can be the model animals used to study and produce Akabane virus preparations.

Keywords: Akabane disease, Akabane virus, cavies, *Culicoides*, reverse transcription polymerase chain reaction, RT-PCR, test-system.

Akabane disease, a transmissible pathology of cattle, sheep and goats, is caused by *Simbu* serotype virus of *Bunyaviridae* family. When epizooties occur the infection results in significant economical losses in livestock due to abortions, unviable and abnormal calves, or dead embryos and calves born (1-3). The disease areal is not yet finally estimated. Since 1959 Akabane disease was registered in Japan, since 1972 in Australia and New South Wales, and since 1969-1970 in Israel, Korea and Kenya (4, 5). The Akabane disease epizooties are characterized by geographic locations and the coincidence with definite seasons, particularly from August to October and also in February, occurring periodically after a few years (1, 2, 6). The virus is transmitted by bloodsucking insects mostly from *Culicoides* genus, particularly *C. milne* and *C. imicola* in Africa, *C. oxystoma* in Japan, and *C. brevitarsis* and *C. wadei* in Australia. It also was isolated from *Aedes vexans* and *Culex triaeniorhynchus* in Japan (6, 7) and from *Anopheles fenestus* in Kenya (2-4, 8-10).

Recently there are a number of reports on Akabane virus detection worldwide (11-13). Its genetic characteristics and pathogenesis are under consideration (14) with serological and immunological research conducted (15, 16). Development of specific vaccines is also discussed (17, 18). Therefore, a special attention should be paid to diagnostics and indication of Akabane disease virus genome and to the test kits (19, 20), and also the model systems to

be used in investigations.

For Akabane disease diagnostics different approaches are commonly used such as fluorescent antibodies, virus neutralization test, delayed hemagglutination, complement binding assay, diffuse precipitation test and enzyme-linked immunosorbent assay. Organs and tissues of aborted animals such as brain, lymphatic gland, spleen, kidney, muscles, placenta and blood, and also the blood samples from sick animals are usually tested (2, 21).

The 1-2 day mice, as the most sensitive system for *Bunyaviridae*, are usually used for isolation preparative quantity of Akabane disease virus (21).

The test system based on real time RT-PCR that we have developed allows to detect the Akabane disease virus genome in the brain probes of experimentally infected mice and in infected cell cultures (22, 23).

Herein we report the results of studying whether it possible to use this system for the Akabane disease virus genome indication in blood of experimentally infected cavies and also estimate the different infectious biomaterial for further use in model experiments.

Technique. For virus production the CV-1 cells were infected with Akabane disease virus strain B8935 and cultivated at 37±0.5 °C for 2-3 days. For titration polystyrene plates with CV-1 cell monolayer in wells were used and the cytopathic effect estimated. The observations were continued for 7 days.

Healthy cavies (n = 20, the animal weight of 400 g; N = 3) were infected with a concentrated viral suspension (B8935 strain) of 7.0 lg $TCID_{50}/cm^3$. The animals were separated into two groups. The group I was infected using 50 μl of virus containing material while in the group II the dose was 500 μl.

From day 2 to day 6 after infecting the blood samples were obtained and analyzed using a developed Test system for Akabane virus RNA real time indication in RT-PCR (3, 22, 23). Additionally, to obtain biolmaterial for further investigation the brain, lung, kidney, lymphatic and hart tissues were sampled on day 4 from animals of the group II.

Viral RNA was extracted by nucleosorption using a TRIzol LS Reagent kit (Invitrogen, Inc., USA) according to the manufacturer's recommendations (23, 24).

For RT-PCR a RotorGene-6000 (Corbett Research, Australia) was used as described in the instruction to the test system (3, 22, 23).

Results. Akabane disease virus can be propagated in hen embryos and also in sucking mice as test animals in which the paralytic symptoms develop for 2-3 days as a result of brain infection. Different continues cell lines such as VNK-21 of newborn hamster kidney, VERO, HmLu-1 are also sensitive to Akabane disease virus with clear cytopathic effect 48 to 72 hours after inoculation.

As far as one of our goals was to find an appropriate model for further investigations, the cavies were chosen due to their size and weight compared to rats and mice, that allows to decrease the number of animals used for biomaterial.

The test developed and applied in this investigation is based on a real time reverse transcription PCR with designed AV d and AV u primers flanking 113 bp fragment complementary to the nucleocapsid N protein gene sequence, and the AV z probe complementary to its internal region according to TagMan technique. The test system is high sensitive at 1.5±0.5 lg $MLD_{50}/см^3$ for tissue and organ assay and 1.0±0.5 lg $TCID_{50}/cm^3$ for cell culture.

In blood the Akabane disease virus genome was detected only 4 days after the cavies were infected (Table). One cavy died a day after infection, probably due to nonspecific reaction as the Akabane disease virus genome was not detected in the post mortem tissue samples of all the organs of the animal tested.

Detection of Akabane disease virus genome in blood of experimentally infected cavies using developed real time RT-PCR test system

Days	Animals	
	group I (n = 12)	group II (n = 8)
2	–	–
3	–	–
4	+	+
5	–	–
6	–	–

Comments. «+» or «–» means detected or undetected genome, respectively.

Figure 1 shows the amplification of nucleic acids extracted from blood of cavies in one of three repeated experiments.

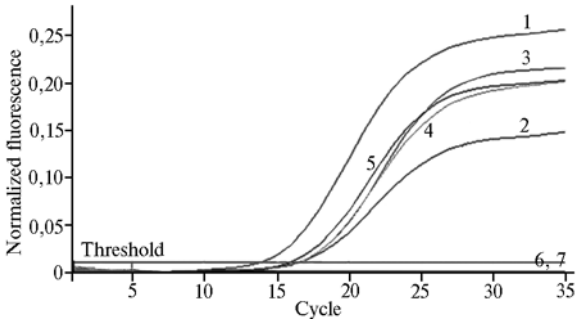

Fig. 1. Detection of Akabane disease virus in blood of experimentally infected cavies using developed real time RT-PCR test system: 1 — recombinant positive control of the amplification; 2-5 — blood of the infected animals №№ 1-4, respectively; 6 — negative extraction control; 7 — negative PCR control (4 days after experimental infection; typical results of amplification are shown).

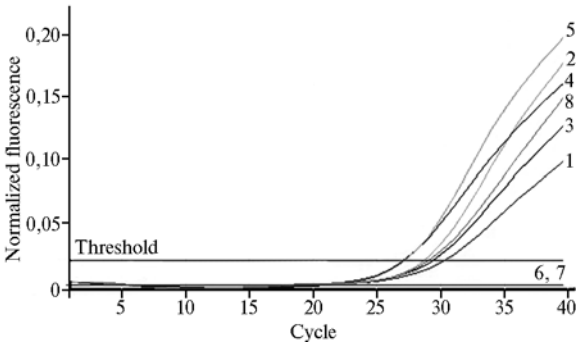

Fig. 2. Detection of Akabane disease virus in organs and tissues of experimentally infected cavy № 4 using developed real time RT-PCR test system: 1 — brain; 2 — lungs; 3 — kidneys; 4 — lymphatic glands; 5 — hart; 6 — negative extraction control; 7 — negative PCR control; 8 — positive control (4 days after experimental infection).

The Akabane disease virus genome was detected in brain, lungs, kidney, lymphatic glands and hart of the animals from the group II (Fig. 2).

Thus, the developed test system based on real time RT-PCR allows detecting the Akabane disease virus genome in blood and organs of experimentally infected cavies. Therefore, these animals may serve a model in validation and approbation of the tests suggested for Akabane disease virus identification.

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DIAGNOSTIC VALUE OF A TEST FOR DETECTION OF SECRETORY IgA FOR CONFIRMATION OF ABSENCE OF FMD VIRUS CIRCULATION

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Abstract

In Russia the preventive vaccination dominates over other protective and preventive measures against foot and mouth disease (FMD). However, the development of the subclinical infection in immune animals should not be ruled out as these animals become virus carriers and pose a potential threat for susceptible animal population. Therefore, the design and reduction to practice of tests for identification of virus-carrier animals continues to be relevant. Secretory immunoglobulins A (sIgA) provide the first line of protection against many infectious agents, they are capable of inhibiting virus intracellular replication and serve as a transmitter of virus neutralization. As reported by foreign authors there is a possibility to identify virus-carrier animals using a rapid ELISA-based test detecting secretory IgA (sIgA-ELISA). Previously we determined sIgA-ELISA optimal conditions for detection virus-specific sIgA in saliva samples in one dilution. In the given paper results of validation and evaluation of the sIgA-ELISA test-system («sandwich» ELISA) are shown as compared with other laboratory methods. The paper presents data on testing bovine biological samples (96 animals with body weight of 200-300 kg at the age of 18-24 months) from FMD-free agricultural enterprises (Vladimir Oblast) collected before and after vaccination from animals, immunized with different batches of FMD vaccine and subject to experimental infection. Samples of saliva, blood sera and esopharyngeal fluid collected with an interval of 3-4 days and up to 94 days after infection were tested using sIgA-ELISA, NP-ELISA (detection of antibodies to virus nonstructural proteins), microneutralization test and Real-Time PCR. Besides, relative sensitivity, specificity and accuracy of the test was evaluated using formulae recommended by the World Organisation for Animal Health (OIE) and compared with results of a reference test conducted by Satya Parida (World Reference Laboratory for Foot and Mouth Disease — WRL, Pirbright Institute, Great Britain) according to a validated method. Out of five preliminary immunized and then infected animals without clinical signs one animal (№ 8895) demonstrated the presence of sIgA in saliva in sIgA-ELISA on day 18 after infection at PP (positivity percent) = 53.00±0.05 % and at other dates (from 21 to 94 days) — at PP from 41.00±0.11 to 115.00±0.41 %. The obtained results were confirmed using the microneutralization test (sIgA titer on day 18 was 1.20±0.05 lg, during a period from day 21 to day 46 the titer was 1.58±0.08-1.90±0.13 lg). At that, the microneutralization test showed significant variations in values and it is undesirable for an identification test. Results of detecting antibibodies to FMD virus nonstructural proteins on day 18-94 were positive. FMD virus RNA was detected using PCR in esopharyngeal fluid samples during a period from day 28 to day 60 after experimental infection, but sIgA-ELISA showed more unambiguous results as compared with PCR. A latent infection was also confirmed in the other animal (№ 8898). Thus, it was demonstrated that the suggested test-system on the basis of indirect «sandwich» ELISA for detection of sIgA to FMD virus in bovine saliva samples made it possible to identify virus-carrier animals according to diagnostic characteristics. After considering results of testing 91 saliva samples the sensitivity of the developed test-system as compared with the reference one (WRL) was 100 %, relative specificity — 97.7 %, accuracy — 99.0 %. The high reproducibility of two test-systems was confirmed on the basis of κ-statistics (κ = 0.99).

Keywords: foot and mouth disease, secretory immunoglobulin A, solid phase immunosorbent assay, virus carrier state, post-vaccination control.

A secretory IgA (sIgA) is essential for the first line of defense against many pathogens and play a critical role in mucosal immunity (1). IgAs can bind and aggregate bacteria and viruses in secretions preventing their adhesion on mucosal surfaces, and at high concentrations IgAs block viruses from attaching to cell membrane. At low concentration sIgAs can inhibit viral replication

though no significant impact on viral adhesive capability is observed. sIgA is a mediator involved in neutralization and probably elimination of viruses, besides, sIgAs stimulate phagocytosis providing local resistance to infection. An increase in sIgA serum concentration is presumably due not only to epithelial lesions but also to sIgA secretion activated in the attacked organs and tissues (2). Development of sIgA specific tests suggests the possibility of their use for foot and mouth disease (FMD) monitoring (3, 4).

In Russia the preventive vaccination dominates over other protective and preventive measures against foot and mouth disease. However, the development of subclinical infection in the immune animals should not be ruled out as these animals become virus carriers and pose a potential threat for susceptible animal population. Therefore, the design of tests for identification of virus-carrier animals remains relevant in order to avoid further development of infection (5).

FMD virus (FMDV) isolation from esophageal-pharyngeal fluid (EPF) using sensitive cell culture is considered reference method (6). However, the sharp phase of viral replication can be short with no clinical symptoms, so a probability of FMDV detection is low. Specific RT-PCR analysis is suggested to identify small FMDV amounts in esophageal-pharyngeal fluid, but positive results can be false because of nonspecific inhibitors. Beside, even at genome degradation when FMDV contagiousness has been lost the PCR amplification of genome fragments is still possible also leading to false positive results (7).

FMDV genome detection in pharyngeal epithelial cells by in situ hybridization also has been reported, however it is inconvenient for large scale screening in case of urgent vaccination. As far as in infected animals the antibodies both to structural and nonstructural FMDV proteins (NSP) are FMDV-induced, whereas at vaccination only the antibodies to structural proteins are vaccine-induced, the serological methods based on NSP identification (NSP-tests) are used in veterinary practice. Nevertheless, their accuracy depends on different factors, e.g. characteristics of commercial vaccine or NSP level, the antigen concentration in the vaccine and frequency of vaccination (8-11). Therefore, development of alternative or additional methods for identification of subclinical FMD among vaccinated animals remains of current interest (12).

As reported by foreign authors, virus-carrier animals can be identified using rapid ELISA-based test to detect secretory IgA (sIgA-ELISA) (3, 4, 12). Previously we designed a protocol for optimal sIgA-ELISA one-dilution detection of virus-specific sIgA in saliva samples (14).

In this paper the results of validation and evaluation of the sIgA-ELISA test-system («sandwich» ELISA) used for virus-carrier identification are shown as compared with other laboratory methods.

Technique. A total of 96 animals with body weight of 200-300 kg at 18-24 months of age from the FMD-free agricultural enterprises with no vaccination against FMDV (Vladimir Province) were used in the experiment carried out under controlled conditions in the FGBU «VNIIZZh» vivarium. Experimental animals were subcutaneously vaccinated in the middle third of the neck with monovalent sorbed anti-FMDV vaccine (VNIIZZh, Russia) based on FMDV O type Pan Asia 2 strain cultivated in the VNK-21 continues line of newborn hamster kidney cells. In prior tests the vaccine immunogenicity in cattle was not less than 6 PD_{50} according to European Pharmacopoeia (15). Both vaccinated and unvaccinated animals, the latter used as a control, were experimentally infected 28 days later by tongue intradermal injection of FMDV O strain № 2108 Zabaikalskii/2010 at 10^4 ID_{50} in 0.2 cm^3 in two portions. Saliva (16), esophageal-pharyngeal fluid (17) and blood sera were sampled with 3-4 day

interval and up to 94 days after infection. Biomaterial from vaccinated animals with no clinical symptoms was sampled at the same periodicity during 2.5 months since day 18 after they have been infected. Data were recorded 8 days after control infection.

Indirect sandwich ELISA test was applied as described (18). The percentage of positivity (PP) was evaluated using ELISA in one dilution (14) at PP ≥ 40 % as positive score.

Antibodies against FMDV nonstructural proteins were assayed as described (19). FMDV was isolated according to the method approved by Veterinary Department of the Ministry of Agriculture of the Russian Federation dated 11.10.2002. The FMDV micro neutralization assay (MNA) was used as recommended (6), and real time PCR analyses was carried out as described (20).

Relative sensitivity, specificity and accuracy of the test were estimated according to the International Epizootic Bureau (IEB) recommendations (12):

$$\text{Relative sensitivity } Se = \frac{a}{a + c} \times 100\ \%, \quad [1]$$

$$\text{Relative specificity } Sp = \frac{d}{b + d} \times 100\ \%, \quad [2]$$

with a as true positives, b as false positives, c as false negatives and d as true negatives.

Common statistical methods according to I.P. Ashmarin et al. (1975) and MS Excel 2003 were used for data processing.

Results. In FMDV-inoculated vaccinated animals no generalaized infection was observed since there were primary aphthous ulcers on tongue with no secondary aphthous ulcers on limbs, meanwhile, in all unvaccinated animals the aphthous ulcers were found both on tongue and limbs.

We surveyed a total of 96 saliva samples from intact cattle, 40 samples from vaccinated cattle, 5 samples from cattle infected 21 days after vaccination and 96 samples from infected cattle. The anti-FMDV sIgAs were not found in saliva of both intact and vaccinated animals prior to experimental FMDV inoculation. In the cattle with FMD clinical symptoms the sIgAs were found after inoculation on days 5-6, 8 and 10-11 that is in line with other published findings (6).

In case of vaccination with FMDV O type Pan Asia 2 strain followed by experimental inoculation with FMDV O strain № 2108 Zabaikalskii/2010 the five animals with no clinical symptoms were found. Notably, a close antigenic similarity was observed between the FMDV O № 2108 Zabaikalskii/2010 and O Pan Asia 2 strain (r_1 = 0.42-0.47) (data not shown). In these animals we failed to isolate FMDV from EPF using sensitive SP primary culture of piglet kidney cells and two continuous cell cultures, the IB-RS-2 of piglet kidney and PSGK-30 of mountain goal kidney. Furthermore, no positive results were also obtained upon blind passages. Nevertheless, in saliva of one of these animals, № 8895, the sIgA was detected in sIgA-ELISA test after experimental FMDV inoculation on day 18 at PP = 53.00±0.05 % and from day 21 up to day 94 at PP from 41.0±0.11 to 115.00±0.41 %. Based on this test, the sIgA concentration in saliva increased upon day 67 (PP = 115.00±0.41 %, OD_{405} = 1.20±0.14), then slightly decreased and remained unchanged until day 94 (PP = 71.00±0.02 %, OD_{405} = 0.91±0.14).

The obtained data were confirmed in MNA. sIgA titer in saliva was 1.20±0.05 lg on day 18, and from 1.58±0.08 to 1.90±0.13 lg on day 21 to day 46 with two peaks at 1.90±0.13 lg (Table 1). Nevertheless, from day 32 to day 53 it decreased to 1.07±0.09-1.40±0.10 lg sometimes indicating negative response (see Table 1). Finally, since day 60 the index increased again, being from 1.50±0.05 to 1.90±0.13 lg with two peaks (see Table 1). Thus, saliva sIgA titer in MNA is an improper identification index because of significant variations.

1. Accumulation of anti-FMDV antibodies and FMDV genome in experimentally infected cattle as identified by different tests ($M \pm m$)

Day after inoculation	Saliva		EPF in PCR	Blood serum		
	sIgA-ELISA, PP %	MNA, lg		NSP-ELISA	ELISA, lg	MNA, lg
			Animal № 8895 (n = 3)			
18	53.00±0.05	1.20±0.05	+	+	2.71±0.15	2.56±0.05
21	54.00±0.19	1.85±0.05	–	+	2.48±0.08	2.70±0.05
28	86.00±0.05	1.58±0.08	+	+	2.56±0.08	2.70±0.15
32	56.00±0.10	1.15±0.25	+	+	2.56±0.05	2.70±0.05
35	41.00±0.11	1.07±0.09	–	+	2.56±0.08	2.70±0.15
39	55.00±0.03	1.90±0.13	+	+	2.40±0.10	2.70±0.05
42	78.00±0.11	1.40±0.10	–	+	1.95±0.08	2.70±0.05
46	65.00±0.10	1.90±0.13	+	+	1.95±0.08	2.56±0.08
49	43.00±0.11	1.20±0.05	–	+	2.26±0.15	2.56±0.05
53	43.00±0.03	1.20±0.05	+	+	1.95±0.08	2.56±0.05
56	Not assayed	Not assayed	+	+	2.26±0.15	2.56±0.05
60	41.00±0.19	1.58±0.08	+	+	2.26±0.15	2.56±0.05
63	65.00±0.08	1.75±0.13	–	+	2.56±0.05	2.56±0.08
67	115.00±0.41	1.85±0.10	–	+	2.41±0.15	2.56±0.05
74	91.00±0.21	1.60±0.10	–	+	2.33±0.38	2.56±0.05
81	56.00±0.10	1.90±0.13	–	+	2.41±0.15	2.56±0.05
88	88.00±0.51	1.80±0.10	–	+	2.56±0.05	2.70±0.05
91	78.00±0.02	1.90±0.13	–	+	2.70±0.05	2.40±0.10
94	71.00±0.02	1.50±0.05	–	+	2.70±0.05	2.56±0.05
			Animal № 8898 (n = 3)			
18	8.00±0.03	0.83±0.15	–	+	3.38±0.08	2.70±0.05
21	10.00±0.05	Not assayed	+	+	3.23±0.07	2.70±0.15
28	26.00±0.09	1.60±0.05	+	+	3.00±0.05	2.70±0.08
32	64.00±0.10	1.27±0.12	+	+	2.86±0.15	2.70±0.05
35	56.00±0.04	1.60±0.15	+	+	3.00±0.05	2.70±0.05
39	68.00±0.05	1.65±0.10	+	+	2.86±0.15	2.70±0.08
42	47.00±0.11	1.20±0.05	+	+	2.26±0.31	2.70±0.15
46	31.00±0.15	1.50±0.05	+	+	2.56±0.08	2.70±0.08
49	83.00±0.13	1.95±0.15	+	+	2.86±0.15	2.70±0.08
53	70.00±0.16	1.95±0.05	–	+	2.40±0.21	2.70±0.08
56	46.00±0.10	1.80±0.10	–	+	2.86±0.15	2.70±0.15
60	77.00±0.02	1.65±0.15	–	+	2.70±0.05	2.70±0.08
63	73.00±0.16	1.73±0.08	–	+	3.46±0.05	2.70±0.05
67	66.00±0.15	1.50±0.05	–	+	Not assayed	2.70±0.08
74	75.00±0.24	1.95±0.15	–	+	2.63±0.37	2.56±0.05
81	68.00±0.15	1.80±0.10	–	+	2.78±0.38	2.56±0.05
88	81.00±0.15	Not assayed	–	+	3.46±0.05	2.70±0.15
91	114.00±0.11	Not assayed	–	+	3.00±0.05	2.40±0.10
94	89.00±0.13	Not assayed	–	+	2.86±0.08	2.40±0.15

Comments. FMDV — foot and mouth disease virus, «+» and «–» — positive and negative test, respectively, NSP — nonstructural proteins, PP — percentage of positivity, MNA — microneutralization assay, EPF — esophageal-pharyngeal fluid.

NSP-ELISA tests were positive from day 18 to day 94, and the FMDV genome was found in EPF, though inconstantly, by PCR from day 28 to day 60. In other words, sIgA-ELISA was more accurate compared to PCR when saliva was tested, and the same conclusion was made by other researchers (5). According to our tests, the № 8895 animal is a FMDV carrier which should have to be immediately isolated in case of a commercial herd.

Compex assay of biomaterial from another animal (№ 8898) of those five revealed as FMD symptom-free clearly indicated the latent infection (see Table 1). Actually, FMDV-specific sIgAs at PP = 64.00±0.10 % were identified in saliva from day 32 after experimental infection, and further tests remained positive at PP from 46.00±0.10 to 114.00±0.11 %. In MNA the sIgA titer was mainly 1.60±0.10 lg, reaching 1.95±0.15 lg on day 49, and further varied from 1.65 to 1.95 lg up to day 63. Then the titers declined together with some decrease in PP, but since day 74 until the end of experiment the sIgA concentration in saliva rose being detected both with ELISA and MNA (see Table 1). In NSP-ELISA sIgAs were constantly detected in all saliva samples. Besides, specific amplification was shown in PCR analysis of EPF 21-49 days after the experimental inoculation. Notably, in blood serum of all in-

fected animals the level of FMDV-neutralizing antibodies was high independently of whether the infection was persistent or typical, and besides no reliable differences appeared during the observation. This emphasizes an inadequacy of virus carrier identification among animals based on tests of neutralizing antibodies in blood serum.

In animal № 8896 the sIgA were detected with ELISA from day 74 to day 94 and with MNA from day 81 to day 94 at 1.80-1.95 lg. However, we failed to identify FMDV genome using PCR analysis of EPF sampled from day 21 to day 94. Probably, it was due to 2 month contact of FMDV-free animals with the FMDV-carrier kept in the same box that resulted in contact re-infection.

Another animals, № 8897 and № 8899, surveyed accordingly the described scheme were not identified as FMDV carriers. In saliva and EPF samples from animal № 8897 the sIgAs were not found in sIgA-ELISA and MNA, and FMDV RNA was detected in PCR only from day 18 to day 32, probably due to experimental inoculation. In saliva of animal № 8899 the sIgAs were not detected by sIgA-ELISA, except day 21, meanwhile in MNA the titer was below 0.9-1.2±0.005 lg. FMDV RNA was detected by PCR analysis of EPF from day 18 to day 32 and from day 46 to day 49.

Thus, based on our testing the animals № 8895 and № 8898 experimentally infected after vaccinated were identified as the FMDV carriers.

As far as we did not isolate FMDV from EPF in a reference sensitive cell culture and our data should be assessed, the same saliva samples, that we tested using sIgA-ELISA system developed in VNIIZZh, were analyzed by Satya Parida (World Reference Laboratory for Foot-and-Mouth Disease — WRLFMD, Pirbright Institute, Great Britain) according to validated method (6) to compare developed and validated tests. Specificity of validated sIgA-ELISA was 97.1 % at threshold of OD_{405} = 0.47 with an increase of specificity up to 99.4 % as threshold rises to 0.60 (6). For accuracy the samples not checked by one of tests were excluded, and questionable samples were considered as positives. Based on analysis of 91 samples the sensitivity of developed test compared to validated test of WRLFMD was 100 % at 97.7 % relative specificity and 99.0 % accuracy.

Besides, based on the comparison (Table 2), we evaluated κ-criterion (κ-statistic) to assess the consistency of these tests.

2. Consistency of sIgA-ELISA tests of World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) **and VNIIZZh** (Russia) **for detection of anti-FMDV sIgAs in bovine saliva**

WRLFMD / VNIIZZh (Russia)	Positive	Negative	Total	Prevalence
Positive	$a = 47$	$b = 1$	$a + b = 48$	$(a + b)/n = 48/91 = 0{,}53$
Negative	$c = 0$	$d = 43$	$c + d = 43$	$(c + d)/n = 43/91 = 0{,}47$
Total	$a + c = 47$	$b + d = 44$		$n = 91$
Prevalence	$(a + c)/n = 47/91 = 0{,}52$	$(b + d)/n = 44/91 = 0{,}48$		

Comments. a — true positives, b — false positives, c — false negatives, d — true negatives, n — sample saze.

The calculated indexes were as follows: absolute consistency $(a + d)/n$ — $(47 + 43)/91 = 0.99$; random consistency of positive results — $0.52 \times 0.53 = 0.28$; random consistency of negative results — $0.48 \times 0.47 = 0.23$; cumulative random probability of consistency of results — $0.28 \times 0.23 = 0.06$; apparent consistency of results without regard to contingent probabilities — $0.99 - 0.06 = 0.93$; nonrandom maximum possible consistency of methods — $1 - 0.06 = 0.94$; κ-criterion (κ-statistics) of $0.93/0.94 = 0.99$. This κ-criterion value indicate good consistency of these two tests.

So, the developed indirect «sandwich» ELISA test for anti-FMDV IgA detection in cattle saliva is suitable for identification of the FMDV carrier animals. Characteristics of the developed test system including its validity indicate strong consistency of our results and the validated test of World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD at κ-criterion = 0.99.

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EXPERIENCE OF USING SEROLOGICAL AND MOLECULAR TESTS TO DETECT EQUINE INFECTIOUS ANEMIA VIRUS IN HORSE

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Abstract

Equine infectious anemia in horses is caused by equine infectious anemia virus (EIAV, *Lentivirus*, *Retroviridae*), affecting hematopoietic organs. The symptoms of the disease are relapsing or continued fever, anemia and a disturbance of cardiovascular functions. Duly virus detection is the only effective way to control infection. Serological methods used to indicate EIAV have some limitations. For instance, they did not allow identifying infected animals prior to seroconversion. Also an immunodeficiency can really occur when the content of virus specific antibodies is too low to be indicated. Here we report the results of comparative study of different serological and molecular techniques for diagnosis of equine infectious anemia in experimentally inoculated susceptible 9 month old foal. In the experiment, we used the kits for agar gel immunodiffusion assay (Russia) and ELISA (France) as serological tests, and our own developed test-systems for viral RNA detection by nested reverse transcription polymerase chain reaction (RT-PCR) with electrophoretic control and by real-time RT-PCR. Developed PCR-tests is based on amplification of EIAV *gag*-gene fragment using specific oligonucleotide primers. In blood, the viral RNA was detected by both test-systems from day 2 after animal inoculation until the end of observation at day 35. Specific antibodies were detected in diffuse precipitation reaction from day 30 and in ELISA from day 21 after animal inoculation. Thus, PCR-analysis could be used as an express-test for detecting EIAV genome prior to seroconvertion, and then other diagnostic methods should be further applied to verify the RCR data. The developed Russian test-systems and investigation techniques confirm the benefits of the PCR diagnosis in the early stages of the EIA.

Keywords: equine infectious anemia, experimental infection, PCR, serodiagnostics.

Equine infectious anemia in horses affects hematopoietic organs with relapsing or continued fever, anemia and a disturbance of cardiovascular functions (1-4). Horses of all ages, ponies, asses, mules can be naturally infected (5, 6). Diseased growing horses often die (3, 7). Because of the consequences of outbreaks the national programs are implemented to eradicate equine infectious anaemia (8, 9). The etiological agent of the disease is RNA containing equine infectious anemia virus (EIAV) from *Lentivirus* genus of *Retroviridae* family (3, 4, 10).

At EIAV infection the nonsterile immunity is developed, meaning the immunity is exists only in the presence of the infectious agent in the organism. In horses attacked by EIAV the antibodies to its different antigenic determinants appear in blood serum due to immune response to viral infection (3).

The EIAV diagnostics is currently based on a detection of specific antibodies. Special kits are developed and commercially distributed for a diffuse precipitation test (DPT) and ELISA test. Of serological methods, the diffuse precipitation test (DPT, or the Koggin's test, is considered the golden standard (7, 11).

Nevertheless, some disadvantages of the these techniques should also be noted. Of them the first is inability to detect sick animals prior to seroconversion. Moreover, the immune deficient status is described resulting in low virus specific antibodies production, so that their small quantity prevents detec-

tion. In these cases the obtained negative immunological results must be considered false (12).

Additionally to indirect serological indication the EIAV genome can be detected directly. PCR diagnostics of EIA disease is most common prior to seroconversion occurs. Abroad different protocols based on RT-PCR are developed and successfully applied (2, 4, 13). A number of researchers and official experts consider the EIAV molecular detection essential at early diagnostics of equine infectious anemia (12, 14), and the molecular methods are also recommended by the World Organization for Animal Health (Paris) (14).

Importantly, the duly virus detection is the only effective way to control infection, as to date there are no preparations for its specific treatment and prevention (1, 3).

Here we report the results of comparative study of different serological and molecular techniques for diagnosis of experimentally developed equine infectious anemia.

Technique. A susceptible 9 month old foal was intravenously injected with 10 ml of 10 % suspension of spleen tissue from a seropositive horse slaughtered at EIA outbreak in Nizhegorodsakay Province in 2011. Then the animal was kept in the vivarium under daily clinical control including thermometry.

Blood was sampled daily from day 1 to day 4 after infection and then each 2 days until the end of experiment on day 35. Blood serum was sampled on days 2, 7, 14, 21, 28, 30 and 35.

EIAV RNA was detected by nested RT-PCR with electrophoretic separation of amplified fragments and by real time RT-PCR assay (15, 16). To perform nested RT-PCR a Palm Cycler (Corbett Research, Australia) was used, and the real time RT-PCR amplification was carried out on a RotorGene-6000 (Corbett Research, Australia) according to recommended protocols (15, 16).

For serological diagnostics a commercial Kit for diagnostics of equine infectious anemia by diffuse precipitation (Russia) and a Test system for detection of antibodies against EIAV in blood serum by indirect ELISA (IDvet, France) were used according to the manufacturers' recommendations.

Results. Prior to inoculation the animal was EIAV-seronegative as confirmed by a diffuse precipitation test.

During our experiment the characteristic synptomes of equine infectious anemia were observed such as fever, hyperthermia, strong thirst, depression and weigh loss. From day 10 a hyperthermia was observed, with the body temperature reached 40.9 °C on day 15 and then decreased to a physiological norm.

Developed PCR-tests is based on amplification of EIAV *gag*-gene fragment using specific oligonucleotide primers (10, 11).

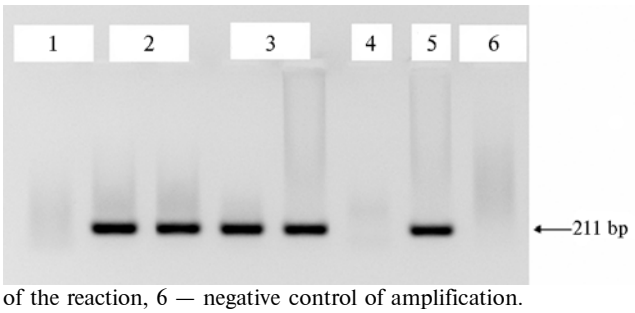

Fig. 1. Amplification of specific genome fragment of equine infectious anemia virus from blood of 9 month old experimentally infected foal in nested RT-PCR: 1 — day 1 after inoculation, 2 — day 2 after inoculation, 3 — day 35 after inoculation, 4 — negative control of the reaction, 5 — positive control of the reaction, 6 — negative control of amplification.

In blood the viral RNA was found from day 2 after animal inoculation by both nested RT-PCR with electrophoretic detection (Fig. 1) and by real time RT-PCR (Fig. 2). Further, EIAV RNA was detected by both test systems until

the end of observation at day 35.

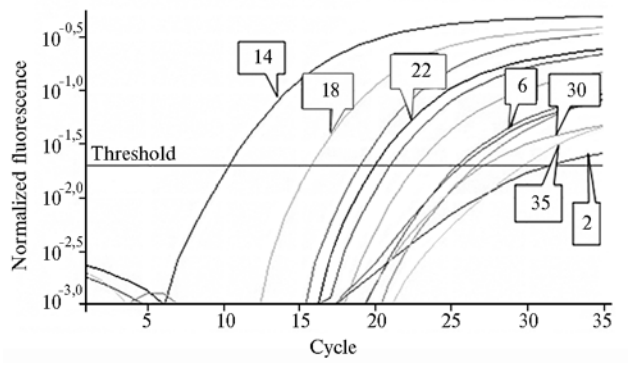

Fig. 2. Amplification of specific genome fragment of equine infectious anemia virus from blood of 9 month old experimentally infected foal by real time RT-PCR (the curves for day 2, 6, 14, 18, 22, 30, and 35 of the experiment shown).

Specific antibodies against EIAV were identified by diffuse precipitation test from day 30 after inoculation and by indirect ELISA test from day 21 after inoculation until the end of our observation on day 35. These data are in line with the reported findings that the concentrations of specific antibodies in blood serum enough to be serologically indicated cannot be reached prior to day 14 to 35 period of infection (3).

Thus, PCR-analysis could be used as an express test for detecting EIAV genome prior to seroconvertion, and other diagnostic methods should be further applied to verify the RCR data. The developed Russian test-systems and investigation techniques confirm the benefits of the PCR diagnosis in the early stages of the EIA. Particularly, EIAV can be effectively detected by PCR analysis from day 2 of infection.

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Gene pools

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CHANGES OF VECTORS OF SELECTION FOR PIGLET'S NEWBORN WEIGHT DURING POPULATION FORMATION IN NEW ENVIRONMENT CONDITIONS

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Abstract

Studying changes of gene pools of populations of the domestic animals caused by selection and reflecting micro evolutionary processes, represents both practical and theoretical interest. As a rule, in such works the qualitative traits controlled by the principle «one genotype — one phenotype» are used that significantly simplifies the analysis. We investigated dynamic processes in Landrace population of domestic pigs (*Sus scrofa domesticus*), having estimated a variation of one of continuous quantitative traits, the newborn piglet weight, for which similar relationship is not unambiguous. Statistical analysis of the zootechnical register data was carried out on more than 26 thousand pigs that were born within 23 years at an experimental farm (Novosibirsk Province), being the regional authorized Landrace breed nucleus. Ancestors of the formed population were delivered from Latvia in the early 1960s. They were the elite young animals estimated on a standard complex of selection traits. The entire period of existence of population the formation of its breeding nuclear was carried out on a complex of traits according to existing Instruction for estimation of breeding value of pigs. As the Instruction didn't contain standards on a large newborn weight, the selection on the specified trait wasn't made. The changes of statistical parameters of the newborn weight in piglets were estimated for each year of the observation, because the analyzed livestock was not a model laboratory population, but a typical breeding commercial herd in which continuous variability of a trait is interfaced to continuous «sliding» alternations of generations and the variability of age structure. During long-time analysis of the dynamics of this unselected trait, the directional (moving) selection on genotypes for the loci controlling growth rate of pigs in ontogenesis (in pre- and post-natal periods) is revealed. In the populations where such selection works, the newborn weight of piglets can be used for forecasting pig weight during the postnatal period. Application of «parent—descendant» regression on the newborn weight allowed to estimate the duration of a population gene pool adaptation to new environment. It appeared that process of adaptation lasted nearly two decades that made five full changes of the generations. In the same population at the same time the stabilizing selection optimized an individual animal weight at birth, cutting both minimum and maximum values. The described mechanism includes cyclic vector changes towards driving selection against the stabilizing selection vectors and thus maintains the population polymorphism on loci which control prenatal growth and large weight in the newborns. An observed unevenness of wavy change of these cycles should be also noted.

Keywords: pigs, *Sus scrofa domesticus*, Landrace, population, adaptation, piglet's newborn weight, regression, the vector of selection, directional (moving) selection, stabilizing selection, microevolution processes.

Changes in gene pools of population caused by selection or occurred due to a random gene drift are constantly attracting attention of the researchers (1-3), and in many works the domestic pigs were studied in this connection (4-12). However, the qualitative traits are mostly estimated as more simple because of a strong concordance of each specific genotype to a single phenotype according to the «one genotype—one phenotype» principle. For long time of our studying variations of qualitative traits the results of both practical and theoretical interest have been obtained (13-17). Importantly, once the quantitative trait changes were first statistically analyzed, a number of genetic determinations peculiar spe-

cifically to domestic animals were found out (18). Besides, the traits used in pig breeding were shown to reflect trends related to artificial selection rather then to natural. So we tried to find the trait convenient for estimation of the natural selection vectors. The number of nipples was first used, but it was good to indicate random factors affecting quantitative traits (19) and poor reflected the intrapopulation changes because of discreteness and a narrow variation range (18, 19). A newborn piglet weight was next tested. This is a continual quantitative trait with indefinite relationship of a genotype to the phenotype, meaning the same phenotype appeared in different genotypes and multiple trait values are observed in the same genotype (20). As far as a piglet weight at birth is a perinatal growth trait its variation in heterozygotes contains the ranges of variations in homozygotes on each allele (21). Reasonably, a piglet birth weight, being a continuous quantitative trait with rather wide variation range and known genetic control, was used as a quantitative trait (22-26).

In this research our goal was to find out whether a piglets' weight at birth is influenced by artificial or by natural selection whereas the population of domestic pigs are being newly formed due to adaptation to unusual environmental conditions.

Technique. Landrace domestic pigs (*Sus scrofa domesticus*) born from 1964 to 1965 and from 1967 to 1986 at an experimental farm (Novosibirsk Province) were analyzed. Ancestors of the formed population were delivered from Latvia in the early 1960s. They were the elite young animals with an estimated standard complex of selection traits (27). During the observation the rearing technologies and feeding were as described (28, 29). Twice a day the animals were fed with standard SK compound feeds (Russia) according to the recommended rations (30).

The entire period of existence of the population the breeding for a complex of traits was carried out according to the Instruction for estimation of pigs' breeding value which didn't contain standards of a large animal weight (9) or a newborn weight. At a breed herd formation the piglets were twice selected, particularly in 2 month of age when weaned and in 5 months of age when a remount group of 25 % of breed nucleus was formed.

To evaluate the trait dynamics, the statistical parameters were annually compared. An average piglets' birth weight in each year was compared to those of their parents, exactly 21461 piglets from 312 paternal boars and 1181 sows were estimated. Additionally, to evaluate the upper limit of the trait values a total of 26086 newborn piglets from 2587 litters were weighed.

The data were processed by common statistical methods (31).

Results. The experimental piggery was the regional authorized Landrace breed nucleus. In the population adapted to Siberian environment no targeted selection for an above standard animal weight was used. In created population its value was increasing for 23 years, mostly in the parent animals from the reproductive herd nucleus and in much less extent in their offspring.

A year was the least period for comparison since the analyzed livestock was not a model laboratory population, but a typical breeding commercial herd. In such a herd the reproductive nucleus consists of animals of different ages and is constantly renewing due to some new boars and sows included while the others discarded because of age or other reasons. Thus, a change of generations in the herd is a continuous process extended in time. At that an annual variability of the trait is linked to a continued change of generations and the age structure of breeding stock. As the latter was almost the same among years, being subject only to random variations, a calendar year as a time unit was reasonably used in a

comparative study of the trait dynamics.

A birth (kg) **variation in piglets and their parents in a Landras pig population during long adaptation to unusual environmental conditions** (experimental farm, Novosibirsk Province)

Year	Descendants		Boars		Sows		Regression coefficient (b_{xy})	
	n	*M*±*m*	*n*	*M*±*m*	*n*	*M*±*m*	boar— descendant	saw— descendant
1964	524	0.98±0.007	9[p]	1.40±0.116	40[p]	1.24±0.040	–0.006	0.091
1965	405	1.14±0.012	14	1.08±0.074	31	1.13±0.032	–0.140	–0.403
1967	262	1.16±0.013	10	1.19±0.038	28	1.15±0.041	–0.670	–0.163
1968	355	1.20±0.007	6	1.18±0.048	30	1.14±0.046	–0.111	–0.123
1969	592	1.29±0.019	10	1.38±0.092	56	1.36±0.046	0.212	0.098
1970	1027	1.26±0.004	22	1.48±0.063	100	1.35±0.030	–0.007	0.032
1971	1116	1.38±0.009	17	1.37±0.051	94	1.45±0.032	–0.189	0.231
1972	1506	1.47±0.007	24	1.38±0.074	106	1.46±0.030	–0.063	0.034
1973	1652	1.33±0.012	14	1.39±0.075	122	1.46±0.025	–0.067	0.018
1974	1255	1.42±0.005	15	1.37±0.064	98	1.43±0.025	–0.030	0.037
1975	1066	1.35±0.009	15	1.39±0.070	73	1.44±0.025	–0.070	–0.042
1976	748	1.42±0.010	16	1.44±0.055	74	1.51±0.025	–0.056	0.029
1977	1329	1.18±0.008	22	1.56±0.056	92	1.49±0.017	0.038	0.024
1978	1451	1.28±0.007	26	1.18±0.076	109	1.24±0.026	0.052	–0.028
1979	1720	1.35±0.007	30	1.47±0.040	115	1.35±0.023	0.007	0.029
1980	1187	1.31±0.008	32	1.48±0.047	101	1.36±0.023	–0.045	–0.141
1981	1663	1.34±0.007	33	1.56±0.063	128	1.34±0.024	–0.009	0.085
1982	1114	1.34±0.010	29	1.51±0.039	79	1.44±0.033	0.224	–0.059
1983	724	1.45±0.012	24	1.59±0.062	67	1.47±0.037	0.046	0.134
1984	562	1.30±0.014	15	1.55±0.061	53	1.49±0.046	0.110	0.191
1985	853	1.24±0.010	23	1.55±0.058	74	1.47±0.034	0.203	0.171
1986	350	1.12±0.009	10	1.32±0.053	31	1.37±0.038	–0.150	–0.022
Total	21461	1.32±0.002	312	1.44±0.017	1181	1.38±0.008		
r	0.33; unreliable		0.61; P < 0.01		0.57; P < 0.01			

C o m m e n t s. 1964, 1969, 1973, 1977, 1981 and 1985 are the years of complete change of the parental animals in the breed nucleus); p in upper index marks offspring of the animals delivered from Latvia; *M*±*m* is an average value and the error; *r* is a correlation coefficient between year and the birth weight.

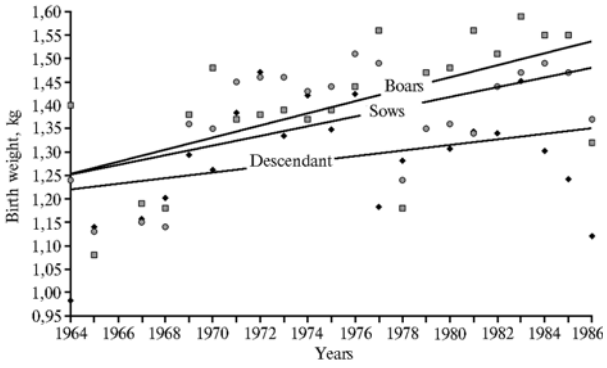


Fig. 1. Birth weight trends in Landras pig population and its reproductive nucleus during long adaptation to unusual environmental conditions: ♦ — descendant, □ — boars, ○ — sows (experimental farm, Novosibirsk Province).

In the basic experiment only piglets individually weighed as well as both their parents were considered (Table) that allow to indicate a correct trends in the trait changes regarding random variation and annual succession of the breed nucleus.

For the trait a positive trend was found reliable ($P < 0.01$) in parents and unreliable in the population as a whole, being probably observed due to expression in the parent animal groups (Fig. 1).

Thus, the animals from reproductive nucleus were selected for an increased birth weight while its influence on the population was insufficient. Herewith, the changes resulted from three factors, namely natural selection for the early postnatal viability, a positive correlation between the weights at birth and at weaning, as demonstrated by the curves (Fig. 2) constructed according to published data (29), and a standardizing selection for total weight specific to domestic animals (18). The first two factors significantly increased probability of more large animals inclusion into effective nucleus of the population. Providing both prenatal and postnatal growth is genetically controlled by the same loci, a standardizing selection for total weight could influence a newborn weight despite the fact that artificial selection for weigh at remount herd formation first occurred in 2 month

old animals, as thereat required (27).

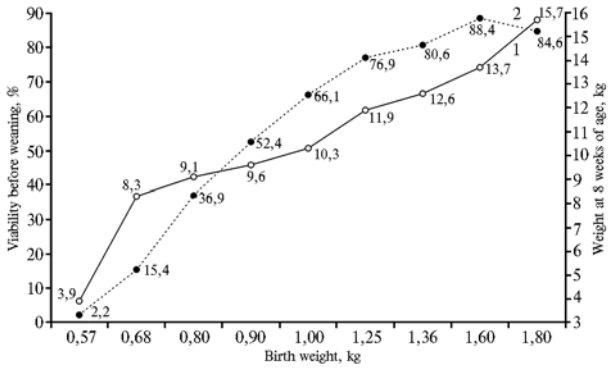


Fig. 2. Viability of sucking piglets (1) **and their weight at weaning in 8 weeks of age** (2) **as related to birth weight** (consorted according to published data) (29).

The observed trends on the birth weight in parental groups and reliable differences between the breeding males and females ($t_a = 3.06$, $P < 0.01$) resulted from the standardizing selection. More clear positive trend (see Fig. 1) and higher birth weight (see Table) in boars resulted from much more strong selection compared to sows. A summarizing vector of the discussed three factors obviously was directed not to the birth weight increase, but to the high rate of prenatal and postnatal growth. Therefore, in the effective part of the population there are the individuals not just large at birth but also fast growing during postnatal period.

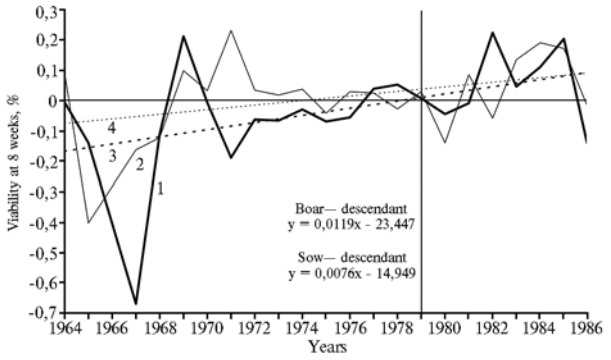


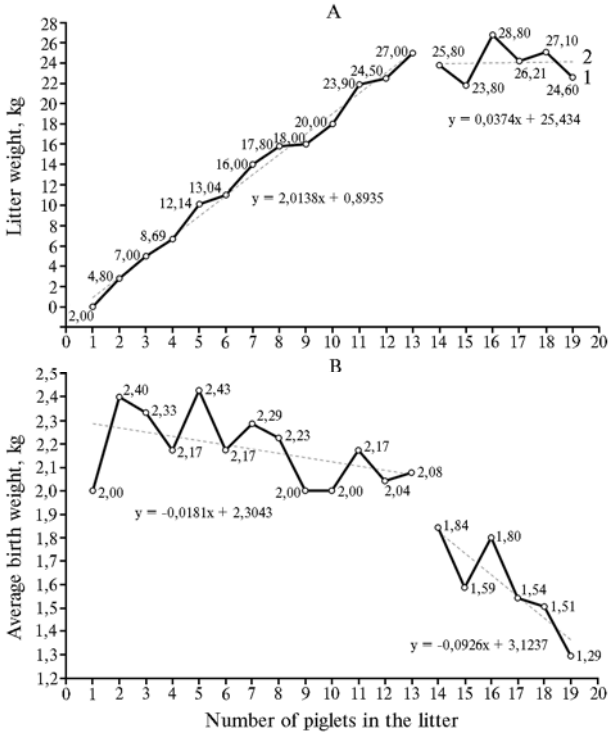
Fig. 3. Birth weight regression coefficient in Landras pig population during long adaptation to unusual environmental conditions: 1 — boar—descendant, 2 — sow—descendant, 3 — trend of boar on descendant regression coefficient, 4 — trend of sow on descendant regression coefficient (experimental farm, Novosibirsk Province).

Piglet's birth weight is polygenically controlled (20, 21) with an expressed indefinite relationship between genotype and phenotype, meaning not only effects of randomnicity but also probable adequate response to external impact. The influence of both these factors may be described by the regression of a parental phenotype on the descendant phenotype. Thus, the both ambiguities, i.e. for the genotype-phenotype relationships in a parent and its offspring, could be considered. The Landras pigs delivered from Baltic region to West Siberia for introduction had to adapt to unusual conditions. As a result, a gene pool has changed during formation of a new population. According to the analyzed trait, these changes were estimated by a linear regression trends for both boars' and sows' phenotypes on that of their offspring (see Table, Fig. 3).

On the curves the longest initial segments of the negative regression coefficient should be noted (see Fig. 3). Suggestible, under unusual feeding and housing conditions the piglets born larger were more viable during sucking period and also gaining greater weight by weaning (see Fig. 2) (29). As a result, the population was subjected to selection for the high rate of prenatal and postnatal growth. Obviously, initially in the population the analyzed phenotype was poor related to genotype. Therefore, in the offspring the birth weight did not exceed an average value in the population even if the parents were larger at birth, resulting in negative regression coefficients. Selection for high growth rate during ontogenesis gradually decreased indetermination of the relationship between genotype and phenotype to a positive parent—descendant regression level occurred 15 years after the adaptation started (see Fig. 3). It probably means that the animals

with relatively stable and locally adapted relationship of the genotype to phenotype exceeded in rate, if compared to those with unstable relationship.

Hereby, a selection for increased birth weigh was really found in the studied population. Nevertheless, the weigh of newborns remained relatively unchanged due to genetic and physiological mechanisms acting towards stabilization within the limits of sows' and offspring viability. In other words, birth weigh can vary in different population as well as in the same population among the years (see Table) but within definite range, so that the selection for the trait can not be effective in the essence. The lowest values are obviously limited by the high death rate in small piglets. Optimization of the birth weight in the population should correlate with some other traits. Particularly in pig as a prolific animal there is a collision between birth weight of a piglet and the number of piglets in the litter resulting in negative correlation of these traits (32). Similar correlation was also observed in the population studied ($r = -0.82$; $P < 0.001$).



Figc. 4. Litter weight (A) **and averages birth weight** (B) **depending on number of piglets in a litter in Landras pig population during long adaptation to unusual environmental conditions:** 1 — actual distribution, 2 — trend line (experimental farm, Novosibirsk Province).

A probable factor limiting birth weigh is a total offspring weight. Abstractly, a curve of its dependence on piglets' number must contain two segments, of which the first tended to a population maximum, looking as an upward sloping line, and the second fluctuated around the horizontal line somewhat below the maximum level. The piglets with no data on their parents' weight, a total of 26086 newborns including those stillborn from 2587 litters, were additionally analyzed to verify this suggestion. The highest weight in the sample was 28.8 kg (Fig. 4, A). We compared a reliability of approximation R^2 (Microsoft Excel) for the most frequent numbers of piglets in the litter to find the end of the first segment. In case of 12, 13 and 14 piglets R^2 was 0.9880, 0.9905 and 0.9823, respectively. So the ascending and horizontal segments corresponded to the numbers from 1 to 13 and from 14 to 19, respectively. For the first and second segments the correlation coefficients between size of the litter and weight of the litter at birth were +0.995 ($P < 0.001$) and +0.039 (unreliable), in the other words, the curve quite well described a relationship between the litter weight at birth and the number of piglets per litter (see Fig. 4, A). The litter weight at birth is related both to the number of piglets per litter and the piglet's weight at birth, in turn, both being physiologically limited by the maternal physiological capabilities. As a result, a negative correlation occurs between fetus number and weight. Thus the second curve of relationship between birth weight and number of piglets in the litter should obviously be

similar in character but reversed, so that the horizontal segment was followed by inclined line (see Fig. 4, B). Correlation coefficients (r) between the number and an average birth weight of piglets in the litter were –0.467 (unreliable) and –0.856 ($P < 0.05$) for two segments, respectively, being in line with our suggestion. Consequently, it can be asserted that the vector reducing birth weight is a factor of stabilizing natural selection towards optimization of the correlation between piglet weight and number.

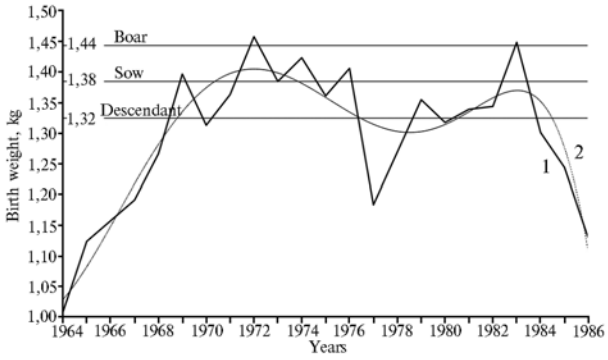


Fig. 5. Changes in birth weight in Landras pig population during 23 year adaptation to unusual environmental conditions:: 1 — actual, 2 — trend line (experimental farm, Novosibirsk Province).

As far as the litter size negatively correlates with birth weight per piglet, and standardizing selection for higher litter size as an important economic trait is constantly occurring in the commercial pig populations, its intensification presumably intensifies the natural selection for lower weight of the newborns. After fixation of the alleles encoding optimal number of piglets in the population the selection for an increased birth weight which provides higher viability of sucking piglets should be resumed and continued until the piglet number fall. Then the next cycle should begin, and so it goes on all time during the population existence. Because of a collision between selection for higher birth weight and its upper limitation, the curve should show wavy fluctuations around optimum value for rather long time that exactly was observed in the studied population (Fig. 5). During 23 years there were two waves, when an average birth weight increased from 1.01 to 1.46 in 1964 to 1972, then decreased to 1.18 kg in 1977, and again increased to 1.45 kg in 1983 and dropped to 1.13 kg in 1986. Generally, the birth weight as a quantitative trait seems to be influenced by the limitations resulting in elimination of the largest individuals (see Fig. 4) rather then by selection towards higher weight parameters, probable due to some other factors reducing piglet's weight at birth to an optimal value.

It should be noted that at long-term adaptation the classical population research based on annual dynamics estimation (33) is more effective compared to constructing series based on the limited data (see Table) for the years of complete change of generations in the reproductive herd nucleus. In fact, exactly classical approach is appropriate to studying real but not model populations. Our findings contribute to current knowledge about genetic and breeding aspects of newborn's weight in pigs (20, 34).

Thus, in Landras pig population formed in the course of adaptation to unusual conditions the directional (moving) selection for the loci controlling growth rate of pigs in ontogenesis, i.e. in prenatal and postnatal periods, is revealed. In the populations where such selection works, the newborn weight of piglets can be used for forecasting pig weight during the postnatal period. A regression of parent—descendant birth weight in the population allows to estimate the time of gene pool adaptation to new environment. Adaptation in the studied population took nearly two decades that made five full changes of generations, and there were two key events observed. First, a predominance of the individuals with reduced ambiguity in genotype—phenotype relationship has been reached

after 15-year adaptation, and secondly, optimization admittedly considered the final adaptation of the population has been achieved 5 years later. In the same population at the same time the stabilizing selection optimizes an individual animal weight at birth, cutting both minimum and maximum values. The described cyclic changes of driving and stabilizing selection vectors seem to provide the population polymorphism on loci which control prenatal growth and large weight in the newborns. An observed unevenness of wavy changes in these cycles should also be noted.

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HIGH SPERM PRODUCTION AS RELATED TO MACRO- AND MICROELEMENT LEVELS IN BLOOD SERUM IN SERVICING BULLS OF THE MODERN SELECTION

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Abstract

Nowadays in commercial animal husbandry the intensive metabolizer genotypes are basically used. In such a case the animal health and performance should be characterized neither by the presence or absence of clinical manifestations of diseases, nor by decreased productivity or reproductive ability, but by the parameters of metabolism and its early violations at a subclinical level. Herein we summarize original data on the levels of the essential macroelements, in particular Ca, P, Mg, Fe and chlorides, and microelements, the Cu, Zn, Se and Mn, in blood serum of the superior sires as influenced by their age, beef or milk specialization and RED-factor expression. In our experiment the 2-7 year meat Aberdeen Angus, Herefordm, Lymousine cattle, milk Red-and-White Holsteine, Black-and-White Holsteine, Ayrshire cattle, and also universal Brown Swiss cattle were assessed (n = 49 in total). The following parameters were investigated: an ejaculate volume, the spermatozoid number per ml, the percentage of spermatozoid motility in the native, diluted, frozen and thawed semen, and in the samples frozen, thawed and then incubated at 38 °C for 5 hours. The total spermatozoid number per ejaculate, semen dose number per each sire, frozen semen dose number sampled during 24 hour, and the percentage of culled native ejaculates were also assessed. It was shown the microelements to influence the semen quality and quantity. Macro- and microelement levels in the sires' blood serum were shown to depend manly on the diet and should be constantly controlled and adjusted. According to the obtained data, the differences in macro- and microelement levels due to specialization and RED-factor expression are unreliable, while the changes in Ca:P ratio and Cu, Zn and chloride levels in blood serum influence the semen quality in the bull sires. Our data suggest that in each cattle breed the physiological standard levels of macro- and microelements in blood serum should be revised with respect to age, sex, physiological condition, a season, livestock farming technology and specialization. Besides, the data confirm the need for an integrated control and adjustment of animal supply with macro- and microelements referring to their deficit or excess in different geochemical provinces.

Keywords: microelements, macroelements, bull sires, sperm production, breeding.

Nowadays in commercial animal husbandry animal health considered the most essential for the their exploitation.

In milk production the significant genetic progress for 20 past years is due mainly to wide use of artificial insemination and multiplication of the valuable genotype with specifically high metabolic activity. In such a case the animal health and performance should be characterized neither by the presence or absence of clinical manifestations of diseases, nor by decreased productivity or reproductive ability, but by the parameters of metabolism and its early violations at a subclinical level (1). Biochemical assays allow to control early disturbances in metabolism of proteins, carbohydrates, lipids, vitamins, macro- and microelements, and the more violation found, the more crucial disorders occur (1).

Verification of performing and reproductive health in cows of new genotypic formation aimed to intensification of their commercial use necessitates the

detailed metabolic examinations of both parents, and moreover, the physiological status of the most valuable bull sires must be under special consideration (2). Besides, slight metabolic disorders in young animals can indicate the genetic abnormalities (3).

The 15 microelements, namely Fe, I, Cu, Zn, Co, Se, Mn, Cr, Ni, V, Mo, F, Li, Si and As, are essential. They are involved in cell receptors and transfer proteins, influence the activity of enzymes and hormones as well as their synthesis, have an antioxidant effect and an impact on immune response, etc. (4, 5).

Metabolic disorders may occur both at deficit or excess of some elements. The same or very similar skeletal diseases were detected at Ca, P, Cu, Mn, Zn, Si, vitamin A and D deficit, as well as at Mo, F, Sr and vitamin D excess. Anemia is caused by deficit of Fe, Cu, Co or some vitamins or by Mn, Vo, Zn, Cu, Pb, Se excess in the animal diet (6).

It is commonly known that micro- and macroelements are involved in the regulation of basic physiological processes. Particularly, Ca is accumulated in skeletal tissues, but also is involved into regulation and different vital processes in cells and tissues. Ca level in blood of healthy animals depends on Ca, P, Mg and vitamin D in the ration. Of total mineral compounds in animal body Ca and P make 70-75 %. At that about 90-99 % of Ca and 80-87 % of P are in the skeleton, while the soft tissues contain 1-2 % and 13-20 %, respectively. Ca and P deficit leads to osteomalacia or osteoporosis in adults. Calcium ions are essential for blood coagulation, colloid structure of proteins and membrane permeability, resistance to infectious diseases and toxic agents. It contributes to a decreased nervous irritability, regulation of hart function and activation of adrenocortical hormones, pituitary hormones and pancreatic hormones (7). P is involved in different biochemical reactions, particularly in energy transfer, as well as in metabolism and transfer of lipids and carbohydrates. P is a component of nucleic acids, phospholipids and phosphorproteins, it is essential in keeping up the acid-base balance in ruminants and activation of fermentation in the rumen (7, 8). The diet deficient in P leads to a decreased weight gain and milk production, disorexia, reproductive disorders and weak underdeveloped newborns.

Excess of P leads to Mg deficit observed at high concentrate feeding and ketosis (6). About 60-70 % of total Mg in the skeleton, of which one third is bound to phosphates and two third are absorbed on bone crystals. In soft tissue Mg serves for normalization of neuromuscular stimulation and enzyme activation. Mg ions activate phosphatase, peptidase, etc. (7). In case of Mg deficit the degenerative and necrotic changes are observed in kidneys, also Ca content in blood vessel walls, heart and skeletal muscles increases and violation of hormonal secretion occur (9). Mg deficit is mainly caused by imbalanced diet, starvation, an increased amount of P-containing food, intensive Mg excretion due to increased physical activity or endocrine dysfunction (8).

Se is important for reproductive function, and its level in liver depends on vitamin E content. At Se deficit in cattle there are delivery complications, detention of the afterbirth and endometritis.

Deficit of I results in increased infertility in cows, aborts, weak libido in bulls, and low semen quality. I is mainly know to be essential for thyroid function and its hormonal activity. In case of its deficit the interaction between pituitary and ovaries become weak and follicular cysts occur.

Zn multiply affects different processes as it is the basic microelement for animal growth and development, productive and reproductive functions, osteogenesis and hemopoesis. Presence of Zn in anterior pituitary is probably related to its role in production of gonadotropins which control sexual function. At Zn deficit the organic disorders develop in semeniferous tubules and spermatogenesis disturbance occurs. A disordered secretion of gonadotropins leads to deficit in

testosterone and probably causes atrophy of testes. Excess of Zn may result in anemia, growth depression and toxemia.

Several thousands metalloproteins are shown to be involved in animal metabolism. For Zn there are more then 200 protein-containing compounds, including 160 metalloenzymes. Particularly, Zn citrate is an essential component secreted by prostate which improves motility of the spermatozoids in ejaculate. Besides, Zn is a powerful antioxidant agent able to stabilize cell membranes, and also it has the immunomodulating effect on T cells (10).

Zn stimulates reproductive function directly or via pituitary-gonadotropins. The insular function of pancreas is shown to be related to Zn content. Zn bound to insulin and glucagon is involved in metabolism of carbohydrates (11). Importantly, an increase Ca input causes an increased Zn requirement in animals.

Fe is essential for hemopoesis and intercellular metabolism. It is contained in blood hemoglobin, cytochromes and enzymes involved into oxidative and reductive processes. About 55-60 % of Fe in the body is hemoglobin-bound, about 24 % is hemoproteins-bound and involved into myoglobin formation, and 21 % is deposited in liver and spleen. Fe is important for enzymatic activity, immune functions and cholesterol metabolism. Both deficit and excess of Fe negatively affects animal health. At Fe deficit anemia occurs because of deficit in hemoglobin synthesis. An increased Fe content could result from long-term administration of Fe-containing preparations. Animals obtain Fe from feed and about 1 mg Fe is daily excreted with urine and faeces. Fe excess leads to gastrointestinal dysfunction, weight loss, kidney failure, liver diseases, arthritis, depression of cell and humoral immune responses, and increased risk of infections. Excess of Fe as an oxidative agent can generate free radicals able to destroy tissues and negatively affect spermatogenesis. An increased level of Fe in feeds and water depresses utilization of fodder protein and animal productivity. For better Fe and Ca assimilation they should be administrated separately, due to that the Fe bioavalibility increases 2-fold while the side effects of its preparations decreases $^1/_3$ times (12).

At chronic Fe excess a bloodletting and hepatoprotectors must be used. Zn and Ca preparations provide Fe assimilation, while phosphates prevent it. Vitamin E and high Zn doses decrease Fe assimilation and vitamin A deficit represses it. Vitamins C and B_{12} in combination with Cu contribute to Fe assimilation. Low acidity of gastric juice leads to low Fe assimilation, and Fe excess prevents Cu and Zn assimilation (13).

Deficit of essential microelements mostly leads to a decreased meat, wool and milk production, disturbed digestion and metabolism, repressed reproduction and weak newborns (14). Deficit of a single and especially several microelements causes endocrine disturbances and can decrease hormonal biosynthesis and activity thus negatively influencing reproduction (15).

Undoubtedly, biochemical monitoring of metabolic parameters in high-producing animals is actual, particularly in bull series of modern breeding, derived from mother cows with annual 15000-20000 kg milk production or daily 1500 g weight gain for milk and meat breeds, respectively.

Herein we summarize original data on the levels of the essential macroelements, in particular Ca, P, Mg, Fe and chlorides, and microelements, the Cu, Zn, Se and Mn, in blood serum of the superior sires as influenced by their age, beef or milk specialization and RED-factor expression.

Technique. In our experiment the 2-7 year meat Aberdeen Angus, Herefordm, Lymousine cattle, milk Red-and-White Holsteine, Black-and-White Holsteine, Ayrshire cattle, and also universal Brown Swiss cattle were assessed (n = 49). The surveys were carried out from 2011 to 2013 in Head Center for

Farm Animal Reproduction (Moscow Province). Feeding, housing and sampling were in accordance with National technology for freezing and using semen of bull series (16). Blood for analysis was traditionally sampled immediately after semen sampling before feeding according to planned veterinary and sanitation measures (16).

Ca, P, Mg, Fe macroelements and chlorides were tested in blood serum on a ChemWell 2902 analyzer (AwareneessTehnology, USA), Cu, Zn, Se, Mn microelements were analyzed in blood using an atomic absorption spectrometer Kvant-2A (Russia).

The investigated parameters were an ejaculate volume, the spermatozoid number per 1 ml, the percentage of spermatozoid motility in the native, diluted, frozen and thawed semen and in the samples frozen, thawed and then incubated at 38 °C for 5 hours. A total spermatozoid number per ejaculate, semen dose number per each sire, frozen semen dose number sampled during 24 hours, and the percentage of culled native ejaculates were also assessed.

All technological steps corresponded to National technology for freezing and using semen of bull series (16).

MS Excel was used for data statistical processing.

Results. We studied micro and macroelement levels in blood of bull series as influenced by age, type of productivity, RED-factor and parameters of sperm production.

Among the years of observation (Table 1) the electrolytic exchange (Ca, P, Mg, Fe) and Cu, Zn, Se, Mg microelements level in blood serum in all bull series were within physiological limits while in Fe, Se contents and Ca:P rate there were some discrepancies, particularly Ca:P = 1.54 compared to physiological norm ranged from 1.70-2.00, so 10-30 % decrease observed).

1. Content of macro- and microelements in blood serum of bull sires (n = 49, $X \pm x$, Head Center for Farm Animal Reproduction, Plc., Moscow Province)

Parameter	2011, spring	min-max	2012, spring	min-max
Ca, mmol/l	2.61±0.14	2.22-2.82	2.45±0.25	2.10-3.36
P, mmol/l	2.64±0.34	2.01-3.89	2.11±0.40	1.72-3.72
Mg, mmol/l	0.94±0.13	0.70-1.49	0.88±0.09	0.69-1.15
Fe, mmol/l	31.46±9.25	18.83-59.45	31.14±5.33	20.62-43.77
Se, μmol/l	2.04±0.41	1.29-3.41	1.13±0.25	0.53-1.63
Cu, μmol/l	14.39±2.04	11.76-21.44	16.00±2.11	13.02-23.48
Zn, μmol/l	33.00±6.72	22.21-52.37	40.16±7.24	24.65-55.67
Ca: P	–	–	1.54±0.25	0.85-1.92
Chlorides, mmol/l	103.64±4.66	87.58-110.87	105.54±4.09	97.5-113.82

C o m m e n t s. Dashes means no parameters detected.

In 2011 Ca level in 46 bulls (94.00 %) was close to upper limit ranging from 2.22 to 2.82 mmol/l compared to physiologically normal values from 2.22 to 3.33 mmol/l. Nevertheless, next year the parameter became worse and ranged from 2.10 to 3.36 mmol/l including normal values in 30 bulls and decreased values in 36.70 % bulls. We suggest this fact being caused by more intensive exploitation and predominance of concentrate feed that was clearly indicated by P value in blood serum (see Table 1).

In 2011 P content in blood serum, because of concentrate feeding, in all bulls (100 %) exceeded the norm ranging from 2.10 to 2.50 mmol/l in 36.73 % bulls, from 2.51 to 3.00 in 51.00 % bulls and from 3.01 mmol/l in 12.46 % bulls. Therefore, the parameter was exceeded in 18 animals by 19.0-60.0 %, in 25 animals by 42.80-100 %, and in 6 animals more then 2 times. In 2012 after the diet was changed according to concentrate ratio the P level improved in 32 animals (65.00 %), but in 16 bulls (32.00 %) the parameter remained exceeded.

The Mg concentration in blood serum in both years was below norm in 9 bulls, and in 2012 a general trend to minimization was observed in all animals.

Because of high local Fe content in fodder and water, it is constantly above the norm in the bulls from Head Center for Farm Animal Reproduction, Plc., particularly in 2011 and 2012 51.00 % and 67.00 % animals possessed an excess of Fe in blood serum. An excess of phosphates is known to inhibit Fe absorption (13). According to our data (see Table 1), P content was inversely proportional to Fe content. In 2011 the parameter value was the same as or more then normal in 18 and 25 bulls, respectively, and next year in 32 animals the P content was stabilized but Fe content increased (see Table 1).

Selenium, being toxic when exceeded in level, could be additionally obtained only from feed premixes. In 2011 in 85.70 % bulls its concentration in blood serum was above the norm, particularly 1.52-2.00 μmol/l in 17 sires (35.00 %), 2.00-2.50 μmol/l in 22 sires (45.00 %) and above 3.00 μmol/l in 3 sires (6.00 %). Next year the indexes decreased significantly, ranging from 1.54 to 1.63 μmol/l in 10.00 % bulls.

A physiological antagonism exists between Cu and Mn, Zn, Ca, Cd. Fe also can depress Cu assimilation (13). In our experiment the blood Cu concentration exceeded the norm in 4 bulls in 2011 and in 11 bulls in 2012.

Zn deficit in animals is mainly manifested in weight loss with specific clinical symptoms (17). During investigation in 2011 the index ranged form 22.20 to 38.50 μmol/l in 41 bulls (84.00 %) being Zn deficient, but next year increased about 2 times due to special diet, nevertheless, 21 bull remained Zn deficient.

Chlorides in both years were in fact the same (see Table 1), being increased in level in 2011 in 9 bulls and in 2012 in 12 bulls with the indexes from 108.72 to 110.87 mmol/l and from 108.84 to 113.72 mmol/l, respectively.

Thus in bull sires blood serum concentration of macro and microelements depends mainly on fodder amount and quality, so the animal diet must be corrected based on blood biochemical monitoring.

To estimate the intensity of macro and microorganism metabolism as influenced by a productive type, the data were compared with respect to milk, meat or universal types of the breeds (Table 2).

2. Content of macro- and microelements in blood serum of bull sires depending on a type of breed productivity (n = 49, $X \pm x$, Head Center for Farm Animal Reproduction, Plc., Moscow Province, 2011-2012)

Parameter	Type		
	meat, n = 10	milk, n = 36	universal, n = 3
Ca, mmol/l	2.38±0.18	2.49±0.26	2.32±0.25
P, mmol/l	2.00±0.29	2.15±0.42	1.99±0.60
Mg, mmol/l	0.87±0.08	0.88±0.10	0.91±0.05
Ca: P	1.55±0.23	1.53±0.25	1.59±0.46
Fe, mmol/l	30.28±6.31	31.41±5.23	31.10±4.08
Chlorides, mmol/l	105.37±3.85	105.32±4.14	108.23±4.97
Se, μmol/l	1.27±0.25	1.10±0.24	0.96±0.14
Cu, μmol/l	15.77±1.62	15.85±2.20	18.49±0.45
Zn, μmol/l	41.72±6.21	40.64±7.02	30.64±6.24

No reliable differences in blood serum macro and microelement levels were shown between the types at the same housing and feeding. Despite the Ca to P balance in the diet and admissible levels in animal blood the Ca: P ratio fluctuated among the groups from 1.16 to 1.19, being below norm of 1.70-2.00. Therefore, at excess of concentrates the bulls were deficient on motion. In universal breed the Zn and Ca levels were 20 and 3-30 % below the norm, respectively.

In all groups the Fe level of 28.64 mmol/l was 10 % above the physiological maximum. As far as Fe excess is obtained from plant fodder and water the premixes should be corrected based on blood chemistry and antagonism of elements, particularly Fe and Ca, Fe and Zn, Mn and Fe, Cu and Zn, etc.

Animal coloration is commonly known to result in different response to insolation, that in turn affects vitamin D production and finally may impact on hormonal activity, definite exchange processes and metabolism in general (18). We studied the impact of RED factor on blood macro and microelements in Holsteine bull sires homozygous on coloration (Table 3).

3. Content of macro- and microelements in blood serum of RED factor homozygous bull sires depending on sensitiveness to insolation (n = 33, $X \pm x$, Head Center for Farm Animal Reproduction, Plc., Moscow Province)

Parameters	Holsteine bulls		Difference, %
	Red-and-White (n = 13)	Black-and-White (n = 21)	
Ca, mmol/l	2.56±0.37	2.46±0.17	–3.9
P, mmol/l	2.29±0.50	2.08±0.35	–10.2
Mg, mmol/l	0.91±0.09	0.86±0.09	–5.5
Fe, mmol/l	32.13±5.09	31.39±5.44	–2.3
Se, μmol/l	1.14±0.23	1.03±0.21	–9.6
Cu, μmol/l	15.48±1.67	16.13±2.56	+4.2
Zn, μmol/l	41.11±5.45	40.56±8.07	–1.4
Ca: P	1.49±0.30	1.53±0.21	+2.7
Chlorides, mmol/l	105.00±3.17	105.68±4.83	+0.4

No reliable relationships were found between the mineral exchange and honozygosity on RED factor affecting sensitivity to insolation, however in Black-and-White bulls compared to Red-and-White bulls the average values of the main indexes were somewhat lower.

4. Content of macro- and microelements in blood serum of bull sires depending on age (n = 49, $X \pm x$, Head Center for Farm Animal Reproduction, Plc., Moscow Province)

Parameters	Age, months	
	< 30 (n = 28)	≥ 30 (n = 21)
Ca, mmol/l	2.48±0.24	2.41±0.26
P, mmol/l	2.12±0.35	2.09±0.46
Mg, mmol/l л	0.88±0.09	0.88±0.10
Fe, mmol/l	32.57±5.64	29.24±4.41
Se, мкмμmol/l	1.10±0.23	1.16±0.29
Cu, μmol/l	15.87±2.20	16.26±2.03
Zn, μmol/l	38.60±6.70	42.65±7.39
Ca: P	1.54±0.28	1.53±0.24
Chlorides, mmol/l	105.61±4.39	105.51±3.78

The age of bull also had no reliable effect on the parameters studied (Table 4). Noteworthy, in young bulls up to 30 months of age an average Zn and Fe levels were 10 % below and above those in adults, respectively. This fact is worth special attention as even the Zn level enough for growth is insufficient for spermatogenesis, and Zn deficit during growth leads to infertility and skeleton abnormalities such as dwarfism or elongation of tubular bone characteristic to infantile development.

In order to evaluate the influence of main macro and microelements on sperm production, the bulls were conditionally divided into groups with deficient, normal or excess concentrations of some studied elements (Tables 5, 6). Grouping animals exclusively deficient or excessive on a single element was impossible as a deficit in any one element is usually compensated by an excess of the other, so the definite conclusions can be hardly made.

The high quality ejaculates were 10 % more in number among bulls with a stable Ca:P rate, i.e. in the animals with no hidden ketosis. Sperm content and number of frozen semen doses per bull were a little bit higher among the animals with Ca:P of 1.01-1.59 due to excessive protein diet. Nevertheless, their sperm was more sensitive to freezing and, thus, less viable. The percentage of rejected ejaculates in bulls with violation of Ca:P was 6.74 % higher compared to that in animals without Ca:P abnormalities. Besides, Ca:P normalization contributed to 9.1 % more viability of sperm during incubation at 38 °C for 5 hours. Best sperm quality was observed in bulls with normal Cu level as the number of rejected ejaculates was 10 % lower, while the semen content and dose number were 6 % and 19 % higher, respectively, compared to bull excessive on Cu. Zn

affected on the ejaculate volume, being a component of prostate secretion. In bulls with normal Zn level the ejaculate volumes were 4.3±0.9 ml compared to 3.9±0.9 ml in bulls with a decreased Zn level ($P < 0.05$). In our investigation the bulls were found out to be mostly deficient on Zn, while in the normal group the index was close to the lower limit value, and in the same group there were the animals excessive on Cu and Fe. At that the rejected semen number was 15 % higher then in clearly Zn deficient animals. Spermatozoid motility after thawing and incubation at 38 °C for 5 hours was higher then in animals with normal Zn level in blood serum. An excess of chlorides in blood had a negative impact on semen quality and viability due to influenced electrolytic balance in generative cells.

5. Quantitative parameters of sperm production as related to the level of macro and microelements in blood serum of bull sires ($n = 49$, $X \pm x$, Head Center for Farm Animal Reproduction, Plc., Moscow Province)

Parameters	*n*	Number of ejaculates per bull	Ejaculate volume, ml	Spermatozoids in an ejaculate	
				content, $\times 10^9$/ml	total, $\times 10^9$
Ca: P					
Below normal level (1.01-1.59)	26	11.4±2.0	4.3±1.0	1.28±0.27	5.4±1.4
Normal level (1.70-2.00)	12	10.8±2.7	4.2±0.9	1.23±0.22	5.0±0.5
Zn					
Below normal level	12	11.8±1.4	3.9±0.9	1.34±0.24	5.2±0.9
Normal level	36	11.0±2.2	4.3±0.9	1.26±0.25	5.3±1.4
Chlorides					
Normal level	38	11.4±2.0	4.1±0.9	1.30±0.30	5.2±1.3
Above normal level	10	11.0±2.9	4.5±1.1*	1.20±0.20*	5.1±1.3
Cu					
Normal level	33	11.2±2.3	4.1±1.0	1.26±0.24	5.1±1.1
Above normal level	11	11.5±2.0	4.3±0.7	1.33±0.30	5.5±1.3

* $P < 0.05$.

6. Qualitative parameters of sperm production as related to the level of macro and microelements in blood serum of bull sires ($n = 49$, $X \pm x$, Head Center for Farm Animal Reproduction, Plc., Moscow Province)

Parameters	*n*	Number of ejaculates per bull	Rejected ejaculates		Frozen doses per bull	Spermatozoid motility, %
			total	%		
Ca: P						
Below normal level (1.01-1.59)	26	11.4±2.0	3.7±2.8	32.32	1293	9.2±3.6
Normal level (1.70-2.00)	12	10.8±2.7	2.3±2.1	25.58	1238	10.1±5.7
Zn						
Below normal level	12	11.8±1.4	2.5±2.3	21.28	1514	8.6±4.4
Normal level	36	11.0±2.2	4.0±2.6	36.73	1127	10.6±4.0
Chlorides						
Normal level	38	11.4±2.0	3.7±2.8	33.64	1209	10.9±5.3
Above normal level	10	11.0±2.9	3.8±3.2	33.64	1259	8.6±2.9
Cu						
Normal level	33	11.2±2.3	3.3±2.5	29.81	1196	11.2±5.3
Above normal level	11	11.5±2.0	4.6±3.7	39.68	1473	7.6±1.5

Comments. Spermatozoid motility was assessed after incubation at 38 °C for 5 hours.

Obtained data indicate the necessity of complex study of macro and microelements interaction. Deficient element should be compensated by premixes and additives considering local biogeochemical conditions (19).

The higher productivity and weight gain, the more intensive metabolism occurs and more macro and microelements are required (1). Consequently, their optimal values in cattle blood serum should be revised according to breed specialization, animal sex, age, physiological state, seasons, rearing technology and exploitation.

Thus, in bull sires the blood level of macro and microelements depends on balanced diet, mode of exploitation, age, breed specialization, and unreliably on RED-factor. In case the levels of elements change towards deficit or excess, the spermatogenesis is disturbed both qualitatively and quantitatively.

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FREE RADICAL LIPID OXIDATION AND REPRODUCTIVE HEALTH OF COWS

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Abstract

Free radical lipid oxidation is currently considered as one of the dominant metabolic processes of physiological system functional activity. When it goes beyond regulated limits it is considered as an inductor of free radical pathology oxidative stress. In the conditions of a big dairy cattle breeding farm (Voronezh Province), specialized in Red-and-White breed, the functioning peculiarities of the peroxidation system lipid-oxidant defense in cows at the normal course of gestation and at gestosis, at the normal course of postpartum period and at puerperal endometritis, uterus subinvolution and ovarian dysfunction were studied. The state of lipid peroxidation processes and antioxidant system were evaluated by determining total lipid content, cholesterol, triglycerides, malonic dialdehyde (MDA), nitrogen oxides ($NO^{\bullet}$), E and C vitamins concentrations, glutathione peroxidase (GPO), glutathione reductase (GR), catalase, superoxide dismustase (SOD) activity in blood. It is demonstrated that the high activity of lipid peroxidation reactions and the system of nitrogen oxide at decrease of antioxidant defense non-enzymic link capacity is the basis of gestosis and acute postnatal complications. Thus, an increase of MDA concentration by 42.3-43.0 %, $NO^{\bullet}$ by 31.9-38.0 % was observed in cows with gestosis. At mild gestosis the glutathione peroxidase activity, catalase activity and vitamin C concentration increased by 11.0 %, 14.3 %, 38.0 %, and 24.1 %, respectively, while vitamin E concentration decreased by 11.7 % due to more consumption for neutralization of the toxic peroxidation products. At more sever pathology, the glutathione peroxidase activity and catalase activity in blood increased by 26.0 % and 17.3 %, respectively, when compared to the healthy animals, while vitamins E and C concentrations decreased by 33.3 % ($p < 0,01$) and 17.2 %, respectively. As free radical oxidation intensified, an anaerobic degradation of carbohydrates was activated to supply the tissues of developing fetus with energy under oxygen deficit occurred because of violation of the blood circulation. Postpartum inflammation in the genital organs in cows developed against the background of increase of MDA concentration by 76.0 %, GPO and GR activity by 65.8 % and 14.6 %, respectively, SOD by 46.0 %, catalase by 45.7 %, 2.9 times increase of $NO^{\bullet}$ concentration and reduction of vitamin E content by 35.5 %. Infertile animals with ovarian dysfunction were characterized by high activity of lipid peroxidation processes and by low level of nitrogen oxide generation. This is indicated by an increased concentration of MDA by 57.0 %, activity of GPO by 27.6 %, GR by 10.5 %, SOD by 31.9 %, catalase by 24.3 %, with a reduced content of $NO^{\bullet}$ and vitamin E by 56.9 % and 31.6 %, respectively, in comparison with healthy animals. A decrease in $NO^{\bullet}$ concentration in blood could result from a sharp depression of hormone synthesizing function in ovaries, and low $NO^{\bullet}$ production could disturb functions of the gonads. The high level of peroxidation is peculiar to animals with chronic uterus pathology, however, it is less expressed than in cows with acute course of the pathological process.

Keywords: cows, blood, lipid peroxidation, gestation, postpartum period, norm, pathology.

Free radical oxidation or lipid peroxidation (FRO, LPO) are currently considered as the dominating metabolic processes, which provide regulation of functional activity of any organism's physiological systems. The reactions of peroxidation have a universal character, being the source of basic energy mass necessary for vital activity and stability index of metabolic transformations in the organism.

The initiators of free radical oxidation are the active forms of oxygen (AFO), forming in oxidase (mitochondrial) and oxygenase (microsomal) reac-

tions of aerobic oxidation, realizing with the participation of molecular oxygen (O_2). During these reactions O_2 is subjected to sequential univalent reduction with the formation of so-called free radical compounds, possessing an unpaired electron. When the oxygen loses one electron at the beginning, we can observe formation of a superoxide anion radical ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2), the subsequent repair of which is accompanied by water (H_2O) formation and hydroxyl radical ($OH^\bullet$). The last one differs by its high reactivity and it is one of the main initiators of LPO (1-4). Under certain conditions non-enzymatic dismutation of superoxide anion can produce singlet oxygen ($^1O_2$) possessing high reactive and biological activity as well as a hydroxyl radical.

AFO undergo oxidation reaction with polyunsaturated lipids, including fatty-sour residues of phospholipids, the main structural components of biological membranes. AFO also activate the formation of a number of molecular products of LPO (peroxide radicals $RO_2^\bullet$): hydroperoxides of saturated fatty acids, aldehydes and dialdehydes, ketones, lactones, epoxides, substances like Schiff bases, etc. All of them play an important role in the processes of structural modifications of biomembranes and changes of their physicochemical properties (5). Excessive production of the active forms of oxygen and redundant in vivo accumulation of LPO products lead to the changes of physicochemical biomembrane properties, activity of many membrane-bound enzymes, penetrability disorder and then structural integrity and genotoxic oxidative DNA damages (6).

Formation, accumulation and utilization of FRO products are controlled by the system of antioxidant protection, including non-enzymatic and enzymatic links. The system of antioxidant protection (AOP) limits the processes of free radical lipid oxidation practically in all its links and maintains this class of reactions at a relatively fixed level. It controls the content of active forms of oxygen, free radicals and molecular products of LPO (5) in the organism and plays a very important role in the homeostasis maintenance.

In the enzyme link of AOP system the central part is occupied by copper-, zinc- and manganese-containing enzymes of superoxide dismutase (SOD), which catalyzes the dismutation reaction of superoxide anion radical with the formation of molecular oxygen and hydrogen peroxide, that is also capable of taking a toxic effect on the cells. The destruction of H_2O_2 molecules is done by catalase enzymes and glutathione peroxidase (8). Catalase is haematin-containing enzyme, destroying H_2O_2 without participation of oxygen acceptors. The hydrogen peroxide itself is the electron donor. Catalase continuously preserves its activity and does not require energy activation and the speed of hydrogen peroxide decomposition is only limited by the speed of substrate diffusion to the active center of the enzyme. Glutathione peroxidase is one of the components of the anti-peroxide complex, including glutathione and glutathione reductase, catalyzes the transformation of hydrogen peroxide and fatty acids of hydroxides into nontoxic compounds. The efficiency of glutathione peroxidase mechanism of hydroperoxide reduction depends on the hydrogen- glutathione content in the principal donor's organism. The support of sufficient amount of reduced glutathione, oxidizing under functioning of glutathione-dependent anti-peroxide systems, is done by the enzyme of glutation reductase (GR).

In the non-enzymatic link of the AOP system the central part is occupied by tocopherols (9), among which α- tocopherol (vitamin E) possesses the highest biological activity. It enters the organism with vegetable and animal feed. It realizes its antioxidant function at the expense of forming solid membrane architecture, preventing the attack of unsaturated fatty-sour residues of membrane phospholipids by active forms of oxygen and at the expense of local damages of oxygen and lipid peroxide radicals. α-Tocopherol acts as an active

«quenching agent» of singlet oxygen and «interceptor» of free radicals, reacting directly with them at the stage of chain cut off (10, 11).

It is considered that only reduced (phenolic) form of vitamin E, possessing free hydroxyl group, can actively react with peroxide radicals. Among the substances capable of reducing oxidated kinone form into a phenolic one and by this to regenerate antiradical activity of vitamin E, the greatest importance belongs to ascorbic acid, standing as a donor of protons and synergist of vitamin E (12, 13). In addition, it itself can interact with singlet oxygen, hydroxyl radical and superoxide anion radical, and destroy hydrogen peroxide (14, 15). Reduction of the ascorbic acid is realized at the expense of reduced glutathione. Close interconnection of the ascorbic acid with tocopherol and glutathione makes it an important component of the biological non-enzymatic system of the antioxidant protection.

Recently $NO^{\bullet}$ was reported also to be involved into the oxidative stress and antioxidation defense mechanisms (16-22). Its protective effect presumably is due to capability to enhance activity of antioxidative enzymes (23, 24), interact with superoxide anion radical and provide detoxication of potentially harmful reactive oxygen species (5, 17, 22).

Under initial insufficiency of the AOP system, its capacity decrease, because of extreme external factors or due to the internal physiological causes, LPO processes outlet over the regulated limits, redundant accumulation of its toxic products, development of oxidative stress and free radical pathology (1, 25, 27) with the structural-metabolic changes in the reproductive organs and occurrence of such diseases as fetoplacental insufficiency, late gestosis, placentitis, antenatal fetal hypoxia, retained placenta, postnatal subinvolution of uterus and endometritis (28-32), chronic pathologies of uterus and ovaries accompanied by infertility (33-35) are marked.

That is why modern scientific literature actively discusses the role of LPO in the molecular mechanisms of adaptive reactions and in the genesis of many diseases of reproductive animals.

The aim of this work was to study the functioning peculiarities of lipid peroxidation—antioxidant protection system and nitric oxide in highly productive dairy cows under physiological and pathological course of pregnancy and puerperal period.

Technique. The experiment has been run during winter keeping cattle stalled period in 2009 at a pedigree farm «Druzhba» (Pavlovskiy region, Voronezh Province). A total of 96 cows of Red Motley breed with average annual productivity of 6.5 thousand kg in the herd under tethered-pasture keeping participated in the experiment. Their ration included corn silage, meadow hay, spring corn straw, concentrated food, treacle and table salt. Common nutritiousness of the ration was 100 % with provision of 98 % digestible protein, 98 % sugar, 73 % calcium, 72 % phosphorus, 92 % carotene; sugar-protein ratio was 1:1 and calcium-phosphorus ratio was 1.8:1. There were seven groups of animals formed as follows: I (n = 9) with a normal gestation course, II (n = 9) with the signs of light gestosis, III (n = 9) with the signs of severe gestosis, IV (n = 17) with a normal course of puerperal period, V (n = 28) with an acute postnatal endometritis, VI (n = 12) with a chronic uterus subinvolution and VII (n = 12) with ovarian hypofunction. Functional state of the reproductive organs was examined by transrectal palpation. The gestosis was diagnosed on the basis of visual detection of pathological subcutaneous edema in the area of hind limbs, abdominal wall and dewlap, measurement of blood pressure using clinical thermometer, detection of protein concentration in urine using test strips AlbuPHAN (Lachema, Czechia).

Blood was obtained from the jugular vein during morning hours. Heparin was as an anticoagulant. The amount of malonic dialdehyde (MDA), activity of glutathione peroxidase (GPO), glutathione reductase (GR), catalase, superoxide dismutase (SOD) (36), the sum of nitric oxide ($NO^{\bullet}$) stable metabolites (37) were evaluated in the blood. E and C vitamins content was determined in blood serum using spectrophotometric method (38), common lipids, cholesterol and triglyceride were determined using Vital Diagnostica (Russia) and Lachema (Czechia) sets, blood lactic acid was determined according to the reaction with paroxy diphenyl (36).

Statistical treatment was done using program Statistica v. 6.0. The validity of the differences was evaluated by paired comparison method using Student's *t*-criterion.

Results. Gestation pathology, clinically revealing as gestosis symptom complex in cows, developed against the background of lipid peroxidation activation under simultaneously increased activity of antioxidant protection system as a compensatory reaction to damaging action of LPO products (Table 1).

1. Indices of lipid peroxidation- antioxidant protection system in Red motley cows under physiological and pathological gestation course ($M \pm m$; pedigree farm «Druzhba», Pavlovskii region, Voronezh Province; winter keeping cattle stalled period 2009)

Index	Clinically healthy ($n = 9$)	Light gestosis ($n = 9$)	Severe gestosis ($n = 9$)
MDA, μmol/l	1.04±0.140	1.49±0.120	1.48±0.140
GPO, mmol GSH/(l · min)	14.6±1.54	17.2±2.11	18.4±2.58
Catalase, mmpl H_2O_2/(л · min)	30.1±1.26	34.4±0.93	35.3±2.44
Vitamin E, μmol/l	11.2±0.89	9.9±1.20	7.7±0.93
Vitamin C, mmol/l	14.5±5.73	18.1±4.02	12.0±1.69
$NO^{\bullet}$, μmol/l	60.1±8.02	83.0±7.87	79.3±8.19

C o m m e n t s. MDA — malonic dialdehyde, GPO — glutathione peroxidasea, $NO^{\bullet}$ — sum of nitric oxide stable metabolites.

In terms of malonic dialdehyde blood concentration increase, the POL product level in animals with slight gestosis increased by 43.0 % in comparison with that in healthy cows ($p < 0.05$). Along with it, the activity of GPO and catalase, concentration of stable $NO^{\bullet}$ metabolites and vitamin C increased by 11.0 %, 14.3 %, 38.0 % and 24.1 %, respectively. Meanwhile, the amount of vitamin E, which is not synthesized in the organism, decreased by 11.7 %, that was connected with the increase of its expenditure on the neutralization of LPO toxic products.

The intensification of the pathological process severity caused the subsequent increase of enzymatic link of AOP system and decrease of non-enzymatic one. Compared to clinically healthy animals, activity of GPO and catalase increased by 26.0 % and 17.3 %, respectively, and the amount of vitamin E and C decreased by 33.3 % ($p < 0.01$) and 17.2 %, respectively.

Against the FRO intensity increase the decrease of total lipid concentration in blood from 4.1±0.35 to 3.1±0.18 g/l or by 24.4 % ($p < 0,05$), and increase of the level of triglycerides from 0.5±0.02 to 1.1±0.04 mmol/l or 2.1 times ($p < 0.001$) and lactic acid from 1.8±0.07 to 4.5±0.13 mmol/l or 2.5 times ($p < 0,001$) were shown. The last one confirms the enhancement of anaerobic decay of carbons for developing fetus's tissue provision with the energy in case of oxygen scarcity, which arises in connection with uteroplacental blood circulation disorder.

Sufficiently active LPO was found under the development of inflammatory processes in reproductive organs after parturition (Table 2). This was proved by a high MDA blood concentration, exceeding analogous index in clinically healthy animals by 76.0 %. Such a tendency was determined by an abrupt in-

crease of neutrophilic and macrophage production of active forms of oxygen, observed under an inflammatory process. At the same time such animals have compensatory inclusion of the enzymatic link of the antioxidant protection. GPO blood activity in sick animals appeared to be higher by 65.8 % ($p < 0.001$), GR by 14.6 % ($p < 0.05$), SOD by 46.0 % ($p < 0.001$), catalase by 45.7 % ($p < 0.001$). However, not high increase of GR activity compared GPO can confirm insufficiency of the functional potential of glutathione link of the AOP system and impossibility of the adequate replenishment of the reduced glutathione pool (39).

2. Indices of lipid peroxidation-antioxidant protection system in Red motley cows under physiological and pathological course of puerperal period ($M \pm m$; pedigree farm «Druzhba», Pavlovskii region, Voronezh Province; winter keeping cattle stalled period 2009)

Показатель	Physiological course of the puerperal period ($n = 17$)	Acute endometritis ($n = 28$)	Chronic subinvolution ($n = 12$)	Ovarian hypofunction ($n = 12$)
MDA, μmol/l	1.00±0.050	1.76±0.400	1.45±0.030	1.57±0.060
GPO, mmol GSH/(l · min)	9.4±0.32	15.8±0.44	13.7±0.45	12.0±0.64
GR, μmol G-SS-G/(l · min)	293.1±10.88	336.2±9.06	299.0±7.11	324.0±8.24
SOD, standard unit/mg of haemoglobin	0.72±0.030	1.05±0.030	0.97±0.040	0.95±0.050
Catalase, mmol H_2O_2/(l · min)	25.9±0.57	37.6±0.63	33.0±1.28	32.2±0.84
Vitamin E, μmol/l	23.7±3.48	15.3±0.93	28.3±2.79	16.2±2.78
$NO^{\bullet}$, μmol/l	47.8±0.29	138.7±7.14	–	20.6±2.21

Comments. MDA — malonic dialdehyde, GPO — glutathione peroxidasea, GR — glutathione reductase, SOD — superoxide mutase, $NO^{\bullet}$ — of nitric oxide stable metabolites. Dash indicates the absence of data.

The decrease of AOP non-enzymatic link activity was simultaneously detected in the animals, which have become sick. Vitamin E content in their blood appeared to be lower by 35.5 % ($p < 0.01$). The imbalance in AOP system didn't allow to support LPO processes at a relatively fixed level, which could be a background of endometrium cell structures damage by means of FRO toxic products accumulation and postnatal pathology development. At the same time production of nitric oxide in the sick cows' increased 2.9 times ($p < 0.01$). Immunocompetent cells, the macrophages and neutrophils, were the source of its generation (16). Possessing antioxidant and muscle relaxing effect, $NO^{\bullet}$, on the one hand, restricted the intensity of the peroxide reactions, and on the other hand, it depressed uterine activity and caused the breakdown in postnatal involutional processes of the reproductive organs.

Lipid peroxidation in cows with uterus inflammatory diseases was accompanied by the decrease of total lipid concentration in blood by 17.9 % (2.71±0,04 versus 3.30±0.18 g/l, $p < 0.001$), cholesterol by 44.0 % (2.68±0.12 versus 4.78±0.33 mmol/l, $p < 0.001$).

The high activity of LPO reaction remained in cows under the development of chronic reproductive organs pathology (see Table 2). So, the animals with chronic uterus subinvolution had higher MDA blood concentration compared to healthy animals by 45.0 %, GPO activity by 45.7 %, SOD by 34.7 % and catalase by 27,4 % ($p < 0.01$-0.001). In cows with ovarian dysfunction the difference according to the same indices was 57.0, 27.6, 31.9 and 24.3 % ($p < 0.001$), respectively. There were no evident differences in GR activity and vitamin E content under chronic uterus pathology. The activity of this enzyme in animals with sexual glands hypofunction exceeded the index in healthy animals by 10.5 %, but vitamin E concentration was lower by 31.6 %, and the cows were characterized by a lower generation of nitric oxide. The concentration of its stable blood metabolites was 56.9 % lower ($p < 0.001$).

Therefore, $NO^{\bullet}$ formation in the organism was connected with the activity of sex steroids biosynthesis (40, 41), and the decrease of its concentration

must be referred to an abrupt reduction of hormone-synthesizing ovarian function. At the same time low $NO^{\bullet}$ production under a mentioned pathology may be the basis for gonads generative function disorder, because this compound is included into the control of hypothalamus secretion of gonadotropin-releasing hormone and hypophysis secretion of luteinizing hormone responsible for the gonad ovulatory function (41-44).

Thus, the activation of free radical oxidation, development of oxidation stress and free radical pathology against the background of imbalanced changes in nitric oxide generation and glutathione link of antioxidant protection (AOP) should be referred to the main mechanisms, leading to reproductive function disorders in highly productive cows. The regularities ascertained in connection with reproductive health of animals, functions of AOP-lipid peroxidation and nitric oxide may be used in developing treatment-prophylactic measures.

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MYCOTOXIN CONTAMINATIONS IN COMMERCIALLY USED HAYLAGE AND SILAGE

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Abstract

Improvement of sanitary control of grass fodder, taking into account the whole variety of factors that have a negative impact on the animal, is the most important task of agricultural science. In the present study, we investigated mycotoxin contamination of hayage and silage by the method of indirect competitive enzyme-linked immunosorbent assay. In a survey of 30 commercial feed batches from the livestock farms located in the central regions of the European Russia, namely Bryanskaya, Lipetskaya, Moskovskaya, Smolenskaya and Tverskaya provinces, a multiple contamination pattern was shown. In all the samples eight or more mycotoxins were found, and more than half of samples contained 14-15 components. Alternariol was found in all samples of the both types of feeds in amounts from 50 to 1260 μg/kg, while aflatoxin B_1, ochratoxin A, citrinin and ergot alkaloids had extensive distribution with low intensity. In haylage with high incidence of all analyzed fusariotoxins, mass concentration of T-2 toxin was the lowest (4 to 30 μg/kg), and the levels of diacetoxyscirpenol, deoxynivalenol, zearalenone and fumonisins were in the range of 100-1000 μg/kg. Sterigmatocystin, emodin, cyclopiazonic and mycophenolic acids, PR-toxin occurred everywhere, wherein emodin, cyclopiazonic acid and PR-toxin often present in amounts up to 1000 μg/kg. Silage prepared mainly from corn grass, with frequent detection of T-2 toxin and deoxynivalenol in significant content (to 350 and 2820 μg/kg, respectively) revealed similarities with corn grain from the center of European Russia. Sterigmatocystin and emodin were detected in all silage samples, whereas PR-toxin, cyclopiazonic and mycophenolic acids were slightly inferior to them in frequency. Levels of accumulation of these mycotoxins were lower than those found in silage, and for mycophenolic acid they generally remained the same. A botanical composition of herbage and features of toxin-producing fungi complexes, accompanying the growing season and the subsequent process of fermentation, are discussed among possible reasons for the differences in mycotoxin contamination of haylage and silage.

Keywords: haylage, silage, mycotoxins, immunoassay.

Haylage and silage are very close to green fodder in biological value and widely used in animal husbandry. In fact, due to haying during plant growing season the animals are provided with grass feeding at indoor housing. Haylage is mainly prepared from mixed legumes and cereals. At preservation the grasses are chopped, cured, pressed and kept in CO_2 and N_2 evolved by plant cells. By that a high degree of preservation may be achieved on fodder nutrients, particularly proteins and carotene. At ensilaging the corn plants at stages of milk to wax or wax ripeness of grain as well as legumes and cereals or their mix with corn are subjected to lactic fermentation after chopping, pressing, treating with preservative chemical additives and then sealing to keep without air access. Each technological step must be in strict accordance with the approved protocols to provide a high sanitation quality of haylage and silage (1).

Annual or perennial plant type, the terms of sawing and mowing and application of fertilizers are usually considered to estimate the suitability of raw material for preservation (2), and we recognize the absolute importance of these factors. Nevertheless, due attention also should be paid to initial plant contamination with mycotoxins formed in field conditions, as well as their change during feed storage.

It is known that vegetating plants are in contact with phytopathogenic and parasitic fungi, whereupon a mycotoxin contamination can often occur. During fermentation of plant biomass and its further storage at anaerobic conditions a specific mycobiota appears which consists of active toxin-producing species (3-5). Besides, at air access because of improper storage or removing the mold is developed on the surface or in deep layers of herbal substrate that often leads to animal poisoning (6).

Safe use of green fodders was mostly considered in view of definite groups of toxic fungal metabolites, such as fusariotoxins in corn silage (7), or detection of extremely dangerous substances, particularly mycophenolic acid, cyclopiazonic acid, alternariol (8-10). However, the high risks for multiple mycotoxin contamination are increasingly reported in recent years (11).

In the present study we carried out a mycotoxicologic survey of commercial haylage and silage batches from livestock farms based on assay of toxic metabolites produced by fungi which can grow both on plants during vegetation and on herbal substrate preserved.

Technique. A total of 30 commercial batches of haylage and silage were tested, including 9 probes of haylage and 14 probes of silage sampled in 2011-2012 on the livestock farms of Bryanskaya Province (Bryanskii, Zhiryatinskii, Kletnyanskii, Pochepskii, Trubchevskii regions), and the rest received in 2006-2012 from the livestock farms located in Lipetskaya, Moskovskaya, Smolenskaya and Tverskaya provinces. The batches differed in terms of harvesting and storage. The haylage was made from alfalfa, cereals and grass mix (5, 2 and 4 batches, respectively, a total of 11 batches), and the silage was prepared from corn, grasses and the mixes of corn and grasses (8, 2 and 9 batches, respectively, a total of 19 batches). For each batch an average sample was tested.

A total of 15 mycotoxins, namely aflatoxin B_1 (AB_1), T-2 toxin (T-2), ergot alkaloids (EA), sterigmatocystin (STE), ochratoxin A (OA), mycophenolic acid (MPA), citrinin (CIT), alternariol (AOL), zearalenone (ZEN), deoxynivalenol (DON), emodin (EMO), fumonisins (FUM), diacetoxyscirpenol (DAS), cyclopiazonic acid (CPA), PR-toxin (PR), were analyzed according to the procedure described in our previous publication (12). Samples were air-dried, chopped and placed into tubes. The acetonitrile-water mixture (84:16) was added at 1:10 (w/v) ratio of a sample to extragent, and the tubes were shaken. After 12-14 hour extraction they were shaken again, and then the samples were 1:10 diluted with buffer and used in indirect competitive enzyme-linked immunosorbent assay (ELISA).

Results. Our data indicate (Table) that the frequencies of T-2, DAS, DON, ZEN and FUM peculiar to fusariosis agents in legumes and cereals were high in tested haylage and comparable to those in hay from the same areas (12). Among fusariotoxins, the T-2 accumulation was the lowest and much lower compared to hay. In the T-2 positive samples its content was not more then 30 μg/kg and mostly ranged from 4 to 10 μg/kg (see Table), while in hay it varied from 5 to 2240 μg/kg at an average value of 450 μg/kg (12). Besides, in haylage the accumulation of DON and ZEN above a significant level of 1000 μg/kg was not found (see Table), while in hay it, though rare, was detected. The observed peculiarities may result from a botanical composition of herbage and different time before plant harvesting, a partial hydrolysis of the toxins long exposed to acidic conditions at pH 4.4-5.6 in plant biomass, and also from toxin biotransformation by microaerofilic fungi (13).

Other studied mycotoxins were found almost in all tested haylage samples (see Table). The AOL and EMO contamination patterns remained the same as in hay. Probably, the frequency of AOL and EMO producing fungi is the

same in herbage of cereals and legumes. Actually, alternariosis with *Alternaria tenuis* is described for oats and peas (14), and *Cladosporium*, the EMO producing fungi (15), as well as *Alternaria* can develop on different plants (16). Besides, the similarities were revealed in OA and CIT contamination which not exceeded 100 μg/kg in all positive samples, and in AB_1 ranged from 2 to 9 μg/kg, while the clear differences were found for STE, CPA, PR, MPA and EA.

Mycotoxin frequency and content in Haylage and silage samples from the livestock farms located in European Russia

Mycotoxins	n^+						Minimal—average—maximal mycotoxin content, μg/kg
	totla/n	mycotoxin content, μg/kg					
		< 10	≥ 10	≥ 100	≥ 1000	≥ 10 000	
				Haylage			
T-2	11/11	7	4	–	–	–	4—13—30
DAS	10/11	npe	npe	10	–	–	100—245—420
DON	9/11	npe	–	9	–	–	125—220—330
ZEN	9/11	npe	7	2	–	–	25—135—630
FUM	5/11	npe	–	5	–	–	125—180—245
AOL	10/10	npe	–	9	1	–	110—545–1260
STE	11/11	–	7	3	1	–	20—185—1000
EMO	11/11	npe	–	3	5	3	270—8160—31600
CPA	11/11	npe	npe	3	8	–	130—2480—8900
OA	9/11	–	9	–	–	–	10—23—30
CIT	10/11	npe	8	2	–	–	40—82—160
MPA	11/11	npe	1	10	–	–	35—330—840
PR	9/11	npe	npe	6	3	–	100—660—1500
AB_1	9/11	9	–	–	–	–	2—4—9
EA	10/11	1	5	4	–	–	6—105—420
				Silage			
T-2	19/19	2	9	8	–	–	8—95—350
DAS	11/19	npe	npe	11	–	–	165—265—490
DON	17/19	npe	–	6	11	–	100—1270—2820
ZEN	16/19	npe	10	6	–	–	25—135—740
FUM	15/19	npe	1	14	–	–	80—350—960
AOL	16/16	npe	4	12	–	–	50—315—860
STE	19/19	–	16	3	–	–	10—85—420
EMO	16/16	npe	1	11	4	–	70—1075—5010
CPA	17/19	npe	npe	8	9	–	170—1200—3160
OA	14/19	1	13	–	–	–	9—17—22
CIT	14/19	npe	12	2	–	–	40—75—110
MPA	15/17	npe	5	9	1	–	30—335—2000
PR	12/19	npe	npe	12	–	–	100—310—625
AB_1	11/19	11	–	-	–	–	3—4—6
EA	13/16	5	6	2	–	–	4—50—270

Comments. Aflatoxin B_1 (AB_1), T-2 toxin (T-2), ergot alkaloids (EA), sterigmatocystin (STE), ochratoxin A (OA), mycophenolic acid (MPA), citrinin (CIT), alternariol (AOL), zearalenone (ZEN), deoxynivalenol (DON), emodin (EMO), fumonisins (FUM), diacetoxyscirpenol (DAS), cyclopiazonic acid (CPA), PR-toxin (PR), Dashes mean no positive probe found; n — tested probes, n^+ — positive probes; npe — no possible estimation.

A decreased EA level in haylage is probably due to low rate of cereals predisposed to development of ergot and specifically colonized by endophytes, however, a mechanical removal of ergot sclerotia during prestorage treatment should not be underestimated. The STE, CPA, MPA and PR were extensively distributed with the above 1000 μg/kg levels of the CPA and PR often detected. A higher STE, CPA, MPA and PR levels may result from activity of microaerofilic fungi able to grow during preservation. Unfortunately, no data about grass haylage microbiota are currently available. In all haylage samples 8 or more mycotoxins were found and more than half of samples contained 14-15 components.

For silage the same frequencies of mycotoxins were characteristic (see Table). In all samples 8 or more mycotoxins were found and 9 samples contained 14-15 toxic metabolites.

According to the batch certificates attached, 2 and 8 of 19 silage batches were made from grasses and corn, respectively, and the rest of batches contained corn as a very easy crop to ensile. It is also indicated by frequent occurrence of FUM as well as other fusariotoxins, including T-2, DAS, DON and ZEN.

Fusarium are known to attack lower parts of corn stems since initial stages of plant development, and after flowering the entire colonization of stems occurs resulting in browning lower and then the upper stem nodes. A long term investigations showed the infection to be continued during plant vegetation at 10 % level (17). *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. sporotrichioides*, *F. crookwellense*, *F. oxysporum*, *F. sambucinum* var. *coeruleum* are considered the most frequent causative agents for stem rots, and *F. graminearum* and *F. moniliforme* Sheldon, recently attributed as *F. verticillioides* (Sacc.) Nirenberg, are also found (17).

According to fusariotoxin contamination patterns, the highest frequency of T-2 and somewhat less frequencies of DON, FUM and ZEN indicate a similarity between silage and corn grain from the center of European Russia (18-20). Unfortunately, a comparative study on DAS is still not possible. High ZEN levels in silage were not shown. Perhaps ZEN was not significantly accumulated in the raw material. As reported by P. Lepom et al. (21), the amount of ZEN remained unchanged at ensilaging during 12 weeks of observation.

AOL, a toxin of *Alternaria* fungi, was detected in all silage batches tested. As reported by M. Muller (22), in corn before harvesting a prevalence of *Alternaria* on leaves and cobs is quite significant, accounting for 18 % of the total mycobiota. Most *A. alternata* and *A. tenuissima* isolated strains can produce AOL in vitro, however, the toxin was not analyzed directly in the silage samples the fungi were isolated from (23).

Frequency and accumulation of the rest mycotoxins are obviously determined by fungal colonization of herbage and ensilaging conditions. STE and EMO presented in all tested samples with no exception, and MPA and PR were quite frequent. However, there were no the same high rates as revealed in haylage. CPA contamination in silage was rarer then in haylage, but of the same content rang. MPA contamination remained generally the same, but in one sample reached 2000 μg/kg (see Table). MPA have been found in 32 % silage samples in Germany (24). *Penicillium roqueforti* (24) and *Byssochlamys nivea* (8) are suggested as candidate MPA-producing fungi.

OA and CIT contamination in the silage were the same as in the haylage (see Table). Quite possibly, they are synthesized by *Aspergillus ochraceus*, as its isolates can produce OA (5), and *Monascus ruber*. In the strains of *A. ochraceus*, isolated from silage in Moscow Province a capability to produce AO has been shown (5), and *M. ruber* producing CIT has been identified in 16 % corn silage and 21 % grass silage samples (24). In 13 samples of 16 tested EA was probably produced by definite *Aspergillus* species and subspecies (25), as well as by *Claviceps purpurea* from occasional herbage components. Particularly, quitch and annual bluegrass predisposed to ergot development are the most common weeds in corn crops in Germany (17).

The final stage of ensilaging was detailed studied for past few decades. Despite of clear differences in mycobiota of silages due to year-to-year effects, region and technology of preparation, three microaerophilic fungi are shown to predominate, namely *Penicillium roqueforti*, able to grow at pH 3 in 80 % CO_2 and 4-5 % O_2, *Aspergillus fumigatus*, and *Monascus ruber* which can develop at pH 2.5 and 3.5 % lactic acid concentration (26).

Importantly, these fungal species can produce a combination of toxic metabolites. Particularly, *P. roqueforti* produces MPA and PR (8, 27), and *A. fumigatus* produces EMO, CPA and EA (9, 28). Moreover, they participate in the competitive relationship (26). A mycological study of silages from the livestock farms located in Moscow Province indicated their successful development under anaerobic conditions and also uneven distribution in silage (5). *M. ruber*

and *A. fumigatus* contaminate entire volume, being more characteristic for deeper and surface layers, respectively, while *P. roqueforti* which was found in 22 % samples prevailed in deep layers and close to the walls being rarer on the surface. Therefore, the mycotoxin contamination may differ depending on the part of the silo.

At the same time an influence of plants as main raw materials for haylage and silage preparation on feed contamination is still remains poor studied. Nevertheless, a few available data indicate relevance of such a research. Recently in a plot field experiment with rye grass and two *Festulolium* species the clear dependence of T-2, DON and ZEN levels on harvesting terms was shown. Besides, their sharp increase occurred after the silo sealing at short initial anaerobic phase of ensilaging when the temperature rose (29).

In Russia both natural grasslands and crops such as corn, clover, alfalfa, vetch, oats, timothy grass, hedgehog, or their mixes are traditionally used for preservation. Besides, sunflower, topinambour, soybean, sorghum, galega, peculiar in their biochemical composition and relationship with micromycetes are also involved. Unfortunately, these extremely important fodder crops have not been yet mycotoxicologically assessed. It necessitates their step-by-step multilevel research ranged from plot field experiments to a wide monitoring in the regions of intensive forage production.

Thus, by a mycotoxicological survey of commercial haulage and silage batches first performed in Russia a multiple contamination pattern and accumulation of mycotoxins have been shown at the levels that can present a serious threat to the animal health. Further studies will contribute to prophylaxis of mycotoxicosis in domestic animals whose diet is based on preserved grass feeds.

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DYNAMICS OF MYCOTOXIN ACCUMULATION IN SILAGE DURING STORAGE

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Abstract

The presence of mycotoxins in the feeds is a big problem in the world. Result of improper silaging is the occurrence of mycotoxins, the metabolic products of molds. Today there is too little information on the accumulation of mycotoxins in silage during ensiling, and there is no answer to the question how to resolve this problem. This article includes results of mycotoxins' analysis (aflatoxins, ochratoxin A, T-2 toxin, zearalenone, deoxynivalenol) in the original forage plant material of cocksfoot *Dactylis glomerata* L. and in the silage at various stages of experimental ensilaging. Also the influence of biological preparations Biotrof and Biotrof-111 (Biotrof+ Ltd, Russia) and chemical preparations AIV 3 Plus and AIV 2000 Plus (KEMIRA OYJ, Finland) for the preservation of silage on reducing the amount of toxic fungal metabolites was investigated. Analysis of the amount of mycotoxins in the samples was performed by the enzyme immunoassay method using ELISA test kit AgraQuant^{TM} (Romer Labs, Inc., Austria). Analysis of the accumulation of mycotoxins in feed plant material and silage showed that mycotoxins already occurred in forage plants as a result of fungal attacks during the growing season and later in the silages if conditions were suitable for mold growth. The biological preparations for ensiling effectively decrease the accumulation of mycotoxins in the silage compared to the variant without supplementation. At the end of silage storage the amount of aflatoxins in the variants with Biotrof and Biotrof-111 was lower by 17.7 and 9.1 %, respectively, compared to the control, with a decrease in the value of ochratoxin A by 21.4 and 34.9 %, T-2 toxin by 20.1 and 32.8 %, zearalenone by 17.7 and 10.4 %, and deoxynivalenol by 0.8 and 55.8 %, respectively. So far as in Russia no maximum permissible concentrations are specified for mycotoxins in silage, the values for feed grain of oats, wheat and barley taxonomically close to perennial cocksfoot grass were used for comparison (corn, one more cereal crop, is not a traditional forage plant in the North West region of Russia). The greatest deterrent effect on accumulation of mycotoxins had the preparation based on *Bacillus subtilis.* Chemical preparation decreased the accumulation of some mycotoxins in the storage silage. However, the silage' total toxicity in presence of the chemical preparation was quite high relatively the maximum permissible concentrations used in this study as the reference values. It significantly surpassed control in the second half of storage. As it is well known, due to changes in environmental conditions the production of mycotoxins by molds increases. In this regard, in our experiment, the influence of chemical preparation has become a stress factor that caused the active synthesis of mycotoxins by molds.

Keywords: mycotoxins, forage plants, silage, maximum limit of mycotoxins content, the biological preparation for ensiling, the chemical preparation for ensiling.

Silage is considered essential in cattle diet. In case of its low quality because of nutrient losses the animals suffer from deficit of nutrients that has a negative impact on production and animal health, and eventually on the profitability in livestock. Ensured full feeding is first related to proper growing forage plants and raw material storage. Additionally to decreased biochemical parameters, an improper ensilaging results in contamination with mycotoxins, the mold metabolic products (1).

Mycotoxins, moldy feed and raw material control problems are relevant worldwide. Mycotoxins causing mycotoxicosis adversely affect production, reproductive and immune functions in animals (2). At little amount the mycotoxins repress production and weigh gain, and increase an incidence of infections. At

long intake the immunosuppressive, cancerogenic, mutagenic, allergenic, neurotoxic and teratogenic effects, as well as repression of reproductive functions occur. Besides, different mycotoxins being usually present together can act as synergists (3).

Traditionally, mycotoxin-related problems are considered relevant to poultry and pigs rather then cattle. However, some mycotoxins possess clear antimicrobial activity thus decreasing number of helpful microorganisms such as cellulolytic bacteria, bacilli, lactate-utilizing bacteria. It may negatively influence the digestion and metabolic activity, as well as defense functions of gut microbiota. Importantly, cows with high milk production are the most sensitive to mycotoxins being less tolerant to stresses. Besides, mycotoxins being fed to the animals with moldy feeds can penetrate into milk or other human foods, so there is an increased public concern over mycotoxins related to human health. Particularly, in cow milk a residual aflatoxin M_1, the aflatoxin B_1 metabolite, reached 0.3-6.0 % of its intake by animals (1).

To date there is too little information on the accumulation of mycotoxins in silage during ensiling, and there is no answer to the question how to resolve this problem.

This article includes the results of testing mycotoxins during an experimental ensilaging as influenced or not influenced by bacterial preparations and chemical preservant.

Technique. In model laboratory experiments we used *Dactylis glo-merata* L. cockfoot grass clipping harvested at stem-extention stage and 65 % moisture level. Two commercial bacterial additives, the Biotrof and Biotrof-111 (Biotrof+ Ltd, Russia, 0.007 ml/kg, or a liter per 150 tons of green biomass), and chemical preservants AIV 3 Plus and AIV 2000 Plus (KEMIRA OYJ, Finland, 4 ml/kg, or 4 liters per ton of green biomass) were used for ensilaging. Ensilaging biomass (320 g) was kept at 26±1 °C, and the probes for mycotoxin testing were sampled before the ensilage start and on days 3, 7, 14 and 30.

Mycotoxins, particularly aflatoxins, ochratoxin A, T-2 toxin, zearalenone, deoxynivalenol, were ELISA-tested (4) using a AgraQuant™ kit (Romer Labs, Inc., Austria) according to the manufacturer's recommendations with standard preparations of five mycotoxins as a control. For mycotoxin extraction, except deoxynivalenol, 70 % methanol was used, and deoxynivalenol was extracted with distilled water. The tests for zearalenone and T-2 toxin were terminated by adding 10 % HCl, and for aflatoxins, ochratoxin A and deoxynivalenol 10 % phosphoric acid was used to stop the reaction. OD_{450} were measured on a microstrip photometer Stat Fax 303+ (Awareness Technology, Inc., USA), comparing samples to the standards. As far as in Russia the allowed level of mycotoxins in silage are not still specified and regulated, we used maximum permissible concentrations (MPCs) for forage grain of wheat, oats and barley most close taxonomically to perennial pants like cockfoot grass as referent values. Based on veterinary and sanitation requirements approved by EuriAsEU Custom Commission (№ 137 dated June 18, 2010), they were as follows: 0.004 mg/kg for aflatoxins, 0.005 mg/kg for ochratoxin A, 0.06 mg/kg for T-2 toxin, 0.1 mg/kg for zearalenone and 1.0 mg/kg for deoxynivalenol (5).

Data were processed by dispersion analysis (6) in Microsoft Excel 2010.

Results. Biotrof is a biopreparation of helpful bacteria identified as *Lactobacillus plantarum* according to 16SrRNA gene sequence (7). It was designed for preserving different crops, including cured plant biomass with reduced moisture. At ensilaging an acidification is rapidly achieved due to Biotrof because of lactic acid production and repression of undesirable microbial activity (8). Biotrof-111 is a 16SrRNA gene sequence identified *Bacillus subtilis* biopreparation applicable

for ensilaging any crop, including those hard to silage such as legumes mixed with cereals, galega, alfala, etc. Due to high antagonistic activity it depresses putrid bacteria, mycotoxin-producing molds and yeasts providing accelerated preservation, so the silage is ready to use in less time (9). AIV chemicals contain a mix of organic acids such as formic, propionic, benzoic.

Before ensilaging, in forage plant raw material we identified aflatoxins at 0.0053±0.00025 mg/kg, zearalenone at 0.0115±0.00048 mg/kg and deoxynivalenol at 1.3±0.0062 mg/kg (Table), the aflatoxins and deoxynivalenol levels being 1.3 times as much as those we used as referent values. Therefore, forage plant contamination by mycotoxins is formed in field conditions during plant vegetation and resulted from activity of phytopatogenic fungi. Of note, the ochratoxin A and T-2 toxin levels were below the reliability of ELISA estimation.

Mycotoxin content (mg per 1 kg of dry matter) **in cockfoot grass *Dactylis glomerata* L. silage depending on used preparations** (*M*±*m,* laboratory experiments)

Days	Control (C), mg/kg	Biopreparations				Chemical preservant	
		lactobacilli-based		*bacilli*-based			
		mg/kg	to C, %	mg/kg	to C, %	mg/kg	to C, %
		Aflatoxins (MPC = 0.004 mg/kg)					
0	0.0053±0.00025	0.0053±0.00025		0.0053±0.00025		0.0053±0.00025	
3	0.0101±0.00040	0.0088±0.00040	87.1	0.0057±0.00026	56.4	0.0110±0.00040	108.9
7	0.0113±0.00047	0.0086±0.00042	76.1	0.0092±0.00043	81.4	0.0152±0.00065	134.5
14	0.0213±0.00800	0.0184±0.00070	86.4	0.0198±0.00080	93.0	0.0270±0.00094	126.8
30	0.0209±0.00100	0.0172±0.00060	82.3	0.0190±0.00047	90.9	0.0144±0.00071	68.9
		Ochratoxin A (MPC = 0.005 mg/kg)					
0	< bre	< bre		< bre		< bre	
3	0.0068±0.00023	0.0053±0.00020	77.9	0.0060±0.00025	88.2	0.0089±0.00040	130.8
7	< bre	< bre		0.0003±0.00001		0.0009±0.00003	
14	0.0127±0.00059	0.0059±0.00023	46.5	0.0031±0.00015	24.4	0.0739±0.00310	581.9
30	0.0295±0.00140	0.0232±0.00090	78.6	0.0192±0.00058	65.1	0.2720±0.01200	922.0
		T-2 toxin (MPC = 0.06 mg/kg)					
0	< bre	< bre		< bre		< bre	
3	0.0937±0.02300	0.0930±0.00410	99.3	0.1025±0.00370	109.4	0.1089±0.00410	116.2
7	0.0391±0.01600	0.0396±0.00170	101.3	0.0140±0.00048	35.8	0.0336±0.00160	85.9
14	0.1221±0.00370	0.1118±0.00520	91.6	0.0965±0.00220	79.0	0.0834±0.00420	68.3
30	0.1116±0.00510	0.0892±0.00320	79.9	0.0750±0.00350	67.2	0.1191±0.00480	106.7
		Zearalenone (MPC = 0.1 мг mg/kg)					
0	0.0115±0.00048	0.0115±0.00048		0.0115±0.00048		0.0115±0.00048	
3	0.0213±0.00100	0.0144±0.00036	67.6	0.0168±0.00065	78.9	0.0142±0.00061	66.7
7	0.0475±0.00170	0.0893±0.00410	188.0	0.0909±0.00380	191.4	0.1167±0.00350	245.7
14	0.1514±0.00360	0.1407±0.00670	92.9	0.1345±0.00630	88.8	0.0977±0.00320	64.5
30	0.1290±0.00610	0.1062±0.00051	82.3	0.1156±0.00460	89.6	0.0790±0.00290	61.2
		Deoxynivalenol (MPC = 1.0 mg/kg)					
0	1.3000±0.00620	1.3000±0.00620		1.3000±0.00620		1.3000±0.00620	
3	0.6800±0.02800	0.4200±0.01800	61.8	0.6900±0.03450	101.5	0.8100±0.02400	119.1
7	5.1200±0.22000	4.6600±0.20000	91.0	3.8100±0.12000	74.4	4.1200±0.19000	80.5
14	1.4000±0.04100	3.1900±0.12000	227.9	0.9900±0.03900	70.7	4.1700±0.20000	297.9
30	1.2900±0.05200	1.2800±0.03500	99.2	0.5700±0.02600	44.2	1.5000±0.06500	116.3

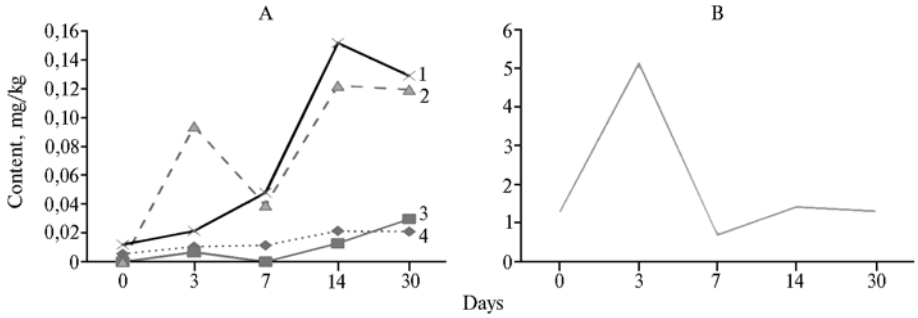
Comments. < bre — below the reliability of ELISA estimation.; AIV 3 Plus chemical preservant used («KEMIRA OYJ», Finland).

On days 3, 7, 14 and 30 a significant accumulation of all tested mycotoxins was found (see Table).

The graphs (Fig., A) illustrate a sharp increase in zearalenone and T-2 toxin levels at ensilaging while aflatoxins and ochratoxin A levels increased slightly compared to those in raw material. Interestingly, all tested mycotoxins, except ochratoxin A, decreased in amount in the second half of the observation, and deoxynivalenol after a sharp increase on day 3 dropped on day 7 (Fig., B).

Of note, a contamination by zearalenone, T-2 toxin and deoxynivalenol produced by *Fusarium* fungi is formed in field conditions during plant growth and can rise at feed storage (10). A rise of mycotoxin production is affected by changes in temperature and moisture or by chemicals (11-15). In our experiments environmental conditions, being a stress factor, probably activated synthe-

sis of zearalenone and T-2 toxin by microscopic fungi during 0 to 14 days of storage. Thereafter, fungal growth was terminated and death occurred with further destruction of toxic metabolites due to microbial activity in the silage. Deoxynivalenol-producing fungi are likely unable to develop in silage, and after 3 day storage the deoxynivalenol was destructed by other microorganisms.



Zearalnone (1)**, T-2 toxin** (2), **ochratoxin A** (3), **aflatoxins** (4) (A) **and deoxynivalenol** (B) **accumulation at ensilaging cockfoot grass *Dactylis glomerata* L. with no additives** (laboratory experiments).

With that, the ensilaging conditions are suitable for the development of aflatoxins and ochratoxin A producing fungi of *Aspergillus* and *Penicillium* genera (10), so that they should not activate any defense mechanisms, including significant amount of mycotoxins synthesized. As a result the aflatoxins and ochratoxin A synthesis during the observation was not high.

The values of mycotixins we used as those referent were exceeded 1.4-6.8 times for aflatoxins, 1.2-54.4 times for ochratoxin A, 1.4-1.8 times for T-2 toxin, 1.2-1.5 times for zearalenone and 1.3-5.1 times for deoxynivalenol (see Table). Obviously, the preparation used for ensilaging should provide high preservation of nutrients and balanced protein, energy and biological value in feeds, and also restrict the accumulation of mycotoxins due to powerful antifungal properties. Some strains of lactobacilli and bacilli are known as potent repressors of microscopic fungi and destructors of their toxic metabolites (16).

Actually, during ensilaging the lacrobacilli-based Biotrof and bacilli-based Biotrof-111 biopreparations provided 12.9-23.9 and 7.0-43.6 % decreased levels for aflotoxins, respectively, if compared to control, and to the end of storage the amount of aflotoxins was lower by 17.7 and 9.1 %, respectively, compared to that in absence of preparations. In case the chemical preservant was used the aflatoxins levels exceeded control by 8.9, 34.5 and 26.8 % on days 3, 7 and 14, respectively, while on day 30 aflatoxins dropped in amount by 31.1 % compared to control.

The Biotrof and Biotrof-111 decreased significantly the ochratoxin A accumulation at ensilaging, except day 7, when the toxic metabolite was detected at 0.0003±0.000007 mg/kg in the presence of Biotrof-111, while in control its level was below reliable detection in ELISA-test. To the end of storage the ochratoxin levels were 21.4 and 34.9 % lower with Biotrof and Biotrof-111, respectively, compared to control. In presence of the chemical preservant the ochratoxin A content was increased throughout the investigation, being in the end 822 % increased compared to control.

Biotrof-111 based on bacilli and chemical preservant caused some increase in T-2 toxin level on day 3, but since day 7 to the end of experiment it significantly decreased, except day 30 in the presence of preservant, when T-2 toxin content exceeded control by 6.7 %. A permanent decrease in T-2 toxin level was also observed from the middle to the end of storage period when Biotof, the lactobacilli based biopreparation, was used.

Zearolenone significantly decreased in amount being influenced by both biopreparations and the chemical preservant, except day 7, when it exceeded the control level in all variants.

Biotrof-111 based on bacilli decreased deoxynivalenol level by 25.6-55.8 % from day 7 to day 30 of the silage storage, while in presence of lactobacillii based Biotrof the values were about the same as in control. However, the application of chemical preservant led to a significantly increased deoxynivalenol level on days 3, 14 and 30, particularly by 16.3-197.9 %.

Toxic fungal metabolites in feeds, due to synergism, reciprocally complement and enhance their negative effects (17-20). So far as a combination of toxins was found, the sum of their excesses over the levels accepted hereinabove as referent values was conditionally considered as «the silage toxicity» parameter and used for estimating and comparing complex effect of the preparation tested.

During ensilaging these sums in presence of Biotrof and Biotrof-111 biopreparation were below control. The lowest value was estimated at Biotrof-111 application. The maximal value, being much higher compared to control after 14 days of preservation, was found at chemical preservant use. As mentioned, stresses, including chemicals, could activate mycotoxin production (21, 22), so chemical preservant could be a chemical stressor activating mycotoxin synthesis.

Thus, for the first time in Russia the mycotoxin accumulation was studied at different stages of experimental ensilaging of cereal perennial grass under the influence of different bio- and chemical additives. It was found out that the mycotoxin contamination was formed during plant vegetation, and mycotoxin production occurred during silage storage. Biopreparations based on *Lactobacillus plantarum* (Biotrof) and *Bacillus subtilis* (Biotrof-111) decreased contamination below the control level (with no additives used), and Biotrof-111 was the most effective. Chemical preservant caused a decrease in some mycotoxin levels, nevertheless «the silage toxicity» as a conditional parameter we suggested to compare a total effect of a preparation used on all mycotoxins tested was higher, and after 14 days much more higher compared to control. Stimulation of mycotoxin production in microscopic fungi under environmental stress is well known. In our investigation chemical preservan can perform as a stress agent and instigate the mycotoxin synthesis. So the development of bacilli-based biopreparation destroying microscopic fungi and destructing mycotoxins in silage is obviously relevant, since chemical preservants could not provide the control of mycotoxin contamination.

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