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HORIZONTAL AND VERTICAL BIOLOGICAL AND BIO SOCIAL EFFECTS OF INCREASED IONIZING RADIATION AS EXAMPLIFIED BY CHERNOBYL AND FUKUSHIMA NUCLEAR ACCIDENTS

(review)

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Abstract

In the review, our own studies of increased ionizing radiation effects on agricultural animals (nutria, rabbits, pigs, cattle) and small rodents of bio-indicator species after Chernobyl NPP accident are compared with published data of human populations' survey after the accidents at Chernobyl and Fukushima nuclear power plants. Similarity is noted of main targets for ionizing radiation (the cardiovascular system and kidneys) identified in humans and agricultural animals. Effects of ionizing radiation and post-traumatic syndromes are also comparable. Biomarkers of damages caused by ionizing radiation are considered. Differences in the estimates of the thyroid gland papillary carcinoma frequency after nuclear accidents at Chernobyl and Fukushima NPPs are discussed. Apparently, this inconsistency is mainly due to genogeographic factors, iodine deficit in geochemical province, and natural selection affecting number of generations in the populations from naturally radioactive provinces or under enhanced radionuclide pollution after technological accidents (T.I. Bogdanova et al., 2015; V.M. Drozd et al., 2015; M.B. Zimmermann, V. Galetti, 2015). A nonlinear dependency of biological effects of irradiation in the low dose range was analyzed with its possible mechanisms discussed (i.e. damage accumulation until the level necessary to induce DNA reparation, changes in young to old cell proportion in the populations, mitochondrial dysfunction) (E. Markievicz et al., 2015). A concept of «horizontal» and «vertical» effects of ionizing radiation on biological objects is introduced. It was shown that in different species of rodents (*Microtus arvalis*, *Clethrionomys glareolus*), and in the laboratory mice of C57BL/6, CC57W/Mv, and BALB/c lines the irradiation of bone marrow cells induced an increase of only those cytogenetic anomalies, in comparison of control groups, the increased variability of which was typical for the studied objects in a relatively clean areas. The main and, apparently, underestimated vertical consequence of raised ionizing radiation is the decrease in reproductive success of irradiated animals. Importantly, a transgenerational transmission of post-traumatic syndrome and its mechanisms, including transmission of microRNAs, the mediators of the stress response, through the spermatozoa (K. Gapp et al., 2014), changes in microbiota of parents and their children, as well as cultural inheritance are involved to explain a complexity of observed radiobiological effects and their inheritance revealed in recent years.

Keywords: NPP accident, ionizing radiation, «horizontal» and «vertical» effects, reproductive success, transgeneration transmission.

Though the Chernobyl Nuclear Rower Plant accident happened 30 years ago in May 1986, it still remains the source of radionuclides affecting people on the polluted areas with the consequences poorly prognosticated and the defense measures not studied and substantiated enough to protect people and environments [1]. Since that time, there is a great number of reports on population genetic consequences of such large-scale man-made disaster, the first to cover the entire globe. Importantly, these studies have been performed on different

animal and plant species, and on human populations. The latter has expectedly attracted special attention of experts and the public concern. However, only the research of biological and biosocial effects of ionizing radiation and a aggregate data accumulated allows us to analyze the observed effects comprehensively and to identify the mechanisms underlying them, on the one hand, and also contributes to understanding the fundamental processes in living things under external stresses, on the other hand. Nevertheless, there still are numerous inconsistencies between the findings [2-5]. Uniform biomarkers of ionizing radiation damage seem to help in finding consensus [6]. European Network of Biological and Retrospective Dosimetry (RENEB, Germany; http://www.reneb.eu/), in particular, is expected to develop the uniform protocol for the reliable radiation dose estimation, the high-risk group identification and prognostication of diseases [7].

In particular, new ionizing radiation biomarkers are developed due to the methods for assessing mutation patterns. However, even for such seemingly reliable statements as a link between the absorbed dose of radioactive iodine and thyroid cancer, details vary. For example, according to the Ukrainian-American Registry of patients with papillary thyroid carcinoma who have received up to 18 doses of radioactive iodine from the Chernobyl accident (UkrAm), a significant increase is found in the incidence of copy number variability (CNV) of genomic DNA short fragments [8]. Based on the same register, the dependence is found between the development of carcinomas of the thyroid gland and the dose of ionizing radiation received at a young age [9]. Regardless of the dose received, the more pronounced invasive phenotype of papillary thyroid carcinomas was observed in the patients with elevated chromosomal instability [10]. In other studies, a direct relationship was not found, and, according to authors, the number of environmental factors can affect it significantly [11].

After the accident at the Fukushima-1 nuclear power plant the thyroid cancer incidence rate among 0-18 year old children of the same age and a variety of observed mutations have been analyzed. These data indicate a significant distribution of papillary thyroid cancer in the surveyed population with the pattern of identified mutations significantly different from the papillary carcinomas resulted from the Chernobyl accident [12]. According to the authors, an increased incidence of thyroid papillary carcinoma in the region is spontaneous rather then induced by ionizing radiation.

Convincing evidences are accumulated that an increased incidence of papillary thyroid carcinoma after the Chernobyl accident are more often detected in iodine-deficient areas [13]. Currently, it seems clear that in the population the effects of ionizing radiation will be directly related to the absorbed dose (i.e. less than or greater than 100 mSv per year), ecological and geographical conditions, age, sex and genotype, and also it will differ in the generation got directly under the irradiation (horizontal effects), and in the descendants (vertical effects) [2, 14].

A special need for classification of consequences has arisen after the Fukushima-1 accident, as it became clear that, despite an increased frequency of nuclear accidents the uniform methods for diagnosis and prediction of the radioactive contamination have not yet been developed. One can only hope that an accident at the Fukushima-1 will allow us to collect and summarizes relevant observations and experimental results more successfully.

In this regard, in the present review the available data about the peculiar effect of different doses of ionizing radiation on human population, farming animals, bioindicator animal species of small rodents, some age-based and species-specific responses, as well as transfer of induced disorders from parents to offspring are summarized.

Nonlinear responses to low ionizing radiation. The lack of a linear relationship between the actual genetic damage and low doses of ionizing radiation is known for long time though its mechanisms remained unclear. In recent years, in several studies it have been shown that this nonlinearity may be due to the DNA repairing induction only after mammalian cell exposure to a dose exceeding 100 mSv [15-17]. A DNA doublestrand breaks (DSBs) is reported which occurs within 30 days after the animals were inoculated once with $^{137}CsCl$ intraperitoneally, or with a conditionally high (1.55±0.1 MBq) and low (200±0.3 kBq) doses of $^{85/90}SrCl_2$ intravenously [18]. Based on biokinetics the authors calculated that after $^{137}CsCl$ removal the total 30-day dose amounted to 10 Gy, while the total absorbed dose after $^{85/90}SrCl_2$ removal, as estimated by the isotope incorporation in skeleton, was from 0 to 5 Gy at low irradiation reaching 49 Gy at the high one. In this, two picks of accumulation of lymphocytes with DSBs, during first week and in 3 weeks after the radionuclide inoculation, were observed. So far as a daily absorbed radiation dose was considered small, long term damage in lymphocytes was surprising. As to authors, the firs pick is primary due to damage of mature, almost completely differentiated lymphocytes while the second one is related to prolyferation of young progeny of these damaged cells.

An increased sensitivity to genotoxic agents and decreased DNA reparation in aging cells are documented [19, 20]. It is a constant rejuvenation of bone marrow cell population to which it seems to be due an active cell divisions and decline in cytogenetic abnormality rate found by us in 16-18-month old CC57W/Mv mice exposed to 0.5-0.6 Gy per year in the experimental vivarium in the Chernobyl exclusion zone as compared to control animals of the same age [2]. Note, in 2-3-month old mice CC57W/Mv from the vivarium the rate of cytogenetic abnormalities was considerably higher while the cell division rate was lower compared to the control. But if an activated cell division and decreased number of damaged cells in old mice may be due to more rapid renewing bone marrow cell population induced by ionizing radiation, a periodic rise and decline in the percent of cytogenetically abnormal cells in CC57W/Mv mice of different generations which were exposed to the same doses and kept under the same conditions in the experimental vivarium in the Chernobyl exclusion zone, still remain unclear.

Mismatch in estimation of low dose effects could also be due to dysfunction of mitochondria. Thus, a decrease in activity of mitochondrial superoxide dismutase 2 (Sod2) can lead to appearance of classical biomarkers of ionizing radiation, e.g. dicentric and ring chromosomes [21]. A decrease in its activity is reported to be involved directly in cell aging and increase of cell sensitivity to ionizing damages [22]. So a designation of DAN as the main target molecules of ionizing radiation seems not to reflect real events, and the observed effect may be due to a functional heterogeneity of mitochondria.

Besides, stresses cause genome instability in dividing cells of a multinuclear organism, affect significantly the crossing-over rates in meiosis, and transposition of mobile elements. On laboratory mice and rats, since Hans Selye's works, a deep suppression of immune function, a durable increase in blood cortical steroids level, and disorders of cell division (a triad of Hans Selye) are known as the consequences of stresses. In rats, changes in photoperiodicity, temperature and noisiness resulted in rise of peripheral blood erythrocytes with micronuclei [23]. It was shown that methylation influenced by the environment may cause genome instability and mutagenesis due to copy number variation (CNV) in short DNA fragments [24]. In living things there is a clear genetic component in variability on stress reactivity and genome instability. Particularly, a

comparison of the micronuclei-containing cell frequency in human identical twins showed that its variability is significantly determined genetically, the same as a response to ionizing radiation detected with a micronuclei test [25].

In our study, in different small rodent (voles), and laboratory mice exposed to a total of < 1 Gy per year in Chernobyl exclusion zone it was shown the increase in the cytogenetic abnormalities in bone marrow cells the same as are characteristic of these species and reordered in control animals which were not exposed to irradiation [26]. That is, our experiments have shown that more than 100-fold increase in the dose of ionizing radiation did not induce new variants of mutational spectra in bone marrow cells in voles and laboratory strains of mice, but only strengthens those specific to a species or lines manifestations of genomic instability that in the control conditions occur in the studied animals spontaneously. For example, there was an increased frequency of aneuploid cells in the C57BL/6 mice, metaphases with chromosomal aberrations in SS57W/Mv line, the proportion of binuclear lymphocytes in BALB/c line, the percentage of aneuploid cells in common vole (*Microtus arvalis*), and the proportion of metaphases with interchromosomal Robertsonian-like translocations in the bank vole (*Clethrionomys glareolus*).

Obviously, very special responses to ionizing irradiation will be observed in human populations, reproducing in the natural radioactive provinces [27, 28], or in many generations of animals in areas with high radionuclide contamination, particularly after the Chernobyl accident [26].

The horizontal effects of ionizing radiation. To date, the most comprehensive long-term survey of the impact of ionizing radiation on human health is summarized by Japanese researchers studying the effects of the atomic bombings of Hiroshima and Nagasaki and accidents at nuclear power plants, including Fukushima [29, 30].

Effects of ionizing radiation on the human body has been studied in more detail in long-term and large-scale epidemiological survey targeted to formation of a database (registry) of the Japan residents, who are he survivors of the atomic bombings of Hiroshima and Nagasaki. Due to a large amount of information, the representation of both sexes and all ages, and a wide variety of doses evaluated individually, the results on the life expectancy, oncological and non-oncological diseases (Life Span Study — LSS) in the Japanese from the register is considered the most reliable information about the effect of ionizing radiation on human populations. For this reason, LSS research was fundamental to risk assessments in radiological protection system, developed by the International Commission on Radiological Protection (International Commission on Radiological Protection — ICRP, GB). Overall, the data suggest that survivors of radiation exposure have a clear excess relative risk (ERR) of cancer compared to the control groups. For all leukemias in all age groups, this figure is 3-5 times higher when the absorbed doses are about 1 Gy per bone marrow cells [31, 32]. A statistically reliable rise in the incidence of solid tumors was observed in 6-10 years at the absorbed doses of 0.1-0.2 Gy. At that, there was an increase in tumors in the bladder, breast, lung, brain, thyroid, colon, ovaries, stomach, liver, but not in pancreas, rectum, uterus, prostate, and kidney parenchyma. Multiple non-cancerous diseases, mainly cardiovascular, respiratory and immune pathologies, and kidney damage, were found in the atomic bombing survivors.

In our research, using standard electrophoresis of proteins in the starch gel and histochemical staining, we compared the isozyme patters of lactate dehydrogenase (LDH), malat dehydrogenase (MDH), malic enzyme (ME) and esterases (ES) in liver, kidney, spleen, heart muscle, lymph nodes in pigs, rabbits, nutria and cattle from 30-km Chernobyl zone of high radionuclide

contamination, and in the control farm animals from Kiev province [25]. In the animals from the Chernobyl exclusion zone the ^{137}Cs accumulation was 7-25-fold when compared to the control and reached 160 Bq/kg.

No differences in organ-specific isozyme patterns were found between nutria from experimental and control groups.

In irradiated and control pigs the LDH spectra were the same in patterns but clearly differed in the relative activity of bands. For example, the staining of the LDH bands 1-2 with the highest electrophoretic mobility was similar in both groups whereas it was much higher in band 3 in irradiated pigs compared to control pigs. No differences were found between the groups on MDH and ME patterns. However, the ES patterns of liver and kidneys in irradiated animals differed significantly. Particularly, two fast migrating bands characteristic of liver in control animals were absent in irradiated ones, while in their kidneys there was the most electrophoretically mobile ES form which was not found in the kidney of control pigs and in any other organs of both control and irradiated animals.

In irradiated rabbits, when compared to the control animals, the ES patters were not the same in different organs. Like in pigs, in rabbits exposed to ionizing radiation some minor ES bands specific to the kidneys of intact animals were absent while new ones appeared. Interestingly, the ES pattern has changed significantly in cardiac muscle. In intact rabbits, there was a characteristic isozyme pattern with a specific band of intermediated electrophoretic mobility not found in other organs. In the cardiac muscle of irradiated rabbits this band was not observed while two new bands, fast and slow migrating, appeared which were also characteristic of ES spectra in some other organs and tissues, particularly in striated muscle. Besides, in rabbits the spleen ES isozyme spectrum has also been changed, and, as in kidneys, some bands disappeared while new ones appeared.

In cattle exposed to ionizing radiation the ESs in kidneys and cardiac muscle also differed markedly from those of intact animals.

The organ specific ES patterns of irradiated cattle and pigs were reproducible in all animals in experimental groups.

Thus, these data indicate that ES spectra are most informative to study the impact of radionuclide pollution on fundamental organ-specific biochemical processes in different animal species. In all three species studied, we observed a marked change of ES isozyme spectra in kidneys of irradiated animals. Moreover, the modified ES spectrum was also observed in the liver of pigs; in the kidneys and in the cardiac muscle of cattle and rabbits, and in spleen of rabbits. These data allows to suggest that in all three species the excretory system such as kidneys (and also the liver in swine), cardiovascular system (in cattle and rabbits), and also lymphoid tissue (in rabbits) are the main target organs damaged under irradiation.

Interestingly, our results are consistent with data on organ specificity of non-cancerous human diseases for which damage was mainly found in the cardiovascular, immune system and in the kidney [33].

Noteworthy is the fact that in the study of the effects of the earthquake and increasing exposure to ionizing radiation in human populations after the Fukushima accident, the marked deviations in carbohydrate and lipid metabolism were observed in the first few days and persist for months after exposure [34]. The same character of changes after the earthquake and at higher levels of ionizing radiation is emphasized. Thus, for 3 years in Fukushima Prefecture after the earthquake and exposure to ionizing radiation the number of patients with neuro otolaringological disease complicated by depression and other mental defects has increased significantly [34, 35].

Polycythemia is found in people who were evacuated from the zone is

elevated ionizing radiation for 2 years after the accident at the Fukushima-1, indicating significant changes in hematopoiesis [35]. The frequency of various somatic diseases was higher in liquidators in comparison with the residents of Latvia. Diseases of the nervous, digestive, cardiovascular, endocrine, respiratory and immune systems were mostly recorded at a 1.3-fold morbidity among liquidators compared to the rest of population in 1986 which reached a 10.9-fold level in 2007 [36].

A lot of evidences of non-specific health problems in people resettled from areas with elevated levels of radiation after the Chernobyl and Fukushima accidents are also accumulated. After the Chernobyl accident the mothers relocated together with children rated their health 2 times less than in the control group [37]. After the Fukushima accident almost 30 % of mothers were diagnosed clinically depressed [38]. It was shown that among people who were evacuated after the nuclear disaster at the Fukushima-1, the concern about radiation risk was associated with psychological disorders [39].

The survey of liquidators even 24 years after the Chernobyl accident showed a decrease in all three indicators (physical, mental health and social well-being) used by the World Health Organization for health monitoring in human populations [40, 41]. The survey carried out in 1999-2002 evidenced of a significant increase in the incidence of thyroid tumors, as well as depression, suicidal ideation and attempts among people living in the most contaminated regions of Ukraine [42].

The damaging effects of ionizing radiation on the brain and cognitive functions are known for a long time. Thus, A.I. Nyagu and K.N. Loganovskii [43] historically summarize the observations of the neurophysiological effects of ionizing radiation in humans which were first described in 1896. In the post-Chernobyl period they also reported about multiple abnormalities in the central nervous system associated with radionuclide contamination after the Chernobyl accident [44, 45]. Evidences are being accumulated on a significant contribution of an oxidative stress, including a decrease in superoxide dismutase activity in the mitochondria, to the neurophysiological disorders induced by ionizing radiation [46, 47].

Additionally, a detailed study of public health after the earthquake of 2011 in Japan in different regions shows that a decrease in the objectively and subjectively assessed health indicators closely depends on the strength of the earthquake and the remoteness from its epicenter [48]. Note, the fact of resettlement from the territory with elevated levels of ionizing radiation around the Fukushima-1 nuclear power plant could itself lead to an increase in mortality among the evacuated population, and this was clearly detected during the first 4 years after the accident [49]. Moreover, an increased mortality was not directly related to the dose of external and internal exposure. It is assumed that this effect is due to post-traumatic syndrome which provokes non-specific, especially chronic, diseases rather than to actual damaging effect of ionizing radiation.

Vertical damage induced by ionizing radiation. From our point of view, the main and underestimated «vertical» consequence of exposure to ionizing radiation for living things is a reduced reproductive function with elimination of individuals possessing alleles and gene systems associated with relatively increased radiosensitivity. It is this limitation of reproduction that explains an increase in the number of radioresistant individuals among the trapped voles and the shift of the genetic structure in progeny of irradiated specialized dairy cattle toward more primitive forms described in Chernobyl exclusion zone [26], and also an increased radioresistance of blood cells in the inhabitants of the radioactive provinces. The observed changes correspond to the

statement of I.I. Schmalhausen that under any environmental changes the most stable but the least specialized forms possess a privilege in reproduction [50].

The negative impact of post-traumatic syndrome on health is widely studied in recent years. There is evidence suggesting that increased sensitivity to stressors is observed in children born from parents with this syndrome. In Denmark in 4-9 year old children which were not subjected to injury, but born in refugee families where the post-traumatic symptom was diagnosed in one or both parents, the transmission of this syndrome has been found [51]. The manifestations of post-traumatic stress syndrome in children born from parents who survived the Holocaust were being studied for more than 50 years [52]. It was found that among the people resettled after the Fukushima accident, and among the grandchildren of Japanese survivors of the Hiroshima and Nagasaki atomic bombings the frequency of the post-traumatic stress syndrome manifestations increased relative to the control group [53]. Detailed studies are being conducted on transmission mechanisms of hypersensitivity to the expression and the induction of post-traumatic syndrome symptoms in generations, and the dependence of this phenomenon on the cultural characteristics and closeness of contacts between parents and children has been revealed [54].

People are subjected to many stress factors adversely affecting health, depressing the immune system, increasing susceptibility to infection and the risk of many other diseases. Stress can cause genetic, epigenetic and genotoxic changes in humans and animals. Ionizing radiation, as is known, may also induce the whole range of such changes. But so far the combined effect of stress factors and ionizing radiation remain poor studied. In rare works, there were the attempts to separate the effects of stress and ionizing radiation. In one of these studies the authors showed that in linear mice it was possible to separate the effects of stress from the damaging effects of ionizing radiation (55).

Note that the actual accidents at the nuclear power plants are always accompanied by the combined effect of a stress factors, and ionizing radiation. The Chernobyl disaster demonstrated that its psychosocial consequences are quite long-term.

Possible mechanisms for transgenerational effects of ionizing radiation. Transmission of changes caused by environmental factors, from a parent to further generation is described in several studies [56, 57].

In human and model species such as laboratory linear mice and rats, the various mechanisms of epigenetic variability affecting genomic reprogramming in gametes have bee described. These are a hormonal control of DNA methylation profile, changes in chromatin packaging and the histone code, an accumulation of regulatory microRNAs in the gametes that may be involved in transgenerational inheritance [58]. Induction of epigenetic changes in spermatogenesis by external factors is described in several studies [59, 60]. Thus, in male mice the traumatic stress during spermatogenesis induced an expression of certain miRNAs, which also persisted in the zygote that led to changes in behavior and metabolism in the offspring [61, 62]. This miRNA, being isolated and then introduced into normal zygote, caused the same changes in born offspring [63, 64]. In the primordial stem cells populating gonads of the embryos prenatally exposed to vinclozolin, the endocrine disregulator, there were changes in synthesis of a number of miRNAs that regulate cell differentiation [65]. To date, a large number of miRNAs are found, in which the changes in metabolism can directly contribute to epigenetic variability of offspring via the influence of the environment on the parents [66].

Another pathway of epigenetic inheritance may be associated with the intergenomic relationship between multicellular organisms and its microbiome

(metagenomics) [67]. Multicellular organisms, such as plants and animals, are not an autonomous units, but a biomolecular networks of the host cells and associated microbiome. In opinion of some researchers, such a set of organisms may be defined as a holobionts, and their genomes are considered as hologenomes [68]. Multiple molecular mechanisms of microbiome action on the various functions of multicellular organism are revealed, including raising its epigenetic variability, and the evidence experimentally obtained on the model objects indicates that the changes in the microbiome can result in modifications of behavior [69, 70].

Thus, the evidence obtained to date, suggests that the transgenerational transmission of responses to environmental stress from parents to their progeny may be due to cultural inheritance (54), or result from induced epigenetic changes in the parental gametes (or in the gametes of descendants during embryogenesis), in embryos during early development, as well as in the microbiome of parents and the offspring.

So, an increase of ionizing radiation leads to multiple changes in living things, which can be divided into «horizontal» in those ones subjected to the irradiation directly, and the «vertical» detected in their offspring. In mammals, not only actively proliferating immune system, but also cardiovascular and excretory systems, as well as brain are among the main targets. Changes depend on the species, the genotypic features of exposed organisms, the region of their reproduction, the feeding, as well as on the absorbed doses, the lowest of which do not induce the intracellular reparative mechanisms thus «preserve» occurred damage for a long time. Expression of «vertical» genetic effects in population is determined by selection of radioresistant forms whereas the inheritance of post-traumatic syndrome in model organisms (linear mice) is due to the transspermal transmission of miRNAs involved in the regulation of many changes in gene systems during ontogenesis. Biosocial consequences for human populations result not only from the radiation-induced changes in central nervous system and the transmission of microRNAs involved in stress syndrome formation, but are also related to the cultural inheritance and modification of microbiome. Eexpectedly, the registers (database) of parents and their descendants of different generations, which takes into account not only the doses of ionizing radiation, but also the eco-geo-biochemical factors of habitats, and the ethnic features, will help to understand peculiarities of radiobiological response. In turn, this will speed up developing methods for diagnostics and radioprotection, and also allow to suggest techniques for targeted compensation of radiation damage in cells, organs or systems of multicellular organisms.

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Varroa destructor AND A THREAT OF VIRAL INFECTIONS OF THE HONEYBEE (*Apis mellifera* L.)

(review)

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Abstract

Bee viral infections worldwide leading to colonies' depopulation have emerged as a threat to bee keeping. To date, nearly 20 RNA viruses, of *Dicistroviridae*, *Iflaviridae*, *Nodaviridae* families mostly, were detected in honeybee *Apis mellifera* (O.F. Grobov et al., 2006; C. Runckel et al., 2011). Also DNA viruses have been found, e.g. iridovirus (*Iridoviridae*), potentially causing losses of bee colonies (J.J. Bromenshenk et al., 2010), *Aphid Lethal Paralysis virus* (*Dicistroviridae*), *Big Sioux River virus* (*Dicistroviridae*), *Lake Sinai Virus strain* 1 and 2 (*Nodaviridae*) (C. Runckel et al., 2011) however, their role in bee mortality has yet to be understood. The most important bee viruses known to date are deformed wing virus (DWV), acute bee paralysis virus, (ABPV), chronic bee paralysis virus (CBPV), Kashmir bee virus, (KBV), sacbrood virus, (SBV), Black queen cell virus (BQCV). These viruses can persist in honey bees (*Apis mellifera* L.) without apparent symptoms, however, *Varroa destructor* infestation causes a viral epidemic, diminishing bee colonies. The range of *V. destructor*, the main viral infections' vector (D. Tentcheva et al., 2004), was confined to that of *A. cerana* being ecologically balanced. However, not long ago this mite crossed the species barrier from the Asian hive bee *A. cerana* to our own Western honey bee *A. mellifera* (R.S. Poltorzhitskaya, 2008). The introduction of *V. destructor* into the *A. mellifera* population has become one of the major contributing factors to colony collapse disorder (D. van Engelsdorp et al., 2008; R.M. Johnson et al., 2009; F. Nazzi et al., 2012). Moreover, the mite Varroa affects the immune response and metabolism of honey bees and allows its vectored viruses to propagate to high viral loads. At present there is an objective need for a closer look into bee viruses implicated in bee colony losses reported worldwide. So far as Varroa mite is an obligate parasite of *A. mellifera* during whole ontogenesis, the Varroa control notably ensures the well-being of bee colonies. In this review, an overview of the world distribution and the impact of the major viruses (DWV, ABPV, CBPV, SBV, BQCV, KBV) on bee health and colony survival is presented. We also discuss approaches to virus control. Overall, the strategy combining new Varroa management practices (A.A. Fedorova et al., 2011), selection of Varroa-resistant bees and novel treatments against viruses will help sustain the honey bee population.

Keywords: *Apis mellifera*, *Varroa destructor*, viruses, viral infection transmission, vectors.

To date, nearly 20 RNA viruses, of *Dicistroviridae*, *Iflaviridae*, *Nodaviridae* families mostly, were detected in honeybee *Apis mellifera* [1-3]. DNA viruses also have been found — iridovirus (*Iridoviridae*), potentially causing losses of bee colonies [4], *Aphid Lethal Paralysis virus* (*Dicistroviridae*), *Big Sioux River virus* (*Dicistroviridae*), *Lake Sinai Virus strain* 1 and 2 (*Nodaviridae*) [2]. However, their role in bee mortality has yet to be understood.

Active replication of most viruses in insects usually results in negative changes of physiological parameters and behavior up to honeybee colony losses [5-8]. Latent viruses can persist in some individuals or in bee family as a whole and not cause the death [9-11]. Importantly, some viruses when influenced by definite but not clearly understood factors can provoke a decrease in bee cold resistance, queen bee reproductive activity, flying activity of bees and nectar collection [12].

Undoubtedly, viral infections are dangerous under bee infestation with *Varroa destructor* mite, the vector of most harmful viral infection [10]. When biting, an infected mite transmits a causal agent to bee. Varroa mite is an obligate ectoparasite of *A. mellifera* at all stages of ontogenesis. Initially, Asian bee *A. cerana* was the host species, but not long ago this mite crossed the species barrier, and now *A. mellifera* bees are also infected in natural habitats [13, 14].

A. cerana and *V. destructor* were ecologically balanced so that the mite did not cause death in the bees. First, the mite can not reproduce in cells with worker *A. cerana* bees [15, 16], and the reproduction occurs in drone cells only. Secondly, the *A. cerana* imagoes effectively remove Varroa mites due to active grooming [16]. Thirdly, at high infection load the drone pupas are less viable, not able to break cocoon and dye, and the bees then ignore these cells [17, 18]. Thus about 25 % of mites in the bee colonies are eliminated.

However, lack of defensive behavior in *A. mellifera*, with the exception of less effective grooming, leads to low immunity, a decreased body weight, disorders in water balance and carbohydrate metabolism, and reduced the life expectancy. Finally, bee viability decreases causing colony death or collapse [19-23].

Viral infections in *A. mellifera* were underestimated until *Varroa* introduction [21]. Nevertheless, high viral infection rate (up to 90 % of bees in some colonies) has been detected by RT-PCR. A combined infection of 5-6 different viruses can be found in highly mite-infested bee colony [29]. A definition of «bee parasitic mite syndrome» is used for symptoms of mite-infested bees with viral pathology [25]. It was shown experimentally that inoculation of virus particles in the bee hemolymph and suppression of the immune response by the Varroa mite reduce the defenses both in a bee and the bee clony, and activate latent viral infections [26].

Of those found to date, the most important bee viruses are deformed wing virus (DWV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), Kashmir bee virus (KBV), sacbrood virus (SBV), and black queen cell virus (BQCV).

Deformed wing virus. DWV (*Iflaviridae*) is found worldwide [27, 28] including Russia [29, 30] and Azerbaijan [31]. DWV is genetically close to *Kakugo virus* found in aggressive bees in Japan [32, 33]. In *Kakugo virus* and DWV RNAs are 97-98 % homologous [32]. Currently, *A. mellifera* is considered the main host species for DWV disseminated due to *V. destructor* [10, 34]. DWV also was detected in *A. cerana*, *A. florea* [35, 36] and in bumblebees (*Bombus terrestris*, *B. pascuorum*) with deformed wings [3]. DWV was isolated from *V. destructor* [21, 37-39] and *Tropilaelaps mercedesae* [40, 41], the ectoparasitic mites of honeybees. These are the vectors, especially *V. destructor* which plays important role in DWV transmission [21, 26, 42, 43].

DWV is also detected in *Aethina tumida*, the small hive beetle providing DWV replication. There are reports of DWV transmission from *A. mellifera* to *A. tumida* [44] and bumblebees [28]. DWV is avirulent in the bees not infested by *V. destructor*. Latent DWVs do not cause clinical symptoms [21, 45-47]. DWV can be transmitted transovarially via drone sperm, and between colonies at swarming [45, 48].

Characteristic DWV symptoms under high mite infestation are rudimentary and crumpled wings, bloated bellies, and discoloration. These bees are not viable and die 67 hours after they emerged from their cells that leads to weakening bee colonies [25, 49, 50]. Evidence for the *V. destructor* as a vector for DWV has been obtained [21, 51]. In recent experiments it was shown that the presence of viral (–)RNA (the replicative form of DWV genome) in mite causes clinical DWV symptoms in pupas. In the body of a mite from died bee with DWV pa-

thology the viral particles can reach 10^{10}-10^{12} in number. Thus the manifestation of DWV syndrome in bees depends on DWV amplification and accumulation in mites not less then on the virus transmission [43]. When studying DWV location in the mites, the viral particles were found only in the intestinal lumen but no evidence for replication was obtained. Probably, in mite population the DWV is replicatively inactive. For elucidation, the mites must be investigated in the colonies where the bees with deformed wings emerge.

In Europe, depending on the season, up to 100 % of honeybee colonies may be infected with DWV [10, 52]. The presence of DWV together with *V. destructor* in bee colonies before winter period is likely to cause the death in winter time [53-55]. In the absence of mites the DWV infection is symptomless [45], but under the Varroa infestation DWV is activated causing death in honeybees. Before the *V. destructor* attacked Europe in 1970-1980, no manifestation of DWV infection was reported [36]. But nowadays the DWV is considered one of the main reasons for the collapse in the mite-infested bee colonies.

DWV infection is always associated with the presence of *V. destructor* as its vector [22, 37, 51]. In Thailand and France the DWV monitoring has shown that the virus can constantly be present in all mite individuals [10, 56]. The presence of DWV was revealed in 69 % and 90 % of infested bees in Poland and England, respectively [57].

It is statistically proven that bee infestation by *V. destructor* in the summer leads to DWV appearance in the autumn. In 4 year survey of 1,250 honeybee colonies in Germany a relationship was found between winter death rate, *Varroa* infestation level and the DWV titer in bee body [47, 58, 59].

DWV can also infect wild bees *A. florea* and *A. dorsata* [60]. It is assumed that the DWV and Varroa destructor virus-1 (VDV-1) can cause degeneration of the ovaries in queen bees [61]. When laying DWV infected eggs, the queen bees transmit the virus to offspring causing symptomless carrier state [45, 62]. DWV was detected in faeces of queen bees [63]. Obtained data [64] are in line with another report about DWV detection in the faeces of worker bees [65].

Varroa mite also is a carrier of VDV-1 virus [66], RNA of which is 84 % identical to the RNA of DWV [66, 67]. Moreover, the recombination between VDV-1 and DWV is possible [68], producing recombinant viruses such as VDV-1_{DVD}, which are better adapted to the horizontal transfer via mite biting and effectively avoid the immune control of the bee host. They also can replicat to larger loads than DW or VDV-1 that may be indicative of higher virulence of the recombinants for the bees [68].

Acute bee paralysis virus. ABPV is genetically close to KBV and Israeli acute paralysis virus (IAPV) (*Dicistroviridae*) [69, 70]. ABPV can infect both brood and worker bees. Naturally, ABPV persists in bees without clinical manifestation and does not cause death [8]. Presumably, ABPV can be transmitted via the saliva when feeding larvae and producing beebread. At high infection rate the larvae usually die before sealing, but in case of surviving no clinical manifestation of ABPV occurs in the adults.

ABPV detection in *Varroa* suggests an involvement in ABPV transmission and dissemination [9, 10, 56]. In addition to acting as a carrier, the mite is able to activate latent infections caused by ABPV. Significant virus accumulation in dead bees with ABPV syndrome and high rate of mite infestation suggests that Varroa can activate the virus replication so that it causes death of the bees [71]. Importantly, an injection of phosphate buffer into bee hemolymph also activate ABPV replication, hence the Varroa mites are not the only agent activating latent ABPV [72].

Both ABPV and Varroa mite seem to cause the mass losses of bee colonies in Europe [9, 73]. ABPV is one of two the most common viruses in Austria [52]. For a long time the ABPV was detected in apparently healthy honeybees in Britain [35]. ABPV was found in honeybees in France, Italy, Canada, Chinese, United States [72], and in New Zealand. Currently, ABPV is expanding worldwide due to the *A. mellifera* expansion [35, 36].

Since the *V. destructor* appeared in Europe, the high rate of ABPV infection was detected in the heavily infested bee colonies with dead sealed and unsealed brood. This was first considered in Russia and Germany in the late 1970s and early 1980s [74]. Then similar data were reported from Netherlands, Italy, former Yugoslavia, France [71], Hungary, Austria [52], Denmark [37] and USA [72]. In Germany and Holland in dead honeybees from the colonies of low, medium and high mite infestation the ABPV frequency was 3 %, 44 % and 80 %, respectively.

In France ABPV was found in 58 % of adult bees, and in 23 % of pupas and apparently healthy bees, while the frequency of ABPV-infected *Varroa* mites was 36 %. Viral infection was more frequent in infested colonies in the late summer and autumn that correlated with the peak in Varroa population [10]. In Denmark [75] the ABPV infection was detected in 14 % of bee colonies, in contrast to data obtained in France and Austria for clinically healthy and sick bees (58 % and 68 %, respectively) [52]. In the opinion of Danish researchers, these may be due to organic acids used against the mite, which are less effective against varraosis so that bees must withstand higher infestation resulting in higher natural death among ABPV infected bees. In Denmark the ABPV frequency was 73 % in 2004 and 80 % in 2005 [76]. In this investigation the relationship was found between infection, infestation and winter death in the colonies. A reliable dependence of bee winter death on ABPV frequency before hibernation was shown for 2005-2006 season but not for 2004-2005 season. However, the bee death in winter correlates with the rate of bee infestation in both seasons. Reasonably, the latent ABPV can be transmitted and activated by the *Varroa* mite that is a cause of bee death in winter [76].

Kashmir bee virus and Israeli acute paralysis virus. KBV was first described in 1977 in *A. cerana* [77]. KBV, a RNA virus, when inoculated into the bee hemolymphm, replicates to high titers [78]. Bees infected via hemolymph died within 6 days, but insects which ate KBV preparation remained apparently healthy [78]. Hence, the lethal effect of KBV depends on transmission routes [78, 79]. In these the *Varroa* mite is of key importance. KBV was first isolated from homogenate of worker bees *A. mellifera*, experimentally infected with extract of *A. cerana* bees from Kashmir (India) [78]. Detection of KBV in *A. mellifera* Australian population is extremely interesting as *A. cerana*, which seems to be the main host bee for KBV, does not inhabit this continent. Then KBV was discovered in *A. mellifera* from Canada and New Zealand [80], on Fiji [81], in USA [49], Europe and Oceania [35, 36, 82]. Currently, KBV is reported in *A. cerana* [35, 77, 78], *A. mellifera*, bumblebees (*B. terrestris*, *B. pascuorum*) and wasps (*Vespula germanica*). In honeybee populations of North America and New Zealand the KBV infection prevails compared to other viruses [72, 83, 84], though in Europe KBV is rarely found [10, 52, 82, 85].

KBV attacks the bees during whole ontogenesis and usually persists latently in the brood.

IAPV which was isolated in Israel from larvae inoculated with homogenized dead bee, is another dangerous virus closely related to KBV [86]. As its symptoms were similar to those under acute paralysis the virus was called Israeli acute bee paralysis virus (IAPV). The virus is widespread in the Middle East

[86], Australia, USA [87-89] and rarely found in Europe [90].

KBV and IAPV (*Dicistroviridae*) are closely related. Together with ABPV they form a common genetic complex with similar transmission routes. Remaining latent in bee larvae, they cause rapid death of adult insects with characteristic clinical symptoms. Genetic analysis of KBV and ABPV revealed about 70 % homology but also some differences [91].

ABPV, KBV and IAPV at low titers can permanently persist in bees without clinical manifestations [72, 89]. When KBV and IAPV titers reach a critical level the infected bees die [77, 86, 88]. An increase in titers in bee hemolymph under natural infection is related to *Varroa* mite as both vector and activator of virus replication [49, 92, 93].

Research of KBV and IAPV transmission routs [42, 51, 94] showed an important role of *V. destructor* in KBV pathogenicity, but the exact mechanisms of disease development still remained unstudied [42]. It is believed that *V. destructor* suppresses the immune defense in bees, resulting in the activation of resident viruses [19]. In other opinion, the *V. destructor* is a vector, which directly transmits KBV to pupas, as KBV was found in the saliva of the mite [42]. Furthermore, mite infection with KBV and transmission to brood has been proved experimentally [95]. Estimated efficiency of KBV transmission to brood by *V. destructor* amounted to 70 %, when the efficiency of mite-to-mite KBV transfer or the rate of infecting mite from brood was 51 %. KBV plays a key part in death of the mite-infested bee colonies [72, 77]. Also IAPV virulence is probably due to *V. destructor*. The IAPV presence is a marker of bee colonies collapse in USA. The collapse signs are as follows: there are no dead bees at the bottom of the hive, or next to it, bees left brood and queen, weight of worker bees in the hive critically reduced despite of enough feed [96]. Note, the IAPV is also common in Australia, but there is no collapse of bee colonies. Study of bee collapse in USA [97] and bee death in winter with no signs collapse in Germany [76] revealed a sophisticated relationship in ABPV-KBV-IAPV complex in which *V. destructor* is involved as the activator of latent infection. These three viruses differ in geographic distribution [98], and that is why IAPV or KBV can cause bee death in USA while ABPV has lethal effect in Europe.

IAPV was shown to integrate into bee genome. A fragment of IAPV genome is found in the genome of 30 % of Israel bees [86]. IAPV-specific sequences are also detected in *Varroa* genome. Moreover, the bees with integrated IAPV genome possess resistance to this virus [86].

Black queen cell virus. BQCV was first isolated from in blackened larvas and pre-pupas from black cells [78]. BQCV affects mainly larvae and pupae queens in sealed cells. Affected larvae are pale-yellow colored and covered with sac-like skin similar to sacbrood. BQCV replication in queen pupas causing blackening and rapid death. Worker bees also can become infected, but with no symptomes. Moreover, when BQCV enters the bee body via alimentary tract it does not replicate.

BQCV is found in North America, Europe, Oceania, Asia, Africa, Midle East ad Azerbaijan [31, 35, 36]. After 5 year studies in Beltsville (MD, USA) the BQCV was shown to be the second of mostly common bee viruses after DWV [27]. In Australia BQCV is the most frequent causal agent leading to queen death during development.

When adults and pupas were compared [10], the BQCV was found in adult bees, preferably in spring and summer.

A relationship occurs between BQCV infection and infestation by microsporidia *Nosema apis* so that the BQCV frequency in the territory increases as the *N. apis* expands. BQCV replication rate in the presence of *N. apis* infesta-

tion is much higher.

It is assumed that BQCV is transmitted to the queen via royal jelly from bee-nurse. *N. apis* affects intestine providing BQCV infection. In all parts of England and Wales the worker bees infected by BQCV were co-infected by *N. apis* [8]. In Austria, the *N. apis* was found in 78 % of BQCV-positive samples from apiaries, and in 75 % bee colonies infected by *N. apis* the BQCV was also detected. Similar data were obtained in France [10].

It has been suggested that the Varroa mite can serve as a vector for BQCV. BQCV detection in the mite body on apiaries in Thailand confirms this hypothesis [56], while in other studies [10] the virus in varroa mite has not been found.

Chronic bee paralysis virus. Earlier tracheal mite *Acarapis woodi* was considered the cause of the bee paralysis, but at the end of the 1960s a viral etiology of adult bee paralysis has been shown with the CBPV as causal agent. Currently CBPV is revealed in worker bees practically worldwide except South America [35, 36]. CBPV at low loads is often found, together with ABPV, in apparently healthy bees. Chronic paralysis virus is less virulent compared to ABPV, so that at CBPV infection it takes several days to cause the death of the bee, whereas at ABPV infection the insect dies for one day.

Under CBPV infection there are usually two types of symptoms. The most pronounced ones are trembling body and wings, crawling along the ground because of the inability to fly, bloated belly and wings kept apart. Other symptoms are loss of hair and a darker appearance. Such individuals are expelled from the hive. Both types of the symptoms can be observed in the same bee colony.

When assessing CBPV infectivity, the virus was injected into the bee hemolymph, applied on the body surface, or added to the feed. It was found that CBPV can easily penetrate through the cuticle in the absence of hair, but in the case of oral infection the virus propagation in the body is minimal and does not lead to death in bees. Tightness in the hive makes the penetration easier. CBPV infection occurs in the presence of two factors. The first factor is bad weather when bees do not fly out of the hive. This explains the higher prevalence of CBPV in France [99]. The second factor is related to used beekeeping technology. Thus, the increase in the area of sunflower crop in France and, as a result, abundant monofloral pollen, led to a rapid decrease in internal hive space that has not been extended timely resulting in the colony death with the typical chronic paralysis symptoms [100]. CBPV is distributed in the world unevenly. In Britain CBPV is very frequent and causes bee colony losses [8], in Austria CBPV is found in 10 % of bee colonies [52], and in France the virus persists in 4 % of bee colonies. Importantly, seasonal dynamics is not characteristic of CBPV [10]. Transcuticular penetration of CBPV is common. In Thailand and France, when the bees and Varroa mites were surveyed to estimate the presence of CBPV, the virus was found in the bees but not in the mites [27]. In another study carried out in France, CBPV was detected in ants (*Camponotus vagus* and *Formica rufa*) and in the mite *V. destructor*, with the CBPV replicative RNA form detected in *C. vagus* and *V. destructor*, which proves the latter's role as a CBPV vector. CBPV detection in *C. vagus* suggests the possible transmission by the ants, though with no direct evidence. Ants can be infected by eating infected bees, and bees and ants can also be infected by eating honeydew of green fir aphids *Cinara pectinatae*. However, to date CBPV has not been found in aphids. Thus, to infect ants, there are still unknown CBPV reservoirs [101].

Sacbrood virus. SBV is widespread in honey bee populations on all continents [35, 36]. The virus affects the bees at all stages of the life cycle, but the 2-day brood is most affected. Viral infection in adult bees develops without

clinical manifestations, but shortens the life of individuals. SBV spreads within the hive as the bees, attempting to remove the infected larvae, accumulate the virus in salivary glands and then contaminate food. The food can be used to feed brood or exchanged between individuals. Young larvae are infected by eating infected food. SBV replication begins in larvae and results in its yellowing after sealing brood. With the progression of the disease the cuticle gets tougher and the larva is unable to pupate. The large amounts of virus accumulate under the larval cuticle [1, 102].

Usually the SBV infection is seasonally dependant with domination in spring and summer [8, 10]. At SBV infection, similar to foulbrood, worker bees usually remove or eat the dead larvae thus promoting further SBV expansion in the hive. In some colonies the hygienic behavior may be less strict, and healthy insects can recognize the latent SBV carriers prior to an increase of viral loads in the mummified bees and manifestation of the symptoms [103].

In numerous publication the mite Varroa involvement in SBV transmission is reported based on identification of the virus in flying bees with high infestation by the mite [52, 73], and also in the mites themselves [10, 42, 56].

Prevention of viral diseases in bees. Experimentally, ABPV, BQCV, CBPV, DWV, KBV and SBV are detected in the pollen (BQCV and DWV are also found in honey), ABPV, CBPV, KBV and SBV are present in beebread but absent in the saliva of bees and royal jelly [63]. As to other data, KBV and SBV were reported in royal jelly, honey and pollen [42].

Detection of viruses in *V. destructor* indicates its key role in the transmission of the causal agent in bee colonies [10, 21, 32, 51, 56, 66]. Harmful pathologies due to ABPV, CBPV, slow paralysis virus (SPV), BQCV, KBV, cloudy wing virus (CWV), SBV and DWV, leading to clinical symptoms and death have been recoreded in bee colonies under high level of mite infestation [10, 104]. Additionally, the rate of virus transmission was shown to depend on mite number in the cell and, consequently, on the total infestation level in the colony. Moreover, in case of uninfected mites in the cell they become infected via hemolymph of infected pupa. Undoubtedly, high mite infestation threatens beekeeping worldwide.

Currently, the development of methods to control mites, bee (*A. mellifera*) breeding for resistant to the mite, and bee treatment against the viral infections are considered as effective ways. These approaches should be used in combination, as in the metagenomic studies of collapsed and survived bee colonies the *V. destructor* together with IAPV and DWV were proved to be the main markers of the collapse [91].

Varroa abundance must be under control to decrease the losses of honeybee colonies [105]. As the mite activates latent viruses, affects the immunity, and causes metabolic disorders, the Varroa control provides the wellbeing of bee colonies. In the absence of the mite and and favourable conditions viral infections in bees are harmless [106]. Bee viruses are found in drone body [95] and sperm [63, 107], and in queen spermatheca (68), thus indicating vertical rout for virus transmission to offspring and probable infection of queen at mating. Since the bee queen during the life lays tens of thousands of eggs, vertical transmission of infection can be a serious risk to bee colonies, threatening its survival and indicating the importance of a regular and timely replacement of queens.

Attempts to breed Varroa-resistant bees are not yet successful. A 6 year survey of 150 honeybee colonies, with no acaricides applied and frees swarming, revealed that 3 years after the observation began the bee death in winter reached 80 %, but decreased to 12-18 % over the next 2 years. Finally, only 11 bee colonies

remained [108]. In France during 7 year monitoring of 82 bee colonies those treated with acaricides and not treated were compared. According to mortality during the winter the colonies did no differ significantly, but the bees from untreated colonies collected less nectar (by 41 %). Thus, in mite-resistant bees the honey production is lower that are not economically profitable [109].

Recently, the treatment of viral infections in bees using small interfering RNA (miRNA, siRNA) due to posttranscriptional silencing seems to be promising. Small interfering RNAs, binding to viral RNA, lead to virus degradation [110, 111]. The viral RNA, when replicating with dsRNA formation, is a source of miRNA [112, 113]. Recently, the gene knockout was used against IBPV infection good effect with a decrease of percent of bet colony losses [114). It was found that viral dsRNA, when fed to bees, activates the degradation of IBPV RNA. However, such products are costly, and it is difficult to provide their targeted delivery to the organs and tissues of the insect. A similar approach was used for the relief of the DWV symptoms. In the bees fed with relevant dsRNA the DWV replication was repressed [115]. RNAases are another potentially effective agent to combat viral infections in bees. For example, RNase can inactivate ABPV when the virus suspension is pre-incubated in vitro. This suspension became harmless to larvae when inoculated [115]. However, a complicated problem of delivering these preparations to bee hemolymph still remains actual.

So, the most important of viruses found in honey bees are deformed wing virus (DWV), the acute paralysis virus (ABPV), chronic paralysis virus (CBPV), Kashmir virus (KBV), the sacbrood virus (SBV), and black queen cell virus (BQCV). ABPV, BQCV, CBPV, DWV are detected in pollen, BQCV and DWV can also be found in honey, and ABPV, CBPV, KBV and SBV are reported in beebread, honey, pollen and royal jelly. Correlation between high virus loads and *Varroa destructor* infestation indicates that the mite serves as agent predisposing bees to viral infection or activation of latent viruses. Under per or inoculation the viruses in most cases are not lethal for bees, whereas they lead to mass death in a short time when vectored by *V. destructor*. Bedides, to prevent the manifestation of bee parasitic mite syndrome, it is necessary to take into account local climate condition and used beekeeping rechnologies.

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BASIC AND PRACTICAL ASPECTS OF VETERINARY ACUPUNCTURE FOR PHYSIOLOGICAL CORRECTION IN ANIMALS

(review)

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Abstract

An urgent task of modern biological science is the development of efficient, reliable and safe methods of body physiological correction in changing environment. This problem covers almost all living things, including humans and farm animals, which in turn affect environment and the humans through animal products. This is the most characteristic of modern industrial animal husbandry being complicated by the increase in the operating loads on the animals. The survey shows grounded and biologically possible use of veterinary acupuncture method to normalize body functions. The advantage of acupuncture is due to therapeutic and economic efficiency, versatility, safety, and wide range of application (G.V. Kazeev, 2000). This method is based on the concept of body integrity in normal state and pathology. The material basis and specific elements of acupuncture are proved (N.I. Verzhbitskaya, 1981; G.V. Kazeev 2000; J.C. Darras et al., 1993; D.J. Mayer, 2000; A.R. Liboff, 2004; R.H. Bosma et al., 2006.). It is found that when animals are subjected to acu-points' treatment, different changes occur, e.g. in biochemical parameters such as concentration of opioid peptides and monoamines in blood (J.S. Han, 2004; C.H. Zhao et al., 2005), levels of hormones (T. Aso et al., 1976; P.V. Malven et al., 1984; D.F. Bossut et al., 1986), catecholamines and growth factors (H. Chang et al., 1983; P.J. Battista et al., 1986), in cellular immunity activation (T. Kuan et al., 1986; AP Sánchez, M.D. Ángel, 2012). Clinical parameters are stabilized due to the acupuncture analgesic and sedative effects (J.G. Lin, W.L. Chen, 2008; J. Lin, Y. Chen, 2012). An uniqueness of acupuncture lies in its regulatory action (G.V. Kazeev, 2000; K.T. Freudenberg, 2010). Acupuncture is finding increasing application in veterinary practice in musculoskeletal diseases (W.W. Chan et al., 1996; G. Habacher et al., 2006), gastroenterological (Y.C. Hwang, E.M. Jenkins, 1988; K. Watanabe et al., 1998; K.T. Freudenberg, 2010) and reproductive disorders (J.H. Lin et al., 2001; W.A. Schofield, 2008), and also it is used for sedation and analgesia (A.M. Klide, B.B. Martin, 1989; J. Still et al., 1998). Acupuncture increases tissue sensitivity to medications and blends well with medicines (G.V. Kazeev, 2000; D. Souza et al., 2007; J.H. Lin J.H. et al., 2001). The therapeutic effect is increased when medications and biologically active substances are introduced into the acupoints (S. Altman, 2003; S. Ben-Yakir, 2006; T.E. Taradaynik et al., 2011), moreover, their doses can be reduced significantly (G.V. Kazeev et al., 2001; P.L. Stelio et al., 2008; T.E. Taradaynik et al., 2012). Finally, the use of acupuncture in the animal husbandry makes it possible to get eco-friendly and safe livestock products.

Keywords: acupuncture, biologically active points, domestic animals, reproduction.

Pharmaceuticals, including antibiotics and hormones, used to correct physiological state of animals, including their reproductive function, do not always lead to the desired results. In addition, a number of drugs have contraindications and restrictions, especially in cases where animal products are used by people. In this regard, the use of acupuncture (AP) seems relevant as this is a drug-free, environmentally friendly and cost-effective method. In AP technique a disease is regarded as a pathological process, inevitably affecting the entire body as an indivisible whole, associated with the environment, in which a dysfunction in one organ will naturally destroy processes in other organs and systems [1-3].

The main elements of the AP in the classical eastern (primarily Chinese) medicine are acupuncture points (APP), channels and circulating energy called, according to the tradition, the life energy. The energy of the internal organs is in constant circulation forming a system. Specialized APP are arranged on channels, through which, according to certain traditionally established rules, one can affect in different ways the body in order to restore the energy balance between the organs [3, 4]. In other words, the acupuncture is a method of regulation of living functions, which works through a dynamic balance between energy intake and expenditure [3].

AP efficacy was observed earlier under violation of thyroid function [5], thyroid-pituitary-ovarian function [6], the pituitary-adrenal axis [7], and AP influence on hypothalamic nucleus was shown [6, 8].

Being originated from China, the method was adapted to the specific conditions of a particular region and has become unique to each country. According to the World Health Organization (WHO), this branch of traditional Chinese medicine is now widely used throughout the world and is one of those kinds of medical practice, in which a considerable progress has been made. According to the reports submitted by 129 countries, 80 % of them now recognize the benefits of acupuncture, and in 30 % of countries which are the WHO members, there are training programs on so-called alternative, or complementary, medicine (including acupuncture) with graduates at sufficiently high professional level, including bachelor's, master's and doctorate [9].

Despite the apparent effectiveness, the theoretical foundations of classical oriental medicine for a long time remained rejected by western experts. Therefore, for AP a theory has been proposed based on morphological, physico-chemical and functional characteristics of the biologically active points of the body [10].

The studies have shown that the elements of the classical AP are quite tangible; AP points (APP) have a specific structure and are activated by appropriate stimuli, including magnetic and electromagnetic fields, having definite effect [3, 10, 11]. In experiments on animals G.V. Kazeev has shown the failure of the reflex mechanism of AP action in contrast to validity of ideas about energy and information system of the body [2, 4], in a certain sense approximated to the modern concepts of energy and signaling in biology.

Under pathology the APP is converted into the zone, and disorders in the organs and systems change biophysical, biochemical, histological parameters in the acupuncture zones [2, 12]. Living cells emit weak electromagnetic wave and photons generating endogenous field of the body, which affects the transmission of intracellular and intercellular signals and, therefore, the physiological functions [13-16]. Experiments with radioactive isotopes proved the presence of channels extending from the APP [17].

The clinical effect of AP is due to changes in biochemical processes and the synthesis of bioactive substances at local, organ-specific and system-specific levels in response to the APP activation. This leads to the normalization of the functioning cells, tissues, organs, systems and organism as a whole. Thus, the AP increases the concentrations of opioid peptides and monoamines in the blood [18-22], has a direct effect on gonadal paracrine and autocrine control of steroidogenesis through stimulation of production and release of adrenaline, catecholamines and growth factors [23, 24]. The role of nitric oxide, which mediates a response of the cardiovascular system to PPA activation, and involvement of hypothalamic nuclei in this process are established [25]. Efficiency of AP as immuno-stimulating, anti-depressant and analgesic factor is proved [26, 27]. More strong immunity response due to AP was reported in experimentally infected animals [28]. Interestingly, in this not only the number

of natural killer cells, but their activity increases [29]. There is experimental evidence of changes occurring at the cellular level when APP activated [30].

Acupuncture improves memory and learning ability in laboratory rats [31]. The authors explain the therapeutic effects by an increased B, Ca, Cu, Fe, K, Mg, Na, and P levels in animal brain tissues due to APP activation. A notable reduction of blood pressure was observed in animals due to significant decrease in the plasma renin activity after PA session [32]. An increase in liver and spleen blood flow was recorded when APP was subjected to current with a frequency of 2 Hz [33].

AP increases the tissue response to chemical substances, including medicines [34]. The method becomes popular in veterinary medicine under diseases of the musculoskeletal system, gastrointestinal tract, the reproductive system, due to sedative and an analgesic effect it is widely used under spinal injuries, intoxications, intervertebral hernia and dysplasia [35, 36]. The results obtained on dogs prove the favor of AP and its combination with pharmacological agents in the pathology of the nervous system, spine, feet and as an anesthetic [37-40]. The effect comparable to the influence of medication, was achieved in the normalization of the gastrointestinal tract in horses [1, 41]. There is evidence of the analgesic effect of electroacupuncture on sheep [19]. Bone regeneration in rats after prolonged APP activation was reported [42]. Efficiency of the method is described for the treatment of pigs [43, 44] and calf [45] with diarrhea. AP sessions (5 days, for 20 minutes daily) normalized liver function in dogs with hepatitis [46]. Positive results were obtained with rumen acidosis [47], left abomasum displacement, and normalization of the proventriculus function [48] in dairy cattle.

Currently, reproduction becomes one of the most pressing problems of biology. A special place takes the problem of high embryonic mortality due to a number of factors [49, 50]. For the modern world agriculture a marked decline in reproductive function at highly productive cattle is the most important (50, 51].

In medical practice, there are enough examples of AP successfully used in reproduction, particularly in extracorporal fertilization (IVF) to reduce fetal mortality [52-56]. Here are the following effects: increased uterine blood flow, improves metabolism and nutrition of the embryo and fetus [57, 58], reduction of uterine activity [59], the weakening of the total stress and mother's anxiety in the absence of negative effects of the procedure [60, 61]. There is evidence on the effectiveness of the procedures under infertility, induced by polycystosis, stress and immunological disorders [62, 63], and for induction of ovulation [64].

AP influences positively on implantation in animals, suggesting that acupuncture affects the activity of endometrium receptors promoting the secretion of leukemia inhibitory factor (LIF) and interleukins, necessary for successful implantation [65]. AP facilitates normalization of cortisol and prolactin levels, which, in turn, affects the quality of oocytes and embryo implantation [54]. Differences were shown in the activity of certain APPs under various pathologies of organs and systems [66-68]. Certain APPs are important to produce combined or opposite effect [30, 69]. For example, in dogs the electroencephalograms with certain APPs show a pronounced calming effect, which is amplified by the combined use of the AP and sedation [70].

Activation of APPs associated with reproductive sphere considerably changes the concentration of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol and progesterone in blood plasma [18, 19, 71-73] and enhances the pituitary response to gonadotropin-releasing hormone (GnRH) [71]. Activation of lumbar APP suppressed cyclooxygenase-2 in the endometrium and myometrium [59].

It is found that the effect arises only when the APPs are activated while a similar action on the adjacent areas of the body does not lead to significant changes [2, 17, 74-76]. Moreover, electropuncture of APPs remote from the stimulated organ led to more tangible results compared to the points locate next to it. It is confirmed, for example, that the APPs remote from the intestine stimulate peristalsis, while the next-located ones slow it down [77]. Data on stimulation and inhibition, contradictory at first glance, can be explained by the initial state of the organ so that high function will be decreased, while low function will be increased. This is the unique regulatory effect of AP [2, 4, 48]. For example, acupuncture of the point located between the nostrils results in 90-100 % revival in animals under apnea for 10-30 s, and in 40-50 % revival under cardiac arrest, when the procedure is performed within 5-10 minutes after stopping [78], whereas delicate prolonged activation of this point results in lasting calming effect [79].

AP-treatment in animals showed sufficient effectiveness at anestrus and multiple unsuccessful insemination in pigs and cows, for controlling the brooding instinct in hens, and also is a method complementary to conventional therapy [2, 36, 80-83]. When affecting AP points located in the thoracic, lumbar and caudal spine, the positive effect is reported of restoring potency in boars, and cervical dilation and coordination of uterine contractions during parturition [84]. The effectiveness of the method was mentioned at anestrus and infertility in mares, and at cryptorchidism and low potency of stallions [85].

It is known that in animal husbandry for normalization of reproduction, in particular for synchronizing the sexual cycle, a variety of hormones and their analogs are widely used throughout. However, the results do not always meet expectations, especially because of the poor management, high environmental temperatures and, most importantly, reproductive dysfunction. In such circumstances, the AP makes a tangible beneficial effect. For example, the combination of AP with the injection of 50 % glucose bilaterally between the transverse-rib processes of the 5^{th} and 6^{th} lumbar vertebrae significantly increased the proportion of pregnant cows (84, 86). A higher effect was observed by combining the AP with an injection of human chorionic gonadotropin, or prostaglandin with follicular or luteal cysts and persistent yellow bodies in cows, respectively [84]. The positive results were obtained under placenta detention, uterine atony, induction of labor and preventing abortions in cows, analgesia and stop bleeding, overcoming agalactia syndrome in sows, reducing the interval before the estrus after giving birth; In addition, there was an increase in weight gain in young animals [87].

APPs are the best place for administration of medicines. Moreover, their effective dose can be considerably reduced, at that possible side effects are absent [2, 88, 89]. The positive therapeutic effect was noted when administering vitamins, homeopathic medicines and electrolytes in APPs [90, 91].

We proposed a method of APPs activation in cows and heifers by means of microdoses of the medium conditioned by embryos in vitro. The injection of 0.2 ml of the medium in the APP of sacral spine makes it possible to increase the effectiveness of artificial insemination by 22 % and reduce mortality in transplanted embryos by 18 % [92-94].

About 150 APPs are known in veterinary practice, with one to 20 used per session [2, 95, 96]. In Russia a unique topographical atlas has been recently developed by G.V. Kazeev and AV Kazeeva for acupuncture points of home animals and formulation of APP application at various diseases. This makes possible the practical use of AP method by veterinary specialists, including industrial animal husbandry [2].

In addition to traditional acupuncture, there are electropuncture, laserpuncture, acupressure, cryopuncture; besides, ultrasound and other factors can be used for APP activation. Any of them are effective [2, 97].

Thus, the modern view on the ancient method of the eastern-based medicine, the acupuncture, and studying its mechanisms at physiological, biochemical and other levels allows to develop science-based approaches to the optimization of therapeutic techniques and to increase the effectiveness of preventive measures in the veterinary practice, using acupuncture along with recognized techniques of veterinary medicine.

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GENOME-WIDE ASSOCIATION STUDY FOR MILK PRODUCTION AND REPRODUCTION TRAITS IN RUSSIAN HOLSTEIN CATTLE POPULATION

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Abstract

Genome-wide association study (GWAS) has been proven as a powerful tool for identifying genomic variants associated with economically important traits in domestic animal species. Development of the methods for genomic evaluation opens the new opportunities in improvement of milk production and fertility traits of livestock. The objective of our study was to evaluate the whole-genome associations between single nucleotide polymorphisms (SNPs) and estimated breeding values (EBVs) for milk production and reproduction traits in Russian Holsteins. SNPs screening was performed in 195 progeny-tested and 61 young bulls using Illumina Bovine SNP50 v2 BeadChip. EBVs were calculated for milk production traits (305-d milk yield (MY), milk fat content (FC), milk protein content (PC), milk fat yield (FY) and milk protein yield (PY)) and reproduction performances (age at fist calving (CA), calving difficulty (CD), conception rate (CR), days open (DO), gestation length (GL) and interval between calving (CI)) using BLUP Sire Model approach. In total, 41370 SNPs were selected for the association analysis based on the quality control results. Direct genomic values (DGV) were calculated by GBLUP approach using genomic relationship matrix (G). Genomic EBVs (GEBVs) were calculated as combination of residual polygenic effects (EBV) with the DGV. To increase the probabilities of GWAS values we used the GEBV values for young bulls, whereas deregressed DGV values were used for progeny-tested bulls. The Bonferroni correction test for detection of significant associations and local false discovery rate (LocFdr) were used to check a type I errors in null-cases hypothesis. Heritability coefficient values for reproduction traits ranged from 0.035 for CR to 0.221 CA, whereas for milk production traits they were higher, i.e. from 0.250 for MY to 0.401 for PC. According to the Bonferroni and LocFdr tests, we have identified several high-significant SNPs, which were associated both with milk production and with fertility traits. Two SNPs with the most significant effect on MY were located on BTA17 (*ARS-BFGLNGS-50172*) and BTA13 (*Hapmap54246-rs29017970*). The association analysis for milk components revealed four SNPs at conservative regions, which were significantly associated with FC, i.e. *BTA-104917-no-rs* and *BTB-01604502* (58 Mb, BTA9), *ARS-BFGL-NGS-107379* and *ARS-BFGL-NGS-4939* (1.8-2.0 Mb, BTA14), and one SNP, which was significantly associated with PC — *Hapmap 43278-BTA-50082* (BTA20). Polymorphisms *ARS-BFGL-BAC-7205* (BTA1) and *Hapmap48395-BTA-58382* (BTA5) were associated with PY. Several SNPs were found to be associated with reproduction traits, i.e. *BTB-01622929* on BTA1 for CA, *ARS-BFGL-NGS-89711* on BTA27 for CR, *ARS-BFGL-NGS-117881* on BTA5 for DO, *BTA-31636-no-rs* on BTA1 and *Hapmap26774-BTA-163037* on BTA27 for CI. The significant effects of SNPs explained up to more than 9.0 % of additive genetic variances. Some of the referent mutations with most significant effect were located within or close to the genes TRAFD1, DGAT1, SLC16A7, DUSP26 and CCDC58. Thus, application of a genome-wide analysis allows with high accuracy to detect the QTL for medium and low heritable productive and reproductive traits in Russian Holsteins.

Keywords: genome-wide association analysis, heritability, milk production, fertility traits

Studying productive qualities in farm animals in traditional breeding pro-

grams is performed without identifying the impact of any functional genes. In the last quarter of the century, molecular genetic methods are developed at a faster rate — from identifying particular genes that control individual physiological processes to the quantitative trait loci (QTL) and single nucleotide polymorphism (SNP) which mark a set of productive qualities in animals [1-3]. Magnitude of the effect of a single gene responsible for the quantitative and qualitative traits in an animal or in blood groups has a low proportion of genetic variation that contributes to the phenotypic expression of the trait [4, 5]. Therefore, animal selection does not guarantee the precise inheritance of productive values in generations. Application of short tandem repeats (STR), the microsatellites, does not provide the desired QTL mapping effect as well, as even in the possible presence of siblings and half-siblings in animal groups, QTL and STR chromosome linkage analysis is performed in the interval of 20 cM or greater, which makes it difficult to determine the main mutations [6].

Genome-wide association study (GWAS) is a developed marker-assisted selection (MAS) method, when genetic markers used are distributed through the genome in such a way that all QTLs are in linkage disequilibrium (LD) for at least with one of them. MAS studies are focused on precise mapping of certain QTLs provided that the gene belonging to QTL may be identified. In turn, GWAS effectiveness depends on the number of SNPs. If SNPs are located too far apart on the chromosome, then a QTL can not be in sufficient linkage disequilibrium with at least one of the markers and, therefore, the locus would not be found. An increase in the density of SNP genome covering improves the probability of QTL determination and, largely, the mapping precision [7, 8].

Thus, significant associations with economically important traits have been found in cattle breeds using 10,000 SNPs, but according to M.E. Goddard [6], 300,000 SNPs are required for interbreed analysis in *Bos taurus*. The number of SNPs of about 50,000 may be quite enough for a single breed, such as Holstein, however the preference is given to the samples of several breeds and high density SNP panels for more precise mapping of QTL and improved reliability of genomic evaluations [9-11].

The number of animals required to study the associations depends on the degree of the effects which is reflected in the proportion of trait variation disclosed by point mutations. This parameter combines the allele frequency and the average difference between SNP genotypes for a quantitative trait. In practice, some SNPs constitute over 4 % of the genetic variance and above, which is a sufficient prediction value in a small sample size even for low inheritable traits [12-14].

GWAS provides an efficient search for relevant polymorphisms both among livestock breeds and within various populations of Holstein origin [15-17]. In addition, mapping of economically important traits is performed for the bull and breeding stocks, which is primarily associated with the search for reliable associations for milk fat and protein production [18, 19].

Conservative cattle genome regions with significant associations with milk production traits have been defined: casein cluster (*CSN*), diacylglycerol O-acyltransferase (*DGAT1*), growth hormone receptor (*GHR*) and others, as well as a number of not previously described population-wide mutations [20-22]. Studies of associations for low inheritable traits make it possible to improve breeding effectiveness by detection or elimination of genes responsible for the health and reproduction of animals, and to fix valuable genotypes in the population [23-27]. The research to find the loci responsible for qualitative animal traits is also of interest [28].

Is necessary to note that the assessment of each marker effects in the

prediction of genomic breeding value or in associative analysis is performed concurrently with other markers. Every mutation carries a certain proportion of the component of genetic variation, while it is assumed that, under the effects of a plurality of SNPs, the total effect should be low on the average. However, this fact does not prevent a clear picture of associations for the parameters studied [29-32].

The search and study of associations with economically important traits for point genetic mutations in Holstein dairy cattle population have not been performed in Russia earlier. Performing GWAS analysis, we combined the samples for the previously proven and young dairy bulls to confirm the effectiveness and the possibility of using models to predict genomic breeding values for dairy Holstein cattle breeding. These first results of genome-wide analysis will be the basis for the mapping of quantitative trait loci responsible for the formation of productive and reproductive qualities of animals.

The purpose of this study was to evaluate the whole-genome associations between single nucleotides and estimated breeding values for milk production and reproduction traits in Holstein bulls.

Technique. Collecting and analyzing information on the economically important traits of Holstein bull daughters was performed based on breeding databases in 77 farms in the Moscow region (primary databases were provided by the Regional Information and Breeding center Mospleminform). Using Bovine SNP50 v2 BeadChip (Illumina Inc., США) with a density of 54,609 SNPs, we genotyped 256 bulls owned by JSC Head Center for Reproduction of Farm Animals (Bykovo, Moscow Province) and OJSC Moskovskoe Breeding Farm (Noginsk, Moscow Province). The sample included 195 progeny-tested bulls (n = 47998) with an average count of 246 daughter first heifers per bull, and 61 young bulls valued by ancestry (Parent Average, PA). The following phenotypic traits were studied in bull daughters: 305-day milk yield (MY), milk fat content (FC), milk protein content (PC), milk fat yield (FY), and milk protein yield (PY), first calving age (CA), calving easy (CE), conception rate (CR), days open (DO), gestation length (GL), and calving interval (CI).

Bull estimated breeding value (EBV) was calculated using BLUP Sire Model approach, after which the resulting estimates were deregressed, i.e. these were adjusted for the impact of systematic environmental effects (drpEBV) [33].

The equation of mixed type models for estimating bull breeding value was implemented in the SAS IML statistical programming language package (SAS University Edition) [34]:

$$y_{ijk} = \mu + HYS_i + b_1A_k + b_2DO_k + sire_j + e_{ijk}, \quad (1)$$

where y_{ijk} is the k-th first heifer trait index; μ is population constant; HYS_i is fixed effect of the i-th «herd-year-season» calving; b_1 and b_2 are linear regression coefficients; A_k is first calving age of the k-th heifer; DO_k is days open of the k-th first heifer; $sire_j$ is randomized effect of the j-th bull with normal distribution with a mean of 0 and a variance of $A\sigma_a^2$, where A is additive relationship matrix; e_{ijk} is unaccounted factor effect.

Genotyping quality control and regression analysis for associations was performed using Plink v. 1.07 software [35]. The resulting number of SNPs was 41370. Previously, we have found that genomic enhanced breeding value (GEBV) was by 9-45 % greater compared to the estimation on the ancestors [36]. In this regard, GEBV values for both young and progeny-tested bulls were used in the analysis of the associations

Estimation of genomic relationship matrix (G) was performed according to the algorithm developed by P.M. VanRaden (37) in the R programming language environment [38]. The matrix consisted of elements — homozygous and

heterozygous loci estimations: $AA = 1$, $AB = 0$, $BB = -1$. Heritability coefficients, genetic and paratypic variances were obtained based on two-factor variance hierarchical complex. GEBVs were calculated as combination of SNP marker direct genomic value (DGV) and EBV (PA) according to the GBLOP approach using the following linear model [37]:

$$DRP = Xb + Zu + e, \quad (2)$$

where DRP is known drpEBV vector of pseudo phenotypic data located on diagonal matrix W, with the weighted effective daughter contribution coefficients: $EDC = k \times r^2/(1 - r^2)$ with the variation ratio $k = (4 - h^2)/h^2$ (here, r^2 is a significance of estimation for trait, h^2 is the value heritability); Xb is a matrix containing one common constant in the model; u is known prediction vector for DGV (G is a genomic relationship matrix), where Z is a single diagonal matrix.

The system of normal equations is as follows:

$$\begin{bmatrix} X'WX & X'WZ \\ Z'WX & Z'WX + kG^{-1} \end{bmatrix} \begin{bmatrix} b \\ u \end{bmatrix} = \begin{bmatrix} X'WDRP \\ Z'WDRP \end{bmatrix}. \quad (3)$$

To confirm the significant impact of SNP and identify significant regions in cattle genome, a number of tests were used to check for null-cases hypotheses, including the Bonferroni correction test (threshold value of $P < 1.2 \times 10^{-6}$, $^{0.05}/_{41370}$), the detection of local false discovery rate (LocFdr) (in B. Efron modification) which was estimated by maximum likelihood method implemented in the locfdr package, R language [39], and the Fdr criterion value according to the Y. Benjamini and Y. Hochberg approach [40] with an empirical estimate threshold value of $P < 0.05$.

To search for the genes closely associated with economic traits, the National Center for Biotechnology Information (NCBI) database was used. Functional gene identification was performed using the Discover EggNOG 4.1 database (http://eggnogdb.embl.de/#/app/home). Data visualization was conducted using the qqman package and R programming language [41].

Results. Heritability coefficient values (h^2) for milk production and reproduction traits indicated the possibility for various breeding in a dairy cattle population. So, to improve reproductive and fertility traits with low h^2 in the range from 0.035±0.005 to 0.068±0.007, a reduction of generation interval and(or) an increase in the intensity of animal selection are required. At the same time, it is possible to predict a higher breeding response based on milk production traits with moderate heritability (Table 1).

1. Heritability and variability parameters for milk production and reproduction traits in Holstein cattle population (Moscow region)

Parameter	$h^2 \pm m_h^2$	σ_P	Cv_P	σ_A	Cv_A	σ_U	Cv_U
305-day milk yield, kg	0.281±0.014	1317	21	697	11	986	16
Milk fat content, %	0.250±0.013	0.37	9	0.19	5	0.27	7
Milk fat, kg	0.328±0.015	57.8	23	33.1	13	39.6	16
Milk protein content, %	0.401±0.016	0.19	6	0.12	4	0.13	4
Milk protein, kg	0.370±0.016	43.8	22	26.6	13	29.6	15
First calving age, months	0.221±0.012	3.8	14	1.8	6	2.9	11
Calving easy, score	0.068±0.007	1.03	242	0.27	63	0.89	210
Conception rate, units	0.035±0.005	1.27	63	0.24	12	1.23	61
Days open, days	0.059±0.006	86.5	56	20.9	14	83.4	54
Gestation length, days	0.036±0.005	12.7	5	2.4	1	12.5	5
Calving interval, days	0.063±0.007	82.4	19	20.6	5	79.2	19

N o t e. h^2 — heritability coefficient, m_h^2 — heritability coefficient error, σ_P, σ_A and σ_U — estimations of phenotypic, genetic, and paratypic standard deviations, respectively; *Cv* — coefficient of variation, %.

Constancy of FC, PC, CA, GL, and CI traits was typical for the coefficients of genetic variability. Regardless of the nature of economically important qualities inheritance for FY, PY, DO, CR, and CE traits, an increase in the values of coefficient of variation was observed which was associated with a signifi-

cant proportion of the effects of paratypic factor.

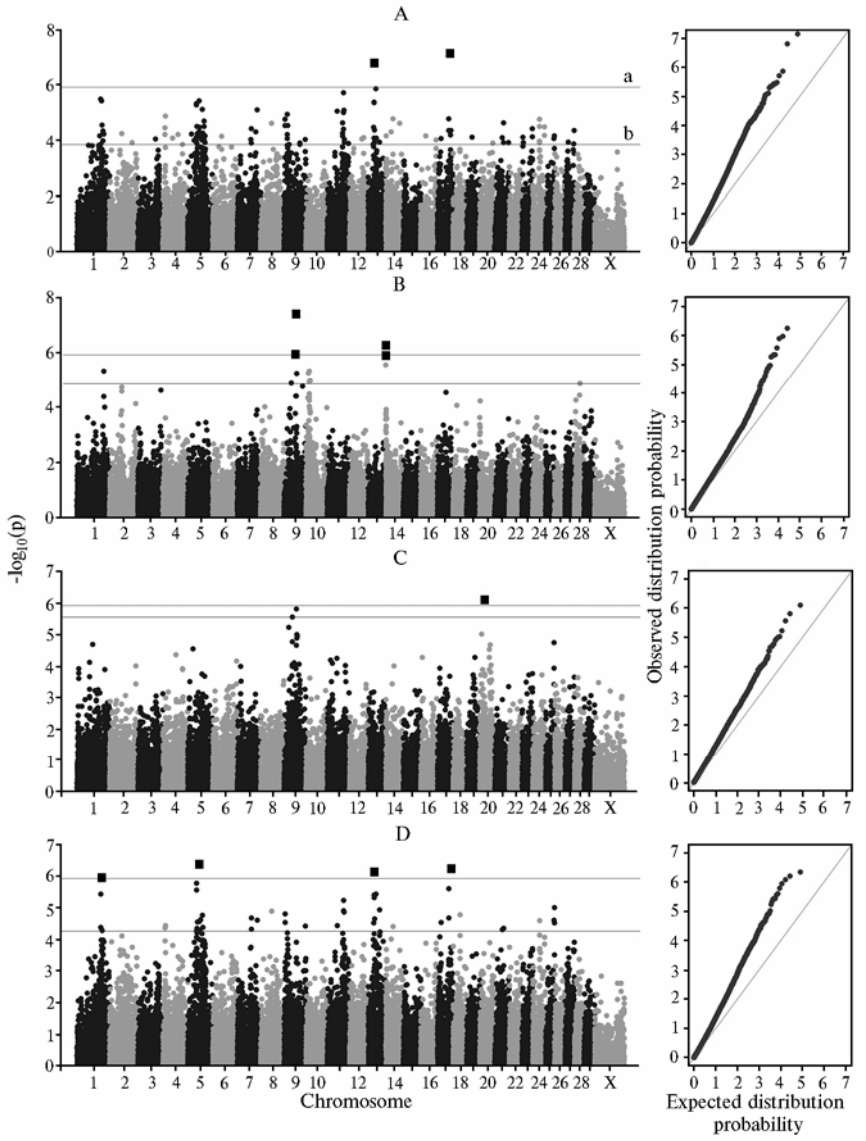

Fig. 1. GWAS results (genome-wide association study, on the left) **and relevant probabilities of estimation significance distribution** (q-q plot, on the right) **for milk yield** (A), **milk fat content** (B), **milk protein content** (C), **milk protein yield** (D) **in Holstein cattle population** (Moscow Province): a and b — thresholds according to Bonferroni and Fdr, respectively. Squares signify compliance in significance for the null hypothesis testing according to Bonferroni (highly significant results for associations).

According to the analysis of milk production traits, significant associations with EBV were defined in the bulls. According to Fdr criterion with a theshold value of $P < 0.05$, 124 SNPs were defined for MY, while LocFdr and Bonferroni tests identified only 2 SNPs of high significance value ($P < 1.2\times10^{-6}$) — *ARS-BFGL-NGS-50172* (*Bos taurus autosome*, BTA17) and *Hapmap54246-rs29017970* (BTA13) (Fig. 1). Conservative loci ($P = 1.2\times10^{-6}$-4.1×10^{-8}) *BTA-104917-no-rs* and *BTB-01604502* (58 Mb, BTA9), *ARS-BFGL-NGS-107379* and *ARS-BFGL-NGS-4939* (1.8-2.0 Mb, BTA14), *Hapmap 43278-BTA-50082* (BTA20) were revealed for FC and PC, with the maximum possible number of significant point mutations, according to Fdr criteria, ranging from 3 to 13.

No associations were identified for FY, while 48 SNPs mostly located

on the 5-th and 13-th autosomes were obtained for PY (Fdr, $P < 0.05$). However, the number of highly significant polymorphisms decreased to 4, according to Bonferroni and LocFdr criteria: *ARS-BFGL-BAC-7205* (119 Mb, BTA1), *Hapmap48395-BTA-58382* (54 Mb, BTA5), *Hapmap54246-rs29017970* (30 Mb, BTA13), and *ARS-BFGL-NGS-50172* (64 Mb, BTA17). For the last two SNPs, there were typical associations with milk yields per standard lactation, suggesting a close genetic relationship and the efficiency of breeding for milk protein.

2. Significant associations of single nucleotide polymorphisms (SPNs) **and estimated breeding values against point mutations and their genomic localization** (Holstein cattle population, Moscow Province)

SNP	BTA	Position	Allele	Allele frequency, $X \pm m$	Effect, $X \pm m$	P	R^2, %	Closest gene	Genetic distance, bp
305-day milk yield, kg									
ARS-BFGL-NGS-50172	17	64342186	*C*	0.275±0.020	–132.6±23.9	7.6×10^{-8}	10.9	*TRAFD1*	10778
Hapmap54246-rs29017970	13	30878341	*G*	0.126±0.015	+172.3±32.0	1.7×10^{-7}	10.4	*LOC104973750*	Within gene
Milk fat content, %									
BTA-104917-no-rs	9	58113021	*A*	0.304±0.020	+0.036±0.006	4.1×10^{-8}	11.3	*EPHA7*	279992
ARS-BFGL-NGS-107379	14	2054457	*G*	0.387±0.022	+0.030±0.006	5.9×10^{-7}	9.5	*LOC786966*	298
BTB-01604502	9	58145538	*A*	0.368±0.021	+0.033±0.007	1.1×10^{-6}	9.0	*EPHA7*	312509
ARS-BFGL-NGS-4939	14	1801116	*G*	0.347±0.021	+0.029±0.006	1.2×10^{-6}	8.9	*DGAT1*	Within gene
Milk protein content, %									
Hapmap43278-BTA-50082	20	25185940	*A*	0.053±0.010	+0.033±0.007	8.0×10^{-7}	9.3	*ARL15*	Within gene
Protein yield, kg									
Hapmap48395-BTA-58382	5	54120342	*G*	0.382±0.022	–3.7±0.7	4.6×10^{-7}	9.7	*SLC16A7*	Within gene
ARS-BFGL-NGS-50172	17	64342186	*PD*	0.275±0.020	–3.9±0.8	6.4×10^{-7}	9.5	*TRAFD1*	10778
Hapmap54246-rs29017970	13	30878341	*G*	0.127±0.015	+5.1±1.0	8.4×10^{-7}	9.3	*LOC104973750*	Within gene
ARS-BFGL-BAC-7205	1	119976661	*PD*	0.416±0.022	+3.5±0.7	1.2×10^{-6}	9.1	*HPS3*	Within gene
First calving age, months									
BTB-01622929	1	117345968	*A*	0.445±0.022	–0.49±0.09	3.7×10^{-7}	9.8	*LOC104970999*	56
Conception rate, units									
ARS-BFGL-NGS-89711	27	29231663	*G*	0.376±0.022	+0.041±0.008	2.1×10^{-7}	10.2	*DUSP26*	261502
Days open, days									
ARS-BFGL-NGS-117881	5	82631679	*A*	0.302±0.020	+2.1±0.4	1.7×10^{-7}	10.4	*PPFIBP1*	Within gene
Calving interval, days									
Hapmap26774-BTA-163037	27	42578430	*A*	0.136±0.015	+0.7±0.1	1.1×10^{-6}	9.0	*LOC101905415*	6930
BTA-31636-no-rs	1	67399528	*PD*	0.380±0.022	–0.5±0.1	1.2×10^{-6}	9.0	*CCDC58*	Within gene
Gestation length, days									
BTA-31636-no-rs	1	67399528	*C*	0.380±0.022	–0.4±0.1	1.3×10^{-6}	9.0	*CCDC58*	Within gene

Note. BTA — cattle chromosome, P — null-case hypothesis probability error, R^2 — coefficient of determination (proportion of marker effect). To identify genes, officially accepted abbreviations were used.

GWAS for reproductive qualities and fertility of bull daughters identified genomic regions with 9 (for GL) to 66 (for DO) SNPs localized, with the $P < 0.05$ value of the expected proportion of false deviation (Fig. 2). Using more powerful null hypothesis assessment criteria made it possible to precisely localizate the mutations for CA (*BTB-01622929*, 117 Mb, BTA1), CR (*ARS-BFGL-NGS-89711*, 29 Mb BTA27), DO (*ARS-BFGL-NGS-117881*, 82 Mb, BTA5) и CI (*BTA-31636-no-rs*, 67 Mb, BTA1; *Hapmap26774-BTA-163037*, 42 Mb, BTA27). A significant nucleotide substitution (*BTA-31636-no-rs*) was identified for GL trait, which was similar to the one found for CI. We could not de-

tect any significant associations for CE trait which, in our opinion, is due to a high impact of paratypic factors on the trait.

Significance of association estimations for economically important traits was close to normal distribution and had a slight deviation from the theoretically expected distribution, as shown in the quantile-quantile plots (q-q plot, Fig. 1, 2).

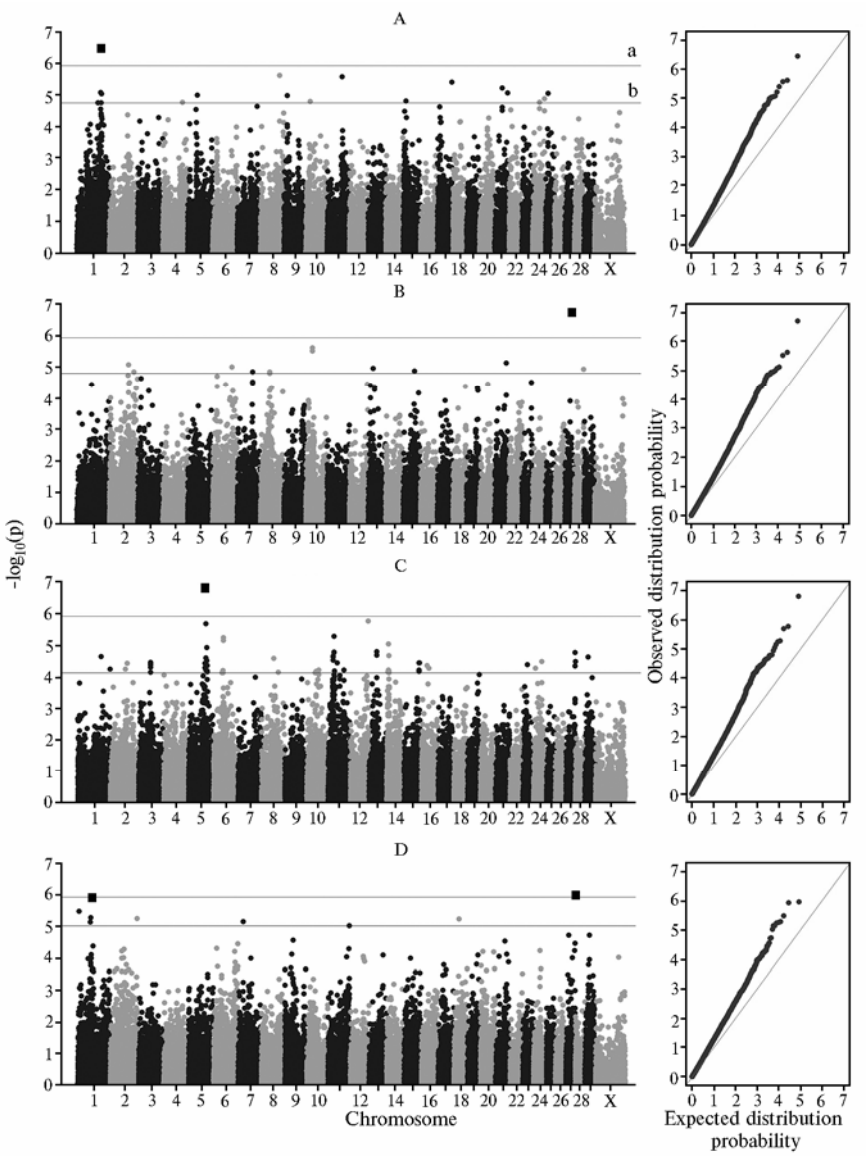

Fig. 2. GWAS results (genome-wide association study, on the left) **and relevant probabilities of estimation significance distribution** (q-q plot, on the right) **for first calving age** (A)**, conception rate** (B)**, days open** (C)**, and calving interval** (D) **in Holstein cattle population** (Moscow region)**:** a and b — thresholds according to Bonferroni and Fdr, respectively. Squares signify compliance in significance for the null hypothesis testing according to Bonferroni (highly significant results for associations).

Significant single nucleotide polymorphisms were identified both within individual genes and in the closest regions. The additive proportion of each marker effect ranged from 8.9 to 11.3 % of the total trait variability. The marker regression effect figures for the estimation of breeding values were both positive and negative, which indicates the possibility of their use for selecting the best animals and searching and eliminating deleterious mutations. Significance of as-

sociations ranged from 1.2×10^{-6} to 7.6×10^{-8}. The frequencies of minor alleles for loci were 0.053-0.445 (Table 2).

Analysis of SNPs associated with qualitative trait estimation in Holstein cattle population identified a number of genes involved in various biological processes (Table 2): *TRAF* is responsible for the immune response, bioregulation, and protein synthesis; *EPHA7* controls cell signaling mechanism and regulation of metabolic processes; *LOC786966* is associated with cytoskeleton and protein structures of epithelial cells; *DGAT1* controls lipid transport and their metabolism; *ARL15* is responsible for intracellular metabolism, secretion, and vesicular apparatus; *SLC16A7* affects metabolism and carbohydrate transport; *HPS3* is associated with cellular processes and pigmentation; *DUSP26* is responsible for protective mechanisms at the molecular level and for metabolism of phosphate containing compounds; *CCDC58* controls cell cycle, cell division, and the phase of meiosis associated with chromosome division. Three genes (*LOC786966*, *DGAT1*, *SLC16A7*) of the above ones were reference for Holstein cattle, as shown in similar studies performed by J.B. Cole [20] in the North American animal population. Generally, it is noteworthy that associations for most traits may be correlated, being a part of the overall relationship of biological processes in the animal body.

Thus, we have obtained data on the mapping of quantitative trait loci (milk production and reproduction) in Holstein cattle. Highly significant associations for 14 SNPs have been defined in chromosomes 1, 5, 9, 13, 14, 17, 20, and 27 with the additive effect of more than 9 % for a number of parameters. Genes *TRAFD1*, *LOC786966*, *DGAT1*, *SLC16A7*, *DUSP26*, and *CCDC58* were most conservative in the genome for reference mutations. Genome-wide studies performed for the traits with different heritability make it possible to quite precisely define the associations for genetic mutations. Using the null hypothesis assessment criterion for the expected proportion of false deviations in accordance with the maximum likelihood function allows to expand detection by SNPs. Bonferroni test has a higher capacity of statistical hypothesis testing.

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AGROBIOLOGICAL BASES OF VETCH (*Vicia sativa* L.) CULTIVATION FOR SEEDS IN THE CENTRAL RUSSIA USING HETEROGENEOUS AGROCENOSES

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Abstract

Common vetch (*Vicia sativa* L.) is widely cultivated in the Russian regions with different climate and edaphic conditions, whereupon a significant plant polymorphism and specific adaptation to environment and cultivation technologies are observed. Recently, a new breeding approach is being developed to created vetch varieties for grain forage use. Since 2002, a new such variety, the Lugovskaya 98, is recorded in the State Register of Selection Achievements of the Russian Federation. This variety differs significantly from those cultivated for green fodder. Particularly, there is no hydrocyanic acid which is the main anti-nutrienent, and the level of trypsin inhibitors is reduced allowing the seeds of Lugovskaya 98 vetch to be used as protein source in animal feeds with no extra processing. In vetch pure stands a significant lodging occurs due to peculiar architectonics and biological peculiarities of plant growth under temperate climate. This necessitates vetch cultivation in the mixes with support crops. A complementary support crop and its stand density must be selected specifically in accordance with local soil and climatic conditions. We compared vetch Lugovskaya 98 seed yield formation in binary agrocenoses with different crops under stand density gradients and found those mostly complementary to vetch with regard to seed yield and quality. Plant allelopathic interactions were shown to begin during early ontogenesis. At high sowing rate of oats (*Avena sativa* L.) the vetch plant death increased up to 7-9 % compared to 3-4 % in the vetch pure stands. Vetch young growth was also 6 to 7 % suppressed in the mixes with white mustard (*Sinapis alba* L.). In contrary, *Phacelia tanacetifolia* had no effect on vetch young growth and juvenile plants. Generally, the vetch generative development was the best in the mixes with oat and mustard plants when the sowing rates of the support crops were low (i.e., not more than 1.00-2.00 and 1.50-2.25 million seeds per hectare, respectively). In these cenoses the bean number averaged 8.8-9.1 per plant, or 714-758 per square meter. As sowing rates of a support crop increased, the bean number reduced by 7-12 %. The highest vetch seed biological yield (283.6 g per square meter) was discovered in the mixes with the white mustard at sowing rate of 1.50 million seeds per hectare, and rather high yield of 260.5-267.5 g per square meter was recorded with phacelia and oat plants when their sowing rates were the lowest. Completeness of harvesting and seed quality in vetch are known to depend significantly on lodging. Before harvesting, in vetch pure stands the lodging was 74 %, and in mixes with oat, mustard and phacelia plants it was 22-42 % (depending on the cereal sowing rates), 37-45 %, and 54-56 %, respectively. Actual vetch seed yield was the highest (1.51-1.57 ton per hectare) when the white mustard or oat plants were used in mixes at sowing rates of 1.50 and 3.00 million seed per hectare, respectively. Under excess rainfall during vegetation vetch/oat ensured a complete harvesting vetch seeds due to less lodging, and at low rainfalls the vetch/mustard mixes were more convenient. According to vetch seed germination of 93-96 % and vigor of 77-83 %, the vetch/oat and vetch/mustard mixes ensured the highest seed quality. In lodged pure vetch these parameters were reliably lower (85 % and 65 %, respectively).

Keywords: common vetch (*Vicia sativa* L.), mixed stands, supporting crops, sowing rate, yield, seeds, seed quality

Common vetch (spring cultivar) (*Vicia sativa* L.) has a long, thin, easily lodging stem. Architectonic specificity and biological features of common vetch necessitates vetch cultivation in the mixes with support crops. Selection of complementary support crops and their proportions in vetch stands are defined by competitiveness of species and varieties, soil and climatic conditions, and herb-

age purposes. Abroad, common vetch is cultivated with grain cereals. Mixes with oat (*Avena sativa* L.) are most common [1-6]. In this, the mixing ratio varies considerably — from 25 % (oat) + 75 % (vetch) [6] to 75 % (oat) + 25 % (vetch) [4]. There is a tendency of the vetch proportion in the mix to be the greater the drier the climate is. Vetch cultivation in the mixes with (*Hordeum vulgare* L.), wheat (*Triticum aestuvum* L.), triticale (*Triticosecale* Wittm. ex A. Camus) is practiced as well [1, 7-9].

In Russia, regional methods have been developed for vetch cultivation for seeds in the mixes with various crops — spring cereals (oat, barley, triticale, wheat); cabbage crops (white mustard *Sinapis alba* L., rapeseed *Brassica napus* var. *napus*); legumes (broad bean *Vicia faba* L., narrowleaf lupin *Lupinus angustifolius* L., pea *Pisum sativum* L.) [10-12]. In commercial crops common vetch is mainly cultivated in oat mixes (the recommended seeding rate for pure live seeds for the Central Black Earth Region is 3.7-4.5 million pcs/ha) [12]. The advantage of oat as support crop against other cereals is, in particular, due to the fact that the use of sorting machines to separate vetch, barley, and wheat seeds is difficult because of their close biometric parameters [13].

The main objective of using mixed agrophytocenoses of vetch cultivated for seeds with support crops is crop regulation and formation of herbage suitable for high-quality mechanical harvesting, which requires learning the basics of such cenoses functioning. In turn, the state of mixed cenoses is defined by the quantitative ratio of their components based on agro-biological and agrocenose properties, as well as technological and varietal characteristics of the crops used.

Due to the wide distribution and cultivation in all 12 agricultural regions of Russia, that results in heterogeneous genotypes, common vetch varieties are considerably polymorphic in traits with individual responses to biotic and abiotic factors, including machinery, and unequal plasticity and homeostasis in the years of different (including extreme) weather conditions [10, 14-17]. There is evidence that vetch genotypes are adapted to place of origin and differ in the yields, with the realization of their potential depending on the growing conditions [18-22]. The reason is that, due to directional selection for a particular phenotype, the genotypic variability of cultivated species is narrowed and adapted to the breeding conditions [23].

Along with traditional vetch breeding for green fodder, specialized varieties for grain forage with high yield, increased protein levels and the absence or low levels of anti-nutrients in grain is currently under development in Russia [24]. Thus, since 2002, variety Lugovskaya 98 (originated from All-Russian Research Institute of Forages) was first registered as a grain forage variety. In this variety, the grain contains no hydrocyanic acid, and the trypsin inhibitory activity is 24-40 mg per 100 g dry matter only, being several times lower compared to MPC. The protein level in seeds is 31.8 %, protein biological value is 59.4 % indicating good quality [24, 25]. The variety Lugovskaya 98 was developed by hybridization and has specific morphological and biological features and economically important traits which distinguish it from a green forage variety. In particular, the plant architectonics has been changed to generative development in the upper part of the stem with converged fertile nodes, also the intensive growth and drought resistance until bloom, enhanced competitiveness in mixed stands, larger seeds of 80 g or more in weight per 1000 seeds are characteristic [13, 24]. All the above requires variety specific cultivation.

We first studied the biological peculiarities of plant development and herbage formation in grain forage vetch Lugovskaya 98 grown in binary cenoses at different abundance of support components. The most complementary support crops and the optimum proportion of the basic and support crops for the highest

seed yield were estimated under the Central Black Earth Russia. A certain support crop was evaluated using argophytocenoses approach based on the study of plant competitiveness in heterogeneous agrophytocenosis and estimation of the support crop edificator ability by quantitating parameters of both components with special attention to yielding and yield structure of the legume component.

The purpose of this study was to determine the effect of support crop sowing density and the vetch grain forage variety on plant growth, yield, and seed quality in the main binary agrocenosis crop.

Technique. The field experiments have been conducted in 2006-2012 (All-Russian Research Institute of Forages) with oat variety Skakun, white mustard variety Lugovskaya, and lacy facelia (*Phacelia tanacetifolia* Benth.) variety Ryazanskaya used as support crops for common vetch variety Lugovskaya 98 at various seeding rates. Vetch was grown according to the recommendation for mixed crop in the Central region, at seeding rate of 1.3 million viable seeds per 1 ha [12]. Conventional row seeding was used for all crops studied. Vetch seeds were pre-inoculated with nodule bacteria *Rhizobium leguminosarum* bv. *viceae*, strain 145 (rizotorfin formulation).

Seed yields were assessed after threshing the entire plot area using Sampo 130 (Sampo Rosenlew Ltd., Finland) (2006-2009) and Classik (Wintersteiger AG, Austria) harvesters (2010 and 2012). Records and monitoring were carried out according to the accepted Guidelines for the Conduct of Field Studies with Forage Crops (Moscow, 1997). Crop phenology, sprouting of seedlings, plant growth dynamics, stand height, and crop lodging were studied, and the values of individual crop structure components, biological yield and sowing quality of harvested seed were estimated. An experimental plot area was 25 m^2. Experiments were conducted in four replications at a andomized crop location.

Data statistical processing was performed by variance analysis [26].

Results. Compatibility of vetch varieties with different grain cereals varies. Compared to wheat and barley, oat was a stronger vetch competitor [13]. In mixed crops, coexisting species share the habitat with a specific ecological capacity. As a result of allelopathic effects of biologically active substances excreted by viable seeds since the early ontogeny, oat may inhibit sprouting some vetch varieties [13]. Different vetch tolerance is manifested in the change of biometric parameters of seedlings, juvenile plants and, ultimately, in crop yielding. To diminish the negative impact on the vetch plants, it is recommended to reduce the oat seeding rate in proportion to the decrease of sprouting growth in a legume [13].

A comparison of the copmleteness of sprouting showed that vetch stand density decreased from 86 to 78 pcs/m^2 as the oat seed rate increased from 1 to 4 million pcs/ha (Table). In this, considerable crop deterioration and elimination of vetch plants, with sprout death increased up to 7-9 % compared to 3-4 % in the control, was observed with oat seeding rates of 3.00 and 4.00 million/ha, that is, at a higher cereal crop sowing density.

Compared to vetch, white mustard seeds germinated faster impacting negatively the legume sprouting. Thus, in the years of adequate soil moisture in post-seeding period, i.e., 120 % of normal rainfall in May decade II (2007), 141 % monthly rainfall in May (2008), and 166 and 121 % rainfall in May decades II and III, respectively (2009), the mustard plants entered the full seedling phase 3-4 days earlier than vetch plan ts. As a result of advanced mustard development, vetch sprouting reduced to 6-7 % (sample area 5 m^2) compared to pure stands (see Table).

Phacelia, with its longer sprouting period and decelerated growth in the first month as compared to mustard plants, had no negative impact on vetch stand density.

Yield structure and seed yielding in common vetch (*Vicia sativa* L.) variety Lugovskaya 98 depending on the support crop (Moscow Province, field experiment, mean values for the years of 2006-2010 and 2012)

Crop, viable seed seeding rate, million pcs/ha	1	Beans		4	5	Seed yield			9	10
		2	3			6	actual, tons/ha			
							7	8		
Pure stand										
Vetch	69	786	76	6.5	74	281.0		0.70	74.74	85
Vetch mixed with support crop										
Phacelia (*Phacelia tanacetifolia* Benth.)										
2.10	69	747	82	6.4	54	260.5	0.13	1.16	74.10	88
3.10	67	667	84	6.3	54	245.8	0.20	1.21	73.32	88
4.10	68	684	85	6.2	56	219.9	0.22	1.02	73.11	90
Mustard (*Sinapis alba* L.)										
1.50	63	744	84	6.5	37	283.6	0.25	1.51	78.56	94
2.25	62	714	86	6.4	39	252.5	0.28	1.37	77.85	94
3.00	63	694	86	6.2	45	230.9	0.35	1.20	78.42	96
Oat (*Avena sativa* L.)										
1.00	66	758	83	6.6	42	267.5	0.65	1.25	76.55	93
2.00	65	744	84	6.5	36	240.2	0.94	1.42	74.95	95
3.00	62	708	86	6.4	27	221.3	1.32	1.57	73.62	94
4.00	60	670	86	6.4	22	206.4	1.60	1.40	73.58	94
LSD_{05}	–	60.20	–	0.30	–	23.72	–	0.11	2.77	3.4

Note. 1 — sprouting, %; 2 — total per m^2; 3 — mature, %; 4 — seeds per bean, 5 — lodging by harvesting, %; 6 — biological, g/m^2, 7 — support crop; 8 — vetch, 9 — weight of 1000 seeds, g; 10 — seed germination, %. The dashes indicate that statistical processing was not performed because of the large sample size.

Heterogeneous sowing which included different crops provided a much more complicated phytocenotic system in agrocenose hierarchy compared to pure stand crop cenoses. Vetch stand yields and structure in the mixes with support crops are determined by their growth biological characteristics and dependent on the specific soil and climatic, agronomic and phytocenotic factors, among which sowing densities of support crops and their ratio to that of the legume component are the most significant ones.

Quantitative parameters of vetch seed herbage elements in mixed crops varied in different ways depending on the abundance of the support crop. Thus, the bean number changed substantially (from 667 to 758 pcs/m^2, or by 14 %), whereas their seed yields changed to a lesser extent (6.2 to 6.5 seeds, or within 5 %) (Table). At the same time, generative development in the legume component was greatly influenced by support crop species and proportion [4, 13].

In general, the mixed stands with mustard and oat plants at their reduced sowing rates (1.00-2.00 and 1.50-2.25 million/ha, respectively) were the most favorable to develop generative organs in vetxh. This was manifested in a larger number of beans per plant (8.8-9.1 pcs.) and total bean number per unit area (714-758 pcs/m^2). As seeding rates of a support crop increased, the vetch bean number reduced by 7-12 % (see Table).

Completeness of harvesting and seed quality in common vetch are known to depend significantly on lodging: potential crop yield losses can reach 50 % or more with greater lodging [30]. According to our data, the timing and extent of mixed stand lodging varied considerably depending on the plant species, seeding rate, and weather. So, the growth of vetch variety Lugovskaya 98 was relatively slow from the phase of full shoots to the beginning of budding, with an average daily growth of 1.2-1.5 cm. Then, entering the reproduction period, the growth was accelerated to 2.5 cm/day or more, until the green bean formation. High crop lodging is most likely in the same period. In particular, lodging in vetch pure stands before harvesting was 74 % (see Table). At the same time, the beginning of the lodging was observed as early as the branching occurred. As a result, despite high biological yield in pure crops (281 g/m^2), vetch seed harvesting completeness was minimal and amounted just to 25 %.

Linear daily growth gain in oat plants in the first 5 weeks averaged about 1.7 cm. And in the first two decades after the full shoot phase, oat was superior to vetch in its height by 50-76 %, and then in the flowering phase, vetch was 12 % superior to the support crop. In oat paniculation phase, both components were aligned in height, but later the growth in vetch was superior to oat in case of enough soil moisture. The most intensive growth of vetch plants was observed in 2008, when rainfall was over 142 % of norm during the growing season, including 110-138 % in June and July. Active vetch growth was observed in 2009 under the 2.1-fold rainfall excess in July decade I compared with the average annual value.

The mixed stand lodging decrease with an increase of cereal crop proportion in vetch/oat mixes was found as a general regularity (see Table). In vetch/oat stands, the lodging was 22-42 % depending on the abundance of bluegrass support component, i.e. it differed by 2 times. Stem type lodging prevailed in the seasons of typical weather conditions, and additional root lodging was observed under excessive moisture.

The growth of white mustard plants was decelerated to 0.8 cm/day, being 38-39 % less than in vetch, in the decade I. Starting from pentad III and throughout the following month, mustard plants had the highest growth of about 2.7 cm/day, and by the vetch budding the supporting plants were superior to vetch in stem height by 34 %.

Crop resistance to stem lodging is determined by the development of intercalary meristem, mechanical tissue layer and vascular bundles in the parenchyma, and by the thickness of sclerenchyma ring, which affects the diameter of internodes and the thickness of stem walls [27]. Lodging depends on the morphological and anatomical parameters of support crop stem corresponding to the load the stem is exposed to. Mustard stem stiffness was 26.9 kg/cm^2, with bending strength of 26.3 kg/cm^2 and fracture resistance of 1.45 kg/cm^2, and in oat plants the values were 16.3; 20.3, and 1.0 kg/cm^2, respectively [28]. In vetch-mustard mixed stands lodging was 37-45 % increasing with the increased cabbage component proportion. A disadvantage of vetch/mustard mixes was their high lodging at excessive moisture due to mustard bolting and thin stem formation. Under these conditions, bolting and reflorescence were observed in vetch, too. This was most pronounced in 2008, when due to an increased rainfall in June and July (110-138 % of norm), crop mix lodging reached 68-73 %. Higher lodging (42-52 %) was observed in 2006 with secondary vetch growth in the maturity phase due to excessive rainfall in August at 75.3 % excess compared to the average long-term value.

Compared to other species studied, in phacelia the growth was the slowest in the first 2-3 weeks with an increase in plant length of 1.1 cm/day, being by 58-70 % behind vetch in the first post-sprouting decade. Then, phacelia growth accelerated (first up to 1.8 cm/day, then to 2.6 cm/day), and by the beginning of vetch budding, the components of the mix were aligned in the plant length. Phacelia has upright ribbed stems up to 80-100 cm, and, therefore, seems to be a promising support crop for vetch. However, compared with mustard and oat, phacelia/vitch mixes were most affected lodging which, under temperate climate with enough and excessive moisture, reached 54-56 % with more pronounced root type.

In the mixed crop, plants interact physiologically and biochemically due to root excretions. As a result of *Rhizobium leguminosarum* bv. *viceae* nodule bacteria activity on the root system, common vetch contributes to soil enrichment with biological nitrogen up to 74-109 kg/ha, thus increasing soil fertility [29]. An important feature of annual legumes is their active symbiotrophic nitro-

gen nutrition which is 1.7-2.5 times higher under mixed cultivation with cereals. The reason is that a cereal crop which absorbs the available soil nitrogen intensely stimulates nodule bacteria activity. Moreover, the cereal component absorbs part of nitrogen fixed by rhizobia, which is indirectly indicated by an increased nitrogen levels in seeds and straw in mixed crop [30]. Vetch inoculation with specific symbiotic strains enhances symbiotic nitrogen fixation, thus considerably increasing the yield and its quality [31]. Here, vetch varieties are genotypically differentiated for their responses to inoculation. Lugovskaya 98 variety is characterized by wide amplitudes and high efficiency of complementary symbiotic interaction with natural and selected root nodule bacteria strains [32, 33].

The effectiveness of bluegrass interaction with legumes in mixed stands depended on its abundance. Thus, vetch had a favorable effect on oat growth in a mixed stand at a ratio of 1:1-1:2 only. In such stands, compared to pure cereal crops, an increase in oat plant height of 9 %, in the number of leaves of 15-17 %, in the number of spikelets per inflorescence of 10-13 %, and in the average absolute dry mass per plant of 18-20 % were observed [34]. With the vetch/oat abundance ratio to 1:3-1:4, the positive impact of legumes on morphological and biological traits of cereals was negligible and within the experimental error, compared to those in pure cereal crops [34]. Consequently and as a result of the increasing intraspecific competition in denser crops, oat reproduction rate in the mixed stand was 20 and 15 % at seeding rates of 1.00 and 2.00 million pcs/ha, respectively, even at higher lodging (42 and 36 %, respectively), and 14 and 12 % only when the seeding rates were 3.00 and 4.00 million pcs/ha with 27 and 22 % lodging (see Table).

The actual and biological grain yields are the most objective criteria for evaluating biological effectiveness of mixed crops and the reflection of the aggressiveness of competitive component interference [35]. A comparison of these figures in two-component agrocenoses demonstrated the highest vetch seed biological yield (283.6 g/m^2) in the mixes with white mustard plants at the seeding rate of 1.50 million pcs/ha, and with phacelia and oat plants (260.5-267.5 g/m^2) when the support crop seeding rates were the lowest (see Table).

The main purpose of creating mixed agrophytocenoses is managing the crop quality and yield, and technological suitability for mechanical harvesting. As a result of support crop seeding rate and lodging balance, actual vetch seed yield was the highest (1.51-1.57 ton/ha) when mustard or oat plants were used in mixes at seeding rates of 1.50 and 3.00 million pcs./ha, respectively. Compared to mustard, more intensive transpiration is characteristic of oat plants. Mustard is less resistant to lodging at excessive moisture. The efficiency of support crops varied depending on the moisture during the growing seasons and the associated crop lodging: in 2006 with 175 % of normal rainfall in August, and in 2008 with 130 % rainfall in June and July, vetch/oat mix was by 22-51 % superior to vetch/mustard mix for actual yields. In 2007 and 2009, at low, 72 and 91 %, rainfalls from June to August, respectively, and elevated temperatures, the vetch/oat mix yield was, on the contrary, by 18-50 % inferior to the vetch/mustard mix.

Seed sowing qualities are integrated indicators which reflect the peculiarities of physiological and biochemical processes of seed formation depending on the fluctuations of exogenous and endogenous imperative factors. Cultivation and harvesting technology has a great influence on vetch seed sowing qualities [36, 37]. When threshing at full maturity of 82-86 % of beans, the most mature seeds with a significant excess in the weight of 1000 seeds (by 3.08-3.82 g at LSD_{05} of 2.77 g) compared to control were obtained in vetch/mustard mixes (see Table). As to seed germination (93-96 %), the highest vetch seed quality conformed the state standards for the original and elite seeds (GOST 11230-95),

was provided in heterogeneous agrocenoses with mustard and oat plants (see Table). In this, seed vigor was 77-83 %. At the same time, lodged pure vetch seeds, though met GOST conditioning requirements, had significantly lower germination (85 % at LSD_{05} of 3.4) and vigor (65 % at LSD_{05} of 5.0).

Thus, common vetch variety Lugovskaya 98 is advisable to be sown for seeds in the Central Black Earth Region of Russia at seeding rate of 1.30 million pcs/ha in mixes with support crops differentially selected for the species and seeding rates. The use of white mustard support crop at seeding rate of 1.50 million live seeds per ha is more efficient in the areas with less rainfall and in upland areas. Vetch/oat crops at a bluegrass seeding rate of 3.00 million pcs/ha should be used in the lower areas and at excessive moisture.

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ALFP-FINGERPRINTING FOR CERTIFICATION OF *Aspergillus niger* L-4, THE CITRIC ACID COMMERCIAL PRODUCER

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Abstract

Citric acid plays an important role in cellular respiration, and participates in oxidation processes as a natural antioxidant and synergist antioxidant, inhibiting plant oxidoreductase, together with ascorbic acid. Citric acid is effective against east and bacterial pathogens, and can be used in ensilaging. Citric acid is an antimicrobial agent alternative to fodder antibiotics prohibited in European countries. It is metabolized in plants, animals and humans with no adverse effect. *Aspergillus niger* is the citric acid common producer. So far as cultural and morphological features are not enough to confirm strain authenticity essential for effective commercial product manufacturing, we used genetic certification of the strain. Note, in 2015 in wide range genetic study of *Aspergillus flavus* diversity the specific ALFP patterns were found though their practical aspects were not discussed (D. Singh et al., 2015). Herein, we studies osmophilic commercial strain *Aspergillus niger* L-4, the citric acid producer derived due to chemical and UV mutagenesis and spontaneous mutations, using optimized AFLP-fingerpinting with 12 different primers. Specific AFLP patterns were obtained for *Aspergillus niger* L-4 authentification. Mse_cc GATGAGTCCTGAGTAACC and Eco_ac (FAM) GACTGCGTACCAATTAC primers were shown to be optimal providing maximum number of DNA fragments in the range of 33.68 to 593.78 bp (89 fragments) subject to the described method of fingerprinting and computer processing. The primers are effective regardless of sample volume. The resulting profiles can be used to authenticate the strain *Aspergillus niger* L-4 from different sources.

Keywords: *Aspergillus niger*, citric acid producer, genetic profiling, AFLP-fingerprinting

Citric acid plays the important role in biochemical reactions of cellular respiration, the biological significance of which is most weighty compared to other biochemical processes in cells. It is involved in oxidation as natural antioxidant and a synergist of antioxidants, inhibiting, along with ascorbic acid, oxidoreducteses in plant tissue [1, 2]. Citric acid binds Fe^{+3} ions in colorless potato tubers improving taste. In the course of long-term storage of tuber crops at low temperature (–4 ºC and –8 ºC) malic, fumaric and tartaric acids levels decrease where as citric acids increases [3]. At positive temperatures the antioxidative activity in plant tissues is not enough to slow down oxidation, so this necessitates using preparations with antioxidant activity. Citric acids is effective in control of contamination with east and bacterial pathogens in food products [4, 5]. Due to acidic conditions citric acid provides binding bivalent cations of bacterial oxidoreductases to chelates [5], it can be easy applied and removed from seeds, and is used to treat seeds for neutralization of fungal and bacterial pathogens for safe long-term storage [6). So citric acid is considered effectively alternative to antimicrobial preparations with other acting mechanism, particularly, feed antibiotics prohibited in Europe. Products of citric acid metabolism in the body have no adverse effect.

Since citric acid synthesis occurs in each living things, it seems biotechnologically reasonable to consider tricarbonic acid cycle (or Krebs cycle, TCC)

as universal source of citric acid. In microorganisms biochemical reactions are highly labile. Naturally microbes do not produce an excess of metabolites, though it may be due to changed parameters of incubation, or a proportion of gene induction and repression. At that, a resulting shift in the character of biochemical pathways, including TCC, occurs.

Micromycete *Aspergillus niger* is a common producer of citric acid, and its authenticity must be under control to provide stable production parameters. For commercial use the high producing strains of *A. niger* are derived which meets normative technological indicators [7]. However, these are not enough to ascertain the strain authenticity. A set of cultural and morphological traits is not sufficient to provide a complete assessment of individual strain within a biological species. Genetic uniformity is characteristic of commercial aspergillus strains producing citric acid, so that necessitate producing periodically pure cultures (elite lines) from a typical conidia or colony [7]. Thus, a genetic authenticity becomes of special importance. AFLP (amplified fragments length polymorphism) fingerprinting is a common technique in molecular genetics of microorganism. Using AFLP-fingerprinting, the differences have been successfully found between close related strains in different taxonomic groups including rhizobia [8-12], lactobacteria [13-16], and myceliar fungi *Trichoderma* and *Aspergillus* [17-20]. AFLP-fingerprinting is high reproducible, so it may be considered promising to authenticate microorganisms [21]. Each strain produces a unique pattern of amplified DNA fragment (AFLP-pattern), which is suitable for computerized processing to compare with other strain and can be used as «genetic passport». Earlier we have optimized the AFLP-fingerprinting protocol for strains of agricultural microorganisms [22].

Cultural and morphological features of *A. niger* strains have been shown not enough to identify individual specific traits [7]. RAPD-PCR (random amplification of polymorphic DNA) with universal primers AS15inv, L 15/AS19 and AA2c producing specific sets of genome DNA amplicons, was more effective for genotyping selected strains in this species [7].

Herein, the AFLP-patterns for osmophilic commercial strain *Aspergillus niger* L-4, the commercial producer of citric acids, are first reported. Specific primers, Mse_cc GATGAGTCCTGAGTAACC and Eco_ac (FAM) GACTGCGTACCAATTAC, are optimized to produce 89 fragments ranged from 33.68 to 593.78 bp in size.

Our aim was a genome AFLP-fingerprinting of commercial citric acid producer *Aspergillus niger* L-4 using 12 combination of primers to obtain unique «genetic passport» for strain authentication.

Technique. Conidia of osmophilic commercial strain *Aspergillus niger* L-4 (collection of All-Russian Research Institute for Food Additives) were the biological material.

For DNA isolation conidia were thoroughly homogenized in mortar with pestle, then frozen, thawed and homogenized again (the procedure was repeted 4 times). After an aliquot 500 μl 2× CTAB buffer (2 % CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) was added, it was placed in 1.5 ml eppendorf and incubated 60 min at 65 °C using vortex for periodical shaking. For extraction repeated twice the chloroform:isoamyl alcohol (24:1, v/v) was used. DNA was precipitated with an equal volume of isopropanol (5 min at room temperature), centrifuged (2 min, 14000 min^{-1}), the supernatant was air dried. Then the precipitate was dissolved in 200 μl 70 % ethanol, centrifuged 7 s, and alcohol was removed. Air dried DNA precipitate was suspended in 50 μl H_2O(MQ). Isolated DNA was stored at –20 °C.

DNA purification was performed by 1 % agarose gel electrophoresis in

a Sub Cell GT (Bio-Rad, USA) chamber for 60 min at 100 V. λ/HindIII Marker (0.5 μg/μl, Fermentas, USA; 1 μl) and DNA Gel Loading Dye (Thermo Scientific, USA; 5 μl) was added to the probes. DNA-containing slice was cut out of the gel and placed into eppendorf. Three volumes (to gel slice volume) of agarose solvent (3 M GITS, 20 mM EDTA, 10 mM Tris-HCl, pH 6.8; Triton X-100 to 40 mg/ml) was added to final volume of 500 μl). Tubes were incubated in thermostat at 65 °C for 5 min, stirring occasionally. To the dissolved gel slice the Silica reagent (Fermentas, USA; 40 μl) was added, and the probe was vortexed for 15 min and then centrifuged for 1 min at 2700 min^{-1}. After the first centrifugation the supernatant was poured out, after second it was removed using needle, and finally it was completely dried. Dry precipitate was rinsed in a 250 μl solution containing 25 % ethanol, 25 % isopropanol, 100 mM NaCl, pH 8.8) and centrifuged 1 min at 2700 min^{-1}. Liquid was poured out dry, and the precipitate was rinsed in 200 μl 96 % ethanol with the supernatant removing. The residue was dried for 10-15 min to evaporation of the alcohol and dissolved in 15 μl eluting solution (10 mM Tris-HCl, pH 8.0). After 5 min incubation at 65 °C and 15 min vortexing at room temperature, followed by one more incubation for 5 min at 65 °C, the samples was centrifuged 1 min at 14000 min^{-1} with removing supernatant to eppendorfs. To assay DNA concentration the 1 % agarose gel electrophoresis at 100 V for 60 min was used. A 3 μl DNA sample with 1 μl loading dye were poured per well; λ/HindIII Marker (0.5 μg/μl) was a marker (1 μl marker and 5 μl dye were mixed).

Aliquot 10 μl of purified DAN was subjected to restriction and ligation using 2.5 U EcoRI and MseI (Thermo Scientific, USA); 2.5 U T_4 ligase (Thermo Scientific, USA); two oligonucleotide adapters for each site adEco1 CTCGTAGAC-TGCGTACC and adEco2 AATTGGTACGCAGTCTAC for EcoRI, adMse1 GACGAGAGTCCTGAG and adMse2 TACTCAGGACTCAT for MseI (5 pmol each); the mixture was allowed for 18 hours at 37 °C. T_4 DNA ligase reaction buffer (Thermo Scientific, USA) was used.

For final fingerprinting, to aliquot 4 μl reaction mixture at the stage of restriction and ligation the 2.5 μl 10× polymerase buffer, 2.5 μl 1,5 мМ dNTPs, two selective primers (10 pmol each), of which one was FAM-marked, 1 U Taq DNA polymerase (Fermenta», USA) were added. All possible primer combinations were tested — Mse_0 GATGAGTCCTGAGTAA, Mse_c GATGAGTCCTGAGTAAC, Mse_g GATGAGTCCTGAGTAAG, Mse_t GATGAGTCCTGAGTAAT, Mse_ct GATGAGTCCTGAGTAACT, Mse_cc GATGAGTCCTGAGTAACC for MseI; Eco_0 (FAM) GACTGCGTACCAATT, Eco_ac (FAM) GACTGCGTACCAATTAC for EcoRI. Aplification was cirreied out in a T100 Thermal Cycler (Bio-Rad, USA) according to the protocol: 50 °C 5 s, 60 °C 5 s, 70 °C 2 min, 95 °C 1 min 30 s; 94 °C 30 s, 55 °C 30 s, 72 °C 1 min 30 s (34 cycles); 72 °C 2 min.

Preliminarily, a 3 μl DNA sample with 1 μl loading dye were analyzed by 3 % agarose gel electorphoresis (100 V for 3 hours, GeneRuler 100bp DNA Ladder, 0.5 μg/μl, Fermentas, USA, as molecular weight marker).

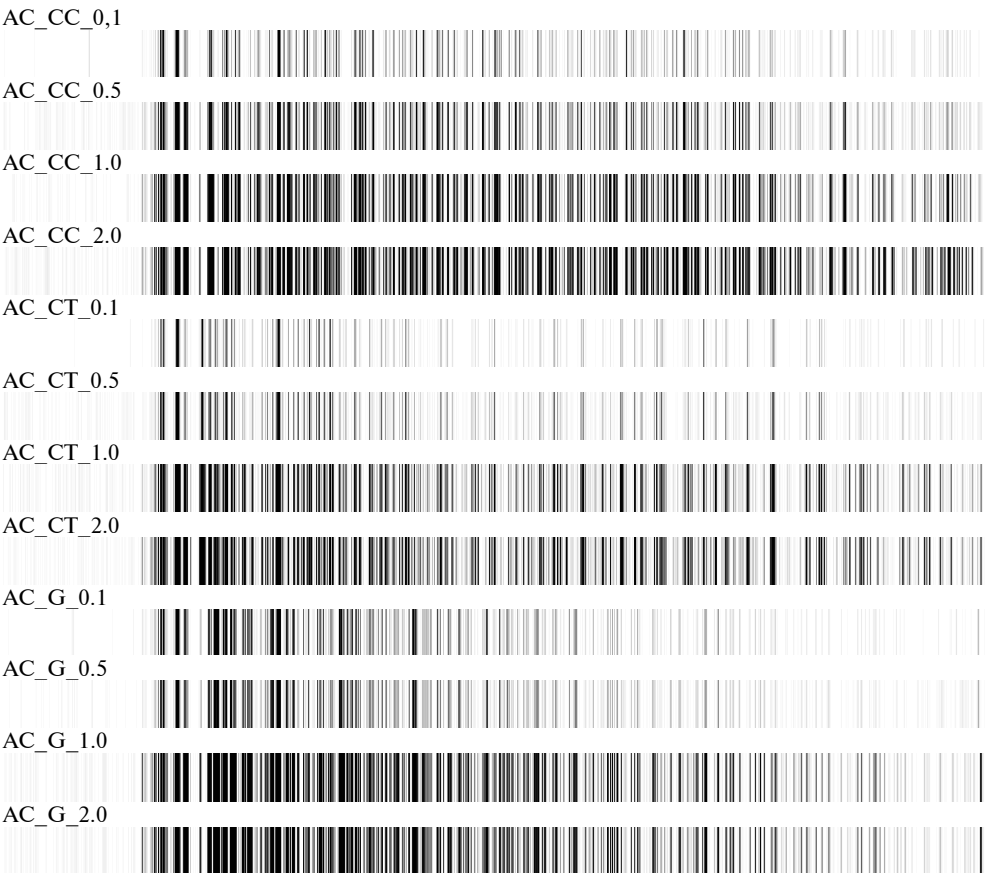
Capillary electrophoresis was performed using a genetic analyzer ABI3500xl (Applied Biosystems, USA) with an integral molecular weight marker GeneScan-600 LIZ Size Standard (Applied Biosystems, USA). DNA aliquots 0.1, 0.5, 1.0 and 2.0 μl were analyzed. After electrophoretic separation of fragments data were processed using BIONUMERICS 7.5 program (Applied Maths, USA).

Results. Strain L-4 was isolated after combined treatment of *A. niger* L-1 (collection of All-Russian Research Institute for Food Additives) with 1,4-bis-diazoaxcetil butane (13 % solution, 3 hours) and UV-irradiation at 3.3000 erg

per mm [23]. The strain produces citric acid at no less then 10 $g \cdot dm^{-3} \cdot day^{-1}$ on molasses-containing medium, highly acidize sucrose-mineral medium under fermentation (no less then 16 $g \cdot dm^{-3} \cdot day^{-1}$) and media containing corn, potato, wheat, rye or sorghum starch hydrolisate (no less then 18 $g \cdot dm^{-3} \cdot day^{-1}$). The strain is permanently (ones a year) re-cultured on must-agar, and additionally stored as dry conidia preparation (a 10 % residual moisture) for 9 months at 16-20 °C, and as dry conidia (a 10 % residual moisture) with sporulating mycelium at –18 to –20 °C with re-culturing each 2 years. Long-term storage at –80 °C is provided using Station of low temperature automatic storage of biological samples (Liconic Instruments, Liechtenstein) at RCAM collection (All-Russian Research Institute for Agricultural Microbiology) [24].

Based on preliminary estimation of the obtained patterns, the Mse_ct GATGAGTCCTGAGTAACT and Eco_ac (FAM) GACTGCGTACCAATTAC; Mse_cc GATGAGTCCTGAGTAACC and Eco_ac (FAM) GACTGCGTACCAATTAC; Mse_g GATGAGTC-CTGAGTAAG and Eco_ac (FAM) GACTGCGTACCAATTAC were the most effective primers of those 12 tested as producing the highest number of DNA fragments.

The results of the automatic capillary electrophoresis (Fig.) and results of analysis formalized on amplified fragments' size for each variant (summarized in the table) are shown hereinbelow.



AFLP patterns of *Aspergillus niger* L-4 DNA obtained with the best three pairs of primers (automatic capillary electrophoresis): AC_CC; AC_CT and AC_G — primers Mse_cc/Eco_ac, Mse_ct/Eco_ac and Mse_g/Eco_ac, respectively; 0.1, 0.5, 1.0 and 2.0 — volume of tested DAN sample (in μl). An analyzer ABI3500xl, Applied Biosystems, USA) with an integral molecular weight marker GeneScan-600 LIZ Size Standard (Applied Biosystems, USA).

Results of *Aspergillus niger* L-4 AFLP-fingerptining formalized on amplified fragments' size for the best three pairs of primers

Fragment, bp	Mse_cc/Eco_ac		Mse_ct/Eco_ac		Mse_g/Eco_ac	
	AC_CC_0.1	AC_CC_2.0	AC_CT_0.1	AC_CT_2.0	AC_G_0.1	AC_G_2.0
593.78	1	1	1	1	1	1
590.69	1	1	1	1	1	1
588.22	1	1	1	1	1	1
580.43	1	1	1	1	1	1
578.20	1	1	0	1	1	1
573.78	1	1	1	1	1	1
571.43	1	1	1	1	1	1
569.00	1	1	1	1	1	1
565.78	1	1	1	1	1	1
562.58	1	1	0	0	1	1
556.26	1	1	0	0	1	1
549.43	1	1	0	0	1	1
545.95	1	1	0	0	1	1
543.39	1	1	0	0	1	1
540.70	1	1	0	0	1	1
534.38	1	1	1	1	1	1
528.60	1	1	1	1	1	1
522.89	1	1	1	1	1	1
518.91	1	1	0	0	1	1
517.94	1	1	1	1	1	1
516.98	1	1	1	1	1	1
515.58	1	1	1	1	1	1
509.15	1	1	1	1	1	1
497.64	1	1	1	1	1	1
493.11	1	1	1	1	1	1
489.14	1	1	1	1	1	1
486.67	1	1	0	0	1	1
483.85	1	1	1	1	1	1
482.60	1	1	1	1	1	1
478.11	1	1	1	1	1	1
471.57	1	1	0	1	1	1
468.29	1	1	0	1	1	1
465.09	1	1	0	0	1	1
461.17	1	1	0	0	1	1
457.84	1	1	1	1	1	1
453.14	1	1	0	0	1	1
449.77	1	1	0	0	1	1
443.78	1	1	1	1	1	1
439.16	1	1	0	0	1	1
435.39	1	1	1	1	1	1
429.90	1	1	0	0	1	1
424.21	1	1	1	1	1	1
418.49	1	1	1	1	1	1
417.20	1	1	0	0	1	1
415.40	1	1	0	0	1	1
409.27	1	1	0	0	1	1
399.10	1	1	0	0	1	1
392.88	1	1	0	0	1	1
388.44	1	1	0	1	1	1
384.48	1	1	1	1	1	1
375.76	1	1	0	0	1	1
356.98	1	1	0	0	1	1
346.87	1	1	0	0	1	1
339.88	1	1	0	0	1	1
334.86	1	1	0	0	1	1
326.17	1	1	0	0	1	1
320.14	1	1	1	1	1	1
318.12	1	1	0	0	1	1
316.96	1	1	0	0	1	1
316.00	1	1	0	0	1	1
313.58	1	1	0	0	1	1
310.47	1	1	0	0	1	1
298.28	1	1	0	0	1	1
285.23	1	1	0	0	1	1
273.72	1	1	0	0	1	1
268.38	1	1	1	1	1	1
263.05	1	1	0	0	1	1
256.30	1	1	0	1	1	1
252.72	1	1	1	1	1	1

Table continued

250.88	1	1	0	0	1	1
247.40	1	1	0	0	1	1
244.32	1	1	0	0	1	1
238.09	1	1	1	1	1	1
230.24	1	1	0	1	1	1
219.86	1	1	1	1	1	1
217.75	1	1	0	0	1	1
216.46	1	1	1	1	1	1
212.77	1	1	1	1	1	1
192.39	1	1	0	0	1	1
183.90	1	1	0	0	1	1
172.12	1	1	1	1	1	1
152.41	1	1	0	1	1	1
141.73	1	1	1	1	1	1
130.64	1	1	1	1	1	1
117.54	1	1	1	1	1	1
116.81	1	1	1	1	1	1
53.37	1	1	1	1	0	0
34.85	1	1	0	0	0	0
33.68	1	1	0	0	0	0

Note. AC_CC, AC_CT and AC_G — primer pair Mse_cc/Eco_ac, Mse_ct/Eco_ac and Mse_g/Eco_ac, respectively; 0.1 and 2.0 — sample volume, μl.

Thus, the optimal pair of primers was Mse_cc GATGAGTCCTGAGTAACC and Eco_ac (FAM) GACTGCGTACCAATTAC, which produce the highest fragment number — 89 fragments, from 33.68 to 593.78 bp in size, provided the suggested technique and processing results. For each pair of primers the AFLP patterns do not depend on the volume of the sample. Note, in 2015 in large scale investigation of biodiversity of *Aspergillus flavus* strain the unique AFLP patterns have been found, however, the practical aspects of this phenomenon was not under consideration [25].

Thus, for the first time the AFLP-fingerptinting was optimized and used for molecular authentication of micromycete *Aspergillus niger* L-4 commercial strain. Obtained AFLP patterns can be used for authentication of this strain form different sources provided the place and protocol of assessment are the same as described herein. Developed method may be applied to produce «genetic passports» for other *Aspergillus* strains.

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FEED ADDITIVE VINIVET OF APICULTURAL PRODUCTS AS AN ALTERNATIVE FOR ANTIBIOTIC GROWTH PROMOTERS IN BROILER CHICK DIETS — BACTERICIDAL AND BIOSTIMULATING EFFECT

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Abstract

Preservation of national gene pool requires the maintenance of quality standards of life including healthy nutrition. In this relation there is a particular public concern about the use of antibiotic growth promoters (AGPs) as an essential part of intensive technologies of animal production, the remnant of the past when it was a commonplace. WHO reported the constant growth of microbial resistance partly due to the transfer of AGPs into animal food products; it could enhance the distribution of bacteria resistant to antibacterial drugs and drug-resistant diseases hazardous for human race. The distribution of these infections among animals (especially breeding flocks) is also hazardous. This hazard is probably underestimated as a result of the lack of knowledge on composition and possible changes in animal and human microbiome including microorganisms undetectable by classic microbiological methods but forming the basis of the microbiota. Another underestimated possibility is the transfer of beneficial biologically active substances to human via animal food products when undesirable ingredients of animal feeds are simply substituted by harmless and biologically active ones, while more costly approaches to production of functional animal foodstuffs are widely discussed. Problem solving must be rapid, economically and biologically effective and safest for human, animals, and environment. In our trials conducted on 3 groups of Cobb-500 broiler chicks (from 1 to 35 days of age) the possibility of substitution of feed additive Vinivet for AGPs in diets for broiler chicks was studied. This additive based on apicultural products, i.e. bee-bread (ambrosia) and slumgum, was produced by no-waste technology (JSC Rusoborotexport, Kazan, Russia) preserving biologically active compounds from these products. It was found that inclusion of Vinivet into broiler diets (5000 ppm) improved live bodyweight in group 3 compared to control group 1 (no Vinivet) by 0.61 and 0.86 % at 28 and 35 days of age, respectively; feed conversion rate (FCR) in group 3 was better by 3.15 % compared to control. AGP Stafac 110 (Phibro Animal Health Corp., USA) fed to group 2 (180 ppm) was found to improve growth rate of broilers compared to groups 1 and 3. Live bodyweight in group 2 was higher than in control at 6, 14, 21, 28 and 35 days of age by 2.63; 1.99; 3.22; 0.51 and 2.04 %, respectively, while FCR in group 2 was better by 3.53 %. The analysis of intestinal microbiota with the use of T-RFLP (Terminal Restriction Fragment Length Polymorphism) method showed substantial decline in pathogenic and opportunistic populations in broilers of Vinivet-fed group 3. The counts of *Staphylococcaceae*, *Fusobacterium* spp., *Pertococcaceae* and *Pasteurellaceae* were significantly lower compared to control (by 0.91; 0.79; 1.85 and 3.37 %, respectively) while total count of cellulolytic bacteria was higher by 7.94 %. Histological study of liver and intestine showed structural changes in small intestine of Vinivet-fed broilers improving its absorbing ability, barrier and motor functions. The height of intestinal villi in group 3 was significantly higher compared to control, density and depth of crypts were higher as well as the total absorbing surface area. To the contrary, in Stafac-fed group 2 the decrease in thickness of mucosa of intestinal wall was found which could mean the decrease in intestinal functionality. The activation of trophic processes in liver was also found in Vinivet-fed broilers. The histological analysis of liver showed the absence of hepatotoxic effect of Vinivet for broilers. The results of our trial proved Vinivet to be a promising

and safe feed additive for poultry.

Keywords: broiler chicks, antibiotic growth promoters, productivity, apicultural products

Increasing production, improving quality and reducing the cost of eggs and meat are the most important objectives of poultry. Currently, much attention is paid to provide people with functional food, as nutrition is one of the most important factors in healthy lifestyle and gene pool maintaining. Proper nutrition helps to prevent diseases and prolong life, increases the ability to resist the adverse agents, and provides normal growth and development of children. The dietary properties of poultry products in combination with their enrichment with biologically active components allow controlling essential nutrient levels in consistence with medical and biological requirements. The demand for high-quality and environmentally friendly products is increasing [1-3].

An approach in the functional nutrition concept supposes to limit the use of feed antibiotics in poultry to produce safe meat and eggs without residual amounts of antibiotics [4, 5]. The World Health Organization (WHO) notes the growing microbial resistance (including that due to the transition of antibiotics in animal production and human consumption, consequently), which may result in the spread of bacteria resistant to antibacterial agents and drug-resistant infections that pose a threat to humanity [6-8]. A not less risk lies in the spread of such infections in livestock, in breeding stock especially.

Possibly, the threat is underestimated greatly, since the composition and changes in microbiome have not been actually studied in animals and humans. Thus, it is generally believed that the gastrointestinal tract (GIT) of avian embryos is sterile [9-12], and the formation of digestive system microbiocenosis occurs after hatching as a result of contacts with the environment [13-15]. However, there is evidence obtained by the methods of classical microbiology [16] and using real-time PCR [17] which indicates the ability of microorganisms to colonize the digestive tract of poultry embryos.

Renewable low-demand apicultural products containing an extensive range of vitamins, amino acids, macro-, and micronutrients are a promising source of raw materials for feed additives with a diversified action [18-20], so that they can be natural physiological and biochemical stimulators in poultry, contributing to the improvement of palatability and ecological purity of the final product. It is noteworthy that the bactericidal properties of honey, pollen, propolis have been investigated extensively [21], while the similar effect of slumgum, a byproduct, not been studied actually.

We first demonstrated the possibility of substituting antibiotic growth promoter (AGP) Stafac 110 (Phibro Animal Health Corp., USA) widely used in Russian industrial poultry by the feed additive Vinivet (Rusoborotexport LLC, Kazan, Russia) based on apicultural product. This additive was found not to be inferior to the antibiotic for the parameters studied (microflora composition of intestinal blind processes in chickens, gastrointestinal tract and liver micromorphology) while also helping to increase poultry growth rate.

Our purpose was to compare biostimulatory and bactericidal effects of Vinivet feed additive and AGP.

Technique. Three groups of Cobb-500 broiler chickens from 1 to 35 days of age (FSUE Zagorskoe EPH VNITIP vivarium, Moscow Province) were fed ad libitum with dry complete feed according to recommended standards (All-Russian Scientific Research and Technology Poultry Institute — VNITIP, 2014) [22]. In control group 1 a balanced complete feed (basic diet — BD) was used. Group 2 poultry within the observation period were fed similar diet supple-

mented with AGP Stafac 110 (Phibro Animal Health Corp., USA) at a dose of 180 g/t of complete feed as recommended by manufacturer, and in experimental group 3 the antibiotics were replaced by apiculture product based feed additive Vinivet (Rusoborotexport LLC, Kazan, Russia) at a dose of 5 kg/t of complete feed. In the first 3 days, the broilers of all groups were given the same pre-start complete feed, further fed according to the experimental scheme. Veterinary measures were in accordance with the vaccination plan accepted at the farm. Broilers were kept in R15 cages (Germany), 35 broilers in each group with no gender separation. Housing conditions complied with VNITIP recommendations. Feed was given manually.

Main zootechnical parameters recorded were live bodyweight at the age of 7, 21, and 35 days (individual weighing), mortality rate, and average daily weight gain. Feed conversion rate per 1 kg of live weight gain were calculated, and the levels of vitamins and carotenoids in the liver, chemical composition of the liver, pectoral and femoral muscles were assayed by conventional methods [23].

35-Day-aged (n = 21) broilers were used for histological studies. Liver and disemboweled intestinal canal were sampled within 1 h after slaughter and fixed in 10 % formalin for one day. After washing in running water, samples were dehydrated in alcohol (70-96 %). Paraffin-embedded tissue sections were prepared using a HM-325 universal automated microtome (Microm international GmbH, Germany). General morphology was studied using a light microscope (Nikon, Japan) after staining with hematoxylin and eosin. Photomicrography and microscopic morphometry with statistical processing were performed using certified ImageScope C microscope combined image analysis software (Systems for Microscopy and Analysis LLC, Russia).

Cecum microbiota was studied using T-RFLP (Terminal Restriction Fragment Length Polymorphism) analysis [24].

Statistical processing of zootechnical parameters and T-RFLP-analysis data was performed by standard methods [25].

Results. It is noteworthy that zootechnical parameters in the course of the experiment were high: in all the groups loss of broilers through natural death was not registered and the feed per unit of gain (feed efficiency) complied with general standards not exceeding 1.6 kg per 1 kg. Average daily weight gain in control broilers was 60.21 g (Table 1).

The obtained data showed AGP Stafac 110 to provide higher growth rate. Thus, the live weight in group 2 broilers exceeded control at the ages of 6, 14, 21 and 28 days by 2.63, 1.99, 3.22, and 0.51 %, respectively, and by the end of growing period, the difference in average live weight in groups 2 and 1 was 2.04 %. In this, feed conversion rate was better compared to control (by 3.53 %).

Our data are consistent with the results of numerous studies [26] which prove that the use of antibiotic growth promoters, due to inhibition of gastrointestinal tract (GIT) pathogenic microflora, not only contributes to poultry survival rate, but also has a growth stimulating effect in chicken which is especially noticeable in their early stages.

Apicultural products are known to have a pronounced antibiotic effect on gastrointestinal pathogenic microflora [21]. However, their efficiency is still inferior to antibiotic growth promoters, as evidenced by the zootechnical parameters we obtained in the experiment. In fact, AGP Stafac 110 at a dose of 180 g/t improved live weight in group 2 broilers compared both to the parameters of control and Vinivet-fed (5 kg/t of feed) groups.

Despite the lower efficiency of Vinivet additive, its application in a dose

of 5 kg/t still had a growth promoting effect on broilers. Group 3 chicken live weight was by 0.61 and 0.86 % greater compared to control at the age of 28 and 35 days (differences in male chickens were significant at $p < 0.05$), and feed consumption per 1 kg gain was 3.15 % lower, which confirms the usefulness of Vinivet in feeding meat poultry. In the early growing (at the age of 14 and 28 days), Vinivet use resulted in a slight growth retardation (3.38 and 0.58 %, respectively) in group 3 broilers compared to control.

1. Zootechnical parameters in Cobb-500 broiler chickens with the use of AGP Stafac 110 and apicultural product based feed additive Vinivet ($M \pm m$, FSUE Zagorskoe EPH VNITIP vivarium, Moscow region)

Parameter	Group		
	1 (control)	2 (Stafac 110)	3 (Vinivet)
Stock preservation, %	100	100	100
Live weight at various ages, g:			
day 1	40.0	40.0	40.0
day 6	152.54±8.60	156.54±1.83	156.37±1.80
day 14	435.37±7.85	444.03±10.07	420.64±8.75
day 21	873.71±14.12	901.82±20.86	868.64±13.99
day 28	1462.51±19.45	1470.00±29.40	1471.48±21.23
Average live bodyweight, kg:			
at the age of 35 days	2086.8	2129.42. or +2.04 %	2104.81
including			
male chickens	2120.00±26.78	2232.15±56.47	2233.86±35.45*
female chickens	2053.60±34.28	2026.68±33.64	1975.75±26.33
Feed conversion rate per broiler, kg	3.195	3.098	3.100
Feed conversion rate per 1 kg of live weight gain, kg	1.557	1.502	1.508
Daily live weight gain, g	60.21	61.45	60.73

N o t e. Basic diet was supplemented with Stafac 110 (Phibro Animal Health Corp., USA) and Vinivet (Rusoborotexport LLC, Kazan, Russia), 180 g/t and 5 kg/t, respectively.
* Differences are significant in group 3 male chickens at $p < 0.05$.

2. Vitamin levels (μg/g) **in 35-day-old Cobb-500 broiler chicken liver with the use of AGP Stafac 110 and apicultural product based feed additive Vinivet** (FSUE Zagorskoe EPH VNITIP vivarium, Moscow region)

Parameter	Group		
	1 (control)	2 (Stafac 110)	3 (Vinivet)
Vitamin A	205.03	191.53	180.81
Vitamin E	9.20	5.73	10.03
Vitamin B_2	10.77	10.14	10.11
Carotenoids	4.69	3.03	4.77

N o t e. The basic diet was supplemented with Stafac 110 (Phibro Animal Health Corp., USA) and Vinivet (Rusoborotexport LLC, Kazan, Russia) at the doses of 180 g/t and 5 kg/t, respectively. According to the method used, the differences are considered significant if the difference in the levels of vitamins A and E exceeds 20 % and 15 % for vitamin B_2 and carotenoids, i.e. there was a significant decrease in vitamin E and carotenoids levels in group 2.

It was found (Table 2) that the use of additives with an antiseptic effect may result in decreased vitamin deposition in broiler liver. Thus, vitamin E and carotenoids levels in group 2 broilers treated with Stafac 110 antibiotic reduced significantly compared to control (by 37.70 and 35.40 %, respectively), while with the use of feed additive Vinivet, the differences in vitamin A and B_2 deposition did not exceeded the method error (11.80 and 6.12 %, respectively), and accumulation of vitamin E and carotenoids increased slightly (by 9.02 and 1.71 %, respectively) which indicates a decrease in the negative impact of this natural antiseptic on GIT microbial community in group 3 broilers.

T-RFLP-analysis of intestinal microbiota demonstrated a substantial decrease of pathogenic and opportunistic microoranisms in broiler cecum in Vinivet-fed group. The counts of *Staphylococcaceae*, *Fusobacterium* sp., *Pertococcaceae,* and *Pasteurellaceae* decreased significantly ($p \leq 0.05$) by 0.91; 0.79; 1.85, and

3.37 % compared to control, while total count of cellulolytic bacteria was higher by 7.94 % (Table 3).

Light-optical histological study of intestinal wall showed the presence of common structural organization features in all the groups studied; the intestinal wall was differentiated into a number of layers: mucous (inner) layer which consists of epithelial cover, lamina propria, muscularis mucosa, and submucosal layer; muscle (intermediate) layer consists of two layers of smooth muscle cells (inner circular and outer longitudinal muscles); serous (outer) layer which includes mesothelial cells and a connective tissue formation (Fig. 1-3, A, B).

3. T-RFLP analysis of microorganism composition (%) in cecum contents in 35-day-old Cobb-500 broiler chickens with the use of AGP Stafac 110 and apicultural product based feed additive Vinivet ($X \pm x$, FSUE Zagorskoe EPH VNITIP vivarium, Moscow Province)

Microorganism group	Group		
	1 (control)	2 (Stafac 110)	3 (Vinivet)
Normal microflora			
Total cellulolytic bacteria	14.28±0.30	12.78±0.40	22.22±0.50
Family *Eubacteriaceae*	7.78±0.04	8.35±0.05	9.49±0.08
Family *Ruminococcaceae*	2.32±0.01	3.10±0.01	2.53±0.01
Family *Lachnospiraceae*	4.18±0.02	1.26±0.00	10.20±0.03
Peptostreptococcus sp.	–	0.07±0.00	–
Order *Selenomonadales*	10.29±0.20	12.05±0.20	13.38±0.30
Family *Lactobacillaceae*	6.15±0.04	6.47±0.03	5.50±0.04
Family *Bacillaceae*	7.89±0.03	7.27±0.04	5.77±0.01
Order *Bacteroidales*	8.78±0.02	6.98±0.02	8.59±0.02
Bifidobacterium sp.	1.49±0.00	1.92±0.01	0.94±0.00
Adverse microorganisma			
Order *Actinomycetales*	6.12±0.02	2.70±0.01	6.04±0.02
Order *Enterobacteriaceae*	4.68±0.02	3.08±0.01	1.23±0.01
Family *Clostridiaceae*	9.52±0.03	5.85±0.02	12.06±0.02
Pathogenic microorganisms			
Family *Staphylococcaceae*	1.38±0.00	1.58±0.01	0.47±0.00
Fusobacterium sp.	2.71±0.01	1.09±0.01	1.92±0.01
Family *Peptococcaceae*	3.31±0.01	0.61±0.00	1.46±0.00
Family *Campylobacteraceae*	0.17±0.03	0.16±0.01	0.20±0.00
Family *Pasteurellaceae*	3.37±0.20	1.99±0.01	–
Non-culturable microorganisms			
Total	18.56±0.30	33.34±1.20	17,46±0,60
Transient microflora			
Family *Pseudomonadaceae*	1.30±0.01	2.14±0.01	2,78±0,01

Note. The basic diet was supplemented with Stafac 110 (Phibro Animal Health Corp., USA) and Vinivet (Rusoborotexport LLC, Kazan, Russia) at the doses of 180 g/t and 5 kg/t, respectively. The dash means that the count of microorganisms was below the limit of reliable determination by T-RFLP.

Micromorphologically, in the liver there were hepatocyte rows separated by blood capillaries with a clear differentiation of the central vein and hepatic triads (Fig. 1-3, B).

In group 3 chickens fed basic diet supplemented with natural apicultural based product, the structural changes improving the absorbing capacity, barrier and motor functions were identified in the small intestine. They included a significant ($p \leq 0.05$) increase in mucosal thickness, lengthened intestinal villi, greater crypt density and depth, increased total absorptive surface area (Fig. 3, A, Table 4). In Stafac 110-fed group 2, mucosal thickness was decreased which may indicate a decrease in intestinal functionality (Fig. 2, 3, A, B).

Micromorphological analysis of intestinal muscular membrane revealed its thickening in experimental groups versus control (in particular, by 40.3 % in the small intestine and by 30.1% in large intestine in group 3) indicating its motor function enhancement in chickens fed basic diet supplemented with feed additive Vinivet.

No significant intergroup differences were found in the thickness of intestinal serous membrane.

In group 3 chickens, liver parenchyma was penetrated with a larger number of blood capillaries closely associated with hepatic beams, a moderate blood supply was characteristic of it, no destructive changes were found in the liver (Fig. 3, C). Thus, activation of trophic processes in the liver caused by supplementing diet with Vinivet was not followed by toxic effects on the liver tissue as evidenced by its structure and suggests safety of the study product in poultry when used as a feed additive.

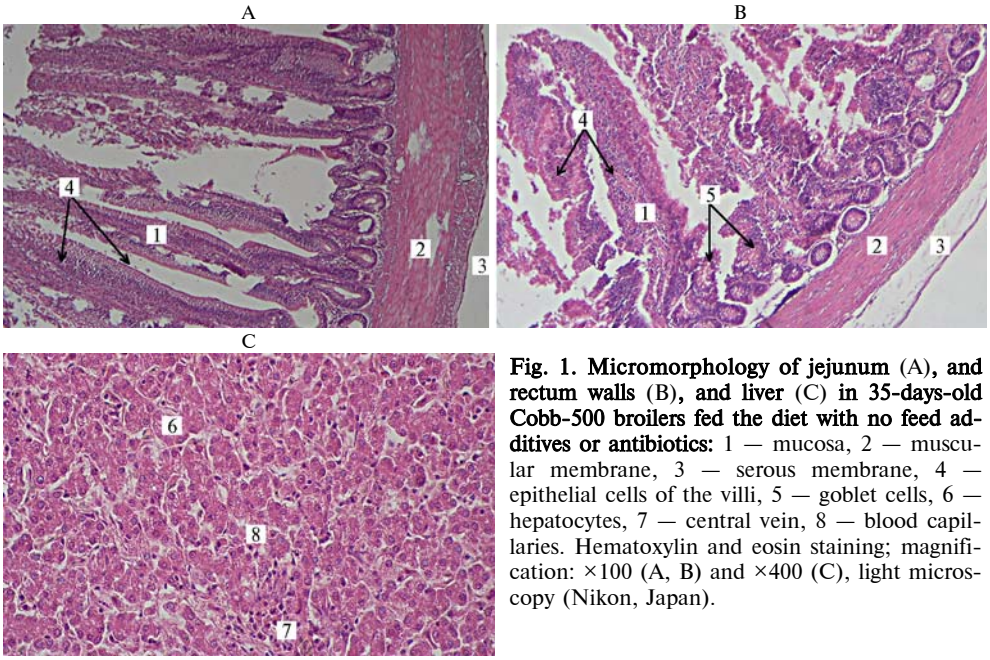

Fig. 1. Micromorphology of jejunum (A)**, and rectum walls** (B)**, and liver** (C) **in 35-days-old Cobb-500 broilers fed the diet with no feed additives or antibiotics:** 1 — mucosa, 2 — muscular membrane, 3 — serous membrane, 4 — epithelial cells of the villi, 5 — goblet cells, 6 — hepatocytes, 7 — central vein, 8 — blood capillaries. Hematoxylin and eosin staining; magnification: ×100 (A, B) and ×400 (C), light microscopy (Nikon, Japan).

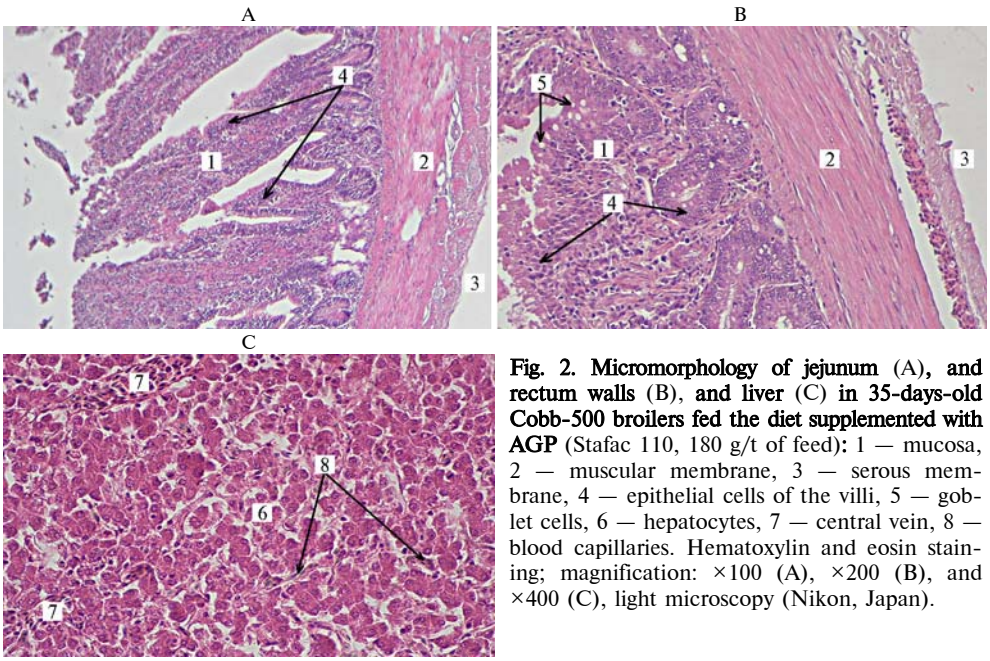

Fig. 2. Micromorphology of jejunum (A)**, and rectum walls** (B)**, and liver** (C) **in 35-days-old Cobb-500 broilers fed the diet supplemented with AGP** (Stafac 110, 180 g/t of feed)**:** 1 — mucosa, 2 — muscular membrane, 3 — serous membrane, 4 — epithelial cells of the villi, 5 — goblet cells, 6 — hepatocytes, 7 — central vein, 8 — blood capillaries. Hematoxylin and eosin staining; magnification: ×100 (A), ×200 (B), and ×400 (C), light microscopy (Nikon, Japan).

Our results are consistent with the data on activating the functions of hepatocytes and immune cells of the liver stroma, and on improving the state of glandular stomach tissue in turkeys with the use of the diet supplemented with feed additive Vinivet [21].

4. Comparative characteristics of intestine morphometric parameters in 35-day-old Cobb-500 broiler chickens with the use of AGP Stafac 110 and apicultural product based feed additive Vinivet (*M*±*m*, FSUE Zagorskoe EPH VNITIP vivarium, Moscow region)

Group	Large intestine (membranes), m		Small intestine (membranes), m	
	mucosa	muscular membrane	mucosa	muscular membrane
1 (control, basic diet — BD)	646,00±3,23	68,40±1,43	732,00±5,14	101,00±6,02
2 (BD + Stafac 110, 180 g/t)	632,00±2,11	72,20±1,54	697,00±3,54	138,00±6,78
3 (BD + Vinivet, 5 kg/t)	695,00±3,54	89,00±2,14	837,00±3,54	141,00±6,02

N o t e. Basic diet was supplemented with Stafac 110 (Phibro Animal Health Corp., USA) and Vinivet (Rusoboroteksport LLC, Kazan, Russia) at the doses of 180 g/t and 5 kg/t, respectively.

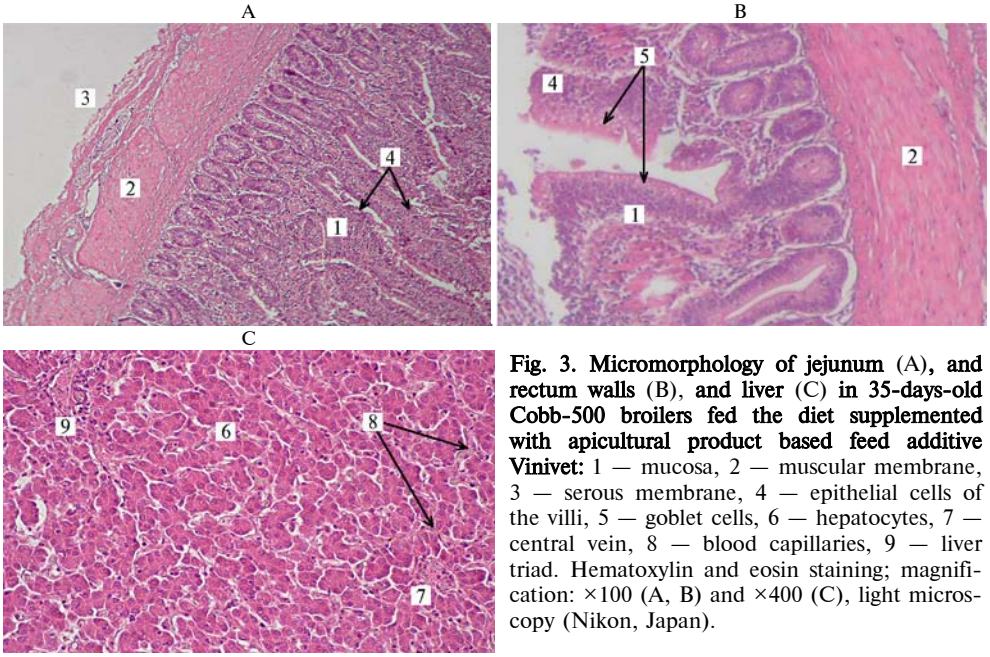

Fig. 3. Micromorphology of jejunum (A)**, and rectum walls** (B)**, and liver** (C) **in 35-days-old Cobb-500 broilers fed the diet supplemented with apicultural product based feed additive Vinivet:** 1 — mucosa, 2 — muscular membrane, 3 — serous membrane, 4 — epithelial cells of the villi, 5 — goblet cells, 6 — hepatocytes, 7 — central vein, 8 — blood capillaries, 9 — liver triad. Hematoxylin and eosin staining; magnification: ×100 (A, B) and ×400 (C), light microscopy (Nikon, Japan).

Thus, histological studies and GIT microflora analysis in broilers are consistent with zootechnical parameters and indicate that apicultural product based feed additive Vinivet has growth stimulating effects due to providing a complex of biologically active substances. It influences beneficially on the gastrointestinal tract, increases absorptive surface area of the small intestine mucosa due to elongation of the villi and deepening of wrinkling and crypts, and thickening of the large intestine muscle mebrane. Moreover, the product possess antiseptic properties, therefore it can be used to substitute antibiotic growth promoters in broilers.

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VISCOSIMETRIC ASSAY OF ENDOGLUCANASE ACTIVITY IN ENZIME FODDER ADDITIVES

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Abstract

Worldwide a large number of enzymes, including those with endo-β-glucanase activity are produced by different companies as fodder additives. In Russia, there is no uniform method for endo-β-glucanase activity analysis that causes difficulties in assessing quality of the enzyme preparations. The aim of the study was to develop such uniform method. In this, we optimized a viscometric determination based on change of the relative viscosity of a substrate mixed with enzyme preparation using Ostwald capillary viscometer. The following regime has been accepted: the diameter of the capillary is 0.73 mm; the temperature of hydrolysis is 30 °C; the solvent is the acetate buffer (pH 4.7); time of incubation is 5, 10, 15 min in accordance with the kinetics of the enzymatic reaction; and β-glucane (0.4 %) was used as substrate. The carboxymethyl cellulose is shown not to be relevant so far as it gives results incomparable with those obtained with β-glucane due to estimation of the cellulase but not the β-glucanase activity, that does not correspond to the objectives of forage production. The unit used in the developed method is the amount of enzyme which increases the relative substrate flow rate in 1 min^{-1} under standard conditions of hydrolysis. The measurement of accuracy of the method was carried out according to GOST R ISO 5725-2002 (the state standards). In this, 5 certified samples with different activity were prepared from a commercial sample of β-glucanase (Sigma-Aldrich, USA) with known activity (GLA/g). The activity of the standard was expressed in GLA/g as glucose equivalents corresponding to the amount of enzyme which acts on β-glucane, releasing 1 μmol of sugars in terms of glucose. Certified samples were analyzed under intermediate precision conditions with three factors changed (i.e., operator, matrix, reagents). The matrices were mineral fillers, the calcium carbonate and zeolite. To calculate the accuracy of the method the viscosity of certified samples was expressed in GLA units using the calibration curves. Expanded uncertainty of measurements ranged within 10-22 % for the coverage factor k = 2.

Keywords: endoglucanase activity, non-starch polysaccharides, hemicellulose, viscosity, viscosimetry, β-glucane, carboxymethyl cellulose, matrix, intermediate precision, expanded uncertainty

Currently, a variety of enzymatic additives used to reduce content of fodder antinutrients have been developed for animal husbandry. Xylanase, glucanase, cellulose, the cytolytic cleaving enzymes, are added in the fodder for monogastric animals, including poultry in which non-starch core polysaccharides (cellulose, hemicellulose, lignin, pectin) are poorly digestable. A high content of soluble non-starch polysaccharides, such as hemicelluloses (xylans and glucans), results in fodder high viscosity, when using fresh harvest or in drought years, especially. Widespread practical use of cellulase and hemicellulase preparations in recent years is due to the maize substitution with cereals (barley, rye, wheat, oats, triticale, and meal and cake) in diets, and to an increase in the levels of non-starch polysaccharides in fodder. Foreign manufacturers estimated such additive activity using their own methods (Rovabio, BASF, Biovet AD, Kemin). This causes difficulties, as it is not possible to compare the effects of different enzyme feed additives with one other and versus the products for similar purposes declared by manufacturers [1-3].

In 2012, the interstate standards regulating enzymatic feed additive β-

xylanase and cellulase activity were developed in Russia (GOST 31488, GOST 31662) and starting from June 1, 2013, a national standard (GOST R 54905-2012) was enacted to describe a unified method of endoglucanase activity assay. However, more and more foreign companies and laboratories are recently switching to the endo-β-glucanase activity measurement because of the requirements of feed production.

It is endoglucanases that make it possible to reduce chyme viscosity in the course of passage through the gastrointestinal tract of monogastric animals, such as pigs and poultry [4, 5], as well as young cattle and small ruminants. The ability of enzymes added to fodder to cleave carbohydrate polymers into smaller fragments makes it possible to solve the problem of improving digestibility of non-starch polysaccharides such as cellulose and hemicellulose which are actually not cleaved by animal glycolytic enzymes. Enzymatic additives allow the wider use of grain diets as an energy source for poultry and pigs [6, 7]. There are over 60 enzyme preparations with different activity that are considered authorized [8-11]. However, unified methods of estimating and comparing these preparations have not been set which results in a problem for the control of their production and use [12].

The methods described can be divided into two groups, colorimetry and viscosimetry [13]. Viscometry provides direct activity measurement as based on determining the rate of a polysaccharide substrate viscosity decrease. Colorimetric methods are indirect, and based on enzyme hydrolysis of stained substrates (polysaccharides) with chromophore elimination and estimation of thus developing color. The results of colorimetric assay have to be assessed in specific optical density (OD) units or expressed in the units adopted for measuring endoglucanase activity (GLA) as glucose equivalents with standard enzyme. However, as there is no unified standard endoglucanase activity assay method, declared activity of standard enzyme depends on the manufacturer (including the method for the measurement of enzyme activity and specificity). Therefore, in our opinion, viscometry, of the two approaches, is the more promising for the standardization of endoglucanase activity research methods and for obtaining comparable results, and is used in a number of laboratories [14-16]. The activity unit used in viscosimetric studies is the amount of enzyme which increases the relative substrate flow rate equal to 1 min^{-1} under standard β-glucan hydrolysis conditions.

Thus, there have been no uniform methods for animal endo-β-glucanase activity analysis in Russia which caused difficulties in certification and regulations for use of these feed additives. We first optimized the viscometric assay of endoglucanase activity based on the assessment of substrate relative viscosity measurement using a capillary viscometer.

Our purpose was to develop a method which allows to standardize the measurement of endo-β-glucanase activity in enzyme preparations used as additives to reduce feed antinutrient content.

Technique description. Viscosity of samples was analyzed using a viscosimetric assay method [17] with our modification. Relative viscosity of substrates was measured using an Ostwald capillary viscometer with capillary diameter of 0.73 mm according to liquid outflow rate (outflow viscometer) as compared with the solvent (acetate buffer, pH 4.7) at 30 °C. The solvent was poured into viscometer, incubated at 30 °C for 5 min, and passed 3 to 5 times sequentially to estimate the average flow time (t_p). Further, viscometer was emptied, 8 cm^3 of substrate and 1 cm^3 of buffer solution were added with a pipette (the volume is viscometer-specific, 9 cm^3 in our case), the mixture was incubated for 5 min at 30 °C and passed 3-5 times to indicate start outflow time τ_0. Viscometer was

emptied again, washed thoroughly, and dried, then 8 cm^3 of substrate were added, incubated at 30 °C for 5-7 min, then 1 cm^3 of pre-heated (30 °C) enzyme solution (sample) was added. The contents of viscometer was mixed by air bubbling. Time of substrate-enzyme mixture outflow (t_i) was registered (τ_i) for 5, 10, and 15 min of incubation, in accordance with the kinetics of the enzymatic reaction (M.L. Rabinovich, A.A. Klesov, I.V. Beresin, 1977).

Relative viscosity of incubation mixture was calculated for each incubation period (τ_i, including τ_0) using the following formula:

$$\eta_i = \frac{t_i - t_p}{t_p}, \quad (1)$$

where η_i is relative viscosity in the τ_i incubation period; t_i is the time of incubation mixture outflow in each incubation period, sec; t_p is the time of solvent outflow, sec. Based on the resulting values, relative flow was calculated as the reciprocal relative viscosity ($1/\eta_i = b_i$).

The change in relative viscosity per minute was estimated for each time interval:

$$A_i = (b_i - b_0)/\tau_i, \text{ min}^{-1} = \Delta b/\tau, \text{ min}^{-1}, \quad (2)$$

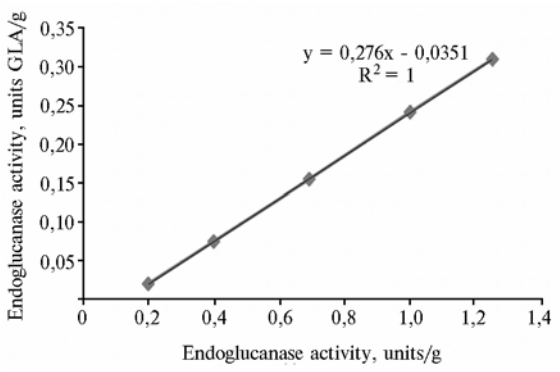
where b_0 is the baseline relative viscosity at τ_0.

Endo-β-glucanase activity (units/g) was calculated as follows:

$$EndoGLA = A/C, \quad (3)$$

where A is relative viscosity change per min; C is amount of enzyme in reaction mixture, g. A was calculated as the A_i arithmetic mean for each incubation period (τ_i) using formula (2).

A series of standard dilutions (standard solutions) were prepared for which the viscosity (fluidity) of the hydrolyzed substrate were estimated after 10 min incubation by outflow time.



Example of calibration curve to convert viscometry in GLA units in the assay of endo-β-glucanase activity based on substrate fluidity changes.

To express endo-β-glucanase activity in GLA units, calibration curves were constructed in each assay series (Fig.).

β-Glucanase (*Trichoderma longibrachiatum*) of 3100 units/g (units GLA) activity (Sigma-Aldrich, USA) was used as standard. The viscosimetric results expressed in units/g, were converted to GLA units.

Method accuracy was assessed by analyzing certified samples with different β-glucanase levels under interlaboratory precision with the following variable factors: operator (№ 1 and № 2), reagent kit (№ 1 and № 2), and matrix. Calcium carbonate (CP, Russia) and zeolite (Nov' NPF, Novosibirsk), the mineral fillers, were the matrices. Each certified sample activity was calculated as standard (enzyme) concentration in certified mixture containing mineral filler. Thus, 5 samples of 93, 310, 620, 1033, and 1550 units GLA/g activity were prepared for each filler, and 8 experiments (total of 40 measurements) were performed considering the three factors for each activity (Table 1).

The 0.1 to 0.5 % β-glucan and carboxymethyl cellulose (CMC) solutions in acetate buffer (0.1 M, pH 4.7) were tested as substrates.

Data processing for each certified sample, with regard to individual test

for 8 variants, included calculation of mean values and mean value dispersion S^2. Based on the resulting values, relative expanded uncertainty (%), repeatability limit (%), and relative uncertainty accuracy indices (%) were calculated.

1. Experimental scheme to assess the accuracy of the method proposed to standardize viscometric endo-β-glucanase activity assay

Experiment No.	Variable factor		
	matrix	reagent kit	operator
1	Calcium carbonate	1	1
2	Calcium carbonate	2	2
3	Zeolite	2	2
4	Calcium carbonate	2	1
5	Zeolite	1	2
6	Calcium carbonate	1	2
7	Zeolite	1	1
8	Zeolite	2	1

N o t e. 1 and 2 are the variable factor variants.

When determining the optimum substrate concentrations, attention was paid to the time of initial solution outflow compared to the solvent. With the solvent (buffer solution) flow rate of 26-30 sec, the outflow rate of baseline solutions of tested concentrations was from 40 sec (for 0.1 % CMC) to 114 sec (for 0.5 % β-glucan). The outflow time of 60-80 seconds can be regarded optimal. At higher rates (as for 0.1 % CMC), a reduced viscosity of the mixture as a result of enzyme activity may cause a reduction in its outflow time to that of the solvent. As the outflow time increases to 80 sec, the controlled period of substrate-enzyme contact decreases.

Accordingly, we chose 0.4 % for β-glucan and 0.2 % for CMC as the optimal substrate concentrations to be further used in this study.

Testing optimal concentrations of various substrates showed a mismatch between the final results for viscosity, which is associated with enzyme specificity. Endo-β-glucanase cleaves 1,3- and 1,4-β-glycosidic bonds in glucose residue chain, and hydrolyzes both glucan (hemicellulose) and cellulose for these bonds. For this reason, some authors use these substrates as interchangeable ones [17, 18], and the β-glucanase and cellulase enzyme names as synonyms. Both substances are glucose polymers in which monomers are β-1.3 and β-1.4 bonds linked. They belong to non-starch polysaccharides and antinutrients as not cleaved by enzymes in animal gastrointestinal tract and preventing assimilation of other fodder nutrients.

However, these compounds differ both in their composition and physical and chemical properties. Glucans are hemicelluloses with a molecular weight up to 50,000 Da and a branched structure. They are soluble in weak acids and alkalis, and are contained in barley, rye, and wheat seeds. Cellulose is insoluble linear glucose polymers with a molecular weight from 50,000 to 1,800,000 Da. They are not soluble in acids and alkalis, and are part of cereal cell walls. It is hemicelluloses (xylans and glucans) that determine chyme viscosity and cause major problems of digestion and assimilation of fodder nutrients.

To compare substrate effects on the final assay results, the Hostazim (Biovet, Bulgaria) viscometric endoglucanase activity was assayed with two substrates. It was found that sample viscosity was 12125±1819 U/g for β-glucan and 56500±475 U/g for CMC.

β-Glucan makes it possible to detect β-glucanase activity, while CMC provides data on cellulase (more precisely, on CM-cellulase) activity, which may vary considerably for the same sample. In this case, the latter was 4.5 times higher which was apparently determined by the specificity of the enzyme wherein cellulase activity predominates, and depends on the properties of the

enzyme producing fungus.

In this connection, we used β-glucan only as a substrate in subsequent tests and performed studies with the three factors — operator, matrix (calcium carbonate and zeolite), and reagent kit. Based on the findings, we estimated the indices of method accuracy in accordance with domestic GOST R ISO 5725-2002 [19] and RMG 61-2010 [20] (Table 2).

2. Calculated parameters for the accuracy of the method proposed to standardize viscometric endo-β-glucanase activity assay

Parameter	Standard sample activity, units GLA/g				
	93	310	620	1033	1550
Relative expanded uncertainty for coverage factor k = 2, %	22.1	11.3	9.6	12.2	11.3
Repeatability limit, %	12.8	7.3	13.3	2.6	8.0
Relative uncertainty accuracy index, %	4.6	3.2	3.2	3.4	3.4

The results allow to accept the range of endoglucanase activity of 93-1550 U GLA/g, as converted to glucanase equivalent, when using viscometric method. Best results may be obtained in the range of 310-1550 units GLA/g which corresponds to 1240-6200 U/g commonly used in viscometric method, with an expanded uncertainty of 10-12%.

Thus, the assessment of fodder additive endo-β-glucanase activity based on a reduced relative viscosity (or increased relative fluidity) of enzyme-substrate mixture was optimized. A capillary viscometer of 0.73 mm diameter is used. The temperature of hydrolysis is 30 °C, and the acetate buffer (pH 4.7) serves as solvent which corresponds to the optimal enzyme activity when incubating for 5, 10, and 15 min. Of the two possible substrates, β-glucan and carboxymethyl cellulose, the β-glucan 0.4 % solution was preferable as mostly appropriate for the specific enzyme activity. The β-glucanase activity units can be converted to glucose equivalents (units GLA) using calibration curves with a standard enzyme of known activity. Parameters of the accuracy of the developed method were assessed. Expanded uncertainty of measurements ranges within 10-22 % (10-12 %) for the coverage factor k = 2.

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METABOLIC STATUS OF THE COWS UNDER INTRAUTERINE GROWTH RETARDATION OF EMBRYO AND FETUS

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Abstract

Intrauterine growth retardation of embryo and fetus (IUGR) in cows is a polyfactorial syndrome which is defined as an inconsistency between the sizes of forming embryos and fetuses and gestation periods. It is thought that the processes of embryo and fetus growth and development in cows are determined by morphofunctional integrity of gametes entering into the process of insemination and are mainly regulated by the character of maternal nutrition, state of metabolic homeostasis and maternal genitals. This work was devoted to the study of cows' metabolic status under intrauterine growth retardation of embryo and fetus. In 2013 at a large dairy complex (Agrotekh-Garant Ltd. Nashchekino, Anninskii Region, Voronezh Province) a total of 53 Red-motley Holstein cows with average annual productivity of 6.0-6.5 thousand kg were studied for protein metabolism indices (content of total proteins, protein fractions, serum urea), carbohydrates (blood concentration of glucose, lactic and pyruvic acids), vitamins (A, E, C), hormonal homeostasis (blood serum content of progesterone, dehydroepiandrosterone sulfate, testosterone, estradiol, cortisol, triiodothyronine), endogenous intoxication (concentration of middle molecular peptides, urea, creatinine, transaminase blood serum activity) and nitric oxide system on the days 38-40, 60-65, 110-115 and 230-240 of gestation. The impact of these indices on the development of embryo and fetus was also studied. Blood was collected from the jugular vein in the morning before feeding. The evaluation of genitals and metrics of embryo and fetus were done by the method of transrectal palpation and sonography with the use of bovine ultrasound scanner Easi-Scan-3 with 4.5-8.5 MHz linear transducer (BCF Technology Ltd, United Kingdom). The diameter of the fertilized horn, placenta size, body diameter and coccyx-parietal size of the fetus were determined. Coccyx-parietal size of 12-16 mm and body diameter of 7-9 mm were the criteria of development at the age of 38-40 days, 25-45 mm and 12-16 mm — at the age of 60-65 days, respectively. The diameter of the fertilized horn of 9-15 cm and placenta of 10-17 mm were the criteria of development at the age of 110-115 days. The animals with IUGR, diagnosed during 38-40 days of gestation, were included into the experiment on the days 230-240. It is stated that during early stages of fetus formation (38-40 days) the retardation of its growth and development is connected with hypoprogesteronemia determined by hypoplasia of the yellow body. The cows with IUGR also demonstrated blood serum decrease of cortisol by 36.9 % ($p < 0.01$) and increase of triiodothyronine by 35.4 % ($p < 0.005$) in comparison with the animals with physiological gestation course that proves total hormonal imbalance. At the stage of placentation (60-65 days) the cows with IUGR demonstrated evident deficit of nitric oxide that was proved by the decrease of its stable metabolite concentration ($NO^{2-} + NO^{3-}$) in blood serum by 23.9 % ($p < 0.05$) in comparison with the level of the cows with physiological gestation. Authentic decrease of vitamin C content in blood serum, increase of middle molecular peptides level and activity of γ-glutamyl transferase by 42.9-51.0 %, 32.6-67.7 % and 22.1-54.0 % ($p < 0.01$), respectively, in comparison with the animals with physiological gestation were observed in cows with IUGR throughout the research. The article discusses the role of metabolic disorders in pathogenesis of intrauterine growth retardation of embryo and fetus in cows.

Keywords: cows, gestation, intrauterine growth retardation of embryo and fetus, metabolism, hormones, nitric oxide, vitamin C, middle molecular peptides, γ-glutamyl transferase

Intrauterine growth retardation of fetus (IUGR) is quite widespread in farm animals and has a negative impact on the viability of the fetus, the resulting offspring, the act of birth and postpartum period in parturient and postpartum

cows [1-3]. In addition, IUGR syndrome adversely affects postnatal ontogenesis [4, 5] and morphological and functional formation of digestive [5], respiratory [6], and reproductive organs [7]. Predisposition to metabolic and endocrine diseases [5, 8], reduced fertility and productivity [7, 9] are observed in the animals and their offspring. Therefore, IUGR remains one of the main problems of productive animal reproduction, being a considerable reserve to increase the efficiency of modern animal husbandry.

It is known that the normal course of pregnancy, growth, and development of embryo and fetus are determined by morphological and functional usefulness of gametes involved in fertilization, and by the state of hormonal and metabolic homeostasis of the mother's body [5, 8, 10-12].

For the first time, we performed a comparative analysis of the metabolic profile of Red-motley cows at physiological gestation and intrauterine growth retardation of embryo and fetus at days 38-40, 60-65, 110-115, and 230-240 of gestation. We studied the main parameters of protein (total protein, protein fractions, serum urea), carbohydrate (blood concentrations of glucose, lactic and pyruvic acids), and vitamin (A, E, C) metabolism, hormonal homeostasis (serum progesterone, DHEAS, testosterone, estradiol, cortisol, triiodothyronine), endogenous intoxication (serum concentrations of middle molecular weight peptides, urea, creatinine, and transaminases), and nitric oxide system.

The purpose of this research was to study the metabolic status of cows with the syndrome of intrauterine growth retardation of fetus during gestation.

Technique. Research was performed in the winter-stall period of 2013 at Agrotekh-Garant LLC Nashchekino (Annino Region, Voronezh Province) on Red-motley leashed cows with an average annual productivity of 6.0-6.5 thousand kg. A total of 53 cows were examined, including 11 on days 38-40, 18 on days 60-65, 11 on days 110-115, and 13 (of those with IUGR diagnosed at days 38-40) on days 230-240 of gestation.

Examination of genitals and metrics of embryo and fetus was performed by transrectal palpation and sonography using Easi-Scan-3 ultrasound scanner (BCF Technology Ltd., United Kingdom) with 4.5-8.5 MHz linear transducer. Fertilized horn diameter, placenta size, fetal body diameter and parietal-coccygeal size were measured. The criteria of growth retardation were the coccyx-parietal size of 12-16 mm and body diameter of 7-9 mm at the age of 38-40 days, and 25-45 mm and 12-16 mm at 60-65 days, respectively; the diameter of fertilized horn of 9-15 cm and placenta of 10-17 mm were the criteria of growth retardation in 110-115 day old fetus [2].

Blood was sampled from the jugular vein in the morning. Serum concentrations of sex (progesterone, dehydroepiandrosterone sulfate — DHEAS, testosterone, estradiol-17β), corticosteroid (cortisol), and thyroid (triiodothyronine) hormones were measured by ELISA using Hema-Medica (Hema-Medika LLC, Russia) test systems and an Uniplan AIFR-1 immunoassay reaction analyzer (Pikon JSC, Russia).

Serum and whole blood proteins, total immunoglobulins, urea, creatinine, vitamins A, E, C, glucose, lactic and pyruvic acids, inorganic phosphorus, total nitric oxide stable metabolites ($NOx = NO_2^- + NO_3^-$), middle molecular weight peptides (MWP), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyl transferase (GGT) activity were measured using a Hitachi-902 biochemical analyzer (Roche Diagnostics, Japan) and UV-1700 spectrophotometer (Shimadzu, Japan) by standardized assay of metabolic indicators [13-15].

Correlation analysis and *t*-test for independent variables (Statistica 8.0, Stat Soft Inc., USA) were used for data statistical processing.

Results. Intrauterine embryo growth retardation was observed in cows against the sexual and adrenal hormone synthesizing dysfunction and the hormonal homeostasis system imbalance (Table). First of all, this applies to the secretion of progesterone, the main pregnancy hormone which provides endometrium transformation and the trophic function of endometrial glands in relation to the growing fetus. Its blood concentration in these animals was 2.40 times lower (8.0±0.9 nmol/l versus 19.2±1.7 nmol/l, p <0.001) as compared with normal gestation.

Corpus luteum is the main producer of progesterone in early pregnancy. Its insufficient activity manifested in hypoprogesteronemia does not provide an optimal secretory response of uterine glands and enough embryo nutrition, but contributes to the aggressive reaction of peripheral mononuclear cells (monocytes, lymphocytes) against the tissues of the growing placenta and the embryo itself [16-18].

The functional failure of ovarian corpus luteum in cows was followed by the decreased function of adrenal glands which produce glucocorticoid and androgenic hormones so that serum cortisol concentration in the animals with IUGR syndrome was 1.58 times lower as compared to those with normal gestation (5.34±0.49 versus 8.46±3.29 nmol/l, p <0.05).

Hormonal and biochemical blood parameters in Red-motley cows at different gestation periods and at intrauterine growth retardation of embryo and fetus (IUGR) ($X \pm x$, Agrotekh-Garant LLC Nashchekino, Annino Region, Voronezh Province, 2013)

Parameter	Days 38-40		Days 60-65		Days 110-115		Days 230-240	
	norm (n = 5)	IUGR (n = 6)	norm (n = 8)	IUGR (n = 10)	norm (n = 5)	IUGR (n = 6)	norm (n = 7)	IUGR (n = 6)
Progesterone, nmol/l	19.2±1.7	8.0±0.9*	24.9±1.6	18.7±1.8*	7.48±1.19	7.59±0.51	9.13±0.61	15.50±1.20*
Testosterone, nmol/l	1.54±0.11	1.48±0.08	1.57±0.12	1.41±0.05	1.33±0.06	1.42±0.12	2.28±0.38	1.67±0.10*
Estradiol-17β, nmol/l	0.28±0.02	0.29±0.02	0.49±0.04	0.23±0.01*	0.33±0.02	0.44±0.05*	0.75±0.05	0.52±0.02*
DHEAS, μg/ml	0.11±0.01	0.13±0.01	0.19±0.01	0.13±0.01*	0.14±0.01	0.16±0.02	0.33±0.02	0.25±0.01*
Cortisol, nmol/l	8.46±3.29	5.34±0.49*	15.70±1.22	3.44±0.32*	2.46±0.20	2.72±0.19	17.73±1.23	2.95±0.21*
Triiodothyronin, nmol/l	2.85±0.23	3.86±0.27*	7.98±0.64	2.56±0.22*	4.19±0.26	5.13±0.33*	7.67±0.46	3.48±0.24*
Total protein, g/l	80.4±1.6	85.4±3.2	79.7±1.5	78.6±1.6	76.2±1.1	81.0±2.9	81.3±2.9	81.5±3.3
Albumin,%	36.2±3.2	37.4±1.9	38.6±2.0	41.2±2.4	50.1±1.5	46.9±2.1	51.2±0.9	48.9±1.9
α-Globulin, %	13.1±1.1	15.6±1.3	13.4±0.6	13.5±0.6	10.6±0.3	12.4±0.7	9.4±0.6	10.2±0.8
β-Globulin, %	21.6±0.7	21.3±0.8	21.9±0.8	22.4±0.7	20.5±0.2	19.1±1.2	18.5±0.8	19.2±0.6
γ-Globulin, %	29.1±2.4	26.6±1.8	26.1±1.2	22.9±1.6*	18.8±1.4	21.6±1.5	20.9±0.9	21.7±1.5
Total Ig, g/l	32.5±2.4	26.8±1.8*	28.6±2.1	28.4±2.1	27.0±1.4	32.6±1.9*	34.9±2.9	29.4±2.4*
Urea, nmol/l	2.98±0.26	3.29±0.38	3.54±0.40	3.70±0.35	2.40±0.28	2.64±0.20	2.27±0.26	3.28±0.23
Creatinine, mcmol/l	84.0±6.7	95,8±5,9	93,3±6,7	90,5±3,0	92,4±4,6	92,5±3,8	101,0±3,5	102,5±4,4
Glucose, mmol/l	3.72±0.31	4,27±0,21	4,15±0,29	3,89±0,19	2,98±0,22	3,04±0,21	3,24±0,11	3,37±0,19
Lactate, mmol/l	1.24±0.07	1,28±0,05	1,25±0,04	1,20±0,04	1,10±0,02	1,12±0,02	0,85±0,04	0,94±0,09
Pyruvate, mcmol/l	73.6±3.3	61,5±4,5	72,1±6,4	74,4±6,7	117,4±16,1	117,3±14,9	211,0±10,7	198,0±12,6
Vitamin A, mcmol/l	1.58±0.11	1,74±0,29	1,34±0,22	1,60±0,27	1,30±0,15	1,20±0,11	1,31±0,09	1,04±0,11
Vitamin E, mcmol/l	37.8±3.1	31,9±3,4	33,9±4,6	34,9±3,6	25,3±2,3	25,8±2,8	29,4±2,7	28,4±2,0
Vitamin C, mcmol/l	22.2±3.4	12,4±2,7*	19,6±1,1	11,2±0,9*	9,7±0,7	17,2±1,3*	15,1±1,3	7,4±0,6*
ALP, U/l	105.0±8.5	112,3±12,5	146,6±17,2	120,4±8,9	109,8±7,6	96,8±10,9	83,7±7,2	62,8±5,3
AST, U/l	65.6±5.3	66,1±6,1	64,5±4,6	68,6±4,2	70,3±3,9	59,6±2,2	53,1±3,4	57,8±5,3
ALT, U/l	24.0±2.3	21,2±1,7	20,2±1,1	24,5±1,0	25,4±1,9	26,3±2,9	19,0±0,6	20,9±1,9
GGT, U/L	16.3±1.1	19,9±1,1*	15,5±0,6	20,7±2,1*	16,8±1,3	18,2±1,4*	10,0±0,3	15,4±1,2*
Phosphorus, mcmol/l	2.07±0.12	2,63±0,14*	2,10±0,13	2,20±0,13	2,03±0,14	2,40±0,21	2,29±0,07	2,18±0,09
NOx, mcmol/l	102.7±8.3	112,7±10,3	132,4±10,8	100,7±8,7*	108,5±3,7	129,1±5,2*	87,2±6,8	102,3±7,3*
MWP, standard units	0.43±0.04	0,57±0,03*	0,51±0,03	0,71±0,04*	0,31±0,03	0,52±0,08*	0,23±0,02	0,32±0,02*

N o t e. DHEAS — dehydroepiandrosterone sulfate, ALP — alkaline phosphatase, AST — aspartate aminotransferase, ALT — alanine aminotransferase, GGT — γ-glytamyl transferase, NOx — total nitric oxide stable metabolites, MWP — middle molecular weight peptides.
* p < 0.05-0.001 as compared to similar parameters in the cows in physiological gestation.

It is believed that reduced corticosteroid production may manifest in hypothalamic-pituitary dysfunction in relation to the incretion of luteinizing hormone [19] which performs a luteotropic function in pregnant animals.

The transition from embryo to the fetal stage (days 60-65) in physiologi-

cal gestation was followed by the increased functional activity of all endocrine glands, as evidenced by the increase in the serum levels of progesterone by 29.7 % ($p < 0.05$), estradiol-17β by 75.0 % ($p < 0.001$), cortisol by 85.6 %, DHEAS by 72.7 % ($p < 0.001$), and 2.80-fold rise in triiodothyronine level ($p < 0.001$). In IUGR cows progesterone concentrations increased 2.33 times ($p < 0.001$) but remained 33.2 % ($p < 0.05$) lower compared to that of animals in physiological gestation. The levels of other hormones responsible for the synthesis of proteins, bone tissue formation in the fetus and proliferative changes in the tissues of the uterus, decreased compared to baseline values as follows: testosterone by 4.7 %, estradiol-17β by 20.7 %, cortisol by 35.6 % ($p < 0.05$), triiodothyronine by 33.7 % ($p < 0.05$). As compared to the animals in normal gestation, serum concentrations of testosterone, DHEAS, estradiolβ were lower by 10.2 %, 31.6 % ($p < 0.001$), and 53.1 % ($p < 0.001$), respectively, and cortisol and of triiodothyronine levels declined 4.56 times ($p < 0.001$) and 3.11 times ($p < 0.001$), respectively.

In cows with IUGR, immunotrophic interaction of the growing embryo and its mother proceeded against the reduction of serum immunoglobulins by 17.5 % ($p < 0.05$) and increased middle molecular weight peptides by 32.6 % ($p < 0.001$) compared to physiological gestation. Increased concentrations of serum middle molecular weight peptides, on the one hand, indicates activation of serum and tissue protein proteolysis, on the other hand it is the evidence of detoxification processes violation. As molecular analogues of regulatory peptides, MWP are able to block cell membrane receptors, decrease albumin transport capability, and break many metabolic processes in pregnant animals [20].

GGT is known to provide energy dependent amino acid transport into cells thus regulating the serum level of total protein and its fractions. We associate the GGT activity increase by 22.1-33.5 % ($p < 0.05$) in IUGR cows compared to physiological gestation with the enzyme involvement in the processes of detoxification and amino acid pool stabilization, the imbalance of the latter being the most important pathogenetic factor of endogenous intoxication [21].

At placentation phase (60-65 days), IUGR cows demonstrated reduced vitamin C and NOx levels compared to the animals in physiological gestation (by 42.9 and 23.9 %, respectively, $p < 0.001$). Low vitamin C were found in the IUGR cows at days 38-40 — 12.4 ± 2.7 versus 22.2 ± 3.4 nmol/l ($p < 0.001$) in normal embryo growth. Since vitamin C provides antioxidant embryo protection [22] and is involved in the formation of fetal connective tissue [23, 24], its reduced levels in pregnant animals should be regarded as a very unfavorable factor for embryo and fetus development.

Nitric oxide is the key regulator of placental angiogenesis and placental-fetal blood flow [3, 25-27]. Reduced nitric oxide synthesis during pregnancy is associated with delayed formation of placental blood flow, violation of the transfer of nutrients and oxygen, which results in embryo and fetus growth retardation.

At the stage of fetoplacental complex formation (days 110-115), the differences in the hormonal status in IUGR cows and in physiological gestation were less pronounced (see Table). We attribute this to a decline in the endocrine gland hormone synthesizing function necessary for normal growth of a cow fetus [8, 10] which was not observed in IUGR cows. In this period, a start of compensatory mechanisms was registered in the IUGR cows: serum total immunoglobulin increased by 14.8 % ($p < 0.05$), vitamin C increased by 53.6 % ($p < 0.001$), stable nitric oxide metabolites increased by 28.2 % ($p < 0.05$) compared to the previous period. The levels of these substances exceeded that in the cows with normal fetus development by 20.7 % ($p < 0.05$), 77.3 % ($p < 0.001$), and 19.0 % ($p < 0.05$), respectively. However, the expected reduction in endogenous intoxication indicators

was not observed. The blood levels of middle molecular weight peptides in these animals remained relatively high, being 67.7 % greater compared to physiological gestation ($p < 0.05$). GGT activity was also 8.3 % greater ($p < 0.05$).

At the final gestation (days 230-240), hormone producing reserves of endocrine glands, fetoplacental complex and hormone synthesizing function were much higher in the cows with normal embryo development than in the animals with IUGR diagnosed at days 38-40 (see Table). In the latter ones, the levels of main serum hormones that form the parturition dominant — cortisol, estradiol, testosterone and DHEAS were 6.01 times ($p <0.001$), 1.44 times ($p <0.001$), 1.37 times, and 1.32 times ($p <0.001$) lower, respectively. In contrast, in these cows the progesterone which blocks the uterine contractility and prolongs gestation was 1.70 times higher ($p < 0.001$). Progesterone-estradiol ratio was 29.8 in IUGR cows versus 12.2 in cows in physiological gestation. Furthermore, serum triiodothyronine levels reduced 2.20 times ($p < 0.001$) in the IUGR cows, which was followed by a deceleration in all metabolic processes. During this period, the IUGR cows demonstrated a decrease in immunoglobulins (15.8 % lower, $p < 0.05$) and vitamin C levels (2 times lower, $p < 0.01$), while middle molecular weight peptides were 39.1 % higher ($p < 0.01$), stable nitric oxide metabolites were 17.3 % higher ($p < 0.05$), and GGT activity increased by 54.0 % ($p <0.01$). Activated synthesis of placental nitric oxide which has muscle relaxant effects, and progesterone is probably targeted at the uterine contractility restriction to prolong the gestation in IUGR cows.

Thus, realization of the genetic program of fetus formation and development is largely determined by the synthesis and metabolism of sex, corticosteroid and thyroid hormones which are specific regulators of biochemical and biophysical processes in the organisms of mother cow and the fetus. The key point in the growth retardation of embryo and fetus (IUGR) syndrome is the violation of embryo nutrition at implantation and early placentation associated with incomplete secretory endometrium transformation and a delay in the formation of placental-fetal blood flow which is due to an imbalance of sex steroids and decrease in the nitric oxide synthesis. Endogenous intoxication should be considered as one of the determining factors of functional insufficiency in biological mother-embryo-fetus system. The revealed pathogenetic mechanisms causing violations in formation, growth and development of embryo and fetus may be the basis for the development of effective strategies for IUGR prevention and therapy in farm animals.

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BLOOD ENZYMATIC ACTIVITY IN SAANEN GOATS DURING DIFFERENT PERIODS OF THE REPRODUCTIVE CYCLE AND THEIR ASSOCIATION WITH THE COMPLETION OF PREGNANCY

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Abstract

Metabolic processes in milk-producing cows are substantially modified that is the main reason of different abnormalities in the reproductive function. At the same time the pattern of metabolic changes and its influence on the reproductive capacity in high-producing dairy goats (*Capra hircus*) are yet unknown. The aim of the present work was to study the activity of metabolic enzymes in the blood of Saanen goats depending on the period of the reproductive cycle and the competence for pregnancy maintenance. We analyzed for the first time alterations in the serum activity of enzymes, regulating the intensity of protein-carbohydrate and energy metabolism including integration of metabolic processes, at different stages of goat gestation as well as prior to the mating period in animals with the negative outcome of the subsequent pregnancy. The enzyme status of animals was assessed during the pre-mating period, the first half of pregnancy (1.5-2.5 months), and the second half of pregnancy (3.5-4.0 months). Goats were divided into two groups — with the completed reproductive cycle (the birth of viable offspring, $n = 15$) and with the interrupted reproductive cycle (abortions in the second half of pregnancy, $n = 6$). Samples of blood serum were tested to determine concentrations of total protein and activities of several enzymes: aspartate aminotransferase (AST, EC 2.6.1.1), alanine aminotransferase (ALT, EC 2.6.1.2), gamma glutamyltransferase (GGT, EC 2.3.2.2), creatine phosphokinase (CPK, EC 2.7.3.2), alkaline phosphatase (ALP, EC 3.1.3.1), lactate dehydrogenase (LDH, EC 1.1.1.27), and alpha hydroxybutyrate dehydrogenase (isoenzymes of LDH: LDH-1 and LDH-2). In goats with the completed reproductive cycle, the blood activity of AST in the second half of pregnancy was found to be 1.2 times lower ($p < 0.05$) than during the pre-mating period or the first half of pregnancy. The activity of ALT in the blood serum of the animals increased 1.3 times ($p < 0.01$) by 1.5-2.5 months of pregnancy as compared with the pre-mating period and then decreased 1.9 times ($p < 0.001$) by 3.5-4.0 months of pregnancy. Furthermore, there was a decline in the blood activities of GGT (1.3 times, $p < 0.05$), CPK (1.9 times, $p < 0.01$), and isoenzymes LDH-1 and LDH-2 (1.3 times, $p < 0.05$) and a rise in the activity of ALP (1.4 times, $p < 0.05$) between the first and the second half of pregnancy. During the pre-mating period, a lower activity of ALT and GGT was revealed in goats with the interrupted reproductive cycle as compared with animals retained pregnancy (14.4 ± 2.9 vs. 20.1 ± 0.7 U/l, $p < 0.05$ and 43.8 ± 2.4 vs. 54.7 ± 4.2 U/l, $p < 0.05$, respectively). The results of this research suggest a reduction in the intensity of some metabolic processes in the goats by the fourth month of pregnancy to maintain increased fetus demands. Analysis of the findings also indicates that the activity of enzymes, regulating the coupling of protein and carbohydrate metabolism, in the blood of goats during the pre-mating period may be related to their subsequent capacity for fetus bearing.

Keywords: goat, saanen breed, pregnancy, metabolism, blood enzymatic activity, transferases

Currently, development of dairy goat breeding in both Russian Federation and foreign economically developed countries is aimed at the creation of large farms of industrial type [1]. At the same time, herd formation at large goat breeding farms is widely practiced in Russia as a result of the purchase of Saanen goats belonging one of the most highly productive breeds in the world [2]. Improvement of milk production is known to potentially violate a number of physiological functions, including reproduction, which is primarily due to meta-

bolic modifications [3-6]. However, the pattern of metabolic changes and its effects on the reproductive capacity in dairy goats are yet unknown. Attempts have been made to define a metabolism-reproduction association in dairy goats. Thus, blood levels of certain metabolites and metabolic hormones have been studied during postpartum period and depending on goat gestation, gestation toxemia, and the number of lambings [7-10]. In addition, a comparative analysis of metabolic enzyme activity was performed in meat and dairy breeds with the different numbers of kids in the litter [11]. However, the available data do not allow to conclude on the association between metabolic types and the presence of reproductive disorders in dairy goats.

Earlier, we have studied biochemical blood parameters and metabolic interrelations in high yielding cows at different physiological periods and in connection with the service period [12-15]. In particular, we have found that under similar feeding and housing conditions, animals have varying ability to adapt to the metabolic imbalance. In the dry period and at the end of milking period, metabolism was characterized by an increased blood alanine aminotransferase activity (ALT, EC 2.6.1.2) in the cows of higher reproductive potential. Considering the role of ALT in the glucose-alanine cycle, the results indicated the involvement of protein and carbohydrate metabolism in maintaining cows' reproductive capacity in the critical physiological periods.

In dairy goats, reproduction intensity was largely determined by the capability to maintain the pregnancy [16, 17]. However, infections of various etiology are not the only factor resulting in abortion as they were revealed in less than 40 % of abortion [17-19]. The causes of the above pathology have not been determined almost in the half of examined animals. This suggests that a change in the nature or the intensity of metabolism may be one of the reasons for adverse pregnancy outcomes in high yielding goats. Indeed, one of the actual problems of high-yield dairy cattle breeding is gestational toxicosis, which is manifested in the violation of the immune, endocrine, anti-oxidant, and metabolic status of maternal organism [20, 21]. The failure of the mother's adaptive systems to provide the fetus requirements results in various negative consequences, including abortion, premature birth and reduced viability of offspring.

For the first time, we analyzed the alterations in the serum activity of enzymes which regulate the intensity of protein-carbohydrate and energy metabolism including integration of metabolic processes, at different stages of goat gestation and prior to the mating period in the animals with negative outcomes of subsequent pregnancies.

Our purpose was to study the blood metabolic enzymes' activity in the of Saanen goats depending on the period of reproductive cycle and the competence for pregnancy maintenance.

Technique. Studies were performed in the Prinevskoe JSC stud farm (Leningrad region) in 2010-2011. Saanen goats (*Capra hircus*) Saanen (n = 21) aged 3.5 years with an average annual milk productivity of 690-750 kg were the examined. Animal diet complied with the zootechnical standards accepted at the stud farm.

Biochemical parameters were assessed during the reproductive cycle: prior to the mating season (10 days before thee male goat mating), in the first (1.5-2.5 months) and second (3.5-4.0 months) halves of gestation which coincided with the dry period. Blood was sampled by jugular vein puncture in 3-4 hours after feeding. Based on pregnancy outcomes, goats were divided into 2 groups: I — with the completed reproductive cycle (birth of viable offspring, n = 15), II — with interrupted reproductive cycle (abortions in the second half of gestation, n = 6). Blood biochemistry parameters were not studied in

3.5-4.0 months (second half of gestation) in group II animals because of interrupted pregnancies.

Blood serum samples were tested for total protein and activities of several enzymes: aspartate aminotransferase (AST, EC 2.6.1.1), alanine aminotransferase (ALT, EC 2.6.1.2), γ-glutamyltransferase (GGT, EC 2.3.2.2), creatine phosphokinase (CPK, EC 2.7.3.2), alkaline phosphatase (ALP, EC 3.1.3.1), lactate dehydrogenase (LDH, EC 1.1.1.27), and γ-hydroxybutyrate dehydrogenase (isoenzymes of LDH: LDH-1 and LDH-2). Total proteins and enzyme activity were measured using commercial kits (DIALAB, Austria; Randox Laboratories, UK). The results were registered photometrically using a biochemical analyzer RX Daytona (Randox Laboratories, UK).

Biochemical parameters obtained were processed by one-way ANOVA using SigmaStat software (Systat Software Inc., USA). The significance of differences was estimated using Tukey's test for normal distribution or Dunn's test for the absence of normal distribution, the accepted significance level was $p < 0.05$. Correlations were calculated using Spearman's rank correlation coefficient

Results. According to most researchers, the energy balance is the main factor determining the realization of reproductive potential in dairy cattle [22]. Earlier, we have obtained the data on the role of protein and carbohydrate metabolism in maintaining reproductive ability of cows during the critical physiological periods [12-15]. Therefore, the enzymes that regulate carbohydrate and protein, and energy metabolism in animals were subjected to the study, particularly ALT and AST the activity of which was related to the reproduction intensity in high yielding cows [12, 15]. In addition, differences in GGT, CPK, ALP, and AST activity were found in meat and dairy goats with different number of kids per litter [11], which was also the basis for the study of these enzymes.

A comparison of biochemical parameters revealed differences in blood enzyme activity in Saanen goats in the course of a complete reproductive cycle (Table 1).

Activity of blood AST which catalyzes the reversible reaction of aspartate transition to oxaloacetate was found to be lower in the second half of gestation compared to pre-mating period or the first half of gestation ($p < 0.05$). The activity of serum ALT involved in the mutual transformations of alanine and pyruvate increased by gestation months 1.5-2.5 versus pre-mating period ($p < 0.05$), followed by a decrease by gestation months 3.5-4.0 ($p < 0.001$). In addition, there was a negative correlation between the activity of both enzymes and gestational age ($r = –0.690$ and $r = –0.634$ for AST and ALT, respectively, $p < 0.001$). De Ritis ratio (AST/ALT) which characterizes the ratio of catabolic and anabolic processes in the body [23], was 2 times higher in the second half of gestation versus the first half ($p < 0.01$) which was the result of a 2-fold reduce in ALT activity. These findings indicate a simultaneous reduction in the intensity of tricarboxylic acid and glucose-alanine cycles with a shift of metabolic processes towards catabolism in maternal organism by the end of gestation. This conclusion is consistent with the data on total blood protein level which changed with the change in ALT activity unidirectionally, although the change was less significant and was negatively associated with the gestational age ($r = –0.444$, $p < 0.05$). Earlier, in the investigation of blood protein and carbohydrate metabolism enzyme activity in Black-and-White heifers, we found a similar decrease in ALT and AST in gestation months 7-8, which is obviously due to the need to meet the increased fetal requirements in energy and proteins [15]. However, de Ritis ratio was decreased or remained unchanged in heifers (unlike goats) by the end of pregnancy, which is consistent with the data on their ana-

bolic metabolism until the last 2-3 weeks of gestation [24].

1. Blood biochemical parameters in Saanen goats (*Capra hircus*) during the completed reproductive cycle (X±SEM, n = 15, Prinevskoe JSC stud farm, Vsevolzhsk region, Leningrad Province, 2010-2011)

Parameter	Pre-mating period	1-st half of gestation	2-nd half of gestation
AST, U/l	95.8 ± 3.7^{a}	98.3 ± 4.6^{a}	80.0 ± 4.9^{b}
ALT, U/l	20.1 ± 0.7^{c}	25.9 ± 1.0^{d}	13.4 ± 1.9^{e}
AST/ALT	4.9 ± 0.3	3.8 ± 0.2^{f}	7.5 ± 1.2^{g}
GGT, units/l	54.7 ± 4.2	57.5 ± 4.6^{h}	43.2 ± 2.9^{i}
CPK, U/l	202.0 ± 15.0^{j}	253.0 ± 69.0^{j}	131.0 ± 22.0^{k}
ALP, units/l	43.6 ± 2.3^{l}	63.8 ± 4.9^{m}	89.5 ± 6.4^{n}
LDH, U/l	724.0 ± 28.0	709.0 ± 50.0	754.0 ± 40.0
LDH-1 + LDH-2, U/l	309.0 ± 11.0	321.0 ± 21.0^{o}	252.0 ± 11.0^{p}
Total protein, g/L	71.1 ± 2.6	77.8 ± 2.0	71.9 ± 2.7

N o t e. AST — aspartate aminotransferase, ALT — alanine aminotransferase, GGT — γ-glutamyltransferase, CPK — creatine phosphokinase, ALP — alkaline phosphatase, LDH — lactate dehydrogenase, LDH-1 and LDH-2 — isoenzymes of lactate dehydrogenase.

Significant differences between parameters for physiological periods: $^{ab}p < 0,05$; $^{cd}p < 0,01$; $^{ce}p < 0,01$; $^{de}p < 0,001$; $^{fg}p < 0,01$; $^{hi}p < 0,05$; $^{jk}p < 0,01$; $^{lm}p < 0,01$; $^{ln}p < 0,001$; $^{mn}p < 0,05$; $^{op}p < 0,05$.

Blood GGT activity in the first half of gestation was not different from that in pre-mating period, but decreased sequentially by gestation months 3.5-4.0 ($p < 0.05$). In addition, it correlated with gestational age negatively ($r = -0.588$, $p < 0.01$). One of GGT functions is known to be related to the regulation of amino acid transport into the cells via γ-glutamine cycle. Therefore, the reduced activity of this enzyme in the second half of gestation may indicate a decrease in the availability of amino acids in the mother's body both for protein synthesis and as glucogenic substrates.

The activity of CPK which catalyzes the reversible transfer of macroergic phosphates from ATP to creatine and from creatine phosphate to ADP decreased in goat blood ($p < 0.01$) in the second half of gestation and was negatively related to the gestational age ($r = -0,574$, $p < 0.01$). This change in CPK activity indicates a decrease in the intensity of energy metabolism by the end of pregnancy.

On the contrary, the activity of ALP which is involved in nonspecific dephosphorylation and phosphorus transport through cell membranes was increased by 1.5-2.5 months of gestation ($p < 0.05$), and then continued to rise to 3.5-4.0 months ($p < 0.01$). At the same time, we found a positive correlation between ALP activity and the gestational age ($r = 0.456$, $p < 0.05$). Increased ALP activity in goats during pregnancy was apparently due to one of its isozymes, the placental ALP [25]. We can not exclude a compensatory role of this increase in the total energy metabolism against a decrease in activity of tricarboxylic acid cycle, glucose-alanine cycle, etc. Indeed, no increases in blood AP activity are observed in Black-and-White heifers in which the anabolic character of metabolism is maintained in the late gestation [15].

Activity of blood glycolytic enzyme LDH which catalyzes the reversible conversion of lactate to pyruvate reaction did not differ in goats during the reproductive cycle. Lack of relevant changes for LDH in different gestation phases has also been noted earlier in heifers [15]. At the same time, the activity of LDH-1 and LDH-2 isozymes which support aerobic glycolysis, decreased in the second half of gestation ($p < 0.05$). Data analysis revealed the presence of a significant negative correlation between the gestational age and the total activity of the two above LDH isoforms ($r = -0.546$, $p < 0.01$). These isozymes that are present mainly in the heart muscle, erythrocytes, platelets, brain and kidney tissues, have a high affinity for lactic acid and convert it efficiently into pyruvate for subsequent inclusion in tricarboxylic acid cycle. A decrease in LDH-1 + LDH-2 activity indicates a reaction shift toward formation of lactate and, thus, a

reduction in the Krebs cycle intensity, at least in cells of the above types, which agrees with the detected decrease in AST activity in the second half of gestation. In addition, these findings give reason to believe that the lactic acid excess in goats in this period is transported to the bloodstream of the fetus and can be used by the latter for the synthesis of non-essential amino acids and lipids and as an energy substrate (after oxidation to pyruvate). The same is known to take place in sheep [26]. This assumption is also evidenced by the data on the increased fetal lactate uptake rate in horses by the end of pregnancy [27].

Thus, activity of some enzymes related to protein-carbohydrate and energy exchange decreased in Saanen goats by gestation month 4. Decreased activity of certain blood enzymes was also observed in the second trimester of pregnancy in women [28] and in the third trimester of gestation in heifers [15]. This suggests that at certain stages of pregnancy (specific of mammal species), maternal organisms start to function in the mode of saving own resources to maintain increased fetal requirements

When comparing blood enzyme activity in pre-mating period, differences in ALT and GGT activity were found in goats with different capacity to maintain pregnancy (Table 2). Blood ALT and GGT activity in the animals with interrupted reproductive cycle was considerably lower compared to the goats with retained pregnancy ($p < 0.05$). At the same time, there were no significant differences in blood biochemical parameters in the animals of compared groups in the first half of gestation (see Table 2). The increase in blood ALT activity by gestation months 2-3 had different intensities in the two groups. It increased 1.3 times ($p < 0.001$) in the goats with completed reproductive cycle and 1.8 times in the goats with interrupted cycle. At the same time, blood GGT activity in the first half of gestation did not differ from that in pre-mating period in both goat groups.

2. Blood biochemical parameters in Saanen goats (*Capra hircus*) in pre-mating period and in the first half of gestation in further completed and interrupted reproductive cycles (X±SEM, Prinevskoe JSC stud farm, Vsevolzhsk district, Leningrad region, 2010-2011)

Parameter	Group I (n = 15)	Group II (n = 6)
Pre-mating period		
AST, U/l	95.8±3.7	89.2±8.0
ALT, U/l	20.1±0.7	14.4±2.9*
AST/ALT	4.9±0.3	11.3±5.7
GGT, U/l	54.7±4.2	43.8±2.4*
CPK, U/l	202.0±15.0	172.0±8.0
ALP, U/l	43.6±2.3	38.1±5.1
LDH, U/l	724.0±28.0	679.0±60.0
LDH-1 + LDH-2, U/l	309.0±11.0	307.0±21.0
First half of gestation		
AST, U/l	98.3±4.6	95.7±2.9
ALT, U/l	25.9±1.0	25.9±1.6
AST/ALT	3.8±0.2	3.8±0.3
GGT, U/l	57.5±4.6	48.0±3.7
CPK, U/l	253.0±69.0	216.0±17.0
ALP, U/l	63.8±4.9	73.4±8.7
LDH, U/l	709.0±50.0	627.0±73.0
LDH-1 + LDH-2, U/l	321.0±21.0	283.0±18.0
Total protein, g/l	77.8±2.0	80.1±3.4

Note. AST — aspartate aminotransferase, ALT — alanine aminotransferase, GGT — γ-glutamyltransferase, CPK — creatine phosphokinase, ALP — alkaline phosphatase, LDH — lactate dehydrogenase, LDH-1 and LDH-2 — isoenzymes of lactate dehydrogenase; group I — animals with completed reproductive cycle (birth of viable offspring), group II — animals with interrupted reproductive cycle (abortions in the second half of gestation).
* Intergroup differences are significant at $p < 0.05$.

Thus, the enzyme status of goats with interrupted reproductive cycle (abortion in the second half of gestation) is characterized by reduced ALT and GGT activity in pre-mating period. This demonstrates the reduced intensity of

glucose-alanine and γ-glutamine cycles and hence the decreased gluconeogenesis rate in the formation of mature oocytes in the ovary. To date, adequate glucose provision for maturing mammalian oocytes has been found to be crucial for their capacity of further embryonic development [29, 30]. In this, glucose entry into the blood in ruminants is mainly provided by gluconeogenesis. Earlier, we have demonstrated that decreased blood ALT activity in high yielding cows at the end of milking period (before the onset of pregnancy) is associated with the increased duration of service period [12, 13] which is known to be largely depend on embryonic and early fetal mortality [31]. Data obtained in dairy goats point to a possible link between the intensity of gluconeogenesis in pre-mating period and oocyte quality characteristics that determine fetal capacity to survive in the later gestation period.

Is necessary to note that female fertility may be affected not only by metabolic, but also by immune, endocrine, oxidative and other factors that were beyond the scope of this study.

Thus, the comparative study of metabolic blood enzymes in the high productive Saanen goats in pre-mating period and during gestation suggest a reduction in the intensity of some metabolic processes in the animals by the fourth month of pregnancy to maintain the increased fetal requirements in energy and metabolic substrates. Our findings also demonstrate that the activity of enzymes which regulate relationship of protein metabolism to carbohydrate metabolism in goat during the pre-mating period may be related to the subsequent fetus maintenance.

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ADAPTIVE ABILITY OF HOLSTEIN CATTLE INTRODUCED INTO NEW HABITAL CONDITIONS

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Abstract

Use of artificial insemination technologies and a purebred animal international trading led to global spreading commercial American and West-European breeds, which possess high productivity potential, but are very demanding to the feed quality, stuff experience and zoohygiene conditions. The highest genetic potential is characteristic of the Holstein cattle from the USA and Canada. Wide use of the Holstein breed in the Russian Federation resulted in rise of dairy productivity and improvement of dairy cattle technological parameters, but some problems of imported animals' health and adaptation have been revealed. The high dairy productivity inevitably leads to the weakening immunity, decreased fertility and less stress resistance. Disease susceptibility, in its turn, also ultimately decreases the productivity, fertility and the time of farming use in highly-productive cows. Nowadays a great importance is given to breeding for production traits, while the lack of resistance to the external factors as a cause of diseases and reduced performance is still remaining less studied. Our main aim was to study the adaptive abilities of the Holstein cattle of Russian and the US origin which were moved to Kabardino-Balkarian Republic in comparison with Black-motley animals long reared under the local conditions of Kabardino-Balkaria. The investigations were carried out on the farms of a pre-mountain zone (Agro-Concern Golden Kolos LLC, Soyuz-Agro LLC). The heifers were divided into 3 groups, 30 animals per each, with regard to age, physiological state, origin and productivity. The Black-motley heifers were the control group, the Russian Holstein heifers were the group 1, and the American Holstein heifers were the group 2. In the groups we studied blood bactericidal, lysozyme, complement and phagocyte activity. The dairy production was estimated monthly during the first two lactations. Fat and protein levels in milk, and cow weight were recorded, and the milkiness index was calculated. The superiority of Black-motley heifers and first-calf cows in blood bactericidal activity (by 5.7-8.3 %, $P > 0.999$, and 5.4-7.5 %, $P > 0.999$, respectively), blood lysozyme activity (by 2.2-3.1 %, $P > 0.999$, and 1.8-4.5 %, $P > 0.999$, respectively), blood complement activity (by 0.4-0.6 %, $P > 0.95$-0.99, and 3.2-5.0 %, $P > 0.99$-0.999) was found, whereas the Russian and American Holsteins were shown to possess more intensive phagocytosis (i.e., 4.9-7.7 % higher in heifers, $P > 0.99$-0.999, and 2.6-3.8 % higher in first-calf cows, $P > 0.95$-0.99). There was a true milk yield priority of 2227 kg ($P > 0.999$) in the first lactation and 2465 kg ($P > 0.999$) in the second lactation in the Holsteins originated from the US when compared to domestic Black-motley cows of the same age. However, the Black-motley cows surpassed the Holstein coevals of foreign origin due to higher milk fat and protein. Note, for the whole observation in all breeds studied the milk fat and protein were higher compared to the breed standards. Though milk quality was higher in the Black-motley cows, the total milk fat and protein yield was higher in the Holsteins, so that during the first lactation the difference in milk fat and protein output between the first-calf cows averaged 47.9-74.8 kg ($P > 0.999$) and 41.0-63.3 kg ($P > 0.999$), respectively, and the same trend was found in the second lactation. The maximum milking index was observed in Holstein cows of the US breeding which were superior to Black-motley cows and Russian Holstiens of the same age on average by 366-373 kg ($P > 0.999$) and 135-141 kg ($P > 0.95$), respectively. Thus Holstein cows bred in Russia and the US are quite successfully adapted to the conditions of Kabardino-Balkaria.

Keywords: Black-motley cattle, selection, adaptation, dairy productivity

The criteria for cows' adaptation to milk production technology are the realization of the genetic potential of productivity and its retaining under extreme stimuli, the ability to reproduce healthy offspring, the time of economic use, and the resistance to diseases [1].

Identification of commercially valuable individuals, introduction of modern reproduction techniques (artificial insemination, embryo transplantation,

genetic engineering) and international trade of breeding material resulted in the global spread of a number of so-called commercial American and West-European breeds [2-4]. They possess a high productivity potential, but are very demanding to the feed quality, stuff experience, zoohygienic factors (temperature, humidity, light conditions), the environment, and veterinary services [5-6].

The diets imbalanced for essential nutrients, inconsistency of animal living conditions with the physiological needs of the organism under increasing productive potential lead to appearing and developing reproductive pathological processes, violation of reproductive function, and early culling [7]. The greatest losses of offspring (57 %) are associated to embryo death, and the problems of placentation (16 %) are in the second place [8].

Close interrelation of the reproductive function of highly productive cattle with a genetic component, adequate nutrition and a positive energy balance has been shown in the research of foreign authors [9-11]. Their studies examine the dependence of herd reproduction on particular factors, but the problem necessitates a comprehensive approach [12, 13]. One of the main causes of the problem of highly productive cattle reproduction is so-called long-term negative energy balance, at the peak of lactation especially [14, 15].

It is believed that increasing natural resistance of dairy cows is a way to increase their long-term use [16]. This is based on the understanding of the role of genetic factors in determining diseases and the possibility of a corresponding change in the genetic structure of farm populations. In this, the complex relationship between the productivity and resistance (adaptability) must be accounted for which complicates the direct estimation and forecasting the manifestations of the specified traits [17].

Wide use of imported Holstein bulls in Russia resulted in a rise of dairy productivity in crossbred milk cows, but at the same time, previously absent hereditary diseases appeared [18] as associated with a high milk production genes [19, 20]. According to N.P. Sudarev et al. [21], importing breeding stock indicates the limited own resources in Russia due to low herd reproduction.

Assessing adaptive qualities of Holstein cattle when moving it in different ecological and geographical conditions, domestic and foreign scientists disagree on the adaptive abilities, productive qualities, duration of economic use, and lifetime milk production.

Breeding cattle is based historically on the selection of phenotypes, that is a set of genes is selected that contribute to the particular phenotype manifestation. Strong returns require higher body resources which are not endless. High milk yielding inevitably results in the immunity and fertility weakening, a decrease in stress resistance, diseases, and a reduction of productive longevity [22, 23].

Among dairy cows, the highest genetic potential is characteristic of the Holstein cattle from the USA and Canada [24]. However, an introduction and continued use of highly productive cattle under new climatic, environmental and feeding conditions necessitate estimation of their adaptation ability [25, 26].

For the first time, we studied the productive features and immunobiological mechanisms of resistance in imported Holstein cattle under the conditions of Kabardino-Balkarian Republic, and confirmed its high acclimatization.

In this paper we summarize obtained data on the adaptive abilities of Holstein cattle of Russian and American origin introduced to Kabardino-Balkarian Republic versus Black-motley cows long reared under the local conditions of the area.

Technique. The research was performed in 2013-2015 at the farms of the pre-mountain Kabardino-Balkarian Republic area (Agro-Concern Golden

Kolos LLC, Soyuz-Agro LLC) specialized in breeding Black-motley and Holstein cattle. Three heifer groups were formed, 30 animals per group regarding age, physiological state, origin, and productivity, with calving occurred during the experiment. Black-motley heifers were the control group, Russian-bred Holstein heifers were group I, American-bred Holstein heifers were group II.

The cellular and humoral factors of immunity was studied at the republican blood transfusion station (RSPK KBR, Nalchik) using conventional methods. We assessed blood bactericidal, lysozyme, complement activity, and neutrophil phagocytic activity [27, 28]. Dairy production (milk yield, milk fat and protein) was estimated monthly during the first two lactations. Milk fat and protein levels were estimated as described [29]. Live weight was monitored. Milking index was calculated as the ratio of the milk yield to the animal live weight.

Blood was sampled from 20 animals of each group at the time when the animals were heifers, and during lactation. Milk productivity values were evaluated in all cows of each group.

Within the study, the animals were kept under the similar feeding and living conditions. Feeding was carried out using conventional farm diets regarding the actual feed nutritional value, lactation period, milk productivity, body weight, and physiological state [30].

The data were processed biometrically [31].

Results. Experimental groups of animals differed in their immunity parameters in different age periods (Table 1).

Blood bactericidal activity (BBA) characterizes the humoral immunoreactivity. BBA maximum was reported in experimental livestock during pregnancy and averaged 54.5-62.8 % which was 5.6-6.4 % greater ($P > 0.99$) compared to first-calf cows. Higher BBA in Black-motley cows compared to Holsteins was apparently due to their lasting breeding in the climatic and feeding conditions of the area.

1. Humoral and cellular immunity parameters (%) in Black-motley and Holstein heifers and first-calf cows of different origin ($X \pm m_x$, Kabardino-Balkarian Republic, 2013-2015)

Parameter	Group		
	control ($n = 20$)	experimental I ($n = 20$)	experimental II ($n = 20$)
Heifers			
BBA	62.8±1.0	57.1±1.2	54.5±1.4
BLA	27.3±0.3	25.1±0.4	24.2±0.4
NPA	65.7±1.2	70.6±1.4	73.4±1.3
BCA	13.6±0.1	13.2±0.2	13.0±0.2
First-calf cows			
BBA	56.4±0.8	51.0±0.9	48.9±1.1
BLA	28.2±0.4	26.4±0.3	23.7±0.4
NPA	54.0±0.7	56.6±0.9	57.8±1.0
BCA	13.3±0.2	12.4±0.2	11.9±0.2

Note. BBA - blood bactericidal activity, BLA - blood lysozyme activity, NPA - neutrophil phagocytic activity, BCA - blood complement activity. Group description is given in the Methods section.

Lysozyme is an important humoral nonspecific immunity factor, in particular it has a stimulating effect on phagocytosis and bactericidal effects on some microorganisms. Depending on the physiological state of the animals, they differed in lysozyme activity. Thus, while in Russian-bred Black-motley and Holstein cows this value increased somewhat during lactation compared to pregnancy (by 0.9 % at $P < 0.95$ and 1.3 % at $P > 0.95$, respectively), then it decreased by 0.5 % ($P < 0.95$) in American animals of the same age. Irrespective of age, the highest lysozyme activity was characteristic of Black-motley animals, as during pregnancy they surpassed Holsteins of the same age of different origin by an average of 2.2-3.1 % ($P > 0.999$), and in the milk production period by 1.8-4.5 % ($P > 0.999$).

Phagocytosis is one of the efficient cellular defense mechanisms. Blood phagocytic activity was higher in American-bred Holsteins which provided them with the protection from adverse environmental factors when moving into new agroclimatic, technological, and feeding conditions. The differences versus Black-motley heifers characterized by minimum indexes, amounted to 7.7 % during pregnancy

(P > 0.999) and 3.8 % (P > 0.99) during lactation. It should be noted that domestic Holsteins were intermediate for this trait. Along with the breed differences, we observed an age-related decline of phagocytosis, mostly notable in Holstein cattle (14.0-15.6 %, P > 0.999) and the lowest in the same age Black-motley animals (11.7 %, P > 0.999).

Blood complement activity and neutrophil phagocytic activity decreased with age, with the greatest decline in Holstein cows — by 0.8-1.1 % (P > 0.99-0.999). During pregnancy and lactation, this value in Black-motley cows was greater compared to American-bred Holstein cows by 0.6 and 1.4 % (P > 0.99 and P > 0.999), respectively.

For a more complete characterization of Holstein cattle adaptive abilities, we studied the dairy traits in the experimental herds (Table 2).

In the first lactation, the milk yields were by 2227 kg (P > 0.999) and 1381 kg (P > 0.999) greater in American-bred and domestic first-calf Holsteins compared to Black-motley cows of the same age. Similar differences were observed in the 2-nd lactation: in Holstein cattle, it was by 2465 (P > 0.999) and 1532 kg (P> 0.999) greater, respectively. Higher coefficients of variation in milk yields in Black-motley cows (Cv = 16-17 %) compared to Holsteins of different breeds (Cv = 13.6-14.9 %) are noteworthy, which indicates the possibility of increasing milk production in Black-motley cattle populations through the appropriate screening and selection of animals.

2. Milk productivity and weight in Black-motley and Holstein animals of different origin ($X \pm m_x$, Kabardino-Balkarian Republic, 2014-2015)

Parameter	Group		
	control (n = 30)	experimental I (n = 30)	experimental II (n = 30)
1-st lactation			
Yield of milk within 305 days of lactation in kg	5937±184	7318±198	8164±217
Fat level in milk in %	3.73±0.02	3.68±0.02	3.63±0.03
Protein level in milk in %	3.39±0.02	3.31±0.02	3.24±0.02
Milk fat yield in kg	221.40±6.80	269.30±7.30	296.20±7.80
Milk protein yield in kg	201.20±6.20	242.20±6.50	264.50±6.90
Weight at the 2-nd to 3-rd months of lactation in kg	526.00±4.10	538.00±3.80	546.00±3.40
Milking index in kg	1129±34.6	1360±36.8	1495±38.7
2-nd lactation			
Yield of milk within 305 days of lactation in kg	6724±195	8256±211	9189±232
Fat level in milk in %	3.76±0.02	3.71±0.03	3.65±0.03
Protein level in milk in %	3.41±0.02	3.34±0.02	3.27±0.02
Milk fat yield in kg	252.80±7.20	306.30±7.70	335.40±8.40
Milk protein yield in kg	229.30±6.50	275.70±6.90	300.50±7.50
Weight at the 2-nd to 3-rd months of lactation in kg	554.00±4.30	571.00±4.00	579.00±3.70
Milking index in kg	1214±35.2	1446±36.9	1587±39.5

Age-related changes in milk yields demonstrated the greatest increase in Holstein cows (average of 938-1025 kg), that is, the environmental conditions were comfortable to realize high productive qualities. However, greater milk fat yields were characteristic of Black-motley cows that were superior to Holsteins of foreign origin of the same age by an average of 0.10 % (P > 0.99) in the 1-st lactation and by 0.11 % (P > 0.99) in the 2-nd lactation, indicating lower milk fat yields in the ancestors of American-bred Holstein bulls. Total milk protein yields were by an average of 0.07-0.15 % (P > 0.95-0.999) higher in Black-motley cattle than in Holsteins. An increase of fat and protein levels of an average of 0.02-0.03 % was observed in experimental groups in the 2-nd lactation versus the 1-st one.

The daughters of Black-motley and Holstein bulls exceeded the minimum requirements for milk production parameters. Thus, in the 1-st lactation, milk yields exceeded standard values by 2437 kg in Black-motley cows, by 2818 kg in domestic Holstein cows, and by 3664 kg in American-bred Holsteins; in the

2nd lactation the exceeding amounted 2924, 3256, and 4189 kg, respectively. However, the 13.3 % increase in milk production from the 1-st to the 2-nd lactation was the greatest in Black-motley cows while it was 12.8 % in domestic cows of Holstein origin and 12.5 % in North American animals. Different increases in milk yields in experimental cows was apparently due to different realization of the productive traits in Holstein cattle in the new geographical, technological and feeding conditions.

Fat and protein levels in milk in all lactations studied exceeded standards for the respective cattle breeds. The qualitative parameters of milk exceeded the minimum requirements in domestic-bred Holsteins greater than in the population of foreign origin which indicates the use of the bulls of domestic origin of higher fat-and-protein lines in the breeding process.

Despite the higher milk characteristics in Black-motley cows, milk fat and protein yields were higher in the groups of Holstein origin which was due to their higher milk productivity. So, the milk fat yield differences between the first-calf cows of the control and experimental groups averaged 47.9-74.8 kg ($P > 0.999$), and milk protein yield differences were 41.0-63.3 kg ($P > 0.999$). The similar tendency was in the second lactation.

The body weight is important parameter in cow breeding. This breed and constitutional trait characterizes animal development and is associated with milk and meat productivity. The body weight of experimental foreign-bred cows at the 2-nd to 3-rd months of lactation was by 20-25 kg ($P > 0.999$) higher as compared to that in domestic Black-motley cattle. This parameter was intermediate in Holstein cows of Russian origin and close to American-bred cows of the same age.

Milking index is a parameter of milk production per center of cow body weight. The maximum milking index was observed in Holstein cows bred in USA that were superior to Black-motley cows and Russian Holstiens of the same age by 366-373 kg ($P > 0.999$) and 135-141 kg ($P > 0.95$) on average, respectively. The obtained milking index characterize all experimental cows as a herd of dairy production type.

Thus, Holstein cows bred in Russia and USA are quite successfully adapted to the conditions of Kabardino-Balkarian Republic. In particular, the lack of humoral protective factors in Holsteins compared to Black-motley cows is compensated by more intense phagocytosis which is a compensatory response to the new agro-climatic, technological and feeding conditions. The high qualities of both domestic- and American-bred Holstein cattle is proved by their considerable superiority over local Black-motley cows of the same age in milk yields, milk fat yields, and milkling indices

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MOLECULAR AND BIOLOGICAL PROPERTIES OF PATHOGENIC NEWCASTLE DISEASE VIRUSES ISOLATED IN KAZAKHSTAN

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Abstract

Currently, Newcastle disease (ND) is highly contagious viral infection of birds, characterized by pneumonia, encephalitis, multiple pointed hemorrhages and defeat of internals is spread in various regions of the world. To the present, all isolated ND viruses (*Paramyxoviridae*, *Paramyxovirus*) are divided into two classes, representing the diverse and constantly developing group of viruses. Despite universal vaccination, the disease is difficult to control, and in connection with this the ND causal agent is listed among the most important pathogens. In recent years, studies on the genetic variability of the ND strains in Kazakhstan were not conducted, although many transcontinental migratory routes of wild birds, the main carriers of the pathogens, are crossed exactly here. The present study was conducted to examine the characteristics of the circulation, as well as the isolation and characterization of isolates of Newcastle disease virus that caused the disease of poultry in different regions of Kazakhstan in 2010, 2012 and 2013. Using 10-day-old developing chicken embryos (DCE), we studied virus isolates from dead hens at ND outbreaks in poultry farms and private yards in Almatinskaya, North Kazakhstan and Zhambylskaya provinces of Kazakhstan. The mean death time of embryos (MDT) and intracerebral pathogenicity index (ICPI) were estimated. Viral RNA was isolated and used for PCR. Amplified products were further detected, purified and sequenced. The obtained nucleotide sequences were analyzed using Sequencher v. 4.5 (Gene Codes Corporation, USA). A set of nucleotide sequences from an international database GenBank was used to construct the dendrogram and determine the genotype. Phylogenetic analysis of the sequences was performed using Mega 6.06 and the following parameters: Statistical Method — Neighbor-joining; Test of Phylogeny — Bootstrap method; No. of Bootstrap Replications — 500; Model/Method — Kimura 2-parameter model. Studies have shown that the ND virus causes outbreaks both among vaccinated and non-vaccinated poultry. The ND isolates belong to velogenic strains. All of them had a proteolytic cleavage site ^{110}GGRRQKRF^{117} in the fusion protein, which is characteristic of the V-pathotype. The sequencing and phylogenetic analysis of the *F*-gene showed that the virus from dead birds of those vaccinated at the poultry farm in Almatinskaya Province belongs to VIId genotype, while the isolates from non-vaccinated birds of the private farms in Almatinskaya, Zhambylskaya and North Kazakhstan provinces belong to VIIb genotype. According to the obtained information, despite the geographical distance of outbreaks, the same ND virus genotypes are circulating in the territory of Northern Kazakhstan and in the southern regions of the country. Wide spread of the virus in Kazakhstan requires from veterinary services to develop effective control measures with regard to ND molecular epidemiology.

Keywords: Newcastle disease, strain, pathogenicity index, PCR, sequencing, phylogenetic analysis

Newcastle disease (ND) is a highly contagious poultry viral infection characterized by pneumonia, encephalitis, multiple pointed hemorrhages and failure of internal organs [1]. It was first registered in the island of Java in 1926 [2]. The causative agent (RNA virus belonging to family *Paramyxoviridae*, genus *Paramyxovirus*) was isolated and described as a filterable virus during an outbreak in the city of Newcastle (UK, 1926) [3], and the disease was called Newcastle disease. ND virus was found in 241 species from 27 orders of the *Aves* [4].

All ND viruses isolated to date are divided into two classes representing the diverse and constantly developing groups. Class I viruses are distributed worldwide among wild birds, are low virulent, and are currently divided into nine genotypes [5]. Class II viruses have been divided into 10 genotypes until recently. However, in 2012, D.G. Diel et al. [6] proposed a new genetic classification of ND viruses. The authors divided the class II viruses into 15 genotypes which included 10 previously known and 5 new ones. Later, having examined the viruses isolated in the Dominican Republic and Mexico, S.C. Courtney et al. [7] demonstrated the existence of genotype XVI, and C.J. Snoeck et al. [8] classified the viruses isolated in the West and Central Africa as genotypes XVII and XVIII.

Currently, phylogenetic analysis has been performed for a large number of ND virus strains, and the main genotypes circulating in different parts of the world have been identified, a total of 18 [6, 7]. Group of scientists from the CIS countries have found that all ND virus isolates from poultry and commensal birds in the territory of Russia, Kazakhstan, Kyrgyzstan, and Ukraine from 1993 to 2007, belonged to genotypes VIIa, VIIb, and VIId [9-11]. It has also been shown that in the territory of Russia, class II genotype VI viruses circulate in pigeon populations [12, 13], genotype I viruses circulate in waterfowl populations [13, 14], and genotype VII viruses circulate in poultry [15, 16]. However, despite the studies of the virus in different countries, including the Republic of Kazakhstan, there are still mass poultry diseases followed by high mortality.

In recent years, genetic variability studies in ND strains have not been conducted in Kazakhstan. In this study, we studied the variations of ND virus circulating within the territory of the Republic of Kazakhstan that have been changed in the process of evolution, which was of particular interest as it is here many transcontinental migration ways of wild birds, the main carriers of the infection, intersect.

This work was aimed to study the specificity of circulation, isolation and characterization of Newcastle disease virus isolate that cause poultry disease in different regions of the Republic of Kazakhstan.

Technique. Virus isolates from dead poultry were studied at ND in farms and private yards in Kazakhstan in 2010, 2012, and 2013

10-Day-old developing chicken embryos (DCE) were used as a culture system. Mean death time (MDT) was estimated by dividing the sum of all embryos' death hours caused by the minimal lethal dose, by the number of embryos [17]. Intracerebral pathogenicity index [ICPI] was estimated using the standard method (18).

RNA was isolated using QIAAmp Viral RNA mini kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. For PCR products, a primer pair [19] (Fwd-upper-f1 — TTGCTTATAGTTAGTTCGCCTGTC, Rev-down-f2 — ACCCGTGTATTGCTCTTTGG) and One-step RT-PCR Kit (Qiagen GmbH, Germany) were used.

PCR products were detected in 1 % Tris-acetate buffer supplemented with ethidium bromide in Bio-Rad gel documentation system (Bio-Rad Laboratories Inc., USA). PCR products were purified using QIAquick PCR purification kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. Sequencing of PCR products was performed using BigDye terminator v.3.1 cycle sequencing kit (Applied Biosystems Inc., USA) and an automatic 3130xl Genetic Analyzer (Applied Biosystems Inc., USA; Hitachi, Japan)

The obtained nucleotide sequences were analyzed using Sequencher v. 4.5 (Gene Codes Corporation, USA). Mega 6.0 software [20] was used for nucleotide sequence alignment. A set of nucleotide sequences from GenBank was used to construct the dendrogram and determine the genotype. Phyloge-

netic analysis of sequences was performed using Mega 6.06 and the following parameters: Statistical Method — Neighbor-joining; Test of Phylogeny — Bootstrap method; No. of Bootstrap Replications — 500; Model/Method — Kimura 2-parameter model.

Results. Epizootic welfare in poultry farms in the Republic of Kazakhstan is maintained due to intensive poultry vaccination from the first days of life. Vaccination regimes are practiced in many farms to maintain a high titer of post-vaccination antibodies required for poultry immunity to Newcastle disease. But despite all the efforts, the Newcastle disease epizootic outbreaks damage the poultry industry in Kazakhstan

In November 2010, there was a mass death of 30-40-day-old broiler chickens at the Allele Agro poultry farm (Ili district, Almaty region). The farm had experienced a preventive vaccination program, poultry were vaccinated with a live vaccine (Nobilis ND Clone 30, Intervet international B.V., Netherlands). Despite vaccination, over 2,000 birds died within one week. In October 2012, mass poultry death was registered at the private yards of Aksuat settlement (Timiryazevskiy district, North-Kazakhstan region) where more than 900 individuals died. In June 2013, mass poultry death was observed at the private yards of Otar (Korday district, Zhambyl region) and Matybulak (Zhambyl district, Almaty region) settlements as well. Poultry of the private yards have not been vaccinated against ND.

We found that the disease and the death of poultry were caused by ND virus in all cases (Table).

Biological characteristics of Newcastle disease virus isolates from different regions of the Republic of Kazakhstan ($X \pm m$, n = 3, culture on 10-day-old developing chicken embryos)

Isolate (ID GenBank)	Site (year of isolation), host	BA	HA	MDT	ICPI	CS
Chicken/KZ/Almaty/11/2010 (KT719396)	Almaty region (2010), domestic chicken	9.20±0.047	8.06±0.12	56	1.76	^{110}GGRRQKRF^{117}
Chicken/KZ/SKO/12/2012 (KT719397)	North-Kazakhstan region (2012), domestic chicken	8.80±0.080	8.53±0.12	52	1.82	^{110}GGRRQKRF^{117}
Chicken/KZ/Kordai/06/2013 (KT719398)	Zhambyl region (2013), domestic chicken	9.26±0.074	8.20±0.08	56	1.82	^{110}GGRRQKRF^{117}
Chicken/KZ/Almaty/07/2013 (KT719399)	Almaty region (2013), domestic chicken	9.58±0.069	8.73±0.05	56	1.80	^{110}GGRRQKRF^{117}

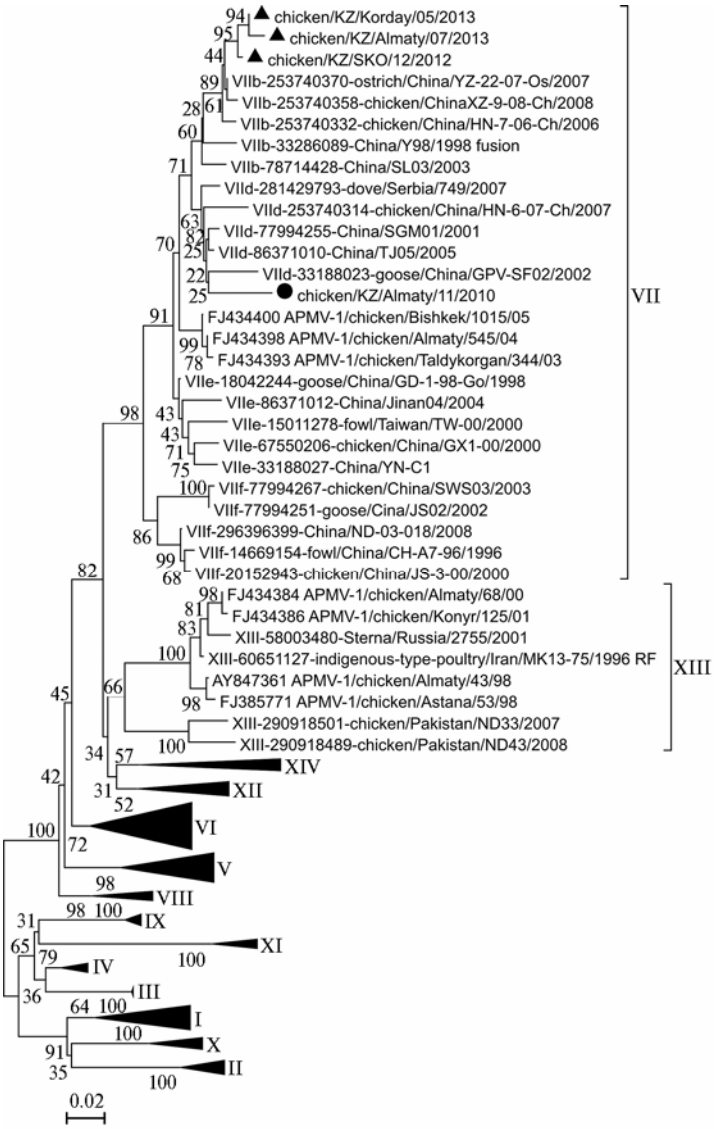
N o t e. BA — biological activity, lg $EID_{50}/см^3$ (embryo infectious dose); HA — hemagglutinating activity, log_2; MDT — mean chicken embryo death time, h; ICPI — intracerebral pathogenicity index; CS — gene *F* product proteolytic cleavage site.

Mean embryo death time for isolated ND was 52-56 hours which corresponds to the velogenic ND virus strains. ICPI was in the range of 1.76-1.82 and confirmed the virulence of ND viruses isolated in Kazakhstan.

Analysis demonstrated the presence of the similar sequence in the proteolytic cleavage site (^{110}GGRRQKRF^{117}) in the fusion protein F, which is characteristic of the velogenic ND V-pathotype [21]. Consequently, the results of genetic studies were consistent with those of biological tests (ICPI and MDT) and confirmed the virulence of ND isolates studied.

An in-depth study of genetic relationships between the viruses isolated in different various areas provided important information on ND molecular epidemiology. It was necessary to find out the genotype belonging of the strains that have caused epizootic Newcastle disease outbreaks in Almaty, Zhambyl, and North Kazakhstan regions. For this purpose, we performed alignment of nucleotide sequences of isolated viruses and the sequences of ND virus available in GenBank, and constructed a phylogenetic tree. All ND viruses isolated in Kazakhstan were close to the ones circulating in Asia and belonged to genotype VII. The VII genotype viruses were first isolated in Taiwan in 1984 [22]. Later, they were found in Europe [23], China [24], Africa [25, 26], and in the CIS

countries — in Russia, Ukraine, Kazakhstan, and Kyrgyzstan [9-11, 27].



Phylogenetic tree of gene *F* which encodes the fusion protein of Newcastle disease virus class II: I-XIV — genotypes; ▲ and ● — isolates from Kazakhstan (VIIb and VIId genotype viruses, respectively; dendrogram constructed using nucleotide sequences deposited in GenBank, Neighbor-joining method, bootstrap = 500).

Using the reference strains proposed by D.G. Diel et al. [6], ND virus Chicken/KZ/Almaty/11/2010 isolate obtained from vaccinated poultry that have died at the poultry farm was assigned to the genotype VIId, and Chicken/KZ/SKO/12/2012, Chicken/KZ/Kordai/06/2013, Chicken/KZ/Almaty/06/2013 isolates from non-vaccinated poultry from private yards in Almaty, Zhambyl, and North Kazakhstan regions were assigned to genotype VIIb.

So, despite the geographical distance of outbreaks, the same genotypes were circulating in the territory of Northern Kazakhstan and in the southern regions of the country. Our data confirm that geographically, any genetic group had its own area, and its distribution may have been due to the migration routes of wild birds. The same opinion is shared by I.S. Korotetskiy et al. [11] who found that the presence of a short migration route between the North Europe

countries and Ukraine resulted in the local spread of genotype VIIa ND viruses within the territory of Ukraine only, and massive migration routes from Southeast Asia through Russia, Kazakhstan, and Kyrgyzstan to the Caspian and beyond resulted in the spread of genotype VIIb and VIId viruses.

The research has demonstrated that ND caused outbreaks among both vaccinated and non-vaccinated poultry. As noted above, preventive vaccination with live vaccine was carried out at the Allele Agro poultry farm (Almaty region). It is noteworthy that ND virus strain used for vaccine preparation is assigned to genotype II, according to the available publications [28]. Perhaps, the outbreak was due to low immunity, as with well-established vaccination regimes the classical vaccines related to genotype II provide 100 % poultry protection irrespective of antigenic differences in the epizootic strain [24, 29, 30]. Vaccination against ND is assumed to provide immunity against infection and suppression of viral replication. But according to some authors, the existing vaccines prevent the disease, but can not stop the replication and spread of the virus [31-33]. Analysis of these studies demonstrates that virus production was significantly lower only when using a particular genotype based vaccine [34, 35].

Thus, the disease and death in poultry in Almaty, Zhambyl, and North Kazakhstan regions of the Republic of Kazakhstan in 2010, 2012, and 2013 were caused by the Newcastle disease (ND) virus. Our findings have shown that ND virus causes the outbreaks both in vaccinated and non-vaccinated poultry. An outbreak of the disease in vaccinated poultry may have occurred as a result of a decrease in immunity. Biological characteristics (mean death time, intracerebral pathogenicity index), and the sequence of proteolytic F protein activation site in the ND viruses studied correspond to those of velogenic strains. Phylogenetic analysis demonstrated that the virus isolated from poultry that died at the poultry farm among the vaccinated population belonged to genotype VIId, and the isolates obtained from non-vaccinated poultry of private yards in Almaty, Zhambyl, and North Kazakhstan regions belonged to genotype VIIb. Wide spread of Newcastle disease virus in the Republic of Kazakhstan requires to develop effective control measures with regard to ND molecular epidemiology.

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RECENT ACHIEVEMENTS AND CHALLENGES IN FARM ANIMAL BIOTECHNOLOGY (10th Anniversary Scientific Conference with international participation — analytical review)

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Abstract

10th All-Russian scientific conference and school «Recent achievements and challenges in farm animals biotechnology — BioTechZh-2015» (http://www.vij.ru/index.php/ru/konferentsii/) was held on December 8-11, 2015 at the L.K. Ernst All-Russian Research Institute of Animal Husbandry (Moscow Province, Russia) with the support of the Federal Agency of Scientific Organizations of the Russian Federation, Russian Foundation for Basic Research (RFBR) and the Ministry of Innovations and Investments of the Moscow region. A series of these scientific conferences dates back to 2001, when on the basis of the All-Russian Research Institute of Animal Husbandry (VIZh) a scientific event on farm animal biotechnology as a new research area in biology of that time has been initiated due to Professor Lev K. Ernst, the Vice-President of the Russian Academy of Agricultural Sciences. In 2015, the anniversary conference brought together experts and young scientists (207 young scientists, graduate students and students of a total of 281 participants) to discuss promising and rapidly developing areas in biotechnology of farm animals. The conference was attended by scientists from 16 Russian regions as well as scientists from Austria, Belarus, Kazakhstan, Kyrgyzstan, Tajikistan.

Keywords: farm animals, game animals, gene pool, molecular and genetic research, molecular genetic studies, genetic profiling, genetic engineering.

The conferences BioTehZh started in 2001, when the innovative event on farm animal biotechnologies was first held in All-Russian Research Institute of Livestock (VIZH) on the initiative of Vice-President of the RAAS professor Lev K. Ernst. Research areas discussed then and there and reflected in the resolutions of the first conference today became a priority of science and technology development of the country [1, 2].

The next 15 years were characterized by the rapid development of molecular genetics, making it possible to study not only individual genes but entire genomes. Full genome sequencing have been reported in animals, from Btau 4.0 whole-genome resequencing in cattle (*Bos taurus*) in 2009 [3] to CHIR_1.0 domestic goat (*Capra hircus*) genome assembly in 2013 [4]. SNPs were identified in comparative studies of different animal breeds to be further used in genomic analysis. A genotyping technology was developed based on BeadArray platform enabling researchers to assay simultaneously several hundreds to several hundred thousands SNPs [5]. To date, it is the most informative to study species domestication and phylogeny, the structure and dynamics of a population, QTL mapping in farm animals [6-10]. Significant progress has been achieved in genome engineering, in which further development is associated with active transgenesis strategy [11, 12].

The 10th Anniversary Scientific Conference, which has become a platform

for discussing the prospects in farm animal biotechnology, has brought together both 207 junior scientists, graduate and undergraduate students, and 84 senior scientists from 16 Russian regions, and also from Austria, Belarus, Kazakhstan, Kyrgyzstan and Tajikistan.

All current aspects of farm and game animal's biotechnology were considered at the plenary session. Professor Dr. Gottfried Brem, the Member of the German and the Austrian Academy of Sciences, a foreign member of the Russian Academy of Sciences (Institut für Tierzucht und Genetik, VMU, Vienna, Austria) made a presentation «Y-chromosomal markers in the study of the origin of stallions» giving a new view of the process of horse domestication and genealogy.

Dr. M.E. Mikhailova (Institute of Genetics and Cytology of the National Academy of Sciences of Belarus, Minsk, Republic of Belarus), in the report «Investigation of gene pools of domesticated and wild species in the Republic of Belarus» told about the experience of studying the gene pool of bison population of the Bialowieza Forest with the use of molecular-genetic methods.

In the reports «Genetic resources of Tajikistan animals» (Dr. F.S. Amirshoev, TAAS Institute of Animal Husbandry, Dushanbe, Republic of Tajikistan), and «The state of livestock genetic resources of the Kyrgyz Republic» (Dr. E.M. Luschihina (Kyrgyz Research Institute of Animal Husbandry, Bishkek, Kyrgyz Republic) the aspects of gene pool of the local animal breeds were considered.

The genomic approach in cattle breeding were discussed by Dr. T.N. Karymsakov (Kazakh Research Institute of Husbandry and Forage Production, Astana, Republic of Kazakhstan) in the report «Perspectives of molecular genetic methods for breeding meat cattle in Kazakhstan», and Dr. K.V. Plemyashov (All-Russian Research Institute of Animal Genetics and Breeding, St. Petersburg) in the report «Verification of breeding value of Holstein Black-and-White sires and cows in Leningrad region».

The current state of species populations and breed populations of farm animals characterized with the use of DNA markers was considered by Dr. Yu.A. Stolpovskii (N.I. Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow) who summarized data on domesticated species with special attention to gene pool evaluation and effective use. Dr. M.I. Selionova (Stavropol Research Institute of Animal Husbandry and Forage Production, Stavropol) reviewed sheep and goat genetic recourses in Russia with regard to the most prospective biotechnologies, and in the presentation of Dr. A.M. Zaitsev (All-Russian Research Institute of Horse Breeding, Ryazan) the intra- and interbreed genetic variability in horses used for molecular genetic certification was considered.

Characterization of pedigree resources, strategy of biodiversity preservation, and gene pool standards were considered by Dr. L.A. Kalashnikova (All-Russian Research Institute of Animal breeding, Moscow Province) in the report «Management and methodology of genetic examination and certification of pedigree animals».

Studies of gene pools of reindeer and bighorn sheep attracted special interest of the participants. These animals are essential in the ecosystems of the North and an integral part of life and culture of indigenous peoples in the region. These presentations were «The current state of bighorn *Ovis nivicola* and *Rangifer tarandus* populaions in Yakutia and perscectives of their use» (Dr. I.M. Okhlopkov, Institute of Biological Problems of Cryolithozone, the RAS Siberian Branch, Yakutsk) and «Genetic monitoring of Nenets Breed reindeer in the Nenets Autonomous Okrug» (Dr. T.M. Romanenkova, Naryan-Mar Agricultural

Experimental Station, Naryan-Mar).

Resent achievements in poultry biotechnology was summarized by Dr. N.F. Volkova (L.K. Ernst VIZh) in the presentation «Modern approach to poultry genetic modification».

In the presentation «Actual problems in cryopreservation of cattle oocytes and embryos» (Dr. G.P. Malenko from the Center of Experimental Embryology and Reproduction Biotechnologies, Moscow) the methodological aspects of cryotechnologies with regard to farm animal germplasm were under consideration.

Based on the competition the best nine presentations of junior scientists were chosen for publication in two journals, the Agricultural Biology and the Achievements in Science and Technology in Agro-industrial Complex.

D.V. Beloglazov (http://www.agrobiology.ru/6-2015beloglazov-eng.html) studied cell proliferation in hen oviduct during ontogenesis to optimize a retrovirus-mediated gene transfer. The efficiency of local transgenesis in an oviduct was shown to be increased more than 3.3-fold due to epithelial cell stimulation using estrogens [13]. T.E. Denisksova obtained whole-genome SNP profiles of local Russian sheep breeds and evaluated the informativeness of an internationalal reference SNPs panel (http://www.agrobiology.ru/6-2015deniskova-eng.html) [14]. O.V. Kostyunina found out a quantitative effect of DNA marker genotype *IGF2* on meat production and nutrient utilization in pigs. The marker genotypes were also gender-influenced (http://www.agrobiology.ru/6-2015kostyunina-eng.html) [15]. A.A. Sermyagin conducted a study of whole-genome SNP associations with breeding value in the Holstein sires (http://www.agrobiology.ru/2-2016sermyagin-eng.html). It was found out that a whole-genome analysis allowed to map quite precisely the productivity and reproduction trait QTLs of heritability from low to moderate [16]. M.S. Fornara compared an informativeness of morphometric parameters and high polymorphic microsatellite DNA markers is evaluation of diversity and differentiation of maintain grey Caucasian honeybees (*Apis mellifera caucasica*) (http://www.agrobi-ology.ru/6-2015fornara-eng.html) [17]. V.P. Kharzinova (http://www.agrobiology.ru/6-2015kharzinova-eng.html) has developed the world's first miltilocus panel for STR-marker analysis of reindeers (*Rangifer tarandus*). Its functionality was shown both in origin authentication and biodiversity study of Russian reindeer population [18].

M.A. Zhilinskii found out (http://agroapk.ru/70-archive/12-2015/1204-2015-12-27-ru) the variations in qualitative and quantitative semen parameters in cocks due to integration of recombinant DNA that should be considered when creating transgenic chicken lines [19]. O.S. Pomanenkova has developed the system for direct molecular genetic testing LoF mutations in *SMC2* gene associated with HH3 haplotype impacting fertility (http://agroapk.ru/68-archive/11-2015/1136-2015-11-27-ru) and causing early embryonic death in cows. The HH3 wide spreading among Russian Holsteins was shown [20]. Comparative data on metabolism in heifers as influenced by ovarian function depression during the first third of lactation were presented by A.A. Solomakhin (http://agroapk.ru/68-archive/11-2015/1137-2015-11-28-ru) [21].

All these young scientists presented the results of their research at sessions or workshops of the conference.

The conference helped identifying the world's research priorities in farm animal biotechnology and cell technologies. It has become an information platform for sharing relevant experience among Russian agrobiotechnologists. It should be especially noted that the conference presented and discussed the results of the research projects supported by Russian Science Foundation (14-36-00039, 15-16-00020), Russian Foundation for Basic Research (№ 13-04-01888,

№ 14-48-03681, № 15-08-99473), Ministry of Education and Science of the Russian Federation (14.604.21.0141, 14.604.21.0062). The conference contributed to establishing alliances between Russian and foreign researchers' teams to address fundamental problems in such a dynamic field of science as biotechnology.

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In memory of Academician J.A. Aliyev

Jalal Alireza oglu Aliyev, breeder scientist and statesman, academician and member of the Presidium of the National Academy of Sciences of Azerbaijan, deputy of Milli Majlis of the Azerbaijan Republic died on February 1, 2016.

Jalal Alireza oglu Aliyev was born on 30 June 1928 in the city of Nakhchivan of Azerbaijan Republic. In 1944 he graduated with honors from the Faculty of Natural Sciences of Nakhchivan Pedagogical Institute and worked in secondary schools in remote mountain villages of the Nakhchivan Autonomous Republic. In 1951 he graduated with honors from the biological faculty of the Azerbaijani State University. From the third year, J.A. Aliyev worked as a laboratory assistant at the Department of Plant Physiology and chose this field of biology for future research activities. He defended his PhD thesis in Biology «The impact of trace elements on wheat plant development and productivity» in 1955, and his DSci thesis «Photosynthetic activity, mineral nutrition and productivity of plants» in 1971.

Since 1951, J.A. Aliyev has conducted research in the Department of Plant Physiology (Azerbaijan Institute of Agriculture), and since 1971 he also created and worked in the Department of Molecular and Genetic Bases of Production Processes (at present, the Department of the Fundamental Problems of the Biological Productivity of the Institute of Botany of the National Academy of Sciences of Azerbaijan). In 1976 he was elected a corresponding member, and in 1980 a full member of the Academy of Sciences of Azerbaijan. In 1981 to 1990 J.A. Aliyev worked as academic secretary of Biological Sciences Department of the Academy of Sciences of Azerbaijan, and to the end of his life he headed the Department of Plant Physiology and Biotechnology of the Azerbaijan Institute of Agriculture and the Department of the fundamental problems of the biological productivity of the ANAS Institute of Botany.

More than 65 years of activities J.A. Aliyev devoted to the research of the theory of photosynthetic productivity in crops, mainly wheat. He studied the complex of photosynthesis and photorespiration processes considering physiological, biophysical, biochemical and molecular genetic basis of plant productivity and production processes at all structural and functional levels — from molecular to the whole plant. These studies resulted in developing principles of high production and grain yield for «ideal» wheat. When studying CO_2-exchange, the wide assimilation variability in crops was shown as depended on morphophysiological traits and donor to acceptor relationships. C-metabolism, assimilate transport and distribution were studied in contrasting genotypes using $^{14}CO_2$. In contradiction to common ideas about photorespiration «squandering», in long-term field experiments on contrasting genotypes of wheat he proved it to be an evolutionarily-formed vital metabolic process, so that the pursuit to reduce it in order to increase plant productivity is untenable. The possible role of photosynthetic changes in adaptation of plants to extreme conditions has been studied. The chloroplast DNA library was created with constructed vectors for gene transfer and transgenic plants have ben produced. To identify the *Triticeae* species, CAPS markers have been generated, and their polymorphism was studied. The transduction pathways of outer signals from cell environment were biochemically studied. Using different molecular markers, genes and loci responsible for drought resistance in wheat have been found.

J.A. Aliyev was a founder of molecular biology, molecular genetics, gene and cell biotechnology, biomathematics and bioinformatics as new research areas in Azerbaijan. Combination of these disciplines made it possible for the first time to apply mathematical methods and computer technology in solving theoretical and practical problems of biology and agriculture in the country. Both basic research and applied science attracted attention of D.A. Aliyev. He developed an idea of so-called «good» photosynthesis in «ideal» wheat plants, and a rich gene pool of wheat was created, covering several thousand genotypes. Under the guidance of J.A. Aliyev the National Programme on Plant Genetic Resources was designed, a strategy for conservation and a sustainable use of biodiversity was developed, and Azerbaijan genebank of plant resources was created. D.A. Aliev initiated radiological surveys of the Azerbaijan territory with regard to anthropogenic ^{90}Sr and ^{137}Cs migration among crops.

J.A. Aliyev supervised and guided 85 PhD and 12 DSci research works. He is the author of

over 600 scientific publications, including 25 monographs and books.

J.A. Aliyev was a foreign member of the Russian Academy of Agricultural Sciences, the National Academy of Agrarian Sciences of Ukraine and the Academy of Agrarian Sciences of Belarus. As Chairman of the Problem Council on biological sciences of the ANAS Council of the organization and coordination of scientific research, a member of many scientific and academic thesis defense council, he contributed to proper development of physical and chemical biology.

Editorial activity took the important place in scientific activities of D.A. Aliyev. He was editor in chief of Proceedings of the ANAS (biology and medicine), a member of editorial board of Reports of the ANAS, a member of editorial boards of Bioinformatics and comparative genomics, Plant Biochemistry and Physiology, Computational Biology and Bioinformatics, The Infectious and Non-infectious Diseases journals.

J.A. Aliyev was a member of Federation of the European Biochemical Societies (FEBS), a representative of Azerbaijan in The Federation of European Societies of Plant Biology (FESPB), a member of American Society of Plant Biologists, International Society of Photosynthesis Research, The Japanese Society of Plant Physiologists, Cell Stress Society International, as well as chairman of the National Committee on Bioethics, Ethics of Science and Technology of UNESCO and Azerbaijan Society of Biochemistry and Molecular Biology.

J.A. Aliyev was awarded the medal «For Valiant Labor in the Great Patriotic War of 1941-1945», many other medals, and twice awarded the Order of Red Banner of Labor. J.A. Aliyev was cavalier of the Order of Independence — the highest award of Azerbaijan, the Order of Glory of the Republic of Georgia. He was awarded Honorary Diploma of the President of the Azerbaijan Republic and the Order of Glory of Azerbaijan Republic. He was awarded the Hasan bey Zardabi prize of ANAS. Four times J.A. Aliyev was elected to the Milli Majlis of the Azerbaijan Republic.

J.A. Aliyev has founded a world scientific school. His followings successfully work in Azerbaijan, and in the CIS, USA, Canada, Japan, South Korea, Australia, Because Rail and other countries.

Integrity, the talent of scientific prediction, desire to know the unknown, attention to junior scientists, inherent to J.A. Aliyev, were in a harmony with his personal charm and kindness. His colleagues will be long remembering him.