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

AGRICULTURAL BIOLOGY

Since January, 1966

ANIMAL BIOLOGY

Vol. 51, Issue 6
November-December

2016 Moscow

Covered in Scopus, Web of Science (BIOSIS Previews, Biological Abstracts, Russian Science Citation Index), Agris

Science editors: E.V. Karaseva, L.M. Fedorova

Publisher: Agricultural Biology Editorial Office NPO

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

For citation: Agricultural Biology,
Сельскохозяйственная биология, Sel'skokhozyaistvennaya biologiya

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

SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA
[AGRICULTURAL BIOLOGY], 2016, Vol. 51, № 6

CONTENTS

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 763-774
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Reviews

UDC 636.4:619:578.842.1:616-097:57.083.3

doi: 10.15389/agrobiology.2016.6.763rus
doi: 10.15389/agrobiology.2016.6.763eng

THE HAEMADSORPTION AT AFRICAN SWINE FEVER (review)

A.D. SEREDA[1], A.R. IMATDINOV[1], V.V. MAKAROV[2]

[1]*All-Russian Institute of Veterinary Virology and Microbiology,* Federal Agency of Scientific Organizations, 1, ul. Akademika Bakuleva, pos. Vol'ginskii, Petushinskii Region, Vladimir Province, 601125 Russia, e-mail sereda-56@mail.ru;
[2]*Peoples' Friendship University of Russia, Agro-Technological Institute,* 8/2, ul. Miklukho-Maklaya, Moscow, 117198 Russia, e-mail vvm-39@mail.ru
Acknowledgements:
Supported by Russian Science Foundation (the research project «Design of African swine fever virus candidate vaccine based on chimeric viruses», contract No 16-16-00090)
Received August 29, 2016

Abstract

The capability of causing haemadsorption at African swine fever (ASF) virus (ASFV) reproduction in swine bone marrow cell cultures, leukocytes or continuous cells in the presence of swine erythrocytes is characteristic of the majority of the virus isolates (W.A. Malmquist, D. Hay, 1960). This trait is used for ASF diagnosis based on autohaemadsorption in porcine blood, the virus titration in cell culture, and selection of its attenuated variants in vitro (A.D. Sereda et al., 2014). The haemadsorption inhibition assay (HIA) in tandem with the bioassay using the disease-resistant pigs is applied for seroimmunotype-based classification of ASFV isolates (N.I. Mitin et al., 1985). The heterogeneity of an ASFV population for quantitative haemadsorption characteristic (like «dense», «moderate» or «loose») is a phenotypic trait of ASFV isolates, strains and/or variants (V. Makarov et al., 2016). Also, the proportion of the circumference of red blood cells as observed at their contact with infected macrophages serves as another quantifiable feature of haemadsorption. Some quantitative differences in HIA activity levels of swine blood sera are determined in the assays carried out using virulent reference variants and their attenuated derivatives, and the obtained results require some interpretation. The loss of ability to induce haemadsorption is not critical for ASFV reproduction and often accompanied by a decrease in the pathogen virulence levels. Hence, as a rule, attenuated ASFV variants are prepared through a selection by limiting dilution from populations of virulent isolates of the virus clones that are characterized by a reduced potential to induce haemadsorption (D.V. Kolbasov et al., 2014). In the course of the virus reproduction, haemadsorption precedes the exocytosis. Virions do not play a significant role in the mechanism of haemadsorption, nevertheless, their interaction with erythrocyte membranes promotes the virus dissemination throughout the swine organism and more effective introduction into the gut cells of ticks (L.K. Dixon et al., 2004). ASFV haemadsorbing potentiality is determined by highly glycosylated transmembrane protein CD2v (J.M. Rodríguez et al., 1993). Probably, nonhaemadsorbing avirulent isolates emerge as a result of some shift of the open reading frames for *EP402R* and *EP153R* encoding the CD2v and lectin-like proteins, respectively (D.A. Chapman et al., 2008). An assumption is made that the haemadsorption phenomenon is due to an interaction between carbohydrate residues of glycoproteins of ASFV oligosaccharides and lectin-like receptors of swine red blood cells.

Keywords: African swine fever, haemadsorption, non-haemadsorbing isolates

The pathogen of African swine fever is a large DNA-containing virus of *Asfarviridae* family [1]. Its virulent isolates may cause the disease in domestic swine (*Sus scrofa domesticus*) and wild boars (*Sus scrofa*). In Africa, ASF is supported in the transmission cycle between its natural hosts, wart hogs (*Phacochoerus* spp.) and bushpigs (*Potomochorus porcus*), and its transmitters — soft ticks *Ornithodoros* [2]. The disease is contagious, and the virus is well adapted to its hosts inducing a sub-clinical chronic or unapparent form of infection.

The interest in haemadsorption in the course of the ASF virus (ASFV) reproduction is driven by several factors. So far, different aspects remained un-

clear, particularly, what molecular mechanisms underline ASFV heterogeneity by haemadsorption, why some isolates do not have this feature and how the haemadsorption capacity is related to other phenotypic characteristics of isolates and laboratory variants. In the veterinary practice, haemadsorption is used for ASFV titration (as in most cases, the obtained results coincide with the titration data by the cytopathic effect), in the selection of attenuated variants, and lifetime disease diagnosis by the autohaemadsorption of blood leukocytes in the infected swine. The haemadsorption inhibition test (HIT) is used, together with the bioassay study of the disease-resistant swine, to classify the ASFV isolates by their immunoserotypes [3-5].

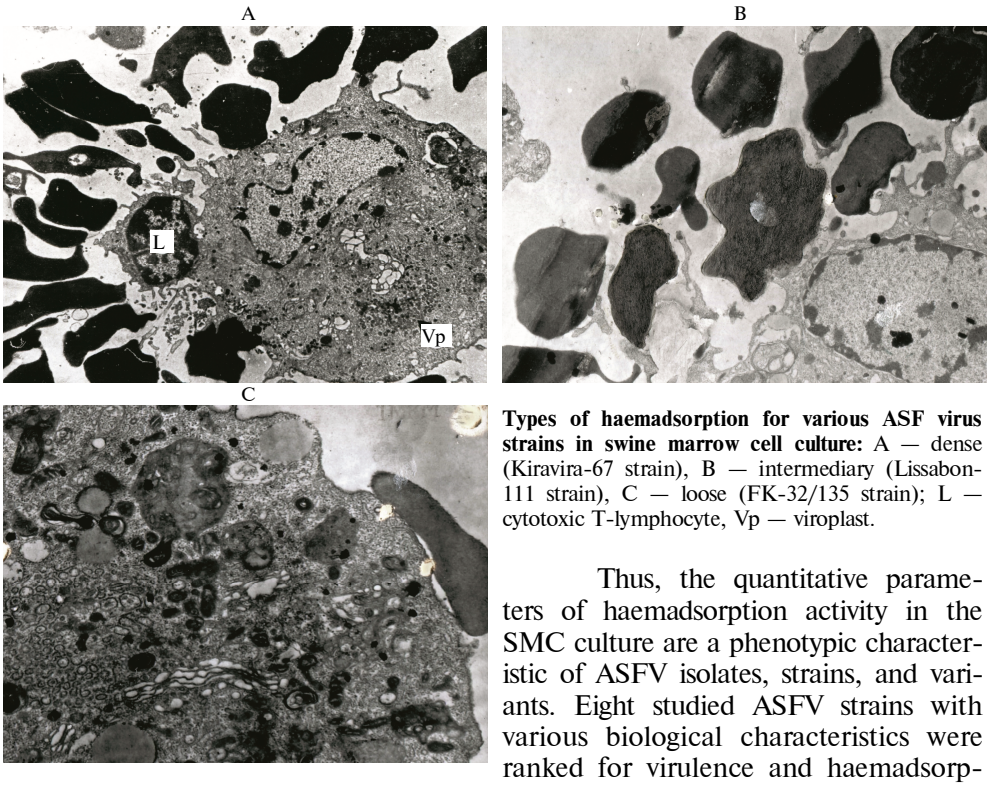
Haemadsorption phenomenon. It is well known that the cell cultures, which are infected with the orthomyxo-, paramyxo-, toga-, rhabdo-, and poxviruses capable of shedding viral progeny via budding, can absorb erythrocytes [6, 7]. Haemadsorption is caused by the inclusion of synthesized virus proteins having an affinity to erythrocytes into the cell membrane. In a number of cases it has common mechanisms with haemagglutination and is observed at early virus reproduction cycle until the development of the cytopathic effect or in its absence or its weak intensity.

Haemadsorption in the course of ASFV reproduction was first identified and described by W.A. Malmquist and D. Hay [3]. By showing that the sera of the swine that had ASF restricted haemadsorption but did not neutralize the cytopathic effect and did not affect the virus reproduction, they assumed that the antigen responsible for haemadsorption is not related to virions. S.S Brees and W.R. Hess [8] ascertained that the integrity of the plasmalemma of the infected cells is an essential condition for haemadsorption in the course of ASFV reproduction. Electronic microscopy revealed two key ways of erythrocytes binding with the cell membrane at haemadsorption, i.e. by attaching to cell filaments protruding out of the plasmalemma, and directly to the membrane of the infected cell [8]. The membrane of the infected cell acquires the capacity to absorb erythrocytes before the virions start migration from the cytoplasmic matrices where they mature. In other words, the virus specific modulation of the cell membrane not just precedes the virus exocytosis, but is chronologically ahead of it, and these stages are not synchronized [9, 10].

The majority of researchers think that the virus particles are not necessarily located at the site of contact between the erythrocytes and the cell [11, 12]. Visually, at ×400 magnification erythrocytes are seen on the macrophage arranged either in one layer or in several layers. Their number per haemadsorbing cell varies from to 80 or more cells. It was shown [13] that the multi-layer appearance in case of the so-called dense haemadsorption results from the changes in the form of erythrocytes and the cytoplasm protuberances of the infected macrophage, contacting erythrocyte, that are located at a significant distance from the main part of cell. The absence of hemagglutination in the course of ASF also evidences that virions do not play any role in the haemadsorption phenomenon. When fluorescent antibodies and the monoclonal antibodies to ASFV-specific 13 kDa and 73 kDa polypeptides were used in the study of interaction between virions and erythrocytes in 30 pigs infected with 17 ASFV isolates, the viral antibodies were in vitro and in vivo observed on the surface of swine erythrocytes [14]. By a transmission microscopy, virions were identified in the invaginations of membranes on the surface of erythrocytes [14]. The virus binds to erythrocytes as soon as they interact with the infected cells. Virions attach to the surface of the erythrocyte cell membrane, but remain in the membrane invaginations without penetrating to the cytoplasm. This mechanism may preserve the virus in blood. The attachment of virions to erythrocytes seems to

contribute significantly to the ASFV penetrating through the intestinal walls in ticks due to the erythrocyte phagocytosis by hemocoel cells [15]. It is noted that haemadsorption does not occur in all cells with virus replication or the presence of virus particles [16].

Quantitative characteristics of haemadsorption. *The number of attached erythrocytes.* Haemadsorption is considered as the ability of infected cells to bind the erythrocytes contained in a cell culture, keeping them when the culture is washed. Comparative analysis of haemadsorption phenomenon showed that ASFV isolates, strains and variants significantly differed in the quantitative haemadsorption parameters and the population structure correlating to the virulence. The number of erythrocytes attached to a cell in swine marrow cell (SMC) culture varied significantly between attenuated strain FK-32/135 and the virulent F-32 and Kiravira-67 strains, and the distribution by this parameter was close to normal. The arithmetic average number of erythrocytes per haemadsorbing cell in the variation series of the studied strains were not the same, being low for FK-32/135 (18.52±0.36), and higher for France-32 and Kiravira-67 (29.32±0.71 and 34.49±0.89, respectively) [17]. Heterogeneity and specificity on qualitative haemadsorption parameters are typical of each strain population, e.g. there was a simultaneous occurrence of dense (41-80 erythrocytes per cell), intermediary (21-40 erythrocytes) and loose (1-20 erythrocytes) haemadsorption in a field of view, but in various proportions (Fig.).



Types of haemadsorption for various ASF virus strains in swine marrow cell culture: A — dense (Kiravira-67 strain), B — intermediary (Lissabon-111 strain), C — loose (FK-32/135 strain); L — cytotoxic T-lymphocyte, Vp — viroplast.

Thus, the quantitative parameters of haemadsorption activity in the SMC culture are a phenotypic characteristic of ASFV isolates, strains, and variants. Eight studied ASFV strains with various biological characteristics were ranked for virulence and haemadsorption activity, that was indicative of genetic control of haemadsorption antigen expression [17, 18]. In the attenuated (avirulent) ASFV variants there were only the subpopulations with loose and intermediary haemadsorption, while in the virulent strains, on the contrary, the subpopulations with intermediary and dense types were observed. These findings allow to assume that antigen modulation of infected mononuclear macrophages is more typical of the avirulent variants [19].

Differences between the strains in the contact circumferencee ratio of erythrocyte at interaction with a macrophage. For the avirulent attenuated strain FK-32/135 with loose one-layer haemadsorption, the ratio of erythrocyte circumference contacting the plasmalemma of the infected macrophages was 34.2±7.3 %, while shorter intermembrane contacts, 7.8±3.1 % for France-32 and 11.2±6.4 % for Kiravira-67, are typical of virulent strains with dense haemadsorption. These results may also be interpreted as the evidence for antigen modulation of the membrane of the infected monocytes (macrophages), which is more typical of avirulent variants, and as an indication of the high affinity of haemadsorbing protein in virulent variants.

The differences in the activity of serums in HIT with virulent and attenuated ASFV strains. A study of the antibodies involved in HIT when testing blood serum of swine, which survived after acute, ASF resulted in interesting findings. In the study, three specific antisera of serotype I were tested with the virulent strain Lisbon-57 and its attenuated derivatives (L-50, LK-111, and LK-200); five antisera of serotype II were tested with virulent strains (Yamba-20, Kongo-49) and attenuated strain KK-202 derived from strain Kongo-49; five antisera of serotype III were tested with virulent strain Mozambique-78 and its attenuated derivatives MK-200 and MK-250; and six antisera of serotype IV were tested with strain France-32 and its attenuated derivative FK-32/135, strain Portuguese-60 and its attenuated variant P60/81, and strain Brazil-80. The titers of antiserf in HIT with virulent strains were 1.7-2.8 $\log_2$ lower than with homological attenuated strains. More differences were observed for attenuated strain FK-32/135 inducing loose haemadsorption, hence France-32 had 29.5-fold lower antiserum titers in HIT compared to FK-32/135 [20]. In other words, substantially fewer amounts of antibodies are required for inhibition at loose haemadsorption than at dense one.

The hypothesis according to which in infected SMC culture or swine leukocyte (SL) culture the lysis of target cells, due to antibody-dependent cellular cytotoxicity (ADCC), can fully or partially compete with the development of haemadsorption and imitate its inhibition was verified experimentally [21). However, in five blood sera the antibody titers in HIT with the variant of FK-135 adapted to cell line coincided both in primary FK-135-infected SMC culture with active ADCC mechanism, and in swine embryo kidney cell line, the PPK-66b, which contained no ADCC effectors. This is the evidence that ADCC and HIT occur simultaneously and independently of each other [21].

ASFV reproduction, as influenced by the glycosilation inhibitors. Inhibition analysis is a very informative tool in studying biosynthesis and morphogenesis of viruses, including processing of their structural and non-structural proteins in an infected cell. Non-cytocide concentrations of inhibitors are determined, in particular, based on the optical and electron microscopy data [22]. To study the role of glycosilation in the ASFV reproduction, its strains, isolates and variants were grouped according to seroimmunotype specificity, common origin and contrasting key biological features [23]. Each group of the isolates included a reference strain of seroimmunotypes I-IV and, as a rule, attenuated laboratory variants derived from the corresponding strains. Reference strains were high virulent and able to induce dense haemadsorption during the reproduction in the SMC culture. Their attenuated variants were either low virulent or avirulent, and haemadsorption during the reproduction in the SMC culture was less dense (even loose). Furthermore, non-haemadsorbing isolates were studied.

It is known that tunicamicin blocks the formation of dolicol-PP-acetyl-D-glycosamine (the first stage in the dolicol-dependent protein glycosylation) [24]. The effect of introducing tuncamicin into the infected SMC culture differed de-

pending on the phenotype of the investigated strains, isolates and variants of the ASF virus. Titers of the reference strains Lissabon-57 (seroimmunotype I), Kongo-49 (seroimmunotype II), and France-32 (seroimmunotype IV) were more reduced than in the laboratory attenuated strains and variants LK-111 and LC (I), FK-32/135, FNG (IV), and non-haemadsorbing isolate Petit-Engre (II). Within non-cytocide concentrations of tuncamicin (0.5-1.0 μg/cm^3) the difference in the accumulated infectious progeny between the virulent and attenuated ASFV strains of seroimmunotype I, II, and IV was 10-fold (about 1.0 lg CPE_{50}). And only the strain Mozambique-78 and its attenuated variant MK-200 (seroimmunotype III) had no such differences. The ionophore monensin equally suppressed the reproduction of all studied virus strains and variants [23].

Electron microscopy showed that tunicamicin, the glycosylation inhibitor, did not affect the structure of virus matrices and the timing of virion budding, however in the presence of tunicamicin budding virions showed a notable association with the vacuole membranes of the Golgi apparatus. At tunicamicin concentration of 0.5 μg/cm^3, the yield of the ASFV strain France-32 in SMC culture reduced by 1.2±0.2 lg HAU_{50}/cm^3 as compared to control (without inhibitor). In the presence of tunicamicin, the number of virions associated with the membranes of the Golgi apparatus and their ratio was 84 (50 %), without the inhibitor — 43 (13 %), the number of virions associated with plasmalemma was 181 (50 %), without the inhibitor — 281 (87 %) [23].

Consequently, when glycosylation is inhibited, the assembly of capsids is not violated and their number does not decrease, but because of the infringed transportation of glycoproteins to the plasma membrane the virion budding into the Golgi vacuole apparatus increases leading to a reduced yield of the extracellular virus. Thus, the production of infectious progeny in haemadsorbing ASFV strains and variants depends on glycosylation.

Effect of monosaccharides, exo- and endoglycosidases on ASFV haemadsorbtion. No haemadsorbtion is observed in swine marrow A-cells (adhesive cells) treated with tuncamicin and monensin inhibitors and then infected with haemadsorbing ASFV strains [23]. This allowed us to assume that haemadsorption results from an interaction between carbohydrate components of ASFV glycoproteins and the lectin-like receptors of erythrocytes. For elucidation, we have studied whether the treatment with exo- and endoglycosidases, and the presence of various monosaccharides in the culture medium could influence haemadsorption in ASFV-infected swine marrow A-cells (Table) [23].

Haemadsorption in swine marrow A-cells infected with African swine fever virus (strain France-32), as influenced by agents affecting carbohydrate component of the viral glycoproteins [23]

Agent	In 1 hour	In 2 hours	In 3 hours
Effect of xo- and endoglycosidases			
Enzymes, units/cm^3:			
neuraminidase, 10	+	+	+
β-galactosidase, 150	–	+	+
β-glycosidse, 50-100	+	+	+
α-mannosidase, 8.5	–	-	-
endoglycosidase D, 0.1	+	+	+
endoglycosidase F, 0.1	–	-	-
Effect of monosaccharides			
Monosaccharides, %:			
D-glucose			
1.25	+	+	+
2.50	+	+	+
5.00	+	+	+
L-fructose			
1.25	+	+	+
2.50	+	+	+
5.00	+	+	+

Table (continued)

D-galactose			
1.25	+	+	+
2.50	–	+	+
5.00	–	+	+
methyl-α-D-mannopyranoside			
1.25	–	+	+
2.50	–	+	+
5.00	–	-	+
N-acetyl-glucosamine			
1.25	+	+	+
2.50	+	+	+
5.00	+	+	+

N o t e. Swine marrow A-cells are adhesive swine marrow cells; "+" and "–" mean the presence or absence of haemadsorption.

Since exoglycosidases split out only monosaccharide residues, the non-regenerated ends of ASFV oligosaccharides in haemadsorbing protein evidently contain α-mannose. It is confirmed by haemadsorption inhibition with methyl-α-D-mannopyranoside, and by its sensitivity to endoglycosidase F, specific to high-mannose glycans [25]. The obtained results evidence that high-mannose oligosaccharides of the ASFV glycoproteins are involved into haemadsorption.

Non-haemadsorbing ASFV isolates, strains, and variants. In Africa, non-haemadsorbing ASFV variants were isolated from chronically infected wild (wart hogs, bushpigs) and domestic, including indigenous, pigs, as well as from ticks *Ornitodoros moubata* [26-32]. In the course of passaging on domestic swine or in primary SMC culture some isolates recovered their haemadsorbing activity, while others did not [33]. From 1972 to 1985, 79 ASFV isolates were found in the survey of over 7,000 pigs of European and domestic breeds in the People's Republic of the Congo. It was shown that under natural epizooties and experimental selection the cultural and pathogenic characteristics of ASFV were substantially modified, i.e. ASFV could lose haemadsorbing capacity or reduce virulence up to its complete loss. Among local animals, the ASFV often circulated in a non-haemadsorbing form causing no clinical signs and creating a positive immune profile [34, 35].

In Europe, non-haemadsorbing ASFV strains are generally isolated from chronically ill domestic swine and ticks *Ornitodoros erraticus*. For example, 206 non-haemadsorbing isolates were collected in Spain from 1965 to 1974 [26]. In Portugal, 10 ASFV isolates derived from the ticks *Ornitodoros erraticus*, collected from 1988 to 1993, six of which were haemadsorbing and pathogenic, and four were non-haemadsorbing and non-pathogenic [27]. With passages on intact domestic swine, the ASFV genome is little subject to modification: its similarity was shown after 20 passages on swine, and after 17-100 passages in swine alveolar macrophage culture [36, 37]. At the same time, it was reported that the virulent and (or) haemadsorbing capacity were lost after long passaging of haemadsorbing isolates in primary cell cultures and in cell line cultures [38, 39].

It is considered that the loss of haemadsorbing phenotype does not affect the pathogenicity of the ASFV isolates. Non-haemadsorbing isolates sampled from swine in the centers of disease in South Africa and in Madagascar caused death in 80-90 % of animals [40]. However, many researchers pointed to the low virulence and protective features of non-haemadsorbing natural isolates and the obtained laboratory variants [11, 28, 41, 42]. By direct and selective passages in the swine marrow cell culture from highly reactogenic strain Katanga-105 (seroimmunotype I), causing death in 87 % of domestic pigs, after 44 direct passages in the swine marrow cells, a non-haemadsorbing variant KC-149 was obtained that became avirulent for the swine. It kept its features up to passage 190, did

not reverse after three passages on the swine, and protected the animals from death after subsequent intramuscular infection with the virulent reference strain Lissabon-57, seroimmunotype I, at dosage of 10^4 HAU_{50} [43, 44].

When studying the effect of γ-radiation on the ASFV, we obtained unexpected results. The preparations of haemadsorbing and non-haemadsorbing ASFV isolates, strains and variants (non-concentrated and concentrated) that lost their infectivity after being exposed to radiation of 25 kGr (γ-radiated preparations, γ-RP) induced haemadsorption when being introduced into the swine marrow cell culture. In sub-passages (3 to 8), this effect was not reproduced and the infectious ASFV was not isolated in the tested samples. By the character of erythrocytes location, haemadsorption caused by γ-RP practically did not differ from the loose one that was observed in the SMC culture infected, for instance, with attenuated strain FK-32/135. Based on data on i) haemadsorption development, ii) identification of virus-specific antigens in swine marrow A-cells infected with γ-RP by immunofluorescence and immunoblotting, and iii) the effect of inhibitors of RNA synthesis and glycolization, it was proved that in this case haemadsorption was caused by de novo synthesis of virus-specific proteins encoded by intact regions of the ASFV genome. We demonstrated the serologic type specificity of haemadsorption induced by γ-RP of non-haemadsorbing ASFV isolates, which made it possible their serotyping in vitro [45].

Proteins involved in haemadsorption in case of ASFV. I.D. Vigario et. al [46] suggested that the existence of non-haemadsorbing ASFV isolates and strains is related to the deficiency of the protein, responsible for their haemadsorption, at reproduction. Later it was ascertained that the haemadsorbing features of the ASFV are determined by its transmembrane protein that is similar to the protein of the CD2 host cells [47, 48]. The virus-specific CD2-like protein was designated as CD2v. Its mRNA is transcribed due to the open reading frame (ORF) in gene *EP402R* [49]. The extracellular domain CD2v is similar to that of the host CD2 binding protein and contains two Ig-like domains and 15 potential sites for N-glycolization unlike 3-4 in CD2 [48, 50, 51]. The molecular weight of the full-size glycosylated CD2v in the isolate Malawi LIL20/1 is 105-110 kDa [52]. In the absence of gene encoding CD2v protein in the virulent ASFV genome impeded the viremia and dissemination of the virus in swine body, but did not reduce the death from the virus. In the blood of an infected swine, the expression of CD2v protein of extracellular virus particles correlated to the activity of binding erythrocyte. Therefore, according to the authors, the loss of haemadsorbing phenotype may be the factor of low or sporadic viremia as observed in case of infections caused by non-haemadsorbing isolates. However, other features of CD2v may also be the reason for reduced virus reproduction in the host's organism. For expamle, CD2v expression suppresses lymphocyte proliferation in response to mitogens in vitro, which indicates that CD2v plays an important role in ASFV escaping from the host's immune system [53, 54]. It is considered that the interaction between CD2v and its ligand on erythrocytes is stabilized as a result of the expression of virus-specific lectin-like protein of C-type coded by ORF *EP153R*, as the deletion of *EP153R* leads to a decreased haemadsorption around the ASFV infected cells [55-57]. It was shown that EP153R protein inhibits apoptosis and decreases expression of antigen of the main histocompatibility complex class I in swine SLA I [58, 59]. It was ascertained that the reading frames *EP153R* and *EP402R* are shifted in the genome of non-haemadsorbing avirulent isolates OURT88/3 and NHV, which ultimately causes this phenotype [60-62]. Interestingly, the recovery of the haemadsorbing phenotype in the strain NHV led to an increased virus reproduction in ticks, but not in swine [63].

In the swine marrow A-cells infected with haemadsorbing ASFV strains and marked with ^{3}H-glycosamine a serotype specific 110-140 kDa glycoprotein was identified by radio immunoprecipitation assay with HIT-active homological antisera [64]. The authors considered this glycoprotein to be involved in haemadsorption due to the fact that this glycoprotein could not be identified when the swine marrow A-cells were infected with non-haemadsorbing isolates and strains, or when using in immunoprecipitation the swine sera non-active in HIT, in particular, those obtained after the animals were infected with attenuated ASFV strains [64].

So, haemadsorption at African swine fever (ASF) has quantiative characteristics. The loss of capability to induce haemadsorption is followed by a reduced virulence. The ability of binding membrane of erythrocytes absorbed on macrophages helps the virus to disseminate in swine organism and contributes to more efficient penetration to the intestinal cells in ticks. The haemadsorbing features of the ASF virus are determined by transmembrane high glycosylated protein CD2v. Based on the inhibition analysis, it can be assumed that haemadsorption is caused by the interaction between the oligosaccharides of ASF virus glycoproteins and the lectin-like receptor of swine erythrocytes.

REFERENCES

1. Dixon L.K., Costa J.V., Escribano J.M., Rock D.L., Vinuela E., Wilkinson P.J. Family *Asfarviridae*. In: *Virus taxonomy: seventh report of the International Committee on Taxonomy of Viruses*. M.H.V.V. Regenmortel, C.M. Fauquet, D.H.L. Bishop, E.B. Carstens, M.K. Estes, S.M. Lemon, J. Maniloff, M.A. Mayo, D.J. McGeoch, C.R. Pringle, R.B. Wickner (eds.). Academic Press, San Diego, CA, 2000: 159-165.
2. Sanchez-Vizcaıno J.M., Mur L., Martınez-Lopez B. African swine fever: An epidemiological update. *Transbound. Emerg. Dis.*, 2012, 59(1): 27-35 (doi: 10.1111/j.1865-1682.2011.01293).
3. Malmquist W.A., Hay D. Hemadsorbtion and cytophatic effect produced by African swine fever virus in swine bone marrow and buffy coat culture. *Am. J. Vet. Res.*, 1960, 21: 104-108.
4. Malogolovkin A., Burmakina G., Titov I., Sereda A., Gogin A., Baryshnikova E., Kolbasov D. Comparative analysis of African swine fever virus genotypes and serogroups. *Emerg. Infect. Dis.*, 2015, 21(2): 312-315 (doi: 10.3201/eid2102.140649).
5. Kolbasov D.V., Balyshev V.M., Sereda A.D. *Veterinariya*, 2014, 8: 3-8 (in Russ.).
6. Uhlendorff J., Matrosovich T., Klenk H.D., Matrosovich M. Functional significance of the hemadsorption activity of influenza virus neuraminidase and its alteration in pandemic viruses. *Arch. Virol.*, 2009, 154: 945-957 (doi: 10.1007/s00705-009-0393-x).
7. Saburi Y., Okuda K., Takhashi T. Electron microscopic study of gemadsorbtion on vaccinia virus infected cells. *Microbiol. Immunol.*, 1977, 21(10): 593-600.
8. Breese S.S., Hess W.R. Electron microscopy of African swine fever virus hemadsorbtion. *J. Bacteriol.*, 1966, 92(1): 272-274.
9. Makarov V., Nedosekov V., Sereda A., Matvienko N. Immunological conception of African swine fever. *Zoology and Ecology*, 2016, 26(3): 236-243 (doi: 10.1080/21658005.2016.1182822).
10. Makarov V.V. *Afrikanskaya chuma svinei* [African swine fever]. Moscow, 2011 (in Russ.).
11. Coggins L. Segregation of a non-hemadsorbing African swine fever virus in tissue culture. *The Cornell Veterinarian*, 1968, 58(1): 12-20.
12. Coggins L. African swine fever virus. Pathogenesis. *Prog. Med. Virol.*, 1974, 18: 48-65.
13. Malakhova M.S. *Vzaimodeistvie virusa ACHS s immunokompetentnymi kletkami svin'i. Kandidatskaya dissertatsiya* [ASF virus interaction with immune cells in pigs. PhD Thesis]. Pokrov, 1987 (in Russ.).
14. Quintero J.C., Wesley R.D., Whyard T.C., Gregg D., Mebus C.A. In vitro and in vivo association of African swine fever virus with swine erythrocytes. *Am. J. Vet. Res.*, 1986, 47(5): 1125-1131.
15. Rowlands R.J., Duarte M.M., Boinas F., Hutchings G., Dixon L.K. The CD2v protein enhances African swine fever virus replication in the tick vector, *Ornithodoros erraticus*. *Virology*, 2009, 393: 319-328 (doi: 10.1016/j.virol.2009.07.040).
16. Sierraj M.A., Gomez-Villamanld C., Rasco O., Fernandeez A., Jover A. In vivo study of hemadsorption in African swine fever virus infected cells. *Vet. Pathol.*, 1991, 28: 178-181.

17. Makarov V.V., Vishnyakov I.F., Vlasov N.A., Serova A.M. *Voprosy virusologii*, 1991, 4: 321-324 (in Russ.).
18. Makarov V.V., Sukharev O.I., Tsvetnova I.V. *Veterinarnaya praktika*, 2013, 60(1): 6-16 (in Russ.).
19. Makarov V.V. *Veterinarnaya praktika*, 2013, 62(3): 7-22 (in Russ.).
20. Mitin N.I., Shevchenko A.A., Balyshev V.M., Elisova S.N. Differentsiatsiya virulentnykh i attenuirovannykh shtammov virusa ACHS v RZGA. *Tezisy nauchnoi konferentsii VNIIVViM, posvyashchennoi 40-letiyu Velikoi Pobedy «Voprosy veterinarnoi virusologii, mikrobiologii i epizootologii»* [Proc. Conf. «Veterinary virology, microbiology and epizootology»]. Pokrov, 1985: 39-41 (in Russ.).
21. Shubina N.G., Kolontsov A.A., Malakhova M.S., Makarov V.V. *Byulleten' eksperimental'noi biologii i meditsiny*, 1996, 122(10): 418-424 (in Russ.).
22. Šereš M., Cholujová D., Bubenčíkova T., Breier A., Sulová Z. Tunicamycin depresses P-glycoprotein glycosylation without an effect on its membrane localization and drug efflux activity in L1210 cells. *Int. J. Mol. Sci.*, 2011, 12(11): 7772-7784 (doi: 10.3390/ijms12117772).
23. Makarov V.V., Sereda A.D., Pirya A.A., Malakhova M.S. *Voprosy virusologii*, 1992, 5-6: 267-270 (in Russ.).
24. Reszka N., Krol E., Patel A.H., Szewczyk B. Effect of tunicamycin on the biogenesis of hepatitis C virus glycoproteins. *Acta biochimika Polonica*, 2010, 57(4): 541-546.
25. Shi X., Brauburger K., Elliott R.M. Role of N-linked glycans on bunyamwera virus glycoproteins in intracellular trafficking, protein folding, and virus infectivity. *J. Virol.*, 2005, 79(21): 13725-13734 (doi: 10.1128/jvi.79.21.13725-13734.2005).
26. Gonzague M., Roger F., Bastos A., Burger C., Randriamparany T., Smondack S., Cruciere C. Isolation of a non-haemadsorbing, non-cytopathic strain of African swine fever virus in Madagascar. *Epidemiol. Infect.*, 2001, 126(3): 453-459 (doi: 10.1017/s0950268801005465).
27. Boinas F.S., Hutchings G.H., Dixon L.K., Wilkinson P.J. Characterization of pathogenic and non-pathogenic African swine fever virus isolates from *Ornithodoros erraticus* inhabiting pig premises in Portugal. *J. Gen. Virol.*, 2004, 85: 2177-2187 (doi: 10.1099/vir.0.80058-0).
28. Oura C.A.L., Denyer M.S., Takamatsu H., Parkhouse R.M.E. In vivo depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus. *J. Gen. Virol.*, 2005, 86(9): 2445-2450 (doi: 10.1099/vir.0.81038-0).
29. Souto R., Mutowembwa P., van Heerden J., Fosgate G.T., Heath L., Vosloo W. Vaccine potential of two previously uncharacterized African swine fever virus isolates from Southern Africa and heterologous cross protection of an avirulent European isolate. *Transbound. Emerg. Dis.*, 2016, 63: 224-231 (doi: 10.1111/tbed.12250).
30. Kolbasov D.V., Sereda A.D. *Veterinariya*, 2013, 1: 19-23 (in Russ.).
31. Jori F., Bastos A.D.S. Role of wild suids in the epidemiology of African swine fever. *EcoHealth*, 2009, 6(2): 296-310 (doi: 10.1007/s10393-009-0248-7).
32. Roger F., Cruciиre C., Randriamahefa N., Zeller H., Uilenberg G., Randriamparany T., Gonzague M., Rousset D., Benkirane A., Diallo A. African swine fever in Madagascar: epidemiological assessment of the recent epizootic. *Proc. 9th Int. Symp. on Veterinary Epidemiology and Economics.* Breckenridge, Colorado, USA, 2000.
33. Sereda A.D., Balyshev V.M., Morgunov Yu.P., Kolbasov D.V. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2014, 1: 64-69 (doi: 10.15389/agrobiology.2014.4.64rus) (in Russ.).
34. Badaev F.A., Rudobel'skii E.V., Chevelev S.F., Kiselev A.V., Nikishin I.V., Badaeva N.V., Burlakov V.A., Balabanov V.A., Zakharov V.M., Baibikov T.Z., Burdov A.N. *Materialy nauchnoi konferentsii VNIIVViM «Voprosy veterinarnoi virusologii, mikrobiologii i epizootologii»* [Proc. Conf. «Veterinary virology, microbiology and epizootology»]. Pokrov, 1992, 1: 24-27 (in Russ.).
35. Badaev F.A., Cheryatnikov L.L., Rudobel'skii E.V., Chevelev S.F., Kiselev A.V., Badaeva N.V., Zakharov V.M., Baibikov T.Z. *Materialy nauchnoi konferntsii VNIIVViM «Voprosy veterinarnoi virusologii, mikrobiologii i epizootologii»* [Proc. Conf. «Veterinary virology, microbiology and epizootology»]. Pokrov, 1992, 1: 44 (in Russ.).
36. Rodríguez J.M., Moreno L.T., Alejo A., Lacasta A., Rodríguez F., Salas M.L. Genome sequence of African swine fever virus BA71, the virulent parental strain of the nonpathogenic and tissue-culture adapted BA71V. *PLoS ONE*, 2015, 10(11): e0142889 (doi: 10.1371/journal.pone.0142889).
37. Portugal R., Coelho J., Hoper D., Little N.S., Smithson C., Upton C., Martins C., Leitro A., Keil G.M. Related strains of African swine fever virus with different virulence: genome comparison and analysis. *J. Gen. Virol.*, 2015, 96: 408-419 (doi: 10.1099/vir.0.070508-0).
38. Morgunov Yu.P., Malogolovkin A.S., Morgunov S.Yu., Burmakina G.S., Kushnir S.D., Yurkov S.G., Tsybanov S.Zh., Kolbasov D.V. *Veterinariya*, 2015, 10: 53-57 (in Russ.).

39. Krug P.W., Holinka L.G., O'Donnell V., Reese B., Sanford B., Fernandez-Sainz I., Gladue D.P., Arzt J., Rodriguez L., Risatti G.R., Borca M.V. The progressive adaptation of a Georgian isolate of African swine fever virus to Vero cells leads to a gradual attenuation of virulence in swine corresponding to major modifications of the viral genome. *J. Virol.*, 2015, 89(4): 2324-2332 (doi: 10.1128/JVI.03250-14).
40. Neser J.A., Pidllips T., Thomson G.R., Gainaru M.D., Coetzee T. African swine fever. I. Morphological changes and virus replication in blood platelets of pigs infected with virulent haemadsorbing and non-haemadsorbing isolates. *Onderstepoort J. Vet.*, 1986, 53: 133-141.
41. Reis A.L., Parkhouse R.M., Penedos A.R., Martins C., Leitão A. Systematic analysis of longitudinal serological responses of pigs infected experimentally with African swine fever virus. *J. Gen. Virol.*, 2007, 88(9): 2426-2434 (doi: 10.1099/vir.0.82857-0).
42. Takamatsu H.H., Denyer M.S., Lacasta A., Stirling C.M.A., Argilaguet J.M., Netherton C.L., Oura C.A.L., Martins C., Rodríguez F. Cellular immunity in ASFV responses. *Virus Res.*, 2013, 173(1): 110-121 (doi: 10.1016/j.virusres.2012.11.009).
43. Bredikhina T.T., Kolosov V.M., Arkhipova S.N. *Tezisy nauchno-teoreticheskoi konferentsii VNIIVViM, posvyashchennoi 60-letiyu obrazovaniya SSSR* [Proc. Conf. of ARRIVV&M]. Pokrov, 1983: 120-123 (in Russ.).
44. Bredikhina T.G., Kolosov V.M. *Tezisy nauchno-teoreticheskoi konferentii VNIIVViM, posvyashchennoi 60-letiyu obrazovaniya SSSR* [Proc. Conf. of ARRIVV&M]. Pokrov, 1983: 123-124 (in Russ.).
45. Sereda A.D. *Nauchnyi zhurnal KubGAU*, 2010, 62(8). Available http://ej.kubagro.ru/2010/08/pdf/23.pdf. No date (in Russ.).
46. Vigario I.D., Terrinha A.M., Moura Nunes J.F. Antigenic relationships among strains of African swine fever virus. *Archiv fur die gesamte Virusforschung*, 1974, 45(3): 272-277.
47. Borca M.V., Kutish G.F., Afonso C.L., Irusta P., Carrillo C., Brun A., Sussman M., Rock D.L. An African swine fever virus gene with similarity to the T-lymphocyte surface-antigen Cd2 mediates hemadsorption. *Virology*, 1994, 199(2): 463-468 (doi: 10.1006/viro.1994.1146).
48. Rodríguez J.M., Yácez R.J., Almazán F., Vicuela E., Rodriguez J.F. African swine fever virus encodes a Cd2 homolog responsible for the adhesion of erythrocytes to infected-cells. *J. Virol.*, 1993, 67(9): 5312-5320.
49. Kay-Jackson P.C., Goatley L.C., Cox L., Miskin J.E., Parkhouse R.M., Wienands J., Dixon L.K. The CD2v protein of African swine fever virus interacts with the actin-binding adaptor protein SH3P7. *J. Gen. Virol.*, 2004, 85(1): 119-130 (doi: 10.1099/vir.0.19435-0).
50. Mima K.A., Burmakina G.S., Titov I.A., Malogolovkin A.S. African swine fever virus glycoproteins p54 and CD2v in the context of immune response modulation: bioinformatic analysis of genetic variability and heterogeneity. *Agricultural Biology*, 2015, 50(6): 785-793 (doi: 10.15389/agrobiology.2015.6.785eng) (in Engl.).
51. Ruiz-Gonzalvo F., Rodríguez F., Escribano J.M. Functional and immunological properties of the baculovirus-expressed hemagglutinin of African swine fever virus. *Virology*, 1996, 218(1): 285-289 (doi: 10.1006/viro.1996.0193).
52. Goatley L.C., Dixon L.K. Processing and localization of the African swine fever virus CD2v transmembrane Protein. *J. Virol.*, 2011, 85(7): 3294-3305 (doi: 10.1128/JVI.01994-10).
53. Tulman E.R., Delhon G.A., Ku B.K., Rock D.L. African swine fever virus. *Curr. Top. Microbiol. Immunol.*, 2009, 328: 43-87.
54. Dixon L.K., Abrams C.C., Bowick G., Goatley L.C., Kay-Jackson P.C., Chapman D., Liverani E., Nix R., Silk R., Zhang F. African swine fever virus proteins involved in evading host defense systems. *Vet. Immunol. Immunopathol.*, 2004, 100(3-4): 117-134 (doi: 10.1016/j.vetimm.2004.04.002).
55. Galindo I., Almazán F., Bustos M.J., Vicuela E., Carrascosa A.L. African swine fever virus *EP153R* open reading frame encodes a glycoprotein involved in the hemadsorption of infected cells. *Virology*, 2000, 266(2): 340-351 (doi: 10.1006/viro.1999.0080).
56. Burmakina G., Malogolovkin A., Tulman E.R., Zsak L., Delhon G., Diel D.G., Shobogorov N.M., Morgunov Y.P., Morgunov S.Y., Kutish G.F., Kolbasov D., Rock D.L. African swine fever virus serotype-specific proteins are significant protective antigens for African swine fever. *J. Gen. Virol.*, 2016, 97(7): 1670-1675 (doi: 10.1099/jgv.0.000490).
57. Neilan J.G., Borca M.V., Lu Z., Kutish G.F., Kleiboeker S.B., Carrillo C., Zsak L., Rock D.L. An African swine fever virus ORF with similarity to C-type lectins is non-essential for growth in swine macrophages in vitro and for virus virulence in domestic swine. *J. Gen. Virol.*, 1999, 80(10): 2693-2697 (doi: 10.1099/0022-1317-80-10-2693).
58. Hurtado C., Granja A.G., Bustos M.J., Nogal M.L., Buitrago G.G., Yébenes V.G., Salas M.L., Revilla Y., Carrascosa A.L. The C-type lectin homo-

logue gene (*EP153R*) of African swine fever virus inhibits apoptosis both in virus infection and in heterologous expression. *Virology*, 2004, 326(1): 160-170 (doi: 10.1016/j.virol.2004.05.019).
59. Hurtado C., Bustos M.J., Granja A.G., Leyn P., Sabina P., Lypez-Viñas E., Gymez-Puertas P., Revilla Y., Carrascosa A.L. The African swine fever virus lectin EP153R modulates the surface membrane expression of MHC class I antigens. *Arch. Virol.*, 2011, 156: 219-234 (doi: 10.1007/s00705-010-0846-2).
60. Chapman D.A., Tcherepanov V., Upton C., Dixon L.K. Comparison of the genome sequences of nonpathogenic and pathogenic African swine fever virus isolates. *J. Gen. Virol.*, 2008, 89: 397-408 (doi: 10.1099/vir.0.83343-0).
61. Leitro A., Cartaxeiro C., Coelho R., Cruz B., Parkhouse R.M., Portugal F., Vigario J.D., Martins C.L. The non-haemadsorbing African swine fever virus isolate ASFV/NH/P68 provides a model for defining the protective anti-virus immune response. *J. Gen. Virol.*, 2001, 82(3): 513-523 (doi: 10.1099/0022-1317-82-3-513).
62. Malogolovkin A., Burmakina G., Tulman E.R., Delhon G., Diel D.G, Salnikov N., Kutish G.F., Kolbasov D., Rock D.L. African swine fever virus CD2v and C-type lectin gene loci mediate serological specificity. *J. Gen. Virol.*, 2015, 96: 866-873 (doi: 10.1099/jgv.0.000024).
63. Rowlands R.J., Duarte M.M., Boinas F., Hutchings G., Dixon L.K. The CD2v protein enhances African swine fever virus replication in the tick vector, *Ornithodoros erraticus*. *Virology*, 2009, 393(2): 319-328 (doi: 10.1016/j.virol.2009.07.040).
64. Sereda A.D., Anokhina E.G., Fugina L.G., Makarov V.V. *Veterinariya*, 1993, 1: 26-28 (in Russ.).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 775-781
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Marker-assisted selection, transgenesis — fundamentals

UDC 636.2.034:636.082:575.162

doi: 10.15389/agrobiology.2016.6.775rus
doi: 10.15389/agrobiology.2016.6.775eng

ASSOCIATIONS OF *BoLA-DRB3* GENOTYPES WITH BREEDING VALUES FOR MILK PRODUCTION TRAITS IN RUSSIAN DAIRY CATTLE POPULATION

A.A. SERMYAGIN[1], N.V. KOVALYUK[2], A.N. ERMILOV[1], I.N. YANCHUKOV[1], V.F. SATSUK[2], A.V. DOTSEV[1], T.E. DENISKOVA[1], G. BREM[1, 3], N.A. ZINOVIEVA[1]

[1]*L.K. Ernst All-Russian Research Institute of Animal Husbandry,* Federal Agency of Scientific Organizations, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail alex_sermyagin85@mail.ru;
[2]*North-Caucasian Institute of Animal Production*, Federal Agency of Scientific Organizations, 4, ul. Pervomayskaya, pos. Znamenskii, Krasnodar, 350055 Russia, e-mail nvk1972@yandex.ru;
[3]*Institut für Tierzucht und Genetik, University of Veterinary Medicine (VMU),* Veterinärplatz, A-1210, Vienna, Austria, e-mail gottfried.brem@agrobiogen.de
Acknowledgements:
Supported by Federal Agency of Scientific Organizations and Ministry of Education and Science of the Russian Federation (project № 14.604.21.0062, RFMEFI60414X0062)
Received September 14, 2016

Abstract

Breeding for genetically resistant cattle is an attractive approach to eradicate infectious disease in livestock. Bovine leukaemia is one of the most common infectious diseases of dairy cattle, which causes significant economic losses. Numerous studies have demonstrated that polymorphisms in the *DRB3* gene at the bovine leukocyte antigen (*BoLA*) locus are associated with cow resistance to persistent lymphocytosis (PL), a clinical sign of leukaemia. Alleles associated with PL susceptibility are generally prevalent in dairy cattle populations and may reflect their association with milk productivity. Our objective was to evaluate the associations between genotypes at the *BoLA-DRB3* locus and milk production traits in dairy cattle in Russia. In total, 171 Holstein sires were genotyped for *DRB3* using the PCR-RFLP technique. Detected *BoLA-DRB3* alleles were assigned to three categories according to their PL susceptibility. The *DRB3.2*08*, *DRB3.2*16*, *DRB3.2*22* and *DRB3.2*24* alleles were defined as susceptible (S), whereas alleles *DRB3.2*11*, *DRB3.2*23* and *DRB3.2*28* were identified as resistant (R), and the remaining alleles were denoted as neutral (N). We calculated the estimated breeding values (EBV) for milk production traits, including milk yield (MY), fat yield (FY) and protein yield (PY), using genotype categories at the *BoLA-DRB3* locus as a fixed effect. We detected fifteen alleles, forty-nine genotypes and six genotype categories for the *BoLA-DRB3* gene. The PL-susceptibility alleles were prevalent in Russian dairy sires (ranging from 0.0877 for *BoLA-DRB3.2*08* to 0.2135 for *BoLA-DRB3.2*22*), assuming 0.6258. The bulls carrying the SS genotype category were characterized by significantly higher EBVs for MY and PY compared to the other genotype categories: 89.3 kg for MY ($p < 0.001$ vs. NN, NR, SN, $p < 0.01$ vs. SR, $p < 0.05$ vs. RR), 2.15 kg for FY ($p < 0.001$ vs. NN, NR, $p < 0.05$ vs. SN); 4.81 kg for PY ($p < 0.001$ vs. all other genotype categories). Our findings indicate that high frequencies of PL-susceptibility alleles in *BoLA-DRB3* may be a result of their association with milk production traits in dairy cattle in Russia. Therefore, to prevent the development of clinical forms of bovine leukaemia in dairy herds in Russia, the impact of the genotypes at the *BoLA-DRB3* gene locus on milk production traits must be considered.

Keywords: *BoLA-DRB3* genotypes, BLV susceptible alleles, milk production traits, genetically resistant cattle

Breeding to develop a livestock population genetically resistant to infection diseases is very important step toward the eradication of infection disease in farm animals and the sustainable food production. Bovine leukemia is an economically important infection disease of dairy cattle, which is caused by bovine leukemia virus (BLV). Economic losses due to BLV infection can come from reduced milk production, reduced reproductive efficiency, increased replacement costs, and increased veterinary costs [1]. High degree of BLV prevalence in dairy

herds worldwide has been shown [2]. In Russia, despite the combating this disease, its ration in the structure of infectious diseases of cattle is more than 50 % [3]. It has been reported, that highly polymorphic DRB3 locus, which is located in the Bovine leukocyte antigen (BoLA) class II region on BTA23 [4] is involved in susceptibility and resistance to clinical signs of leucosis in the form of persistent lymphocytosis (PL). Molecular basis for gene control of the resistance and susceptibility to PL is the presence of the amino acids Glu-Arg at putative Ag binding residues 70 and 71 of the BoLA-DRB3 [5]. It has been demonstrated that carriers of the *DRB3.2*11*, *DRB3.2*23* and *DRB3.2*28* alleles containing the Glu-Arg at residues 70-71 are resistant (R), i.e. they are not prone to the transition of leukemia to PL. Conversely, carriers of the *DRB3.2*08*, *DRB3.2*16*, *DRB3.2*22* and *DRB3.2*24* alleles are susceptible (S) to PL, i.e. they are more often affected by hematological diseases than other genotypes. The remaining alleles are neutral (N), i.e. they are not associated with either resistance or susceptibility to PL [5-9].

The intense studies to characterize the genetic variability at BoLA-DRB3 were performed in Holsteins [reviewed by 10] — the most widely used dairy cattle breed. Up to 29 alleles were identified in regional Holstein populations and S alleles were the most common in majority of them [11-15]. We hypothesized that prevalence of S alleles of *BoLA-DRB3.2* may reflect the effect of the selection pressure for milk production traits. There is experimental evidence that some of the *BoLA-DRB3* alleles are associated with milk productivity in cattle, but the associations are in some cases antagonistic [16-19]. No studies were performed to evaluate the effect of *BoLA-DRB3* genotypes assigned to categories according to susceptibility to PL on milk production traits.

Our objective was to study the effect of the genotype categories at *BoLA-DRB3* locus defined according their susceptibility to PL on estimated breeding values (EBVs) for milk production traits in dairy bulls in Russia.

Technique. The samples were collected from 171 sires having 62096 data records in 77 herds. Genomic DNA was extracted from semen samples, using Diatom Prep 100 kit (Izogen, Russia) according to recommendation by manufacture. The *BoLA-DRB3.2* genotypes were analyzed using PCR-RFLP technique as described by M.J.T. Van-Eijk et al. [20]. Genotype variants were identified by comparing the restriction patterns previously described in literature [20; BoLA nomenclature, ISAG]. Six categories of genotypes (SS, SR, SN, NR, NN, RR) were assigned according the presence of S, R and N alleles, whereas *DRB3.2*11*, *DRB3.2*23* and *DRB3.2*28* alleles are resistant (R), *DRB3.2*08*, *DRB3.2*16*, *DRB3.2*22* and *DRB3.2*24* alleles are susceptible (S) and the remaining alleles are neutral (N).

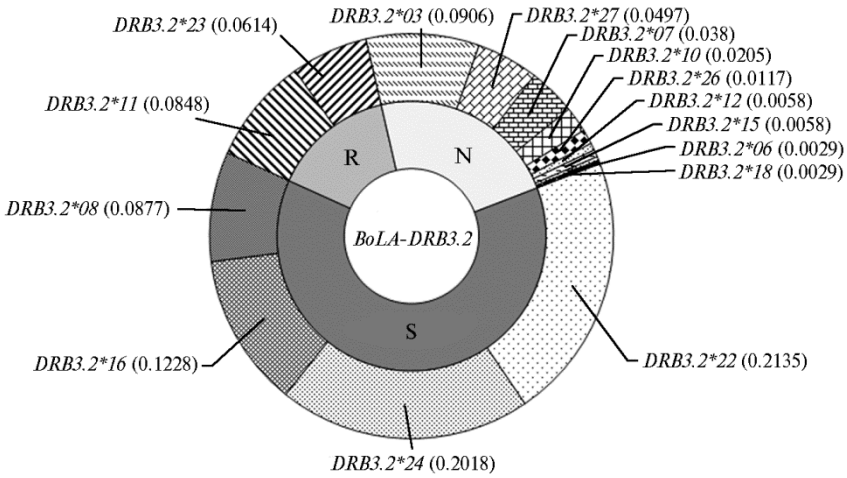
Association study was conducted using estimated breeding values (EBVs) for milk production traits including milk yield (MY), fat yield (FY) and protein yield (PY). EBVs were calculated by best linear unbiased estimator procedure (BLUP) written in the SAS IML software (SAS Inst., Inc., Cary, NC, USA). The heritability values calculated by ANOVA based on the estimated variance and covariance components were 0.390 for 305-d MY, 0.459 for FY and 0.316 for PY. To evaluate the association between the categories of genotypes (SS, SN, SR, NN, NR, RR) and EBVs for milk production traits the following mixed model was used:

$$Y_{ijkl} = \mu + HYS_i + \sum_k b_1 A_k + \sum_k b_2 DO_k + \sum_l \alpha_l BoLA_l + Sire_j + e_{ijkl},$$

where Y_{ijkl} is the dependent variable of production traits (MY, FY, and PY), μ is the population mean of the analyzed traits, HYS_i is the fixed effects of herd-year-season of the parity i (I = 1, ..., 2075 subclasses), $b_{1,2}$ are the linear regres-

sion coefficients, A_k and DO_k is respectively the age of fist calving and days open of the k_{th} cow, $_l$ is the regression coefficient on the number of copies of the l_{th} BoLA-DRB3.2 allele (0, 1 or 2), $BoLA_l$ is the fixed effect of l_{th} BoLA-DRB3 genotype category (l = 1, ..., 6 subclasses), $Sire_j$ is the random permanent sire effect of j_{th} bull assumed being distributed normally and independently with mean 0 and variance $A\sigma_a^2$,where A is the pedigree additive relationship matrix (j = 1, ..., 171 sires) and finally e_{ijk} is the random residual effect with mean 0 and variance σ_e^2.

Results. We identified fifteen *BoLA-DRB3* alleles in the investigated bull population. The *DRB3.2*08*, *DRB3.2*16*, *DRB3.2*22* and *DRB3.2*24* alleles, which are susceptible (S) to PL comprised 62.6 %. The frequency of resistant (R) to PL alleles (*DRB3.2*11*, *DRB3.2*23*) was 14.6 %. Nine alleles were neutral (N) to PL with the frequency ranged from 0.3% for *DRB3.2*06* to 9.1 % for *DRB3.2*03* assuming 22.8 % (Fig.).



Allele frequencies of BoLA-DRB3.2 in dairy bulls in Russia. The BoLA-DRB3.2 alleles are shown according their categories (S — susceptible, R — resistant and N — neutral) descending their frequencies within each of categories; alleles frequency of the each of alleles is indicated after the symbol of allele.

In total, we identified 49 genotype variants of *BoLA-DRB3.2* (**03/03 ... *24/27*) in studied sires and assigned them to six genotype categories according to presence of S, N and R alleles: SS (9 genotypes), SN (17 genotypes), NN (6 genotypes), SR (8 genotypes), NR (8 genotypes) and RR (1 genotype). Results of association studies are summarized in Table.

Effects of *BoLA-DRB3.2* genotype categories on breeding values for milk production traits in dairy bulls in Russia

Bull genotype	Number of daughters	EBV_{MY}±SEP	EBV_{FY}±SEP	EBV_{PY}±SEP
SS	25937	89.3±11.7	2.15±0.51	4.81±0.36
SR	12524	25.1±16.8**	1.62±0.74$^{\times\times}$	2.14±0.52^{a}
SN	11601	–0.7±17.5***	0.21±0.76$^{\times}$	–0.31±0.54^{a}
NR	7273	–2.0±22.1***	–2.14±0.96$^{\times\times\times}$	–0.18±0.69^{a}
NN	4145	–20.9±29.3***	–2.92±1.28$^{\times\times\times}$	–1.18±0.91$^{a, b}$
RR	616	–90.7±75.9*	1.08±3.32	–5.28±2.36$^{a, b, c}$

N o t e. EBV — estimated breeding value (calculated using *BoLA-DRB3.2* genotype category as a fixed effect, 6 subclasses), SEP — standard error prediction.
*** $p < 0.001$ (SS vs. NN, SS vs. NR, SS vs. SN); ** $p < 0.01$ (SS vs. SR); * $p < 0.05$ (SS vs. RR); $^{\times\times\times}$ $p < 0.001$ (SS vs. NN, SS vs. NR); $^{\times\times}$ $p < 0.01$ (SR vs. NN); $^{\times}$ $p<0.05$ (SS vs. SN, SN vs. NN); ^{a} $p < 0.001$ (SS vs. all other genotype categories); ^{b} $p < 0.01$ (SR vs. NN, SR vs. RR); ^{c} $p < 0.05$ (NR vs. RR).

The bulls carrying SS genotypes showed the highest EBVs for MY (89.3 kg), FY (2.15 kg) and PY (4.81 kg). The significant differences were observed be-

tween SS and all other genotype categories for MY (+64.2 kg vs. SR ($p < 0.01$), +90.0 kg vs. SN ($p < 0.001$), +91.3 kg vs. NR ($p < 0.001$), +110.2 kg vs. NN ($p < 0.001$) and +180.0 kg vs. RR ($p<0.05$)) and for PY (+2.67 kg vs. SR ($p < 0.001$), +5.12 kg vs. SN ($p < 0.001$), +4.99 kg vs. NR ($p < 0.001$), +5.99 kg vs. NN ($p < 0.001$) and +10.09 kg vs. RR ($p<0.001$)). Bulls carrying SS genotype category have significantly higher EBVs for FY comparing to three of the five remaining genotypes: +1.94 kg vs. SN ($p < 0.05$), +4.29 kg vs. NR ($p < 0.001$) and +5.07 kg vs. NN ($p < 0.001$).

The S alleles of *BoLA-DRB3* (*DRB3.2*08*, *DRB3.2*16*, *DRB3.2*22* and *DRB3.2*24*) have been shown to be prevalent in the majority of the Holstein population. Twenty-seven and fourteen alleles were identified in two studies of Canadian Holstein cows and S alleles were the most frequent assuming 62.2 and 59.3 %, respectively [11, 12]. S alleles were the most common (56.8 %) among sixteen alleles detected in Japanese Holstein cows [21]. Among 28 alleles identified in Iranian Holsteins 48.3 % were S alleles [13]. Twenty-nine *DRB3.2* alleles were identified in two herds of Polish Holstein-Frisian cattle and the frequencies of S alleles were 47.3 and 48.0 % [15]. S alleles comprised 44.7 % of all the alleles of *BoLA-DRB3.2* distributed in populations of American Holsteins [6]. The altered patterns of BoLA-DRB3.2 allele distribution was observed in Chinese regional populations of Holsteins, that is probably the result of specific breeding strategy [22]. We observed the highest frequencies of S alleles in Russian dairy bulls (62.5 %) among all of the regional Holstein populations analyzed.

Numerous studies have been performed to find the association between *BoLA-DRB3* alleles and milk production traits. A significant positive effect of allele *DRB3.2*11* on MY and PY and negative effect of allele *DRB3.2*08* on MY, FY and PY were observed in American dairy cows [16]. In similar study, R. Rupp et al. [18] have also reported a positive effect of allele *DRB3.2*11* on MY, FY and PY in a Canadian dairy population. Conversely, S. Sharif et al. [17] reported a positive effect of allele *DRB3.2*08* and negative effect of *DRB3.2*22* on PY in Canadian cow population. Allele *DRB3.2*24* has been shown to be associated with increased FY in American dairy cows [16]. Cows of Black-and-White and Red Gorbatov breeds, carrying homozygote genotypes for resistant alleles *DRB3.2*11*, *DRB3.2*23* и *DRB3.2*28*, were characterized by low milk yield comparing to cows carrying other genotypes [23]. In contrast, M. Pashimi et al. [13] observed a significant association of allele *DRB3.2*22* with an increase in PY. Using *BoLA-DRB3* genotype as a fixed effect we did not observed significant associations of alleles with EBVs for milk production trait in Russian dairy bulls, probably due the small number of animals carrying the certain genotypes (49 different BoLA-DRB3 genotypes were detected in 171 sires) and high variability degree of milk production traits (data not shown). But association studies performed for BoLA-DRB3 genotype categories assigned according their susceptibility to PL revealed significant association of SS genotypes (carrying ER motif at residues 70-71) with increased breeding values for MY and PY comparing to all other genotypes and for FY comparing to three of the five remaining genotypes. It became evident that the eradication programs for bovine leukemia based on the breeding genetically resistant cattle using *BoLA-DRB3* as a marker should be developed for one population of animals and not across populations and breeds, and presumable associations of this genetic marker with milk production traits should be previously evaluated.

Thus, based on our data, we conclude, that high frequencies of *BoLA-DRB3* alleles, which are susceptible to clinical signs of leucosis, observed in Russian dairy cattle, may reflect to their association with milk production traits. We detected significant higher breeding values for milk production traits in sires car-

rying two alleles of *BoLA-DRB3* susceptible to PL comparing to all other genotype categories for MY and PY and to three of five other genotype categories for PY. Thus, development of eradication programs for bovine leukemia based utilizing *BoLA-DRB3* locus as a genetic marker has to be taken into account the pleiotropic effects of this gene on milk production traits and has to be based on the balance between these important health- and economically related traits.

REFERENCES

1. Ott S.L., Johnson R., Wells S.J. Association between bovine-leukosis virus seroprevalence and herd-level productivity on US dairy farms. *Prev. Vet. Med.*, 2003, 61: 249-262 (doi: 10.1016/j.prevetmed.2003.08.003).
2. Nekouei O., Van Leeuwen J., Sanchez J., Kelton D., Tiwari A., Keefe G. Herd-level risk factors for infection with bovine leukemia virus in Canadian dairy herds. *Prev. Vet. Med.*, 2015, 119: 105-113 (doi: 10.1016/j.prevetmed.2015.02.025).
3. Ivanov O.V., Ivanova O.Yu., Fedotov V.P., Balandina M.V., Verkhovskii O.A., Fedorov Yu.N. Age dynamics in number of antibodies against leucosis virus in calves born from seropositive cows. *Agricultural Biology*, 2008, 6: 87-90 (in Engl.).
4. Sigurdardottir S., Lunden A., Andersson L. Restriction fragment length polymorphism of DQ and DR class II genes of the bovine major histocompatibility complex. *Anim. Genet.*, 1988, 19: 133-150 (doi: 10.1111/j.1365-2052.1988.tb00799.x).
5. Xu A.L., Vaneijk M.J.T., Park C., Lewin H.A. Polymorphism in *BoLA-DRB3* exon 2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia virus. *J. Immunol.*, 1993, 151: 6977-6985.
6. Kelm S.C., Deitilleux J.C., Freeman A.E., Kehrli M.E. Jr., Dietz A.B., Fox L.K., Butler J.E., Kasckovics I., Kelley D.H. Genetic association between parameters of innate immunity and measures of mastitis in preparturient Holstein cattle. *J. Dairy Sci.*, 1997, 80: 1767-1775.
7. Lewin H.A., Russell G.C., Glass E.J. Comparative organization and function of the major histocompatibility complex of domesticated cattle. *Immunol. Rev.*, 1999, 167: 145-158 (doi: 10.1111/j.1600-065X.1999.tb01388.x).
8. Juliarena M.A., Poli M., Sala L., Ceriani C., Gutierrez S., Dolcini G., Rodríguez E.M., Marico B., Rodríguez-Dubra C., Esteban E.N. Association of BLV infection profiles with alleles of the *BoLA-DRB3.2* gene. *Anim. Genet.*, 2008, 39(4): 432-438 (doi: 10.1111/j.1365-2052.2008.01750.x).
9. Forletti A., Juliarena M.A., Ceriani C., Amadio A.F., Esteban E., Gutiérrez S.E. Identification of cattle carrying alleles associated with resistance and susceptibility to the Bovine Leukemia Virus progression by real-time PCR. *Res. Vet. Sci.*, 2013, 95(3): 991-995 (doi: 10.1016/j.rvsc.2013.07.018).
10. Behl J.D., Verma N.K., Tyagi N., Mishra P., Behl R., Joshi B.K. The major histocompatibility complex in bovines: a review. *ISRN Vet. Sci.*, 2012, Article ID 872710 (doi: 10.5402/2012/872710).
11. Sharif S., Mallard B.A., Wilkie B.N., Sargeant J.M., Scott H.M., Dekkers J.C., Leslie K.E. Associations of the bovine major histocompatibility complex *DRB3* (*BoLA-DRB3*) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. *Anim. Genet.*, 1998, 29: 157-160.
12. Ledwidge S.A., Mallard B.A., Gibson J.P., Jansen G.B., Jiang Z.H. Multiprimer target PCR for rapid identification of bovine *DRB3* alleles. *Anim. Genet.*, 2001, 32: 219-221.
13. Pashmi M., Qanbari S., Ghorashi S.A., Sharifi A.R., Simianer H. Analysis of relationship between bovine lymphocyte antigen *DRB3.2* alleles, somatic cell count and milk traits in Iranian Holstein population. *J. Anim. Breed. Genet.*, 2009, 126: 296-303 (doi: 10.1111/j.1439-0388.2008.00783.x).
14. Kovaljuk N.V., Satsuk V.F., Matviets A.V., Machulskaja E.V. Possible causes and consequences of the spread of individual allelic variants of the *BoLA–DRB3* locus in groups of Holstein and Ayrshire cattle. *Russ. J. Genet.*, 2010, 46(3): 379-382.
15. Oprządek J., Urtnowski P., Sender G., Pawlik A., Łukaszewicz M. Frequency of *BoLA-DRB3* alleles in Polish Holstein-Friesian cattle. *Anim. Sci. Paper Rep.*, 2012, 30(2): 91-101.
16. Starkenburg R.J., Hanses L.B., Kehrli Jr.M.E., Chester-Jones H. Frequencies and effects of alternative *DRB3.2* alleles of bovine lymphocyte antigen for Holsteins in milk selection and control lines. *J. Dairy Sci.*, 1997, 80: 3411-3419 (doi: 10.3168/jds.S0022-0302(97)76316-1).
17. Sharif S., Mallard B.A., Wilkie B.N., Sargeant J.M., Scott H.M., Dek-

kers J.C., Leslie K.E. Associations of the bovine major histocompatibility complex DRB3 (*BoLA-DRB3*) with production traits in Canadian dairy cattle. *Anim. Genet.*, 1998, 30(2): 157-160 (doi: 10.1046/j.1365-2052.1999.00459.x).

18. Rupp R., Hernandez A., Mallard B.A. Association of bovine leukocyte antigen (BoLA) DRB3.2 with immune response, mastitis, and production and type traits in Canadian Holstein. *J. Dairy Sci.*, 2007, 90: 1029-1038 (doi: 10.3168/jds.S0022-0302(07)71589-8).
19. Duangjinda M., Buayai D., Pattarajinda V., Phasuk Y., Katawatin T., Vongpralub T., Chaiyotvittayakul A. Detection of bovine leukocyte antigen *DRB3* alleles as candidate markers for clinical mastitis resistance in Holstein × Zebu. *J. Anim. Sci.*, 2009, 87: 469-476 (doi: 10.2527/jas.2007-0789).
20. Van Eijk M.J.T., Stewart-Haynes J.A., Lewin H.A. Extensive polymorphism of the *BoLA-DRB3* gene distinguished by PCR-RFLP. *Anim. Genet.*, 1992, 23: 483-496 (doi: 10.1111/j.1365-2052.1992.tb00168.x).
21. Yoshida T., Mukoyama H., Furuta H., Kondo Y., Takeshima S.N., Aida Y., Kosugiyama M., Tomogane H. Association of *BoLA-DRB3* alleles identified by a sequence-based typing method with mastitis pathogens in Japanese Holstein cows. *Anim. Sci. J.*, 2009, 80(5): 498-509 (doi: 10.1111/j.1740-0929.2009.00663.x).
22. Wu X.X., Yang Z.P., Wang X.L., Mao Y.J., Li S.C., Shi X.K., Chen Y. Restriction fragment length polymorphism in the exon 2 of the *BoLA-DRB3* gene in Chinese Holstein of the south China. *J. Biomed. Sci. Eng.*, 2010, 3: 221-225 (doi: 10.4236/jbise.2010.32030).
23. Gladyr' E.A., Zinov'eva N.A., Bykova A.S., Vinogradova I.V., Ernst L.K. *Dostizheniya nauki i tekhniki APK*, 2012, 8: 46-48 (in Russ.).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 782-787
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.52/.58:573.6.086.83:636.082:577.21

doi: 10.15389/agrobiology.2016.6.782rus
doi: 10.15389/agrobiology.2016.6.782eng

INFLUENCE OF OVALBUMIN GENE REGULATORY ELEMENTS ON TISSUE SPECIFICITY AND LEVEL OF TRANSGENE EXPRESSION

N.A. VOLKOVA, I.K. FOMIN, A.V. DOTSEV, T.E. DENISKOVA, N.A. ZINOVIEVA

L.K. Ernst All-Russian Research Institute of Animal Husbandry, Federal Agency of Scientific Organizations, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail natavolkova@inbox.ru
Acknowledgements:
Supported by Federal Agency of Scientific Organizations
Received September 14, 2016

Abstract

The use of lentiviral vectors for the genetic modification of embryonic chicken cells is regarded as one of the promising methods for producing transgenic poultry. In this case, it is very important to determine the regulatory elements of the ovalbumin gene, providing tissue-specificity and high transgene expression in cells of chicken oviduct. The aim of this work was to study the effect of the intron sequences and promoter of ovalbumin gene on tissue specificity and level of the transgene expression. For this purpose constructs based on lentiviral vector pWpxl, containing eGFP marker gene under control of the modified ovalbumin gene regulatory elements were obtained. Vector pW2.8 included a chromosomal DNA fragment of 2.8 kb comprising a first exon, intron sequence and part of the second exon of the ovalbumin gene; vector pW1.2 — chromosomal DNA fragment of 1.2 kb comprising a promoter and ovalbumin gene sequence (without the intron and the first exon) to the transcription initiation point; vectors pW131, pW225, pW315 — chromosomal DNA fragment, similar to the fragment of 1.2 kb in pW1.2 vector in which promoter (80 bp) was replaced by highly structured sequence of the birds β-actin gene promoter region of 131, 225 or 315 bp respectively. The control vector pWCAGgfp included constitutive hybrid regulatory element comprising human cytomegalovirus early gene enhancer and birds' β-actin gene promoter (CAG). Primary culture cells of chick oviduct and human fibrosarcoma cells 293T (control) were used as target cells for transfection. Viral preparation was added after a monolayer of cells reached concentration of 1-3×10^7 CFU/ml. eGFP expression was determined by fluorimetry in 72 hours after transfection. Low level of expression of eGFP gene controlled by chromosomal fragment of 2.8 kb leader region of the ovalbumin gene was confirmed in vitro using culture of chicken oviduct cells and 293T cell line: in vector pW2.8 recombinant protein expression level was up to 25 times lower compared to pWCAGgfp vector with a constitutive promoter CAG. Yet the eGFP expression levels for pW1.2 and pW2.8 constructs were identical, indicating the absence of introns' influence on the expression level of the recombinant DNA using this regulatory element. When the ovalbumin gene promoter was replaced by highly structured elements of β-actin constitutive gene promoter the increase in the expression of eGFP in 2-3 times was observed, as well as the increased expression occurred with lengthening of the promoter region of β-actin gene (vectors pW131, pW225, pW315). The achieved levels of expression with the use of exogenous β-actin gene promoter were comparable with expression levels controlled by a constitutive promoter CAG, however when the promoter part of the ovalbumin gene was replaced by exogenous promoter (gene β-actin), the deregulation of tissue-specific expression of eGFP was observed, indicating that transcription with tissue-specific ovalbumin promoter gene can be modulated or activated with exogenous enhancers.

Keywords: transgenesis, chickens, lentiviral vectors, oviduct, ovalbumin gene regulatory elements

The use of viral vectors derived from recombinant retroviruses and lentiviruses for genetic modification of poultry is regarded as one of the promising methods for producing transgenic animals. The high efficacy of these vector systems for poultry transgenesis has been shown [1-6]. One of the main directions in the transgenesis of poultry is the development of effective vector systems, which provide the high expression of recombinant protein in the oviduct cells to produce a transgenic product.

A significant expression of recombinant protein in the oviduct cells (up

to several mg per ml) in transgenic animals (G_0) was achieved using gene constructs comprising constitutive promoters/enhancers, in particular the CMV (human cytomegalovirus) immediate-early gene enhancer/promoter and chicken β-actin gene promoter [7]. However decreased expression of recombinant proteins in oviduct cells in subsequent generations (G_1 and G_2) was observed. A direct correlation between the expression of the recombinant product and a "gene dose" (the number of virus copies per cell genome) has been established, i.e. the intensive production of the transgenic product in organs and tissues of genetically modified animals increases the risk of physiological defects [8-10].

A solution to the problem may be the use of promoters providing tissue-specific expression of recombinant genes as a part of gene constructs. As a promising approach is considered the use of regulatory elements that control egg ovalbumin synthesis, in particular the 7.5 and 2.8 kbp chromosomal DNA fragments flanking the 5'-end of the ovalbumin gene [11-15]. A stable tissue-specific expression of the recombinant gene in several generations of transgenic birds was observed when these DNA fragments were included in the gene constructs. However, the synthesis of the recombinant product was 20-50 times lower compared to the results obtained when using constitutive promoters [16]. It indicates the effect on the ovalbumin synthesis regulation system from several regulatory elements located both inside and next to or outside the structural gene.

We first attempted to investigate the extent of tissue-specific gene expression using various modifications of the 2.8 kbp chromosomal DNA fragment that included about 1.2 kbp located upstream of the initiation point of transcription and 1.6 kbp, including the first two exons and an intron of the ovalbumin gene.

The aim of the present work was to study the effect of the intron sequences and promoter of ovalbumin gene contained in the gene constructs on the tissue specificity and expression of recombinant DNA.

Technique. We used a modified lentiviral vector system of the second generation, which included three plasmids, such as psPAX2 (encodes *gag* and *pol* genes, is a classic packer), pLPG (encodes a surface glycoprotein G of vesicular stomatitis virus VVS-G) and pWPXL (contains *eGFP* gene, the enhanced green fluorescence protein), under the control of a promoter of human RNA polymerase II elongation factor-1 *hEF1α*, serves as a self-inactivating lentiviral vector) [17]. The pWCAGgfp, pW2.8, pW1.2, pW131, pW225 and pW315 vectors were constructed based on the pWPXL plasmid, lentiviral vectors are standard molecular cloning techniques [18].

To obtain a recombinant virus and determine viral titers, the cell line 293T was used. Cells were cultured in the DMEM medium (Dlbecco's Modified Eagle's Medium), containing fetal calf serum (10 %), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) in an atmosphere of 5 % CO_2 at a temperature of 37 °C. Viral preparations were concentrated, subjecting the culture supernatants to ultracentrifugation (70,000 g, 120 min, +4 °C), pellets were resuspended in the TNE buffer (50 mM Tris-HCl, pH 7.8; 130 mM NaCl; 1 mM Na_2-EDTA). The physical titers of viral vectors were determined by a quantitative real-time PCR using the MiniOpticon™ analyzer (Bio-Rad, USA), and the biological titre of the virus — according to G. Tiscornia et al. [19]. These viral preparations used for primary culture of chicken oviduct cells infected according to the procedure [20].

The primary culture of chick oviduct cells and human fibrosarcoma cells 293T (control) were used as target cells for transfection. Viral preparation (1-3 CFU/ml) was added after cells reached a monolayer.

eGFP fluorescence was measured using the FACSCanto flow cytometer

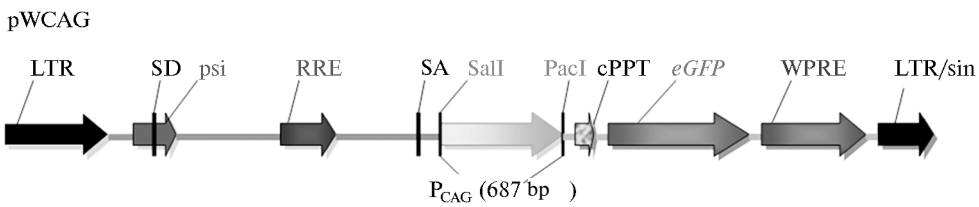
(BD, USA), with a set of filters λ = 480-490 nm (excitation) and λ = 510 nm (emission), 72 hours after transfection. To account the expression of *eGFP*, the mean fluorescence intensity (MFI) of cells with expression constructs was determined compared to the original cells.

Results. Based on the pWpxl lentiviral vector, five gene expression constructs were derived containing the *eGFP* marker gene under control of the modified ovalbumin gene regulatory elements (Table 1).

1. A characterization of gene constructs derived from lentiviral vector pWpxl (*eGFP* used as a structural gene)

Vector	Regulatory elements
pW2.8	2.8 kbp, includes the first exon, first intron and a part of the second exon of the ovalbumin gene
pW1.2	1.2 kbp, includes a promoter and a sequence (without the intron and the first exon) of the ovalbumin gene upstream to the initiation point of transcription
pW131	1.2 kbp, similarly to pW1.2, the promoter part (80 bp) was replaced by 131 bps of a highly structured sequence of the promoter region of the chicken β-actin gene
pW225	1.3 kbp, similarly to pW1.2, the promoter part (80 bps) was replaced by 225 bps of a highly structured sequence of the promoter region of the chicken β-actin gene
pW315	1.4 kbp, similarly to pW1.2, the promoter part (80 bps) was replaced by 315 bps of a highly structured sequence of the promoter region of the chicken β-actin gene

The control was the pWCAGgfp vector we obtained previously (Figure), which contained the *eGFP* gene and a hybrid constitutive regulatory element comprising the CMV (human cytomegalovirus) immediate-early gene enhancer and chicken β-actin gene promoter [21].



The structure of pWCAG lentiviral expression vector. LTR, LTR/sin (long terminal repeat; sin, self-inactivating): 5′-LTR — wild type, 3′-LTR/sin — self-inactivating variant. SD, SA — splice donor and splice acceptor sites; psi — a region responsible for virion packaging of the viral RNA; RRE — Rev-responsible element, a binding site of the Rev-protein, carrying out transport of the genomic RNA molecule from the nucleus to the cytoplasm; cPPT — central polypurine tract participating in the transport of the pre-integration complex to the cell nucleus; *eGFP* — enhanced green fluorescence protein; WPRE — Woodchuck hepatitis virus post-transcriptional regulatory element. P_{CAG} — the hybrid human cytomegalovirus immediate-early gene enhancer/chicken β-actin gene promoter. SalI, PacI — restriction sites that were used to clone regulatory elements.

Plasmid DNA of viral vectors, as well as of the packer and VVS-G was isolated in the preparative amounts, then fine purified and assayed to determine the optimal ratio between the components during transfection. When producing viral preparations, a three-plasmid vector system was injected transiently into human cells (293T line) via $CaPO_4$-precipitation. For each of the expression constructs, we derived preparations with high viral titers ($1\text{-}3\times10^7$ CFU/ml) for their further introduction into target cells.

The mean fluorescence intensity in the chicken oviduct cell culture using pWCAGgfp vector with a constitutive promoter/enhancer CAG was 89 times higher than the background intensity in the control (Table 2). When vectors pW2.8 and pW1.2 were introduced into the target cells along with ovalbumin gene regulatory elements, the *eGFP* expression exceeded the control values by 3.5 times, and was 25 times lower than in the case of a constitutive promoter. The inclusion of highly structured elements of the constitutive promoter β-actin gene (pW131, pW225 and pW313 vectors) in the ovalbumin gene regulatory elements contributed to a 7.8-14.8-fold increase of expression of the recombinant gene.

2. Expression of the *eGFP* marker gene (in mean fluorescence intensity, U) **in the primary chicken oviduct cells and heterologous 293T cells when using different vectors with modified ovalbumin gene regulatory elements**

Cell culture	Control	pWCAGgfp	pW2.8	pW1.2	pW131	pW225	pW315
Primary culture of chicken oviduct cells	128	11433	459	461	3601	4841	6785
293T cell line	542	10936	497	538	1324	1508	1810

N o t e. Control – non-transformed cell culture, background fluorescence.

To assess the tissue specificity of the obtained vectors and identify the regulatory elements in the leader region of the ovalbumin gene that are responsible for tissue-specific expression, the vectors prepared were introduced into heterologous 293T cells (human fibrosarcoma cells) by infection. Expression levels of the *eGFP* marker gene were determined for different expression cassettes. The expression of the marker gene in transfected cells using the pWCAGgfp vector was virtually identical to that obtained in chicken oviduct cells. However, given the relatively high background fluorescence, only a 20-fold excess of control values was found in the test samples. When pW2.8 and pW1.2 vectors, carrying the chromosomal fragments of ovalbumin gene, were introduced, mean fluorescence intensity in the test samples did not differ from the background fluorescence, indicating the lack of expression of the *eGFP* gene as a result of tissue specificity of the constructs. A 2.5-4.0-fold increase of mean fluorescence in the test samples was observed in the case of pW131, pW225 and pW315 vectors containing fragments of the promoter sequence of the β-actin gene in the composition of the regulatory elements of the ovalbumin gene. And the portion of fluorescent cells in this case amounted up to 35 % (for pW2.8 and pW1.2 it was 0.65-1.70 %), which indicates a lack of tissue specificity in the used vectors.

Therefore, the results confirmed the low expression of recombinant genes when using regulatory elements of the 2.8 kb chromosomal DNA fragment from the leader region of the ovalbumin gene. This value was about 25 times lower than for a vector with a constitutive promoter CAG. The level of the *eGFP* expression in the pW2.8 and pW1.2 constructs was identical, suggesting no influence of the intron on the expression if this regulatory element concerned. The substitution of the ovalbumin gene promoter with highly structured elements of the constitutive promoter of the β-actin gene contributed to a significant increase in the expression of the recombinant gene. The effect of the expression gain was identified when the promoter region of the β-actin gene extended. However, the substitution of the promoter part of the ovalbumin gene with an exogenous promoter (the β-actin gene promoter) resulted in the deregulation of the tissue-specific expression of *eGFP*.

REFERENCES

1. Mizuarai S., Ono K., Yamaguchi K., Nishijima K-I., Kamihira M., Iijima S. Production of transgenic quails with high frequency of germ-line transmission using VSV-G pseudotyped retroviral vector. *Biochem. Biophys. Res. Commun.*, 2001, 286: 456-463 (doi: 10.1006/bbrc.2001.5422).
2. Kwon M.S., Koo B.C., Choi B.R., Park Y.Y., Lee Y.M., Suh H.S., Park Y.S., Lee H.T., Kim J.H., Roh J.Y., Kim N.H., Kim T. Generation of transgenic chickens that produce bioactive human granulocyte-colony stimulating factor. *Mol. Reprod. Dev.*, 2008, 75(7): 1120-1126 (doi: 10.1002/mrd.20860).
3. Kwon S.C., Choi J.W., Jang H.J., Shin S.S., Lee S.K., Park T.S., Choi I.Y., Lee G.S., Song G., Han J.Y. Production of biofunctional recombinant human interleukin 1 receptor antagonist (rhIL1RN) from transgenic quail egg white. *Biol. Reprod.*, 2010, 82: 1057-1064 (doi: 10.1095/biolreprod.109.081687).
4. Chapman S.C., Lawson A., Macarthur W.C., Wiese R.J., Loechel R.H., Burgos-Trinidad M., Wakefield J.K., Ramabhadran R., Mauch T.J.,

Schoenwolf G.C. Ubiquitous GFP expression in transgenic chickens using a lentiviral vector. *Development*, 2005, 132: 935-940 (doi: 10.1242/dev.01652).
5. Smith C.A., Roeszler K.N., Sinclair A.H. Robust and ubiquitous GFP expression in a single generation of chicken embryos using the avian retroviral vector, RCASBP. *Differentiation*, 2009, 77(5): 473-482 (doi: 10.1016/j.diff.2009.02.001).
6. Rapp J.C., Harvey A.J., Speksnijder G.L., Hu W., Ivarie R. Biologically active human interferon α-2b produced in the egg white of transgenic hens. *Transgenic Res.*, 2003, 12(5): 569-575 (doi: 10.1023/A:1025854217349).
7. Kamihira M., Ono K., Esaka K., Nishijima K., Kigaku R., Komatsu H., Yamashita T., Kyogoku K., Iijima S. High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using a retroviral vector. *J. Virol.*, 2005, 79(17): 10864-10874 (doi: 10.1128/JVI.79.17.10864-10874.2005).
8. McGrew M.J., Sherman A., Ellard F.M., Lillico S.G., Gilhooley H.J., Kingsman A.J., Mitrophanous K.A., Sang H. Efficient production of germline transgenic chickens using lentiviral vectors. *EMBO Rep.*, 2004, 5: 728-733 (doi: 10.1038/sj.embor.7400171).
9. Scott B.B., Velho T.A., Sim S., Lois C. Applications of avian transgenesis. *ILAR J.*, 2010, 51(4): 353-361 (doi: 10.1093/ilar.51.4.353).
10. Furlan-Magaril M., Rebollar E., Guerrero G., Fernandez A., Moltai E., Gonzalez-Buenda E., Cantero M., Montoliu L., Recillas-Targa F. An insulator embedded in the chicken α-globin locus regulates chromatin domain configuration and differential gene expression. *Nucl. Acids Res.*, 2011, 39(1): 89-103 (doi: 10.1093/nar/gkq740).
11. Scott B.B., Lois C. Generation of tissue-specific transgenic birds with lentiviral vectors. *PNAS*, 2005, 102(45): 16443-16447 (doi: 10.1073/pnas.0508437102).
12. Dougherty D.C., Sanders M.M. Estrogen action: revitalization of the chick oviduct model. *Trends Endocrinol. Metab.*, 2005, 16: 414-419 (doi: 10.1016/j.tem.2005.09.001).
13. Shimizu M., Losos J.K., Gibbins A.M. Analysis of an approach to oviduct-specific expression of modified chicken lysozyme genes. *Biochem. Cell Biol.*, 2005, 83(1): 49-60 (doi: 10.1139/o04-122).
14. Byun S.J., Kim S.W., Kim K.W., Kim J.S., Hwang I.S., Chung H.K., Kan I.S., Jeon I.S., Chang W.K., Park S.B., Yoo J.G. Oviduct-specific enhanced green fluorescent protein expression in transgenic chickens. *Biosci. Biotechnol. Biochem.*, 2011, 75(4): 646-649 (doi: 10.1271/bbb.100721).
15. Lillico S.G., Sherman A., McGrew M.J., Robertson C.D., Smith J., Haslam C., Barnard P., Radcliffe P.A., Mitrophanous K.A., Elliot E.A., Sang H.M. Oviduct-specific expression of two therapeutic proteins in transgenic hens. *PNAS*, 2007, 104(6): 1771-1776 (doi: 10.1073/pnas.0610401104).
16. Kodama D., Nishimiya D., Nishijima K., Okino Y., Inayoshi Y., Kojima Y., Ono K., Motono M., Miyake K., Kawabe Y., Kyogoku K., Yamashita T., Kamihira M., Iijima S. Chicken oviduct-specific expression of transgene by a hybrid ovalbumin enhancer and the Tet expression system. *J. Biosci. Bioeng.*, 2012, 113(2): 146-153 (doi: 10.1016/j.jbiosc.2011.10.006).
17. Schomber T., Kalberer C.P., Wodnar-Filipowicz A., Skoda R.C. Gene silencing by lentivirus-mediated delivery of siRNA in human $CD34^+$ cells. *Blood*, 2004, 103(12): 4511-4513 (doi: 10.1182/blood-2003-07-2397).
18. Sambrook J., Fritsch E.F., Maniatis T. *Molecular cloning: a laboratory manual. 2nd ed.* Cold Spring Harbor, NY, 1989.
19. Tiscornia G., Singer O., Verma I.M. Production and purification of lentiviral vectors. *Nature Protocols*, 2006, 1(1): 241-245 (doi: 10.1038/nprot.2006.37).
20. *DNA cloning. Volume III. A practical approach.* D.M. Glover (ed.). Oxford, Washington DC, IRL Press Ltd., 1987.
21. Volkova N.A., Fomin I.K., Tomgorova E.K., Vetokh A.N., Mennibaeva E.R., Brem G., Zinovieva N.A. The study of factors affected the gene transfer efficiency in chicken embryonic cells by application of lentiviral vectors. *Agricultural Biology*, 2015, 50(4): 458-466 (doi: 10.15389/agrobiology.2015.4.458eng) (in Engl.).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 788-800
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Genome scanning and biodiversity

UDC 636.2:575.174:575.2:51-76

doi: 10.15389/agrobiology.2016.6.788rus
doi: 10.15389/agrobiology.2016.6.788eng

STUDY OF GENETIC DIVERSITY AND POPULATION STRUCTURE OF FIVE RUSSIAN CATTLE BREEDS USING WHOLE-GENOME SNP ANALYSIS

N.A. ZINOVIEVA[1], A.V. DOTSEV[1], A.A. SERMYAGIN[1], K. WIMMERS[2], H. REYER[2], J. SÖLKNER[3], T.E. DENISKOVA[1], G. BREM[1, 4]

[1]*L.K. Ernst All-Russian Research Institute of Animal Husbandry,* Federal Agency of Scientific Organizations, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail n_zinovieva@mail.ru, asnd@mail.ru, alex_sermyagin85@mail.ru;
[2]*Institute of Genome Biology, Leibniz Institute for Farm Animal Biology (FBN),* Mecklenburg-Vorpommern, 18196 Dummerstorf, Germany, e-mail wimmers@fbn-dummerstorf.de, reyer@fbn-dummerstorf.de;
[3]*Division of Livestock Sciences, University of Natural Resources and Life Sciences,* Augasse 2-6, A-1090, Vienna, Austria, e-mail johann.soelkner@boku.ac.at;
[4]*Institut für Tierzucht und Genetik, University of Veterinary Medicine (VMU),* Veterinärplatz, A-1210, Vienna, Austria, e-mail gottfried.brem@agrobiogen.de
Acknowledgements:
We thank Department of Animal Husbandry and Breeding (Ministry of Agriculture of the Russian Federation), Dr V.S. Matyukov (Komi Republic Research Institute of Agriculture) and Dr D.K. Nekrasov (Ivanovo State Academy of Agriculture) for assistance in collecting samples.
Supported by Russian Science Foundation, project № 14-36-00039
Received September 26, 2016

Abstract

With the publication of the complete sequence of a cattle genome, it became possible to trace the history of breed origins and to evaluate genetic relationships between modern breeds, based on the results of genome-wide SNP screening. Whilst numerous studies have been undertaken to characterize the commercial breeds and some local cattle breeds of Europe, North America, Asia and Africa at whole-genome level, little is known about genetic differences, relationships and population genetic structure of the Russian native cattle breeds. The aim of our work was to study the genetic diversity and population structure of five locally-developed Russian cattle breeds, based on genome-wide single nucleotide polymorphisms (SNPs) generated using Illumina Bovine SNP50 BeadChips (Illumina, San Diego, CA, USA). In total, 116 samples (sperm or tissue) collected from five breeds were analyzed, including Bestuzhev (BEST, n = 27), Kholmogor (KHLM, n = 25), Kostromsky (KSTR, n = 20), Red Gorbatov (RGBT, n = 23) and Yaroslavl breeds (YRSL, n = 21). Samples of Holstein cattle (HLST, n = 29) were used for comparison. Quality filtering of genetic markers was performed in PLINK v 1.07. Data processing was performed using software PLINK 1.07, HP-Rare 1.1, STRUCTURE, ver. 2.3.4, Phylip, ver. 3.695, FigTree 1.4.2, Arlequin suite, ver. 3.5.2.2 and R pocket. The final set of markers passed through the quality control and selected for further analysis included 35874 SNPs. Average heterozygosity within breeds ranged from 0.378 in BEST to 0.390 in KHLM and was higher comparing to HLST (0.377). Allelic richness was ranging from 1.914±0.001 in KSTR to 1.955±0.001 in BEST. A slight heterozygote excess was detected in all breeds studied (F_{IS} from –0.015 in BEST to –0.054 in KHLM). The multidimensional scaling (MDS) showed the presence of non-overlapping breed specific clusters, whereas the first principal component (PC1) accounted for 5.46 % and the second principal component (PC2) was responsible for 5.05 % of the genotypic variance. Phylogenetic analysis based on parsimony method grouped individuals into six clusters according to their breeds. The STRUCTURE analyses supported the assumption that the ancestry of the locally developed Russian cattle breeds is distinct from Holsteins and Holstein-related breeds. The highest ΔK showing the assumed number of populations was observed for k = 6. At k = 6, the genetic structure is in agreement with breed origin of individuals: $Q_{1/6}$ = 0.855±0.018 for BEST, $Q_{2/6}$ = 0.818±0.029 for KHLM, $Q_{3/6}$ = 0.923±0.015 for KSTR, $Q_{4/6}$ = 0.816±0.027 for RGBT, $Q_{5/6}$ = 0.873±0.031 for YRSL and $Q_{6/6}$ = 0.935±0.014 for HLST. Analysis of molecular variance (AMOVA) showed highly significant results for genetic differentiation ($p < 0.001$) in studied breeds. AMOVA revealed that most of the genetic variation in cattle breeds was found within populations (91.2 %), and less among populations (8.8 %). The emerging structure of the phylogenetic tree constructed on the Nei genetic distances, is in full concordance with the his-

torical origin of breeds and confirms the MDS and STRUCTURE results. Thus, using the method of genome-wide SNP studies we were able for the first time to study the population structure and genealogical relationships among the five Russian cattle breeds. The received information is the first step towards the evaluation of the value of these breeds regarding their conservation and usage in the agricultural production of the future.

Keywords: Russian cattle breeds, whole-genome SNP screening, biodiversity

The development of intensive livestock farming systems based on a limited number of breeds leads to a decrease of the diversity in livestock species [1, 2]. Identification and maintenance of the unique variability of local breeds in terms of growth of anthropogenic load and climate change can become an indispensable source of new valuable genotypes for future animal breeding. The centuries-old history of the territory of Russia formed cattle populations that are well adapted to the local climatic environment and economic conditions, while foreign cattle breeds were not massively imported to the old Russia in the first quarter of the XVIII century [3]. The chaotic importation of different cattle breeds from the European countries to Russia, with the aim of improving local cattle, began in 1725 with the creation of the Tsarskoe Selo farm. From the eighteenth century to the first quarter or even the half of the nineteenth century, the Holland cattle was the most imported breed. In parallel, with the beginning of the nineteenth century, Tyrolean cattle were imported in large quantities for several decades. From the end of the first half of the XIX century to early XX century (1930s), a large number of Simmental and Brown Swiss cattle were brought to the old Russia [4]. Along with these breeds, which dominated over other imported cattle, a limited number of almost all cattle breeds that were bred in Western Europe were imported to the old Russia [5-7]. The decision on import of a particular breed to improve the local cattle was taken independently by each landowner and was often based on fashion for a particular breed in a certain period of time [3]. Based on this unsystematic use of a variety of breeds in the regions of the old Russia very diverse cattle populations occurred. In cases where the local natural and economic conditions were favorable (as, for example, in Kholmogor district of the Arkhangelsk region), groups of improved and more productive cattle were formed [8]. However, in most areas the importation of foreign cattle had no visible influence and did not affect the development of local livestock farming. In the 20s of the XX century, based on a territorial principle and phenotypic traits, 12 large arrays of cattle arose in the USSR — the so-called Russian spawn (currently defined as breeds), which acquired considerable notoriety, has been of a great economic interest and became the subject of breeding. In addition, there were a few small groups of local cattle [3].

In the 20s to 30s of the XX century, the best part of the population became the basis for the development of breeds. So, such breeds were officially recognized as Yaroslavl (1925), Red Gorbatov (1926), Kholmogor (1927), and Bestuzhev (1928). In the 30s, after the civil war was over and the agriculture was restored, the breed-zoning plan was developed, which defined the target cattle breeds and regions of their breeding due to economic, naturally historical and zootechnical assumptions. In total, 17 breeds, including twelve indigenous and five foreign, were selected as planned breeds. In the European part of Russia the breeding of 11 local breeds has been recommended including Kholmogor, Tagil, Red German, White-headed Colonist, Yaroslavl, Town Caucasian, Bestuzhev, Red Gorbatov, Kalmyk, Kirghiz, and Grey Ukrainian and 5 foreign: Holland, Simmental, Brown Swiss, Shorthorn and Hereford [4]. In subsequent years, crossbreeding of domestic breeds with foreign ones has been practiced, which led to the extinction of many indigenous breeds and the development of new crossbred breeds. For example, based on the local cattle of Kostroma region, which

was previously slightly improved by crossing with Yaroslavl and Algaus cattle, the new Kostromsky breed was created through intensive crossing with Brown Swiss cattle and received the official recognition in 1944 [9]. Starting in the 80s-90s of last century, due to the lack of economic competitiveness, which was mainly based on low yield and unsuitability to industrial scale, the number of domestic cattle began to decline significantly. For example, from 1990 to 2013, the number of Kholmogor cattle decreased from 2137.0 to 285.8 thousand heads, Yaroslavl — 746.2 to 62.4 thousand heads, Bestuzhev — 981.9 to 33.1 thousand heads, Kostromsky — 384.0 to 13.0 thousand heads, Red Gorbatov — 38.0 to 1.5 thousand heads [10, 11]. Taking into account a significant contribution of indigenous breeds and regional cattle populations to the genetic diversity of domesticated species [12, 13], the reduction of their population size and "dilution" of their allele pool may be one of the main reasons for the decline of the biodiversity of domesticated species.

It should be noted that the improvement of local cattle breeds occurred mainly by crossbreeding to obtain crosses with different contributions from improved breeds [9]. Given the significant impact of environmental conditions on the formation of allele pool, it can be assumed that many alleles, which are specific for aboriginal breeds and associated with the adaptation to local climatic conditions, were kept by selection pressure and have survived in modern populations.

Generating new knowledge concerning the structure of animal genome, the improvement of methodical approaches, the development of high-performance technologies of genomic analysis, and the creation of the analytical equipment of the new generation has led to the identification and application of different types of genetic markers in studies of animal diversity [14]. Until recently, mtDNA polymorphisms and microsatellites were most widely used in studies of domestication investigating the origin and demographic history of cattle breeds. Previous studies of the D-loop of mtDNA polymorphisms revealed the strongest evidence for an independent domestication of zebu and taurine cattle [15-17] that was later confirmed by microsatellite analysis [18, 19]. Based on microsatellite diversity, the hybrid origin of the Near East cattle breeds was verified [19, 20] and the different histories of Mediterranean and Northern European cattle populations were proven [21]. Microsatellites were successfully applied to study genetic diversity, genetic structure and diversity of several Russian cattle breeds [22-24). With the publication of the complete sequence of a cattle genome in 2009 [25, 26], it became possible to trace the history of breed origins and to evaluate genetic relationships between modern breeds, based on the results of genome-wide SNP screening [27-31]. Conducting genome-wide studies of the allele pool of the local breeds will contribute to the identification of new polymorphisms and to the understanding of biological mechanisms that allow these breeds to adapt and survive in different local environmental conditions.

In the present work, we were the first to show the uniqueness of the allele fund of domestic dairy breeds at genomic level, which allows us to consider these breeds as a reserve of variability for animal husbandry.

The aim of our work was to study the genetic diversity and population structure of five locally-developed Russian cattle breeds, including Bestuzhev, Kholmogor, Kostromsky, Red Gorbatov and Yaroslavl, based on genome-wide single nucleotide polymorphisms (SNP) generated using Illumina Bovine SNP50 BeadChips.

Technique. In total, 116 samples (sperm or tissue) collected from five locally developed Russian cattle breeds were analyzed, including Bestuzhev (BEST, n = 27), Kholmogor (KHLM, n = 25), Kostromsky (KSTR, n = 20), Red Gorbatov (RGBT, n = 23) and Yaroslavl breeds (YRSL, n = 21). The sam-

ples of North American and German Holstein cattle (HLST, n = 29) were used for comparison.

Genomic DNA was extracted from biomaterial samples using Nexttec column (Nexttec Biotechnology GmbH, Germany) following the manufacturer's instructions. The DNA concentration was estimated by measuring the absorbance at 260 nm and the DNA quality was checked by separation on agarose gels. Whole-genome SNP screening was performed using Bovine SNP50 BeadChip (Illumina, San Diego, CA, USA).

Quality filtering of genetic markers was performed in PLINK 1.07 [32]. During an initial quality check, GenCall (GC) and GenTrain (GT) scores were used to assess the accuracy and efficiency of SNP genotyping. A GC score of 0.5 and GT score of 0.3 cutoff was applied to determine valid genotypes for each SNP [33]. At the next stage, the following filters were used: SNPs with less than 90 % of individuals genotyped (--geno 0.1), MAF less than < 5 % (--maf 0.05), Hardy-Weinberg equilibrium test $p < 10(^{-6})$ (--hwe 1e-6) and in linkage disequilibrium (--indep-pairwise 50 5 0.5) were pruned. SNPs located on sex chromosomes as well as SNPs with unknown map positions were also deleted. Individuals were initially quality controlled in PLINK 1.07: ones with less than 90 % SNPs genotyped (--mind 0.1) were removed.

Observed and expected heterozygosity, test for deviation from Hardy-Weinberg equilibrium were calculated in PLINK 1.07 [32]. Allelic richness (Ar) for each population was calculated using the program HP-Rare 1.1 [34]. In order to compare the allelic richness of samples having different sizes the procedure of rarefication was applied.

F_{IS} was calculated using formula: $F_{IS} = (H_{EXP} - H_{OBS})/H_{EXP}$, where H_{OBS} is the observed heterozygosity and H_{EXP} is the expected heterozygosity.

Multidimensional scaling (MDS) based on pairwise identical-by-state (IBS) distance was performed with PLINK 1.07 (--cluster, --mds-plot 4) and visualized with R package 3.2.3 [35].

Population structure was evaluated using admixture model in STRUCTURE 2.3.4 software [36]. We evaluated k values (the number of assumed populations) from 2 to 9 using a burn-in of 50 000 and 50 000 Markov chain Monte Carlo (MCMC) iterations for each value of K. Ten iterations for each value of K were carried out. Average values of similarity coefficient Q in the i-th cluster for the total number of clusters k (Qi/k) were calculated for each breed.

An unrooted individual phylogenetic tree was constructed using parsimony method with the program Dnapars from the Phylip 3.695 package [37] and visualized with FigTree 1.4.2 (38).

Pairwise Nei's genetic distances [39] were calculated using R package StAMPP [40]. Software Arlequin suite 3.5.2.2 [41] was used to determine pairwise fixation indices (Fst) [42] and to perform an analysis of molecular variance (AMOVA).

A rooted tree was constructed using neighbor-joining analysis using M. Nei distances [39] in the Neighbour program from the Phylip 3.695 package [37] and visualized with FigTree 1.4.2 [38]. Reindeer whole-genome genotyping data (n = 12) generated applying the same Bead Array were used as an outgroup for the tree.

R version 3.2.3 was used as an instrument for creating input files [35].

Results. After filtering for GT and GC, 1764 SNPs on sex chromosomes were excluded from the initial set of 54609 SNPs. At the subsequent step of the quality filtering, 4901 SNPs failed missingness test (GENO > 0.1), 9343 SNPs failed frequency test (MAF < 0.05) and 127 markers were excluded based on HWE test (p <= 1e-006). Afterwards 6228 SNPs in linkage disequilibrium were

deleted resulting in the final set of markers of 35874 SNPs that was used for further analyses. Summary statistics for genetic diversity are shown in Table 1.

1. Summary statistic for the genetic diversity of five Russian indigenous cattle breeds compared to Holsteins

Pops	*n*	Plmph	H_{OBS} (±0.001)	H_{EXP} (±0.001)	F_{IS}	Ar (±0.001)	MAF (±0.001)
BEST	27	33631	0.378	0.372	–0.015	1.955	0.282
KHLM	25	31518	0.390	0.370	–0.054	1.921	0.280
KSTR	20	32115	0.379	0.364	–0.040	1.914	0.275
RGBT	23	31383	0.379	0.368	–0.030	1.922	0.278
YRSL	21	31015	0.381	0.375	–0.018	1.925	0.284
HLST	29	32381	0.377	0.371	–0.016	1.933	0.281

N o t e. Studied cattle breeds: BEST — Bestizhev, HLST — Holstein, KHLM — Kholomogor, KSTR — Kostromsky, RGBT — Red Gorbatov, YRSL — Yaroslavl. Plmph — number of polymorphic SNPs, which pass through quality control, H_{OBS} – observed heterozygosity, H_{EXP} – expected heterozygosity, F_{IS} – fixation index, Ar – rarified allelic richness, MAF – minor allele frequency.

Average heterozygosity within breeds ranged from 0.378 in BEST to 0.390 in KHLM and was higher compared to HLST. The observed tendency of higher heterozygosity values in Russian local cattle breeds could be a result of the less intensive use of artificial insemination sires. Significant higher value of observed heterozygosity in KHLM could be due to the application of a natural mating with own bulls in some cases. Allelic richness is a measure of genetic diversity in either a sample or a population. In our study, it was ranging from 1.914±0.001 in KSTR to 1.955±0.001 in BEST. A slight heterozygote excess was detected in all breeds studied. BEST and YRSL breeds were characterized by smaller heterozygote excess (F_{IS} = –0.015, F_{IS} = –0.018, respectively), which was comparable to HLST (F_{IS} = –0.016), while a greater heterozygote excess was observed in KHLM (F_{IS} = –0.054) and KSTR (F_{IS} = –0.040).

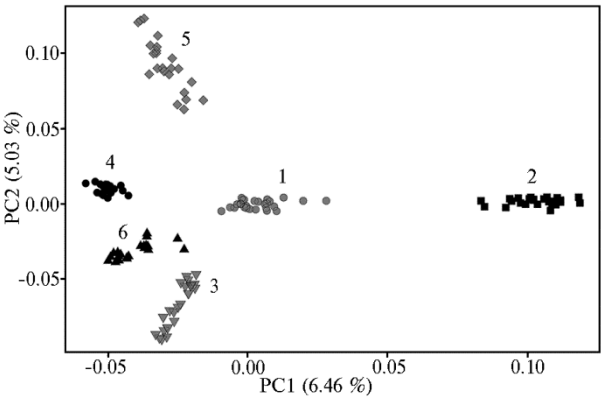


Fig. 1. Genetic variation in 116 individuals of five Russian indigenous cattle breeds and 29 individuals of Holstein breed (comparison group), genotyped at 35874 SNP loci, based on the results of principal component analysis (PCA): 1 — Bestuzhev, 2 — Holstein, 3 — Kholmogor, 4 — Kostromsky, 5 — Red Gorbatov, 6 — Yaroslavl (studied cattle breeds).

The results of multidimensional scaling (MDS) are presented on Figure 1. The principal component 1 (PC1) accounts for 5.46 % of the genotypic variance and splits the Russian domestic breeds from Holsteins. According to G. McVean [43], locations of samples on the principal component analysis plot generated from genome-wide data can be predicted based on the knowledge of the average coalescent time for pairs of samples. The first principal component can be interpreted as the deepest coalescent event in a tree, and the projection of admixed individuals onto this axis can be used to estimate the proportion of mixture between two parental groups [43]. Four out of the five Russian domestic breeds are located at the same region along the first axis indicating that their common ancestry differs from HLST breed. The position of BEST cattle along this axis, between remaining Russian breeds and Holsteins, can be interpreted as the presence of some admixture of Holsteins or Holstein-related cattle in the modern BEST population. The principal component 2 (PC2) is associated with the divergence of KHLM and YRSL (these two breeds have their

origin in the Northern Great Russian cattle) from Red cattle (RGBT), which was formed with great contribution of Tyrolean cattle [44]. The intermediate position of KSTR cattle is probably associated with their crossbred origin and the contribution of both the Northern Great Russian and the Red cattle in their breeding. It is known that the ancestry population of KSTR breed was formed in XIX century by crossing of domestic well adapted to local environmental and forage conditions Northern Great Russian cattle with KHLM bulls and then with Wilstermarsh, Simmental, Ayrshire and Brown Swiss cattle. In the beginning of the XX century, a multiple crossing with Brown Swiss bulls was carried out [9, 44]. In total, the PC2 accounts for 5.03% of the variance in genotypes.

Phylogenetic analysis based on parsimony method grouped individuals into six clusters according to their breeds. All the individuals from the same breeds were consolidated on the neighboring branches of their respective clusters (Fig. 2).

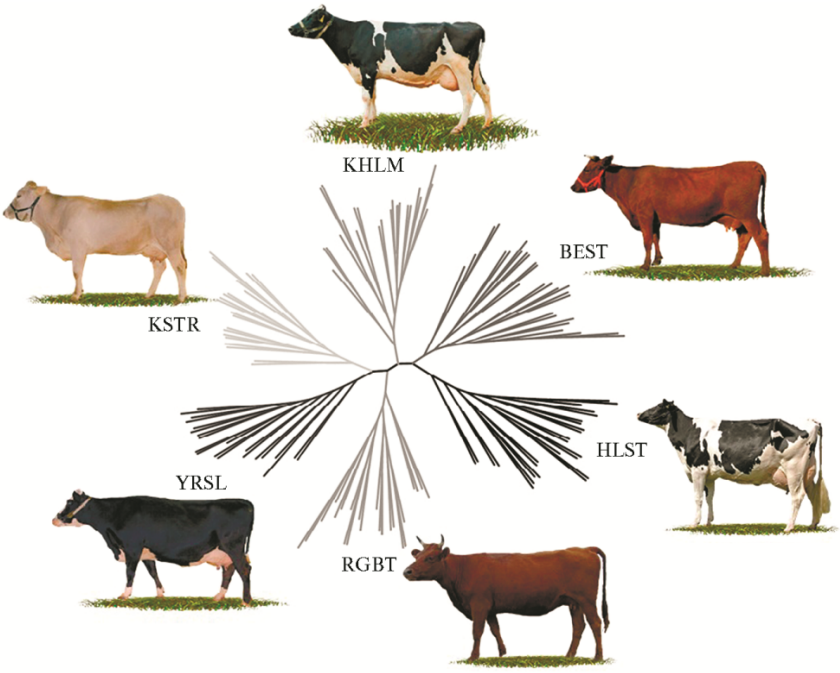


Fig. 2. An unrooted phylogenetic tree of five Russian indigenous cattle breeds (Holstein cattle were included in analysis as comparison group) based on parsimony method: BEST — Bestuzhev, HLST — Holstein, KHLM — Kholomogor, KSTR — Kostromsky, RGBT — Red Gorbatov, YRSL — Yaroslavl (studied cattle breeds).

The STRUCTURE analyses supported the assumption that the ancestry of the locally developed Russian cattle breeds is distinct from Holsteins and Holstein-related breeds (Fig. 3). We found strong split of HLST breed ($Q_{2/2}$ = 0.956±0.011) from the five studied Russian cattle breeds. The HLST contribution in ancestry of four of them is insignificant: $Q_{2/2}$ = 0.057±0.008, 0.003±0.002, 0.043±0.009 and 0.028±0.008 for KHLM, KSTR, RGBT and YRSL, respectively. The introgression of HLST was observed in BEST ($Q_{2/2}$ = 0.300±0.010), probably, due to using Red Holsteins for the improvement of this breed during last decade. Increasing k values from 3 to 6 showed only slight variation in likelihood scores for HLST from 0.935±0.014 at k = 6 to 0.957±0.010 at k = 4. At k = 3, we observe the KHLM clustering ($Q_{3/3}$ = 0.862±0.023). Some degree of KHLM admixture in YRSL ($Q_{3/3}$ = 0.389±0.005) and BEST ($Q_{3/3}$ = 0.206±0.004) reflects the contribution of KHLM cattle in improvement of Yaroslavl and Bestuzhev breeds

2. Proportion of membership of the studied native Russian cattle breeds in each of the six cluster inferred in STRUCTURE

Pops	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
BEST	0.896±0.015[P]	0.004±0.002	0.002±0.001	0.005±0.002	0.039±0.006	0.054±0.012
KHLM	0.072±0.012	0.818±0.029[P]	0.005±0.003	0.013±0.004	0.064±0.009	0.027±0.005
KSTR	0.019±0.007	0.004±0.001	0.923±0.015[P]	0.009±0.003	0.042±0.006	0.003±0.002
RGBT	0.079±0.013	0.014±0.004	0.018±0.005	0.816±0.027[P]	0.047±0.006	0.026±0.006
YRSL	0.060±0.014	0.021±0.007	0.017±0.006	0.013±0.005	0.873±0.031[P]	0.016±0.007
HLST	0.051±0.013	0.002±0.001	0.000+0.000	0.001±0.001	0.011±0.001	0.935±0.014[P]

N o t e. BEST — Bestuzhev, HLST — Holstein, KHLM — Kholomogor, KSTR — Kostromsky, RGBT — Red Gorbatov, YRSL — Yaroslavl (studied cattle breeds); the inferred cluster is marked by [P].

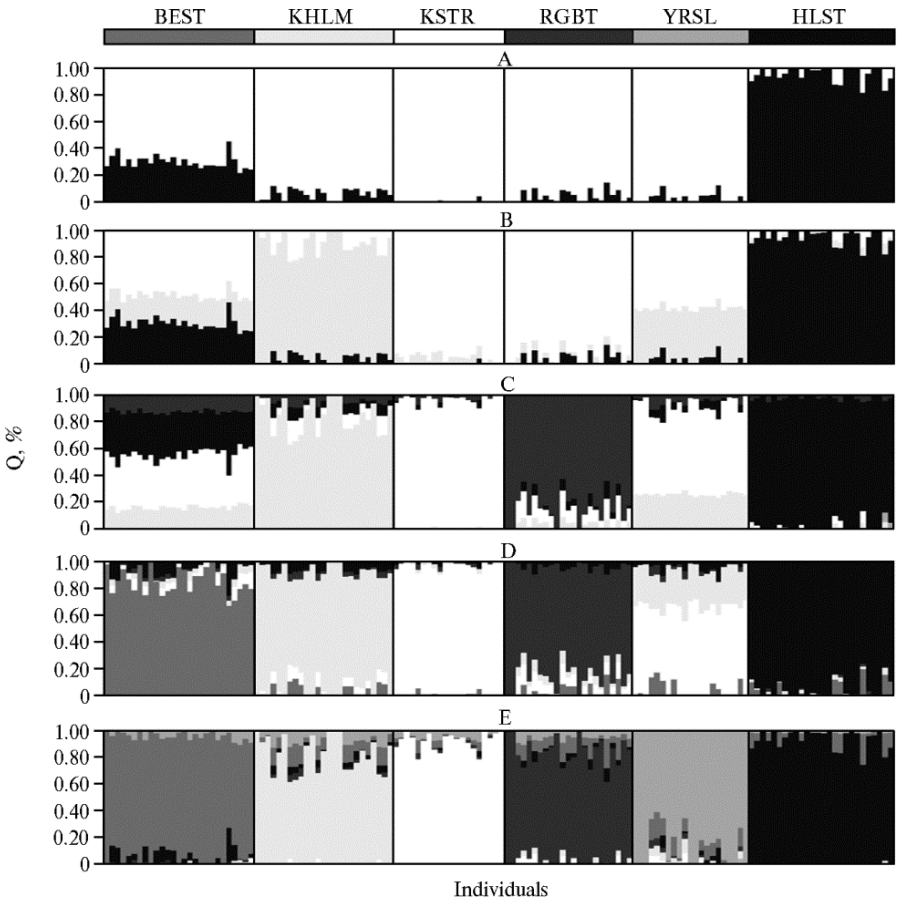


Fig. 3. Population assignment for 145 individuals based on the 35874 markers using STRUCTURE [36] for k = 2 (A)**, k = 3** (B)**, k = 4** (C)**, k = 5** (D)**, k = 6** (E)**:** BEST — Bestuzhev, HLST — Holstein, KHLM — Kholomogor, KSTR — Kostromsky, RGBT — Red Gorbatov, YRSL — Yaroslavl (studied cattle breeds); individuals are represented as thin vertical lines, with the proportion of different shades of gray representing their estimated ancestry deriving from different populations. Breeds are labeled by abbreviation at the top of figure.

in XIX-XX centuries [9]. At k = 4, the STRUCTURE shows the subdivision of RGBT ($Q_{4/4}$ = 0.829±0.026). The presence of slight RGBT admixture in BEST ($Q_{4/4}$ = 0.121±0.003), probably, reflects the contribution of Tyrolean cattle in improvement of ancestor populations in the XIX century [9, 44]. The highest ΔK showing the assumed number of populations was observed for k = 6. At k = 6, the genetic structure is in agreement with breed origin of individuals: $Q_{1/6}$ = 0.855±0.018 for BEST, $Q_{2/6}$ = 0.818±0.029 for KHLM, $Q_{3/6}$ = 0.923±0.015 for KSTR, $Q_{4/6}$ = 0.816±0.027 for RGBT, $Q_{5/6}$ = 0.873±0.031 for YRSL and $Q_{6/6}$ = 0.935±0.014 for HLST (Table 2). In total, across a range of k values

from 3 to 6, all of the five studied Russian local cattle breeds consistently showed complex ancestry (Fig. 3). At higher values of k, observed at k = 6 among-breed genetic structure predominated.

Analysis of molecular variance (AMOVA) (Table 2) showed highly significant results for genetic differentiation ($p < 0.001$) in studied breeds. AMOVA revealed that most of the genetic variation in cattle breeds was found within populations (91.2 %), and less among populations (8.8 %).

Calculation of the pairwise F_{ST} and Nei's distances (D_N) (Table 3) confirmed the most closeness of BEST breed to HLST ($F_{ST} = 0.0661$, $D_N = 0.0496$), whereas the remaining Russian breeds were approximately equally distant from HLST ($F_{S\,T} = 0.0963$-0.1103, $D_N = 0.0675$-0.0783).

3. Genetic distances between studied cattle breeds

Pops	BEST	KHLM	KSTR	RGBT	YRSL	HLST
BEST		0.0504	0.0551	0.0542	0.0508	0.0496
KHLM	0.0684		0.0700	0.0670	0.0603	0.0675
KSTR	0.0740	0.1004		0.0715	0.0661	0.0783
RGBT	0.0743	0.0972	0.1027		0.0684	0.0718
YRSL	0.0671	0.0854	0.0931	0.0980		0.0721
HLST	0.0661	0.0963	0.1103	0.1025	0.1014	

Note. BEST — Bestuzhev, HLST — Holstein, KHLM — Kholomogor, KSTR — Kostromsky, RGBT — Red Gorbatov, YRSL — Yaroslavl (studied cattle breeds); pairwise Fst values are shown below diagonal ($p \leq 0{,}001$); Nei's unbiased distances [39] are shown above diagonal. P-values are given only for F_{ST} values.

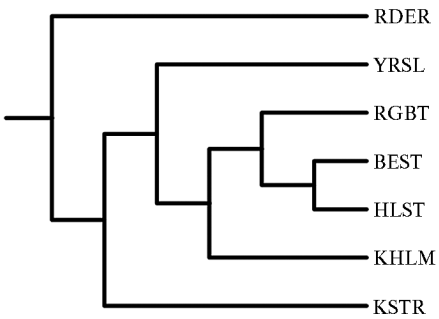


Fig. 4. A rooted phylogenetic tree constructed by neighbor-joining analysis: BEST — Bestuzhev, HLST — Holstein, KHLM — Kholomogor, KSTR — Kostromsky, RGBT — Red Gorbatov, YRSL — Yaroslavl (studied cattle breeds); RDER — reindeer (an outgroup).

The emerging structure of the phylogenetic tree constructed on the Nei genetic distances (Fig. 4), is in full concordance with the historical origin of breeds and confirms the MDS and STRUCTURE results.

The development of the high throughput genotyping technologies has opened new prospective in the evaluation of genetic diversity in livestock species. The application of genome-wide sets of DNA markers may expand our understanding of population genetic structure of livestock, breed origins and genetic relationship between populations. Whilst numerous studies have been undertaken to characterize the commercial breeds and some local cattle breeds of Europe, North America, Asia and Africa at whole-genome level [29, 31, 45, 46], little is known about genetic differences, relationships and population genetic structure of the Russian native cattle breeds. The Russian dairy cattle population consists of 8.9 million cows and 949 thousands of them are breeding cows [47, 11]. The most widely used breeds of dairy cattle in Russia are Black-and-White with high degree of Holstein blood and Holsteins. They account for 66 % of the total number of dairy cattle. Russian native cattle breeds account only 15 % of the total dairy cattle population, including 8 % for Kholmogor breed, 6 % for Yaroslavl breed and less than 1 % for all other breeds [47]. Locally developed breeds could however carry the gene pool, which is important for adaptive traits and may become an indispensable source of genetic variability for the future geographically targeted system of milk production. Furthermore, information about genetic diversity and population structure is essential for utilization and conservation of cattle breeds [48].

We evaluated the genetic diversity and population structure of five native

Russian cattle breeds using a set of 35874 polymorphic SNPs generated by the Bovine SNP50K BeadChip (Illumina Inc, San-Diego, USA). We clearly distinguished Russian breeds from each other and from Holstein breed, which was used as comparison. All the investigated cattle breeds revealed a complex origin. Some signals of admixture were observed between several Russian breeds and Holsteins, and between different Russian breeds. The highest contribution of Holsteins or Holstein-related breeds was observed in Bestuzhev breed. Based on PC1 of the MDS analysis the Bestuzhev breed was positioned between other Russian breeds and the Holstein breed showing some degree of membership in the Holstein specific cluster as revealed by STRUCTURE analysis. These two breeds were characterized by lowest pairwise F_{ST} values and formed a common branch at the Nei's phylogenetic tree. The closeness of Bestuzhev breed to Holsteins is probably due to intensive use of Holstein bulls for improvement of this native cattle breed [49]. We observed some admixture of Holsteins or Holstein-related cattle in Kholmogor and Red Gorbatov cattle. In the Kholmogor breed, it is probably associated with the contribution of Dutch cattle in the development of the ancestral populations of this breed. It is known that Dutch cows were distributed among the inhabitants of fertile meads in Kholmogor in the 60s of the XVIII century caused by Empress Catherine II [8]. Geographically close regions of origin of the ancestral populations of Bestuzhev and Red Gorbatov cattle and the similar climatic conditions of their breeding on the banks of the Volga River were depicted by some admixture signals based on the STRUCTURE analysis and the formation of a common branch on the phylogenetic tree. Genetic closeness of Bestuzhev and Red Gorbatov cattle can reflect the contribution of Tyrolean cattle in improvement of both breeds in the XIX century. Most likely, this contribution comes from Tux-Zillertaler cattle, which was widespread in Tyrol due to their unpretentiousness and good productive capacity in poor forage resources. At the agricultural exhibition of 1855 in Tyrol, they were presented as a Tyrolean breed [50]. The export of Tux-Zillertaler cattle to Russia is dated to 1848 [51]. Close localization of Kholmogor and Yaroslavl cattle at the MDS plot, the presence of admixture, observed at values of k from 3 to 5 on the results of the STRUCTURE analysis and relatively low values of F_{ST} and D_N may reflect their common historical origin from the Northern Great Russian cattle [3] and the contribution of Kholmogor cattle in the improvement of Yaroslavl cattle in the XIX and XX centuries [9].

Thus, using the method of genome-wide SNP studies we were able for the first time to study the population structure and genealogical relationships among the five Russian cattle breeds. The received information is the first step towards the evaluation of the value of these breeds regarding their conservation and usage in the agricultural production of the future.

REFERENCES

1. Notter D.R. The importance of genetic diversity in livestock populations of the future. *J. Anim. Sci.*, 1999, 77(1): 61-69 (doi: 10.2527/1999.77161x).
2. Ajmone-Marsan P., GLOBALDIV Consortium. A global view of livestock biodiversity and conservation—GLOBALDIV. *Anim. Genet.*, 2010, 41(s1): 1-5 (doi: 10.1111/j.1365-2052.2010.02036.x).
3. Liskun E.F. *Russkie otrod'ya krupno-rogatogo skota* [Landraces of Russian cattle]. Moscow, 1928 (in Russ.).
4. Diomidov A.M., Zhirkovich E.F. *Razvedenie porod krupnogo rogatogo skota* [Cattle breeding]. Moscow-Leningrad, 1934 (in Russ.).
5. Armfel'd A.A. *Krupnyi rogatyi skot v usloviyakh russkikh khozyaistv* [Cattel breeding in Russia]. St. Petersburg, 1904 (in Russ.).
6. Liskun E.F. *Kratkie spravochnye svedeniya o zhivotnykh nekotorykh russkikh khozyaistv* [Brief view of the animals from some farms of Russia]. Petrograd, 1915 (in Russ.).

7. Pridorogin M.I. *Krupnyi rogatyi skot. Vazhneishie porody* [Cattle: the most important breeds]. Moscow, 1919 (in Russ.).
8. Tooke W. View of the Russian empire, during the reign of Catharine the second, and to the close of the eighteens century. *The Breeding of Sattle*, 1800, 3: 85-129.
9. Dmitriev N.I. *Porody skota po stranam mira* [Cattle breeds — a geography]. Leningrad, 1978 (in Russ.).
10. Ernst L.K., Dmitriev N.G., Paronyan I.A. *Geneticheskie resursy sel'skokhozyaistvennykh zhivotnykh v Rossii i sopredel'nykh stranakh* [Genetic diversity of farm animals in Russia and neighboring countries]. St. Petersburg, 1994 (in Russ.).
11. *Spravochnik porod i tipov sel'skokhozyaistvennykh zhivotnykh, razvodimykh v Rossiiskoi Federatsii* /Pod redaktsiei I.M. Dunina, A.G. Dankverta [Breeds and types of farm animals in the Russian Federation: a directory. I.M. Dunin, A.G. Dankvert (eds.)]. Moscow, 2013 (in Russ.).
12. Muir W.M., Wong G.K.-S., Zhang Y., Wang J., Groenen M.A.M., Crooijmansd R.P.M.A., Megensd H.-J., Zhange H., Okimotof R., Vereijkeng A., Jungeriusg A., Albersg G.A.A., Lawley C.T., Delany M.E., MacEachern S., Cheng H.H. Genome-wide assessment of worldwide chicken SNP genetic diversity indicates significant absence of rare alleles in commercial breeds. *PNAS USA*, 2008, 105(45): 17312-17317 (doi: 10.1073/pnas.0806569105).
13. Dolmatova I.Yu., Zinovieva N.A., Gorelov P.V., Il'yasov A.D., Gladyr E.A., Traspov A.A., Sel'tsov V.I. Characteristics of the allele pool of the Bashkiria population of Simmental cattle using microsatellites. *Agricultural Biology*, 2011, 6: 70-74 (in Engl.).
14. Yang W., Kang X., Yang Q., Lin Y., Fang M. Review on the development of genotyping methods for assessing farm animal diversity. *Journal of Animal Science and Biotechnology*, 2013, 4: 2-6 (doi: 10.1186/2049-1891-4-2).
15. Loftus R.T., MacHugh D.E., Bradley D.G., Sharp P.M., Cunningham E.P. Evidence for two independent domestications of cattle. *PNAS USA*, 1994, 91(7): 2757-2761 (doi: 10.1073/pnas.91.7.2757).
16. Loftus R.T., MacHugh D.E., Ngere L.O., Balain D.S., Badi A.M., Bradley D.G., Cunningham E.P. Mitochondrial genetic variation in European, African and Indian cattle populations. *Anim. Genet.*, 1994, 25: 265-271 (doi: 10.1111/j.1365-2052.1994.tb00203.x).
17. Bradley D.G., MacHugh D.E., Cunningham P., Loftus R.T. Mitochondrial diversity and the origins of African and European cattle. *PNAS USA*, 1996, 93: 5131-5135 (doi: 10.1073/pnas.93.10.5131).
18. MacHugh D.E., Shriver M.D., Loftus R.T., Cunningham P., Bradley D.G. Microsatellite DNA variation and the evolution, domestication and phylogeography of taurine and zebu cattle (*Bos taurus* and *Bos indicus*). *Genetics*, 1997, 146: 1071-1086.
19. Loftus R.T., Ertugrul O., Harba A.H., El-Barody M.A.A., MacHugh D.E. Park S.D.E., Bradley D.G. A microsatellite survey of cattle from a centre of origin: the Near East. *Mol. Ecol.*, 1999, 8: 2015-2022 (doi: 10.1046/j.1365-294x.1999.00805.x).
20. Edwards C.J., Baird J.F., MacHugh D.E. Taurine and zebu admixture in Near Eastern cattle: a comparison of mitochondrial, autosomal and Y-chromosomal data. *Anim. Genet.*, 2007, 38: 520-524 (doi: 10.1111/j.1365-2052.2007.01638.x).
21. Cymbron T., Freeman A.R., Isabel Malheiro M., Vigne J.D., Bradley D.G. Microsatellite diversity suggests different histories for Mediterranean and Northern European cattle populations. *Proceedings of the Royal Society B: Biological Sciences*, 2005, 272(1574): 1837-1843 (doi: 10.1098/rspb.2005.3138).
22. Gorelov P.V., Kol'tsov D.N., Zinovieva N.A., Gladyr' E.A. The comparative analysis of blood groups and microsatellites in characteristics of new cattle types of brown Swiss and Sychevskaja breeds. *Agricultural Biology*, 2011, 6: 37-40 (in Engl.).
23. Gladyr' E.A., Zinov'eva N.A., Bagirov V.A., Amirshoev F.S., Volkova V.V., Klenovitskii P.M., Karpov A.P., Ernst L.K. *Dostizheniya nauki i tekhniki APK*, 2012, 8: 58-62 (in Russ.).
24. Kiseleva T.Yu., Kantanen J., Vorobyov N.I., Podoba B.E., Terletsky V.P. Linkage disequilibrium analysis for microsatellite loci in six cattle breeds. *Russian Journal of Genetics*, 2014, 50(4): 406-414 (doi: 10.1134/S1022795414040048).
25. Elsik C.G., Bovine Genome Sequencing and Analysis Consortium. The genome sequence of taurine cattle: A window to ruminant biology and evolution. *Science*, 2009, 324(5926): 522-528 (doi: 10.1126/science.1169588).
26. Zimin A.V., Delcher A.L., Florea L., Kelley D.R., Schatz M.C., Puiu D., Hanrahan F., Pertea G., Van Tassell C.P., Sonstegard T.S., Marçais G., Roberts M., Subramanian P., Yorke J.A., Salzberg S.L. A whole-genome assembly of the domestic cow, *Bos taurus. Genome Biol.*, 2009, 10(4): R42 (doi: 10.1186/gb-2009-10-4-r42).
27. Decker J.E., Pires J.C., Contant G.C., McKay S.D., Heaton M.P., Chen K.,

Cooper A., Vilkki J., Seabury C.M., Caetano A.R., Johnson G.S., Brenneman R.A., Hanotte O., Eggert L.S., Wiener P., Kim J.-J., Kim K.S., Sonstegard T.S., Van Tassell C.P., Neibergs H.L., McEwan J.C., Brauning R., Coutinho L.L., Babar M.E., Wilson G.A., McClure M.C., Rolf M.M., Kim J.W., Schnabel R.D., Taylor J.F. Resolving the evolution of extant and extinct ruminants with high-throughput phylogenomics. *PNAS USA*, 2009, 106(44): 18644-18649 (doi: 10.1073/pnas.0904691106).

28. Kuehn L.A., Keele J.W., Bennett G.L., McDaneld T.G., Smith T.P., Snelling W.M., Sonstegard T.S., Thallman R.M. Predicting breed composition using breed frequencies of 50,000 markers from the US Meat Animal Research Center 2,000 Bull Project. *J. Anim. Sci.*, 2011, 89(6): 1742-1750 (doi: 10.2527/jas.2010-3530).
29. McTavish E.J., Decker J.E., Schnabel R.D., Taylor J.F., Hillis D.M. New World cattle show ancestry from multiple independent domestication events. *PNAS USA*, 2013, 110(15): E1398-E1406 (doi: 10.1073/pnas.1303367110).
30. Decker J.E., McKay S.D., Rolf M.M., Kim J., Molina Alcalá A., Sonstegard T.S., Hanotte O., Götherström A., Seabury C.M., Praharani L., Babar M.E., Regitano L.C.A., Yildiz M.A., Heaton M.P., Wan-Sheng L., Lei C.-Z., Reecy J.M., Saif-Ur-Rehman M., Schnabel R.D., Taylor J.F. Worldwide patterns of ancestry, divergence, and admixture in domesticated cattle. *PLoS Genetics*, 10(3): e1004254 (doi: 10.1371/journal.pgen.1004254).
31. Decker J.E., Taylor J.F., Kantanen J., Millbrooke A., Schnabel R.D., Alexander L.J., MacNeil M.D. Origins of cattle on Chirikof Island, Alaska, elucidated from genome-wide SNP genotypes. *Heredity*, 2016, 116(6): 502-505 (doi: 10.1038/hdy.2016.7).
32. Purcell S., Neale B., Todd-Brown K., Thomas L., Ferreira M.A.R., Bender D., Maller J., Sklar P., de Bakker P.I., Daly M.J., Sham P.C. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.*, 2007, 81: 559-575 (doi: 10.1086/519795).
33. Fan J.B., Oliphant A., Shen R., Kermani B.G., Garcia F., Gunderson K.L., Hansen M., Steemers F., Butler S.L., Deloukas P., Galver L., Hunt S., McBride C., Bibikova M., Rubano T., Chen J., Wickham E., Doucet D., Chang W., Campbell D., Zhang B., Kruglyak S., Bentley D., Haas J., Rigault P., Zhou L., Stuelpnagel J., Chee M.S. Highly parallel SNP genotyping. *Cold Spring Harbor Symposia on Quantitative Biology*, 2003, 68: 69-78.
34. Kalinowski S.T. HP RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Mol. Ecol. Notes*, 2005, 5: 187-189 (doi: 10.1111/j.1471-8286.2004.00845.x).
35. *R Development Core Team. R: A language and environment for statistical computing. 2009. R Foundation for statistical computing.* Vienna, Austria, 2005 (ISBN 3-900051-07-0). Available http://www.Rproject.org. No date.
36. Pritchard J.K., Stephens M., Donnelly P. Inference of population structure using multilocus genotype data. *Genetics*, 2000, 155(2): 945-959.
37. Felsenstein J. *PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author.* Department of Genetics, University of Washington, Seattle, 1993.
38. *FigTree.* Available http://tree.bio.ed.ac.uk/software/figtree/. No date.
39. Nei M. Genetic distance between populations. *Am. Nat.*, 1972, 106(949): 283-292.
40. Pembleton L.W., Cogan N.O.I., Forster J.W. StAMPP: an R package for calculation of genetic differentiation and structure of mixed-ploidy level populations. *Mol. Ecol. Resour.*, 2013, 13(5): 946-952 (doi: 10.1111/1755-0998.12129).
41. Weir B.S., Cockerham C.C. Estimating F-Statistics for the analysis of population structure. *Evolution*, 1984, 38(6): 1358-1370 (doi: 10.2307/2408641).
42. Excoffier L., Lischer H.E.L. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.*, 2010, 10: 564-567 (doi: 10.1111/j.1755-0998.2010.02847.x).
43. McVean G. A genealogical interpretation of principal components analysis. *PLoS Genetics*, 2009, 5(10): e1000686 (doi: 10.1371/journal.pgen.1000686).
44. Liskun E.F. *Otechestvennye porody krupnogo rogatogo skota* [Cattle — Domestic breeds]. Moscow, 1949 (in Russ.).
45. The Bovine HapMap Consortium. Genome-wide survey of SNP variation uncovers the genetic structure of cattle breeds. *Science*, 2009, 324: 528-532 (doi: 10.1126/science.1167936).
46. Makina S.O., Muchadeyi F.C., Marle-Köster E., Mac Neil M.D., Maiwashe A. Genetic diversity and population structure among six cattle breeds in South Africa using a whole genome SNP panel. *Frontiers in Genetics*, 2014, 5: 333 (doi: 10.3389/fgene.2014.00333).
47. Labinov V.V. *Rezul'taty i osnovnye napravleniya sovershenstvovaniya selektsionno-plemennoi raboty v molochnom skotovodstve Rossiiskoi Federatsii. Ministerstvo sel'skogo khozyaistva RF* [The achievements and improvement of cattle breeding in the Russian Federation. Ministry of Agriculture of the Russian Federation. Available http://www.mcx.ru/documents/file_document/v7_show/33370.133.htm. Ac-

cessed May 28, 2016.
48. Groeneveld L.F., Lenstra J.A., Eding H., Toro M.A., Scherf B., Pilling D., Negrini R., Finlay E.K., Jianlin H., Groeneveld E., Weigend S., The GLOBALDIV Consortium. Genetic diversity in farm animals — a review. *Anim. Genet.*, 2010, 41: 6-31 (doi: 10.1111/j.1365-2052.2010.02038.x).
49. Katmakov P.S., Gavrilenko V.P., Bushov A.V., Sten'kin N.I. *Vestnik Ul'yanovskoi GSKHA*, 2014, 1(25): 126-132 (in Russ.).
50. Moser C., Reiter M. *Die Rinderrasse der Tux-Zillertaler — Ein Stück Tiroler Kultur.* Verlag Edition Tirol, Reith im Alpbachtal, 1996.
51. Tux Zillertaler. Available http://en.zar.at/Cattle_breeding_in_Austria/Cattle_breeds/Additional_Breeds/Tux_Zillertaler.html. No date.

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 801-810
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.32/.38:575.174.015.3:577.2.08:51-76

doi: 10.15389/agrobiology.2016.6.801rus
doi: 10.15389/agrobiology.2016.6.801eng

VARIABILITY OF MICROSATELLITES IN SHEEP BREEDS RACED IN RUSSIA

T.E. DENISKOVA[1], M.I. SELIONOVA[2], E.A. GLADYR'[1], A.V. DOTSEV[1], G.T. BOBRYSHOVA[2], O.V. KOSTYUNINA[1], G. BREM[3], N.A. ZINOVIEVA[1]

[1]*L.K. Ernst All-Russian Research Institute of Animal Husbandry,* Federal Agency of Scientific Organizations, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail tandeniss@rambler.ru;
[2]*All-Russian Research Institute of Sheep and Goat Breeding,* Federal Agency of Scientific Organizations, 15, per. Zootechnicheskii, Stavropol, 355017 Russia;
[3]*Institut für Tierzucht und Genetik, University of Veterinary Medicine (VMU),* Veterinärplatz, A-1210, Vienna, Austria, e-mail gottfried.brem@agrobiogen.de
Acknowledgements:
We thank Dr R.V. Ivanov (Yakut Research Institute of Agriculture) for assistance in collecting samples
Supported by Russian Science Foundation (project № 14-36-00039)
Received September 26, 2016

Abstract

At the current stage of biological development is impossible to establish conservation programs and to monitor genetic resources of sheep without a preliminary study by DNA markers. The Russian sheep breeding is represented by wide variety of breeds, including all productivity and wool types. However, until recently only some sheep breeds, which belong to the same breeding zone or productivity type, were investigated by DNA markers including microsatellites. We studied 25 Russian sheep breeds (n = 751), including fine-fleeced — Dagestan Mountain (DAG), Grozny (GRZ), Kulunda (KUL), Manych Merino (MNM), Salskaya (SAL), Stavropol (STA), Soviet Merino (SVM), Volgograd (VOL), Baikal's fine-fleeced (ZBL); semi fine-fleeced — Altay Mountain (ALT), Kuibyshev (KUI), North Caucasian (NC), Russian long-haired (RLH), Tsigai (TSIG); coarse-wooled — Andean (AND), Buubey (BUB), Edilbai (EDL), Karachaev (KAR), Kuchugur (KCH), Kalmyk (KLM), Karakul (KRK), Lezgin (LEZ), Romanov (ROM), Tushin (TSH), Tuvan short fat-tailed (TUV). The research was conducted using 11 microsatellite loci (OarCP49, INRA063, HSC, OarAE129, MAF214, OarFCB11, INRA005, SPS113, INRA23, MAF65 и McM527). The data were processed using GenAIEx 6.5 and PAST software. In general, the studied breeds were characterized by moderately high allelic diversity. The average number of alleles per locus is varied from 7.20±0.98 in KUL and 10.30±0.99 in TSIG. The values of Na 10.0 were found in TSIG, TUV, BUB and KRK, values of Na 8.0 were identified in KUL, RLH and SVM. The effective allele number was the highest in the KRK and TUV (Ne 5.7) and the minimum was detected in KCH, ALT, RLH and NC (Ne 4.3). The level of the observed heterozygosity in 21 of the 25 studied breeds ranged from 0.489±0.095 in TUV to 0.651±0.050 in ROM and 0.651±0.060 in SVM, and four other breeds (BUB, TSIG, ZBL and TUV) it varied from 0.798±0.023 in BUB up 0.977±0.017 in TUV. There was a substantial deficit of heterozygotes in 21 of the 25 studied breeds (F_{IS} values ranged from 0.13 in ROM to 0.36 in KAR and SAL), in the other four (BUB, TSIG, ZBL and TUV) an excess of heterozygotes (F_{IS} values ranged from –0.04 to –0.22) was detected. The analysis of molecular variance (AMOVA) showed that 5.02 % of genetic variation is composed of differences among breeds and 94.98 % is explained by within breeds' component. Analysis of the structure of the UMPGA phylogenetic tree, based on the matrix of pairwise genetic distances by M. Nei (1972), showed that the nature of the identified relationships is mainly related with the wool type, productivity type and breeding region. Thus, the identified polymorphism of eleven microsatellite loci is quite powerful for differentiating sheep of various breeds. For a better understanding population structure and obtaining new information on the genetic diversity at the genomic level the application of DNA microarrays, based on the multiple SNPs-markers, is required.

Keywords: sheep breeds, microsatellites, genetic diversity

Thanks to advancing DNA-based technology, various genetic markers have been detected and applied, including microsatellites, which became an important source of information about the current status of animal genetic resources [1, 2]. Choosing microsatellites as genetic markers is determined by their

unique features, such as the widespread and uniform distribution in the genome, allelic diversity, great informative value, codominant Mendelian-type inheritance, high reproducibility, and ease of analysis automation [3-5]. In the global and domestic practice of sheep rearing, microsatellite analysis is used to assess the genetic diversity of breeds and study phylogenetic relationships between them [6-8], proof of origin [9] and pedigree of animals [10], to investigate the population structure and genetic drift [11, 12], and establish the level of inbreeding in specific groups and herds [13].

Currently, high-performance genotyping methods are increasingly used, such as genome-wide SNP (single nucleotide polymorphism), scanning using DNA microarrays with different densities [14-17] and genotyping-by-sequencing (GBS) [18]. Despite this, microsatellites remain relevant as highly informative DNA markers in conducting population genetic studies [5]. A microsatellite-based analysis is still an indispensable tool for the routine animal testing on the origin authenticity and the breed. This is primarily due to the fact that the panels for origin diagnostics based on highly informative SNP markers are rather expensive, and blood groups are much inferior to the microsatellites in the testing accuracy [19].

In Russia, there are 37 sheep breeds, including 13 fine-fleeced, 10 semifine-fleeced, 12 coarse-wool and 2 medium-wool breeds [20]. They are also represented by all known types based on productivity (wool, mutton-wool, wool and meat, fur, fat-tailed, mutton-wool and dairy, hair and meat). This diversity is due to the peculiarities of climate, feed and social factors in the regions where the breeds were developed.

In recent years, genetic studies have been performed on some Russian breeds of sheep using the microsatellite markers [21-23]. Within the framework of the genetic diversity study program and the establishment of the population structure of sheep breeds in North Eurasia, the Russian breeds were compared with breeds from Norway, Denmark, Sweden and Estonia [24]. For the genetic classification of semifine-fleeced breeds, the Kuibyshev, Russian long-haired and North Caucasian mutton-wool sheep breeds were involved [25]. However, until now genetic studies of sheep based on the microsatellite markers in Russia have involved only several breed groups, combined by the productivity type or a breeding region.

In this paper, we first studied and compared 25 Russian sheep breeds representing all known types by their microsatellite markers. In general, polymorphism in 11 microsatellite loci was quite informative for the interbreed differentiation. Our findings most completely reflect the state of the allele pool and genetic diversity of most of the major sheep breeds, commonly spread in Russia.

The purpose of the research was the study of variability of microsatellites, the assessment of diversity and degree of genetic differentiation among different sheep breeds, raised in Russia.

Technique. Tissue samples were taken from sheep of 25 Russian breeds, such as fine-fleece, semifine-fleece, coarse-wool, with different types of meat, wool and dairy productivity (n = 751).

To isolate the DNA the Nexttec columns (Nexttec Biotechnologie GmbH, Germany) were used, as well as the DNA Extran test kit (CJSC Syntol, Russia) and the extraction method using sodium perchlorate [26]. For the genetic research, we selected 11 microsatellite loci grouped into two multiplex panels including the following loci: OarCP49, INRA063, HSC, OarAE129, MAF214, OarFCB11, INRA005, and SPS113, INRA23, MAF65 and McM527 loci. The reactions were performed in a final volume of 10 μl in the PCR buffer containing 2 mM dNTPs, 1.0 mM of $MgCl_2$, 0.5 mM of the primer mixture, 1 unit of

Taq-polymerase (Dialat Ltd., Russia), and 50-100 ng of genomic DNA. The composition of the PCR buffer was as follows: 16.6 mM $(NH_4)_2SO_4$, 67.7 mM of Tris-HCl (pH = 8.8), 0.1 vol of Tween 20. After the initial denaturation (95 °C, 4 min) we performed 41 (Panel 1) and 35 (Panel 2) amplification cycles in the following temperature-time regime: 95 °C, 20 sec; 63 °C (Panel 1) and 55 °C (Panel 2), 30 sec; 72 °C, 1 min. Fragments were examined using the ABI3130xl genetic analyzer (Applied Biosystems, USA) and GeneMapper 4 software (Applied Biosystems, USA).

The GenAIEx 6.5 software [27] was used to calculate the following statistical parameters: mean number of alleles per locus (Na), the effective number of alleles (Ne), the number of informative alleles or alleles with a frequency of > 5 % (Na 5 %), the expected (H_e) and observed (H_o) heterozygosity, and inbreeding coefficient (F_{IS}). The degree of genetic differentiation of breeds was assessed by the F_{ST} parameter [28] and genetic distances by M. Nei (D_N) [29] for paired comparison. The F_{ST} values were visualized by analyzing the principal coordinates (Principal Coordinates Analysis, PCoA) using GenAIEx 6.5 software. Based on the genetic distance matrix by M. Nei [29] in the PAST software [30], a phylogenetic tree was built by the unweighted pair-group method using arithmetic averages (UPGMA).

Results. Table 1 provides a summary of sheep population involved in the study.

1. The characteristics of 25 breeds of sheep (*Ovis aries*), most common in Russia and assessed based on 11 microsatellite loci (n = 751)

Breed	Code	n	Region
Fine-fleece breeds			
Wool			
Grozny	GRZ	30	Republic of Kalmykia
Stavropol	STA	32	Stavropol Krai, Kalmykia
Manych Merino	MNM	30	Stavropol Krai
Soviet Merino	SVM	23	Stavropol Krai
Salskaya	SAL	30	Rostov Province
Mutton-Wool			
Volgograd	VLG	30	Volgograd Province
Dagestan Mountain	DAG	30	Republic of Dagestan
Wool and meat			
Transbaikalian fine-fleece	ZBL	30	Republic of Sakha (Yakutia)
Kulunda	KUL	30	Altai Krai
Semifine-fleece breeds			
Mutton-Wool			
Russian long-haired	RLH	30	Voronezh Province
Kuibyshev	KUI	30	Samara Province
North Caucasian Mutton-Wool	NC	30	Stavropol Krai
Wool and meat			
Tsigai	TSIG	30	Saratov Province, Rostov Province
Altay Mountain semifine-fleece	ALT	30	Altai Krai
Coarse-wool breeds			
Hair and meat			
Romanov	ROM	30	Yaroslavl Province, Ryazan Province, Moscow Province.
Fur-bearing			
Karakul	KRK	32	Republic of Kalmykia, Astrakhan Province
Fat-tailed			
Edilbai	EDL	30	Volgograd Province
Kalmyk fat-rumped	KLM	30	Republic of Kalmykia
Mutton-Wool			
Buubey	BUB	30	Republic of Sakha (Yakutia)
Tuvan short fat-tailed	TUV	30	Republic of Tyva
Kuchugur	KCH	34	Voronezh Province
Dual-purpose and dairy			
Karachaev	KAR	30	Karachay-Cherkessia
Lezgin	LEZ	30	Republic of Dagestan
Andean	AND	30	Republic of Dagestan
Tushin	TSH	30	Republic of Dagestan

The average number of alleles per locus ranged from 7.20±0.98 in KUL

to 10.30±0.99 in TSIG (Table 2). The highest values of this parameter were characteristic of four breeds (TSIG, TUV, BUB, KRK with Na 10.0), the lowest — of three breeds (KUL, RLH, SVM with Na 8.0). The effective number of alleles was highest in KRK and TUV breeds (Ne 5.7), and lowest — in KCH, ALT, RLH and NC breeds (Ne 4.3). The range of variability in terms of the number of informative alleles per locus (Na 5 %) varied from 4.70 in STA to 6.40 in TSIG.

2. The characteristics of the allele pool and genetic diversity parameters in the Russian breeds of sheep (*Ovis aries*) by 11 microsatellite loci

Breed	Na	Ne	Na 5 %	H_o	H_e	F_{IS}
EDL	9.30±1.21	4.66±0.71	5.30±0.72	0.557±0.073	0.730±0.043	0.27±0.07
VLG	8.90±1.22	5.08±0.70	5.80±0.63	0.525±0.082	0.751±0.047	0.33±0.08
SAL	8.50±0.92	5.05±0.63	5.90±0.64	0.512±0.089	0.764±0.036	0.36±0.09
KAR	9.20±1.10	5.25±0.72	5.70±0.63	0.516±0.087	0.764±0.040	0.36±0.09
KLM	9.50±0.96	5.07±0.63	5.90±0.50	0.577±0.071	0.771±0.030	0.27±0.08
GRZ	9.00±1.14	4.92±0.62	5.40±0.67	0.540±0.089	0.761±0.033	0.33±0.09
DAG	9.00±1.07	5.45±0.82	5.40±0.86	0.560±0.079	0.774±0.032	0.30±0.08
TSH	9.60±1.13	5.02±0.79	5.70±0.60	0.507±0.081	0.748±0.042	0.35±0.08
AND	8.70±0.98	4.80±0.58	5.40±0.65	0.550±0.074	0.757±0.033	0.29±0.08
LEZ	8.60±0.72	4.74±0.74	5.80±0.71	0.510±0.070	0.730±0.044	0.33±0.06
ALT	8.50±0.99	4.28±0.70	4.80±0.61	0.509±0.084	0.678±0.065	0.26±0.09
KUL	7.20±0.98	4.42±0.72	5.30±0.70	0.489±0.095	0.701±0.053	0.33±0.12
KRK	10.00±0.91	5.75±0.86	5.40±0.76	0.634±0.047	0.785±0.033	0.20±0.04
KUI	8.50±1.06	5.32±0.80	5.90±0.69	0.646±0.052	0.767±0.036	0.16±0.05
KCH	9.20±1.15	4.24±0.53	4.80±0.39	0.574±0.059	0.729±0.032	0.22±0.08
RLH	8.00±0.79	4.28±0.51	4.90±0.41	0.555±0.066	0.726±0.041	0.26±0.07
NC	8.50±0.90	4.32±0.59	5.00±0.42	0.586±0.053	0.726±0.041	0.20±0.04
STA	9.20±0.92	4.88±0.63	4.70±0.45	0.575±0.061	0.765±0.027	0.26±0.06
MNM	8.20±0.90	4.54±0.51	5.00±0.45	0.647±0.055	0.752±0.029	0.15±0.05
TSIG	10.30±0.99	5.53±0.42	6.40±0.48	0.873±0.014	0.807±0.019	–0.09±0.03
TUV	10.10±1.16	5.74±0.58	6.30±0.52	0.977±0.017	0.808±0.020	–0.22±0.04
BUB	10.00±1.13	5.11±0.69	6.30±0.76	0.798±0.023	0.774±0.026	–0.04±0.04
ZBL	8.90±0.77	5.32±0.53	6.00±0.45	0.891±0.018	0.794±0.021	–0.13±0.03
ROM	9.80±1.04	5.27±0.81	5.20±0.57	0.651±0.050	0.758±0.043	0.13±0.06
SVM	8.00±0.75	4.95±0.44	5.20±0.42	0.651±0.060	0.782±0.020	0.17±0.07
Mean	8.99±0.20	4.96±0.13	5.50±0.59	0.616±0.015	0.756±0.007	0.20±0.02

N o t e. Na — mean number of alleles per locus; Ne — the effective number of alleles; Na 5 % — the number of informative alleles per locus (with a frequency of > 5 %); H_o — the observed heterozygosity; H_e — the expected heterozygosity; F_{IS} — coefficient of inbreeding. See Table 1 for the explanation of the abbreviations for the sheep breeds.

The observed heterozygosity in 21 of 25 studied breeds varied from 0.489±0.095 in TUV to 0.651±0.050 in ROM and 0.651±0.060 in SVM. In four breeds (BUB, TSIG, ZBL, TUV), heterozygosity was significantly higher, such as from 0.798±0.023 in BUB to 0.977±0.017 in TUV. A comparison of the observed and expected heterozygosity revealed a significant deficit of heterozygotes in 21 of 25 studied breeds which was from 10.5 % (ROM) to 24.9 and 25.1 % (in KAR and SAL, respectively). The deficit of heterozygotes was confirmed by the positive values of the coefficient of inbreeding F_{IS}, which ranged from 0.13 in ROM to 0.36 in KAR and SAL. A slight (2.5 to 16.8 %) excess of heterozygotes was detected in BUB, TSIG, ZBL and TUV. The F_{IS} values for these four breeds ranged from –0.04 to –0.22.

The analysis of molecular variance (AMOVA) showed that 5.02 % of the genetic variability of breeds accounted for the differences between breeds and 94.98 % — for the intrabreed component.

The genetic relationship between the studied breeds based on the F_{ST} index is presented in Figure 1 as the PCoA plot. The lowest values of the index were observed for the following pairs: GRZ-STA (F_{ST} = 0.013), MNM-SVM (F_{ST} = 0.014), STA-SVM (F_{ST} = 0.014), ZBL-SVM (F_{ST} = 0.014), and the highest for KUL-EDL (F_{ST} = 0.071) and ALT-ROM (F_{ST} = 0,070) pairs.

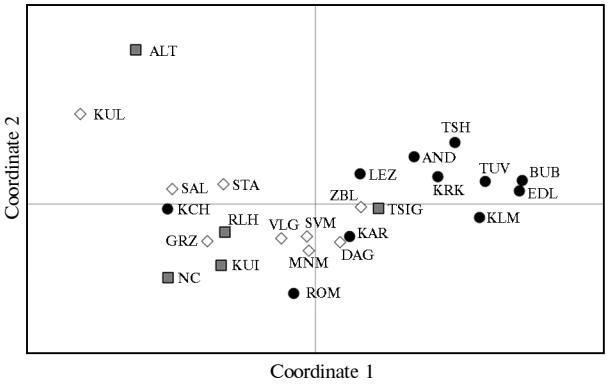


Fig. 1. Genetic differentiation of 25 Russian breeds of sheep (*Ovis aries*), belonging to different classes according to the wool and productivity types, in the space of the first two principal coordinates, calculated by the F_{ST} index based on PCoA in paired comparison for 11 microsatellite loci: ● — coarse-wool, ■ — semifine-fleece, ◇ — fine-fleece sheep breeds. See Table 1 for the explanation of the abbreviations for the sheep breeds.

According to D.L. Hartl and A.G. Clark [31], the F_{ST} values < 0.05 indicate a negligible genetic differentiation, 0.05 to 0.15 — a moderate, and 0.15 to 0.25 — a significant genetic differentiation. Most of the studied Russian breeds were characterized by negligible to moderate genetic differentiation. It is noteworthy that KUL and ALT breeds were at some genetic distance from most others. This was confirmed by F_{ST} = 0.05. M.Yu. Ozerov et al. [25] found that the F_{ST} values between Russian semifine-fleece sheep breeds averaged to 0.03. In general, this is consistent with our findings: the F_{ST} values between TSIG-KUI and TSIG-NC pairs were 0.026 and 0.031, respectively, and only for a NC-KUI pair this value was lower (F_{ST} = 0.015).

The analysis of genetic distances [29] showed the greatest remoteness of KCH-BUB, ROM-TUV, KCH-TUV, ALT-ROM and KUL-TUV breeds, for which the D_N values were 0.490, 0.457, 0.448, 0.431 and 0.430, respectively. This could be due to the geographical remoteness of breeding regions, which prevented the exchange of genetic material. The minimum genetic distances were detected mainly between breeds belonging to the same productivity type: GRZ-STA (D_N = 0.087), MNM-SVM (D_N = 0.099), STA-SVM (D_N = 0.102). In general, it can be concluded that the genetic relationships between the studied breeds, assessed by the D_N and F_{ST} parameters were similar.

The phylogenetic tree (Fig. 2) built on the basis of the genetic distances (D_N) demonstrated marked independent branches formed by Kuchugur and Romanov breeds, which probably was the result of their genetic uniqueness. The remaining breeds formed two clusters, the first of which was presented exclusively with fine-fleece and semifine-fleece breeds, and the second — with all coarse-wool breeds as well as Tsigai and Transbaikalian fine-fleece breeds.

Four subclusters were distinguished in the first cluster, each of which included several breeds. Thus, a general subcluster (1-1) was formed by fine-fleeced breeds, such as Grozny, Stavropol, Manych merino and Soviet merino, which could be explained by one productivity type, breeding habitat, and the long-term (over 25 years) use of the gene pool of Australian merino sheep for their improvement [32]. Semifine-fleece breeds, such as Kuibyshev, North Caucasian mutton-wool and Russian long-haired, formed a separate subcluster (1-2). It is known that rams of the Romney Marsh breed originated in England were used to improve the first two breeds [33, 34], while Lincoln sheep were involved in deriving the Russian long-haired breed (at the initial stage) and North Caucasian mutton-wool breed (at the final stage of breed development) [35]. Combining breeds of the Mutton-Wool productivity type (Dagestan Mountain and Volgograd) in a separate cluster (1-3) could be due to the use of local inbred coarse-wool fat-rumped and fat-tailed sheep (as a maternal base) at the initial stage of their breeding, and Caucasian and Grozny merino breeds at the final

stage [36]. The fourth subcluster (1-4) included Salskaya, Kulunda and Altay Mountain semifine-fleece breeds.

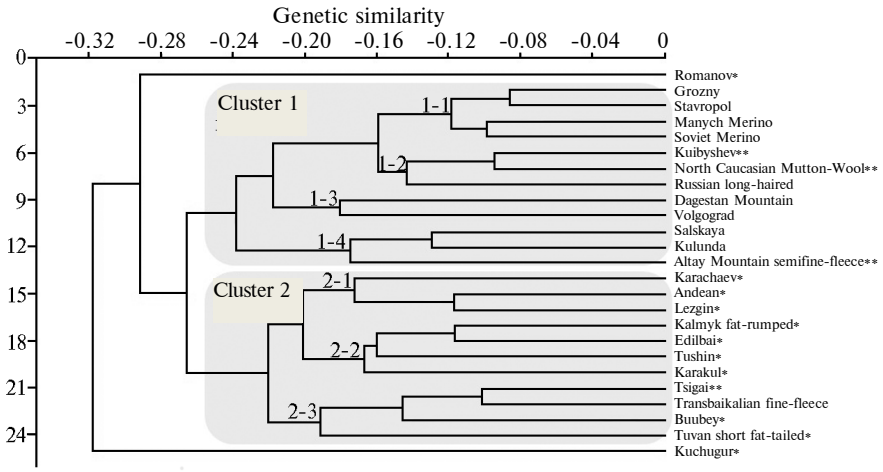


Fig. 2. The phylogenetic tree built based on the matrix of pairwise genetic distances between the studied Russian breeds of sheep (*Ovis aries*) by M. Nei [29]**:** 1-1, 1-2, 1-3 и 1-4 — subclusters of the cluster 1; 2-1, 2-2 and 2-3 — subclusters of the cluster 2. One asterisk denotes coarse-wool, and two asterisks — semifine-fleece breeds; all the remaining are fine-fleece breeds. The UMPGA method was used to construct a dendrogram.

Three subclusters stood out in the cluster 2. The first one (2-1) was formed by sheep of Karachaev, Andean and Lezgin breeds developed in different mountainous areas of the North Caucasus through the long-term sustained selection of local coarse-wool sheep by local inhabitants. A distinctive feature of these breeds is an equally weighted ratio of meat, wool and dairy productivities, as well as an exceptional adaptability to keeping on the mountainous and lowland pastures [37, 38], which apparently determined their genetic proximity.

The second subcluster (2-2) included Edilbai, Kalmyk fat-rumped, Tushin and Karakul breeds, characterized by increased deposits of fat in the tail area. The first two breeds are of meat-fat productivity type, well adapted to grazing over large distances in semi-deserts and deserts, and have the fat tail. Furthermore, it is known that Edilbai breed was the improver at the initial stage of Kalmyk breed development [36]. The Tushin and Karakul breeds belong to fat-tailed sheep by the morphological classification, and also tend to fat deposition. Given the zoological classification, the productivity type, the area of spreading, the history of breeding and phenotypic similarity of breeds, an isolation of the two subclusters within the second cluster can be considered reasonable.

The third subcluster included Tsigai, Transbaikalian fine-fleece, Buubey and Tuvan short fat-tailed breeds of different productivity types and historical origins. The presence of the Transbaikalian fine-fleece, Buubey and Tuvan breeds in one subcluster is possibly due to the fact that indigenous coarse-wool Buryat-Mongolian sheep are found in their genealogy. Thus, Buubey breed was developed through a long-term improvement of indigenous Buryat sheep, Transbaikalian fine-fleece breed — through a long transformation of inbred coarse-wool Buryat-Mongolian sheep using the gene pool of the North Caucasian fine-fleece breeds [39]. Previously, the genetic similarity of the Tuvan indigenous breed of sheep, raised by the local tribes in the Republic of Tuva, and the Mongolian sheep has been already pointed out [40]. The explanation for the clustering of these breeds may be in the fact that all of them were derived and are still being raised in the extreme climatic conditions of Transbaikalia and Western Siberia. Probably, adaptive mechanisms, developed over hundreds

of years, affected many loci, including microsatellite ones, and led to the similarity of the genetic profile. However, this assumption requires further investigation and validation.

Therefore, our findings give the most complete presentation of the state of the allele pool and genetic diversity of the majority of sheep breeds, the most commonly spread in Russia. In general, polymorphism in 11 microsatellite loci was quite informative for the differentiation sheep belonging to different breeds, while the nature of the identified relationships were mainly due to the wool type, productivity type and a breeding region. For a more profound research of the population structure and additional information on the genetic diversity at the genomic level, the investigation of Russian sheep breeds is anticipated with the application of DNA microarrays based on the multiple SNP markers.

REFERENCES

1. Crispim B., Seno L., Egito A., Vargas Junior F., Grisolia A. Application of microsatellite markers for breeding and genetic conservation of herds of Pantaneiro sheep. *Electronic Journal of Biotechnology*, 2014, 17: 317-321 (doi: 10.1016/j.ejbt.2014.09.007).
2. Stolpovskii Yu.A. Concept and principles of genetic monitoring for the purpose of preservation in situ of domestic animals kinds. *Agricultural Biology*, 2010, 6: 3-8. Available http://www.agrobiology.ru/6-2010stolpovskiy-eng.html. No date (in Engl.).
3. Tautz D., Renz M. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucl. Acids Res.*, 1984, 12: 4127-4138.
4. Litt M., Luty J.M. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.*, 1989, 44: 397-401.
5. Putman A.I., Carbone I. Challenges in analysis and interpretation of microsatellite data for population genetic studies. *Ecology and Evolution*, 2014, 4(22): 4399-4428 (doi: 10.1002/ece3.1305).
6. Dalvit C., Sacca E., Cassandro M., Gervaso M., Pastore E., Piasentier E. Genetic diversity and variability in Alpine sheep breeds. *Small Ruminant Res.*, 2008, 80: 45-51 (doi: 10.1016/j.smallrumres.2008.09.005).
7. Kusza S., Dimov D., Nagy I., Bõsze Z., Jávor A., Kukovics S. Microsatellite analysis to estimate genetic relationships among five bulgarian sheep breeds. *Genet. Mol. Biol.*, 2010, 33(1): 51-56 (doi: 10.1590/S1415-47572010005000003).
8. Ferrando A., Goyache F., Parés P.-M., Carriyn C., Miry J., Jordana J. Genetic relationships between six eastern Pyrenean sheep breeds assessed using microsatellites. *Spanish Journal of Agricultural Research*, 2014, 12(4): 1029-1037 (doi: 10.5424/sjar/2014124-6173).
9. Yilmaz O. Power of different microsatellite panels for paternity analysis in sheep. *Animal Science Papers and Reports*, 2016, 34(2): 155-164.
10. Baumung R., Cubric-Curik V., Schwend K., Achmann R., Sölkner J. Genetic characterization and breed assignment in Austrian sheep breeds using microsatellite marker information. *J. Anim. Breed. Genet.*, 2006, 123: 265-271 (doi: 10.1111/j.1439-0388.2006.00583.x).
11. Tapio I., Tapio M., Grislis Z., Holm L-E., Jeppsson S., Kantanen J., Miceikiene I., Olsaker I., Viinalass H., Eythorsdottir E. Unfolding of population structure in Baltic sheep breeds using microsatellite analysis. *Heredity*, 2005, 94: 448-456 (doi: 10.1038/sj.hdy.6800640).
12. Neubauer V., Vogl C., Seregi J., Sáfár L., Brem G. Genetic diversity and population structure of Zackel sheep and other Hungarian sheep breeds. *Archives Animal Breeding*, 2015, 58: 343-350 (doi: 10.5194/aab-58-343-2015).
13. Pariset L., Savarese M.C., Cappuccio I., Valentini A. Use of microsatellites for genetic variation and inbreeding analysis in Sarda sheep flocks of central Italy. *J. Anim. Breed. Genet.*, 2003, 120(6): 425-432 (doi: 10.1046/j.0931-2668.2003.00411.x).
14. Kijas J.W., Lenstra J.A., Hayes B., Boitard S., Porto Neto L.R., San Cristobal M., Servin B., McCulloch R., Whan V., Gietzen K., Paiva S., Barendse W., Ciani E., Raadsma H., McEwan J., Dalrymple B. Genome wide analysis of the world's sheep breeds reveals high levels of historic mixture and strong recent selection. *PLoS ONE*, 2012, 10: e1001258 (doi: 10.1371/journal.pbio.1001258).
15. Heaton M.P., Leymaster K.A., Kalbfleisch T.S., Kijas J.W., Clarke S.M., McEwan J., Maddox J.F., Basnayake V., Petrik D.T., Simpson B., Smith T.P.L., Chitko-McKown C.G., the International Sheep Genomics Consortium. SNPs for parentage testing and traceability in globally diverse breeds of sheep. *PLoS ONE*, 2014, 9(4): e94851

(doi: 10.1371/journal.pone.0094851).
16. Clarke S.M., Henry H.M., Dodds K.G., Jowett T.W.D., Manley T.R., Anderson R.M., McEwan J.C. A high throughput single nucleotide polymorphism multiplex assay for parentage assignment in New Zealand sheep. *PLoS ONE*, 2014, 9(4): e93392 (doi: 10.1371/journal.pone.0093392).
17. Deniskova T.E., Sermyagin A.A., Bagirov V.A., Okhlopkov I.M., Gladyr E.A., Ivanov R.V., Brem G., Zinovieva N.A. Comparative analysis of the effectiveness of STR and SNP markers for intraspecific and interspecific differentiation of the genus *Ovis*. *Russian Journal of Genetics*, 2016, 52(1): 79-84 (doi: 10.1134/S1022795416010026).
18. Elshire R.J., Glaubitz J.C., Sun Q., Poland J.A., Kawamoto K., Buckler E.S., Mitchell S.E. A Robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS ONE*, 2011, 6(5): e19379 (doi: 10.1371/journal.pone.0019379).
19. Ernst L.K., Zinov'eva N.A. *Biologicheskie problemy zhivotnovodstva v XXI veke* [Biological aspects of animal husbandry in the XXI century]. Moscow, 2008 (in Russ.).
20. Amerkhanov Kh.A. *Ovtsevodstvo i kozovodstvo Rossiiskoi Federatsii v tsifrakh* [Goats and sheep breeding in Russia: statistics]. Stavropol', 2015 (in Russ.).
21. Gladyr' E.A., Zinov'eva N.A., Burylova S.S., Selionova M.I., Moiseikina L.G., Ernst L.K., Brem G. *Dostizheniya nauki i tekhniki APK*, 2012, 11: 34-37 (in Russ.).
22. Zinovieva N.A., Selionova M.I., Gladyr E.A., Petrovic M.P., Caro Petrovic V., Ruzic Muslic D., Petrovic M.M. Investigation of gene pool and genealogical links between sheep breeds of southern Russia by blood groups and DNA microsatellites. *Genetika*, 2015, 47(2): 395-404 (doi: 10.2298/GENSR1502395Z).
23. Deniskova T.E., Kaneva L.A., Gladyr' E.A., Selionova M.I., Zinov'eva N.A. *Dostizheniya nauki i tekhniki APK*, 2016, 30(8): 75-78 (in Russ.).
24. Tapio M., Ozerov M., Tapio I., Kantanen J., Toro M.A., Marzanov N., Ćinkulov M., Goncharenko G., Kiselyova T., Murawski M. Microsatellite-based genetic diversity and population structure of domestic sheep in northern Eurasia. *BMC Genetics*, 2010, 11: 76 (doi: 10.1186/1471-2156-11-76).
25. Ozerov M.Yu., Tapio M., Kantanen Yu., Marzanova S.N., Buzerov V.V., Andryukhin A.P., Sharlapaev B.N., Petrov S.N., Marzanov N.S. *Problemy biologii produktivnykh zhivotnykh*, 2013, 3: 16-24 (in Russ.).
26. Zinov'eva N.A., Popov A.N., Ernst L.K., Marzanov N.S., Bochkarev V.V., Strekozov N.I., Brem G. *Metodicheskie rekomendatsii po ispol'zovaniyu metoda polimeraznoi tsepnoi reaktsii v zhivotnovodstve* [PCR analysis in animal husbandry — guidelines]. Dubrovitsy, 1998 (in Russ.).
27. Peakall R., Smouse P.E. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, 2012, 28(19): 2537-2539 (doi: 10.1093/bioinformatics/bts460).
28. Weir B.S., Cockerham C.C. Estimating F-Statistics for the analysis of population structure. *Evolution*, 1984, 38(6): 1358-1370 (doi: 10.2307/2408641).
29. Nei M. Genetic distance between populations. *Am. Nat.*, 1972, 106(949): 283-292 (doi: 10.1086/282771).
30. Hammer O., Harper D.A.T., Ryan P.D. PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, 2001, 4(1): 4-9.
31. Hartl D.L., Clark A.G. *Principles of population genetics*. Sunderland, United Kingdom, 1997.
32. Moroz V.A. *Ovtsy, kozy, sherstyanoe delo*, 2015, 2: 45-48 (in Russ.).
33. Sel'kin I.I., Sokolov A.N. *Ovtsy, kozy, sherstyanoe delo*, 2002, 3: 10-12 (in Russ.).
34. Medvedev M.V., Erokhin A.I. *Ovtsy, kozy, sherstyanoe delo*, 2004, 1: 29-30 (in Russ.).
35. Sel'kin I.I. *Severokavkazskaya myasosherstnaya poroda* [A dual-purpose North Caucasian meat and wool sheep breed]. Stavropol, 2007 (in Russ.).
36. Erokhin A.I., Kotarev V.I., Erokhin S.A. *Ovtsevodstvo* [Sheep breeding]. Voronezh, 2014 (in Russ.).
37. Gadzhiev Z.K. *Grubosherstnye ovtsy Dagestana* [Dagestan coarse-wool sheep]. Makhachkala, 2010 (in Russ.).
38. Murzina T.V., Vershinina V.A. *Informatsionnyi byulleten'*, 2016, 1(11): 35-41 (in Russ.).
39. Biltuev S.I. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Znachenie i perspektivy razvitiya ovtsevodstva v Agrarnoi ekonomike Sibiri i Dal'nego Vostoka»* [Proc. Int. Conf. «The value and prospects of sheep farming in the economy of Siberia and the Far East»]. Chita, 2016: 52-57 (in Russ.).
40. Stolpovskii Yu.A., Shimiit L.V., Kol N.V., Evsyukov A.N., Rusina M.N., Churgui-ool O.I., Sulimova G.E. Analysis of genetic variability and phylogenetic relations in populations of Tuvinian short-fat-tailed sheep with ISSR-markers usage. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2009, 6: 34-43 (in Russ.).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 811-823
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.294:575.174

doi: 10.15389/agrobiology.2016.6.811rus
doi: 10.15389/agrobiology.2016.6.811eng

STUDY OF THE ALLELE POOL AND THE DEGREE OF GENETIC INTROGRESSION OF SEMI-DOMESTICATED AND WILD POPULATIONS OF REINDEER (*Rangifer tarandus* L., 1758) USING MICROSATELLITES

V.R. KHARZINOVA[1], A.V. DOTSEV[1], A.S. KRAMARENKO[2], K.A. LAYSHEV[3], T.M. ROMANENKO[4], A.D. SOLOV'EVA[1], T.E. DENISKOVA[1], O.V. KOSTYUNINA[1], G. BREM[1, 5], N.A. ZINOVIEVA[1]

[1]*L.K. Ernst All-Russian Research Institute of Animal Husbandry,* Federal Agency of Scientific Organizations, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail veronika0784@mail.ru, asnd@mail.ru, anastastasiya93@mail.ru, horarka@yandex.ru, kostolan@mail.ru, n_zinovieva@mail.ru;
[2]*Mykolayiv National Agrarian University*, 9, vul. Georgiya Gongadze, Mykolayiv, 54020 Ukraine, e-mail kssnail@mail.ru;
[3]*North-West Center of Interdisciplinary Food Research,* 7, sh. Podbelskogo, St. Petersburg—Pushkin, 196608 Russia, e-mail layshev@mail.ru;
[4]*Naryan-Mar Agricultural Experimental Station,* Federal Agency of Scientific Organizations, 1a, ul. Rybnikova, Naryan-Mar, Nenets Autonomous District, 166004 Russia, e-mail nmshos@atnet.ru;
[5]*Institut für Tierzucht und Genetik, University of Veterinary Medicine (VMU),* Veterinärplatz, A-1210, Vienna, Austria, e-mail gottfried.brem@agrobiogen.de
Acknowledgements:
Supported by Russian Science Foundation (project № 16-16-10068)
Received September 5, 2016

Abstract

The coexistance of domestic and wild reindeer populations (*Rangifer tarandus* L., 1758) — is an important feature of this species. Both forms inhabit in conditions, which remain substantially unchanged for a long time. Due to gene flow between domestic and wild populations we observe a relatively high amount of admixture in the gene pool. Biodiversity characteristics of two most numerous reindeer populations (semi-domesticated Nenets breed and wild population of reindeer inhabiting territories of Nenets Autonomous Okrug (NAO) and Taimyr Autonomous Okrug (TAO) based on the analysis of microsatellites are given and the degree of introgression in these populations is determined. Samples of Nenets breed of domestic rein deer were collected in several farms in NAO and TAO (n = 115, four subpopulations). Samples of wild Taimyr population were collected in the course of field research in different geographic regions of TAO (n = 63, five subpopulations). Genomic DNA was isolated using Nexttec columns («Nexttec Biotechnologie GmbH», Germany). Polymorphism of 9 STR-loci (NVHRT21, NVHRT24, NVHRT76, RT1, RT6, RT7, RT9, RT27, RT30) was determined according to the previously developed technique for DNA analyzer ABI3130xl («Applied Biosystems», US). To estimate the allele pool of each population average number of alleles (Na), the effective number of alleles (Ne) based on the locus, rarified allelic richness (Ar), private allelic richness (PrAr), observed (H_o) and expected (H_e) heterozygosity and inbreeding coefficient (F_{IS}) were calculated. The degree of genetic differentiation of populations was assessed using pairwise F_{ST} values and Nei's genetic distances. We calculated the degree of migration of genes between populations based on microsatellite allele frequencies. Distribution of genetic variation between and within populations was studied by analysis of molecular variance (AMOVA). It was found that the wild population of reindeer is characterized by a higher level of genetic diversity: the average number of alleles per locus was 10.00±0.78 vs. 8.44±0.80, the observed heterozygosity — 0.633±0.060 vs. 0.589±0.049. STRUCTURE analysis revealed the formation of two independent clusters corresponding to the wild and domestic populations with high values of the membership coefficient in own clusters: Q_{WLD} = 0.940±0.013 and Q_{DOM} = 0.938±0.010. However, a few individuals (4.4-4.8 %) carrying a mixed genetic origin were found. The degree of introgression between the populations was around 6 %. Cluster analysis of genetic structure performed separately for wild and domestic populations at the level of subpopulations for the number of cluster k ranged from 2 to 5 did not reveal a clear clustering between subpopulation. It's confirmed the homogeneity of genetic structure within populations. Examination of overall genetic diversity with AMOVA procedure indicated that most of the variation was observed within populations (95.4 %, $p < 0.001$). Principal component analysis (PCA) revealed clear differentiation of the studied domestic and wild popula-

tions along the axis 1 with their slight overlapping; herewith the principal component 1 was responsible for 5.15 % of variability. Evaluation of differentiation degree between subpopulations of rein deer, performed by calculation of the pairwise values of F_{ST} and Nei's genetic distances (DN) showed relatively higher degree of genetic differentiation between subpopulations within wild population comparing to domestic population (maximal F_{ST} and DN values were 0.046 vs 0.023 and 0.353 vs 0.151, respectively). The obtained results of genetic diversity and population structure of reindeer will be used to develop the breeding program with Nenets breed of domestic rein deer and to organize the measures for protection and sustainable use of wild reindeer bioresources.

Keywords: allele pool, introgression, populations, reindeer, microsatellites

Reindeer breeding is one of the oldest animal industries that takes the leading place in agriculture and commercial industry of the Far North. This is the only agricultural industry in which 18 indigenous minorities numbering over 130,000 people, mostly rural dwellers, are directly or indirectly involved [1]. Due to reindeer in the tundra and forest tundra zones, millions of hectares of pastures unavailable to other animals are effectively used [2]. According to the Arctic Council's project "Sustainable Reindeer Breeding" [3)], Russia has about $^2/_3$ of the world's domestic reindeer stock browsing in tundra, forest tundra, boreal forest, and mountain regions throughout the territory of over 3 mln km^2.

Reindeer (*Rangifer tarandus* L., 1758) is the only representative of *Rangifer* C. H. Smith, 1827. For a historically long period, a large group of domestic reindeer was bred with clearly expressed morpho-biological and useful sustainably inherited traits. In 1985, four reindeer breeds were approved and entered into the State Register: Nenets, Chukotka, Evenk, and Even. For the last decade, the number of domestic reindeer in Russia reduced 2-fold and as of 1 January 2012 was assessed at 1,583,000 livestock units, which amounted only to 70.0 % as compared to 1990 [4].

The Nenets indigenious reindeer breed is the largest in Russia by the number (950,000 livestock units) and pasture territory (110 million ha) [5]. The reindeer of this breed are of an average size with rather well-defined working characgeristics [6]. They are perfectly adapted to the local climate conditions [7]. The Nenets reindeer are widely spread in the Nenets, Yamalo-Nenets, Khanty-Mansi, and Tamyr Autonomous Okrugs, Murmansk and Arkhangelsk regions, and the Republic of Komi. According to the breeding records as of 1 January 2015, there are about 170,000 reindeer bred by 23 farms in the Nenets Autonomous Okrug (NAO) [8]. Despite the existence of several ecological geographic types, the Nenets indigenous reindeer are the most homotypic and consolidated. Larger species can be found only on the arctic islands (Kolguev, Vaigach, etc.), which is explained by favorable feeding conditions, rather than genetic peculiarities [9].

The number of wild reindeer has also reduced for the said period [10-12]. Currently, their number is 1.4 million livestock units, with over 70 % concentrated in the north of Middle Siberia. The largest population is the Taymyr population which is of exclusive value as a key component of Taymyr biodiversity. Wild reindeer largely determine the conditions for vegetation recovery, and are the species that affect the habitat of mammals and birds in the region [13, 14]. The Taymyr reindeer population is a geographic and ecological phenomenon having no analogs by the number, the distance of migrations (up to 1,500 km), and the coverage of zonal types (from the northern tundra to polar deserts) [15].

The co-existence of domestic and wild forms is an important peculiarity of the species. Domestic reindeer does not differ substantially from its wild ancestor. Both forms dwell in almost similar conditions that remain practically unchanged for a long time. In reindeer breeding, no artificial forage is used and there are no

zootechnical methods of feeding or artificial management. Domestic reindeer are range-fed and feed on the same forage as their wild congeners, but consume forage resources more fully as they do not migrate for long distance [3]. Discussing the intraspecific status of the domestic reindeer, some authors believe that in the same geographical areas it forms a common genetic pool with the wild species [16].

For the reindeer breeding industry, the existence of domestic and wild reindeer forms poses a serious problem [11], which, among others, may be caused by the withdrawal of domestic reindeer by wild ones, grazing of pastures and mutual pasture competition, remaining centers of infections, and transfer of diseases. The most serious are the first two reasons resulting in a complete loss of domestic reindeer breeding in the central part of Taymyr Peninsula, and a substantial reduction of domestic reindeer livestock in Eastern Taymyr and some other regions [17]. In this respect, E.E. Syroechkovskii [18] and L.M. Baskin [19] think that a decrease in the wild reindeer population is caused by intensive development of domestic reindeer breeding that extensively ousts the wild population.

It is ascertained that domestic and wild populations exchange genes which mixes their gene pool. Along with evolution factors (gene drift, mutations, natural selection), the population gene pool also changes under the influence of the migration process. The divergence between the populations may be caused by the genetic drift because of full or partial isolation and heterogenetic selection among sub-populations. The animal migrations, no matter how insignificant they are, prevent divergence and reduce the genetic diversity among populations, but at the same time increase this parameter within sub-populations [20].

The key live-stock animals (cattle, sheep, swine, goats, horses, hens, dogs) were domesticated several thousand years ago [21, 22], their genetic variability preserves inside and between the species, and they substantially differ from their wild predecessors [23, 24]. Although reindeer were domesticated 5-10 thousand years ago [25, 26], the wild and domestic populations demonstrate gene exchange [27].

The assessment of genetic divergence and the monitoring of evolution processes may be adequately performed using molecular-genetic methods that provide objective understanding the genetic structure of populations and make it possible to determine the degree of introgression between them [28]. One of the methods widely used to assess the genetic structure of reindeer populations of various subspecies is the analysis of microsatellites (STR, short tandem repeats) [29-33]. Most often, microsatellites are used in population and ecological research to study gene transfer, the effective size of populations, migration, an intraspecific genetic variability and differentiation of populations [34-37].

In this research for the first time ever, the possibility of identifying reindeer species of wild, domestic or mixed forms using highly polymorphic DNA markers has been shown by the example of populations dwelling in the north of Nenets Okrug and Taymyr Autonomous Okrug.

The purpose of this work was to study the allele pool of domestic and wild reindeer populations dwelling in the north of Nenets Okrug and Taymyr Autonomous Okrug and to assess the genetic introgression between them using microsatellites.

Technique. The tissue samples were collected from 178 reindeers (*Rangifer tarandus* L., 1758), including domestic Nenets reindeer (DOM, $n = 115$) and wild Taymyr reindeer (WLD, $n = 63$). The animals of the Nenets breed were from two farms in Nenets Autonomous Okrug (NAO), the SRO Ilebts (ILB, $n = 17$) and SPK Indiga (IND, $n = 64$), and also from two reindeer breeding

brigades in Taymyr Autonomous Okrug (TAO), the № 4 in the Dudinka river region (DUD, n = 26) and № 11 in the Pelyatka river region (PEL, n = 9). The samples of wild species were collected during the field research in various geographic regions of TAO: Portnyagino (POR, n = 7; 74°8'48.12"N, 107°9'54"E), Belogorka (BEL, n = 13; 72°9'33.13"N, 91°18'92"E), Kongudoyar (KON, n = 19; 72°7'62.05"N, 91°36'09"E), Ust-Avam (UAV, n = 20; 71°11'40.71"N, 92°82'07"E) and Volchanka (VOL, n = 4; 70°97'60.83"N, 94°54'13"E). The biomaterial was collected throughout 2016. The sample collection map was built in R-ggmap [38] with visualization in ggplot2 [39].

The genome DNA was isolated using Nexttec columns (Nexttec Biotechnologie GmbH, Germany) in accordance with the manufacturer's recommendations. Polymorphism of 9 STR loci (NVHRT21, NVHRT24, NVHRT76, RT1, RT6, RT7, RT9, RT27, RT30) was determined by the developed methods [33] using a DNA-analyzer ABI3130xl (Applied Biosystems, USA). The allele sizes determined in GeneMapper 4.0 (Applied Biosystems, USA) were converted into numerical expressions used as a basis for the genotype matrix in the MS Excel format.

The allele pool of each population was assessed by determining the average number of alleles (Na) and the effective number of alleles (Ne) per locus [40], the allele diversity estimated by a rarification procedure (Ar), the number of private alleles per locus (PrAr) [41], the observed (H_o) and expected (H_e) heterozygosity, and the inbreeding coefficient (F_{IS}) [42]. Genetic differentiation of the populations was assessed based on paired F_{ST} values [43] and the genetic distances according to M. Nei [44]. GenAIEx 6.5.1 [45], HP-Rare 1.1 [46] and Genetix 4.05 [47] software was used for calculations.

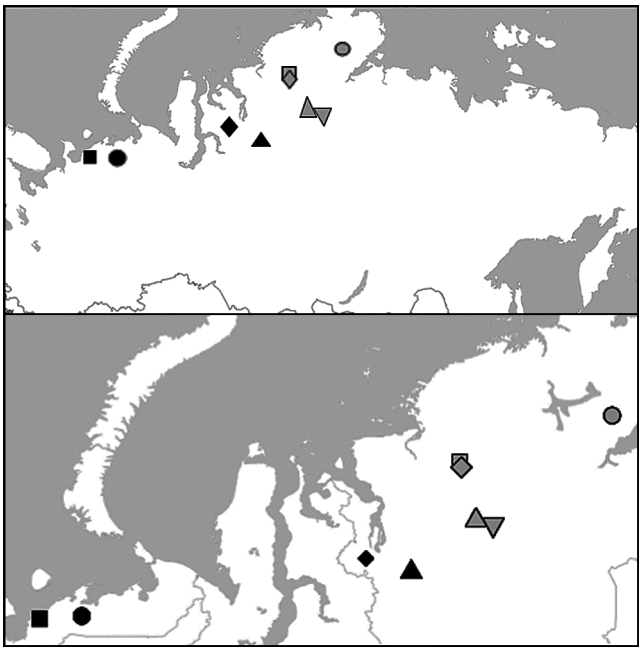


Fig. 1. The points of sampling biomaterial in wild and domestic reindeer (*Rangifer tarandus* L., 1758) populations: ● — ILB, ■ — IND, ♦ — PEL, ▲ — DUD, ● — POR, ■ — BEL, ♦ — KON, ▲ — UAV, ▼— VOL. For the abbreviation, see the *Technique* section for description of the sub-populations.

Gene migration between the populations was calculated by the frequency of alleles using the divMigrate graphic network model [48] in R diveRsity [49]. This model makes it possible to compare the populations based on various assessments of genetic differentiation, such as paired F_{ST} values [45], G_{ST} [50], D_N [46], and D_{Jost} [51]. Principal Component Analysis (PCA) was performed in R adegenet [52] with visualization in R gglot2 [39].

Distribution of general genetic variability between and within the populations was studied by AMOVA in Arlequin suite 3.5.2.2 [53]. The genetic structure of the populations was assessed through clustering in STRUCTURE 2.3.4 [54] using a mixed model, with C from 1 to 6 for the number of expected clusters; 100,000 for the burn-in period, and 100,000 for the Markov chain Monte Carlo model. For each C value, 10 iterations were per-

formed. STRUCTURE HARVESTER [55] was used to determine the optimum number of clusters (ΔC) for the sample assessed by the method of G. Evanno et al. [56]. For each of the samples, an average value of the Q membership coefficient in the i-th cluster was determined for the overall number of clusters (C).

Results. The points of the sample collecting are shown in Figure 1.

The analysis on Na, Ne, Ar, PrAr, H_o and H_e parameters revealed the tendency to higher genetic diversity in the wild population as compared to the domestic one. Both populations demonstrated a deficiency of heterozygotes (Table 1), however, the VOL sub-population had a slight excess of them. A similar pattern is described by K.H. Mager [57] when studying the genetic structure of the wild caribou population in Alaska and the domestic reindeer population in the Seward Peninsula (western Alaska), i.e. Ar from 12.28 to 13.15 and H_e = 0.86 for the wild population, Ar = 10.06 and H_e = 0.75 for the domestic population.

1. Genetic diversity of the wild (WLD) **and domestic** (DOM) **reindeer (*Rangifer tarandus* L., 1758) populations by microsatellite loci**

Population, sub-population	*n*	Na	Ne	Ar	PrAr	H_o	H_e	F_{IS}
POR	7	6.00±0.62	4.47±0.61	4.59±0.39	0.32±0.14	0.730±0.065	0.738±0.036	0.011
BEL	13	7.44±0.56	5.29±0.52	4.76±0.24	0.36±0.11	0.633±0.072	0.790±0.029	0.199
KON	19	8.00±0.60	5.17±0.46	4.66±0.23	0.23±0.08	0.620±0.068	0.787±0.029	0.213
UAV	20	7.89±0.72	4.74±0.69	4.42±0.31	0.29±0.10	0.617±0.080	0.741±0.044	0.167
VOL	4	3.67±0.50	2.80±0.45	3.67±0.50	0.20±0.10	0.611±0.074	0.576±0.056	–0.060
WLD	63	10.00±0.78	5.44±0.63	4.60±0.26	2.29±0.29	0.633±0.060	0.786±0.035	0.195
ILB	16	5.56±0.65	3.39±0.50	3.60±0.35	0.10±0.03	0.563±0.052	0.643±0.056	0.125
IND	64	7.22±0.66	3.97±0.43	3.88±0.21	0.11±0.05	0.603±0.045	0.723±0.030	0.167
DUD	26	6.44±0.56	3.67±0.53	3.74±0.27	0.15±0.04	0.556±0.067	0.684±0.042	0.188
PEL	9	5.89±0.63	4.24±0.56	4.24±0.37	0.21±0.07	0.642±0.076	0.722±0.042	0.111
DOM	115	8.44±0.80	4.12±0.56	3.92±0.26	1.61±0.19	0.589±0.049	0.719±0.037	0.180

N o t e. Na — the average number of alleles per locus, Ne — the average effective number of alleles per locus, Ar — allele diversity, PrAr — the number of private alleles, H_o — observed heterozygosity, H_e — expected heterozygosity, F_{IS} — inbreeding coefficient. See the description of the sub-populations in the *Technique* section.

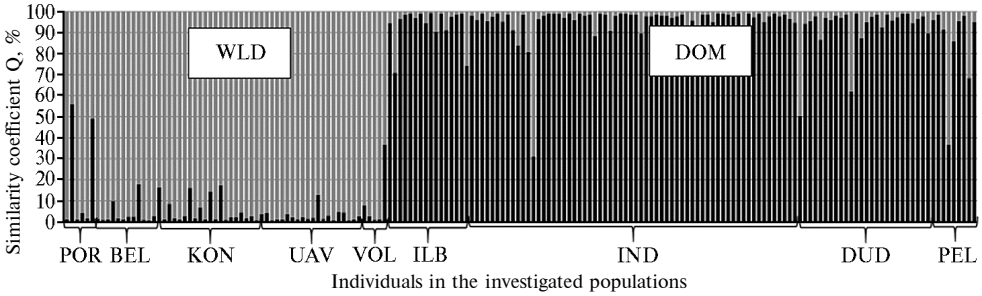


Fig. 2. Cluster analysis of wild (WLD) **and domestic** (DOM) **reindeer (*Rangifer tarandus* L., 1758) populations by nine STR loci using STRUCTURE 2.3.4** [54] **for the number of clusters C = 2**. Q — the contribution of each of the clusters to the species genotype (the probability that the species belongs to this or that cluster). For the domestic population is marked in black, for the wild one — in grey. See the description of the sub-populations in the *Technique* section.

For cluster analysis, we determined the most probable number of clusters (C), or in other words, objectively isolated genetic groups in the sample in accordance to the algorithm based on the ΔC values [56]. The optimum number of clusters for the sample was C = 2 (ΔC = 87.23). The structure analysis with C = 2 (Fig. 2) showed a high genetic isolation both in wild and domestic reindeer populations, which is confirmed by the mean values of the similarity coefficient Q in each population (Q_{WLD} = 0.940±0.013 and Q_{DOM} = 0.938±0.010). At the same time, we revealed three animals in the wild population (Q_{WLD} = 0.441, Q_{WLD} = 0.508, Q_{WLD} = 0.631), and five animals in the domestic population (Q_{DOM} = 0.310, Q_{DOM} = 0.505, Q_{DOM} = 0.621, Q_{DOM} = 0.368, Q_{DOM} = 0.684) (4.4 and 4.8 % respectively) of a mixed genetic origin. Such mixed (crossbred)

animals with a share of the other reindeer population's alleles in the genotype evidence a periodic gene exchange between these populations. The cluster analysis of the domestic population (C = 1-5) showed the homogeneous genetic structure of all the samples. Our results confirm the findings on the key morpho-biological, growth and development parameters, and polymorphic protein blood systems that gave ground to consider the breed consolidated by its origin and genotype [58].

Despite the fact that the wild Taymyr reindeer population is represented by several isolated groups [10], according to N.V. Malygina [59] it should be regarded as a single whole ecological population. Probably that is why all samples of the Taymyr population that we studied were characterized by a common genetic structure without any clear clusterization between them (the analysis for the wild population at C = 1-6).

AMOVA showed that 95.4 % of genetic variability was the variability inside the samples of wild and domestic populations, and 4.6 % was due to the variability between the populations ($p < 0.001$).

The genetic variability in natural populations is generally indicative of the structure that emerges as a result of various processes (geographic isolation, progenitor effect, migration and admixture). One of the methods widely used to identify this structure of populations is the PCA analysis that determines two or three basic axes in the variability and plots tested objects in these coordinates [60]. This analysis based on the frequency of alleles in the studied populations makes it possible to simultaneously characterize the level of variability, to identify the key alleles contributing to the differentiation of animal groups, to track the key patterns of the population-genetic differentiation and to identify their relation to ecological and geographical factors [61]. The obtained results of the PCA are provided in Figure 3.

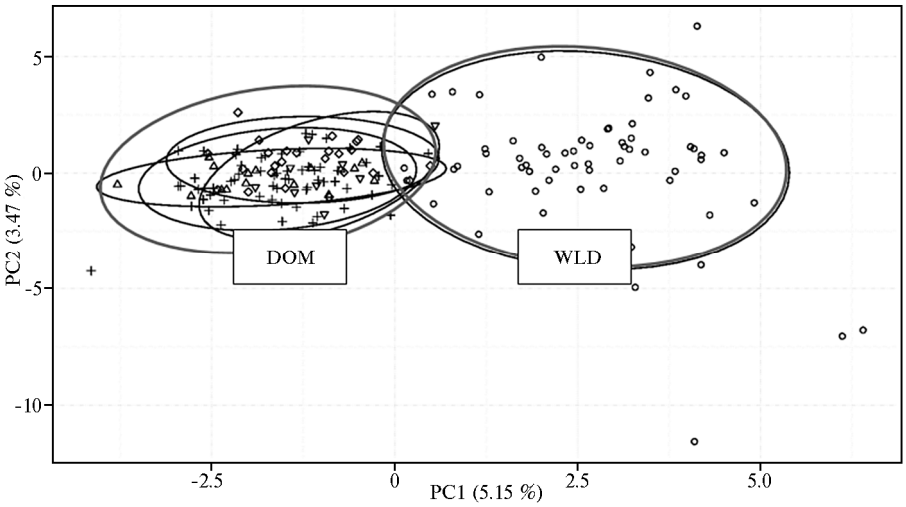


Fig. 3. Distribution of reindeer (*Rangifer tarandus* L., 1758) individuals from the wild (WLD) **and domestic** (DOM) **populations within the space of two principal components:** DOM — domestic population, △ — ILB, **+** — IND, ◇ — DUD, ▽ — PEL. See the description of the sub-populations in the *Technique* section.

The first principal component reflected 5.15 % of the observed variability of populations and clearly divided the domestic and wild reindeer populations. The insignificant overlay of the domestic and wild populations can be regarded as an indication of a non-controlled migration between them. According to A.V. Davydov [62], it is in the period of migrations that the herds of

reindeer take away a part of domestic animals, and wild males that joined the domestic herds during the rutting period mate with females that resulted in up to 3 % of the newborn reindeers. Within populations, reindeer from different sub-populations demonstrated similarity in the frequency of common alleles constituting a single genetic pool.

2. Genetic distances between the studied sub-populations of wild and domestic reindeer p (*Rangifer tarandus* L., 1758) opulations

Sub-population	POR	BEL	KON	UAV	VOL	ILB	IND	DUD	PEL
POR		0.194	0.187	0.241	0.353	0.436	0.387	0.378	0.393
BEL	0.003		0.122	0.191	0.324	0.402	0.369	0.339	0.368
KON	0.004	0.003		0.119	0.230	0.397	0.349	0.328	0.320
UAV	0.024	0.021	0.009		0.118	0.450	0.425	0.326	0.348
VOL	0.046	0.040	0.025	0.002		0.562	0.521	0.400	0.387
ILB	0.097*	0.093*	0.096*	0.116*	0.150*		0.072	0.094	0.151
IND	0.072*	0.074*	0.075*	0.099*	0.120*	0.008*		0.077	0.101
DUD	0.076*	0.073*	0.075*	0.081*	0.097*	0.020*	0.015*		0.113
PEL	0.047*	0.047*	0.045*	0.060*	0.066*	0.023	0.018	0.010	

N o t e. The non-shifted distances according to M. Nei (D_N) are above the diagonal; the F_{ST} values in the paired comparison are under the diagonal. See the description of the sub-populations in the *Technique* section.
* The differences for F_{ST} are statistically significant at $p < 0.05$.

The calculations of F_{ST} values in paired comparison and of the genetic distances according to M. Nei (D_N) [44] (Table 2) generally showed a relatively higher genetic differentiation of sub-populations within a wild population as compared to the domestic one: maximum F_{ST} and D_N values were 0.046 versus 0.023 and 0.353 versus 0.151. Note, the genetically closer sub-populations were distinguished within the wild population than within the domestic population.

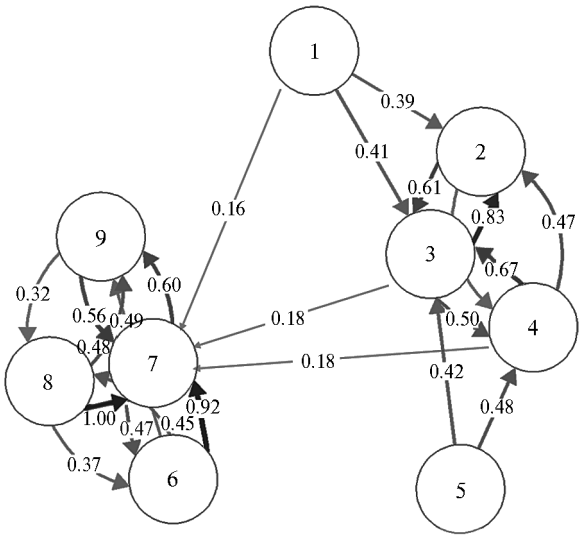


Fig. 4. Relative directed gene migration between reindeer (*Rangifer tarandus* L., 1758) populations visualized using the divMigrate model [48]**:** on the left — domestic population (DOM), on the right — wild population (WLD); 1 — POR, 2 — BEL, 3 — KON, 4 — UAV, 5 — VOL, 6 — ILB, 7 — IND, 8 — DUD, 9 — PEL. See the description of the sub-populations in the *Technique* section.

Figure 4 provides an assessment of gene migration between reindeer populations. Studied samples of the individuals are shown as nods in networks. Each nod is hypothetically connected with every other nod by two lines representing two mutual components of gene flow between any of the population pairs. The length and thickness of each line changes depending on the intensity of the gene flow. Such characteristics are rather informative as they indicate the populations between which genes are exchanged with high intensity locally, but with low intensity outside the populations. The patterns of the population's genetic structure are presented as a single cluster in a network space [63].

The introgression of alleles between the domestic and wild populations was one way, i.e. from wild to domestic one (see Fig. 4). The maximum migration within the domestic population was identified between the sub-populations ILB-IND and DUD-IND, where IND was a recipient of the gene flow from ILB and

DUD. For reindeer, the highest similarity was observed between the subpopulations BEL-KON-UAV, and the maximum migration occurred between UAV and BEL.

According to L. Sundqvis et al. [63], this approach shows graphically integrated network patterns of the gene flow between the populations and estimated the valid difference in the gene flow between the paired samples.

Scientists have always had increased interest in studying genetic diversity and introgressive hybridization in reindeer populations using genetic markers. The relationship between wild and domestic reindeer species was touched upon back in 1989 [64] and in early research works [65]. B. Jepsen et al. [66] found out the introgressive hybridization between wild caribou and domestic reindeer populations dwelling in the territory of Nuuk city (Greenland). M.A. Cronin et al. [67] identified common alleles for several genes present both in the wild and in domestic reindeer populations in Alaska suggesting insignificant genetic introgression in both directions. Hybrid animals are probably less adapted to wild life and captive crossbreeding is comparatively low [64, 68]. When studying the genetic diversity of migrating caribou populations in the Alaska North Slope and their potential hybridization with domestic reindeer, K.H. Mager et al. [69] discovered several individuals of mixed genetic origin (8 % in caribou populations and 14 % among domestic reindeer).

It is expected that expanding the set of STR loci and increasing the sample size in both reindeer populations, including animals from other geographical points of the area, will make it possible to define more precisely the introgression in this species. The new-generation molecular markers based on the analysis of single nucleotide polymorphism (SNP) [70] may give more comprehensive insight into the interaction between wild and domestic reindeer populations on a genetic level. Understanding the genetic variability in reindeer populations within the whole area will help us see the evolution pattern of this species. Moreover, constant monitoring of genetic diversity and population size is required to preserve the wild reindeer.

Thus, based on microsatellite polymorphism we characterized the allele pool in the sample sets of two most numerous reindeer populations, the domestic Nenets reindeer and wild reindeer dwelling in the territory of Nenets Okrug and Taymyr Autonomous Okrug. Although the cluster analysis showed high genetic isolation of both forms, we identified several individuals of mixed genetic origin. The comparison of intrapopulation parameters revealed that wild reindeer have higher genetic diversity than domestic reindeer. Estimation of genetic diversity and the structure of Nenets breed population of reindeer is required for protestation and a sustainable use of its biological resources.

REFERENCES

1. Mukhachev A.D. *Puteshestvie v mir olenevodov* [Journey to the reindeer herders' world]. Novosibirsk, 2001 (in Russ.).
2. Borozdin E.K., Zabrodin V.A., Vostryakov P.N. *Severnoe olenevodstvo* /Pod redaktsiei V.A. Zabrodina [Northern reindeer. V.A. Zabrodin (ed.)]. Moscow, 1979 (in Russ.).
3. Iernsletten I.-L., Klokov K. *Ilin*, 2005, 1(42). Available: http://ilin-yakutsk.narod.ru/2005-1/18.htm. No date (in Russ.).
4. Loginov V.G. *Agrarnyi vestnik Urala*, 2014, 11(129): 74-77 (in Russ.).
5. Yuzhakov A.A. *Nenetskaya aborigennaya poroda severnykh olenei. Avtoreferat doktorskoi dissertatsii* [Indigenous Nenets reindeer. DSci Thesis]. Novosibirsk, 2004 (in Russ.).
6. Yugai V.K. *Agrarnyi vestnik Urala*, 2009, 10(64): 48-51 (in Russ.).
7. Stammler F. *Reindeer nomads meet the market: culture, property and globalisation at the end of the land.* Lit-Verlag Muenster, 2005 (ISBN 3-8258-8046-x).
8. Romanenko T.M., Filippova G.I. *Simvol nauki*, 2015, 11: 44-52 (in Russ.).
9. Ernst L.K., Dmitriev N.G., Paronyan I.A. *Geneticheskie resursy sel'skokhozyaistven-*

nykh zhivotnykh v Rossii i sopredel'nykh stranakh [Genetic diversity of farm animals in Russia and neighboring countries]. St. Petersburg, 1994 (in Russ.).
10. Kolpashchikov L.A. *Taimyrskaya populyatsiya dikogo severnogo olenya (biologicheskie osnovy upravleniya i ustoichivogo ispol'zovaniya resursov). Avtoreferat doktorskoi dissertatsii* [Taimyr population of wild reindeer — biological aspects of control and sustainable use. DSci Thesis]. Noril'sk, 2000 (in Russ.).
11. Syroechkovskii E.E., Rogacheva E.V., Savchenko A.P., Sokolov G.A., Baranov A.A., Emel'yanov V.I. *Krasnaya kniga Krasnoyarskogo kraya. Redkie i nakhodyashchiesya pod ugrozoi ischeznoveniya vidy zhivotnykh* [Red Data Book of Krasnoyarsk region. Rare and endangered animal species]. Krasnoyarsk, 2000 (in Russ.).
12. Mukhachev A.D., Kolpashchikov L.A., Laishev K.A. *Myasnaya produktivnost' severnykh olenei* [Meat production in reindeer]. Novosibirsk, 2001 (in Russ.).
13. Tikhomirov B.A. *Vzaimosvyazi zhivotnogo mira i rastitel'nogo pokrova tundry* [Relationships of wildlife and vegetation of the tundra]. Moscow-Leningrad, 1959 (in Russ.).
14. Tishkov A.A. *Zhurnal obshchei biologii*, 1977, 38(1): 15-23 (in Russ.).
15. Kolpashchikov L.A., Mikhailov V.V. *Sovremennye problemy prirodopol'zovaniya, okhotovedeniya i zverovodstva*, 2012, 1: 108-109 (in Russ.).
16. Semenov-Tyan-Shanskii O.I. *Severnyi olen'* [Reindeer]. Moscow, 1977 (in Russ.).
17. Klokov K.B. *Materialy III Mezhdunarodnoi shkoly po yuridicheskoi antropologii «Olen' vsegda prav»* [Proc. III Int. Workshop on anthropology of law «Reindeer is always right»]. St. Petersburg—Pushkin, 2003: 53-74 (in Russ.).
18. Syroechkovskii E.E. *Wild reindeer*. Amerind Publishing, New Delhi, India, 1995.
19. Baskin L.M. Number of wild and domestic reindeer in Russia in the late 20th century. *Rangifer*, 2005, 25(1): 51-57 (doi: 10.7557/2.25.1.337).
20. Papa R. Gene flow and introgression between domesticated crops and their wild relatives. *Proc. Int. Workshop «The role of biotechnology for the characterization and conservation of crop, forestry, animal and fishery genetic resources»*. Turin, Italy, 2005: 71-76.
21. MacHugh D.E., Bradley D.G. Livestock genetic origins: goats buck the trend. *PNAS USA*, 2001, 98: 5382-5384 (doi: 10.1073/pnas.111163198).
22. Bruford M.W., Bradley D.G., Luikart G. DNA markers reveal the complexity of livestock domestication. *Nat. Rev. Genet.*, 2003, 4: 900-910 (doi: 10.1038/nrg1203).
23. MacHugh D.E., Shriver M.D., Loftus R.T., Cunningham P., Bradley D.G. Microsatellite DNA variation and the evolution, domestication and phylogeography of taurine and zebu cattle (*Bos taurus* and *Bos indicus*). *Genetics*, 1997, 146: 1071-1086.
24. Luikart G., Gielly L., Excoffier L., Vigne J.-D., Bouvet J., Taberlet P. Multiple maternal origins and weak phylogeographic structure in domestic goats. *PNAS USA*, 2001, 98: 5927-5932 (doi: 10.1073/pnas.091591198).
25. Baskin L.M. *Reindeer husbandry in the Soviet Union. Wildlife production systems: economic utilization of wild ungulates.* R.J. Hudson, K.R. Drew, L.M. Baskin (eds.). Cambridge University Press, Cambridge, UK, 1989: 197-206.
26. Staalannd H., Eikelmann I.M.H. *Status of the reindeer industry in Fennoscandia. Wildlife production: conservation and sustainable development.* L.A. Renecker, R.J. Hudson (eds.). AFES Misc. University of Alaska, Fairbanks, 1991, 91(6): 77-88.
27. Davydov A.V., Kholodova M.V., Meshcherskii I.G., Gruzdev A.R., Sipko T.P., Kol N.V., Tsarev S.A., Zheleznov-Chukotskii N.K., Mirutenko V.S., Gubar' Yu.P., Lin'kov A.B., Rozhkov Yu.I. Differentiation of wild and domestic forms of caribou (*Rangifer tarandus* L.) on the results of mtDNA analysis. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2007, 6: 48-53. Available http://agrobiology.ru/articles/6-2007davydov.pdf. No date (in Russ.).
28. Khokhlov A.M. *Teoreticheskoe obosnovanie protsessa domestikatsii, selektsionno-geneticheskii i tekhnologicheskii monitoring v svinovodstve. Avtoreferat doktorskoi dissertatsii* [Theoretical aspects of domestication, genetic and technological monitoring in pig-breeding. DSci Thesis]. Khar'kov, 2006 (in Russ.).
29. Ball M.C., Finnegan L., Manseau M., Wilson P. Integrating multiple analytical approaches to spatially delineate and characterize genetic population structure: an application to boreal caribou (*Rangifer tarandus caribou*) in central Canada. *Conserv. Genet.*, 2010, 11: 2131-2143 (doi: 10.1007/s10592-010-0099-3).
30. Zittlau K.A. *Population genetic analyses of North American caribou (Rangifer tarandus). PhD Thesis.* University of Alberta, Canada, 2004.
31. Cronin M.A., MacNeil M.D., Patton J.C. Variation in mitochondrial DNA and microsatellite DNA in caribou (*Rangifer tarandus*) in North America. *J. Mammal.*, 2005, 86: 495-505 (doi: 10.1644/1545-1542(2005)86[495:VIMDAM]2.0.CO;2).
32. Tyler S.K., Mcfarlane K.A., Pamela Groves P., Mooers A.Ø., Shapiro B. Modern and ancient DNA reveal recent partial replacement of caribou in the southwest Yukon. *Mol. Ecol.*, 2010, 19: 1312-1323 (doi: 10.1111/j.1365-294X.2010.04565.x).
33. Kharzinova V.R., Gladyr' E.A., Fedorov V.I., Romanenko T.M., Shim-

it L.D., Layshev K.A., Kalashnikova L.A., Zinovieva N.A. Development of multiplex microsatellite panel to assess the parentage verification in and differentiation degree of reindeer populations (*Rangifer tarandus*). *Agricultural Biology*, 2015, 50(6): 756-765 (doi: 10.15389/agrobiology.2015.6.756eng) (in Engl.).
34. Pfeiffer A., Olivieri A.M., Morgante M. Identification and characterization of microsatellites in Norway spruce (*Picea abies* K.). *Genome*, 1997, 40: 411-419.
35. Scotti I., Magni F., Paglia G.P., Morgante M. Trinucleotide microsatellite in Norway spruce (*Picea abies*): their features and the development of molecular markers. *Theor. Appl. Genet.*, 2002, 106: 40-50 (doi: 10.1007/s00122-002-0986-1).
36. Fluch S., Burg A., Kopecky D., Homolka A., Spiess N., Vendramin G.G. Characterization of variable EST SSR markers for Norway spruce (*Picea abies* L.). *BMC Res. Not.*, 2011, 4: 401-412 (doi: 10.1186/1756-0500-4-401).
37. Cremer E., Liepelt S., Sebastiani F., Buonamici A., Michalczyk I.M., Ziegenhagen B., Vendramin G.G. Identification and characterization of nuclear microsatellite loci in *Abies alba* Mill. *Mol. Ecol. Notes*, 2006, 6: 374-376 (doi: 10.1111/j.1471-8286.2005.01238.x).
38. Kahle D., Wickham H. ggmap: Spatial visualization with ggplot2. *The R Journal*, 2013, 5(1): 144-161.
39. Wickham H. *ggplot2: Elegant graphics for data analysis.* Springer-Verlag, NY, 2009.
40. Brown A.H.D., Weir B.S. Measuring genetic variability in plant populations. In: *Isozymes in plant genetics and breeding, Part A.* S.D. Tanksley, T.J. Orton (eds.). Elsevier Science Publ., Amsterdam, 1983.
41. Kalinowski S.T. Counting alleles with rarefaction: Private alleles and hierarchical sampling designs. *Conserv. Genet.*, 2004, 5: 539-543 (doi: 10.1023/B:COGE.0000041021.91777.1a).
42. Hartl D.L., Clark A.G. *Principles of population genetics.* Sunderland, Massachusetts, 1997.
43. Weir B.S., Cockerham C.C. Estimating F-statistics for the analysis of population structure. *Evolution*, 1984, 38: 1358-1370.
44. Nei M. Genetic distance between populations. *Am. Nat.*, 1972, 106(949): 283-292 (doi: 10.1086/282771).
45. Peakall R., Smouse P.E. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research — an update. *Bioinformatics*, 2012, 28: 2537-2539 (doi: 10.1093/bioinformatics/bts460).
46. Kalinowski S.T. HP RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Mol. Ecol. Notes*, 2005, 5: 187-189 (doi: 10.1111/j.1471-8286.2004.00845.x).
47. Belkhir K., Borsa P., Chikhi L., Raufaste N., Bonhomme F. *GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations.* Laboratoire Génome, Populations, Interactions, CNRS UMR 5171, Université de Montpellier II, Montpellier (France), 1996-2004.
48. Sundqvist L., Zackrisson M.Z., Kleinhans D. *Directional genetic differentiation and asymmetric migration.* Available https://arxiv.org/abs/1304.0118 [q-bio.PE]. No date.
49. Keenan K., McGinnity P., Cross T.F., Crozier W.W., Prodohl P.A. diveRsity: An R package for the estimation of population genetics parameters and their associated errors. *Methods in Ecology and Evolution*, 2013, 4: 782-788 (doi: 10.1111/2041-210X.12067).
50. Meirmans P.G., Hedrick P.W. Assessing population structure: FST and related measures. *Mol. Ecol. Resour.*, 2011, 11: 5-18 (doi: 10.1111/j.1755-0998.2010.02927.x).
51. Jost L. GST and its relatives do not measure differentiation. *Mol. Ecol.*, 2008, 17: 4015-4026 (doi: 10.1111/j.1365-294X.2008.03887.x).
52. Jombart T. adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, 2008, 24: 1403-1405 (doi: 10.1093/bioinformatics/btn129).
53. Excoffier L., Lischer H.E.L. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.*, 2010, 10: 564-567 (doi: 10.1111/j.1755-0998.2010.02847.x).
54. Pritchard J.K., Stephens M., Donnelly P. Inference of population structure using multilocus genotype data. *Genetics*, 2000, 155: 945-959.
55. Earl D.A., von Holdt B.M. Structure Harvester: A website and program for visualizing Structure output and implementing the Evanno method. *Conserv. Genet. Resour.*, 2012, 4: 359-361 (doi: 10.1007/s12686-011-9548-7).
56. Evanno G., Regnaut S., Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.*, 2005, 14: 2611-2620 (doi: 10.1111/j.1365-294X.2005.02553.x).
57. Mager K.H. *Population structure and hybridization of Alaskan caribou and reindeer: integrating genetics and local knowledge. Dissertation of the degree doctor of philosophy.* Fairbanks, Alaska, 2012.
58. Yuzhakov A.A. *Nenetskaya aborigennaya poroda severnykh olenei. Avtoreferat doktorskoi dissertatsii* [Indigenous Nenets reindeer. DSci Thesis]. Novosibirsk, 2004 (in Russ.).
59. Malygina N.V. *Dikii severnyi olen' (Rangifer tarandus L.) Vostochnogo Taimyra. Avtoreferat*

kandidatskoi dissertatsii [Reindeer (*Rangifer tarandus* L.) of eastern Taimyr. PhD Thesis]. Moscow, 2000 (in Russ.).
60. McVean G. A genealogical interpretation of principal components analysis. *PLoS Genet.*, 2009, 5(10): e1000686 (doi: 10.1371/journal.pgen.1000686).
61. Yurchenko A.A. *Geneticheskaya struktura populyatsii sakhalinskogo taimenya Parahucho perryi brevoort i voprosy prirodookhrannoi genetiki vida. Avtoreferat kandidatskoi dissertatsii* [Genetic structure of *Parahucho perryi brevoort* population and conservation genetics of the species. PhD Thesis]. Moscow, 2015 (in Russ.).
62. Davydov A.V. *Morfologicheskaya i geneticheskaya differentsiatsiya populyatsii severnogo olenya Evrazii. Avtoreferat kandidatskoi dissertatsii* [Morphological and genetic differentiation of the Eurasian reindeer populations. PhD Thesis]. Moscow, 1997.
63. Sundqvist L., Keenan K., Zackrisson M., Prodöhl P., Kleinhans D. Directional genetic differentiation and asymmetric migration. *Ecol. Evol.*, 2016, 6(11): 3461-3475 (doi: 10.1002/ece3.2096).
64. Burns J.J. *Report of the scientific workshop on caribou in the NPRA.* Barrow, Alaska, 1989.
65. Klein D.R. Conflicts between domestic reindeer and their wild counterparts: A review of Eurasian and North American experience. *Arctic*, 1980, 33: 739-756.
66. Jepsen B., Siegismund H., Fredholm M. Population genetics of the native caribou (*Rangifer tarandus groenlandicus*) and the semi-domestic reindeer (*Rangifer tarandus tarandus*) in Southwestern Greenland: Evidence of introgression. *Conserv. Genet.*, 2002, 3: 401-409 (doi: 10.1023/A:1020523303815).
67. Cronin M.A., Renecker L.A., Pierson B.J., Patton J.C. Genetic variation in domestic reindeer and wild caribou in Alaska. *Anim. Genet.*, 1995, 26: 427-434 (doi: 10.1111/j.1365-2052.1995.tb02695.x).
68. Cronin M.A., Patton J.C., Balmysheva N., MacNeil M.D. Genetic variation in caribou and reindeer (*Rangifer tarandus*). *Anim. Genet.*, 2003, 34: 33-41 (doi: 10.1046/j.1365-2052.2003.00927.x).
69. Mager K.H., Colson K.E., Hundertmark K.J. High genetic connectivity and introgression from domestic reindeer characterize northern Alaska caribou herds. *Conserv. Genet.*, 2013, 14: 1111-1123 (doi: 10.1007/s10592-013-0499-2).
70. Kharzinova V.R., Sermyagin A.A., Gladyr E.A., Okhlopkov I.M., Brem G., Zinovieva N.A. A study of applicability of SNP chips developed for *Bovine* and *Ovine* species to whole-genome analysis of reindeer *Rangifer tarandus. J. Heredity*, 2015, 106(6): 758-761 (doi: 10.1093/jhered/esv081).

Events

RNA THERAPEUTICS

(22-23 February, 2017, London, United Kingdom)

SMi Group announces the return of its 8th annual RNA Therapeutics conference to London on the 22nd-23rd of February 2017. Technology in RNA interference, oligonucleotides and mRNA has revolutionised the way infectious disease, cancer and neurological disorders are treated. It is now possible to target previously 'un-druggable' sites, therefore expanding the range of therapeutics that selectively silence genes before the disease develops.

This exciting program for 2017 will showcase new developments through clinical and pre-clinical results in topics such as: messenger RNA-based therapeutics, anti-sense oligonucleotides and new sites for RNA silencing.

The main challenge in RNA therapeutics continues to be the delivery of RNA based drugs to target sites outside the liver. Join us in exploring the different delivery systems in use and in development such as transportation with nanoparticles and the ability to enhance drug stability.

We will be reviewing clinical trial updates in RNA-based therapeutics and discussing the current regulations involved in getting drug approval.

Contacts: https://www.cambridgenetwork.co.uk/news/astrazeneca-and-moderna-therapeutics-announce-new-collaboration

Information: http://www.globaleventslist.elsevier.com/events/2016/04/the-application-of-molecular-and-functional-imaging-in-neurodegenerative-disease/

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 824-829
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Reproductive biotechnologies

UDC 636.2:591.391.1:591.158.1

doi: 10.15389/agrobiology.2016.6.824rus
doi: 10.15389/agrobiology.2016.6.824eng

IN VITRO PRODUCTION OF INTERSPECIES HYBRID EMBRYOS OF CATTLE (*Bos taurus*) AND WISENT (*Bison bonasus*)

G.N. SINGINA, V.A. BAGIROV, S.S. DANCH, T.E. TARADAINIK, A.V. DOTSEV, N.A. ZINOVIEVA

L.K. Ernst All-Russian Research Institute of Animal Husbandry, Federal Agency of Scientific Organizations, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail g_singina@mail.ru

Acknowledgements
Supported by the Program of Presidium of the Russian Academy of Science (project No IV.13.3)
Received September 28, 2016

Abstract

European bison or wisent (*Bison bonasus*) is a rare species that is endangered. The technology for producing in vitro embryos using oocytes of cows and wisent semen can be an effective tool for conservation of genetic resources of this species and their rational use in basic research on the physiology of development and to create the new animals' types. However, the methods for production of *in vitro* embryos using the above-mentioned germ cells are currently not described in the literature. In this work, we first attempted to obtain the hybrid cow-wisent embryos *in vitro* using the IVM/IVF/IVC protocol, developed for domestic cattle (*Bos taurus*). Frozen epididymal sperm derived from wisent was thawed and prepared by the swim-up procedure. Matured oocytes were co-incubated during 18 h together with prepared sperm in Fert-Talp medium, which was replaced then by embrional medium where the embryos were cultured until the blastocyst state. Additionally, for positive control, a part of matured bovine oocytes were fertilized with frozen/thawed ejaculated bull sperm. Fertilizing capacity of male cells of both species was assessed according to the nature of spermatozoa-egg interactions, as well as the capacity of fertilized bovine oocytes to subsequent embryonic development. It was found that the rate of oocytes with sing polyspermy was higher for fertilization with wisent sperm comparing to allogenic insemination (21.6 vs. 8.5 %, $P < 0.05$). There were no differences between two types of fertilizations nether for the sperm penetration rate (90.0±0.3 vs. 93.3±1.7 % for bull and wisent, respectively), nor for the rate of normal fertilization (78.3±1.7 % vs. 73.2±2.3 %). The similar cleavage and blastocyst formation rates were observed for two fertilization types. Our data indicate the similarity in the mechanisms of oocyte activation and embryonic development in cow and wisent females. In addition, it is obvious that IVM/IVF/IVP protocol, in general, allows in vitro producing cattle-wisent hybrid embryos, however, high levels of polyspermy observed in heterogeneous fertilization, indicates the necessity to adjust the method.

Keywords: cattle oocytes, the European bison sperm, fertilization in vitro, hybrid embryos

European bison, or wisent, (*Bison bonasus*) is a rare endangered species. Current European bison population is strongly inbred. All modern wisents come from 12 individuals kept in the zoos and reserves in the early XX century [1]. Low genetic variability is one of the main threats to the long-term maintenance of the species. Assisted reproductive technology such as production of in vitro embryos using oocytes of domestic cattle and wisent semen will not only help to maintain the existing genetic resources of the species, but also to involve this species in basic research on the physiology of development and use in creations of new bred forms [2-4].

Cow oocytes are a universal object for interspecific hybridization. In publications, there is evidence of their in vitro fertilization using semen of gaur [5], antelope [6], horse [7], sheep [8], and donkey [9]. Close relationship between the species, the lack of specific antigens against wisent spermatozoa in the pellucid zone (PZ) of cow oocytes [10], and the previously described possibility to obtain a hybrid offspring by crossing [11] are the prerequisites for extracorpo-

real hybridization between bovine oocytes and wisent sperm.

Current development of in vitro technologies makes the efficient and large-scale obtaining of the embryos of domestic animals possible [12-16]. However, the biological usefulness of oocytes reduces during further embryonic development with interspecific extracorporeal fertilization (even with closely related species) [17]. One solution to the problem is adjustment of the method depending on the specificity of source gametes and embryos.

Typically, the schemes developed for domestic animals are used for extracorporeal hybridization involving the gametes of rare and wild species as basic IVM (in vitro maturation)/IVF (in vitro fertilization)/IVC (in vitro culture) protocols [17-19]. Own technology for producing embryos in vitro for wisent has not been modeled yet. Cases of *Bos taurus* and *Bison bonasus* hybrids under similar conditions have not been described in publications as well.

In this paper, a possibility of formation and development of interspecific hybrids resulting from in vitro fertilization of cow oocytes by wisent sperm until the blastocyst stage is demonstrated for the first time.

The purpose of the study was fertilization of bovine oocytes by wisent sperm and evaluation of their further embryonic development in vitro using a protocol routinely used in vitro for domestic cattle embryos.

Technique. The source of females germ cells were cow ovaries collected after slaughter and delivered to the laboratory within 2-3 hours at 30-35 °C.

Oocyte-cumulus complexes (OCC) were isolated by dissecting visible follicles and washing 3 times in TC-199 medium containing 5 % fetal bovine serum, 10 μg/ml heparin, 0.2 mM sodium pyruvate, and 50 μg/ml gentamicin (Sigma, USA). Round shape oocytes with homogeneous cytoplasm and uniform width pellucid area surrounded by compact cumulus were collected for the experiments. OCC were cultured in groups of 25-35 oocytes in 500 μl of modified TC-199 medium [20] under a layer of light mineral oil (Sigma, USA) for 24 hours. The matures oocyte was transferred to Fert-TALP fertilization medium containing 114 mM NaCl, 3.2 mM KCl, 0.4 mM $NaH_2PO_4 \times 2H_2O$, 2 mM $CaCl_2 \times 2H_2O$, 0.5 mM $MgCl_2 \times 6H_2O$, 25 mM $NaHCO_3$, 10 mM HEPES, 10 mM sodium lactate, 0.25 mM sodium pyruvate, 6 mg/ml bovine serum albumin (BSA), 10 μg/ml heparin, 20 μM penicillamine, 10 μM hypotaurine, 1 μM epinephrine, 0.1 % nonessential amino acids and 50 μg/ml gentamicin (Sigma, USA).

Frozen epididymal semen obtained after the forced shooting of 7 year old wisents was used for fertilization (♀ cow — ♂ wisent fertilization). Bovine semen was the positive control (♀ cow — ♂ bull fertilization). Before the procedure, sperm was thawed for 1 min at 37 °C and prepared by swim-up procedure [21] using Sperm-TALP medium containing 100 mM NaCl, 3.1 mM KC1, 0.3 mM $NaH_2PO_4 \cdot 2H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, 1.5 mM $MgCl_2 \cdot 6H_2O$, 25 mM $NaHCO_3$, 10 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/ml BSA (Sigma, USA). The resulting active spermatozoa fraction was added to the dishes with Fert-TALP medium and mature oocytes at a dose of 1.0×10^6 spermatozoa/ml.

After 18-20 hours of co-culture, cumulus cells and the adhered sperm cells were removed from oocytes, then part of the oocytes was used for the cytological analysis of penetration and fertilization rates. The remaining oocytes were transferred to specialized medium [22] and cultured until day 7. The oocyte cleavage and blastocyst formation rates were determined.

For the cytogenetic studies of the nuclei of inseminated oocytes, they were fixed in 4 % paraformaldehyde, subjected to permeabilization in 0.5 % Triton X-100 solution, stained with 1 μg/ml DAPI (Sigma, USA), then transferred to a degreased glass slide and embedded in Vectashield medium (Vector Labora-

tories, UK). Cytological preparations were examined using fluorescence light microscopy (Axiovert 40 CFL microscope, Carl Zeiss, Germany) at a ×40 magnification.

When calculating penetration rate, the presence of condensed sperm heads and male pronuclei in the cytoplasm of mature oocytes were counted. The oocytes with male and female pronuclei, or condensed sperm heads, as well as the ones in anaphase II or telophase II were considered the cells with normal fertilization. In addition, oocytes that contain two or more condensed sperm heads in the cytoplasm and two or more male pronuclei were defined as the oocytes with polyspermic fertilization.

OCC maturation and fertilization were carried out, and embryo cultures were maintained in 5 % CO_2 atmosphere at a temperature of 38.5 °C and 90 % humidity.

Experiments on OCC culture were performed in 3-4 independent replicates. The data were processed by ANOVA using SigmaStat software (IBM SPSS, USA). The significance of differences of the mean values compared was assessed using Tukey test. Percentages (X) and the error of the mean (m) are provided.

Results. Domestic cattle (*Bos taurus*) is the leader in the efficient and large-scale obtaining of embryos in vitro. There is evidence of extracorporeal fertilization of bovine oocytes by the semen of different species [6-9], including closely related ones: gaur (*Bos gaurus*), banteng (*B. javanicus*) [5], yak (*B. grunniens*) [5, 18], African (*Syncerus caffer caffer*) and Asian (*Bubalus bubalis*) buffalo [17, 19], and American bison (*Bison bison*) [23].

Analysis of the data obtained in this study (Table 1) demonstrated an increase in the proportion of oocytes with polyspermy at interspecific fertilization (by 13.1 %, $P < 0.05$) as compared to intraspecific fertilization, but did not reveal any differences between the two types of fertilization neither in the proportion of oocytes with manifestations of sperm penetration through pellucid zone, nor in the proportion of normally fertilized oocytes. According to the published data, polyspermy is observed in 8-55 % at IVF in cows, depending on the individual characteristics of the bull, sperm concentration and conditions of capacitation and fertilization [24, 25].

As in most other studies, to obtain interspecific hybrid embryos, we used the technological system used in extracorporeal fertilization in cattle [26]. As in the case of bovine oocytes contact with bull sperm a polyspermy was lower than when they interacted with wisent sperm, it can be assumed that a too high concentration of male gametes was used for interspecific fertilization, or the conditions for the interaction of male and female cells proved inadequate. It is worth noting that similar results were observed when bovine oocytes were in vitro fertilized by yak semen [18].

1. Extracorporeal fertilization of cow (*Bos taurus*) oocytes by frozen-thawed bull and wisent (*Bison bonasus*) epididymal semen ($X \pm m$)

Fertilization	Number of experiments	Inseminated oocytes, n	Proportion of oocytes, %		
			with penetration	with normal fertilization	with polyspermic fertilization
♀cow—♂bull	3	77	90.0±0.3	78.3±1.7	8.5±1.8[a]
♀cow—♂wisent	3	81	93.3±1.7	73.2±2.3	21.6±0.8[b]

[a, b] Differences are significant at $p < 0.05$.

In an earlier study, heterogeneous fertilization of bovine oocytes was used to assess the European bison sperm fertility. The effectiveness of insemination was determined by the penetration of sperm through the oocyte PZ and pronuclei formation [10]. However, embryo formation and development is the

best evidence of successful gamete interaction.

In our experiments, the oocyte cleavage and blastocyst formation rates after insemination of bovine oocytes by wisent sperm were high and did not differ from those after bull sperm fertilization (Table 2). Moreover, in both cases, parameters of embryonic development were consistent with the traditional values resulting from IVF in cattle [13, 26].

2. Early embryonic development of bovine (*Bos taurus*) oocytes after in vitro fertilization by frozen-thawed wisent (*Bison bonasus*) epididymal semen ($X \pm m$)

Fertilization	Number of experiments	Inseminated oocytes, *n*	Oocyte cleavage rate, %	Proportion of oocytes developed to the blastocyst stage, %	
				of inseminated	of cleavaged
♀cow—♂bull	4	143	72.4±2.5	26.7±1.9	37.1±2.6
♀cow—♂wisent	4	131	77.1±2.5	27.8±3.5	35.8±3.3

Our findings and the results of other studies [10] suggest that wisent male gametes are capable of recognizing specific carbohydrate sequence of bovine oocyte pellucid zone glycoprotein and overcome the species barrier, and the acrosome enzymes contribute to sperm penetration into xenogenous oocytes. In addition, the fact of two-cell hybrid embryos formation and their development to the blastocyst stage indicates the similarity in the mechanisms of oocyte activation and embryonic development in cow and wisent females.

Thus, bovine oocytes can be fertilized by wisent sperm, and the resulting interspecies zygotes are capable of forming pre-implantation embryos. It is also obvious that IVM/IVF/IVP protocol, in general, allows in vitro producing cattle-wisent hybrid embryos, but the high levels of polyspermy observed in heterogeneous fertilization indicate the necessity to adjust the method.

REFERENCES

1. Olech W. Analysis of inbreeding in European bison. *Acta Theriologica*, 1987, 32: 373-387.
2. Kozdrowski R., Niżański W., Dubiel A., Olech W. Possibilities of using the European bison (*Bison bonasus*) epididymal spermatozoa collected post-mortem for cryopreservation and artificial insemination: a pilot study. *Reprod. Biol. Endocrinol.*, 2011, 9(1): 31 (doi: 10.1186/1477-7827-9-31).
3. Khandoker M.A.M.Y., Reza M.M.T., Asad L.Y., Saha S., Apu A.S., Hoque S.A.M. In vitro maturation of buffalo oocytes and fertilization by cattle spermatozoa. *Bang. J. Anim. Sci.*, 2012, 41(1): 6-12 (doi: 10.3329/bjas.v41i1.11969).
4. Singina G.N., Volkova N.A., Bagirov V.A., Zinovieva N.A. Cryobanking of somatic cells in conservation of animal genetic resources: prospects and successes (review). *Agricultural Biology*, 2014, 6: 3-14 (doi: 10.15389/agrobiology.2014.6.3eng) (in Engl.).
5. McHugh J.A., Rutledge J.J. Heterologous fertilization to characterize spermatozoa of the genus *Bos*. *Theriogenology*, 1998, 50(2): 185-193 (doi: 10.1016/S0093-691X(98)00125-3).
6. Kouba A.J., Atkinson M.W., Gandolf A.R., Roth T.L. Species-specific sperm-egg interaction affects the utility of a heterologous bovine in vitro fertilization system for evaluating antelope sperm. *Biol. Reprod.*, 2001, 65(4): 1246-1251 (doi: 10.1095/biolreprod65.4.1246).
7. Campos-Chillun L.F., Barcelo-Fimbres M., Carnevale E.M., Seidel G.E.J. Use of bovine oocytes to evaluate in vitro fertilizing capacity of equine sperm. *Theriogenology*, 2007, 68(3): 515-516 (doi: 10.1016/j.theriogenology.2007.05.036).
8. Garcia-Alvarez O., Maroto-Morales A., Martínez-Pastor F., Fernández-Santos M.R., Esteso M.C., Pérez-Guzmán M.D., Soler A.J. Heterologous in vitro fertilization is a good procedure to assess the fertility of thawed ram spermatozoa. *Theriogenology*, 2009, 71(4): 643-650 (doi: 10.1016/j.theriogenology.2008.09.036).
9. Taberner E., Morato R., Mogas T., Miro J. Ability of Catalonian donkey sperm to penetrate zona pellucida-free bovine oocytes matured in vitro. *Anim. Reprod. Sci.*, 2010, 118(2-4): 354-361 (doi: 10.1016/j.anireprosci.2009.08.005).
10. Pérez-Garnelo S.S., Oter M., Broque C., Talavera C., Delclaux M., Martinez-Nevado E., Palasz A.T., De la Fuente J. Post-thaw viability of European Bison (*Bison bonasus*) semen frozen with extenders containing egg yolk or lipids of plant origin and examined with a heterologous in vitro fertilization assay. *J. Zoo Wild Med.*, 2006, 37(2): 116-125 (doi: 10.1638/05-039.1).

11. Ernst L.K., Abilov A.I., Sipko T.P., SHumov A.V., Sokolovskaya I.I., Kombarova N.A., Bronskaya A.V. *Molochnoe i myasnoe skotovodstvo*, 1993, 4: 19-21 (in Russ.).
12. Cognie Y., Baril G., Poulin N., Mermillod P. Current status of embryo technologies in sheep and goat. *Theriogenology*, 2003, 59(1): 171-188 (doi: 10.1016/S0093-691X(02)01270-0).
13. Lonergan P., Fair T. In vitro-produced bovine embryos: dealing with the warts. *Theriogenology*, 2008, 69(1): 17-22 (doi: 10.1016/j.theriogenology.2007.09.007).
14. Gil M.A., Cuello C., Parrilla I., Vazquez J.M., Roca J., Martinez E.A. Advances in swine in vitro embryo production technologies. *Reprod. Domest. Anim.*, 2010, 45: 40-48 (doi: 10.1111/j.1439-0531.2010.01623.x).
15. Hinrichs K. In vitro production of equine embryos: state of the art. *Reprod. Domest. Anim.*, 2010, 45: 3-8 (doi: 10.1111/j.1439-0531.2010.01624.x).
16. Dang-Nguyen T.Q., Somfai T., Haraguchi S., Kikuchi K., Tajima A., Kanai Y., Nagai T. In vitro production of porcine embryos: current status, future perspectives and alternative applications. *Anim. Sci. J.*, 2011, 82(3): 374-382 (doi: 10.1111/j.1740-0929.2011.00883.x).
17. Owiny O.D., Barry D.M., Agabac M., Godke R.A. In vitro production of cattle × buffalo hybrid embryos using cattle oocytes and African buffalo (*Syncerus caffer caffer*) epididymal sperm. *Theriogenology*, 2009, 71(6): 884-894 (doi: 10.1016/j.theriogenology.2008.10.016).
18. Zi X-D., Lu H., Yin R-H., Chen S-W. Development of embryos after in vitro fertilization of bovine oocytes with sperm from either yaks (*Bos grunniens*) or cattle (*Bos taurus*). *Anim. Reprod. Sci.*, 2008, 108(1-2): 208-215 (doi: 10.1016/j.anireprosci.2007.08.005).
19. Kochhar H.P.S., Appa Rao K.B.C., Luciano A.M., Totey S.M., Gandolfi F., Basrur P.K., King W.A. In vitro production of cattle-water buffalo (*Bos taurus – Bubalus bubalis*) hybrid embryos. *Zygote*, 2002, 10(2): 155-162 (doi: 10.1017/S0967199402002216).
20. Singina G., Taradajnic T., Taradajnic N., Zinovieva N. Effects of in vitro culture system modification using CR1aa medium on embryo development and pregnancy rate in cattle. *Reprod. Fertil. Dev.*, 2014, 26(1): 154 (doi: 10.1071/RDv26n1Ab81).
21. Parrish J.J., Susko-Parrish J.L., Leibfried-Rutledge M.L., Critser E.S., Eyestone W.H., First N.L. Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology*, 1986, 25(4): 591-600 (doi: 10.1016/0093-691X(86)90143-3).
22. Rosenkrans C.F.Jr., First N.L. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. *J. Anim. Sci.*, 1994, 72(2): 434-437.
23. Krishnakumar S., Whiteside D., Dance A., Elkin B., Thundathil J. Effect of chilling duration on post-thaw characteristics of sperm from the North American bison (*Bison bison*). *Reprod. Dom. Anim.*, 2013, 48(4): 636-642 (doi: 10.1111/rda.12137).
24. Chikamatsu N., Urakawa M., Fukui Y., Aoyagi Y., Ono H. In vitro fertilization and early development of bovine follicular oocytes matured in different culture systems and inseminated with spermatozoa treated by different methods. *Jpn. J. Anim. Reprod.*, 1989, 35: 154-158.
25. Long C.R., Pinto-Correia C., Duby R.T., Ponce de Leon F.A., Boland M.P., Roche J.F., Robl J.M. Chromatin and microtubule morphology during the first cell cycle in bovine zygotes. *Mol. Reprod. Dev.*, 1993, 36(1): 23-32 (doi: 10.1002/mrd.1080360105).
26. Singina G.N., Taradainik T.E., Taradainik N.P. *Problema biologii produktivnykh zhivotnykh*, 2011, 4: 132-133 (in Russ.).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 830-836
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.2:636.018:591.147

doi: 10.15389/agrobiology.2016.6.830rus
doi: 10.15389/agrobiology.2016.6.830eng

BLOOD ESTRADIOL LEVEL IN BULL SIRES INFLUENCES SPERM COUNT AND EFFECTIVENESS OF ARTIFICIAL INSEMINATION

A.I. ABILOV[1], G.V. ESKIN[1], N.A. KOMBAROVA[2]

[1]*L.K. Ernst All-Russian Research Institute of Animal Husbandry,* Federal Agency of Scientific Organizations, pos. Dubrovitsy, Podolsk Region, Moscow Province, 142132 Russia;
[2]*Head Center for Reproduction of Farm Animals OAO*, 3, ul. Tsentralnaya, pos. Bykovo, Podolsk Region, Moscow Province, 142143 Russia, e-mail komnina@list.ru
Received May 13, 2016

Abstract

Wide use of artificial insemination necessitates a deeper understanding of how hormones and immune system influence on semen production in sires. With this regard, the role of follicle stimulating hormone and luteinizing hormone in spermatogenesis is under consideration. Blood testosterone and estradiol levels are related and depend on a testosterone-estradiol binding globulin function. We first examined seasonal effects of blood estradiol levels as a reproduction marker in Holstein bull sires and showed the relationship between the blood estradiol concertation, semen fertilizing ability, semen volume, and semen concentration which, in turn, impact on the results of artificial insemination. Estradiol level in Holstein sires aged 30±6 months (n = 18) was assayed using Immuno-FA-E ELISA kit and a Uniplan equipment (ZAO Pikon, Russia). The effectiveness of artificial insemination was tested in 214 cows. We showed that in the bulls the blood estradiol level varied significantly depending on a season ($P < 0.001$). In spring, the lowest (0.100 nmol/l) estradiol level detected in 78 % of the bulls was mostly characteristic, and only in 17 % of the bulls estradiol was beyond 0.200 nmol/l ($P < 0.001$). At autumn, blood estradiol concentration increased in 94 % bulls ($P < 0.001$). When estradiol level rises two times and more, a 31 % decrease in semen volume per ejaculation is observed (i.e. 3.4 ml vs 4.6 ml) which results in about 50 % decrease in semen dose number (112 vs 171). When low blood estradiol on the day of semen collecting, a 12-17 % success rate occurred in cows after a single insemination ($P < 0.05$), and 17-29 % heifers became pregnant to first insemination ($P < 0.001$). Thus the blood estradiol in bulls additionally indicates a fertilizing ability of the semen and can be used to improve effectiveness of artificial insemination technique.

Keywords: estradiol, bull sires, seasonal changes, semen indices, success rate of insemination

For effective breeding and artificial insemination of farm animals, the rational use of sires' sperm [1] should be employed, which requires, in turn, an in-depth research of the role of the hormonal and immune systems in the sperm production. Even V.K. Milovanov [2] indicated to a major importance of sex hormones for normal functioning of both reproductive organs and the whole body of sires. Sexual function of animals is known to be under neuroendocrine control. Hormones exert their effects on the metabolism and all physiological functions at very low concentrations (10^{-6}-10^{-12} mol/l) [3]. In all mammalians, spermatogenesis is modulated by peptide and steroid hormones, such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), testosterone, estradiol, etc. [4]. Under the influence of LH, which is secreted by the pituitary gland after puberty, Leydig cells begin actively to synthesize testosterone which acts on Sertoli cells. Steroid hormones secreted by the testes are represented by androgens and progesterone. They can easily penetrate the cytoplasm and control the cell function, with the participation of specific high-molecular-weight protein receptors [3, 5-7].

Significant variations in the testosterone concentrations have been documented in animals of different origin. Thus, in the Sverdlovsk region, testos-

terone concentrations in sires imported from abroad were 1.64 times higher than those in domestic bulls (14.28±2.26 vs. 8.72±1.92 nmol/l) [8]. M. Anderson [9] reported a positive correlation between the testosterone concentrations in bulls and the pregnancy occurrence in cows. In addition, there was a high positive correlation between the sperm counts in the semen and the amounts of testosterone, as well as a negative correlation between the semen pH and testosterone blood concentrations [10]. Testosterone levels in the blood of bulls correlate with their age and breed (higher in beef breeds vs. milk breeds) as well as exogenous factors [11). A positive correlation has been identified between the concentrations of cholesterol and testosterone in bulls, where the latter increases when the former is raised [12].

Testosterone-estradiol binding globulin, involved in transport, regulatory and protective functions, plays a major role in the formation of the testosterone/estradiol complex. A certain amount of androgens is converted to DHT and estradiol. Estradiol is synthesized from testosterone by the enzyme aromatase [13, 14]. In mammalian males, the adrenal glands also produce estrogens, moreover, their positive impact on the quality of sperm is observed.

Estrogens influence the development of the genitals and the secondary sexual characteristics, fat metabolism (in particular, increase the plasma concentrations of phospholipids and β-lipoprotein, reduce the levels of cholesterol and α-lipoprotein), stimulate protein anabolism and the growth hormone production, and slow bone growth in mature adult animals. Under influence of estrogens, the reticuloendothelial system is stimulated, the body's resistance to infections increases, and tissue regeneration is enhanced [3]. LH induces the secretion of androgens in the testes, stimulates the development of interstitial tissue and the production of the male hormone testosterone, and, together with FSH, promotes the proliferation of the seminiferous tubules (the initial stages of spermatogenesis). In males and females, FSH promotes the development of sperm and egg cells, respectively [2, 3].

However, at high doses, estrogens can cause the opposite effect (up to necrotic phenomena in the kidneys and liver) [3]. With increases in the concentrations of estradiol over the upper limits, worsening of spermatologic parameters has been revealed [15]. The excess weight in sires may serve as an indirect signal of excessive amounts of estrogens and worsened quantitative and qualitative indicators of the sperm [16, 17].

A.I. Abilov et al. [11] revealed the dependence of the blood concentrations of estradiols in sires on exogenous factors. Furthermore, the quantitative indicators of the sperm production increased with decreasing amounts of estradiol. There is evidence on the relationship between the spermatozoa autoantibodies in sires and the estradiol concentrations [18].

With increasing productivity in cattle, hormonal processes, metabolic rate [19] and, as a consequence, the reproductive capacity are changed. Therefore, it is important to take into account the productive type of the animal when studying its hormonal status.

In view of the discussion on the role of estrogens in the sperm production, papers assessing the impact of phyto-, xenoestrogens and chemically synthesized estradiol on the metabolism and animal reproductive function are also of interest. Xenoestrogens demonstrate activity similar to that of the endogenous estrogens and mimic their properties, therefore, influencing the synthesis, secretion, transport, metabolism, binding and excretion of endogenous hormones involved in the regulation of homeostasis, reproduction and development [20]. Data are available for the negative impact of phytoestrogens on reproductive function. For instance, it has been shown that phytoestrogens may inhibit LH and

FSH synthesis in women [21] and sexual behavior in animals [22].

In this paper, we have first defined the blood estradiol concentrations in sires in connection to the quantity and quality of sperm production, depending on the season, and how the sire's estradiol status on the day of semen collection could affect the performance of artificial insemination.

The aim of the study was to evaluate the influence of serum endogenous estradiol on the sperm productivity in sires and the efficacy of the insemination using the collected sperm.

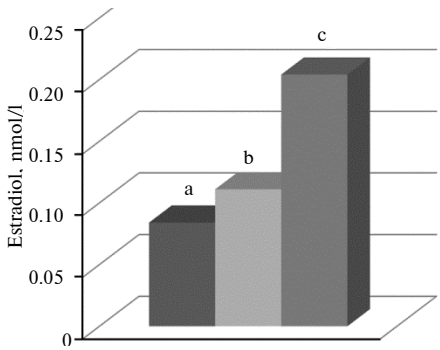
Technique. Eighteen Holstein sires aged 30±6 months were selected to enter the study (Head Center for Reproduction of Farm Animals, 2012-2014). The animals received a diet balanced according to the nutritional standards by L.K. Ernst All-Russian Research Institute of Animal Husbandry (VIZh), and were maintained under the conditions met the established requirements and standards [23].

Blood samples were collected from the jugular vein 1 hour after collecting semen. The blood serum was separated and stored at –18...–20 °C until testing. Serum concentrations of estradiol were determined by enzyme immunoassay (ELISA) using the Immuno-FA-E test kit and a Uniplan AFG-01 analyzer (JSC Picon, Russia).

Estrus in cows (the Klenovo-Chegodaevo experimental farm, New Moscow, 2012-2014) was determined twice, the optimal time for insemination was identified visually by the standing reflex, and by rectal examination, based on the follicle maturity. Animals were inseminated two times per estrus with a 10-12 hour interval. Insemination was considered productive in the absence of re-heat, and based on a rectal examination on days 45-60 after the last insemination.

The significance of differences between the compared options was evaluated by Student's *t*-test. The tables below summarize mean values (X) and mean deviations ($\pm x$).

Results. When evaluating endogenous blood estradiol concentrations in Holstein sires depending on the season, we revealed (Fig.) that in the spring (April 23, 2012) it averaged to 0.084±0.070 nmol/l, and in the autumn (September 5 and October 18, 2012) it significantly, almost 2.5-fold, increased and amounted up to 0.204±0.060 nmol/l (P < 0.001).



Seasonal blood estradiol concentrations in the Holstein sires: a — spring, b — summer, c — autumn (n = 18, the animals aged 30±6 months; P < 0.001; Moscow Province, 2012).

Based on these data, the sires were conventionally assigned to groups by the estradiol levels: low (0.100 nmol/l) for group I, mean (0.101-0.200 nmol/l) for group II, and high (> 0.200 nmol/l) for group III. The distribution of all the animals in groups according to the seasonal estradiol levels is presented in Table 1.

It appeared that most of sires (77.80 % on average in the sample) had minimum estradiol level in the spring (0.100 nmol/l), and only in 16.80 % sires on average the concentrations were significantly (P < 0.001) higher than the maximum value (0.200 nmol/l). In the autumn, a significant increase of the estradiol amount (P < 0.001) was recorded in 94.28 % sires.

The quantitative estimation of the sperm production in sires showed (Table 2) that with a significant (P < 0.001) increase in blood estradiol, as com-

pared to that in group I, the quantitative indices of sperm decreased and, as a consequence, there was a decrease in an output of qualitative semen doses per ejaculate, in a total number of qualitative doses (approximately by 50 %), and in the ejaculate semen volume (by 31 %). We suppose this is due to the fact that when the male experience hyperestrogenization, there is an increase in the level of testosterone-estradiol binding globulin, which inhibits the function of free testosterone and reduces the number of mature spermatozoa.

1. The proportion (%) of Holstein sires conventionally assigned to groups of different estradiol levels by the seasons ($X \pm x$, n = 18, the animals aged 30±6 months, Moscow Province, 2012)

Date of testing (number of animals examined)	Estradiol, nmol/l		
	up to 0.100 (group I)	0.101-0.200 (group II)	> 0.200 (group III)
April 23 (n = 18)	77.80±9.80	5.56±5.40	16.80±8.78
July 24 (n = 18)	61.11±11.49	33.33±11.10	5.56±5.40
September 5 and October 18 (n = 35)	5.71±3.92	48.57±8.45	45.71±8.42

N o t e. Differences between groups are significant at P < 0.001.

2. Quantitative characteristics of sperm production in Holstein sires conventionally assigned to groups of different estradiol levels ($X \pm x$, n = 18, the animals aged 30±6 months, Moscow Province, 2012)

Parameter	Estradiol, nmol/l		
	up to 0.100 (group I)	0.101-0.200 (group II)	> 0.200 (group III)
Number of sires, n	7	4	7
Estradiol, nmol/l	0.06±0.02	0.16±0.03*	0.26±0.04*
Ejaculate volume, ml	4.6±0.9	3.4±1.1	3.5±0.9
Sperm counts in ejaculate, bln/ml	1.3±0.3	1.4±0.3	1.3±0.2
Doses frozen per one ejaculate	170.6±69.4	146.7±37.4	112.1±32.3

* Differences vs. group I are significant at P < 0.001.

In assessing semen fertility, sires were divided into groups depending on the estradiol level on the day of semen collection (autumn 2013), i.e. 0.240-0.320 (min), 0.321-0.360 (mean) and > 0.361 nmol/l (max). We evaluated the pregnancy occurred from the first insemination (cows that were inseminated for the first time after calving) and from singel insemination (regardless of the multiplicity of coming in heat). As seen from the data (Table. 3), the lower the endogenous blood estradiol levels on the day of the semen collection, the more effective the insemination. At the lowest (within this investigation) concentration of estradiol in sires (0.240-0.320 nmol/l), the performance of one insemination by their sperm was the highest (62.03±3.86 %), while at maximum estrogenization level it was the lowest (45.45 %). Similar data were obtained for the first insemination. In both cases, differences vs. minimum values were significant at P < 0.05.

3. Insemination by sperm from Holstein sires having different estradiol levels on the day of semen collection ($X \pm x$, the sires aged 30±6 months, Klenovo-Chegodaevo Experimental Farm, New Moscow, 2013-2014)

Estradiol, nmol/l	Number of cows							
	inseminated		pregnant					
			from one insemination			from first insemination		
	total	first	total	%	vs. control, %	total	%	vs. control, %
0.240-0.320 (control)	79	52	49	62.03±3.86		31	59.62±4.81	
0.321-0.360	91	59	46	50.55±3.71	–11.98*	25	42.37±4.55	–17.25*
> 0.361	44	29	20	45.45±5.31	–16.58*	9	31.03±6.07	–28.59*
Total	214	140	115	53.74±2.41	–8.29	65	46.43±2.98	–13.19*

* Differences vs. control are significant at P < 0.05.

A probable cause of the observed variations in the amount of endogenous estradiol depending on the season might be an unequal hypothalamic response to incoming signals. In some cases, this may result in the deficiency of sex hor-

mone binding globulin, as well as in increased levels of LH and FSH, leading to manifestation of the primary and secondary testicular failure that may cause a drastic change in blood estradiol concentrations [19]. Our findings are consistent with the results showing that hyperestrogenization increases the amount of testosterone-estradiol binding globulin, and this in turn suppresses the free testosterone function and leads to deterioration of spermatologic parameters [15], including reduced sexual activity and number of spermatozoa in the ejaculate. Increased serum estradiol concentrations may also serve as an indicator of the onset of fatty liver disease (especially if sires are fed with concentrates).

Thus, the level of endogenous blood serum estradiol in sires significantly ($P < 0.001$) varies depending on the season, that affects the sperm productivity ($P < 0.001$) and the insemination ($P < 0.05$). Low levels of estradiol exert a positive effect on quantitative characteristics of sperm production with an increase in ejaculate volume by 31 %, and in the output of high-quality doses by 52 %. The number of successful inseminations, resulted in pregnancy from single or from the first insemination, is inversely proportional to the blood estradiol level in sires on the day of semen collection. These data can be used as an additional test in predicting the results of artificial insemination.

REFERENCES

1. Ernst L.K., Subbotin A.D. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Rol' i znachenie metoda iskusstvennogo osemeneniya sel'skokhozyaistvennykh zhivotnykh v progresse zhivotnovodstva XX-XXI vekov»* [Proc. Int. Conf. «Artificial insemination and progress in animal husbandry in XX-XXI»]. Dubrovitsy, 2004: 10-26 (in Russ.).
2. Milovanov V.K. *Biologiya vosproizvedeniya i iskusstvennogo osemeneniya zhivotnykh* [Biology of animal reproduction and artificial insemination]. Moscow, 1962 (in Russ.).
3. Aliev A.A. *Obmen veshchestv u zhvachnykh zhivotnykh* [Metabolism in ruminants]. Moscow, 1997 (in Russ.).
4. Kumanov P., Nandipati K., Tomova A., Agarwal A. Inhibin B is a better marker of spermatogenesis than other hormones in the evaluation of male factor infertility. *Fertil. Steril.*, 2006, 86: 332-338.
5. Sverdloff R., Bkhasin Sh. V knige: *Endokrinologiya* /Pod redaktsiei N. Lavina [In: Endocrinology. N. Lavin (ed.)]. Moscow, 1999: 369-409.
6. Browder L. *Developmental biology*. Philadelphia, Saunders, 1980: 146-231.
7. *Biology of fertilization. V. 3. The fertilization response of the egg*. C.B. Metz, A. Monroy (eds.). Academic Press, Orlando, FL, 1985: 1-44.
8. Mymrin V.S., Khalturina L.V., Shkuratova I.A., Ryaposova M.V. *Molochnoe i myasnoe skotovodstvo*, 2012, 4: 7-9 (in Russ.).
9. Anderson M. Relationship between GnRH-induced sperm motility and fertility in Ayrshire bulls. *Animal Reprod. Sci.*, 1992, 27(2): 107-111.
10. Chacur M.G.M., Mizusaki K.T., Gabriel Filho L.R.A., Oba E., Ramos A.A. Seasonal effects on semen and testosterone in Zebu and Taurine bulls. *Acta Scientiae Veterinariae*, 2013, 41: pub. 1110.
11. Abilov A.I., Kombarova N.A., Mityashova O.S. *Materialy X Konferentsii molodykh uchenykh s mezhdunarodnym uchastiem «Sovremennye dostizheniya i problemy biotekhnologii sel'skokhozyaistvennykh zhivotnykh — BioTekhZH-2015»* [Proc. X Int. Young Scientists Conf. «Recent advances and challenges in farm animals' biotechnology — BioTechAnimals-2015]. Dubrovitsy, 2015: 182-192 (in Russ.).
12. Amerkhanov Kh.A., Abilov A.I., Eskin G.V., Kombarova N.A., Turbina I.S., Fedorova E.V., Varennikov M.V., Gusev I.V. Concentration of testosterone and cholesterol in blood serum of servicing bulls depending on their type of productivity, age and the season. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2014, 2: 59-66 (doi: 10.15389/agrobiology.2014.2.59rus) (in Russ.).
13. Reznikov A.G., Varga S.V. *Antiandrogeny* [Anti-androgens]. Moscow, 1988 (in Russ.).
14. Reznikov A.G. *Polovye gormony i differentsiatsiya mozga* [Sexual hormones and brain differentiation]. Kiev, 1982 (in Russ.).
15. Gutorova N.V., Osadchuk L.V., Kleshov M.A., Kuznetsova N.N., Osadchuk A.V. *Problemy reproduktsii*, 2010, 6: 89-93 (in Russ.).
16. Fejes I., Koloszar S., Zavaczki Z., Daru J., Szollosi J., Pal A. Effect of

body weight on testosterone, estradiol ratio in oligozoospermie patients. *Arch. Androl.*, 2006, 52: 97-102.
17. Qin D.D., Yuan W., Zhou W.J., Cui Y., Wu J., Gao E. Do reproductive hormones explain the association between body mass index and semen quality? *Asian J. Androl.*, 2007, 9: 827-834.
18. Abilov A.I., Amerkhanov Kh.A., Eskin G.V., Fedorova E.V., Zhavoronkova N.V., Kombarova N.A., Varennikov M.V. *Zootekhniya*, 2013, 11: 9-11 (in Russ.).
19. Smirnova E.V., Nezhdanov A.G., Retsky M.I., Bratchenko E.V., Papin N.E., Stepanov A.V., Shushlebin V.I., Chusova G.G. The metabolic profile of late pregnant cows of various ethological activity types. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2014, 2: 67-71 (doi: 10.15389/agrobiology.2014.2.67rus) (in Russ.).
20. Diamanti-Kandarakis E., Bourguignon J.P., Giudice L.C., Hauser R., Prins G.S., Soto A.M., Zoeller R.T., Gore A.C. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr. Rev.*, 2009, 30(4): 293-342 (doi: 10.1210/er.2009-0002).
21. Hooper L., Ryder J.J., Kurzer M.S., Lampe J.W., Messina M.J., Phipps W.R., Cassidy A. Effects of soy protein and isoflavones on circulating hormone concentrations in pre- and post-menopausal women: a systematic revive and meta-analysis. *Hum. Reprod. Update*, 2009, 15: 423-440.
22. Patisaul H.B., Jefferson W. The pros and cons of phytoestrogens. *Front. Neuroendocrin.*, 2010, 31(4): 400-419.
23. *Natsional'naya tekhnologiya zamorazhivaniya i ispol'zovaniya spermy plemennykh bykov-proizvoditelei* /Pod redaktsiei N.M. Reshetnikovoi, A.I. Abilova [National Technique for freezing and usage of sperm from bull sires. N.M. Reshetnikova, A.I. Abilov (eds.)]. Moscow, 2008 (in Russ.).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 837-844
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

African swine fever: virus proteomics, diagnostics

UDC 636.4:619:616.636:578:[577.2.08+51-76

doi: 10.15389/agrobiology.2016.6.837rus
doi: 10.15389/agrobiology.2016.6.837eng

EXPRESSION OF RECOMBINANT GENES ENCODING FRAGMENTS OF THE PROTECTIVE IMPORTANT PROTEINS OF AFRICAN SWINE FEVER VIRUS IN EUCARYOTIC CELLS

A.R. IMATDINOV, A.D. SEREDA, I.R. IMATDINOV, A.S. KAZAKOVA, O.A. DUBROVSKAYA, D.V. KOLBASOV

All-Russian Institute of Veterinary Virology and Microbiology, Federal Agency of Scientific Organizations, 1, ul. Akademika Bakuleva, pos. Vol'ginskii, Petushinskii Region, Vladimir Province, 601125 Russia, e-mail almazlcf@yandex.ru
Acknowledgements:
We thank Prof. V.M. Balyshev, Dr S.P. Zhivoderov and Dr I.A. Titov for assistance in carrying out the work.
Supported by Russian Science Foundation (the research project «Design of African swine fever virus candidate vaccine based on chimeric viruses», contract No 16-16-00090)
Received August 30, 2016

Abstract

Control of African swine fever (ASF) is complicated by the lack of specific prevention medications. The attempts to obtain live attenuated vaccines by conventional methods were not promising, and the inactivated or subunit vaccines have not been developed so far (N.J. Petiska, 1965; D.V. Kolbasov et al., 2014; V. Makarov et al., 2016). The investigation of protective immune response against ASF virus (ASFV) enabled determination of a critical role of cellular defense mechanisms and the most important viral proteins p30, p54 and CD2v (or gp 110-140) involved (P. Gomez-Puertas et al., 1998; J.M. Argilaguet et al., 2012; A.D. Sereda et al., 2015). In view to develop a DNA vaccine against ASFV seroimmunotype 3 we have constructed a set of hybrid plasmids containing fragments of ASFV genes *CP204L*, *E183L* and *EP402R* from attenuated strain MK-200 (pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v). To study expression of the antigenically active polypeptide products for recombinant proteins rp30, rp54 and rCD2v in the eukaryotic cells, we transfected human embryonic kidney cells HEK293T, which stably express the SV40 large T antigen, with recombinant plasmids pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v. By immunoblotting, the polypeptides of the expressed recombinant proteins were identified in the HEK293T cell lysates and characterized for their molecular weights. Regarding size, some antigenically active recombinant polypeptides were as calculated, whereas the other ones apparently resulted from post translational modification. We identified a 21.6 kDa polypeptide after pCI-neo/ASFV/p30 transfection, a major (20.9 kDa) and a minor (36.3 kDa) polypeptides after pCI-neo/ASFV/p54 transfection, and, finally, major polypeptides of 39.8 kDa and 63.1 kDa, together with minor polypeptides of 28.8 kDa and 104.7 kDa when pCI-neo/ASFV/CD2v transfected. These genetic constructions will be helpful to investigate antigenic, immunogenic and protective properties of ASFV recombinant proteins rp30, rp54 and rCD2v.

Keywords: African swine fever, recombinant genes and proteins, transfection, antigenicity

African swine fever (ASF) is a contagious viral septic disease of swine, characterized by fever, toxical signs, hemorrhagic diathesis and high mortality; it may be hyperacute, acute, subacute, chronic and asymptomatic. In acute, the most common form of infection, 100 % of animals die within 5-10 days after onset of clinical signs. The disease affects wild boars and domestic pigs, is transmitted from sick animals and virus carriers through direct contact, alimentary and transplacentally [1]. In the South Eastern Africa, the evolution of ASF virus occurs within the sylvatic cycle, which includes warthogs and ticks of the genus *Ornitodoros* [2, 3]. After outbreaks of ASF reported in Georgia in 2007, the disease has spread to Armenia, Azerbaijan, Nagorno-Karabakh, Iran, Abkhazia, Russia, the Baltic States, Belarus, Poland, and Ukraine [4-6].

The infection control is complicated by the lack of specific prevention.

The attempts to immunize domestic pigs with attenuated strains of ASF in Spain and Portugal in the 1960s were unsuccessful [7, 8]. Later, it was confirmed that due to the high probability of reversion and poor immunobiological characteristics of many attenuated strains the live vaccines based on them were unsuitable for widespread use [9].

The experiments to develop inactivated or subunit vaccines against ASF using conventional methods have also failed because of their inability to induce cell-mediated immunity, which plays a crucial role in the formation of protection at ASF [10-12]. However, the research on the development of safe protective candidate drugs for the temporary protection of swine, ensuring their protection in enzootic areas or the planned slaughter on large pig farms, is ongoing [9, 13]. It has been shown that immunization of swine with recombinant p30 and p54 proteins or a preparation of the GP 110-140 serotype-specific major viral glycoprotein led to partial protection of animals against challenge with homologous virulent isolates [14-17].

The investigation of the protective properties of DNA constructs containing the genes of p30, p54 and CD2v proteins (GP 110-140) suggests a crucial role of the latter in the formation of protection against ASF [18, 19]. Therefore, the research of antigenic and immunogenic characteristics of the expression products of the DNA constructs, encoding genes of the protectively important ASF virus (ASFV) proteins, opens up perspectives for a new generation of drugs against virulent isolates of different immunotypes. The necessary steps of this work are cloning genes in the DNA constructs, their expression in eukaryotic systems, and the confirmation of deriving antigenically active translation products [20]. We believe that attenuated strains of ASF virus, which are characterized by the seroimmunotype and protective properties, should be used as a source of viral genes.

In this work, for the first time we have constructed hybrid plasmids that are suitable for proteomic research and development of the DNA vaccine against ASFV of seroimmunotype III.

Our purpose was to derive DNA constructs containing fragments of the *CP204L*, *E183L*, *EP402R* genes of the ASFV (seroimmunotype III), and identify the corresponding antigenically active translational products of the recombinant rp30, rp54, rCD2v proteins in the transfected eukaryotic cells.

Technique. Mozambique-78 (M-78) and Stavropol 01/08 (highly virulent) ASFV strains of seroimmunotypes III and VIII, as well as the MK-200 strain and the Stavropol 01/08 A_4C_2/9k variant (attenuated) are deposited in the State Collection of Microorganisms (All-Russian Research Institute of Veterinary Virology and Microbiology — VNIIVViM) [21-23].

Protocols to obtain antisera were as follows: No. 1 — twice intramuscular administration of the Stavropol 01/08 A_4S_2/9k attenuated variant at a dose of 10^3 HAU_{50} to a domestic pig (days 0 and 14), intramuscular infection with the Stavropol 01/08 virulent strain at a dose of 10^3 HAU_{50} (day 28) and bleeding the animal (on day 35 after the start of the experiment); No. 2 — blood withdrawal on day 24 after a single intramuscular inoculation of the MK-200 attenuated strain ($10^{6.5}$ HAU_{50}) to a wild boar; No. 3 — a single intramuscular inoculation of the MK-200 attenuated strain at a dose of $10^{6.5}$ HAU_{50} (day 0) to a domestic pig, intramuscular infection with the M-78 virulent strain at a dose of 10^3 HAU_{50} (day 21) and bleeding the animal (on day 35 after the start of the experiment).

The PCR mixture contained the primers, flanking genes *CP204L*, *E183L*, *EP402R* of ASFV MC-200 strain. For the accumulation of PCR products, previously obtained recombinant plasmids pJET1.2/p30-M200/2, pJET1.2/p54-M200/1 and pJET1.2/CD2v-M200/10 were used. Ligation was performed using a commer-

cial kit CloneJET PCR Cloning Kit according to the manufacturer's recommendations (Thermo Fisher Scientific, Inc., USA) and description [24].

Competent cells of the *Escherichia coli* XL-1 strain were transformed by heat shock in the presence of Ca^{2+} ions [25]. Plasmids were isolated from the selected ampicillin-resistant transformants, and the presence of specific inserts was confirmed by the restriction analysis.

Nucleotide sequences of the derived chimerical structures, containing *CP204L*, *E183L* and *EP402R* genes in the pJET1.2 and pCI-neo plasmids, were determined using an Applied Biosystems 3130xl genetic analyzer (Applied Biosystems, Inc., USA).

A human embryonic kidney cell line transformed with the gene of T-antigen of SV40 virus (HEK293T; the Collection of Cell Cultures, VNIIVViM) was transfected with the pCI-neo/ASFV/p30/1, pCI-neo/ASFV/p54/1 and pCI-neo/ASFV/CD2v/1 recombinant plasmids by the calcium-phosphate method according to the guidelines [26], and cultured for 1 to 5 days after the change of the Eagle's MEM (Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products, Russia). Transfection was monitored by the GFP protein expression, with plasmid phMGFP as positive control (Promega, USA) using an Olympus MIT-2 inverted fluorescence microscope (Olympus Corp., Japan). Transfected cells were collected from the substrate mechanically, after thrice washing off from serum proteins with phosphate buffered saline pH 7.2 (FBS), then sedimented by centrifugation (3,000 g, 10 min) and frozen at –70 °C. Further, 10^6 cells were lyzed in 1 cm^3 of RIPA-buffer [24], the cell debris was pelleted by centrifugation (3,000 g, 20 min) and the supernatants were investigated by Western blot analysis. Electrophoresis, electromigration and Western blot analysis were performed by U.K. Laemmle [27], J. Kyhse-Andersen [28] and J.M. Escribano, E. Tabares [29].

The BLASTn program was used to compare the nucleotide sequences of the derived chimerical constructs with those published in GenBank (available on the website http://www.ncbi.nlm.nih.gov/BLAST/). Alignment and analysis were performed using the BioEdit 7.2.5 software (intellectual property of Tom Hall, Freeware) and uGene 1.22 (LLC Novosibirsk Center of Information Technologies UniPro). Prediction of epitopes and structural elements was performed using online servers (Technical University of Denmark, http://www.cbs.dtu.dk/cgi-bin/).

Specific oligonucleotide primers, flanking regions of genes encoding p30, p54 and CD2v proteins

Name	Nucleotide sequence
F-p30Domen	5′-AGTACTGTTAAGTATGATATTGTGAAATCTG-3′
R-p30Domen	5′-AAGTTTAATAACCATGAGTCTTACCACC-3′
F-p54Domen	5′-TCCTCAAGAAAGAAAAAAGCTGCTGCTATTGAG-3′
R-p54Domen	5′-CAAGGAGTTTTCTAGGTCTTTATGCGTATAGG-3′
F-CD2-IgHA	5′-AGTTATAATGAAACAATAATTTTAAATAGTAAT-3′
R-CD2-IgHA	5′-GTGATTTCCTAATAAAAAAGAATATTGATAATA-3′

Results. Bioinformatic analysis of three potentially protective proteins, the p30, p54 and CD2v, of ASFV MK-200 strain revealed the signal sequences, transmembrane regions and potential sites for post-translational modifications. This resulted in identification of extracellular (external to virion) domains of membrane-spanning proteins that carry the highest number of predicted B- and T-cell epitopes in the amino acid sequences.

A clonable region of gene *EP402R* with 49-651 bp (201 residues) excluded native signal and membrane-spanning regions as well as a cytoplasmic domain with 721-1137 bp. Similar regions were localized for *CP204L* (142-546 bp) and *E183L* (160-597 bp) genes. Primers were designed in accordance with the further strategy of "seamless" cloning of DNA constructs by D.G. Gibson et al. [30]. The nucleotide sequences of the specific primers are shown in the Table.

To improve the efficiency of intracellular sorting of these proteins, we used the universal signal elements from heterologous viruses, i.e. the signal se-

quence of fusion (F) glycoprotein of human parainfluenza virus type 1 and a transmembrane region of the HN protein of Sendai virus. To do this, the p30-Domain, p54-Domain and CD2v-IgHA amplicons were subcloned with signal sequences and membrane-spanning regions into the pJET1.2 plasmid vector (Thermo Fisher Scientific, Inc., USA). Based on the PCR screening and restriction analysis, the clones were selected which contained plasmids with the specific nucleotide insertions. Plasmid sequencing showed the integrity of reading frames of the obtained chimerical sequences.

At the next stage, DNA constructs, expressing in eukaryotic cells, were obtained. For this purpose, the corresponding nucleotide sequences were re-cloned into the pCI-neo plasmid vector (Promega, USA) in the NheI and SmaI restriction sites. A CMV promoter in the pCI-neo vector ensures the high expression in eukaryotic systems. The maps of open reading frames of the chimerical genes are shown in Figure 1.

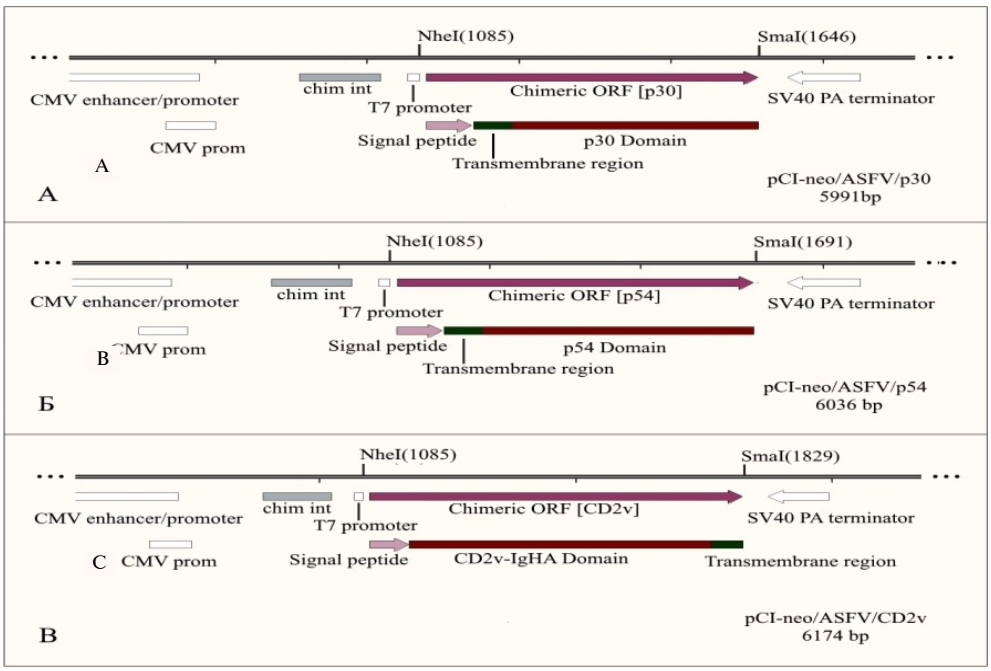


Fig. 1. A diagram of open reading frames for the chimerical genes with cloned sequences of the fragments encoding p30, p54 and CD2v proteins of African swine fever virus in the resulting DNA constructs: A — pCI-neo/ASFV/p30, B — pCI-neo/ASFV/p54, C — pCI-neo/ASFV/CD2v; CMV enhancer/promoter — the human cytomegalovirus immediate early enhancer and promoter, CMV promoter — promoter of human cytomegalovirus, chim intron — a chimerical intron, T7 promoter — promoter of T_7 phage, SV40 PA terminator — the polyadenylation signal and terminator of the SV40 virus DNA, Signal peptide — a signal sequence.

The analysis of the derived recombinant pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v plasmids by PCR with diagnostic primers demonstrated that the amplicons, the sizes of which are consistent with the estimated ones, are synthesized based on these matrices (Fig. 2). The first clones of each construct were chosen for further work.

HEK293T cell line (human embryonic kidney cells transformed with the gene of SV40 virus T-antigen) served as an in vitro expression tool for the obtained recombinant genes. The presence of the virus SV40 replication origin in the pCI-neo plasmid provides for its episomal replication. After by calcium phosphate transfection of the HEK293T cell monolayer using each of the recombinant plasmids (a confluency of 80-90 %) and subsequent culturing for 1-5 days, the trans-

fection efficiency was calculated as a percentage of fluorescent cells from the total number of cells observed with luminescent microscopy. As a control, the same experiments were carried out under identical conditions with the phMGFP plasmid.

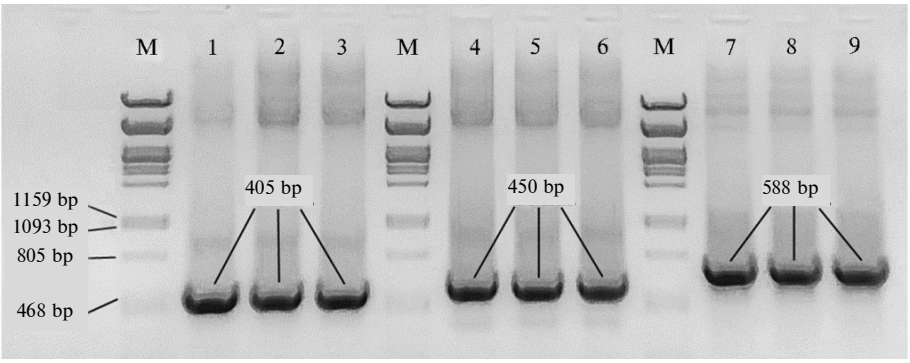


Fig. 2. An electrophoregram of separated PCR products of the chimerical genes in 1.5 % agarose gel: 1, 2, 3 — p30 amplicons (the pCI-neo/ASFV/p30 plasmids derived from *Escherichia coli* were used as a template); 4, 5, 6 — p54 amplicons (the pCI-neo/ASFV/p54 plasmids derived from *E. coli* were used as a template); 7, 8, 9 — CD2v amplicons (the pCI-neo/ASFV/CD2v plasmids derived from *E. coli* were used as a template); M — Lambda DNA/PstI Marker (DNA Sizer 247-11501 bp, GeneOn GmbH, Germany).

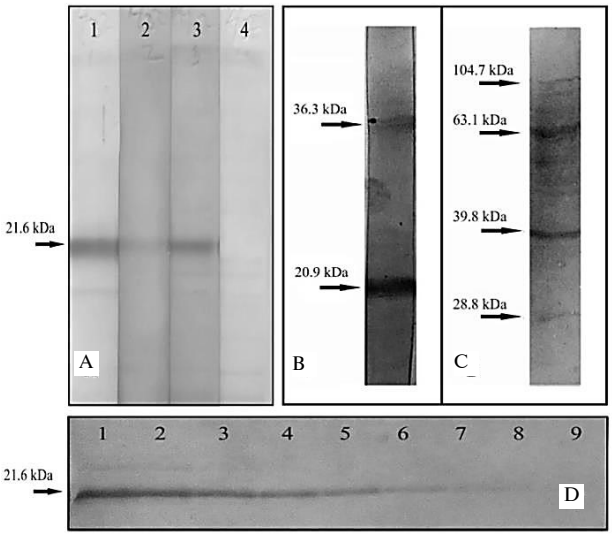


Fig. 3. Western blot analysis showing translation of recombinant rp30 (A), **rp54** (B) **and rp CD2v** (C) **proteins in HEK293T cells transfected with pCI-neo/ASFV/p30/1, pCI-neo/ASFV/p54, pCI-neo/ASFV/CD2v plasmids, and antigenic activity of rp30 protein** (D): 1-4 — numbers of antisera, 5-13 — dilutions (from 1:2 to 1:512 with 2-fold increments) of the lysate of HEK293T cells expressing the rp30 protein. Left arrows indicate the location and molecular weights of virus-specific polypeptides.

The obtained results shown in Figure 3 (A, D) reflecr the antigenic activity of the recombinant (r) rp30 protein which was synthesized in HEK293T cells transfected with the pCI-neo/ASFV/p30/1 plasmid. Western blot analysis with antibodies against ASFV-positive antisera of the domestic pig and wild boar (a 1:20 dilution) showed that its molecular weight is 21.6 kDa. We did not identify virus-specific antigens when testing the blood serum of the intact pig. Western blot analysis of the lysate proteins from the non-transfected HEK293T cells using all antisera gave negative results (data not shown). Titration of a lysate of the HEK293T cells transfected with the pCI-neo/ASFV/p30/1 plasmid (1:2 to 1:512 with 2-fold increments) (see Fig. 3, D) revealed antigenic activity up to a 1:128 dilution, which indicated a high rp30 expression.

Western blot analysis using antiserum No. 3 in a lysate of the HEK293T cells transfected with the pCI-neo/ASFV/p54 plasmid demonstrated the presence of a 20.9 kDa major polypeptide and a 36.3 kDa minor polypeptide (see Fig. 3, B); and if the cells were pCI-neo/ASFV/CD2v transfected, the 39.8 and 63.1 kDa major polypeptides and the 28.8 and 104.7 kDa minor polypeptides were identified (see Fig. 3, C).

The calculated molecular weights of the unmodified recombinant proteins were 21.6 kDa (rp30), 18.7 kDa (rp54) and 28.6 kDa (rCD2v). The Western blot analysis revealed that the actual molecular weights of the obtained recombinant polypeptides matched or were close to the estimated ones, i.e. 21.6 kDa for rp30, 20.9 kDa and 36.3 kDa for rp54 (the latter probably is a rp54 dimer). According to P. Gómez-Puertas et al. [16] and F. Rodriguez et al. [31], the weight of a full-length p54 monomer is 24-28 kDa. In the HEK293T cells transfected with the pCI-neo/ASFV/CD2v plasmid, translated virus-specific polypeptides were 28.8; 39.8; 63.1 and 104.7 kDa. The first of these matched the size of the estimated unmodified molecule rCD2v. The remaining were apparently the forms, which had been variously modified during glycosylation. These results are consistent with the findings from L.C. Goatley and L.K. Dixon [32], who identified in the Vero cells transfected with the SV5CD2vHA plasmid the polypeptides of the recombinant CD2v with molecular weights of 26, 63, 89 and 104 kDa. The authors additionally identified the 42 and 47 kDa polypeptides following the exposure to tunicamycin, a glycosylation inhibitor, or endoglycosidases D and F [32].

Therefore, we obtained DNA constructs with fragments of the *CP204L*, *E183L*, *EP402R* genes of African swine fever virus (ASFV). Western blot analysis using ASFV-specific antisera demonstrated the antigenic activity of the polypeptides expressed as a result of transfecting HEK293T cells with the recombinant pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v plasmids which contained the nucleotide sequences for the recombinant proteins (respectively, for rp30, rp54 and rpCD2v). The next stage of research should determine the immunogenic properties of the recombinant plasmids and improve the obtained gene constructs to develop appropriate protective ones.

REFERENCES

1. Sanchez-Vizcaíno J.M., Mur L., Gomez-Villamandos J.C., Carrasco L. An update on the epidemiology and pathology of African swine fever. *J. Comp. Pathol.*, 2015, 152(1): 9-21 (doi: 10.1016/j.jcpa.2014.09.003).
2. Makarov V.V., Sukharev O.I., Tsvetnova I.V. *Veterinarnaya praktika*, 2013, 1(60): 6-16 (in Russ.).
3. Kolbasov D.V., Sereda A.D. *Veterinariya*, 2013, 1: 19-23 (in Russ.).
4. Rowlands R.J., Michaud V., Heath L., Hutchings G., Oura C., Volsoo W., Dwarka R., Onashvili T., Albina E., Dixon L.K. African swine fever virus isolate Georgia, 2007. *Emerg. Infect. Dis.*, 2008, 14(12): 1870-1874 (doi: 10.3201/eid1412.080591).
5. Gogin A., Gerasimov V., Malogolovkin A., Kolbasov D. African swine fever in the North Caucasus region and the Russian Federation in years 2007-2012. *Virus Res.*, 2013, 173(1): 198-203 (doi: 10.1016/j.virusres.2012.12.007).
6. Śmietanka K., Woźniakowski G., Kozak E., Niemczuk K., Frączyk M., Bocian Ł., Kowalczyk A., Pejsak Z. African swine fever epidemic, Poland, 2014-2015. *Emerg. Infect. Dis.*, 2016, 22(7): 1201-1207 (doi: 10.3201/eid2207.151708).
7. Petisca N.J. Quelques aspects morphologiques a la suite de la vaccination contre la peste porcine Africaine (Virose L) au Portugal. *Bull. Off. Int. Epizoot.*, 1965, 63: 199-237.
8. Vigario I.D., Terrinha A.M., Nunes J.F.M. Antigenic relationships among strains of African swine fever virus. *Archiv für die gesamte Virusforschung*, 1974, 45(3): 272-277 (doi: 10.1007/BF01249690).
9. Kolbasov D.V., Balyshev V.M., Sereda A.D. *Veterinariya*, 2014, 8: 3-8 (in Russ.).
10. Makarov V., Nedosekov V., Sereda A., Matvienko N. Immunological conception of African swine fever. *Zoology and Ecology*, 2016, 26(3): 236-243 (doi: 10.1080/21658005.2016.1182822).
11. Takamatsu H.H., Denyer M.S., Lacasta A., Stirling C.M.A., Argilaguet J.M., Netherton C.L., Oura C.A.L., Martins C., Rodríguez F. Cellular immunity in ASFV responses. *Virus Res.*, 2013, 173(1): 110-121 (doi: 10.1016/j.virusres.2012.11.009).
12. Oura C.A.L., Denyer M.S., Takamatsu H., Parkhouse R.M.E. In vivo depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus. *J. Gen. Virol.*, 2005, 86(9): 2445-2450 (doi: 10.1099/vir.0.81038-0).
13. King K., Chapman D., Argilaguet J.M., Fishbourne E., Hutet E., Cariolet R., Hutchings G., Oura C.A.L., Netherton C.L., Moffat K., Taylor G., Le

Potier M.F., Dixon L.K., Takamatsu H.H. Protection of European domestic pigs from virulent African isolates of African swine fever virus by experimental immunization. *Vaccine*, 2011, 29(28): 4593-4600 (doi: 10.1016/j.vaccine.2011.04.052).
14. Bachmann M.F., Kundig T.M., Freer G., Li Y., Kang C.Y., Bishop D.H., Hengartner H., Zinkerna R.M. Induction of protective cytotoxic T cells with viral proteins. *Eur. J. Immunol.*, 1994, 24: 2228-2236 (doi: 10.1002/eji.1830240944).
15. Gomez-Puerta P., Rodriguez F., Oviedo J.M., Ramiro-Ibanez F., Ruiz-Gonzalvo F., Escribano J.M. Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. *Virology*, 1996, 70(8): 5689-5694.
16. Gómez-Puertas P., Rodriguez F., Oviedo J.M., Brun A., Alonso C., Escribano J.M. The African swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response. *Virology*, 1998, 243: 461-471 (doi: 10.1006/viro.1998.9068).
17. Sereda A.D. *Aktual'nye voprosy veterinarnoi biologii*, 2013, 4(20): 31-35 (in Russ.).
18. Argilaguet J.M., Perez-Martin E., Nofrarias M., Gallardo C., Accensi F., Lacasta A., Mora M., Ballester M., Galindo-Cardiel I., Lopez-Soria S., Escribano J.M., Reche P.A., Rodrıguez F. DNA vaccination partially protects against African swine fever virus lethal challenge in the absence of antibodies. *PLoS ONE*, 2012, 7(9): e40942 (doi: 10.1371/journal.pone.0040942).
19. Sereda A.D., Kazakova A.S., Imatdinov A.R., Kolbasov D.V. Humoral and cell immune mechanisms under African swine fever (review). *Agricultural Biology*, 2015, 50(6): 709-718 (doi: 10.15389/agrobiology.2015.6.709eng) (in Engl.).
20. Mima K.A., Burmakina G.S., Titov I.A., Malogolovkin A.S. African swine fever virus glycoproteins p54 and CD2v in the context of immune response modulation: bioinformatic analysis of genetic variability and heterogeneity. *Agricultural Biology*, 2015, 50(6): 785-793 (doi: 10.15389/agrobiology.2015.6.785eng) (in Engl.).
21. Sereda A.D., Balyshev V.M. *Voprosy virusologii*, 2011, 4: 38-42 (in Russ.).
22. Malogolovkin A., Burmakina G., Titov I., Sereda A., Gogin A., Baryshnikova E., Kolbasov D. Comparative analysis of African swine fever virus genotypes and serogroups. *Emerg. Infect. Dis.*, 2015, 21(2): 312-315 (doi: 10.3201/eid2102.140649).
23. Balysheva V.I., Prudnikova E.Yu., Gal'nbek T.V., Balyshev V.M. *Voprosy virusologii*, 2015, 2: 43-47 (in Russ.).
24. Green M.R., Sambrook J. *Molecular sloning: a laboratory manual.* Cold Spring Harbor Laboratory Press, NY, 2012.
25. Maniatis T., Fritsch E. F., Sambrook J. *Molecular cloning: a laboratory manual.* Cold Spring Harbor, NY, 1982.
26. Graham F.L., van der Eb A.J. Transformation of rat cells by DNA of human adenovirus 5. *Virology*, 1973, 54(2): 536-539 (doi: 10.1016/0042-6822(73)90163-3).
27. Laemmle U.K. Clevage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*, 1970, 227: 680-685 (doi: 10.1038/227680a0).
28. Kyhse-Andersen J. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods*, 1984, 10(3/4): 203-209.
29. Escribano J.M., Tabares E. Proteins specified by African swine fever virus. V. Identification of immediate early, early and late proteins. *Arch. Virol.*, 1987, 92: 221-238.
30. Gibson D.G., Young L., Chuang R.Y., Venter J.C., Hutchison C.A. III, Smith H.O. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, 2009, 6: 343-345 (doi: 10.1038/nmeth.1318).
31. Rodriguez F., Alcaraz S., Yanez R.J., Rodriguez J.M., Alonso C., Rodriguez J.F., Escribano J.M. Characterization and molecular basis of heterogeneity of the African swine fever virus envelope protein p54. *J. Virol.*, 1994, 68(11): 7244-7252.
32. Goatley L.C., Dixon L.K. Processing and localization of the African swine fever virus CD2v transmembrane protein. *J. Virol.*, 2011, 85(7): 3294-3305 (doi: 10.1128/jvi.01994-10).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 845-852
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 619:616.98:578.842.1:57.083.3

doi: 10.15389/agrobiology.2016.6.845rus
doi: 10.15389/agrobiology.2016.6.845eng

VALIDATION OF AN ELISA KIT FOR DETECTION OF ANTIBODIES AGAINST ASF VIRUS IN BLOOD OR SPLEEN OF DOMESTIC PIGS AND WILD BOARS

O.M. STRIZHAKOVA, V.M. LYSKA, A.S. MALOGOLOVKIN, M.B. NOVIKOVA, M.V. SIDLIK, I.V. NOGINA, A.E. SHKAEV, E.A. BALASHOVA, V.V. KURINNOV, A.P. VASIL'EV

All-Russian Institute of Veterinary Virology and Microbiology, Federal Agency of Scientific Organizations, 1, ul. Akademika Bakuleva, pos. Vol'ginskii, Petushinskii Region, Vladimir Province, 601120 Russia, e-mail OMStr@yandex.ru
Received September 25, 2016

Abstract

The causal agent of African swine fever (ASF) is a DNA virus belonging to *Asfaviridae* family which affects both wild boar *Sus scrofa* and domestic pig *Sus scrofa domestica*. Special features of the course of (ASF and its forms should be considered for the effective use of various ASF diagnostic methods aimed at the pathogen or specific antibody identification. ASF diagnosis in the wild boar is of special importance. The wild boar susceptibility to ASF virus is well known, and the disease has been repeatedly reproduced in experiments and detected in European wild boars in natural conditions. It is not unfrequently that when shooting wild boars, only organ samples are delivered to laboratories, so we decided to estimate the diagnostic value of the antibodies detected in tissues and evaluate an earlier developed test system for its efficacy when used for the assay. This report represents the results of validation of an indirect ELISA (a commercial kit «VNIIVViM ASF-ELISA Ab/Ag») ASF virus specific antibody detection in blood serum and spleen tissue extracts. For comparison, an indirect immunofluorescence assay (indirect IFA) was used. To estimate the obtained results, ROC analysis was applied. Examination of positive (n = 66) and negative (n = 410) porcine blood serum samples using indirect ELISA showed high sensitivity and specificity of the method with reference to IFA. Among the 476 serum samples examined in indirect ELISA, only 8 sera (1.6 %) were within the positive/negative cutoff area. The highest sensitivity (100 %) and specificity (99.27 %) for indirect ELISA when examining both domestic pig and wild boar blood sera were determined at a cutoff value of 0.264. The samples of spleen extracts to be used for the study were collected from clinically healthy wild boars in ASF-affected Smolensk region in 2013 to 2014. In view of an acute form of the disease, we can suspect that the animals exhibiting positive reaction were in a latency period of the pathogeny. When examining the positive (n = 59) and negative (n = 678) spleen extracts, we also determined high sensitivity and specificity levels of indirect ELISA with reference to indirect IFA. Among the samples examined (n = 737), 10 (1.3 %) samples of spleen extracts were within the positive/negative cutoff area. The highest sensitivity (100 %) and specificity (98.82 %) were observed at a cutoff value of 0.284. Thus, we confirmed the assay to be effective for porcine blood sera with 100 % sensitivity (94.6 to 100 %) and 99.27 % specificity (97.90 to 99.80 %) and for spleen extracts with 100 % sensitivity (93.90 to 100 %) and 98.82 % specificity (97.70 to 99.50 %).

Keywords: African swine fever, specific antibody, indirect ELISA, ROC analysis

African swine fever (ASF) is a highly contagious disease with 100 % mortality when acute course, leading to significant economic losses [1]. ASF is characterized by fever, hemorrhagic diathesis and necro-degenerative changes in parenchymal organs [2-5]. The causative agent of ASF is a DNA-containing virus of *Asfarviridae* family [6]. There are hyperacute, acute, subacute, chronic, and latent ASF [7-9]. Special features of the ASF course and its forms should be considered for the effective use of various ASF diagnostic methods [10] aimed at the identification of the pathogen or specific antibodies. If hyperacute and acute forms, detection of virus-specific antibodies is possible only in spleen samples, as specific antibody producing cells and, respectively, the very antibodies appear there on day 2 to 3 post-infection, whereas the animals die on days 3-7 [11, 12].

In subacute and chronic disease, virus-specific antibodies are found in blood on days 7-10 [13-16], so earlier a detection of the viral antigen or genome in blood is advisable [17], since viremia is typical of this ASF period [17-19].

ASF diagnosis in the wild boar is of special importance as their susceptibility to ASF virus (ASFV) is well known. The disease has been repeatedly reproduced in experiments and detected in European wild boars under natural conditions [20].

In the Russian Federation, ASFV has been circulating in the populations of domestic pigs (*Sus scrofa domestica*) and wild boars (*Sus scrofa*) since November 2007 until present. High virulence of ASFV, circulating in the Russian Federation [21], and the acute ASF proven indicates basic importance of direct virus detection by PCR and direct immunofluorescence test (DIFT) in the scheme of the laboratory diagnosis [15). At the same time, long-time persistence of ASFV in wild boar populations in the territory of Russia suggests the possibility of endemic infection, so serological survey could be carried out more widely, especially in the disease-affected areas of the Russian Federation. When shooting wild boars, only organ samples are often delivered to laboratories, so we decided to estimate the diagnostic value of the antibodies detected in tissues and to validate for this a VNIIVViM ASF-ELISA Ab/Ag kit. Earlier, we used random samples of spleen extracts from dead ASFV-infected domestic pigs and wild boars when studying diagnostic value of specific tissue antibodies [16]. Indirect immunofluorescence test (IIFT) detects tissue anti-ASFV antibodies in spleen extracts of 49 % domestic pigs and 33 % wild boars (PCR- and DIFT-positive) died of acute ASF. IIFT is considered a reference test for the detection of anti-ASFV antibodies [22], but, along with high sensitivity and specificity, it has a low performance and some technological inconveniences. These necessitate the development, validation and practical use of ELISA methods which make it possible to detect specific antibodies not only in blood, but also in tissue extracts of infected animals.

In recent years, the ROC (receiver operating characteristic) analysis [23-25] is often used in medicine and veterinary science, which allows, in the presence of positive and negative reference samples, to determine cut-off points, both numerically and graphically, and validate protocols by true positive rate/false positive rate of the objects classified as possessing a trait. When processing data using ROC curves, it is necessary to determine the cut-off level. Without this value it is impossible to perform calculations and interpret the positive and negative results [26, 27].

We have performed validation of a VNIIVViM ASF-ELISA Ab/Ag kit (All-Russian Research Institute of Veterinary Virology and Microbiology, VNIIVViM) for the detection of ASFV specific antibodies both in the blood serum and the tissue using ELISA test and ROC-analysis.

The purpose of the study was to estimate the operational characteristics of the developed commercial kit for indirect immunoassay (ELISA), VNIIVViM ASF-ELISA Ab/Ag, versus indirect immunofluorescence assay (IIFT) when detecting specific anti-ASFV antibodies in blood and tissue extracts of infected animals and to find an optimum cutoff point providing high reliability of the test.

Technique. Reference positive samples were collected randomly and independently from domestic pigs and wild boars in the experiment or in ASF outbreaks. The reference positive samples included 43 blood sera from 10 pigs experimentally infected with avirulent ASFV strain PSA-1-NH ($10^{-4.5}$ TCD_{50}/ml) collected during infection development, and 23 sera from slaughtered domestic pigs and wild boars shot at ASF outbreaks. For tissue extracts, 59 spleen samples were collected from clinically healthy wild boars shot in 2013-2014 in ASF-

affected Smolensk region (the presence of specific antibodies was confirmed in IIFT). Absence of anti-ASFV antibodies in all reference negative samples was confirmed by IIFT (the reference test recommended by the World Organization for Animal Health, OIE, France), PCR and DIFT to be sure of no viral infection. The reference negative samples included 410 blood sera of domestic pigs and shot wild boars from historically ASF-free Russian farms and territories, and 678 PCR-, DIFT- and IIFT-negative spleen extracts from shot wild boars (ASF-affected Smolensk region, 2013-2014).

Blood sera were centrifuged at 2000 rpm. The samples for ELISA were diluted (1:50) with Tris-HCl buffer containing casein (0.01 M Tris-HCl, 0.154 M NaCl, 0.5 % casein, pH 7.8), as recommended in the VNIIVViM ASF-ELISA Ab/Ag kit instructions.

For tissue extracts, the spleen samples (1 g) were homogenized in a sterile 0.85 % NaCl (5 cm^3). The resulting 20 % homogenates (weight/volume) were frozen twice at –18±2 °C, thawn at room temperature, and subjected to centrifugation (3000 rpm, 10-15 min). The supernatant (extract) was used in indirect IIFT and indirect ELISA (without additional dilution) to detect tissue-specific anti-ASFV antibodies

IIFT was performed with a commercial Kit for differential immunofluorescence diagnostics of African swine fever, classical swine fever, Aujeszky's disease (VNIIVViM). For test, we used African green monkey kidney cell culture CV-1 (Collection of VNIIVViM cell cultures) infected with avirulent nonhemesorbing ASFV strain 691/88 (State VNIIVViM Collection of Microorganisms). a fluorescence microscope Eclipse E200 (Nikon Corp., Japan) was used for luminescent microscopy with documentation.

A commercial VNIIVViM ASF-ELISA Ab/Ag kit for ELISA diagnostics was used in accordance to the recommended protocol. The results interpretation was based on a standard deviation principle (2.5-fold difference). For the MedCalc program, S/P, the ratio of optical density in the test sample to the positive control, was calculated using OD at λ = 405 nm (spectrophotometer Sunrise™, Tecan Austria GmbH, Austria) as follows:

$$\frac{S}{P} = \frac{OD_{mean}\ \text{test sample} - OD_{mean}\ \text{negative control}}{OD_{mean}\ \text{positive control} - OD_{mean}\ \text{negative control}},$$

ROC curves for assessment of the cutoff point and statistical analysis were constructed by S/P values. The S/P positive and negative samples in IIFT served as Diagnosis 1 and Diagnosis 0, respectively. The data were entered in the MedCalc program according to the procedure for the current version (available at http://www.medcalc.org).

1. ELISA and IIFT detection of anti-ASFV antibodies in blood of experimentally infected pigs and healthy animals

ELISA	IIFT		
	positive	negative	total
Positive	43	2	45
Negative	0	98	98
Total	43	100	143

N o t e. Commercial diagnostic kits (All-Russian Research Institute of Veterinary Virology and Microbiology) were used.

Results. Relative sensitivity and relative specificity of indirect ELISA (VNIIVViM ASF-ELISA Ab/Ag kit) were determined vs. IIFT ("gold standard" test). When analyzing blood sera (Table 1) from experimentally infected domestic pigs (n = 43) and healthy animals (historically ASF-free farms, n = 100), the relative sensitivity was 100 % (43 samples out of 143 were positive both in ELISA and IIFT, and 2 samples were positive in ELISA only), relative specificity was 98 % at 95 % confidence interval.

When checking the value of positive/negative cutoff for indirect ELISA based on the results of testing 66 positive and 410 negative serum samples from do-

mestic pigs and wild boars, a diagram was plotted (Fig. 1) for the frequency of distribution of the values obtained. A total of 5 reference positive and 19 reference negative samples were co-located in the S/P interval from 0.2 to 0.4, that is the optical densities of the samples within this range could be classified both as false negative and false positive.

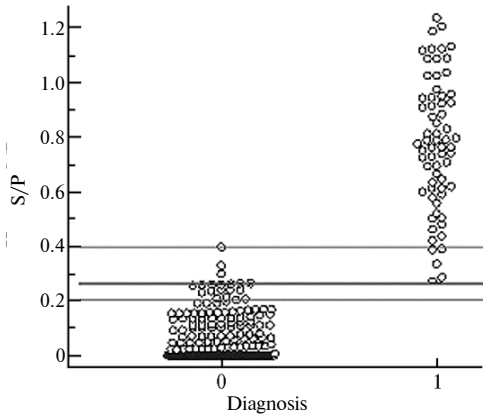


Fig. 1. Distribution of 410 reference negative (Diagnosis 0) **and 66 reference positive for African swine fever** (Diagnosis 1) **samples of pig blood serum in indirect ELISA by S/P index** (the ratio of the test sample to positive control optical density). Each point represents the average of individual sample analysis duplicates. A sensitivity of 100 % and a specificity of 99.3 % correspond to the cutoff line at S/P > 0.264. The S/P area boundaries are marked in which both positive and negative samples are co-located.

Sensitivity and specificity of indirect ELISA blood test were calculated for different cutoff values (Table 2). In the samples from domestic pigs and wild boars, the highest diagnostic sensitivity (100 %) at 95 % confidence interval (CI of 94.6 to 100 %) along with the highest specificity of 99.27 % (95 % CI: 97.9-99.8 %) was observed at the cutoff value of 0.264.

2. Criteria and ROC curve coordinates for indirect ELISA tests of serum anti-ASFV antibodies depending on the cutoff value

Criterion	Sen, %	95 % CI	Sp, %	95 % CI	+LR	95 % CI	–LR	95 % CI
> 0.258	100.00	94.6-100.0	98.54	96.8-99.5	68.33	30.9-151.2	0.000	
> 0.261	100.00	94.6-100.0	98.78	97.2-99.6	82.00	34.3-196.0	0.000	
> 0.263	100.00	94.6-100.0	99.02	97.5-99.7	102.50	38,7-271,8	0.000	
> 0.264	100.00	94.6-100.0	99.27	97.9-99.8	136.67	44.3-422.0	0.000	
> 0.271	98.48	91.8-100.0	99.27	97.9-99.8	134.60	43.6-415.8	0.015	0.002-0.1
> 0.285	96.97	89.5-99.6	99.27	97.9-99.8	132.53	42.9-409.5	0.031	0.008-0.1
> 0.298	96.97	89.5-99.6	99.51	98.2-99.9	198.79	49.9-792.7	0.030	0.008-0.1
> 0.388	93.94	85,2-98,3	99.76	98.6-100.0	385.15	54.3-2730.4	0.061	0.020-0.2

N o t e. A total of 66 blood serum samples from ASFV-infected (positive group, Diagnosis = 1) and 410 samples from non-infected pigs and wild boars (negative group, Diagnosis = 0) have been tested; 476 samples in total). Sen — sensitivity, Sp — specificity, CI — confidence interval, +LR and –LR mean the likelihood ratio: +LR = Sen/(100 – Sp), –LR = (100 – Sen)/Sp. The table shows part of ROC analysis criteria and coordinates calculated in the MedCalc program for the most relevant cutoff values in the range of optimal sensitivity and specificity.

Area under the ROC curve (AUC): area under the ROC curve — 1.000; standard error — 0.000269; 95 % confidence interval — from 0.992 to 1.000; Z-statistics — 1857.961; significance level P (area = 0.5) < 0.0001.

Youden index: index J — 0.9927; associative criterion > 0.264.

When testing 59 reference positive and 678 reference negative spleen tissue extracts (Fig. 2), the S/P overlap frequencies of false positive (51 samples) and false-negative (3 samples) results were in the same range (0.2-0.4) as for the serum antibodies. The optimal parameters were at a cutoff value of 0.284: method sensitivity was 100 % (95 % CI: 93.9-100 %), specificity was 98.82 % (95 % CI: 97.7-99.5 %) (Table 3).

Currently, ELISA is approved by OIE for primary screening anti-ASFV antibodies [26], but in general all of the known tests allow detecting specific antibodies in blood only [10, 12, 14]. The validation of ELISA kit by ROC analysis showed the possibility to use this kit for the detection of specific anti-ASFV antibodies in blood and tissue extracts in infected domestic pigs and wild boars. Examination of positive (n = 66) and negative (n = 410) pig blood samples confirmed high sensitivity and specificity of the method compared to IIFT. Only 8 (1.6 %) of the 476 samples were within the positive/negative cutoff area (see Fig. 1).

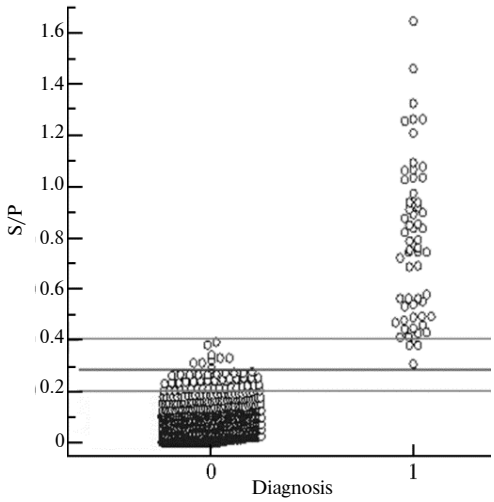


Fig. 2. Distribution of 678 reference negative (Diagnosis 0) **and 59 reference positive for African swine fever** (Diagnosis 1) **pig tissue samples (spleen) in indirect ELISA by S/P index** (the ratio of the test sample to positive control optical density). Each point represents the average of individual sample analysis duplicates. A sensitivity of 100 % and a specificity of 98.8 % correspond to the cutoff line at S/P > 0.284. The S/P area boundaries are marked in which both positive and negative samples are co-located.

When testing positive (n = 59) and negative (n = 678) spleen extracts from clinically healthy wild boars shot in ASF-affected areas, sensitivity and specificity of indirect ELISA also was high compared to IIFT. Only 10 samples (1.3 %) were within the positive/ne-gative cutoff area (see Fig. 2).

Considering the acute disease character, we can assume a state corresponding to the incubation period of infection in the animals with positive reactions.

3. Criteria and ROC curve coordinates for indirect ELISA tests of serum anti-ASFV antibodies in spleen tissue depending on the cutoff value

Criterion	Sen, %	95 % CI	Sp, %	95 % CI	+LR	95 % CI	–LR	95 % CI
> 0.263	100.00	93.9-100.0	97.94	96.6-98.9	48.43	28.8-81.3	0.000	
> 0.268	100.00	93.9-100.0	98.08	96,7-99,0	52.15	30.4-89.4	0.000	
> 0.269	100.00	93.9-100.0	98.23	96.9-99.1	56.50	32.2-99.0	0.000	
> 0.270	100.00	93.9-100.0	98.53	97.3-99.3	67.80	36.6-125.4	0.000	
> 0.276	100.00	93.9-100.0	98.67	97.5-99.4	75.33	39.4-144.2	0.000	
> 0.284	100.00	93.9-100.0	98.82	97.7-99.5	84.75	42.6-168.8	0.000	
> 0.304	98.31	90.9-100.0	98.82	97.7-99.5	83.31	41.8-166.1	0.017	0.002-0.1
> 0.310	98.31	90.9-100.0	99.12	98.1-99.7	111.08	50.0-246.6	0.017	0.002-0.1
> 0.320	98.31	90.9-100.0	99.26	98.3-99.8	133.30	55.6-319.4	0.017	0.002-0.1
> 0.330	98.31	90.9-100.0	99.56	98.7-99.9	222.17	71.8-687.5	0.017	0.002-0.1
> 0.380	94.92	85.9-98.9	99.85	99.2-100.0	643.53	90.7-4566.1	0.051	0.020-0.2

N o t e. A total of 59 spleen samples from ASFV-infected (positive group, Diagnosis = 1) and 678 samples from non-infected pigs and wild boars (negative group, Diagnosis = 0) have been tested; 737 samples in total. Sen — sensitivity, Sp — specificity, CI — confidence interval, +LR and –LR mean the likelihood ratio: +LR = Sen/(100 – Sp), –LR = (100 – Sen)/Sp. The table shows part of ROC analysis criteria and coordinates calculated in the MedCalc program for the most relevant cutoff values in the range of optimal sensitivity and specificity.

Area under the ROC curve (AUC): area under the ROC curve — 1.000; standard error — 0.000243; 95 % confidence interval — from 0.994 to 1.000; Z-statistics — 2055.362; significance level P (area = 0.5) < 0.0001.

Youden index: index J — 0.9882; associative criterion > 0.284.

The results of indirect ELISA ROC analysis obtained in the study of blood and tissue samples allow choosing cutoff points in a wide range with the possibility of optimizing method performance. In both cases, alternative options are possible depending on the planned purposes, such as diagnosis, monitoring, or confirmation of the absence of ASF infection in the population of domestic pigs (farm) or wild boars (hunting facilities). Thus, if it is necessary to prevent the introduction of infection, high sensitivity is more important and specificity is of less significance (e.g. in diagnosis or screening in an affected area). In this case, the set cutoff level is 0.264 (blood) or 0.284 (tissue). If a false positive result is inadmissible, that is, specificity is important (e.g. when screening animals for ASF in an ASF-free area), the cutoff threshold can be increased to 0.380 with a minimal effect on sensitivity.

Thus, the validation indicates that the VNIIVViM ASF-ELISA Ab/Ag kit allows reliable detection of anti-ASFV antibodies both in the blood serum and spleen tissue extracts at S/P cutoff values of 0.264 (blood) or 0.284 (tissue), re-

spectively. This is especially important for monitoring wild boars after shooting, when only organs are often delivered to the laboratory. The commercial set developed and validated by us is expedient for using to evaluate the presence of anti-ASFV antibodies and the ASF status of an animal and the population as a whole.

REFERENCES

1. Kurinnov V.V., Kolbasov D.V., TSybanov S.Zh., Vasil'ev A.P., Shendrik A.G., Balyshev V.M., Mikolaichuk S.V., Belyanin S.A., Kalantaenko Yu.F., Zhukov A.N. *Zhizn' bez opasnostei*, 2010, 3: 82-87 (in Russ.).
2. Atuhaire D.K., Afayoa M., Ochwo S., Mwesigwa S., Mwiine F.N., Okuni J.B., Olaho-Mukani W., Ojok L. Prevalence of African swine fever virus in apparently healthy domestic pigs in Uganda. *BMC Vet. Res.*, 2013, 9: 263-271 (doi: 10.1186/1746-6148-9-263).
3. Gallardo C., Reoyo A.T., Fernández-Pinero J., Iglesias I., Mucoz J., Arias L. African swine fever: a global view of the current challenge. *Porcine Health Management*, 2015, 1: 21 (doi: 10.1186/s40813-015-0013-y).
4. Carrasco L. An update on the epidemiology and pathology of African swine fever. *J. Comp. Pathol.*, 2015, 152: 9-21 (doi: 10.1016/j.jcpa.2014.09.003).
5. Guinat C., Reis A.L., Netherton C., Goatley L., Pfeiffer D., Dixon L. Dynamics of African swine fever virus shedding and excretion in domestic pigs infected by intramuscular inoculation and contact transmission. *Vet. Res.*, 2014, 45: 93 (doi: 10.1186/s13567-014-0093-8).
6. *Diseases of swine*. D.J. Taylor, J.J. Zimmerman, S. D'Allaire (eds.) Iowa State University Press, 2006.
7. Gallardo C., Soler A., Nieto R., Cano C., Pelayo V., Sánchez M.A., Pridotkas G., Fernandez-Pinero J., Briones V., Arias M. Experimental infection of domestic pigs with African swine fever virus Lithuania 2014 genotype II field isolate. *Transbound. Emerg. Dis.*, 2015 Mar 22: 1-5 (doi: 10.1111/tbed.12346) [Epub ahead of print].
8. Boinas F.S., Hutchings G.H., Dixon L.K., Wilkinson P.J. Characterisation of pathogenic and non-pathogenic African swine fever virus isolated from *Ornithodoros erraticus* inhabiting pig premises in Portugal. *J. Gen. Virol.*, 2004, 5(8): 2117-2187 (doi: 10.1099/vir.0.80058-0).
9. Howey E.B., O'Donnell V., de Carvalho Ferreira H.C., Borca M.V., Arzt J. Pathogenesis of highly virulent African swine fever virus in domestic pigs exposed via intraoropharyngeal, intranasopharyngeal, and intramuscular inoculation, and by direct contact with infected pigs. *Virus Res.*, 2013, 178: 328-339 (doi: 10.1016/j.virusres.2013.09.024).
10. Charleston B., Bankowski B.M., Gubbins S., Chase-Topping M.E., Schley D., Howey R., Barnett P.V., Gibson D., Juleff N.D., Woolhouse M.E. Relationship between clinical signs and transmission of an infectious disease and the implications for control. *Science*, 2011, 332: 726-729 (doi: 10.1126/science.1199884).
11. Botija S., Ordas A. Rapid diagnosis by identification of antibodies extracted from tissues using indirect immunofluorescence. Hog Cholera. In: Classical swine fever and African swine fever. *Luxembourg*, 1977: 658-659.
12. Reis A.L., Parkhouse R.M., Penedos A.R., Martins C., Leitro A.. Systematic analysis of longitudinal serological responses of pigs infected experimentally with African swine fever virus. *J. Gen. Virol.*, 2007, 88: 2426-2434 (doi: 10.1099/vir.0.82857-0).
13. Pérez J., Fernández A.I., Sierra M.A., Herraez P., Fernández A. Serological and immunohistochemical study of African swine fever in wild boar in Spain. *Vet Rec.*, 1998, 143: 136-139.
14. Gallardo C., Soler A., Nieto R., Carrascosa A.L., De Mia G.M., Bishop R.P., Martins C., Fasina F.O., Couacy-Hymman E., Heath L., Pelayo V., Martín E., Simyn A., Martín R., Okurut A.R., Lekolol I., Okoth E., Arias M. Comparative evaluation of novel African swine fever virus (ASF) antibody detection techniques derived from specific ASF viral genotypes with the OIE internationally prescribed serological tests. *Vet. Microbiol.*, 2013, 162: 32-43 (doi: 10.1016/j.vetmic.2012.08.011).
15. Kurinnov V.V., Vasil'ev A.P., Belyanin S.A., Strizhakova O.M., Nogina I.V., Sidlik M.V., Gazaev I.Kh., Tsybanov S.Zh., Mironova L.P., Alikova G.A., Dzhailidi G.A., Chernykh O.Yu. *Veterinariya Kubani*, 2014, 3: 5-9 (in Russ.).
16. Kurinnov V.V., Belyanin S.A., Vasil'ev A.P., Strizhakova O.M., Lyska V.M., Nogina I.V., Zubairova S.N., Balyshev V.M., Tsybanov S.Zh., Kolbasov D.V., Mironova P.L., Chernykh O.Yu., Alikova G.A. *Veterinariya Kubani*, 2012, 4: 9-11 (in Russ.).
17. Cubillos C., Gymez-Sebastian S., Moreno N., Nuñez M.C., Mulumba-Mfumu L.K., Quembo C.J., Heath L., Etter E.M.C., Jori F., Escribano J.M., Blanco E. African swine fever virus serodiagnosis: A general review with a focus on the analyses of African serum samples. *Virus Res.*, 2013, 173(1): 159-167 (doi: 10.1016/j.virusres.2012.10.021).
18. Nieto-Pelegr E., Rivera-Arroyo B., Sanchez-Vizcaıno J.M. First detection of antibodies against African swine fever virus in faeces samples. *Transbound. Emerg. Dis.*, 2015,

62(6): 594-602 (doi: 10.1111/tbed.12429).
19. Zsak L., Borca M., Risatti G., Zsak A., French R., Lu Z., Kutish G., Neilan J., Callahan J., Nelson W. Preclinical diagnosis of African swine fever in contact-exposed swine by a real-time PCR assay. *J. Clin. Microbiol.*, 2005, 43: 112-119 (doi: 10.1128/JCM.43.1.112-119.2005).
20. Makarov V.V., Sukharev O.I., Boev B.V., Gavryushkin D., Kolomytsev A.A., Litvinov O.B. *Veterinariya*, 2010, 9: 24-28 (in Russ.).
21. Khomenko S., Beltrán-Alcrudo D., Rozstalnyy A., Gogin A., Kolbasov D., Pinto J., Lubroth J., Martin V. African swine fever in the Russian Federation: risk factors for Europe and beyond. *EMPRES Watch*, 2013, 28: 1-14.
22. *Manual of diagnostic tests and vaccines for terrestrial animals. 7th Ed. OIE, France. 2012.* Available http://www.oie.int/manual-of-diagnostic-tests-and-vaccines-for-terrestrial-animals/. No date.
23. Gardner A., Greiner M., Dubey J. Statistical evaluation of test accuracy studies for *Toxoplasma gondii* in food animal intermediate hosts. *Zoonoses and Public Health*, 2010, 57: 82-94 (doi: 10.1111/j.1863-2378.2009.01281).
24. Greiner M., Sohr D., Gijbel P. A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. *J. Immunol. Methods*, 1995, 185: 123-132.
25. *The ELISA guidebook*. J.R. Crowther (ed.). Vienna, 2009.
26. Jacobson R.H. Validation of serological assays for diagnosis of infectious diseases. *Rev. Sci. Tech. OIE*, 1998, 17(2): 469-486.
27. Tignon M., Gallardo C., Iscaro C., Hutet E., Van der Stede Y., Kolbasov D., De Mia G.M., Le Potier M.-F., Bishop R.P., Arias M. Development and interlaboratory validation study of an improved new real-time PCR assay with internal control for detection and laboratory diagnosis of African swine fever virus. *J. Virol. Methods*, 2011, 178: 161-170 (doi: 10.1016/j.jviromet.2011.09.007).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 853-860
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Veterinary virology, microbiology, parasitology

UDC 619:578:57.083.2

doi: 10.15389/agrobiology.2016.6.853rus
doi: 10.15389/agrobiology.2016.6.853eng

PERMISSIVITY OF LAMB SYNOVIAL MEMBRANE CELL CULTURE FOR AKABANE DISEASE VIRUS

E.A. BALASHOVA, E.I. BARYSHNICKOVA, V.M. LYSKA, O.L. KOLBASOVA, A.V. LUNITSIN, D.V. KOLBASOV

All-Russian Institute of Veterinary Virology and Microbiology, Federal Agency of Scientific Organizations, 1, ul. Akademika Bakuleva, pos. Vol'ginskii, Petushinskii Region, Vladimir Province, 601125 Russia, e-mail balashowa.l@yandex.ru
Received July 31, 2016

Abstract

Presently, due to the variety and diversity of economic and tourist ties of Russia, episodes of accidental or maybe purposeful (i.e., biological terrorism) entry of exotic infectious pathogens including Akabane disease to the Russian Federation should not be excluded. Akabane disease is a viral transmissible infection. Its recurrent outbreaks are characterized by abortions, still or premature births, or malformations (e.g. arthrogryposis and/or hydrocephaly) for calves, lambs and kids. Akabane disease virus can persist both in animal body and in vitro (e.g., in continuous cell lines). A study of the sensitivity of African green monkey kidney cell line to Akabane virus carried out earlier showed that Akabane virus caused definite cytopathic changes resulting in cell rounding followed by cytolysis and detachment of the cell monolayer within 48 hours post infection. In this paper we first reported on the cytomorphological changes caused by Akabane virus in the primary lamb synovial membrane diploid cell culture (LSMCC) prepared according to an earlier developed procedure, and on a suitability of this culture for the virus accumulation in titers sufficient for study and making diagnosis. It has been formerly determined that the lamb synovial membrane cell culture is sensitive to small ruminant lentiviruses like caprine arthritis encephalitis virus or Visna-Maedi virus in sheep. LSMCC was prepared using a method for tissue explant culture. On day 4 post inoculation of the cell monolayer with Akabane virus the cytopathic effect appeared which manifested as formation of symplasts that grew larger due to their fusion on day 5 to 6. The Akabane virus activity was 6.0±0.05 lg $TCID_{50}/cm^3$ for strain V8935, and 5.0±0.05 lg $TCID_{50}/cm^3$ for strain P. As many as three passages and also the primary cell culture (after freezing) kept the virus-producing activity, and the Akabane virus retained its infective properties. The lamb synovial membrane cells can be re-cultured, and excessive diploid culture can be frozen to preserve and thawed as required. It is expedient to use a strain of diploid lamb synovial membrane cells deposited and patented earlier. One more advantage of the primary LSMCC as compared to monkey cell lines is that the latter ones may be a source of simian herpes B virus.

Keywords: lamb synovial membrane cell culture, Akabane disease, *Orthobunyavirus*

Due to the expansion of international economic relations and the development of tourism, accidental entry of exotic infectious pathogens to the territory of Russia is possible. Moreover, because of episodes of international terrorism, there is a real threat of biological terrorism, the focused introduction of the dangerous infections pathogens. These include the virus of Akabane disease, a vector-transmitted disease with the recurrent outbreaks followed by abortions, still or premature births, or malformations (arthrogryposis, hydrocephaly) in calves [1-4], lambs and goatlings [5]. Anti-Akabane virus disease antibodies have been found in buffalo, horses, camels, pigs, and monkeys [6-8]. Encephalitis effects of the Akabane disease virus have been described in mice, hamsters, guinea pigs, and chicken embryos [9, 10]. Virus has no pathological effects on the humans, however anti-Akabane virus antibodies have been found in a number of subjects in Japan [11]. Infectious agent belongs to Simbu serogroup of the genus *Orthobunyavirus*, family *Bunyaviridae,* which includes Aino, Chamond, and Schmallenberg diseases viruses [12].

Akabane disease virus is transmitted by ticks and mosquitoes which ensures the formation of stable natural foci in harsh environments and creates the possibility of expanding the range of vertebrate hosts. Long-term preservation of the viral population in susceptible vertebrates contributes to the rapid spread of the disease among wild and domestic animals in a favorable for mosquitoes climate period [13]. Akabane disease virus has been isolated from the animals in Japan [14, 15], Israel [16], Korea [17-19], Australia [20], Turkey [21], Cyprus [22], Syria [23], Sudan [24], and Kenya [25]. The widespread, high contagiousness and economic losses are the reasons for which the study and control of this pathogen are necessary. Continuous cell cultures are the main laboratory model for the study of animal viruses, and the primary culture is the model in the absence of continuous cell lines.

Akabane disease virus can persist both in animal and in continuous cultures in vitro. At various times, a number of foreign papers reported that hamster lung (HmLu-1), African green monkey kidney (Vero), newborn Syrian hamster kidney (BHK-21-W12), pig kidney (PK-15), rabbit kidney (RK-13), and fetal calf kidney (BEK) continuous cultures were sensitive to Akabane disease virus [26-28].

In the Russian Federation, Akabane disease virus has been studied only at the All-Russian Scientific Research Institute of Veterinary Virology and Microbiology (VNIIVViM). The pathogen morphology has been described [29], a method of reverse transcription PCR (RT-PCR) was developed to detect Akabane virus genome [30], and the sensitivity of Vero [31] and African green monkey kidney CV-1 [32] cultures to the virus was studied.

Russian collections include continuous cell cultures sensitive to Akabane virus. Alternatively, however, it is important to have sensitive primary cell cultures (with a proof of their advantages or detection of disadvantages) which can be prepared in any equipped laboratory. A strain of lamb *Ovis aries* synovial membrane diploid cells has been patented and deposited in VNIIVViM [33] which is used to derive a primary culture. It is noted that this culture, and also its subcultures are stable in its biological characteristics [33] and sensitive in the early passages to small ruminant lentiviruses, such as caprine arthritis encephalitis virus and Visna-Maedi virus in sheep [33, 34].

In this paper, we first reported the cytomorphological changes caused by Akabane disease virus in lamb synovial membrane diploid cell culture prepared according to an earlier developed procedure [34]. This culture is suitable for the virus accumulation in titers sufficient for the study and diagnosis.

We evaluated primary lamb synovial membrane cell culture as a laboratory model for the accumulation and titration of Akabane disease virus.

Technique. Akabane disease virus (strains B8935 and P) was obtained from State VNIIVViM collection of microorganisms. Primary synovial membrane cell culture donor was a 3-day old lamb (grown in VNIIVViM experimental animals sector).

For cell culture, we used minimal Dulbecco's Modified Eagle's Medium (DMEM, HyClone Laboratories, Inc., USA) with a double amount of amino acids and vitamins, the fetal bovine serum (HyClone Laboratories, Inc., USA), Benzylpenicillin sodium salt (150 U/cm^3) and gentamicin (100 μg/cm^3). A mixture of 0.02 % Versene (Sigma, USA) and 0.25 % trypsin (Sigma, USA) at a ratio of 2:1 heated to 37 °C was applied for cell dispersion.

Cell culture was derived from a tissue explant [34]. Hock and wrist joints were collected aseptically, the skin was removed and the joints treated with 96 % alcohol for 15-30 seconds. The isolated synovial membrane was transferred into a Petri dish with nutrient DMEM medium containing 2 % fetal bovine serum

and the antibiotics, crushed mechanically into 1-2 mm fragments, then washed thrice with the medium of the same composition. Explants were placed into culture flasks with DMEM, 10 % fetal bovine serum and the antibiotics, and incubated at 38 °C, 90 % relative air humidity and 5 % CO_2. Lamb synovial membrane diploid cells resulted from passaging primary culture.

Virus containing culture liquid of the continuous CV-1 cell line (the infectious activity of $10^{4.0}$ lg $TCID_{50}/cm^3$, a dilution of 1:100) was used for the lamb synovial membrane cell culture inoculation. The added culture liquid level was 3-4 mm above the cell monolayer. The inoculum was stored at –60±0.5 °C before using. Akabane disease virus was cultured without absorption on cells and change of the culture medium using standard methods. Intact lamb synovial membrane cell culture was a control. Infected and intact lamb synovial membrane cell culture incubation continued for 8 days at 37±0.5 °C under daily observation.

In Akabane disease virus titration performed in 3 replicates the lamb synovial membrane cells were cultured in 96-well polystyrene plates. A 150 µl aliquot of virus-containing DMEM diluted from 1:10 to 1:10 000 000 was added in each well. The plates were kept at 5 % CO_2 and 90 % relative humidity in an incubator. To compare sensitivity, Vero and CV-1 cultures from VNIIVViM Collection of Cell Cultures were used as well.

Statistical analysis included estimation of the mean and standard deviation.

Results. In continuous Vero [31] and CV-1 cultures (Fig. A, B), Akabane virus caused cytopathic changes resulting in cell rounding followed by cytolysis and detachment of the cell monolayer within 48 hours post infection. Cytomorphological transformation took place in lamb synovial membrane cell culture as well (see Fig., C-E).

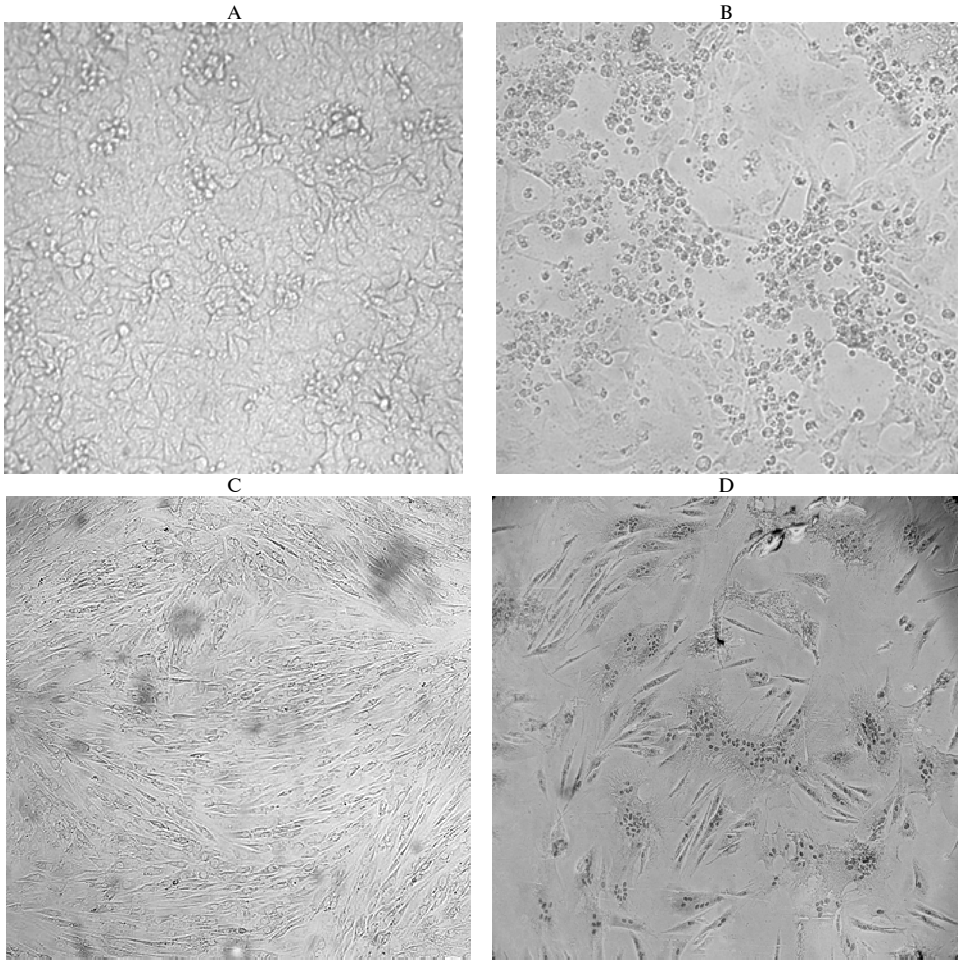
In subculture, lamb synovial membrane cell population transformed from primary culture to cell subculture. At day 6, we observed extensive cell colonies which merged with each other turning into a confluent monolayer. The cells formed a clearly defined multidirectional chords typical of fibroblast-like cultures (see. Fig., C). Lamb synovial membrane cell culture was viable and not subjected to morphological changes.

Akabane disease virus was able to replicate in lamb synovial membrane cell culture without prior adaptation. In the infected culture, unlike the intact one, we found significant cytopathic changes. The culture retained its sensitivity to the Akabane disease virus up to 12 passages (the observation period).

In primary culture infected with Akabane virus the apparent cytopathic effect was manifested in cell rounding and formation of symplasts recorded 72 hours post-infection (see Fig., D). Some cells increased in sizes and were destroyed, and "windows" formation was followed by expansion of intercellular space. Probably, particles were released from the infected cells by endocytosis and cell lysis. Symplasts grew larger in 96-120 hours due to their fusion. After 120-144 hours, progressive detachment of cells from the walls occurred, then the most part of the monolayer was destroyed. These changes were not recorded in the intact culture.

At 80-90 % cytopathic effect in lamb synovial membrane cell culture in 96-120 hours (see Fig., E), virus containing culture fluid was frozen at –60±0.5 °C for the release of the intracellular virus after thawing. Thawed culture fluid was titrated in 96-well polystyrene plates (Table). Thus, after three passages of the Akabane virus in lamb synovial membrane cell culture, virus activity was 5.0±0.05 lg $TCID_{50}/cm^3$ for strain P, and 6.0±0.05 lg $TCID_{50}/cm^3$ for strain V8935. Therefore, up to as many as three passages (observation period), primary cell culture retained its virus-producing activity, and the Akabane virus

retained its infective properties. Cytomorphological changes in lamb synovial membrane cell culture are a technical test to obtain qualitative results in the evaluation of Akabane disease virus activity by titration.



Cytopathic effect of the Akabane disease virus (strain B893) **in continuous culture of African green monkey kidney cell line CV-1 and in primary lamb synovial membrane cell culture:** A and B — intact and infected culture CV-1 72 hours after inoculation; C — intact primary lamb synovial membrane cell culture in 72 hours, D and E — infected primary lamb synovial membrane cell culture 72 and 96 hours after inoculation (light microscopy, magnification ×100).

We compared Vero, CV-1 and lamb synovial membrane cell cultures sensitivity to Akabane disease virus and found virus activity which differed insignificantly. The obtained parameters were 5.85±0.05; 5.8±0.09 and 6.0±0.05 lg ТЦД$_{50}$/cm^3, respectively.

Therefore, we can state that all the above cultures are suitable as a laboratory model for the Akabane disease virus research. However, the advantage of the primary diploid cell culture is that it is more sensitive, prepared in a special-

ized laboratory independently of available donor tissue, that is a referral to a culture museum is not required. The lamb synovial membrane cells can be re-cultured, and excessive diploid culture, like the passaged cells, can be frozen to preserve and thawed if necessary. It is expedient to use a strain of diploid lamb synovial membrane cells but not the primary culture, as the physiological state of diploid lines is better. Preference is usually given to continuous cell lines for the reasons of preservation of donor animals, reducing the cost and a possibility to control cell quality. However, in the case of monkey cells, for example, lack of these animals, their high cost and the fact that they are a source of potential infectious danger as carriers of herpes B virus should be considered.

Dynamics of Akabane disease virus cytopathic effect in primary lamb synovial membrane cell culture

Virus dilution	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Strain P								
1:10	–	–	+	+	+	+	+	+
1:100	–	–	–	+	+	+	+	+
1:1000	–	–	–	–	+	+	+	+
1:10 000	–	–	–	–	+	+	+	+
1:100 000	–	–	–	–	–	+	+	+
1:1 000 000	–	–	–	–	–	–	–	–
1:10 000 000	–	–	–	–	–	–	–	–
Strain B8935								
1:10	–	–	+	+	+	+	+	+
1:100	–	–	+	+	+	+	+	+
1:1000	–	–	–	+	+	+	+	+
1:10 000	–	–	–	–	+	+	+	+
1:100 000	–	–	–	–	–	+	+	+
1:1 000 000	–	–	–	–	–	+	+	+
1:10 000 000	–	–	–	–	–	–	–	–

Note. «+» — effect, «–» — no effect. Titration was performed in three replicates.

Lamb synovial membrane cell culture is close to the cells of one of the animal species with natural susceptibility to the pathogen, so it can subsequently be used to obtain attenuation and live vaccine. Furthermore, the suitability of this culture for the primary isolation of the virus from pathological material should be studied. Note, primary cell cultures are more sensitive and are better suited for such purposes.

Thus, lamb synovial membrane cell culture, as permissive to Akabane disease virus, may be used, along with Vero and CV-1 cell lines, to produce the culture antigen for serological tests to diagnose the disease. Akabane virus causes a characteristic cytopathic effect in the infected monolayer of the above cultures, all of them can serve as a laboratory models for the study, accumulation and titration of the virus, and produced viral raw materials may be used in virology and molecular genetic studies. At the same time, the advantage of the proposed primary diploid cell culture is in its greater sensitivity and accessibility for independent preparation. It may be re-cultured and the excessive culture can be frozen to preserve and thawed as required.

REFERENCES

1. Omori T., Inaba Y., Kurogi H., Miura Y., Nobuto K., Motumoto M. Viral abortion arthrogryposis-hydranencephaly syndrome in cattle in Japan 1972-1974. *Bull. Off. Int. Epiz.*, 1974, 81: 447-458.
2. Kono R., Hirata M., Kaji M., Goto Y., Ikeda S., Yanase T., Kato T., Tanaka S., Tsutsui T., Imada T., Yamakawa M. Bovine epizootic encephalomyelitis caused by Akabane virus in southern Japan. *Vet Res.*, 2008, 13: 4-20.
3. Oem J.K., Yoon H.J., Kim H.R., Roh I.S., Lee K.H., Lee O.S., Bae Y.C. Genetic and pathogenic characterization of Akabane viruses isolated from cattle with encephalomyelitis in Korea. *Vet. Microbiol.*, 2012, 158(3-4): 259-266 (doi: 10.1016/j.vetmic.2012.02.017).
4. Della-Porta A., Murray M., Cybinski D. Congenital bovine epizootic artrogryposis

and hydranencephaly in Australia. Distribution of antibodies to Akabane virus in Australian cattle after the 1974 epixootic. *Aust. Vet. J.*, 1976, 52: 496-501 (doi: 10.1111/j.1751-0813.1976.tb06983.x).
5. Kalmar E., Peleg B., Savir D. Arthogryposis-hydranencephaly syndrome in newborn cattle, sheep and goats. Serological survey for antibodies against the Akabane virus. *Ref. Vet.*, 1975, 32: 47-54.
6. Al-Busaidy S.M., Hamblin C., Taylor V. Neutralising antibodies to Akabane virus in free-living wild animals in Africa. *Tropical Animal Health and Production*, 1987, 19: 197-202.
7. Al-Busaidy S.M., Mellor P.S., Taylor W.P. Prevalence of neutralizing antibodies to Akabane virus in Arabian Peninsula. *Vet. Microbiol.*, 1988, 17(2): 141-149.
8. Oya A. *Annual reports of WHO Regional Reference Center for Arbovirus.* National Institute of Health, Tokyo, 1971.
9. Nakajima Y., Takahashi E., Konno S. Encephalomyelitis in mice experimentally infected with Akabane virus. *Natl. Inst. Anim. Health Q. (Japan)*, 1979, 19: 47-52.
10. Miah A., Spradbrow P. The growth of Akabane virus in chicken embryos. *Research in Veterinary Science*, 1978, 25: 253-254.
11. Nakamura T., Matsuyama T., Okuno T., Oya A. Arbovirus antibody survey. *Igaku no Ayumi (in Japanese)*, 1967, 60: 72-73.
12. Nikitina E.G., Sal'nikov N.I., Balashova E.A., Tsybanov S.Zh., Kolbasov D.V. Akabane and Schmallenberg diseases: similarities and differences. *Agricultural Biology*, 2013, 4: 48-52 (doi: 10.15389/agrobiology.2013.4.48eng) (in Engl.).
13. Matumoto M., Inaba Y. Akabane disease and Akabane virus. *Kitasato Arch. Exp. Med.*, 1980, 53(1-2): 1-21.
14. Kato T., Shirafuji H., Tanaka S., Sato M., Yamakawa M., Tsuda T., Yanase T. Bovine arboviruses in *Culicoides* biting midges and sentinel cattle in Southern Japan from 2003 to 2013. *Transbound. Emerg. Dis.*, 2015, 63(6): 160-172.
15. Motumoto M., Oya A., Ogata T., Kobayashi I., Nakamure T., Takahashi H., Kitacka M. Isolation of arbor viruses from mosquitoes collected at live-stook pens in Gumma Prefecture in 1959. *Jap. J. Med. Sci. Biol.*, 1960, 13: 191-198.
16. Brinner J., Tsuda T., Yadin H., Chai D., Stram Y., Kato T. Serological and clinical evidence of a teratogenic Simbu serogroup virus infection of cattle in Israel 2001-2003. *Veterinaria Italiana*, 2004, 40(3): 119-123.
17. Yang D.K., Kim B.H., Kweon C.H., Nah J.J., Kim H.J., Lee K.W., Yang Y.J., Mun K.W. Serosurveillance for Japanese encephalitis, Akabane, and Aino viruses for Thoroughbred horses in Korea. *J. Vet. Sci.*, 2008, 9(4): 381-385.
18. Oem J.K., Kim Y.H., Kim S.H., Lee M.H., Lee K.K. Serological characteristics of affected cattle during an outbreak of bovine enzootic encephalomyelitis caused by Akabane virus. *Trop. Anim. Health Prod.*, 2014, 46: 261-263.
19. Oem J.K., Lee K.H., Kim H.R., Bae Y.C., Chung J.Y., Lee O.S., Roh I.S. Bovin epizootic encephalomyelitis caused by Akabane virus infection in Korea. *J. Comp. Pathol.*, 2012, 147(2-3): 101-105 (doi: 10.1016/j.jcpa.2012.01.013).
20. Murray M.D. Akabane epizoonotis in New South Wales: evidence for the long-distance dispersal of the biting midge *Culicoides brevitarsis*. *Australian Veterinary Journal*, 1987, 64(10): 305-308.
21. Yilmaz H., Hoffmann B., Turan N., Cizmecigil U.Y., Richt J.A., Van der Poel W.H. Detection and partial sequencing of Schmallenberg virus in cattle and sheep in Turkey. *Vector-Borne and Zoonotic Diseases*, 2014, 14: 223-225.
22. Sellers R., Herniman K. Neutralising antibodies to Akabane virus in ruminants in Ciprus (transmitted by *Culicoides* midges). *Trop. Anim. Health Prod.*, 1981, 13(1): 57-60.
23. Sellers R., Pedgley D. Possible windborne spread to western Turkey of bluetongue virus in 1977 and of Akabane virus in 1979. *Journal of Hygiene*, 1995, 1: 149-158.
24. Elhassan A.M., Mansour M.E., Shamon A.A., El Hussein A.M. A serological survey of Akabane virus infection in cattle in Sudan. *ISRN Veterinary Science*, 2014: Article ID 123904 (doi: 10.1155/2014/123904).
25. Metselaar D., Robin Y. Akabane virus isolated in Kenya. *Vet. Rec.*, 1976, 99(5): 86 (doi: 10.1136/vr.99.5.86-a).
26. Anderson A.A., Campbell C.H. Experimental placental transfer of Akabane of virus in the hamster. *Am. J. Vet. Res.*, 1978, 39: 301-304.
27. Kurogi H., Inaba Y., Takahashi H., Sato K., Akashi H., Satoda K., Omori T. An attenuated strain of Akabane virus: a candidate for live virus vaccine. *Natl. Inst. Anim. Health Q (Tokyo)*, 1979, 19(1-2): 12-22.
28. Kurogi H., Inaba Y., Takahashi H., Sato K., Omori T., Miura Y., Goto Y.M., Fujiwara Y., Hatano Y., Kodama K., Fukuyama S., Sasaki N., Matumoto M. Epizootik congenital artrogryposis-hydranencephaly syndrom in cattle of Akabane virus from affected fetuses. *Arch. Virol.*, 1976, 51: 67-74.
29. Malakhova M.S., Balashova E.A. *Tezisy konferentsii «Voprosy veterinarnoi virusologii,*

mikrobiologii i epizootologii» [Proc. Conf. «Veterinary virology, microbiology and epizootology»]. Pokrov, 1990: 83-84 (in Russ.).

30. Nikitina E.G., Sal'nikov N.I., Katorkin S.A., Balashova E.A., Tsybanov S.Zh., Kolbasov D.V., Lunitsin A.V. Detection of Akabane virus genome in organs and blood of experimentally infected cavies. *Agricultural Biology*, 2014, 6: 67-72 (doi: 10.15389/agrobiology.2014.6.67eng) (in Engl.).
31. Kotova O.Yu., Khan E.O., Kushnir S.D., Ponomarev V.N., Yurkov S.D., Balashova E.A. *Nauchnyi zhurnal KubGau (Krasnodar)*, 2012, 83(09). Available http://www.ej.kubagro.ru/2012/09/pdf/52.pdf. No date (in Russ.).
32. Kotova O.Yu., Suvorova Yu.A., Kushnir S.D., Yurkov S.G., Balashova E.A. *Veterinariya*, 2015, 3: 57-59 (in Russ.).
33. Baryshnikova E.I., Kolbasova O.L. *Shtamm diploidnykh kletok sinovial'noi membrany yagnenka Ovis aries, ispol'zuemyi dlya virusologicheskikh issledovanii. Pat. RF 2507255. GNU NIIVViM Rossel'khozakademii. Zayavl. 23.07.2012. Opubl. 20.02.2014* [A strain of diploid cells of lamb *Ovis aries* synovial membrane for virological research. Patent RF 2507255. GNU NIIV-ViM RAS. Appl. July 23, 2012. Publ. February 20, 2014] (in Russ.).
34. Sidel'nikov G.D., Kolbasova O.L., Zhigaleva O.N., Tsybanov S.Zh., Kolbasov D.V. *Veterinariya*, 2009, 4: 52-55 (in Russ.).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 861-866
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.32/38:619:578.821.6

doi: 10.15389/agrobiology.2016.6.861rus
doi: 10.15389/agrobiology.2016.6.861eng

IDENTIFICATION AND ISOLATION OF ORF VIRUS FROM SHEEP IN THE REPUBLIC OF TUVA IN 2015

D.V. YANZHIEVA[1], N.I. SAL'NIKOV[1], T.R. USADOV[1], S.P. ZHIVODEROV[1], L.K. SARYGLAR[2], A.V. LUNITSIN[1]

[1]*All-Russian Institute of Veterinary Virology and Microbiology,* Federal Agency of Scientific Organizations, 1, ul. Akademika Bakuleva, pos. Vol'ginskii, Petushinskii Region, Vladimir Province, 601125 Russia, e-mail darima.yanzhieva.90@mail.ru, nikolai.salnikov2010@yandex.ru, usadov.tr@mail.ru, zhivoderov-serg@mail.ru, lunicyn@mail.ru;
[2]*Tuvan State University,* 36, ul. Lenina, Kyzyl, Tuva Republic, 667000 Russia, e-mail saryglar.1959@mail.ru
Received October 4, 2016

Abstract

Orf is infectious disease of sheep and goats, characterized by lesions of mucosa of the oral cavity, skin of lips, head, mammary glands and limbs. The causal agent (virus of contagious ecthyma — Orf virus) is a member of genus *Parapoxvirus*, subfamily *Chordopoxvirinae*, family *Poxviridae*. Genome of the Orf virus consists of linear double stranded DNA (138 kbp) and contains 132 open reading frames. Its antigenic structure is poorly understood, and strains are not serologically identical. That is why the investigation of novel isolates of Orf virus is actual. However, we are not aware of such research in Russia possibly due to rare outbreaks recognized. We first identified Orf virus in sheep from Tuva Republic and studied biological properties of the isolate. Disease of sheep, characterized by erythema, vesicles, pustules and scabs on ears and legs has been registered in August 2015 in Tuva Republic. The scabs were sampled for isolation and identification of the causative agent. For this result three lambs were experimentally infected with the scab suspension. The mucocutaneous borders of the lips along the labial commissures were scarified and inoculum was loaded by cotton swabs. The lip vesicles appeared at days 4-5 and then formed pustules and scabs. After 2 weeks the lesions healing occurred. The suspension of scabs from lips of infected lambs was used to inoculate sheep kidney cell culture. In 7 days post inoculation the cell monolayer was harvested for second passage. However, we did not observe specific cytopathic effect in the monolayers during these passages. The skin swabs from experimentally infected lambs were examined by Real-Time PCR in accordance to the protocol developed by G. Venkatesan et al. (2014), and Orf virus DNA was detected at days 7 to 28 post infection. We also detected Orf virus DNA in scabs from the infected lambs and in lysates of the monolayers harvested after second passage. The PCR test was positive for reference strain IA82 of Orf virus unlike other viruses (nodular dermatitis vaccine strain, sheep pox strains Mongolian and B5/96 or goatpox strain QA/A-04) that confirmed specificity of the PCR system. Its analytical sensitivity to detect Orf virus genomic DNA was 1.3±0.03 lg TCD_{50}/cm^3. Thus, the pathogen which caused the disease of sheep in Tuva Republic in 2015 was isolated from experimentally infected lambs using sheep kidney cell culture and identified as Orf virus.

Keywords: sheep, Orf virus, PCR, cell culture

Orf is an infectious disease, which is characterized by lesions of the oral mucosa, skin on lips, head, mammary glands and limbs forming nodules, vesicles, pustules and crusts, with a primary involvement of any single area of the body. The disease is widely spread around the world. In many countries with advanced sheep and goat breeding it is a stationary infection [1]. Its viral etiology was proven in experimentally, and a secondary role of the causative agent of necrobacillosis was established. The causal agent of contagious pustular dermatitis (Orf) is a contagious ecthyma virus (CEV; Orf virus, the genus *Parapoxvirus*, subfamily *Chordopoxvirinae* and family *Poxviridae*). Its virions are oval in shape with a size of 200-300×140-170 nm. The genome is represented by a linear double-stranded DNA molecule (138 kbp) containing 132 open reading frames [2]. The virus causes disease in sheep, goats, chamois and tours of all ages, as well as in other cloven-hoofed bovid animals. The disease is more severe in young animals. Humans get

infected very rarely, mostly through contact with sick animals, and only if there are cuts and scratches on the skin. Lesions develop at the site of the virus penetration, usually on hands [3].

Sick animals are the main source of the infection, as the virus reproduces in their bodies. It is excreted with scabs, crusts and the flux from the oral cavity, which causes contamination of pastures, bird feeders, water, feed, sheepyards, inclosures, pens, and litter. The virus spreads to sheep coat, care items, and clothing of shepherds. Sheep can get infected when grazing on infected pastures, eating contaminated hay, feed, while taking water from contaminated watering sites, as well as through direct contact with sick animals [1, 4]. The disease is also spread by recovered virus carriers, which have been taken into good operating farms [1, 4, 5], therefore, the virologic control on the animal movement is very important.

The antigen structure of CEV is not well understood, and not all the strains are serologically identical, and therefore the detection and investigation of its isolates is topical [3, 6-9]. Abroad, a conventional scheme for studying the properties of new CEV isolates includes pathogen isolation in ovine cell cultures, sequencing of the variable regions of the genome and phylogenetic analysis. This analysis has been performed for the strains obtained from goats in South Korea, India, the Himalayas and Taiwan [10-14]. The biological properties of the five strains of CEV isolated in goats were investigated (Texas, 2003-2004) [15]. Based on B2L gene sequencing [16], a method for the identification and phylogenetic analysis of the CEV isolates was characterized, which was then applied to the study of the strains from sheep in India and Brazil [17, 18]. The methods to identify the virus genome by PCR have been developed and validated [19-21], with a comparison of diagnostic features of various PCR protocols and CEV isolation in cell culture carried out [22]. In the domestic literature, there are no such papers that, apparently, is due to the lack of attention to the infection from animal owners and veterinary professionals, and, therefore, to a small number of reported outbreaks of contagious ecthyma in Russia.

We first isolated CEV from sheep in the Republic of Tyva, using the collected biological material to experimentally infect lambs, and studied the biological properties of the isolate.

The purpose of the work was to identify the pathogen that caused in 2015 the disease with symptoms of Orf in sheep in the Republic of Tyva.

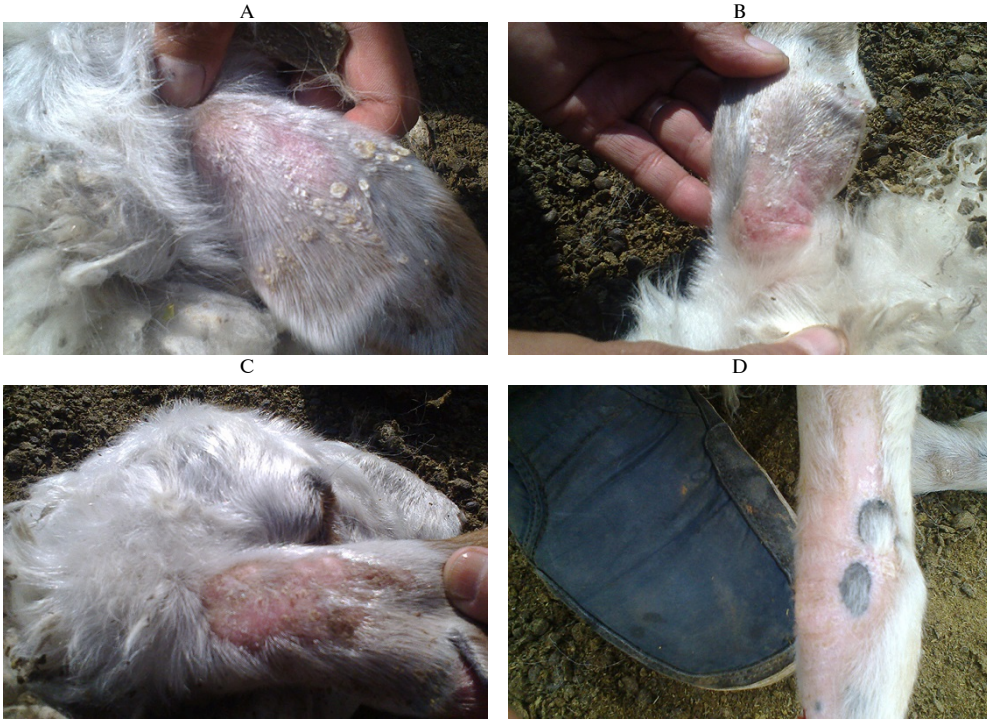
Technique. Skin scabs from limbs and ears, sampled from the sheep with signs of Orf, were placed into the Eddington's liquid and transported in the cold box. The weighed portions of biomaterial was used to prepare a 10 % suspension in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM NaH_2PO_4, pH 7.4) at a ratio of 1:9 (w/v) and homogenized in a porcelain mortar. The suspension was clarified by centrifugation (3 min at 7,000 rpm). The biological properties of the virus were studied by the infection of three lambs in a vivarium using the suspension of skin scabs from the limbs of sick animals. The infectious inoculum was loaded with a cotton swab on the scarified surface of the lips on the mucocutaneous borders. Clinical examination of animals was carried out daily, with obtaining swabs from the sites of virus introduction.

The virus was isolated in a passaged culture of ovine kidney cells (the PO-VNIIVViM line; the museum of the All-Russian Research Institute of Veterinary Virology and Microbiology), grown in flasks with the landing area of 150 cm^2. The suspension of scabs from infected animals was passed through a 45 μm filter (Millipore, USA), then diluted with Eagle's MEM with antibiotics (50 μg/ml gentamicin, 2.5 μg/l Amphotericin B) at a ratio of 1:1. Next, 1 ml of

suspension was added to cell culture flasks which were then kept for 30 minutes in the MCO-18AC CO_2 incubator (Sanyo Electric Co., Ltd, Japan) at 37 °C. The culture medium was decanted, then 10 ml Eagle's MEM was added, containing 2 % fetal calf serum (Biolot, Russia), and cultured under the same conditions for 7 days. Then the monolayer was frozen and thawed thrice and used for the 2nd passage (there were no subsequent passages).

The samples were tested for the Orf virus DNA using the real-time PCR. PCR was performed on a Rotor Gene 6000 thermocycler (Corbett Research Pty, Ltd, Australia) according to the following protocol: 10 min at 95 °C — a preliminary denaturation; 30 secs 95 °C, 1 min at 60 °C — detection by "Green" channel. The reaction mixture contained 10 pmol of each primer, 3 pmol of a fluorescent probe, 2.5 µl of 10× DNA buffer, 10 mM triphosphates mixture and 1.5 Taq units of recombinant DNA polymerase (Thermo Fisher Scientific, USA). OV RT-F (5′-TACACGGAGTTGGCCGTGATCTTGTA-3′) and OV RT-R (5′-CGCCAA-GTACAAGAAGCTGATGA-3′) primers, and a hybridization probe OV Probe (5′- [FAM]TGCATCGAGTTGTAGATCTCGCGGT[BHQ-1]-3′) developed in 2014 [23] were used. The system specificity was tested with DNA samples of the viruses of nodular dermatitis, sheep pox, goat pox and Orf (The VNIIVViM Collection of Microorganisms), the test analytical sensitivity was controlled using 10-fold dilutions of the primary cell culture of lamb synovial membrane (VNIIVViM Museum) infected with the Orf virus. The values with the relative standard deviation at ±0.03 are given for the titres [24].

Results. In August 2015, in the Buy-Khol APC of the Bay-Tal sumon



The clinical signs of Orf in sheep: A, B — scabs on the skin of ears, C — skin pustules on the ears, D — pustules on the leg skin (Republic of Tyva, 2015).

(Erzin district, Republic of Tyva) in sheep and lambs born in late March-early April, a disease was reported involving lesions of the skin in the form of scabs on the limbs, ears and nose. In lambs, the redness of the skin around the ears was first observed, then it appeared on the limbs and in the nostrils, the hair fell out,

and the formation of vesicles and long healing ulcers was seen (Figure). In the experimental infection of healthy animals with the biomaterial from sick animals, on day 4-5 at the site of virus introduction vesicles appeared that turned into pustules 1 day after. Drying out, pustules formed grayish-brown scabs. Two weeks after the experimental infection lesions healed.

After two passages (for 7 days each) specific cytopathic effect of the Orf virus in the primary PO-VNIIVViM cell culture was not found. The virus reproduction in cell culture was confirmed by the identification of its genomic DNA by real-time PCR in the lysate of the cell monolayer after the 2nd passage (7 days). A used system of oligonucleotide primers and Taq-man probe are complementary to the DNA polymerase gene of this virus [9]. When studying the reference DNA samples of CEV (IA82 strain), nodular dermatitis (vaccine strain), sheep pox (Mongolian and B5/96 strains), goat pox (OK/A-04 strain), positive results were obtained only with DNA of CEV that confirmed the system specificity. The lysate of the PO-VNIIVViM cell culture infected with CEV (2nd passage) was PCR positive. It means that only samples of CEV DNA were PCR positive, additionally indicating the specificity of the primers and probes used. The highest dilution of the culture material containing CEV (the reference strain IA82, the infectious activity of 5.3±0.03 lg TCD_{50}/cm^3), for which a positive PCR result was obtained, was 10^{-4} (Table 1). Therefore, the analytical sensitivity of real-time PCR to detect the Orf virus genome was 1.3±0.03 lg TCD_{50}/cm^3.

1. The analytical sensitivity of real-time PCR in detecting Orf virus genome

Dilution	Viral titer, lg TCD_{50}/cm^3	Threshold cycle
10^{-1}	4.3±0.03	17.81
10^{-2}	3.3±0.03	22.09
10^{-3}	2.3±0.03	25.59
10^{-4}	1.3±0.03	30.46
10^{-5}	0.3±0.03	N/A

Note. Threshold cycle is a cycle in which the fluorescence curve intersects the threshold line.

Testing of the material from experimentally infected sheep demonstrated that on day 7-28 the virus was accumulated in the epithelium of oral mucosa and in the saliva in the amounts sufficient to be identified by real-time PCR (Table 2). Testing of pustules formed at the site of the virus infection, as well as the culture material, also gave positive results (Ct 8.1-9.3).

2. The real-time PCR threshold cycles for Orf virus genome detection in oral smears after experimental infection

Day after infection	Lamb 1	Lamb 2	Lamb 3
7	18.23	19.32	18.51
14	25.98	22.27	24.21
21	35.59	29.21	30.43
28	Negative	Negative	Negative

Note. Threshold cycle is a cycle in which the fluorescence curve intersects the threshold line.

Therefore, we first detected the Orf virus in the sheep manifesting sings of this disease, occured in the Republic of Tyva in 2015, and studied the biological properties of the isolate. In the experimental infection, the field isolate induced vesicular-pustular rash at the site of viral inoculation (on the mucocutaneous border of the lips). The causative agent was isolated from cutaneous scabs in the experimentally infected animals using ovine kidney cell line culture and identified by real-time PCR analysis.

REFERENCES

1. Kolomytsev A.A., Zakutskii N.I., Guzalova A.G., Mikolaichuk S.V., Morgunov Yu.P. *Veterinarnaya patologiya*, 2008, 4: 31-37 (in Russ.).
2. Delhon G., Tulman E.R., Afonso C.L., Lu Z., de la Concha-Bermejillo A., Lehmkuhl H.D., Piccone M.E., Kutish G.F., Rock D.L. Genomes of the parapoxviruses Orf virus and bovine papular stomatitis virus. *J. Virol.*, 2004, 78(1): 168-177 (doi:

10.1128/JVI.78.1.168-177.2004).
3. Bayindir Y., Bayraktar M., Karadag N., Ozcan H., Kayabas U., Otlu B., Durmaz R., Doganay M. Investigation and analysis of a human orf outbreak among people living on the same farm. *New microbiologica*, 2011, 34: 37-43.
4. Hosamani A., Scagliarini A., Bhanuprakash V., McInnes C.J., Singh R.K. Orf: an update on current research and future perspectives. *Expert Review on Anti-infective Therapy*, 2009, 7(7): 879-893 (doi: 10.1586/eri.09.64).
5. Bhanuprakash V., Venkatesan G., Balamurugan V., Hosamani M., Yogisharadhya R., Chauhan R.S., Pande A., Mondal B., Singh R.K. Pox outbreaks in sheep and goats at Makhdoom (Uttar Pradesh), India: evidence of sheep pox virus infection in goats. *Transbound. Emerg. Dis.*, 2010, 57(5): 375-382 (doi: 10.1111/j.1865-1682.2010.01158.x).
6. Abubankr M.I., Abu-Elzein E.M., Housawi F.M., Abdelrahman A.O., Fadlallah M.E., Nayel M.N., Adam A.S., Moss S., Forrester N.L., Coloyan E., Gameet A., Al-Afaleq A.I., Gould E.A. Pseudo cow poxvirus: the etiological agent of contagious ecthyma (Auzdyk) in camels (*Camelus dromedarius*) in the Arabian peninsula. *Vector Borne Zoonotic Diseases*, 2007, 7(2): 257-260 (doi: 10.1089/vbz.2006.0627).
7. Abdelrabman K., Soliman H. Molecular and virological studies on contagious pustular dermatitis isolates from Egyption sheep and goats. *Research in Veterinary Science*, 2010, 89(2): 290-294 (doi: 10.1016/j.rvsc.2010.02.019).
8. Wise L.M., Savory L.J., Dryden N.H., Whelan E.M., Fleming S.B., Mercer A.A. The development of oral lesions in lambs naturally infected with orf virus. *Veterinary Journal*, 2007, 174(3): 663-664 (doi: 10.1016/j.tvjl.2006.10.024).
9. Venkatesan G., Balamurugan V., Bhanuprakash V. TaqMan based real-time duplex PCR for simultaneous detection and quantitation of Capri pox and Orf virus genomes in clinical samples. *J. Virol. Methods*, 2014, 201: 44-50 (doi: 10.1016/j.jviromet.2014.02.007).
10. Oem J.-K., Chung J.-Y., Kim Y.-J., Lee K.-K., Kim S.-H., Jung B.-Y., Hyun B.-H. Isolation and characterization of orf viruses from Korean black goats. *J. Vet. Sci.*, 2013, 14(2): 227-230 (doi: 10.4142/jvs.2013.14.2.227).
11. Bora D.P., Barman N.N., Das S.K., Bhanuprakash V., Yogisharadhya R., Venkatesan G., Kumar A., Rajbongshi G., Khatoon E., Chakraborty A., Bujarbaruah K.M. Identification and phylogenetic analysis of orf viruses isolated from outbreaks in goats of Assam, a northeastern state of India. *Virus Genes*, 2012, 45: 98-104 (doi: 10.1007/s11262-012-0740-y).
12. Chan K.W., Lin J.W., Lee S.H., Liao C.J., Tsai M.C., Hsu W.L., Wong M.L., Shih H.C. Identification and phylogenetic analysis of orf virus from goats in Taiwan. *Virus Genes*, 2007, 35: 705-712 (doi: 10.1007/s11262-007-0144-6).
13. Mondal B., Bera A.K., Hosamani M., Tembhurne P.A., Bandyopadhyay S.K. Detection of orf virus from an outbreak in goats and its genetic relation with other parapoxviruses. *Veterinary Research Community*, 2006, 30: 531-539 (doi: 10.1007/s11259-006-3270-z).
14. Hosamani M., Yadav S., Kallesh D.J., Mondal B., Bhanuprakash V., Singh R.K. Isolation and characterization of an Indian orf virus from goats. *Zoonoses Public Health*, 2007, 54: 204-208 (doi: 10.1111/j.1863-378.2007.01046.x).
15. Musser J.M.B., Taylor C.A., Guo J., Tizard I.R., Walker J.W. Development of a contagious ecthyma vaccine for goats. *Am. J. Vet. Res.*, 2008, 69: 1366-1370 (doi: 10.2460/ajvr.69.10.1366).
16. Hosamani M., Bhanuprakash V., Scagliarini A., Singh R.K. Comparative sequence analysis of major envelope protein gene (B2L) of Indian orf viruses isolated from sheep and goats. *Vet. Microbiol.*, 2006, 116: 317-324 (doi: 10.1016/j.vetmic.2006.04.028).
17. Venkatesan G., Balamurugan V., Bora D.P., Yogisharadhya R., Prabhu M., Bhanuprakash V. Sequence and phylogenetic analyses of an Indian isolate of orf virus from sheep. *Veterinaria Italiana*, 2011, 47(3): 323-332.
18. Abrahro J.S., Campos R.K., Trindade G.S., Guedes M.I., Lobato Z.I., Mazur C., Ferreira P.C., Bonjardim C.A., Kroon E.G. Detection and phylogenetic analysis of orf virus from sheep in Brazil: a case report. *Virol. J.*, 2009, 6: 47 (doi: 10.1186/1743-422X-6-47).
19. Chan K.W., Hsu W.L., Wang C.Y., Yang C.H., Lin F.Y., Chulakasian S., Wong M.L. Differential diagnosis of orf viruses by a single-step PCR. *J. Virol. Methods*, 2009, 160(1-2): 85-89 (doi: 10.1016/j.jviromet.2009.04.025).
20. Gallina L., Dal Pozzo F., Mc Innes C.J., Cardeti G., Guercio A., Battilani M., Ciulli S., Scagliarini A. A real-time PCR assay for the detection and quantification of orf virus. *J. Virol. Methods*, 2005, 134: 140-145 (doi: 10.1016/j.jviromet.2005.12.014).
21. Nitsche A., Buttner M., Wilhelm S., Pauli G. Meyer H. Real-Time PCR detection of parapoxvirus DNA. *Clin. Chem.*, 2006, 52(2): 316-319 (doi: 10.1373/clinchem.2005.060335).
22. Kottaridi C., Nomikou K., Lelli R., Markoulatos P., Mangana O. Laborato-

ry diagnosis of contagious ecthyma: comparison of different PCR protocols with virus isolation in cell culture. *J. Virol. Methods*, 2006, 134: 119-124 (doi: 10.1016/j.jviromet.2005.12.005).
23. Venkatesan G., Balamurugan V., Bhanuprakash V. TaqMan based real-time duplex PCR for simultaneous detection and quantitation of Capri pox and Orf virus genomes in clinical samples. *J. Virol. Methods*, 2014, 201: 44-50 (doi: 10.1016/j.jviromet.2014.02.007).
24. Lakin G.F. *Biometriya* [Biometry]. Moscow, 1990 (in Russ.).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 867-874
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.2:637.12.04/.07:579.62:[579.861.2+615.33

doi: 10.15389/agrobiology.2016.6.867rus
doi: 10.15389/agrobiology.2016.6.867eng

ANTIBIOTIC-RESISTANCE PROFILE OF *Staphylococcus aureus* STRAINS ISOLATED FROM MILK OF HIGH YIELD COWS IN CENTRAL RUSSIA

O.A. ARTEM'EVA, D.A. NIKANOVA, E.N. KOTKOVSKAYA, E.A. GLADYR', A.V. DOTSEV, N.A. ZINOVIEVA

L.K. Ernst All-Russian Research Institute of Animal Husbandry, Federal Agency of Scientific Organizations, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail vijmikrob@mail.ru, DAP2189@gmail.com, asnd@mail.ru, elenagladyr@mail.ru, n_zinovieva@mail.ru
Acknowledgements:
Supported by Russian Science Foundation (project № 15-16-00020)
Received September 9, 2016

Abstract

The development of microbial resistance to antibiotics determines the effectiveness of the veterinary measures. The study and monitoring, well-designed treatment schemes are those measures that help to reduce the risk of spread of pathogenic and conditional-pathogenic microorganisms. One of the problems in the dairy farming is mastitis of high producing cows which is mainly caused by *Staphylococcus aureus.* Here we first report findings on the development of resistance in *S. aureus* strains isolated from milk of clinically healthy dairy cows during treatment with antibiotics of different classes. The study was carried out using high yield holsteinized black-and-white cows (n = 1321, Kaluga region, 2016) which were vaccinated twice with anti-mastitis drug Mastivak («Ovejero», Spain). Milk was sampled during the lactation period individually from each cow. For the species identification of *S. aureus* isolates we used the following criteria: (i) morphology and microscopy of the colonies grown on the Baird Parker Agar medium («HiMedia Laboratories Pvt., Ltd», India); (ii) the presence of hemolysis zones on the Azide Blood Agar Pronadisa medium («Conda», Spain) supplemented with 5 % defibrinated sheep blood; (iii) a coagulation of dry citrated rabbit plasma (CJSC «EKOlab», Russia); (iv) biochemical characterization with API 20 STAPH panel («BioMerieux», France). *S. aureus* ATCC 25923 was a reference strain. The sensitivity of the isolated microorganisms to ten antibiotics («Pharmacotherapy Research Center», Russia) including penicillin (PEN, 10, ED), oxacillin (OX, 10 μg), gentamicin (GN, 10 μg), erythromycin (ER, 15 μg), lincomycin (LN, 15 μg), rifampicin (RF, 5 μg), ciprofloxacin (CP, 5 μg), vancomycin (VA, 30 μg), fuzidin (FZ, 10 μg) and novobiocin (NB, 5 μg) was determined by disk diffusion method. A total of 104 strains among 155 isolates (67.1 %) showed resistance to one or more antibiotics. The majority of the strains were resistant to novobiocin (49.7 %) while the smallest part (2.6 %) exhibited resistant to vancomycin. Importantly, the antibiotic resistance to next-generation antibacterial agents (novobiocin and vancomycin) which are currently widely used in medicine should be noted. Gentamicin, rifampicin and vancomycin had the highest efficiency among the tested antibiotics (81.9 %, 86.5 % and 97.4 %, respectively). In order to evaluated the effectiveness of antibiotic treatment strategy, the cows (n = 87) with pre-detected *S. aureus* were divided into four groups. Gentamicin was administrated in group I (n = 26), erythromycin was administrated in group II (n = 22), rifampicin was administrated in group III (n = 12), and penicillin and amoxicillin were administrated in group IV (n = 27). The animals were considered cured when no *S. aureus* after the treatment. Rifampicin application was the most effective (91.7 %) whereas gentamicin showed the lowest effectiveness (53.8 %). Moreover, the number of multidrug resistant strains reached 55.6-61.5 % after the treatment comparing to 33.3-43.8 % before treatment, thus a tendency of developing multiple drug resistance has been shown.

Keywords: high-yield dairy cows, *Staphylococcus aureus*, antibiotic resistance, mastitis, antibiotics

In recent years, a significant increase in resistance of causative agents of acute and chronic bacterial infection to antibiotics has been observed worldwide. Two problems arise in the course of antibiotic therapy: increase in frequency of resistant microorganism strains isolation and the lack of antibiotics capable of inhibition of their development and spreading [1[. Between 2013 and 2015, the

sensitivity of microorganisms to fluoroquinolones has decreased by 27 %, the decrease in aminoglycosides group amounted to 42 %, in tetracyclines group — to 67 % [2-4].

Microbial resistance, i.e. resistance of a causative agent strain to one or several antibiotics, determines the effectiveness of the veterinary measures to a large extent. At that, only joint activities aimed at inhibition of increase in microbial resistance in each country will ensure positive results in solving this problem.

There are several levels of antibiotic resistance, i.e. global, regional and local [5]. Changing properties of strains and isolates of staphylococci, pneumococci, salmonellas and other opportunistic microorganisms isolated in humans and animals provide an example of rapid globalization of antibiotic resistance. *Staphylococcus aureus* is a typical representative of strains resistant to antibiotics, which is the main cause of mastitis and endometritis in high-yield dairy cows in 42-73 % of cases [6, 7]. Mastitis is one of the best understood diseases, but effectiveness of its management in the dairy farming is still low [8-12]. Economic losses due to mastitis include decrease in milk yield and quality, decline in milk production and early cow disposal (before completion of genetically predetermined lactation period and productive use term) [13, 14]. Mastitis also poses a hazard to human health due to possible presence of pathogens and their toxins in the milk [15-18].

Failure to take sanitary and hygienic measures, irregular screening of animals for latent mastitis due to significant labor and time expenditure required for diagnostics, and haphazard veterinary drug treatment regimens lead to development of local antibiotic resistance at a farm [19, 20[. Vaccines, chemotherapeutic drugs and antibiotics of various generations are currently used for prevention and treatment of mastitis in cattle [21-23].

Detoxicative antibiotic resistance of gram-positive bacteria, including *S. aureus,* is less effective due to absence of periplasmatic space in the cell wall [24]. All cells of the population may carry genetic information on resistance coded by *mecA* gene, but in vitro resistance phenotype is only observed in a few of them. This phenomenon is known as heteroresistance [25]. Effectiveness of clinical mastitis treatment depends on immune resistance of an animal, infection duration and bacterial genotypes of a causative agent [26]. For selection of a treatment regimen and mastitis prevention measures in cows it is important to study the mechanism of antibiotic resistance and spreading of infection caused by *S. aureus* [27-30].

Use of various drugs of natural origin, as well as virulent bacteriophages, is an alternative to antibiotic therapy [31]. The prospects of use of bacteriophages for treatment and prevention of mastopathy and genitourinary diseases in cows depend on safety of use, sanitizing activity of bacteriophages, improvement of microbial balance in vivo and general immune system reinforcement [32]. However, antibiotic therapy is currently a method of choice for treatment of mastitis. Evaluation of its effectiveness for a certain dairy herd, as well as on national level, requires studies of in vitro sensitivity of *S. aureus* strains to antibiotics.

Here we first reported findings focused on the range of antibiotic resistance in *S. aureus* strains associated with subclinical mastitis in high-yield dairy cows.

Our objective was to study the development of resistance in *Staphylococ aureus* strains isolated from milk of clinically healthy dairy cows during treatment with antibiotics of different groups.

Technique. The tests were performed in 2016 on clinically healthy high-yield holsteinized black-and-white cows (n = 1321), loose-housed at a farm in Kaluga region (the Central region of Russia) and preliminary vaccinated twice with anti-mastitis drug Mastivak (Ovejero, Spain).

Milk was sampled from each cow individually during milking. Salt Meat Broth (HiMedia Laboratories Pvt., Ltd, India) was inoculated with the milk samples at a ratio of 1:9, and kept at 37±1 °C for 18-24 houts. Then streak inoculation of differential diagnostic media was performed. Identification of *S. aureus* strains was performed according to the following criteria: evaluation of morphology and microscopy of the colonies grown on the Baird Parker Agar medium (HiMedia Laboratories Pvt., Ltd, India); the presence of hemolysis zones on the Azide Blood Agar Pronadisa medium (Conda, Spain) supplemented with 5 % defibrinated sheep blood; a positive coagulation of dry citrated rabbit plasma (ZAO EKOlab, Russia); biochemical characterization using microbiological media (The State Science Centre for Applied Microbiology & Biotechnology, Moscow region) and API 20 Staph panel (BioMerieux, France). Identified strains were stored in test tubes with Tryptic Soy Broth (Merck, Germany) and 30 % sterile glycerin at –18 °C.

The sensitivity of the isolated microorganisms to ten antibiotics (Pharmacotherapy Research Center, Russia) including penicillin (PEN, 10, ED), oxacillin (OX, 10 μg), gentamicin (GN, 10 μg), erythromycin (ER, 15 μg), lincomycin (LN, 15 μg), rifampicin (RF, 5 μg), ciprofloxacin (CP, 5 μg), vancomycin (VA, 30 μg), fuzidin (FZ, 10 μg) and novobiocin (NB, 5 μg) was determined by disk diffusion method [33]. The dishes with inoculated cultures were incubated under aerobic conditions at 37±1 °C for 18-24 hours. The sensitivity was evaluated by the diameters of the inhibition zones in accordance with the instructions of ZAO Research Centre of Pharmacotherapy (St. Petersburg, Russia). *S. aureus* ATCC 25923 (The Federal Culture Collection of Pathogen Microorganisms, Russia) was a reference strain for the control of sensibility determination.

In order to evaluate the effectiveness of antibiotic treatment strategy, the cows (n = 87) with pre-detected *S. aureus* content in milk were divided into groups. Antibiotics of the same class, responsible for pre-detected sensitivity of strains identified in the milk, were administered in each group in accordance with the regimen developed. The animals in which no *S. aureus* was detected during the repeated study were considered as cured.

Biometric processing was performed using Microsoft® Office Excel 2010, SAS 6.12 software (SAS Institute, USA). The diagrams depict percentage ratios of resistant *S. aureus* strains, the tables show average values (X) and error (x). The differences were considered statistically significant at $p < 0.05$.

Results. The study of 1321 cow with no clinical signs of mammary gland pathology has demonstrated the presence of 155 (in 11.7 % of the total number of animals) hemolytic strains identified as *S. aureus*. Of 155 strains studied, 104 ones (67.1 %) were resistant to one or several antibiotics (Table 1).

1. Distribution (%) of antibiotic resistance among *Staphylococcus aureus* strains (n = 155) isolated from milk of clinically healthy holsteinized black-and-white dairy cows (X±x, Kaluga region, 2016)

Antibiotics	Dose	Resistant	Moderately resistant	Sensitive
NB	5 μg/disk	49.7±4.0	–	50.3±4.0
PEN	5 ED/disk	32.3±3.8	27.7±3.6	40.0±3.9
LN	15 μg/disk	30.3±3.7	29.0±3.6	40.7±3.9
FZ	10 μg/disk	27.1±3.6	40.0±3.9	32.9±3.8
OX	10 μg/disk	25.2±3.5	7.1±2.1	67.7±3.8
GN	10 μg/disk	18.1±3.1	–	81.9±3.1
ER	15 μg/disk	14.2±2.8	43.9±4.0	41.9±4.0
CP	5 μg/disk	11.6±2.6	34.2±3.8	54.2±4.0
RF	5 μg/disk	8.4±2.2	5.1±1.8	86.5±2.7
VA	30 μg/disk	2.6±1.3	–	97.4±1.3

Note. NB — novobiocin, PEN — penicillin, LN — lincomycin, FZ — fucidin, OX — oxacillin, GN — gentamicin, ER — erythromycin, CP — ciprofloxacin, RF — rifampicin, VA — vancomycin. Dashes mean absence of moderately resistant strains.

The majority of the strains were resistant to novobiocin (49.7 %) while the smallest part (2.6 %) exhibited resistant to vancomycin (Fig. 1).

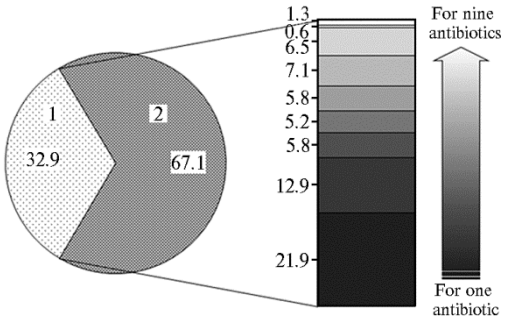


Fig. 1. Distribution (%) **of** ***Staphylococcus aureus*** strains (n = 155) **strains isolated from milk of clinically healthy holsteinized black-and-white cows dairy cows, by antibiotic resistance.** Distribution of the strains sensitive (1) and resistant to at least one of the tested antibiotic (2) is shown on the left (circle diagram); distribution of strains by resistance to various number of antibiotics in increasing order (from the bottom up) is shown on the diagram on the right (Kaluga region, 2016).

The presence of antibiotic resistance to novobiocin and vancomycin, new generation drugs that are widely used in modern medicine, should be particularly noted. Gentamicin (81.9 %), rifampicin (86.5 %), and vancomycin (97.4 %) had the greatest effectiveness among the antibiotics tested.

According to the detailed analysis of antibiotic resistance of isolated strains (see Fig. 1), 45.2 % were resistant to two and more drugs. Interestingly, the percentage of strains resistant to five and more antibiotics was relatively high (21.3 % of the total tested number).

Four groups of cows (n = 87) resulted from the evaluation of therapy effectiveness according to the classes of antibiotics used: aminoglycosides (group I, n = 26, gentamicin), macrolides (group II, n = 22, erythromycin), ansamycins (group III, n = 12, rifampicin), penicillins (group IV, n = 27, amoxycillin and penicillin). Rifampicin application was the most effective (91.7 % animals cured) whereas gentamicin showed the lowest effectiveness (53.8 %) (Table 2).

2. Comparative effectiveness of various groups of antibiotics in treatment of holsteinized black-and-white dairy cows with ***Staphylococcus aureus*** **found in milk** (Kaluga region, 2016)

Parameter	Antibiotic group				
	aminoglycosides	macrolides	ansamycins	penicillins	
Cow group	I	II	III	IV	
Number of cows, n	26	22	12	27	
Antibiotic	Gentamicin	Erythromycin	Rifampicin	Amoxycillin	Penicillin
Dose	3 mg/kg body weight	20 ml/animal	0.2 ml/kg body weight	0.1 ml/kg body weight	1 ml/25 kg body weight
Application	3 times (every 24 hours)	3 times (every 24 hours)	4 times (two times daily, every 24 hours)	2 times (every 48 hours)	2 times (every 24 hours)
Cows cured, %	53.9±9.8[a]	59.1±10.5[b]	91.7±8.0[a, b, c, d]	63.0±9.3[c]	53.9±9.6[d]

N o t e. The differences between the groups marked with similar letters are significant: at $p < 0.01$ for [a, d], at $p < 0.05$ for [b, c].

Isolates form milk of group I and II cows were obtained after treatment and tested with regard to antibiotic resistance in order to identify possible reasons of negative antibiotic treatment effect. We have discovered a tendency to polyresistance in such newly isolated *S. aureus* strains (Fig. 2), but the changes in resistance to the active substance of the drug that was used in a corresponding treatment regime were not observed. The total resistance was 22.3 % higher after erythromycin and 17.7 % higher after gentamicin treatment (see Fig. 2).

In recent years attempts were made to develop new complex antibiotics for treatment of clinical and subclinical forms of mastitis in cattle during various lactation periods. Russian patents for invention, conforming positive results of treatment, have been obtained. However, several researchers have studied anti-

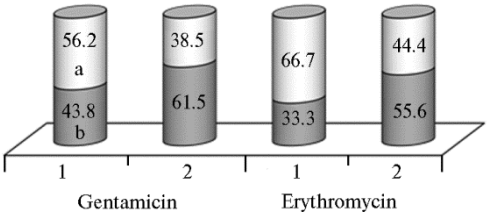


Fig. 2. The proportion (%) of *Staphylococcus aureus* strains isolated from milk of holsteinized black-and-white dairy cows, which are resistant and moderately resistant (a) **or sensitive** (b) **to gentamicin and erythromycin:** 1 — before treatment, 2 — after treatment (Kaluga region, 2016). See the treatment regime in Table 2.

biotic resistance development upon performance of therapeutic activities and evaluated the development of resistance to various antibiotic groups [34, 35]. The solution of this problem must be an integral part of effective programs for mastitis prevention in high-yield dairy cows.

Thus, the studies of *Staphylococcus aureus* isolates from milk of clinically healthy cows have shown a relatively high proportion of the strains characterized by resistance to one and more antibiotics. Additionally, we have found development of polyresistance against antibiotics used for treatment of clinical mastitis. For improving effectiveness of treating mastitis in high-yield dairy cows, consistent monitoring of antibiotic resistance in *S. aureus* persistent in the herds is required in order to prevent spreading strains from local (farm) to regional level.

REFERENCES

1. Tanzin T., Nazir K.H.M.N.H., Zahan M.N., Parvej M.S., Zesmin K., Rahman M.T. Antibiotic resistance profile of bacteria isolated from raw milk samples of cattle and buffaloes. *Journal of Advanced Veterinary and Animal Research*, 2016, 3(1): 62-67 (doi: 10.5455/javar.2016.c133).
2. Shchepetkina S.V. *Sel'skokhozyaistvennye vesti*, 2016, 2: 55-57 (in Russ.).
3. Thaker H.C., Brahmbhatt M.N., Nayak J.B. Isolation and identification of *Staphylococcus aureus* from milk and milk products and their drug resistance patterns in Anand, Gujarat. *Veterinary World*, 2013, 6(1): 10-13 (doi: 10.5455/vetworld.2013.10-13).
4. Anueyiagu K.N., Isiyaku A.W. Isolation, identification of *Staphylococcus aureus* from bovine milk and its antibiotics susceptibility. *International Journal of Livestock Production*, 2015, 6(6): 74-77 (doi: 10.5897/IJLP2015.0248).
5. Lysenko V.A., Orlova E.V., Litvinova T.I., Babich M.V. *Byulleten' fiziologii i patologii dykhaniya*, 2004, 18: 17-20 (in Russ.).
6. Talukder A.A., Rahman H.H., Jamil Mahmud S.M., Alam F., Dey S.K. Isolation, identification and resistance pattern of microorganisms associated with mastitis in Buffalo. *J. Microbiol.*, 2013, 30(1-2): 1-5.
7. Sayed R.H., Salama S.S., Soliman R.T. Bacteriological evaluation of present situation of mastitis in dairy cows. *Global Veterinaria*, 2014, 13(5): 690-695.
8. Hussain R., Javed M.T., Khan A., Muhammad G. Risks factors associated with subclinical mastitis in water buffaloes in Pakistan. *Trop. Anim. Health Prod.*, 2013, 45: 1723-1729 (doi: 10.1007/s11250-013-0421-4).
9. Srinivasan P., Jagadeswaran D., Manoharan R., Giri T., Balasubramaniam G.A., Balachandran P. Prevalence and etiology of subclinical mastitis among buffaloes in Namakkal, India. *Pakistan J. Biol. Sci.*, 2013, 16: 1776-1780.
10. Kryukov N.I. *Effektivnoe zhivotnovodstvo*, 2016, 2(123): 22-24 (in Russ.).
11. Modin A.N., Klimov N.T., Efanova L.I. *Zootekhniya*, 2010, 10: 27-28 (in Russ.).
12. Abeer A.M., Zakia A.M., Muna E.A., Sabiell Y.A. Bacteriological and pathological studies of mammary glands affections in camels (*Camelus dromedarius*) at Tumbool Abattoir, Sudan. *British Microbiology Research Journal*, 2016, 15(5): 1-8 (doi: 10.9734/BMRJ/2016/25966).
13. Ashfaq M., Razzaq A., Shamsheer-ul-Haq, Muhammad G. Economic analysis of dairy animal diseases in Punjab: a case study of Faisalabad district. *J. Anim. Plant Sci.*, 2015, 25(5): 1482-1495.
14. Singh D., Kumar S., Singh B., Bardhan D. Economic losses due to important diseases of bovines in central India. *Veterinary World*, 2014, 7(8): 579-585 (doi: 10.14202/vetworld.2014.579-585).
15. Rola J.G., Czubkowska A., Korpysa-Dzirba W., Osek J. Occurrence of *Staphylococcus aureus* on farms with small scale production of raw milk cheeses in Poland. *Toxins*,

2016, 8(3): 62 (doi: 10.3390/toxins8030062).
16. Hennekinne J.A., de Buyser M.L., Dragacci S. *Staphylococcus aureus* and its food poisoning toxins: characterization and outbreak investigation. *FEMS Microbiol. Rev.*, 2012, 36: 815-836 (doi: 10.1111/j.1574-6976.2011.00311.x).
17. Rola J.G., Korpysa-Dzirba W., Osek J. Prevalence of *Staphylococcus aureus* and staphylococcal enterotoxins at different stages of production of raw milk cheeses — preliminary results. *Bulletin of the Veterinary Institute in Pulawy*, 2013, 57: 341-345 (doi: 10.2478/bvip-2013-0059).
18. Bianchi D.M., Gallina S., Bellio A., Chiesa F., Civera T., Decastelli L. Enterotoxin gene profiles of *Staphylococcus aureus* isolated from milk and dairy products in Italy. *Lett. Appl. Microbiol*, 2014, 58: 190-196 (doi: 10.1111/lam.12182).
19. Tassew A., Negash M., Demeke A., Feleke A., Tesfaye B., Sisay T. Isolation, identification and drug resistance patterns of methicillin resistant *Staphylococcus aureus* from mastitis cow's milk from selected dairy farms in and around Kombolcha, Ethiopia. *J. Vet. Med. Anim. Health*, 2016, 8(1): 1-10 (doi: 10.5897/JVMAH2015.0422).
20. Hezil N., Baazize-Ammi D., Kebbal S., Saadaoui R., Brahim E.M., Guetarni D. Principal germs causing clinical mastitis in dairy cattle farms in governorate of Blida (Algeria). *Journal of Animal Science Advances*, 2013, 3(1): 19-26
21. Sankar P. New therapeutic strategies to control and treatment of bovine mastitis. *Vet. Med. Open J.*, 2016, 1(2): e7-e8 (doi: 10.17140/VMOJ-1-e004).
22. Gomes F., Henriques M. Control of bovine mastitis: old and recent therapeutic approaches. *Curr. Microbiol.*, 2016, 72: 377-382 (doi: 10.1007/s00284-015-0958-8).
23. Sun H., Xue F., Qian K., Zhang X., Yin Z. Intramammary expression and therapeutic effect of a human lysozyme-expressing vector for treating bovine mastitis. *J. Zhejiang Univ. - Sci. B*, 2006, 7: 324-330 (doi: 10.1631/jzus.2006.B0324).
24. Supotnitskiy M.V. Mechanisms of antibiotics resistance in bacteria. *Biopreparats (Biopharmaceuticals)*, 2011, 2: 4-11.
25. Al-Ashmony A.L., Al-Sawy A.A.F., Torky H.A. Genotypic molecular detection of certain genes encoding virulence determinates and atibiotic resistance in *Staphylococcus aureus* isolates from mastitis cows. *Alexandria Journal of Veterinary Sciences (AJVS)*, 2016, 49(2): 90-98 (doi: 10.5455/ajvs.230403).
26. Maram A., Mamu G., Birhanu T. Prevalence and antibiotic resistance of *Staphylococcus aureus* mastitis in Holeta area, Western Ethiopia. *Global Veterinaria*, 2016, 16(4): 365-370.
27. Villanueva San Martin M., Jousselin A., Baek K.T., Prados J., Andrey D.O., Renzoni A., Ingmer H., Frees D. Rifampicin resistance *rpoB* alleles or multicopy thioredoxin/thioredoxin reductase suppresses the lethality of disruption of the global stress regulator spx in *Staphylococcus aureus. J. Bacteriol.*, 2016, 198(19): 2719-2731 (doi: 10.1128/JB.00261-16).
28. Jousselin A., Manzano C.S., Biette A., Reed P., Pinho M.G., Rosato A.E., Kelley W.L., Renzoni A.M. The *Staphylococcus aureus* chaperone prsA is a new auxiliary factor of oxacillin resistance affecting penicillin-binding protein 2A. *Antimicrob. Agents Chemother.*, 2016, 60(3): 1656-1666 (doi: 10.1128/AAC.02333-15).
29. Artem'eva O.A., Pereselkova D.A., Fomichev Yu.P. Dihydroquercetin, the bioactive substance, to be used against pathogenic microorganisms as an alternative to antibiotics. *Agricultural Biology*, 2015, 50(4): 513-519 (doi: 10.15389/agrobiology.2015.4.513eng) (in Engl.).
30. Artem'eva O.A., Pereselkova D.A., Vinogradova I.V., Kotkovskaya E.N., Gladyr' E.A., Sivkin N.V., Zinovieva N.A. Screening of dairy cows' herd for presence in milk of hemolytic microorganisms in relation to somatic cell content. *Agricultural Biology*, 2015, 50(6): 810-816 (doi: 10.15389/agrobiology.2015.6.810eng) (in Engl.).
31. Mašlaňová I., Doška J., Varga M., Kuntová L., Mužík J., Malúšková D., Růžičková V., Pantůček R. Bacteriophages of *Staphylococcus aureus* efficiently package various bacterial genes and mobile genetic elements including SCC*mec* with different frequencies. *Environ. Microbiol. Reports*, 2013, 5: 66-73 (doi: 10.1111/j.1758-2229.2012.00378.x).
32. Verstappen K.M., Tulinski P., Duim B., Fluit A.C., Carney J., van Nes A. The effectiveness of bacteriophages against methicillin-resistant staphylococcus aureus ST398 nasal colonization in pigs. *PLoS ONE*, 2016, 11(8): e0160242 (doi: 10.1371/journal.pone.0160242).
33. *Opredelenie chuvstvitel'nosti mikroorganizmov k antibakterial'nym preparatam. Metodicheskie ukazaniya. MUK 4.2.1890-04* (*utv. Glavnym gosudarstvennym sanitarnym vrachom RF 04.03.2004*) [Assay of the sensitivity of microorganisms to antibacterial agents — guidelines (approved by Chief State Sanitary Physician of the Russian Federation, March 4, 2004]. Available http://docs.cntd.ru/document/1200038583 [No date] (in Russ.).
34. Komarov V.Yu., Belkin B.L. *Izvestiya Orlovskogo GAU*, 2015, 3(53): 100-102 (in Russ.).
35. Komarov V.Yu., Belkin B.L. *Vestnik Altaiskogo gosudarstvennogo agrarnogo universiteta*, 2015, 5(127): 107-110 (in Russ.).

Events

26th INTERNATIONAL CONFERENCE OF THE WORLD ASSOCIATION FOR THE ADVANCEMENT OF VETERINARY PARASITOLOGY (WAAVP 2017)

(4-8 September 2017, Kuala Lumpur, Malaysia)

Organization: Malaysian Society of Parasitology & Tropical Medicine (MSPTM) and Department of Veterinary Services (DVS)

Disciplines: Life Science, Health Science

The World Association for the Advancement of Veterinary Parasitology (WAAVP) is the largest international veterinary parasitology organization in the world, which is affiliated with the World Veterinary Association, cooperates with various national, international and affiliated organizations, such as WHO and FAO, in all matters concerning veterinary parasitology. WAAVP conferences are held bi-annually in various major cities around the world for the past 52 years.

The Conference theme is: Combating Zoonoses: Strength in East–West Partnerships.

The Conference provides an opportunity for scientists who study the parasites of animals, encompassing helminthology, protozoology and entomology to meet with each other to discuss the matters of common interest and benefit from a comprehensive and ambitious scientific programme designed by the organising committee, which will include presentations by a range of knowledgeable speakers from within and beyond the region.

The Conference will also provide maximum opportunity for discussion, networking and informal engagement to promote exchange of information and material amongst researchers, veterinary practitioners and animal health investigators. It is intended that a number of priority recommendations and strategies will emerge and that these will be endorsed by the Conference for intended action by individual member states.

Contacts: http://www.waavp2017kl.org/

Information: http://www.globaleventslist.elsevier.com/events/2017/09/waavp-2017-the-26th-international-conference-of-the-world-association-for-the-advancement-of-veterinary-parasitology/

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 875-882
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 619+61]:615.284:615.45:616-098

doi: 10.15389/agrobiology.2016.6.875rus
doi: 10.15389/agrobiology.2016.6.875eng

DERIVATIVES OF 16-MEMBERED MACROCYCLIC LACTONES: ANTIPARASITIC PROPERTIES AND INTERACTION WITH $GABA_A$ RECEPTORS

M.Kh. DZHAFAROV[1], F.I. VASILEVICH[1], G.I. KOVALEV[1, 2], K.S. KRIVONOS[1], I.I. TSEPILOVA[1], I.V. ZAVARZIN[3], E.V. VASIL'EVA[2]

[1]*Moscow State Academy of Veterinary Medicine and Biotechnology—K.I. Skryabin Moscow Veterinary Academy,* 23, ul. Akademika Skryabina, Moscow, 109472 Russia, e-mail mxd123@mail.ru;
[2]*V.V. Zakusov Research Institute of Pharmacology,* Federal Agency of Scientific Organizations, 8, ul. Baltiiskaya, Moscow, 125315 Russia, e-mail kovalev@academpharm.ru;
[3]*N.D. Zelinsky Institute of Organic Chemistry,* Federal Agency of Scientific Organizations, 47, Leninskii prosp., 119991 Russia, e-mail zavi@ioc.ac.ru
Acknowledgements:
Supported by Russian Science Foundation, grant № 15-16-00019
Received June 8, 2016

Abstract

Searching for antiparasitics with a different mode of action than existing drugs, and (or) with the same but much more effective mechanisms is necessary to periodically update the applicable protection chemicals. For the first time we here present data on the biocidal action of new semisynthetic derivatives of avermectin B1 that we have synthetized earlier. These are the 16-membered macrocyclic lactones, the representatives of an important class of anthelmintics. In 2015 S. Omura (Japan) and W. Campbell (USA) who discovered this avermectin group, were awarded the Nobel Prize in physiology and medicine. In our study the oligochaetes *Tubificidal tubifex* were used as a test-object. The original chemicals and synthetized derivatives tested were avermectin B1 (abamectin), ivermectin, monosaccharide analogues of abamectin and ivermectin, namely abamectin, ivermectin, 5-O-succinyl avermectin B1, methyl ester of 5-O-succinyl avermectin B1, ethyl ester of 5-O-succinyl avermectin B1, diethyl ester of 5,4″-di-O-succinyl avermectin B1, ethyl ester of 5-O-malonyl avermectin B1, diethyl ester of 5,4″-di-O-dimalonyl avermectin B1, monosaccharide hemisuccinate of avermectin B1 (5-O-succinyl-4′-dezoleandrozyl-4′-hydroxyavermectin B1), ethyl ester of 5-O-succinyl-4-O-chloroacetyl avermectin B1, 5-O-succinyl ivermectin, ethyl ester of 5-O-succinoyl ivermectin, 5,4″-di-O-succinyl ivermectin, diethyl ester of 5,4″-di-O-succinyl-ivermectin, ethyl ester of 5-O-malonylivermectin, diethyl ester of 5,4″-di-O-dimalonyl ivermectin, monoavermectin-5-yl ester of 4-[2-(4-nitrophenyl)-2-oxoethoxy]-4-oxobutanoic acid, monoavermectin-5-yl ester of 4-[2-(4-chlorophenyl)-2-oxoethoxy]-4-oxobutanoic acid, monoavermectin-5-yl ester of 4-[(4-nitrophenyl)-methoxy]-butanoic acid, monoavermectin-5-yl ester of 4-[1-methyl-2-(4-methylphenyl)-2-oxoethoxy]-4-oxobutanoic acid, monoavermectin-5-yl ester of 4-[2-(4-chlorophenyl)-1-methyl-2-oxoethoxy]-4-oxobutanoic acid, monoavermectin-5-yl ester of 4-[3-chloro-1-(4-chlorbenzoil)-propoxy]-4-oxobutanoic acid, monoavermectin-5-yl ester of 4-{2-[(4-methylphenyl)-amino]-2-oxoethoxy}-4-oxobutanoic acid and monoavermectin-5-yl ester of 4-{2-[(4-bromophenyl)-amino]-2-oxoethoxy}-4-oxobutanoic acid. The acute toxicity (LD_{50}) of the most effective ones, 5-O-succinyl avermectin B1, 5-O-ethylsuccinyl avermectin B1 and 5,4″-di-O-ethylsuccinyl avermectin B1, for intraperitoneally challenged white mice was 37.85; 41.37 and 45.82 mg/kg, respectively. We also used membrane preparations of rat brain as in vitro model for screening and studying activity of natural and semi-synthetic avermectins. A radioligand [G-3H]SR 95531 binding assay of avermectin B1, ivermectin, and 5-O-succinyl avermectin B1 interaction with GABA-receptors (the biotargets for these compounds) showed a 30 % increase of maximal inhibition (Imax) of specific binding by hemisuccinate derivative of avermectin B1 when compared to original avermectin B1.

Keywords: 16-membered macrocyclic lactones, avermectins, avermectin monosaccharides, 5-O-succinyl avermectin B1, 5-O-ethylsuccinyl avermectin B1, 5,4″-di-O-ethylsuccinyl avermectin B1, antiparasitics, oligochaeta *Tubifex tubifex*, $GABA_A$-receptor, radioligand binding assay

Chemotherapy and prophylaxis remain among the most effective and cheap methods of controlling parasites. The most important classes of anthelmintics are benzimidazoles, imidazoles and thiazoles, pyrazine isoquinolines,

macrocyclic lactones [1], especially 16-membered macrocyclic lactones with a broad spectrum of the antiparasitic activity. Adaptation of parasites (the development of resistance) to the applied aagents, and focus on high-performance and environmentally safer remedies promote the search for substances with both a novel mechanism of antiparasitic action and a known, but more effective one [2].

The 16-membered macrocyclic lactones (avermectins and other, similar in structure, natural macrolides called milbemycins or their semi-synthetic analogues) are widely used to control parasites (nematodes, insects and mites) in humans, animals and plants. For the discoveries of a novel avermectin-based therapy of infections caused by roundworm parasites, S. Omura (Japan) and W. Campbell (USA) were awarded 2015 Nobel Prize in Physiology or Medicine [3].

Evolution of pharmaceutical substances based on macrocyclic lactones suggests in particular the chemical modification of the natural metabolites and derivation of analogues that are the most suitable for practical purposes (Fig. 1) [4-8].

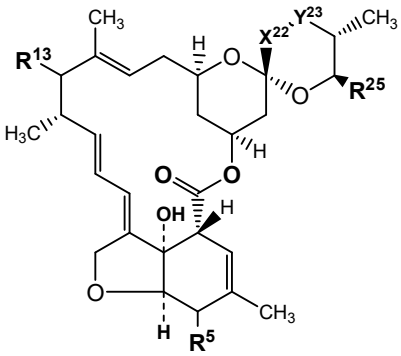


Fig. 1. The general formula of natural and semi-synthetic avermectins: R^5 — a hydroxyl group or a modified functional group; R^{13} — L-oleandrosyl-L-oleandroside residue; R^{25} — various hydrocarbon radicals [2].

Various avermectins and related milbemycin-based substances have been introduced in the medical, veterinary and agronomic practices, such as a mixture of avermectin B1a and B1b (abamectin, 1979), ivermectin (1981), doramectin (1993), avermectin B1 benzoate (1997), eprinomectin (1997), and selamectin (2000), as well as close to them Milbemectin, a mixture of A3 (a3) and A4 (a4) milbemycins (1990), lepimectin (a derivative of milbemectin, 2004), a derivative of nemadectin (Moxidectin, 1989), and Gemax [2, 4]. Antiparasitic action of all these macrolides is due to the pharmacophore group, i.e. a unique 16-membered lactone [2], specifically interacting with glutamate- [9] and GAB_A-dependent Cl^--ion channels [10].

Most studies on the mechanism of avermectins' action, i.e. membrane hyperpolarization resulted from activation of glutamate- and $GABA_A$-dependent channels, were made with ivermectin, as the most frequent in practice [11-14]. In general, findings of such studies are applicable to all members of this class, although lactones are not similar in structure, and their effect may vary [15-17].

In the present study, we first report biocidal action of new semi-synthetic avermectin B1 derivatives on oligochaetes. These findings allowed to select several promising compounds, such as 5-O-succinyl avermectin B1, 5-O-ethylsuccinyl avermectin B1 and 5,4″-di-O-ethylsuccinyl avermectin B1. A decrease in toxicity (LD_{50} of 37.85, 41.37 and 45.82 mg/kg, respectively) was revealed compared to avermectin B1 (15-20 mg/kg) [18]. We compared avermectin B1, ivermectin and 5-O-succinyl avermectin B1 interaction with $GABA_A$-receptors, which are a biotarget for this class of compounds, using rat brain membrane preparations as a model for in vitro screening and evaluation of natural and semi-synthetic avermectins. It was revealed that maximal inhibition of specific binding for these synthesized derivatives, estimated by radioligand binding assay, increased.

The aim of this work was to study biocidal properties of the semi-

synthetic derivatives of 16-membered macrocyclic lactones and their interaction with the $GABA_A$-receptors.

Technique. The 5-O- and 5,4″-di-O-derivatives of avermectin B1, ivermectin, and monosaccharide analogues have been previously synthesized [2, 19]. Their antiparasitic activity at a dose of 15 μg/ml was studied in a rapid assay using *Tubifex tubifex* oligochaetes. Acute toxicity of 5-O-succinyl-, 5-O-ethylsuccinyl-, 5,4″-di-O-ethylsuccinyl avermectins B1 was determined according to the recommendations on preclinical studies of drugs using white outbred mice weighing 18-21 g (the drug was administered intraperitoneally) [20].

For the radioreceptor assay, avermectin B1, ivermectin and a hemisuccinate derivative of avermectin B1 were dissolved in dimethylsulfoxide (DMSO). In vitro effects of the substances on the $GABA_A$-receptors were investigated at the range of concentrations of 10^{-10}-10^{-4} mol/l.

Membrane preparations containing $GABA_A$-receptors of rat cerebral cortex were isolated by a modified method of J.E. Hawkinsonet al. [21]. Biomaterial was taken after decapitation. Frontal cortex was separated and homogenized (a Potter S homogenizer, Sartorius AG, Germany) in 20 volumes of ice-cold sucrose (0.32 M, pH 7.1). A dense fraction was separated using the Optima L-70K ultracentrifuge (Beckman Coulter, Inc., USA) for 10 min at 1 000 g. The supernatant was re-centrifuged (20 000 g, 20 min). The precipitate was resuspended in 20 ml of cold distilled water and centrifuged (8 000 g, 20 min), the supernatant and the yellow supernatant were centrifuged once again at 48 000 g for 20 min. The precipitate was suspended in a freshly prepared 0.05M Tris-citrate buffer (TCB) (pH 7.1) and centrifuged (48 000 g, 20 min). The resulting membrane fraction was frozen and stored at –85 °C. On the day of testing, the membranes were suspended in 40 volumes of 0.05M TCB (pH 7.1) and centrifuged at 48 000 g for 20 min. The resulting precipitate was suspended in 40 volumes of 0.05M TCB (pH 7.1), incubated at 24 °C for 30 min, and centrifuged again at 48 000 g for 20 min. The final precipitate was resuspended in the fresh buffer.

Tritium-labeled [G-^{3}H]SR 95531 (Perkin Elmer, USA) with a specific activity of 49.5 Ci/mmol was used in radioligand binding assays. The incubation mixture (0.5 ml final volume) contained 50 μl of [G-^{3}H]SR 5531, 250 μl of buffer and 200 μl of membrane protein suspension; for non-specific binding, 50 μl of unlabeled SR 95531 ligand (1 mM) was added. The reaction mixture was incubated at 4 °C for 1 hour. After incubation samples were passed through the GF/B glass fiber filters (Whatman PLC, UK) previously immersed in 0.3 % polyethylenimine for 2 hours. Each tube was washed twice with cold buffer and then the filters were washed twice with the same volume of buffer. The filters were dried out in air, transferred to scintillation vials and poured over with 5 ml of scintillation liquid (4 g of PPO, 0.2 g of POPOP per liter of toluene). Radioactivity was determined on a Tri-Carb 2900TR counter (Perkin Elmer, USA) with 42-46 % counting efficiency . Specific binding was calculated as the difference between total and non-specific binding. The results of radioligand binding assay were expressed as the concentration corresponding to 50 % inhibition of specific radioligand binding (IC_{50}) and the maximal degree of inhibition (I_{max}), reflecting the difference between the lower and upper plateau [21]. Protein concentration was measured by a standard procedure [22].

Animal experiments were performed in accordance with the provisions of the Geneva Convention and the principles of Good Laboratory Practice (National Standard of the Russian Federation, GOST R 53434-2009), as well as recommendations set out in The Guide for the Care and Use of Laboratory Animals (Na-

tional Academy Press Washington, DC 1996).

GraphPad Prizm 4 Demo (https://www.graphpad.com, GraphPad Software, Inc., USA) was used to process binding displacement curves. Statistical processing was performed using Statistica 6 software package (http://www.statsoft.ru, Statsoft Inc., USA). The arithmetic mean (*m*) and standard error of the mean (SEM) are given, the significance of differences was assessed by the Student's *t*-test at $p < 0.05$.

Results. We examined the biological effects of the synthesized 5-O- and 5-O-4″-O-derivatives as well as ivermectin and a number of monosaccharide analogues derived previously (Table 1).

1. A qualitative comparison of the biocidal action of the derived 16-membered lactones (concentration of 15 μg/ml) on *Tubifex tubifex* oligochaetes

Substance	Time, min		
	30	60	180
Abamectin (control)	+++	++++	++++
Ivermectin	+++	++++	++++
Tap water (control)	0	0	0
Tap water:isopropanol 9:1 (control)	0	0	0
5-O-succinyl avermectin B1	++	+++	++++
Methyl ester of 5-O-succinyl avermectin B1	++	+++	++++
Ethyl ester of 5-O-succinyl avermectin B1	++	+++	++++
Diethyl ester of 5,4″-di-O-succinyl avermectin B1	++	+++	++++
Ethyl ester of 5-O-Malonyl avermectin B1	++	+++	++++
Diethyl ester of 5,4″-di-O-Dimalonate avermectin B1	++	+++	++++
Hemisuccinate derivative of avermectin B1 monosaccharide (5-O-succinyl-4'-Desoleandrosyl-4'-Hydroxy avermectin B1)	++	+++	++++
Ethyl ester of 5-O-Hemisuccinyl-4″-O-Chloroacetyl avermectin B1	++	+++	++++
5-O-Hemisuccinate ivermectin	++	+++	++++
Ethyl ester of 5-O-Hemisuccinate ivermectin	++	+++	++++
5,4″-di-O-succinyl ivermectin	++	+++	++++
Diethyl ester of 5,4″-di-O-succinyl ivermectin	++	+++	++++
Ethyl ester of 5-O-Malonyl ivermectin	++	+++	++++
Diethyl ester of 5,4″-di-O-Malonyl ivermectin	++	+++	++++
Monoavermectin-5-yl ester 4-[2-(4-Nitrophenyl)-2-oxoethoxy]-4-oxobutanoic acid	++	+++	++++
Monoavermectin-5-yl ester 4-[2-(4-Chlorophenyl)-2-oxoethoxy]-4-oxobutanoic acid	+++	+++	++++
Monoavermectin-5-yl ester 4-[(4-Nitrobenzyl)-methoxy]-4-oxobutanoic acid	++	+++	++++
Monoavermectin-5-yl ester 4-[1-methyl-2-(4-Methylphenyl)-2-oxoethoxy]-4-oxobutanoic acid	++	+++	++++
Monoavermectin-5-yl ester 4-[2-(4-chorophenyl)-1-methyl-2-oxoethoxy]-4-oxobutanoic acid	++	+++	++++
Monoavermectin-5-yl ester 4-[3-chloro-1-(4-chlorobenzoyl)-propoxy]-4-oxobutanoic acid	++	+++	++++
Monoavermectin-5-yl ester 4-{2-[(4-methylphenyl)-amino]-2-oxoethoxy}-4-oxobutanoic acid	+++	+++	++++
Monoavermectin-5-yl ester 4-{2-[(4-bromophenyl)-amino]-2-oxoethoxy}-4-oxobutanoic acid	++	+++	++++

N o t e. 0 — no action; "+" — paralysis of < 50 % of individuals; "++" — paralysis of 50-60 % of individuals; "+++" — paralysis of 60-80 % of individuals; "++++" — paralysis of 80-100 % of individuals.

Antiparasitic activity parameters of the compounds we selected (see Table 1) evidenced a strong biocidal action: after a 30-minute exposure of oligochaetes to the 1.5 % isopropanol solution of the tested compounds (15 μg/ml), half of the specimens lost their motor activity (see Table 1). Three of the tested compounds (5-O-succinyl-, 5-O-ethylsuccinyl- and 5,4″-di-O-succinyl avermectin B1) were selected for the acute toxicity evaluation.

LD_{50} of 5-O-succinyl avermectin B1 for mice was 37.85 mg/kg, and LD_{100} was 70.35 mg/kg, while of 5-O-succinyl avermectin B1 these were 41.37 and 74.27 mg/kg, respectively, and of 5,4″-di-O-ethylsuccinyl avermectin B1 — 45.82 and 78.23 mg/kg, respectively. These values are comparable to LD_{50} for currently used avermectin-based products, such as 20 and 30 mg/kg for abamectin and ivermectin [18].

The clinical manifestations of the toxic effects of the compounds were similar (muscle tremors, convulsions). All animals demonstrated discoordination of movements followed by strong depression: mice were lying, reactions to external stimuli were absent, and the death occurred within 20-30 min. Autopsy of the dead mice and the animals sacrificed at the end of the experiments showed no visible pathological changes. Therefore, according to the regulations of the

State Standard GOST 12.1.007-76, these compounds can be regarded as substances of toxicity class II.

In vitro study of the influence on $GABA_A$-receptors showed that unlabeled SR 95531, the bromic 2-(3-carboxy prolyl)-3-amino-6- (4-methoxyphenyl)pyridazine (i.e. gabazine, a competitive antagonist of $GABA_A$-receptors), almost completely replaced [G-^{3}H]SR 95531 actively in the binding sites (IC_{50} of 145 nM). Avermectin B1 under the same conditions inhibited the binding of [G-^{3}H]SR 95531 only partially (I_{max} 22.0±1.2 %) (Fig. 2, Table 2).

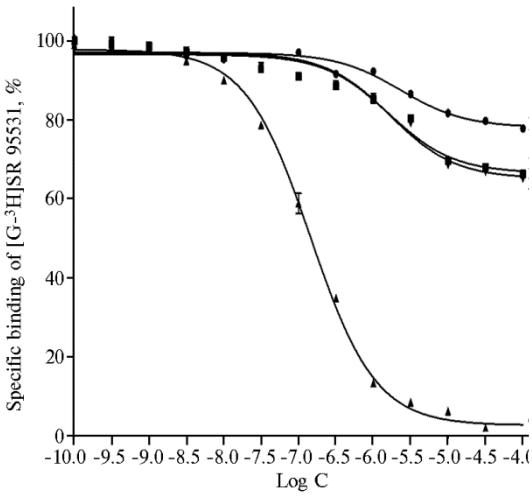


Fig. 2. Radioligand replacement with unlabelled SR 95531 and avermectins at in vitro binding [G-^{3}H]SR 95531 to membranes of rat cerebral cortex: 1 — avermectin B1; 2 — ivermectin; 3 — a hemisuccinate derivative of avermectin B1; 4 — SR 95531; Log C — logarithm of the molar concentration of the compound.

A hemisuccinate of avermectin B1, i.e. an original derivative of avermectin B1, and ivermectin were shown to be more active inhibitors. Their I_{max} increased by one third (up to 35.9±1.4 and 33.5±1.4 %, respectively; $p < 0.05$). However, all three studied substances had similar IC_{50} values in the micromolar range (Table 2).

2. Efficacy of avermectins in replacement of [G-^{3}H] SR 95531 specifically bond to membrane receptors of rat cerebral cortex (*m*±SEM)

Compound	IC_{50}, μmol/l	I_{max}, %
Avermectin B1	2.31±0.18	22.0±1.2
Ivermectin	1.61±0.14	33.5±1.4
Hemisuccinate of avermectin B1	1.69±0.13	35.9±1.4

N o t e. IC_{50} — the concentration that causes 50 % inhibition of specific radioligand binding; I_{max} — the highest inhibition of ligand binding, which reflects the difference between the upper and lower plateau.

The results suggest that chemical modification of the avermectin B1 molecule significantly increased the competitive activity of the original compound, i.e. 5-O-succinyl avermectin, for the binding sites of [G-^{3}H]SR 95531 with $GABA_A$-receptors. The use of labelled antibodies against GABA in the experiments with nematodes showed that 26 of 302 neurons in *Caenorhabditis elegans* were GABA-ergic [23], and genetic screening allowed to identify six genes which are required to perform the neural functions of GABA [24].

It is assumed that all three avermectins act as allosteric modulators of a specific $GABA_A$-receptor competitive ligand SR 95531 (gabazine) [25]. This is a GABA-derivative of arilaminopyridazine, which is a competitive receptor antagonist in mammals, whereas in invertebrates the substance SR 95103, i.e. 2-(3-carboxy prolyl)-3-amino-4-methyl-6-phenylpyridazine, is more active [26]. Recent investigations in several laboratories have shown that the primary targets for macrocyclic lactones are glutamate-dependent chloride channels [4, 27]. These channels are not detected in mammals, but belong to the so-called family of Cys-loop channel receptors that includes GABA-, glycine, nicotine and serotonin-3 receptors. This, in particular, allowed using $GABA_A$-receptor of rat brain as a model to screen and study the mechanism of action of natural and semi-synthetic avermectins.

Thus, the study of the biocidal action of a number of 5-O-substituted

avermectins found that the 1.5 % solution of 5-O-succinyl, 5-O-ethylsuccinyl and 5,4″-di-O-ethylsuccinyl avermectin B1 after a 60-minute exposure completely paralyzed oligochaetes. The LD_{50} values of these compounds, when administered intraperitoneally to mice at a dose of 37-47 mg/kg, are comparable to or lower than those of the known analogues. Moreover, based on the effects according to State Standard GOST 12.1.007-76, these substances belong to toxicity class II, i.e. can be considered as promising antiparasitic agents.

REFERENCES

1. Arkhipov I.A. *Antigel'mintiki: farmakologiya i primenenie* [Anthelmintics: pharmacology and use]. Moscow, 2009 (ISBN 978-5-85941-305-8) (in Russ.).
2. Dzhafarov M.Kh. Evolution in chemotherapy of human and animal helminthiases (review). *Agricultural Biology*, 2013, 4: 26-44 (doi: 10.15389/agrobiology.2013.4.26eng) (in Engl.).
3. Nobelforsamlingen. *The Nobel Assembly at Karolinska Institutet. The 2015 Nobel Prize in Physiology or Medicine.* Available http://www.nobelprize.org/nobel_prizes/medi-cine/laureates/2015/press.pdf. No date.
4. Campbell W.C. History of avermectin and ivermectin, with notes on the history of other macrocyclic lactone antiparasitic agents. *Curr. Pharm. Biotechnol.*, 2012, 13(6): 853-865 (doi: 10.2174/138920112800399095).
5. Omura S. Ivermectin: 25 years and still going strong. *Int. J. Antimicrob. Agents*, 2008, 31: 91-98 (doi: 10.1016/j.ijantimicag.2007.08.023).
6. Pitterna T., Cassayre J., Huter O., Jung P.M., Maienfisch P., Kessabi F.M., Quaranta L., Tobler H. New ventures in the chemistry of avermectins. *Bioorg. Med. Chem.*, 2009, 17: 4085-4095 (doi: 10.1016/j.bmc.2008.12.069).
7. Dzhafarov M.Kh., Mirzaev M.N., Urazaev D.N., Maksimov V.I. Antiparasitic activity of adermectin and compounds of a steroid nature. *Russian Agricultural Sciences*, 2010, 36(2): 130-132 (doi: 10.3103/S1068367410020163).
8. Dzhafarov M.Kh., Myrzaev M.N., Zavarsin I.V. Antiparasitic activity of famectin and some compounds of different chemical nature. *Agricultural Biology*, 2011, 2: 108-111. Available http://www.agrobiology.ru/2-2011dzhafarov-eng.html. No date (in Eng.).
9. Raymond V., Sattelle D.B. Novel animal-health drug targets from ligand-gated chloride channels. *Nat. Rev. Drug Discov.*, 2002, 1: 427-436 (doi: 10.1038/nrd821).
10. Estrada-Mondragon A., Lynch J.W. Functional characterization of ivermectin binding sites in α1β2γ2LGABA(A) receptors. *Front. Mol. Neurosci.*, 2015, 8: 55 (doi: 10.3389/fnmol.2015.00055).
11. Wolstenholme A.J., Rogers A.T. Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. *Parasitology*, 2005, 131: S85-S95 (doi: 10.1017/S0031182005008218).
12. Lynagh T., Lynch J.W. Molecular mechanisms of Cys-loop ion channel receptor modulation by ivermectin. *Front. Mol. Neurosci.*, 2012, 5: 60 (doi: 10.3389/fnmol.2012.00060).
13. Yoluk Ö., Brömstrup T., Bertaccini E.J., Trudell J.R., Lindahl E. Stabilization of the GluCl ligand-gated ion channel in the presence and absence of ivermectin. *Biophys. J.*, 2013, 105: 640-647 (doi: 10.1016/j.bpj.2013.06.037).
14. Pang S., Qi S., Ran Z., Song X., Li X., Wang C., Duan L. Synergistic effect of gamma-aminobutyric acid with avermectin on Bombyx mori. *J. Food Agric. Environ.*, 2013, 11(1): 1022-1024.
15. Cobb R., Boeckh A. Moxidectin: a review of chemistry, pharmacokinetics and use in horses. *Parasites Vectors*, 2009, 2(Suppl 2): S5 (doi: 10.1186/1756-3305-2-S2-S5).
16. Prichard R., Ménez C., Lespine A. Moxidectin and the avermectins: Consanguinity but not identity. *International Journal of Parasitology: Drugs and Drug Resistance*, 2012, 2: 134-153 (doi: 10.1016/j.ijpddr.2012.04.001).
17. Menez C., Sutra J.-F., Prichard R., Lespine A. Relative neurotoxicity of ivermectin and moxidectin in Mdr1ab (2/2) mice and effects on mammalian GABA(A) channel activity. *PLOS*, 2012, 6(11): e1883 (doi: 10.1111/jnc.13644).
18. Lankas G.R., Gordon L.R. Toxicology. In: *Ivermectin and avermectin*. W.C. Campbell (ed.). Springer-Verlag, NY, 1989: 89-112.
19. Chernoburova E.I., Lishchuk V.A., Ovchinnikov K.L., Kolobov A.V., Dzhafarov M.Kh., Vasilevich F.I., Zavarzin I.V. *Izvestiya RAN. Seriya khimicheskaya*, 2016, 12: 2965-2969 (in Russ.).
20. Mironov A.N., Bunyatyan N.D., Vasil'ev A.N., Verstakova O.L., Zhuravleva M.V., Lepakhin V.K., Korobov N.V., Merkulov V.A., Orekhov S.N., Sakaeva I.V., Uteshev D.B., Yavorskii A.N. *Rukovodstvo po provedeniyu doklinicheskikh issledovanii lekarstvennykh sredstv. Chast' I* [Preclinical studies of drugs — guidelines. Part I]. Moscow, 2012: 13-25 (in Russ.).

21. Hawkinson J.E., Acosta-Burruel M., Kimbrough C.L., Goodnough D.B., Wood P.L. Steroid inhibition of [^{3}H]SR 95531 binding to the $GABA_A$ recognition site. *Eur. J. Pharmacol.*, 1996, 304: 141-146 (doi: 10.1016/0014-2999(96)00090-8).
22. Lowry O.H., Rosenbrough N.J., Farr A.L., Randall R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 1951, 193: 265-275.
23. Schuske K., Beg A.A., Jorgensen E.M. The GABA nervous system in *C. elegans. Trends Neurosci.*, 2004, 27: 407-414 (doi: 10.1016/j.tins.2004.05.005).
24. Wolstenholme A.J. Surviving in a toxic world. *Science*, 2012, 335: 545-546 (doi: 10.1126/science.1218166).
25. Maksay G. Differential effects of thiocyanate on the binding thermodynamics of bicuculline methiodide versus SR95531 (Gabazine) to the γ-aminobutyric acid receptor-ionophore complex. *Biochem. Pharmacol.*, 1998, 56: 729-731 (doi: 10.1016/S0006-2952(98)00064-1).
26. Duittoz A.H., Martin R.J. Effects of the arylaminopyridazine-GABA derivatives, SR95103 and SR95531 on the Ascaris muscle GABA receptor: the relative potency of the antagonists in the Ascaris is different to that at vertebrate GABAa receptors. *Comp. Biochem. Physiol.*, 1991, 98C: 417-422 (doi: 10.1016/0742-8413(91)90227-K).
27. Nakao T., Banba Sh., Hirase K. Comparison between the modes of action of novel meta-diamide and macrocyclic lactone insecticides on the RDL GABA receptor. *Pestic. Biochem. Phys.*, 2015, 120: 101-108 (doi: 10.1016/j.pestbp.2014.09.011).

Events

BACTERIOPHAGE 2017

(17-19 January 2017, online event, United Kingdom)

Organization: Euroscicon

Subdisciplines: biology, microbiology, molecular biology, biochemistry, medicine, biotechnology, virology

This is the FIRST live streamed only, three day professional conference discussing bacteriophages. This annual event will discuss emerging research relating to bacteriophage structure and mechanism of action, and their application in medical and industrial biotechnologies.

As one of the most abundant biological entities on earth, bacteriophages are major drivers of bacterial adaptive evolution through the predator-prey roles of the phage-bacterium interaction and through the adaptive impacts of lysogeny and lysogenic conversion. Bacteriophage biology underpins many biochemical reagents and technologies, indispensible for modern molecular biology, and phages continue to be exploited in several areas of biotechnology, including diagnostics, prophylaxis and other aspects of food microbiology. Furthermore, the use of bacteriophages as natural alternatives to antibiotics (known as phage therapy) are of increasing interest for the treatment of human and animal disease in the face of rising levels of antibiotic resistance. This meeting will bring together researchers working with phages across these disciplines to encourage collaboration and knowledge sharing. This event is aimed at those interested in the biology of bacteriophages and their applications in biotechnology, including research scientists, academics and pharmaceutical professionals.

Contacts: http://lifescienceevents.com/phage2017/

Information: http://www.globaleventslist.elsevier.com/events/2017/01/bacteriophage-2017/

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 883-890
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Microbiome and productivity

UDC 636.52/.58:591.3:579.64

doi: 10.15389/agrobiology.2016.6.883rus
doi: 10.15389/agrobiology.2016.6.883eng

POULTRY GASTROINTESTINAL MICROBIOME CHANGES DURING ONTOGENESIS

V.I. FISININ[1], G.Yu. LAPTEV[2], I.N. NIKONOV[2], L.A. IL'INA[2], E.A. YILDIRIM[2], V.A. FILIPPOVA[2], N.I. NOVIKOVA[2], A.A. GROZINA[1], T.A. EGOROVA[1], T.N. LENKOVA[1], V.A. MANUKYAN[1], I.A. EGOROV[1]

[1]*All-Russian Research and Technological Poultry Institute,* Federal Agency of Scientific Organizations, 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail olga@vnitip.ru;
[2]*JSC «Biotrof»*, Kolpino, St. Petersburg, 192288 Russia, e-mail nikonov@biotrof.ru
Acknowledgements:
Supported by grant from Russian Science Foundation (project № 14-16-00140 «Modern views on the intestinal microflora of poultry in different diets: molecular genetic approaches»)
Received September 13, 2016

Abstract

Microorganisms which inhabit gut play great role in providing with nutrients, antibiotics, hormones and vitamins necessary for poultry health and performance. Therefore study of gut microbiome changes during ontogenesis seems to be essential. The structure of gut microflora in poultry embryos is of particular interest and debated because of very few publications on the problem. Despite embryo intestine is commonly considered sterile there are several reports on gut colonization by microorganisms in embryos during ontogenesis. Using T-RFLP (Terminal Restriction Fragment Length Polymorphism) analysis to generate a fingerprint of a microbial community we compared gut flora in chick embryos on days 6 and 17 to those in 26-day, 150-day and 300-day old Hisex White layers. Unlike accepted view, a high biodiversity was seen in embryo gut with *Enterobacteriaceae* (*Escherichia coli* mainly) predominated. *Clostridia*, *Bacteroides*, *Negativicutes*, *Actinomycetales*, *Bifidocteriales* were also found in contrast to earlier reports of their presence only in chicks at hatching and in adult poultry gut. Moreover, in the embryo gut we found the causal agents of dangerous animal disease, *Burkholderia* sp., *Pseudomonas* sp., *Salmonella* sp., *Klebsiella* sp. and *Rickettsiales* bacteria. Interestingly, the embryo gut biodiversity on day 6 was higher as compared to day 17 (75±2.75 phylotypes vs 30±1.20 phylotypes). In the layers aged 26, 150 and 300 days the diversity was much higher (over 175±8.12 phylotypes) as compared to embryos due to new members involved into gut bacterial community. Moreover, the poultry aged 300 days was lower both in the total diversity and in the percentage of unidentified microorganisms when compared to 26-day and 150-day old hens. In the adults, the predominating microbial taxa changed, in particular, *Clostridia* and *Negativicutes* became more abundant whereas *Bacillales* and *Bifidobacteriales* were depressed. Our findings indicate gut colonization by *Lactobacilales* and pathogenic *Listeria* sp., *Pantoea* sp., *Enterobacter* sp., *Mycoplasma* sp., *Acinetobacter* sp., *Pasteurellaceae*, *Campylobacteraceae*, *Fusobacteria* which occurred during ontogenesis. Thus the gut microbiome formation starts in embryo which is important for hatching and growing healthy poultry.

Keywords: gut microflora, caecum, ontogenesis, hens, embryo, T-RFLP

Microorganisms which inhabit the gastrointestinal tract (GIT), by using their own cellulosolytic and amylolytic enzymes, completely absent in poultry, provide the host with nutrients, vitamins, antibiotics, proteins, hormones and other compounds involved in metabolism [1-4]. According to traditional concepts, microbiocenosis of embryonal digestive system is sterile, and its colonization by microorganisms takes place after chicken hatching [5-7]. Bacteria inhabiting poultry intestine normally include bifidobacteria, streptococci, lactobacilli, lactate fermenting bacteria, eubacteria, bacteroides and enterobacteria [1, 8, 9].

Bacterial community of digestive tract undergoes subsequent changes throughout life of a bird, related to a number of factors, the main of which are growth and development of digestive tract, feeding regime and feed composition.

At that, intestinal microorganisms act as a highly sensitive indicator system. It should be noted that the change of ecological balance between obligatory microorganisms of digestive tract do not always have a positive impact on metabolic processes and health of a bird [10]. In this context, studies of qualitative and quantitative composition of GIT microbiota in ontogenesis are worthwhile.

Up to the 1990s, studies of microorganisms in various ecosystems were limited to examination of strains cultivated on artificial media. Development of metagenomic methods, without a necessity to cultivate microorganisms, allowed us to broaden our understanding of composition of microbiota [11, 12]. This is of crucial significance, as up to 99 % of biosphere microorganisms are unculturable on artificial media, but may play an important ecological role. We failed to find any data on composition of chicken embryo GIT microbiome in available literature.

For the first time, we have analyzed the composition of chicken GIT bacterial community in ontogenesis, from an embryo to an adult bird, using T-RFLP (terminal restriction fragment length polymorphism) method. A wide taxonomic composition of embryonal intestine bacteria has been demonstrated, including opportunistic strains, pathogens and unculturable microorganisms.

The work focuses on examination of succession in a bacterial community of poultry GIT in ontogenesis, using T-RFLP method.

Technique. Three samples of GIT content of 6 and 12-day old chicken embryos and three samples from cecum of Hisex White adult 26, 150 and 300-day old laying hens were collected (hatching house of the All-Russian Research and Technological Poultry Institute — VNITIP, Zagorskoe EPH VNITIP vivarium, Moscow region). The poultry were fed manually, ad libitum with dry complete feed according to the cross standards. The birds were kept in cage batteries (Big Dutchman, Germany) in groups of 35 individuals, with no gender separation, in compliance with all technological parameters according to the norms of VNITIP. Sampling and sample preparation was performed in strict compliance with sterility in accordance with the established requirements [13].

Total DNA was isolated from the samples using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to manufacturer's recommendations. T-RFLP analysis was used for examination of bacterial community composition. PCR was performed using a Verity DNA amplificator (Life Technologies, Inc., USA) and 63F primer (CAGGCCTAACACATGCAAGTC) with the 5' end marked (fluorophore WellRed D4, Beckman Coulter, USA), and 1492R primer (TACGGHTA-CCTTGTTACGACTT). Fluorescence-labelled amplicons of 16S rRNA gene were purified by a standard methodology [14]. The obtained amplicons (30-50 ng) were treated with endonucleases HaeIII, HhaI and MspI, according to the manufacturer's recommendation (Fermentas, Lithuania). Restriction products were analyzed on a CEQ 8000 sequencer (Beckman Coulter, USA). Phylogenetic status of bacteria was determined using Fragment Sorter software (http://www.oardc.ohiostate.edu/trflpfragsort/index.php).

Statistical processing of the results was performed by means of analysis of variance using Microsoft Excel 2010.

Results. It is generally believed that the GIT of avian embryos is sterile [1, 15, 16], and the formation of digestive system microbiocenosis takes place after hatching as due to a contact with the environment [5-7]. However, there is evidence obtained by classical microbiology methods [17, 18] and real time PCR [19] which indicates the ability of microorganisms to colonize GIT of birds still inside an egg, at the embryonic development stage.

Using T-RFLP analysis, we have established that embryonal GIT bacterial community was characterized by significant taxonomic diversity (Table). Bi-

odiversity of microorganisms in microbiocoenosis of chicken embryo GIT content on day 6 of incubation was much more significant than that on day 16. Members of family *Enterobacteriaceae*, typical of poultry intestine microflora, were predominant, mainly *Escherichia coli*. Interestingly, their number was twice as much as that of 16-day old embryos (46.90±1.87 % vs. 21,30±1,03 %). Representatives of class *Clostridia* (families *Lachnospiraceae*, *Eubacteriaceae*, etc.), phylum *Bacteroidetes*, orders *Negativicutes*, *Actinomycetales*, *Bifidobacteriales* were also identifies among the embryonal GIT indigenous microflora, which were previously detected in hatched and adult chickens [20]. Amylo- and proteolytic bacteria of order *Actinomycetales*, cellulolytic microorganisms of class *Clostridia*, phylum *Bacteroidetes*, inhabiting GIT of hatched chickens and adult birds, play an important role in metabolism due to active participation in fermentation of protein, starch and polysaccharides of feeds.

Microorganisms of order *Bifidobacteriales*, producing organic acids and bacteriocins in GIT of birds, can ensure colonization resistance of a microbiotopes to pathogens, and produce essential nutrients [1]. We heve observed 130-fold increase in bifidobacteria amount in embryonal intestine during incubation

Interestingly, metagenomic community of GIT of 16-day old embryos has shown absence of autochthonous symbiotic intestine microflora, typical in birds, i.e. facultative anaerobic bacteria of order *Lactobacillales*, while the proportion of these bacteria in GIT of 6-day old chicken embryos was high (10.11±0.42 %). Most bacteria of order *Lactobacillales* are capable of active acid production, so they ensure competitive exclusion of pathogens in GIT of hatched chickens and adult birds [1]. With regard to order *Lactobacillales*, the proportion of microorganisms of genus *Lactobacillus* was 9.30±0.39 %, of genus *Pediococcus* — 0.66±0.02 % and *Trichococcus* — 0.150±0.006 %. A high percentage of order *Bacillales* representatives, mostly bacteria of genus *Bacillus*, were detected in GIT of embryos. The majority of them are capable of poultry digestive tract colonization, synthesis of organic acids, bacteriocins, antibiotics and enzymes, active participation in metabolic processes of various nutritious substrates [18, 19]. Apart from that, several unidentified bacterial genotypes were observed.

Pathogenic and opportunistic bacteria, causative agents of dangerous diseases in animals of genera *Burkholderia*, *Pseudomonas*, *Salmonella*, *Klebsiella*, order *Rickettsiales*, etc. (Table) were detected in metagenomic community of 6-day old embryo intestine; on day 16 of incubation a colonization of GIT with new microorganisms of genera *Staphylococcus*, *Morganella*, *Bordetella* was observed, which have possibly entered through eggshell pores.

It should be noted that *E. coli* and bacteria of genera *Staphylococcus* and *Pseudomonas*, detected by us in GIT of embryos during incubation, may cause omphalitis (i.e. inflammation of the navel and yolk sac), a dangerous disease which is the main reason of death in chickens from hatching to day 14 of life [21]. Microorganisms of genus *Bordetella* cause respiratory tract diseases in poultry, mostly in young chickens [22]. Bacteria of order *Rickettsiales* are pathogens, transmitted by representatives of *Arthropoda*. Presumably, development of the said diseases is caused by unfavorable composition of GIT microflora, i.e. increase in the number of the pathogens during embryonic development.

The results obtained by us using T-RFLP analysis correspond to the data of Z. Babaca [18]. Using classical microbiological methods, the author has examined more than 3000 samples of chicken embryo GIT content from incubators of three poultry farms, in order to identify the reasons of the mass death of embryos, and has detected pathogenic bacteria *E. coli* (18.28 %), *Staphylococcus* (14.10 %), *Pseudomonas* (11.75 %) and *Klebsiella* sp. in the samples. It has been also reported that microflora of laying hens plays a key role in formation of em-

bryo GIT pathogenic microflora [17)]. Using the real time PCR, it has been shown that content of these microorganisms in GIT of laying hens, artificially contaminated with *Campylobacter coli*, amounts to 4.35-5.65 thous. cells/g of body weight [17]. Our results are indicative of GIT colonization by microorganisms as early as at the embryonic development stage.

The proportion and number of bacterial taxa in embryo GIT and cecum of Hisex White laying hens of various age ($X \pm x$, FSUE Zagorskoe EPH VNITIP vivarium, Moscow region)

Taxonomic group of bacteria	Frequency (%) and total number of taxa in a group				
	embryo, age		bird, age		
	day 6	day 17	day 26	day 150	day 300
Phylum *Firmicutes*	25.01±1.03	10.01±0.40	55.13±2.58	56.39±2.79	61.02±2.88
Class *Clostridia*	5.5±0.17	1.10±0.03	31.82±1.27	20.37±0.98	17.35±0.84
Family *Lachnospiraceae*	0.08±0.01	1.10±0.03	4.61±0.18	1.96±0.08	1.85±0.07
Family *Eubacteriaceae*	0.32±0.01	N/D	5.43±0.20	5.58±0.23	3.61±0.12
Family *Ruminococcaceae*	0.38±0.01	N/D	8.72±0.35	5.18±0.20	5.95±0.22
Family *Clostridiaceae*	1.70±0.06	N/D	11.40±0.45	6.19±0.28	5.20±0.21
Family *Peptococcaceae*	N/D	N/D	1.66±0.04	1.46±0.06	0.74±0.03
Family *Syntrophomonadaceae*	2.30±0.04	N/D	N/D	N/D	N/D
Class *Negativicutes*	0.70±0.03	N/D	12.70±0.58	7.91±0.39	8.77±0.39
Class *Bacilli*	18.81±0.84	8.91±0.36	10.61±0.76	28.11±1.35	34.90±1.25
Order *Lactobacillales*	10.11±0.42	N/D	7.28±0.33	22.56±0.95	32.48±1.22
Lactobacillus sp.	9.30±0.39	N/D	5.14±0.15	16.00±0.75	26.10±1.10
Enterococcus sp.	N/D	N/D	0.61±0.03	4.18±0.18	3.06±0.14
Leuconostoc sp.	N/D	N/D	1.11±0.04	1.11±0.03	0.49±0.02
Pediococcus sp.	0.66±0.02	N/D	0.22±0.01	0.67±0.02	1.75±0.05
Weissella sp.	N/D	N/D	0.20±0.01	0.60±0.03	1.08±0.04
Trichococcus sp.	0.15±0.01	N/D	N/D	N/D	N/D
Order *Bacillales*	8.70±0.41	8.91±0.36	3.35±0.15	5.08±0.19	2.33±0.11
Alicyclobacillus sp.	0.16±0.01	0.50±0.02	0.10±0.01	N/D	0.08±0.00
Brevibacillus sp.	0.34±0.01	N/D	N/D	N/D	N/D
Bacillus sp.	7.10±0.33	6.79±0.28	2.05±0.09	2.46±0.11	1.37±0.05
Paenibacillus sp.	1.10±0.04	1.21±0.03	0.55±0.02	0.99±0.02	N/D
Staphylococcus sp.	N/D	0.41±0.01	0.65±0.03	1.46±0.05	0.88±0.01
Listeria sp.	N/D	N/D	N/D	0.17±0.01	N/D
Phylum *Actinobacteria*	4.53±0.21	34.2±1.02	4.12±0.17	3.18±0.12	2.21±0.11
Order *Actinomycetales*	4.38±0.19	13.58±0.65	3.74±0.14	2.63±0.12	2.05±0.09
Order *Bifidobacteriales*	0.15±0.01	20.62±0.89	0.38±0.02	0.55±0.02	0.16±0.01
Phylum *Proteobacteria*	34.83±1.62	49.60±2.32	9.88±0.44	9.24±0.36	9.44±0.41
Family *Enterobacteriaceae*	22.35±0.98	47.26±1.98	1.07±0.04	3.94±0.14	2.63±0.10
Pantoea sp.	N/D	N/D	0.17±0.01	0.76±0.03	0.73±0.03
Salmonella sp.	0.26±0.01	N/D	0.24±0.01	0.21±0.01	0.34±0.01
Morganella sp.	N/D	0.36±0.02	N/D	N/D	N/D
Enterobacter sp.	N/D	N/D	0.11±0.01	1.39±0.05	1.49±0.06
Escherichia coli	21.30±1.03	46.90±1.87	0.29±0.01	0.98±0.04	0.06±0.01
other (*Citrobacter* sp., *Kluyvera* sp., *Rahnella* sp., *Serratia* sp., *Yersinia* sp.)	0.79±0.01	N/D	0.26±0.01	0.60±0.02	0.01±0.00
Order *Burkholderiales*	0.31±0.01	2.00±0.08	N/D	N/D	N/D
Burkholderia sp.	0.31±0.01	0.94±0.04	N/D	N/D	N/D
Bordetella sp.	N/D	1.09±0.03	N/D	N/D	N/D
Order *Pseudomonadales*	2.62±0.10	N/D	7.42±0.29	3.69±0.14	2.7±0.12
Acinetobacter sp.	N/D	N/D	2.90±0.13	0.50±0.02	0.55±0.02
Pseudomonas sp.	2.62±0.10	N/D	4.52±0.20	3.19±0.14	2.15±0.12
Family *Caulobacteraceae* (*Brevundimonas* sp.)	9.44±0.42	0.31±0.02	N/D	N/D	N/D
Family *Pasteurellaceae* (*Pasteurella* sp., *Haemophilus* sp.)	N/D	N/D	0.48±0.03	0.92±0.02	0.69±0.02
Family *Campylobacteraceae* (*Campylobacter* sp., *Arcobacter* sp.)	N/D	N/D	0.91±0.04	0.69±0.03	3.42±0.60
Order *Rickettsiales*	0.11±0.01	N/D	N/D	N/D	N/D
Phylum *Tenericutes* (*Mycoplasma* sp.)	N/D	N/D	1.16±0.05	1.01±0.03	1.13±0.04
Phylum *Bacteroidetes*	5.42±0.22	N/D	8.88±0.35	9.68±0.44	9.24±0.39
Phylum *Fusobacteria*	N/D	N/D	1.39±0.04	2.21±0.12	2.71±0.33
Unidentified bacteria	30.21±1.42	6.19±0.28	19.44±0.56	18.29±0.86	14.25±0.69
Total number of phylotypes, pcs	75±2.75	30±1.20	224±10.23	255±12.46	175±8.12

N o t e. N/D (not detected) means the amounts below the limit of reliable determination by T-RFLP analysis.

We found the taxonomic composition of poultry cecum content to be much more diverse and significantly different from that of embryo GIT. First of

all, regardless of the age of chickens, representatives of phylum *Firmicutes* (above 55.13 %), the proportion of which increased with age, rather than microorganisms of family *Enterobacteriaceae*, were taxonomic dominants. In 26-day old chickens and 150 and 300-day old laying hens the representatives of class *Clostridia* predominated, which mostly includes bacteria of families *Eubacteriaceae*, *Ruminococcaceae*, *Peptococcaceae*, *Clostridiaceae*, *Lachnospiraceae* with cellulose- and amylolytic properties, content of which decreased with age. The number of bacteria with similar properties from phylum *Bacteroidetes* was not related to the age of laying hens and was higher than that in embryo GIT. Birds have little, if any, digestive enzymes of their own, required for cellulose and other non-starch polysaccharides cleavage, so the role of the said microorganisms in digestion of chickens is extremely important [1, 2].

As compared to embryos, the number of bacteria from class *Negativicutes*, which play an important role in digestion by fermentation of organic acids, including lactate, with formation of volatile fatty acids necessary for energy supply [2], increased significantly in adult chickens.

The proportion of microorganisms of orders *Bacillales* and *Bifidobacteriales*, characterized by high antagonistic activity towards pathogenic microflora, decreased with age and was significantly lower than that of embryo GIT. An inverse trend was observed for lactobacilli of order *Lactobacillales*, the largest proportion of which was detected in 300-day old chickens. Diversity of lactobacilli in adult chickens was higher than in enbryos, and included the representatives of genera *Weissella*, *Leuconostoc* and *Enterococcus* as well.

The diversity of pathogens in 26-day chickens and 150 and 300-day old adults was significantly higher than that in embryos. Poultry GIT was colonized by new pathogens of genera *Listeria*, *Pantoea*, *Enterobacter*, *Mycoplasma*, *Acinetobacter*, families *Pasteurellaceae*, *Campylobacteraceae*, phylum *Fusobacteria*. Some microorganisms of the listed taxa (*Pantoea*, *Enterobacter*, *Acinetobacter*) cause intestine dysbiotic disorders in mammals and birds, while the other (genus *Mycoplasma*, family *Pasteurellaceae*) are mostly detected in the respiratory tract in birds and are considered as causative agents of respiratory diseases. The fact that bacteria of phylum *Fusobacteria*, which were previously considered as typical inhabitants of the rumen of ruminants, are found in poultry cecum is of significant interest [23]. It should be noted that the presence of several listed pathogens in poultry GIT was previously reported only based on molecular genetic studies [24, 25].

Interestingly, some pathogens detected in embryo GIT, including bacteria of orders *Burkholderiales* and *Rickettsiales* and genus *Brevundimonas*, have not been observed in 26-day old chickens, as well as in 150 and 300-day old laying hens. At that, the number of unidentified bacteries decreased with age of a bird.

Thus, using T-RFLP analysis, highly abundant taxonomic diversity of bacteria has been detected in the gastrointestinal tract (GIT) of chicken embryos, including the representatives of indigenous GIT normal flora of hatched and adult chickens, opportunistic and pathogenic microflora, and unculturable microorganisms. Taxonomic diversity in chicken GIT content on day 6 of incubation was much more significant than that on day 16. The bacterial community of poultry GIT develops during ontogenesis, and new microorganisms appear. Cecum community in 26-day old chicken, as well as 150 and 300-day old poultry is represented by a wide variety of microorganisms, including opportunistic and pathogenic ones. In conclusion, development of microbioecological system, as GIT content with inhabiting microflora, starts in poultry as early as at the embryonic development stage. Presumably, the structure of embryo GIT microbiotope forms under the influence of a laying hen microflora by vertical transfer due to bacterial trans-

location. Presumably, microflora that colonizes chicken embryo GIT enters through eggshell pores. At that, microorganisms in embryo GIT act as a basis of the forming initial intestine biocenosis in hatched chickens, which in many ways determines their viability, resistance to pathogens and development.

REFERENCES

1. Timoshko M.A. *Mikroflora pishchevaritel'nogo trakta sel'skokhozyaistvennykh zhivotnykh* [Microflora of alimentary canal in farm animals]. Kishinev, 1990 (in Russ.).
2. Tarakanov B.V. *Metody issledovaniya mikroflory pishchevaritel'nogo trakta sel'skokhozyaistvennykh zhivotnykh i ptitsy* [Study of microflora in alimentary canal of farm animals — methods]. Moscow, 2006 (in Russ.).
3. Salanitro J., Fairchilds I., Zgornicki Y. Isolation, culture characteristics, and identification of anaerobic bacteria from the chicken cecum. *Appl. Microbiol.*, 1974, 27: 678-687.
4. Stanley D., Hughes R.J., Moore R.J. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Appl. Microbiol. Biotechnol.*, 2014, 98: 4301-4309 (doi: 10.1007/s00253-014-5646-2).
5. Mead G.C. Microbes of the avian cecum: types present and substrates utilized. *J. Exp. Zool. Suppl.*, 1989, 3: 48-54.
6. AmitRomach E., Sklan D., Uni Z. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poultry Sci.*, 2004, 83: 1093-1098.
7. Dibner J.J., Richards J.D., Knight C.D. Microbial imprinting in gut development and Health. *J. Appl. Poultry Res.*, 2008, 17: 174-188 (doi: 10.3382/japr.2007-00100).
8. Barnes E. The intestinal microbiota of poultry and game birds during life and after storage. *J. Appl. Bacteriol.*, 1979, 46: 407-419.
9. Mead G.C. Microbes of the avian cecum: types present and substrates utilized. *J. Exp. Zool.*, 1989, 3: 48-54 (doi: 10.1002/jez.1402520508).
10. Torok V., Ophel-Keller K., Loo M., Hughes R. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. *Appl. Environ. Microbiol.*, 2008, 74(3): 783-791 (doi: 10.1128/AEM.01384-07).
11. Park S.H., Lee S.I., Ricke S.C. Microbial populations in naked neck chicken ceca raised on pasture flock fed with commercial yeast cell wall prebiotics via an Illumina MiSeq Platform. *PLoS ONE*, 2016, 11(3): e0151944 (doi: 10.1371/journal.pone.0151944).
12. Amann R.I., Ludwig W., Schleifer K.H. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.*, 1995, 59: 143-169.
13. *Instruktsiya po sanitarno-mikrobiologicheskomu kontrolyu tushek, myasa ptitsy, ptitseproduktov, yaits i yaitseproduktov na ptitsevodcheskikh i pererabatyvayushchikh predpriyatiyakh* [Instruction for the sanitary-microbiological control of poultry carcasses, meat, eggs and egg products in the poultry production and processing enterprises]. Moscow, 1990 (in Russ.).
14. Maniatis T., Fritsch E. F., Sambrook J. *Molecular cloning: a laboratory manual.* Cold Spring Harbor, NY, 1982.
15. van der Wielen P.W.J.J., Keuzenkamp D.A., Lipman L.J.A., van Knapen F., Biesterveld S. Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. *Microbiol. Ecol.*, 2002, 44: 286-293.
16. Maiorka A., Dahlke F., de Azevedo Morgulis M.S.F. Broiler adaptation to post-hatching period. *Ciencia Rural*, 2006, 36: 701-708.
17. Kizerwetter-Swida M., Binek M. Bacterial microflora of the chicken embryos and newly hatched chicken. *J. Animal Feed Sci.*, 2008, 17: 224-232 (doi: 10.22358/jafs/66602/2008).
18. Babaca Z. Isolation of bacterial pathogens from dead in-shell chicken embryos from local hatcheries. *J. Vet. Sci. Technol.*, 2014, 5: 170-171.
19. Rossi D.A., Fonseca B.B., de Melo R.T., da Silva Felipe G., da Silva P.L., Mendonça E.P., Filgueiras A.L., Beletti M.E. Transmission of *Campylobacter coli* in chicken embryos. *Brazil. J. Microbiol.*, 2012, 43(2): 535-543 (doi: 10.1590/S1517-83822012000200014).
20. Nakphaichit M., Thanomwongwattana S., Phraephaisarn C., Sakamoto N., Keawsompong S., Nakayama J., Nitisinprasert S. The effect of including *Lactobacillus reuteri* KUB AC5 during post hatch feeding on the growth and ileum microbiota of broiler chickens. *Poultry Sci.*, 2011, 12(90): 2753-2765 (doi: 10.3382/ps.2011-01637).
21. Lowder B.V., Fitzgerald J.R. Human origin for avian pathogenic *Staphylococcus aureus. Virulence*, 2010, 4(1): 283-284 (doi: 10.4161/viru.1.4.11838).
22. Biberstein E.L., Hirsh D.C. *Bordetella in veterinary microbiology.* Reino Unido, Blackwell Sci., Oxford, 1999: 148-150.
23. Gong J., Forster R.J., Yu H., Chambers J.R., Sabour P.M., Wheatcroft R.,

C h e n S. Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen. *FEMS Microbiol. Lett.*, 2002, 208(1): 1-7 (doi: 10.1111/j.1574-6968.2002.tb11051.x).

24. T o r o k V.A., H u g h e s R.J., M i k k e l s e n L.L. Identification and characterization of potential performance-related gut microbiota in broiler chickens across various feeding trials. *Appl. Environ. Microbiol.*, 2011, 77(17): 5868-5878 (doi: 10.1128/AEM.00165-11).
25. R i n t t i l a T., A p a j a l a h t i J. Intestinal microbiota and metabolites — implications for broiler chicken health and performance. *J. Appl. Poultry Res.*, 2013, 22(3): 647-658 (doi: 10.3382/japr.2013-00742).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 891-902
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.52/.58:636.084:579.64

doi: 10.15389/agrobiology.2016.6.891rus
doi: 10.15389/agrobiology.2016.6.891eng

THE *Saccharomyces* sp. AND *Bacillus subtilis* BASED PROBIOTICS INFLUENCE ON CHICKEN BROILER PRODUCTIVITY AND CAECUM MICROBIOME COMMUNITY

T.A. EGOROVA[1], T.N. LENKOVA[1], L.A. IL'INA[2], E.A. YILDIRIM[2], I.N. NIKONOV[2], V.A. FILIPPOVA[2], G.Yu. LAPTEV[2], N.I. NOVIKOVA[2], A.A. GROZINA[1], V.A. MANUKYAN[1], V.I. FISININ[1], I.A. EGOROV[1]

[1]*All-Russian Research and Technological Poultry Institute,* Federal Agency of Scientific Organizations, 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail olga@vnitip.ru;
[2]*JSC «Biotrof»*, Kolpino, St. Petersburg, 192288 Russia, e-mail nikonov@biotrof.ru
Acknowledgements:
Supported by grant from Russian Science Foundation (project № 14-16-00140 «Modern views on the intestinal microflora of poultry in different diets: molecular genetic approaches»)
Received September 26, 2016

Abstract

Study of probiotic microorganisms which can produce enzymes and amino acids is important to develop biologicals to prevent disease and increase productivity in poultry. *Lactbacillus* sp. and *Bifidobacterium* sp. are widely used as probiotics due to their adhesive ability and antipathogenic activity. *Bacillus* sp. and yeasts *Saccharomyces* sp. are less examined but considered perspective as probiotic agents due to antibiotic activity and some other helpful features. Using T-RFLP (Terminal Restriction Fragment Length Polymorphism) and Real-Time PCR we compared number and composition of caecum microbiome in 37-day old Cobb 500 broiler chicken. In group I the chickens were fed with balanced combined fodder. In group II the chickens were fed with the same combined fodder supplemented with a probiotic which contained *Saccharomyces* sp. living cells, and in group III this probiotic was replaced by a probiotic product Cellobacterin-T. Chickens' caecum microbiome contained various taxa including several unidentified phylotypes in addition to commonly found gut microorganisms. Phylum *Firmcutes* (mainly cellulolytic and amylolitic *Clostridia*) and *Bacillus* sp., *Lactobacillus* sp., *Enterococcus* sp. which possess anti-bacterial activity are identified as predominating taxa. In addition, various opportunistic and pathogenic microorganism were found including causative agents of respiratory diseases (*Pasteurellaceae*, *Mycoplasma* sp., etc.). Both probiotics resulted in an increase of total caecum microbiome and a decreased of its biodiversity. The most remarkable changes we found in the chickens fed with yeast probiotic. Caecum microbiome community of the broilers from group II showed the lowest Shannon index and Simpson index. Cellobacterin-T had the highest probiotic effect. In the broilers from group III the microbiome *Bacillus* sp. counts increased 1.38-fold, *Lactobacillus* sp. number was 1.47 times higher whereas the *Campylobacter* sp. number was 3.00 times lower and the family *Enterobacteriaceae* number was 1.44 times lower as compared to the control chicks. Yeast probiotic resulted in positive effect on cellulolytic *Clostridia* microorganisms but also led to rise of *Campylobacter* sp., *Pasteurella* sp. and *Mycoplasma* sp. counts. Poultry growth rate and productivity were influenced positively by both probiotics. The highest growth rate, weight gain, digestibility coefficient and vitamin A, E, B_2 and carotinoid accumulation in liver were characteristic of the chickens from group III. Yeast probiotic promoted feed consumption. Chemical composition of pectoral and leg muscles of the chicks fed with probiotics remained unchanged.

Keywords: microflora, caecum, broiler chickens, bacterial community, T-RFLP, real time PCR, probiotic, Cellobacterin-T, yeast, productivity

Nowadays the studies of effect of probiotics based on beneficial microorganism strains, producing enzymes, amino acids and other biologically active substances, on the intestinal microbiome content of poultry, are of fundamental scientific interest [1, 2)]. It is known that normal intestinal microflora affects the macroorganism immune system formation, contributes to inactivation of some harmful decomposition products, and prevents reproduction of opportunistic bacteria [3, 4]. In poultry, gastrointestinal tract (GIT) caecum, where the intes-

tine content remains for the longest time, and the basic processes of microbial proteolysis and cellulose and starch cleavage take place, is usually studied [5, 6].

Bacteria of the genus *Bacillus* are promising subjects for development of probiotics. For example, the species *Bacillus subtilis*, *B. licheniformis*, *B. coagulans*, *B. clausii*, *B. pumilus*, and *B. cereus* have been tested as probiotics for human use [7]. The properties of bacilli differ significantly from those of classic probiotic microorganisms, such as lactobacilli or bifidobacteria; however, they have a number of benefits. First of all, the bacilli are more resistant to GIT conditions because of spore formation [8]. It has been shown that the baccilli are able to colonize the digestive tract of poultry, interacting both with intestine epithelium of the host organism and directly with microorganisms that inhabit GIT. Most bacilli are capable of synthesis of organic acids, bacteriocins, and antibiotic substances, so they may act as antagonists forcing pathogens (salmonellas, proteus, staphylococci, *E. coli*, and streptococci) out of the intestine [8, 9]. Apart from that, most bacilli strains show significant enzyme activity and contribute to processes of metabolism of various nutritious substrates.

Yeasts *Saccharomyces* sp. are of interest as a probiotic for poultry because of production of various antibiotic compounds, inhibiting the growth of pathogens [10, 11]. Antibiotic resistance is the key competitive advantage of yeasts. Positive effect of yeasts on intestine mucosa (i.e. increase in villi size and density of goblet cells), and on performance of various monogastric animals and ruminants has been reported (12, 13). A positive effect of yeasts is related to both metabolites produced and components of their cell walls [14, 15]. The studies also confirm positive effect of probiotic microorganisms on immunity in poultry [16-18]. For example, ability of two strains, *Saccharomyces boulardii* and *B. subtilis*, to activate the immunity due to interaction with toll-like receptors (TLR) of poultry cells has been demonstrated recently [18-20].

Despite of the widespread interest in the subject, the mentions microorganisms are relatively new probiotics in poultry farming. The effect of baccilli and yeasts on intestine microbiocoenosis and productivity performance is still not fully understood. The work becomes more complicated due to nearly complete absence of methodological base for studies of facultative anaerobic and strictly anaerobic microorganisms inhabiting the digestive tract of poultry.

According to traditional concepts based on classical microbiology methods, bifidobacteria, lactobacilli, nonspore-forming anaerobes, and bacteroides prevail among the intestine microorganisms [21, 22]. The advances in microbiology of the recent decades allow for analysis of intestine microorganism composition using molecular-genetic methods and determination of microbial diversity without the culturing stage [23-24]. As a result, the presence of significantly larger amount of species was identified, comparing with that previously suggested, which lead to reevaluation of classical concepts. Molecular-genetic methods, such as T-RFLP analysis, ensure detailed characterization of microbial community, identification of taxonomic dominants and minor components, including uncultured microorganisms, the proportion of which in various ecosystems may be as high as 90 % [25]. Real time PCR method ensures determination of quantity of microorganisms within an ecosystem [26]. However, there are only a few examples of molecular genetic studies of intestine microbiome [24-28], and the data on effect of bacilli and yeast probiotic strains on microbiocenosis of intestine content are nearly almost absent.

In this study we first have demonstrated the effect of live *Saccharomyces* yeast product and cellobacterine-T, a probiotic based on *B. subtilis* bacteria, on caecum bacterial community, using T-RFLP and real time PCR. By a biodiversity coefficient analysis, the yeast biological was shown to have the most signifi-

cant effect on the bacterial community. However, probiotic effect on pathogenic microorganisms was higher when using the bacterial product.

This work focused on caecum bacteria characteristics and productivity in broiler chickens, as influenced by dietary probiotics based on *Saccharomyces* (yeasts) and *Bacillus subtilis* (bacteria).

Technique. The study was performed in 3 groups of Cobb 500 broiler chickens from 1 to 37 days of age (Zagorskoe EPH VNITIP vivarium, Moscow region). Chickens were fed manually, ad libitum with dry complete feed according to the cross standards. Group I (control) chickens were fed with balanced complete feed (BD). In group II the same feed was enriched with foreign-manufactured probiotic based on live *Saccharomyces* yeast cells at a dose of 1 kg/t. Group III broilers were provided with the same BD with addition of cellobacterine-T probiotic (JSC Biotrof) at a dose of 1 kg/t. The broilers consumed the same pre-starter granulated feed during the first 5 days; later they were fed according to the experimental design.

The broilers were kept in cage AviMax batteries (Big Dutchman Livestock Equipment Co., Ltd, China) in groups with no gender separation, in compliance with all technological norms of the All-Russian Poultry Research and Technology Institute (VNITIP).

The following parameters were estimated: stock preservation, live weight of broilers at the age of 7, 21, and 37 days (individual weighing), average daily live weight gain, feed consumption and conversion rate per 1 kg of live weight, the levels of vitamins and carotenoids in the liver, chemical composition of pectoral and femoral muscles, digestibility and utilization of the feed nutrients based on a physiological balance between their intake and excretion [29].

Bacterial community composition was examined by T-RFLP and real time PCR methods. Caecum content for molecular studies was collected aseptically from slaughtered 37-day old poultry in accordance to requirements [30].

Total DNA was isolated using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to manufacturer's manual. PCR was performed using a Verity DNA amplificator (Life Technologies, Inc., USA) and eubacterial primers, the 63F (CAGGCCTAACACATGCAAGTC) marked on 5' end (fluorophore WellRed D4, Beckman Coulter, USA), and 1492R (TACGGHTACCTTGTTACGACTT). Fluorescence-labelled products of 16S rRNA gene amplification were purified as described [31]. The amplicons (30-50 ng) were digwsted with restriction endonucleases HaeIII, HhaI and MspI following the manufacturer's protocol (Fermentas, Lithuania). Restriction products were analyzed on a CEQ 8000 (Beckman Coulter, USA) according to the attached manual. Phylogenetic status of bacteria was determined using Fragment Sorter software (http://www.oardc.ohiostate.edu/trflpfragsort/in-dex.php). Real time PCR to account for the total number of bacteria was performed on a DT Lite-4 amplifier (OOO NPO DNA Technology, Russia) with the Reagents for qPCR in the presence of intercalating EVA Green stain Kit (JSC Syntol, Russia) and Eub338/Eub518 primers (5′-ACTCCTACGGGAGGCAGCAG-3′ and 5′-ATTACCGCGGCTGCTGG-3′) by the following protocol: 95 °C for 3 min; 95 °C for 13 sec, 57 °C for 13 sec, 72 °C for 30 sec (40 cycles).

MS Excel 2010 dispersion analysis software was applied for quantitative data processing. Shannon and Simpson biodiversity coefficients were calculated using Past software (http://folk.uio.no/ohammer/past/).

Results. It is known that bacteria act as taxonomic dominants in caecum microbial community and, according to traditional concepts, play an important role in provision of nutritional components, vitamins and other vital substances in poultry. The total number of bacteria in poultry caecum in the control group

amounted to $2.43\times10^8\pm8.31\times10^6$ genome equivalents/g (Table 1). Addition of live microorganism cells to the diet of broilers lead to significant increase in this parameter in both experimental groups — 2.88-fold for dietary cellobacterine-T and 1.77-fold for dietary yeast probiotic.

The caecum bacterial community was taxonomically diverse. We observed maximum biodiversity in chickens of the control group. When dietary probiotics were administrated, this parameter decreased by 12.32 % ($P < 0.05$) and 21.09 % for groups II and III, respectively, as compared to the control (see Table 1). Taxonomic analysis also revealed a significant proportion of DNA sequences (from 18.92±0.67 to 24.19±1.12 %, depending on the experimental group) that could not be identified. The presence of unidentified microorganisms in caecum has been previously observed [32, 33], which is indicative of complete absence of knowledge on their existence.

1. The number and proportion of bacterial taxa in caecum of 37 day-aged Cobb 500 broilers, as influenced by dietary cellobacterine-T probiotic and a yeast-based probiotic ($X\pm x$, Zagorskoe EPH VNITIP vivarium, Moscow region).

Parameter	Group I (control, n = 3)	Group II (n = 3)	Group III (n = 3)
Total number of bacteria, genome eq./g	$2.43\times10^8\pm8.31\times10^6$	$4.31\times10^8\pm3.15\times10^6$***	$7.02\times10^8\pm1.42\times10^6$***
Shannon biodiversity index	4.28±0.20	4.00±0.19	4.21±0.17
Simpson biodiversity index	0.98±0.04	0.96±0.03	0.98±0.03
The number of phylotypes, units	155±6.24	128±5.93*	138±5.45
Taxon frequency, %			
phylum *Bacteroidetes*	22.00±1.09	21.18±0.87	20.69±1.01
phylum *Firmicutes*	39.09±1.85	43.05±1.99	42.85±2.11
class *Clostridia*	19.49±0.79	23.90±1.20*	21.60±0.88
family *Lachnospiraceae*	2.96±0.12	2.20±0.10**	2.32±0.10**
family *Eubacteriaceae*	6.06±0.28	9.97±0.38***	8.50±0.09***
family *Ruminococcaceae*	3.50±0.16	4.80±0.21**	4.09±0.13*
family *Clostridiaceae*	6.63±0.21	6.29±0.24	5.64±0.35
genus *Peptococcus*	0.34±0.02	0.64±0.03***	1.05±0.04***
genus *Lactobacillus*	3.58±0.16	4.31±0.19*	5.29±0.22***
genus *Enterococcus*	2.81±0.11	1.44±0.06***	1.57±0.08***
genus *Bacillus*	4.55±0.21	3.84±0.16*	6.30±0.28**
genus *Staphylococcus*	1.22±0.05	0.90±0.04**	1.31±0.06
order *Negativicutes*	7.44±0.32	8.66±0.38	6.78±0.31
phylum *Actinobacteria*	4.81±0.23	3.96±0.17*	4.53±0.21
genus *Bifidobacterium*	1.55±0.06	1.07±0.04***	1.26±0.12
other	3.26±0.12	2.86±0.11*	3.27±0.10
phylum *Proteobacteria*	6.14±0.28	8.08±0.39*	7.95±0.31*
family *Enterobacteriaceae*	2.70±0.09	2.00±0.04***	1.87±0.05***
family *Campylobacteriaceae*	0.33±0.01	0.49±0.02***	0.11±0.01***
family *Pseudomonadaceae*	2.52±0.16	4.86±0.22***	5.21±0.19***
family *Pasteurellaceae*	0.59±0.03	0.73±0.03*	0.66±0.06***
phylum *Tenericutes* (genus *Mycoplasma*)	3.02±0.14	3.84±0.10**	3.06±0.16
phylum *Fusobacteria*	0.75±0.12	0.97±0.15	0.87±0.14
unclassified sequences	24.19±1.12	18.92±0.67**	20.15±0.98*

N o t e. For description of the groups with regard to administration of the probioitics see the *Technique* section.
* $P < 0.05$; ** $P < 0.01$; *** $P< 0.001$.

The identified microorganisms included six phyla, mainly phylum *Firmicutes*, including bacteria of families *Bacillaceae*, *Lactobacillaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Clostridiaceae*, *Eubacteriaceae*. Microorganisms of phylum *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Tenericutes* and *Fusobacteria* were less abundant.

The numbers of detected opportunistic and pathogen microorganisms of genus *Staphylococcus* and families *Campylobacteriaceae* и *Enterobacteriaceae*, normally observed in GIT of poultry, were small. The presence of these microorganisms is usually related to dysbiotic GIT disorders in mammals and poultry. Apart from that, pathogens of families *Pasterellaceae* and *Actinobacteriaceae* and phylum *Fusobacteria* were identified, which were discovered in poultry GIT only due to molecular methods [24, 34]. The presence of bacteria of family *Pas-*

terellaceae and genus *Mycoplasma* in GIT of broiler chickens, which are mostly found in the respiratory tract of poultry and are considered as respiratory pathogens, is of significant interest.

Our findings are generally consistent with the present-day concepts of bacteria quantity and composition in the poultry caecum. The monograph by M.A. Timoshko [22] describes the following bacteria in poultry intestine, found by traditional methods of microbial detection: bacteroides, eubacteria, peptococci, lactic acid bacteria, bifidobacteria, streptococci, enterobacteria, staphylococci, bacilli. Domestic and foreign publications based on next-generation sequencing (NGS) and T-RFLP analysis confirm the presence of numerous microorganisms, including various pathogenic bacteria of genera *Campylobacter*, *Arcobacter*, *Shigella*, *Salmonella*, *Enterobacter*.

We have shown that dietary yeast and bacterial probiotics contributed to the change of qualitative and quantitative composition of bacterial microbiota in broilers' caecum.

In the chickens from group III, fed with dietary cellobacterine-T, the *Bacillus* sp. counts increased 1.38-fold ($P < 0.01$) as compared to the control, which was evidently related to successful colonization and reproduction of the introduced baccilli. These results are in line with data that some bacilli species, including *B. subtilis*, are capable of adhesion to intestine mucosa. It allows them to colonize the digestive tract and occupy free ecological niches of intestine microbiome, that resulted in a probiotic effect [9, 35]. In contrast, in the broilers from group II the proportion of bacilli decreased by 18.49 % ($P < 0.05$).

The number of bacteria from genus *Lactobacillus* increased by 20.39 % ($P < 0.05$) and 47.77 % ($P < 0.005$) in groups II and III, respectively. These microorganisms are known to be capable of competitive exclusion of pathogens due to production of organic acids and bacteriocins. The amount of other microorganisms of genus *Enterococcus* and family *Bifidobacteriaceae*, which are similar in properties, decreased by 95.13 % ($P < 0.005$) and 78.98 % ($P < 0.005$), and by 44.86 ($P < 0.005$) and 23.02 % for groups II and III, respectively.

Dietary probiotics had practically no effect on the phylum *Bacteroidetes* bacteria with cellulolytic and amylolytic enzymes but directly affected bacteria of phylum *Firmicutes* with similar properties. Interestingly, the effect of bacterial and yeast products on the said microorganisms was similar. An increase in proportion of families *Eubacteriaceae* and *Ruminococcaceae*, genus *Peptococcus*, and a decrease in *Clostridiaceae* and *Lachnospiraceae* counts occurred in both experimental groups, as compared to the control. The total proportion of the listed microorganisms from class *Clostridia* increased by 22.63 % ($P < 0.05$) and 10.83 %, when using probiotic products in groups II and III, respectively, as compared to the control.

Inclusion of probiotics in the diet had a positive effect on decrease in content of pathogens in caecum. The probiotics decreased the number of opportunistic enterobacteria in groups II and II by 35.00 % and 45.94 % compared to control. Apart from that, cellobacterine-T has reduced 3-fold ($P < 0.005$) the count of campylobacteria of family *Campylobacteriaceae*, pathogenic for both poultry and humans. Administration of yeast product lead to a decrease in proportion of staphylococci by 35.56 % ($P < 0.01$), with increase in other pathogenic bacteria, i.e. by 48.50 % ($P < 0.005$) for campylobacteria, by 23.72 % ($P < 0.05$) for pasteurella, and by 27.15 % ($P < 0.05$) for mycoplasmas.

The difference in broiler caecum microbiome composition resulted from feeding bacterial and yeast probiotics was apparent from biodiversity indices (see Table 1). Shannon indices were lower in chickens fed with dietary probiotics, which indicates more stable and uniform microbiocoenosis composition. The

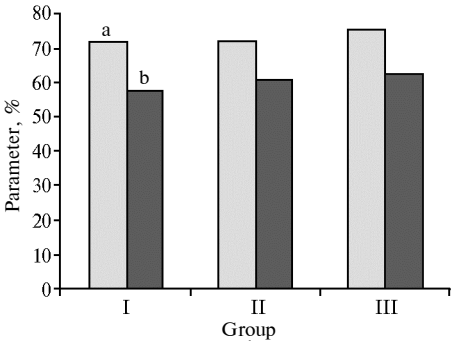
difference between caecum bacterial community of these chickens and microbiome of other groups appeared to be the most significant for yeast probiotic, and was characterized by the smallest Shannon index (4.00±0.19) and Simpson dominance index (0.9±0.03), which is indicative of more expressed effect of this product.

In all groups, the viability amounted to 100 %, and poultry performance was associated with the changes in the bacterial community (Table 2).

2. Productivity in Cobb 500 broiler chickens, as influenced by dietary cellobacterine-T probiotic and a yeast-based probiotic ($X \pm x$, Zagorskoe EPH VNITIP vivarium, Moscow region)

Parameter	Group I (control, n = 3)	Group II (n = 3)	Group III (n = 3)
Viability, %	100.0	100.0	100.0
Live weight, g:			
broilers aged 1 day	42.8±0.25	42.1±0.31	41.9±0.27
broilers aged 7 day	193.9±2.27	195.6±2.52	196.3±2.94
broilers aged 21 day	779.7±11.81	786.0±11.77	809.5±13.41
Average weight at the age of 37 days, g:			
overall	2096.2±68.15	2128.9±71.21	2188.2±59.30
in hens	1980.5±16.65	2004.3±17.09	2062.9±20.45**
in cockerels	2211.9±14.86	2253.5±11.54*	2313.4±16.09***
Average daily live weight gain, g:	55.5	56.4	58.0
Feed conversion rate			
per broiler, kg	3.46	3.59	3.45
per 1 kg of live weight gain, kg	1.69	1.72	1.61

N o t e. For description of the groups with regard to administration of the probioitics see the *Technique* section.
* P < 0.05; ** P < 0.01; *** P < 0.001.



Digestibility of the feed dry matter (a) **and utilization of nitrogen** (b) **by Cobb 500 broiler chickens, as influenced by dietary cellobacterine-T probiotic and a yeast-based probiotic** (Zagorskoe EPH VNITIP vivarium, Moscow region). For description of the groups with regard to administration of the probioitics see the *Technique* section.

The live weight gain at the age of 7, 21, and 97 days was higher than that of the control, i.e. by 0.9, 0.8, and 1.6 % for group II, and by 1.2, 3.8, and 4.4 % for group III. So, the chickens consuming compound feed supplemented with cellobacterine-T probiotic had maximum growth rate. In group III, the live weight at the age of 37 days was above the control values by 4.2 % (P ≤ 0.01) for female chickens, and by 4.6 % (P ≤ 0.001) for male chickens.

When using the yeast probiotic, the feed consumption rate increased by 3.8 %, as compared to the control. Feed conversion rate per 1 kg of live weight gain in group II was higher than that in the control and group III by 1.8 % and 4.7 %, respectively.

The results of physiological balance test were consistent with the data on broiler productivity (Fig.). The digestibility of feed dry matter in all experimental groups was higher than that in the control. The highest digestibility, with the difference of 3.7 %, was in group II. This group also differed from other groups in greater average daily live weight gain of 58.0 g. A similar pattern of the feed nitrogen utilization was in group III compared to other groups, i.e. the nitrogen utilization was significantly higher by 4.7 % than that in the peers from the control group.

The probiotic additives have affected vitamin accumulation in broiler liver (Table 3). In groups II and III, as compared to the control, the level of vitamin A increased by 4.7 % and 8.9 %, of vitamin E — by 7.7 % and 22.1 % (P < 0.01),

of vitamin B_2 — by 7.3 % and 12.4 %, and the amount of carotenoids was 4.8 % and 9.2 % higher, respectively.

3. Vitamins and carotenoids (μg/g) **in 37-day-old Cobb 500 broiler chicken liver, as influenced by dietary cellobacterine-T probiotic and a yeast-based probiotic** (Zagorskoe «EPH VNITIP vivarium, Moscow region)

Bioactive substances	Group I (control, n = 3)	Group II (n = 3)	Group III (n = 3)
Vitamin A	139.85±5.35	146.40±6.12	152.23±4.95
Vitamin E	5.71±0.12	6.15±0.17	6.97±0.09*
Vitamin B_2	10.96±0.42	11.76±0.18	12.32±0.32
Carotenoids	4.37±0.18	4.58±0.02	4.77±0.07

N o t e. For description of the groups with regard to administration of the probioitics see the *Technique* section.
* P < 0.01.

The differences by chemical composition of pectoral and femoral muscles in broilers were not observed.

It shall be noted that broiler growth significantly depend on the intestine bacterial community composition. According to other reportes, some obligate intestine inhabitants are able to affect the poultry productivity directly. V.A. Torok et al. [24)] have found the correlation between caecum microorganism composition and the effectiveness of feed energy utilization. As researchers thought, the bacteria can produce various acids, first of all butyric acid, which acts as a preferable energy source and increases the intestine epithelium size [36, 37], thus these bacteria can play an important role by creating a barrier for toxic agents [38]. The representatives of phylum *Firmicutes* [39], including *Eubacterium rectale* (family *Eubacteriaceae*), genus *Roseburia* (family *Lachnospiraceae*), *Faecalibacterium prausnitzii* (family *Ruminococcaceae*) are the main butyric acid producers [27, 28, 40]. When analyzing both probiotics, we observed an increase in proportion of *Eubacteriaceae* and *Ruminococcaceae* microorganisms in broiler caecum. This fact may indicate that the increase in productivity is related to increase in butyric acid content in the intestine.

Additionally, some authors note the dependence of poultry productivity on the intestine content of lactic acid produced by lactobacilli of genera *Lactobacillus*, *Enterococcus*, etc. It is known that lactate has more expressed antimicrobial properties with regard to pathogens, as compared to other acids (acetic, propionic, etc.) produced by intestinal microflora. As a rule, significant amounts of lactic acid do not accumulate in the intestine due to rapid absorption and assimilation by lactate-fermenting microorganisms of genera *Anaerostipes*, *Veillonella* and *Megasphaera* of order *Negativicutes*. According to our findings, administration of cellobacterine-T lead to an increase in proportion of lactate-producing bacterial of genus *Lactobacillus* and a decrease in the number of lactate-fermenting bacteria of order *Negativicutes*, which could contribute to greater accumulation of lactic acid in the broiler intestine in group III and, consequently, to more expressed antimicrobial effect on pathogens as compared to group II. Administration of live yeast resulted in an increase in the proportion of lactate-fermenting bacteria of order *Negativicutes* in the intestine. It could lead to better assimilation of lactic acid and, consequently, to a decrease in antimicrobial properties, as compared to group III [41, 42].

Thus, cellobacterine-T shows high probiotic activity and has a positive effect on the composition of the caecum bacterial community in broiler chickens. As a result, the number of normal flora (representatives of genera *Bacillus* and *Lactobacillus*) increases significantly, whereas the number of microorganisms traditionally related to intestine dysbiosis in humans and animals (families *Enterobacteriaceae* and *Campylobacteriaceae*) decreases. Yeast-based probiotic had a positive effect on the content of cellulosolytic microorganisms of class *Clostridia*; however, its effect on pathogenic microorganisms was less significant, e.g. lead to a significant increase in proportion of campylobacteria, pasteurella, and mycoplasmas.

Both probiotics positively influenced on poultry productivity, feed digestibility, utilization of nutrients and the level of vitamins in liver. The maximum live weight gain, accelerated growth and higher digestibility of the feed nutrients, as well as accumulation of vitamins A, E, B_2 and carotenoids in the liver were characteristic of the chickens fed with cellobacterine-T.

REFERENCES

1. Czerwinski J., Hojberg O., Smulikowska S., Engberg R.M., Mieczkowska A. Influence of dietary peas and organic acids and probiotic supplementation on performance and caecal microbial ecology of broiler chickens. *Brit. Poultry Sci.*, 2010, 51(2): 258-569 (doi: 10.1080/00071661003777003).
2. Malik N.I., Panin A.N. *Veterinariya*, 2001, 1: 27 (in Russ.).
3. Stanley D., Hughes R.J., Moore R.J. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Appl. Microbiol. Biotechnol.*, 2014, 98: 4301-4309 (doi: 10.1007/s00253-014-5646-2).
4. Rodriguez-Lecompte J.C., Yitbarek A., Brady J., Sharif S., Cavanagh M.D., Crow G., Guenter W., House J.D., Camelo-Jaimes G. The effect of microbial-nutrient interaction on the immune system of young chicks after early probiotic and organic acid administration. *J. Anim. Sci.*, 2012, 90(7): 2246-2254 (doi: 10.2527/jas.2011-4184).
5. Stanley D., Hughes R.J., Moore R.J. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Appl. Microbiol. Biotechnol.*, 2014, 98: 4301-4310 (doi: 10.1007/s00253-014-5646-2).
6. Rehman H., Vahjen W., Awad W., Zentek J. Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. *Arch. Anim. Nutr.*, 2007, 61: 319-335 (doi: 10.1080/17450390701556817).
7. Urdaci M.C., Bressollier P., Pinchuk I. *Bacillus clausii* probiotic strains: antimicrobial and immunomodulatory activities. *J. Clin. Gastroenterol.*, 2004, 38: 86-90.
8. Mazza P. The use of *Bacillus subtilis* as an antidiarrhoeal microorganism. *Boll. Chim. Farm.*, 1994, 133: 3-18.
9. Hong H.A., Huang J.M., Khaneja R., Hiep L.V., Urdaci M.C., Cutting S.M. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. *J. Appl. Microbiol.*, 2008, 105: 510-520 (doi: 10.1111/j.1365-2672.2008.03773.x).
10. Mountzouris K.C., Dalaka E., Palamidi I., Paraskeuas V., Demey V., Theodoropoulos G., Fegeros K. Evaluation of yeast dietary supplementation in broilers challenged or not with *Salmonella* on growth performance, cecal microbiota composition and *Salmonella* in ceca, cloacae and carcass skin. *Poultry Sci.*, 2015, 94(10): 2445-2455 (doi: 10.3382/ps/pev243).
11. M'Sadeq S.A., Wu S.B., Choct M., Forder R., Swick R.A. Use of yeast cell wall extract as a tool to reduce the impact of necrotic enteritis in broilers. *Poultry Sci.*, 2015, 94(5): 898-905 (doi: 10.3382/ps/pev035).
12. DeVries T.J., Chevaux E. Modification of the feeding behavior of dairy cows through live yeast supplementation. *J. Dairy Sci.*, 2014, 97(10): 6499-6510 (doi: 10.3168/jds.2014-8226).
13. Zhang Z., Cao L., Zhou Y., Wang S., Zhou L. Analysis of the duodenal microbiotas of weaned piglet fed with epidermal growth factor-expressed *Saccharomyces cerevisiae*. *BMC Microbiol.*, 2016, 28: 161-166 (doi: 10.1186/s12866-016-0783-7).
14. Pizzolitto R.P., Armando M.R., Combina M., Cavaglieri L.R., Dalcero A.M., Salvano M.A. Evaluation of *Saccharomyces cerevisiae* strains as probiotic agent with aflatoxin B_1 adsorption ability for use in poultry feedstuffs. *J. Environ. Sci. Health. B*, 2012, 47(10): 933-941 (doi: 10.1080/03601234.2012.706558).
15. Slizewska K., Piotrowska M. Reduction of ochratoxin A in chicken feed using probiotic. *Ann. Agric. Environ. Med.*, 2014, 21(4): 676-680 (doi: 10.5604/12321966).
16. Amit-Romach E., Uni Z., Reifen R. Multistep mechanism of probiotic bacterium, the effect on innate immune system. *Mol. Nutr. Food. Res.*, 2010, 54: 277-284 (doi: 10.1002/mnfr.200800591).
17. Pagnini C., Saeed R., Bamias G., Arseneau K.O., Pizarro T.T., Cominelli F., Amit-Romach E., Uni Z., Reifen R. Probiotics promote gut health through stimulation of epithelial innate immunity. *PNAS USA*, 2010, 107: 454-459 (doi: 10.1073/pnas.0910307107).
18. Rajput I.R., Hussain A., Li Y.L., Zhang X., Xu X., Long M.Y., You D.Y., Li W.F. *Saccharomyces boulardii* and *Bacillus subtilis* B10 modulate TLRs mediated signaling to induce immunity by chicken BMDCs. *J. Cell. Biochem.*, 2014, 115(1): 189-198 (doi: 10.1002/jcb.24650).

19. Alizadeh M., Rogiewicz A., McMillan E., Rodriguez-Lecompte J.C., Patterson R., Slominski B.A. Effect of yeast-derived products and distillers dried grains with solubles (DDGS) on growth performance and local innate immune response of broiler chickens challenged with *Clostridium perfringens*. *Avian Pathol.*, 2016, 45(3): 334-345 (doi: 10.1080/03079457.2016.ne).
20. Alizadeh M., Rodriguez-Lecompte J.C., Yitbarek A., Sharif S., Crow G., Slominski B.A. Effect of yeast-derived products on systemic innate immune response of broiler chickens following a lipopolysaccharide challenge. *Poultry Sci.*, 2016, 95(10): 2266-2273 (doi: 10.3382/ps/pew154).
21. Tarakanov B.V. *Metody issledovaniya mikroflory pishchevaritel'nogo trakta sel'skokhozyaistvennykh zhivotnykh i ptitsy* [Study of microflora in alimentary canal of farm animals — methods]. Moscow, 2006 (in Russ.).
22. Timoshko M.A. *Mikroflora pishchevaritel'nogo trakta sel'skokhozyaistvennykh zhivotnykh* [Microflora of alimentary canal in farm animals]. Kishinev, 1990 (in Russ.).
23. Park S.H., Lee S.I., Ricke S.C. Microbial populations in naked neck chicken ceca raised on pasture flock fed with commercial yeast cell wall prebiotics via an Illumina MiSeq Platform. *PLoS ONE*, 11(3): e0151944 (doi: 10.1371/journal.pone.0151944).
24. Torok V.A., Hughes R.J., Mikkelsen L.L. Identification and characterization of potential performance-related gut microbiota in broiler chickens across various feeding trials. *Appl. Environ. Microbiol.*, 2011, 77(17): 5868-5878 (doi: 10.1128/AEM.00165-11).
25. Witzig M., Camarinha-Silva A., Green-Engert R., Hoelzle K., Zeller E., Seifert J., Hoelzle L.E., Rodehutscord M. Correction: spatial variation of the gut microbiota in broiler chickens as affected by dietary available phosphorus and assessed by T-RFLP analysis and 454 pyrosequencing. *PLoS ONE*, 2015, 10(12): e0145588 (doi: 10.1371/journal.pone.0145588).
26. de Boer P., Rahaoui H., Leer R.J., Montijn R.C., van der Vossen J.M. Real-time PCR detection of *Campylobacter* spp.: a comparison to classic culturing and enrichment. *Food Microbiol.*, 2015, 51: 96-100 (doi: 10.1016/j.fm.2015.05.006).
27. Hold G.L., Schwiertz A., Aminov R.I., Blaut M., Flint H.J. Oligonucleotide probes that detect quantitatively significant groups of butyrate-producing bacteria in human feces. *Appl. Environ. Microbiol.*, 2003, 69: 4320-4324.
28. Bjerrum L., Engberg R.M., Leser T.D., Jensen B.B., Finster K., Pedersen K. Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and cellular-based techniques. *Poultry Sci.*, 2006, 85: 1151-1164.
29. *Metodika provedeniya nauchnykh i proizvodstvennykh issledovanii po kormleniyu sel'skokhozyaistvennoi ptitsy. Molekulyarno-geneticheskie metody opredeleniya mikroflory kishechnika* /Pod redaktsiei V.I. Fisinina [Poultry feeding: research and practical study. Molecular methods for the analysis of gut microflora. V.I. Fisinin (ed.)]. Sergiev Posad, 2013 (in Russ.).
30. *Instruktsiya po sanitarno-mikrobiologicheskomu kontrolyu tushek, myasa ptitsy, ptitseproduktov, yaits i yaitseproduktov na ptitsevodcheskikh i pererabatyvayushchikh predpriyatiyakh* [Instruction for the sanitary-microbiological control of poultry carcasses, meat, eggs and egg products in the poultry production and processing enterprises]. Moscow, 1990 (in Russ.).
31. Maniatis T., Fritsch E. F., Sambrook J. *Molecular cloning: a laboratory manual.* Cold Spring Harbor, NY, 1982.
32. Gong J., Forster R.J., Yu H., Chambers J.R., Sabour P.M., Wheatcroft R., Chen S. Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen. *FEMS Microbiol. Lett.*, 2002, 208: 1-7 (doi: 10.1016/S0378-1097(01)00521-3).
33. Amit-Romach E., Sklan D., Uni Z. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poultry Sci.*, 2004, 83: 1093-1098 (doi: 10.1093/ps/83.7.1093).
34. Rinttila T., Apajalahti J. Intestinal microbiota and metabolites — Implications for broiler chicken health and performance. *J. Appl. Poultry Res.*, 2013, 22(3): 647-658 (doi: 10.3382/japr.2013-00742).
35. Sanchez B., Arias S., Chaignepain S., Denayrolles M., Schmitter J.M., Bressollier P., Urdaci M.C. Identification of surface proteins involved in the adhesion of a probiotic *Bacillus cereus* strain to mucin and fibronectin B. *Microbiology*, 2009, 155: 1708-1716 (doi: 10.1099/mic.0.025288-0).
36. Le Blay G., Blottiere H.M., Ferrier L., Le Foli E.C., Bonnet J.P., Galmiche C., Cherbut C. Shortchain fatty acids induce cytoskeletal and extracellular protein modifications associated with modulation of proliferation on primary culture of rat intestinal smooth muscle cells. *Dig. Dis. Sci.*, 2000, 45: 1623-1630.
37. Yasuoka T., Tsujikawa Y. Fujiyama, Bamba T. Effects of the soluble fibre pectin on intestinal cell proliferation, fecal short chain fatty acid production and microbial population. *Digestion*, 2003, 67: 42-49.
38. Niba A.T., Beal J.D., Kudi A.C., Brooks P.H. Bacterial fermentation in the gastroin-

testinal tract of non-ruminants: Influence of fermented feeds and fermentable carbohydrates. *Trop. Anim. Health Prod.*, 2009, 41: 1393-1407 (doi: 10.1007/s11250-009-9327-6).
39. Pryde S.E., Duncan S.H., Hold G.L., Stewart C.S., Flint H.J. The microbiology of butyrate formation in the human colon. *FEMS Microbiol. Lett.*, 2002, 217: 133-139.
40. Louis P., Young P., Holtrop G., Flint H.J. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environ. Microbiol.*, 2010, 12: 304-314 (doi: 10.1111/j.1462-2920.2009.02066.x).
41. Belenguer A., Duncan S.H., Holtrop G., Anderson S.E., Lobley G.E., Flint H.J. Impact of pH on lactate formation and utilization by human fecal microbial communities. *Appl. Environ. Microbiol.*, 2007, 73: 6526-6533.
42. Harmsen H.J.M., Raangs G.C., He T., Degener J.E., Welling G.W. Extensive set of 16S rRNAbased probes for detection of bacteria in human feces. *Appl. Environ. Microbiol.*, 2002, 68: 2982-2990.
43. Swiqtkiewicz S., Arczewska-Wlosek, Jozetiak D. Immunomodulatory efficacy of yeast cell products in poultry: a current review. *World's Poultry Science Journal*, 2014, 70(1): 57-68.
44. Birgit K. Hefen in der Nutztierfutterung. *Feed Mag.*, 2015, 98(1-2): 17-21.
45. Fanelli A., Agazzi A., Alborali G.L., Pilotto A., Pilotto A., Bontempo V., Dell'Orto V., Demey V., Caputo J.M., Savoini G. Prevalence reduction of pathogens in poultry fed with Saccharomyces cerevisiae. *Biotechnol. Agron. Soc. Environ.*, 2015, 19(1): 3-10.

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 903-911
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Nanoparticles of metals: bioeffects

UDC 636.52/.58:636.087.72: 546.56-022.532

doi: 10.15389/agrobiology.2016.6.903rus
doi: 10.15389/agrobiology.2016.6.903eng

MORPHOLOGICAL AND BIOCHEMICAL BLOOD PARAMETERS IN BROILERS AT CORRECTION WITH DIETARY COPPER SALTS AND NANOPARTICLES

E.A. SIZOVA[1, 2], V.L. KOROLEV[1], Sh.A. MAKAEV[1], E.P. MIROSHNIKOVA[2], V.A. SHAKHOV[3]

[1]*All-Russian Research Institute of Beef Cattle Breeding,* Federal Agency of Scientific Organizations, 29, ul. 9 Yanvarya, Orenburg, 460000 Russia, e-mail sizova-178@ya.ru, vniims.or@mail.ru;
[2]*Orenburg State University*, 13, prosp. Pobedy, Orenburg, 460018 Russia;
[3]*Orenburg State Agrarian University,* 18, ul. Chelyuskintsev, Orenburg, 460014 Russia
(ORCID: Sizova E.A. orcid.org/0000-0002-5125-5981)
Acknowledgements:
Chemical analysis was performed in the Laboratory of Agroecology of Nanomaterials and Test Center of All-Russian Research Institute of Beef Cattle Breeding (accreditation certificate RA. RU.21PF59 of 12/02/15). Chemical analysis was performed in the laboratory of ANO Center for Biotic Medicine, Moscow (accreditation certificate GSEN.RU.TSAO.311, registration number in the State Register ROSS RU. 0001.513118)
Supported by Russian Science Foundation (project № 14-36-00023)
Received July 11, 2016

Abstract

Diets of modern crosses and breeds of farm animals require mandatory correction of mineral nutrition. Ionic forms commonly used for correction are characterized by low bioavailability, prooxidant effect, and high toxicity in vivo. That is why low-toxic sources of essential chemical elements are of particular interest, including nanocrystalline metals. We firstly assessed the efficient of nanosized copper on the model of broiler chickens. A comparative study of productive and biological effects of copper nanoparticles (CuNPs) and copper sulfate was carried out on Smena 7 chickens of 14-42 days of age using different administration methods. Hematologic and biochemical parameters and elemental composition of tissues were assessed. We revealed an increase in red blood cells and hemoglobin and decrease of platelets at the highest doses of Cu NPs administrated orally or intramuscularly. The biochemical blood parameters assessed indicated an enhancement of plastic processes in the body and, as a consequence, an increase in the growth rate under Cu NPs administration. The difference of the total protein compared to the control (33.6 %, $p < 0.05$) was the highest when Cu NPs injected at a dose of 2 mg/kg which increased the supply of protein synthesis. The albumin level was higher in 28-day old chicks injected with Cu NPs (2 and 0.2 mg/kg). A replacement of dietary $CuSO_4$ with dietary Cu NPs led to an increase in the growth rate. When Cu NPs fed, the found biochemical parameters indicated strengthening of energy and synthetic processes in the body, and therefore, an increase in the poultry growth. At 1.7 mg/kg of dietary Cu NPs the gained weight was 13.5-23.8 % ($p < 0.01$ to $p < 0.001$) more as compared to the control. Dietary Cu NPs at 0.7 mg/kg was not enough to highly stimulate the poultry growth and resulted in the weight gain which was lower than in the poultry fed with Cu NPs at 1.7 mg/kg but higher (by 8.5-18.4 %, $p < 0.01$ to $p < 0.01$) as compared to the poultry not fed with Cu NPs. Thus, the promoting effect depends on the form of copper source. The Cu NPs effectiveness under enteral administration at 1.7 mg/kg or intramuscular double injections at 0.2 mg/kg was superior to that of dietary copper sulfate.

Keywords: nanoparticles, cooper, broiler chicks, growth intensity, chemical elements, blood, biochemical and morphological parameters

Diets in the modern animal and poultry farming are subject to mandatory adjustment of mineral composition, which is determined by an increase in genetic potential [1], the specific properties of biogeochemical provinces [2, 3], and impact of regional components on availability of mineral components [4-6], etc. Generally, ionic forms act as the sources of essential chemical elements. They are characterized in vivo by low bioavailability, pro-oxidant effect and high toxicity [7]. In particular, this explains controversial attitude to metallotherapy

and special interest to low-toxic sources of essential chemical elements, which may include nanocrystalline metal forms [8-10].

Unique properties of nanomaterials may ensure the possibility of their wide use in medicine and biology [11, 12] due to low toxicity and high bioavailability at a size of about 100 nm [13]. Unique properties of nanomaterials have objectively determined the emergence of new nanoparticle-based sources of microelements, with feasibility of application in feeding animals and poultry demonstrated in several studies [14, 15].

Here we first assessed effectiveness of various copper forms ($CuSO_4$ and nanocrystalline compiund) in poultry farming, and have revealed the tendency towards increase in erythropoeisis, albumin synthesis in liver and growth intensity due to Cu nanoparticles.

Our objective was a comparative evaluation of cooper nanoparticle and mineral salt product with regard to bioavailability and effect on growth, development, hematological and biochemical parameters in broiler chickens.

Technique. Cu nanoparticles (Cu NPs) were synthesized by high-temperature condensation method (Migen-3, Institute for Energy Problems of Chemical Physics, RAS, Moscow) in accordance with the description [16]. The material attestation of the preparations included electronic scanning and transmission microscopy (JSM 7401F and JEM-2000FX, JEOL, Japan). The X-ray phase analysis was conducted using a diffractometer DRON-7 (Burevestnik, Russia). The size (d) of obtained nanoparticles is 103±2 nm. Nanoparticle lysozoles in saline solution were prepared in an ultrasonic disperser UZDN-2T (NPP Akadempribor, Russia) (35 kHz, 300/450 W, 10 μA), with UV sterilization.

The studies were performed on Smena 7 broiler chickens (Orenburg State University vivarium). The keeping conditions and test procedures met the recommendations provided for by the national regulations (Order of the USSR Ministry of Health No. 755 d/d August 12, 1977) and The Guide for Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996). The day-old chickens (n = 270) were assigned individual tags and placed in the same conditions. At the age of 2 weeks, based on individual daily weighing and food consumption data, three control and five test groups were formed (n = 30 in each group). Chickens received a complete compound feed recommended by the All-Russian Scientific Research and Technology Poultry Institute (VNITIP) [17, 18]. Copper content in starting basic diet (BD) amounted to 11.79 mg/kg, in growth BD — to 9.51 mg/kg, including that due to introduction of copper sulphate (1.7 mg/kg) [18]. Basic diet only (BD, K_1), BD without $CuSO_4 \cdot 5H_2O$ (K_2) and BD with double intramuscular injections of saline solution at the age of 14 and 28 days (K_3) were the controls. The difference between the groups was as follows: group I — BD without $CuSO_4 \cdot 5H_2O$ plus Cu nanoparticles fed at a dose of 1.7 mg/kg, group II — BD without $CuSO_4 \cdot 5H_2O$ plus Cu nanoparticles fed at a dose of 0.7 mg/kg (oral consumption of nanoparticles); group III — BD without $CuSO_4 \cdot 5H_2O$ plus Cu nanoparticles (2 mg/kg of live weight) injected into femoral muscles, group IV — BD without $CuSO_4 \cdot 5H_2O$ plus Cu nanoparticles (0.2 mg/kg) injected into femoral muscles, group V — BD without $CuSO_4 \cdot 5H_2O$ plus Cu nanoparticles (0.02 mg/kg of live weight) injected into femoral muscles (double parenteral input at the age of 14 and 28 days). All chickens received distilled water. From day 14 to day 28 the BD formulation contained 320 g/kg wheat, 10 g/kg barley, 184 g/kg sunflower cake, 200 g/kg soy bean meal, 40 g/kg fish flour, 60 g/kg vegetable oil, 163 g/kg corn grain, 10 g/kg wheat bran, 10 g/kg limestone, and 3 g/kg common salt. From day 28 to day 42 the BD contained 182 g/kg wheat, 50 g/kg barley, 180 g/kg sunflower cake, 75 g/kg soy bean meal, 45 g/kg fish flour, 45 g/kg vegetable oil, 400 g/kg corn

grain, 10 g/kg wheat bran, 10 g/kg limestone, and 3 g/kg common salt.

The tested chickens were daily weighed individually. Blood was sampled from the axillary vein, before slaughter in the morning in a fasting state, at the age of 28 and 42 days. Samples for examination of morphological parameters were placed in vacuum tubes with anticoagulant (EDTA), for biochemical studies — into vacuum tubes with a coagulation activator (thrombin). Morphological parameters were determined using an automatic hematology analyzer URIT-2900 Vet Plus (URIT Medial Electronic Co., Ltd, China). The biochemical blood serum test was made using an automatic biochemical analyzer CS-T240 (DIRUI Industrial Co., Ltd, China) and commercial veterinary kits (DiaVetTest by ZAO DIAKON-DS, Russia; Randox Laboratories Ltd., Great Britain). The blood serum was analyzed within 2 hours after blood was sampled.

When determining Cu pool in the body in the beginning and in the end of the reference period, average samples (n = 15) were formed after slaughtering at the age of 28 and 42 days for muscle tissue, skin, internal organs, i.e. gastrointestinal tract (GIT), heart, lung, liver, kidney, spleen, genital tissues, bone tissue, central nervous system (CNS) and visceral fat. The samples were frozen and stored at –18 °C. The overall pool of a chemical element was determined by summarizing organ and tissue parameters. Elementary content of biosubstrates and compound feed was analyzed by inductively coupled plasma atomic emission spectrometry (Optima 2000 V, Perkin Elmer, USA) and mass spectrometry (Elan 9000, Perkin Elmer, USA). The biosubstrates were ashed using a microwave decomposition system Multiwave-3000 (Anton Paar, Austria).

When calculating the bioavailability of copper from various sources (nanoparticles, salt), it was assumed that transformation coefficient of copper from BD (without regard to copper sulphate) remains unchanged in all test and control groups. Transformation coefficient of copper [18] from BD (without regard to copper sulphate) was calculated based on the total intake of Cu with feeds and the growth of Cu pool in the body in groups K_1 (BD) and K_2 (BD without $CuSO_4 \cdot 5H_2O$).

The data are presented as the arithmetic mean (M) with the standard error of the mean (m). Statistical processing was performed using Statistica 10.0 software package (StatSoft Inc., USA). The differences were considered statistically significant at $p < 0.05$.

Results. Productivity of broilers was related to copper content in the diet (Fig. 1). Thus, exclusion of copper sulphate from the premix (group K_2) led to a decrease in body weight by 8 %, as compared to the complete diet (K_1). At the same time, intramuscular injection of saline solution (K_3) during normalization of the diet with regard to Cu had no negative consequences, and live weight in this group was similar to that in K_1 (differences within the error value).

Replacement of $CuSO_4 \cdot 5H_2O$ with dietary Cu NPs was followed by more intensive growth of chickens. By the end of the experiment their weight in group I exceeded the weight in K_1 by 13.5 % ($p < 0.01$) and in K_2 by 23.8 % ($p < 0.001$). Cu NPs dose of 0.7 mg/kg was insufficient to meet the poultry demand in copper and ensure the maximum growth promoting effect, so live weight was lower than that in group I, but significantly higher than that in K_1 (by 8.5 %, $p < 0.01$) and K_2 (by 18.4 %, $p < 0.01$).

Similar growth promoting effect resulted from an increase of parenteral dose of Cu NPs (from 0.02 mg/kg to 2 mg/kg). Presumably, the dose of 0.02 mg/kg was insufficient to provide the poultry with this element that was confirmed by weighing results. By the end of the experiment, the difference in live weight amounted to 13.5 % as compared to K_1 in case of dietary Cu NPs at 1.7 mg/kg, and to 9.5 % as compared to K_3 in case of Cu NPs intramuscularly injected at 2 mg/kg.

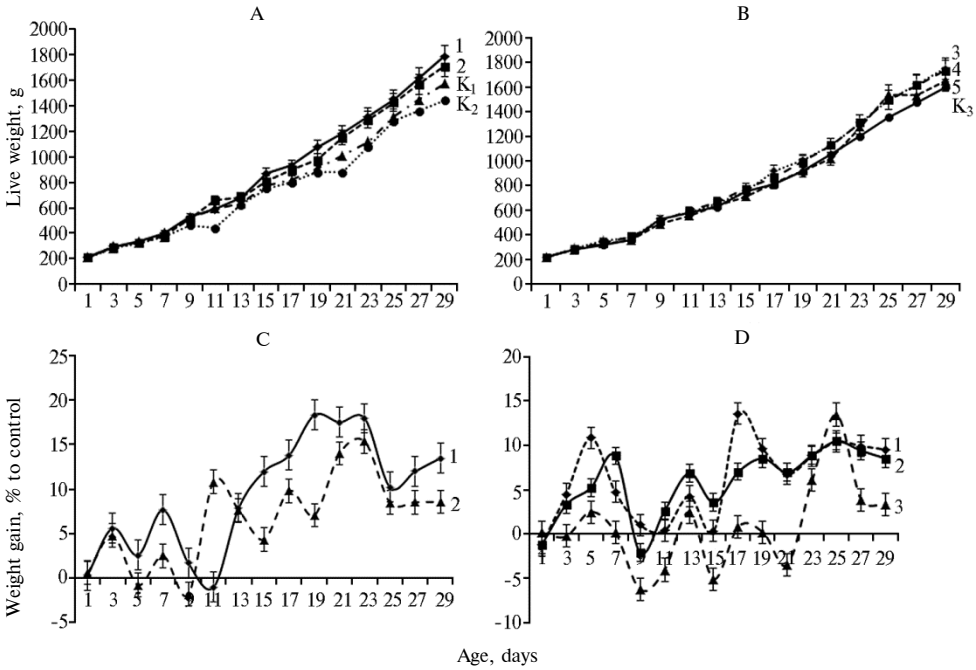


Fig. 1. Growth (A, B) **and relative live weight gain** (C, D) **in Smena 7 broiler chickens fed** (A, B) **or intramuscularly injected** (C, D) **with Cu NPs:** 1, 2, 3, 4, 5 — groups I, II, III, IV and V (see description in section *Technique*); K_1, K_2 and K_3 (controls) — basic diet (BD), BD without $CuSO_4 \cdot 5H_2O$ and BD with intramuscular injections of saline solution. Gain values are given with regard to K_1 (C, intake of Cu NPs with the feed) and K_3 (D, intramuscular injection). By the end of the observation period, the differences between groups I and II vs. K_1, group I vs. K_2, as well as groups III and IV vs. K_3 are significant at $p < 0.05$, between group II and K_2 — significant at $p < 0,01$ ($M \pm m$, $n = 30$, vivarium).

Blood morphological parameter under a replacement of copper sulphate with Cu NPs in the diet and in intramuscular injections had a number of specific features (Table 1).

1. Blood morphological parameters in Smena 7 broiler chickens at various doses and ways of Cu NPs and Cu salts intake ($M \pm m$, $n = 15$, vivarium)

Groups	Hematocrit, %	Erythrocytes, $\times 10^{12}$/l	Hemoglobin, g/l
Day 28			
Dietary consumption			
I (BD without $CuSO_4 \cdot 5H_2O$, Cu NPs 1.7 mg/kg)	25.0±0.41[a]	2.05±0.02[a]	110.0±2.62[a]
II (BD without $CuSO_4 \cdot 5H_2O$, Cu NPs 0.7 mg/kg)	23.4±1.10[a]	2.93±0.09[a]	102.7±4.71[a]
Parenteral injections			
III (BD without $CuSO_4 \cdot 5H_2O$, Cu NPs 2 mg/kg)	25.9±1.20[ac]	3.04±0.12[a]	102.3±5.82[a]
IV (BD without $CuSO_4 \cdot 5H_2O$, Cu NPs 0.2 mg/kg)	25.5±0.27[ac]	3.01±0.03[ac]	103.2±1.90[a]
V (BD without $CuSO_4 \cdot 5H_2O$, Cu NPs 0.02 mg/kg)	23.7±0.81[a]	2.89±0.09[ab]	94.5±4.75
K_1 (BD)	18.5±1.03	2.83±0.14	96.0±4.84
K_2 (BD without $CuSO_4 \cdot 5H_2O$)	22.3±1.68	2.50±0.09	86.2±8.80
K_3 (BD with double administration of saline solution)	21.3±0.88	2.75±0.08	93.0±4.76
Day 42			
Dietary consumption			
I (BD without $CuSO_4 \cdot 5H_2O$, Cu NPs 1.7 mg/kg)	28.4±0.43[ab]	3.29±0.02[ab]	125.5±4.18[ab]
II (BD without $CuSO_4 \cdot 5H_2O$, Cu NPs 0.7 mg/kg)	21.9±2.47	2.91±0.02	102.0±9.80
Parenteral injections			
III (BD without $CuSO_4 \cdot 5H_2O$, Cu NPs 2 mg/kg)	27.55±1.43	3.23±0.12	118.2±7.30
IV (BD without $CuSO_4 \cdot 5H_2O$, Cu NPs 0.2 mg/kg)	26.6±0.49	3.22±0.06	117.0±2.62
V (BD without $CuSO_4 \cdot 5H_2O$, Cu NPs 0.02 mg/kg)	28.4±0.69[b]	3.41±0.06[b]	128.2±2.44[b]
K_1 (BD)	23.9±0.98	3.98±0.12	119.3±3.36
K_2 (BD without $CuSO_4 \cdot 5H_2O$)	22.5±0.62	3.13±0.06	110.3±2.49
K_3 (BD with double administration of saline solution)	24.6±0.57	3.68±0.11	120.3±3.01

Note. BD — basic diet; [a] — differences with K_1 are significant at $p < 0.05$, [b] — differences with K_2 are significant at $p < 0.05$, [c] — differences with K_3 are significant at $p < 0.05$.

Thus, the number of erythrocytes amounted to the maximum value in the

2. Blood biochemical parameters in Smena 7 broiler chickens fed with Cu NPs ($M \pm m$, n = 15, vivarium)

Parameter	Group I	Group II	K_1
	Day 28		
Total protein, g/l	25.8±1.85	20.8±1.57	20.1±1.99
Albumin, g/l	12.3±0.99*	8.8±0.54	10.2±0.81
Bilirubin, μM/l	3.29±0.070*	3.20±0.070*	2.13±0.080
ALT, U/l	10.3±0.84	7.5±0.57	8.8±0.62
AST, U/l	277.5±6.06**	196.8±8.12	179.1±5.11
Urea, mmol/l	1.10±0.011	1.13±0.070	1.25±0.020
Creatinine, mmol/l	74.0±3.63	67.7±2.11	61.9±2.94
Cholesterol, mmol/l	3.28±0.042	2.57±0.027	2.98±0.011
Glucose, mmol/l	11.13±0.120**	8.90±0.840*	6.11±0.500
Magnesium, mmol/l	0.87±0.031	1.13±0.027	1.05±0.090
Calcium, mmol/l	0.64±0.009	0.54±0.001*	1.62±0.091
Phosphorus, mmol/l	1.14±0.020	0.59±0.030	1.44±0.070
Iron, mmol/l	43.20±2.690	33.19±0.880*	60.20±3.140
	Day 42		
Total protein, g/l	37.8±2.61	38.8±2.81	32.3±2.12
Albumin, g/l	15.8±0.45	15.7±0.15	13.2±0.13
Bilirubin, μM/l	3.94±0.092	4.74±0.080	3.8±0.091
ALT, U/l	9.50±0.620*	10.50±0.160*	7.9±0.320
AST, U/l	310.7±13.73	238.7±18.35	265.1±10.21
Urea, mmol/l	1.34±0.090	1.49±0.020	1.12±0.020
Creatinine, mmol/l	54.3±1.11*	55.8±2.60*	63.3±4.12
Cholesterol, mmol/l	3.30±0.016	2.90±0.029	3.13±0.015
Glucose, mmol/l	8.95±0.440	9.09±0.110	11.09±0.970
Magnesium, mmol/l	0.32±0.009	0.68±0.005	0.91±0.006
Calcium, mmol/Ll	1.13±0.050	1.68±0.040	1.84±0.086
Phosphorus, mmol/l	1.03±0.030	1.70±0.070	1.62±0.090
Iron, mmol/l	21.7±1.64	28.5±1.16	23.1±1.15

Note. Groups I and II — basic diet (BD) without $CuSO_4 \cdot 5H_2O$ with Cu NPs added (1.7 and 0.7 mg/kg feed, respectively); K_1 — BD; ALT — alanine aminotransferase, AST — aspartate aminotransferase.
*, ** Differences with K_1 are significant at $p < 0.05$ and $p < 0.01$, respectively.

3. Blood biochemical parameters in Smena 7 broiler chickens intramuscularly injected with Cu NPs ($M \pm m$, n = 15, vivarium)

Parameter	Group III	Group IV	Group V	K_3
	Day 28			
Total protein, g/l	25.2±1.76	30.5±1.14*	38.5±1.70	19.5±0.18
Albumin, g/l	14.8±0.76*	14.2±0.47*	15.2±0.83	9.2±0.11
Bilirubin, μM/l	1.13±0.010	2.78±0.190	1.46±0.080	1.91±0.050
ALT, U/l	8.0±0.48	4.0±0.51**	8.5±0.62	9.8±0.72
AST, U/l	228.3±6.62	277.5±8.17**	191.2±4.40	183.6±9.36
Urea, mmol/l	1.47±0.011	1.25±0.021	1.47±0.035	1.27±0.020
Creatinine, mmol/l	71.3±4.14	69.5±3.11	72.5±3.49	62.8±3.54
Cholesterol, mmol/l	3.13±0.025	3.67±0.024**	3.87±0.034**	2.78±0.021
Glucose, mmol/l	4.79±0.480	3.88±0.190	3.47±0.170	5.19±0.500
Magnesium, mmol/l	0.50±0.060	0.78±0.020	0.94±0.025	1.02±0.018
Calcium, mmol/l	1.20±0.032	0.92±0.03*	0.65±0.03	1.42±0.018
Phosphorus, mmol/l	1.88±0.090	1.14±0.050	2.47±0.080	1.34±0.017
Iron, mmol/l	96.49±1.65*	49.31±1.92	72.73±3.88	58.0±4.94
	Day 42			
Total protein, g/l	44.5±1.33**	43.8±1.54**	40.3±1.99**	33.3±1.16
Albumin, g/l	15.7±0.95	16.2±0.31	16.5±0.67	14.2±0.25
Bilirubin, μM/l	4.37±0.081	2.79±0.070	4.77±0.077	3.5±0.028
ALT, U/l	10.83±0.35	15.33±0.60	10.33±0.61	6.9±0.27
AST, U/l	298.0±9.46	354.0±12.17	310.3±11.84	273.1±10.25
Urea, mmol/l	1.46±0.090	0.81±0.080	1.09±0.090	1.32±0.020
Creatinine, mmol/l	59.3±4.01	66.0±2.24	81.7±3.84	61.3±3.66
Cholesterol, mmol/l	3.33±0.037	2.95±0.046	2.53±0.027	2.93±0.018
Glucose, mmol/l	10.10±0.140	14.79±0.150*	8.48±0.520	10.08±0.270
Magnesium, mmol/l	0.65±0.012	0.78±0.020	0.81±0.020	0.86±0.076
Calcium, mmol/l	1.73±0.040	1.81±0.020	1.77±0.040	1.74±0.015
Phosphorus, mmol/l	1.38±0.080	1.44±0.320	0.90±0.010	1.51±0.050
Iron, mmol/l	56.1±2.13	35.9±1.09	29.8±2.08	25.2±1.04

Note. Groups III-V — basic diet (BD) without $CuSO_4 \cdot 5H_2O$ with Cu NPs administration (injections at 2; 0.2 and 0.02 mg/kg live weigt, respectively); K_3 — with double administration of saline solution; ALT — alanine aminotransferase, AST — aspartate aminotransferase.
*, ** Differences with K_3 are significant at $p < 0{,}05$ and $p < 0{,}01$, respectively.

groups with the maximum dose of Cu NPs, both oral and intramuscular. For example, in group I the deference, as compared to the control, was 12.6 % ($p < 0.001$) at

the age of 28 days, and 75.0 % ($p < 0.05$) at the age of 42 days. Hemoglobin content (regardless of the Cu NPs administration route) exceeded the same of the control (with maximum difference of 8.9 % in group I). Similar effect of nanoparticles on hematological parameters was described earlier [19].

The highest level of total protein was found in chickens which received Cu NPs intramuscularly (Table 2, 3). At that, maximum difference with the control (33.6 %, $p < 0.05$) was at a dose of 2 mg/kg, which improved protein synthesis. Significant differences in concentrations of albumins in groups received Cu NPs at the doses of 2 and 0.2 mg/kg, were observed in chickens aged 28 days. Intake of 0.7 mg/kg Cu NPs with the feed promoted albumin synthesis in liver to a lesser extent. Dynamics of urea and creatinine concentrations also confirmed high activity of metabolic processes in broiler chickens. Content of creatinine (a dehydrated form participating in energy metabolism in muscles) [20] has decreased by 8.96-11.41 % ($p < 0.05$), as compared to the control, in case of oral intake of Cu NPs, which is quite natural due to active transformation of creatinine to creatine phosphate and delivery to muscles in the form of energy during activation of biochemical processes. It was reported that the amount of creatinine decreases by 12-27 % under arabinogalactan added to the broilers diet, which is followed by intensification of metabolic processes [21].

Dietary Cu NPs at 0.7 mg/kg was not sufficient to compensate for complete absence of copper sulphate in the diet and did not meet the requirements of the body. An increase in blood glucose level under Cu intake with feed is indicative of intensification of synthesis and energy processes, which leads to more intensive growth (especially at the age of 28 days).

The amount of bilirubin formed in liver reticuloendothelial system as a result of catabolism of heme containing proteins, tended towards increase in case of Cu NPs intake with an increase in control groups as the chickens were growing. The amount of bilirubin in chickens aged 42 days, which received Cu NPs, slightly exceeded the control values.

Due to a metabolic shift under Cu NPs intake, catalytic activity of aminotransferases differed from the control. The ALT activity increased significantly in broilers aged 42 days under oral intake of Cu NPs at various doses, e.g. by 36.9 % ($p < 0.05$) in group I and by 51.3 % ($p < 0.05$) in group II. Intramuscular administration lead to higher activity of enzymes, up to the difference of 56-120 % ($p < 0.05$), as compared to the control.

4. Chemical composition of tissues in Smena 7 broilers which received Cu NPs ($M \pm m$, $n = 15$, vivarium)

Parameter	K_1	Group I	Group IV
Dry matter (DM)	41.6±0.53	37.3±0.45[ab]	40.7±0.82[b]
Protein	16.2±0.67	18.0±0.16[ab]	16.8±0.32[b]
Lipids	22.6±1.50	18.1±1.32[a]	21.0±1.74
Ash	2.2±0.07	2.4±0.08[a]	2.7±0.09
Energy, MJ/kg DM	25.7±0.92	30.9±0.67	31.4±1.52

N o t e. Group I — basic diet (BD) without $CuSO_4 \cdot 5H_2O$ with Cu NPs added at 1.7 mg/kg feed; group IV — BD without $CuSO_4 \cdot 5H_2O$ with Cu NPs intramuscularly injected at 0.2 mg/kg live weigt; K_1 — OP; [a] — differences with K_1 are significant at $p < 0.05$, [b] — differences between groups I and IV are significant at $p < 0.05$.

Dietary Cu NPs decreased the amount of Ca more significantly than intramuscular injections. Blood Ca concentration increased with age and was close to control values. It is known that excessive Cu intake may inhibit iron absorption [22]. In our experiments iron concentration in serum reached maximum in the chickens aged 28 days, which received high doses of Cu NPs (2 mg/kg). The period from injection to slaughter (2 weeks) ensured prolonged release of copper to the bloodstream. At the age of 42 days the broilers of this group still showed higher iron level, but its amount decreased comparing to that at the age of 28 days. In

28 day-old chickens which received Cu NPs with the feed these parameters were below the control values (by 25 % and 42 % in group I and II, respectively).

We have observed a decrease in fat accumulation in chickens from test groups, e.g. by 4.5 % ($p < 0.05$) in group I, which took place with simultaneous increase in protein content (by 1.8 %, $p < 0.05$) (Table 4).

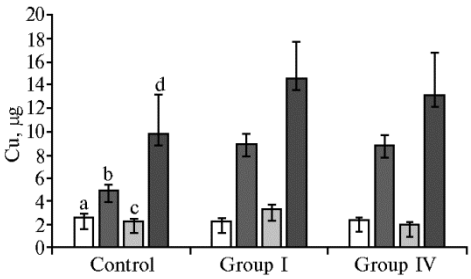


Fig. 2. Cu pool in organs and tissues of Smena 7 broiler chickens aged 42 days when fed with Cu NPs (group I, 1.7 mg/kg feed) **and intramuscularly infected with Cu NPs** (group IV, 0.2 mg/kg live weight)**:** a — muscles, b — internal organs, c — skin, d — total (control — K_1, basic diet; $M \pm m$, $n = 30$, vivarium).

Examination of Cu accumulation in biosubstrates discovered inter-group dynamics for internal organs only (Fig. 2). The differences for skin and muscles were insignificant, with relatively constant copper content. The way and multiplicity of NPs intake, as well as their dose played the key role. In case of daily chronic intake copper content was stable during the whole experiment, which was not observed in case of double intramuscular administration. An increase in copper pool in the body when using nanoparticles was previously observed by other researchers [23].

Calculated Cu bioavailability for the compared additives amounted to 20.08 mg per broiler (including 4.05 mg with copper sulphate) in K_1 and 30.56 mg per broiler (including 4.13 mg with nanoparticles) in group I, respectively. Taking into account that Cu transformation coefficient in the basic diet without $CuSO_4$ (group K_2) was 32.5 %, apparent bioavailability of Cu from mineral additives amounted to 46.1 % for copper sulphate, and to 71.0 % for dietary Cu NPs.

Thus, the effectiveness of copper preparations as a micronutrient depends on their form. Cu NPs when added to diet at a dose of 1.7 mg/kg of feed and injected intramuscularly at 0.2 mg/kg of live weight turned out to be optimal for broiler chicken farming.

REFERENCES

1. Fisinin V.I., Egorov I.A. *Ptitsa i ptitsevodstvo*, 2015, 3: 27-29 (in Russ.).
2. Notova S.V., Miroshnikov S.A., Bolodurina I.P., Didikina E.V. *Vestnik Orenburgskogo gosudarstvennogo universiteta*, 2006, 2: 59-63 (in Russ.).
3. Miroshnikov S.A., Burtseva T.I., Golubkina N.A., Notova S.V., Skal'nyi A.V., Burlutskaya O.I. *Vestnik Orenburgskogo gosudarstvennogo universiteta*, 2008, 12: 97 (in Russ.).
4. Sokolova O.Ya., Miroshnikov S.A., Drozdova E.A., Kholodilina T.N. *Vestnik Orenburgskogo gosudarstvennogo universiteta*, 2006, 12(62-2): 230-232 (in Russ.).
5. Bykov A.V., Miroshnikov S.A., Mezhueva L.V. *Vestnik Orenburgskogo gosudarstvennogo universiteta*, 2009, 12: 77-80 (in Russ.).
6. Miroshnikov S.A., Muslyumova D.M., Bykov A.V., Rakhmatullin Sh.G., Bykova L.A. *Vestnik myasnogo skotovodstva*, 2012, 3(77): 7-11 (in Russ.).
7. Albert A. *Selective toxicity — the physico-chemical basis of therapy*. London, NY, 1985. V. 2.
8. Sizova E., Miroshnikov S., Yausheva E., Polyakova V. Assessment of morphological and functional changes in organs of rats after intramuscular introduction of iron nanoparticles and their agglomerates. *BioMed Research International*, 2015, 2015: Article ID 243173 (doi: 10.1155/2015/243173).
9. Sizova E., Yausheva E., Miroshnikov S., Lebedev S., Duskaev G. Element status in rats at intramuscular injection of iron nanoparticles. *Biosci. Biotechnol. Res. Asia*, 2015, 12: 119-127 (doi: 10.13005/bbra/2018).
10. Sizova E., Miroshnikov S., Yausheva E., Kosyan D. Comparative characteristic of toxicity of nanoparticles using the test of bacterial bioluminescence. *Biosci. Biotechnol. Res. Asia*, 2015, 12: 361-368 (doi: 10.13005/bbra/2211).
11. Patel D., Kell A., Simard B., Deng J., Xiang B., Lin H.Y., Gruwel M., Tian G.

Cu^{2+}-labeled, SPION loaded porous silica nanoparticles for cell labeling and multifunctional imaging probes. *Biomaterials*, 2010, 31(10): 2866-2873 (doi: 10.1016/j.biomaterials.2009.12.025).
12. Ruparelia J.P., Chatterjee A.K., Duttagupta S.P., Mukherji S. Strain specificity in antimicrobial activity of silver and copper nanoparticles. *Acta Biomaterialia*, 2008, 4: 707-716 (doi: 10.1016/j.actbio.2007.11.006).
13. Bogoslovskaya O.A., Sizova E.A., Polyakova V.S., Miroshnikov S.A., Leipunskii I.O., Ol'khovskaya I.P., Glushchenko N.N. *Vestnik Orenburgskogo gosudarstvennogo universiteta*, 2009, 2: 124-127 (in Russ.).
14. Nikonov I.N., Folmanis Yu.G., Folmanis G.E., Kovalenko L.V., Laptev G.Yu., Egorov I.A., Fisinin V.I., Tananaev I.G. *Doklady akademii nauk*, 2011, 440(4): 565-569 (in Russ.).
15. Miroshnikova E.P., Arinzhanov A., Kilyakova Y., Sizova E.A., Miroshnikov S.A. Antagonism metal alloy nanoparticles of iron and cobalt: impact on trace element metabolism in carp and chicken. *Hum. Vet. Med.*, 2015, 7(4): 253-259.
16. Zhigach A.N., Leipunskii I.O., Kuskov M.L., Stoenko N.I., Storozhev V.B. *Pribory i tekhnika eksperimenta*, 2000, 6: 122-127 (in Russ.).
17. Fisinin V.I., Egorov I.A., Lenkova T.N., Okolelova T.M., Ignatova G.V., Shevyakov A.N., Egorova T.V., Andrianova E.N., Egorova T.A., Panin I.G., Grechishnikov V.V., Panin A.I., Sergachev P.A., Ryasnoi P.V., Afanas'ev V.A. *Metodicheskie ukazaniya po optimizatsii retseptov kombikormov dlya sel'skokhozyaistvennoi ptitsy* [Guidelines for the optimization of animal feed recipes for poultry. VNITIP]. Moscow, 2009 (in Russ.).
18. Fisinin V.I., Egorov I.A., Okolelova T.M. *Nauchnye osnovy kormleniya sel'skokhozyaistvennoi ptitsy* [Scientific basis for poultry feeding]. Sergiev Posad, 2008 (in Russ.).
19. Vishnyakov A.I., Ushakov A.S., Lebedev S.V. *Vestnik myasnogo skotovodstva*, 2011, 2(54): 96-102 (in Russ.).
20. Hofmann E. *Dinamicheskaya biokhimiya* [Dynamic biochemistry=Dynamische Biochemie]. Moscow, 1971 (in Russ.).
21. Torshkov A.A. *Fundamental'nye issledovaniya*, 2011, 9(3): 583-587 (in Russ.).
22. Skal'nyi A.V., Rudakov I.A. *Bioelementy v meditsine* [Bioelements in medicine]. Moscow, 2004 (in Russ.).
23. Cuillel M., Chevallet M., Charbonnier P., Fauquant C., Pignot-Paintrand I., Arnaud J., Cassio D., Michaud-Soret I., Mintz E. Interference of CuO nanoparticles with metal homeostasis in hepatocytes under sub-toxic conditions. *Nanoscale*, 2014, 6(3): 1707-1715 (doi: 10.1039/c3nr05041f).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 912-920
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.52/.58:636.087.7:[546.72-022.532+577.112.3

doi: 10.15389/agrobiology.2016.6.912rus
doi: 10.15389/agrobiology.2016.6.912eng

NANOPARTICLES IN COMBINATION WITH AMINO ACIDS CHANGE PRODUCTIVE AND IMMUNOLOGICAL INDICATORS OF BROILER CHICKEN

E.V. YAUSHEVA[1], S.A. MIROSHNIKOV[1], D.B. KOSYAN[1, 2], E.A. SIZOVA[1, 2]

[1]*All-Russian Research Institute of Beef Cattle Breeding,* Federal Agency of Scientific Organizations, 29, ul. 9 Yanvarya, Orenburg, 460000 Russia, e-mail sizova-l78@ya.ru, vniims.or@mail.ru;
[2]*Orenburg State University*, 13, prosp. Pobedy, Orenburg, 460018 Russia
(ORCID: Sizova E.A. orcid.org/0000-0002-5125-5981)
Acknowledgements:
We thank Dr N.N. Glushchenko (Institute of Energy Problems of Chemical Physics, RAS, Moscow) for kindly providing the Fe nanoparticles used in the study.
Hematological studies were performed using standard techniques in the Laboratory of Agroecology of Nanomaterials and Test Center of All-Russian Research Institute of Beef Cattle Breeding (accreditation certificate RA. RU.21PF59 of 12/02/15)
Supported by Russian Science Foundation (project № 14-16-00060)
Received September 1, 2016

Abstract

The prospects of using metal nanoparticles to stimulate productivity of farm animals are widely discussed. However, nano-sized materials exhibit various negative properties, such as pro-oxidant effects, and can provoke apoptosis and kidney damage. A possible approach is the use of ultrafine materials in combination with agents leveling adverse effects of nanoparticles. For the first time we studied the prospects of joint use of iron and arginine nanoparticles, the mechanism of their interaction and influence on the productivity of poultry and demonstrated that their simultaneous application promote live weight gain. We formed 6 groups (n = 30) of 11-day old broilers of the cross Smena 8. The poultry was injected twice (after 2 week intervals) with iron nanoparticles and fed either with dietary arginine (the amino acid which is known to influence metabolism and immune response and considered as conditionally essential for inflammatory and oxidative stress), or the mixture of arginine, lysine and methionine. Our experiments showed that the joint use of iron nanoparticles and arginine increased the weight gain up to 9.2 % as compared to the control, and moreover, the iron nanoparticles together with a mixture of amino acids provided an increase up to 20 %. Withal, the nanoparticles and amino acids when applied separately resulted in lower weight gain, and at the end of the experiment the body weight of broilers fed with dietary arginine (group II) and those injected with iron nanoparticles (group III) increased by 6.1 and 5.9 % ($P \leq 0.05$), respectively. Intramuscular administration of iron nanoparticles (the poultry groups III, IV and VI) promoted the immune response that was manifested in enhanced level of leukocytes — by 8.12; 10.50 and 3.88 % ($P \leq 0.05$), respectively, on the day 1, and by 7.3; 8.19 and 4.00 % ($P \leq 0.05$), respectively, in a week. The study of NO-metabolites showed an increased level in blood and liver (by 3-4 %) only in groups III, IV and VI. Singly injected iron nanoparticles (group III) changed metabolism of arginine and increased its level by 3.83 % ($P \leq 0.05$). Thus the joint use of iron nanoparticles and the complex of arginine with other amino acids is most likely to be helpful in the poultry meat production.

Keywords: nanoparticles cooper, broiler chicks, growth intensity, chemical elements, biochemical and morphological parameters of blood

The search for substances with growth-stimulating action is one of the current trends in agricultural biology. A number of studies indicate the prospects of using metal nanoparticles as preparations to increase productivity of farm animals [1, 2]. Essential metal-based nanoparticles have been reported to significantly exceed the analogues in the form of mineral salts in their bioavailability [3], are characterized by less pronounced toxic effect (4), with which the promise of their use as sources of microelements is associated [5].

However, nanoparticles have a number of disadvantages. They stimulate

the production of active forms of oxygen [6, 7], apoptosis [8], and structural and functional reorganization of tissues [9], cause kidney damage [10], affect the elemental status of organs and tissues [11]. Nevertheless, a set of measures for leveling the negative effects of micronutrient nanoparticles can make the practical application of such ultradisperse substances possible.

Previously, we have demonstrated that the intake of iron nanoparticles by animals is associated with an increase in the arginine level in the liver [12]. The mechanism of this is unclear yet. Arginine is known to exhibit various metabolic and immunological effects and is considered conditionally essential for inflammatory and oxidative stress [13, 14]. At the same time, arginine is one of the factors involved in the regulation of animal growth [15]. Mechanisms for triggering arginine synthesis are closely related to the proliferation of white blood cells and NO synthase activation. It is noteworthy that iron homeostasis is closely related to the homeostasis of NO metabolites [16]. The use of arginine to reduce the negative effects of zinc nanoparticles intake has been reported by L.M. Faddah et al. [17].

In this paper, we are for the first time demonstrating that inclusion of arginine in the diet in combination with intramuscular injections of iron nanoparticles increases productivity of agricultural poultry more efficiently than each of these techniques used individually.

Our purpose was to study the prospects for the joint use of the preparations of iron nanoparticles with arginine and other amino acids, the mechanism of their interaction and the impact on productivity in poultry.

Technique. Iron nanoparticles (NP) of d = 80±5 nm were used (the particles are a core of crystalline iron with an oxide shell of Fe_3O_4 on the surface). Preparations of nanoparticles were obtained by high-temperature condensation at the MiGen assembly (Institute for Energy Problems of Chemical Physics, RAS, Moscow). Material certification of preparations included electronic scanning and translucent microscopy using a JSM 7401F and JEM-2000FX (JEOL, Japan), X-ray diffraction analysis using a diffractometer DRON-7 (NPP Burevestnik, Russia), atomic force microscopy using a multi-microscope SMM-2000 (OJSC PROTON-MIET, Russia). For injection, iron nanoparticles were prepared by mixing with saline solution, after which they were subjected to UV sterilization and processed for 30 minutes using ultrasonic disperser UZDN-2T (NPP Akadempribor, Russia) (35 kHz, 300-450 W, amplitude of oscillation of 10 μm).

Dietary methionine (JSC Volzhsky Orgsintez, Russia); lysine monochlorohydrate (Ha-ngzhou Greensky Biological Tech Co., Ltd., China), and arginine hydrochloride (Tianjin Tiyanyan Pharmaceutical Co., Ltd., China) were used as amino acid preparations.

Experiments were performed in the vivarium (Orenburg State University) with broiler chicken cross Smena 8. The keeping conditions and experimental procedures met the instructions and recommendations provided for by the Russian regulations (Order of the USSR Ministry of Health No. 755 of August 12, 1977) and The Guide for Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996). All efforts were taken to minimize the suffering of animals and to reduce the number of samples used. The 11-day old chickens (n = 168) were selected for the experiment and divided into six groups by analogous pair method (n = 28). At the age of 15 and 29 days, birds from groups III, IV, and VI were injected with Fe nanoparticle (NP) lysozoles (2 mg/kg live weight) intramuscularly [18]. Starting from the age of 15 days, daily arginine at a dose of 7 g/kg of feed [19] was added to the basic diet (BD) in groups II and IV. Arginine (7 g/kg), lysine (6 g/kg), and methionine (2 g/kg) were administered in group V and VI. Control poultry (group I) received BD. Diets were

formed taking into account feeding recommendations [20].

During the experiment, all poultry were kept in the same conditions, the microclimate in the room conformed VNITIP recommendations and requirements [21]. Poultry were fed twice daily, feed consumption was controlled daily. Water was provided ad libitum. The growth and development of chickens (examination and individual weighing) were evaluated. The birds were decapitated under nembutal ether at days 1, 7, and 14. Blood samples for hematological studies were collected into vacuum tubes with anticoagulant (EDTA-K3), for biochemical studies into vacuum tubes with a coagulation activator (thrombin).

Hematological parameters (number and type of leukocytes) were estimated using an automatic hematological analyzer URIT-2900 Vet Plus (URIT Medical Electronic Group Co., Ltd, China).

Concentration of plasma NO metabolites (the total amount of nitrate and nitrite ions) was determined spectrophotometrically with the Griss reagent using the microplate reader Infinite PROF200 (Tecan Austria GmbH, Austria) at $\lambda = 540$ nm. Blood was pre-deproteinized by adding a 2-fold excess of 96 % ethyl alcohol to 1 ml of blood. Since the method allows only nitrite ions to be determined, vanadium chloride (III) was added to the plasma to reduce nitrates, after which the tubes were placed in a water bath (37 °C, 30 min). The resulting optical density values of the samples were compared with the calibration curve. To construct it, a series of $NaNO_2$ solution delutions (5 to 100 μM) was prepared, and the samples were processed similarly to the experimental samples [21]. Similarly to test samples NO metabolites were also measured in tissue samples. In this case, sample preparation included processing biomaterial in phosphate buffer solution (pH 7.45) using a homogenizer TissueLyser LT (Qiagen N.V., Germany).

Weight proportions of arginine, lysine, methionine, tyrosine, phenylalanine, histidine, leucine-isoleucine, valine, proline, threonine, serine, alanine and glycine were estimated in the study of amino acid composition of poultry tissues and feeds. In the preparation of liver tissue and feed samples, the material was homogenized (TissueLyser LT, Qiagen N.V., Germany), dried at 60-70 °C and milled. Test samples were subjected to acid or alkaline (for tryptophan determination only) hydrolysis at a temperature of 110 °C for 14-16 hours. After acid hydrolysis, the sample was filtered (decalcified slowly filtering blue ribbon filters, Melior XXI LLC, Russia), after alkaline hydrolysis, filtration was not performed. Hydrolyzates were mixed with reagents (sodium carbonate, JSC Bashkir soda company, Russia; phenyl isothiocyanate, Shandong Hailan Chemical Industry Co., Ltd, China) and evaporated in a warm air stream. The dry residue was diluted in distilled water (0.5 ml) and centrifuged (5 min, 5000 rpm). The resulting supernatant was examined by capillary electrophoresis using the Kapel system (Lumex-Marketing Ltd, Russia; GOST 55569-2013).

The data are presented as the arithmetic mean (*M*) with the standard error of the mean (*m*). Statistical analysis was performed using ANOVA (Statistica 10.0 software package, StatSoft Inc., USA). Differences were considered statistically significant at $P \leq 0.05$.

Results. Increased arginine content in group II diet resulted in an increase in the live weight of the birds versus control by 4.0 % in 1 day, by 5-6 % in 2 weeks, and by 6.1 % ($P \leq 0.05$) at the end of the study (Table 1). Intramuscular Fe NP injection in group III chickens resulted in an increase in live weight by 6.2 % ($P \leq 0.05$) versus control at day 1 and by 9.4 % ($P \leq 0.05$) by day 4. At week 2, a decrease in growth down to 3.5 % versus control was observed. A repeated injection of nanoparticles after 2 weeks, like the first one, increased the living weight in group III by 5.9 % ($P \leq 0.05$) versus control in 1 day. This difference was practically maintained for 2 weeks, and by the end of the experi-

ment, the corresponding parameter in group III exceeded the control one by 7.1 % ($P \leq 0.05$). In group IV, a combination of Fe NP injections with additional arginine in the diet promoted similar changes in the live weight in the first week of studies, as in group III. Thus, in group IV, these parameters exceeded the control ones by 7.8 % in 1 day ($P \leq 0.05$), and by 7.5 % ($P \leq 0.05$) after 1 week. During week 2, group IV chickens exceeded control ones in the weight gain (increase by 6.0 %, $P \leq 0.05$). Repeated administration of Fe NP increased the live weight gain, and by the end of the study the difference between group IV and control broilers in the weight reached 9.2 % ($P \leq 0.05$).

1. Changes in the live weight (g) **in cross Smena 8 broilers with intramuscular Fe nanoparticle** (NP) **injections against the background of amino acid feed additives** ($M \pm m$, $n = 7$, vivarium)

Groups by experimental embodiment	Week 1	Week 2	Week 3
I (control, BD)	886.6±14.9	1268.0±17.4	1608.1±23.6
II (BD + arginine)	835.1±9.21	1347.9±19.5	1663.6±17.9*
III (BD, Fe NP injections)	868.8±8.69*	1312.8±12.7*	1672.7±20.3
IV (BD + arginine, Fe NP injections)	866,2±6,32*	1344.1±11.5*	1693.3±15.4*
V (BD + arginine + lysine + methionine)	905.9±8.39*	1392.2±10.8*	1775.0±16.3*
VI (BD + arginine + lysine + methionine, Fe NP injections)	924.0±10.3*	1431.6±14.7*	1937.2±13.8*

N o t e. BD — basic diet.
* Differences versus control are significant at $P \leq 0.05$.

2. Changes in WBC numbers ($\times 10^9$/l) **in cross Smena 8 broilers with intramuscular Fe nanoparticle** (NP) **injections against the background of amino acid feed additives** ($M \pm m$, $n = 7$, vivarium)

Groups	Leucocytes	Lymphocytes	Monocytes	Granulocytes
		D a y 1		
I (control)	22.2±0.58	12.1±0.02	1.37±0.040	8.69±0.700
II	21.7±1.07	12.7±0.32	1.34±0.030	7.63±1.360
III	23.9±0.08*	12.6±0.02*	1.58±0.010*	9.83±0.030*
IV	24.5±0.12*	12.8±0.01*	1.61±0.050*	10.05±0.110*
V	22.6±0.31	12.3±0.23	1.35±0.020	8.98±0.430
VI	23.0±0.03*	12.4±0.03*	1.50±0.020*	9.15±0.050*
		Day 7		
I (control)	25.2±0.62	13.2±0.29	1.62±0.070	10.50±0.440
II	25.8±0.63	13.2±0.22	1.77±0.020	10.80±0.410
III	27.1±0.06*	14.6±0.10*	1.69±0.020*	10.80±0.130*
IV	27.3±0.08*	14.6±0.09*	1.66±0.010*	11.00±0.050*
V	25.9±0.63	13.4±0.40	1.76±0.050	10.80±0.180
VI	26.2±0.04*	13.8±0.10*	1.66±0.020*	10.70±0.060*
		D a y 1 4		
I (control)	25,5±0,29	12.9±0.26	2.01±0.020	10.50±0.100
II	25,0±0,37	13.2±0.23	1.89±0.010	9.94±0.260
III	26.1±0.28	13.8±0.27	2.01±0.030	10.30±0.100
IV	26.2±0.29	13.7±0.39	2.04±0.020	10.50±0.090
V	26.0±0.12	13.5±0.14	2.00±0.030	10.50±0.150
VI	25.2±0.35	13.3±0.26	2.03±0.030	9.83±0.130

N o t e. Group I control (basic diet, BD), group II — BD + arginine, group III — BD, Fe NP injections, group IV — BD + arginine, Fe NP injections, group V — BD + arginine + lysine + methionine, group VI — BD + arginine + lysine + methionine, Fe NP injections (see details in section *Technique*).
* Differences versus control are significant at $P \leq 0.05$.

Combined use of iron nanoparticles and a mixture of amino acids proved to be optimal: at the end of the experiment, the live weight of group VI chickens exceeded this parameter in the control group by 20.5 % ($P \leq 0.001$) and was by 9.1 % ($P \leq 0.01$) greater compared to group V, which demonstrates a pronounced synergy in the effects of these substances on poultry growth. Methionine is known to be actively involved in metabolic processes, in particular, it increases iron absorption in the gastrointestinal tract. A combination of methionine and arginine is effective for productivity growth [22]. Lysine and arginine are antagonists, but when combined, they stimulate production of growth hormone. Part of the energy required for protein synthesis is formed due to lysine

oxidation [23]. This explains the pronounced effects in the combined use of Fe NP with a complex of arginine, lysine, and methionine amino acids.

Analysis of morphological and biochemical parameters of the blood revealed changes only in the groups that received preparations of iron nanoparticles (Table 2). Thus, in groups III, IV, and VI, the number of leukocytes increased, versus control, respectively, by 8.12, 10,5, and 3.88 % ($P \leq 0,05$) at day 1, by 7.30, 8.19, and 4.00 % ($P \leq 0.05$) after 1 week, and no significant differences versus control were observed after 2 weeks. Similar changes in the parameters were observed for certain types of leukocytes.

In groups III, IV, and VI, the number of lymphocytes increased versus control, respectively, by 3.63. 5.91, and 2.20 % ($P \leq 0.05$) after 1 day, the number of monocytes increased by 15.40, 17.90, and 10.20 % ($P \leq 0.05$), the number of granulocytes — by 13.10, 15.60, and 5.24 % ($P \leq 0.05$). After 1 week, in these groups, the number of lymphocytes significantly increased by 10.90. 10.90, and 5.40 % ($P \leq 0.05$), the number of monocytes increased only by 4.30, 2.47, and 2.47 % ($P \leq 0.05$), the number of granulocytes — by 3.61, 5.25, and 2.55 % ($P \leq 0.05$).

These data are consistent with the results of our earlier assessment of the effects of iron nanoparticles on the productivity and physiological status of broiler chickens [12]. A similar effect of iron and amino acid nanoparticles on productivity has been described by other authors [24, 25]. The data on the morphological composition of the blood presented in this paper are consistent with the conclusions about the ability of metal nanoparticles and their compounds to promote the immune response [26, 27]. Probably, the observed change in the composition of leukocytes upon administration of nanoparticles is a short-term "physiological leukocytosis" which is especially evident against the background of amino acid additives (protein food). Our findings also indicate the close relationship between the gain of leukopoiesis and the growth stimulating effect of the preparations. Arginine metabolism is known to be closely related to the proliferation of monocytes and lymphocytes and the development of oxidative stress through the synthesis of polyamines and protein [28-32].

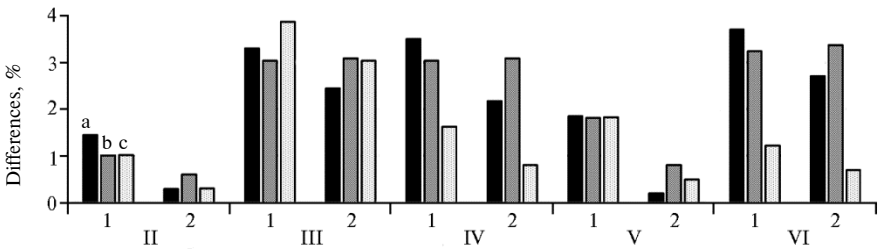


Fig. 1. Differences (%) **versus control** (group I) **in the blood** (1) **and liver** (2) **levels of NO metabolites in cross Smena 8 broilers of various ages with intramuscular Fe nanoparticle injections against the background of amino acid feed additives:** II, III, IV, V, and VI — groups by experiment embodiment (see details in section *Technique*); a, b, c — poultry age of 16, 21, and 35 days (experimental day 1, week 1, and week 2) ($n = 7$, vivarium).

The blood levels of NO metabolites increased in 1 day after administration of nanoparticles in groups III, IV, and VI (by 3.3, 3.5, and 3.7 %) (Fig. 1). In 1 week, this parameter was increased in the same groups (by 3.0-3.4 % versus control), in 2 weeks, increased NO metabolites (by 3.85 % versus control) were maintained in group III only. The changes of the level of NO metabolites in the liver were similar: regular changes were observed in groups III, IV, and VI only. Thus, in group III, as a result of nanoparticle administration, this parameter was increased by 2.0-3.0 % in group III versus control, and this difference persisted

throughout the observation period. In group IV, Fe NP injections combined with arginine dietary additives increased the level of liver NO metabolites in the poultry in the first 7 days only (by 2.0-3.4 % versus control). Similar changes were observed in the same time frame in group VI (level increase by 2.7-3.7 % versus control).

Liver amino acid composition was changed in group III only: by the end of the study, arginine levels were increased versus control by 3.83 % ($P \leq 0.05$) (Fig. 2).

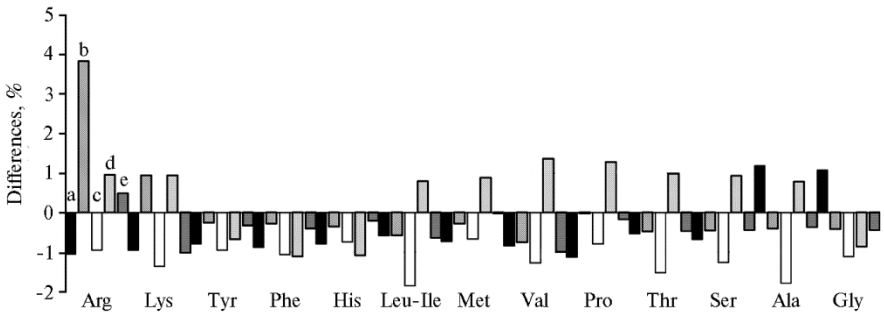


Fig. 2. Differences (%) **versus control** (group I) **in the liver amino acid composition in cross Smena 8 broilers aged 42 days old with intramuscular Fe nanoparticle injections against the background of amino acid feed additives:** a, b, c, d, e — groups II, III, IV, V, VI by experiment embodiment (see details in section *Technique*) (n = 7, vivarium).

Formation of arginine can be initiated by several mechanisms, including activation of the metabolism, as well as through the synthesis of nitric oxide (NO), as shown experimentally [33, 34[. We have found an association between increased formation of nitric oxide and the entry of iron nanoparticles, as well as its dependence on the amount of arginine present in the diet. In particular, with combined use of nanoparticles and amino acids, the blood and liver levels of NO metabolites was increased in chickens in the first week of the experiment only, whereas in the absence of amino acid additives, nanoparticles caused an increase in the amount of NO metabolites throughout the observation period. Additionally, we note that an increase in the amount of arginine in the diet, eliminating the need for its synthesis in the body resulted in a considerable and significant increase in the productivity of the poultry.

Thus, intramuscular injections of iron nanoparticles are followed by a change in the arginine metabolism in the poultry body. Combined use of a preparation of iron nanoparticles and arginine complex with other amino acids results in an increase in the productivity of broiler chickens.

REFERENCES

1. Abramyan A., Beklemyshev V., Solodovnikov I., Letov A., Filippov K., Makhonin I. *Nanoindustriya*, 2007, 6: 24-25 (in Russ.).
2. Il'ichev E., Nazarova A., Polishchuk S., Inozemtsev V. *Molochnoe i myasnoe skotovodstvo*, 2011, 5: 27-29 (in Russ.).
3. Nikonov I.N., Folmanis Yu.G., Folmanis G.E., Kovalenko L.V., Laptev G.Yu., Egorov I.A., Fisinin V.I., Tananaev I.G. *Doklady akademii nauk*, 2011, 440(4): 565-569 (in Russ.).
4. Bogoslovskaya O.A., Sizova E.A., Polyakova V.S., Miroshnikov S.A., Leipunskii I.O., Ol'khovskaya I.P., Glushchenko N.N. *Vestnik Orenburgskogo gosudarstvennogo universiteta*, 2009, 2: 124-127 (in Russ.).
5. Aslam M.F., Frazer D.M., Faria N., Bruggraber S.F.A., Wilkins S.J., Mirciov C., Powell J.J., Anderson G.J., Pereira D.I.A. Ferroportin mediates the intestinal absorption of iron from a nanoparticulate ferritin core mimetic in mice. *FASEB J.*, 2014, 28(8): 3671-3678.

6. Li N., Sioutas C., Cho A., Misra C., Sempf J., Wang M., Oberley T., Froines J., Nel A. Ultrafine particulate pollutants induce oxidative stress and mitochrondrial damage. *Environmental Health Perspectives*, 2003, 111: 455-460.
7. Møller P., Jacobsen N.R., Folkmann J.K., Danielsen P.H., Mikkelsen L., Hemmingsen J.G., Vesterdal L.K., Forchhammer L., Wallin H., Loft S. Role of oxidative damage in toxicity of particulates. *Free Radical Research*, 2010, 44(1): 1-46 (doi: 10.3109/10715760903300691).
8. Sizova E.A., Miroshnikov S.A., Polyakova V.S., Lebedev S.V., Glushchenko N.N. *Morfologiya*, 2013, 144(4): 47-52 (in Russ.).
9. Polyakova V.S., Sizova E.A., Miroshnikov S.A., Notova S.V., Zavaleeva S.M. *Morfologiya*, 2015, 148(6): 54-58 (in Russ.).
10. BeruBe K., Balharry D., Sexton K., Koshy L., Jones T. Combustion-derived nanoparticles: mechanisms of pulmonary toxicity. *Clinical and Experimental Pharmacology and Physiology*, 2007, 34(10): 1044-1050 (doi: 10.1111/j.1440-1681.2007.04733.x).
11. Sizova E., Glushchenko N., Miroshnikov S., Skalny A. Influence of Cu10x copper nanoparticles intramuscular injection on mineral composition of rat spleen. *Journal of Trace Elements in Medicine and Biology*, 2011, 25: 84-89.
12. Sizova E., Yausheva E., Kosyan D., Miroshnikov S. Growth enhancement by intramuscular injection of elemental iron nano- and microparticles. *Modern Applied Science*, 2015, 9(10): 17-26 (doi: 10.5539/mas.v9n10p17).
13. Huang C.C., Tsai S.C., Lin W.T. Potential ergogenic effects of L-arginine against oxidative and inflammatory stress induced by acute exercise in aging rats. *Exp. Gerontol.*, 2008, 43(6): 571-577 (doi: 10.1016/j.exger.2008.03.002).
14. Mostafavi-Pour Z., Zal F., Monabati A., Vessal M. Protective effects of a combination of Quercetin and vitamin E against cyclosporine A-induced oxidative stress and hepatotoxicity in rats. *Hepatol. Res.*, 2008, 38(4): 385-392 (doi: 10.1111/j.1872-034X.2007.00273.x).
15. Flynn N.E., Meininger C.J., Haynes T.E., Wu G. The metabolic basis of arginine nutrition and pharmacotherapy. *Biomed. Pharmacother.*, 2002, 56: 427-438.
16. Nairz M., Schleicher U., Schroll A., Sonnweber T., Theurl I., Ludwiczek S., Talasz H., Brandacher G., Moser P.L., Muckenthaler M.U., Fang F.C., Bogdan C., Weiss G.J. Nitric oxide-mediated regulation of ferroportin-1 controls macrophage iron homeostasis and immune function in *Salmonella* infection. *J. Exp. Med.*, 2013, 210(5): 855-873 (doi: 10.1084/jem.20121946).
17. Faddah L.M., Abdel Baky N.A., Al-Rasheed N.M., Al-Rasheed N.M., Fatani A.J., Atteya M. Role of quercetin and arginine in ameliorating nano zinc oxide-induced nephrotoxicity in rats. *BMC Complementary and Alternative Medicine*, 2012, 12: 1062 (doi: 10.1186/1472-6882-12-60).
18. Sipailova O.Yu., Lebedev S.V., Sizova E.A. Voprosy biologicheskoi, meditsinskoi i farmatsevticheskoi khimii, 2011, 9(8): 43-46 (in Russ.).
19. Sakomura N.K., Ekmay R.D., Mei S.J., Coon C.N. Lysine, methionine, phenylalanine, arginine, valine, isoleucine, leucine, and threonine maintenance requirements of broiler breeders. *Poultry Sci.*, 2015, 94(11): 2715-2721 (doi: 10.3382/ps/pev287).
20. Fisinin V.I., Egorov I.A., Lenkova T.N., Okolelova T.M., Ignatova G.V., Shevyakov A.N. et al. *Metodicheskie ukazaniya po optimizatsii retseptov kombikormov dlya sel'skokhozyaistvennoi ptitsy* [Guidelines for the optimization of animal feed recipes for poultry. VNITIP]. Moscow, 2009 (in Russ.).
21. Mazhitova M.V. *Sovremennye problemy nauki i obrazovaniya*, 2011, 3: 2-9 (in Russ.).
22. Chamruspollert M., Pesti M., Bakalli R.I. Dietary interrelationships among arginine, methionine, and lysine in young broiler chicks. *Brit. J. Nutr.*, 2002, 88(6): 655-660.
23. Ekmay R.D., De Beer M., Mei S.J., Manangi M., Coon C.N. Amino acid requirements of broiler breeders at peak production for egg mass, body weight, and fertility. *Poultry Sci.*, 2013, 92(4): 992-1006 (doi: 10.3382/ps.2012-02554).
24. Szaby J., Andrásofszky E., Tuboly T., Bersényi A., Weisz A., Hetényi N., Hullár I. Effect of arginine or glutamine supplementation on production, organ weights, interferon gamma, interleukin 6 and antibody titre of broilers. *Acta Vet. Hung.*, 2014, 62(3): 348-361.
25. Bautista-Ortega J., Cortes-Cuevas A., Ellis E.A., Ruiz-Feria C.A. Supplemental L-arginine and vitamins E and C preserve xanthine oxidase activity in the lung of broiler chickens grown under hypobaric hypoxia. *Poultry Sci.*, 2014, 93(4): 979-988.
26. Mohammadi V., Ghazanfari S., Mohammadi-Sangcheshmeh A., Nazaran M.H. Comparative effects of zinc-nano complexes, zinc-sulphate and zinc-methionine on performance in broiler chickens. *Brit. Poultry Sci.*, 2015, 56(4): 486-493.
27. Džarová A., Dubničková M., Závišová V., Koneracká M., Kopčanský P., Gojzewski H., Timko M. The influence of magnetite nanoparticles on human. *Journal of Life Sciences*, 2010, 4(5): 37-43.

28. Yu S.S., Lau C.M., Thomas S.N., Jerome W.G., Maron D.J., Dickerson J.H., Hubbell J.A., Giorgio T.D. Size- and charge-dependent non-specific uptake of PEGylated nanoparticles by macrophages. *Int. J. Nanomedicine*, 2012, 7: 799-813.
29. Suchner U., Heyland D.K., Peter K. Immune-modulatory actions of arginine in the critically ill. *Brit. J. Nutr.*, 2002, 87: 121-132.
30. Huang C.C., Lin T.J., Lu Y.F., Chen C.C., Huang C.Y., Lin W.T. Protective effects of L-arginine supplementation against exhaustive exercise-induced oxidative stress in young rat tissues. *Chinese J. Physiol.*, 2009, 52(5): 306-315.
31. Lin W.T., Yang S.C., Chen K.T., Huang C.C., Lee N.Y. Protective effects of L-arginine on pulmonary oxidative stress and antioxidant defenses during exhaustive exercise in rats. *Acta Pharmacologica Sinica*, 2005, 26(8): 992-999 (doi: 10.1111/j.1745-7254.2005.00155.x).
32. Ahamed M., Akhtar M.J., Siddiqui M.A., Ahmad J., Musarrat J., Al-Khedhairy A.A., AlSalhi M.S., Alrokayan S.A. Oxidative stress mediated apoptosis induced by nickel ferrite nanoparticles in cultured A549 cells. *Toxicology*, 2011, 283(2-3): 101-108 (doi: 10.1016/j.tox.2011.02.010).
33. Weiss G., Werner-Felmayer G., Werner E.R., Grünewald K., Wachter H., Hentze M.W. Iron regulates nitric oxide synthase activity by controlling nuclear transcription. *J. Exp. Med.*, 1994, 180: 969-976.
34. Dlaska M., Weiss G. Central role of transcription factor NF-IL6 for cytokine and iron-mediated regulation of murine inducible nitric oxide synthase expression. *J. Immunol.*, 1999, 162: 6171-6177.

Events

3D CELL CULTURE
TECHNOLOGICAL INNOVATION AND CLINICAL SUCCESS

(22-23 February 2017, London, United Kingdom)

3D Cell Culture 2017 will address the latest developments of 3D cell culture techniques; the ways in which 3D methods are presently paving the way to future technologies, and the ways in which they are currently revolutionising cancer research, stem cell and regenerative medicine. The 3D cell culture market is predicted to reach $3702.2 million by 2021 with main increase seen in novel technologies and culture methods. This event will highlight emerging technologies, like 3D and 4D bio imaging, and their application to furthering research and medical practice.

In addition, we aim to focus on the involvement of 3D culture methods in drug development and screening, a topic of great current commercial interest to pharmaceutical and research bodies. 3D Cell Culture 2017 will bring together leading professionals and researchers in the industry to discuss the latest developments and future potential of this technique.

We aim to provide a unique and exciting conference on 3D cell culture, covering cutting edge technologies like CRISPR. There will also be emphasis on the success and bright future of 3D methods in cancer research and stem cell research, the potential of organoids, and drug development besides.

Contacts: http://www.smi-online.co.uk/pharmaceuticals/uk/3D-Cell-Culture?utm_medium=www.3d-cellculture.com&utm_source=P-220&utm_campaign=glob

Information: http://www.globaleventslist.elsevier.com/events/2016/03/paediatric-clinical-trials/

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 921-928
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 639.2/.3:574.5:57.084.1:[546.28+546.28]-022.532

doi: 10.15389/agrobiology.2016.6.921rus
doi: 10.15389/agrobiology.2016.6.921eng

ASSESSMENT OF GENERAL TOXICITY AND PROOXIDANT EFFECTS OF CeO_2 AND SiO_2 NANOPARTICLES ON *Danio rerio*

E.P. MIROSHNIKOVA[1], D.B. KOSYAN[1, 2], A.E. ARIZHANOV[1], E.A. SIZOVA[1, 2], V.V. KALASHNIKOV[3]

[1]*Orenburg State University*, 13, prosp. Pobedy, Orenburg, 460018 Russia;
[2]*All-Russian Research Institute of Beef Cattle Breeding,* Federal Agency of Scientific Organizations, 29, ul. 9 Yanvarya, Orenburg, 460000 Russia, e-mail sizova-178@ya.ru, vniims.or@mail.ru;
[3]*All-Russian Research Institute of Horse Breeding*, Federal Agency of Scientific Organizations, pos. Divovo, Rybnovskii Region, Ryazan Province, 391105 Russia
(ORCID: Sizova E.A. orcid.org/0000-0002-5125-5981)
Acknowledgements:
Supported by Russian Science Foundation (project № 14-36-00023)
Received May 25, 2016

Abstract

A diversified use of nanomaterials leads to their accumulation in the environment and involvement into remediation. In water biocoenosis, nanomaterials influence fishes. Lipid peroxidation (LPO) in aquatic bioindicators is considered the parameters generally used to assess an impact of man-caused water pollution. It should be taken into account that the level of LPO products can be due not only to anthropogenic pollution, but also to the presence of peroxide substrates in fish tissues. We firstly showed the effect of silica and cerium nanoparticles in water environment with direct assay of the enzyme activity of the bioindiator used. Our purpose was to evaluate the prooxidant effects of CeO_2 (15.8 nm) and SiO_2 (40.9 nm) nanoparticles (NPs) on the *Danio rerio* model, to study LPO as influenced by the NPs doses, and to find out if there are any adaptive mechanisms in *Danio rerio* to withstand the NPs in the habitat. Complete death of the test objects occurred on days 80 and 84 when CeO_2 NPs used. The first signs of the CeO_2 NPs toxic effect at a dose of 10 mg/dm^3 in the feed appeared on day 45, on day 56 the test-organism number was 33 % lower, and on day 65 a more than 54 % decline occurred. SiO_2 NPs led to 33 % reduced survival. The presence of the nanoparticles in the habitat depressed the antioxidant system of *Danio rerio* but the signs of adaptation were manifested by the end of week 2, and a significant increase in catalase (CAT) and superoxide dismutase (SOD) activity proceeded by the end of the test. At 10 and 100 mg/dm^3 of CeO_2 NPs the malonic dialdehyde (MDA) level decreased by 11.0 % and 61.0 %, respectively. For SiO_2 NPs the changes were similar with the MDA level decrease of 50.0 and 41.5 % at 10 and 100 mg/dm^3 dosage, respectively. SOD activity when influenced by CeO_2 NPs (10 mg/dm^3 and 100 mg/dm^3) decreased by 75 and 69 %, respectively, and for SiO_2 NPs the indexes were 50 and 26 % lower as compared to control. Similar changes were characteristic of CAT activity. Thus, the investigated nanoparticles possess sufficient toxic properties that necessitates their further study.

Keywords: *Danio rerio*, survival, catalase, superoxide dismutase, nanoparticles of silicone and cerium dioxide, mass spectrometry

According to some estimates, by 2020, nanotechnology advances will provide the establishment of industries, which will employ about 6 million people with a combined production of goods by $ 3 trillion [1]. Naturally this will increase the flow of ultrafine materials into the environment, where they will be involved in the biological processes. In the aqueous environment, nanomaterials may be incorporated into various processes, such as become the components of effluents or emissions; they are not biodegradable, and leave the biological cycle very slowly [2]. This necessitates the study of the bodies of terrestrial and water (natural and anthropogenic) ecosystems upon exposure of nanomaterials, primarily, of those the forms of which have significant potential applications. These include nanopreparations containing cerium and silicon and used in many biotechnological and medical productions [3]. Research has shown that the toxic effect of CeO_2

and SiO_2 is detected only when particles are up to 10 nm in size, but there is no exact evidence confirming their low toxicity to humans and animals. A limited number of landmark works on the effects of nanoparticles (NPs) of CeO_2 and SiO_2, the inconsistency of the data, as well as the expansion of the applications of materials based on the CeO_2 and SiO_2 NPs necessitate the biological assessment of these nanomaterials, including in the environmental objects [4, 5].

It should be noted that information on the effects of cerium- and silicon-containing nanoparticles is ambiguous. Using the freshwater fish *Catostomus commersonii*, it was shown that these nanoparticles were characterized by unstable manifestation of their activity [6]. The investigation on the *Danio rerio* model revealed no toxic and damaging effects [7], while demonstrated the perspectives of using cerium-containing nanoparticles for therapeutic purposes [8]. Furthermore, the data on severe toxicity of these materials have gained widespread, particularly, the ability of the CeO_2 NPs has been found to cause pulmonary inflammations when tested in rats [9], induce oxidative stress and, as a result, the breaks of single-strand DNA [10, 11].

In its assessment, the studied parameters are the products of lipid peroxidation (LPO) in the tissues of hydrobionts considered as biological indicators of anthropogenic pollution of water bodies. However, when interpreting these results, it is important to consider that revealed values can be related not only to the reaction to anthropogenic pollution, but also to endogenous substrates of peroxidation in the tissues.

We were the first to study the effect of nanoparticles of silicon and cerium dioxide in an aquatic environment with immediate assessment of enzyme system of the bioindicator organism.

Our purpose was to determine the biological effects of the CeO_2 and SiO_2 NPs in an aquatic environment depending on the preparation dose and routes of contamination.

Tecnique. The investigations were performed on a model of *Danio rerio* aged 1 month and selected by weight.

The preparations of SiO_2 (d = 40.9 nm) and CeO_2 (d = 15.8 nm) nanoparticles, used in the research, were synthesized by a vapour-phase method in the Shared Knowledge Center at The A.N. Tupolev Kazan National Research Technological University. Materials research certification of the preparations included electronic scanning and transmission microscopy using JSM 7401F and JEM-2000FX microscopes (JEOL, Japan), as well as X-ray diffraction analysis (DRON-7 X-ray diffraction meter, NPO Burevestnik, Russia). Atomic force microscopy was performed using SMM-2000 microscope (OJSC PTOTON-MIET, Russia). The scanning used MSCT-AUNM (Park Scientific Instruments, USA) cantilevers with a spring constant at k = 0.01 N/m and the needle radius of curvature of 15-20 nm. Quantitative morphometric analysis of the derived images was performed using regular software microscope.

The aquarium fish *Danio rerio* (a species of ray-finned freshwater fish in the *Cyprinidae* family) aged 1 month (n = 75) were kept in a single aquarium stand (V = 300 l) for 21 days. Next, five groups (n = 15 each) were allocated using the analogue method, placing each group in a separate fish tank (V = 10 l, stocking density of 15 individuals), in the water of which the studied nanoparticles were added: CeO_2 NPs (10 mg/dm^3) in group I; CeO_2 NPs (100 mg/dm^3) in group II; SiO_2 NPs (10 mg/dm^3) in group III, SiO_2 NPs (100 mg/dm^3) in group IV; and group V as a control (without addition of nanoparticles). After adaptation of the model object within 21 days (a preliminary experiment stage) the nanoparticles of SiO_2 (SiO_2 NPs) and CeO_2 (CeO_2 NPs) as lyosols were administered with feed (*Chironomidae* larvae) every 7 days (10 and 100 mg/dm^3

feed by groups according to the design of experimen; in control, the nanoparticles were not added). To prepare lyosols, the nanoparticles were dispersed in water and sterilized by sonication (UZDN-2T, NPP Akadempribor, Russia; f-35 kHz, 300 W, A-10 μA, 30 min). The experiment lasted for 84 days.

The toxic effect of nanopreparations was evaluated by the survival of the test object calculated as the percentage of animals alive at the end of the experiment from the baseline number. The concentrations of the nanopreparations were distributed to the following groups of toxicity: for bioindicator survival 0-39 % — Tox, 50 % — LC_{50}, 40-69 % — LOEC and 70-100% — NOEC.

To identify the products of lipid peroxidation (LPO) and the status of antioxidant protection systems, on day 7, 14 and 84, five *Danio rerio* fish were homogenized (TissueLyser LT, Qiagen N.V., Germany). To prepare an extract, nine volumes of Tris buffer (Tris-HCl 50 mmol/l, dithiothreitol DTT 1.0 mmol/l, EDTA 1.0 mmol/l, sucrose 250 mmol/l, pH 7.5) was added to one volume of the homogenate. After centrifugation (10 min at 15000 rpm), the supernatant was collected, and the content of malondialdehyde (MDA), as well as the activity of key antioxidant enzymes, such as catalase (CT, EC 1.11.1.6) and superoxide dismutase (SOD, EC 1.15.1.1), were measured in it, using a CS-T240 automatic biochemistry analyzer (Dirui Industrial Co., Ltd, China) and commercial biochemical test kits for veterinary use (Randox Laboratories, Ltd, UK).

Throughout the experiment the following conditions were maintained: average temperature 22±2 °C, pH 7.3±0.07, dissolved oxygen concentration 5±0.2 mg/l; 12/12 hours (day/night). The fish were fed every 2 days. Conditions for growth and maintenance of test objects were in compliance with the rules of OECD (Organisation for Economic Co-operation and Development) [12]. The experiments were performed in accordance with the provisions of the Geneva Convention and the principles of Good Laboratory Practice (National Standard of the Russian Federation GOST R 53434-2009), as well as recommendations set out in The Guide for the Care and Use of Laboratory Animals (National Academy Press Washington, DC 1996). Animal care was carried out according to Good Laboratory Practice as per regulations on conducting preclinical research in the Russian Federation (GOST 3 51000.4-96).

The results are provided as mean (*M*) and standard error of mean (*m*). Statistical analysis was performed using ANOVA standard methods (Statistica 10.0 software package, StatSoft Inc., USA), and subsequent safety evaluation using a Tukey's test of additivity in SPSS 17.0 (IBM Corporation, USA). The differences were statistically significant at $p < 0.05$.

Results. In our experiment, fish mortality in the presence of nanoparticles of both elements on day 1 was not observed (data not shown). A longer exposure to nanoparticles affected the number of *Danio rerio* (Fig. 1). The first signs of toxicity of CeO_2 NPs at a dose of 10 mg/dm^3 feed were observed on day 45, while on day 56 the number of fish was reduced by 33 %, on day 65 already by 54 %, and by day 84 deaths of 100 % test objects were recorded. At a CeO_2 NPs concentration of 100 mg/dm^3, the death of *Danio rerio* was noted on day 80.

On day 7 and 28, both doses of CeO_2 NPs and SiO_2 NPs (10 and 100 mg/dm^3) were classified as NOEC (survival of the test object within 70-100 %). On day 56, both doses of CeO_2 NPs moved into the LOEC group (the concentration maintaining 40-69 % survival of the test object), while those of SiO_2 NPs remained in the NOEC group.

Finally, by day 84 both doses of CeO_2 NPs were toxic (Tox means concentrations at which the survival of the subject is 0-39 %), while for SiO_2 NPs the 10 mg/dm^3 dose effect was still ranked as NOEC, and only for the 100 mg/dm^3 dose toxicity category was changed to LOEC.

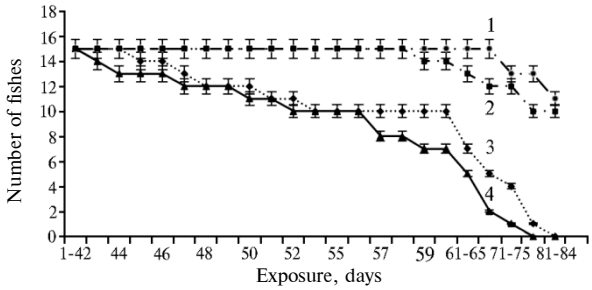


Fig. 1. Dynamics of the bioindicator *Danio rerio* population depending on the dose of SiO_2 NPs (1, 2) **and CeO_2** (3, 4) **added with feed, and time of exposure:** 1, 3 — 10 mg/dm^3, 2, 4 — 100 mg/dm^3 (n = 75).

It should be noted that the high toxicity of cerium nanoparticles which has been revealed in these experiments contradicted our working hypothesis, based on a series of papers about the ability of CeO_2 NPs to exhibit antioxidant properties, acting as an analogue of superoxide dismutases (SOD) and catalases. These reported neutralizing free radicals and protecting cells from oxidative damage [13]. On these grounds, the authors suggested the use of CeO_2 NPs in the treatment of neurological disorders and for the cell radioprotection [13, 14]. However, the ability of CeO_2 to trigger oxidative stress was shown in cell culture [15, 16] and model objects, such as rats [17, 18] and the nematode *Caenohabditis elegans* [19].

Our data also showed the invalidity of the assumptions made [20] on the biological inertness of the silicon dioxide nanoparticles. Thus, contact of the SiO_2 NPs with the test object manifested in a decreased survival of *Danio rerio* by 7 %, and by 33 % at the end of the experiment, although the total loss of the test object was not observed. Previously, against application of the SiO_2 NPs chromosomal aberrations were identified, as well as the development of the oxidative stress [21, 22]. In a model of *Carassius auratus gibelio* (crucian carp), the ability of the Si/SiO_2 nanoparticles to induce an inflammatory response was demonstrated [23]. Similar results were also obtained in other test objects [24-26].

1. The content (μmol/l) **of malondialdehyde in homogenates of *Danio rerio* depending on dose of and time of exposure to nanoparticles** (NPs) **of CeO_2 and SiO_2** ($M \pm m$, n = 75)

Nanopreparation	10 mg/dm^3	100 mg/dm^3
	Day 7	
CeO_2 NPs	0.615±0.011*	0.269±0.006*
SiO_2 NPs	0.346±0.008**	0.404±0.003**
Control	0.691±0.005	
	Day 14	
CeO_2 NPs	0.245±0.003**	0.251±0.004*
SiO_2 NPs	0.252±0.005*	0.269±0.006*
Control	0.461±0.008	
	Day 84	
SiO_2 NPs	4.100±0.105*	4.500±0.095**
Control	0.693±0.001	

*, ** Differences vs. control are statistically significant at $P < 0.05$ and $P < 0.01$, respectively.

The contact of *Danio rerio* with CeO_2 NPs and SiO_2 NPs during the first two weeks of the experiment was accompanied by a decreased content of MDA in all groups compared to the control (Table 1). For example, on day 7 the MDA content decreased by 11 and 61 % compared to control when the CeO_2 NPs were used at doses of 10 and 100 mg/dm^3. For SiO_2 NPs the dynamics was similar, i.e. a decrease in the MDA content on day 7 by 50.0 and 41.5 %, respectively, at doses of 10 and 100 mg/dm^3. On day 14 of the experiment, the same dynamics was observed. An increase in terms of exposure up to 84 days resulted in the elevated MDA production and, consequently, an increased lipid PO. Thus, in the presence of SiO_2 NPs at a dose of 10 mg/dm^3 the MDA concentration was 11-fold higher than that in the control, and at a dose of 100 mg/dm^3 — 12-fold. Similar results were obtained when assessing the effect of silicon nanoparticles in crucian carp [23].

The presence of nanoparticles in feed and water affected the activity of antioxidant enzymes (SOD and CT) in the test object. On day 7, the SOD value in *Danio rerio* in all study groups was lower than in the control. For example, in

the option with CeO_2 NPs, it decreased by 75 % at a nanopreparation dose of 10 mg/dm^3 and by 69 % at a dose of 100 mg/dm^3, and with SiO_2 NPs by 50 and 26 %, respectively, at a dose of 10 and 100 mg/dm^3. On day 14, the SOD activity in the presence of nanoparticles differed depending on their type. CeO_2 NPs induced a decrease in the SOD values, such as by 41 % for 10 mg/dm^3, and by 29 % for 100 mg/dm^3 (Table 2). When SiO_2 NPs were added to the feed, the SOD activity parameters exceeded those in the control samples only on day 20, reaching values 1.96 and 1.48 times as much as control ones at doses of 10 and 100 mg/dm^3, respectively.

2. The catalase (CT) **and superoxide dismutase** (SOD) **activity in homogenates of *Danio rerio* depending on dose of and time of exposure to nanoparticles** (NPs) **of CeO_2 and SiO_2** ($M \pm m$, $n = 75$)

Nanopreparation	CT, μmol/L		SOD, % of epinephrine inhibition	
	dose of 10 mg/dm^3	dose of 100 mg/dm^3	dose of 10 mg/dm^3	dose of 100 mg/dm^3
Day 7				
CeO_2 NPs	93.1±0.7*	51.4±0.5**	17.6±2.8*	48.5±1.7*
SiO_2 NPs	15.9±0.4	46.9±1.0*	35.6±1.2**	52.4±2.6*
Control	16.4±0.5		70.6±0.5	
Day 14				
CeO_2 NPs	97.4±2.1**	83.3±2.9*	28.1±1.5	33.7±1.1
SiO_2 NPs	77.1±2.8**	29.3±1.4	93.6±3.7*	70.6±2.1*
Control	16.2±0.3		47.6±0.6	
Day 84				
SiO_2 NPs	15.9±0.9*	20.2±1.1	6.1±0.2**	10.8±1.1*
Control	23.1±0.8		59.3±1.8	

*, ** Differences vs. control are statistically significantat $P < 0.05$ and $P < 0.01$, respectively.

A significant decrease in SOD activity by the end of the experiment was quite expected, and is determined by the development of toxic reactions similar to those described previously in the presence of xenobiotics and cadmium [27]. We also observed similar dynamics for catalase (see Table 2).

Therefore, our results indicate that at the initial stages of NPs exposure to the body there is a pronounced reduction in the function of cell protection systems against oxidative stress. This is probably a consequence of the NPs ability to act as an analogue of catalase, and exert activity, to some extent similar to the effect of SOD [28]. It is also specific that the CT and SOD activity, which changes in the presence of CeO_2 NPs and SiO_2 NPs, is recovered with time [29]. Therefore, we believe it is natural that the changes in SOD and CT values in *Danio rerio* under the influence of nanoparticles are opposite to the dynamics previously described for toxic substances, such as the insecticide imidacloprid, whose action first manifested in the increased activity of CT and SOD, and then in its significant decrease [30].

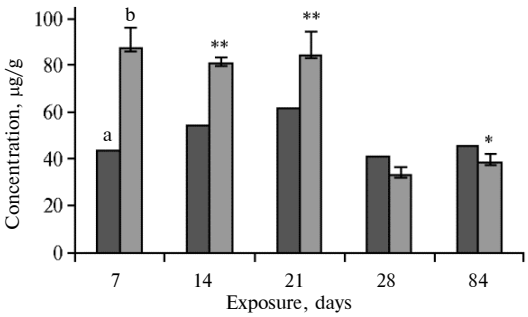


Fig. 2. The amount of silicon in tissues of *Danio rerio* depending on time of exposure to SiO_2 NPs (100 mg/dm^3 water)**:** a — control; b — experiment.
*, ** Differences vs. control are statistically significant at $P < 0.05$ and $P < 0.01$, respectively.

It is therefore difficult to explain the death of *Danio rerio* in the experiment. The reason could be that the ultradispersive nature of oxide particles may lead to their accumulation in the tissues of fish. It has previously been shown in several studies [24, 27, 30]. Such a fact was also observed in our experiments, especially in the first weeks, which is well seen on the example of silicon (Fig. 2). However, further on the silicon content in the body of *Danio rerio* decreased, probably due to homeostasis activity [25]. In

addition, SiO_2 NPs do not prevent the regeneration of tissues [23], but can cause both prothrombotic effects and increased concentrations of fibrinogen, as well as of anti-inflammatory cytokines in the blood plasma [22, 31]. Moreover, the markers of oxidative stress (SOD, CT) are not affected [22]. An interesting explanation of the death of the test organism after the contact with NPs was proposed by S.N. Petrache et al. [26], who linked it with the influence of CeO_2 on *Escherichia coli*, which is accompanied by a decrease in feed intake and subsequent changes in metabolism [32]. Similar data were reported by M.C. Arnold et al. [33].

Thus, the investigated nanoparticles (NPs) possess toxic effects, because their entry into the body of *Danio rerio* (a bioindicator) is accompanied by a total (in case of CeO2 NPs) or partial (in case of SiO2 NPs) loss of the test object at the end of the experiment. In the initial period of exposure, there is a depression of the antioxidant system, but by the end of the 2nd week the signs of adaptation develop. The content of malondialdehyde at a SiO_2 NPs dose of 10 mg/dm^3 increased by 11 times, and at a dose of 100 mg/dm^3 by 12 times; and similar changes were found for CeO_2 NPs.

REFERENCES

1. Roco M.M. The long view of nanotechnology development: the National Nanotechnology Initiative at 10 years. *J. Nanopart. Res.,* 2011, 13: 427-445 (doi: 10.1007/s11051-010-0192-z).
2. Keller A.A., McFerran S., Lazareva A., Suh S. Global life cycle releases of engineered nanomaterials. *J. Nanopart. Res.*, 2013, 15: 1692 (doi: 10.1007/s11051-013-1692-4).
3. Gerashchenko I.I. *Mediko-biologicheskie aspekty poverkhnostnykh yavlenii*, 2009, 1(16): 288-306 (in Russ.).
4. Jackson P., Raun Jacobsen N., Baun A., Birkedal R., Kühnel D., Alstrup Jensen K., Vogel U., Wallin H. Bioaccumulation and ecotoxicity of carbon nanotubes. *Chem. Cent. J.*, 2013, 7(1): 154.
5. Li Y., Li P., Yu H., Bian Y. Recent advances (2010-2015) in studies of cerium oxide nanoparticles' health effects. *Environ. Toxicol. Pharmacol.*, 2016, 9(44): 25-29 (doi: 10.1016/j.etap.2016.04.004).
6. Rundle A., Robertson A.B., Blay A.M., Butler K.M., Callaghan N.I., Dieni C.A., MacCormack T.J. Cerium oxide nanoparticles exhibit minimal cardiac and cytotoxicity in the freshwater fish *Catostomus commersonii. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 2016, 181-182: 19-26 (doi: 10.1016/j.cbpc.2015.12.007).
7. Piccinetti C.C., Montis C., Bonini M., Laura R., Guerrera M.C., Radaelli G., Vianello F., Santinelli V., Maradonna F., Nozzi V., Miccoli A., Olivotto I. Transfer of silica-coated magnetic (Fe_3O_4) nanoparticles through food: a molecular and morphological study in zebrafish. *Zebrafish*, 2014, 11(6): 567-579 (doi: 10.1089/zeb.2014.1037).
8. Ouyang Z., Mainali M.K., Sinha N., Strack G., Altundal Y., Hao Y., Winningham T.A., Sajo E., Celli J., Ngwa W. Potential of using cerium oxide nanoparticles for protecting healthy tissue during accelerated partial breast irradiation (APBI). *Phys. Medica*, 2016, 32(4): 631-635 (doi: 10.1016/j.ejmp.2016.03.014).
9. Morimoto Y., Izumi H., Yoshiura Y., Tomonaga T., Oyabu T., Myojo T., Kawai K., Yatera K., Shimada M., Kubo M., Yamamoto K., Kitajima S., Kuroda E., Kawaguchi K., Sasaki T. Pulmonary toxicity of well-dispersed cerium oxide nanoparticles following intratracheal instillation and inhalation. *J. Nanopart. Res.*, 2015, 17(11): 442 (doi: 10.1007/s11051-015-3249-1).
10. Dogra Y., Arkill K.P., Elgy C., Stolpe B., Lead J., Valsami-Jones E., Tyler C.R., Galloway T.S. Cerium oxide nanoparticles induce oxidative stress in the sediment-dwelling amphipod *Corophium volutator. Nanotoxicology*, 2016, 10(4): 480-487 (doi: 10.3109/17435390.2015.1088587).
11. *OECD Guideline for Testing of Chemicals. Guideline 203. Fish, Acute Toxicity Test.* Organization of Economic Cooperation Development, Paris, France, 1992.
12. Ramesh R., Kavitha P., Kanipandian N., Arun S., Thirumurugan R., Subramanian P. Alteration of antioxidant enzymes and impairment of DNA in the SiO_2 nanoparticles exposed zebra fish (*Danio rerio*). *Environment Monitoring and Assessment*, 2013, 185(7): 5873-5881 (doi: 10.1007/s10661-012-2991-4).
13. Tarnuzzer R.W., Colon J., Patil S., Seal S. Vacancy engineered ceria nanostructures for protection from radiation-induced cellular damage. *Nano Lett.*, 2005, 5(12): 2573-2577 (doi: 10.1021/nl052024f).

14. Schubert D., Dargusch R., Raitano J., Chan S.W. Cerium and yttrium oxide nanoparticles are neuroprotective. *BBRC*, 2006, 342(1): 86-91 (doi: 10.1016/j.bbrc.2006.01.129).
15. Park E.J., Choi J., Park Y.K., Park K. Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells. *Toxicology*, 2008, 245(1-2): 90-100 (doi: 10.1016/j.tox.2007.12.022).
16. Kim I.S., Baek M., Choi S.J. Comparative cytotoxicity of Al_2O_3, CeO_2, TiO_2 and ZnO nanoparticles to human lung cells. *J. Nanosci. Nanotechnol.*, 2010, 10(5): 3453-3458 (doi: 10.1166/jnn.2010.2340).
17. Das M., Patil S., Patil S., Bhargava N., Kang J.F., Riedel L.M., Seal S., Hickman J.J. Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons. *Biomaterials*, 2007, 28(10): 1918-1925 (doi: 10.1016/j.biomaterials.2006.11.036).
18. Srinivas A., Rao P.J., Selvam G., Murthy P.B., Reddy P.N. Acute inhalation toxicity of cerium oxide nanoparticles in rats. *Toxicol. Lett.*, 2011, 205(2): 105-115 (doi: 10.1016/j.toxlet.2011.05.1027).
19. Rogers S., Rice K.M., Manne N.D., Shokuhfar T., He K., Selvaraj V., Blough E.R. Cerium oxide nanoparticle aggregates affect stress response and function in *Caenorhabditis elegans*. *SAGE Open Medicine*, 2015, 3: 2050312115575387 (doi: 10.1177/2050312115575387).
20. Erogbogbo F., Yon K.-T., Roy I., Xu G., Prasad P.N., Swihart M.T. Biocompatible luminescent silicon quantum dots for imaging of cancer cells. *ACS Nano*, 2008, 2(5): 873-878 (doi: 10.1021/nn700319z).
21. Rajiv S., Jerobin J., Saranya V., Nainawat M., Sharma A., Makwana P., Gayathri C., Bharath L., Singh M., Kumar M., Mukherjee A., Chandrasekaran N. Comparative cytotoxicity and genotoxicity of cobalt (II, III) oxide, iron (III) oxide, silicon dioxide, and aluminum oxide nanoparticles on human lymphocytes in vitro. *Hum. Exp. Toxicol.*, 2016, 35(2): 170-183 (doi: 10.1177/0960327115579208).
22. Nemmar A., Beegam S., Yuvaraju P., Yasin J., Shahin A., Ali B.H. Interaction of amorphous silica nanoparticles with erythrocytes in vitro: role of oxidative stress. *Cell Physiol. Biochem.*, 2014, 34(2): 255-265 (doi: 10.1159/000362996).
23. Stanca L., Petrache S.N., Radu M., Serban A.I., Munteanu M.C., Teodorescu D., Staicu A.C., Sima C., Costache M., Grigoriu C., Zarnescu O., Dinischiotu A. Impact of silicon-based quantum dots on the antioxidative system in white muscle of *Carassius auratus gibelio*. *Fish Physiol. Biochem.*, 2012, 38: 963-975 (doi: 10.1007/s10695-011-9582-0).
24. Serban A.I., Stanca L., Sima C., Staicu A.C., Zarnescu O., Dinischiotu A. Complex responses to Si quantum dots accumulation in carp liver tissue: Beyond oxidative stress. *Chem-Biol. Interact.*, 2015, 239: 56-66 (doi: 10.1016/j.cbi.2015.06.015).
25. Nemmar A., Albarwani S., Beegam S., Yuvaraju P., Yasin J., Attoub S., Ali B.H. Amorphous silica nanoparticles impair vascular homeostasis and induce systemic inflammation. *Int. J. Nanomed.*, 2014, 9(1): 2779-2789 (doi: 10.2147/IJN.S52818).
26. Petrache S.N., Stanca L., Serban A.I., Sima C., Staicu A.C., Munteanu M.C., Costache M., Burlacu R., Zarnescu O., Dinischiotu A. Structural and oxidative changes in the kidney of crucian carp induced by silicon-based quantum dots. *Int. J. Mol. Sci.*, 2012, 13(8): 10193-10211 (doi: 10.3390/ijms130810193).
27. Asagba S.O., Eriyamremu G.E., Igberaese M.E. Bioaccumulation of cadmium and its biochemical effect on selected tissues of the catfish (*Clarias gariepinus*). *Fish Physiol. Biochem.*, 2008, 34: 61-69 (doi: 10.1007/s10695-007-9147-4).
28. Pirmohamed T., Dowding J.M., Singh S., Wasserman B., Heckert E., Karakoti A.S., King J.S., Seal S., Self W.T. Nanoceria exhibit redox state-dependent catalase mimetic activity. *Chem. Sommun.*, 2010, 46: 2736-2738 (doi: 10.1039/b922024k).
29. Heckert E.G., Karakoti A.S., Seal S., Self W.T. The role of cerium redox state in the SOD mimetic activity of nanoceria. *Biomaterials*, 2008, 29: 2705-2709 (doi: 10.1016/j.biomaterials.2008.03.014).
30. Ge W., Yan S., Wang J., Zhu L., Chen A., Wang J. Oxidative stress and DNA damage induced by imidacloprid in zebrafish (*Danio rerio*). *J. Agric. Food Chem.*, 2015, 63(6): 1856-1862 (doi: 10.1021/jf504895h).
31. Xie G., Sun J., Zhong G., Shi L., Zhang D. Biodistribution and toxicity of intravenously administered silica nanoparticles in mice. *Arch. Toxicol.*, 2010, 84: 183-190 (doi: 10.1007/s00204-009-0488-x).
32. Miroshnikov S.A., Lebedev S.V. *Vestnik Orenburgskogo gosudarstvennogo universiteta*, 2009, 6(112): 241-243.
33. Arnold M.C., Badireddy A.R., Wiesner M.R., Di Giulio R.T., Meyer J.N. Cerium oxide nanoparticles are more toxic than equimolar bulk cerium oxide in *Caenorhabditis elegans*. *Arch. Environ. Contam. Toxicol.*, 2013, 65(2): 224-233 (doi: 10.1007/s00244-013-9905-5).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 929-936
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 615.9:546.77-022.532]:57.084.1

doi: 10.15389/agrobiology.2016.6.929rus
doi: 10.15389/agrobiology.2016.6.929eng

MORPHOLOGICAL AND BIOCHEMICAL PARAMETERS IN Wistar RATS INFLUENCED BY MOLYBDENUM AND ITS OXIDE NANOPARTICLES

E.A. SIZOVA[1, 2], S.A. MIROSHNIKOV[1], V.V. KALASHNIKOV[3]

[1]*All-Russian Research Institute of Beef Cattle Breeding,* Federal Agency of Scientific Organizations, 29, ul. 9 Yanvarya, Orenburg, 460000 Russia, e-mail sizova-178@ya.ru, vniims.or@mail.ru;
[2]*Orenburg State University*, 13, prosp. Pobedy, Orenburg, 460018 Russia;
[3]*All-Russian Research Institute of Horse Breeding*, Federal Agency of Scientific Organizations, pos. Divovo, Rybnovskii Region, Ryazan Province, 391105 Russia
(ORCID: Sizova E.A. orcid.org/0000-0002-5125-5981)
Acknowledgements:
Studies were performed using standard techniques in the Laboratory of Agroecology of Nanomaterials and Test Center of All-Russian Research Institute of Beef Cattle Breeding (accreditation certificate RA. RU.21PF59 from 12/02/15). Analysis of chemical elements was performed in the laboratory of ANO Center for Biotic Medicine, Moscow (accreditation certificate GSEN.RU.TSAO.311, registration number in the State Register ROSS RU. 0001.513118)
Supported by Russian Science Foundation (project № 14-36-00023)
Received July 11, 2016

Abstract

Despite widespread use of nanoparticles in industry and medicine, there is very little information about how the newly developed nanomaterials interact with biological objects. Certain properties of the Mo-containing nanoparticles (NPs) suggest their possible toxic effect on warm-blooded animals. In this paper we compared the effect of Mo NPs (at 1 and 25 mg/kg) and its oxide MoO3 NPs (at 1.2 and 29 mg/kg), when administrated parenterally, on metabolic parameters and the exchange of chemical elements in Wistar laboratory rats. There, we assessed the red and white blood cell counts, the hemoglobin level, the activity of catalase (CAT) and superoxide dismutase (SOD) (for oxidative status), the ALT, AST, LDH, GGT, creatine kinase activity, blood creatinine, bilirubin and urea concentrations (for metabolic status) at days 1, 7 and 14. A day after Mo NPs and MoO_3 NPs administration the number of blood leukocyte lowered by 11.3 % ($P < 0.05$) and 58.5 % ($P < 0.01$), respectively. Also, a decrease in monocyte number by 18.9 ($P < 0.05$), 41.9 ($P < 0.01$), 51.7 ($P < 0.05$) and 83.3 % ($P < 0.001$) as depending on NPs chemical composition and doses was characteristic, though on day 14 a significant difference to control (54.5 %, $P < 0.05$) was found only for MoO_3 NPs at a dose of 29 mg/kg. The number of thrombocytes was the highest on day 14 for the maximum dosage of both NPs leading to hindered blood microcirculation. The experiments also showed an increase in serum aminotransferases, GGT and LDH activity. In sum, we observed manifestations of oxidative stress, anemia and capillary-trophic insufficiency in the animals administrated with high doses of molybdenum and Mo oxide NPs. These signs were progressing and the most apparent for molybdenum oxide NPs. Given the comparable doses used, the molybdenum nanoparticles exhibit lower toxicity as compared to its oxide.

Keywords: catalase, superoxide dismutase, glutamyl transferase, lactate dehydrogenase, aminotransferase, nanoparticles of molybdenum, nanoparticles of molybdenum trioxide

Molybdenum nanoforms are widely used in modern technologies [1, 2], e.g. for multifunctional electrocalalysis [3] and production of lubricants [4]. Apart from that, ultradisperse products containing molybdenum and its compounds show unique biological properties, which allows using them for oncotherapy [5], as antibiotics [6, 7] and antifungal agents [8], and for blue-green algae growth promotion [9].

Molybdenum is a well-studied essential microelement. Its participation in enzymatic systems is well-known [10]. Ranges of molybdenum deficiency, sufficiency and toxicity have been described [11]. Background [12], threshold and toxic concentration of molybdenum for invertebrates in soils have been reported [13]. However, the knowledge on consequences of interaction of newly developed molybdenum-based nanomaterials with biological objects is still ex-

tremely poor. At the same time, according to investigative studies, biological effects of nanoforms of molybdenum are much more expressed. Various models have been used to demonstrate this. For example, entry of molybdenum oxide nanoparticles into soil leads to high mortality rate, adaptive changes of antioxidant enzyme activity and inhibition of Cr, Fe, Mg, Mn, Ni, Si, V metabolism in *Eisenia fetida* [14]. The presence of molybdenum nanoparticles in water has a negative impact on cell membrane permeability in *Stylonychia mytilus* and leads to processes, accompanying the damage. Analysis of collected data on biological effects of molybdenum-based nanoparticles suggests that they may have a toxic effect on warm-blooded animals.

Here we for the first time compared the biological effects of molybdenum nanoparticles and molybdenum oxide on rats, the homeothermic mammals.

The objective of this work was the examination of morphological and biochemical blood parameters, morphological and functional characteristics of tissue and chemical element metabolism in model objects due to the effects of molybdenum nanoparticles and molybdenum oxide.

Technique. Molybdenum and molybdenum oxide nanoparticles (NPs) (Mo NPs and MoO_3 NPs) were obtained by plasma chemical synthesis (OOO Platina, Moscow). Mo NPs products (d = 50 nm, specific surface area 14 m^2/g, Z-potential **–43**±0,52 mV) contained 99,7 % Mo and 0,3 % O_2, and MoO_3 NPs (d = 92 nm, specific surface area 12 $м^2/г$, Z-potential –43±0,21 mV) contained 99,8 % Mo and 0,2 % O_2. The material attestation (determination of particle size, polydispersity, voluminosity, fraction content, surface area) included electronic scanning, transmission and atomic force microscopy using LEX T OLS4100, JSM 7401F и JEM-2000FX (JEOL, Japan). Particle size distribution was examined using a Photocor Compact analyzer (OOO Photocor, Russia). Nanoparticle samples were dispersed in saline solution using UZDN-2T (NPP Akadempribor, Russia) (35 kHz, 300 W, 10 uA, 30 min).

The studies were performed in 75 Wistar white male rats, with the weight of 150-180 g in standard vivarium conditions (experimental biological clinic, Orenburg State University).The diet of animals (State Standard GOST R 50258-92) complied with the requirements of the Good Laboratory Practice in conducting preclinical research in the Russian Federation (State Standard GOST 51000.4-96). The experiments were performed in accordance with the provisions of the Geneva Convention and the principles of Good Laboratory Practice (National Standard of the Russian Federation GOST R 53434-2009), as well as recommendations set out in The Guide for the Care and Use of Laboratory Animals (National Academy Press Washington, DC 1996). After the preliminary period (1 month) the animals were divided into 5 groups (n = 15 per group). Mo NPs were administered intraperitoneally in a single dose of 1 and 25 mg/kg of live weight in groups I and II, respectively, and in groups III and IV MoO_3 NPs were administered at the dose of 1.2 and 29 mg/kg, respectively. Saline solution was injected to control animals.

Biomaterial for the study was obtained after decapitation of rats under Nembutal anesthesia (5 species for each option of the experiment and control in 1, 7, and 14 days after administration of nanopreparations). Blood for examination of morphological parameters was placed in vacuum tubes with anticoagulant, for biochemical studies — into vacuum tubes with a coagulation activator (thrombin). Morphological blood composition and hemoglobin concentration were estimated using an nautomatic hematological analyzer URIT-2900 Vet Plus (URIT Medical Electronic Group Co., Ltd, China). The biochemical blood serum test was performed using an automatic biochemical analyzer CS-T240 (DIRUI Industrial Co., Ltd, China) and commercial veterinary kits (DiaVetTest

by DIAKON-DS, Russia; Randox Laboratories Ltd., Great Britain). The content of chemical elements in the examined samples studied was measured using a mass spectrometer Elan 9000 and an atomic emission spectrometer Optima 2000V (Perkin Elmer, USA). The samples were ashed using microwave decomposition system Multiwave-3000 (Anton Paar, Austria).

For liver microstructure studies, samples were fixed in 10 % neutral formalin and embedded in paraffin mixture HISTOMIX® (OOO BioVitrum, Russia). 5-6 μm thick histological sections were prepared using a semi-automatic microtome (01 MW, Tekhnom, Russia), stained with Mayer's Haematoxylin and Eosin and examined under a light microscope MT 5300L (Meiji Techno Co., Ltd, Japan, ×400).

The data are presented as the arithmetic mean (*M*) with the standard error of the mean (*m*). Statistical analysis was performed using ANOVA (Statistica 10.0 software package, StatSoft Inc., USA) and Microsoft Excel. The validity of differences in the indicators compared was determined by Student's *t*- test. The values were considered statistically significant at $P < 0.05$.

Results. Significant morphological changes of blood were observed as early as 1 day after administration of molybdenum and molybdenum oxide nanoparticles (Table 1). For example, leukocyte counts in groups II and IV have decreased by 11.3 % ($P < 0.05$) and 58.5 % ($P < 0.01$).

1. Morphological blood parameters in Wistar rats upon intraperitoneal administration of Mo and MoO_3 NPs at various doses ($M \pm m$, $n = 75$)

Parameter	Control	Mo NPs		MoO_3 NPs	
		group I	group II	group III	group IV
Day 1					
Leucocytes, $\times 10^9$/l	8.80±0.180	8.85±0.095	7.90±0.080	7.25±0.550	6.55±0.150
Erythrocytes, $\times 10^{12}$/l	8.59±0.120	8.90±0.155	8.65±0.155	9.50±0.380	8.52±0.165
Hemoglobin, g/l	172.5±1.50	189.5±5.50	162.5±4.50	184.0±6.00	133.5±1.50
Platelets, $\times 10^9$/l	170.50±9.500	176.00±12.000	250.00±5.130*	188.50±6.500	184.50±6.500
Lymphocytes, $\times 10^9$/l	4.10±0.010	3.75±0.015	4.35±0.015	2.90±0.030	2.30±0.090
Monocytes, $\times 10^9$/l	2.20±0.090	1.85±0.050*	1.55±0.035*	1.45±0.035**	1.20±0.070**
Granulocytes, $\times 10^9$/l	2.50±0.090	3.25±0.015*	2.00±0.050	2.90±0.090*	2.05±0.055
Day 7					
Leucocytes, $\times 10^9$/l	7.10±0.120	6.50±0.160	7.85±0.195	6.60±0.110	7.95±0.450
Erythrocytes, $\times 10^{12}$/l	8.12±0.430	7.60±0.360	7.43±0.110	6.62±0.230	7.55±0.050
Hemoglobin, g/l	165.0±10.20	156.0±6.00	143.0±3.00	126.0±5.0	145.0±8.0
Platelets, $\times 10^9$/l	159.5±3.50	162.0±8.00	182.5±2.50*	183.0±8.00	179.5±7.50
Lymphocytes, $\times 10^9$/l	3.80±0.01	3.50±0.07	3.70±0.09	2.90±0.01*	3.40±0.07
Monocytes, $\times 10^9$/l	2.10±0.210	2.35±0.150	2.65±0.300	1.95±0.020	1.40±0.060*
Granulocytes, $\times 10^9$/l	2.50±0.090	1.65±0.035	2.50±0.070	2.75±0.085	3.15±0.015*
Day 14					
Leucocytes, $\times 10^9$/l	8.15±0.350	8.95±0.150	8.75±0.150	6.26±0.043	7.95±0.250
Erythrocytes, $\times 10^{12}$/l	8.78±0.430	8.62±0.165	6.86±0.105	5.91±0.139	6.76±0.160
Hemoglobin, g/l	166.5±9.50	157.5±5.50	122.5±5.50	108.8±5.25	128.0±8.00
Platelets, $\times 10^9$/l	188.5±7.50	154.5±3.50	352.0±6.00**	149.88±5.13	311.5±9.50**
Lymphocytes, $\times 10^9$/l	4.50±0.012	3.05±0.015	2.20±0.020	2.10±0.099	2.75±0.035
Monocytes, $\times 10^9$/l	2.55±0.015	2.05±0.015	2.10±0.012	2.42±0.038	1.65±0.015*
Granulocytes, $\times 10^9$/l	2.10±0.020	3.85±0.025	4.45±0.035	4.74±0.066	3.55±0.045

Note. Groups I and II — doses of 1 and 25 mg/kg live weight, respectively; groups III and IV — 1.2 and 29 mg/kg.
*, ** Differences vs. control are statistically significant at $P < 0.05$ and $P < 0.01$, respectively.

The effect of molybdenum-based nanoparticles was characterized by decrease in monocyte counts on day 1 by 18.9 ($P < 0.05$), 41.9 ($P < 0.01$), 51.7 ($P < 0.05$) and 83.3 % ($P < 0.001$) in groups I, II, III and IV, respectively. In 7 days this difference amounted to 55.6 ($P < 0.05$), 27.3 ($P < 0.05$), 7.7 and 50.0 % ($P < 0.05$). At the time of completion of the experiment significant differences were only observed between the control and group IV (54.5 %, $P < 0.05$). The nanoparticle effects with regard to the monocytes observed by us differed dramatically from those reported for polystyrene nanoparticles [16] and Cu and Fe nanoforms [17]. Similar dynamics was observed for lymphocytes, the number

of which in the blood of animals has decreased by 78.6 % ($P < 0.001$) on day 1 and by 63.6 % ($P < 0.001$) on day 14. In other groups significant decrease in the number of lymphocytes was only observed on day 14. Similar dynamics of the number of lymphocytes was previously reported in humans when molybdenum oxide inhalation [18].

On day 7 and 14 upon administration of nanoparticles the rats demonstrated the signs of molybdenum intoxication (decrease in erythrocyte and hemoglobin counts in blood), which were more expressed for MoO_3 NPs due to higher toxicity of the oxide compared to metal [19].

In the groups where the highest doses of molybdenum and molybdenum oxide NPs were administered, the number of platelets has increased by day 14, which led to blood sludging, an increase in viscosity and impaired perfusion through microcirculation vessels. Similar signs have been described earlier for molybdenum-based nanoparticles [20, 21].

2. Biochemical blood parameters in Wistar rats upon intraperitoneal administration of Mo and MoO_3 NPs at various doses ($M \pm m$, $n = 75$)

Parameter	Control	Mo NPs		MoO_3 NPs	
		group I	group II	group III	group IV
Day 1					
ALT, U/l	59.75±5.890	97,00±7,000	70,65±3,750	80,12±2,720	44,35±1,550*
AST, U/l	140.50±2.200	434,80±16,500*	452,95±14,950*	376,50±16,600	218,00±17,120
LDH, U/l	279.00±12.200	232,00±15,700	272,50±10,150	250,00±13,000	324,50±14,500
GGT, U/l	1.40±0.014	7,00±0,030*	1,00±0,010	15,00±0,120*	3,00±0,150
Catalase, μmol $H_2O_2 \cdot l^{-1} \cdot min^{-1}$	2668±104	10983±418	6550±148*	8225±295	5664±231
SOD, %	110.0±5.12	130,0±8,01	128,0±7,81	140,0±2,13*	140,0±3,11*
Creatinine, μmol/l	48.50±1.200	42,45±1,450	49,95±2,850	44,95±0,850	75,60±1,700**
Bilirubin, μmol/l	5.31±0.025	5,13±0,031	5,25±0,018	25,80±0,640**	19,70±0,920*
Urea, mmol/l	5.35±0.051	4,25±0,053	6,30±0,050	4,40±0,070	7,00±0,080**
Creatine kinase, U/l	2948±220	3556±134	5227±107	2693±114	2902±172
Day 7					
ALT, U/l	67.25±3.450	128,85±17,350	147,60±6,800	111,50±4,800	132,20±3,100
AST, U/l	148.35±7.450	567,60±19,100	664,95±11,950	531,20±25,700	699,25±13,550
LDH, U/l	305.50±9.500	374,50±9,500	309,00±11,000	305,00±14,000	433,00±15,800
GGT, U/l	1.50±0.050	1,50±0,050	5,00±0,025	2,00±0,100	4,50±0,050
Catalase, μmol $H_2O_2 \cdot l^{-1} \cdot min^{-1}$	2413±131	3792±147	7486±248	8865±212	22029±931**
SOD, %	136.8±16.20	666,2±6,80**	470,4±14,90	367,0±12,00	396,8±3,00*
Creatinine, μmol/l	47.75±0.650	58,20±1,100	32,15±1,250	44,20±3,500	42,65±3,150
Bilirubin, μmol/l	5.96±0.042	5,86±0,028	5,49±0,067	8,05±0,040	6,90±0,097
Urea, mmol/l	5.70±0.012	4,15±0,050	5,00±0,100	5,80±0,150	5,55±0,170
Creatine kinase, U/l	2726±110	5429±298	4394±115	4278±283	4178±148
Day 14					
ALT, U/l	65.80±4.900	182,50±8,900	242,25±9,750	142,00±8,300	128,60±7,600
AST, U/l	142.05±11.150	459,70±28,800	629,25±37,850*	147,00±4,280	367,55±15,850*
LDH, U/l	243.00±12.000	236,00±14,000	294,50±8,500	177,88±5,120	291,50±8,500*
GGT, U/l	1.50±0.050	12,50±0,500	8,50±0,120	1,58±0,042	5,50±0,050
Catalase, μmol $H_2O_2 \cdot l^{-1} \cdot min^{-1}$	2532±151	4122±195	6846±285	8764±256*	20021±725*
SOD, %	130.2±8.84	146,5±12,93	108,1±5,82	94,0±5,05	166,3±2,09*
Creatinine, μmol/l	42.60±0.140	48,30±0,240	51,40±0,200*	46,07±0,535	77,75±0,650**
Bilirubin, μmol/l	5.64±0.425	5,25±0,545	12,02±0,615	2,40±0,171	4,58±0,125
Urea, mmol/l	5.60±0.016	5,50±0,030	6,00±0,017	4,21±0,099	10,60±0,080*
Creatine kinase, U/l	2670±68	4090±175	3148±103	3225±118	3971±163

Note. Groups I and II — doses of 1 and 25 mg/kg, respectively; groups III and IV — 1.2 and 29 mg/kg live weight. ALT, AST, LDH, GGT, SOD — alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, γ-glutamyl transferase, superoxide dismutase.

*, ** Differences vs. control are statistically significant at $P < 0.05$ and $P < 0.01$, respectively.

Thus, the signs of anemia, leukopenia, sludge, local inflammatory reactions reflect the development of capillary-trophic insufficiency in case of MoO_3 NPs application. The doses of molybdenum and molybdenum oxide nanoparticles used are comparable, so we can conclude that the former are less toxic.

An increase in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in animals was indicative of cytolysis, which became

more severe in the course of time (Table 2). For example, with regard to ALT and total bilirubin, the difference as compared to control amounted to 18.2-62.0 % on day 1, 119.0 % on day 7, and 272.0 % on day 14. The difference was even more significant for AST, i.e. 222.0-352,0 %; with regard to bilirubin, significant (4.8-fold) increase was observed for MoO_3 NPs as early as on day 1. Significant ($P < 0.05$) decrease in ALT activity was observed for maximum dose of MoO_3 NPs (29 mg/kg) on day 1. This can be indicative of glomerular filtration impairment, which is confirmed by high values for creatinine (14.3-fold difference with the control, $P < 0.01$) and urea (30.8 % higher, $P < 0.01$).

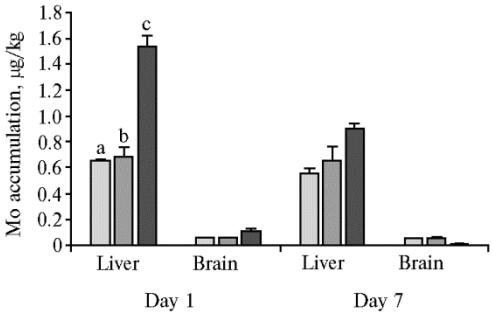
We did not detect an increase in serum γ-glutamyl transferase (GGT) and lactate dehydrogenase (LDH) activity. The values close to upper limit of normal were detected on day 1 for minimum doses of Mo NPs and MoO_3 NPs, as well as on day 7 and 14 at the minimum doses of both nanoparticles. Such dynamics of GGT and LDH activity may be indicative of membrane destruction in a small part of cell population and weak microsomal oxidation induction due to nanoparticles of transition metals [22]. At the same time, with increase in duration of impact up to 14 days at the minimum dose of Mo NPs GGT activity increased 5-fold, which may be considered as a sign of oxidative stress due to effect of Mo NPs. This phenomenon was previously reported for mouse fibroblasts (line L929) when generation of active oxygen forms with subsequent decrease in glutathione content and catalase activity [23]. LDH activity only increased at high doses of the agent (mostly MoO_3 NPs), which may be considered as moderately toxic, as compared to other transition metals [24].

The development of oxidative stress was also confirmed by the dynamics of catalase (CT) and superoxide dismutase (SOD) activity. The peak values for CT were recorded at the high dose of MoO_3 NPs (29 mg/kg) on day 7 and day 14, with 9.0-fold ($P < 0.01$) and 7.9-fold ($P < 0.05$) difference, respectively, compared to control. Minimum doses on day 1 caused a rapid increase in catalase activity (4.1-fold compared to control); on day 7 the values decreased to 1.5-fold difference, and by day 14 increased again, but not up to the initial level. Presumably, catalase activation takes place in response to increase in lipid peroxidation and accumulation of hydrogen peroxide and other oxidative stress products, as catalase metabolizes them and prevents their accumulation in cells. However, according to some researchers, Mo NPs are not toxic and can act as antioxidants, e.g. by showing protective effect in contact with peroxide compounds (H_2O_2) and ZnO-NPS, which has been demonstrated for cell lines of human mammary gland adenocarcinoma (MCF-7) and fibrosarcoma (HT-1080). Mo NPs have been reported to significantly increase glutathione content in MCF-7 line (1.6-fold) and HT-1080 line (1.3-fold), which could be compared to the effect of antioxidant drug N-acetylcysteine (NAC) [25].

Both minimum microstructural changes (granular degeneration, hepatocyte hypertrophy and hyperchromia of their nucleus) in case of low doses of Mo NPs and MoO_3 NPs, and significant pathological changes (large areas of hepatosis and necrosis) in case of high doses of MoO_3 NPs were observed in liver.

Antagonistic interactions of molybdenum with other microelements could contribute to effect of molybdenum nanoparticles in animals [26]. Analysis of liver composition, muscle tissues and brain of animals for 25 chemical elements has revealed significant changes related to three of them, i.e. Mo, Fe and Ca. Thus, in liver in groups I and II a decrease in Fe content by 31.1 ($P < 0.01$) and 38.9 % ($P < 0,001$) was observed on day 1, and by 24.0 ($P < 0.01$) and 76.1 % ($P < 0.001$) on day 7, respectively. The difference for brain tissues amounted to 48.3 ($P < 0.001$) and 90.1 ($P < 0.001$), and 21.1 ($P < 0.01$) and

41.5 % (P < 0,001), respectively. Application of MoO_3 NPs was accompanied by similar changes. With regard to Ca, significant increase in accumulation in liver was only observed on day 1, by 17.1 % (P < 0.05) in group I and by 26.3 % (P < 0.01) in group II. Mo level analysis has demonstrated the same dynamics (Fig.). Peak values were observed on day 1 and were directly dependent on Mo administration dose with clinical difference (136.9 %) for liver. During the next 7 days the amount of Mo in liver decreased in animals of group II by 41.55 %, of group I — by 4.41 %, as compared to the value on day 1.



Mo accumulation in organs of Wistar rats upon single administration of Mo nanoparticles at various doses: a — control, b — 1 mg/kg, c — 25 mg/kg.

Accumulation in brain at a dose of 1 mg/kg was comparable with that of intact animals. Increase of the dose up to 25 mg/kg led to an increase in Mo content by 83.3 % on day 1 and its decrease on day 7 up to the values below control ones.

The obtained results are generally expectable. It is known that molybdenum is one of the essential microelements and its deficiency is accompanied by development of a number of pathologies in humans and animals [27]. However, reports exist on toxic effect of molybdenum in the body, a strong connection between excessive Mo content and development of asthma [28], alveolar and bronchial adenomas and carcinomas [29], etc.

So, administration of Mo nanoparticles in rats is accompanied by capillary-trophic insufficiency, signs of oxidative stress, with more clear manifestation in case of molybdenum oxide nanoparticles. Considering the comparable doses of the agents, we may suggest that Mo nanoparticles are less toxic than nanoforms of its oxide.

REFERENCES

1. Chen Y.X., Wu C.W., Kuo T.Y., Chang Y.L., Jen M.H., Chen I.W. Large-scale production of large-size atomically thin semiconducting molybdenum dichalcogenide sheets in water and its application for supercapacitor. *Sci. Rep.*, 2016, 26(6): 26660 (doi: 10.1038/srep26660).
2. Naylor C.H., Kybert N.J., Schneier C., Xi J., Romero G., Saven J.G., Liu R., Johnson A.T. Scalable production of molybdenum disulfide based biosensors. *ACS Nano*, 2016, 10(6): 6173-6179 (doi: 10.1021/acsnano.6b02137).
3. Tadi K.K., Palve A.M., Pal S., Sudeep P.M., Narayanan T.N. Single step, bulk synthesis of engineered MoS_2 quantum dots for multifunctional electrocatalysis. *Nanotechnology*, 2016, 27(27): 275402 (doi: 10.1088/0957-4484/27/27/275402).
4. Parenago O.P., Bakunin V.N., Kuz'mina G.N., Suslov A.L., Vedeneeva L.M. *DAN*, 2002, 383(1): 84-86 (in Russ.).
5. Liu Q., Sun C., He Q., Liu D., Khalil A., Xiang T., Wu Z., Wang J., Song L. Ultrathin carbon layer coated MoO_2 nanoparticles for high-performance near-infrared photothermal cancer therapy. *Chem. Commun. (Camb.)*, 2015, 51(49): 10054-10057 (doi: 10.1039/c5cc02016f).
6. Fakhri A., Nejad P.A. Antimicrobial, antioxidant and cytotoxic effect of Molybdenum trioxide nanoparticles and application of this for degradation of ketamine under different light illumination. *J. Photochem. Photobiol. B*, 2016, 159: 211-217 (doi: 10.1016/j.jphotobiol.2016.04.002).
7. Zhang W., Shi S., Wang Y., Yu S., Zhu W., Zhang X., Zhang D., Yang B., Wang X., Wang J. Versatile molybdenum disulfide based antibacterial composites for in vitro enhanced sterilization and in vivo focal infection therapy. *Nanoscale*, 2016, 8(22): 11642-11648 (doi: 10.1039/c6nr01243d).
8. Qureshi N., Chaudhari R., Mane P., Shinde M., Jadakar S., Rane S., Kale B., Bhalerao A., Amalnerkar D. Nanoscale Mo-MoO_3 entrapped in engineering thermoplastic: inorganic pathway to bactericidal and fungicidal action. *IEEE Transactions on NanoBioscience*, 2016, 15(3): 258-264 (doi: 10.1109/TNB.2016.2535285).

9. Sam J.S., Yuvakkumar R., Suriya P.R., Karunakaran G., Rajendran V., Hong S.I. Facile and novel synthetic method to prepare nano molybdenum and its catalytic activity. *IET Nanobiotechnology*, 2015, 9(4): 201-208 (doi: 10.1049/iet-nbt.2014.0015).
10. Mendel R.R., Bittner F. Cell biology of molybdenum. *BBA*, 2006, 1763: 621-635 (doi: 10.1016/j.bbamcr.2006.03.013).
11. McBride M.B., Richards B.K., Steenhuis T., Spiers G. Molybdenum uptake by forage crops grown on sewage sludge-amended soils in the field and greenhouse. *J. Environ. Qual.*, 2000, 29: 848-854.
12. He Z.L.L., Yang X.E., Stoffella P.J. Trace elements in agroecosystems and impacts on the environment. *J. Trace Elem. Med. Bio.*, 2005, 19: 125-140 (doi: 10.1016/j.jtemb.2005.02.010).
13. Van Gestel C.A.M., Borgman E., Verweij R.A., Diez-Ortiz M. The influence of soil properties on the toxicity of molybdenum to three species of soil invertebrates. *Ecotox. Environ. Safe.*, 2011, 74: 1-9 (doi: 10.1016/j.ecoenv.2010.10.001).
14. Lebedev S., Yausheva E., Galaktionova L., Sizova E. Impact of molybdenum nanoparticles on survival, activity of enzymes, and chemical elements in *Eisenia fetida* using test on artificial substrata. *Environ. Sci. Pollut. Res. Int.*, 2016, 23(18): 18099-18110.
15. Kosyan D., Rusakova E., Sizova E., Miroshnikov S., Skalniy A. Impact of nanoparticles of heavy metals and their oxides on *Stylonychia mytilus. Ecology, Environment and Conservation*, 2015, 21: 113-119.
16. Fernández-Urrusuno R., Fattal E., Rodrigues J.M., Féger J., Bedossa P., Couvreur P. Effect of polymeric nanoparticle administration on the clearance activity of the mononuclear phagocyte system in mice. *J. Biomed. Mater. Res.*, 1996, 31(3): 401-408.
17. Yausheva E.V., Miroshnikov S.A., Kvan O.V. *Vestnik Orenburgskogo gosudarstvennogo universiteta*, 2013, 12(161): 203-207 (in Russ.).
18. Ott H.C., Prior C., Herold M., Riha M., Laufer G., Ott G. Respiratory symptoms and bronchoalveolar lavage abnormalities in molybdenum exposed workers. *Wien Klin Wochenschr.*, 2004, 116(1): 25-30.
19. Khanturina G.R., Ibraeva L.K., Lebedeva E.A. *Uspekhi sovremennogo estestvoznaniya*, 2012, 12: 11-13 (in Russ.).
20. Ostroushko A.A., Danilova I.G., Medvedeva S.Yu., Gette I.F., Tonkushina M.O. *Ural'skii meditsinskii zhurnal*, 2010, 9(74): 114-117 (in Russ.).
21. Ostroushko A.A., Gette I.F., Danilova I.G., Medvedeva S.Yu., Tonkushina M.O., Prokof'eva A.V. *Ural'skii meditsinskii zhurnal*, 2011, 11(89): 75-79 (in Russ.).
22. Asadi F., Mohseni M., Dadashi Noshahr K., Soleymani F.H., Jalilvand A., Heidari A. Effect of Molybdenum nanoparticles on blood cells, liver enzymes, and sexual hormones in male rats. *Biol. Trace Elem. Res.*, 2016: 1-7 (doi: 10.1007/s12011-016-0765-5).
23. Siddiqui M.A., Saquib Q., Ahamed M., Farshori N.N., Ahmad J., Wahab R., Khan S.T., Alhadlaq H.A., Musarrat J., Al-Khedhairy A.A., Pant A.B. Molybdenum nanoparticles-induced cytotoxicity, oxidative stress, G_2/M arrest, and DNA damage in mouse skin fibroblast cells (L929). *Colloids and Surfaces B: Biointerfaces*, 2015, 1(125): 73-81 (doi: 10.1016/j.colsurfb.2014.11.014).
24. Hussain S.M., Hess K.L., Gearhart J.M., Geiss K.T., Schlager J.J. In vitro toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicol. in Vitro*, 2005, 19(7): 975-983 (doi: 10.1016/j.tiv.2005.06.034).
25. Akhtar M.J., Ahamed M., Alhadlaq H.A., Alshamsan A., Khan M.A., Alrokayan S.A. Antioxidative and cytoprotective response elicited by molybdenum nanoparticles in human cells. *J. Colloid Interf. Sci.*, 2015, 457: 370-377 (doi: 10.1016/j.jcis.2015.07.034).
26. Zhou S., Zhang C., Xiao Q., Zhuang Y., Gu X., Yang F., Xing C., Hu G., Cao H. Effects of different levels of molybdenum on rumen microbiota and trace elements changes in tissues from goats. *Biol. Trace Elem. Res.*, 2016, 174(1): 1-8 (doi: 10.1007/s12011-016-0706-3).
27. Bourke C.A. Molybdenum deficiency produces motor nervous effects that are consistent with amyotrophic lateral sclerosis. *Front. Neurol.*, 2016, 7: 28 (doi: 10.3389/fneur.2016.00028).
28. Huang X., Xie J., Cui X., Zhou Y., Wu X., Lu W., Shen Y., Yuan J., Chen W. Association between concentrations of metals in urine and adult asthma: a case-control study in Wuhan, China. *PLoS ONE*, 2016, 11(5): e0155818 (doi: 10.1371/journal.pone.0155818).
29. Chan P.C., Herbert R.A., Roycroft J.H., Haseman J.K., Grumbein S.L., Miller R.Al. Lung tumor induction by inhalation exposure to molybdenum trioxide in rats and mice. *Toxicol. Sci.*, 1998, 45(1): 58-65.

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 937-945
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Mycotoxicology: feeds, fodder crops

UDC 636.085.19:636.086.1/.3:632.4(470)

doi: 10.15389/agrobiology.2016.6.937rus
doi: 10.15389/agrobiology.2016.6.937eng

AFFECTION OF COARSE FODDERS BY TOXIGENIC *Fusarium* FUNGI

E.A. PIRYAZEVA, G.P. KONONENKO, A.A. BURKIN

All-Russian Research Institute of Sanitary, Hygiene and Ecology, Federal Agency of Scientific Organizations, 5, Zvenigorodskoe sh., Moscow, 123022 Russia, e-mail kononenkogp@mail.ru
Acknowledgements:
We thank Dr T.Yu. Gagkaeva (All-Russian Research Institute of Plant Protection, St. Petersburg—Pushkin) for fungal identification and consultations
Received April 27, 2016

Abstract

Lack of information about the distribution of toxigenic *Fusarium* fungi in hay and cereal straw which is used for bedding and feeding animals inhibits preventive measures against animal toxicoses. Multiple combined fusariotoxin contamination of hay was recently established during advanced mycotoxicological survey of commercially used batches from the livestock farms in European Russia (G.P. Kononenko, A.A. Burkin, 2014). The aim of this work was to study the species complex of fusaric fungi in fodders from Chelyabinsk, Bryansk and Moscow regions of Russia in different years and to evaluate the toxin-producing capacity of their populations. All 60 samples of hay and straw from Chelyabinsk region (1992) were colonized by fusaric fungi of 6 species — *F. sporotrichioides* Sherb, *F. avenaceum* (Corda ex Fr.) Sacc., *F. poae* (Peck) Wr., *F. tricinctum* (Corda) Sacc., *F. culmorum* (W.G. Smith) Sacc. and *F. sambucinum* Fuckel. Unidentified *Fusarium* species were detected in hay of bromegrass, galega, annual (a mixture of wheat, oats and peas) and perennial grasses, and a complex of these fungi was found in rye straw. The majority of samples (90 %) contained *F. sporotrichioides* (a single species, or this species along with other, but *F. sporotrichioides* mostly predominated). Of 19 samples of the hay and straw harvested in the Bryansk region (2011), 16 were infected by *Fusarium* fungi belonging to the same 8 species. *F. sporotrichioides* was found in 11 samples and prevailed, and the other 7 detected species were less frequent — *F. tricinctum* (4 samples), *F. equiseti* (Corda) Sacc. (3 samples), *F. poae* (2 samples), *F. solani* (Mart.) Sacc. (2 samples), *F. culmorum*, *F. graminearum* Schw. and *F. semitectum* Berk. et Rav. (each in one sample). In fodders from Moscow region (2013) *Fusarium* fungi were found in 171 samples of 239 tested (71.5 %) and the affection was often very high. Isolated fungi belonged to the same 8 species as in other regions mentioned. By the frequency of occurrence they were ranged in the following order: *F. sporotrichioides*, *F. tricinctum*, *F. avenaceum*, *F. poae*, *F. culmorum*, *F. graminearum*, *F. semitectum*, *F. solani*, with *F. sporotrichioides* found in 85.7 % of the samples. In addition, we identified three isolates as *F. cerealis* (Cooke) Sacc., *F. sambucinum* and *F. torulosum* (Berk. & M. A. Curtis). Fungal cultures grown on rice grain substrate at 23 °C for 7 days were first used to test the toxin production. *F. sporotrichioides* was shown to possess 100 % potential for T-2 toxin (122-1078 μg/g) and diacetoxyscirpenol (0.7-20 μg/g) production, *F. culmorum*, *F. graminearum*, *F. cerealis*, *F. torulosum* — for deoxynivalenol and zearalenone production, and in two *F. semitectum* isolates the level of zearalenone was high. Extensive distribution of *F. sporotrichioides* producing T-2 toxin and its highly toxic analog diacetoxyscirpenol, necessitates special attention to control the level of these trichothecenes at harvesting and storage of roughage. Specific features of toxin-production of other *Fusarium* species and hay contamination with deoxynivalenol and zearalenone are discussed.

Keywords: hay, straw, *Fusarium* fungi, mycotoxins

High-quality hay and straw are critical for the successful dairy and beef farmers both in temperate climatic zone and tropical areas. There is a significant progress in formation and optimal exploiting grasslands in many countries, but their safe use remains understudied. The reports on roughage contaminated with microscopic fungi capable of toxin production are of great concern. The abundant *Fusarium*, *Aspergillus*, *Mucor*, and *Absidia* microbiota has been found at surveys in Canada and Ireland [1, 2]. When harvesting bermudagrass (*Cynodon* sp.) hay, the major fodder cereal in tropics and subtropics, *Fusarium population* ac-

companied by *Penicillium*, *Aspergillus*, *Rhizopus* and *Cladosporium* was the most abundant [3].

Until 1980-1990, the feed-caused fusariotoxicoses with sharp positive skin test occurred among calves and sheep in many regions and republics of the USSR, as well as sporadic outbreaks of acute intoxication of ruminants and horses related to "highly toxic fungi" in hay and straw [4-6]. The response measures were unprecedented. Moldy hay and straw with ≥ 10 % contamination, or those infected by *Dendrodochium toxicum* fungus were subject to destroy; the roughages toxic in skin test were prohibited; straw and chaff affected by *Stachybotrys alternans* could only be used after neutralization, and the roughage low-toxic in skin test should not exceed 25 % of animal diets [7].

Fusarium fungi were carefully studied in Ukraine [8, 9], western Belorussia where these species predominated in clover hay [10], in Lithuania and Armenia [11, 12], in the south of Kazakhstan where *Fusarium* fungi were the most abundant on hay and straw [13], in lowland and foothill areas of Dagestan [14]. Nineteen *Fusarium* species and subspecies were found in hay from Northern Ossetia [15], and 13 species were identified on wild fodder cereals from southern Big Caucasus in Azerbaijan [15]. In the Russian Federation the surveys were carried out in Ryazan Province, Volgograd Province [17, 18], Altai [17-19], and then suspended. However, cattle fusariotoxicoses remained a threat. At 1985-1989 Fusarium epiphytotics in crops in Krasnodar Territory, the level of trichothecenes from deoxynivalenol group in the straw exceed that in grain [20]. In the late autumn 1998, acute T-2 toxicosis among calves grazed in the unharvested corn fields in several districts of the Kursk region was due to severe plant infection by fungi (data not published).

Lack of progress in understanding toxigenic fungi prevalence in hay and straw used for bedding or feeding holds back the preventive measures against fusariotoxicoses. Note, the latest data indicate that multiple combined fusariotoxic contamination is characteristic of herbaceous feeds [21-24].

We first in Russia targetedly sought for toxigenic fungi producing trichothecenes and zearalenone in coarse fodders. In the work rice grain substrate was inoculated and cultured for 7 days. This original approach allowed us to identify six *Fusarium* species of 11 fungal isolates as toxigenic ones. Among these, *F. cerealis*, *F. torulosum* and *F. semitectum* are of special interest as toxigenic properties of these species remain rather poorly studied.

Our objectives were to examine fusarium species composition in hay and straw harvested at different times in three Russian regions and to estimate toxigenicity of the revealed fungal populations.

Techniques. Hay and straw batches were sampled from commercial farms in 5 districts of Chelyabinsk Province, 9 districts of Bryansk Province, and 31 districts of Moscow Province in 1992, 2011 and 2013 (Table 1). Each batch was cut into ~2 cm segments, and mixed thoroughly. Part of the batch was placed on Czapek agar supplemented with medical bile (10 %), penicillin (50,000 U/l) and streptomycin (100,000 U/l) in three petri dishes. After incubation at 25 °C for 5 to 7 day the fragments with Fusarium attack were counted, and the portion of affected segments was calculated. For pure isolates, the colonies characteristic of the genus Fusarium, were grown on the same nutrient medium in petri dishes for 5-7 days and then used individually for inoculation of potato glucose agar (PGA) slants. For identification we used taxonomic keys [25] and manuals [26, 27].

To estimate toxin producing activity, approximately equal portions of 10-day PGA fungal culture were individually placed in three 10 ml vials (bottom diameter of 18 mm) with 1 g sterile crushed rice grain pre-wetted with 1 ml H_2O. The vials were closed with cotton-gauze plugs, which were tightly wrapped with

a layer of Parafilm M® («Pechiney Plastic Packaging», USA). The vials were kept in the dark for 7 days at 23 °C, then 5 ml mix of acetonitrile:water (v/v 86:14) was added. At the beginning and the end of stationary 14-hour extraction the vials were shaken vigorously.

T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), and zearalenone (ZEN) in the extracts were estimated using certified ELISA test systems [28]. The lower limit of quantification was 0.2 μg/g for T-2, and 0.5 μg/g for the rest fusatiotoxins. We tested *Fusarium* isolates from hay harvested in 2014 in Moscow Province, including *F. graminearum*, *F. culmorum*, *F. semitectum*, *F. solani*, four more isolates with a revised taxonomic identification, randomized sample of *F. sporotrichioides*, *F. poae*, *F. tricinctum*, and *F. avenaceum* (10 isolates of each species), and 12 isolates of *F. sporotrichioides* found in hay with high ZEN contamination.

Data were analyzed in descriptive statistics program Microsoft Excel 2013.

Results. Table 1 shows the characteristics of the samples.

1. Characterization of feeds from different Russian regions tested for *Fusarium* infection

Location, year of harvesting	Feed type, number of batches	Botanical composition (according to batch certificate)
Chellaybinsk Province (Argayashskii, Kaslinskii, Krasnoarmeiskii, Kunashakskii, Sosnovskii regions), 1992	Hay, n = 56	Bromegrass, goat's rue, a mixture of wheat, oats, peas, timothy, fescue, perennial grass mixture, seeded grass mixture, alfalfa
	Straw, n = 4	Cereals (including rye)
Bruansk Province (Bryanskii, Vygonichskii, Zhiryatinskii, Zlynkovskii, Karachaevskii, Klinstovskii, Pochepskii, Trubchevskii regions), 2011	Hay, n = 14	Cereals, meadow grass, ryegrass, herbs
	Straw, n = 5	Not specified
Moscow Province (31 regions, except Krasnogorskii, Naro-Fominskii, Lyuberetskii, Egor'evskii, Stupinskii, Lukhovitskii, Balashikhinskii regions), 2013	Hay, n = 230	Herbs, perennials, meadow mix, timothy, cereals, legumes, bromegrass, alfalfa, fescue, vetch, goat's rue, clover
	Straw, n = 9	Cereals (including wheat and oats), vetch-oats

We found *Fusarium* fungi in all 60 batches of hay and straw from Chelyabinsk Province among which we have identified *F. sporotrichioides* Sherb., *F. avenaceum* (Corda ex Fr.) Sacc., *F. poae* (Peck) Wr., *F. tricinctum* (Corda) Sacc., *F. culmorum* (W.G. Smith) Sacc. and *F. sambucinum* Fuckel. Unidentified Fusaria were revealed in the hay of bromegrass, goat's rue, annual mix (wheat, oats and pea mix), perennial grasses, and in the rye straw. *F. sporotrichioides* was detected in 90 % probes either as a single species, or in combination with with others, but as a rule, the *F. sporotrichioides* prevailed.

In Bryansk Province, among 19 probes of hay and straw tested the 16 ones were attacked by *Fusarium* fungi of 8 species, of these *F. sporotrichioides* was the most frequent (11 probes), while others were more rare, e.g. we identified *F. tricinctum* in 4 probes, *F. equiseti* (Corda) Sacc. in 3 probes, *F. poae* in 2 probes, *F. solani* (Mart.) Sacc. in 2 probes, *F. culmorum*, *F. graminearum* Schw. and *F. semitectum* Berk. et Rav. in one probe each.

In Moscow regions we have identified *Fusarium* fungi in 171 batches form 239 those tested (71.5 %). The degree of sample destruction ranged from 1 to 100 %, making 1-20 % in 47.3 % of the samples and exceeding 50 % in 20 % of the samples.

Isolates from *Arthrosporiella*, *Sporotrichiella*, *Discolor* and *Martiella* sections were allocated to 8 species (Table 2). All these species, also mentioned in two previous reports, were arranged on their predominance in a descending order as *F. sporotrichioides*, *F. tricinctum*, *F. avenaceum*, *F. poae*, *F. culmorum*, *F. graminearum*, *F. semitectum*, *F. solani*.

Similarly to *Fusarium* fungi in Chelyabinsk and Bryansk provinces, the

prevalence rate for *F. sporotrichioides* was high and reached 85.4 % vs. 8-15 % for *F. tricinctum*, *F. avenaceum* and *F. poae*, 2.3 % for *F. culmorum* and *F. graminearum*, 1.2 % for *F. semitectum*, and 0.6 % for *F. solani*. For some species no compliance of their occurrence with the botanical composition of hay and the place of harvesting was traced, however, in three cases of four *F. graminearum* detection this species was found in timothy hay from western areas of the region (see Table 2).

2. Prevalence of *Fusarium* fungi species in hay and straw (Moscow Province, 2013)

Section	*Fusarium* species	Prevalence, % (n = 171)	Number of isolates
Arthrosporiella	*F. sporotrichioides* Sherb.	85,4	265
	F. avenaceum (Corda ex Fr.) Sacc.	11,1	20
	F. semitectum Berk. et Rav.	1,2	2
Sporotrichiella	*F. tricinctum* (Corda) Sacc.	15,2	28
	F. poae (Peck) Wr.	8,2	15
Discolor	*F. culmorum* (W.G. Smith) Sacc.	2,3	4
	F. graminearum Schw.	2,3	4
Martiella	*F. solani* (Mart.) Sacc.	0,6	1
-	*Fusarium* spp.	4,7	9

N o t e. n — the number of isolates tested.

Four of the found *Fusarium* spp. isolates (see Table 2) were attributed according to modern taxonomic systems and approaches. As a result, the isolate No 252/3, earlier morphologically described as *F. culmorum* according to Booth system, was identified as *F. cerealis* (Cooke) Sacc., and the isolate No. 392/1, previously attributed to *F. graminearum*, was identified as *F. torulosum* (Berk. & M.A. Curtis). Of two fungal species which could not be initially identified the isolate No. 79/4a belonged to the *F. incarnatum-equiseti* species complex (FIESC), and the isolate No. 79/4b was attributed to *F. sambucinum* Fuckel. The remaining five *Fusarium* spp. isolates (see Table 2) were contaminated by *Penicillium* fungi, which complicated the research and eventually led to loss of the fungal cultures.

Toxigenicity was assessed in *F. sporotrichioides*, *F. tricinctum*, *F. avenaceum* and *F. poae* (10 isolates of each species), and in all other species cultures. For rapid evaluation, a procedure which provided stable active growth of compact culture was developed: fungal cultures grew for 7 days at 23 °C on crushed rice grain as a substrate (1 g), sterilized after moistening (1 ml of water), in 10 cm^3-vials with cotton plugs, tightly wrapped with a layer of laboratory film to prevent drying out.

3. Toxigenicity (μg/g of substrate) **of *Fusarium sporotrichioides* after 7-day growth on crushed rice grain at 23 °C** (n = 3, $X \pm s$)

Isolate No.	T-2	DAS	ZEN
9/1	327±48	1.7±0.4	–
50/3	348±154	1.4±0.3	0.5±0.1
54/1	290±70	1.4±0.2	1.2±0.4
236/1	408±76	1.8±0.4	–
276/2	396±8	3.6±0.2	–
324/2	1078±353	20.0±3.0	–
349/4	446±110	2.5±0.6	–
501/1	387±146	2.1±0.7	–
614/3	122±66	0.7±0.2	0.7±0.2
647/3	362±141	2,3±1,0	–

N o t e. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, ZEN — zearalenone; n — number of replications in the experimrnt, X — arithmetical mean, s — sample mean error. Dashes mean the mycotoxin is not detected.

All 10 *F. sporotrichioides* isolates produced T-2 (122-1078 μg/g) and DAS (0.7-20 μg/g), and only a few ones could produce ZEN (3 of 10 isolates, 0.5-1.2 μg/g) (Table 3). Apparently, the cases of acute dermatitis in cows, when the *F. sporotrichioides*-infected oat straw has been used, were caused by the T-2 toxin [29]. We have revealed the extensive hay lesion by *F. sporotrichioides*, the

active producer of T-2 and its highly toxic analogue, in different territories and in different years. Hence, a special attention should be paid to control of the T-2 content during harvesting and feed storing.

4. Toxigenicity (μg/g of substrate) **of *Fusarium culmorum*, *F. cerealis*, *F. graminearum*, and *F. torulosum* after 7-day growth on crushed rice grain at 23 °C** (n = 3, $X \pm s$)

Isolate No.	DON	ZEN
	F. culmorum	
28/3	5±1	57±55
258/4	55±5	256±95
266/1	10±3	88±26
505/1	9±1	12±7
	F. cerealis	
252/3	16±4	14±7
	F. graminearum	
9/3	37±21	18±8
109/2	3±1	3±1
389/1	23±14	80±79
398/1	49±29	2±1
	F. torulosum	
392/1	62±21	101±65

Note. DON — deoxynivalenol, ZEN — zearalenone; n — number of replications in the experimrnt, X — arithmetical mean, s — sample mean error.

F. poae, *F. avenaceum*, *F. tricinctum* did not produce T-2 and ZEN. Importantly, the toxigenicity of *F. sporotrichioides* and *F. poae* in a rapid test on a grain substrate coincided with the results under prolonged fungal culture [30-32].

In most *F. culmorum* and *F. graminearum* cultures isolated from hay, as well as in sporadic isolates of *F. cerealis* and *F. torulosum*, DON and ZEN amounts were more than 10 μg/g (Table 4).

However, it is unlikely that the total contribution of the trichothecenes of the DON and ZEN groups to the contamination of coarse fodder can be significant due to the weak fungal affection. Nevertheless, the researchers in Germany [33] and Canada (2) reported about a combined hay contamination. An extensive T-2, HT-2, DON and ZEN contamination of cereals and straw occurred at Swedish farms in 2011-2012 [34].

Low DON production (0.6±0.1 μg/g) was characteristic of isolate No. 79/4a from *F. incarnatum-equiseti* complex, whereas ZEN was not found. No toxins were not detected in *F. solani* (No. 537/4) and *F. sambucinum* (No. 79/4б), however, *F. semitectum* isolates No. 252/4 and No. 373/4 of different samples actively produced ZEN (77±13 and 110±5 μg/g, respectively).

Grain substrate for fungal culture, first applied in this work, allowed a comparative assessment of the toxicity of representative samples, which is especially important when working with natural populations. Our results showed (see Tables 3 and 4) that the relative sample mean error at 3-fold replications was 34 %, and for T-2 and DAS it was less (28 %) than for ZEN (44 %). Thus, it is necessary to improve this methodology.

Finding the agents caused ZEN contamination in hay remains very relevant. In Ireland, this toxin was found in 21 % of hay samples [2]. Previously we found high ZEN accumulation in the hay, harvested in the European Russia, including the Moscow region [21-24]. Six ZEN-contaminated probes contained 12 *F. sporotrichioides* strains, and two of these probes contained *F. culmorum* (No. 266/1), *F. tricinctum* (No. 266/3) and *F. semitectum* (No. 373/4). Only 3 of 12 *F. sporotrichioides* isolates excreted low amounts of ZEN, *F. tricinctum* (No. 266/3) did not produce ZEN, the toxin-producing activity of *F. culmorum* (No. 266/1) was similar to that of other members of the genus (see Table 4), and only *F. semitectum* (No. 373/4) was highly active. Interestingly, in the hay from which the No. 373/4 was isolated the ZEN amount reached 3160 μg/kg,

and, when compared to the collection *Fusarium* strains on potato-sucrose agar, this species was classified as a possible ZEN producer [24].

Obviously, the mycological analyses of plants and feeds can not exhaustively reflect complex and dynamic processes in mycobiota, so the probability of identifying the causal relationships leading to an increased risk of fusariotoxicosis, is not so great. Nevertheless, the determination of the species composition and the toxigenic potential of the entire complex of *Fusarium* fungi affecting coarse fodder remains important, since high-toxic isolates are found among the new species [35]. Mycotoxicological evaluation of roughage should be continued not only for fusarium fungi. The ability to produce ZEN and its derivatives is known for the phytopathogenic fungus *Drechslera portulacae* [36] and fungi of the genus *Stachybotrys* [37]. Using a moist chamber technique, we did not find fungi of highly toxic cellulose destructors — *Stachybotrys alternans* and *Dendrodochium toxicum* (= *Myrothecium roridum*) in hay and straw samples from the Chelyabinsk region, nevertheless, fodder contamination by toxic metabolites is of special relevance. In recent years, new information about the prevalence of such fungi inhabiting soils and plant residues has appeared. Recently, mycobiota of maize phylloplans in the Primorsky Territory have been replenished with the *Myrothecium verrucaria* fungus, which can infect plants in the early ontogenesis [38].

Thus, among the fusarium fungi affecting hay and straw, six species are able to provide a combined contamination with mycotoxins — T-2 and DAS (*Fusarium sporotrichioides*), DON and ZEN (*F. graminearum*, *F. culmorum*, *F. cerealis*, *F. torulosum*), and ZEN (*F. semitectum*). The extensive *F. sporotrichioides* infection, found in different territories and in different years, specially indicates the need to control the amount of T-2 and its highly toxic analogue, DAS, during harvesting and storage. It is rational to elucidate the causes of ZEN contamination in hay, taking into account the effects of biogenic and anthropogenic factors, as well as the possible contribution of fungi belonging to other taxonomic groups. The rapid fungal culture on grain substrate, first applied in this work and allowing a comparative assessment of the toxicity of fungi in a representative sample sets, seems to be promising for the investigation of natural populations.

REFERENCES

1. Undi M., Wittenberg K.M., Holliday N.J. Occurrence of fungal species in stored alfalfa forage as influenced by moisture content at baling and temperature during storage. *Canadian Journal of Animal Science*, 1997, 77: 95-103 (doi: 10.4141/A96-024).
2. Buckley T., Creighton A., Fogarty U. Analysis of Canadian and Irish forage, oats and commercially available equine concentrate feed for pathogenic fungi and mycotoxins. *Irish Veterinary Journal*, 2007, 60: 231-236 (doi: 10.1186/2046-0481-60-4-231).
3. Taffarel L.E., Mesquita E.E., Castagnara D.D., Costa P.B., Neres M.A., Horn M.B., Oliveira P.S.R., Meiners C.C. Dehydration curve, fungi and mycotoxins in Tifton 85 hay dehydrated in the field and in shed. *Revista Brasileira de Zootecnia*, 2013, 42(6): 395-403 (doi: 10.1590/S1516-35982013000600003).
4. Kurmanov I.A. *Veterinariya*, 1961, 11: 30-31 (in Russ.).
5. Vertinskii K.I., Dzhilavyan Kh.A., Koroleva V.P. *Byulleten' Vsesoyuznogo instituta eksperimental'noi veterinarii*, 1967, issue 2: 86-90 (in Russ.).
6. Khmelevskii B.N., Pilipets V.I., Malinovskaya L.S., Kostin V.V., Komarnitskaya N.P., Ivanov V.G. *Profilaktika mikotoksikozov zhivotnykh* [Prevention of animal mycotoxicoses]. Moscow, 1985 (in Russ.).
7. Ivanova L.G., Golovina N.L. V sbornike: *Otsenka zagryazneniya pishchevykh produktov mikotoksinami. Tom III. Kontrol' za zagryazneniem prodovol'stvennogo syr'ya i pishchevykh produktov mikotoksinami* [In: Evaluation of food contamination with mycotoxins. V. III. Control of raw materials and food contamination]. Moscow, 1985: 33-37 (in Russ.).
8. Starchenko L.E. *Toksichnye griby na grubykh kormakh i ikh mikologicheskii kontrol' v usloviyakh L'vovskoi oblasti. Avtoreferat kandidatskoi dissertatsii* [Toxigenic fungi affecting coarse feed and mucological control under the conditions of Lvov region. PhD Thesis]. Lvov, 1967 (in Russ.).

9. Rukhlyada V.V. *Fuzario-T-2-toksikoz sel'skokhozyaistvennykh zhivotnykh. Avtoreferat doktorskoi dissertatsii* [T-2 fusariotoxicosis in farm animals. DSci. Thesis]. Moscow, 1991 (in Russ.).
10. Ivanov A.T. *Sanitarno-mikologicheskie issledovaniya bobovykh kul'tur v usloviyakh Belorusskoi SSR. Avtoreferat kandidatskoi dissertatsii* [Sanitary and mycological study of legume crops in the Byelorussian SSR. PhD Thesis]. Moscow, 1967 (in Russ.).
11. Gustas A.P. *Sanitarno-mikologicheskie issledovaniya kormov v usloviyakh Litovskoi SSR. Avtoreferat kandidatskoi dissertatsii* [Sanitary and mycological study of fodder under conditions of the Lithuanian SSR. PhD Thesis]. Kaunas, 1968 (in Russ.).
12. Lugina Zh.A. *Trudy Vsesoyuznogo nauchno issledovatel'skogo instituta veterinarnoi sanitarii*, 1970, 35: 73-79 (in Russ.).
13. Zhakhanov A. *Mikotoksikologicheskaya kharakteristika grubykh kormov i osobennosti toksinoobrazovaniya griba Fusarium sporotrichioides Scherb. na yuge Kazakhstana. Avtoreferat kandidatskoi dissertatsii* [Mycological characterization of coarse fodder and *Fusarium sporotrichioides* Scherb. toxigenesis in southern Kazakhstan. PhD Thesis]. Alma-Ata, 1975 (in Russ.).
14. Salikhova N.M. *Trudy Vsesoyuznogo nauchno-issledovatel'skogo instituta veterinarnoi sanitarii*, 1973, 47: 43-46 (in Russ.).
15. Kaloev S.S. *Trudy Kubanskogo sel'skokhozyaistvennogo instituta*, 1975, 97(125): 3-7 (in Russ.).
16. Ibragimov R.G. *Mikoflora zernofurazha i vegetiruemykh kormovykh zlakov v yuzhnoi chasti Bol'shogo Kavkaza Azerbaidzhana i ikh toksikologicheskaya kharakteristika. Avtoreferat kandidatskoi dissertatsii* [Mycoflora of grain fodder and cereals grown in southern Big Caucasus and their toxicologial characterization. PhD Thesis]. Kiev, 1980 (in Russ.).
17. Lychagin G.Ya. *Sbornik nauchnykh trudov Ryazanskogo SKHI*, 1970, 24(2): 56-60 (in Russ.).
18. Konovodova T.V. *Materialy po izucheniyu aflatoksikoza ptits i toksicheskikh gribov na kormakh Volgogradskoi oblasti. Avtoreferat kandidatskoi dissertatsii* [Aflatoxicosis in birds and feed contamination with toxigenic fungi in Vlgograd region. PhD Thesis]. Moscow, 1974 (in Russ.).
19. Kurmanov I.A., Osipov A.N. *Trudy Vsesoyuznogo nauchno-issledovatel'skogo instituta veterinarnoi sanitarii*, 1970, 35: 80-87 (in Russ.).
20. Leonov A.N., Kononenko G.P., Soboleva N.A. *Mikologiya i fitopatologiya*, 1989, 23(2): 147-151 (in Russ.).
21. Kononenko G.P., Burkin A.A. Mycotoxin contaminations in commercially used hay. *Sel'skokhozyaistvennaya biologiya* [*Agricultrural Biology*], 2014, 4: 120-126 (doi: 10.15389/agrobiology.2014.4.120rus) (in Russ.).
22. Kononenko G.P., Burkin A.A. *Uspekhi meditsinskoi mikologii*, 2014, 13: 305-306 (in Russ.).
23. Kononenko G.P., Burkin A.A. *Materialy III Mezhdunarodnogo kongressa veterinarnykh farmakologov i toksikologov «Effektivnye i bezopasnye lekarstvennye sredstva v veterinarii»* [Proc. III Int. Conr. «Effective and safe drugs for veterinary»]. St. Petersburg, 2014: 123-125 (in Russ.).
24. Burkin A.A., Kononenko G.P., Gavrilova O.P., Gagkaeva T.Yu. About zearalenone levels in grass fodders and toxin producing activity of *Fusarium* fungi. *Agricultural Biology*, 2015, 50(2): 255-262 (doi: 10.15389/agrobiology.2015.2.255eng) (in Engl.).
25. Vooth C. *The genus Fusarium*. Commonwealth Mycological Institute, Kew Surrey, 1971.
26. Gerlach W., Nirenberg H. *The genus Fusarium — a pictorial atlas*. Berlin, 1982.
27. Bilai V.I. *Fuzarii* [Fusarium fungi]. Kiev, 1977 (in Russ.).
28. Burkin A.A., Kononenko G.P. Mycotoxin contamination of meadow grasses in European Russia. *Agricultural Biology*, 2015, 50(4): 503-512 (doi: 10.15389/agrobiology.2015.4.503eng) (in Engl.).
29. Wu W., Cook M.E., Chu F.S., Buttles T., Hunger J., Sutherland P. Case study of bovine dermatitis caused by oat straw infected with *Fusarium sporotrichioides*. *Veterinary Records*, 1997, 140(15): 399-400 (doi: 10.1136/vr.140.15.399).
30. Burkin A.A., Soboleva N.A., Kononenko G.P. *Mikologiya i fitopatologiya*, 2008, 42(4): 354-358 (in Russ.).
31. Tokarev S.V., Kononenko G.P., Soboleva N.A., Burkin A.A. Toxin production in population Fusarium poae on corn of cereals in Ural and Western Siberia. Sel'skokhozyaistvennaya biologiya [Agricultrural Biology], 2009, 1: 89-92. Available http://www.agrobiology.ru/1-2009tokarev.html. No date (in Russ.).
32. Kononenko G.P., Burkin A.A., Soboleva N.A. *Trudy VIEV (Moskva)*, 2009, 75: 364-371 (in Russ.).
33. Schollenberger M., Müller H.M., Rüfle M., Suchy S., Plank S., Drochner W. Natural occurrence of 16 *Fusarium* toxins in grains and feedstuffs of plant origin from Germany. *Mycopathologia*, 2006, 161: 43-52 (doi: 10.1007/s11046-005-0199-7).
34. Nordkvist E., Häggblom P. *Fusarium* mycotoxin contamination of cereals and bedding straw at Swedish pig farms. *Anim. Feed Sci. Tech.*, 2014, 198: 231-237 (doi: 10.1016/j.anifeedsci.2014.10.002).
35. Yli-Mattila T., Ward T.J., O'Donnell K., Proctor R.H., Burkin A.A., Kononenko G.P., Gavrilova O.P., Aoki T., McCormick S.P., Gagkaeva T.Yu.

Fusarium sibiricum sp.nov, a novel type A trichothecene-producing *Fusarium* from northern Asia closely related to *F. sporotrichioides* and *F. langsethiae*. *Int. J. Food Microbiol.*, 2011, 147(1): 58-68 (doi: 10.1016/j.ijfoodmicro.2011.03.007).

36. Sugawara F., Kim K.-W., Kobayashi K., Uzawa J., Yoshida S., Murofushi N., Takahashi N., Strobel G.A. Zearalenone derivatives produced by the fungus *Drechslera portulacae*. *Phytochemistry*, 1992, 31(6): 1987-1990 (doi: 10.1016/0031-9422(92)80346-G).
37. Zaichenko A.M., Andrienko E.V., Tsyganenko E.S. *Makrotsiklicheskie trikhotetsenovye mikotoksiny* [Macrocyclic trichothecene mycotoxins]. Kiev, 2008 (in Russ.).
38. Martynyuk T.D., Egorova L.N. *Mikologiya i fitopatologiya*, 2009, 43(5): 457-459 (in Russ.).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 946-950
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.085.19:615.917:579.64:579.222

doi: 10.15389/agrobiology.2016.6.946rus
doi: 10.15389/agrobiology.2016.6.946eng

STUDY OF AFLATOXIN B1-DESTROYING ACTIVITY OF *Gliocladium roseum* AND *Trichoderma viride* AND THEIR ANTAGONISM TOWARD TOXIGENIC *Aspergillus flavus*

L.A. SHCHERBAKOVA, O.D. MIKITYUK, T.A. NAZAROVA, V.G. DZHAVAKHIYA

All-Russian Research Institute of Phytopathology, Federal Agency of Scientific Organizations, 5, ul. Institute, pos. Bol'shie Vyazemy, Odintsovskii Region, Moscow Province, 143050 Russia, e-mail dzhavakhiya@yahoo.com, larisa@vniif.ru
Acknowledgements:
Supported financially by Russian Science Foundation (RSF project № 14-16-00150)
Received February 9, 2016

Abstract

Aflatoxin B1-destroying activity and antagonistic potential of *Gliocladium roseum* GRZ7 and *Trichoderma viride* TV35 strains isolated from natural substrates colonized by aflatoxigenic *Aspergillus flavus* were studied in vitro. Submerged cultures of *G. roseum* grown on liquid Czapek's medium with casein hydrolizate (Czapek-CasH) at 28 °C and 200 rpm for 7 days were able to destroy 80-90 % of aflatoxin B1 (AFB1), which was added in the nutrient medium before inoculation. *T. viride* grown under the same conditions destroyed only 48 % of initial AFB1 during the same time of cultivation. The tested *T. viride* strain effectively suppressed the growth of toxigenic *A. flavus* strain A11 on Czapek-CasH agar. Co-cultivation of A11 with *T. viride* TV35 resulted in 64 % diminution of the average colony diameter of the aflatoxigenic strain. The strain GRZ7 of *G. roseum* was ineffective as an antagonist of A11. AFB1-destroyimg activity was detected in samples of high-molecular weight metabolites (> 5 kDa) isolated from culture liquid of *G. roseum* grown without AFB1. In addition, *T. viride* ability to degrade the mycotoxin was shown to be inducible. Obtained results were supposed to be of interest for further investigation on decontamination of feeds, which are contaminated with AFB1 or AFB1-producers.

Keywords: aflatoxin B1, biological decontamination, *Gliocladium roseum*, *Trichoderma viride*

Contamination of forage grains and plant materials with aflatoxins, the secondary metabolites of aspergillus fungi, is a serious problem. Because of hepatotoxicity, carcinogenicity and teratogenicity, aflatoxins are dangerous for the vast majority of warm-blooded organisms [1]. Feed contamination with aflatoxins can cause animal death or decreased production, and also lead to food stuff contamination [2]. Due to wide spread of aspergillas, their ability to evolve as facultative parasites on vegetating plants and, at the same time, to keep up saprophytic growth, it is almost impossible to completely exclude the contamination of grain and fodder grass by aflatoxin producers during harvesting and storage. Therefore, the approaches to solving the problem are mainly aimed at decontamination of raw materials [3, 4] or biocontrol of potentially aflatoxigenic *Aspergillium* species, in particular *Aspergillus flavus*, by microbial antagonists [5]. Due to the high stability of aflatoxins, their chemical or physical degradation is carried out under stringent conditions [6, 7]. This often reduces the feed quality making such treatment economically unprofitable. The biological method of decontamination [8-10] is based on the use of secondary metabolites of some plants toxic to *A. flavus* [11], and also on the search for natural inhibitors of aflatoxinogenesis or microorganism that could serve as a source of enzymes destroying aflatoxins or transform them to non-hazardous derivatives [12-16].

For example, when some micromycetes colonizing natural substrates are grown together with the toxigenic isolates of *A. flavus*, aflatoxin B1 (AFB1), typ-

ical for this fungus, decomposes in the culture liquid (CL) [17]. The enzymatic nature of the detoxifying activity of CL in a such biodestructor, *Phoma glomerata* (strain PG41), has been confirmed [18].

Here we first investigated the ability of two other micromycetes, *Gliocladium roseum* and *Trichoderma viride*, previously isolated from the toxigenic *A. flavus* consortium, to destroy AFB1 in view to assess the prospects of their use as sources of the AFB1-catabolizing enzymes or as antagonists of the of AFB1 producer.

Technique. Destroying activity of GRZ7 (*Gliocladium roseum* Bainier) and TV35 (*Trichoderma viride* Pers.) strains from the collection of Laboratory of Pathophysiology (All-Russian Research Institute of Phytopathology) was studied in 7-day cultures grown in liquid Czapek's medium with casein hydrolizate (Czapek-CasH) at 28 °C and 200 rpm (Excella^{TM} E-25/25R, New Brunswick Scientific Co., Inc., USA). Prior to fungi inoculation, AFB1 (5-10 μg/ml, Sigma», USA) was added under sterile conditions. High molecular weight metabolites with nominally cut off molecular weight of > 5 kDa were isolated from CL of *G. roseum*, grown under the same conditions in the absence of toxin (intact CL), by precipitation of the filtrate with ammonium sulfate and ultrafiltration of aqueous solution of the precipitate. AFB1 was added to sterile samples of the high molecular weight fraction and the mixture was incubated at 27-28 °C for 3 days. Residual amounts of B1 in CL or in the sample of the fraction were determined using high performance liquid chromatography [18].

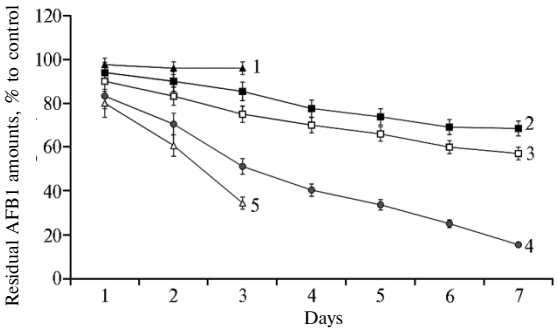
The effect of *T. viride* and *G. roseum* on the growth of toxigenic *A. flavus* (strain A11) was studied by the method of double cultures (co-cultivation on potato-glucose agar at 20-22 °C for 6 days). The minimum and maximum diameters were measured, and the area of the colonies was calculated based on the maximum diameter. Control cultures of strain A11 were grown under the same conditions in the absence of the putative antagonists.

Data were processed using Statistica 6.0 (StatSoft, USA). The significance of the differences between control and experimental values was confirmed by a *t*-test for independent variables ($p \leq 0.05$). The table and the figure show the mean (*M*) and standard error of the mean (*m*). Each experiment included at least 6 replicates with 3-fold reproduction.

Results. A study of the dynamics of destruction of aflatoxin by *G. roseum* showed that after 3 days of its growth on medium with AFB1 the content of the latter in CL was halved, and by the end of cultivation it did not exceed 10-20 % of the initial amount (Fig., curve 4). A significant decrease in the AFB1 concentration also occurred after its incubation in the fraction of high-molecular metabolites isolated from the filtrate of intact CL of G. roseum (see Fig., curve 5). Thermal treatment of the fraction led to the loss of toxin-degrading activity (see Fig., curve 1).

These results suggest that extracellular enzymes of this biodestructor may participate in the decomposition of AFB1, similar to that in *P. glomerata* [18] or other ascomycetes [12, 19]. Earlier, it was found that *G. roseum* produces zearalenone-specific lactonase [20] which hydrolyses the lactone ring in ZEN, the mycotoxin of fusarium fungi dangerous for mammalians, thereby reducing ZEN toxicity [21]. The inhibition of most micromycetes by ZEN significantly reduces the possibility of using antagonistic fungi against toxic fusariums, which affect plants, but *G. roseum* is not sensitive to ZEN [20]. Like ZEN, aflatoxin B1 molecule contains the lactone ring which is associated with its toxicity, mutagenic and carcinogenic effects [22-25]. Aflatoxin-destroying activity that we have discovered in *G. roseum*, makes it even more attractive as a bioagent against fungi

that produce mycotoxins of different chemical nature.



Aflatoxin B1 (AFB1) **biodegradation by the micromycetes** ***Gliocladium roseum*** **GRZ7** (4) **and** ***Trichoderma viride*** **TV35** (2, 3) **grown on the medium with aflatoxin, and intact** (5) **or heat inactivated** (1) **high molecular extracellular metabolites of** ***G. roseum.*** Controls: for 2, 3, 4 — the AFB1 concentration in a nutrient medium not inoculated with micromycetes; for 1, 5 — the AFB1 amount immediately after its adding to analyzed fraction of metabolites (without incubation). Differences between the values in treated and control samples are statistically significant at $P \leq 0.05$; the Y-errors bars show SEM for 3 experiments with 6 replicates per option in each.

The destroying activity of the studied *T. viride* strain was low. The utilization of AFB1 from the nutrient medium occurred slowly, and its concentration in the CL decreased not significantly as the fungus grew (see Fig., curve 2). However, the ability of the strain to destroy AFB1 in LC increased (curve 3) in case of using spore suspension of the fungus previously grown on the Czapek-CasH agar medium supplemented with AFB1 (0.9 μg/ml). When using such an inoculum, the proportion of the destructured toxin in the immersed culture increased by the end of the fermentation by 20 % ($p = 0.03$). Therefore, the AFB1-degrading activity which we detected in *T. viride* was inducible and could be increased by mutagenesis and selection of the most effective clones. Moreover, the tested *T. viride* strain proves to be an effective antagonist of *A. flavus*. When the toxigenic A11 strain was grown together with *T. viride*, the growth of the aflatoxin producer was significantly lower (Table) with a reduction in the colony size by 64 % on average.

Radial growth of toxigenic ***Aspergillus flavus*** **A11 colonies in single culture and under co-cultivation with** ***Trichoderma viride*** **TV35** (*Tv*) **or** ***Gliocladium roseum*** **GRZ7** (*Gr*) (*M*±*m*)

Variant of cultivation	Average colony diameter, cm		Average colony area, cm^2	Growth inhibition, % to control value	p
	min	max			
A11 (control)	6.90±0.03	7.10±0.00	39.50±0.00		
A11 + *Tv*	3.33±0.09	4.26±0.06	14.25±0.56	63.9	0.004
A11 + *Gr*	6.20±0.06	6.89±0.08	37.27±0.12	5.6	0.05

Mycoparasite *G. roseum* did not noticeabley influence the radial growth of *A. flavus* colonies as compared to the control, however, the differences were significant (Table), and in some cases the A11 strain colonies were overgrow with the *G. roseum* mycelium by the end of co-cultivation which could be a sign of hyperparasitism.

Thus, it has been found out that the studied strains of *Tricho-derma viride* and *Gliocladium roseum*, the antagonist species of many pathogenic fungi, competing with *Aspergillus flavus* for natural substrates, were different in their capacity to degrade the aflatoxin B1 in vitro and to inhibit the growth of aflatoxigenic *A. flavus strain*. Additionally, the inducibility of degrading activity, discovered by us, is of undoubted interest both in the theoretical and practical aspects. The obtained results can be used in the development of technologies for biological decontamination of feed contaminated with both aflatoxin B1 and its producer. In this case, *G. roseum*, apparently, can serve as a source of enzymes that decompose this mycotoxin, and *T. viride* can be a bioagent that inhibits the development of a producer.

REFERENCES

1. Bbosa G.S., Kitya D., Lubega A., Ogwal-Okeng J., Anokbonggo W.W., Kyegombe D.B. Review of the biological and health effects of aflatoxins on body organs and body systems. *In: Aflatoxins: recent advances and future prospects.* M. Razzaghi-Abyaneh (ed.). InTech, Rijeka, Croatia, 2013: 239-266 (doi: 10.5772/51201).
2. Filasi A., Sireli U.T. Occurrence of aflatoxins in food. *In: Aflatoxins: recent advances and future prospects.* M. Razzaghi-Abyaneh (ed.). InTech, Rijeka, Croatia, 2013: 143-170 (doi: 10.5772/51031).
3. Miller J.D., Schaafsma A.W., Bhatnagar D., Bondy G., Carbone I., Harris L.J., Harrison G., Munkvold G.P., Oswald I.P., Pestka J.J., Sharpe L., Sumarah M.W., Tittlemier S.A., Zhou T. Mycotoxins that affect the North American agrifood sector: state of the art and directions for the future. *World Mycotoxin Journal,* 2014, 7(1): 63-82.
4. Whitlow L.W. Evaluation of mycotoxin binders. *Proc. 4th Mid-Atlantic Nutrition Conference.* N.G. Zimmermann (ed.). College Park, University of Maryland, 2006: 132-143.
5. Dhramaputra O.S., Putri A.S.R., Retnovati I., Ambarwati S. Antagonistic effect of three fungal isolates to aflatoxin-producing *Aspergillus flavus. Biothropia*, 2003, 21: 19-31.
6. Raters M., Matissek R. Thermal stability of aflatoxin B1 and ochratoxin A. *Mycotoxin Research*, 2008, 24(3): 130-134 (doi: 10.1007/BF03032339).
7. Womack E.D., Brownab A.E., Sparksa D.L. A recent review of non-biological remediation of aflatoxin-contaminated crops. *J. Sci. Food Agric.*, 2014, 94(9): 1706-1714 (doi: 10.1002/jsfa.6520).
8. Wu Q., Jezkova A., Yuan Z., Pavlikova L., Dohnal V., Kuca K. Biological degradation of aflatoxins. *Drug Metab. Rev.*, 2009, 41(1): 1-7 (doi: 10.1080/03602530802563850).
9. Kabak B., Dobson A.D.W., Var I. Strategies to prevent mycotoxin contamination of food and animal feed: A review. *Critical Reviews in Food Science and Nutrition*, 2006, 46: 593-619 (doi: 10.1080/10408390500436185).
10. Mishra H.N., Das C. A review on biological control and metabolism of aflatoxin. *Critical Reviews in Food Science and Nutrition*, 2003, 43: 245-264 (doi: 10.1080/10408690390826518).
11. Shukla R., Kumar A., Prasad C.S., Srivastava B., Dubey N.K. Antimycotic and antiaflatoxigenic potency of *Adenocalymma alliaceum* Miers. on fungi causing biodeterioration of food commodities and raw herbal drugs. *Int. Biodeter. Biodegr.*, 2008, 62: 348-351 (doi: 10.1016/j.ibiod.2007.11.006).
12. Sardjono E., Rahayu S., Raharjo S., Rahayu K. Detoxification of aflatoxin B1 by extracellular enzymes of *Aspergillus oryzae* KKB4. *Indonesian Food and Nutrition Progress*, 2004, 11(1): 30-34.
13. Motomura M., Toyomasu T., Mizuno1 K., Shinozawa1 T. Purification and characterization of an aflatoxin degradation enzyme from *Pleurotus ostreatus. Microbiol. Res.*, 2003, 158: 237-242 (doi: 10.1078/0944-5013-00199).
14. Alberts J.F. *Microbial degradation of mycotoxins.* PhD Thesis. University of Stellenbosch, 2007.
15. Guan S., Ji C., Zhou T., Li J.X., Ma Q.G, Niu T.G. Aflatoxin B1 degradation by *Stenotrophomonas maltophilia* and other microbes selected using coumarin medium. *Int. J. Mol. Sci.*, 2008, 9: 1489-1503 (doi: 10.3390/ijms9081489).
16. Cao H., Liu D., Mo X., Xie C., Yao D. A fungal enzyme with the ability of aflatoxin B1 conversion: purification and ESI-MS/MS identification. *Microbiol. Res.*, 2011, 166: 475-483 (doi: 10.1016/j.micres.2010.09.002).
17. Zhemchuzhina N., Mikityuk O., Shcherbakova L., Campbell B., Dorofeeva L., Dzhavakhiya V. Isolation of aflatoxin B1-degrading fungi among the mycoflora coincidental with toxigenic *Aspergillus flavus. Proc. 14th Int. Congr. on Molecular Plant-Microbe Interactions.* Quebec City, 2009: 158.
18. Shcherbakova L., Statsyuk N., Mikityuk O., Nazarova T., Dzhavakhiya V. Aflatoxin B1-degrading activity by *Phoma glomerata* PG41 isolated from natural substrate colonized by aflatoxigenic *Aspergillus flavus. Jundishapur Journal of Microbiology*, 2015, 8(1): 1-5 (doi: 10.5812/jjm.24324).
19. Forootanfara H., Faramarzia M.A., Shahverdia A.R., Yazdia M.T. Purification and biochemical characterization of extracellular laccase from the ascomycete *Paraconiothyrium variabile. Bioresource Technol.*, 2011, 10(2): 1808-1814 (doi: 10.1016/j.biortech.2010.09.043).
20. Utermark J., Karlovsky P. Role of zearalenone lactonase in protection of *Gliocladium roseum* from fungitoxic effects of the mycotoxin zearalenone. *Appl. Environ. Microb.*, 2007, 73(2): 637-642 (doi: 10.1128/AEM.01440-06).
21. Utermark J., Karlovsky P. Detoxification of zearalenone by *Gliocladium roseum*: biological function and regulation of zearalenone lactonase synthesis. *Proc. 29th Mycotoxin Workshop.* Fellbach, 2007: 54.
22. Lee L.S., Dunn J.J., De Lucca A.J., Ciegler A. Role of lactone ring of aflatoxin B1

in toxicity and mutagenicity. *Experientia*, 1981, 37: 16-17 (doi: 10.1007/BF01965543).
23. Guan S., Zhou T., Ym Y., Xie M., Ruan Z., Young J.C. Microbial strategies to control aflatoxins in food and feed. *World Mycotoxin Journal*, 2011, 4: 413-424 (doi: 10.3920/WMJ2011.1290
24. Nicolás-Vázquez I., Méndez-Albores A., Moreno-Martínez E., Miranda R., Castro M. Role of lactone ring in structural, electronic, and reactivity properties of aflatoxin B1: a theoretical study. *Arch. Environ. Con. Tox.*, 2010, 59(3): 393-406 (doi: 10.1007/s00244-010-9501-x).
25. Iram W., Anjum T., Iqbal M., Ghaffar A., Abbas M. Mass spectrometric identification and toxicity assessment of degraded products of aflatoxin B1 and B2 by *Corymbia citriodora* aqueous extracts. *Scientific Reports*, 2015, 5: 14672 (doi: 10.1038/srep14672).

AGRICULTURAL BIOLOGY,
2016, V. 51, № 6, pp. 951-960
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.087.26:633.39:581.145

doi: 10.15389/agrobiology.2016.6.951rus
doi: 10.15389/agrobiology.2016.6.951eng

BIOLOGY OF FLOWERING, DIVERSITY OF SEXUAL TYPES AND SEED PRODUCTION IN *Salsola orientalis* S.G. Gmel.

E.Z. SHAMSUTDINOVA

W.R. Williams All-Russian Fodder Research Institute, Federal Agency of Scientific Organizations, korp. 1, ul. Nauchnii Gorodok, Lobnya, Moscow Province, 141055 Russia, e-mail daplant@mtu-net.ru
Received March 21, 2016

Abstract

Reproductive biology of many cultivated plants has been studied in detail whereas in newly introduced, especially arid fodder species, different aspects of flowering, pollination, reproduction, seed production, etc., should be understood. *Salsola orientalis* S.G. Gmel. is a dwarf semi shrub haloxerophyte plant vegetated for a long period (250-254 days), 35-55 cm in height, with the root system of the generic type that penetrates the soil to a depth of 5-8 m. The species is extremely drought-resistant and tolerant to salt stress so this is a valuable fodder pasture plant in the deserts of Central Asia, well eaten by sheep and camels, especially in autumn and winter. *Salsola orientalis* is a polymorphic species consisting of a plurality of intraspecific ecotypes, populations and biotypes which differ in environmental sustainability and fodder productivity. *S. orientalis* is considered promising for breeding due to intraspecific plant polymorphism on adaptiveness and productivity. Here we report the data on flowering, intraspecific diversity of sexual types in population, and seed reproductive function of *S. orientalis.* The seeds were collected in arid, geographically and environmentally distinct areas of Tajikistan, Kyrgyzstan, Uzbekistan, and sown in the Desert Karnabchul (Uzbekistan, Samarkand region, Karnab). Karnabchul is characterized by hot summers (+40-45 °C in June-July) and dry climate (annual precipitation of 180 mm with 100-250 mm fluctuations). The soil is grey-brown, middle-saline. This study was carried out in the nursery (each plot of 100 m^2 in size) by the method of A.N. Ponomarev. The number of functionally female flowers on each plant in all populations was estimated. The type of pollination was determined in experiments with isolation of individual plants and flowers. Embryonless seeds were found among the specimen collected in different eco-geographical areas and in three tested *S. orientalis* varieties (Pervenets Karnaba, Solnechnyi and Salang). The seed of these varieties were divided into three fractions by size (large, medium and small) and weight of 1000 seeds. We found that the flowers in *S. orientalis* were cross-wind-pollinated. Self-pollination does not occur, since by the time of stamen appearance in bisexual flowers the pistil turns brown and dry. *S. orientalis* flowering begins in June and lasts until mid-July, the hottest season. However, the disclosure and the dusting of the flowers occur in the cooler morning hours (at a temperature of 25-26 °C and relative humidity of 26-65 %). In *S. orientalis*, self-incompatibility to avoid self-fertilization is not limited to dichogamy (i.e., non-simultaneous maturation of male and female generative structures of hermaphrodite individuals), and also combined with other adaptations to cross-pollination. These are the presence of female flowers and intermediate flowers with long stigma lobes of the pistil, and emergence of the plants with only functionally female flowers. The cross-pollination in *S. orientalis* is provided by both genetic incompatibility and structure of the flower. We attributed *S. orientalis* species to trioecious plants because i) only staminate flowers, ii) hermaphrodite and pistillate flowers, and iii) only pistillate flowers were characteristic of the individuals in the population. A wide variation on embryoless seed ratio (from 0-2 % to 98-100 %) was found in *S. orientalis.* Hence, selection makes it possible to increase the number of plant with low embryoless seed ratio in the population. Similar result can be achieved when using the parental forms with low percentage of embryoless seeds in breeding to obtain population with more frequent female forms. In both cases, you can get perspective parental forms for breeding varieties with low percentage of embryoless seeds and increased seed germination. Our findings are essential for development of *S. orientalis* breeding and seed production in the arid zones of Central Asia and Russia.

Keywords: *Salsola orientalis*, flowering, sex type, intrapopulation diversity, pollination type

The Russian scientists have notably contributed to plant reproductive biology [1-3]. Special attention was paid to embryogeny [4], polyembryony [5], genetic heterogeneity of seeds [6, 7], embryo autonomy [8], embryological bases of androclinic haploidy [9, 10]. These fundamental knowledges were the bases

for effective technologies used to create wheat, barley, and sunflower varieties and lines [11-13], and to reproduce valuable genotypes of rare plant species for preserving their biodiversity and extending resource reproduction [14-16]. Further studies concerned the origin of different types of embryonic sacs and ovules, fertilization processes, the peculiar morphogenetic structures, seed development, and the role of heterospermia in seed reproduction [17-19]. The data elucidated the role of position control in the specialization of female gametophyte cells [20, 21], their capacity for polyembryony, and the cytoembryological mechanisms of interspecific and interlinear hybridization in economically important species of cultivated plants [22, 23]. The achievements in reproductive biology have become the theoretical basis for effective (innovative) methods in breeding [23, 24], reproduction of biological resources [25], biodiversity conservation [26] and the biotechnologies used to massively reproduce valuable genotypes [27, 28].

Though the reproductive biology of many cultivated plants has been quite well studied, in introduced species, especially arid fodder plants, bloom biology, types of pollination, seed reproduction, and other related aspects have hardly been investigated [29]. Eastern salsola (*Salsola orientalis* S.G. Gmel.), a haloxerophytic semi-shrub which is widely used to restore the forage productivity of degraded pastures in the arid regions of Central Asia and Russia, is among these species [29]. Eastern salsola is a perennial plant with a root system of a universal type deeply penetrating into the soil, which possesses exceptionally high drought tolerance and tolerance to salt stress, serves as a valuable fodder plant on the arid pastures of Central Asia, is well eaten by sheep and camels, especially in autumn and winter seasons [29].

Eastern salsola is a polymorphous species consisting of a multitude of intraspecific ecotypes, populations and biotypes [29]. Its ecotypes and population forms that appeared during evolution in ecologically distinct climatic and edaphic conditions of arid zones are endowed with different adaptive and productive potentials. Such ecotype and population diversity on ecological and economically valuable traits predetermines the possibility of its improvement toward more productive drought and salt tolerant varieties.

Domestic and foreign experience of breeding forage plants indicates the success to be largely depending on the knowledge of reproductive biology. In this paper we first experimentally established that the Eastern salsola is a cross-wind-pollinated trioecious species. Cross-pollination is ensured by the system of genetic incompatibility and a peculiar structure of the flower. The daily and seasonal rhythms of flowering were studied under extremely harsh xerothermal conditions of the arid climate of the Central Asia, with the stages of flowers' opening and dusting described. Manifestation of the absence of corcule and its dependence on the conditions of the year were established. Based on that, we revealed a wide variability of the seeds on this trait and proposed a method for selecting plants in which the absence of corcule is low and the rate of seed germination is high.

Our aim was the study of daily and seasonal flowering rhythms, as well as estimation of the intraspecific population diversity on sexual types and seed reproduction in Eastern salsola plants in view of its breeding and seed production in arid regions of Central Asia and Russia.

Technique. Eastern salsola seeds which differed in ecological and geographical origin were collected in arid regions of Tajikistan, Kyrgyzstan, Uzbekistan, and then sown under the conditions of Karnabchul Desert, Uzbekistan, Samarkand region, Karnab) in 1998-1999. Plant reproductive biology was studied in the collection nursery as described by A.N. Ponomarev [30]. A plot size was 100 m^2 [30].

Since June, the flower structure was studied, and the dynamics of flower

opening was estimated under field conditions daily from 6.00 a.m. to 8.00 p.m. with 15-20 minute interval using loops of different magnification. Simultaneously, the temperature and the relative air humidity were recorded. Inflorescences (25 ones in each of 4 repetitions) and all the flowers in the lower and upper parts of the inflorescence were measured in length and width with a millimeter tape. The number of flowers per day was counted on 3 medium-sized plants of each ecotype. When studying the seasonal flowering rhythm, the number of flowers was counted daily from the beginning of flowering to the last flower appearance 4 times a day (i.e. twice from 6.00 a.m. to 12.00 noon and twice from 1.00 p.m. to 8.00 p.m.). Flowers of different sexual types were counted on all the plants of each ecotype during the entire flowering period.

The yield of normal seeds was estimated by the number of functionally female flowers on each plant in all the populations. First all the flowers, then only the female ones were counted upward along the longest second-order generative shoot. The portion of male and bisexual plants with functionally female flowers was calculated.

When studying pollination type, the whole and half of the plant were enclosed in a frame, covered on all sides by unbleached calico, and the inflorescences were isolated in parchment sacs. After maturing, the seeds were counted. To determine the rate of germless seeds in each population, the seeds of the longest generative shoot cut from each plant were crumbled into an individual paper sachet specifying the sample and plant numbers, and four samples of 100 seeds from each sachet were placed into separate bags. The bags were left in water for a day, then removed and dried. Embryos were squeezed out on the glass by light compression. The germless seed percentage was calculated for 100 seeds. In the study of germless seeds in the varieties Pervenets Karnaba and Senokosniy (All-Union Karakul Research Institute, Samarkand) and Salang (W.R. Williams All-Russian Fodder Research Institute), seeds were divided by size into the fractions (large, medium and small), and 1000 seeds of each fraction were weighted. Dependence of the germless seed occurrence on the variety genotype and plant age was studied in the collection nursery laid in 1990.

Statistical processing was carried out by dispersion and correlation analyses [31].

Results. The climate in the region is continental, with hot and dry summers and cold winters. Soils are gray-brown, sandy loamy, medium saline of chloride-sulfate type. The average annual air temperature is +16 °C, in June-July in the shade it reaches +40-45 °C, in January it drops to –18 ...–20 °C. The annual atmospheric precipitation amounts 180 mm with fluctuations of 160-250 mm in years. The origin of the samples is also shown in Table 1.

1. Samples (ecotypes) of Eastern salsola (*Salsola orientalis* S.G. Gmel.) used in the experiments, and growing conditions

Accession number	Origin	Soils	Average annual precipitation, mm
k-9	Tajikistan, Leninabad region, settlement Farab, foothill semi-desert	Light gray, loamy, slightly saline soils	290
k-10	Tajikistan, the town of Kanibadam, settlement Kutkan, foothill semi-desert	Gray, loamy, slightly saline soils of sulfate-chloride type	300
k-11	Tajikistan, the suburbs of Kurgan-Tube (point 1), foothill semi-desert	Light gray, loamy, medium saline soils	280
k-12	Tajikistan, the suburbs of Kurgan-Tube (point 2), foothill semi-desert	Light gray soils, sandy loamy, slightly saline soils	280
k-13	Kyrgyzstan, the suburbs of Tash-Kumyr, foothill plains	Light gray, loamy-gravelly soils	270
k-14	Uzbekistan, Namangan region, Chust district, the foothill semi-desert	Light gray loamy soils	290
k-15	Tajikistan, Aini region, settlement Asht, foothill plains	Light gray, loamy, slightly saline soils	230

Table 1 (Continued)

k-16	Kyrgyzstan, the town of Talas, settlement Bulak, foothill semi-desert	Middle gray loamy soils	310
k-17	Kyrgyzstan, Osh region, settlement Haydarkan, foothill semi-desert	Typical gray, loamy, slightly saline soils	320
k-18	Tajikistan, the suburbs of the town of Isfara, settlement Gorka, foothill plains	Light gray loamy, medium saline soils	285

Flowers of the Eastern salsola form paniculate inflorescences, one by one, are bisexual, sessile, with a spiral arrangement. A pistil appears a little bit earlier then the stamens. The calyx consists of five membranous light-green sepals, folded like in corn cob, partly overlapping one another, and having hairs. Calyx is not opening, has five free stamens, located around the base of the pistil. When the stamens appear, the anthers are considerably removed from the pistil. Stamens are the same in size. Stamen filaments are white in color, and round in cross section. Anthers are yellow, cream and pink. There is one pistil (without a post) per flower. Stigma is bilobate with a lot of papillae. Ovary has the upper location. When flowering, the calyx remains closed, resembling a cone in the neck of which the stamen filaments are grouped. In windless weather, the pollen fell on the stigma of its own pistil. On the pistil of not blossoming buds, one could see numerous papillae with pollen grains. Therefore, the pistil, even before the release of the stamens in its flower, possessed a sufficient amount of pollen from the flowers of neighboring plants.

The flowering of the Eastern salsola in the Karnabchul desert began in May and lasted until mid-July, in the hottest period when the air temperature rose to 40-41 °C, and the relative humidity dropped to 6-7 %. However, the opening and dusting of flowers occurred in the morning, at an air temperature of 25-26 °C and a relative humidity of 26-65 %. On the growing generative branches, along with acropeatel flowering, new buds continued to appear. In October, mature winged seeds with a developed spiral-twisted embryo appeared. The peak flowering was from the end of June to July. The flowers were protoginic, and flowering proceeded as follows. First, a bilobate, strongly pubescent stigma was seen from the perianth. On days 2 to 3 from 6.30 to 7.30 a.m., stamen filaments began to grow rapidly, carrying brightly colored anthers out of perianth. Flowering proceeded at an increasing rate, with a maximum opening of flowers at 8.30-9.30 a.m., and ended with the appearance of single flowers by 12.30 to 1.00 p.m. The extension of anthers and dusting occurred 40-50 minutes after the opening of floral scales. Each anther burst with a longitudinal crack and began to dust. The dusting was copious and fleeting, i.e. 10-15 minutes on warm days, 30-40 minutes in cloudy ones. The flower remained open for 5-6 hours.

We distinguished the following subphases in the flowering of Eastern salsola: the appearance of the stigma of the pistil (female subphase) (2-3 days); stamens dusting (1.0-1.5 hours); the end of flowering, the flower closing (4-7 hours). On the shoots there were flowers in different stages of flowering: some just exposed stamens, some began to dust, some intensively dusted, some were completely free from pollen, and some were closed (returned to their original state) and grouped pollen-free brown stamens in its center. Thanks to this character of blossoming, a more effective pollination and, consequently, fertilization can be achieved.

Based on flower structure and the presence of plants with a complete absence of bisexual flowers, the Eastern salsola can be referred to wind-pollinated species with cross pollination. Self-pollination did not occur, because by the time the stamens were released from the bisexual flowers, their pistil has turned brown and dry. It is known that in angiosperms the homomorphic incompatibil-

ity of species is most common, when populations are homogeneous on the flower characteristics. Homomorphic incompatibility is reported in such large groups as the family *Compositae*, *Cruciferae*, *Gramineae*, *Leguminosae*, *Rosaceae* and *Solanaceae*; in the genus *Salsola* (family *Chenopodiaceae*) the homomorphic incompatibility has also been found [1, 4].

The experiments in which we isolated the whole plant or a half of the plant indicated cross-pollination in the Eastern salsola, as the seeds were not produced in both variants (Table 2).

2. Seed formation in Eastern salsola (*Salsola orientalis* S.G. Gmel.) plants cultivated under the conditions of wormwood-ephemeral of Karnabchul Desert (*M*±*m*, Uzbekistan, Samarkand region, arid year with average annual precipitation of 130 mm)

Variant	Bud number	Normal seed number
	No isolation	
Whole bush	6.60±0.60/100	5.40±0.49/100
Half of bush	4.37±0.50/100	3.80±0.34/86.9
	Under isolation	
Whole bush	8.90±1.40/100	0
Half of bush	2.13±0.29/100	0

Note. The absolute values (pcs), and relative values (%) are shown before and after the slash, respectively.

Most studies of plant reproduction are focused on self-incompatibility as the key point in the genetic control of reproduction in cross-pollinated species. However, pollination mode, the life cycle of a flower, its sexual type and structure are of no less importance in providing cross-pollination and reliable function of incompatibility systems in wind-pollinated species. Obviously, only combination of all these features makes the base for cross-pollination and, as a consequence, provides complex heterozygosity in the population [1, 12, 18].

It is known that in some arid fodder plants [29], e.g. *Kochia prostrata* (L.) Schrad. and *Salsola orientalis*, the self-incompatibility which prevents self-fertilization, is not limited to dichogamy (non-simultaneous maturation of male and female generative structures), but is combined with other adaptations to cross pollination, i.e. the presence of female and transitional forms of flowers with long lobes of the pistil stigma.

At flowering, in the Eastern salsola female plants, unlike hermaphroditous ones, the calyces were intensively green and did not open. In case of plant bisexuality, the bisexual flowers were light yellow and orange in the morning, and only after a complete pollination, when the sepals closed, they again turned greenish. These differences made it easy to detect sterile plants. In addition, the flower of a sterile plant was more like a bud having a bilobate pistil which was 1.0-1.5 times larger than the pistil of a fertile plant. In sterile forms, anthers inside the calyx did not crack and remained unclosed until the seeds ripened. Pollen coloration was from light yellow to white. The anthers of sterile plants were smaller in size and shrunken, the pollen was not spherical in shape and less in diameter as compared to fertile pollen (7-9 mm vs. 29-31 mm). Thus, a distyly may be assumed in some Eastern salsola populations, when there are plants of the first type with short stamen filaments and very elongated lobes of the pistil stigma, and those of the second type with relatively high stamen filaments, large anthers and short stigma lobes. According to available data [20, 32], heterostyly in other flowering plant species is related to dioeciousness. The short-pistil plants with short stigma lobes and the long-pistil plants (or those with oblong lobes) are analogs of male and female forms, respectively.

A significant increase in the number of pistillate flowers of the Eastern salsola was observed in a wetter year (Table 3). The lack of moisture inhibited

the formation and development of female flowers and led to the predominance of male flowers. Optimum hydration, on the contrary, contributed to an increase in the number of female flowers and a change in the flower proportion on the plant in their favor. This led to a significant increase in the seed production per plant and, consequently, in the total seed yield.

3. Percentage of functionally female flowers in the populations of Eastern salsola (*Salsola orientalis* S.G. Gmel.) of different ecogeographic origin in contrasting years (collection nursery, Uzbekistan, Samarkand region)

Accession number	Plant density, psc per 100 m^2	Year 1 (arid, average annual precipitation of 130 mm)		Year 2 (humid, average annual precipitation of 283 mm)	
		on average	limits	on average	limits
k-9	18	28	15-50	36	20-55
k-10	82	36	8-65	44	26-58
k-11	82	41	10-63	49	12-90
k-12	14	24	10-48	34	19-60
k-13	13	24	13-41	32	20-60
k-14	58	47	19-71	55	32-86
к-15	26	36	15-63	47	36-70
k-16	66	47	9-82	53	20-98
k-17	56	43	15-72	52	30-89
k-18	30	40	13-73	44	28-64

As cultivars are usually more homogeneous in morphological and eco-biological characteristics, we studied the occurrence of germless seeds in three varieties of Eastern salsola. The seed size in the Pervenets Karnaba plants ranged from 6.2 (large) to 3.8 mm (small), and the 1000 seed weight was 7.4-5.4 g. A similar relationship between the seed size and weight was observed in the Seno-kosniy and Salang varieties. Fractions of middle-size seeds with intermediate 1000 seed weight value had a greater field germination rate as compared to large- and small-size seed fractions (Table 4). Mainly, this is due to the fact that in large-size fractions low-quality seeds were quite frequent, while among the relatively small seeds one could find those of good quality.

4. Seed germination in Eastern salsola (*Salsola orientalis* S.G. Gmel.) varieties, as influenced by seed size (collection nursery, Uzbekistan, Samarkand region, in the arid year with an average annual precipitation of 130 mm)

Variety	Fraction	Seed size, mm	1000 seed weight, g	Field seed germination, %
Pervenets Karnaba	Large	6.2	7.4	13.5
	Middle	5.3	6.2	20.0
	Small	3.8	5.4	4.3
Senokosniy	Large	5.7	7.0	11.7
	Middle	4.6	6.6	13.5
	Small	3.8	5.0	6.8
Salang	Large	6.5	8.4	14.5
	Middle	5.8	7.2	15.8
	Small	4.0	6.4	7.0

One of the causes influencing relationship between seed size and weight, on one side, and their germless character, on the other side, could be the seeds insufficiently filled during growing and have a shrunken or shriveled appearance. This feature of seeds, despite the importance, has been little studied due to the lack of fairly simple and reliable tests.

In our experiments, the mean rate of germless seeds in the wild East-ern salsola plants varied in two years from 48 to 76 % and from 46 to 64 % (Table 5). In the humid year, the percentage in all the samples was 2-19 % lower than in the arid year, at that, the plants could be found in which all the seeds contained germs, while in some other plants all the seeds were germless. Plants with zero level of germless seeds belonged to k-10, k-11 and k-18 populations (Tajikistan), k-14 population (Uzbekistan) and k-13, k-17 populations (Kyrgyzstan).

5. Percentage of germless seeds in Eastern salsola (*Salsola orientalis* S.G. Gmel.) plants of different ecogeographic origin in contrasting years (collection nursery, Uzbekistan, Samarkand region)

Accession number	Plant density, psc per 100 m^2	Year 1 (arid, average annual precipitation of 130 mm)		Year 2 (humid, average annual precipitation of 283 mm)	
		on average	limits	on average	limits
k-9	18	66	52-85	64	19-96
k-10	82	64	17-95	52	0-100
k-11	82	69	27-90	51	10-90
k-12	14	76	57-90	57	26-80
k-13	13	48	49-77	62	26-88
k-14	58	56	29-90	48	4-98
k-15	26	69	29-75	55	34-74
k-16	66	56	18-91	52	8-98
k-17	56	57	28-98	46	0-100
k-18	30	54	27-87	50	18-84

6. Seed yield (SY, g) **and germless seed rate** (GLR, %) **in Eastern salsola (*Salsola orientalis* S.G. Gmel.) varieties during plant ageing** (collection nursery, Uzbekistan, Samarkand region)

Variety	Plant age								LSD_{05}
	1 year		2 years		3 years		8 years		
	SY	GLR	SY	GLR	SY	GLR	SY	GLR	
Senokosniy	4.7	41.9	13.7	49.5	48.7	52.0	154.7	57.8	3.9
Salang	3.8	40.9	19.3	45.0	40.7	52.3	136.5	62.2	2.5
Pervenets Karnaba	5.5	46.9	–	–	49.7	47.7	144.7	65.5	3.6

N o t e. Dashes mean no data.

With the age of plants, the germless seed rate elevated from 41.9 to 65.5 %, and the differences between extreme values in most varieties were significant (Table 6). The lowest value was found in Senokosniy variety, with 41.9 % germless seeds in 1-year old plants and 57.8 % germless seeds 8-year old plants. Also, individual plant seed productivity in all the varieties increased constantly, depending on age (see Table 6). In 1-year old plants, it ranged from 3.8 to 5.5 g per plant, and increased 26-fold and 35-fold in 8-year old plants of Pervenets Karnaba and Salang variety, respectively. An inverse relationship was found out between the seed productivity and the presence of embryo in seeds. With the rise of seed productivity as plants grew older, the germless fraction of their seeds increased.

Thus, Eastern salsola (*Salsola orientalis* S.G. Gmel.) plants are cross-wind-pollinated. Self-pollination is not observed, since by the time the stamens are released from bisexual flowers, the pistil becomes dry. Blossoming of Eastern salsola begins in May and lasts until mid-July in the hottest period, however, the flower opening and dusting occur in the morning hours at a temperature of 25-26 °C and relative humidity of 26-65 %. Self-incompatibility, which prevents self-fertilization, is provided by dichogamy, and the presence of female and transitional flowers, as well as the plants with only functionally female flowers. Reliable cross-pollination is due to both incompatibility systems and the peculiar features of flowers. The detection of female plants among the Eastern salsola populations allows us to classify this species as trioecious, since in the population of this semi-shrub plants there are individuals with only staminate flowers, with hermaphroditic and pistillate flowers, and with the pistillate flowers only. The average amount of germless seeds varies from 48 to 76 %. Variability in this feature (from 0-2 % to 98-100 %) makes a wide background for section of plants with very low percent of germless seeds to increase their number in the population. A similar result can be achieved by crossing plants with low germless seed proportion to obtain populations enriched with female forms. In both cases, it is possible to create promising parental plants for breeding varieties low in germless

seed percent and high in laboratory and field germination rate

REFERENCES

1. *Embryology of flowering plants. Terminology and concepts. V. 3. Reproductive systems.* T.B. Batygina (ed.). USA, Enfield (NH), 2009.
2. L'vova I.N., Murashev V.V. *Vestnik Moskovskogo universiteta. Seriya 16: Biologiya*, 2005, 3: 38-43 (in Russ.).
3. Anisimova I.N., Gavrilova V.A., Timofeeva G.I. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Sovremennye problemy nauchnogo obespecheniya proizvodstva podsolnechnika», posvyashchennoi 120-letiyu so dnya rozhdeniya akademika V.S. Pustovoita.* [Proc. Int. Conf. «Current scientific aspects of sunflower production»]. Krasnodar, 2006: 162-174 (in Russ.).
4. Bragina E.A., Batygina T.B., Looks Yu.A. Reproduction system and repatriation in orchids. *Proc. I Int. School for young scientists «Embryology and biotechnology»*. St. Petersburg, 2005: 33.
5. Batygina T.B. Sexyal and asexsual processes in reproductive systems of flowering plants. *Acta Biologica Cracoviensia*. Ser. Bot., 2006, 47(1): 51-60.
6. Batygina T.B., Vinogradova G.Yu. *Ontogenez*, 2007, 38(3): 1-26 (in Russ.).
7. Voronova O.N., Gavrilova V.A. *Botanicheskii zhurnal*, 2007, 92(10): 1535-1544 (in Russ.).
8. Harrison C.J., Alvey E., Henderson I.R Meiosis in flowering plants and other green organisms. *Journal of Experimental Botany*, 2010, 61(11): 2863-2875 (doi: 10.1093/jxb/erq191).
9. Anisimova G.M., Shamrov I.I., Yakovleva O.V. *Botanicheskii zhurnal*, 2005, 90(10): 1499-1516 (in Russ.).
10. Kruglova N.N., Batygina T.B., Gorbunova V.Yu., Titova G.E., Sel'dimirova O.A. *Embriologicheskie osnovy androklinii pshenitsy. Atlas* /Pod redaktsiei I.I. SHamrova [Embryological bases of wheat androclinia: Atlas. I.I. Shamrov (ed.)]. Moscow, 2005.
11. Sel'dimirova O.A., Titova G.E., Galin I.R., Kruglova N.N. *Izvestiya Samarskogo NTS RAN*, 2013, 15(3): 1676-1679 (in Russ.).
12. Batygina T.B., Titova G.E., Vasil'eva V.E. *Innovatsii*, 2007, 2: 39-46 (in Russ.).
13. Vinogradova G.Yu. *Botanicheskii zhurnal*, 2013, 98(8): 959-974.
14. Babro A.A., Anisimova G.M., Shamrov I.I. *Rastitel'nye resursy*, 2007, 43(4): 1-13 (in Russ.).
15. Torshilova A.A., Titova G.E., Batygina T.B. Female reproductive structures and seed development in *Dioscorea nipponica* Makino (*Dioscoreaceae*). *Acta Biologica Cracoviensia. Ser. Bot.*, 2003, 45(1): 149-154.
16. Titova G.E. *Botanicheskii zhurnal*, 2012, 97(9): 1-26 (in Russ.).
17. Vinogradova G.Yu. *Materialy Mezhdunarodnoi konferentsii «Biotekhnologiya kak instrument sokhraneniya bioraznoobraziya rastitel'nogo mira»* [Proc. Int. Conf. «Biotechnology as a tool in saving flora biodiverdity»]. Simferopol, 2014: 264-265 (in Russ.).
18. Voronova O.N. Integumentary embryony in CMS sunflower line. *Russ. J. Dev. Biol.*, 2010, 41(6): 394-399 (doi: 10.1134/S106236041006007X).
19. Voronova O.N. Developmental of female reproductive structures and apomixes in some CMS lines of sunflower. *Helia*, 2013, 6: 47-60 (doi: 10.2298/HEL1358047V).
20. Batygina T.B., Osadtchiy J.V. Polyembryony: Twins — the result of vegetative propagation. *The International Journal of Plant Reproductive Biology*, 2013, 5(1): 21-27.
21. Brundrett M.C. Mycorrhizal associations and other means of diversity of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant Soil*, 2009, 320(1-2): 37-77 (doi: 10.1007/s11104-008-9877-9).
22. Munafo Jr. J.P., Gianfagna T.J. Quantitative analysis of steroidal glycosides in different organsof Easter lily (*Lilium longiflorum* Thunb.) by LC-MS/MS. *J. Agr. Food Chem.*, 2011, 59(3): 995-1004 (doi: 10.1021/jf1036454).
23. Essl F., Biry K., Brandes D., Broennimann O., Bullock J.M., Chapman D.S., Chauvel B., Dullinger S., Fumanal B., Guisan A., Karrer G., Kazinczi G., Kueffer C., Laitung B., Lavoie C., Leitner M., Mang T., Moser D., Müller-Schärer H., Petitpierre B., Richter R., Schaffner U., Smith M., Starfinger U., Vautard R., Vogl G., Lippe M., Follak S. Biological flora of the British isles: *Ambrosia artemisiifolia. J. Ecol.*, 2015, 103(4): 1069-1098 (doi: 10.1111/1365-2745.12424).
24. Habben J.E., Bao X., Bate N.J., Debruin J.L., Dolan D., Hasegawa D., Helentjaris T.G., Lafitte R.H., Lovan N., Mo H., Reimann K., Schussler J.R. Transgenic alteration of ethylene biosynthesis increases grain yield in maize under field drought-stress conditions. *Plant Biotechnol. J.*, 2014, 12(6): 685-693 (doi: 10.1111/pbi.12172).
25. Van Dijk H., Hautekиete N.C. Evidence of genetic change in the flowering phenology of sea beets along a latitudinal cline within two decades. *J. Evolution. Biol.*, 2014, 27(8): 1572-1581 (doi: 10.1111/jeb.12410).
26. Dem'yanova E.I. *O postanovke antiekologicheskikh issledovanii v usloviyakh introduktsii.*

Mat. Mezhd. nauch. konf. «Biologicheskoe raznoobrazie. Introduktsiya rastenii» [Proc. Int., Conf. «Biodiversity. Plant introduction»]. St. Petersburg, 2011: 211-213 (in Russ.).
27. Russell S.D., Gou X., Wei X., Yuan T. Male gamete biology in flowering plants. *Biochemical Society Transactions*, 2010, 38(2): 598-603 (doi: 10.1042/BST0380598).
28. Barrett S.C.H. Understanding plant reproductive diversity. Philosophical Transactions of the Royal Society of London. *Series B: Biological Sciences*, 2010, 365(1537): 99-109 (doi: 10.1098/rstb.2009.0199).
29. Shamsutdinov Z.Sh., Kosolapov V.M., Savchenko I.V., Shamsutdinov N.Z. *Ekologicheskaya restavratsiya pastbishch (na osnove novykh sortov kormovykh galofitov)* [Eco-restoration of pastures using new vrieties of fodder halophytes]. Moscow, 2009 (in Russ.).
30. Ponomarev A.N. V knige: *Polevaya geobotanika,* tom 2 /Pod redaktsiei E.M. Lavrenko, A.A. Korchagina [In: Field geobotany. E.M. Lavrenko, A.A. Korchagin (eds.)]. Moscow-Leningrad, 1960, V. 2: 9-19 (in Russ.).
31. Dem'yanova E.I. *Vestnik Permskogo universiteta. Seriya: Biologiya*, 2014, 2: 10-17 (in Russ.).
32. Dospekhov B.A. *Metodika polevogo opyta (s osnovami statisticheskoi obrabotki rezul'tatov issledovanii)* [Methods of field trials (with statistical analysis)]. Moscow, 1985 (in Russ.).