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PRODUCTION OF AVERMECTINS: BIOTECHNOLOGIES AND ORGANIC SYNTHESIS

(review)

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Abstract

The proposed review analyzes the results of research on various aspects of improving the technology of obtaining avermectins, the 16-membered macrocyclic lactones which have a wide spectrum of antiparasitic action with a high therapeutic index and harmlessness for mammals (W.C. Campbell, 2012). According to published data, the unique ability of avermectins to suppress the development of insects, nematodes and ticks is associated with the ability to block the transmission of nerve impulses in the neuromuscular synapse. The essence of this mechanism of action, leading to paralysis and death of parasites, is to stimulate the release of chlorine ions, depolarization of the cell membrane and pathological disorders of its functions (A.J. Wolstenholme et al., 2016). Of the known 8 components (A1a, A1b, A2a, A2b, B1a, B1b, B2a and B2b) of the avermectin complex produced by the microorganism *Streptomyces avermitilis*, the avermectin B1 is the most active against parasite pathogens (S. Omura, 2002; W.C. Campbell, 2012). Therefore, the main studies on the production of avermectins are associated with the selection of highly productive strains which predominantly synthesize avermectins B1 (S.S. Ki et al., 2005; H. Gao et al., 2010; W. Liu et al., 2015; L. Meng et al., 2016), and the preparation of semi-synthetic analogs of avermectins B1 with improved physicochemical and pharmacological properties (J. Vercruysse et al., 2001; A. Awasthi et al., 2012). Attempts to develop a technology for the complete chemical synthesis of avermectins have not yet yielded significant results due to the low yield of the target product and the complexity of the synthesis scheme (S. Yamashita et al., 2016). Considerable attention has been paid to the biochemical aspects of the diversity of 16-membered macrocyclic lactones and their producers, as well as to semisynthetic analogues, and prospects for searching for new highly efficient and environmentally friendly semisynthetic analogues of avermectin B1 have been defined. Main streams of researches on genetics, biochemistry and physiology of the producer of avermectins, ways of regulated culture of *S. avermitilis* strains and biosynthesis of required components of avermectin complex are discussed (S. Kitani et al., 2009; J. Guo et al., 2018). The data on the problem of emerging resistance in some species of parasites to long-used avermectin-containing drugs are analyzed. This phenomenon is shown to have a multifactor nature, including mutation of genes determining GluCl subunits and increased P-glycoprotein expression (J.H. Gill et al, 1998; R.K.Prichard, 2007; F.D. Guerrero et al., 2012; P.C. Pohl et al., 2014; P. Godoy et al, 2016). For the successful control of nematodes, insects and mites of agricultural, sanitary and medical importance, it seems appropriate to create drugs based on natural avermectins and their new semi-synthetic derivatives, for example, 5-O-succinylavermectin B1 and C2017 compounds.

Keywords: avermectins, milbemycins, nemadectins, doramectin, abamectin, moxidectin, ivermectin, moxidectin, milbemycin oxime, 5-O-succinylavermectin B1, compound C2017, avermectin oximes, *Streptomyces avermitilis*, organic synthesis, antiparasitic drugs, nematicides, insectoacaricides

Avermectins (16-membered macrolides produced by *Streptomyces avermitilis*) [1, 2] have extensive nematicide and insectoacaricide effects; for over 35

years, they have been effectively used to treat and prevent parasitic diseases in humans, animals, and plants [3-7]. Annual sales of avermectins exceed $850 million [8, 9]. The integral antiparasitic effect of this class of drugs is pertaining to their ability to affect glutamate-dependent (the main target) Cl^--ion channels specific to invertebrates [10], as well as $GABA_A$ (γ-aminobutyric acid)-dependent receptors [11]. Besides, avermectins have an affinity to various ion channels and receptors of the Cys-loop superfamily, P2X4 and farnesoid receptors, G protein-coupled inwardly rectifying potassium channels, GIRK receptors) and sundry channels, making this class pharmacologically promising [12, 13]. Ivermectin, which is an avermectin, has been found capable of blocking PAK1-dependent growth of benign and malignant tumor cells [14, 15]. Antitumor effects have been described for other 16-membered antiparasitic macrolides, too [16-19]. Ivermectin has recently been found to inhibit the replication of yellow fever virus [20] and the sporogony of *Plasmodium falciparum* in *Anopheles gambiae* [21]; avermectins have been found to curb tuberculosis [22] and to reduce the cellular absorption of ethanol [23]; ivermectin has demonstrated a curative effect in experimental pathologies, e.g. remyelination in autoimmune encephalitis resulting from allosteric activation and restoration of the disordered functions of ATP-dependent (purinergic) P2X4Rs ionophore receptors [24-27].

This review mainly covers the methods for the production of natural avermectins and their semisynthetic derivatives.

Avermectin synthesis technologies conventionally [2] imply obtaining highly productive strains that preferably synthesize B1 avermectins; the nutrient media must be optimized to grow the producer; the final step is to produce semisynthetic analogs of avermectins B1 with improved physicochemical and pharmacological properties [28-30]. In recent years, there has emerged another area of focus, which is to use synthetic biology methods to synthesize the required products, e.g. ivermectin or milbemycins [31-34]. In the 1980s and 1990s, researchers attempted a fully chemical synthesis of some avermectins, B1a and A1a [35]; however, they proposed multistage technologies that had limited output of 0.08% at max, making the microbiological method clearly advantageous. Research into more efficient fully chemical synthesis of avermectins is a work in progress [36-39].

Selection of producers, microbiological synthesis, and biotechnologies. The main focus in upgrading the producer of avermectins, *Streptomyces avermitilis* (ex Burg et al. 1979) Kim and Goodfellow, 2002 [40] is to obtain productive strains generating the avermectin complex or one of its components, mainly B1, while suppressing the synthesis of oligomycins that adversely affect the growth and development of the producer. State-of-the-art industrial strains genealogically trace back to wild-type *S. avermitilis* MA-4680 (strains NRRL 8165; NCIMB 12804; http://gcm.wfcc.info), which is a Japanese soil isolate that has deworming effects. The strain is deposited in the microbiological collections of many countries, although labeled differently (ATCC 31267, VKM Ac-1301, etc.) [41]. VKM Ac-1301 from the All-Russian Collection of Microorganisms is the ancestor of all Russian avermectin producers (http://www.vkm.ru/contact.htm) [42, 43]. The further step was to select mutants, both spontaneous and induced by physical (UV and X-ray irradiation) and chemical (nitric mustard, methyl-methane sulfonate, etc.) agents, as well as to genetically improve the producer [44, 45]. One of such strains, an *S. avermitilis* MA-4848 derivative, produces eight known avermectins; it was first obtained in the United States by UV mutagenesis using a lyophilized suspension of the MA-4680 (ATCC 31267) parent strain coupled with optimized nutrient medium and growth environment. As a result, the avermectin complex output rose from 9 to

500 rg/ml with a relative B1 content of about 35%. This composition, named C-076, has nematicide, acaricide, and insecticide effects. The lyophilized and frozen MA-4848 strain is deposited by the names of ATSS 31271 and ATP 31272, respectively (Patent US 4285963; 1981), its productivity was further raised to > 9,000 g/ml at B1 content of 95% and more [33, 46, 47]. Russia has also obtained *S. avermitilis* strains that produce a full 8-component avermectin complex (A1a, A1b, A2a, A2b, B1a, B1b, B2a, and B2b) with an intensive biocide effect [48, 49]. The productivity of the *S. avermitilis* VNIISKhM 56 strain is 500 rg/ml in terms of the avermectin complex, where avermectins B (B1 + B2) account for up to 50-70% (Patents RU No. 2087535, No. 2125609). The producers obtained by selection did not synthesize toxic oligomycin that the mycelium extract of the original VKM Ac-1301 strain was rich in. The first Russian drug, Aversect-1 (MGP Bifidum, part of Biotekhnologiya R&D, Moscow), registered by the Directorate General for Veterinary, Ministry of Agriculture of the Russian Federation, in 1992, contained avermectins produced by *S. avermitilis* 198 (VNIISKhM 50) and VNIISKhM 51 strains step-selected from *S. avermitilis* VKM Ac 1301 (Patent RU No. 2087535). Selection from these strains produced the VNIISKhM 54 (Patent RU No. 2054483) and VNIISKhM 56 (Patent RU No. 2087535) strains with a productivity of 400 to 500 μg/ml. These strains became fundamental to selecting even more active producers; they are still in use. In particular, directed selection of *S. avermitilis* VNIISKhM 54 via a series of intermediate variants produced *S. avermitilis* CCM 4697, which has an avermectin production of up to 2,300 μg/ml and a relative B1 content of about 50%; see Patent RU No. 2156301. NITsB 132 (Patent RU No. 2147320) features a biosynthesis of avermectins at a minimum of 3,500 μg/ml, including 1,500 μg/ml of B1, with a B1a content of about 80%. Ukrainian and Belorussian researchers have isolated avermectin producers: *S. avermitilis* UKM Ac-2179 and *S. avermitilis* X-1 [50, 51]. Reports have been published on *S. avermitilis* that produce natural (C-076) and artificial avermectins based on recombinant strains (Patent RU No. 2096462). Biosynthesis of avermectins by *S. avermitilis* UKM Ac-2179 was found to rise drastically when exposed to pyruvate, L-threonine or L-methionine, whereby the cultural fluid also accumulated amino acids, lipids, and phytohormones [52], which is consistent with the earlier data [53] and can be used to develop a waste-free avermectin biosynthesis technology.

To select highly active producers, Streptomyces spores were exposed to the mutagenic effect of a short-pulse X-ray with a quantum energy of 80 to 160 keV (Patent RU No. 2074256), UV radiation, nitrous acid, N-methyl-N″-nitro-N-nitrosoguanidine, ethyl methanesulfonate, sundry conventional or novel mutagens [54, 55].

Industrial production of avermectin-based drugs currently relies upon *S. avermitilis* strains: the abamectin-producing G8-17, SA-01, AV-LP, A-144, A-178, NA-108 (China); VKPM S-1440, VNIISKhM 56 (Russia) that synthesize the known avermectin complex, etc. Thus, intensive selection of avermectin producers basically cut the range of strains down to just a few.

Advancements in the Russian avermectin biosynthesis technology resulted in the invention of Aversect-1 (TU 10.07090-92 Aversect-1) based on the avermectin complex (*S. avermitilis* VNIISKhM 51 strain, Patent RU No. 2048520). One peculiar feature of avermectin biosynthesis is that avermectins are accumulated in the Streptomyces biomass rather than released into the environment. Leaving the process uncontrolled, especially when mycelium lysis begins, may result in the loss of the target product [56].

Standard avermectin-complex production technology elaborated for the Russian strains of *S. avermitilis*: VNIISKhM 50, VNIISKhM 51, VNIISKhM 56,

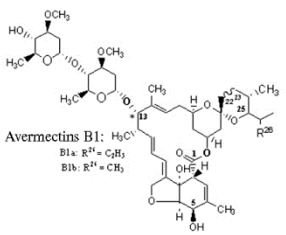
implies growth in shake flasks (250 and 750 ml) as well as in bioreactors (250 l) [56]. The conventional technology is being improved by target changes in the strain genome [45-47] or by adding metabolism-affecting components to the medium [52, 57-59]. Thus, in the presence of sinefungin, which inhibits the conversion of avermectins B into avermectins A, the proportion of avermectins B in the output of *S. avermitilis* NRRL 8165 reached 77% [60, 61]. The regulatory role of amino acids in the biosynthesis of avermectins and adjusting the B/A ratio of the avermectin complex has been demonstrated when growing a number of *S. avermitilis* strains [62, 63]. Some papers describe the biosynthesis, release, and purification of avermectins: the product concentrate is extracted by a water-immiscible organic solvent, e.g. ethyl acetate, or a mixture of solvents containing water and water-miscible low- (ethanol or propanol) or high-boiling (e.g. PEG-200) solvents [64, 65]. The basic principles behind the biosynthesis of avermectins have been researched by isotope methods and mutagenesis [64].

Cloning the genes of the avermectin complex and sequencing the genome of *S. avermitilis* enabled researchers to predict and experimentally verify the biosynthesis of sundry secondary metabolites, e.g. the polyene macrolide filipin III [66]. *S. avermitilis* has a genome sized 9,025,608 base pairs that contains at least 7,582 possible open reading frames and 38 clusters of secondary metabolite biosynthesis genes [67]. Avermectin biosynthesis is determined by 17 genes. Four of them (*aveA1 to aveA4*) encode multifunctional protein subunits (AveA1 to AveA4) comprising 3,973, 6,239, 5,532 or 4681 amino-acid residues, respectively, and forming the avermectin polyketide synthase complex [48]. AveA is a Type I, 12-module polyketide synthase [48]. The enzymes AveBI to AveBVIII (glycosyltransferase, thymidylyl transferase, TDF-4-keto-6-desoxy-L-hexose-3-ketoreductase, TDF-4-ketohexulose-reductase, TDF-TDF-4-keto-6-deoxyglucose-3-epimerase, TDF-4-keto-6-deoxy-glucose-2,3-dehydratase, TDF-6-deoxy-L-hexose-3-O-methyltransferase, TDF-4-keto-6-dexosy-L-hexose-3-ketoreductase, respectively) [33] synthesize the disaccharide of L-oleandrose from D-glucose-6-phosphate and bind to aglycone, AveE and AveF for the furan cycle, while the rest are involved in forming the spiroketal fragment (AveC), 5-O-methylation (AveD); AveR serves as the factor of positive biosynthesis regulation [64, 67].

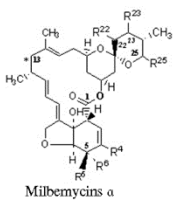
To synthesize avermectins, the producer first biosynthesizes the monomer structural units used in the polyketide avermectin synthesis, then assembles the precursor of the pentacyclic structural avermectin frame, tridecaketide, which involves the polyketide mechanism, then invokes post-polyketide transformations [1]. The former include converting tridecaketide into the avermectin intermediate with a 16-membered lactone ring: 6,8a-seco-6,8a-deoxy-5-oxoavermectin; converting 6,8a-seco-6,8a-deoxy-5-oxoavermectin into avermectin aglycone (at oxidative cycling, reduction and/or methylation); synthesizing modified L-oleandrose; glycosylation of aglycone with deoxythymidine-diphosphate-L-oleandrose (dTDP-L-Ole), whereby avermectins are synthesized [64].

When initiating the whole process, the first step is to biochemically configure (i.e. charge or recharge with the starting unit) the loading module (Module 0) for polyketide synthesis: the substrate center of acyltransferase activity (domain AT_0) of the polyfunctional synthase captures the available residue of the monocarbonic acid from the pool of acyl~S-CoA by acylating the thiol group of the cysteine in this enzyme domain [68-72]. The captured AT_0 acyl (2-methylbutyryl or isobutyryl) residue is transferred to the thiol group (substituting hydrogen in –SH) of the phosphopantetheinyl (Ppant) fragment bound to the serine residue of this domain, which carries the properties of acyl carrier proteins (ACP_0) and functions as the Ppant "sleeve" or the ACP manipulator. Thus, Module 1 prepares to receive the starting unit; similarly, AT1 activates dicarbon-

ic methylmalonic acid (methylmalonyl~S-Ppant-ACP) in the module. Upon condensation, the β-ketosyntase domain (KS_1) of Module 1 catalyzes the formation of a head-to-tail C-C-bond between the acyl residues of Modules 0 and 1, see the Claisen mechanism [1]. At the same time, the dicarbonic acid residue is decarboxylated [73], which produces diketide [68, 70] anchored at the ACP_1 domain, Module 1. This diketide is reduced by the ketoreductase domain (KR_1) to β-hydroxy-diketide ready for further condensation in Module 2. The structural diversity of the condensation products depends on the set of catalytically active domains in each module [74, 75]. The polyketide chain is lengthened step-by-step in 12 modules (one condensation per module) by 12 consecutive ester reactions of condensing 7 units of malonic acid and 5 units of methylmalonic acid activated as acyl~S-CoA, which produces the tridecaketide precursor of avermectin aglycone [1]. At each condensation cycle, the methylmalonic or malonic residue from methylmalonyl-CoA or malonyl-CoA is transferred to the phosphopantetheinyl group of the acyl carrier protein (ACP), which is stereochemically controlled by the acyltransferase (AT) of the next module [68, 76]. As the last chain growth cycle is over (Module 12, polyketide chain synthesis terminating), there occur biochemical reactions of unknown sequence that result in separating the acyclical aglycone from ACP_{12} and forming 16-membered lactone (6,8a-seco-6,8a-deoxy-5-oxoavermectin) and aglycone. The papers [77, 78] show that the spiroketalization occurs after closing the cyclohexene cycle and the 16-membered macrolide cycle BEFORE the hexahydrobenzofuran fragment is formed. A number of further transformations produce the A and B components of the avermectin complex [64].



Avermectins	R^5	R^{25}	22~23
A1a	CH_3	C_2H_5	-CH=CH-
A1b	CH_3	CH_3	-CH=CH-
B1a	H	C_2H_5	-CH=CH-
B1b	H	CH_3	-CH=CH-
A2a	CH_3	C_2H_5	$-CH_2-CH(OH)-$
A2b	CH_3	CH_3	$-CH_2-CH(OH)-$
B2a	H	C_2H_5	$-CH_2-CH(OH)-$
B2b	H	CH_3	$-CH_2-CH(OH)-$



Milbemycins	R^5	R^{25}	R^{22}	R^{23}	R^4	R^6
α1	OH	CH_3	H	H	CH_3	H
α2	OCH_3	CH_3	H	H	CH_3	H
α3	OH	C_2H_5	H	H	CH_3	H
α4	OCH_3	C_2H_5	H	H	CH_3	H
α5	OH	CH_3	OH	X	CH_3	H
α6	OCH_3	CH_3	OH	X	CH_3	H
α7	OH	C_2H_5	OH	X	CH_3	H
α8	OCH_3	C_2H_5	OH	X	CH_3	H
α9	OH	CH_3	H	H	Y	H
α10	OH	C_2H_5	H	H	Y	H
D	OH	$CH(CH_3)_2$	H	H	CH_3	H
F	OH	$CH(CH_3)_2$	H	H	Y	H
G	OCH_3	$CH(CH_3)_2$	H	H	CH_3	H
J	$R^5 = R^6 = O$	CH_3	H	H	CH_3	$R^5 = R^6 = O$

X: $OCOCH(CH_3)C_4H_9$ Y: CH_2OCO-

Fig. 1. Diversity of avermectin and milbemycin α molecular structures (milbemycins β with an open 5-membered cycle are not shown).

There is a significant similarity in the assembly of linear tridecaketide avermectin precursors and similarly structured milmemycins that have similar antiparasitic properties [79]. However, the acyltransferase of the milbemycin

synthase loading module (MilA, also consists of 12 modules), *S. hygroscopicus* ssp. *aureolacrimosus*, ssp. *noncyanogenus* is different from AveA in the sense that it is specific to acetyl~S-CoA, propionyl~S-CoA and isobutyryl~S-CoA [73]. There are also some differences in the set of catalytic activities of avermectin synthase and milbemycin synthase: unlike MilA, AveA does not have the enoyl reductase (ER) domain in Modules 2 and 7; the dehydratase (DH) domain it has is inactive, which is what determines the somewhat different structure of aglycones in avermectins and milbemycins. Milbemycins feature an open 5-membered tetrahydrofuran cycle at C22, C23, and C25 [1, 80] (see Figure 1 and Appendix on http://www.agrobiology.ru).

Avermectin synthesis is regulated consistently with the general patterns of polyketide biosynthesis [69] in Streptomyces [81, 82]. Avermectin biosynthesis regulation factors are classified as general and specific [83-87]. In the cluster of genes synthesizing this class of macrocyclic lactones, *aveR,* which determines the generation of the specific regulatory protein AveR, functions as a specific positive regulator and controls the expression of genes for both polyketide condensation and post-polyketide modification [85]: a mutant with an *aveR* deletion will not synthesize avermectins, but will produce oligomycins in larger amounts than a wild strain. *aveR* is believed to encode a specific activator that is necessary for avermectin biosynthesis [86]. *aveI* is a gene identified as a negative regulator of the biosynthesis of these macrolides, as inactivating it results in a 16-fold production of avermectin B1a in *S. avermitilis* NRRL 8165 [33]. It has been found out that the increased expression of *aveT and sav_4189* that encode the regulatory factors SAV3619 (AveT) from the TetR (Tet Repressor Protein) family of repressor protein, and SAV4189, which is homologous to the MarR (multiple antibiotic resistance regulator) family, will increase the avermectin output [87, 88]. Among the general polyketide biosynthesis regulators found in other actinomycetes of the genus *Streptomyces* (e.g. in the actinorodine-synthesizing *S. coelicolor* M145), factors SAV3818 and AvaR3 are positive regulators, whereas AvaR1 is a negative regulator of avermectin biosynthesis [33].

Melingmycin produced by *S. nanchangensis* (structurally similar to milbemycin α11) [89], the tetracyclic milbemycin-like compound in *S. microflavus,* neau3 Y-3 [90], and avermectin B1 homologs in *Anthogorgia caerulea* (Beibu Bay, China), have been added to the group of the described avermectin-like natural substances; like any other compound of this group, these new members are nematicides and insectoacaricides [91].

The diversity of organisms producing avermectin-like compounds (avermectins, milbemycins, and other similar substances) indicates the commonness of the combinatorial synthesis that uses the polyketide mechanism [89-91]. The aglycone structure of these natural lactones is based on the same tridecaketide. What makes natural avermectin-like compounds so diverse is the fact that biosynthesis involves various source units for tailing the carbon chain (2-R-derivatives of malonic acid [73] and a set of catalytically active polyketide synthase domains in various streptomycetes [92, 93].

Application of synthetic biology [32, 34, 73] and organic synthesis [93, 94] is an important trend in expanding the range and improving the production of avermectin-like substances [32]. Most commercial substances of this class are known to be semisynthetic derivatives of native abamectin [1, 2]. The basic approach to improving the production of abamectin and other avermectins consists in optimizing the growth conditions combined with directed biosynthesis in selected productive strains [32]. Thus, one research team attempted substituting the *ave*DH2-KR2 site in the cluster of avermectin biosynthesis genes in the

S. avermitilis NA-108 industrial strain with the *mil*DH2-ER2-KR2 fragment from the milbemycin biosynthesis cluster of the *S. bingchenggensis* strain; this effectively created the highly productive *S. avermitilis* AVE-T27 strain that produces 3,450±65 μg of ivermectin per ml [95]. The well-known semisynthetic ivermectin is produced by hydrogenating the 22,23-double bond of abamectin in the presence of the Wilkinson catalyst $[(PH_3P)_3RCl]$ [1, 2]. Substituting *ave*LAT-ACP and *ave*DH2-KR2 with *mil*LAT-ACP and *mil*DH2-ER2-KR2, respectively, produced the *S. avermitilis* AVE-H39 strain that synthesizes two new ivermectin-like metabolites that contain methyl radical (output of 2,093±61 μg/ml) and ethyl radical (output of 951±46 μg/ml) at C25. Those kill *Caenorhabditis elegans* 2.5 times more efficiently than milbemectin [95]. The productive mutant milbemycin-synthesizing *S. avermitilis* SAMA1M7 strain was obtained by substituting the genes *aveA1* and *aveA3* (AveA3 Module 7) in the productive *S. avermitilis* SA-01 industrial strain with templates for the genes *milA1* and *milA3* (MilA3 Module 7) of the milbemycin producer *S. hygroscopicus* subsp. *aureolacrimosus* NRRL 5739 [79]. The *S. avermitilis* SAMA1M7 strain produced milbemycins α3, α4, D (in small amounts), as well as their 5-O-methyl derivatives (about 292 μg/ml) [79]. Subsequent inactivation of 5-O-methyltransferase (AveD) in *S. avermitilis* SAMA1M7 and introducing the *aveD* stop codon with plasmid pΔAveD produced the *S. avermitilis* SAMA1M7ΔD strain that synthesizes milbemycins α3 and α4 (the basic components of commercial milbemectin) at 377 μg/ml [79]. One research team has successfully performed heterologous expression of the cluster of avermectin biosynthesis genes, *ave*, in *S. lividans* 1326, which produced A2a, B1a, and A1a [96]. *S. avermitilis* or its mutant devoid of branched-chain α-ketoacid dehydrogenase (bkdF) are able to synthesize avermectin-like compounds with differently structured C25 radicals if carbonic acids (precursors of the starting units for the synthase loading module) are added to the nutrient medium; this is due to the inactivation of the gene *bdkF*. In the presence of cyclohexane carboxylic (CHC) acid, the producer synthesizes a compound similar to avermectin B1 that has a cyclohexyl radical at C25; this is known as doramectin [1, 96, 97]. Mutant *S. avermitilis* TG2002 was constructed by substituting the avermectin synthase loading module (aveATL-ACPL) of *S. avermitilis* M1 with the CHC-synthesizing module (pnATL-ACPL) of phoslactomycin synthase (Pn) from *S. platensis* SAM-0654 using plasmid pTG2002 [99]. The recombinant *S. avermitilis* TG2002 has a doramectin output of 58±2 μg/ml, which is six times that attained by fermenting the parent strain, *S. avermitilis* M1 (9±1 μg/ml), with a doramectin/avermectin ratio of 300 [99].

Avermectin structure, action, and resistance. All the known avermectins and similar milbemycins, as well as the recently discovered melingmycin [89], 28-homo-avermectin B1a, and 28-isopropyl-avermectin B1a [91], are efficient against parasites even at very low concentrations of about 1 nmole/l [100]. Nevertheless, these compounds are not identical; varying the substitutes at different sites of the pentacyclic nucleus (C4, C5, C13, C22 to C23, C25) will modulate their biological activity only to a certain degree. In avermectins, deleting one (the remote one) oleandrose residue will somewhat lower the antinematode effect; deleting the disaccharide residue (aglycones of avermectins with 13-OH groups) will considerably reduce this effect while preserving the insectoacaricide effect. Substituting the 13-OH group with hydrogen (which is observed in milbemycin) will restore the antiparasitic effect [76]; lepimectin, which is a derivative of milbemycin that has a polar structural fragment at C13, is an efficient parasiticide. In general, avermectins and milbemycins with lipophilic groups at C13 are more active, whereas polar substitutes make such compounds less active. Similar correlation of structure and insecticide/ixodicide activity is observed in

avermectin B1: substituting the 4″-OH group with a 4″-epi-methylamine group will greatly amplify the effect the substance has on various lepidopterans while reducing the ixodicide effects [101, 102].

Avermectins and sundry 16-membered macrocyclic lactones target glutamate-dependent chloride ion channels (GluCl channels) that are common in invertebrates (nematodes, arthropods such as insects or ticks) but not in vertebrates. These channels are activated by nanomolar lactone concentrations. The irreversible activation of GluCl channels results in hyperpolarization of membranes incompatible with neural conductivity in neuromuscular synapses, which causes a strong and stable paralysis of the muscles in the pharyngeal system, musculocutaneous sac, and ovipositors [103, 104]. Invertebrates commonly have GABA receptor proteins that are related to, but have evolved differently from, GluCl channel-forming proteins (subspecies A) (g-butyric acid; $GABA_A$) ($GABA_A$-dependent Cl^- channels), which are also targeted by avermectins, as GABA is the most important inhibitory neurotransmitter in the central nervous systems of mammals, human included. However, avermectins are safe for mammals as they cannot penetrate the blood-brain barrier and reach the $GABA_A$-sensitive Cl channels of the CNS [105]. As known, GluCl channels and $GABA_A$ receptors both belong to Cys-loop receptors that also include glycine, nicotine, and serotonin (5-HT3) ionotropic receptors [101, 106, 107] also affected by avermectins and milbemycins, although to a lesser affinity. Ivermectin has been found to affect the P2X4 receptor [108].

Testing the sensitivity to and resistance to ivermectin shows that parasitic resistance to ivermectin is associated with mutations in genes that determine the synthesis of GluCl subunits (*glc-1*, *avr-14,* and *avr-15*) coupled with increased expression of P-glycoprotein genes [109]. The greater sensitivity of collie dogs to ivermectin and moxidectin is due to an *MDR1* mutation that affects the gene responsible for the synthesis of P-glycoprotein, a mandatory component of the blood-brain barrier that keeps the barrier intact and prevents harmful substances from entering the brain. This mutation enables lactones to penetrate the blood-brain barrier in mammals [110].

16-membered macrocyclic lactones have a certain specific correlation of resistance and the compound structure. Thus, cross-resistance to ivermectin and doramectin is possible; however, doramectin is often more efficient against parasites resistant to avermectins [101]. One interesting phenomenon has been discovered [111]: a higher concentration of GABA at feeding *Tetranychus cinnabarinus* with an exogenous GABA or suppressing the expression of the GABA transaminase gene (GABA-T) makes the experimental pest specimens resistant to abamectin. Another interesting phenomenon that was described recently is the direct interaction of avermectins and epidermal growth factor receptor (EGFR). This factor activates EGFR/AKT/ERK pathways and induces superexpression of P-glycoprotein in the thickened chitin layers in *Drosophila melanogaster* larvae in a resistant population [112].

Semisynthetic avermectins. Quite often, the secondary metabolites need to be modified for use as an agent to improve their bioavailability, quality, physical and chemical properties, adverse effects, etc. This process creates more efficient counterparts to the original natural substances, including 16-membered avermectins [2, 113-115].

From the chemical point of view, avermectins can be presented as the derivatives of the corresponding milbemycin complex components obtained by tailing the latter with a 4-α-L-oleandrosyl-L-oleandrosyloxy group at C13 lactone nucleus. Consider examples of creating crucial pharmaceuticals, as well as prospective areas of research, e.g. synthesis of 5-O derivatives that the team behind

this paper has been researching since the mid-1990s.

The chemical modification strategy depends on the biological activity data available for the components of the antiparasitic avermectin and milbemycin complex. Let us illustrate this by data on how avermectin B1 derivatives affect mature spider mite females in direct application [116]. 96-hour mortality rate was 100% at 0.05 ppm for avermectin B1 (abamectin), 8,9-epoxy-avermectin B1, 10,11-dihydroavermectin B1, and 10-fluoro-10,11-dihydroavermectin B1; 92% for 22,23-dihydroavermectin B1 (ivermectin); 72% for 10-hydroxy-10,11-dihydroavermectin B1; 20% for 3,4-cyclopro-pylavermectin B1 and 8,9-epoxy-milbemycin (25-sec-butyl); 18% for 3,4,8,9,10,11,22,23-octahydroavermectin B1; 15% for 8,9-cyclopropylavermectin B1 and 11% for 3,4,10,11,22,23-hexahydroavermectin B1 [116]. The production of avermectin and milbemycin derivatives, the efficiency, antiparasitic spectrum, and eco-friendliness of which make them crucial for agriculture, is a multistep process that includes microbiological synthesis and chemical modification [1], see Figure 2 and Appendix on http://www.agrobiology.ru. The team behind this research is studying the suitability of 5-O and 5-C derivatives of avermectin B1, ivermectin, and other avermectins and milbemycins as antiparasitic drugs; some of them have already been patented [117]:

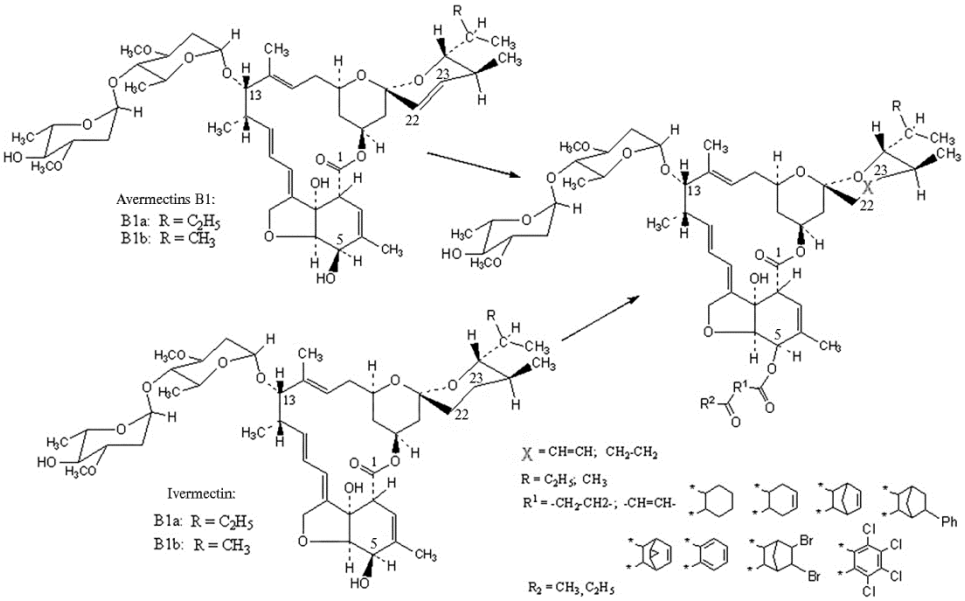


Fig. 2. Production of 5-O derivatives of avermectin B1 (22 and 23 are, respectively, the structural components $-CH_2-$ or $=CH-$ for single or double bond for X).

We have also synthesized a series of 5-O, 5-O,4″-O, and 4″-O acyl derivatives and their esters, methyl carbamates, 5-O-sulfate sodium salt, etc. [118-122]. It has been found that 5-O-succinoylavermectin B1 (sumectin) and the compound code-named C2017 have pronounced antiparasitic effects; they have thus been used to develop liquid and solid drugs for topical and oral administration (Patents RU No. 2629600 and 2661615). Researchers from other countries are also trying to obtain similar compounds, in particular, 5-oxime derivatives and chitosan derivative [123, 124]. When testing the antiparasitic effects of sumectin, C2017, and abamectin on laboratory mice infested with *Aspiculuris tetraptera*, the authors found that an oral dose of 0.25 mg/kg had a 100% anti-helminthic effect regardless of the substance; however, unlike abamectin and

sumectin, C2017 also had a repellent effect (data unpublished). The authors have also found that C2017 far exceeds abamectin in affecting the binding of radioligand [G-^{3}H]SR 95531 with the membranes containing the $GABA_A$ receptors of cerebral cortex in rats, raising the maximum inhibition of specific binging I_{max} by 86% (in-lab unpublished data).

To sum it up, one can state that natural and semisynthetic avermectins are broadly used to treat and prevent nematodiasis and arachnoentomosis in animals, humans, and plants. The most popular drugs are abamectin, ivermectin [1], doramectin [2, 3], selamectin [2], avermectin B1 benzoate [4], eprinomectin [1], as well as the similar milbemectin (a mixture of milmemycins α3 and α4) [1]. These substances are contained in various veterinary and medicinal preparations under various brand names; such preparations are used to treat onchocerciasis, dermatitis, etc. In recent years, ivermectin-based anti-rosacea creams [125], avermectin B1 hemisuccinate-based ointments and liquid against arachnoentomosis, etc. have been patented in Russia, including Russian oral drugs [126-129] as well as VEIS granules designed to combat synanthropic insects, see Certificate No. RU.77.99.88.002. E007964.09.14).

Thus, *Streptomyces avermitilis*-produced 16-membered macrolides (avermectins) and similar macrocyclic lactones are efficient against nematodes, insects, and ticks, as they affect the glutamate-dependent channels of Cl ions in invertebrates while also somewhat affecting the GABA-dependent Cys-loop receptors. Studies into fully chemical synthesis of avermectins have so far been futile due to too complex synthesis and low output. Chemical modification of natural macrolides: avermectin B1 (produced by *S. avermitilis*), milbemycin α3/α4 (*S. hygroscopicus* ssp. *aureolacrimosus*), nemadectin (*S. hygroscopicus* ssp. *noncyanogenus*), and similar biosynthetic drugs (e.g. doramectin produced by a mutant *S. avermitilis* strain with a defective gene of branched α-ketoacid dehydrogenase) has produced similar substances for use in veterinary medicine (ivermectin, eprinomectin, selamectin, and moxidectin), human medicine (ivermectin), botany (abamectin, emamectin benzoate, milbemycin oxime α3/α4); 5-O-succinoyl avermectin compounds B1 and C2017 are being researched as promising medications.

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VACCINES AGAINST EQUINE INFLUENZA
(review)

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Abstract

Equine influenza is a highly infectious disease that can rapidly spread and induce high morbidity in susceptible horse populations (K.P. Yurov, 2009; S.P. Waghmare et al., 2010). Equine influenza is caused by RNA viruses are belonged to the genus *Influenzavirus A* of the family *Orthomyxoviridae* (A.D. Zaberezhnyi et al., 2017). Two different equine influenza virus (EIV) subtypes have been recognized based on antigenic properties of the envelope glycoproteins (HA and NA), the H7N7 subtype (equi-1) and the H3N8. The H7N7 subtype was first isolated in Czechoslovakia in 1956 (prototype strain: A/eq/Prague/1/56). The last confirmed outbreak occurred in 1979 in Italy. The H3N8 subtype of EIV is still circulating in the most countries of the world and has caused outbreaks of disease US and Europe (R. Paillot, 2014; B. Cowled et al., 2009; C.O. Perglione et al., 2016; A.I. Kydyrmanov et al., 200;). Vaccination is one of the most effective tools, alongside isolation, movement restriction and basic biosecurity measures, to prevent EIV infection or to limit its consequences (S.S. Wong et al., 2013). The main goal of vaccination against equine influenza is a significant reduction in clinical signs of disease, virus replication and shedding. Potent EIV vaccines reduce virus transmission and increase resistance to infection (D.J. Baker, 1986). Because of effectiveness EIV vaccines depends on antigenic homology between vaccines and circulates strains of EIV all equine influenza vaccines should contain epidemiologically relevant strains recommended by the OIE (OIE Expert Surveillance Panel on Equine Influenza Vaccine Composition, 2017; R. Paillot, 2014). In accordance with last OIE recommendations EIV vaccines should contain both clade 1 and clade 2 viruses of the Florida sublineage. Clade 1 continues to be represented by A/eq/South Africa/04/2003-like or A/eq/Ohio/2003-like viruses. Clade 2 continues to be represented by A/eq/Richmond/1/2007-like viruses. It is not necessary to include an H7N7 virus or an H3N8 virus of the Eurasian lineage in vaccines (R. Paillot, 2014; OIE Headquarters, 2017). This review gives actual data about the types of licensed vaccines against equine influenza. Whole inactivated/sub-unit, live-attenuated and viral-vector based vaccines are considered. Numerous experimental EIV vaccines developed with modern molecular biology technique have been reported. Reverse genetics techniques which provide a good tool for the generation of recombinant influenza viruses and develop both inactivated and live-attenuated influenza vaccines are also discussed (E.-J. Jung et al., 2010; E. Hoffmann et al., 2010; Y. Uchida et al., 2014). Reverse genetics allows generation of artificial recombinant influenza viruses and provides the possibility to rapidly and easily modify the antigenic characteristics of the vaccine strain by genetic manipulation.

Keywords: equine influenza, vaccines, vaccination, whole inactivated vaccines, sub-unit vaccines, live-attenuated vaccines, viral-vector based vaccines, recombinant vaccines, reverse genetics

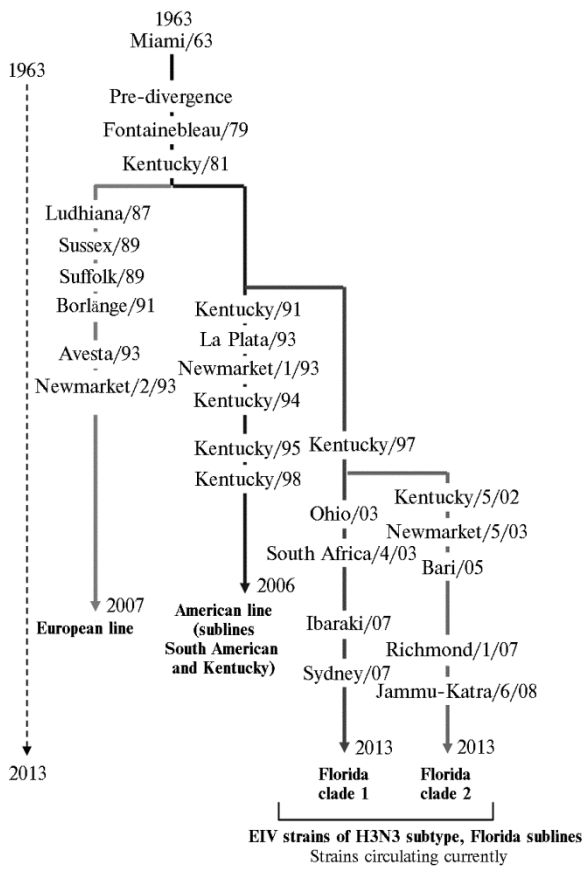
Equine influenza is an infectious disease that affects horses, donkeys, and mules, and causes a body temperature rise to 41 °C, catarrh of the upper respiratory tracts, painful dry cough, rhinotracheitis, or even pneumonia in severe cases. Other notable clinical signs include myalgia, lack of appetite, and enlarged submaxillary lymph nodes [1, 2]. Lethal outcomes mainly occur among younger animals (colts, foals) and donkeys. Death of an adult specimen is usually the result

of a general morbid condition and/or secondary bacterial infection, where pleuritis and pneumonia ensue [3].

Preventive action is the best way to avoid influenza infection; vaccination against equine influenza remains an important tool to control the disease. This makes it imperative to continue research to develop more effective vaccines. The only Russian-made vaccine veterinaries use against equine influenza is a first-generation inactivated vaccine.

This review presents up-to-date information on horse vaccination against influenza; it also covers the types of vaccines, both existing and under development. Today, there exist multiple ways to produce candidate influenza virus strains; one relies upon reverse genetics. The team behind this research used this method to produce Russia's first reassortant recPR8-H3N8eq virus strain, which can be considered a candidate for synthesizing an equine influenza vaccine.

The pathogen is an RNA virus that belongs to the family *Orthomyxoviridae*, genus *Influenzavirus* [4]. The severity depends on the equine influenza virus (EIV) strain as well as on the animal's immunity status. EIV is classified into two subtypes based on the antigenic differences of two envelope glycoproteins: hemagglutinin (HA) and neuraminidase (NA). The first subtype, H7N7, equi-1, is less virulent; its prototype strain is A/equine/1/Prague/56, first isolated in Czechoslovakia in 1956. The last confirmed outbreak occurred in Italy in 1979; however, this subtype was also isolated in India in 1987 [5], as well as in Egypt in 1989 [6]. The other subtype (H3N8, equi-2) is represented by the prototype strain A/equine/2/Miami/63, first isolated in North America in 1963 [7]. It continues circulating in most countries except Australia, New Zealand, and Iceland. Since the late 1980s, H3N8 EIV is classified into the American line and the European line [8]. The American line has three sublines of different antigenic characteristics: South American, Kentucky, and Florida sublines [9]. Further genetic evolution of the Florida subline caused two new viral groups to emerge (Florida clade 1 sublineage, Florida clade 2 sublineage), which contain all the viral isolates that have recently been isolated in the Americas [10, 11], Europe [12-14] and Asia [15, 16] (Fig.).



Equine influenza virus (EIV) **evolution (**main sublines and strains) **[3].**

Note that EIV vaccines have a history of 50 years. Nevertheless, H3N8 viruses are still able to cause enzootics in North America and Europe. Over the last 10 years, multiple major outbreaks occurred in: Sweden (2007), Australia (2007) [17], Japan (2007)

[18, 19], India (2008-2009) [20], South America (2012) [11, 21], Kazakhstan (2007) [22], and Mongolia (2007-2008) [23]. In 2017, an outbreak of influenza caused by A/donkey/Shandong/1/2017(H3N8) EIV was observed in donkeys in the Chinese province of Shandong [24]. In the Russian Federation, EIV epizootics have been registered in the Republics of Khakassia (2007), Buryatia, and Tywa (2008-2009) [25].

Influenza A viruses feature a considerable variability of the virion envelope glycoproteins (HA and NA). Therefore, the more identical are the *HA* genes in the vaccine strain and in the field strain, the more efficiently vaccination will reduce the replication of the influenza virus in the respiratory tract or its release into the environment in case of infection. An efficient vaccine against equine influenza must contain the actual circulating EIV strains [26].

The Vaccine Panel of the World Organisation for Animal Health, OIE, France, publishes annual reports on the laboratory and epidemiological data on EIV strain circulation; the reports present recommendations on vaccine composition. Since 2010, OIE has been recommending that representative H3N8, Florida clade 1 (South Africa/03 or Ohio/03) and Florida clade 2 (Richmond/1/07) strains be included in vaccines. Adding H7N7 and H3N8 (European line) strains is not necessary [3, 27].

Like quarantine and restrictive actions, vaccination against equine influenza is one of the key tools to control the disease [28]. Vaccination mainly seeks to reduce the clinical manifestations of the disease, which improves the animal well-being, shortens the re-convalescence period, and lowers the risk of secondary infections. Besides, vaccination helps reduce the release of the virus into the environment, which curbs the spread of infection [29, 30]. In the Russian Federation, vaccinating horses against influenza is regulated by the Guidelines for Prevention and Tackling of Equine Influenza as approved by the Chief Veterinary Department of the USSR Ministry of Agriculture on September 1, 1980; and by the Veterinary Rules of Transport of Sporting Horses in the Russian Federation as approved by the Ministry of Agriculture of the Russian Federation on May 30, 2003. These documents state the vaccinating horses against influenza is recommendatory rather than mandatory. Subject to preventive vaccination with an inactivated polyvalent vaccine are horses owned by horse farms, sports organization, and circuses; sporting and pedigree horses leaving their farms; horses from any farm or household at risk of influenza. Sporting horses, i.e. horses issued a passport by the Russian Equestrian Federation (REF) or the Russian National Research Institute of Horse Breeding (RNRIHB), as well as circus and theater horses, must be vaccinated at least once every 6 months.

EIV vaccines currently in use can be classified by the production technology in three groups: inactivated whole-virion vaccines, subunit live attenuated vaccines, and vector vaccines (Table) [3].

The first generation of EIV vaccines veterinaries used for decades comprised whole-virion inactivated vaccines that contained aluminum hydroxide as an adjuvant [31]. The primary advantage of this type is that the virus does not replicate, and the vaccine cannot render a horse sick [32]. The immunity this type of vaccines induces is mainly based on stimulating the humoral response in horses. No cytotoxic cell response is induced [33]. The immune system produces antibodies specific not only to variable envelope antigens (HA and NA) but also to more conservative EIV proteins such as NP or M, which are supposedly responsible for cross-protective immunity [3]. Humoral immunity induced by inactivated EIV vaccines does not last long. It has been shown that the conventional inactivated vaccine stimulates the production of short-lived (<100 days) IgG(T) antibodies that cannot fix the complement, whereas a natural EIV infection

mainly causes the production of virus-specific IgA, IgG2a, and IgG2b antibodies. As a result, a horse will need double or multiple vaccinations to have an immune response lasting 12 months [34].

Veterinary-used EIV vaccines classified by the production technology [3]

Name	Produced by	Adjuvant	Antigen	EIV strains
Inactivated virions/cubunits				
DuvaxynTm IE Plus	Elanco (USA)	Carbopol	Whole EIV virions	Newmarket/1/93 (H3N8) Suffolk/89 (H3N8) Prague/56 (H7N7)
Calvenza®-03 EIV	Boehringer Ingelheim Animal Health (Germany)	Carbopol	Whole EIV virions	Newmarket/2/93 (H3N8) Kentucky/2/95 (H3N8) Oiho/03 (H3N8)
Equilis Prequenza (updated 2013)	MSD Animal Health (USA)	ISCOM-Matrix	Whole EIV virions	Newmarket/2/93 (H3N8) South Africa/4/03 (H3N8)
Equilis Prequenza	MSD Animal Health (USA)	ISCOM-Matrix	HA subunits	Prague/56 (H7N7) Newmarket/1/93 (H3N8) Newmarket/2/93 (H3N8)
EquipTM F	Pfizer Ltd. (USA)	ISCOM	HA and NA subunits	Newmarket/77 (H7N7) Borlänge/91 (H3N8) Kentucky/98 (H3N8)
Inactivated Polyvalent Horse Flu Vaccine	Kurskaya biofabrika (Russia)	Гидроокись алюминия	Whole EIV virions	Cambridge-63 (H7N7) France-98 (H3N8)
Live attenuated cold-adapted strains				
Flu Avert® I.N.	Intervet/Schering-Plough Animal Health (The Netherlands)	Нет	Whole EIV virions	Attenuated cold-adapted Kentucky/91 (H3N8)
Vector vaccines				
PROTEQ FLU™	Merial Animal Health Ltd. (France)	Carbomer	HA	Ohio/03 (H3N8) Newmarket/2/93 (H3N8)
PROTEQ FLU™ (updated 2014)	Merial Animal Health Ltd. (France)	Carbomer	HA	Ohio/03 (H3N8) Richmond/1/07 (H3N8)

Note. EIV stands for equine influenza virus; HA stands for hemagglutinin; NA stands for neuraminidase.

Subunit EIV vaccines contain purified viral proteins (HA and/or NA). The adjuvant is an ISCOM or ISCOM-Matrix immunity-stimulating complex. ISCOM consists of 35-nm spherical structures formed by the hydrophobic interaction of amphiphilic antigen (HA and/or NA) molecules with cholesterol, phospholipids, and *Quillaja* saponins. These complexes are more immunogenic than the original proteins [35]. Since ISCOM consists of microparticles, they are easily absorbed by macrophages, where antigens are subsequently processed and presented. ISCOM-Matrix based vaccines are similar, but their viral proteins are not part of the lipid-saponin complex [36]. It has been shown that using inactivated EIV vaccines with a carbomer adjuvant stimulates greater production of protective antibodies [37].

Over the last 15 to 20 years, there has emerged a new generation of EIV vaccines (see Table 1) that trigger both the humoral and the cellular immune response. One example is the live attenuated vaccines based on temperature-sensitive (ts) and cold-adapted (ca) strains. Vaccine EIV strains effectively reproduce in vivo at suboptimal (25 °C) upper respiratory tract temperatures, whereby they induce local and systemic immune response; at the same time, they do not replicate in the lower respiratory tracts at 38-39 °C, where the wild-type virus replication is usually associated with bronchitis, bronchiolitis, interstitial pneumonia, and edema [33].

The first intranasal live attenuated vaccine against EIV (Flu Avert® I.N., Heska Corporation) was developed and licensed in 1999 in the US. The ca strain it was based on had been produced by serial passages of A/Equine/Kentucky/1/91 in chicken embryos (CE) at lower temperatures (34 °C, 30 °C, 28 °C, and 26 °C) [38]. Single vaccination was shown to protect ponies against clinical manifestations of the disease for 3 to 6 months or more in the case of control infection with a homologous wild-type virus [39] or with a heterologous European-line

H3N8 IEV whereas the antiviral antibodies were low [40].

Although such vaccines are clearly advantageous, they pose a risk of vaccine virus reversion or reassortment to the circulating wild-type virus in the horse body, which will cause new pathogenic viruses to emerge. Nevertheless, an experiment, in which five direct consecutive horse-to-horse passages were performed, did not identify any reversions of the Flu Avert® ca strain to the wild type, which indicated stable attenuation and temperature-sensitive phenotype [40].

After a major outbreak of equine influenza caused by H3N8 EIV in Kazakhstan (2007), the St. Petersburg Influenza Research Institute cooperated with Kazakhstani researchers to develop the first Kazakhstan-produced live modified ca vaccine against equine influenza. Classical genetic reassortment methods were used to obtain a ca strain, A/HK/Otar/6:2/2010, which carries genes encoding the envelope proteins (HA, NA) of the A/equine/Otar/764/2007 wild strains (H3N8, American Florida line, clade 2) as well as genes encoding the internal proteins (PB2, PB1, PA, NP, M, NS) of the attenuation donor, the A/Hong Kong/1/68/162/35 (H3N2) ca strain. The safety and efficacy of this vaccine have been studied in horses. As of today, Kazakhstan's horse farms are testing the vaccine in the field [41, 42].

The emergence and advancement of reverse genetics in the late 20th century revolutionized the development of prototype viral strains for inactivated and live attenuated vaccines. When applied to the influenza virus, reverse genetics can quickly produce vaccine candidate strains of any HA or NA subtype. Recombinant vaccines are produced by cloning individual kDNA molecules encoding the eight segments of the virus A RNA into a special plasmid. Then the plasmids are transfected into eukaryotic cells [43, 44]. Reverse genetics can not only produce reassortant viruses of required antigenic properties and lower virulence but also modify them to match the changing antigenic properties of the circulating field strains. Veterinary research has used this method to produce vaccine candidate strains for swine [45] and avian influenza [33, 46].

Mutating the genes of the internal viral proteins and removing the virulence factors of highly pathogenic influenza viruses can produce attenuated strains [45, 47]. Quinlivan et al. [48] thus obtained three recombinant EIV with *NS1* deletions, which attenuated them in relation to the original wild-type A/eq/Kentucky/5/02 (H3N8) and rendered them unable to replicate in interferon-competent cells in vitro. Testing recombinant EIV as candidate vaccines confirmed that vaccinating horses with a mutant NS1-126 virus provided effective clinical protection of animals infected with a wild-type virus. Vaccinated horses had far weaker and fewer clinical manifestations, as well as a shorter viral release period, as compared to the controls [49].

In early 2018, Rodrigueza et al. [50] reported developing a temperature-sensitive (ts) live attenuated vaccine against equine influenza. The researchers applied reverse genetics to make mutations in the polymerase genes *PB1* and *PB2,* A/equine/Ohio/1/2003 H3N8 (Florida sub-lineage clade 1); the mutations they made were responsible for the ts/ca/att phenotype of the attenuation donor A/Ann Arbor/6/60 H2N2 from the human live attenuated vaccine FluMist (MedImmune, US). The resultant recombinant virus was able to effectively replicate at lower temperatures (33 °C); both in vivo and in vitro, it had a phenotype similar to that of the live attenuated vaccine Flu Avert®. Single intranasal administration of the recombinant virus effectively protected the horses against a control exposure to a homologous wild-type virus [50, 51].

Vector vaccines are produced by inserting the required gene of a particular pathogen along with a set of regulatory elements in the viral vectors. Viral antigens are expressed and synthesized de novo in the infected cells [52, 53].

ProteqFlu® (Merial Ltd., France) is the only live recombinant vector vaccine against equine influenza that has been in use by horse breeders since 2003 [33]. The vaccine uses recombinant canarypox virus, ALVAC, as a vector to express HA genes of the A/equine/Ohio/03 (H3N8) and A/equine/Richmond/1/07 (H3N8) equine influenza viruses [54, 55]. The vaccine is safe to use, as the recombinant canarypox virus causes an abortive infection in mammalian cells [56]. Vaccinated horses develop a humoral and cellular immune response. One-vaccinated ponies had far fewer and weaker clinical manifestations of the test infection as compared to the controls. The viral release was significantly lesser but not shorter. After double vaccination with a 35-day interval, the virus-neutralizing IgGa and IgGb antibodies preserved their protective level for 4 months. Therefore, triple vaccination is necessary to induce a 12-month protective immune response [57, 58].

Van de Walle et al. [59] synthesized a recombinant vaccine viral vector based on the abortive NYO3 EHV-1 strain that expresses the *HA* EIV A/eq/Ohio/03 gene. Double immunization with this recombinant virus with a 5-week interval produced specific IEV antibodies in horses, which were detectable for 18 weeks after the second vaccination. However, the authors did not research the protectiveness of the potential vaccine against experimental IEV infection; the safety of the viral vector was not tested either [59].

Reverse genetics is a state-of-the-art and efficient approach to synthesizing vaccine strains of required properties [60]. The Russian Ministry of Health's Gamaleya National Research Center for Epidemiology and Microbiology is also researching the use of reverse genetics to produce recombinant influenza viruses. Thus, they produced a reassortant recPR8-H5N1strain that contains the *HA* gene from the highly pathogenic A/Kurgan/05/2005 (H5N1) avian influenza virus isolated in Russia. The Institute has researched the reproductive, antigenic, and virulent properties of the reassortant. The lab-synthesized recPR8-H5N1-based inactivated emulsified vaccine was shown to protected chicks, aged 6 weeks, against control administration of A/Kurgan/05/2005 (H5N1), which is a highly pathogenic virus [46]. As of today, the Institute's Molecular Diagnostics Laboratory is trying to obtain a reassortant IEV strain with *HA* and *NA* genes from A/equine2/Bitza/07 (H3N8), other genes from the highly productive A/Puerto Rico/8/34 (H1N1) strain.

Thus, equine influenza is a highly infectious disease that tends to spread fast and have high incidence in sensitive specimens. Its outbreaks may significantly impact horse breeding, especially that of sporting horses. Vaccination against equine influenza is an effective prevention tool. First-generation vaccines were inactivated whole-virion and subunit vaccines that induced the production of protective antibodies. Then they invented the second generation (live attenuated and vector vaccines), which could stimulate a humoral and cellular immune response to imitate the protective immune response a natural virus would cause in horses. Reverse genetics is one of the promising methods for the production of recombinant live attenuated vaccines; it can obtain reassortant viruses of the required antigenic properties. It can also modify such viruses to tailor them to the changing antigenic properties of the circulating field strains.

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THE STUDY OF EFFECT OF GENOTYPES FOR DNA MARKER ON REPRODUCTIVE QUALITIES OF SOWS OF LARGE WHITE AND LANDRACE BREEDS

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Abstract

The genetic progress by low-inherited reproduction traits in pigs can be increased by integrating into breeding programs the DNA markers, which are associated with quantitative trait loci (QTL) of reproductive qualities (marker selection, MAS). The aim of the present study was to assess the effect of DNA markers *IGF2* (insulin-like growth factor 2), *ECR F18/FUT1* (*Escherichia coli* F18 receptor), *ESR* (estrogen receptor) and *MUC4* (mucin 4) on the fertility traits of Large White and Landrace sows. The studied traits included the total number of piglets born per litter (TNB); number of piglets born alive per litter (NBA); average birth weight (BW) and adjusted birth weight (BW_{adj}); weight at weaning at 21 days (WW) and adjusted weight at weaning at 21 days (WW_{adj}). The genotypes frequencies of the analyzed markers were determined. Besides we identified significant deviations of the genotype frequencies from the population equilibrium for Large White breed by the *IGF2* gene ($p < 0.01$) and Landrace breed by the *IGF*2 ($p < 0.01$), *ECR F18/FUT1* ($p < 0.01$) and *MUC4* ($p < 0.001$). The homozygosity coefficient according to Robertson (Ca) was the highest for genotypes for *IGF2* and *ECR F18/FUT1*. The values of this parameters reached 0.76 and 0.65 for Large White breed against 0.60 and 0.72 for Landrace breed, respectively. We calculated the heritability coefficients for the analyzed traits, which were 0.165-0.179 for TNB, 0.100-0.155 for NBA, 0.232-0.338 for BW, and 0.010-0.115 for WW. Based on the developed equations, breeding values of pigs were determined using the BLUP AM method. The *IGF2* marker showed a significant effect on the weight at weaning for Landrace sows (PHE_{WW}, PHE_{WWadj}, EBV_{WW}); individuals with genotypes *AA* and *AG* were the best. The genotype for *ECR F18/FUT1* significantly influenced the phenotype and breeding value of sows for the number of piglets born and for the birth weight of piglets. Sows with the *AA* genotype were characterized by a lower number of piglets born (by 8.0-8.5 %), and by a higher average birth weight (by 2.0-3.0 %). The significant effects of the *ESR* on TNB and NBA and on EBV values for birth weight were revealed: the sows of both breeds with *CC* genotype for *ESR* were characterized by highest average piglet weight at birth. We found the significant effect of *MUC4* on birth weight of piglets for both breeds. Sows with *CC* and *CG* genotypes were superior comparing to individuals, which are homozygous for the *G* allele. Thus, using the marker assisted selection along with traditional methods for assessing the genetic potential of pigs (BLUP AM) will significantly improve the efficiency of breeding measures on the fertility traits.

Keywords: pigs, Large White breed, Landrace, *IGF2, ECR F18/FUT1, ESR, MUC4*, linear regression, fertility traits, estimated breeding values, marker assisted selection

Improving reproduction traits is one of the key goals of improving ma-

ternal breeds [1]. However, the low inheritance, significant variability, and sex-limited manifestation of such reproduction traits in sows as prolificacy, the number and weight of live piglets upon birth and at weaning, and the milk yield [2] limit the effectiveness of traditional breeding. Thus, the heritability coefficients of fertility traits in Large White sows vary from 0.02 to 0.21 [3]. In this regard, it would be interesting to use DNA markers associated with quantitative trait loci (QTLs) of reproduction traits in the selection programs, i.e. to perform the so-called marker-assisted selection, or MAS [4, 5]. MAS facilitates reproducing the existing genetic diversity in the breeding populations and can be used to improve the desirable traits [6].

ESR was one of the first DNA markers of reproduction traits (the number born alive) recommended for use in breeding programs [7]. The association of this marker with reproduction traits has been confirmed by numerous studies both abroad [8-10] and in Russia [11-13]. Such DNA markers as *ECR F18/FUT1* (*Escherichia coli F18/FUT1* receptor), the genetic variants whereof are associated with resistance to post-weaning diarrhea [14, 15], and *MUC4* (mucin 4) [16], the genetic variants whereof are associated with resistance to colibacteriosis [17, 18], do have an indirect effect on the reproduction traits of sows. Besides, today's genetic engineering programs for pigs seek to improve not only the reproduction traits but also the meat and feedlot productivity. This is where breeders apply a number of DNA markers, including the insulin-like growth factor 2 (*IGF2*) gene. Using *IGF2* is primarily related to its paternalistic properties [19], as well as its effects on pig meatiness [20-22].

One important part of integrating MAS in pig breeding programs is to study the possible antagonistic effects of DNA markers on various productive traits. Scientific literature presents very limited data on how the DNA markers of meat and feedlot traits could negatively affect the reproduction traits of sows, or how the markers of reproduction traits could affect the meat and feedlot productivity traits.

This paper is the first to analyze how a group of genetic markers could affect the determination of reproduction traits in Large White and Landrace sows of Russian reproduction. The results indicate that the *IGF2* gene does not significantly affect sow fertility. The contingency of *ECR F18/FUT1*, *ESR* and *MUC4* genes with the breeding value in terms of sow productivity has proven significant and confirmed the correlation of genetic and physiological mechanisms behind the reproduction traits of sows.

This paper seeks to evaluate how the DNA markers *IGF2*, *ECR F18/FUT1*, *ESR,* and *MUC4* affect the reproduction traits in Large White and Landrace sows.

Techniques. Studies were carried out in 2018 and 2019. Research data comprised the primary records of reproduction traits as observed in the first three litters of *Sus scrofa* Large White (n = 894, 2008 to 2018) and Landrace (n = 513, 2010 to 2018) sows at OOO Selection and Hybrid Center, Voronezh Region. The array of data for Large White sows (born of 66 boars and 291 sows) comprised 2,250 entries (2.52 litters per sow on average); for Landrace sows (born of 63 boars and 503 sows), the data contained 1,360 entries (2.65 litters per sow on average). The authors analyzed the absolute values and adjusted phenotypic indices, as well as the estimated breeding value (EBV) in terms of the total number born (TNB) per litter, the number born alive (NBA) per litter, the average birth weight (BW), the adjusted birth weight (BW_{adj}), sow milk yield (weight at weaning, Day 21) (WW), and adjusted sow milk yield (adjusted weight at weaning, Day 21) (WW_{adj}). Phenotypic data on the studied traits followed a normal distribution.

Adjusted BW and WW phenotypic values were calculated by estimating the significance of the paratypic factor effects per Fisher's *F*-test by linear regression equations. The adjusting factors, presumably influencing the variability of the analyzed traits, included the number born alive, the number after transfer, the number at weaning (NW), and the perinatal and lactation period (PLP). When computing the adjusted milk yield, the researchers considered the factor NW in connection with the more significant coefficient of determination, which characterizes the linearity of trait dependency on the analyzed factor. How factors affected the variability of traits was evaluated by multi-factor analysis of variance (ANOVA).

Linear regression equations for the two breeds for the adjusted BW trait (R^2 is the coefficient of determination):

adjusting factor	Large White pig	Landrace
NBA	$y = -0.03x + 1.83$, $R^2 = 0.23$	$y = -0.03x + 1.84$, $R^2 = 0.19$

Linear regression equations for the two breeds for the adjusted WW trait:

adjusting factor	Large White pig	Landrace
NAT	$y = 2.01x + 51.22$, $R^2 = 0.14$	$y = 1.11x + 60.55$, $R^2 = 0.054$
NW	$y = 7.12x + 0.45$, $R^2 = 0.55$	$y = 6.60x + 5.19$, $R^2 = 0.48$
PLP	$y = 2.89x + 4.41$, $R^2 = 0.42$	$y = 2.46x + 13.22$, $R^2 = 0.41$

Genomic DNA was isolated from tissue samples (earmarks) using a DNA-Extran-2 kit by OOO NPF Sintol, Russia. To evaluate the quality and measure the concentration of DNA, a Qubit 2.0 fluorimeter (Invitrogen/Life Technologies, USA) and a NanoDrop8000 spectrophotometer (Thermo Fisher Scientific, USA) were used.

Genotypes by the DNA marker *IGF2* (G→A at 16144, Accession No. AY242112, GenBank, https://www.ncbi.nlm.nih.gov/genbank/) were determined as described by Melnikova et al. [22]. Genotypes by the DNA Markers *ESR* (GG→AT at 65-68, Accession No. HF947272.1, No. AY242112), *ECR* (G→A at 915, Accession No. AY242112), *ECR* (G→A at 915, Accession No. DQ848681) were detected by multiplexed PCR with fluorescent amplification-based specific hybridization (FLASH) on the endpoint using a Fluidigm EP1 high-performance genotyping system (Fluidigm Corporation, USA).

The authors have developed and tested models that evaluate how DNA marker genotypes could affect the variability of absolute and adjusted phenotypic pig reproduction indices; the models use least square means (LSM) and the following equation:

$$y = \mu + YMCG + b_1Par + G_l + e, \quad (1)$$

where y are the considered phenotypic indices of the traits TNB and NBA, μ is the population mean, YMCG is the factor of temporary environmental conditions (year, month, and comparison group), b_1Par is the coefficient of regression and the regression factor (sow litter No.), G_l is the genotype effect for each of the markers *IGF2, ECR F18/FUT1, ESR, MUC4*, e is the residual (unaccounted for) model effects. The factor YMCG must be made part of the model equation as this factor significantly affects the variability of all the analyzed traits in both breeds (*F*-test returns significance at $p < 0.01$), which proves that paratypic effects significantly influence the variability of reproduction traits in pigs. White Large sows were grouped into 115 groups by the factor YMCG (19.6 entries per group on average); Landrace sows were grouped into 163 groups (8.3 entries per group).

The breeding value (EBV) was estimated by the BLUP Animal Model, which takes into accounts kinships and uses an additive kinship matrix.

The model contained the following equations:

$$\text{for TNB and NBA: } y = \mu + YMCG + b_1Par + animal + e, \quad (2)$$

for BW: $y = \mu + YMCG + b_1Par + b_2NBA + animal + e$, (3)
for WW: $y = \mu + YMCG + b_1Par + b_2PLP + animal + e$, (4)

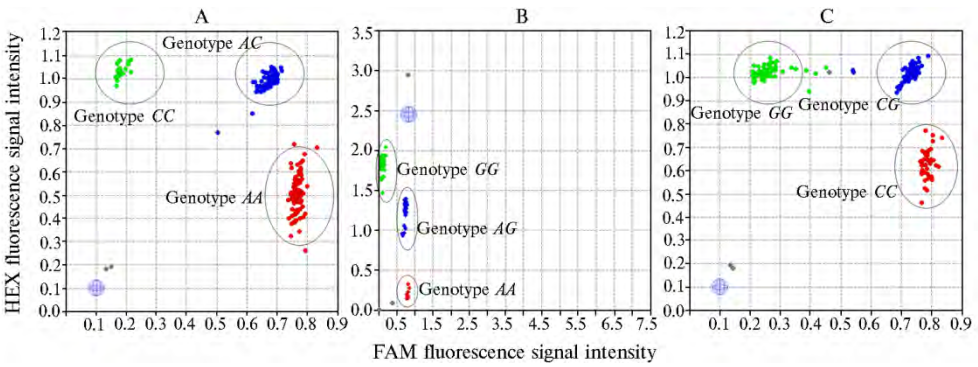
where y is the absolute phenotypic indices, μ is the population mean for TNB and NBA (2), BW (3), WW (4), YMCG is the factor of temporary environmental conditions (year, month, and comparison group), b_1Par is the coefficient of regression and regression factor (sow litter No.), b_2NBA is the coefficient of regression and regression factor (the number born alive), b_2PLP is the coefficient of regression and regression factor (perinatal and lactation period), animal is the animal's additive genetic effect, e is the residual (unaccounted for) model effects.

Heritability coefficients were calculated by restricted maximum likelihood (REML): $h^2 = varA/(varA + varE)$, where varA is the variance of the additive effects of the entire animal genotype, varE is the variance of the residual (unaccounted for) model effects.

ANOVA and LSM calculations were run in STATISTICA 10 (StatSoft, USA). For EBV and ANOVA calculations, BLUPF90 software was used [23]. To characterize the input data arrays, the researchers determined the arithmetic mean of the phenotype in terms of the trait in the sample (μ), the error of the mean ($\pm m_\mu$), and the standard deviation of the trait in the sample (σ).

Results. The figure below shows the results of genotype detection.

Large White sows had 3% to 7% greater values than their Landrace counterparts by all the traits except BW. Attention should be paid to the phenotypic standard deviations, which indicated that the studied populations were promising in terms of genetic progress in fertility. Thus, the observable variability of the analyzed traits indicated that the bred population was likely to demonstrate the theoretical effects of selection, see Table 1. The inheritance of the analyzed traits did not vary too much: 0.115 to 0.232 for Large White sows and 0.010 to 0.338 for Landrace sows. Heritability coefficients were the highest for birth weight ($h^2 = 0.232$ for Large White piglets and $h^2 = 0.338$ for Landrace piglets) due to lower phenotypic variability, which in its turn was due to taking into account the effects of NBA, see Table 1.



Detection of Large White and Landrace pigs (*Sus scrofa*) by the DNA markers *ESR* (A), ***ECR F18/FUT1*** (B) **and *MUC4*** (C)**.** Method: **PCR,** FLASH**,** endpoint detection (Fluidigm EP1, Fluidigm Corporation, USA) (OOO Selection and Hybrid Center, Voronezh Region, 2017-2018).

The population was χ^2-tested to find significant deviations of genotypic occurrences from the population equilibrium for Large White sows in terms of the *IGF2* ($p < 0.01$) gene, as well as for Landrace sows in terms of the *IGF2* ($p < 0.01$), *ECR F18/FUT1* ($p < 0.01$), and *MUC4* ($p < 0.001$) genes, see Table 2. The Robertson homozygosity coefficient (Ca) was the highest for *IGF2* and *ECR F18/FUT1* genotypes: 0.76 and 0.65 for Large White sows vs 0.60 and 0.72 for Landrace sows. At the same time, the distribution of allele frequencies for *IGF2* and *ESR* genes differed radically in the studied samples, which might indicate

breed-specific phenotype manifestations.

1. Reproduction traits in the studied population of Large White and Landrace pigs (*Sus scrofa*) (OOO Selection and Hybrid Center, Voronezh Region, 2017-2018)

Breed	Trait	Values				
		n, sows	*n*, litters	$\mu \pm m_\mu$	σ	h^2
Large White	TNB	894	2250	14.6±0.1	4.1	0.179
	NBA			13.5±0.1	3.8	0.155
	BW			1.45±0.01	0.22	0.232
	WW			76.6±0.3	13.5	0.115
Landrace	TNB	513	1360	13.7±0.1	3.5	0.165
	NBA			12.6±0.1	3.2	0.100
	BW			1.45±0.01	0.23	0.338
	WW			74.6±0.3	12.6	0.010

N o t e. TNB is the total number born; NBA is the number born alive (both values per litter); BW is the average birth weight; WW is the weight at weaning, Day 21; μ is the phenotypic arithmetic mean of the trait in the sample, m_μ is error of the mean, σ is the standard deviation of the trait in the sample, h^2 is the trait heritability coefficient.

2. Distribution of genotype and allele frequencies for *IGF2*, *ECR F18/FUT1*, *ESR*, and *MUC4* genes in Large White and Landrace sows (*Sus scrofa*) ($\mu \pm m_\mu$, OOO Selection and Hybrid Center, Voronezh Region, 2017-2018)

DNA marker	GFD	Genotype			Allele frequency		χ^2	Ca
		11	12	22	1	2		
				Large White				
IGF2	O	0.73±0.01	0.26±0.01	0.01±0.00	0.86±0.01	0.14±0.01	10.7	0.76
	E	0.74	0.24	0.02				
ECR F18/FUT1	O	0.06±0.00	0.34±0.01	0.60±0.01	0.23±0.01	0.77±0.01	2.7	0.65
	E	0.05	0.35	0.60				
ESR	O	0.06±0.00	0.36±0.01	0.58±0.01	0.24±0.01	0.76±0.01	0.0	0.64
	E	0.06	0.36	0.58				
MUC4	O	0.39±0.01	0.46±0.01	0.15±0.01	0.62±0.01	0.38±0.01	1.1	0.53
	E	0.39	0.47	0.14				
				Landrace				
IGF2	O	0.06±0.01	0.44±0.01	0.50±0.01	0.28±0.01	0.72±0.01	7.6	0.60
	E	0.08	0.40	0.52				
ECR F18/FUT1	O	0.02±0.00	0.30±0.01	0.68±0.01	0.17±0.01	0.73±0.01	8.3	0.72
	E	0.03	0.28	0.69				
ESR	O	0.56±0.01	0.36±0.01	0.08±0.01	0.74±0.01	0.26±0.01	3.6	0.62
	E	0.55	0.38	0.07				
MUC4	O	0.38±0.01	0.53±0.01	0.09±0.01	0.64±0.01	0.36±0.01	28.7	0.54
	E	0.41	0.46	0.13				

N o t e. GFD stands for genotype frequency distribution; O stands for observed; E stands for expected. Alleles 1 and 2, and genotypes 11, 12, 22 correspond to alleles *A, G* and genotypes *AA, AG, GG* for *IGF2* and *ECR F18/FUT1*; to alleles *A, C* and genotypes *AA, AC, CC* for *ESR*; and to alleles *C, G* and genotypes *CC, CG, GG* for *MUC4*.

3. Significance of the marker genotypes of Large White and Landrace sows (*Sus scrofa*) for the variability of phenotypic and genetic reproduction traits (OOO Selection and Hybrid Center, Voronezh Region, 2017-2018)

Index	*F*-test							
	Large White (n = 894)				Landrace (n = 513)			
	IGF2	*ECR F18*	*ESR*	*MUC4*	*IGF2*	*ECR F18*	*ESR*	*MUC4*
PHE_{TNB}	0.24	5.79*	6.27*	0.43	1.78	3.08	0.54	0.49
PHE_{NBA}	0.16	10.79*	6.80*	0.23	0.78	1.24	1.85	0.46
PHE_{BW}	1.13	4.28*	2.62	8.75*	0.72	2.10	2.00	3.21*
PHE_{BWadi}	1.44	2.79	1.20	12.59*	1.51	2.63	2.44	4.75*
PHE_{WW}	1.18	0.21	1.84	0.36	6.01*	0.21	0.68	1.47
PHE_{WWadi}	0.30	0.30	1.80	1.00	5.31*	1.14	0.10	0.30
EBV_{TNB}	1.04	2.95	5.30*	3.70*	1.65	3.01	0.87	0.44
EBV_{NBA}	0.02	8.96*	7.90*	4.93*	0.85	0.47	4.51*	0.02
EBV_{BW}	1.44	3.46*	10.90*	22.84*	1.00	2.80	10.69*	3.19*
EBV_{WW}	2.05	3.58*	0.32	2.43	14.99*	2.86	2.30	2.76

N o t e. PHE stands for the phenotypic index, EBV stands for the estimated breeding value; TNB stands for the total number born; NBA stands for the number born alive (both per litter); BW stands for the birth weight, BW_{adj} stands for the mean adjusted birth weight, WW stands for milk yield (weight at weaning, Day 21), WW_{adj} stands for adjusted milk yield (weight at weaning, Day 21).
* The *F*-value is significant at $p < 0.05$ for the corresponding number of degrees of freedom.

Testing the significance of genetic factors (*F*-testing) for each of the ana-

lyzed markers identified marker genotypes, each of which significantly affected the phenotypic manifestations of reproduction traits as well as the variability of EBV, see Table 3.

Thus, F-test returned significance for the marker *IGF2* when applied to the phenotypic and genetic milk-yield indices (PHE_{WW}, PHE_{WWadj}, EBV_{WW}) of Landrace sows. The authors believe that this correlation could be due to the better milk yield of sows featuring the preferable genotype in terms of this marker, see Table 4. Earlier studies [22] found that specimens featuring *IGF2* alleles *A* had the thinnest speck and matured faster. At the same time, sows of this genotype had better milk yield than their alternative-allele heterozygous and homozygous counterparts. Notably, the milk yield measured as the piglet weight at weaning (Day 21) could be affected by the piglet growth rate, i.e. animals carrying the desirable alleles of this marker had greater growth rate, resulting in greater weight at weaning. No significant difference in this marker was identified for Large White sows.

4. Phenotypic means of the studied traits in Large White and Landrace sows (*Sus scrofa*) as a function of marker genotypes ($\mu \pm m_\mu$, OOO Selection and Hybrid Center, Voronezh Region, 2017-2018)

Marker genotype		*n*, farrows	Mean phenotypic indices (LS) for traits					
			TNB	NBA	BW	BW_{adj}	WW	WW_{adj}
Large White (*n* = 2250 farrows)								
IGF2	*AA*	1649	14.9±0.2	13.6±0.1	1.47±0.01	1.46±0.01	76.8±0.5	60.2±0.1
	AG	576	14.8±0.2	13.7±0.2	1.47±0.01	1.46±0.01	76.4±0.7	60.2±0.1
	GG	25	15.2±0.8	13.7±0.8	1.40±0.05	1.39±0.04	73.2±2.6	59.9±0.4
ECR F18/FUT1	*AA*	128	13.8±0.4*	12.2±0.3*	1.50±0.02*	1.45±0.02	76.5±1.2	60.3±0.2
	AG	759	15.0±0.2	13.7±0.2	1.45±0.01	1.44±0.01	76.8±0.6	60.2±0.1
	GG	1363	15.0±0.2	13.8±0.2	1.47±0.01	1.47±0.01	76.5±0.5	60.2±0.1
ESR	*AA*	129	14.7±0.4	13.4±0.3	1.44±0.02	1.42±0.01	75.0±1.2	60.3±0.2
	AC	817	14.6±0.2	13.3±0.2	1.48±0.01	1.46±0.01	77.1±0.6	60.2±0.1
	CC	1304	15.2±0.2*	13.9±0.2*	1.45±0.01	1.45±0.01	76.6±0.5	60.1±0.1
MUC4	*CC*	884	14.9±0.2	13.6±0.2	1.49±0.01	1.48±0.01	75.9±0.6	60.2±0.1
	CG	1034	14.7±0.2	13.5±0.2	1.46±0.01	1.44±0.01	76.3±0.6	60.3±0.1
	GG	332	15.0±0.3	13.6±0.3	1.43±0.01*	1.41±0.01*	76.6±0.8	60.1±0.1
Landrace (*n* = 1360 farrows)								
IGF2	*AA*	87	14.0±0.4	13.0±0.4	1.51±0.03	1.51±0.02	79.5±1.5*	62.3±0.3*
	AG	591	14.0±0.2	12.8±0.2	1.46±0.01	1.46±0.01	75.4±0.7	61.8±0.1
	GG	682	13.6±0.1	12.6±0.2	1.47±0.01	1.46±0.01	74.1±0.7	61.5±0.1
ECR F18/FUT1	*AA*	23	14.3±0.8	13.0±0.7	1.46±0.05	1.46±0.04	73.6±2.7	61.0±0.6
	AG	407	14.2±0.2	12.9±0.2	1.44±0.01	1.44±0.01	75.1±0.8	61.7±0.2
	GG	930	13.7±0.2	12.6±0.2	1.48±0.01	1.47±0.01	75.3±0.6	61.7±0.1
ESR	*AA*	759	13.8±0.2	12.6±0.2	1.46±0.01	1.45±0.01	75.1±0.7	61.7±0.1
	AC	496	14.0±0.2	12.9±0.2	1.48±0.01	1.47±0.01	75.6±0.7	61.7±0.2
	CC	105	13.9±0.4	12.9±0.3	1.51±0.02	1.51±0.02	74.0±1.3	61.7±0.3
MUC4	*CC*	517	13.8±0.2	12.7±0.2	1.49±0.01	1.48±0.01	74.4±0.8	61.7±0.2
	CG	715	13.9±0.2	12.8±0.2	1.47±0.01	1.46±0.01	75.7±0.7	61.8±0.1
	GG	128	13.6±0.3	12.5±0.3	1.43±0.02*	1.42±0.02*	75.0±1.2	61.6±0.2

Note. TNB stands for the total number born; NBA stands for the number born alive (both per litter); BW stands for the birth weight, BW_{adj} stands for the mean adjusted birth weight, WW stands for milk yield (weight at weaning, Day 21), WW_{adj} stands for adjusted milk yield (weight at weaning, Day 21).
* Difference in relation to the alternative homozygous genotype group deemed significant at $p < 0.05$.

ECR F18/FUT1 genotype was found to significantly affect the phenotype in terms of the total number born, the mean birth weight, and the number born alive. The impact of the DNA marker on the genetic value of specimens was confirmed for such indices as prolificacy and mean birth weight. Thus, *AA* (*ECR F18/FUT1*) sows had significantly lower TNB per litter (8.0% to 8.5% negative), but the mean birth weight was significantly larger by 2.0% to 3.0% at $p < 0.05$. According to the mean estimated genetic value in terms of TNB and NBA, AA sows were significantly inferior to heterozygous and homozygous animals with the alternative allele ($X_{EBV(AA)} = -0.50$ and -0.55). This pattern was not confirmed in Landrace sows, as no significant difference in terms of these traits was identified between genotypes.

Results were ambiguous when it came to *ESR* effects on reproduction traits. In Large White sows, ESR genotype had a significant ($p < 0.05$) effect on TNB, NBA, as well as on EBV in terms of mean birth weight. *CC* sows had better phenotypic and genetic characteristics. *ESR* effects on the reproduction phenotype of Landrace sows were not identified; however, mean EBV in terms of TNB and NBA was significantly higher in heterozygous animals: $\bar{X}_{EBV(AC)} = +0.10$, whereas $\bar{X}_{EBV(CC)} = 0.00$ and $\bar{X}_{EBV(AA)} = -0.10$. *CC* sows were the best in terms of mean birth weight.

MUC4 genotypes proved to be significant factors of variability in terms of mean birth weight in both breeds; genotypic effects were significant for both phenotypic (absolute and adjusted) and genetic estimates ($p < 0.05$) *CC* and *CG* sows were superior to *G*-homozygous sows in terms of the absolute and adjusted phenotypic index of piglet birth weight (2.0% to 5.0% for Large White sows and 4.0% to 4.5% for Landrace sows). EBV was significantly higher in *CC* sows than in heterozygous or *GG* sows: $\bar{X}_{EBV(CC)} = +0.01$ for Large White sows, $\bar{X}_{EBV(CC)} = 0.00$ for Landrace sows at negative values in compared groups.

Earlier studies did not identify any effects of *IGF2* polymorphism on the reproductive traits of Large White sows; *ESR-CC* genotype positively correlated with meat and feedlot qualities [24]. Some papers devoted to finding the correlation of *ESR* variants with productivity traits demonstrated the superiority of allele *C* in Large White sows in terms of reproduction traits [25, 26], which is consistent with the authors' data.

ECR F18/FUT1 polymorphism is associated with resistance to colibacteriosis. Horak et al. [27] studied this polymorphism and reported a far lower prolificacy and TNB in black-motley *AA* sows. In turn, Bao et al. [28, 29] found that in terms of litter size, AA sows were superior to AG or GG sows of Duroc and Sutai pigs. This research has identified that AA carriers had greater TNB and NBA, which is consistent with the reports of Bao et al. Fontanesi et al. [30] studied *MUC4* polymorphism and found that allele *G* associated with susceptibility to ETEC (enterotoxigenic *Escherichia coli* K88) did speed up the maturation of Large White (P = 6.66E–04) and Landrace (P = 7.23E–12) pigs, indicating an antagonistic association of alleles in *MUC4* g.8227C>G in terms of growth and ETEC susceptibility. Bannikova [31] discovered the superiority of Large White pigs of CC genotype in terms of prolificacy; however, this research identified no significant correlation of this trait in either breed.

The obtained data have confirmed that some *IGF2*, *ECR F18/FUT1*, *ESR*, *MUC4* genotypes do affect the variability of phenotypic indices of pig reproduction traits and breeding value. *AG* and *GG* genotypes (*ECR F18/FUT1),* and *CC* genotype (*ESR*, White Large sows only) were the best in terms of prolificacy; *CC* and *CG* sows (*MUC4*, both breeds) had the best mean birth weight; *AA* Landrace sows (*IGF2*) had best milk yield. It should be noted, however, that animal fertility traits are largely attributable to paratypic factors (>90%) and the additive effects of a significant number of genes and their combinations that may both positively and negatively affect the biology of reproduction; any factor can turn out to be dominant in affecting the outcome. Besides, pig reproduction traits feature genetic correlations, including negative ones. For NBA and BW, $r_g = -0.33$. This complicates evaluating the effect of genetic markers on the manifestation of the analyzed traits in selecting animals for reproduction, as specimens that carry the desirable alleles of one marker are not guaranteed to have the desirable alleles of other markers.

Thus, it can be recommended to apply marker-assisted selection in combination with conventional BLUP AM methods to decide on whether to select a specimen for breeding or to cull it; this is applicable to sows and boars alike.

When selecting parental genotypes, data on the animals' genetics in terms of *IGF2*, *ECR F18/FUT1*, *ESR*, *MUC4* will help increase the occurrence rate of the desirable alleles and genotypes to improve the genetics of Large White and Landrace pigs in terms of fertility.

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TESTICULAR HISTOMORPHOMETRY AND SPERM CHARACTERISTICS IN Lori RAMS (*Ovis aries* L.)

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Abstract

Lori sheep breed is one of the important genetic resources in the Zagros region of Iran. Accurate knowledge of reproduction of Lori sheep is an essential step for optimal utilization of its potential. The present research aimed to study the testis histology and identify the sperm parameters in Lori rams. Ten healthy rams 2-3 years, were selected. After slaughtering, the testes were removed and epididymis minced to release sperm. The averages of weight, volume, length and diameter of testis were 237.00±6.56 g, 235.30±3.77 cm^3, 9.81±0.32 cm and 7.63±0.42 cm, respectively. Study of testicular sections was shown that the mean diameter, lumen and height of epithelium of seminiferous tubules were 220.04±8.73 μm, 125.10±13.99 μm and 65.68±13.54 μm, respectively. The mean number of Sertoli cells, spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids per each section were 1.90±0.87, 6.02±1.44, 15.30±5.90, 9.65±3.82 and 7.60±2.63, respectively. Rate of motility, viability and normal morphology of released spermatozoa from epididymis were 64.42 %, 83.81 % and 90.5 %, respectively. The knowledge generated in the present study can be used in reproduction for comparative reproductive biology of mammalian species.

Keywords: testis, Sertoli cells, Lori sheep, morphometry

Sheep production is considered as a main economic activity for maximizing income and providing better income to the rural poor of the tropical countries. More than 1000 distinct sheep breeds are found in different parts of the world accounting 1172 million sheep population [1]. Lori sheep (*Ovis aries*) deemed, as dual-purpose sheep is suitable sources of wool and meat, Therefore, is of desirable economic efficiency in nomadic and rural systems [2].

Irrespective of animal species, understanding of reproductive parameters is an important factor in herd expansion [3]. Applied studies on spermatogenesis can be effective in preserving and improving genetics and hence increasing the reproductive capacity of livestock animals [4]. Estimation of reproductive parameters in male animals vary between different area such as the caliper method [5], orchidometry and ultrasonography in human, and goat [6, 7]. Seminiferous tubular length in goat occupied about 85% of parenchyma testis [8]. Testis weight in feral pig was estimated as 23.7±1.8 g [9]. In Arabian ram, seminiferous tubule diameter estimated in early winter is 220.97±12.15 μm [10].

There has been little research reported on reproductive parameters of Iranian breed sheep [11]. The results presented by us in this report supplement this data.

The present research aimed to study histomorphometry of testicular tissues and some sperm characteristics in Lori rams.

Techniques. Sperm parameters. The experiment was conducted at the Agriculture and Natural Resources collage of Lorestan University on ten Lori rams with 2-3 years. The weight of rams was 70.0±5.0 kg. After weighting,

slaughtering (Approved by Institutional Animal Ethics Committee, Lorestan University, Khorram abad 02.2018) and removing the testis from the scrotum, epididymis was incised in multiple locations with a scalpel to release the sperm. The part of epididymis agitated to encourage release of the sperm from the tissue into the surrounding culture media. Sperm collected from the epididymis, diluted with sodium citrate 2.9% at 37 °C. Sperm motility was evaluated by computer assisted semen analysis (CASA, Pro, Way Bulb Type Microscope), Straight movement, zigzag movement (Wavy movement), Non-progressive motile (vibrate movement) and Non-motile were registered by CASA (Computer-aided sperm analysis). The Eosin-Nigrosin staining method, for the differentiation of live and dead spermatozoa was applied on Sperm samples for determination of viability and abnormality rate. Nigrosin stain increases the contrast between the background and head of sperm, making sperm easier to visualize. Eosin stains only the dead sperm, turning them a dark pink, whereas live sperm appears white. The 5 μl of semen was mixed with 20 μl of eosin/nigrosin solution on glass microscope slide [12]. The prepared sperm samples were smeared on microscope slides and fixed by air-drying at room temperature for 10 min before observation. Viable sperm remained unstained and dead cells were totally or partially pink to red/brown. Viable sperms were further classified as morphologically normal or abnormal, depending on the head, midpiece, and tail morphology. At least 200 sperm cells were examined using a light microscope at ×400 magnification [13].

The hypo-osmotic swelling test (HOS) estimates the functional integrity of the sperm's plasma membrane. The HOS test estimates membrane integrity by determining the ability of the sperm membrane to maintain equilibrium between the sperm cell and its environment. Entry of the fluid due to hypo-osmotic stress causes the sperm tail to coil and balloon or "swell." A higher percentage of swollen sperm indicates the presence of sperm having a functional and intact plasma membrane. The 100rL incubated semen was added to the 1ml HOST solution, mixed gently with the pipette and kept at 37 °C for at least 30 minutes. Evaluated 200 spermatozoa by microscopy at ×400 magnification. All forms of swollen tails calculated as live spermatozoa [14, 15].

Immediately after slaughtering, the testes were removed from the scrotum. The diameter and length of testes were measured using a digital caliper (10-rm precision). The weight of testes was determined by digital electrical balance. Volume of testis with mass measurement were estimated using water displacement method (Archimedes' Principle) [10, 16]. To determine the testicular volume, formula (1), was used where m and v show testis weight (grams) and testicular volume (cm^3) respectively:

$$\rho = \frac{m}{V}. \quad [1]$$

Histological sections. The preparation of tissue sections were modified Culling 1974 [17], Fixation (buffer formalin 10%), dehydration (ethylic alcohol with increasing concentrations of 50, 70, 90, and 100%), clearing (xylene), saturation and embedding in paraffin. The embedded samples in paraffin cut using a microtome, such that the sample thickness was 6 μm. Histological sections were stained with hematoxylin-Eosin. Seminiferous epithelium was measured at ×400 magnification using a calibrated ocular micrometer (IS capture-Tucsen). The diameter of seminiferous tubules was taken in 5-10 randomly selected cross sections of apparently round seminiferous tubules per section [18]. Epithelial height was measured in the same tubules. The Germ cells with round nuclei were characterized spermatogium, whereas irregular nuclei were characterized Sertoli cells [19]. The number of Sertoli cells, and the germ cells (spermatogonia, the primary and secondary spermatocytes, spermatids) were deter-

mined using an Olympus microscope (Olympus Corporation, Japan) ×400 magnification and test grid (15×15 cm). The number of tubules in each tissue sections was calculated under a microscope with ×100 magnification and formula (2):

$$V_v = \frac{P(y) \times 100}{P(ref)}, \quad (2)$$

where $P(y)$ stands for sum of points following on the tissue section, and $P(ref)$ denotes total number of points in the test grid [20].

The number of seminiferous tubules per each grid, the number of primary spermatogonia, number of secondary spermatogonia, number of Sertoli cells and the number of spermatid were counted in 10 round or nearly round cross sections of seminiferous tubules chosen randomly.

For the obtained indicators, mean values (M) and standard deviations (±SD) were calculated.

Results. The results related to semen characteristics and testicular morphometry are shown in Table 1 and 2. The seminiferous tubules consist of a multilayered germinal epithelium containing spermatogonia and Sertoli cells (Fig. 1). Spermatogenesis starts with the production of primary spermatocyte on the basement membrane and continues to the production of spermatozoid in the center of the tubules. The spermatogonia in tissue sections were observed as spherical and large, around the seminiferous tubules. Moreover, the Leydig and myoid cells were found normally with blood vessels in the interstitial tissue of the testes (Fig. 2). Number of Sertoli cells in tissue section was low near to spermatogonia (see Fig. 2).

1. Different parameters of testis morphometry and semen in Lori rams (*Ovis aries* L.) (n = 10, Agriculture and Natural Resources College of Lorestan University, Iran)

Sperm classes, %	M±SD	Sperm parameters	M±SD	Parameter of motility	M±SD	Testis parameter	M±SD
Straight movement	64.42±4.22	Cell integrity, %	61.70±11.88	VAP, мкм/с	92.67±1.40	Weight, g	237.0±6.56
Zigzag movement	29.45±5.85	Normality, %	5.90±0.96	VCL, мкм/с	126.76±8.50	Testis weight/body weight, %	0.33±0.10
Vibrate movement	24.88±1.12	Viability, %	83.81±4.61	VSL, мкм/с	74.72±5.13	Volume, cm^3	235.3±3.77
Non-motile	14.24±0.84	Motility, %	86.30±4.73	BCF, Hz	14.46±60	ρ, g/cm^3	1.01±0.02
		Concentration, $\times 10^9$	2.68±0.17	STR, %	80.63±4.60	Diameter, cm	7.63±0.42
				LIN, %	58,94±1,11	Length, cm	9,81±0,32

N o t e. VCL stands for velocity of curved line, VAP for velocity of average path, VSL for velocity of straight line, STR for straightness: STR = (VSL/VAP) × 100; LIN for linearity: LIN = (VSL/VCL) × 100, and BCF for beat frequency.

2. Functional parameters of tissue section of testis in Lori rams (*Ovis aries* L.) (n = 10, Agriculture and Natural Resources College of Lorestan University, Iran)

Parameter	M±SD	Parameter	M±SD
Diameter of seminiferous tubules, μm	220.04±8.73	Density of seminiferous tubules	87.66±1.99
Epithelium length, μm	65.68±13.54	Number of vessels/mm^2	1.37±0.65
Lumen diameter, μm	125.11±13.99	Length of seminiferous tubules in mm^3	10.09±2.18
Number of tubule	4.95±1.07	Number of Sertoli cell/mm^2	1.90±0.87
Number of primary spermatocyte/mm^2	15.30±5.90	Number of Spermatogonium/mm^2	6.02±1.44
Number of secondary spermatocyte/mm^2	9.65±3.82	Number of Spermatid/mm^2	7.60±2.63

The diameter of the seminiferous tubules and epithelial height of seminiferous tubules in Lori ram was recorded 220.04±8.73rm and 65.68±13.54rm, respectively, which was similar to that reported in some domestic ram. The average diameters of seminiferous tubules and epithelial height of seminiferous tubules in goat were 197.20, and 39.2 μm, respectively [5]. Akosman et al. [21] found that the mean diameters of seminiferous tubules in Holstein-Friesian and Simmental breeds of cattle were 226.68 μm and 223.44 μm, respectively. Arrighi et al. [13] reported that the mean diameter of seminiferous tubules in buffaloes

was 243.19 μm. In general, the diameter of seminiferous tubules in most mammals varies from 180 to 350 μm [18, 22].

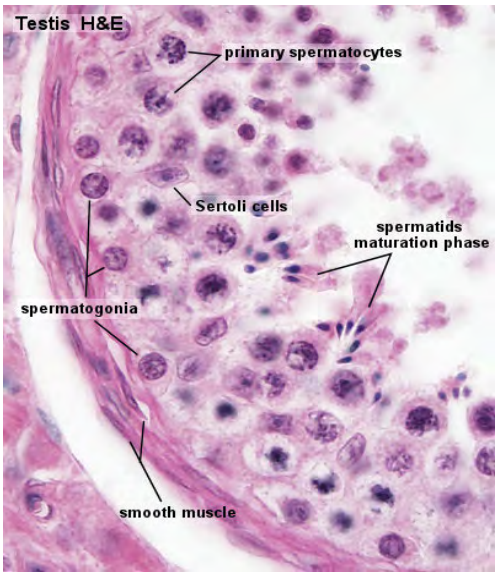

Fig. 1. Different cells of seminiferous tubules in testis of Lori rams (*Ovis aries* L.). Primary spermatocytes, Sertoli cells, spermatogonia, spermatids (maturation stage) and smooth muscles are visible. Histological sections, hematoxylin and eosin staining, light microscopy (Olympus Corporation, Japan), magnification ×400.

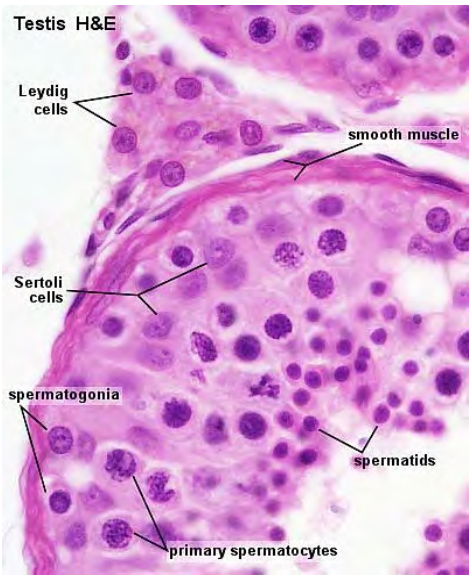

Fig. 2. Interstitial tissue, germ cells and sertoil cell in testis of Lori rams (*Ovis aries* L.). Leydig and Sertolli cells, spermatogonia, primary spermatocytes, spermatids and smooth muscles are visible. Histological sections, hematoxylin and eosin staining, light microscopy (Olympus Corporation, Japan), magnification ×400.

The determination of testis volume and characteristics of seminiferous tubules is an important index in identifying maturity and evaluating spermatogenesis [23, 24]. In this experiment, the testis parameters in Lori rams were similar to the results found by Martins et al. [25] and Mohammadzadeh et al. [5]. These findings were reported for Santa Ines rams [26] and European sheep breeds such as Merino, Corriedale, Suffolk, and Lori rams [27].

The Sertoli cell was the major controller of testis development and efficiency of spermatogenesis [28].). Sperm production capacity in testes is adjusted with the help of events related to proliferation of Sertoli cells that happens before the beginning of sexual maturity. Since Sertoli cells are referred as the nourishing for sperm production, number of Sertoli cells is closely correlated with spermatogenesis process [29]. In the present research, the Sertoli cells with triangular nuclei and expanded cytoplasm were observed at ×400 magnification.

Testis size in the mature males is correlated with reproductive capacity and total number of Sertoli cells in various species of mammals [30]. The mean number of Sertoli (Sustentacular) cells recorded in the Lori ram was 1.90±0.87 (see Fig. 2).

The seminiferous density comprised the main compartment of the testis which changed from 70 to 90% of the testis parenchyma in most mammals [30]. In this study, the seminiferous density obtained was 87.66±1.99% in Lori ram at 2-3 year of ages. In domestic cat, the volume density of the seminiferous tubule was reported to be 90% [18]. Result of this experiment were shown that, large part of the testis was occupied by seminiferous tubules. The percentage of seminiferous tubules in Lori goats was re 80-85 percent [5]. Franca and Godinho [18] reported that the percentages of seminiferous tubules in mammals were not substantially different: 87, 83, 80-87, 73, 88, and 81 percent in rabbits, pigs,

rams, stallions, cats, and bulls respectively. In the present research, the mean numbers of Sertoli cells, spermatogonia, initial spermatocytes, and secondary spermatocytes per mm^2 were estimated as 1.90±0.87, 6.02±1.44, 15.30±5.90, 9.65±3.82, and 7.60±2.60, respectively. Relevant information pertaining to this parameter was found to be very scant in the available literature.

Thus, quantitative study of the number of cells, tubule diameter, testis volume and its other parameters can provide effective help in better understanding of the spermatogenesis process in native breed. Since there is a high correlation between seminiferous density and spermatogenesis, several parameters such as leydig cell, size of nucleus of spermatogonia are need to determined.

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MULTIPLE EFFECTS OF PREECLAMPSIA IN COWS ON POSTNATAL GROWTH AND HEALTH OF OFFSPRING

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Abstract

Scientific interest in such pathologies of pregnancy as functional deficiency of the fetoplacental system, intrauterine growth retardation of embryo and fetus, and preeclampsia (gestosis), is caused not only by their wide spread among productive animals, but also by negative impact on postnatal growth and the health of offspring. The influence of a preeclampsia on incidence of newborn calves of anemia, an omphalitis, inflammatory diseases of gastrointestinal and respiratory tracts and also intensity of growth in the first two months of life was studied on red-motley cows in 2016 in the conditions of a large dairy complex (Voronezhpishcheprodukt Co Ltd, Novousmansky Rregion, Voronezh Province). Total of 45 cows (*Bos taurus taurus*) with a gestation period of 248-255 days were examined, including 31 with clinical signs of preeclampsia and 14 with the physiological course of pregnancy, and calves obtained from them (n = 45). The cows were assessed for clinical signs of preeclampsia (pathological swelling of mammary gland, ventral abdominal wall, dewlap, pelvic extremities, arterial hypertension and proteinuria), the blood concentrations of progesterone, estradiol and dehydroepiandrosterone sulfate (DHEA-S) were determined, the endogenous intoxication features were examined, i.e. concentration of medium size peptides (MSP), effective (ECA) and total (TCA) serum albumin concentration, the toxicity index TI = (TCA/ECA) – 1 and the intoxication coefficient IC = (MSP/ECA) × 1000 were calculated. Within 1-3 hours after birth, the diameter of the calves' navel was measured; an increase in the diameter of the navel of more than 18.0 mm was considered as a retrospective indicator of the fetoplacental blood circulation disorder. On day 1, day 30 and day 60 the calves' bodyweight was measured; the absolute, relative and average daily weight gain for the first and second months of life was calculated. During the first 60 days of life, the calves underwent constant clinical observation, the incidence of anemia, an omphalitis, gastroenteritis, bronchitis, bronchopneumonia and severity of disease course were considered. Frequency of anemia cases in calves from mother cows with preeclampsia was 1.80-fold, of omphalitis 9.40-fold, of gastroenteritis 1.80-fold, of bronchitis 1.75-fold, and of bronchopneumonia 2.71-fild as compared to animals from cows with the physiological course of pregnancy. For the first month of life, an average daily bodyweight gain of the calves received from the cows suffered from a preeclampsia was 46.2 % ($p < 0.01$) less than that of offspring of healthy cows. We revealed significant individual differences in the protein concentration in urine, endogenous intoxication parameters (ECA/TCA, MSP, IC) and the blood content of steroid hormones (progesterone, estradiol, DHEA-S) among the cows with the symptoms of preeclampsia. An inverse relationship was established between the rate of intoxication in cows with preeclampsia and the average daily bodyweight gain in their calves during the first month (r_S = –0.79, $p < 0.01$) and the second (r_S = –0.58, $p < 0.01$) month of life. As proteinuria in cows increases, the probability of omphalitis progression ($r_{\tau\text{-}K}$ = +0.32, $p < 0.05$) and anemia ($r_{\tau\text{-}K}$ = +0.33, $p < 0.05$) of calves increases too. Statistically significant links are found between accumulation of MMP in blood of cows suffering from preeclampsia, and probability of omphalitis ($r_{\tau\text{-}K}$ = +0.36, $p < 0.01$) and bronchopneumonia ($r_{\tau\text{-}K}$ = +0.35, $p < 0.05$) progression in their offspring and also between coefficient of intoxication and probability of progression of these diseases (with τ-Kendall's coefficients +0.35, +0.35 and +0.38 respectively at $p < 0.05$). The functional disorder of fetoplacental system is associated with progression of omphalitis ($r_{\tau\text{-}K}$ = +0.33, $p < 0.05$), gastroenteritis ($r_{\tau\text{-}K}$ = +0.49, $p < 0.01$) and also early (within the first week of life) manifestation of bronchitis ($r_{\tau\text{-}K}$ = +0.48, $p < 0.01$) in calves, and fetoplacental blood circulation dis-

order is associated with gastroenteritis ($r_{\tau\text{-}K}$ = +0.77, p < 0.01) and bronchopneumonia ($r_{\tau\text{-}K}$ = +0.75, p < 0.01). ROC-analysis showed that the factor of intoxication and the content of MSP in the blood serum of cows suffering from preeclampsia may serve as predictors of omphalitis and bronchopneumonia progression in newborn calves. Blood concentration of estradiol and the ratio of progesterone/estradiol in mother cows are valuable indicators to predict a severe course of gastroenteritis and anemia in their calves. High specificity (94.7 %) for predicting anemia progression for newborn calves is detected for proteinuria of more than 2.0 g/l.

Keywords: preeclampsia, pregnancy, *Bos taurus taurus*, cows, calves, arterial hypertension, proteinuria, anemia, omphalitis, gastroenteritis, respiratory diseases, average daily bodyweight gain, ROC-analysis

Preeclampsia (gestosis) is a pathology of pregnant cows and heifers that manifests as multiple-organ functional failure, systemic endotheliosis, disordered uterine-placental and fetus-placental circulation, endogenous intoxication, and disintegration of the mother-fetus hormonal status [1-4]. According to Kolchina [4], preeclampsia occurs in 42.0 to 69.4% of all cows and heifers aged 32 to 36 weeks.

Despite considerable interest in the problem, the pathogenesis of preeclampsia in cows remains understudied [1, 4, 5]. Health researchers believe [6-8] that preeclampsia is a result of maternal exposure to a number of neurogenic, hormonal, immunologic, placental, and genetic factors during pregnancy. The disease has two stages of progression [7]. Stage One includes disordered trophoblast invasion and remodeled spiral arteries in the endometrium, vascular spasm, reduced uterine-placental perfusion [6]. Stage Two includes systemic inflammatory response and multiple-organ failure [6, 8]. Numerous attempts to reproduce preeclampsia experimentally in lab animals have failed [9]. When ligating the uterine vessels, placental ischemia and fetal growth delays were identified in pregnant rats, rabbits, and monkeys; however, changes in the rheological and coagulation properties of blood, microcirculation disorders, and arterial hypertension, all of which are characteristic of preeclampsia, did not develop [9].

Two groups of causative factors of preeclampsia are identified in dairy cows. The first group is associated with extragenital pathology (hepatopathy and nephropathy) accompanied by endogenous intoxication, metabolic disorders (changed protein and lipid composition of blood, oxidative stress), affected immune status (activation of lysozyme and the complement system), increased aggregation of erythrocytes and thrombocytes, changes in the rheological and coagulation properties of blood, microcirculation disorders and damaged capillary endothelium, especially in the emerging placental tissue [1, 5, 10]. The second group is associated with an endocrine deficiency that adversely affects uterine-placental circulation and causes a diffusion-perfusion failure in the placenta [4, 10]. According to Nezhdanov et al. [10], negative factors may overlap and have a cumulative effect in early preeclampsia.

Classic signs of preeclampsia in cows are arterial hypertension, proteinuria (nephropathy), pathological edema of the mammary gland, ventral abdominal wall, dewlap, and pelvic limbs [1, 4, 10]. These symptoms may manifest individually or in combinations proportionally to the severity of the disease [2, 4, 10]. According to Kolchina [4], edemas are noted in 35.3% of preeclampsia-affected cows and heifers, nephropathy in 4.7%, and polysystemic forms in 40.2%. The disease is characterized by the functional failure of the antioxidant protection system and fetoplacental complex [4].

Morphological studies of maternal and fetal parts of the placenta in preeclampsia-affected cows [4, 11] identified deep vascular changes with elements of congestive hyperemia, hemorrhage, extravasates, and signs of dystrophy. Calves born of such cows have blood-filled umbilical vessels with enlarged lumen and diameter, a partially loosened and interrupting intima endothelium,

blood-filled muscular-shell vessels, scattered myocytes and vacuoles in-between, enlarged and loosened adventitia collagen fibers [12].

These morphofunctional changes in pregnant cows are accompanied by significant changes in metabolism [1, 4] as well as in the structure and functional status of fetal organs and systems [2]. Such changes result in less viable offspring [4, 12, 13] with organ and system maldevelopment at cellular and subcellular levels [14-17], lower natural resistance [2, 12] and lesser adaptive capacities [15, 18, 19].

This paper is the first to demonstrate the diagnostic value of identifying the markers of endogenous intoxication, nephropathy, disordered fetoplacental circulation and endocrine function in preeclampsia-affected cows so as to predict neonatal diseases in their offspring. The researchers have found a correlation between the severity of endogenous intoxication in preeclampsia-affected cows and the daily average weight gain of their calves over the first and the second months of life; another correlation is between intoxication and the probability of omphalitis and bronchopneumonia. Functional failure of the fetoplacental system in cows is shown to be related to omphalitis, gastroenteritis, and early (Week 1) manifestation of bronchitis in calves, while malfunctioning fetoplacental circulation is shown to correlate with gastroenteritis and bronchopneumonia in the offspring. It has been found that a higher concentration of protein in mother cows' urine is associated with a greater likelihood of anemia in newborn calves.

The goal was to study how morphofunctional disorders in preeclampsia-affected cows could affect newborn calves' susceptibility to anemia, omphalitis, inflammatory gastrointestinal or respiratory tract diseases, as well as their growth rate in the first two months of life.

Techniques. The research was carried out in 2016 during the winter housing season at OOO Voronezhpishcheprodukt facilities, Novusmansky District, Voronezh Province. The research team examined a total of 45 *Bos taurus taurus* red-motley cows with a gestation age of 248 to 255 days, including 31 cows with clinical manifestations of preeclampsia (Group I) and 14 animals with a normal pregnancy (Group II), as well as their calves (n = 45). Over the previous lactation, the cows had had a milk yield of 6,278 to 9,796 kg with a fat content of 3.54 to 3.96%.

Clinical tests evaluated habitus; nutritional status; edemas in mammary glands, ventral abdominal walls, dewlap, and pelvic limbs; rectal temperature; heart rate and respiratory rate (HR and RR, respectively); systolic and diastolic blood pressure (SP and DP). Researchers also considered the course of delivery (normal delivery, assisted delivery and why it was necessary). Each calf's navel diameter was measured at the base within 1 to 3 hours of life. The navel diameter in excess of 18.0 mm was considered a retrospective marker of fetoplacental circulation disorders [12].

Calves were weighed on Days 1, 30, and 60 to calculate absolute, relative, and daily average body weight gain over the first and the second months of life. For the first 60 days, calves were subject to continuous clinical monitoring with the following readings: rectal temperature, HR and RR, condition of visible mucosa, laryngeal/tracheal/intercostal sensitivity to palpation, time and nature of cough, wheezing, and shortness of breath, nasal bleeding and ocular secretions. Pulmonary ventilation (respiratory volume and minute volume) were measured using an SSP spirometer (KPO Medapparatura, Ukraine) and a valve mask. When diagnosing omphalitis, the researchers paid attention to the presence or absence of edemas in the umbilical area, in the lateral or lower abdominal wall; thickened umbilical cord and navel; skin redness at the navel base; umbilical cord stump color and consistency; navel and umbilical ring tenderness; local

temperature increase. The intestinal inflammatory process was indicated by the fecal presence of soluble protein, hemoglobin, leukocytes, and pH imbalance (too acidic, i.e. <7.0, or too alkaline, i.e. >7.5) [20]. In the case of diarrhea, researchers paid attention to the frequency of defecation, the odor, color, and consistency of feces, the skin turgor, the eyeball retraction, the anal sphincter tone, and the abdominal wall sensitivity to palpation. In the case of the respiratory syndrome, calve condition was WI-scored [21] with due account of when the first clinical signs manifested, when bronchitis manifestations peaked, how severe the disease was, and whether bronchopneumonia complications occurred.

For laboratory tests, blood was sampled from cows 30 days before expected calving, or 24 hours after birth from calves. Samples were taken from the jugular vein using a commercial vacuum pump and EDTA as an anticoagulant. Serum was obtained by an anticoagulant-free centrifuge at 4,000 rpm (UC-1612, ULAB, China) at room temperature over 10 minutes. Serum samples were frozen and stored in liquid nitrogen at –196 °C before biochemical testing.

Cow urine samples were collected in sterile polypropylene containers 30 days before expected calving. The urine concentration of protein was estimated (a PocketChem PU-4210 automatic analyzer, Arkrey, Japan).

Hematological tests used a Micros-60 analyzer (Horiba ABX, France); a leukogram was computed by the standard procedure applied to Romanovsky-stained blood smears. The concentration of progesterone, estradiol, and dehydroepiandrosterone sulfate (DHEA-S) in cow serum was determined by solid-phase immunoassay (ELISA) using a Uniplan AIFR-01 analyzer (Zao Pikon, Russia) and commercial test kits by ZAO NVO Immunotech, Russia. Medium-sized peptide (MSP) content of blood serum was determined by the author's methodology using a Shimadzu UV-1700 spectrophotometer (Shimadzu, Japan). Effective and total albumin concentration (EAC, TAC) in the blood serum was measured (a Shimadzu RF-5301 PC spectrofluorophotometer, Shimadzu, Japan) and kits made by the Physico-Chemical Medicine Research Institute, Russia.

Integral endogenous intoxication indices were calculated by the formulas [23]:

$$\text{Toxicity index (TI)} = (\text{TAC}/\text{EAC}) - 1,$$

$$\text{Intoxication coefficient (IC)} = (\text{MMP}/\text{EAC}) \times 1000.$$

Severity was scored as follows: 0 for healthy cows; 1 for mild disease; 2 for moderate disease; 3 for severe disease. ROC analysis per DeLong et al. [24] was used to determine the diagnostic value of identifying the markers of endogenous intoxication, nephropathy, disordered fetoplacental circulation and endocrine function in preeclampsia-affected cows so as to predict neonatal diseases in their offspring (anemia, omphalitis, gastroenteritis, and bronchopneumonia). To that end, the authors analyzed the ROC parameters, in particular, the area under curve (AUC), which characterizes the diagnostic value of an indicator: excellent at 0.9 to 1.0; very good at 0.8 to 0.9; good at 0.7 to 0.8; average at 0.6 to 0.7; unsatisfactory at 0.6 or lower. Other parameters were sensitivity (%), specificity (%), and cut-off points.

Data were processed statistically by Statistica 8.0 (StatSoft Inc., USA) and IBM SPSS Statistics 20.0.0.1 (IBM Corp., USA). Arithmetic means and standard deviations ($M \pm SD$), minima (min), maxima (max), and the median (*Me*) were calculated. Since the distribution of most indicators was not Gaussian as indicated by Shapiro-Wilk W-test, the significance of inter-group difference was found by comparing the medians by the nonparametric Wilcoxon test. Indicator correlation was identified by nonparametric Spearman (r_S) and τ-Kendall ($r_{\tau\text{-}K}$) correlation tests. After applying all the methods, the null hypothesis would be rejected at $p < 0.05$.

Results. Clinical tests of preeclampsia-affected cows revealed higher heart

rates (96.8±5.5; *Me* = 98.0) and respiratory rates (26.7±4.2; *Me* = 26.0), an increase of 45.2 ($p < 0.01$) and 44.4% ($p < 0.05$) compared to the normal-pregnancy group. Systolic blood pressure was within 120.0 to 152.0 mmHg (*Me* = 138.0 mmHg), diastolic within 92.0 to 112.0 mmHg (*Me* = 96.0 mmHg), which was 30.2 ($p < 0.01$) and 52.4% ($p < 0.01$) higher than in Group II. Pathological edema of ventral abdominal wall, dewlap, and pelvic limbs was noted in 80.6% of cows with arterial hypertension.

Preeclampsia-affected animals had significant interindividual differences in the urine protein concentrations, endogenous intoxication indices (EAC, TAC, MSP, and IC), and in the steroid hormones concentration in the serum, see Table 1. The urine protein concentration was 1.0 to 3.0 g/l (*Me* = 1.0 g/l) in Group I and 0.0 to 0.3 g/l (*Me* = 0.0 g/l) in Group II. Higher proteinuria was associated with greater likelihood of omphalitis ($r_{\tau\text{-}K} = +0.32$ at $p < 0.05$) and anemia ($r_{\tau\text{-}K} = +0.33$ at $p < 0.05$) in the offspring.

No significant difference in the total and effective albumin concentrations or the EAC/TAC ratio was identified between the groups. The concentration of medium-sized peptides (0.55±0.16 cu; *Me* = 0.55) and intoxication coefficient (23.4±7.4 cu; *Me* = 22.5) in preeclampsia-affected cows was above the median values of the normal-pregnancy group, a difference of 71.9 ($p < 0.05$) and 67.9% ($p < 0.05$), respectively; significant intra-sample variation was observed. Correlation analysis identified statistically significant correlation between the accumulation of medium-sized peptides in the serum of Group I cows and the likelihood of omphalitis ($r_{\tau\text{-}K} = +0.36$ at $p < 0.01$) and bronchopneumonia ($r_{\tau\text{-}K} = +0.35$ at $p < 0.05$) in the offspring; the intoxication coefficient, too, correlated with the likelihood of these diseases (τ-Kendall of +0.35 and +0.38, respectively, at $p < 0.05$).

1. Clinical and laboratory markers of preeclampsia in red-motley cows (Novousmansky District, Voronezh Province, 2016)

Indicator	*M*±SD	min-max	*Me*
RR min	26.7±4.2	26.0-32.0	26.0*
	18.9±2.0	16.0-22.0	18.0
HR min	96.8±5.5	86.0-104.0	98.0**
	68.5±5.3	60.0-75.0	67.5
systolic BP, mmHg	139.9±10.0	120.0-152.0	138.0**
	105.2±6.8	95.0-112.0	106.0
diastolic BP, mmHg	100.8±7.3	92.0-112.0	96.0**
	65.4±8.7	56.0-78.0	63.0
Proteinuria, g/l	1.7±0.9	1.0-3.0	1.0***
	0.1±0.1	0.0-0.3	0.0
EAC, g/l	23.1±4.2	14.8-30.2	23.7
	26.1±4.6	18.3-36.0	24.9
TAC, g/l	37.0±5.0	27.5-48.9	37.0
	39.3±4.2	33.3-48.3	38.1
EAC/TAC, %	62.2±6.3	53.7-74.9	60.8
	66.5±10.2	54.7-93.7	63.0
MSP, cu	0.55±0.16	0.30-0.78	0.55*
	0.35±0.11	0.23-0.56	0.32
CI	23.4±7.4	11.1-33.9	22.5*
	13.5±4.0	7.2-19.4	13.4
Progesterone, nmol/l	47.7±13.8	21.6-82.4	43.6*
	67.3±22.2	50.7-110.7	52.5
Estradiol, pmol/l	96.8±53.4	32.4-245.1	90.4***
	435.9±114.1	245.1-582.9	443.1
DHEA-S, μmol/l	0.31±0.14	0.11-0.46	0.30*
	0.49±0.23	0.14-0.85	0.63

N o t e. Values above the line are those of preeclampsia-affected cows ($n = 31$); values below the line are those of the normal-pregnancy group ($n = 14$) HR = heart rate; RR = respiratory rate; BP = blood pressure; EAC = effective albumin concentration; TAC = total albumin concentration; MSP = medium-sized peptides; IC = intoxication coefficient; DHEA-S = dehydroepiandrosterone sulfate.
*, **, and *** Inter-group difference is statistically significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

The blood level of progesterone (47.7±13.8 nmol/l; *Me* = 43.6 nmol/l),

estradiol (96.8±53.4 pmol/l; *Me* = 90.4 pmol/L), and DHEA-S (0.31±0.14 μmol/l; *Me* = 0.30 μmol/l) in Group I was 17.0 ($p < 0.05$), 79.6 ($p < 0.001$), and 52.4% ($p < 0.05$) lower than in Group II. A functional failure of the fetoplacental system we identified in 71.0% of preeclampsia-affected cows. In Group I, a functional failure of the fetoplacental system was associated with omphalitis ($r_{\tau\text{-}K} = +0.33$ at $p < 0.05$) and gastroenteritis ($r_{\tau\text{-}K} = +0.49$ at $p < 0.01$), as well as early (Week 1) manifestation of bronchitis ($r_{\tau\text{-}K} = +0.48$ at $p < 0.01$) in the offspring.

In calves born of preeclampsia-affected cows, the navel diameter was 17.0 to 21.00 mm (18.4±1.1 mm; *Me* = 18.0 mm), which was 33.3% ($p < 0.01$) bigger than that of the calves born from normal pregnancy: 13.0 to16.0 mm (13.9±1.1 mm; *Me* = 13.5 mm). In 38.9% of calves, it was > 18.0 mm, a sign of perinatal fetoplacental circulation disorder [7]. The research team identified a statistically significant correlation of fetoplacental circulation disorders in Group I and the likelihood of gastroenteritis ($r_{\tau\text{-}K} = +0.77$ at $p < 0.01$) and bronchopneumonia ($r_{\tau\text{-}K} = +0.75$ at $p < 0.01$) in their offspring. The severity of gastroenteritis and pneumonia did correlate with the navel diameter in the first three hours of life at Spearman rank correlation coefficients of +0.82 and +0.72, respectively, at $p < 0.01$.

Group I calves had 9.40 times the occurrence of omphalitis, 1.80 times the occurrence of bronchitis, 2.71 times the occurrence of bronchopneumonia, and 1.80 times the occurrence of anemia in Group II, see Table 2. In the group of calves born of preeclampsia-affected cows, omphalitis was severe in 38.7% of all cases, anemia (hemoglobin < 70 g/l) was in 9.7% of all cases, with no such complications in the group of normal pregnancy.

2. Incidence of neonatal diseases in calves born of red-motley cows: normal pregnancy vs preeclampsia (Novousmansky District, Voronezh Province, 2016)

Indicator	Normal pregnancy (*n* = 14)	Preeclampsia (*n* = 31)
Omphalitis, animals (%):		
total	1 (7.2)	21 (67.7)
severe	0 (0)	12 (38.7)
Gastroenteritis, animals (%)		
total	6 (42.9)	24 (77.4)
severe	4 (28.6)	20 (64.5)
Bronchitis, animals (%)	8 (57.1)	31 (100)
Bronchopneumonia, animals (%)	2 (14.3)	12 (38.7)
Anemia, animals (%)		
total	2 (14.3)	8 (25.8)
severe	0 (0)	3 (9.7)

Omphalitis in calves was largely attributable to endogenous intoxication, endocrine placenta dysfunction, and disordered fetoplacental circulation in the perinatal period. ROC analysis showed that the intoxication coefficient, MSP and estradiol concentration in the serum of preeclampsia-affected cows could be used as neonatal omphalitis predictors. These indices featured a good diagnostic value (AUC = 0.747, 0.753, 0.706), high sensitivity (76.9% for all indicators), and specificity (52.9; 70.6, and 88.2%). Omphalitis risk cut-off points were >15.92, 0.412 cu or < 83.6 pmol/l.

The diagnostic value of an enlarged navel (a marker of perinatal fetoplacental circulation disorder in cows) for prediction of calf omphalitis was excellent (AUC = 0.953): sensitivity = 84.6%, specificity 100.0% at a cut-off point of 17.5 mm.

Respiratory diseases in calves born of preeclampsia-affected cows were due to endogenous intoxication, functional failure of the fetoplacental system, and circulatory disorders. Neonatal bronchopneumonia predictors are the intoxi-

cation coefficient, the MSP and estradiol content of the maternal blood serum, and the navel diameter within three hours post-birth. These indices had a very good, good, or excellent diagnostic value (AUC = 0.812; 0.782; 0.707 and 0.907), high sensitivity (85.7; 85.7; 77.8; and 88.9%), and specificity (59.1; 81.8; 77.3 and 77.3%). Bronchopneumonia risk cut-off points were 18.08 and 0.555 cu, < 71.2 pmol/l and > 17.5 mm. The greatest value for diagnosing early (Week 1) bronchitis in calves was attached to the estradiol (AUC = 0.729) and progesterone/estradiol ratio (AUC = 0.750) in maternal blood serum: the sensitivity was 70.8 and 54.2%; the specificity was 85.7 and 85.7%; cut-off points were < 116.8 pmol/l and > 571.0:1.

For predicting severe gastroenteritis in calves, the concentration of estradiol (AUC = 0.710) and the progesterone/estradiol ratio (AUC = 0.734) in cow serum, as well as the calf navel diameter within three hours post-birth (AUC = 0.782), were of greatest value: these indicators had a sensitivity of 77.8, 77.8, and 61.1%; a specificity of 77.3, 71.4, and 85.7%; and cut-off points of < 103.7 pmol/l, > 413.9:1, and > 17.5 mm.

Anemia in calves was mainly associated with the functional failure of the fetoplacental system. ROC analysis showed that the concentration of estradiol, DHEA-S, and the progesterone/estradiol ratio in preeclampsia-affected cows could predict severe neonatal anemia in calves at hemoglobin < 70 g/l. These indices had good diagnostic value (AUC = 0.782; 0.750, and 0.750), sensitivity (100.0% for all indices) and specificity (61.3; 71.0; and 51.6%); cut-off points for severe anemia in calves were < 78.0 pmol/l, < 0.229 μmol/l, and > 510.1:1.

Urine protein concentrations in preeclampsia-affected cows also had a good diagnostic value (AUC = 0.758) for prediction of neonatal anemia in calves. At low sensitivity (20.0%), this indicator had high specificity (94.7%); the cut-off point was > 2.0 g/l.

3. Body weight of red-motley calves: normal pregnancy vs preeclampsia (Novousmansky District, Voronezh Province, 2016)

Indicator	*M*±SD	min-max	*Me*
Body weight, Day 1, kg	39.4±6.4	27.0-50.0	40.0
	40.3±3.7	35.0-47.0	40.0
Body weight, Day 30, kg	47.2±7.9	28.0-59.0	48.0*
	52.7±5.0	44.0-60.0	53.0
Absolute body weight gain over Month 1, kg	7.7±3.3	1.0-13.0	7.0**
	12.3±2.2	7.0-15.0	13.0
Relative body weight gain over Month 1, %	19.6±8.8	3.7-38.2	17.5**
	30.6±5.3	17.9-36.1	30.6
Daily average body weight gain over Month 1, g	254.7±106.6	33.0-433.0	233.0**
	410.8±72.6	233.0-500.0	433.0
Body weight, Day 60, kg	62.6±8.4	38.0-75.0	64.5
	68.6±6.4	59.0-78.0	68.0
Absolute body weight gain over Month 2, kg	15.1±2.5	10.0-19.0	16.0
	17.2±1.9	13.0-20.0	18.0
% Relative body weight gain over Month 2, %	32.1±5.9	22.8-43.9	31.4
	33.6±3.1	28.2-36.7	35.1
Daily average body weight gain over Month 2, g	490.5±79.0	333.0-633.0	483.0
	573.2±63.3	433.0-660.0	660.0

N o t e. Values above the line are those of preeclampsia-affected cows (n = 31); values below the line are those of the normal-pregnancy group (n = 14)
* and ** Inter-group difference is statistically significant at $p < 0.05$ and $p < 0.01$, respectively.

Groups I and II did not differ significantly in body weight on Day 1. On Day 30, calves from preeclampsia-affected cows weighed 9.4% less ($p < 0.05$) than those born after a normal pregnancy; by Day 60, the difference in live mass leveled, see Table 3. The absolute body weight gain over the first month of life was 46.2% lower in Group I calves ($p < 0.01$), while the relative and daily average gain was 42.8% ($p < 0.01$) and 46.2% ($p < 0.01$) than the median values of

Group II offspring. Correlation analysis identified a statistically significant correlation of daily average body weight gain over the first month of life and endogenous intoxication indices: intoxication coefficient ($r_S = -0.79$ at $p < 0.01$), blood MSP ($r_S = -0.73$ at $p < 0.01$), estradiol concentration ($r_S = +0.37$ at $p < 0.05$) and progesterone/estradiol ratio ($r_S = -0.50$ at $p < 0.01$) in maternal serum, which reflects the functional status of the fetoplacental system. An inverse correlation of the daily average weight gain in calves and endogenous intoxication indices in preeclampsia-affected cows: intoxication coefficient ($r_S = -0.58$ at $p < 0.01$) and blood MSP concentration ($r_S = -0.57$ at $p < 0.01$) was observed over the second month, too.

Researchers' interest in such pregnancy pathologies as a functional failure of the fetoplacental system, intrauterine embryo maldevelopment and preeclampsia is attributable not only to the high incidence of these in productive animals [2, 4, 25] but also to their negative impact on the postnatal growth and health of the offspring [9, 13, 16]. According to the concept of developmental origins of health and disease, DOHaD, this or that factor present during critical intrauterine fetal development may have remote consequences in the postnatal ontogenesis [26-28]. Other researchers have earlier noted that preeclampsia-affected cows give birth to less viable calves [2, 29, 30] that have a higher incidence of omphalitis [12], gastrointestinal diseases [31], and respiratory diseases [18, 32]. However, it is not clear yet how maternal preeclampsia actually affects the morphofunctional status of newborn calves [2, 30].

The data presented herein suggest that preeclampsia-affected cows differ significantly in terms of urine protein concentration, endogenous intoxication indices (EAC/TAC, MSP, IC) and steroid hormone concentrations (progesterone, estradiol, DHEA-S) in serum, which affects the neonatal condition of their calves. ROC analysis proves that the intoxication coefficient and MSP concentration in the serum of preeclampsia-affected cows can be used as predictors of neonatal omphalitis and bronchopneumonia in calves. This data is consistent with earlier reports [33, 34] and indicates a close pathogenetic correlation of neonatal omphalitis and respiratory diseases in calves. It is known that MSPs can block cell receptors, load the active centers of the albumin molecule, and compete against regulatory peptides, which adversely affects humoral regulation and is toxic for the developing fetus [23, 25, 35]. The authors have shown that intrauterine intoxication negatively affects the daily average body weight gain in calves within the first two months of life: calves born of normal pregnancy had 46.2% gain over the first month ($p < 0.01$) than their counterparts born of preeclampsia-affected cows. The research has identified an inverse correlation of intoxication coefficient in preeclampsia-affected cows and the daily average body weight gain of their calves over the first month of life ($rS = 0.79$ at $p < 0.01$) or second month of life ($rS = 0.58$ at $p < 0.01$).

Finding the content and ratio of steroid hormones in the serum of deep-bone cows can identify the functional failure of the fetoplacental system [25, 36] to predict neonatal diseases in the offspring [4, 37]. Finding the estradiol concentration and progesterone/estradiol ratio in the serum has proven useful for predicting severe gastroenteritis and anemia in calves. Earlier, Lavrijsen et al. [38] experimented in vitro to demonstrate the dose-dependent progesterone effects on the fetal erythropoiesis in cattle, while Safonov et al. [37] showed how neonatal respiratory diseases in calves could be predicted on the basis of the progesterone and estradiol content and ratio in maternal serum.

Proteinuria in cows approaching labor indicates a functional disorder of the kidneys [2, 4, 10, 39]. This research has identified a high specificity of urine protein concentration in deep-bone cows (94.7%) for predicting anemia in calves

(cut-off at >2.0 g/l); this indicator is recommendable for use at farms.

It is known that preeclampsia in cows is associated with fetoplacental circulation disorders and the altered diameter and structure of umbilical vessels [4, 10-12]. Zolotaryov et al. [12] found that if a calf has a navel diameter of > 18.00 mm at the base within the first three hours post-birth, this retrospectively indicates fetoplacental circulation disorders and a high probability of omphalitis. In this study, this parameter did differ significantly in normal-birth vs preeclampsia groups ($p < 0.01$). The diagnostic value of an enlarged navel for prediction of calf omphalitis was excellent (AUC = 0.959): sensitivity = 84.6%, specificity 100.0% at a cut-off point of 17.5 mm. Note that the correlation of the navel diameter in newborn calves and the likelihood of gastroenteritis or bronchopneumonia, as well as the severity of these diseases as described in this report, was first identified by this research and is highly significant ($p < 0.01$). This research proves that calves are rendered susceptive to neonatal gastrointestinal and respiratory diseases not by omphalitis, but by deep morphofunctional changes in the mother-placenta-fetus system in preeclampsia, which precedes omphalitis.

Thus, determining the endogenous intoxication indices (medium-sized peptide content, effective albumin concentration, and the intoxication coefficient), the concentration of steroid hormones (progesterone, estradiol, and dehydroepiandrosterone sulfate) in the serum, and protein concentration in urine in preeclampsia-affected cows 25 to 32 days before expected calving helps not only objectively evaluate the animal's condition but also predict neonatal diseases and body weight gain in their offspring. Measuring the navel diameter at the abdominal wall base within three hours post-birth must be a mandatory part of the clinical examination protocol, as exceeding 17.5 mm in this value indicates a high probability of omphalitis (84.6% sensitivity and 100.0% specificity), severe gastroenteritis (61.1% and 85.7%), and bronchopneumonia (88.9% and 77.3%). The viability and health of calves born of dairy cows cannot be improved without timely prevention and treatment of preeclampsia, which must include metabolism corrections, reducing the endogenous intoxication and oxidative stress, and treating extragenital diseases.

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INTERFERON-TAU AND FORMATION OF PREGNANCY IN COWS

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Abstract

Coordinated action of steroid and peptide hormones and interferons ensure the formation of pregnancy and embryo-fetal development in cows. A special class of such interferons is interferon-tau (INFT) which is synthesized by embryo trophoblast cells. This interferon is responsible for preserving the progesterone synthesis by the ovary yellow body and embryo implantation. This paper is the first in which we report a significance of INFT under impaired embryonic development and data on evaluation of biological and clinical efficacy of bovine recombinant INFT administered to cows after artificial insemination. The aim of this work was to study the dynamics of the blood content of INFT and progesterone (P_4) during early pregnancy of Black-and-White cows (*Bos taurus taurus*) and to identify the possibility of using a bovine recombinant INFT preparation to prevent embryonic losses and to increase the effectiveness of insemination. Recombinant INFT was obtained at the Belarusian State University (V.A. Prokulevich, M.I. Potapovich). Blood content of INFT and P_4 was determined by ELISA test using Bovine Interferon-Tau Elisa Kit (USA) and Immuno-Fa-PG (Russia) 7, 14, 21 and 35 days after artificial insemination. Animals with physiological formation of embryos (n = 15) and with embryonic death (n = 3) were tested. The sensitivity of the analyses was 2.9 pg/ml for INFT and 0.4 nmol/l for P4. The phagocytic activity of leukocytes, the content of serum immunoglobulins, and the bactericidal activity of blood serum were also determined. The presence/absence of the embryo in the uterus was judged by the concentration of blood progesterone on day 21 and day 35 and by double ultrasound examination on day 35 and day 50. The evaluation of the efficacy of prescribing different doses and schemes of recombinant interferon to increase the effectiveness of insemination and to prevent delayed embryo-fetal development syndrome was carried out on 87 cows. INFT was administered parenterally once, three times, or five times in doses of 5 and 10 ml from day 12 to day 16 after insemination. Intact animals and those subjected to Progestamage administration were used as control groups. It was found that the blood concentration of INFT increased by 23.2 % from day 7 to day 14, and decreased by 30.8 % on day 35, P_4 content increased 32 times from day 7 to day 14 of embryo formation. The concentration of INFT was 7.7 % lower on day 14 and 25.2 % lower on day 35 when the embryo died. The blood P_4 level of these animals was 26.5 % lower by day 21 and 9.3 times lower by day 35. This suggests that hypointerferonemia and associated hypoprogesteronemia are among the reasons for the delay in the development and death of embryos in the early pregnancy. It was revealed that the optimal scheme of the recombinant INFT use to improve the pregnancy formation in cows is its three-fold parenteral administration in the dose of 5 ml on days 12, 14 and 16 after insemination. As compared to the intact animals, the effectiveness of insemination increased from 38.9 to 75.0 %, or by 36.1 %, and the delayed embryo-fetal development syndrome decreased from 28.6 to 16.7 %, or by 11.9 %. Metric indexes of developing embryos exceeded those of the intact animals by 32.2 % on days 28-30 of pregnancy, and by 55.3 % on days 60-65 of pregnancy, and birth weight of the calves was 14.2 % greater. This occurred along with a 33.9 % increase in INFT blood concentration, and 2.3 times increase in P_4. Direct replenishment of progesterone deficiency in animals by Progestamage administration provided 38.1 % increase in preservation of pregnancy. It is also shown that INFT

preparation has an immunomodulatory effect on the cows. The phagocytic activity of lymphocytes increased by 8.7 %, phagocytic number by 35.1 %, phagocytic index by 25.1 %, bactericidal activity of blood serum increased by 5.9 %, and immunoglobulin content by 14.3 % after the INFT triple administration. The conclusion is made about expediency of using the recombinant INFT to increase the fertility of cattle pedigree stock.

Keywords: *Bos taurus taurus*, cows, blood, interferon-tau, progesterone, early embryogenesis, embryonic death, fertility

Modern achievements in immunoendocrine control of pregnancy in animals indicate that the intrauterine formation and development of embryos and fetuses is provided by the overlapping action of progesterone, somatotropin, placental prolactin, cortisol, and interferons produced by immunocompetent cells of the uterine and placental complex [1-3]. The output and action of the latter constitute the most important mechanism of maternal-fetal relationships [4, 5].

Interferon-tau (INFT) is a special class of interferons that is directly related to the formation of pregnancy, has a common property with all interferons and provides implantation and elongation of the embryo [6-8]. It was first discovered in 1982 in sheep [9-10] and somewhat later in cows and goats. INFT is produced by mononuclear trophoblastic cells of the embryo from the first day of pregnancy, reaches a maximum content during the implantation period, and contributes to the inclusion of all mechanisms of embryo adoption by the maternal organism into this process [11-13]. First of all, it ensures the preservation and prolongation of the progesterone-synthesizing function of the ovarian corpus luteum. The mechanism of its anti-luteolytic action is associated with blockade of oxytocin and estrogen receptors in the endometrium and inhibition of the production of luteolytic prostaglandin $F_2\alpha$ ($PGF_2\alpha$). In addition, INFT inhibits key luteolytic genes induced by $PGF_2\alpha$ in the yellow body (corpus luteum) itself [14]. Having an indirect and direct anti-luteolytic effect, it increases the life expectancy of luteocytes in early pregnancy and the active synthesis of progesterone, which provides an active endometrial secretory response and nutrition of the embryo. It has also been shown [15] that INFT stimulates the expression of interleukin IL-8 mRNA within the corpus luteum, activates the migration of neutrophils into it and thereby increases the secretion of progesterone by the luteocytes [16]. INFT has a direct effect on the synthesis of other cytokines (INF-γ, IL-2, IL-4) [17-19], has an antiviral effect and acts as one of the mediators in the induction of an anti-inflammatory reaction in the uterus of cows. This reaction to the presence of the embryo manifests itself already in the first 4 days of its life in the uterus [20, 21].

In the aggregate, the biological effect of INFT in the organism of animals is aimed at creating physiologically necessary conditions for the intrauterine development of the embryo and fetus. Its insufficient production leads to a loss of pregnancy in the early stages of gestation. It should be noted that in the domestic literature there are practically no publications on this very relevant and promising area of research.

In this paper, for the first time, the authors determined the blood levels of INFT and P_4 in cows during embryogenesis during the physiological formation of the embryo, retardation of its development and death. The pathogenetic significance of INFT in impaired embryonic development was shown. The biological and clinical efficacy of bovine recombinant INFT was evaluated for the first time when it was administered to cows after artificial insemination.

The authors' goal was to study the dynamics of blood content in cows of interferon-tau and progesterone in the early period of gestation and to identify the possibilities of using bovine recombinant INFT as a means of preventing embryonic losses and increasing the effectiveness of insemination.

Techniques. The research was carried out in 2017 in the conditions of JV Vyaznovatovka LLC (Nizhnedevitsky District, Voronezh Region) with a tie barn on Black-and-White cows (*Bos taurus taurus*) (105 cows) of 4-7 years old with an average annual milk yield of 6.5-7.6 thousand kg. The animals were fed according to the norms of the All-Russian Research Institute of Livestock.

In the first series of experiments on 18 cows, the dynamics of the content of interferon-tau and progesterone in the serum during the physiological formation of the embryo and its death were investigated. Their content was determined by enzyme immunoassay (ELISA) using the Bovine Interferon-Tau Elisa Kit test systems (Clod Clone Corp., USA) and Immuno-F-PG (Immunotech, Russia) on the 7th, 14th, 21st days and 35 days after artificial insemination of animals with cryopreserved sperm. Blood was obtained from the tailgate vein. The sensitivity of the INFT assay was less than 2.9 pg/ml, and for progesterone (P_4) 0.4 nm/l. The presence or absence of an embryo in the uterus was judged by the serum concentration of progesterone on the 21st and 35th days and on the basis of a double ultrasound on the 35th and 50th days. For ultrasound, an Easi-Scan-3 ultrasound scanner (BCF Technology, UK) with a 7.5 MHz linear probe was used.

In the second series of experiments performed on 87 cows, the authors studied the effectiveness of bovine recombinant interferon-tau for the prevention of fetal mortality and increasing the effectiveness of insemination. Interferon-tau was obtained at the Belarusian State University. The bovine interferon-tau (INFT) gene sequence optimized for expression in *Escherichia coli* cells was designed using the DNAStar program (https://www.dnastar.com/software/). The synthesis of the optimized sequence was performed by Integrated DNA Technologies (USA), the sequence was cloned as part of the pIDTSmart vector at the restriction sites Nde I and Eco RI. After that, the bovine INFT gene was recloned as part of the experimental vector pET24b(+) for the same restriction sites. For visualization of the results, the gel documentation system Fusion FX (Vilber Lourmat, France) was used. Calcium transformation, restriction analysis, and ligation were performed according to standard protocols [22]. Next, the *E. coli* strain BL21CodonPlus(DE3)-RIPL was transformed with recombinant plasmid pET24-cow INFT, in the cells of which the inducible expression of the bovine INFT gene was performed. *E. coli* BL21CodonPlus (DE3)-RIPL-pET24-cow INFT was grown in a 10-liter Biotron F15L bioreactor (Biotron, Korea) at 37 °C for 7 hours in LB medium supplemented with kanamycin and chloramphenicol. Then, induction was performed by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG), continued cultivation for another 4 hours, after which the cells were collected by centrifugation in an Avanti J30I flow centrifuge (Beckman Coulter, USA), destroyed with a Panda Plus 2000 homogenizer (Gea, Italy) under a pressure of 1000 bar, and the cell homogenate was separated by centrifugation.

The inclusion bodies containing INFT were washed and solubilized in a buffer with guanidine hydrochloride. After that, INFT was refolded and the recombinant protein was purified by chromatography (desalination, ion exchange, and size exclusion chromatography) with an NGC Scout Plus medium pressure chromatograph (Bio-Rad, United States). The purity of the protein according to HPLC (Ultimate 3000 HPLC chromatograph, Thermo Fisher, USA) and protein electrophoresis was > 99%; antiviral activity was 1.02×10^9 IU/mg. Antiviral activity was measured on the MDBK cell line with vesicular stomatitis virus, Indiana strain.

Enzymes and buffer systems from Thermo Scientific (USA) were used in the work. Plasmid DNA was isolated using a Nucleospin Plasmid reagent kit

(Macherey-Nagel, Germany), and agarose gel DNA using a Nucleospin Gel PCR Clean-up reagent kit (Macherey-Nagel, Germany) according to the attached protocols. Electrophoresis of proteins in a polyacrylamide gel was carried out according to the method described by Laemmli [23].

The animals were divided into seven groups. The cows were inseminated with frozen-thawed sperm at a dose of 0.25 ml, containing 15 million spermatozoids with active forward movement. Sperm was injected through the cervical canal into the body cavity of the uterus during the manifestation of the immobility reflex. Cows from group I (n = 18) were not prescribed the drug (negative control). On the 5th and 12th day after artificial insemination, animals from group II (n = 25) were injected with the drug Progestamag − progesterone of prolonged action (Mosagrogen CJSC, Russia) at a dose of 2 ml (positive control). Group III cows (n = 8) were injected once on day 12 with bovine recombinant INFT (NPU ProBioTech, Belarus) at a dose of 5 ml, group IV (n = 8) with INFT at a dose of 10 ml, V group (n = 8) with INFT at a dose of 5 ml on day 12 and day 14, Group VI (n = 8) with INFT at a dose of 5 ml on days 12, 14 and 16, Group VII (n = 8) with INFT in a dose of 10 ml daily from day 12 to day 16. The time of interferon injection was timed to the period of nidation and implantation of the embryo (days 12-17).

Clinical evaluation of the efficacy of exogenous interferon-tau and the selection of the optimal variant of its administration was carried out according to the results of ultrasound diagnostics of pregnancy and metric indicators of developing embryos on the 28th and 30th and 60th and 65th days. Before setting up the experience and in diagnosing pregnancy or infertility in the blood of experimental cows, the content of INFT, progesterone, as well as the phagocytic activity of leukocytes, the blood serum bactericidal activity (BSBA) and the number of total immunoglobulins in it were determined [24]. At the end of pregnancy, the nature of the course of labor, the postpartum period in cows, and the body weight of newborn calves were taken into account.

The data obtained in the experiment were subjected to statistical processing using the Statistica 8.0 application program (StatSoft Inc., USA). Results were expressed as arithmetic mean (M) and standard deviation (±SD). Statistical significance was determined using the paired Wilcoxon W-test. Differences were considered statistically significant at $p < 0.05$.

Results. In the first series of experiments, the physiological formation of an embryo was recorded in 15 cows, its death in 3 animals. It was established that during embryo formation, the blood INFT concentration in cows on the 7th day was 925±35.7 pg/ml, by the 14th day it increased by 23.2% ($p < 0.05$), by the 21st day, declined by 13.7%, and by the 35th day, by 30.8% ($p < 0.001$) (Table 1). According to Kose [25], the content of interferon-tau in the blood of ruminants reaches maximum values by day 17 of gestation and then decreases by days 20-22. The P_4 concentration during this period increased 3.2 times ($p < 0.001$). Consequently, the trophoblastic interferon production peak in cows falls on the period of the embryo implantation, which ensures high progesterone-synthesizing activity of the corpus luteum, normal feeding and the formation of the embryo. When the embryo died, the content of INFT on the 14th day was 1052±36.1 pg/ml, which was lower than in healthy animals by 7.7%, and the progesterone concentration on day 21 decreased by 26.5% ($p < 0.01$) up to 22.5±1.18 nmol/l. Hypoprogesteronemia, which is formed in animals, caused a compensatory increase in INFT production by day 21, its concentration increased 1.32 times ($p < 0.01$) compared to healthy cows. However, the delayed increase in the amount of interferon did not ensure the prolongation of the function of the corpus luteum and the preservation of the emerging embryo. By the time of embryo death, the

blood INFT content decreased to 679±31.4 pg/ml, and that of progesterone to 4.0±0.21 nmol/l.

1. The concentration of interferon-tau and progesterone in serum of Black-and-White cows (*Bos taurus taurus*) during the physiological formation of the embryo and its death (*M*±SD, Vyaznovatovka JV, Nizhnedevitsky District, Voronezh Province, 2017)

The physiological state of the cows	Days after insemination			
	7	14	21	35
	Interferon-tau, pg/ml			
Pregnancy	925±35.7	1140±54.2	984±27.5	800±33.4
Embryonic death	–	1052±36.1	1297±48.9*	679±31.4
	Progesterone, nmol/l			
Pregnancy	11.8±0.21	15.8±0.98	30.6±1.16	37.3±1.67
Embryonic death	–	17.5±1.12	22.5±1.18*	4.0±0.21**

Note. A dash means that no embryonic mortality was noted during these periods.
* and ** The differences with the index at physiological pregnancy are statistically significant at $p < 0.01$ and $p < 0.001$. respectively.

Direct replenishment of progesterone deficiency through parenteral administration of progesterone-containing drugs increased the safety of pregnancy in such animals by 33.1% compared with intact cows (Table 2). The efficiency of interferon-tau administration depended on the dose and frequency of administration. The optimal was 3-fold administration at 48-hour intervals at a dose of 5 ml (on days 12, 14, and 16 after insemination). At the same time, the rates of pregnancy preservation exceeded those of animals from the intact group by 36.1%, and the incidence of embryo development retardation syndrome decreased by 11.9%. Increasing the dose and frequency of administration of interferon-tau did not provide an improvement in the clinical effect.

2. Results of the use of interferon-tau for the prevention of embryonic mortality and increasing the effectiveness of insemination in of Black-and-White cows (*Bos taurus taurus*) (Vyaznovatovka JV, Nizhnedevitsky District, Voronezh Province, 2017)

Group	Inseminated cows	Diagnosed pregnancy		Fetal developmental delay syndrome, number/%
		number	%	
I (negative control)	18	7	38.9	2/28.6
II (positive control)	25	18	72.0	4/22.2
III	8	3	37.5	1/33.3
IV	8	5	62.5	1/20.0
V	8	6	75.0	1/16.7
VI	8	4	50.0	1/25.0
VII	8	5	62.5	1/20.0

Note. For a description of the groups, see theTechniques" section.

In cows of group V, the blood INFT concentration increased from 818±42.0 to 1079±46.8 pg/ml, or by 31.9% ($p < 0.01$) from 12 to 20 days after insemination, and decreased to 694 ± 24.1 pg/ml ($p < 0.01$) by day 35. At the same time, the blood P_4 content within the same period increased 2.7 times from 17.1±1.16 to 46.9±2.41 nmol/l ($p < 0.001$) and exceeded that in the intact animals with physiological pregnancy by 25.7% ($p < 0.01$). In group II, the blood INFT increased from 858±27.0 to 919±38.5 pg/ml, or by 7.1%, while the content of progesterone increased from 15.8±0.87 to 41.8±2.64 nmol/l, or 2.6 times ($p < 0.001$). Therefore, exogenous progesterone reduces the interferon-producing function of the embryo trophoblast via negative feedback.

The positive effect of bovine recombinant INFT on the morphofunctional state of the ovarian corpus luteum and the emerging embryo and fetus confirmed their metric indices during ultrasound scanning (Figs. 1, 2).

The size of the ovarian corpus luteum in cows in group V compared to the intact animals was 25.2% ($p < 0.01$) higher at days 28-30 after insemination

and 32.1% ($p < 0.01$) higher at days 60-65. The metric indices of developing embryos under INFT administration were 32.2% ($p < 0.01$) higher than those of intact cows on the 28th-30th day of pregnancy, and 55.3% ($p < 0.01$) higher on the 60th-65th days.

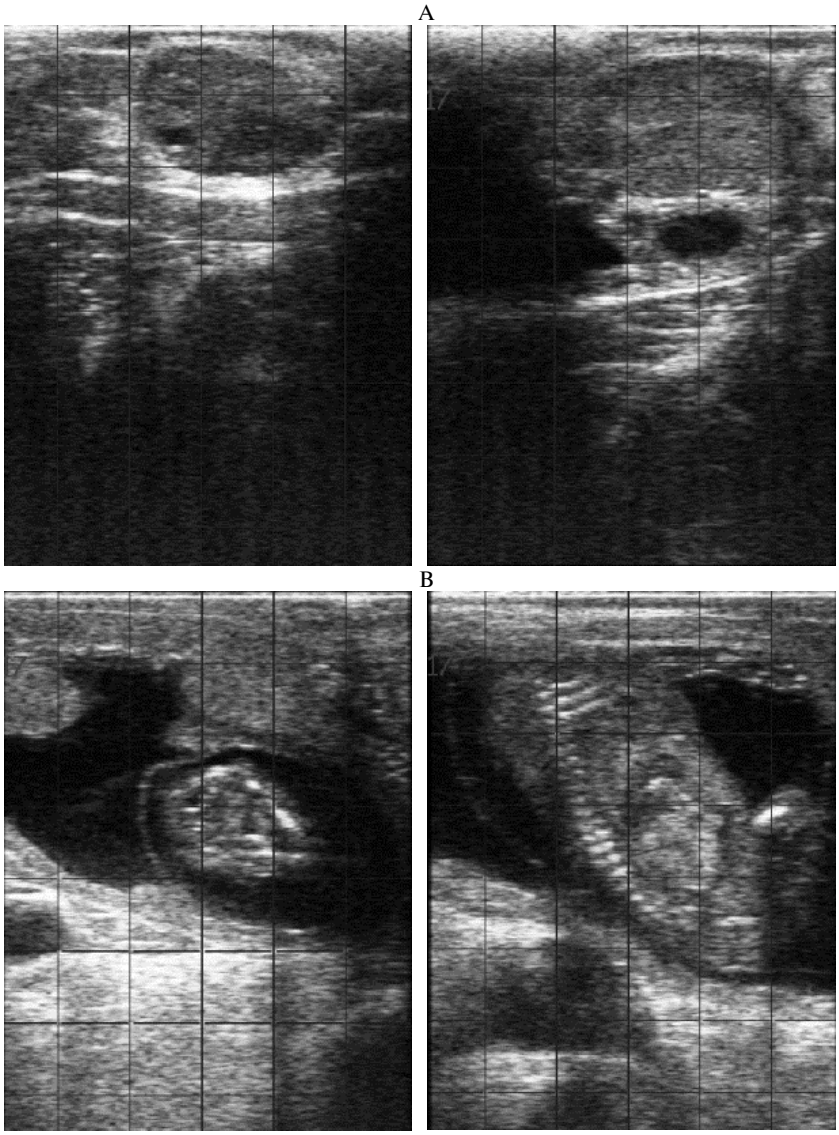

Fig. 1. The ovarian corpus luteum (A) **and the embryo** (B) **in intact** (left) **and treated with bovine recombinant interferon-tau** (right) **Black-and-White cows (*Bos taurus taurus*) on day 60 of pregnancy** (Vyaznovatovka JV, Nizhnedevitsky District, Voronezh Province, 2017).

Evaluation of the immune status of cows before and after the recombinant INFT administration showed that this interferon also has an immunomodulatory effect. This was evidenced by changes in the phagocytic activity of leukocytes (PAL), the bactericidal activity of blood serum and the content of serum immunoglobulins (Table 3). Two weeks after 3-fold injections of interferon, the PAL increased by 8.7%, phagocytic number (PN) by 35.9%, the phagocytic index (PI) by 25.4%. The blood bactericidal activity increased by 5.9% ($p < 0.05$), the content of immunoglobulins by 14.3% ($p < 0.01$). In intact animals, no such changes were observed. Differences between animals of groups I

and V were for PAL 9.3% ($p < 0.001$), for PN 15.2%, for PI 6.2%, for blood bactericidal activity 10.8% ($p < 0.001$), and for immunoglobulins 18.9%.

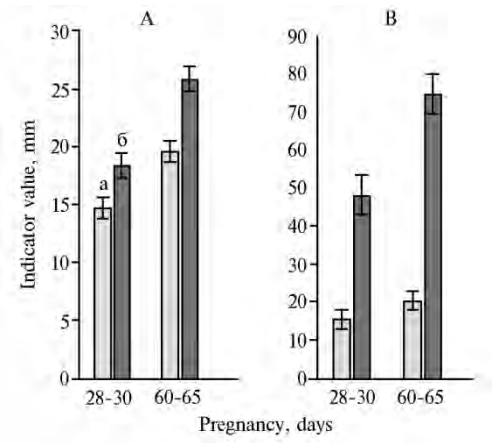

Fig. 2. The diameter of the ovarian corpus luteum (A) **and the coccygeal-parietal size of the embryo and fetus** (B) **in intact** (a) **and treated with bovine recombinant interferon-tau** (b) **Black-and-White cows (*Bos taurus taurus*) during pregnancy** (Vyaznovatovka JV, Nizhnedevitsky District, Voronezh Province, 2017).

At birth, the body weight of calves from cows treated with INFT exceeded the corresponding figure in intact animals by 14.2% (respectively, 33.7±1.9 kg and 29.5±1.5 kg). In mother-cows, when interferon was used, the number of postpartum complications decreased 1.5 times.

3. Indicators of the immune status of intact and interferon-treated Black-and-White cows (*Bos taurus taurus*) (*M* ± SD, Vyaznovatovka JV, Nizhnedevitsky District, Voronezh Region, 2017)

Indicator	Group			
	I (negative control)		V	
	before the administration	2 weeks after the administration	before the administration	2 weeks after the administration
PAL, %	69.3±1.2	70.9±0.8	71.3±1.5	77.5±1.0**
PN, microbial cells per phagocyte	4.5±0.28	4.6±0.19	3.9±0.22	5.3±0.31**
PI, microbial cells per active phagocyte	6.5±0.39	6.5±0.18	5.5±0.21	6.9±0.42*
General immunoglobulins, g/l	24.9±1.5	24.9±1.4	25.9±1.1	29.6±1.5**
BSBA, %	58.6±1.2	58.4±0.7	61.1±0.8	64.7±0.8*

N o t e. PAL is phagocytic activity of leukocytes, PN is phagocytic number, PI is phagocytic index, BSBA is blood serum bactericidal activity. For a description of the groups, see the Techniques section.
* and ** Differences with background values are statistically significant at $p < 0.05$ and $p < 0.01$, respectively.

The pleiotropic effects of IFN-tau provide for the physiological formation of pregnancy in cows [16, 26]. It has been shown that INFT not only prolongs the progesterone-synthesizing function of the corpus luteum but also, together with progesterone, provides for the synthesis and secretion of endometrial histotroph, including amino acids, enzymes, glucose, cytokines, and growth factors critical for the embryo's nutrition, implantation, and placentation [27]. However, the threshold value of the concentrations of INFT in the blood of animals, necessary to maintain pregnancy, has not yet been established. In the authors' experiments during the nidation period of the embryo, they were within 1090-1200 pg/ml. The death of the embryo was recorded at a concentration of 1000-1080 pg/ml. Continuing research to assess the interferon status of animals in the early stages of gestation, in the authors' opinion, will allow proposing a standard indicator of the INFT content and using it as an indicator of the emerging pregnancy state. In experiments performed in mice [28], it was also shown that exogenous INFT prevents lipopolysaccharide-induced implantation failure and increases the number of implanted embryos in such animals by suppressing the production of proinflammatory cytokines (IL-1, TNF).

Thus, the obtained results showed that the concentration of INFT in the serum of cows from the 7th to the 14th day of embryo formation increased by 23.2%, and by the 35th day it decreased by 30.8%. With the death of the embryo, the concentration of INFT on the 14th day was lower by 7.7%, on the

35th day – by 25.2%. Normal embryo formation, the course and preservation of pregnancy in cows in early gestation are largely determined by the production of ovarian progesterone and trophoblastic interferon-tau as one of the autocrine regulators of early embryogenesis and implantation. The interferon preparation used by the authors meets the stated requirements for biological and therapeutic qualities. Its parenteral injections to animals during the implantation of the embryo provide prolongation and activation of the progesterone-synthesizing function of the ovarian corpus luteum by 25.7%, insemination efficiency – by 36.1%, reduction of the manifestation of intrauterine growth retardation syndrome – by 11.9%, as well as an increase in the natural resistance of the organism of mother-cows by increasing the intensity of phagocytosis, the synthesis of serum immunoglobulins and the bactericidal activity of the blood serum. The pharmacological control of interferon, progesterone and cytokine statuses of inseminated animals during the period of blastogenesis and implantation can be the basis for preventing embryonic losses and increasing the fertility of cows in highly productive dairy herds.

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HISTOBIOCHEMICAL ASPECTS OF THE EFFECT OF A COMBINATION OF SOME NATURAL METABOLITES ON GENERAL RESISTANCE IN EGG CHICKS

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Abstract

Industrial incubation of eggs accompanied by various stresses often leads to early embryonic death. For this reason, the ecologically safe methods to prevent negative stressful effects in embryogenesis are still important. The purpose of this work was to study the effect of natural metabolites on the histological structure of the Bursa fabricii and general resistance of chickens. Studies were performed on eggs of egg cross Shaver 2000 chickens (*Gallus gallus*) (Ptichnoe Enterprise, Moscow Province, 2011). 3-4 hours before the eggs were put into the incubator, their shells were treated with an aqueous solution of ethanolamine (colamine), succinic acid and serine (0.1; 0.1 and 0.2 %, respectively) using a spray gun, and on day 19 of incubation 0.1 % aqueous colamine solution was applied. The preparations were pre-dissolved in distilled water at 18-22 °C. The untreated eggs serve as control. Each batch included 544 eggs. The recorded indicators were hatching rates, hatchability and incubation losses, also blood samples were collected to determine the content of immunoglobulins, lysozyme activity and bactericidal activity. Bursa fabricii was collected for histological analysis ($n = 5$) from chickens aged one day. Transovarian treatment of eggs with a complex of biologically active substances containing ethanolamine, succinic acid and serine has positively influenced the viability of embryos and young hens due to leveling of oxidative stress reactions and stimulation of the central components of resistance, i.e. the immune response and non-specific defense of chicks aged one day. Ethanolamine turns into phosphatidylcholine and phosphatidylethanolamine, and serine is metabolized into phosphatidylserine, which are able to neutralize the destruction of cell membranes inevitably arising under free-radical oxidation. Succinic acid, an energy substrate and an antihypoxant, contributes to prevention of the lack of energy (the lack of ATP) in the body, which occurs during critical periods of chick embryogenesis. Applied biologically active substances can optimize the embryo's living conditions during incubation accompanied by technological stresses. The higher viability of chicks of the test group resulted in a decrease of all incubation loss categories with an increase in the chick hatching values and egg hatchability. The content of immunoglobulins responsible for the primary and secondary immune response also increased, which is a demonstration of the immunomodulatory action of the composition used. Immunoglobulin E level remained unchanged, which indicates the absence of hypersensitivity reactions. Ethanolamine, succinic acid and serine also promoted the activation of non-specific defense factors, i.e. blood bactericidal activity and lysozyme, which increased in the test group of chicks too. We also revealed that the composition of biologically active substances positively influence the histoarchitecture of the bursal sac in chicks of the test group, which was confirmed by our histological data. In this, signs of physiological intracellular hyperplasia, as well as the absence of involution processes in the organ have been noted. As the aftereffect of the composition use, the viability of young chicks during the first 60 days of growing increased due to the reduction in mortality. Thus, ethanolamine, succinic acid and serine can prevent oxidative stress and also have a positive effect on general resistance in egg chicks.

Keywords: chicks, embryogenesis, antioxidants, lipoperoxidation, active immunity, bursal sac, chick hatching, ethanolamine, succinic acid, serine

High production in poultry, including the hatching rate, is achievable

only at adequate, timely formation of antioxidant systems and resistance in birds [1]. Uneven heating of eggs in the batch, egg candling, the absence of air ionization, bioacoustics, possible failures in the operation of incubators and other technological stresses prevent the full formation of embryos and lead to mass early embryo death or suppressed, weak young chicks [2-4]. Many papers prove that the biological indicators are affected by the content of gases [5-7], temperature [8, 9], and humidity in the incubator [10].

A developing and growing body is much more sensitive to the effects of environmental factors in comparison with adults because its antioxidant, immune and other systems, as well as non-specific protection, are still underdeveloped and unable to resist the effects of stress factors to the full [11-15]. The development of embryos includes critical periods; therefore, young chicks obtained in inadequate production conditions often have a low potential of resistance, as well as viability and productivity [16].

The peripheral immune organs of chicks (spleen and lymphoid diverticulum) develop morphofunctionally throughout postembryogenesis, and, by day 42, this process is not completed. By the hatching time, only the central immune organs, thymus and bursal sac, are formed morphologically and functionally [17]. Since the bursal sac plays a key role in the formation of poultry immunity, the study of its morphological and functional characteristics in connection with the search for effective methods to prevent the effects of stress arising during industrial incubation of eggs is considered one of the most important tasks [18]. The stem cells of the bone marrow form the population of clones of B-lymphocytes in the bursal sac. Then, they leave bursa and colonize thymus-independent zones of peripheral immune organs and structures, where under the influence of antigens they are differentiated and turned into plasma cells that synthesize antibodies [19].

Extreme stress states of various origins are characterized by premature involution and atrophy of the bursal sac, which can be considered as the morphological equivalent of the secondary immunodeficiency [20]. According to Turitsyna [20], histostructure modifications in the bursal sac may also be connected with an antigen-induced immune response to the conducted vaccinations and evolution of infections (while changes in the number of immunoglobulins in chicks' blood serum are also observed). The changes in the size and shape of a bursa, a decrease in the number of lymph follicles, thinning of the cortical layer, cirrhosis, and abundant infiltration of the organ by macrophages and granulocytes are possible in the case of immunization and infections [20].

Immunomorphological studies are informative to evaluate immune function [21]. This determines the both practical and fundamental interest in studying the histostructure of immune-competent organs that are directly involved in immunological response, in particular, the bursal sac.

Technological factors have a significant impact on the bursal sac histostructure. According to Travnikova [22], a complex of pathological processes is observed among chicks in conditions of the high cage density. Leukocytes, pseudo-eosinophils, macrophages, and piocytes are accumulated in the bursal lumens. The same infiltrating cells are found in the bursal subepithelial layer, as in the lumen [22]. Various fodder boosters promoting immune activation, which positively affects the histologic pattern of immune-competent organs [23]. Thus, when using probiotic products, the mass of immune protection organs increases due to physiological hyperplasia [24]. The defects of bursal sac microstructure might have been neutralized in the embryogenesis already. For example, the introduction of the immunomodulator (methisoprinol) inside the egg promotes positive changes in the histological structure of this organ [25].

Note, the morphological, histological, and biochemical indicators of the

bursal sac state were studied during late ontogeny mainly, and a comprehensive study of the organ in early postembryogenesis, i.e., immediately after the hatching, is a matter of special interest.

The composition of biologically active substances (BASs) studied in the previous paper had a positive effect on the number of blood cells, some of which were producers of nonspecific protection factors of the body, for example, a lysozyme [26]. To investigate the functioning of the immune system deeper, it is necessary to assess not only the histological state of the bursal sac when using the developed composition but also to determine the content of immunoglobulins in the blood of chicks, which will allow detecting the presence or absence of an immunomodulatory effect.

The role of the immunoglobulin M (IgM) in immune responses is short-term and represents the body's primary response to any pathogen. IgY of chickens is largely similar to that of mammals in structure and function, both being determined by the same immunoturbidimetric method, which allows the latter to be used to assess the immunity of poultry [27]. IgA is the main form of antibodies in the body's secretions [28]. It should be noted that birds do not have immunoglobulin D because the genes encoding it are absent in their organisms [29].

It is also known that among all species of birds chickens have the strongest humoral response [30]. It has been reported that even chickens that had not been immunized have natural antibodies [31]. It was concluded that they can play an important role in both activation and regulation of specific humoral immune responses of poultry [32]. The half-life period of immunoglobulins at day-old chicks ranges within 3 days [33].

Therefore, it is interesting to determine the quantitative changes in natural antibodies under the influence of various compounds during embryogenesis to identify their potential immunomodulatory effect. A method to stimulate the chicks' embryonic development, increasing non-specific resistance, which includes transovarian treatment of eggs with a composition of natural metabolites, consisting of colamine, succinic acid, and serine, has been developed for this study. It is well known that abnormal intensification of free radical reactions leads to excessive activation of lipid peroxidation (LP) and damages of bilipid membranes. Colamine in animals and poultry is easily converted into phosphatidylethanolamine and phosphatidylcholine; serine is rarely used for the synthesis of ethanolamine [34] and primarily serves as a component of phosphatidylserine or a donor of the carbon skeleton in biochemical processes. The effectiveness of these elements in the composition is practically confirmed in a series of experiments [35]. Since the synthesis processes are energy-consuming, succinic acid is added to the composition as the main intermediate of the tricarboxylic acid cycle and the substrate of biological oxidation, the natural pool of which is quickly wasted under any medium and severe stresses. Succinic acid supports ATP synthesis both in the Krebs cycle and in the mitochondrial respiratory chain, compensating a decrease in the amount of ATP, typical for the stressed state. It is important to note that not only succinate but also colamine (with the help of choline) in the body are able to prevent disturbances in the mitochondrial respiratory chain function, preventing energy losses and excessive synthesis of reactive oxygen species [34, 36].

The immune system of birds is the most vulnerable to stress and the first to suffer the consequences of excessive generation of free radical particles. The use of antioxidant preparations prevents the formation of destructive changes in the bilipid membrane skeleton [37]. The proposed composition of metabolites, which has a significant antioxidant effect [35], is able to prevent the excessive genera-

tion of free radicals effectively and reduce the intensity of lipid peroxidation in chicks' bodies, while maintaining the structural integrity of internals, including the bursal sac.

The peculiarities of changes in the specific and non-specific resistance (the contents of immunoglobulins, lysozyme, and the bactericidal activity of blood serum) and histological architectonics of the bursal sac when using the proposed composition of the metabolites are revealed in the present paper for the first time.

The work objective was to study the effects of natural metabolites on the histological structure of the bursal sac and the total resistance of the obtained chicks.

Techniques. Studies (Ptichnoe Enterprise, Moscow Province, 2011) were performed on hatching eggs of the egg cross Shaver 2000 (Hendrix Genetics Company, Holland) chickens (*Gallus gallus*). The optimal composition of natural metabolites and the treatment multiplicity were selected earlier [26, 37]. The experimental batch of eggs was treated twice. 3-4 hours before the eggs were put into the incubator, their shells were treated with an aqueous solution of colamine, succinic acid and serine (0.1; 0.1 and 0.2%, respectively) using a spray gun, and on day 19 of incubation, 0.1% aqueous colamine solution was applied. The preparations were previously dissolved in distilled water at 18-22 °C. A batch of eggs, which was not subjected to treatment, was used as a control ("dry" control), as it was proved that, in this case, the results do not differ from the "wet control" [38].

Each batch consisted of 544 eggs. The recorded indicators were hatching rates, hatchability, and incubation losses. To determine the safety of chicks, 100 animal units were grown from each group under standard conditions. In whole blood taken from day-old chicks after decapitation, the immunoglobulin content was determined by the immunoturbidimetric method, in serum by lysozyme activity nephelometrically, and bactericidal activity (BASC) by the photoelectric nephelometric method [39].

Samples of bursal sac tissue were taken from one-day chicks for histological analysis ($n = 5$). The organ samples were embedded in paraffin and the histological sections with a thickness of 5-7 microns were prepared on a microtome MPS-2 (OAO HZ Tochmedpribor, Russia) according to standard techniques [40] followed by staining with Meyer's hemalum and eosin. Microscopy of histological sections was performed using a biological microscope ScienOpBP-52 (ScopeTec, China) with the magnification of ×7, ×10 eyepieces and ×4, ×10 and ×40 lenses. The photos were shot with a digital camera-eyepiece for a microscope DCM800 (ScopeTec, China, 8000 pixels, USB 2.0). Morphometry was performed using an eyepiece micrometer using the program ImageJ (National Institutes of Health, USA) with a set of modules for medical morphometry. Measurements were carried out at a total increase of ×70 in 30 consecutive fields equal to the area of the ocular grid.

The data were processed statistically in the Microsoft Excel program. The mean values (M) and standard errors of means (±SEM) were calculated. The significance of differences was assessed according to Student's t-criterion.

Results. Penetrance of eggshells for biologically active substances was based scientifically by Karmoliev et al. [41]. The use of natural metabolites led to a decrease in the abnormal intensity of free-radical reactions, as well as the processes of lipid peroxidation among young chicks of the experimental group and, consequently, to the optimization of metabolic processes [42]. It determined a significant increase in embryonic viability, reflected in the reduction of incubation losses at a significant increase in hatching by 9.38% ($p \leq 0.001$) (in control and experience, 75.0 and for 84.38% respectively) and hatchability by

9.26% ($p \leq 0.001$) (81.27 and 90.53%).

It should be noted that the high viability of the individuals of the experimental group remained for a long period of post-embryonic development. While breeding the group of 100 young chicks during 60 days, covering the main critical periods of post-embryonic development, the death loss in the experimental group decreased by 1.2 times with an increase in viability by 1% (in control and experience, 94 and 95% respectively).

These positive effects were observed against the background of increased active immunity, i.e., immunoglobulin levels, recorded in the experimental group, were optimal for the studied individuals. Indicators of specific body protection of day-old chicks in the experimental group were higher than in the control (Table 1).

1. Blood immunoglobulins (g/ml) **in cross Shaver 2000 day-old chicks (*Gallus gallus*) under egg treatment with the composition of metabolites** (Ptichnoe Enterprise, Moscow Province, 2011)

Immunoglobulin	Group	
	control (n = 5)	test (n = 5)
IgM	0.300±0.007	0.330±0.008*
IgG (IgY)	3.000±0.100	3.300±0.070
IgA	0.250±0.009	0.260±0.007
IgE	1.1000±0.01	1.100±0.020

N o t e. See the description of groups in the section Techniques.
* Differences form control are statistically significant at $p < 0.05$.

The content of IgM, responsible for the primary immune response, in chicks from the experimental group, was significantly higher (10%, $p < 0.05$), while the content of IgG (IgY), which determines the secondary immune response, also increased by 10% (see Table 1). The IgA content increased slightly (by 4%), while the content of IgE remained unchanged. These data indicate the immunomodulatory effect of the used composition against the background of oxidative stress during incubation. Manifestations of hypersensitivity were absent, as evidenced by the equal IgE indicators in the control and experiment. The immunomodulatory synergistic effect of the studied BAS was also indicated by an increase in the content of non-specific protection factors − BASC (3.8%) and lysozyme (4.8%).

High body reactivity of chicks from the experimental group was a consequence of the positive effects of natural metabolites on the histologic architectonics of the bursal sac (Table 2, Fig.).

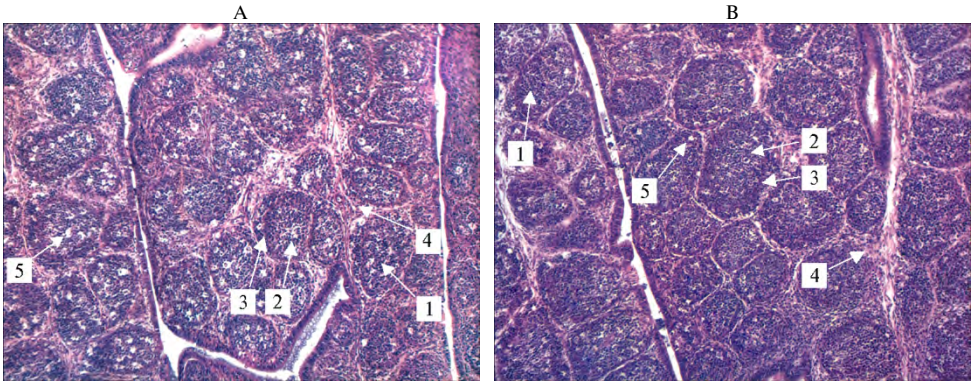
2. Morphometric bursal sac indicators in day-old cross Shaver 2000 chicks (*Gallus gallus*) under egg treatment with the composition of metabolites (Ptichnoe Enterprise, Moscow Province, 2011)

Indicator	Control (n = 5)	Test (n = 5)
Bulk density, %:		
stroma	35.00±1.22	23.60±1.08*
follicles cortical zone	30.00±1.00	59.00±2.35**
follicles medullar zone	35.00±1.38	18.00±0.89**
Area, $\times 10^6$ rm^2:		
follicle	7291.138±440.780	9360.420±798.910
cortical zone	3680.440±349.870	7818.922±602.680**
brain zone	3703.168±387.320	1374.432±83.240**
Cortical brain index	99.39	568.88

N o t e. See the description of groups in the section "Techniques."
*, ** Differences form control are statistically significant at $p < 0.05$ and $p < 0.01$, respectively.

As can be seen from the data of Table 2, the volume density of the bursal sac stroma of chicks from treated eggs is by 11.4% significantly less, and the bulk density of the brain zone of the follicles is by 29% more than in the control. The density of the brain zone of follicles significantly decreased (by 17%); the area of the cortical zone increased and the brain zone decreased (by 2.12 and 2.69 times, compared with the control, respectively). As a result, there was an increase in the cortical brain index (CBI), which reflects the functional ac-

tivity of the organ: the higher the index is, the better the organ functions [43]. In the authors' study in the experimental group, CMI exceeded the control indicators by 5.72 times. The data obtained are consistent with the micromorphological results of histological examination of the organ (see Fig.).



The histological structure of the bursal sac in day-old cross Shaver 2000 chicks (*Gallus Gallus*) of the control (A) **and test** (B) **groups:** 1 — lymphatic node, 2 — medullary part, 3 — cortical part, 4 — stroma connective-tissue elements, 5 — small cystic cavities (hematoxylin and eosin staining; microscope ScienOpBP-52, ScopeTec, China; eyepiece ×10, lens ×10).

Among the chicks of the control group, a significant involutional process was observed in the bursal sac. The capsule of the organ was thickened; the trabecular pattern was sharply expressed by increasing the connective-tissue elements of the stroma (fibrocytes, fibroblasts, and intercellular fibrous structures). The lumen of the bursal sac is fitted with a plural-row cylindrical epithelium with no signs of damage. Directly behind the epithelial layer in the depth of the mucous membrane, there were multiple closed lymphoid follicles mainly of medium and small size, having a rounded, oval or trapezoidal shape and an indistinct division into cortical and brain zones. Lymph knots were adjacent to each other and located in the folds in one or two rows. The cortical zone was thinned, had the form of a narrow strip lying on the periphery of the follicles on the border with the organ stroma, and was represented by a small accumulation of small lymphocytes. The lighter brain zone consisted of rarely located large lymphocytes and separate plasma cells, as well as a large number of macrophages and granulocytes. The apoptotic cells were often found among the lymphocytes. In the follicles, the numerous small cystic cavities were clearly expressed. At the same time, the organ showed no significant signs of hemodynamic disturbances.

In the chicks of the control group, the bursal sac capsule was not thickened; the trabecular pattern was poorly expressed due to the weak development of the connective-tissue elements of the stroma. The lumen of the bursal sac was fitted with a plural-row cylindrical epithelium with no signs of damage. Directly behind the epithelial layer in the depth of the mucous membrane, there were multiple closed lymphoid follicles mainly of big and medium size, having a rounded, oval or trapezoidal shape. Lymph knots were adjacent to each other and located in the folds in three or four rows. The cortical zone occupied a large area of the follicle, spreading from the center to the periphery, and was represented mainly by a relatively dense cluster of small lymphocytes. The centrally located brain zone was lighter, relatively poorly developed, included large lymphocytes, plasma cells, as well as a small number of macrophages and individual granulocytes. In the follicles, rare individual places of the polycystic process were observed, apoptotic bodies were rare.

The histological studies reveled a number of positive changes in test

chicks compared to control. So, young chicks of the control group had signs of a decrease in the functional activity of the bursal sac. They were characterized by an increase in the proportion of connective tissue in the organ, a small size of lymphoid follicles with the development of the processes of delimitation in the cortical and medullar layer. At the same time, the formation of microcystic cavities and an increase in the number of macrophages in the brain layer of the follicles clearly indicated the death of lymphocytes. The absence of a significant inflammation in the organ gave a reason to assume that the process of lymphocyte death was due to apoptosis, not necrosis, which allows us to consider non-infectious factors as the cause of this process [43]. It is also confirmed by the increase in the number of apoptotic cells, which were found in lymphoid follicles during the histological examination of the bursal sac.

The literature data [44, 45] show that the influence of non-specific environmental factors (imperfection of egg incubation, feeding, and layer housing) on the formation of organs among chicks during embryonic development is quite high. As a result of such adverse effects, the process of formation of immunocompetent organs is disrupted, which is manifested by a decrease in non-specific resistance and immunological reactivity.

Chicks have several stages of the bursal sac development: growth (up to 2 weeks of age), maturity (up to 5-8 weeks), early involution (up to 9-15 weeks), late involution (up to 25-30 weeks), and rudimentary (after 30 weeks) until complete disappearance [46]. Taking into account that one-day chicks were used in the experiment, it can be affirmed that the processes of atrophy of the bursal sac, or the so-called accidental involution of the organ started early. At the same time, the rapid decrease in its mass and volume, primarily under the influence of glucocorticosteroids in various stressful situations, was observed. In these studies, the mass of the bursal sac in the experiment was 0.084 ± 0.002 g vs. 0.069 ± 0.001 g in the control, with the 21.7% difference ($p < 0.001$) between the test and control.

The impact of stress factors on the embryo, and then in the postembryonic period on the chick leads to the formation of secondary immunodeficiency, expressed by the inferiority of the morphological structure of immunocompetent organs [46].

Histological examination of bursal sac in the test group showed signs of a moderately developed physiological hyperplasia of the organ in the absence of the processes of involution. Hyperplasia was expressed in an increase in the size of the lymphoid follicles of the organ by increasing the number of lymphocytes while maintaining the shape and structural elements that make up the follicles. The increase in the size of the follicles was primarily due to an increase in the cortical layer, while the brain layer was characterized by the small size and rather rare processes of apoptosis among lymphocytes. Together, these data indicate the full development of the organ, typical for chicks in this age period.

Thus, the treatment of chicken eggs with the composition of natural metabolites which contributes to a more complete timely formation of all the structures of bursal sac reduces embryonic death, positively influences the overall resistance of chicks, and determines higher quality and safety of young birds. The physiological intracellular hyperplasia occurs in the bursal sac of day-old chicks, while the involutive processes are not fixed. In other words, the complex of natural metabolites optimizes the histoarchitectonics of the bursal sac.

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POULTRY DIETS WITHOUT ANTOBIOTICS. I. INTESTINAL MICROBIOTA AND PERFORMANCE OF BROILER (*Gallus gallus* L.) BREEDERS FED DIETS WITH ENTEROSORBENT POSSESSING PHYTOBIOTIC AND PROBIOTIC EFFECTS

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Abstract

Recent trend of rejection of the in-feed antibiotics in animal and poultry production launched the search for reliable alternative growth stimulators, primarily probiotics, or phytobiotics (most commonly essential oils) rendering antimicrobial and antioxidant properties to improve the digestibility of dietary nutrients, suppress the growth of pathogens, etc. Another important problem is the contamination of feeds with mycotoxins which can negatively impact the productive performance in poultry. The growth efficiency and composition of intestinal microbiota were studied in growing broiler (*Gallus gallus* L.) breeders of preparental lines B5 and B9 (selected at the Center for Genetic Selection "Smena", Moscow Province) fed vegetable diets supplemented with complex preparation Zaslon 2+ (JSC Biotrof+, Russia), containing an intestinal adsorbent, a mixture of essential oils, and a strain of *Bacillus* sp. (10^5 CFU/g). Zootechnical and physiological experiments were carried out in in 2017 (Smena Center, Zagorsk EPH, Sergiev Posad, Moscow Province). Control poultry fed the same vegetable diets with dietary antibiotics Bacitracin 30 (42 IU/mg, a dosage of 100 g/t). Test and control groups contained 50 birds each. There were no significant differences between the control and experimental treatments in live bodyweight at 21 weeks of age: 3168 g in males and 2317 g in females in B5 line (vs. 3171 and 2307 g in control), 2592 and 1930 g in B9 line (vs. 2574 and 1924 g in control), in the development of the reproductive organs (testicles in males, ovary and oviduct in females), and in the digestibility of dietary nutrients. In the duodenal microbiota from 18 to 110 bacterial phylotypes, with statistically significant differences from control for several taxonomic groups ($p < 0.05$), were found using terminal restriction fragment length polymorphism (T-RFLP) analysis. The calculated indices revealed taxonomic diversity and complexity of the intestinal bacterial communities in both control treatments (B5 and B9); in both experimental treatments more unidentified bacterial phylotypes were found in compare to the respective control treatments. An increase in the number of bacteria of the *Bacillaceae* and cellulolytic bacteria of the *Clostridiaceae* in the duodenum of the B9 line birds and an increase in the number of bacteria of the genus *Bifidobacterium* and the order *Bacteroidales*, along with a decrease in genus *Campylobacter* counts, in the duodenum of the B5 line birds occurred as a result of administration of the Zaslon 2+ preparation. Therefore, an increase in the number of bacteria in these groups and a decrease in the proportion of pathogenic microorganisms may indicate correction of dysbiotic disorders in the intestines of birds.

Keywords: preparental lines, broiler chicken, phytobiotics, intestinal adsorbent, live bodyweight, intestinal microbiota.

Providing food, in particular, high-value dietary meat is based on use of

high-productive poultry crosses. The genetic potential of broiler productivity is quite high and corresponds to average daily gain of 60-70 g, feed consumption of 1.4-1.8 kg per 1 kg of live weight, and 97-98% safety of livestock during the growing. The maximum realization of genetic potential under the production conditions depends on the norms and modes of feeding, management of broilers, as well as on the quality of the breeding bird and the initial lines [1, 2].

A macroorganism and its microflora represent a single ecological system in a dynamic equilibrium. Microorganisms are involved in metabolic processes, so the composition of the organism's microbiome is relatively constant. Meanwhile, the microbiota colonizing the digestive tract of poultry is influenced by such factors as age, feed composition, antibiotics, mycotoxins, and other substances [3, 4]. Mycotoxins are the most dangerous ones, which are secondary metabolites of mold fungi *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp. (aflatoxins, ochratoxins, zearalenone, fumonisins, and deoxynivalenol). Potential threats to poultry from mycotoxins include the high probability of contamination of grain with mycotoxins, the absence of standards for maximum permissible concentrations (MPCs) for most of them, and synergies of action [5]. To prevent mycotoxicosis in poultry, specialized feed additives are used (mycotoxin sorbents, probiotics, immunomodulators, enzymes, etc.) [6].

At present, the method of enterosorption is used to reduce the concentration of mycotoxins in animal and poultry feeds. However, the used sorbents (activated carbon, hydrated sodium-calcium aluminosilicate) have significant drawbacks. New sorbents with high and irreversible sorption capacity are sought, which lack a binding capacity with respect to essential micro- and macroelements, vitamins, and other nutrients. The modification of sorbent carriers with biologically active substances is used to correct the immune reactivity of animals, as well as to introduce probiotic properties [7, 8].

It was established that the natural resistance of birds to mycotoxins always depends on the state of the microbiota of the gastrointestinal tract. With its maximum stabilization and high resistance, the pathological effect of the toxins of the fungal microflora can be significantly decreased and practically reduced to zero. Also, the competing normal microflora produces a set of its own enzymes acting at pH characteristic of the host organism. These enzymes can biotransform groups of mycotoxins of different mycotoxin producers [9].

Because of the prohibition of antibiotics inclusion in feed in most European countries, feed additives that could replace antibiotics are studied [10]. It was proved that essential oils and probiotic strains of bacteria can be a worthy alternative to antibiotics [11, 12]. Essential oils are a variety of biologically active substances, i.e. terpenes and terpenoids, aromatic compounds, saturated and unsaturated hydrocarbons, aldehydes, organic acids and alcohols, their esters, as well as heterocyclic compounds, amines, ketones, flavones, phenols, quinones, organic sulfides, and oxides [13-15]. The potential therapeutic use of essential oils in poultry is due to their immunomodulatory properties, antimicrobial activity against pathogens, the ability to enhance the production of nutritional secretion, stimulate blood circulation, exert an antioxidant effect, and increase nutrient absorption of feed [16-18]. In this case, the microflora of the digestive tract acts as a highly sensitive indicator system that responds to occurring changes [19]. Using molecular genetic methods, up to 140 genera of bacteria are found in the intestinal microbiome of poultry, of which only 10% are identified by the 16S rRNA gene, and the rest belong to new species and even genera [20-22].

Due to the success of genetic selection, metabolic processes in modern meat poultry crosses intensifies, so that the ability of the poultry digestive system

to ensure the physiological efficiency of nutrient metabolism of diets becomes the limiting factor for the development of poultry farming. Over the past 50 years, significant progress has been made in the world in terms of the growth rate of broilers and their effective use of feed [23]. Moreover, genetic progress has led to changes in the physiology of nutrition of modern meat birds and the composition of the microflora of its gastrointestinal tract (GIT) [24]. To realize the genetic potential of poultry productivity, adequate functional support of the digestive tract is required, in particular, regulation of microflora composition.

The role of gastrointestinal microorganisms in the digestion and metabolism is of increased interest in the context of substantiating more rational and complete feeding, increasing productivity and improving the health status of poultry as a whole [25-28]. The intestinal microbiome of poultry is one of the first barriers to pathogens, coming with feed [29], and plays a huge role in the functioning of the macroorganism.

A significant part of the research on the intestinal microflora of poultry was conducted by sowing cultured strains on artificial nutrient media, which made it possible to study less than 20% of the actual number of microorganisms. Since the 1990s, the understanding of the composition of cicatricial microbiota has significantly expanded, molecular and biological methods for studying microorganisms have evolved, based on information about their genome [30-32].

In the present work, for the first time, the data are presented comparing the productivity of young females of the initial lines B9, B5 of a new domestic meat cross, as well as the effectiveness of their use of feed nutrients in connection with the composition of the intestinal microbiota when using the in-feed antibiotics and the complex preparation Zaslon 2+, consisting of sorbent, a mixture of essential oils and probiotic bacteria.

The goal of the paper was to assess the effect of the complex preparation of enterosorbent, containing bioactive substances and probiotic bacteria, on live weight, the development of reproductive organs, digestibility and the use of nutrient components of feed, as well as the composition of the microbiome in the intestines of meat chickens.

Techniques. The studies were conducted on two initial lines of meat chickens (*Gallus gallus* L.) (obtained at the Smena Center for Genetic Selection, Moscow Province) [33]. B5, the paternal line of paternal parental breed of Cornish form, is quick-growing with feed conversion, higher growth rate, and meat quality as the main selectable traits. B9 is the maternal line of maternal parental breed of Plymouth Rock form selected for egg production, hatchability, growth rate, feed conversion, and viability.

During the period of zootechnical and physiological experiments (Centre for Genetic Selection Zagorsk Experimental Breeding Economy, Sergiev Posad, Moscow Province, 2017), 1-day old to 21-weeks old birds were kept in special cages of 50 birds each in a group. The light, temperature and humidity regimes, the front of the feed and watering corresponded to the recommendations of the All-Russian Research and Technological Institute of Poultry [34].

Up to 1 week of age, the poultry of all groups consumed combined feed from vegetable components ad lib; from week 2, the daily amount of feed was limited. The control group received combined feeds of the vegetable type, balanced in all nutrients in accordance with age periods, with the addition of the in-feed antibiotics Bacitracin-30 (activity 42 IU/mg) in the amount of 100 g/t throughout the raising. The poultry of the experimental group instead of the in-feed antibiotics received 1000 g/t feed of complex enterosorbent Zaslon 2+ (Technical Specifications 9291-028-50932298-2016 of November 18, 2016, JSC Biotrof +, St. Petersburg).

The poultry of the initial lines were fed with crumbled combined feed as follows: during days 1-21 of raising the 280 kcal/100 g exchange energy, 20% crude protein, 1.0% calcium, 0.7% phosphorus, 1.15% total lysine, 0.95% available lysine, 0.45% total methionine, and 0.39% available methionine; during days 22-35 — 275 kcal/100 g, 18, 1.0, 0.7, 0.9, 0.76, 0.38, and 0.32%, respectively; during days 36-105 — 265 kcal/100 g, 14, 1.0, 0.65, 0.65, 0.58, 0.30, and 0.26%, respectively; and during days 106-147 — 270 kcal/100 g; 15, 1.5, 0.7, 0.64, 0.57, 0.30, and 0.26%.

The poultry of all groups were weighed weekly. At 4 and 7 weeks of age, balance experiments were conducted, for which 3 birds from each group with an average live weight were placed in special balance cages. The preliminary period of the experiment lasted 5 days, the test period lasted 3 days. The consumption of feed, and during the test period also the amount of poultry manure were recorded. The live weight of the bird at the beginning and the end of the experiment was recorded. Ammonia in the average sample of the manure was fixed by 0.1 N oxalic acid solution (4 ml per 100 g manure). At the end of the balance experiment, the samples were dried at 60-70 °C and stored in a container with a ground lid in a refrigerator. According to the data of daily accounting of the feed and manure weight and composition, the number of released and assimilated substances was counted.

After the birds were slaughtered at 21 weeks of age, the testicles of males, ovaries, and oviducts of females were weighed ($n = 3$ from each group). The composition of the microbiome in the intestines of these birds was also studied.

The composition of the microflora of the blind processes of the intestine was investigated by the T-RFLP method (terminal restriction fragment length polymorphism). Total DNA from the samples was isolated using the DNA Purification Kit (Fermentas, Inc., Lithuania), following the manufacturer's recommendations. PCR was performed with 63F 5′-CAGGCCTAACACATGCAAGTC-3′ eubacterial primers labeled at the 5′-terminus (D4 WellRED fluorophore, Sigma-Aldrich, Inc., USA) and 1492R 5′-TACGGHTACCTTGTTACGACTT-3′ (Verity DNA Amplifier; Life Technologies, Inc., USA). These primers allow amplifying a 63 to 1492 fragment of the 16S rRNA gene (the positions are numbered according to 16S rRNA *Escherichia coli* gene) in the mode: 3 min at 95 °C (1 cycle); 30 s at 95 °C, 40 s at 55 °C, 60 s at 72 °C (35 cycles); 5 min at 72 °C. Fluorescently labeled amplicons were purified by standard methods [35]. Concentration of purified 16S rRNA gene fragments was determined on a Qubit 2.0 fluorometer (Invitrogen, Germany). The amplicons (30-50 ng) were restricted with HaeIII, HhaI, and MspI endonucleases (Fermentas, Lithuania). The obtained fragments were analyzed (a CEQ 8000 sequencer, Beckman Coulter, USA). The phylogenetic groups of bacteria were determined using Fragment Sorter software and a database (http://www.oardc.ohiostate.edu/trflpfragsort/index.php).

The results were statistically processed in the Microsoft Excel program. The mean values (M) and standard errors of the mean (±SEM) were determined. The significance of differences was assessed by Student's *t*-test. The differences were considered statistically significant at $p < 0.05$. When estimating taxonomic diversity, Shannon, Simpson, Margalef and Berger-Parker indices were calculated.

Results. The preparation Zaslon 2+ has multifunctional properties that are caused by the adsorption characteristics of diatomite and the biologically active properties of the essential oils of thyme, lemon, garlic, and sage. In addition, its composition includes a modifier − the strain of the bacterium *Bacillus* sp. in the amount of 10^5 CFU/g.

1. Live weight (g) **of males and females, feed intake** ($g \cdot bird^{-1} \cdot day^{-1}$) in **two lines of poultry of different age in using dietary antibiotics or a complex enterosorbent** (*M*±SEM; Centre for Genetic Selection Zagorsk EPH, Sergiev Posad, Moscow Province, 2017)

Возраст, нед	Line									
	B5					B9				
	♂		♀		CF	♂		♀		CF
	C	T	C	T		C	T	C	T	
1	230±4.8	234±5.3	225±5.0	231±4.2	272	214±2.6	220±2.8	210±4.2	207±3.8	251
2	337±5.6	342±6.2	328±5.5	337±4.7	294	290±4.4	280±5.2	277±6.0	280±4.9	280
3	621±7.5	630±8.6	600±7.0	610±7.5	350	522±6.2	501±5.8	490±7.2	494±6.8	336
4	784±9.6	777±10.3	710±11.3	687±10.8	434	677±10.3	650±9.5	599±8.8	600±9.0	420
5	941±15.3	939±14.6	801±17.9	815±16.5	469	835±14.6	840±13.3	715±12.4	720±11.6	455
6	1107±18.6	1118±20.0	975±17.6	990±16.9	476	990±17.3	989±15.9	845±14.6	849±14.0	462
7	1255±21.3	1269±20.5	1015±18.3	1009±19.2	504	1225±20.4	1218±18.6	1042±17.1	1034±16.8	490
8	1447±24.8	1427±22.6	1165±20.0	1148±18.4	511	1384±22.6	1370±20.4	1130±18.6	1125±16.2	497
9	1599±22.4	1560±20.1	1233±20.2	1240±21.3	518	1445±24.5	1482±22.7	1162±20.8	1217±20.0	504
10	1784±26.6	1782±25.4	1435±23.3	1437±22.7	525	1581±25.0	1590±26.2	1290±24.1	1280±23.6	511
11	1893±28.0	1919±27.2	1544±26.6	1566±25.1	546	1610±25.3	1609±24.4	1381±25.5	1374±22.4	532
12	2011±30.4	2010±28.2	1710±25.4	1710±27.0	553	1803±28.1	1810±26.6	1464±26.8	1459±25.5	539
13	2107±32.2	2118±30.8	1735±28.7	1736±29.0	560	1881±30.4	1884±28.5	1527±28.1	1510±26.8	546
14	2270±34.3	2269±33.7	1864±32.4	1868±31.6	574	1935±32.6	1937±33.3	1590±30.4	1613±28.3	560
15	2490±36.6	2490±36.0	1907±34.5	1910±33.3	602	1981±34.8	1980±35.5	1680±33.6	1671±33.2	588
16	2525±34.2	2537±32.6	1936±28.6	1949±28.0	623	2133±35.3	2141±34.9	1708±30.7	1715±32.2	609
17	2647±36.4	2650±35.8	2001±34.3	2014±35.2	630	2270±36.8	2271±35.6	1761±32.2	1772±31.8	616
18	2688±35.0	2778±34.3	2187±36.8	2190±34.6	658	2361±37.7	2361±37.1	1802±34.6	1804±35.5	644
19	2933±34.3	2945±35.7	2209±38.4	2217±35.8	714	2484±38.5	2482±38.8	1840±35.2	1850±34.8	672
20	3066±36.5	3082±37.8	2230±36.5	2234±36.8	728	2510±40.2	2509±40.0	1890±37.6	1887±38.2	682
21	3171±38.8	3168±40.1	2307±40.6	2317±38.4	770	2574±44.6	2592±42.8	1924±38.6	1930±39.8	730

N o t e. C and T are, control and test, respectively, CF means consumption of feed per week. For the description of the groups, see the Techniques section.

The conducted experiments showed that the live weight of males and females of B5 and B9 lines from the control and experimental groups practically did not differ (Table 1). At 21 weeks of age, the males and females of the B5 line of the experimental group had a live weight of 3172 and 2318 g, the control group − 3169 and 2316 g; for the B9 line, these figures were 2590 and 1917, respectively (experimental), 2589 and 1920 g (control). The preservation of the birds in all groups was high and amounted to 100%. Over the entire period of growth, feed consumption per bird for the B5 line was 11.311 kg, for the B9 line 10.924 kg. The feed conversion in the experimental groups when using the preparation Zaslon 2+ was not significantly different from the control.

The results of the physiological (balance) experiment, conducted at 4 and 7 weeks of age, are consistent with the data on live weight of the young chickens. Significant differences in digestibility of dry matter of feed, fat, and the use of nitrogen between the birds of the control and experimental groups were not found. However, the males and females of the B5 line digested better by 4.14% the dry matter of the feed with a greater (by 3.04%) use of nitrogen. At the same time, fat digestibility was also higher (by 3.12%).

The weight of testicles in males at 21 weeks of age in the control and experimental group had no significant differences and was in the range of 8.3-9.4 g (B5 line) and 7.5-8.8 g (B9 line) in the control group, 8.1-9.3 g (B5 line) and 7.6-9.4 g (B9 line) in the test group. There are no significant differences in the mass of ovaries and ovaries with oviducts as well. The weight of the ovaries was 1.82-1.89 g (B5 line) and 1.71-1.88 g (B9 line) in the control, and 1.82-1.99 g (B5 line) and 1.788- 1.97 g (B9 line) in the test group; the weight of the ovaries with oviduct was 5.44-5.72 g (B5 line) and 5.68-5.67 g (B9 line) in the control, and 5.67-5.80 g (B5 line) and 5.63-5.77 g (B9 line) in the test group.

2. Microbial community in the duodenum of meat chickens of two lines under the use of dietary antibiotics or a complex enterosorbent (*M*±SEM; Centre for Genetic Selection Zagorsk EPH, Sergiev Posad, Moscow Province, 2017)

Микроорганизм, индекс доминирования	Line B5		Line B9	
	control	test	conttrol	test
Number of phylotypes	110.0±5.4	49.0±2.5*	57.0±2.5	18.0±0.8*
Simpson index	0.91±0.36	0.87±0.03	0.82±0.03	0.72±0.03
Shannon index	3.39±0.23	2.62±0.12***	2.53±0.13	1.70±0.07***
Margalef index	22.55±1.10	9.76±0.47*	11.19±0.51	3.33±0.15*
Berger-Parker index	0.21±0.01	0.27±0.01	0.34±0.02	0.39±0.01
Phylum *Firmicutes*				
Family *Lactobacillaceae*, %	89.05±5.30	39.19±2.30*	74.26±3.60	64.53±3.36**
Family. *Veillonellaceae*, %	B.l.r.d.	3.82±0.23*	B.l.r.d.	B.l.r.d.
Family. *Bacillaceae*, %	B.l.r.d.	12.50±0.58*	3.59±0.15	4.32±0.23***
Staphylococcus sp., %	B.l.r.d.	0.83±0.03	0	B.l.r.d.
Family. *Clostridiaceae*, %	0.31±0.02	0.38±0.02	B.l.r.d.	0.97±0.04
Clostridium novyi and/or *Cl. perfringens*, %	0.48±0.04	B.l.r.d.	B.l.r.d.	B.l.r.d.
Phylum *Bacteroidetes*				
Order *Bacteroidales*, %	B.l.r.d.	4.02±0.25	0.48±0.03	1.43±0.07**
Phylum *Actinobacteria*				
Order *Actinomycetales*, %	B.l.r.d.	0.82±0.03	0.69±0.02	B.l.r.d.
Bifidobacterium sp., %	B.l.r.d.	0.56±0.03*	B.l.r.d.	B.l.r.d.
Phylum *Proteobacteria*				
Campylobacter sp., %	1.04±0.06	0.26±0.01*	0.51±0.01	B.l.r.d.
Family *Pseudomonadaceae*, %	B.l.r.d.	1.66±0.07	B.l.r.d.	B.l.r.d.
Phylum *Fusobacteria*				
Fusobacterium sp. , %	0.40±0.02	1.01±0.04*	B.l.r.d.	B.l.r.d.
Phylum *Tenetricutes*				
Mycoplasma sp., %	B.l.r.d.	B.l.r.d.	0.53±0.01	B.l.r.d..
Uncultivated bacteria				
	3.98±0.28	27.94±1.50*	4.06±0.26	9.22±0.36**

Note. For the description of the groups, see the "Techniques" section.
*, ** and *** Differences from control are statistically significant at $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$, respectively; B.l.r.d. means below the limit of reliable T-RFLP determination.

T-RFLP method revealed from 18.0±0.8 to 110.0±5.4 bacterial phylotypes in the duodenum of B5 and B9 poultry (Table 2). Shannon, Simpson and Margalef indices showed a more pronounced taxonomic diversity and complexity of the structure of the microbial communities of the duodenum in the control variants of both lines. This indicates the uncertainty and heterogeneity of the compositions of the microbiocenosis, the accumulation of entropy and some disorganization compared to the test groups.

A significant proportion of microorganisms detected in the blind processes of the intestine of chickens in all the studied groups could not be attributed to any existing taxon. In chickens of both lines, the number of unidentified bacteria in the experimental groups was significantly higher ($p \leq 0.01$) than in the control.

The composition of the intestine microorganisms of meat chickens in both lines was similar. In the majority of the studied samples, the bacteria in the structure of the intestinal microbiota were assigned to 5 phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria*. In the control variant, the representatives of the phylum *Tenericutes* (genus *Mycoplasma*) were found in chickens of the B9 line. Bacteria belonging to the *Firmicutes* phylum were dominant in all the examined variants. These results are partially consistent with the data previously obtained by other authors [30, 31, 36]. Thus, of the 13 bacterial phyla, identified in the intestines of chickens and turkeys, *Firmicutes*, *Bacteroidetes* and *Proteobacteria* dominated, with more than 90% of all analyzed sequences [37].

Despite the general similarity in the composition of the intestinal microbiocenosis of meat chickens of two lines, some differences were observed in the structure of the microflora. For example, in the intestine of chickens in the control group of the B5 line, there were bacteria of the family *Clostridiaceae*, genera

Fusobacterium, *Clostridium novyi/Cl. perfringens*, whereas in the B9 line these microorganisms were not identified. It is known that representatives of the *Clostridiaceae* family can play a positive role in the digestion of birds, since they are able to form a number of digestive enzymes, including cellulases, which allow the macroorganism to effectively use the energy of feeds, rich in fiber. In contrast, species of *Clostridium novyi* and *Clostridium perfringens* are often associated with gastroenteritis and claudication in chickens [38, 39], and pathogenic forms are also found among bacteria of the genus *Fusobacterium*.

The administration of complex enterosorbent with phytobiotic properties in the diet of the poultry of both lines contributed to the change in the qualitative composition of the duodenal microbiome. As a result of the use of the preparation Zaslon 2+ in the duodenum of the poultry of the B5 line, there was an increase in the number of bacteria of the genus *Bifidobacterium* and an order *Bacteroidales*, as well as a decrease in the content of bacteria of the genus *Campylobacter*. This indicates a normalization of the microflora balance in the gastrointestinal tract, since bacteria of the genus *Bifidobacterium*, living in the intestinal lumen, have antimicrobial and immunomodulatory activity, synthesize vitamins and some essential amino acids, and microorganisms of the order *Bacteroidales* are capable of synthesizing enzymes that break down complex polysaccharides and starch of feed. Among the representatives of the genus *Campylobacter*, pathogenic forms are often found, such as *Campylobacter jejuni* [40]. Probably, positive changes in the composition of the microflora under the influence of Zaslon 2+ were associated with the antimicrobial activity of essential oils and the probiotic bacterial strain in the composition of the preparation. On the one hand, the mechanism of action of probiotic bacterial strains was to synthesize biologically active substances with antimicrobial activity, on the other, bacterial strains could colonize the gastrointestinal tract through adhesion, forming a protective layer, covering the surface of the mucous epithelium, which simultaneously mechanically and functionally blocked colonization of the intestine by pathogenic microorganisms [41].

The data of the studies are consistent with the results obtained earlier. So, Wang et al. [42] showed that in the microbiome of the blind processes of the intestine of chickens, in the diet of which probiotic strains of bacteria were introduced, bacteria of the genus *Bacteroides,* belonging to the order *Bacteroidales*, dominated. Despite the positive changes in the composition of the microflora under the influence of the preparation in the experimental group of the authors' study, in the duodenum of the poultry of the B5 line, in the diet of which Zaslon 2+ was included, the number of bacteria of the *Lactobacillaceae* family decreased. Lactic acid bacteria of the *Lactobacillaceae* family play an important role in the intestines of birds, because they synthesize lactate as the main product of metabolism, which reduces the pH of chyme, leading to the suppression of pathogenic forms.

With the administration of the preparation Zaslon 2+ of the B9 line, the number of bacteria of the *Bacillaceae* family and cellulolytic bacteria of the *Clostridiaceae* family increased in the duodenum of poultry. The representatives of the *Bacillaceae* family are able to synthesize a wide range of bacteriocins [43] that effectively inhibit the development of pathogenic bacteria [44]. Therefore, an increase in the number of bacteria in these groups may indicate the correction of dysbiotic disorders in the intestine.

Thus, the use of the feed additive Zaslon 2+ in combined feeds for young chickens of the domestic initial lines B5 (Cornish breed) and B9 (Plymouth Rock breed) instead of dietary antibiotics does not significantly change the live weight of poultry. The development of the reproductive organs of males (tes-

ticles) and females (ovaries and oviducts) in both groups remaines within the normal range without statistically significant differences. The digestibility and the use of nutrient substances of feed do not differ significantly between groups of young chickens. The T-RFLP method reveales some differences in the composition of the microbiocenosis of the duodenum in the test groups. The number of unidentified bacteria in the test groups exceeded that in the control. Zaslon 2+ administering results in an increase in the number of *Bacillaceae* family and cellulolytic *Clostridiaceae* bacteria in the duodenum of the B9 birds, and in the B5 line the number of bacteria of the genus *Bifidobacterium* and the order *Bacteroidales* increase, and the counts of the genus *Campylobacter* bacteria decrease. An increase in the bacteria in these groups and a decrease in the portion of pathogenic microorganisms may indicate a correction of dysbiotic disorders in the intestines of poultry.

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THE EFFECTS OF FEED ADDITIVES BASED ON THE HYDROLYSATES OF KERATIN- AND COLLAGEN-CONTAINING WASTE MATERIALS ON THE INTESTINAL MICROBIOTA AND PRODUCTIVITY PARAMETERS IN BROILER CHICKS (*Gallus gallus* L.)

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Abstract

The use of new animal-derived protein ingredients in commercial diets for poultry as a substitute for the expensive fishmeal is an urgent direction of the nutritional research. At present a range of new additives is designed based on the wastes of poultry slaughter and processing. In this paper, we first report the results of comparative analysis of the bacterial community in blind processes of the intestines of broiler chickens fed with dietary protein-rich additives derived from keratin- and collagen-containing waste products after short-term high-temperature enzymatic hydrolysis in a thin layer. These findings show possibility of using new feed additives to increase the productivity and quality of broiler meat. Our study was aimed at the evaluation of the effects of the feed additives based on keratin- and collagen-containing wastes on the intestinal microbiota counts and composition in conjunction with productive performance of broiler chicks. The chicks (*Gallus gallus* L.) of Ross 308 cross were reared at the Vivarium of All-Russian Research and Technological Institute of Poultry (Moscow Province) on the floor until 38 or 49 days of age. Control Treatment (Trt) 1 was fed standard diet with fishmeal as the main protein source. In the diet for Trt 2 the fishmeal was substituted by a hydrolysate of keratin-containing wastes (poultry feathers); in Trt 3 by a hydrolysate of collagen-containing wastes of poultry deboning; in Trt 4 by a mixture of these additives with additional supplementation with probiotic Bacell-M (containing *Bacillus subtilis, Lactobacillus paracasei, Enterococcus faecium*). The counts and composition of cecal microbiota in broilers were determined using Terminal Restriction Fragment Length Polymorphism (T-RFLP) technique. The live weight at 38 and 49 days of age (individual weighing), average daily weight gain, livestock safety, feed costs per 1 kg of live weight, grade and weight of gutted carcasses, meat yield of carcasses and meat qualities, digestibility and use nutrient feed were recorded. The protein additives based on the hydrolysates of poultry wastes do not compromise the composition of cecal microbiota in broilers, and the obligate species were abundant with all diets studied. Cellulolytic *Clostridia* class (phylum *Firmicutes*) including families *Ruminococcaceae*, *Eubacteriaceae*, *Lachnospiraceae*, *Clostridiaceae* etc. dominated in the cecal microbial communities. The additives beneficially affected the productive performance in broilers. The best productivity parameters were in broilers fed diets with the hydrolysate of keratin-containing material (Trt 2) and a mixture of the hydrolysates of keratin- and collagen-containing wastes with the probiotic (Trt 4). Average live bodyweight at 38 and 49 days of age in Trt 2 was significantly higher, by 9.2 % ($p < 0.01$) and 10.1 % ($p < 0.001$), respectively, as compared to control Trt 1. Mortality level in Trt 2 was 0 % while feed conversion ratio (FCR) at 38 and 49 days of age was 6.32 and 7.28 % better compared to control. Average live bodyweight in Trt 4 at 38 days of age was 5.3 % higher, and FCR was 2.87 % better compared to control; at 49 days of age these parame-

ters were better in compare to control by 4.96 and 4.37 %, respectively, while mortality in Trt 4 during 38 and 49 days of rearing was 0 %.

Keywords: broiler chicks, Ross 308 cross, keratin- and collagen-containing materials, enzymatic hydrolysis, cecal microbiota, productive performance, meat quality

The optimal functioning of the segments of bird digestive tract depends largely on the feeding system. The microflora of the gastrointestinal tract (GIT) is directly affected by the structure of the feed [1, 2]. Disturbances in feeding cause undesirable changes in the microbiocenosis, which negatively affects the productivity of poultry and leads to diseases.

The first days of chickens after hatching are considered critical for their further growth and development. During this period, their organisms undergo a metabolic and physiological transition from feeding on the residual yolk of an egg to the combined feed. The intestine develops rapidly to efficiently absorb the nutrients of the feed. Slow formation of the intestinal microflora can be a consequence of the unfavorable state of feed, water and maintenance conditions, which, in turn, jeopardizes the proper development of an organism [3].

The contents of the gastrointestinal tract of poultry represent a favorable environment for the growth of many bacteria [4]. Harmful groups of bacteria may be involved in the development of infections and the production of toxins. Populations of useful bacteria are involved in the production of vitamins and also suppress harmful bacterial populations [5, 6]. Microflora metabolizes several nutrients that an organism cannot digest and turns them into final products (for example, fatty acids). It not only protects the body from exogenous pathogens capable of colonizing cells and tissues but also plays an important role in providing immunity. Any changes in the functions of the gastrointestinal tract lead to a deterioration in the absorption and assimilation of nutrients, an increase in secretion, the development of dysbacteriosis, a decrease in resistance to diseases and productivity of a bird as a whole [7].

It is known that the largest accumulation of microorganisms (10^{10}-10^{11} per 1 g of the content) as compared to other parts of the gastrointestinal tract is typical of the blind processes of the intestine [8]. The microflora, which is present there, plays an important role in digesting feed and performs multiple functions to maintain the homeostasis of the macroorganism as a whole [9-12]. The structure of the microbial community of the cecum is quite complex and not yet fully characterized. The advantage of microbial technologies is that they can improve the understanding of the functions and structure of the intestinal microbiota, the relationships between the organism and the microbiota, and also help to choose alternative products that contribute to the health state of the intestine [13, 14]. New molecular technologies make it possible to conduct a detailed molecular and physiological assessment, including the quantitative determination of individual types of microorganisms and their metabolites [15].

The elaborated and improved molecular genetic methods allow differentiation of any bacteria. One of the effective approaches to determining microflora is the use of a polymerase chain reaction, in particular, an express method based on T-RFLP analysis (terminal restriction fragment polymorphism). It includes DNA extraction, amplification of target fragments and sequencing, followed by a study of the obtained T-RFLP-grams involving databases using the Fragment Sorter program and comprehensive analysis, based on statistical (correlation and cluster), taxonomic and ecological approaches [16].

The use of molecular genetic methods significantly expanded the concept of biodiversity of microorganisms in the gastrointestinal tract of poultry. Currently, the gastrointestinal tract has more than 900 species of bacteria, methanogenic ar-

chaea, and fungi. In a number of works, a multilateral characterization of the intestinal microbiota of broiler chickens was given, which made it possible to investigate in detail the important patterns in the functioning of this complex microbio-ecosystem [17, 18]. The intestinal microbiota of broiler chickens has been studied for many years, since it is inherently involved in many physiological processes and affects both feed and health of poultry [19, 20]. Using molecular technologies, the mechanisms of action of new feed additives to diets are studied, and the changes in the intestinal microbiome and immune function are assessed. The use of such products in the broiler industry is necessary to improve the health of broiler chickens' intestines and minimize risks [21].

In Russia, T-RFLP analysis has been used relatively recently; in particular, in 2008, JSC Biotrof modified this method to study GIT microflora of poultry. It allows assessing the effect of various components of the diet on the microbial background and identifying the relationship between its composition, structure, and indicators of bird productivity. In addition, the method is considered promising for the early diagnosis of bacterial diseases. Therefore, this helps to rationally choice the feed additives [22].

In this paper, the authors conducted for a first time a comparative analysis of the bacterial community of the contents of the blind processes of the intestines in broiler chickens with the inclusion of protein feed additives in the diet from keratin- and collagen-containing raw materials obtained by short-term high-temperature hydrolysis in a thin layer. The possibility of using new feed additives for increasing the productivity and quality of meat in broiler chickens is shown.

The purpose of the research was to assess the effect of protein feed additives from keratin- and collagen-containing raw materials obtained by the method of short-term high-temperature hydrolysis in a thin layer, on the microflora of the gastrointestinal tract and the productivity of broiler chickens.

Techniques. In 2018, the experiments were performed on four groups of broiler chickens of the Ross 308 cross (50 birds each), reared on litter in vivarium conditions of the Center for Genetic Selection Zagorsk EPH (Sergiev Posad, Moscow Region) from the first day. Broiler chickens of group I (control) received combined feed, in which fishmeal was the main source of crude protein. In group II, instead of fishmeal, we used feed additive from a hydrolysate of keratin-containing raw materials (feathers), in group III, an additive from a hydrolysate of collagen-containing raw materials (wastes of poultry deboning), in group IV, an additive from a mixture of hydrolysates of keratin- and collagen-containing raw materials with the inclusion of a probiotic preparation based on live bacteria *Bacillus subtilis*, *Lactobacillus paracasei*, *Enterococcus faecium* (Bacell-M, Russia).

Prior to grouping, 1-day-old chickens were individually weighed and distributed by random sampling. The sex ratio of males and females in all groups was determined at the end of poultry rearing. When conducting the experiment, chickens were selected on the basis of analogs – identical in origin, age, general development, and reared from the same batch of eggs. The growing conditions complied with the technological standards of the Federal Science and Technology Center All-Russian Research and Technological Institute of Poultry RAS [23].

The live weight of broilers was recorded at 38 and 49 days of age by individual weighing, average daily gain, preservation of stock, feed “costs” per 1 kg of live weight, grade and weight of eviscerated carcasses, slaughter meat, meat qualities of carcasses, digestibility and use of nutritional substances were estimated in accordance with the methods of physiological (balance) experiments [24]. To study the gastrointestinal microflora in broiler chickens aged 38 and 49 days, the samples of the blind processes of the intestines were collected and examined using

the T-RFLP method.

Total DNA from the samples was isolated using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) in accordance with the recommendation of the manufacturer. The extraction was conducted simultaneously for three samples from each experimental group; then the samples in each group were combined into one sample for further analysis. PCR amplification was performed on a Verity DNA amplifier (Life Technologies, Inc., USA) using eubacterial 63F primers (5′-CAGGCCTAACACATGCAAGTC-3′) labeled at 5′-end (WellRed D4 fluorophore, Beckman Coulter, USA) and 1492R (5′-TACGGHTACCTTGTTACGACTT-3′), which allow amplifying a fragment of the 16S rRNA gene (positions from 63 to 1492, numbering for the 16S rRNA gene *Escherichia coli*) in the following mode: 3 min at 95 °C (1 cycle); 30 s at 95 °C, 40 s at 55 °C, 60 s at 72 °C (35 cycles); 5 min at 72 °C (1 cycle). The final concentration of total DNA in the solution was determined with a Qubit fluorometer (Invitrogen, Inc., USA) using the Qubit dsDNA BR Assay Kit (Invitrogen, Inc., USA) according to the manufacturer's recommendations.

Fluorescently labeled amplicons of the 16S rRNA gene fragments were purified according to standard procedures. 30-50 ng of DNA was treated with restrictases HaeIII, HhaI, and MspI, following the manufacturer's recommendation (Fermentas, Lithuania), for 2 hours at 37 °C. The restriction products were precipitated with ethanol; then 0.2 μl of Size Standart-600 molecular weight marker (Beckman Coulter, USA) and 10 μl of Sample Loading Solution formamide (Beckman Coulter, USA) were added. The analysis was performed using CEQ 8000 (Beckman Coulter, USA); the error of the device CEQ 8000 is no more than 5%. The sizes of peaks and their areas were calculated using the Fragment Analysis program (Beckman Coulter, USA), on the basis of which subtypes (phylotypes) were detected with an error of 1 nucleotide, used in the study, and their relative content in the microbial community was evaluated. The affiliation of bacteria to taxonomic groups was determined using a database (http://mica.ibest.uidaho.edu/trflp.php).

The obtained results were processed by the method of variance analysis in Microsoft Excel 2010. The tables show the mean values (*M*) and standard errors of the mean (±SEM). The statistical significance of differences between the mean values of the studied parameters was evaluated using Student's *t*-test at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

Results. A comparative analysis of the bacterial community of the content of the blind intestinal processes in broiler chickens revealed differences in the composition of the microbiota associated with the period of ontogenesis and the diet of the poultry. The content of normal microflora in the blind processes was high in all groups (Table 1). Bacteria with cellulose and amylolytic properties from the *Clostridia* class of the *Firmicutes* phylum (including the families *Ruminococcaceae*, *Eubacteriaceae*, *Lachnospiraceae*, *Clostridiaceae*) and the *Bacteroidetes* phylum were dominant, which corresponds to the modern concepts regarding the intestinal microbiota of poultry [25, 26]. As follows from the results available in the GenBank databases (https://www.ncbi.nlm.nih.gov/genbank/), Ribosomal Database Project (http://rdp.cme.msu.edu/) and Silva (http://www.arb-silva.de/), the taxonomic composition of the gastrointestinal tract of poultry and turkey is 90% represented by microorganisms from the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria* [27]. In the authors' experiment, in 38-day-old broiler chickens, the proportion of cellulolytic bacteria was more than 59%, in the 49-day-old poultry this indicator decreased by 10% in groups I and III, by 30% in group II, and 2-fold in

1. The content of microorganisms (%) in the blind processes of the intestines in Ross 308 cross broiler chickens depending on age and diet according to T-RFLP analysis (*M*±SEM, Zagorsk EPH vivarium, Moscow Province)

Микроорганизм	Age, group (n = 50)							
	38 days				49 days			
	I (control)	II	III	IV	I (control)	II	III	IV
Normal flora								
Cellulolytic, including:	62.49±2.89	60,05±2,37	59,25±1,41	60,28±3,08	56,82±1,36	45,85±1,89*	54,07±1,37	29,19±1,13
family *Eubacteriaceae*	13.56±0.71	13,24±0,58	24,79±0,94**	11,73±0,59	24,32±0,96	11,31±0,51**	18,32±0,48*	8,67±0,37***
family *Clostridiaceae*	18.57±0.65	24,8±0,97*	6,96±0,27***	12,36±0,43**	13,12±0,37	13,72±0,33	7,27±0,26***	7,11±0,46***
family *Lachnospiraceae*	6.08±0.28	2,99±0,14**	2,67±0,18**	2,14±0,13**	4,68±0,17	4,28±0,14	2,29±0,07***	2,57±0,22**
family *Ruminococcaceae*	8.62±0.32	5,12±0,27**	6,58±0,19*	12,23±0,47**	0,33±0,01	2,30±0,14***	10,19±0,55***	1,14±0,04***
phylum *Bacteroidetes*	15.66±0.48	13,9±0,65	18,23±0,75	21,82±0,69**	14,37±0,48	14,24±0,60	16,00±0,67	9,70±0,30**
Lactobacillus sp., *Enterococcus* sp.	2.56±0.08	5,17±0,14***	1,54±0,05**	1,24±0,01***	1,79±0,03	5,69±0,27***	2,57±0,09**	2,02±0,07
Bacillus sp.	1.31±0.04	0,74±0,03**	0,79±0,01**	2,60±0,09***	1,11±0,02	2,23±0,06***	1,19±0,03	11,69±0,41***
Selenomonas sp., *Veillonella* sp.	8.23±0.25	10,24±0,45*	10,78±0,38*	12,27±0,44**	16,89±0,57	10,51±0,37**	13,35±0,28*	5,26±0,14***
Family *Bifidobacteriaceae*)	0	0,07±0,01	0	0	0,11±0,01	0,16±0,01	0,24±0,01	0,20±0,01
Conditionally pathogenic microflora								
Actinobacteria (order *Actinomycetales*)	1.59±0.03	4,81±0,16***	3,35±0,18**	2,54±0,09**	5,22±0,15	7,18±0,24**	10,68±0,32***	3,16±0,07**
Enterovacteria (family *Enterobacteriaceae*)	0.12±0.01	3,84±0,13***	1,16±0,03***	0,50±0,01***	2,66±0,11	2,28±0,09	3,26±0,12*	1,92±0,08*
Pathogenic microflora								
Staphylococcus sp.	0.23±0.01	0,06±0,01**	1,26±0,05***	1,13±0,03***	0	0,22±0,01	0,35±0,01	0,55±0,01
Clostridium novyi, *Clostridium perfringens*	0.99±0.03	0,11±0,01***	0,79±0,02*	1,51±0,04**	0,59±0,02	1,96±0,08***	1,93±0,12**	0,19±0,01***
Family *Pasterellaceae*)	0.29±0.01	0	0,64±0,02***	0,15±0,01**	0	1,43±0,06	0,75±0,02	1,81±0,06
Fusobacterium sp.	1.48±0.06	1,76±0,04*	7,19±0,28***	3,79±0,15***	0,10±0,01	0,64±0,01***	0,10±0,01	1,67±0,05***
Family *Campylobacteriaceae*)	0.38±0.01	0,41±0,01	0,77±0,03***	0,42±0,01	0,49±0,02	0,74±0,01**	0,48±0,02	6,82±0,29***
Family *Peptococcaceae*)	0.5±0.01	1,26±0,04***	0,89±0,03**	0,78±0,02**	0,59±0,01	1,42±0,04***	1,57±0,07***	4,82±0,19***
Mycoplasma sp.	0.5±0.01	0	0,05±0,01	0	0	0,66±0,01	0	0
Transit microflora								
Family *Pseudomonadaceae*)	5.95±0.23	4,14±0,19**	2,09±0,05***	3,92±0,13***	1,71±0,07	0,33±0,01***	0,63±0,02***	4,64±0,14***
Uncultivated bacteria	13.38±0.41	7,34±0,28**	9,47±0,38**	8,87±0,35**	11,92±0,41	18,7±0,81**	8,83±0,32**	26,06±0,98***

Note. For the description of the groups, see the Techniques section.

*, ** and *** The differences from the control are statistically significant at $p \le 0.05$; $p \le 0.01$ and $p \le 0.001$, respectively.

group IV.

The presence of cellulolytic bacteria of the *Lachnospiraceae* family in birds, the diet of which included the hydrolysate of collagen-containing raw materials (groups III and IV), was the smallest. In addition, in these experimental groups, the presence of bacteria of the family *Clostridiaceae*, which are capable of fermenting starch, fiber, and some other carbohydrates, was the smallest. The proportion of bacteria of the *Negativicutes* class, which utilize organic acids as a result of fermentation of carbohydrate in feed, changed depending on the age and the group of birds. In group I, by the age of 49 days, the relative number of selenomonads decreased by 2 times ($p \leq 0.001$), in group II, it did not change, in group III it increased by 24% ($p \leq 0.01$), and in group IV it decreased 2.3-fold ($p \leq 0.001$).

Interesting changes were noted for the obligate microflora of the poultry intestines, i.e. lactic acid bacteria of the genera *Lactobacillus*, *Enterococcus* and bifidobacteria of the genus *Bifidobacterium*, which, due to the synthesis of various organic acids and bacteriocins, are capable of antagonistically displacing representatives of pathogenic and conditionally pathogenic groups from the intestine (salmonella, Proteus, Staphylococcus, coliform bacillus, pseudomonad, streptococcus) [28, 29]. The number of lactobacilli of the genus *Lactobacillus*, showing significant antagonism against pathogenic species [28], at 38 days of age in groups I, III and IV did not exceed 2.5%. In group II, the lactobacilli reached 5.17%. At day 49, the relative number of lactobacilli in group I decreased 1.5 times ($p \leq 0.01$) and their number increased by 10% in group II, and by 65% ($p \leq 0.01$) in groups III and IV.

The relative number of bacilli in group I did not change with the bird age, abd increased 3 times ($p \leq 0.001$) in group II, 1.5 times ($p \leq 0.01$) in group III, and 4 times ($p \leq 0.001$) in group IV. Bifidobacteria (*Bifidobacteriaceae* family) were practically not detected in the samples of the contents of the blind processes of chickens at the age of 38 days. By day 49 of age, its share increased in all groups to 0.10-0.24%.

Conditionally pathogenic microorganisms were widely represented in the community. Most of them are traditionally associated with the development of gastroenteritis (*Enterobacteriaceae, Pseudomonadaceae* family). The proportion of actinomycetes from the order *Actinomycetales*, the representatives of which are capable of causing actinomycosis, was also high. The relative number of conditionally pathogenic *Actinobacteria* increased with the age of chickens 3.3 times ($p \leq 0.001$) in group I, and 3 times ($p \leq 0.001$) in group III. The enterobacteria in group I increased 22 times ($p \leq 0.001$), in group III 2.8 times ($p \leq 0.001$), in group IV 3.8 times ($p \leq 0.001$). In group II, the relative number of enterobacteria decreased 1.7 times ($p \leq 0.01$).

Among the bacteria capable of causing infectious diseases, we identified causative agents of clostridiosis (*Clostridium novyi*, *Clostridium perfringens*), pasteurellosis (family *Pasteurellacea*, genus *Pasteurella*, genus *Haemophilus*), mycoplasmosis (phylum *Tenericutes*, genus *Mycoplasma*), necrotic enteritis (phylum *Fusobacteria*), and purulent-necrotic infections (genus *Staphylococcus*). The most of the above-listed microorganisms in the intestinal community of poultry was minor.

The proportion of staphylococci was high only in 38-day-old poultry in groups III and IV (more than 1%); by the age of 49 days, the number of staphylococci decreased in these groups 2-2.5 times. The relative number of pathogenic clostridia was high, regardless of the age of the chickens. By day 49 in group I, its share decreased 2 times, in group IV 7.5 times. In group II and III, the number of these bacteria increased with age 18 times and 2.4 times, respectively.

The portion of *Pasteurella* was low at the age of 38 days, but by day 49 it

increased in all groups except the control. Fusobacteria in a significant amount were present in all the studied groups of birds at the age of 38 days. By day 49, its relative number decreased 15-fold in group I, 2.8-fold in group II, 72-fodl in group III, and 2.3-fold in group IV.

The relative number of campylobacters was low in all samples. A large amount of these bacteria was observed only at the age of 49 days in the group IV (6.82%), which was 16 times higher as compared to day 38. The number of peptococci was low both by day 38 and by day 49. In group II, the proportion of peptococci was higher (more than 1%), but did not change with the age of the poultry. In groups III and IV, this indicator on day 38 was less than 1%, but by day 49, it increased to 1.57 and 4.82%, respectively. The share of mycoplasmas in the samples was low and only in group II it increased on day 49 to 0.66%. Transit microflora in all the studied groups was present in insignificant amount.

The results of the research on the number and composition of microorganisms in the blind processes of the intestines of the poultry are generally consistent with the literature data [30, 31]. Cellulolytic bacteria from the class *Clostridia* of the phylum *Firmicutes* (including the families *Ruminococcaceae, Eubacteriaceae, Lachnospiraceae, Clostridiaceae*, etc.) and the phylum *Bacteroidetes* occupied a dominant position in the community. At the same time, the authors found that the inclusion of keratin hydrolysates and collagen-containing raw materials in the diet of broiler chickens did not negatively affect the microflora of poultry gastrointestinal tract.

At one day of age, the live weight of chickens ranged from 45.4 to 45.9 g. At the age of 38 days, the best group in terms of productivity was the second experimental group, in which chickens received fermented feather hydrolysate instead of fishmeal (Table 2). Thus, the average live weight of chickens in this group was ahead of the control indicator by 9.20%. The average weight of males and females was 2391 and 2183 g, respectively, which was 8.78 and 9.70% higher than their peers from group I (basic diet) with a significant difference ($p \leq 0.01$ and $p \leq 0.001$). With 100% preservation of livestock, feed costs per 1 kg of weight gain were lower by 6.32%, and the average daily gain was 9.46% higher than the control. The use of combined feed with the inclusion of fermented feather hydrolysate in it at 49 days of age contributed to an increase in the average live weight, average daily growth of broiler chickens and livestock preservation by 4.0%. At the same time, the feed costs per 1 kg of increase in live weight were 7.28% lower compared with the control. Thus, the average live weight of males and females was significantly ($p \leq 0.001$) higher by 9.35 and 10.89%, respectively.

Broiler chickens, which received a feed additive from fermented collagen hydrolysate (group III), did not significantly differ in productivity from the control poultry at 38 days of age. Both males and females practically did not differ from chickens in the control in terms of average live weight, average daily gain, and preservation of livestock, but feed costs in this group were 0.57% lower. At the age of 49 days, in respect of productivity indicators, with the exception of preservation, group III did not differ from the control. Preservation was 2.0% higher.

The introduction of a mixture of fermented hydrolysates of feathers and collagen into the diet using a probiotic preparation (group IV) made it possible to increase the productivity indicators of broilers. So, on day 38, the average live weight of chickens and the average daily gain in live weight were 5.3% and 5.4% higher compared to the same indicators in group I. The feed “costs” fell to 2.87% with 100% preservation of livestock. At 49 days of age, the average live weight of the chickens exceeded the control by 4.96%, while the live weight of the males was 4.79% higher, and the females 5.19% higher. The average daily gain in chickens on day 49 in group IV was 62.6 g, exceeding the control by 5.03%. Feed conversion

2. Productivity of Ross 308 cross broiler chickens depending on age and diet (Zagorsk EPH vivarium, Moscow Province)

Indicator	Age, group (n = 50)							
	38 days				49 days			
	I (control)	II	III	IV	I (control)	II	III	IV
Average live weight, g /animal unit:								
♂ (M±SEM)	2198±49.8	2391±38.5**	2218±53.1	2296±68.8	3154±70.3	3449±54.0***	3161±82.4	3305±92.4
♀ (M±SEM)	1990±48.1	2183±31.7***	1992±38.7	2114±36.5*	2773±68.5	3075±48.9***	2789±55.8	2917±63.6
Arithmetic mean M	2094	2287	2105	2205	2964	3262	2975	3111
To the control, %		+9.2	+0.52	+5.3		+10.05	+0.37	+4.96
Average daily gain, g (M±SEM)	53.9±0.72	59.0±0.51	54.2±0.65	56.8±0.53	59.6±0.79	65.6±0.61	59.8±0.73	62.6±0.63
Preservation, %	98	100	98	100	96	100	98	100
Feed costs per 1 kg gain, kg	1.74	1.63	1.73	1.69	2.06	1.91	2.02	1.97
Mass of eviscerated carcasses, g (M±SEM)	1514±21.5	1692±16.9	1526±20.9	1607±22.3	2167±31.68	2443±23.6	2181±30.7	2296±25.4
Slaughter yield, %	72.3	74.0	72.5	72.9	73.1	74.9	73.3	73.8
Grade of carcasses, %:								
1st grade	65.3	72.0	67.3	68.0	68.8	76.0	69.4	72.0
2nd grade	34.7	28.0	32.7	32.0	31.2	24.0	30.6	28.0

N o t e. For the description of the groups, see the Techniques section.

*, ** and *** The differences from the control are statistically significant at $p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$, respectively.

3. Digestibility and use of nutrients of combined feed (%) by Ross 308 cross chicken broilers depending on age and diet (Zagorsk EPH vivarium, Moscow Province)

Indicator	Age, group (n = 3)							
	38 days				49 days			
	I (control)	II	III	IV	I (control)	II	III	IV
Digestibility of:								
dry matter	74.8	76.0	75.1	76.2	74.3	75.3	74.8	75.8
protein	91.1	93.2	92.5	93.4	90.2	92.1	91.4	92.4
fat	80.5	83.0	81.2	83.5	79.8	82.4	81.6	83.0
fiber	10.0	12.8	11.9	13.1	11.4	13.5	12.5	13.1
Use of :								
nitrogen	57.9	59.4	58.3	59.9	56.4	58.5	57.1	59.1
calcium	46.5	47.1	46.8	47.0	44.1	46.2	45.4	46.1
phosphorus	29.6	31.4	30.3	31.6	30.8	33.0	32.4	33.2

N o t e. For the description of the groups, see the Techniques section/ Calculations are basedon on the average sample.

was 4.37% higher than in group I.

The inclusion of the hydrolysate of keratin-containing raw materials (group II) in the combined feed provided an increase in digestibility and use of nutrients of the feed compared to the control (Table 3). The digestibility of dry matter in chickens at the age of 38 and 49 days increased by 1.2 and 1.0%, protein by 2.1 and 1.9%, fat by 2.5 and 2.6%, fiber by 2.8 and 2.1%. The use of nitrogen increased by 1.5 and 2.1%, calcium by 0.6 and 2.1%, phosphorus by 1.8 and 2.2%.

In group IV, in poultry aged 38 and 49 days, an increase was also noted in the digestibility of feed dry matter by 1.4 and 1.5%, protein by 2.3 and 2.2%, fat by 3.0 and 3.2% and fiber by 3.1 and 1.7%. The use of nitrogen increased by 2.0 and 2.7%, calcium by 0.5 and 2.0%, phosphorus by 2.0 and 2.4%. The indicators of nutrient utilization were slightly lower in group III. So, compared to the control, the digestibility of dry matter of the feed here increased by 0.3 and 1.0% protein by 1.4% and 2.0%, fat by 0.7% and 1.8%, and fiber by 1, 9 and 1.1%. At the age of 38 days, no significant differences with the control were observed in utilization of nitrogen, calcium, and phosphorus, and only on day 49, these indicators increased by 0.7, 1.3 and 1.6%, respectively

In assessing the meat qualities of broiler chickens, it was found that at the age of 38 days, the highest value of the most valuable part, the pectoral muscles (33.17% of eviscerated carcass weight), was observed in broiler chickens from group II, which was 1.28% higher than in the control. In general, the yield of all the muscles in broiler carcasses was 1.56% higher compared to group I. The highest yield of edible parts in chicken carcasses was in group II, 79.14% vs. 77.47% in the control. This was mainly due to a higher (by 1.56%) muscle yield in broiler carcasses. As to skin with subcutaneous fat and bones, the broiler carcasses in group II did not differ from the control. At the age of 49 days, the yield of the pectoral muscles was 0.55% higher, than in the control. The total muscle yield in broiler carcasses in group II was 1.45%, the yield of edible parts was 1.32% higher, and the bone weight was 1.22% lower compared to the control.

Group III had no significant differences in meat quality of carcasses compared to group I. In group IV, the yield of the pectoral muscles in the carcasses was 0.45% and 0.46% higher compared to the control at the age of 38 and 49 days, respectively. The yield of all muscles in broiler carcasses exceeded the control by 0.77 and 0.97%. Moreover, in group IV, there was a greater yield of edible parts in carcasses at the age of 38 and 49 days, 78.09 and 79.76%, respectively, or 0.62% and 0.96% higher than the control values. The differences in skin content with subcutaneous fat and bones in broiler carcasses were not found.

The highest taste and aromatic advantages of broth and meat on a 5-point

scale were in the groups II and IV. That is, the inclusion of protein feed additives from keratin- and collagen-containing raw materials in the diet of broiler chickens contributed to an increase in the meat and taste qualities of carcasses.

The results obtained in the study, in general, are consistent with the modern understanding of the microbiota of the blind processes of the intestines of poultry. Thus, it was previously reported on the effect of various feed components of the bird diet on the intestinal microflora [32-34], as well as on the possible relationship between the number and species composition of intestinal microorganisms and bird productivity [35-37]. For example, there are data that an increase in the amount of barley, rich in non-starch polysaccharides, in the diet of broilers modifies the intestinal microbiocenosis by species composition and structure. At the same time, the species composition of both useful bacteria and pathogens changes, which is reflected in the main zootechnical indicators of poultry rearing and use of nutrients of combined feed [38]. It was also reported that the replacement of even one protein component in the poultry diet has a significant effect on the structure and abundance of the microbiocenosis of the intestines of broilers [39]. However, the data on the effect of protein feed additives from keratin- and collagen-containing raw materials, obtained by the method of short-term high-temperature hydrolysis in a thin layer, on the microflora of the gastrointestinal tract and the productivity of broiler chickens were absent until now.

Thus, the inclusion of a hydrolysate of keratin- and collagen-containing raw materials in the diet of broiler chickens does not have a negative impact on the microflora of the gastrointestinal tract. The content of normal microflora in the blind processes of the intestines remains high in all groups. Cellulolytic bacteria from the *Clostridia* class of the *Firmicutes* phylum (including the families *Ruminococcaceae*, *Eubacteriaceae*, *Lachnospiraceae*, *Clostridiaceae*) and the *Bacteroidetes* phylum dominate in the community. Feed additives from a hydrolysate of keratin-containing raw materials, as well as from a mixture of a hydrolysate of keratin- and collagen-containing raw materials with the inclusion of a probiotic preparation, provide higher rates of productivity and quality of poultry meat. Thus, in the first case, the live weight of broilers increased compared to the control by 9.2% ($p \leq 0.01$) by day 38 and by 10.1% ($p \leq 0.001$) by day 49, and in the second case, the corresponding values on day 38 and day 49^t were 5.30 and 4.96% with 100% of livestock preservation and lower feed costs per 1 kg of live weight gain.

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DIFFERENT CHROME SOURCES INFLUENCE ON MORPHO-BIOCHEMICAL INDICATORS AND ACTIVITY OF DIGESTIVE ENZYMES IN Wistar RATS

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Abstract

Nowadays, issues of mineral nutrition of humans and animals are quite relevant. High-energy rations, multicomponent feed mixtures and additives in the diets require special attention when optimizing limited microelements. A priori, chromium, being an important trace element in animals, is used to correct carbohydrate, fat and lipid metabolism. Due to its low content in the components of diets, its role in the formation of the microecological status of the body is poorly understood. At the same time, its biological availability in the body depends on the source of chromium. In the present work, using a model object, the Wistar rats, we for the first time compared the biological effects of various chromium sources, i.e. picolinate (CrPic), nanoform (NP Cr_2O_3) and chloride ($CrCl_3$) at doses of 300 and 500 μg/kg feed, according to a set of indicators (feed digestibility, hematological parameters, activity of digestive enzymes, composition of intestinal microflora) and established greater bioavailability and more pronounced positive effect of picolinate and chromium nanoparticles on body weight and hematological parameters and ambiguous influence of the studied forms on the activity of digestive enzymes and intestinal microflora. The purpose of this work was to study the biological effect of chromium in various forms and dosages on Wistar rats. The studies were carried out on 105 white male rats weighing 70-80 g under standard vivarium conditions (Federal Scientific Center for Biological Systems and Agrotechnologies of the Russian Academy of Sciences). Animals were divided into seven groups (n = 15 each). The control group was fed a common diet. The group I diet included Cr_2O_3 NPs at a dose of 300 μg/kg feed (NP 300), group II — $CrCl_3$ at a dose of 300 mg/kg ($CrCl_3$ 300), group III — chromium picolinate (CrPic) at a dose of 300 mg/kg (CrPic 300), group IV — Cr_2O_3 NPs at a dose of 500 μg/kg of feed (NP 500), group V — $CrCl_3$ at a dose of 500 mg/kg ($CrCl_3$ 500), and group VI — CrPic at a dose of 500 mg/kg (CrPic 500). Nanoparticles were introduced into the feed by mixing. The introduction of chromium in the form of CrPic and Cr_2O_3 NPs at a dose of 500 μg/kg, under the same feed consumption, was accompanied by an increase in the body weight of rats by 22.6 and 22.2 % ($p \leq 0.05$). The effect of Cr_2O_3 NPs expressed as the absence of the reaction of lymphocytes, monocytes and granulocytes, in other cases their level exceeded control values by 14 to 45 %. Synthesis of hemoglobin was adequate to stimulation of erythropoiesis, but an increase in the number of platelets resulted in blood sludging, an increase in viscosity and difficulty in perfusion through vessels. This symptom was typical for NP Cr_2O_3 300, $CrCl_3$ 300 and $CrCl_3$ 500 (the difference with the control is from 70 to 90 %, $p \leq 0.05$). High digestibility of CrPic and NPs Cr_2O_3 500 (from 20.2 to 34.0 %), accompanied by manifestation of hepato- and nephrotoxicity, with signs of oxidative stress, decreased activity of amylase and lipase in the blood plasma, indicating a depressant effect of high doses of chromium on enteropancreatic circulation of the digestive enzymes and metabolic disorders of Mg and Fe in the blood. Triglycerides, like true fats, decreased at maximum doses of chromium in the form of chloride and picolinate, confirming their participation in lipid metabolism, causing splitting of excess fat in the body, and reducing the ratio of fat to body weight ratio from 2 (control group) to 0.82 (NP Cr_2O_3 500). The indicators of bilirubin and creatinine clearly demonstrate the absence of toxicity at low doses, i.e. Cr_2O_3 NPs 300 and CrPic 300. Amylase activity in the pancreas is increased at a dose of 300 μg/kg

of Cr_2O_3 NPs. Dietary CrPic in a similar dosage stimulated the activity of lipase and protease, whereas in the 12 duodenal ulcer it led to a decrease in the activity of amylase and lipase. $CrCl_3$ and CrPic at a dosage of 500 μg/kg reduced the activity of lipase in the duodenum. The specific effect of Cr NPs 500 μg/kg on the microecological status of the organism was manifested in a decrease in the number of lactic acid bacteria by 55.9 %. The number of bifidobacteria was significantly higher, by 48.6 % ($p \leq 0.05$), in the PicCr 500-fed group. The number of enterobacteria in the NP 300-fed group was 34.8 % lower than the control, while in the other groups their number increased 24.0-33.7 times ($p \leq 0.05$). From the totality of the estimated parameters of the intestinal microflora, the use of nanoparticles in the composition of the diet is promising due to the lack of resistance. Thus, chromium of CrPic 500, NP Cr_2O_3 300 does not show a toxic effect on the body, and has a stimulating effect on growth, development, digestibility of chromium, the activity of the digestive enzymes and microecological status of the organism, which puts these forms in the category of promising sources of chromium for the correction of metabolism and the microbial composition of the gastrointestinal tract of animals.

Keywords: rats, chromium concentration, productivity, blood biochemical parameters, digestive enzymes, intestinal microflora

Chromium (Cr) is a necessary element for animals and humans, but the mechanism of its biological action is not fully understood. It is known that chromium enhances the function of insulin, as well as stimulates the rate of insulin-induced swelling of isolated mitochondria [1], increases the respiratory factor of epididymal fat pad [2], interacts with the thyroid gland [3], and plays an important role in the metabolism of proteins and nucleic acids by significantly increasing the stimulation of amino acids in liver protein in vitro [4]. Okada et al. [5] found that the direct interaction of Cr with DNA leads to a significant stimulation of RNA synthesis in vitro, and also identified a unique protein containing 5-6 Cr atoms, which is characterized by anabolic functions. A new Cr-binder [6] was found that potentiates the effect of insulin in the conversion of glucose into lipid and carbon dioxide in isolated adipocytes [6].

Although chromium exists in nature in oxidation states from Cr^{2-} to Cr^{6+}, relatively inert complexes of Cr^{3+} may function as structural components that bind ligands with the proper dimensional orientation, contributing to the enzymatic catalysis, and also support the tertiary structure of proteins or nucleic acids [7]. In chickens, the inclusion of additional chromium in the diet improved the physiological status and productivity under cold and heat stress [8, 9], and in pigs, the addition of 200 rg of chromium picolinate (CrPic) in the diet increased the rate of nitrogen absorption [10].

The modern livestock breeding has a tendency to replace traditional mineral sources of trace elements with new organo-mineral or nanoforms, due to their better digestibility, bioavailability, and prolonged action [11, 12]. It is shown that the metabolic response of the body to chromium depends on its chemical form: organic sources have higher bioavailability (10-25%) than inorganic sources (3%) [13]. Reduction of the size of chromium particles may increase the rate of digestion and absorption. Lien et al. [14] found that CrPic nanoparticles were significantly better absorbed than typical CrPic, which led to an increase in the content of chromium in the blood serum of rats. In the case of the inclusion of chromium nanoparticles in the diet of pigs, the area of the longissimus muscle and the chromium content in tissues increased, while the fat ratio and the thickness of back fat decreased [15, 16]. In the studies in rats, nanosized chromium significantly increased the mean increment of body weight, the feed efficiency, the ratio of fat deposits, the insulin concentration in the blood serum, and the content of chromium in the organs [17].

It may be assumed that the change in feed conversion, meat productivity, and biochemical parameters in animals is based on the mechanisms of chromium participation in digestion and metabolism through the stimulation of digestive enzymes. The study of alternative forms of trace elements in the diet of

animals is a necessary tool for the management of digestion, the formation of productivity, and the nutritional value of animal products.

In the present paper, the biological effect of various forms of chromium (picolinate, nanoforms, and chloride) at doses of 300 and 500 rg/kg of feed on a set of indicators (digestibility, the hematological parameters of blood, the activity of digestive enzymes, and the composition of intestinal microflora) was compared for the first time on the model object (Wistar rats); the study showed high bioavailability and a more significant positive effect of picolinate and chromium nanoparticles on body weight and hematological parameters, as well as the ambiguous effect of the studied forms on the activity of digestive enzymes and intestinal microflora.

The work objective was to study the biological effect of chromium in various forms and dosages on Wistar rats.

Techniques. The studies were carried out on 105 white male Wistar rats weighing 70-80 g under standard vivarium conditions (Federal Scientific Center for Biological Systems and Agrotechnologies of the Russian Academy of Sciences). The diet of animals (GOST R 50258-92) corresponded to the rules of laboratory practice during preclinical studies in the Russian Federation (GOST R 51000.3-96 and R 51000.4-96). The experimental part of the work was carried out in accordance with the protocols of the Geneva Convention and the principles of good laboratory practice and "The Guide for Care and Use of Laboratory Animals (National Academy Press Washington, D.C. 1996)." Rats were kept in separate cells with free access to water and feed.

Based on the results of previous tests [16, 17], the doses of chromium in the experiments were 300 and 500 rg/kg of feed. After the preparatory period (7 days), the animals were divided into seven groups (n = 15 each). The control group was fed a common diet. The diet of group I included Cr_2O_3 NPs at a dose of 300 μg/kg feed (NP 300), group II received $CrCl_3$ at a dose of 300 mg/kg ($CrCl_3$ 300), group III received chromium picolinate (CrPic) at a dose of 300 mg/kg (CrPic 300), group IV received Cr_2O_3 NPs at a dose of 500 rg/kg of feed (NP 500), group V received $CrCl_3$ at a dose of 500 mg/kg ($CrCl_3$ 500), and group VI received CrPic at a dose of 500 mg/kg (CrPic 500). Nanoparticles were introduced into the feed by step-type mixing.

Ultrafine particles (UFPs) of chromium were obtained by the method of plasma-chemical synthesis (OOO Platina, Moscow; d = 91 nm, specific surface of 9 m^2/g, Z-potential 93±0.52 mV, the Cr content of 99.8%). Nanoparticle preparations were dispersed in a saline solution using UZDN-2T (NPP Akadempribor, Russia; 35 kHz, 300 W, 10 μa, 30 min). The inorganic form of Cr in the form of chromium chloride $CrCl_3 \cdot 6H_2O$ a.r.g. (19.5% Cr) (AO Reakhim, Russia) and chromium picolinate (contains 10% organic chromium, ZAO Evalar, Russia) were also used.

At the beginning and end of the experiment (day 21), growth rates and feed consumption were taken into account. Blood was taken from the tail vein into vacutainer tubes with the addition of anticoagulant, for biochemical parameters – into vacutainer tubes with a clot activator (thrombin). Blood morphological analysis was carried out on the automatic hematology analyzer URIT-2900 Vet Plus (URIT Medical Electronic Group Co., Ltd, China), biochemical analysis of blood serum on the automatic analyzer CS-T240 (DIRUI Industrial Co., Ltd, China) with commercial veterinary kits (ZAO DIACON-DS, Russia).

After decapitation of rats on day 21 under Nembutal anesthesia (5 individuals from each group), bio-substrates were selected for analysis. The concentration of chromium was determined at the beginning and end of the experiment in milled samples of biomaterial (all tissues and systems of the body), feed and

feces, followed by ignition in the microwave decomposition system Multiwave 3000 (Anton Paar, Austria). The elemental composition was studied by the atomic emission spectrometry (Optima 2000 V, Perkin Elmer, USA) and mass spectrometry (Elan 9000, Perkin Elmer, USA) methods.

The digestibility of chromium was evaluated on the basis of the general collection of biological substrates (feces, urine) that took place during the digestion trial (5 days) in individual metabolic cages (http://цтт-ягпу.рф/dxl-d). The digestibility of Cr (D, %) was calculated by the formula: D = {[(individual feed intake × % Cr) – (amount of feces and urine per period of 5 days in grams × % Cr)] × (individual feed intake for rats × % Cr)} × 100.

The duodenum and pancreas were removed through the abdominal wall cut from the animals after slaughter to assess their enzymatic system. To prepare tissue samples of the studied organs, the sub-samples (1 g) were rubbed in cooled Ringer's solution (4 ml), the homogenate was centrifuged at 3000 rpm for 10 min (CM-12, Fabrika NV-group, Russia). Amylase activity was determined according to the method by Coles [18], lipase activity by Boutwell [19], protease with the use of the technique by Batoev [20]. The activity of the studied enzymes was expressed in conventional units (con. un., the difference between the indications of the sample with the substrate and the blank sample per 1 g of wet sub-sample of intestinal mucosa tissue for 1 min).

Samples of intestinal contents were collected in sterile Eppendorf tubes. For the complex study of microflora, 0.1 ml of each of the 10-fold dilutions was plated on nutrient media according to the standard technique [21]. Endo agar, meat-and-peptone agar (MPA), yolk-salt agar (YSA) (OOO NIFTS, Russia), Rogosa agar, Bifidobacterium Agar, BCA (HiMedia Laboratories Pvt. Ltd, India) were used for the studies. The final result of the quantitative content of bacteria in a gram of feces was expressed as CFU/g.

All experiments were carried out in 3-fold repetitions. Statistical processing of the obtained results included the calculation of the mean value (*M*) and standard errors of the mean (±SEM). The significance of differences between compared indicators was defined according to Student's *t*-criterion. Differences were considered statistically significant at $p < 0.05$. Statistical analysis was performed using ANOVA (Statistica 10.0 software package, StatSoft Inc., USA) and Microsoft Excel.

Results. Against the background of almost the same feed consumption during the experiment period (830±17 g/animal), the addition of 500 μg/kg CrPic and 500 NP to the diet was accompanied by an increase in rat weight by 22.6 and 22.2% ($p \leq 0.05$) and a decrease in the ratio of abdominal fat to body weight. The weight of the liver and kidneys did not differ significantly from the control values. Similarly, in the studies by Lien et al. [14], the addition of Cr to the diet (0.2 mg) increased the average daily growth of the body weight without an increase in feed intake and reduced fat deposits. A similar effect of organic and nanoforms of chromium in comparison with its mineral form has been reported earlier [13].

The positive balance of chromium accumulation with the use of 300 and 500 μg/kg CrPic in the diet was observed; this result may be compared with the results of the investigation by Wang et al. [15]. The bioavailability of Cr NP reached the highest positive balance at a dose of 500 μg/kg and was 81.6% higher than the control. In other groups, chromium digestibility was in the range of 4.3-7.5% and was the lowest when using $CrCl_3$ (Table 1).

Previously in the investigations of Wang et al. [15], the ability of chromium nanoparticles to stimulate growth indicators has been described while increasing metal deposition in muscles, heart, liver, and kidneys at the same time.

1. Morphometric parameters and digestibility of various forms of chromium in Wistar rats (*M*±SEM, vivarium experiment)

Indicator	Group						
	control	I	II	III	IV	V	VI
Initial body weight, g	69.30±2.95	114.20±2.80	75.50±2.88	87.90±1.19	101.80±7.63	81.10±3.13	100.30±5.50
Live weight at the end of the experiment, g	118.60±3.50	149.30±0.10	131.20±9.86	146.70±7.01	152.30±6.30*	123.30±4.17	151.50±6.50*
Liver weight, g	5.55±0.49	5.03±0.43	6.88±0.43	7.90±0.45*	6.50±0.32	6.23±0.67	6.70±0.45
Kidney weight, g	1.20±0.08	1.13±0.06	1.15±0.16	1.43±0.27	1.23±0.19	1.43±0.23	1.53±0.22*
Internal fat, g/weight ratio	1.33±0.21	1.21±0.10	1.80±0.37*	1.63±0.21	1.25±0.09	1.37±0.79	2.10±0.30*
	2	1.06	1.38	1.12	0.82	1.12	1.39
Growth, g	49.30±1.10	35.10±0.90*	55.70±1.20	58.80±2.30*	50.50±1.35	42.20±0.95	51.20±1.32
Cr content in the diet, mg/kg	0.13	0.43	0.43	0.43	0.63	0.63	0.63
Cr content in feces, mg/kg	0.97±0.01	3.18±0.12***	3.27±0.18***	2.75±0.05***	3.30±0.13***	4.83±0.24***	4.02±0.74***
Cr content in the body, mg/kg	0.18±0.05	0.05±0.00*	0.05±0.00*	0.06±0.00	0.21±0.04	0.06±0.00*	0.09±0.00*
Cr digestibility, %	6.7±0.3	7.6±0.4*	4.9±0.6*	20.2±1.1**	34.5±1.3*	4.3±0.9*	20.2±1.6

N o t e. See the description of groups in the section Techniques.
*, **, and ** Differences with control are statistically significant at $p \le 0.05$, $p \le 0.01$, and $p \le 0.001$, respectively.

2. Blood morphology of Wistar rats on day 21 after addition of dietary chromium in various forms and dosages (*M*±SEM, vivarium experiment)

Indicator	Group						
	control	I	II	III	IV	V	VI
Leucocytes, $\times 10^9$/l	7.50±3.50	11.00±1.60*	10.70±1.10	12.20±2.60*	7.10±1.70	8.80±2.10	9.00±2.10
Lymphocytes, $\times 10^9$/l	3.80±1.80	6.50±2.10*	5.80±0.70*	5.10±1.40*	3.80±1.10	4.60±0.90*	5.20±1.20*
Monocytes, $\times 10^9$/l	1.60±0.80	2.10±0.80	1.70±0.10	2.30±0.90	1.60±0.80	1.70±0.01	1.70±0.30
Granulocytes, $\times 10^9$/l	2.10±0.78	2.40±0.90	3.00±0.30	4.80±1.10*	1.70±1.07	2.50±0.08	2.40±0.20
Erythrocytes, $\times 10^{12}$/l	4.69±0.63	5.79±1.10	5.70±0.70	5.64±1.80	5.67±1.70	5.21±0.80	5.36±1.10
Hemoglobin, g/l	103.00±15.60	128.00±22.10*	125.00±3.60*	125.00±23.10*	119.00±12.50	115.00±12.60	119.00±23.60
Hematocrit, %	26.40±7.80	31.40±11.20	30.70±11.50	32.00±9.90*	28.00±9.68	28.10±3.60	29.10±2.40
Thrombocytes, $\times 10^9$/l	120.00±25.30	233.00±35.20*	237.00±23.60*	169.00±25.30	110.00±15.60	204.00±9.80*	186.00±12.50

N o t e. See the description of groups in the section Techniques.
* Differences with control are statistically significant at $p \le 0.05$.

3. Blood biochemical parameters of Wistar rats on day 21 after addition of dietary chromium in various forms and dosages (*M*±SEM, vivarium experiment)

Indicator	Group						
	control	I	II	III	IV	V	VI
Glucose, mmol/l	3.11±1.40	1.99±0.90	2.44±1.10	3.78±1.80	7.00±2.10*	7.74±2.10*	2.32±1.60
Total protein, g/l	63.60±9.80	68.30±10.30	57.90±16.30	63.30±9.70	64.90±11.60	62.50±12.10	61.60±9.90
Total bilirubin, μmol/l	8.90±0.40	8.30±2.10	6.80±1.10*	7.10±0.90	11.60±1.50	10.90±2.10	9.60±1.90
Cholesterol, mmol/l	1.85±1.90	1.59±0.68	1.48±0.78	2.27±0.68	1.85±1.30	1.80±0.80	1.45±0.35
Triglycerides, mmol/l	9.63±1.80	10.80±3.60	8.61±2.80	7.69±2.10	6.84±2.10	5.88±1.20*	5.69±1.60*
Urea, mmol/l	7.20±1.10	7.60±2.40	5.90±2.10	5.30±1.90	4.00±1.40*	6.40±1.30	6.10±1.80
Creatinine, μmol/l	29.70±8.90	27.60±11.30	33.90±9.60	30.80±11.10	19.70±4.30	33.90±2.10	36.10±3.10
Calcium, mmol/l	1.80±0.40	2.00±0.11	1.88±0.01	2.27±0.20	1.98±0.60	1.94±0.27	2.10±0.21
Iron, μmol/l	41.00±7.60	41.10±11.4	27.30±8.70*	29.70±7.70*	49.50±8.10	35.80±4.10	32.40±5.60
Amylase, IU/l	1266.00±42.30	1112.90±45.30	1130.60±39.60	1578.00±23.60	1414.00±19.60	643.80±122.30*	648.00±214.30*
Lipase, IU/l	2.40±0.70	4.80±2.40	0.80±1.10**	2.10±1.10	3.10±0.90	1.10±0.01*	1.60±0.03*
Magnesium, mmol/l	0.90±0.04	0.68±0.04	0.78±0.01	0.45±0.01	1.17±0.07	0.63±0.01	0.52±0.02
Phosphorus, mmol/l	3.65±0.80	3.82±1.40	3.59±1.40	4.04±2.10	1.87±1.30**	5.18±1.20*	3.86±1.60

N o t e. See the description of groups in the section Techniques.
*, ** Differences with control are statistically significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

4. Activity (IU/ml) **of digestive enzymes in the pancreas and duodenum of Wistar rats on day 21 after addition of dietary chromium in various forms and dosages** (*M*±SEM, vivarium experiment)

Group	Duodenum			Pancreas		
	amylase	protease	lipase	amylase	protease	lipase
Contro;	81.8±6.7	2.0±0.4	0.9±0.2	31.5±0.5	2.0±0.2	4.1±0.4
I	90.7±13.2	4.6±1.2	0.3±0.1	88.8±4.7*	1.9±0.1	4.4±0.7
II	58.4±10.8	3.6±0.2	2.3±0.6	32.8±10.2	2.6±0.1	4.3±0.2
III	37.4±5.1*	3.1±0.2	2.7±0.2*	5.8±2.3*	4.1±0.4*	13.7±0.5*
IV	80.7±9.8	2.5±0.3	1.3±0.3	32.3±1.1	2.5±0.4	5.9±0.7
V	72.8±13.3	2.2±0.2	2.8±0.4*	54.4±7.8	2.6±0.4	3.7±0.6
VI	85.7±7.5	2.9±0.7	4.1±0.5*	55.4±9.4	1.7±0.1	5.2±0.3

N o t e. See the description of groups in the section Techniques.
* Differences with control are statistically significant at $p \leq 0.05$.

It happens due to better absorption of Cr nanoform in the intestine and the high penetration of Cr particles through the blood capillaries [23]. The absorbed chromium binds mainly to transferrin, distributes in tissues depending on the chemical state in which the element entered the body [24], and is excreted in the urine and feces in the form of acetate and citrate complexes for 4 days in an amount of 60-90% of the received chromium [25].

To avoid distortion in the interpretation of the data when assessing the effects of different forms of chromium on the body, especially in concentrations exceeding biotic, it is necessary to use markers. They should exclude hidden toxic effects that are associated with metabolic disorders and expressed in the accumulation of fat, changes in hematological and biochemical parameters of blood [27] (Table 2).

In this experiment, the peculiarity of the dose of NP 500 was the absence of reaction of lymphocytes, monocytes, and granulocytes, while at a dose of NP 300, these parameters exceeded the control values by 14-45% ($p \leq 0.05$). The observed effects of chromium on the group of formed elements are similar to those of copper and iron NP addition [28]. The addition of chromium to the rat diet stimulated erythropoiesis; the greatest effect was achieved at a concentration of CrPic 300: the hematocrit index in animals in group III was by 17.5% ($p \leq 0.05$) higher than in the control group. Hemoglobin synthesis corresponded to the stimulation of erythropoiesis, but an increase in the number of thrombocytes led to blood sludge, increased viscosity, and difficulty in perfusion through the vessels. These symptoms were typical for NP 300, $CrCl_3$ 300 and $CrCl_3$ 500 (difference with the control group from 70 to 90%, $p \leq 0.05$). The reason for this effect could be the thrombogenicity of nanoparticles, and for $CrCl_3$ this could be a manifestation of the toxic properties of this compound.

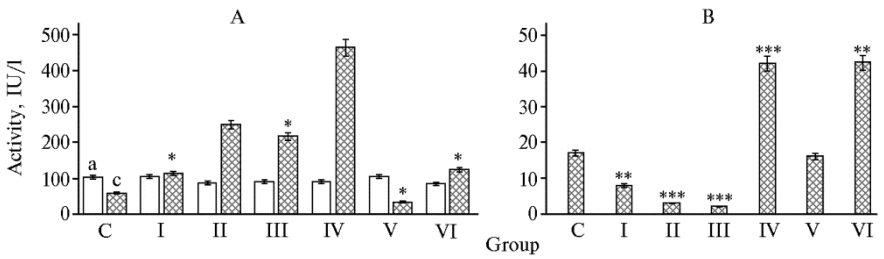
Two aspects of the biocompatibility of nanomaterials, the thrombogenicity and hemolytic activity require special attention, because after penetration through the barrier structures of the body, nanoparticles are in the lymph and blood flow, which implies their contact with both macromolecules of blood plasma and lymph and with shaped elements. It is known that particles with a negative surface charge can trigger the formation of a blood clot by contact activation of the clotting cascade, leading to the formation of fibrin; in other words, they activate the external pathway of blood clotting.

There were the hyper- and hypoglycemic effects of chromium in various forms and dosages on the body of rats (Table 3). Since 65-70% of circulating glucose in the blood is utilized by the central nervous system, this determines the danger of hypoglycemic conditions that significantly change the metabolism of the brain, which ultimately leads to the death of neurons and the disruption of its function [29]. Thus, in experimental groups IV and V, there was a significant increase in the glucose content (by 2.49 and 2.25 times, $p \leq 0.05$). The concentration of total protein between the groups did not differ significantly. The maximum decrease in the creatinine content was observed in the blood of rats from group IV (by 33.7%), which, along with a decrease in the urea content by 44.4% ($p \leq 0.05$), was one of the criteria for disorders in the excretory apparatus [17].

The amount of triglycerides as true fats decreased at the maximum doses of chromium in the form of chloride and picolinate, which indicates the effect of chromium on lipid metabolism, the splitting of excess fat in the body and reducing the ratio of fat to weight from 2.00 (control) to 0.82 (NP 500). The influence of chromium on lipid metabolism is also mediated by its regulatory action on the functioning of insulin.

The activity of pancreatic enzymes of amylase and lipase in blood serum decreased with an increase in the content of $CrCl_3$ and CrPic in the feed, which

indicates the depressing effect of the used forms of chromium on the entero-pancreatic circulation of digestive enzymes. When NP 500 was added, the content of phosphorus decreased by 48.8% ($p \le 0.05$). The data on the blood content of iron serum are interesting. At a low concentration, chromium and iron mainly occupy different binding sites [30, 31], while at a higher level, they compete for the sites, which was manifested in a decrease in iron metabolism in the experimental groups receiving a diet with chloride and chromium picolinate. The analysis of the aminotransferase reaction (Fig.) as an indicator of the presence of damages in the cells indicated a decrease in the activity of AspAT by 42.7% at the dose of NP 500 ($p \le 0.01$) and the increase in NP 300, CrPic 300, and CrPic 500 by 1.97 ($p \le 0.001$); by 3.75 ($p \le 0.001$); and by 2.14 times ($p \le 0.01$) in comparison with the control against the background of a stably high content of bilirubin. This fact can be explained by the massive release of enzymes into the bloodstream after the destruction of cells caused by the onset of oxidative stress and various pathological processes. A significant decrease in the activity of ALT by 10.7% ($p \le 0.05$) under the influence of NP 500 may be indirect evidence of the disruption of glomerular filtration, as evidenced by a decrease in the content of creatinine by 33.7% ($p \le 0.05$) relative to the control group (Fig., A).



The activity of blood transaminases (A) **and γ-glutamyltransferase** (B) **in Wistar rats on day 21 after addition of dietary ultrafine chromium particles of different forms and dosages:** a — alanine aminotransferase, b — aspartate aminotransferase; C — control, I — NP Cr_2O_3 (300 μg/kg feed), II — $CrCl_3$ (300 mg/kg), III — CrPic (300 mg/kg), IV — NP Cr_2O_3 (500 μg/kg), V — $CrCl_3$ (500 mg/kg), VI — CrPic (500 mg/kg) (M±SEM, n = 15, vivarium experiment).
*, **, and ** Differences with control are statistically significant at $p \le 0.05$, $p \le 0.01$, and $p \le 0.001$, respectively.

The analysis of the blood activity of γ-glutamyltransferase (γ-GT) revealed a significant increase in the case of adding NP 500 and CrPic 500 to the diet, which could be due to cell death, stagnation within the ducts or toxic effects against the background of intoxication [32]. The introduction of NP Cr_2O_3, $CrCl_3$, and CrPic at a dose of 300 mg/kg led to a decrease in this indicator. NP Cr_2O_3 at a dose of 500 mg/kg had the greatest impact on hematological parameters. The ambiguous manifestation of the activity of transamination enzymes, on the one hand, may indicate the destruction of cell membranes, on the other − a weak induction of microsomal oxidation under the influence of mixed valence NP [33]. The other forms of chromium in the used dosages did not lead to critical changes in the hemostatic system.

The ability of chromium to penetrate into the intestine quickly (1 h after feeding) and form stable difficult to absorb hydrates in it suggests its participation in the development of digestive enzymes [34]. In this study, the stimulation of amylase activity was observed in the pancreas after addition of NP 300 and the decrease in this indicator with the use of CrPic 300. At the same time, CrPic has a stimulating effect on the activity of lipase and protease. In the duodenum, the reduction of amylase activity by 54.3% ($p \le 0.05$) and increased activity of lipase by 67.7% ($p \le 0.05$) was typical for CrPic 300. Adding of NP CrCl3 and CrPic to the diet of rats at a dosage of 500 mg/kg stimulated the lipase activity (Table 4).

Despite the absence of data in the international scientific literature that may explain the mechanism of NP chromium action on the digestive enzymes, the option to modulate their activity through induction seems to be promising. Adsorption of biological macromolecules on the surface of nanoparticles may change their spatial structure and some functional properties. Thus, the enzyme α-chymotrypsin adsorbed on single-layer carbon nanotubes loses 99% of its activity due to a violation of the secondary structure [35]. When adsorbing proteins on larger nanoparticles, significant changes in the structure and function of proteins are observed due to the formation of additional contacts [36].

Taking into account the multiple effect of chromium on the activity of digestive enzymes, it should be emphasized that nanoparticles and picolinate have a similar effect due to possible direct contact with the active center of the enzyme [37].

5. The number of microorganisms ($\times 10^6$ CFU/g) **in the large intestine of Wistar rats on day 21 after addition of dietary chromium in various forms and dosages** (M±SEM, vivarium experiment)

Микроорганизмы	Group						
	control	I	II	III	IV	V	VI
Lactobacilli	14.3±2.1	9.3±1	10±1.1	12.6±2.1	6.3±0.8*	8.0±1.1	8.6±0.4
Bifidobacteria	14.9±4.1	11.7±0.9	12.3±1.5	13.1±0.9	6.5±0.4	22.0±2.2	29.0±2.5*
Enterobacteria	0.8±0.1	0.5±0.1*	25.0±2.3**	29.0±3.1**	23.0±3.7**	20.6±2.4*	26.0±2.9**

N o t e. See the description of groups in the section Techniques.
*, ** Differences with control are statistically significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

The number of lactobacilli in group IV was 55.9% ($p \leq 0.05$) lower than in the control group; in other groups, it did not change significantly. The number of bifidobacteria in group VI was 48.6% higher than in the control group ($p \leq 0.01$). The number of enterobacteria in the group of NP 300 was lower than in the control group by 34.8% ($p \leq 0.05$), while in the other groups their number increased 24.0-33.7 times (Table 5).

Thus, chromium in the form of nanoparticles and picolinate at a dose of up to 300 μg/kg can be used as a bacteriostatic agent to correct the content of bifidobacteria in the intestine. The content of bilirubin and creatinine, and an increase in the activity of amylase in the pancreas, lipase and protease in the duodenum have demonstrated a lack of toxicity of NP 300 and CrPic 300 for Wistar rats. The use of chromium at a dose of 500 rg/kg, regardless of the form of the element, was accompanied by a manifestation of hepatotoxicity and nephrotoxicity with signs of oxidative stress. High digestibility of CrPic and NP 500 (from 20.2 to 34.0%) had a depressing effect on energy metabolism and enteropancreatic circulation of digestive enzymes, reduced the triglyceride content and the ratio between the amount of fat and body weight. Depression of lipid metabolism was confirmed by decreased activity of amylase in the duodenum. In general, the prospects for the use of nanoscale diets are explained by the predominance of surface interactions. Due to their size, comparable to the size of cells, viruses, proteins, and DNA, nanoparticles can approach the biological object and bind to it, being involved in biochemical processes in the body.

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NUTRITIONAL PROPERTIES OF *Hermetia illucens* L., A NEW FEED PRODUCT FOR YOUNG PIGS (*Sus scrofa domesticus* Erxleben)

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Abstract

In connection with the proven possibility of industrial breeding of black lion larvae *Hermetia illucens* L. on various organic substrates, it is of interest to study their nutritional properties and the possibility of effective use as a new feed product for the Russian livestock in the diets of different farm animal species. This paper is the first report in Russia about *H. illucens* larvae effects on growth of young pigs. The aim of the work was to assess the biochemical composition and nutritional value of *H. illucens* larvae depending on the composition of the substrate on which they were grown and to estimate efficiency of these larvae as a substitution for dietary fish meal in pigs' (*Sus scrofa domesticus* Erxleben) feed. The fly *H. illucens* larvae were reared in the laboratory of the Institute of Ecology and Evolution RAS on different substrates, i.e. distillers dried grains with solubles, feed wheat grain, wheat bran, crushed corn, mix of fruit and vegetable waste with grain bran, mixture of bird manure with litter. Bioconversion of substrates by larvae ranges from 41 % (bird droppings) to 77 % (crushed corn). Also, the use of different substrates leads to a different yield of dry biomass of larvae, from 54 g/kg (bird litter) to 240 g/kg (grain-fruit-vegetable mixture). The most preferable substrates for growing larval biomass are grain and grain-fruit-vegetable mixtures. The nutritional value of fly larvae varies depending on the substrate of culture. The exchange energy is within 15.32-21.41 MJ/kg, the amount of protein is in the range of 35.5-48.3 %, and fat level is 20.6-45.5 %. The larval protein contains a complete set of amino acids characteristic of animal protein, and also depends on the substrate used. The larvae age had a significant impact on their biochemical composition. Dry prepupa (last instar stage), when grown on feed wheat grain, showed higher protein content and lower body fat compared to larvae, i.e. 42.8 and 31.9 % vs. 37.6 and 38.3 %, respectively. The study of the effect of substitution of fish meal (5 %) for flour from larvae (7 %) in feed included estimates of digestibility of nutrients, balance and use of nitrogen by pigs. Physiological tests on animals ($n = 6$) were conducted during rearing pigs (F_1 Large White × Landrace) in the conditions of physiological yard (Ernst Federal Science Center for Animal Husbandry). Compound feeds for animal experimental groups were balanced by nutritional and energy value, the level of minerals. The experiment was performed in two repetitions. It is shown that the replacement of fish meal with dry *H. illucens* larvae contributes to better use and deposition of nitrogen and, as a consequence, a higher bodyweight gain (504,95±17,94 vs. 475,92±22,93 g, $p > 0,05$). Dietary black soldier fly larvae had no statistically significant negative impact on the use of calcium and phosphorus by animals. Thus, the larvae of *H. illucens* fly can be considered as a rational alternative to traditional high-protein feeds that meet the nutritional needs of intensively growing young pigs.

Keywords: larvae, *Hermetia illucens*, feed, young pigs, digestibility, productivity

The development of new components of combined feed for farm animals is one of the relevant trends in the modern combined feed industry. The feed sup-

ply, which determines the largest object of expenditure, is represented by a variety of ingredients of plant and animal origin, as well as feed obtained by microbiological synthesis. At the same time, the search for new non-traditional components with a high concentration of protein is conducted, that can become a worthy alternative to soybean and fish meal. High-protein components are necessary to meet the protein requirements of animals, and therefore, for the full development of the body and obtaining the highest productivity with lower feed costs [1]. In the Russian feed industry, the production of animal feed is reduced; their price increases with deterioration in quality, and falsification products appear in the market. The grain group (barley, oats, wheat, corn, peas) serves as raw material for the production of not only feed but also food products, which causes competition for these sources of protein; large areas of arable land are allocated for the provision of animal husbandry with such feed, which could be used for food production. New alternative protein components can reduce the strain of these problems, which determines the relevance of the authors' experiments [2].

The base of non-traditional feed expands every year. In the works by Russian researchers, the high efficiency of the use of the larvae of synanthropic flies (*Musca domestica*) in feeding farm animals was established [3, 4]. More experiments with other biological objects are relevant to find candidate feed ingredients and to identify the most economically promising ones. Such objects should have high nutritional properties (primarily in protein and fat content), ensure rapid biomass accumulation, serve as sources of biologically active substances and be safe from the point of view of veterinary medicine and ecology. The black lion fly (*Hermetia illucens* L.), the larvae of which many authors propose to use in animal feed [5-7], meets these requirements. *H. illucens* biomass is produced by many companies, e.g. Hermetia Baruth GmbH (Germany), AgriProtein Technologies (South Africa), Enterra Feed Corporation (Canada), Protix (Netherlands), Bühler Insect Technology Solutions (Switzerland). Most of the companies that offer products of black lion *H. illucens* are located in Europe [8].

Various sources serve as the feed substrate for the larvae of this species of insects, such as: manure, unconditioned grain, and products of the agricultural and food industry, as well as food waste [9]. Every day, up to a third of the volume of manufactured products is thrown away as food waste, most of which are of plant origin (residues of fruits and vegetables, which is due to the complexity of their storage and transportation). Products decompose quickly and become unsuitable for further use. Bioconversion of waste when growing larvae on it partially solves the problem of utilization and allows obtaining high-quality protein feed product [10].

Black lion larvae are an economical way to convert residual organic biomass into a valuable source of biomolecules — proteins, lipids, and chitin [11]. It is estimated that by 2050 insect protein may account for 15% of the total protein produced in the world [12]. The appearance of black lion larvae in the market requires an assessment of the biological effectiveness of this feed product. In particular, there are works indicating the promising character of using *H. illucens* larvae in the diets of fish, pigs, and birds [13-15], in feeding calves [16], and also instead of soybean meal in the diets of cows [17].

However, the information on the effectiveness of the use of *H. illucens* larvae in feeding animals, in particular, intensively growing young pigs, is still not sufficient, and such studies have not been carried out in Russia before. In the presented work, the authors found that feeding young pigs with black lion larvae as part of balanced combined feed of complete ration (7.0%, or 70 kg/t of combined feed) positively affects the physiological processes in the animals' organisms, their productivity and reduces the cost of feed. It should be taken into account that in

the authors' experiments, depending on the used substrates, there was an almost two-fold difference in the nutritional value of the obtained additive.

The aim of the study was to assess the nutritional characteristics of *Hermetia illucens* larvae grown on various organic residues, as well as their effect when introduced into the diet of farm animals (using the example of young pigs).

Techniques. In the work, conducted in 2016-2017, the larvae and prepupae (larvae of the last instar stage) of the black lion *Hermetia illucens* L. were used, grown in laboratory conditions (Severtsov Institute of Ecology and Evolution RAS) on distillers dried grains, feed wheat grain, wheat bran, crushed maize, fruit-grain-vegetable mixture (apples, tangerines, wheat, bran, potatoes, carrots in equal quantities) or bird litter. The air temperature during the growth of the larvae was 20-21 °C; the humidity of the substrates in all variants was maintained within 70±5%. The temperature was controlled by a mercury thermometer; the moisture of the feed mass was controlled by an Eleks-7 device (Eleks Group of Companies, Russia). The substrates were colonized with 6-day-old larvae (5 larvae per 1 cm^2). All experiments were performed in 3 replications. The raw biomass was dried at 70 °C to constant weight on a wall-mounted SNOL drying unit (AB UMEGA, Lithuania). The samples were weighed to an accuracy of 0.005 g (CAS XE-300 scales, CAS, South Korea). The total substrate consumption (conversion) was estimated as the ratio of the difference between the initial mass of the dry substrate and the final dry residue (substance consumption) to the initial dry mass.

The chemical composition and nutritional value of dried black lion larvae were analyzed according to standard techniques described in GOST 54951-2012, 31640-2012, 32044.1-2012, 32905-2014, 31675-2012, 26176-91, 32904-2014, GOST R 51420-99, GOST 13496-17-95, GOST 13496.12-98 (Ernst Federal Science Center for Animal Husbandry), the exchange energy (EE) was determined by the calculation method [16]. The amino acid composition was evaluated in dried biomass of larvae grown on crushed corn using a liquid chromatograph LC-20 Prominence (Shimadzu, Japan). The results of repeated measurements were obtained under convergence conditions (confidence coefficient $P = 0.95$) [18].

The physiological experiment on hybrid young hogs (*Sus scrofa domesticus* Erxleben) (F_1 Large White × Landrace, 6 animal units with an average initial body weight of about 15 kg) was performed in the conditions of a physiological yard (Ernst Federal Science Center for Animal Husbandry). According to the principle of analogs (origin, age, and live weight), the animals of F_1 Large White × Landrace formed two groups of 3 animal units in each. The duration of feeding was 27 days, with 5-day reference period of the balance experiment. The animals of group I (control) received a full ration starter combined feed (SF-4) with 5% of bran and the addition of 5.0% of fish meal. The analogs from group II were given SF-4 with 3% of wheat bran and 7.0% of dry *H. illucens* larvae, grown on crushed corn. Antibiotics were not used in the composition of feed. According to the energy and nutritional value indicators, the SF-4 feed met the requirements for the specified age and weight parameters of animals [19]. The experiment was conducted in 2 replications. The main diet and the housing conditions of all groups of animals (temperature, humidity, light conditions and the gas composition of the air in the room) were the same and were within the limits of zoohygienic norms.

Upon completion of feeding, a balance experiment was conducted to assess the digestibility of the combined feed nutrients [20]. At the time of the experiment, the animals were transferred to special cells equipped with individual feeders and means for collecting feces and urine. The duration of physiological studies was 5 days.

At the beginning, at the end of the experiment and weekly in the morn-

ing before feeding, the animals were individually weighed to determine the absolute and average daily weight gain. The influence of the type of feed on its palatability was estimated on the basis of daily individual accounting of the feeds and their residues. After the end of the experiment, the average samples of feed, feces, and urine were subjected to chemical analysis by standard methods [21]. The measure of palatability and the feed-use efficiency of products was feed consumption per unit of increase in live weight.

The rate of exchange energy (EE) was calculated for digestible nutrients. The equation for full ration combined feed (pigs) was as follows [22]:

$$EE = 0.01924 \times CP + 0.03597 \times CF - 0.01430 \times CFi + 0.01494 \times NFES,$$

where EE is exchange energy, MJ per 1 kg of feed; CP is crude protein, g; CF is crude fat, g; CF_i is crude fiber, g; NFES is nitrogen-free extractable substances, g.

The obtained data were processed biometrically by the method of variance analysis (ANOVA) in the program STATISTICA 10 (StatSoft, Inc., USA). The arithmetic mean values (M), the mean square error (±MSE) and the level of statistical significance (p) were calculated.

Results. The composition of the feed in the rations is given in Table 1.

1. The composition and nutritional value of experimental batches of combined feed for young hogs (F_1 Large White × Landrace) (Ernst Federal Science Center for Animal Husbandry, 2016-2017)

Ingredient, %	Group	
	I	II
Larvae *Hermetia illucens*	0	7,0
Fish meal	5.0	0
Wheat	41.8	41,8
Barley	10.0	10,0
Corn	8.0	8,0
Wheat bran	5.0	3,0
Sunflower cake, CP 32%	16.0	16,0
Sunflower oil	4.0	4,0
Nutrient yeast, CP 34%	7.0	7,0
Salt	0.2	0,2
Tricalcium phosphate	2.0	2,0
Premix, P52-3	1.0	1,0
1 kg contains:		
EFU	1.32	1,33
exchange energy, MJ	13.23	13,34
dry matter, kg	0.843	0,845
CP, g	180.5	179,1
digestible protein, g	140.9	140,4
crude ash, g	54.0	53,5
NFES, g	527.7	522,0
starch, g	354.2	346,1
sugar, g	27.9	26,9
lysine, g	7.9	7,4
methionine + cystine, g	5.9	5,3
threonine, g	6.3	5,9
crude fat, g	66.0	71,3
crude fiber, g	54.6	52,7
calcium, g	10.0	8,2
phosphorus, g	8.5	7,5
Mg, g	1.9	1,8
S, g	1.0	0,8
K, g	5.4	5,0
Na, g	1.6	1,1
NaCl, g	4.1	4,1
vitamin A, thousand IU/kg	20.00	20,00
vitamin D_3, thousand IU/kg	2.00	2,00
vitamin E, mg/kg	20.00	20,00
Fe, mg/kg	80.00	80,00
Cu, mg/kg	10.00	10,00
Zn, mg/kg	60.00	60,00
Mn, mg/kg	40.00	40,00
Co, mg/kg	0.30	0,30
I, mg/kg	0.60	0,60
Se, mg/kg	0.20	0,20

N o t e. CP — crude protein, EFU — energetic feed unit, NFES — nitrogen-free extractable substances.

A distinctive feature of the black lion fly *Hermetia illucens* is the ability to exist in controlled artificial conditions. At the same time, the larvae can use various organic substrates as feed, which makes it possible to solve the problems of partial bio-utilization of organic wastes and to obtain the protein biomass of larvae. However, not all types of substrates were used equally by the larvae (Table 2), which is also noted in the literature [23]

2. Bioconversion of experimental substrates by *Hermetia illucens* L. larvae (per dry matter, *M*±MSE, Institute of Ecology and Evolution RAS, 2016-2017)

Substrate	Conversion, %	Larvae biomass yield, kg	
		per 1 kg substrate	per 1 m^2
Bird litter	41.0±1.6	0.054±0.017	0.6±0.2
Distillers dried grains	53.0±1.3	0.084±0.001	0.7±0.4
Crushed corn	77.4±0.9	0.180±0.008	3.4±0.4
Feed wheat grain	74.5±1.7	0.155±0.002	3.1±0.4
Wheat bran	72.2±0.6	0.137±0.001	1.7±0.3
Fruit-grain-vegetable mixture	65.3±0.8	0.240±0.009	3.3±0.5

Conversion of the presented substrates ranged from 41 (bird litter) to 77% (crushed corn). Also on different substrates, there was an unequal yield of dry biomass of larvae: from 54 (bird litter) to 240 g/kg (grain-fruit-vegetable mixture). Grain and grain-fruit-vegetable mixtures turned out to be the most preferred substrates for growing biomass of larvae. The authors did not use high-protein feed mixtures with animal protein in accordance with the recommendations of the EU Standing Committee on Plants, Animals, Food and Feed (SCoPAFF): insect protein is approved for use in the territory of the EU in feed (for example, aqua culture) under the condition that the larvae are grown on a plant substrate [24]. Although the limiting factor for larval biomass accumulation is the increased fiber content in the used feed [25], the introduction of wheat bran increased the biomass yield and the overall conversion of the substrate, apparently by improving the structure of the substrate, its moisture capacity, and aeration.

3. Biochemical composition of dried larvae *Hermetia illucens* L. grown on different feed substrates (*M*±MSE, Institute of Ecology and Evolution RAS, 2016-2017)

Indicator	Feed substrate						
	1	2	3	4	5	6	7
Total moisture, %	6.28	7.45	8.19	2.78	0.88	2.18	7.68
Absolutely dry matter, %	93.72	92.55	91.81	97.22	99.12	97.82	92.32
Protein g/kg	482.9	375.7	427.87	452. 2	365.2	355.38	403.82
Fat, g/kg	205.6	382.9	318.70	194.5	455.4	261.38	221.14
Fiber (chitin), g/kg	81.4	51.9	64.60	70.2	88.8	79.63	36.48
NFES, g/kg	98.6	140.8	46.20	157.0	25.7	172.95	159.12
Ash, g/kg	67.7	36.2	60.7	71.2	38.5	67.40	102.6
Gross energy, MJ/kg	18.85	н/о	24.39	18.70	25.19	20.47	21.53
Exchange energy, MJ/kg	16.45	н/о	18.39	15.32	21.41	15.44	14.63
Energetic feed units	1.6	н/о	1.83	1.53	2.14	1.54	1.46
Digestible protein, g/kg	385.6	н/о	385.01	407.0	328.0	301.70	382.8
Calcium, g/kg	6.69	4.1	4.40	8.44	4.69	11.08	15.1
Phosphorus, g/kg	7.35	3.2	2.64	7.72	3.87	6.98	8.9

N o t e. 1 — distillers dried grains, 2 — feed wheat grain (larvae), 3 — feed wheat grain (prepupae), 4 — wheat bran, 5 — corn, 6 — fruit-grain-vegetable mixture, 7 — bird litter. The calculation of the exchange energy is given for pigs; n/d – not determined. The results of repeated measurements were obtained under convergence conditions (repeatability limit of 5%, confidence coefficient P = 0.95).

The types of substrates, on which the larvae were grown, significantly influenced their biochemical composition. The protein content in dried larvae varied from 35.5% (fruit-grain-vegetable mixture) to 48.3% (distillers dried grains) (Table 3). Digestible protein was 80% for growing the larvae on distillers dried grains, 85% for on the fruit-grain-vegetable mixture, and 90% for on grain, bran. Fat content ranged from 20.6% (distillers dried grains) to 45.5% (crushed corn). Most carbohydrates were present in the larvae grown on grain substrates. Corn

starch contributed to the accumulation of lipids in the bodies of larvae. It should also be noted that there was a significant variation in the content of calcium and phosphorus in dry larvae, depending on the type of the substrate. The age of the larvae also had a significant effect on the biochemical composition of the larvae. In the larvae of the final age (prepupae), the amount of protein increased and the mass fraction of fat decreased: dry larvae grown on feed wheat grain contained 37.6% of protein and 38.3% of fat, while in prepupae, those indicators were respectively 42.8 and 31.9%. This is due to the fact that at the prepupae stage, the larvae lose water, fat, and carbohydrates, thus the proportion of protein increases [26].

4. Amino acid composition of dried larvae *Hermetia illucens* L., soybean and fish meal (average sample, % of total protein)

Indicator	Soybean meal	Fly *Hermetia illucens* larvae	Fish meal
Aspartic acid	11.82	8.25	10.17
Threonine	4.08	3.97	4.57
Serine	5.46	4.49	4.35
Glutamic acid	18.22	12.85	14.35
Proline	5.50	6.02	4.73
Glycine	4.34	5.63	6.67
Alanine	4.42	7.25	6.70
Valine	4.70	5.12	5.32
Methionine	1.26	1.85	3.13
Cystine	1.52	0.85	1.00
Isoleucine	4.56	5.58	4.38
Leucine	7.66	12.24	7.92
Tyrosine	2.56	6.32	3.65
Phenylalanine	4.90	4.35	4.27
Histidine	2.54	4.21	3.17
Lysine	6.26	5.75	8.70
Arginine	4.30	4.20	6.48
Total of amino acids	98.10	98.93	99.56

N o t e. Reference data are given for soybean and fish meal [27]. The results of repeated measurements were obtained under convergence conditions (repeatability limit of 5%, confidence coefficient P = 0.95).

Regardless of the used feed substrate, the protein of fly *H. illucens* larvae contained all amino acids characteristic of animal protein, including irreplaceable ones. Compared to fish meal, the amino acid profile of the black lion larvae contained less aspartic acid, arginine, lysine, methionine and cystine, more proline, leucine, isoleucine, and tyrosine (Table 4). In general, the amount of lysine in the black lion was comparable to the content of this amino acid in soybean meal [27].

5. Growth dynamics of experimental hybrid young hogs (F_1 Large White × Landrace) with the addition of dry *Hermetia illucens* L. larvae to the diet (N = 12, M±MSE, Institute of Ecology and Evolution RAS, 2016-2017)

Live weight	Group	
	I	II
Initial weight, kg	15.72±1.08	15.63±0.95
At the end of the experiment, kg	28.09±1.57	28.76±1.36
Absolute gain, kg	12.37±0.60	13.13±0.47
Average daily gain, g	475.92±22.93	504.95±17.94
To the control, %	100.0	106.1

N o t e. For a description of the groups, see the Techniques section.

Pigs are omnivorous monogastric animals, which especially need animal protein. Depending on age, their combined feed should include 13-21% of crude protein [28]. The introduction of black lion larvae protein into the diet of pigs, containing the whole complex of natural essential and conditionally replaceable amino acids, as well as enzymes, biologically active substances, micro- and macroelements, can be effective with the full or partial replacement of fish or soybean meal. In the authors' experiment, the growth dynamics of young hogs when feeding with a full ration starter combined feed (SF-4) with the addition of 7%

of black lion larvae was positive compared to the control: the average daily weight gain in group II was 6.1% higher than in group I ($p > 0.05$) (Table 5).

6. Feed consumption when adding dry *Hermetia illucens* L. larvae to the diet of young hogs (F_1 Large White × Landrace) (*M*±MSE, Ernst Federal Science Center for Animal Husbandry, 2016-2017)

indicator	Group	
	I	II
The content of EE in 1 kg of combined feed, MJ	11.61	11.98
Feed spent for the period, kg	28.650	28.650
Spent per day:		
combined feed, kg	1.08	1.08
EE, MJ	12.59	13.06
Absolute weight gain for the period, kg	12.60±0.90	13.33±0.47
The cost of combined feed per 1 kg of gain, kg:		
total	2.26	2.11
to the control, %	100.00	93.36
EE costs per 1 kg of gain, MJ:	26.34	25.45
total		
to the control, %	100.00	96.62

N o t e. EE — exchange energy of digestible nutrients. For a description of the groups, see the Techniques section.

In terms of the cost of feed for obtaining a unit of products, the indicators in group II were lower compared to the control by 0.15 kg, or 6.6% (Table 6). That is, the animals from the experimental group better used nutrients of feed for live weight gain. In group I, the digestibility coefficients were 75.13% for the dry matter, 77.81% for organic matter, 72.27% for crude protein, 41.68% for crude fat, 41.85% for crude fiber, 84.77% for nitrogen-free extractable substances; in group II, these figures were 75.19; 77.14; 71.58; 38.52; 37.11 and 85.03%, respectively. Significant differences were not revealed when comparing nutrient digestibility ratios in animals of the experimental and control groups ($p > 0.05$). When 7% meal of fly larvae was included in the diet, crude fat and fiber were somewhat worse digested. Perhaps, this is due to a slightly higher content of fat in the experimental feed (see Table 1) and the presence of poorly digestible chitin of the larvae, determined in the total indicator of fiber content in the feed.

The rate of the utilization of a nutrient shows how efficiently the arrived and digested nutrient in the animal are used in metabolic processes. To study protein metabolism, the authors calculated the balance and use of nitrogen by young hogs during the period of the balance experiment. When comparing the amount of nitrogen in relation to the one which came with the feed and was digested, there is a tendency to an increase in indicators of the animals from the experimental group compared to the counterparts from the control group; however, this difference is slight and not statistically significant. Nevertheless, it was shown that the addition of fly *H. illucens* larvae to the combined feed contributed to better utilization and deposition of nitrogen and, consequently, to a higher live weight gain (504.95±17.94 vs. 475.92±22.93 g, $p > 0.05$) (see Table 5). The inclusion of black lion larvae in the diet of the group II did not have a negative or statistically significant effect on the use of calcium and phosphorus by animals.

The obtained results to some extent correlate with the data when partially defatted meal from *H. illucens* larvae was introduced into the diet of piglets, with 75% replacing soybean meal without balancing and with balancing of the amino acid composition by adding crystalline L-lysine, DL-methionine, and L-threonine [25]. The animals, the diet of which included black lion larvae and amino acid supplements, did not differ in the studied zootechnical parameters with control piglets. When replacing soybean meal with defatted black lion meal with basic amino acid content, there was a tendency for a slightly smaller growth of piglets, feed consumption and protein conversion. However, no significant

negative effect was noted. The authors concluded that partially defatted meal from *H. illucens* may be a promising alternative to soybean meal in the diets of young pigs. In this work, the zootechnical indicators of young hogs from the experimental group were similar to those of control animals. Moreover, there was a tendency to exceed the control indicators with the introduction of complete full-fat larvae flour into the feed without additional enrichment with synthetic amino acids. Larvae lipids seem to play a positive role, increasing the feed efficiency of the product based on the whole insect, since they contain biologically active components [29].

It should be noted that the biometric processing of the obtained data did not reveal statistically significant differences in the analyzed parameters, as well as in relation to the growth of animals and the digestibility of the nutrients of their diets. The absence of such differences suggests that replacing fish meal with dry larvae biomass does not lead to negative consequences and allows maintaining control values when raising young pigs, which should be evaluated positively. Thus, the nutritional value of the fly *Hermetia illucens* larvae is sufficiently high: the content of exchangeable energy in 1 kg, depending on the used feed substrate for growing the larvae, is 15.32-21.41 MJ, the amount of protein varies within 35.5-48.3%, fat is 20.6-45.5%. The amino acid composition of the larval protein contains a complete set of amino acids, including all essential amino acids. Feeding the young pigs with the black lion larvae in the composition of balanced full-ration combined feed (7.0%, or 70 kg/t of combined feed) has a positive effect on the physiological processes in the animals' bodies, their productivity, as well as the costs of feed. Fly *H. illucens* larvae can be considered as a rational alternative to traditional high protein feeds (including fish meal) in the diets of growing young pigs during the nursery period.

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LUPINE IS APPLICABLE IN DIETS FOR LAYER CHICKENS OF PARENTAL FLOCK

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Abstract

Climatic conditions in most Russian regions are unfavorable for the cultivation of soy beans which are considered the best protein source in diets for all types of poultry. Soya yield in Russian soya-producing regions (Far East, Krasnodar Krai, and some other southern territories) cannot provide the growing poultry production with this important protein source. The alternatives for soybean meal and other soya products are therefore in need; domestically selected low-alkaloid cultivars of white and narrow-leaf lupine are increasingly gaining importance as vegetable protein sources. Feed-grade lupine usually contains up to 42 % of crude protein. The disadvantages of lupine are high contents of fiber (12.5-16.0 %) and lignin (0.9 %), and the presence of alkaloids. Alkaloid content in sweet lupine cultivars is 0.008-0.12 %, in bitter cultivars 1-3 %. Chemical and amino acid composition of white lupine modern cultivars was earlier determined, and their efficiency in poultry diets was studied in vivo. It was found that the grain of low-alkaloid cultivars Gamma, Dega, Dikaf 14 can be included into the diets for poultry at 15-20 % dosage. Supplementation of lupine-containing diets with proper enzyme preparations can improve the digestibility of dietary nutrients and poultry performance. Dehulling of lupine grain decreases fiber content and increases protein content in concentrated lupine-based protein feeds; the latter in dehulled lupine is close to that in soybean products. This original research for the first time proves the possibility of soybean and sunflower meals substitution for white low-alkaloid lupine cultivar Dega the in diets for parental flock of laying hens. The trial was performed on 5 groups of parental White Leghorn layers (cross SP 789) from 184 to 365 days of age fed balanced diets containing 0; 5; 7; 10; and 15 % of dehulled Dega lupine grain (39.61 % crude protein, 5.60 % crude fiber). It was found that 5-10 % lupine does not impair livability and productivity parameters in layers. Lupine was found to influence the intensity of lay, egg fertility, hatch of chicks, micromorphology of liver in hens. The substitution of soy for lupine (7, 10, and 15 %) improved egg production and egg weight output per hen by 1.51 and 7.10 %; 3.31 and 1.64 %; 6.56 and 3.64 %, respectively, in compare to control; feed expenses per 1 kg egg weight in these groups was 0.9; 4.07; and 1.81 % lower. The percentages of infertile eggs and early embryonic deaths in using 10 and 15 % lupine were lower compared to control, evidencing the absence of negative impact of lupine Dega on early embryonic development. The highest doses of lupine did not increase the incidence of late embryonic deaths in eggs from layers aged 47 weeks: the percentages of late embryonic deaths and weak chicks from layers fed 15 % lupine were 4 and 5 % vs. 9 and 8 % in control. The histological investigation of liver revealed no significant differences between lupine-fed and control layers; nucleoplasmic ratio in the hepatocytes was similar in all treatments. In all treatments liver had no abnormalities, the connective tissues were poorly developed and located in the peripheral segments of the liver (where it forms a capsule) and near the portal triad. The hepatic plate structure is well developed, and the tortuous plates are radially oriented. The clusters of blood cells are seen in the lumen of the central veins and sinuous capillaries. Hepatocytes are not clearly bordered, polygonal in shape; nuclei centered or sometimes slightly peripherally shifted, round or oviform, has 1-4 nucleoli. Cytoplasm is unevenly stained, granular; lymphoid cells are found in the stroma and parenchyma. The results of histological investigation of liver, productivity parameters in layers, and the efficiency of incubation of eggs obtained evidence that 5-15 % of Dega lupine dehulled grain as a protein source in diets of laying hens from parental flock does not impair

productivity and rendered no cytotoxic effects on the liver of hens. This cultivar of white lupine can be recommended as a dietary protein source both for commercial and parental layer flocks.

Keywords: white lupine, alkaloids, laying hens, productivity, egg fertility, hatchability, liver histomorphology

Climatic conditions in most Russian regions are unfavorable for the cultivation of sufficient quantity of soybeans (i.e., a worthful crop) required for poultry production. Sunflower meal is a common protein feed in Russia. As for pulse crops, pea, field beans, vetch and lupine bear mentioning [1-4]. Due to lack of protein poultry feed, lupine is of greater interest because of high yield and potential cultivation in areas where soybean growth is impossible, or its yield is low. Some Russian low-alkaloid lupine cultivars are developed to substitute soya products in feed production [5, 6]. In terms of global production, lupine occupies about 1% of cultivation areas. Nowadays, Australia, New Zealand, Poland and Belarus are leaders of lupine cultivation [7-9]. Romanian cultivars of low-alkaloid white lupine were developed recently. Climatic conditions are favorable to cultivate narrow-leaf and yellow lupine in Poland, Belarus and Germany. Its impact on egg production and quality [10-12] associated with improved dietary formulation [13], antinutrient effects [14], as well as application of low-alkaloid cultivars [15], whole and dehulled seeds [16] are still studied. Feed value and potential use of blue lupine is an active area of research in Great Britain [17, 18].

Feed-grade lupine usually contains up to 42% of protein. This is a good source to replenish concentrated feed with crude protein that, in turn, is of great importance to manage deficiency of qualified animal feeds and soybean meal. The disadvantages of lupine are high contents of fiber (12.5-16.0%) and lignin (0.9%), and the presence of alkaloids. Namely, sweet and bitter lupine cultivars contain 0.008-0.120% and 1-3%, respectively. Lupine alkaloids are lupinine, lupanine, sparteine and hydroxylupanine. Lupanine is the most toxic alkaloid quantitatively predominant in most of bitter lupine cultivars. Containing an atom of nitrogen and a primary alcohol group, it can be oxidized to lupininic acid. Lupinine is a main toxic factor of lupine intoxication. At the same time, other alkaloids are nothing but accessory factors of toxicosis. TDL0 of lupinine is 25-28 mg/kg, LD is 29-31 mg/kg [9]. As for Russian white low-alkaloid lupine cultivar Dega, mean total content of alkaloids in whole grain is 0.062% where lupanine (37.1%) and dehydroxylupanine (14.3%) are predominant components. The value increases to 0.080% [3] in dehulled grain.

1. Alkaloid content in Dega lupine cultivar grain (on absolutely dry basis, %) (M±SEM) **[3]**

Parameter	Whole grain	Dehulled grain
Alkaloid content	0.062±0.006	0.080±0,008
Alkaloid composition, %		
sparteine	5.9±0.6	8.5±0.8
ammodendrine	8.5±0.9	5.4±0.5
angustifoline	9.5±1.0	8.1±0.8
isolupaine	9.3±0.9	8.8±0.9
aphillidine	4.7±0.5	5.7±0.6
dehydoxylupanine	14.3±1.4	15.0±1.5
lupanine	37.1±3.7	40.1±4.0
13-oxylupanine	8.5±0.8	6.5±0.6
ether 13-oxylupanine	2.2±0.2	1.4±0.1

Previously we [1, 2, 19, 20] and other researchers [21] studied efficiency of Gamma and Dega cultivars consumed by commercial broilers and layers. According to the studies, up to 20% of lupine can be included into their diet [1, 19].

The remarkable thing is that high content of fiber specific for narrow-leaf and white lupine results in restricted application of high doses in poultry diets. To solve the issue, multi-enzyme preparations [1, 4, 22] or lupine bean dehulling are used. The latter enables both decrease in fiber content in concentrated feed and increased protein level that is equivalent in the dehulled lupine grain and soya products [16, 19].

Despite broader industrial use of lupine to feed commercial broilers and layer flocks, there are no similar national studies in breeding poultry in fact.

It is the first time we demonstrated that white low-alkaloid lupine cultivar Dega can be an efficient substitution for soya and sunflower products to produce concentrated feed for laying hens of parental flock. It was found that 5-15% of Dega lupine dehulled grain did not have a negative effect on layer liver and hepatic micromorphology. Also, this resulted in high livability and productivity parameters, as well as 1-day young crop equivalent to ones associated with soya products.

Our purpose was to evaluate potential application of the white lupine cultivar Dega as a substitution for soya and sunflower products to feed layers of a parental flock.

Techniques. The trial was performed on 5 groups of parental layers (cross SP 789; each group included 30 hens) aged 184-365 days in the vivarium of Zagorskoe Genetic Selection Centre of the Federal Scientific Center All-Russian Research & Technology Institute of Poultry RAS (Moscow Province) during the 6-month productive life. Poultry were held on dedicated cages (Pyatigorsksel'mash ZAO, Russia). Feed was given manually. Dega white lupine toasted dehulled grain was included into poultry diet. Feeding and management conditions (concentrated feed nutritional value, seating standards, light, temperature and moisture conditions, feeding and drinking spaces) complied with appropriate guidelines (All-Russian Research & Technology Institute of Poultry, 2015) within all the test. Layers of group I (i.e., controls) consumed balanced crumbled complete concentrated lupine-free feed (primary diet, PD). To replace soya products, groups II, III, IV and V were fed with concentrated feed containing 5%, 7%, 10% and 15% of lupine.

Layers aged 34 and 47 weeks were artificially inseminated to obtain hatching eggs. The insemination was carried out as per a standard method using a new patented medium to dilute cock semen (patent RU no. 2637774 C2, insemination dose – 0.1 ml, dilution – 1:3). 100 eggs collected from each group were placed in an experimental incubator (Danki, Belgium). Using a dedicated probe (accuracy < 0.1 °C), 37.7 °C and 37.2 °C was maintained during pre-incubation (days 1-18) and hatcher period (days 19-21). Relative humidity values were 52-53% and 52-75%, respectively.

We considered main zootechnical parameters such as initial and final weight (individual weighing), livestock livability and egg production. Feed consumption and expenses (per a hen, 10 eggs and 1 kg of eggs) were calculated. Incubation parameters (fertility and hatch), vitamin and carotenoid hepatic levels, hepatic chemical composition, as well as calcium, phosphorus and manganese shinbone levels were assessed as per effective GOST standard methods [23].

At the end of the test hepatic samples were collected from the lateral side of the right hepatic lobe of laying hens (n = 15, 3 hens were selected in each group) within 1 hour after the slaughter. These samples were fixed in 10% aqueous solution of neutral formalin. The material was washed with current water, dehydrated in ethanol of increasing concentration and condensed with paraffin. Paraffin 5-8 μm sections were produced by a RMD-3000 semi-automatic rotary microtome (Kreonika OOO, Russia). Deparaffinized sections were stained with hematoxylin and eosin. General morphological picture was evaluated with a Micromed-3 (LOMO, Russia) light microscope. Micrometry was performed with a DSM 300 ocular camera (Hangzhou Scopetek Opto-Electric Co., Ltd, China) and ScopePhoto 3.1 software (China) (https://scopephoto.software.informer.com/3.1/). Major and minor diameters of hepatocytes and their nuclei, volume of cells, cytoplasm and their nuclei, as well as nucleoplasmic ratio were evaluated. Volume

of hepatocytes and their nuclei was assessed using C. Tasca's formula [24]: $\pi/(6 \times L \times B^2)$, where L is a major cell (nuclear) diameter (μm) and B is a minor cell (nuclear) diameter (μm).

The findings were processed according to Lakin [25] and Plokhinskii [26]. STATISTICA 10 (StatSoft, Inc., USA) software was applied. Means (*M*) and errors of means (±SEM) were assessed. To calculate significance of differences, *t*-test was used. Differences were considered as statistically significant at $p \leq 0.05$.

Results. See primary parameters of Dega chemical composition compared with other white lupine cultivars in the Table 2. Certainly, protein, fat and carotenoid levels can vary depending on climatic conditions. In general, the table demonstrates nutritional value of modern national white lupine cultivars completely.

2. Chemical and amino acid compositions of Russian lupine cultivar grain (on absolutely dry basis, %)

Content, %	Start	Manovitskii	Gamma	Delta	Dega	Deter	Desnyanskii
Moisture	9.68	9.44	8.66	8.45	8.61	8.65	8.78
Protein	34.18	34.93	37.75	33.12	33.81	35.12	32.18
Fiber	10.68	10.92	10.76	10.21	10.86	9.95	10.19
Fat	9.37	9.28	9.95	11.25	9.79	9.90	11.75
Ash	3.37	3.09	3.32	3.12	3.31	3.11	3.05
Calcium	0.308	0.321	0.350	0.335	0.354	0.360	0.380
Phosphorus	0.300	0.359	0.300	0.310	0.310	0.300	0.300
Carotenoids	27.33	27.25	28.77	17.71	28.47	23.17	23.42
Lysine	1.37	1.41	1.44	1.43	1.41	1.57	1.54
Histidine	0.79	0.80	0.94	0.85	0.81	0.92	0.94
Arginine	2.84	2.91	3.34	2.99	2.97	3.17	2.73
Aspartic acid	3.22	3.16	3.10	2.96	2.89	3.35	3.01
Threonine	1.27	1.24	1.34	1.27	1.18	1.23	1.18
Serine	1.66	1.75	1.81	1.72	1.65	1.70	1.55
Glutamic acid	6.42	6.95	7.63	7.28	7.17	6.78	6.22
Proline	0.93	1.13	1.04	1.69	1.46	1.30	1.40
Glycine	1.17	1.22	1.28	1.19	1.18	1.18	1.12
Alanine	0.96	1.03	1.19	1.62	1.02	0.94	0.96
Cystine	0.47	0.39	0.44	0.48	0.45	0.45	0.51
Valine	1.03	1.06	1.19	1.09	1.08	1.08	1.03
Methionine	0.42	0.32	0.39	0.40	0.37	0.39	0.36
Isoleucine	1.05	1.13	1.32	1.17	1.18	1.21	1.02
Leucine	2.01	2.14	2.44	2.16	2.22	2.35	2.18
Tyrosine	1.31	1.40	1.60	1.36	1.44	1.55	1.25
Phenylalanine	1.07	1.11	1.24	1.28	1.09	1.20	1.01

N o t e. Presented findings resulted from the test performed in the Testing Center (Federal Scientific Center All-Russian Research & Technology Institute of Poultry RAS).

See results of chemical and toxicological tests, as well as amino acid composition of Dega dehulled grain in the Table 3.

It was found that 5-15% of Dega lupine grain fed within the 6-month productive life did not decrease productivity and livability of layers. Thus, the livestock livability was 96.67-100 %. Mortality of controls, as well as poultry in groups III and V consumed 7% and 15% of lupine, respectively, was 3.33%. It was not associated with a food factor (see Table 4). Poultry consumed lupine-containing concentrated feed willingly. Despite higher productivity of layers in groups III, IV and V, as well as increased nutrient removal with eggs, weight of these 52-week hens was by 7.25%, 3.52% and 8.69% higher than in controls (differences between test and control poultry are significant if $p \leq 0.5$). Along with this, layers in groups III, IV and V consumed by 0.90%, 4.07% and 1.81% less of feed per 1 kg of eggs. In general, lupine substitution for conventional protein sources in concentrated feed (groups III, IV and V) increased egg production and, in turn, egg output and weight per a layer by 1.51%, 7.10% and 3.31%, respectively, as well as by 1.64%, 6.56% and 3.64%, respectively.

3. Chemical composition of Dega dehulled grains (on air-dried basis, %)

Parameter	Value
Chemical composition	
Moisture content, %	8.20
Crude protein, %	39.61
Crude fiber, %	5.60
Crude fat, %	9.74
Crude ash, %	3.64
Soluble protein, %	88.59
Acid number, mg KOH/g	4.42
Peroxide number, %	0.013
Non-protein nitrogen, %	0.36
Anino acid composition, %	
Aspartic acid	4.18
Threonine	1.42
Serine	1.86
Glutamic acid	8.60
Proline	1.49
Glycine	1.54
Alanine	1.36
Valine	1.65
Isoleucine	1.82
Methionine	0.24
Leucine	2.90
Tyrosine	1.80
Phenyl alanine	1.59
Lysine	2.08
Histidine	0.90
Arginine	4.03
Cystine	0.51
Leucine	2.90
Vitamins, pigments	
Carotenoids, μg/g	32.56
Vitamin E, μg/g	7.30
Minerals	
Calcium, %	0.243
Phosphorus, %	0.410
Manganese, mg/kg	1324.50
Toxic elements	
Lead, mg/kg	0.13
Cadmium, mg/kg	0.002
Arsenic, mg/kg	0.15
Mycotoxins	
Zearalenone, μg/kg	2.06
Ochratoxin, μg/kg	< 1.05
Fumonisin, μg/kg	Not detected
T-2 toxin, μg/kg	3.84
HT2 toxin, μg/kg	9.78
Deoxynivalenol, μg/kg	17.44
Aflatoxin B_1, μg/kg	Not detected
General toxicity	Non-toxic

Note. Presented findings resulted from the test performed in the Testing Center (Federal Scientific Center All-Russian Research & Technology Institute of Poultry RAS).

It is a well-known fact that lupine contains less essential amino acids than soybeans. Although lysine, methionine and threonine deficiency can be compensated with synthetic amino acids, lack of other amino acids can lead to decreased layer productivity (including reduced egg weight). As tabulated in the Table 4, 5-15% of lupine did not result in substantial differences between mean egg weight in control and test groups.

Mineral metabolism intensity providing a stable poultry skeleton, good quality of eggshell and certified hatching eggs is of great importance for parental flock layers. Maximum elastic strain (i.e., a specific parameter of eggshell quality) demonstrated a specified value (23.00 μm) in 34-week test layers. 47-week test layers showed the decreased value as compared with controls (28.05-24.35 μm vs. 28.77 μm). At the same time, group V consumed 15% of lupine demonstrated better eggshell quality. Nevertheless, it worth mentioning that eggshell thickness in all the layers complied with all the specifications of the cross during the observation.

4. Zootechnical parameters of parental flock layers (cross SP 789) consumed different doses of Dega lupine dehulled grain for 6-month productive life (*M*±SEM, vivarium of Zagorskoe Genetic Selection Centre, All-Russian Research & Technology Institute of Poultry RAS, Moscow Province)

Parameter	Group I (контроль)	Group II (5 % lupine)	Group III (7 % lupine)	Group IV (10 % lupine)	Group V (15 % lupine)
Age, days	182-364 сут (26-52 нед)				
Livestock livability, %	96.67	100	96.67	100	96.67
Weight, g:					
start of the test	1621.67±23.54	1613.67±24.43	1592.73±18.19	1590.87±18.02	1599.87±18.02
end of the test	1719.83±40.79	1777.33±41.13	1844.48±51.23*	1780.33±32.59*	1869.31±47.34*
Consumed feed:					
1 hen/day, g	114.8	114.78	114.81	115.68	115.9
per 10 eggs, g	1.403	1.458	1.413	1.356	1.403
per 1 kg of eggs/layer, kg	2.21	2.29	2.19	2.12	2.17
Total number of eggs/layer, pcs	144.13	142.50	146.3	154.37	148.8
Egg production intensity, %	79.19	78.73	80.83	85.29	82.21
Mean egg weight, g	64.44	63.75	64.52	64.11	64.69
Egg output/layer, kg	9.288	9.08	9.440	9.897	9.626
Elastic strain, μm:					
34 weeks	24.10±1.39	23.78±1.37	22.90±1.34	24.67±2.10	22.40±0.98
47 weeks	28.77±2.06	28.05±1.39	27.86±1.83	27.97±1.61	24.35±1.36
Calcium level in eggshell of 47-week hens, %	37.09	37.29	37.39	37.60	37.34
Egg shell thickness, μm:					
34 weeks	418.50±12.15	431.27±9.61	409.74±6.92	411.67±11.70	390.28±6.10
47 weeks	378.72±8.69	343.33±18.47	400.66±15.29	407.73±11.40	394.31±8.08
364-day hen shinbone contains:					
ash, %	58.00	57.30	58.62	60.37	57.75
Ca, %	20.30	20.32	20.80	21.18	20.29
P, %	9.74	9.50	9.88	10.05	9.61
Mn, mg%	1.72	1.80	1.85	2.33	2.99

N o t e. Individual values and mean group values are presented.
* Differences between initial and final test values are statistically significant at $p \leq 0.05$.

Of course, eggshell quality depends on consumption of calcium, phosphorus and vitamin D_3 by laying hens. Also, worse eggshell quality and skeleton status can be caused by manganese deficiency or poor digestibility. We found that lupine is a natural source of manganese organic compounds [27]. Manganese level was assessed in white and narrow-leaf lupine cultivars cultivated in various Russian regions with different level of the element in soil. For example, Gamma white lupine whole, uncoated and coated grains contain 491.1 mg/kg, 941.63 and 115.05 mg/kg of Mn, respectively [27]. Dega cultivar also demonstrates increased accumulation of manganese (1324.5 mg/kg on air-dried basis). Our findings correspond with data reported by other researchers. In particular, manganese content in Dega grain is 1428.0±20 mg/kg on absolutely dry basis [3].

As lupine dosage in layer diet increased (see the Table 4), shinbone manganese pooling rose from 1.72 mg% (controls) to 2.99 mg% (group V, 15% of lupine). There were no significant intergroup differences detected between calcium and phosphorus levels in shinbone and eggshell. However, according to the test, lupine included into the diet induced 0.20%, 0.30%, 0.51% and 0.25% higher calcium levels in eggshell of 47-week hens (groups II, III, IV and V, respectively) as compared with controls.

As known, healthy embryonic development during the early incubation influences on quantitative and, most significantly, qualitative results of incubation. As for our test, all the groups demonstrated high egg fertility (94-100%) for all ages (see the Table 5). Amongst other things, it was associated with cock semen dilutant quality used for insemination [28]. Along with this, groups IV and V showed lower number of infertile eggs than controls. Together with low incubation mortality (blood ring), the result indicates absence of negative impact of

10-15% of Dega lupine dehulled grain on early embryonic development. Moreover, we did not mention increased mortality in the groups during the late incubation of eggs obtained from 47-week layers. Therefore, group V consumed 15% of lupine in the diet demonstrated 4% and 5% of late embryonic deaths and weak chicks vs. 9% and 8% in controls, respectively.

5. Biocontrol parameters (%) of egg incubation in parental flock layers (cross SP 789) consumed different doses of dietary Dega lupine dehulled grain (n = 100, vivarium of Zagorskoe Genetic Selection Centre, All-Russian Research & Technology Institute of Poultry RAS, Moscow Province)

Parameter	Group I (контроль)	Group II (5 % lupine)	Group III (7 % lupine)	Group IV (10 % lupine)	Group V (15 % lupine)
Poultry aged 34 weeks					
Infertile eggs	4	6	1	0	0
Blood rings	5	0	2	7	1
Missed	0	2	1	1	1
Late embryonic death	10	15	9	16	4
Weak	2	5	8	7	2
Hatchability	84.38	81.91	87.88	75.0	94.0
Total hatched	81	77	87	75	94
Condition chicks, pcs	79	72	79	68	93
Poultry aged 47 weeks					
Infertile eggs	6	-	1	5	4
Blood rings	2	1	0	1	3
Missed	2	0	0	0	0
Late embryonic death	9	10	11	7	4
Weak	8	11	2	10	5
Hatchability	86.17	89	88.89	91.58	92.71
Total hatched	81	89	88	87	89
Condition chicks, pcs	73	78	86	77	84

6. Hepatic chemical composition (%) in layers (cross SP 789) consumed different doses of dietary Dega lupine dehulled grain (on air-dried basis, vivarium of Zagorskoe Genetic Selection Centre, All-Russian Research & Technology Institute of Poultry RAS, Moscow Province)

Parameter	Group I (контроль)	Group II (5 % lupine)	Group III (7 % lupine)	Group IV (10 % lupine)	Group V (15 % lupine)
Protein	52.34	49.66	46.20	46.93	48.38
Fat	32.31	33.72	39.66	40.32	36.05
Ash	3.88	3.68	3.90	3.88	3.95

Note. According to the method, differences are significant if variations of protein and fat levels are higher than 1% and 2%, respectively. Thus, increase in fat levels is significant in groups III, IV and V.

As known, decrease in egg production and livability of layers, as well as less qualified incubation eggs observed during the late productive life are associated with higher incidence of fatty liver [23, 29]. Increased hepatic fat level detected in test layers consumed concentrated feed with different lupine content (see the Table 6) resulted in an additional histological liver investigation to determine safe dosage of lupine to be included into a diet.

7. Гистоморфометрические показатели печени у кур-несушек кросса СП 789 при разных дозах обрушенного зерна люпина сорта Дега в рационе (M±SEM, виварий СГЦ «Загорское» ФНЦ ВНИТИП РАН, Московская обл.)

Parameter	Group I (контроль)	Group II (5 % lupine)	Group III (7 % lupine)	Group IV (10 % lupine)	Group V (15 % lupine)
Volume, μm^3:					
hepatocyte	511.73±36.04	494.18±27.61	383.00±24.45	420.56±25.21	496.00±30.59
nucleus	45.07±2.41	41.2±2.30	38.73±2.32	38.57±2.02	41.66±2.48
cytoplasm	466.66±35.31	452.98±27.14	344.62±23.99	381.99±24.46	454.32±30.10
Nucleoplasmic ratio	0.10±0.01	0.09±0.01	0.12±0.01	0.11±0.01	0.10±0.01
Trabecules, μ	18.92±0.53	19.72±0.44	18.43±0.40	18.82±0.48	18.79±0.47
Sinusoids, μ	4.16±0.19	4.63±0.20	4.46±0.19	4.42±0.21	4.69±0.22

The morphometric research of hepatic histostructure did not detect any

significant differences between control and test hens. Nucleoplasmic ratio in hepatocytes was equivalent (see the Table 7).

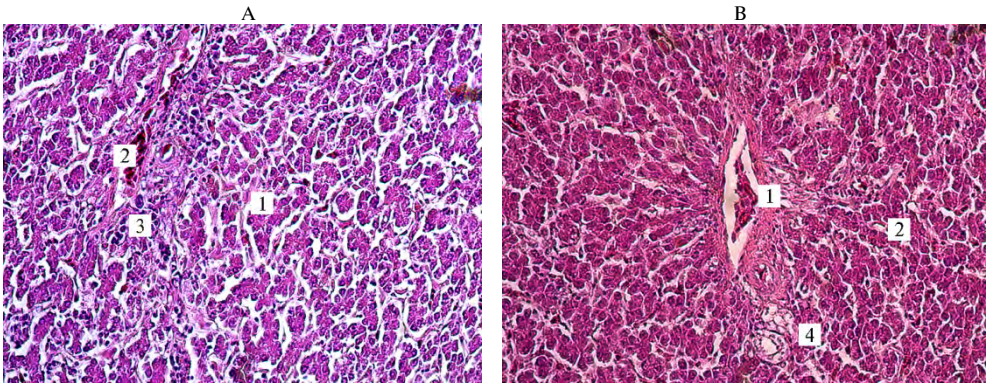

Fig. 1. Hepatic micromorphology in 365-day layers (cross SP 789) consumed dietary Dega lupine dehulled grain: A — lupine-free diet, B — lupine-containing diet (5%); 1 — hepatocytes, 2 — central vein and erythrocytes, 3 — lymphocytes, 4 — sinusoids. Hematoxylin and eosin staining, light microscope (Micromed-3, LOMO, Russia), magnification ×300.

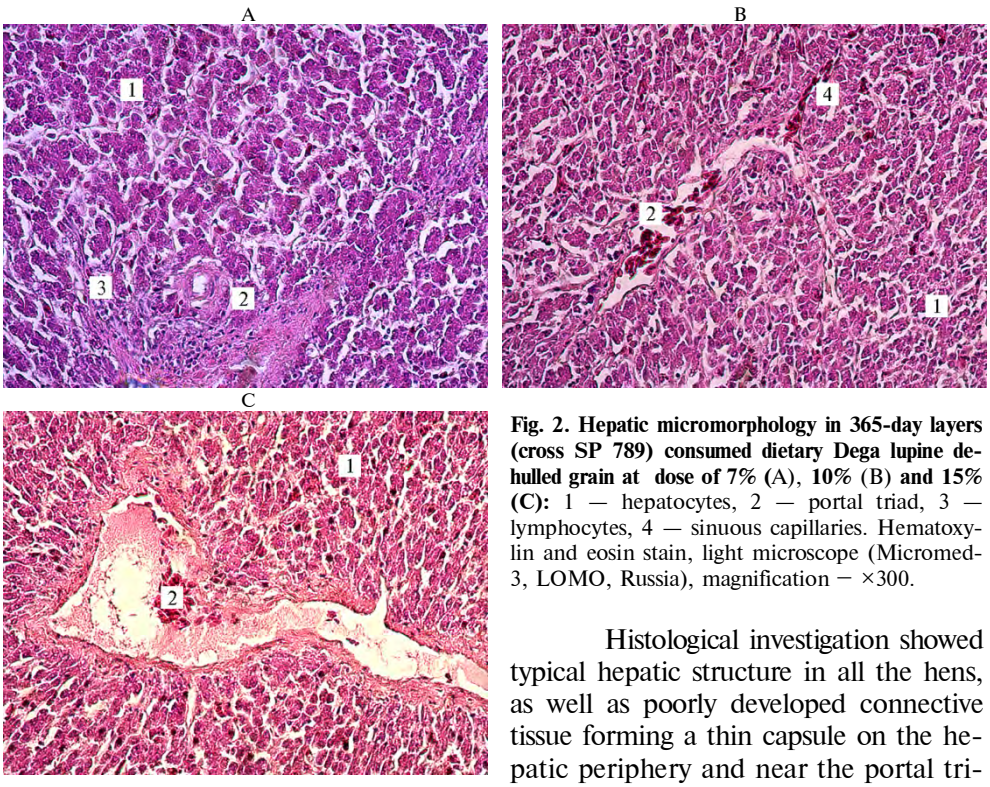

Fig. 2. Hepatic micromorphology in 365-day layers (cross SP 789) consumed dietary Dega lupine dehulled grain at dose of 7% (A), **10%** (B) **and 15% (C):** 1 — hepatocytes, 2 — portal triad, 3 — lymphocytes, 4 — sinuous capillaries. Hematoxylin and eosin stain, light microscope (Micromed-3, LOMO, Russia), magnification – ×300.

Histological investigation showed typical hepatic structure in all the hens, as well as poorly developed connective tissue forming a thin capsule on the hepatic periphery and near the portal triad. The hepatic plate structure is well developed, and the tortuous branchy plates are radially oriented. The clusters of blood cells are seen in the lumen of the central veins and sinuous capillaries. Hepatocytes are not clearly bordered, polygonal in shape; nuclei centered or sometimes slightly peripherally shifted, round or oviform, have 1-4 nucleoli. Cytoplasm is unevenly stained, granular; lymphoid cells are found in the stroma and parenchyma (Fig. 1, 2). Our data on hepatic histological structure comply with findings of other authors [30-34].

Thus, 5-15% of lupine dehulled grain included into the diet of parental flock layers as a protein source did not reduce any zootechnical and incubation pa-

rameters. Poultry demonstrated a long-term egg productivity. Moreover, test livestock livability was similar to controls even after high lupine dose consumption. Histological results confirm absence of evident cytotoxic effect of lupine on hepatic histostructure in layers corresponding with their zootechnical and incubation parameters. This brings us to recommend Dega white lupine to be applied as a feed-grade protein source both in commercial and breeding poultry.

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OBTAINING RECOMBINANT NUCLEOCAPSID PROTEIN OF PPR VIRUS FOR DISEASE SERODIAGNOSTIC

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Abstract

Peste des petits ruminants is a highly contagious, acute or subacute viral disease of sheep and goats, characterized by fever, sores in the mouth, haemorrhagic gastroenteritis, lesions in lymphatic system and pneumonia. (E.P.J. Gibbs et al., 1979; A. Diallo et al., 1989; T.M. Ismail et al., 1995). Because of high morbidity of 50-100 % and mortality of 50-90 %, Peste des petits ruminants belongs to a number of emerging diseases, having a significant threat to livestock production in countries where the disease is notified (R.A. Kock et al., 2015; E.M.E. Abu-Elzein et al., 1990). The etiological agent of PPR is a Morbillivirus (PPRV) of *Paramyxoviridae* family (M.H.V. Van Regenmortel et al., 2000). The PPRV antigens are similar to antigens of other Morbilliviruses (G. Libeau et al., 2014). Severity of the clinical signs depends on different factors, e.g. PPRV line, animal species, breed, and immune status. Because of that, the final diagnosis must be confirmed by laboratory methods. In diagnostics and monitoring serological testing, the preference is given to sensitive and automated Enzyme-linked immunosorbent assay (ELISA). Modern methods of PPRV serodiagnostic are developed on the basis of virus-specific recombinant proteins and primary nucleocapsid N protein (A. Diallo et al., 1994; G. Libeau et al., 1995; M. Munir et al., 2013; N.V. Vavilova et al., 2006.), which is superior to the other Morbilliviruses's proteins in antigenic and immunogenic characteristics (P.C. Lefevre et al., 1991; M. Yunus et al., 2012). The other protein N advantage is that, as an antigen, it is the most conservative of the PPR virus proteins (M. Muhammad, 2013). The purpose of this paper was to obtain recombinant nucleocapsid N protein of PPR virus as an antigen and virus-specific antiserum of pigs as a source of antibodies for serodiagnostic of disease by competitive ELISA. A gene construct was designed which contained a sequence of protein N gene fragment of 1530 kb in length in the express plasmid vector pET32a. After polypeptide screening by SDS-PAGE and immunoblotting we found clones of *Escherichia coli* pET32a/N/10 which express 70 kDa virus-specific major polypeptide. It was shown, that in competitive ELISA the optimal dose of recombinant protein N purified by Immobilized Metal Chelate Affinity Chromatography method is 0.25 μg per well. The ratio of OD_{450} values for negative and positive control goat sera was 11.52. So the electrophoretically purified and immunochemically pure recombinant protein N can be used in competitive ELISA for PPRV serodiagnostic. For obtaining specific antisera, pigs were inoculated with purified PPRV. The antibody titers in antisera samples from the pigs in a neutralization test with PPRV were 1:64-1:128. These values are comparable with antibodies titers in sera of sheep and goats vaccinated against PPR (A.V. Konstantinov et al., 2017). However pigs' antisera were less effective in competitive ELISA than positive goat sera.

Keywords: peste des petits ruminants, *Morbillivirus*, diagnostic, recombinant proteins, immunosorbent assay

Peste des petits ruminants (PPR) is a highly contagious, acute or subacute viral disease of sheep and goats characterized by fever, sores in the mouth, haemorrhagic gastroenteritis, lesions in lymphatic system and pneumonia [1-3]. For the first time, the disease was described on the coast of Cote d'Ivoire in the West Africa [4]. Then, it was reported in the Africa South of the Sahara, on the Arabian Peninsula and in the Southwest Asia [5]. Due to high morbidity (50-100%) and mortality (50-90%), PPR belongs to a number of emerging diseases having a significant threat to livestock production in countries where the disease is notified [6, 7].

The etiological agent is a PPR virus (genus *Morbillivirus*, family *Paramyxoviridae*). The PPRV antigens are similar to antigens of other Morbilliviruses [8, 9]. The virus is transmitted by a close contact of susceptible animals (several species of gazelles, oryxes and white-tailed deers) through secretions and excretions of ill animals. Although cattle, buffaloes and pigs can be naturally or experimentally PPRV-infected, they are dead-end hosts because of absence of the disease clinical signs and impossible viral transmission to other animals [5]. Four PPR virus lines are reported nowadays. One of them (line 4) is common in Asia only, but others are wide-spread in Africa [10, 11]. The line division is based on differences between a nucleocapsid protein gene. As compared to a categorization centered around a glycoprotein gene F alterations, the arrangement highlights a geographical origin more clearly [12, 13]. Intensity of clinical signs depends on PPR virus line, species, breed and immune status of an animal. That is why a final diagnosis should be confirmed by laboratory data.

Coupled with a direct detection of the viral genome by a real-time polymerase chain reaction (RT-PCR), laboratory diagnostics applies some serological tests such as virus neutralization test (NT) [14], diffuse precipitation test [15], counter-immunoelectro-osmophoresis, indirect immunofluorescence test [16, 17], direct and indirect enzyme-linked immunosorbent assay (ELISA) [18], and monoclonal antibody-based competitive ELISA [19-23] to reveal virus-specific antigens and antibodies. NT and ELISA are the most common methods to study serum samples. NT is the most laborious method, which is not suitable for an investigation of great number of samples because of required handling with cell cultures. Therefore, ELISA, an advanced sensitive method, is a technique of choice to conduct diagnostic and monitoring studies.

Initially, an inactive PPR virus (PPRV) purified from infected cell lysates was used in ELISA to detect virus-specific antibodies. However, since the antigen preparations included cell culture proteins, the method specificity was low. The technology related to design of recombinant proteins with affinity labels enables their purification by a chromatographic method on metal chelate sorbents, withdrawal of a living PPRV use and simultaneous increase in ELISA specificity by means of highly purified antigens. Advanced PPR serodiagnostic tools are developed on the basis of virus-specific recombinant proteins. Above all, we mean protein N [24-27] whose antigenic and immunogenic properties are better than in other Morbillivirus proteins [28, 29]. Another protein N advantage is that, as an antigen, it is the most conservative protein of the PPR virus [30]. It worth mentioning that immune responses to protein N generated by susceptible animals during vaccination/disease are not protective tools because of its intravirionic localization.

The paper presents a technology related to production of a recombinant full-size PPRV nucleocapsid protein (N) producer. It was demonstrated that purified recombinant protein N is suitable to detect antibodies to PPRV in a competitive ELISA. We studied a potential use of positive PPRV vaccine strain-immunized pig sera as controls of test systems intended for the disease serodiag-

nosis for the first time ever. It was found that, according to competitive ELISA, they are inferior to positive goat sera.

The purpose of our study was to obtain recombinant PPRV protein N (i.e., an antigen) and virus-specific pig antisera (i.e., antibody source) intended for the disease serodiagnosis by a competitive enzyme-linked immunosorbent assay.

Techniques. Large White domestic pigs (*Sus scrofa domesticus*) weighed 30 kg (n = 2, Animal Preparation Sector, Federal Research Virology and Microbiology Center), green monkey kidney cell line (VERO) and PPRV vaccine strain (45G37/35-K; collections of Federal Research Virology and Microbiology Center) were involved in the study.

PPRV accumulation and infectious activity were evaluated in VERO cell culture in Eagle's Minimum Essential Medium (PanECO, Russia) with 2.5% cattle fetal serum in polystyrene flasks and 96-well plates (Costar, France), respectively. During titration, the virus-containing material underwent a successive 10-fold dilution in quadruplicate. Infected and control VERO cell cultures were exposed at 37 °C (5% of CO_2). The maintenance medium was refreshed every 2-3 days. Results were recorded considering a cytopathogenic effect (CPE) for 10 days. The viral titer was calculated as per Körber's method modified by I.P. Ashmarin. The result was expressed as $TCID_{50}/cm^3$ [31].

A neutralization test involved a 45G37/35-K strain. Antisera were subjected to 2-fold serial dilutions in the cultivation medium. An 100 μl aliquot of the virus (titer 10^3 $TCID_{50}/cm^3$; 10^2 $TCID_{50}/cm^3$ per a well) and 100 μl of diluted antisera test samples (4 wells per a dilution) were mixed. Then, the cultivation medium was removed from 96-well plates containing VERO cell culture and virus-antiserum mixtures were added. Virus-free and PPRV-infected (10^2 $TCID_{50}/cm^3$) wells were used as controls. Plates were incubated at 37 °C for 2 h (5% of CO_2). Liquid phase was removed from wells and cultivation medium was added instead. Antibody titer in serum was calculated according to dilutions neutralized PPRV in 50% of wells.

pJET1.2 (Thermo Fisher Scientific, Inc, USA) and pET32a (Novagen, USA) plasmid vectors were used to clone PCR-products and to create expression constructs, respectively. Sequencing was carried out by an Applied Biosystems 3130 genetic analyzer (Applied Biosystems, Inc., USA). Potential application of a recombinant protein N in a competitive ELISA was evaluated using several components of ID Screen® PPR Competition Kit (IDvet, France) (hereinafter — Kit) intended for detection of antibodies to PPRV nucleoprotein in serum and plasma of sheep and goats.

Some components of ID Screen® PPR Competition Kit (IDvet, France) were used in a competitive ELISA.

Electrophoretic separation of polypeptides was performed in 10% polyacrylamide gel by Laemmli method [32]. Polypeptides were electrotransported from the gel to a nitrocellulose membrane (Sigma, USA) in a semi-dry buffer system by Kyhse-Andersen method [33]. Total protein content in test samples was assayed according to Lowry et al. [34] with a KFK-2 photocolorimeter (Granat PGK, Russia) at λ = 750 nm.

Results. PPRV (45G37/35-K strain) was accumulated in the setting of multiple infection (10^{-1} $TCID_{50}$/cl) and titrated according to TCID in VERO cell culture. The virus demonstrated infectious activity of $10^{4.5}$-$10^{6.5}$ $TCID_{50}/cm^3$ at the fourth passage.

Typically, Paramyxoviruses can reproduce inside the body of most of animal species. For example, the better part of ground carnivores (e.g., *Canidae*, *Mustelidae*, *Procyonidea*, *Ursidae*, *Hyaenidae*, *Ailuridae*, *Viverridae)* infected with canine distemper virus (CDV) die or demonstrate clinical signs of the dis-

ease. Despite cats (*Felidae*) and pigs (*Suidae*) are also susceptible to CDV infection, they do not show its clinical signs [35, 36]. Titers of virus-neutralizing antibodies to CDV found in antisera of experimentally infected pigs are comparable to those in dogs and ferrets who had the disease [37]. Also, PPRV can reproduce in pigs [38]. We studied potential production of PPR-specific pig antiserum intended for serodiagnosis (i.e., competitive ELISA) because pig immunoglobulins can react with conjugates on the basis of protein A.

To reduce possible antibody response to cell components and virus-containing suspension proteins dissolved in the cultivation medium from, virions were purified by a differential centrifugation. PPRV suspension (titer of 10^5 $TCID_{50}/cm^3$, 30 cm^3 volume) was centrifuged at 5,000 g for 40 min. Cell debris was removed. Supernatant was recentrifuged through a 20% (w/v) sucrose cushion at 45,000 g for 4.5 h. At the end, supernatant was decanted, and precipitate was resuspended in phosphate buffer (10 cm^3). Purified virions (titer of 10^5 $TCID_{50}/cm^3$) were administered by 2.5 cm^3 to both pigs intramuscularly and intranasally as a single dose. Clinical signs of the disease were not detected. Animals were exsanguinated in 28 days after infection. In terms of NT, antibody titers in antiserum samples of both PPR-infected pigs were 1:64-1:128. The values were comparable to antibody titers in serum samples of PPR-vaccinated goats and sheep (39).

Nucleotide sequences of PPRV protein N gene were obtained from the GenBank database (NCBI, https://www.ncbi.nlm.nih.gov/). To design primers, Nigeria 75/1 (X74443.2) strain nucleotide sequence was used as a reference one. Primers flanking full-size copies of the viral protein N gene were designed with Oligo 7 and SnapGene v.4.1 software. Primer pair containing SalI and EcoRV restriction sites on 5′-ends was designed. Forward-primer (F-PPRV_N_atc) sequence is 5′-ATCTCGGGTT-CAGGAGGGGCCATCCGGGGG-3′, Revers-primer (R-PPRV_N_SalI) sequence is 5′-GGGTGCGTCGACTTAGCTGA-GGAGATCCTTGTCGTTGTAGATCTG-3′. Primers were designed to amplify a fragment of 1530 bp in length (positions nos. 4-1518 in a protein N gene). Specific hexameric sequences of SalI and EcoRV endonuclease restriction sites were built in oligonucleotide primers. RNA was extracted using TRIzol (Invitrogen Corp., USA). To synthesize the first chain of cDNA with a single-strand RNA matrix, M-MLV reverse transcriptase was used (α-Ferment, Russia). Prior to RT-PCR, annealing of primers was carried out at 70 °C for 5 min.

Protein N gene nucleotide sequence was synthesized by a polymerase chain reaction (PCR) with Pfu-polymerase (α-Ferment, Russia) with a Tertsik amplifier (DNK-Tekhnologii, Russia) as per the manufacturer's guidelines. Previously obtained cDNA was used as a matrix. The program included several stages such as hot start at 95 °C (pre-denaturation); 3 min at 95 °C (denaturation), 30 s at 95 °C (annealing of primers), 185 s at 68 °C (elongation) (25 cycles); 5 min at 72 °C (final elongation). To purify PCR products from agarose gel, GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Inc., USA) was used as per the manufacturer's instructions. The PCR product purified from agarose gel was cloned on the blunt end basis in pJET1.2 vector using T4 DNA ligase (Thermo Fisher Scientific Inc., USA).

Escherichia coli (Rosetta 2 pLysS strain) competent cells were transformed with obtained constructs as per the standard procedure using heat shock and further growing in a solid medium containing a selective antibiotic (i.e., ampicillin). PCR-positive plasmid clone sequencing demonstrated integrity of the recombinant gene open reading frame. The next stage represented EcoRV and XhoI restriction site-based recloning of a protein N gene from pJET1.2/N/4 plasmid to a pET32a expressing plasmid vector (Fig. 1). Presence of thioredoxin in the N-

end of a pET32a vector multicloning site increased solubility of recombinant protein. In turn, two polyhistidine areas enabled nickel sorbent-based purification of the expressed recombinant protein. Single positive clones were screened in PCR. Sequence of positive clones confirmed integrity of protein N gene (clones nos. 8 and 10) open reading frame. Further, plasmid pET32a/N/10 DNA was applied.

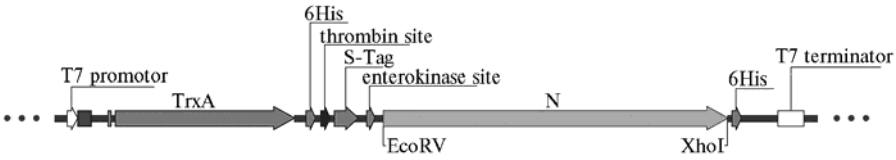

Fig. 1. Scheme of the open reading frame related to the recombinant gene with a cloned sequence of the fragment encoding PPRV protein N: T7 promotor — T7 bacteriophage RNA polymerase promotor; TrxA — thioredoxin gene A; 6His — polyhistidine encoding sequence; thrombin site — nucleotide sequence encoding thrombin cleavage site; S-Tag — RNase A N-end oligopeptide encoding sequence; enterokinase site — nucleotide sequence encoding enterokinase cleavage site; EcoRV and XhoI — restriction sites; T7 terminator — T7 bacteriophage RNA polymerase transcription terminator.

PET32a/N/10 plasmid was transformed into *E. coli*. Then, single clones were induced in different conditions (26, 30 and 37 °C in liquid SOB medium containing ampicillin, the selective antibiotic). Samples were evaluated in 4, 8 and 16 hours, respectively. It was found that the best possible induction was performed at 26 °C for 16 hours with IPTG inducer (isopropyl-β-D-1-thiogalactopyranoside) at 0.4 mM final concentration.

The estimated molecular weight of recombinant protein N fused to thioredoxin A and two 6His was about 70 kDa. SDS-PAGE polypeptide screening revealed clones of *E. coli* pET32a/N/10 nos. 3, 5, 6, 8 expressing a major polypeptide of 70 kDa absent in pET32a plasmid-transformed *E. coli* cell lysate without an insertion.

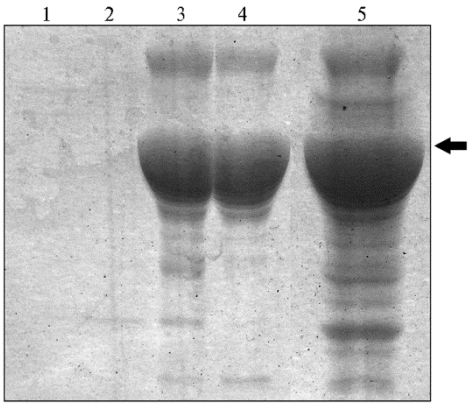

Fig. 2. Electrophoregram of fractions after chromatographic purification of recombinant PPRV protein N in the setting of a discrete increase in imidazole concentration: 1 — 50 mM, 2 — 100 mM, 3 — 250 mM, 4 — 500 mM, 5 — initial lysate of *Escherichia coli* induced cell. Position of purified recombinant protein N fraction is arrowed.

Recombinant protein N was purified by Metal Chelate Affinity Chromatography in native conditions. Ni Sepharose™ 6 Fast Flow (GE Healthcare Bio-Sciences Corp., USA) was used as a sorbent. Then, elution with aqueous imidazole solutions (50, 100, 250 and 500 mM) was performed. Eluates were screened by SDS-PA-GE method (Fig. 2).

After dialysis final concentration of recombinant protein N in eluate fraction with 500 mM imidazole was adjusted to 2 mg/cm^3. Its antigenic activity was confirmed by immunoblotting assay with antibodies of PPR-positive pig antisera. A major virus-specific polypeptide with molecular weight of 70 kDa was detected on the blottogram. Activity of recombinant PPRV protein N and PPRV pig antisera was calculated using several components of ID Screen® PPR Competition Kit (IDvet, France).

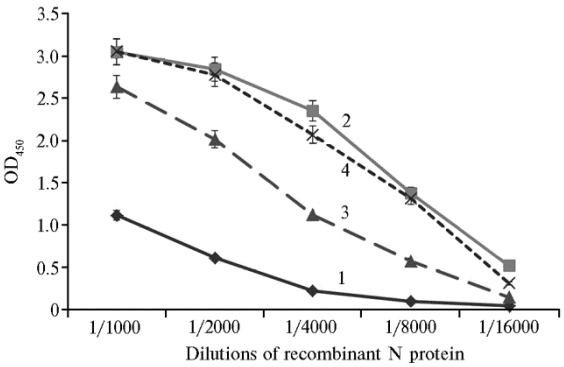

Fig. 3. Competitive enzyme-linked immunosorbent assay of recombinant PPRV protein N immobilized on strip wells (Eppendorf, Germany): 1 and 2 — positive and negative sera (ID Screen® PPR Competition Kit, IDvet, France); 3 and 4 — pig sera (positive and negative ones collected in an experimentally PPRV-immunized animal and an intact animals, respectively).

Instead of ID Screen® PPR Competition Kit strips (IDvet, France), free plastic strips (Eppendorf, Germany) were used in whose wells obtained recombinant protein N (by 50 μl in increasing 2-fold dilutions, 1:1000 - 1:16000) was immobilized. An aliquots of 100 μl of positive/negative sera form the kit or positive/negative pig sera were added to wells and incubated in a shaker at 37 °C for 1 h. The next stages were performed as per the kit manufacturer's guidelines.

The optimal concentration of recombinant protein N was 0.25 μg/well (1:8000 dilution). Considering the concentration, the ratio of OD_{450} values for negative and positive sera included into the kit was 11.52; in turn, the ratio for negative and positive pig sera was 2.22 (Fig. 3). The similar result (0.20 μg/well) was obtained while testing 50 kDa recombinant protein produced in *E. coli* BL21(DE3) pLysS cells transformed with protein N gene 5′-end 838 bp sequence [23].

In using sensibilized strips from the manufacturer's kit, the ratio of OD_{450} values for negative and positive sera was 16.03. Considering objective parameters, obtainer recombinant protein N is suitable for PPR serodiagnosis by competitive ELISA method.

It should be emphasized that absorbance values of negative pig serum reaction were comparable to those obtained with a negative serum included into the manufacturer's kit. Along with this, 5-fold difference was seen between the values resulted from tests involved positive serum included into the manufacturer's kit and positive antiserum collected in immunized pigs when recombinant protein dilution was 1:8000. It is likely to indicate lower concentration of antibodies to protein N in sera of PPRV-immunized pigs than in positive serum included into the kit and collected in recovered goats.

Recombinant PPRV protein N was used as an antigen in indirect/competitive ELISA to provide serodiagnosis for several studies. Previously, a great amount of the protein was extracted from cells of insects infected with a recombinant baculovirus containing PPRV nucleoprotein gene [25]. Competitive ELISA demonstrated activity of shorter and full-size PPRV protein N producer in *E. coli*. The best concentration of protein N to reveal antiviral antibodies was 0.16 μg/well [40]. We note that *E. coli* prokaryotic system is the most common in proteins that do not require post-translational modification because of simple expression and potential production of a great protein amount. Moreover, as compared with other heterologous systems, bacterial production of recombinant antigens is quite simpler and more cost-effective.

Thus, we obtained *Escherichia coli* pET32a/N/10 clone suitable for application as a producer of recombinant protein N of peste des petits ruminants (PPR) virus. A chimeric recombinant protein N contains a thioredoxin fragment to increase solubility in water and two polyhistidine regions to provide an efficient nickel sorbent-based purification. Underwent electrophoretic and immunochemical purification, pure recombinant protein N is suitable for PPR serodiag-

nosis by a competitive enzyme-linked immunosorbent assay (ELISA). Active in the neutralization test, specific anti-PPR pig antisera used in a competitive ELISA were less efficient than a positive goat antiserum (ID Screen® PPR Competition Kit, IDvet, France). At the next stage, we are going to obtain other active components of the developed test system suitable for diagnosis of peste des petits ruminants by a competitive ELISA.

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ONE-RUN REAL TIME PCR ASSAYS FOR THE DETECTION OF CAPRIPOXVIRUSES, FIELD ISOLATES AND VACCINE STRAINS OF LUMPY SKIN DISEASE VIRUS

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Abstract

The cattle and sheep industry is economically important for sustainable growth. However, the increasing demand for livestock products drives animal population growth and risks for infection diseases. Lumpy skin disease (LSD) has recently expanded its historical range northward reaching countries that were never affected before. Prior to 2015 the territory of the Russian Federation was free of lumpy skin disease, whereas by 2017 Turkey, Serbia, Greece, Azerbaijan have reported incursions of this virus. Not only lumpy skin disease but also sheep pox has increased in incidence. Given this scenario, timely detection of these pathogens is key towards successful control and eradication. Moreover, diagnostic tools should detect both LSDV genome in the face of the use of live vaccine LSD virus strains and distinguish between the two. In this paper we report the development of a set of one-run real-time PCR assays to detect and differentiate between *Capripoxvirus* genome, field and vaccine LSD virus genomes. The assay for field LSD virus targets the 27 bp deletion in ORF126, the assay for vaccine LSD virus targets genetic signatures unique to Neethling vaccine strains, and the capripoxvirus assay targets the conserved *P32* gene. The assays proved highly sensitive and specific. The set of PCRs was validated against a panel of 596 samples collected in the field, including whole blood, serum, skin lesions, nasal and ocular discharge, milk, lymph nodes, lungs, trachea, spleen and aborted calves. Using the assays reported here some samples obtained as part of national surveillance for LSD virus from animals exhibiting clinical signs consistent with LSDV turned out to be positive for vaccine LSD virus genome in 2017. This vaccine strain is highly likely to have derived from commercial live-attenuated vaccines against LSD virus. The way of introduction of a vaccine LSD virus strain into Russian cattle remains to be investigated.

Keywords: lumpy skin disease, vaccine, diagnostics, real-time PCR, genome, virus

Bovine lumpy skin disease (LSD) (nodular dermatitis) is a transborder bovine infection manifested by fever and skin nodes (tubercles); generalized infection demonstrates lymphadenitis, as well as conjunctival disorders and respiratory/digestive mucosa conditions [1-4]. LSD causative agent is a DNA-containing enveloped virus (family *Poxviridae*, genus *Capripoxvirus*) involving related sheep and goat pox causative agents [5]. The viral genome represents a double-stranded DNA of 151 kb in length [6].

Cattle and buffaloes are susceptible to the disease [7]. Among them, dairy cows are the most vulnerable. Along with this, morbidity can be 3-80% [3, 8-10] indicating contribution of other potential non-investigated factors that influence on clinical sign severity. According to reported data, blood-sucking insect [11, 12] and tick [13, 14] bites are considered the most common route of infection. Nevertheless, a well-defined vector is not determined yet. All the LSD

outbreaks associated with the infection clinical signs must be notified in the International Epizootic Bureau (IEB, Office International des Epizooties, OIE, France). Nowadays, according to IEB, Turkey, Serbia, Greece, Albania, Bulgaria and other countries have reported incursions of the disease [15-19]. For the first time, Russia reported the infection in 2015 [20]. Then, 16 federal subjects (mainly, the Central Federal District) reported 313 outbreaks in 2016 [21]. In 2017-2018, the Volga Federal District started to report several incursions near the border of the Russian Federation [22].

Living homologous Neethling (i.e., an attenuated vaccine strain)-based LSD vaccines are in active use in near-border countries (e.g., the Republic of Kazakhstan, EU). Therefore, a complex of methods is required to detect and to differentiate causative agents of capripoxvirus infections including identification of Neethling vaccine strain that can induce clinical presentations of the disease [23, 24]. Apart from LSD, sheep pox reported in the Far East sometimes also jeopardizes the Russian Federation. The North Caucasus District and the Central Federal District [22] reported several incursions of the disease in 2015 and 2016-2018, respectively. Considering unexampled LSD virus spread (including subclinical recovery [25, 26], we need highly sensitive diagnostic methods that enable monitoring of latent infected susceptible animals to detect the causative agent's genome rapidly, to combat the infection and to prevent its extension.

The paper proposes sensitive and specific RT-PCR test systems for a single-mode sample testing characterized by a similar temperature profile for capripoxvirus genome (PCR-CAPR), lumpy skin disease virus field isolates (PCR-LSDV) and vaccine strain (PCR-NEE) for the first time ever.

Our purpose was to develop a complex of real-time PCR methods to detect genomes and to differentiate all the *capripoxviruses*, LSDV field isolates and Neethling vaccine strain in various biomaterials.

Techniques. Total DNA was extracted from 100 μl of the biomaterial suspension with a DNA Nucleic Acid Extraction Mini Kit (Qiagen, Germany) as per the manufacturer's instructions.

To select amplification sites and probe annealing regions in PCR, we evaluated several whole-genome sequences of LSDV field isolates (KX683219, KSGP 0240, KY829023 Evros/GR/15, KY702007 SER-BIA/Bujanovac/2016, AF409137 Neethling Warmbaths LW, AF325528 Neethling 2490), vaccine strains (KX764643 SIS-Lumpyvax vaccine, KX764644 Neethling-Herbivac vaccine, AF409138 Neethling vaccine LW 1959, KX764645 Neethling-LSD vaccine-OBP), as well as those related to sheep and goat pox (KX576657 Gorgan, KC951854 FZ, AY077836 G20-LKV, AY077835.1 Pellor, AY077833 Sheeppox virus A, AY077832 Sheeppox virus 10700-99 strain TU-V02127, MG000156 Sheeppox virus strain Jaipur, AY077834 Sheeppox virus NISKHI) available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

Primers and probes developed with Primer3 engine software (http://bioinfo.ut.ee/primer3-0.4.0/) were synthesized by Syntol (Moscow). FAM dye (5′-probe end) and BHQ1 (3′-probe end) were used as a source and a quencher of fluorescence, respectively. Primers and the probe used to detect viral field isolates in PCR-LSDV system we mentioned in the previous work [27].

Real-time PCR (RT-PCR, Rotor Gene Q 6 plex amplifier, Qiagen, Germany) was performed as per the appropriate procedure (activation at 95 °C for 10 min; 40 cycles: 95 °C 15 s, 60 °C 1 min). GoTaq® MDx Hot Start Polymerase reagent kit (Promega Corp., USA) was applied. Reagent mixture (25 μl) included 5 μl of 10× PCR buffer, 3 μl of 25 mM $MgCl_2$, 0.5 μl of 10 nmol dNTP, direct and reverse primers, 12.5 pmol each, and 7.5 pmol of the probe.

The results were interpreted depending on whether a fluorescence curve intercepts a threshold line or not. This corresponds to presence/absence of Ct threshold cycle value. If Ct ≤ 40, a sample was considered to be positive (i.e., a viral DNA was detected).

To assess RT-PCR test system specificity, it was tested with genetic material of homologous and heterologous viruses. Specificity tests were performed analyzing each viral DNA individually and in presence of several viral DNAs. To test and to improve developed methods, we used DNAs of reference strains of heterologous viruses (Microorganism Strain Collection, Federal State Budgetary Institution Federal Animal Health Care Center), as well as DNAs of homologous and heterologous viral isolates obtained from various Russian regions. PCR-LSDV specificity test we mentioned in the previous work [27].

Analytical sensitivity of the test system intended for detection of the vaccine strain genome (PCR-NEE) was evaluated by a series of 10-fold dilutions ($1:10^1$-$1:10^6$) of Neethling LSD vaccine virus genome DNA. Initial titer was 5.21 lg $TCID_{50}$/ml. To analyze the test system designed for capripoxvirus genome detection (PCR-CAPR), sheep pox virus (Afghan strain) was applied; titer was 6.17 lg $TCID_{50}$/ml. In view of statistical verification of the findings and linear regression plotting, 10-fold genome DNA dilution test was conducted in triplicate. Amplification efficiency was calculated as per the formula:

$$E = (10^{slope} - 1) \times 100\ \%,$$

where 10^{slope} is slope coefficient.

Using Ct values, reproducibility (*Cv*, %) of each dilution series was calculated considering standard deviation (±SD). To assess threshold cycle variability, each sample was tested for 3 times in pentaplicate (1 launch — 5 replicates) for 3 days. Mean Ct values, standard deviations (±SD) and coefficient of variation (*Cv*, %) were calculated for each launch (5 replicates) and all the launches (15 replicates).

Diagnostic sensitivity (DS) and diagnostic specificity (DSp) of test systems were tested on samples of blood and nasal discharge collected in naturally infected animals. The results were calculated as per formulae as follows:

$$DS = TP/(TP + FN),$$

where TP is true-positive results, FN is false-negative results;

$$DSp = TN/(TN + FP),$$

where TN is true-negative results, FP is false-positive results.

We analyzed 596 samples of biological materials, i.e. stabilized whole blood, blood serum, skin scrapings (nodules), nasal and ocular discharge, milk, lymph nodes, lungs, trachea, spleen and aborted calves, from animals with clinical signs of the disease. Analyses were hosted by the Disease Reference Laboratory (Federal Animal Health Care Center, 2016-2017).

To assess Ct values, Passing-Bablok Regression was used and Bland-Altman plots were constructed. Lin's concordance correlation coefficient was used to examine agreement between PCR assays [28].

Results. See a brief description of used primers and probes in the Table 1. See description of heterologous and homologous viruses (reference strains, Microorganism Strain Collection, Federal Animal Health Care Center), as well as homologous and heterologous viral isolates obtained from various Russian regions used to assess specificity of RT-PCR test systems in the Table 2.

In virtue of sequence alignment, we identified several sites, which were the most conserved in field isolates only, in vaccine strains only and in all the capripoxviruses. To amplify field isolate genome region, we selected a LSDV126 reading frame for *EEV* gene of nodular dermatitis virus where 27 bp region is deleted in other *Capripoxviridae* and Neethling vaccine viruses, whereas the in-

sertion is observed in field isolates (Fig. 1, A). To amplify a vaccine strain genome fragment, we selected LSDV008 region where unique strain-specific substitutions are available (Fig. 1, B). To amplify the capripoxvirus genome, we selected *P32* gene, which is conserved for all the *Capripoxviridae* (Fig. 1, C).

1. Primers and probes used to detect *capripoxviruses* and to differentiate a genome of the LSDV vaccine strain and field isolates in RT-PCR

Agent (test system)	Nucleotide sequence (5′→3′)	Primers, probes	Gene	Amplicon, bp	Reference
BLSD vaccine virus (NEE)	TGTTTCCATTCTCCACTGCT TACTTACTAAAAAATGGGCGCA TCGCTGACATCGTTAGTCCACTC	fnee3 rnee3 Probe	*LSDV008*	185	The study
Capripoxviruses (CAPR)	ATGAAACCAATGGATGGGATA CGAAATGAAAAACGGTATATGGA ATGAGCCATCCATTTTCCAA	Capr_f Capr_r Probe	*P32*	92	The study
BLSD field isolate (LSDV)	AGAAAATGGATGTACCACAAATACAG TTGTTACAACTCAAATCGTTAGGTG ACCACCTAATGATAGTGTTTATGATTTACC	f2 r33 lsdv probe	*EEV*	96	[27]

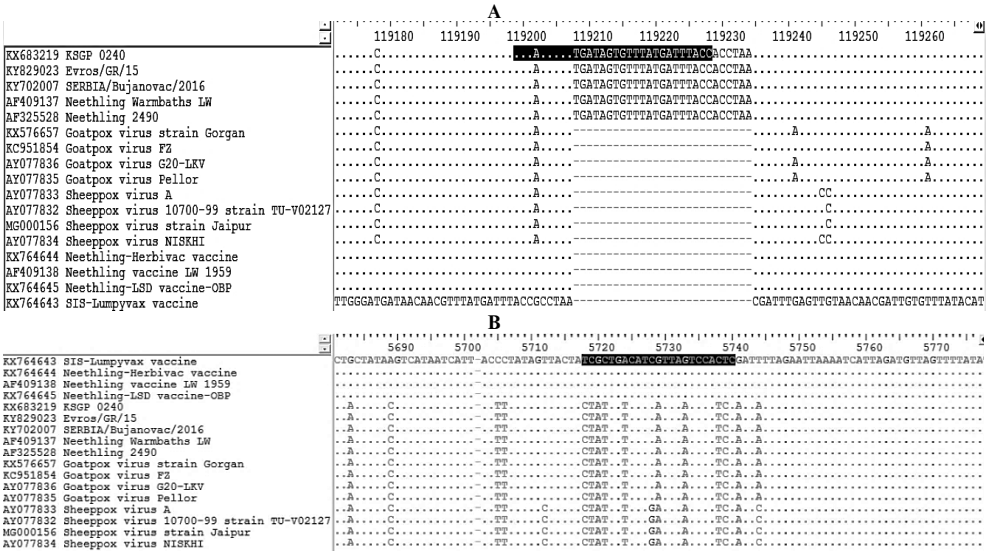
N o t e. BLSD is bovine lumpy skin disease.

2. Studied viral strains and isolates

Virus	Strain/isolate	Origin/collection	PCR	
			CAPR	NEE
BLSD virus	CNDV/Dagestan/2015 (diagnostic strain)	Russia/Federal State Budgetary Institution Federal Animal Health Care Center	+	–
BLSD virus	CNDV E-95 (D)	Africa/Federal State Budgetary Institution Federal Animal Health Care Center	+	–
Attenuated LSD vaccine strain	Neethling	RSA VRI Onderstepoort/Federal State Budgetary Institution Federal Animal Health Care Center	+	+
Sheep pox virus	Afghan strain	Afghanistan/Federal State Budgetary Institution Federal Animal Health Care Center	+	–
Sheep pox virus field isolate		Russia (Yaroslavl' Region)/NA	+	–
Goat pox virus	Primorye 2003	Russia/Federal State Budgetary Institution Federal Animal Health Care Center	+	–
Sheep ecthyma virus field isolate		Russia/NA	–	–

N o t e. BLSD is bovine lumpy skin disease, “+” and “–” are positive and negative RT-PCR.

Evaluating the method specificity, we studied material containing DNA of heterologous viruses (PPR, vesicular stomatitis, sheep ecthyma and cow pox). Finally, we did not reveal any false-positive results of the diagnostic system testing using individual DNA of each virus or mixture of several viral DNAs.



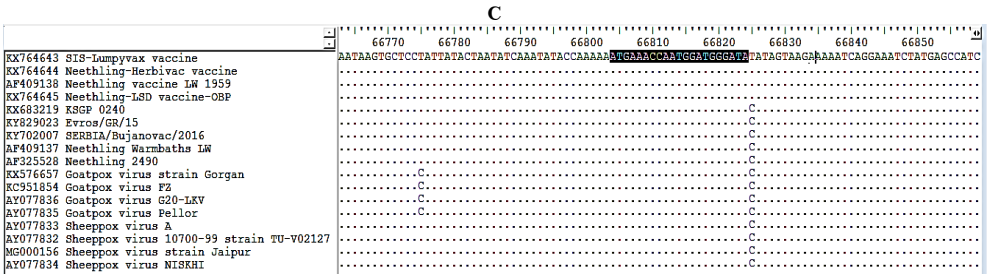


Fig. 1. Probe annealing area to detect bovine lumpy skin disease field isolates (A)**, vaccine strains of the virus** (B) **and capripoxviruses** (C) **in RT-PCR** (also see the journal website: http://www.agrobiology.ru).

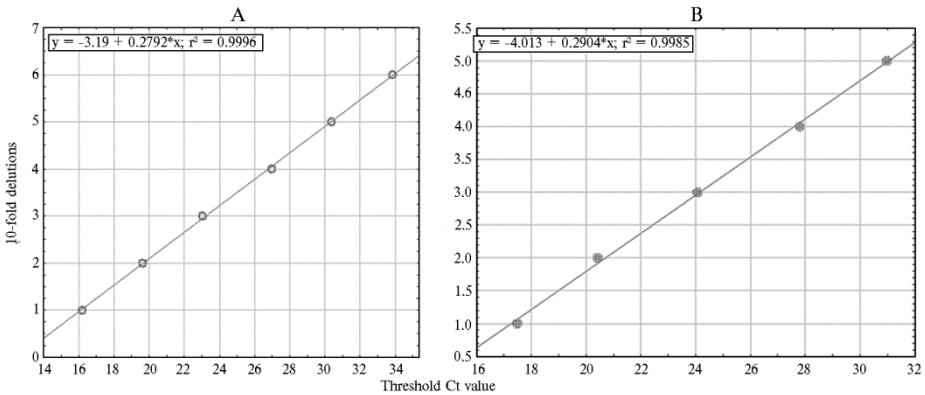


Fig. 2. Linear regressions of Ct values based on RT-PCR testing of 10-fold dilutions of capripoxvirus DNA (A) **and lumpy skin disease vaccine strain** (B) **confirm linearity of the results.**

To evaluate sensitivity of the PCR-CAPR test system, we used DNA of sheep pox virus (Afghan strain) whose infectious activity titer is 6.17 lg $TCID_{50}$/ml. The PCR-CAPR test system (Fig. 2, A) detected viral DNA at 0.17 lg $TCID_{50}$/ml. To assess amplification efficiency, we conducted three replicate tests and obtained Ct values applicable to the efficiency calculation. In virtue of the slope coefficient value resulted from linearity regression (Fig. 2, A), amplification efficiency is E = 90.2%. Reproducibility test (6 successive 10-fold dilutions) demonstrated a standard deviation (SD) of 0.12-0.32.

PCR-NEE test system sensitivity was assessed using DNA of Neethling LSDV vaccine strain whose infectious activity titer is 5.21 lg $TCID_{50}$/ml. The test system detected viral DNA at 0.21 lg $TCID_{50}$/ml. Amplification efficiency (Fig. 2, B) was E = 95.16%. The PCR-NEE test system (5 successive 10-fold dilutions) demonstrated a standard deviation (SD) of 0.03-0.60.

Primary quantitative characteristics of developed test systems are summarized in the Table 3.

3. Efficiency, standard deviation (SD) **and determination coefficient** (r^2) **of RT-PCR test systems to detect and to differentiate bovine lumpy skin disease field isolates, the virus vaccine strains and capripoxviruses**

Test system	Efficiency, %	SD (min-max)	r^2
PCR-CAPR	90.20	0.12-0.32	0.999
PCR-LSDV	98.60	0.11-0.33	0.990
PCR-NEE	95.16	0.03-0.60	0.998

N o t e. Test systems are described in the Techniques section. Reported data of prior PCR-LSDV study are presented [27].

See Ct value variations associated with parameter measurements in the Table 4.

See PCR-CAPR and PCR-LSDV DS and DSp comparison results on

the Figure 3. As per the reported data, there were no any significant differences between Ct values of PCR-CAPR and PCR-LSDV ($p > 0.05$). In virtue of Ct values of two test systems, Lin's concordance correlation coefficient between PCR CAPR and PCR-LSDV tests was 91.3%.

4. Ct variability associated with 1-3 launches of a RT-PCR test system

Test system, launch	Mean Ct value	±SD	*Cv*, %
PCR-CAPR (n = 5):			
1st	28.80	0.23	0.79
2nd	29.05	0.54	1.85
3d	29.15	0.32	1.09
PCR-LSDV (n = 5):			
1st	30.01	0.43	1.43
2nd	30.76	0.41	1.33
3d	29.93	0.16	0.53
PCR-NEE (n = 5):			
1st	29.56	0.37	1.20
2nd	29.53	0.36	1.20
3d	30.15	0.25	0.80
Three launches, mean (n = 15):			
PCR-CAPR	29.00	0.39	1.34
PCR-LSDV	30.27	0.52	1.71
PCR-NEE	29.76	0.50	1.60

N o t e. Test systems are described in the Techniques section. Each launch was performed in pentaplicate.

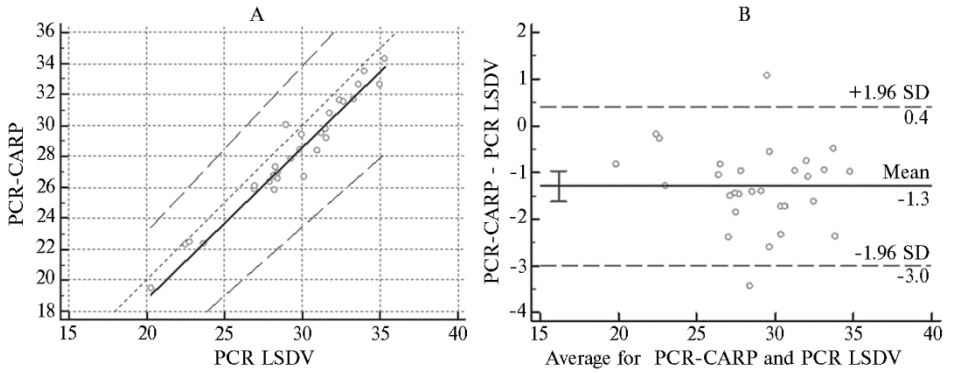


Fig. 3. Passing-Bablok regression (A) **and Bland-Altman scatter diagram** (B) **for Ct values (PCR-CAPR and PCR-LSDV test systems) at 95% CI.** Test systems are described in the Techniques section.

5. Detection of LSD virus genome in field isolates from cattle biomaterial (2015-2017)

Biomaterial	Samples		
	total	positive	
		n	%
Nodules	95	74	78
Whole blood	235	31	13
Blood serum	117	17	14.5
Nasal discharge	104	26	25

We applied three RT-PCR test systems to study 596 bovine biological material samples collected in 2015-2017. LSD virus genome was detected in 155 samples (26.0%) (Table 5). Bovine LSD virus was the most common in a nasal discharge (25.0%), serum (14.5%) and whole blood (13.0%). As a result of pathological material testing, LSDV genome was detected in 78% of affected skin (nodule) samples. LSDV genome was not mentioned in trachea, spleen and aborted calves. Additionally, 3 milk samples (2 LSDV-positive ones) from cattle with clinical signs of LSD, 5 lymph node samples (2 LSDV-positive ones) and 4 lung samples (3 LSDV-positive ones) were tested. PCR-CAPR and PCR-LSDV demonstrated 100% correspondent positive/negative results.

LSD laboratory diagnosis is of importance to confirm a suggested diagnosis and, in turn, to perform timely actions to prevent the viral spread. Also, we note that LSDV biological and epizootological properties are still understudied,

and more comprehensive understanding is required. Lumpy skin disease and sheep pox viruses are urgent threat to global livestock production (including the Russian Federation) [21]. Since LSD was historically restricted by the Africa and the Middle East, Russia was considered to be a LSD-free country before 2015 [25]. As for sheep pox, it was typically reported by the Far East and the North Caucasus. Vaccination is the only way to combat capripoxviruses. In terms of LSD, this is a vaccination with heterologous and homologous (attenuated) vaccines [25]. Sheep pox virus-based heterologous vaccine is safe for cattle whereas living attenuated vaccines, e.g., Lumpyvax (RSA) and equivalent, can induce clinical signs of the disease [26]. That is why the development of differential molecular diagnostic methods is a hot issue.

The paper presents a developed complex of RT-PCR test systems providing simultaneous detection of capripoxvirus genomes, LSDV field isolates and Neethling vaccine strain for the first time ever. The advantage of the approach is that reagent concentrations and temperature profile are similar in all the tests. These test systems are validated successfully with a great number of samples collected in experimentally infected animals or during the disease outbreak. The results are confirmed by sequencing and virus extraction in a cell culture (data are not available). All the samples collected during notified LSD outbreaks in the Russian Federation since 2015 were tested in PCR-CAPR and PCR-LSDV test systems. As a result, 100% similar findings were reported.

PCR-CAPR and PCR-NEE test systems were developed as a part of the trial, and PCR-LSDV test system dealing with field isolates was described [27]. To provide high specificity, all the amplification loci and probe annealing sites were selected considering conservation (all the isolates whose data are available in GenBank) and unicity (see Fig. 1). Unique genetic signatures provided detection of mentioned viruses by each developed RT-PCR test system.

During a launch amplification efficiency of all the test systems is > 90% in a series of 5 10-fold dilutions of the viral material; sensitivity is 0.3 lg $TCID_{50}/cm^3$ (see the Table 3). Moreover, highly sensitive and specific DNA panel testing of homologous/heterologous viruses make the complex irreplaceable to establish a diagnosis while dealing with field material samples. Comparison between PCR-CAPR and PCR-LSDV demonstrated no significant differences between Ct values while testing the same samples ($p > 0.05$); mean difference between the values was 1.3 cycles (Lin's concordance correlation coefficient is 91.3%) (see the Table 4). Unfortunately, it is impossible to compare PCR-CAPR and PCR-NEE test systems because of too small sampling of biomaterial containing vaccine LSDV genome.

Several data on methods to detect genomes of capripoxviruses, field or vaccine LSDV strains are reported. Although the common Ireland and Binepal's method [29] based on a conventional PCR was used previously, it is non-specific for a given virus detecting all the capripoxviruses [27]. At the same time, a standard PCR associated with a cross-contamination risk is less sensitive. As for R-T PCR types, there are several reported papers dealing with duplex studies of capripoxvirus genome [30], field isolates [31], vaccine and field strains (Duplex PCR is a simultaneous detection of two target genes) [32]. It is significant that our PCR-LSDV and PCR-NEE test systems were more efficient (95.16% and 98.60%, respectively) than mentioned [32] duplex PCR (91.3% and 90.7%, respectively).

To assess specificity, we used national isolates, whereas our foreign colleagues evaluated test specificity in foreign strains. To consider a method to be an all-purpose one, a cross-validation must be conducted involving all the strains currently detected worldwide.

Potential differentiation of a field isolate from a vaccine strain in the same tube is both an advantage and disadvantage of the method proposed by Agianniotaki et al. [32]. As distinct from a duplex testing where simultaneous presence of both viruses decreases sensitivity of the response, our test systems can function independently in different tubes. The feature of a duplex PCR is very critical in the beginning of a living vaccine immunization in areas of active spread of field viral strains.

The developed complex of PCR test systems was tested on 596 biomaterial samples (i.e., various tissues and organ samples) collected in naturally LSD-infected cattle in the Russian Federation in 2015-2017 [21] (see the Table 5). We emphasize that correlation between test results in PCR-LSDV and PCR-CAPR is 100%. So, this confirms reliability of these diagnostic methods. It must be noted that suspected LSD-associated sampling should consider clinically healthy animals because they can be latent virus carriers without evident symptoms [24]. Due to the fact that bovine LSD epizootic situation is observed mainly intravitally, this has a special practical importance.

LSDV genome is the most common in nodules (78% samples). The fact complies with other study findings and confirms an evident viral tropism to skin epithelium [33]. Further, it was shown that PCR can detect LSDV genome in blood after a nodule appearance [33]. In terms of our study, 13-25% of other biomaterial samples demonstrated LSDV genome. Although the viral genetic material was detected in lungs, lymph nodes and milk, a precise statistical processing is still impossible due to restricted number of samples. It's important to stress that samples were collected at the onset of clinical signs that can influence on efficiency of LSDV detection. For example, absence of the viral genome in most of discharge samples, serum and whole blood may be associated with insufficient accumulation of the virus and its concentration is lower than a test system sensitivity limit during excretion in biological fluids. Moreover, Babiuk et al. [33] found that after experimental infection mucosal excretion of the virus can be observed after a nodule appearance. Moreover, the low concentrated viral DNA is detected in discharge within a short period (i.e., several days); transient (9 days) viremia is associated with intermittent presence of the virus during the test [33]. These properties may cause weak transmission of the virus between animals without flying transmitters.

It is important to notice that the PCR-NEE test system to detect the vaccine strain validated with the same volume of material revealed several cases of potential illegal use of Neethling-based LSDV-vaccine in number of Russian regions (data are not shown) despite Neethling genome was firstly found in cows with clinical signs of LSD in the Bashkortostan [34]. Although living attenuated LSDV vaccines are banned in the Russian Federation, they are approved in other countries of the Customs Union (e.g., the Republic of Kazakhstan) that, in turn, can lead to LSDV spreading in near-border regions [34]. Sequencing showed 100% homology of the appropriate *RPO30* fragment (data are not available) in the detected vaccine virus and vaccine strains used in commercial attenuated living vaccines. Agreement of PCR-CAPR and PCR-NEE results was 100%.

All the results obtained with proposed test systems are confirmed by virus extraction in a susceptible cell culture (data are not available) indicating reliability of R-T PCR methods used to differentiate the vaccine strain and the field isolate. High-degree certification and validation of proposed test systems was confirmed by the Russian Federation patent application. This is also an evidence of the development novelty [35, 36].

Thus, we developed reliable RT-PCR test systems to perform a single-

mode testing for genome of capripoxviruses, lumpy skin disease virus field isolates and LSDV vaccine strain. Methods demonstrated high specificity and sensitivity of panel testing of biomaterial samples collected in naturally infected animals.

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TEST-SYSTEM FOR DETECTION OF PESTE DES PETITS RUMINANTS VIRUS GENOME BY REVERSE TRANSCRIPTION REAL-TIME PCR

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Abstract

Peste des petits ruminants (PPR) is a highly contagious transboundary infection disease of small ruminants, characterized by fever, anorexia, ocular and nasal discharge, erosions and ulcers in digestive mucosa, diarrhoea and marked leucopoenia with immunosuppression. Because of the complexity of the PPR epizootic situation in neighboring countries (Tajikistan, China, Mongolia, Kazakhstan, Afghanistan) the risk of occurrence of this disease on the territories of Siberian, Ural, Far Eastern, North Caucasian and Southern Federal Districts of Russian Federation is very high. So, the development of molecular-genetic methods for the diagnosis of PPR and methods for stabilizing of biological samples represents scientific relevance. This article presents data on the development of a test system for detecting the PPR virus genome by reverse transcription Real-Time PCR. This technique is based on the amplification of a fragment (148 bp) of hemagglutinin gene of PPR virus using the original oligonucleotide primers and fluorescent-labeled hybridization probe. Analytical specificity of the developed test system was evaluated by testing the strains of PPR virus, rinderpest virus, bluetongue virus, as well as biological samples from clinically healthy animals and intact cell cultures. Positive results were obtained only with samples containing PPR virus (strains Epizooticheskiy, Nigeria 75/1 and 45G37/35-K). The analytical sensitivity of the developed test-system, determined using ten-fold serial dilutions of cultural virus-containing material, is 0.83±0.22 lg $TCID_{50}$/ml. To create a positive amplification control, a fragment of hemagglutinin gene synthesized usign Real-Time PCR was cloned in plasmid pTZ57 R/T in *Escherichia coli*. It was established that the plasmid DNA concentration for amplification linearity ranges from 2.4×10^7 to 24 molecules/μl. To assess the practical suitability of the developed test system for the diagnosis of PPR, the blood samples from sheep experimentally infected with PPR virus (strain Epizooticheskiy) were tested. As a result, the PPR virus genome was detected in blood samples from day 5 to day 12 after infection. Since transportation of biological samples over long distances may require during survey, we have developed "dry" blood method to collect and store blood samples. It has been shown that "dry" blood drops are stable at room temperature for a month and can be used in Real-Time PCR testing.

Keywords: peste des petits ruminants, virus, reverse transcription, PCR, cell culture, recombinant plasmid, experimental infection

Peste des petits ruminants (PPR) is a highly contagious transboundary infection disease of small ruminants, which is endemic for most of African, Asian and Middle Eastern countries. It jeopardizes their livestock industry. PPR elimination is considered to be an essential component of global food security and poverty reduction [1, 2]. PPR is characterized by fever, anorexia, ocular and nasal discharge, erosions and ulcers in digestive mucosa, diarrhea and marked

leucopoenia with immunosuppression. Abortions can be observed in pregnant sheep and goats [3, 4]. Direct viral affection with PPR virus (PPRV), decreased immunity and reproduction of other pathogens (e.g., *Pasteurella* spp., *Escherichia coli*, *Mycoplasma* spp.) can lead to an animal's death. Depending on an infected animal's species, breed, age and extension of secondary infectious agents, mortality varies substantially (10-90%) [5].

A causative agent is a RNA-containing virus (family *Paramyxoviridae*, genus *Morbillivirus*) [6, 7] whose antigens are related to contagious bovine typhus (CBT) (i.e., another *Morbillivirus*) [8]. PPRV genome represents a negative single-strand RNA molecule (15,948 nucleotides). PPRV genome RNA contains open reading frames for 6 structural proteins (i.e., nucleocapsid, phosphoprotein, matrix, fusion protein and haemagglutinin) and 2 non-structural ones (V and C) [9]. Although PPR strains and isolates are not divided into serotypes, they are clustered in 4 genetic lines [10] according to nucleotide genetic sequences of nucleocapsid protein and phosphoprotein. It was found that lines I and II are common in West Africa, line III is typical for East Africa, Middle East and South India; line IV is widespread in Asia [11, 12].

For the first time, PPR was reported in West Africa. Then, the infection got across to Central and East Africa. In 1980 it was reported on the Arabian Peninsula. Nowadays, the disease is wide-spread in Africa and Asia. Moreover, it was mentioned in our neighboring countries (Tajikistan, China, Mongolia, Kazakhstan, Afghanistan, Georgia and Turkey). According to analysis of PPR epizootic situation in Russia, the risk of occurrence of this disease on the territories of Siberian, Ural, Far Eastern, North Caucasian and Southern Federal Districts of the Russian Federation is quite high [13, 14].

As per the International Epizootic Bureau (IEB, Office International des Epizooties, OIE, France) guidelines, PPR can be diagnosed in virtue of the viral genome revealed by a polymerase chain reaction (reverse transcription PCR), virus extraction with permissive cell line, and viral antigen detection by enzyme-linked immunosorbent assay (ELISA), fluorescent antibody test (FAT), agarose gel diffusion test and immunoelectrophoresis. Serological methods of specific antibody detection (neutralization test and competitive ELISA) are applicable to monitoring studies and determination of immune status of a vaccinated animal. IEB points out that reverse transcription PCR and ELISA methods to detect viral antigens are the most suitable for confirmation of PPR clinical cases [15]. Reverse transcription Real-time PCR combined with electrophoresis or hybrid fluorescent detection of amplification products is the most common diagnostic method. Sustainable reverse transcription PCR schedules with electrophoretic detection showed good results. Proposed by Shaila et al. [16] and Couacy-Hymann et al. [17] in 1996 and 2002, respectively, they are based on amplification of gene fragments related to nucleocapsid protein and phosphoprotein. Further, Bao et al. [18], Kwiatek et al. [19] and Batten et al. [20] offered several schedules of highly efficient and more sensitive reverse transcription Real-Time PCR on the basis of amplification of highly conserved regions of nucleocapsid protein gene. Analytical sensitivity of these test systems is 30-10 RNA copies/reaction. They can detect genomes of PPRV strains falling into all the four lines [18-20]. In 2010, Balamurugan et al. [21] published a reverse transcription Real-Time PCR protocol where matrix protein gene fragment is amplified at 0.1-1.0 $TCID_{50}/cm^3$.

To diagnose PPR viral genome, conjunctival, nasal and oral discharges, as well as blood samples are collected in animals. To avoid a non-specific degradation of the viral nucleic acid, biomaterial must be shipped in cooled state [22]. If temperature conditions cannot be met during sample shipping to a diagnostic

laboratory, a paper medium should be used to stabilize blood samples (i.e., "dry" blood method). Dry spot method is very common in veterinary and medical practice to collect samples of various biological fluids intended for virological, serological and biochemical tests in domestic environment and to ship them to a laboratory [23, 24].

Even though molecular-genetic methods are widely used in veterinary practice for PPR diagnosis, alternative target gene-based confirmatory tests must be performed. Validated as per IEB standards, these tests must detect PPR viral genome in samples of biomaterial collected in infected animals.

The paper presents results related to development of a test system to detect PPR viral genome based on amplification of hemagglutinin gene fragment by reverse transcription Real-Time PCR. Sensitivity of the test system is 0.83 ± 0.22 lg $TCID_{50}/cm^3$. It can differentiate PPR viral genome from genomes of contagious bovine typhus and bluetongue that cause similar symptoms in sheep. The proposed test system suitability for practical diagnosis of PPR was verified experimentally.

Our purpose was to develop a reverse transcription Real-Time PCR-based test system to detect genome of PPR virus and to validate it using blood samples of experimentally infected sheep.

Techniques. We used several strains of PPRV (Nigeria 75/1, Epizootichesky and $45G_{37}$/35-K), contagious bovine typhus (Nakamura and LT 67 vaccine strains) and bluetongue (serotype VIII, NET 2007 strain) stored in the National Microorganism Collection of the Federal State Budgetary Scientific Institution Federal Research Virology and Microbiology Center. Cell cultures (Cell Culture Collection, Federal Research Center for Virology and Microbiology) were used as negative samples to assess analytical specificity of the test system.

Viral RNA was extracted with a RNA-Sorb kit (Interlabservis OOO, Russia). Reverse transcription was carried out with a Tertsikl thermocycler (DNK-Tekhnologii ZAO, Russia) and Real-Time PCR was carried out with a DT-Prime detecting amplifier (DNK-Tekhnologii ZAO, Russia). To synthesize a cDNA on the matrix of extracted RNA samples, we used a reaction mixture (20 μl) consisted of a test sample (10 μl), a reverse primer (1 μl; 10 pmol/μl, Evrogen ZAO, Russia), 5× reverse transcription buffer (4 μl; Alfa-ferment OOO, Russia), deoxynucleoside triphosphate mixture (0.3 μl; 10 mmol/ml, Syntol ZAO, Russia), reverse transcriptase (0.2 μl; 200 units/μl; Alfa-ferment OOO, Russia) and bidistilled water (4.5 μl). The reaction mixture was incubated at 42 °C for 30 min. Using obtained cDNA, we conducted Real-Time PCR in the 25 μl reaction mixture containing matrix (5 μl), direct and reverse primers (1 μl each), Taq-man probe (0.3 μl; 10 pmol/μl), 10× PCR buffer (2.5 μl; Alfa-ferment OOO, Russia), deoxynucleoside triphosphate mixture (0.3 μl; 10 mmol/ml), $MgCl_2$ (0.5 μl; 25 mmol/ml, Syntol ZAO, Russia), Taq MS polymerase (0.1 μl; 5 units/μl, Alfa-ferment OOO, Russia) and bidistilled water (14.3 μl). We applied following PCR schedule: pre-denaturation at 94 °C for 2 min and 40 cycles of amplification (94 °C — 15 s, 60 °C — 15 s, 72 °C — 15 s). Fluorescence (FAM) was detected at 60 °C.

To design a recombinant amplification control, PCR products were extracted from the reaction mixture with a Cleanup Standard kit (Evrogen ZAO, Russia). As a part of pTZ57 R/T plasmid vector (Thermo Fisher Scientific, USA), extracted PCR products were cloned in *Escherichia coli* strain Top10 (Invitrogen, USA). A plasmid DNA was extracted with a Plasmid Miniprep kit (Evrogen ZAO, Russia) and its concentration was determined with a NanoDrop Lite microspectrophotometer (Thermo Fisher Scientific, USA).

Three Romanov sheep weighed 45-50 kg were experimentally PPR-

infected. Sheep nos. 1 and 2 were infected with intravenously administered culture containing PPR virus (Epizooticheskiy strain) (3.5 lg $TCID_{50}$). Sheep no. 3 was used as an intact control. All the animals underwent a clinical examination (including daily thermometry and blood sampling). Animal experiments were observed by the Bioethics Committee of Federal Research Center for Virology and Microbiology.

For "dry" blood preparations, we used 0.05 cm^3 of PPRV-contaminated blood stabilized with EDTA on circles ($\varnothing \approx$ 4-5 cm) cut out of Whatman DE-81 filter paper (Whatman, Great Britain). Blood was contaminated with cultural material containing PPRV (Nigeria 75/1 strain) with a titer of 4.83±0.22 lg $TCID_{50}/cm^3$ (1000:1). To extract viral RNA, we placed these preparations into microcentrifuge tubes with 800 μl of lysis buffer containing 4 M guanidine isothiocyanate, and incubated at 56 °C for 10 min. Then, they were centrifugated at 10,000 g for 10 min. Supernatant was used to extract RNA by a nucleosorption method as per instructions of RIBO-Sorb kit (Interlabservis ZAO, Russia).

We performed a statistical processing of the findings as per standard methods. Statistical significance between means was determined as per the Student-Fischer difference method. In this view, in most of bioassays $p > 0.05$ indicates absence of differences between compared values; $p < 0.05$, $p < 0.1$ and $p < 0.001$ mean statistically significant differences. Arithmetic means (*M*) of *n* number of tests and standard deviation (σ) of arithmetic mean were calculated with Microsoft Excel 365 software.

Results. A nucleotide sequence of hemagglutinin (H) gene was selected as a primer annealing target after analysis of nucleotide sequences related to genomes of various PPRV strains and isolates available in the GenBank database (https://www.ncbi.nlm.nih.gov/). Using BioEdit 7.0 and Oligo 6.0 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html; http://www.oligo.net), we aligned nucleotide sequences, searched for conserved regions and calculated structure of oligonucleotide primers. Finally, we chose several oligonucleotide primers (PPRHf (5′- TCAAGATCGGGTCCAACATG-3′) and PPRHr (5′-CAATCGGACTGGGTAGAAGTAAG-3′) flanking a hemagglutinin gene 148 bp fragment. To detect amplification products, we applied a PPRHz 5′-(FAM)TCGCTCCTGGAAACATCATAAGTGGC(BHQ1)-3′ hybridization fluorescent-labeled probe.

Reverse transcription Real-Time PCR conditions were optimized by RNA preparations extracted from PPRV-infected (Nigeria 75/1 strain) Vero cell culture. Since primer melting points determined by Oligo 6.0 software were 58-62 °C, 60 °C was set as an annealing temperature in the temperature-time profile of the reaction.

To evaluate analytical sensitivity of the test system, we applied successive 10-fold dilutions of cultural PPR virus (Nigeria 75/1 strain) whose infectious activity was 4.83±0.22 lg $TCID_{50}/cm^3$.

According to Figure 1 and Table 1, the last dilution of the virus-containing material led to a positive result was 10^{-4}. Thus, analytical sensitivity of reverse transcription Real-Time PCR was 0.83±0.22 lg $TCID_{50}/cm^3$ during detection of PPRV genome. Amplification efficiency was 87%, and approximation accuracy (R^2) was ≈ 0.99. Intratest repetition of PCR results (threshold cycle values) characterized by mean standard deviations was 0.12-0.41. Coefficients of threshold cycle value variation resulted from re-tests of similar threshold samples were < 1.5% indicating high repetition of reverse transcription PCR analytical sensitivity test results.

Analytical specificity of the test system was assessed while testing samples

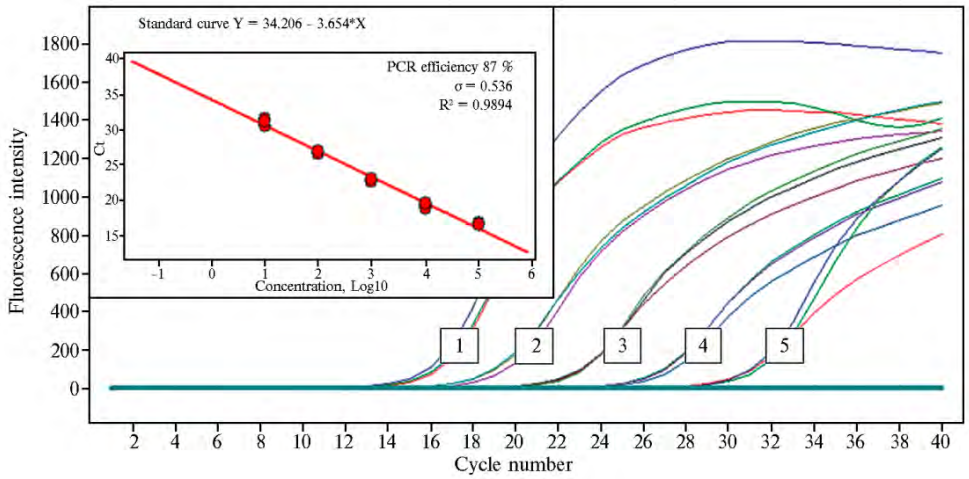

Fig. 1. Evaluation of successive 10-fold dilutions of cultural material containing virus of peste des petits ruminants (PPRV) (Nigeria 75/1 strain) of 4.83±0,22 lg $TCID_{50}/cm^3$ initial activity with the proposed test system: 1 — initial material; 2-5 — dilutions from 10^{-1} to 10^{-4}.

1. Analytical sensitivity parameters of reverse transcription Real-Time PCR in detection of virus of peste des petits ruminants (n = 3)

Dilution	Viral titer, lg $TCID_{50}/cm^3$	Reverse transcription Real-Time PCR result		
		mean Ct value	mean σ value	*Cv*, %
Initial material	4.83±0.22	16.53	0.12	0.75
10^{-1}	3.83±0.22	19.13	0.29	1.50
10^{-2}	2.83±0.22	22.83	0.17	0.74
10^{-3}	1.83±0.22	26.70	0.14	0.53
10^{-4}	0.83±0.22	31.07	0.41	1.35
10^{-5}	–0.17±0.22	Not available		

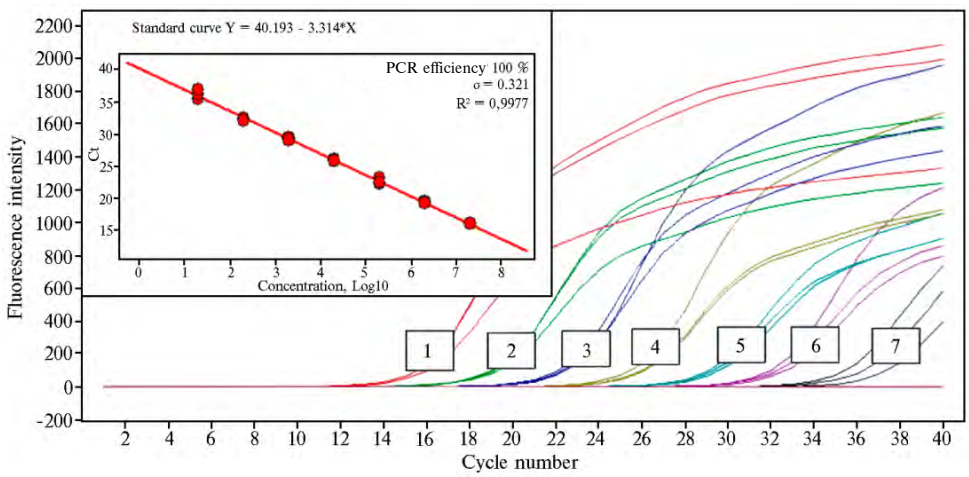

Fig. 2. Determination of working concentrations of positive recombinant amplification control (copies/μl) **to the test system intended for detection of PPRV genome by reverse transcription Real-Time PCR:** 1 — $2{,}4\times10^7$, 2 — 2×10^6, 3 — $2{,}4\times10^5$, 4 — $2{,}4\times10^4$, 5 — $2{,}4\times10^3$, 6 —$2{,}4\times10^2$, 7 — 24.

containing PPR, CBT and bluetongue viruses, blood samples of healthy sheep, as well as intact Vero and VNK-21 cell cultures (Cell Culture Collection, Federal Research Center for Virology and Microbiology). In this view, the test system specificity was confirmed by positive results demonstrated by PPRV-containing samples only.

After construction of a positive amplification control of the test system based on cloning of PPRV hemagglutinin gene 148 bp fragment as a part of

pTZ57 R/T vector and synthesis by reverse transcription PCR we obtained a plasmid DNA preparation whose concentration, according to spectrophotometry, was 80 ng/μl (equivalent to 2.4×10^{10} molecules per 1 μl). To improve quantity of plasmid DNA used as a positive control, 8 10-fold dilutions of the preparation were studied (starting with 10^{-3}) by reverse transcription PCR method. Plasmid DNA concentration range associated with a linear amplification was from 2.4×10^{7} to 24 DNA molecules/μl, with 100% amplification efficiency, 0.99 approximation accuracy (R^2) and 0.32 standard Ct deviation (σ) (Fig. 2). Recommended working plasmid concentrations in the test system correspondent with an analytical sensitivity range were 2.4×10^{7}-2.4×10^{4} DNA molecules/μl.

During the experimental PPRV infection designed to evaluate efficiency of the developed test system infected animals did not demonstrate any specific signs of the disease for 15 days. However, hyperthermia (< 40.2-40.5 °C) was observed in sheep 6-8 days after the infection. Along with this, using the proposed reverse transcription Real-Time PCR test system, PPRV genome was detected in blood samples collected in 5-12 days after the infection (Table 2).

2. Results of thermometry and PPRV genome detection in blood samples of experimentally infected sheep with the proposed reverse transcription Real-Time PCR test system

Post-infection period, days	Sheep no. 1		Sheep no. 1 2		Sheep no. 1 3	
	body temperature, °C	Ct value	body temperature, °C	Ct value	body temperature, °C	Ct value
1	38.6	–	39,5	–	39,0	–
2	39.4	–	39,4	–	39,3	–
3	39.5	–	39,2	–	39,1	–
4	39.6	–	39,5	–	39,0	–
5	39.6	–	39,6	–	39,3	–
6	40.2	+ (20,15)	39,7	–	38,9	–
7	40.5	+ (20,30)	40,2	+ (20,38)	39,2	–
8	40.4	+ (21,45)	40,5	+ (20,57)	39,3	–
9	39.2	+ (21,89)	39,9	+ (21,74)	38,8	–
10	39.0	+ (22,12)	39,5	+ (21,90)	39,0	–
11	38.7	+ (22,90)	39,9	+ (22,25)	38,9	–
12	38.8	+ (23,12)	39,9	+ (22,85)	39,1	–
13	38.7	–	39,8	–	38,8	–
14	38.8	–	39,9	–	38,9	–
15	39.2	–	39,7	–	39,1	–

N o t e. "+" — positive test, "–" — negative test..

Observed viremia period complies with reported data [25, 26]. Absence of clear specific signs can be associated with low infection dose (3.5 lg $TCID_{50}$). For example, in 2012 El Harrak et al. [27] used 5.1 lg $TCID_{50}$ of PPR virus to simulate the infection.

3. Detection of peste des petits ruminants viral genome in "dry" blood preparations on the paper basis by reverse transcription Real-Time PCR

Preparation no.	Native preparation (virus-contaminated blood)	"Dry" blood preparation	
		freshly prepared	1-month storage
1	20.76	20.51	23.67
2	20.15	20.83	24.98
3	21.00	21.43	25.01
4	20.35	21.02	23.73
Average	20.64±0.64	20.99±0.38	24.55±0.62

Nowadays, a "dry" blood method is a common technique to sample and to ship biological fluids. We evaluated applicability of the method in our test system. During development of "dry" blood method to collect and to store samples (PPRV diagnosis) on the paper basis we conducted comparative studies of RNA samples extracted from initial virus-containing material and freshly prepared "dry" blood samples with our test system. The results demonstrated absence

of significant differences between threshold cycles (Ct). Reverse transcription PCR analysis of RNA samples extracted from "dry"' blood preparations stored on the paper basis in airtight polyethylene ZIP-bags at 20±2 °C for 1 month, and initial preparations tested in the beginning of the experiment resulted in differences of 3-5 cycles between Ct values (Table 3).

Thus, we developed the highly specific and sensitive test system based on hemagglutinin gene fragment amplification by reverse transcription Real-Time PCR method to detect a genome of peste des petits ruminants (PPR) virus. Due to high sensitivity (0.83±0.22 lg $TCID_{50}/cm^3$), the test system can differentiate PPRV genome from viruses causing similar clinical signs in sheep (i.e., contagious bovine typhus and bluetongue). Practical application of the test system intended for PPR diagnosis was confirmed by blood testings involving experimentally infected sheep. Also, we developed a method of collection and storage of blood samples on the paper basis (i.e., "dry" blood method) to reveal PPRV genome. It was found that "dry" blood preparations on the paper basis are stable at room temperature for a month. So, they are suitable for reverse transcription Real-Time PCR test.

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MULTIPLEX MULTILOCUS REAL TIME PCR FOR ANALYSIS AND CONTROL OF AVIAN LEUKOSIS VIRUS SUBGROUPS A, B, J AND K IN RUSSIA

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Abstract

The avian leukosis virus (ALV) belongs to genus *Alpharetrovirus* of *Retroviridae* family and has a diploid genome consisting of a single-stranded RNA. ALV subgroups A, B, C, D, E, J and K are specific for chicken. The classification is based on differences in the viral coat protein structure. ALV, in particular ALV subgroup J, causes huge damage to industrial poultry. The gold standard for detecting ALV is virus isolation in CEFs or DF-1 cell cultures. This method has significant disadvantages, i.e. it takes 7-9 days, requires specialized facilities and equipment. Enzyme-linked immunosorbent assay based on detection of the ALV p27 group-specific antigen is the most widely used, but it also has significant deficiencies, the main of which are false positive results due to the expression of p27 by endogenous viruses and lack of sensitivity. In this study, a test system has been developed to detect exogenous viruses of the most common ALV subgroups A, B, J, and K and to control the spread of ALV. To test the developed system, we use 1200 samples of broiler DNA from a poultry farm of the Moscow Province. Analysis of the samples detected ALV subgroup J in 51 % poultry flock and ALV subgroup K in 8 % poultry flock. No viruses of subgroups A and B were found. We also analyzed 97 DNA samples from chickens from the regions of Russia, i.e. Orenburg, Chelyabinsk, Kemerovo, Tyumen, Kaliningrad, Leningrad, Sverdlovsk, Novgorod regions and Krasnodar Territory. ALV subgroups K were found in samples from the Kaliningrad, Leningrad, Sverdlovsk, and Novgorod regions, ALV subgroups A in samples from the Leningrad region, and ALV subgroups J in the Sverdlovsk and Leningrad regions. ALV subgroup B has not been identified, that is, it may indicate that this subgroup of ALV is not common in Russia at the present time. At the next stage, measures were taken to eradicate the ALV of subgroups J and K found in the broiler-type meat cross lines in one of the farms of the Moscow Province. For this, a multiplex multilocus real-time PCR test system was developed and applied for the simultaneous detection of ALV subgroups J and K. Using the proposed test system, several screening cycles of four broiler-type chicken meat cross lines with an initial total of 9029 chickens were performed. Prior to the start of the program for control and eradication of ALV, the proportion of poultry with neoplasia ranged from 17 to 26 % depending on the line of chickens (the maximum was observed in the line with the *ev21* locus). On

the 265th day after the start of the program for the control and eradication of ALV subgroups J and K, only three out of 2621 individuals (0.10 %) were diagnosed with a diagnosis of neoplasia, confirmed by positive results of real-time PCR as ALV subgroup J. Total percentage of individuals' samples containing ALV DNA of subgroups J and K in a sample of 2621 individuals at the age of 265 days were 0.67 % and 0.04 %, respectively.

Keywords: Avian leukosis virus, ALV subgroups A, B, J and K, real time PCR, ALV detection

The avian leukosis virus (ALV) belongs to genus *Alpharetrovirus* of *Retroviridae* family and has a diploid genome consisting of a single-stranded RNA. ALV subgroups A, B, C, D, E [1], J [2] and K [3] are specific for chicken. The ALV classification is based on differences in the GP85 coat protein structure [2, 3]. Exogenous ALV (subgroups A, B, C, D, J and K) spreads horizontally (bird-to-bird) and vertically (from parents to offspring through an egg). These viruses are characterized by greater pathogenicity than endogenous virus E with weak or even absent pathogenicity. Infecting an embryonic cell, endogenous viral genome builds in a host's genome. As per the Mendel's law, it is transmitted vertically [1]. ALV induces lymphoid/myeloid leukosis and other neoplasms [1]. Sometimes chicken morbidity and mortality can be 60% and > 20%, respectively [4]. ALV (subgroups A, B and J) is the most common worldwide whereas ALV-C and D are extremely rare [1]. Since 2007 China implements a National ALV Eradication Programme focused on ALV-J combating. Also, these viruses represent a critical issue in Russia. In particular, antibodies to them are detected in 70% of 223 monitored poultry farms in 46 regions, and general antibodies to ALV were observed in 90% of farms [5].

The gold standard for detecting ALV is virus isolation in CEFs or DF-1 [1] cell cultures. The method has substantial disadvantages because of long duration (7-9 days) and required sterile premises and equipment. Enzyme-linked immunosorbent assay based on detection of the ALV p27 group-specific antigen is the most common method of ALV testing. However, it also has significant disadvantages, the main of which are false positive results caused by p27 expressed by endogenous viruses [6] and lack of sensitivity [1, 7]. Moreover, endogenous viruses cause false positive results in several modern test systems based on the real-time polymerase chain reaction (RT-PCR) intended for ALV detection [6]. Sensitivity of RT-PCR test systems to detect ALV is by 15-20% higher than in cultural and enzyme-linked immunosorbent methods [8].

In the context of the study, a multiplex multilocus real-time PCR (RT-PCR) test system was developed and applied for simultaneous detection of the most common ALV subgroups (A, B, J and K), as well as for simultaneous detection of ALV-J and K.

The purpose was to apply developed test systems to detection and eradication of ALV.

Techniques. Using Blast software together with the GenBank (http://www.ncbi.nlm.nih.gov/BLAST) and ClustallW (http://www.genome.jp/tools-bin/clustalw) databases, we selected conserved regions of ALV genome specific for subgroups A, B, J and K. Further, they were used as targets for DNA amplification and sequencing. To choose specific regions, we used reference sequences related to different ALV subgroups stored in the GenBank such as M37980, HM452341 (ALV-A); AF052428, JF826241 (ALV-B), J02342 (ALV-C); D10652 (ALV-D); EF467236, AY013303, AY013304, KC610517 (ALV-E); Z46390, JF951728, JQ935966, HM776937, JX855935, JF932002, KX058878, DQ115805, KX034517, KU997685, HM235668, HM582657 (ALV-J). Several sequences isolated in various Russian regions are presented among them (i.e., KF746200, KP686143, GD14LZ (ALV-K)). Primers and probes intended for RT-PCR and Sanger sequencing were developed on the basis of reported DNA sequences [10, 11, 15] and synthesized by

Syntol OOO. They were selected in such a way as to amplify ALV DNA without amplification of known endoviruses. 6FAM, 5R6G, 6ROX, Cy5 and Cy5.5 colorants were used as a fluorescent label in R-T PCR hybridization probes. BHQ1 and BHQ2 colorants connected to thymidine inside the hybridization probe with a linker were fluorescence quenchers. A phosphate (P) group was used for 3′-end probe modifications.

DNA was extracted from chicken feathers with a M-Sorb reagent kit (Syntol OOO, Russia). A feather 0.3-0.5 cm fragment or chicken cloaca swab was placed in a 1.5-ml tube. Then, we added 400 μl of lysis solution and incubated a sample at 60 °C for 20 min. Lysate was precipitated in a high-speed microcentrifuge (Cyclotemp-902; Cyclotemp ZAO, Russia) at 13,000 rpm for 1 min. Supernatant was transferred to a 1.5-ml tube. The isolation was proceeded according to the standard schedule of a M-Sorb reagent kit (Syntol OOO, Russia). A real-time polymerase chain reaction involved 1.5 μl of isolated DNA.

Reverse transcription was performed with OT-1 reagent kit (Syntol OOO, Russia). In this, 10 μl of nucleic acid preparation isolated with M-Sorb reagent kit was added to 25 μl of the reaction mixture.

RT-PCR was carried out with ANK-48 (Institute of Analytical Instrument-Making of the Russian Academy of Science, Russia) and DT-96 (DNK-Tekhnologii OOO, Russia) as per the program as follows: 90 °C for 30 s; denaturation at 90 °C for 10 s, annealing at 60 °C for 30 s (50 cycles). To amplify DNA, we used 10 μl of finished 2.5× M-428 RT-PCR reaction mixture (Syntol OOO, Russia). Concentrations of primers and probes in the reaction mixture were 450 nM and 100 nM, respectively.

RT-PCR specificity was verified by sequencing of amplification products obtained with ALVKF, SEQA-KR, SEQJF, SEQJR, ALVAF, SEQA-KR primers with a Nanofor 05 genetic analyzer (Institute of Analytical Instrument-Making RAS, Russia) equipped with DNK Analiz 5.0.2.3 software (Institute of Analytical Instrument-Making RAS, Russia).

Results. A multiplex RT-PCR of the most common avian leukosis virus subgroups. Although ALV-J, A and B are the most common worldwide, C and D subgroups are extremely rare. Comparatively recent [3, 13-16] ALV-K is of interest to study its spread and pathogenic properties. Even subclinical exogenous and endogenous ALVs lead to decreased poultry productivity and great economic losses [1, 17]. That is why, the multiplex test system was designed to detect the subgroup as well (Table 1). A *gp85* coat protein gene of appropriate ALV subgroups was used as a DNA target of test systems. Synthetic DNA fragments correspondent with estimated amplified ALV (A, B, J and K) genome fragments were used as positive controls (see the Table 1).

To evaluate sensitivity of the test system, we used successive 10-fold and 2-fold dilutions of positive control samples. Analytical sensitivity of the test system assessed by dilutions of a positive control whose amount was calculated as per the description [11, 12] was 25 genome equivalents (ALV-A), 10 genome equivalents (ALV-J), 25 genome equivalents (ALV-B) and 10 genome equivalents (ALV-K) per 1 μl of an initial sample.

1. Primers and probes used in the multiplex test system to detect various avian leukosis viruses (ALV)

Primers, probes	Nucleotide sequence (5′→3′)	Amplicon, bp	Subgroup
ALF	AGCACCACTGCTGCCTTGGA	62	ALV A-E
ALR	CTAGCGACCGCTCCTTCCAGA		
ALVPL	(6FAM)CGATGGGACCC(dT-BHQ1)GCCCTGC-P		

Table 1 continued

ALVAF	GCCACACGGTTCCTCCTTAGA	114	ALV A
ALVAR	CGCAGTACTCACTCCCCATGAA		
APL	(5R6G)TACGGTGG(dT-BHQ1)GACAGCGGATAG-P		
JFF1F	GCCCTGGGAAGGTGAGCAAGA	139	ALV J
JJR	GGAAATAATAACCACGCACACGA		
JNP	(6ROX)TCCTCTCGA(dT-BHQ2)GGCAGCAAGGGTGTC-P		
ALBF1	GGCCGAGGCCTCCCCGAAA	77	ALV B
ALVBR	GTCTCATTAATTTCCTTTGATTGA		
BPL1G	(Cy5)CCCATGTACC(dT-BHQ2)CCCGTGCCTTG-P		
ALVKF	CGGAGCATTGACAAGCTTTCAGA	72	ALV K
ALVKR	GTGATTGCGGCGGAGGAGGA		
KPL	(Cy5.5)CCACCTCGTGAG(dT-BHQ2)TGCGGCC-P		

2. Primers and probes used in reference test system to detect avian leukosis virus J (ALV-J)

Primers, probes	Nucleotide sequence (5′→3′)	Amplicon, bp	Subgroup
ALV-JNF	TTGCAGGCATTTCTGACTGG	214	ALV J [8]
ALV-JNR	ACACGTTTCCTGGTTGTTGC		
JCP	(6FAM)CCTGGGAAGGTGAGCAAGAAGGA-BHQ1		
H5	GGATGAGGTGACTAAGAAAG	545	ALV J [9, 10])
H7	CGAACCAAAGGTAACACACG		
Probe	(6FAM)CTCTTTGCAGGCATTTCTGACTGGGC(BHQ1)		

During ALV-J detection additional analytical sensitivity of the probe and primer system was evaluated by comparison of threshold RT-PCR cycle values (Ct) with those in a reference test system to detect ALV-J [8-10] (Table 2) using ALV-J DNA isolates extracted in a poultry farm in the Moscow Province. The results were similar to ones obtained with a Qin L test system [8] whose stated sensitivity was lower than 10 viral copies/sample; RT-PCR test based on conventional H5 and H7 primers was 100 times less sensitive to detect DNA of ALV-J [9, 10].

The 1× PCR buffer containing an exogenous virus-free chicken DNA was used as a negative control in all the tests. Negative controls did not induce any DNA amplification after 50 cycles of RT-PCR. We did not assess analytical sensitivity of a test system involving ALF, ALR, ALVPL probes and primers (see the Table 1) to detect all known ALV subgroups (including endogenous viruses). As a part of a multiplex RT-PCR, it can be applied as an inner amplification control.

Using the test system, we analyzed 1200 samples of chicken DNA collected in a poultry farm in the Moscow Province. ALV-J and ALV-K were detected in 42% and 8% of a chicken flock, respectively. The test did not reveal ALV-A and ALV-B in the tested sample. Moreover, we analyzed 97 DNA samples from chickens from the regions of Russia, i.e. Orenburg, Chelyabinsk, Kemerovo, Tyumen, Kaliningrad, Leningrad, Sverdlovsk, Novgorod regions and Krasnodar Territory. ALV-K was found in samples from the Kaliningrad (3 pcs), Leningrad (2 pcs), Sverdlovsk (5 pcs), and Novgorod regions (1 pc); ALV-A was detected in samples from the Leningrad region (7 pcs), and ALV-J was mentioned in the Sverdlovsk (3 pcs) and Leningrad regions (5 pcs). Since ALV-B was not detected, we can conclude that it is not common in Russia recently. Some positive samples were sequenced with primers mentioned in the Table 3. This verified specificity of the test system to detect ALV (A, J and K).

3. Primers for PCR and *gp85* gene fragment sequencing in various subgroups of avian leukosis virus (ALV)

Primers	Nucleotide sequence (5′→3′)	Amplicon, bp	Subgroup
ALVAF	GCCACACGGTTCCTCCTTAGA	443	ALV A
SEQA-KR	CGCGATCCCCACAAATGAGGAAA		

Table 3 continued

SEQJF	CCCTGGGAAGGTGAGCAAGAA	498	ALV J
SEQJR	CCTTTATAGCACACCGAACCGAA		
ALBF1	GGCCGAGGCCTCCCCGAAA	253	ALV B
SEQA-KR	CGCGATCCCCACAAATGAGGAAA		
ALVKF	CGGAGCATTGACAAGCTTTCAGA	466	ALV K
SEQA-KR	CGCGATCCCCACAAATGAGGAAA		

N o t e. Amplicons are fragments of *gp85* gene encoding GP85 virus coat protein.

A multiplex multilocus RT-PCR test system is intended for simultaneous detection of ALV-J and ALV-K to eradicate the virus. It was developed to eradicate ALV-J and ALV-K detected in lines of broilers from a poultry farm of the Moscow Province. As compared to other methods to detect and to eradicate avian leukosis virus, RT-PCR method is the most sensitive and advanced [8]. Total virus eradication is a quite challenging issue that requires a high-level testing programme. Vertical ALV transmission can be performed in absence of detected gs-antigen in chickens [1]. The infection control in some lines can be more complicated than in others. Presence of *ev21* locus containing an endogenous virus complicates ALV eradication because chickens become more susceptible to the infection [18]. A risk of ALV-J-associated viremia in broiler-type meat cross chickens can also restrict ALV eradication [1]. Extreme variability of the most common pathogenic ALV-J is a one of substantial difficulties associated with the virus spread control [1, 2]. The *gp85* gene of ALV-J coat protein isolated from different organs of the same body can demonstrate 94.9% variability of sequences [19]. A difference in amino acid sequences between the most distal isolates of the coat protein is 86.2% [20].

4. Primers and probes of the multiplex multilocus RT-PCR test system for simultaneous detection of avian leukosis virus (ALV) **J and K**

Primers	Nucleotide sequence (5′→3′)	Amplicon, bp	Subgroup
ALVKF	CGGAGCATTGACAAGCTTTCAGA	72	ALV K
ALVKR	GTGATTGCGGCGGAGGAGGA		(gene *gp85*)
KPL	(6FAM)CCACCTCGTGAG(dT-BHQ1)TGCGGCC-P		
JFF1F	GCCCTGGGAAGGTGAGCAAGA	139	ALV J локус 1
JJR	GGAAATAATAACCACGCACACGA		(gene *gp85*)
JJPLN	(ROX)CAGCAAGGGTG(dT-BHQ2)CTTCTCCG-P		
JNP	(ROX)TCCTCTCGA(dT-BHQ2)GGCAGCAAGGGTGTC-P		
JEF	CCTATTCAAGTTGCCTCTGTGGA	72	ALV J locus 2
JER	GCTTGCTCTATTTGGCCGTCAGA		(LTR)
JEP	(Cy5)CCATCCGAGC(dT-BHQ2)GCCTCCAGTCC-P		

To increase ALV-J detection reliability, we developed RT-PCR test system equipped with an additional JJPLN probe for *gp85* gene encoding the virus coat protein, and JEP probe intended for a long terminal repeat (LTR) fragment in ALV-J genome (Table 4). The test system increased ALV-J detectability by 2.3% as compared with one mentioned above.

A strategy of multilocus RT-PCR equipped with additional probes is also suitable for detection of retroviruses, coronaviruses and other microorganisms characterized by substantially variable genome. At the first stage of the programme, we used cloaca swabs collected in 1-day chickens to detect ALV-J and ALV-K. Further, we used feather pulp containing significantly greater virus amount than plasma and other tissues as a biomaterial. In a point of fact, ALV persists in feather pulp longer than in plasma [21]. As distinct from standard DNA isolation in blood, DNA isolation from feather pulp is a non-invasive labor saving method resulting in greater amount of DNA whose preparation does not contain PCR inhibitors. Aseptic blood sampling requires sterile tubes and needles for each chicken. In turn, only gloves and microcentrifuge tubes are used for a feather test [22]. While testing poultry for Marek's virus and ALV-J,

DNA isolation in feather pulp demonstrated better PCR findings than splenic DNA isolation [23].

After ALV penetration into a cell a reverse transcriptase and two copies of a single-stranded retroviral genome are released from a capsid. Then, a double-stranded DNA intermediate is formed that can built in a host's cell genome in presence of integrase [24]. Similarly to other types of different stages of retroviral development, the virus can be detected by RT-PCR [25. That is why, it would be quite invidiously to draw a conclusion about provirus detection only if a reverse transcription is not carried out. As for avian leukosis virus, detection of nucleic acids rather than viral antigens enables rejection of poultry with temporarily inactive provirus decreasing virus persistence in a flock. Stress is one of factors leading to reactivation of the infection [26]. We compared analytical sensitivity of the RT-PCR test systems with/without a reverse transcription. A reverse transcription resulted in up to 2 orders of magnitude greater increase in ALV-J and ALV-K detection sensitivity in tissues with active infection. This provides several additional opportunities to improve analytical sensitivity of ALV detection.

Using the multiplex multilocus RT-PCR test system, we carried out 7 cycles of screening of 4 initial and 6 experimental lines of boiler-type meat cross chickens (initial number — 9029). In the beginning of the ALV-J and ALV-K eradication program chickens with neoplasia were 17-26% depending on the line (the peak value was observed in a line with *ev21* locus). On day 265 after the start of the program, only three chickens of 2621 birds (0.10%) demonstrated neoplasia. Total percentage of samples containing ALV-J and ALV-K DNA among 2621 individuals at the age of 265 days was 0.67 % and 0.04 %, respectively.

Thus, our multiplex multilocus RT-PCR test systems intended for simultaneous detection of the most common ALV subgroups (A, B, J and K) and simultaneous detection of ALV-J and ALV-K demonstrated highly efficient detection and eradication of ALV.

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DIFFERENTIATION OF *Mycoplasma bovis*, *Mycoplasma bovigenitalium*, *Mycoplasma californicum* AND IDENTIFICATION OF *Ureaplasma diversum* BY REAL-TIME PCR

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Abstract

Mycoplasmas and ureaplasmas are important etiological agents of mastitis, pneumonia and reproductive disorders in cattle, which cause significant economic damage to cattle farming. The most significant species are *Mycoplasma bovis*, *M. bovigenitalium*, *M. californicum* and *Ureaplasma diversum*. Commercial diagnostic PCR systems for the detection of bacteria of the genus *Mycoplasma* in different biological samples are described, but no PCR kits have been developed to address the identification of *Mycoplasma* species. In this work, real time PCR assays for differentiation of pathogenic mycoplasmas (*Mycoplasma bovis, M. bovigenitalium, M. californicum*) and detection of *Ureaplasma diversum* in biological material (semen, milk, vaginal swabs, tissues) are developed. *UvrC* gene for *M. bovis*, 16S rRNA gene for *M. bovigenitalium* and *U. diversum*, and *rpoB* gene for *M. californicum* were chosen as target genes. The PCR assays included a system of primers and probes for detection of exogenous noncompetitive internal control sample. The specificity of the developed techniques was tested on a panel of samples containing viral and bacterial pathogens causing diseases in cattle, as well as cow genomic DNA. To assess the sensitivity of each PCR assay, positive control samples were developed based on genetically engineered constructs containing the region of the corresponding specific DNA. Analytical sensitivity of the PCR assays was evaluated separately for each pathogen, for which we used 10-fold dilutions of the corresponding control samples in negative samples of biological material, i.e. semen, milk, vaginal swabs and tissues. The sensitivity (detection limit) of the assays for different types of biological species was 5×10^3 copies per ml on average. The efficiency of PCR was 99 % for *M. bovis*, 87 % for *M. bovigenitalium*, 94 % for *M. californicum*, and 98 % for *U. diversum*. A total of 410 samples of bovine semen intended for artificial insemination from local and foreign breeding centers were tested to detect *M. californicum*, *M. bovigenitalium*, *M. bovis* and *U. diversum*. DNA of *M. bovis* was found in 2.5 % of semen samples from foreign centers. In samples of Russian origin *M. bovis* DNA was not detected. DNA of *M. bovigenitalium* was identified for 60.7 % of local and 25.1 % of foreign semen samples; DNA of *M. californicum* was detected in 51.7 % and 25.1 % samples, respectively. *Ureaplasma diversum* DNA was found in 55.0 % of semen samples from Russian bulls and in 12.1 % of semen samples of foreign origin. Co-infection of *M. californicum*/*M. bovigenitalium* was detected in 97 samples (23.7 %), *M. bovigenitalium*/*U. diversum* in 86 cases (21.0 %). Simultaneous infection of *M. bovigenitalium*, *M. californicum* and *U. diversum* was observed in 52 samples (24.6 %) of semen from domestic bull sires and in 4 samples (2.0 %) from foreign breeding centers. Novel PCR assay tests can be used for monitoring of semen quality as well as control and prevention of the pathogens distribution.

Keywords: *Mycoplasma bovis*, *Mycoplasma californicum, Mycoplasma bovigenitalium*, *Ureaplasma diversum*, real-time PCR, bovine semen

Mycoplasmas and ureaplasmas are important etiological agents of mastitis, pneumonia and reproductive disorders in cattle, which cause significant economic damage to cattle farming. The most common pathogenic and clinically significant species are *Mycoplasma bovis*, *M. bovigenitalium*, *M. californicum* and

Ureaplasma diversum [1, 2].

M. bovis is one of the most dangerous pathogens, an etiological agent of upper airway diseases, pneumonia, otitis, arthritis, mastitis, endometritis, keratoconjunctivitis and other bovine conditions [3-5]. Running second after *M. bovis*, *M. californicum* inducing arthritis and pneumonia in young animals is associated with mastitis [6, 7]. *M. bovigenitalium* which also can cause bovine mastitis is detected in a reproductive tract. Additionally, it is associated with endometritis, infertility and impaired labor [8, 9]. It was demonstrated that *M. bovigenitalium* is an etiological agent of necrotic vulvovaginitis damaging cattle farms [10]. Moreover, a statistically significant correlation was found between presence of the mycoplasma in bovine semen and decreased sperm motility.

Ureaplasma diversum is another representative of *Mycoplasmataceae* that differs from *Mycoplasmae* with urea hydrolysis. At the same time, it is associated with various bovine reproductive disorders (such as granular vulvovaginitis, endometritis, salpingitis, spontaneous abortions, infertility and weak offspring) [11, 12].

Selective medium cultivation is a conventional method to detect mycoplasmas [13]. However, it has several restrictions. For example, dedicated media and microaerophilic cultivation are required for mycoplasmal growth. The testing takes 7-10 days. Along with this, another bacterial growth substantially inhibits or disables a precise identification of the etiological agent.

Nowadays, a polymerase chain reaction (PCR) with genus-specific primers is a standard method to detect mycoplasmosis. Independently from other microorganisms, it can reveal *Mycoplasmae* in various biomaterials rapidly. Nevertheless, this approach cannot differentiate a genus of the etiological agent.

Using novel PCR-based methods in the context of the paper, we detected and differentiated pathogenic *Mycoplasmataceae* in samples of cryopreserved stud bull semen used for artificial insemination in national farms. We compared mycoplasmal content in the semen products supplied by national and foreign breeding centers. Co-infection of samples with several mycoplasmal species was reported.

Our purpose was to develop methods of identification and differentiation of the most common pathogenic mycoplasmas (*Mycoplasma bovis*, *M. californicum*, *M. bovigenitalium*) and *Ureaplasma diversum* based on a real-time polymerase chain reaction.

Techniques. During our study we used vaginal swabs, milk, parenchymal organs, bovine semen and several strains such as *Mycoplasma bovis* ATCC 25523, *M. bovigenitalium* ATCC 19852, *M. arthritidis* ATCC 19611, *M. bovirhinis* PG43 ATCC 27748, *M. arginine* G230 ATCC 23838-TTR; *Histophilus somni* ATCC 700025; *Campylobacter fetus* 25936; *Brucella abortus* 82; *Yersinia enterocolitica* serotype 03; *Salmonella enterica* subsp. *enterica* Dublin 6; *Pseudomonas aeruginosa* serotype 0-17; *Staphylococcus aureus* VKPMV 6646; *Mycobacterium bovis* AN5 2/5-69-MS-07, *Mycobacterium intracellulare* 13-4; *Leptospira interrogans* Pomona VGNKI-6; *Bacillus cereus* VKPM B-8076; *Arcanobacterium pyogenes* ATCC 8164; *Neospora caninum* ATCC 50977; *Escherichia coli* 0157:H7; *Clostridium perfringens* type C; *Streptococcus pyogenes* ATCC 19615; *Candida albicans* ATCC 10231; *Aspergillus* niger ATCC 16404; *Enterococcus faecalis* ATCC 29212; Bovine Herpesvirus 1 MBA 2; Bovine Herpesvirus ATCC-VR-845; Bovine Herpesvirus 4 DN-599ATCC-VR-631; bovine diarrhea virus (DV) Oregon C24V strain; bovine DV NADL strain; bovine parainfluenza PTK 45/86 virus strain; nodular dermatitis virus DNA; bovine positive samples containing Schmallenberg disease virus RNA.

We tested 410 samples of cryopreserved bovine semen supplied by national and foreign breeding centers.

DNA was extracted with a commercial Ribo-prep kit (Amplisens, Russia). *Mycoplasmae* were detected with a Mik-Kom test system (Amplisens, Russia). A LSI VetMAX™ *Mycoplasma bovis* PCR kit (Thermo Fisher Scientific, France) was also applied.

To identify *M. bovis*, *M. bovigenitalium*, *M. californicum* and *U. diversum*, a real-time PCR was conducted with RotorGene Q (Qiagen, Germany) and CFX (Bio-Rad, USA). Amplification data were interpreted according to presence/absence of interception of a fluorescence curve and a threshold line. Amplification reaction mixture contained 10 μl of DNA matrix, 10 μl of PCR mixture 1 (6 μM specific primers, 3 μM specific probes, 3 μM primers to amplify an exogenous non-competitive inner control sample (ICS), 1.5 μM ICS probe, dNTP solution, deionized water), 0.5 μl of Taq-F polymerase, 5 μl of PCR mixture 2-FRT (Amplisens, Russia). As for *M. bovis*, *M. californicum* and *M. bovigenitalium*, we used following amplification programme: 15 min at 95 °C; 10 s at 95 °C, 20 s at 60 °C, 10 s at 72 °C (10 cycles without detection of a fluorescent signal); 10 s at 95 °C, 20 s at 55 °C, 10 s at 72 °C (35 cycles with detection of a fluorescent signal). *U. diversum* amplification program included stages as follows: 15 min at 95 °C; 10 s at 95 °C, 20 s at 55 °C, 10 s at 72 °C (10 cycles without detection of a fluorescent signal); 10 s at 95 °C, 20 s at 55 °C, 10 s at 72 °C (35 cycles with detection of a fluorescent signal).

Positive control samples (PCS) were obtained by a specific amplification product cloning in pAL2-T plasmid (Evrogen, Russia). PCR products were cloned in a pAL2-T vector by the standard manufacturer's method without pretreatment with restrictases and exonucleases. Plasmid concentration was measured with a spectrophotometer and expressed as a number of copies/ml.

Analytical method sensitivity of each pathogen was assessed separately. 10-fold dilutions of plasmids in known negative samples of semen and milk, vaginal swabs and 10% suspension of inner parenchymal organs were used as samples. Specificity was evaluated on a sample panel consisting of bovine genome DNA, as well as DNA of mycoplasmal and heterologous bacterial/viral strains inducing bovine diseases. Positive samples were verified by PCR fragment sequencing with specific primers. The sequencing was carried out with a Big Dye® Terminator v1.1 Cycle Sequencing Kit, GeneAmp PCR System 2720 amplifier (Applied Biosystem, USA) and ABI PRISM 3130 Genetic Analyzer sequencer (Applied Biosystem, USA).

Results. To detect and to differentiate mycoplasmas with molecular-genetic methods, foreign researchers use PCR schedules with various diagnostic efficiency [1, 14, 15]. To amplify *M. bovigenitalium* and *U. diversum*, most of authors apply primers selected for 16S rRNA gene and 16S-23S rRNA intergenic spacer region [16-18]. As for *M. californicum*, primers selected for *rpoB* gene are applied [1, 2]. The better part of papers is dedicated to *M. bovis* detection in various biological materials. In order to increase sensitivity and specificity, *vsp*, *fusA*, *oppD* gene primer systems are recommended [1, 14, 19]. *uvrC* gene application to *M. bovis* detection is reported [14, 15, 20]. Specific sequences selected for amplification of the genome fragment enable differentiation of *M. bovis* from *M. californicum*, *M. bovigenitalium*, *M. bovirhinis*, *M. bovoculi*, *M. dispar* and *M. agalactiae*.

Analyzing nucleotide sequences available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) with VectorNTI Advanced 11.0 software (InforMax, Inc., USA), we proposed oligonucleotide primers and DNA probes to amplify several gene regions (i.e., *UvrC* for *M. bovis*, 16S rRNA for *M. bovigenitalium* and *U. diversum*). We identified *M. californicum* with oligonucleotide sequences reported by Boonyayatra et al. [1]. Selected primers flank

gene regions with length of 148 bp (positions nos. 697986-698133 of the GenBank CP019639.1 sequence) for *M. bovis*, 96 bp (positions nos. 504837-504932 of the GenBank CP007521.1 sequence) for *M. californicum*, 127 bp (positions nos. 131-257 of the GenBank AY974058.1 sequence) for *M. bovigenitalium*, 114 bp (positions nos. 119-232 of the GU227397.1 sequence) for *U. diversum*. We selected oligonucleotide probes to carry HEX and ROX fluorescent colorants providing simultaneous multiplex detection and differentiation of *M. californicum*/*M. bovigenitalium* and *M. bovis*/*U. diversum*. Inner control sample amplification was detected with a FAM-labeled probe in all the methods.

Oligonucleotide specificity was evaluated with Nucleotide BLAST online (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PA-GE_TYPE=BlastSearch).

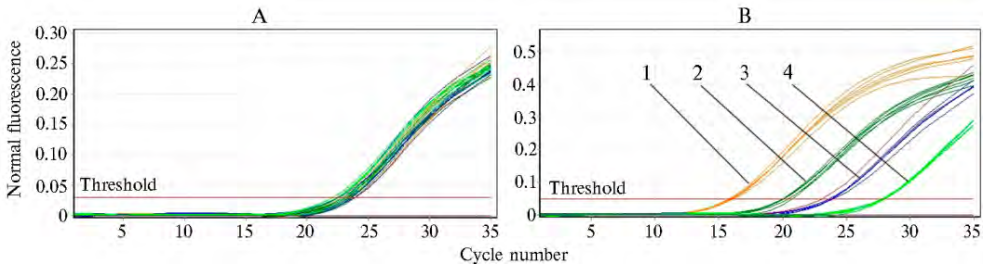
Selected nucleotides with specific targets demonstrated substantial homology and absence of significant homology with nucleotide sequences in other *Mollicutes*, viruses, bacteria or eukaryotes. Primer specificity was experimentally evidenced with a control panel including DNA of 32 strains of different microorganisms and bovine genome DNA. The panel testing showed 100% specificity.

To prevent false negative results, during DNA extraction we added an exogenous non-competitive inner control sample (ICS) amplified simultaneously with a specific target. ICS is a pAL2-T plasmid containing a synthetic DNA fragment. Added ICS enabled control of all the PCR stages for each sample.

To assess absolute primer sensitivity, we used 10-fold PCS dilutions with known concentration of a plasmid DNA containing cloned fragments of specific targets. Also, we amplified serial 10-fold PCS dilutions (5×10^5 to 5×10^2 copies/ml) in negative samples of semen, milk, bovine vaginal swabs and inner parenchymal organ suspension.

Tests were performed by different specialists using different equipment on different days. PCR efficiency was assessed automatically using RotorGene Q amplifier's software. Each sample was tested in 6 replicates. Analytical sensitivity was expressed as the lowest DNA PCS concentration providing a 6/6 positive signals in PCR.

Mean sensitivity of methods developed for different biological materials was 5×10^3 copies/ml. See milk testing data on the figure. Amplification efficiency for *M. bovis, M. bovigenitalium*, *M. californicum* and *U. diversum* was 99%, 87%, 94% and 98%, respectively.



Fluorescent signal accumulation during amplification of target DNA fragments extracted from 10-fold dilutions of positive control DNA samples in negative milk samples: A — ICS amplification (FAM fluorophore), B — *Ureaplasma diversum* amplification (HEX fluorophore); 1, 2, 3, 4 — DNA dilutions; concentration — 5×10^5, 5×10^4, 5×10^3 and 5×10^2 copies/ml, respectively.

We used developed methods to detect pathogenic mycoplasmas in samples of preserved semen collected in stud bulls. In total, 410 samples of semen supplied by national and foreign breeding centers were tested (see the Table). These samples underwent a pre-testing with a Mik-Kom test system (Amplisens, Russia) intended to detect DNA of *Mycoplasmae* in biological material [21].

***Mycoplasmataceae* detection by real-time PCR in samples of bovine semen intended for artificial insemination**

Pathogen	Breeding centers				Total (n = 410)	
	national (n = 211)		foreign (n = 199)			
	DNA detection	%	DNA detection	%	DNA detection	%
Mycoplasma spp.	182	86.3	127	63.8	309	75.4
M. bovis	0	0	5	2.5	5	1.2
M. californicum	109	51.7	44	22.1	153	37.3
M. bovigenitalium	128	60.7	50	25.1	178	43.4
Ureaplasma diversum	116	55.0	24	12.1	140	34.1

N o t e. n — number of samples..

Foreign semen demonstrated lower number of *Mycoplasmae*, in total, and pathogenic mycoplasmas, in particular, than national one. Nevertheless, *M. bovis* was detected in foreign semen only. The data were verified with a LSI VetMAX™ *Mycoplasma bovis* test system. Positive detection and differentiation of *M. bovis*, *M. bovigenitalium*, *M. californicum* and *U. diversum* were verified by sequencing.

Co-infection with several mycoplasmas was detected in 121 national (57.3%) and 31 foreign samples (15.5%). Co-infection with *M. californicum*/*M. bovigenitalium* was seen in 74 national and 24 foreign semen samples (23.7%); *M. bovigenitalium*/*U. diversum* were observed in 79 national and 7 foreign samples (21.0%). Co-infection with *M. bovigenitalium*, *M. californicum* and *U. diversum* was detected in 52 (24.6%) national and 4 (2%) foreign semen samples.

Since mycoplasmas often discharge with semen without any clinical presentations [22], pre-insemination semen test should be done to avoid infection and mastitis [23]. High incidence of bovine mycoplasmal mastitis, reproductive and respiratory disorders indicates an urgent need in verification of an etiological agent's mycoplasmal nature [1)]. According to our findings, we recommend our methods to be applied in a veterinary laboratory, to improve diagnosis and to optimize animal epidemic countermeasures, as well as to monitor quality of bovine semen intended for artificial insemination.

Thus, our methods based on a real-time polymerase chain reaction to identify and to differentiate pathogenic mycoplasmas (*Mycoplasma californicum*, *M. bovigenitalium*, *M. bovis* and *Ureaplasma diversum*), on the average, demonstrated high specificity and sensitivity (i.e., 5×10^3 of a target DNA copies/ml) in various material testings. High-degree mycoplasmal infection was detected in tested samples of frozen bovine semen intended for artificial insemination.

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DYNAMICS OF AMINO ACID PROFILE IN MILK OF BIV- AND BLV-BIV-INFECTED COWS DURING STORAGE

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Abstract

Leukemia and viral immunodeficiency of cattle rank to the number of difficult diagnosed infections of farm animals which increases the probability of virus carrying. At present time there are no legislative approved standards for bovine immunodeficiency diagnosis so the milk from sick cows can infect bulk milk obtained from healthy animals. Frequently bovine immunodeficiency virus (BIV) is detected in animals infected by bovine leukemia virus (BLV). The present work reveals that milk of cows infected by retroviruses has an altered amino acid balance of the casein fraction, as well as lower protein stability when stored in a refrigerator. The aim of our research was to assay the amino acid composition and evaluate its stability in milk of BIV-infected and BLV-BIV-co-infected cows, as compared to milk of healthy animals. A total of 6 samples of pure cow milk obtained from 2.8-6.7-year old black-and-white dairy cows infected by bovine immunodeficiency virus (n = 3) and cows with BLV-BIV coinfection (n = 3) were tested. BIV and BLV-BIV infection was approved by PCR tests. Analysis of amino acid composition of the protein fraction was performed using a capillary electrophoresis system Capel 105M (LLC "Lumex-Marketing", Russia). Milk was tested on day 1, day 3 and day 6 of storage at 4 °C. The standard data on the amino acid balance of the casein fraction of healthy cows' milk was used for the comparative analysis. Our results indicate that milk from BIV and BLV-BIV infected cows differs significantly in amino acid composition from healthy cows' milk. We consider the milk casein fraction to be more illustrative because casein is the main milk protein and its amino acid profile in not changed even under hydrolysis. The weight percentage of the essential and conditionally essential amino acids such as methionine, glutamine, histidine and glycine in infected milk reduces 3-4-fold. The content of arginine, lysine, proline and asparagine are similar to normal, tyrosine is passed into the limiting position under a deficient phenylalanine which content is 6 times lower than normal. There was a comparative excess amount of threonine and serine. The weight percentage of lysine, valine, leucine-isoleucine and glutamine is 9, 13, 17.5 and 22 times lower that in healthy cows' milk. The leucine-isoleucine and valine ratio of infected milk is approximately 1:1 whereas it amounts to 2.5:1 in healthy cows' milk. It was observed that milk of BIV and BLV-BIV infected cows is characterized by expressed dynamic of amino acid profiles during storage in a refrigerator. The essential amino acids arginine, histidine and methionine were not detected in the milk of infected cows on the sixth day of the storage in the refrigerator. The weight percentage of tyrosine increased on day 3 and decreased on day 6 of storage. Moreover, these parameters were significantly changed in BIV infected milk. The weight percentage of phenylalanine, leucine-isoleucine, valine, serine and glycine in BIV and BLV-BIV infected milk increased on day 6 of storage by 27.1 and 2.4 %; 22.3 and 8.9 %; 43.9 and 37.2 %; 25.0 and 27.3 %, and 0 and 60 %. The weight percentage of proline and threonine reduced on day 6 of storage by 10.6 and 13.7 %; 5.3 and 0 %. The weight percentage of alanine on day 6 in BIV infected milk increased by 37.5 % but in BLV-BIV infected milk reduced by 17.8 %. The imbalance and instability of amino acid profile of BLV and BLV-BIV infected milk prove are indicative of development of uncontrolled processes.

Keywords: cow's milk, milk protein, amino acids, immunodeficiency virus, leukemia

According to the Institute of Nutrition of RAMS, annual dietary protein deficiency is more than 1.6 mln tons in Russia. Total global protein deficiency is

10-25 mln tons per year [1]. Along with this, each person accounts for 60 g of protein daily. Nitrogen body balance studies revealed that the best daily dosage of protein consumed by a healthy adult is 0.8 g per kg body weight. The value can vary depending on mental and physical load, sex, age and physiological state. Thus, 88 g per day is considered a normal protein diet for a young healthy man experiencing the minimum physical load [2]. Along with quantity, quality of protein (including balanced amino acid ratio) is also associated with protein nutritive value for a human body. Contrary to plant proteins, animal ones contain a well-balanced ratio of all the necessary amino acids. Plant products contain substantially lower amount of essential amino acid that animal ones. Even soy protein, despite the best possible amino acid composition, is short of methionine (i.e., an essential amino acid) [3].

Typically, each person consumes cow milk regardless of age, and its proper amino acid composition is well-balanced. A human body digests 96-98% of cow milk proteins. It was found that milk proteins stabilize blood pressure in hypertonic patients. Casein fraction represents about 80% of milk proteins. Namely, its average content in cow milk is 2.1-2.9% [4].

Enzymic hydrolysis of milk protein starts during storage of raw milk. It is performed by proteolytic enzymes of milk microflora and a starter, as well as by plasmin enzyme [5, 6]. Peptides are predominant products of enzymic milk hydrolysis. In this view, free amino acid fraction is comparatively small. Milk peptides stimulate secretion of insulin and growth hormone. Also, they have a positive impact on digestion of essential micronutrients (including calcium ions) [7]. Resulted from milk hydrolysis, peptides, apart from high digestibility, inhibit desquamation of intestinal mucosa cells and stimulate production of endogenous enzymes [8]. Low-molecular milk peptides penetrate into muscles rapidly causing a strong insulinotropic effect. Replenishing energy reserves of a human body, they nourish muscles with amino acids leading to promoted synthesis of muscle proteins. Microfractions of low-molecular serum proteins (i.e., glycomacropeptides) reduce risk of viral diseases, have positive influence on digestion and calcium/protein assimilation, and induce development of normal intestinal microflora [9].

Uncontrolled and unpredictable proteolysis induced by proteolytic enzymes of secondary microflora (including putrefactive one) can result in formation of several compounds with foreign odor and flavor in raw milk. Along with this, free amino acids undergo fermentation that causes their interamination, deamination and decarboxylation leading to formation of keto acids, oxy acids, carboxylic acids and aldehydes [10]. Deep degradation of milk proteins induced by foreign microorganisms leads to formation of free amino acids that, in turn, affect organoleptic properties of milk. For example, alanine, glycine, proline and serine are sweet; asparaginic and glutamic acids are sour; arginine, leucine, histidine and tryptophan are bitter; methionine and cystine are sulphuric; threonine, valine and phenylalanine are bittersweet [11]. Moreover, free amino acids are in the running to use amino acid channels. In turn, a great amount of free amino acids induces gastrointestinal disorders [12].

Quality, biological and nutritive values of cow milk depend on a breed, management conditions, feeding and health status of an animal. Most of diseases (including chronic infections) can lead to a persistent decrease in cow milk yields and lower quality of dairy products. It has been established that enzootic bovine leukosis virus (EBLV, bovine leukemia virus, BLV) in dairy cows results in somatic cell content in milk $(4.9\text{-}5.2)\times10^5/cm^3$; milk yields decrease by 13.3-15.5% [13] that, in turn, results in a significant economic damage to livestock production [14]. BLV-infected cows demonstrate decreased ferritin and iron lev-

els in milk. Nevertheless, serum ferritin level increases being a marker of inflammation and malignant processes [15].

BLV-infection is associated with reduction of total body resistance and breast immunity inhibition. This results in less efficient phagocytosis followed by a substantial bacterial count (up to $2\times10^7\pm4\times10^2$ CFU/ml) in milk of an infected cow [16]. A BLV-infected cow shows a significant decrease in apoptotic B cell fraction and CD44 neutrophilic expression (especially, in an animal with persistent lymphocytosis) [17]. Also, levels of total protein and amino acids (including 5 essential ones, i.e., isoleucine, tryptophan, methionine, leucine and phenylalanine) decline. Electrophoresis assay of milk protein demonstrates increased fractions of proteose peptones, lactalbumins and immunoglobulins associated with decreased serum albumin fraction [18].

In general, leukosis is accompanied by another chronic bovine infection (i.e., viral immunodeficiency (BVI)). Similarly to leukosis virus, bovine immunodeficiency virus (BIV) is tropic to lymphocytes and induces persistent immune disorders. Although BVI is a relatively new disease, it is even more incident in a sick flock than EBL [19, 20].

According to our prior studies, milk of BIV- and BLV-infected cows differs significantly from healthy cows' milk in protein composition [16]. Profile of milk proteins and peptides, as well as ratio of free and bound amino acids can vary in cows with immune deficiency. Nowadays, there are no available data on alteration in amino acid composition of refrigerated milk collected from BIV- and BLV-co-infected and BIV-infected cows. Being of great academic interest, the data have a practical significance because this can justify milk biochemical transformations and draw a conclusion about a biological value of the product. From our point of view, data on milk casein fraction are the most meaningful since even hydrolysis cannot change amino acid composition of the protein.

In the work presented here we newly detected that milk of cows infected with retroviruses has altered amino acid balance of a casein fraction; also, the protein demonstrates lower stability when milk is stored in a refrigerator at 4 °C.

Our purpose was to compare and to evaluate stability of amino acid composition of casein fraction in milk of cows infected with bovine immunodeficiency virus, cows co-infected with bovine leukosis and bovine immunodeficiency viruses, and healthy cows' milk.

Techniques. We tested 6 samples of whole milk collected from BLV- and BIV-co-infected (n = 3) and BIV-infected (n = 3) black-and-white dairy cows (*Bos taurus taurus*) aged 2.8-6.7 years.

Nucleic acids were extracted and purified with a DNK Sorb B kit (InterLabServis, Russia). Presence/absence of BLV and BIV proviruses in whole blood samples was established by a conventional PCR method with LEIKOZ kit (InterLabServis, Russia) and by our own multiplex PCR (Patent of the Russian Federation no. 2615465) with specific primers synthesized by Syntol ZAO (Russia), PCR-Mix kit and application buffer (Lytech Research and Production Company, Russia). Amplification was performed in a T100 thermocycler (Bio-Rad, USA). Amplification products were detected by a horizontal electrophoresis in 2% agarose gel with 0.5 μg/l of ethidium bromide with an EP kit (InterLabServis, Russia) under standard conditions with photographic recording. The results were recorded on GelDoc XP PLUS equipment (Bio-Rad, USA). Commercial kits and equipment were used as per guidelines of their manufacturers and developers.

Clinically evident mastitis was not mentioned in cows. Also, milk organoleptic properties remained normal.

During sample preparation whole milk (10 ml) was centrifuged at 5 °C

and 10,000 rpm for 30 min. Then, we separated precipitate and upper lipid fraction. In constant stirring, we added hydrochloride acid dropwise at 37 °C to adjust pH at 4.5. Further, we continued stirring for 30 min. Resultant substance was centrifuged at 25 °C and 5,000 rpm for 10 min, and supernatant was separated. The precipitate pH value was adjusted at 6.5 with 1 M sodium hydroxide solution.

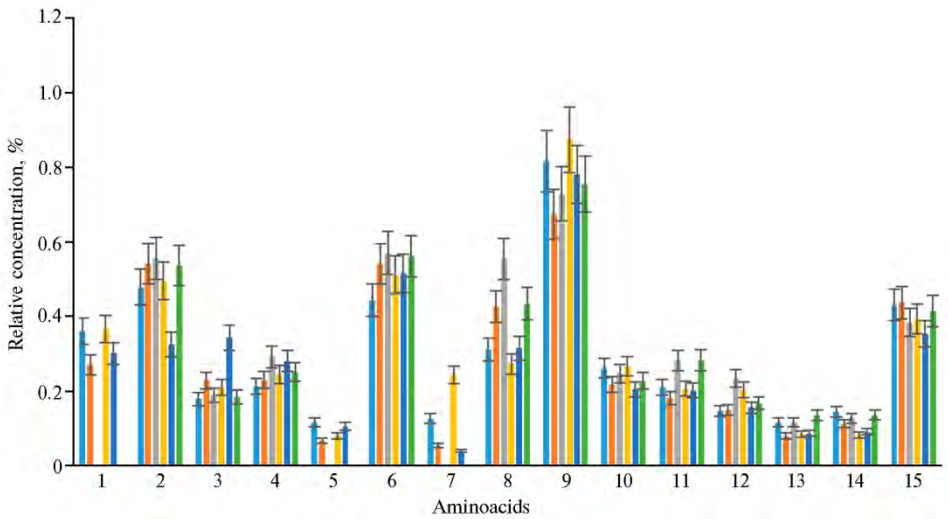
We assessed amino acid composition of protein fractions with Capel 105M capillary electrophoresis system (LLC Lumex-Marketing, Russia; procedure no. M-04-38-2009, amendment no. 1 dated 01.02.2010) as per the manufacturer's guidelines. Milk was tested on days 1, 3 and 6 of storage in a refrigerator at 4 °C.

To perform a comparative analysis, we used common standard data on amino acid balance of casein fraction in healthy cows' milk.

We processed our data in Statistica 8.0 software (StatSoft, Inc., USA). Difference significance was evaluated with Mann-Whitney U test. Statistically significant differences must comply with 95% ($p < 0.05$). The paper presents mean weight percentages of primary amino acids in a milk casein fraction (M) and standard deviations ($\pm\sigma$).

Results. According to follow-up stability studies of amino acid composition of infected cows' milk, amino acid content is very variable (Fig.). This can be caused by active biochemical processes in milk.

Arginine, methionine and histidine demonstrated the most variable weight percentages because they were below the limit of detection on the day 6 of the storage in a refrigerator. Other amino acids showed relatively stable (i.e., threonine, glutamine, asparagine, phenylalanine, proline, leucine-isoleucine) or even more variable levels (serine, glycine, alanine, lysine, valine, tyrosine and glutamine). Most of these changes were random. The content increased on day 3 (tyrosine) or the day 6 (phenylalanine, valine, serine, glycine) of the storage. Equally, it decreased eventually (i.e., threonine, proline).



Following weight percentages of amino acids were mentioned in milk of BIV (bovine immunodeficiency virus)**-infected or BLV** (bovine leukemia virus)**-co-infected black-and-white cows (*Bos taurus taurus*) when stored in a refrigerator at 4°C:** 1 — arginine, 2 — lysine, 3 — tyrosine, 4 — phenylalanine, 5 — histidine, 6 — leucine + isoleucine, 7 — methionine, 8 — valine, 9 — proline, 10 — threonine, 11 — serine, 12 — alanine, 13 — glycine, 14 — glutamine, 15 — asparagine; ■ ■ ■ ■ ■ ■ — on day 1 (BIV), day 3 (BIV), day 6 (BIV), day 1 (BLV-BIV), day 3 (BLV-BIV), and day 6 (BLV-BIV), respectively ($M\pm\sigma$). Differences are statistically significant at $p < 0.05$.

It is known that casein fraction of cow milk has the most stable amino acid composition. Considering a lactation phase, it slightly (0.01-0.15%) varies in black-and-white cows depending on amino acid concentration [21].

Relative content of amino acids (normal percentage, %) **in milk of BIV** (bovine immunodeficiency virus)**-infected or BLV** (bovine leukemia virus) **+ BIV-co-infected black-and-white cows after storage in a refrigerator at 4 °C** ($M \pm \sigma$)

Amino acid	BIV			BLV-BIV		
	day 1	day 3	day 6	day 1	day 3	day 6
Arginine	8.8±0.9	5.6±0.6*	–*	8.9±0.9	6.6±0.6*	–*
Lysine	5.8±0.6	6.6±0.6*	6.8±0.7*	6.1±0.6	6.6±0.7	6.5±0.6
Phenylalanine	4.2±0.4	4.6±0.4	5.8±0.6*	4.9±0.5	4.6±0.4*	5.0±0.5*
Tyrosine	2.1±0.2	2.8±0.3*	2.3±0.2	2.6±0.2	4.2±0.4	2.2±0.2
Histidine	3.7±0.3	2.1±0.2*	–*	2.4±0.2	2.1±0.2*	–*
Leucine + Isoleucine	2.9±0.3	3.5±0.3*	3.7±0.3*	3.3±0.3	3.5±0.3	3.6±0.3
Methionine	4.5±0.5	1.4±0.1*	–*	8.6±0.8	1.9±0.2*	–*
Valine	4.3±0.4	4.4±0.4	7.7±0.7*	3.8±0.3	5.9±0.6*	6.0±0.6*
Proline	7.2±0.7	6.9±0.7	6.5±0.6*	7.7±0.7	6.0±0.6*	6.7±0.7*
Threonine	5.3±0.5	4.2±0.4*	5.1±0.5	5.4±0.5	4.4±0.4*	4.6±0.4*
Serine	3.3±0.3	3.2±0.3	4.4±0.4*	3.3±0.3	2.9±0.3*	4.5±0.4*
Alanine	4.8±0.4	5.1±0.5	7.8±0.8*	6.7±0.6	4.9±0.5*	5.5±0.5*
Glycine	4.3±0.4	3.1±0.3*	4.3±0.4	3.1±0.3	2.9±0.3	5.0±0.5*
Glutamine	0.6±0.1	0.4±0.1*	0.6±0.1	0.4±0.1	0.5±0.1*	0.6±0.1*
Asparagine	6.1±0.6	6.2±0.6	5.4±0.5*	5.5±0.5	5.9±0.6	5.8±0.6

N o t e. Dashes stand for amino acid level below the limit of detection (arginine and histidine level < 0.50%; methionine < 0.25%).

* Differences with the values on day 1 are statistically significant at $p < 0.05$.

The table illustrates amino acid levels in milk of BLV-BIV- and BIV-infected cows as compared with reference means in healthy animals [22]. Mean amino acid levels in milk of BIV-infected cows were 5-20 times lower than in healthy ones (see the Table), and better part of essential amino acids (such as methionine, glycine, histidine, glutamine) demonstrated trace levels. Substantial changes were mentioned in amino acid balance of milk. Even though infected cows showed substantially lower levels of tyrosine, leucine and serine than healthy ones, arginine, lysine, proline and asparagine levels were closer to normal values.

As for humans, reference amino acid dietary intake, in general, is calculated considering dietary features and individual needs. While evaluating amino acid balance, content of 11 essential amino acids (i.e., lysine, methionine, tryptophan, valine, arginine, histidine, leucine, isoleucine, phenylalanine, threonine and glycine) is considered as a limitation. Other 10 amino acids (asparagine, alanine, aspartate, glutamate, glycine, glutamine, serine, proline, tyrosine and cysteine) are regarded as replaceable ones. However, this is an uncertain division [23]. In particular, milk of BIV-infected cows demonstrated deficiency of arginine and histidine. At the same time, due to lack of phenylalanine, tyrosine became a limitative factor despite a sufficient content.

Several persons need increased reference daily intake of amino acids. Concurrently, excessive amino acids can have an adverse impact because a body must spend more energy to dispose them [24]. An excessive amino acid can replace a deficient one in metabolism. In turn, this can lead to skeletal disorders, toxicosis and increased adipopexia. We detected relatively excessive serine and threonine levels in milk of BIV-infected cows. Similar trend was mentioned in cows co-infected with BIV and BLV (see the Table).

To prevent metabolic disorders, amino acid balance index (i.e., dietary amino acid ratio) should be maintained. This is caused by antagonism between amino acids with similar structure (i.e., threonine-tryptophan, arginine-lysine, leucine-isoleucine) during metabolism [25]. We revealed a balance conflict between leucine-isoleucine and valine because their weight percentage must be

about 2.5:1 [22] in healthy cows' milk whereas it was 1:1 in stored milk of infected cows. Altered amino acid metabolism (i.e., their relative ratio) leads to change in whole body homeostasis. Leucine is a significant essential amino acid because it is required to stimulate protein synthesis and to inhibit cell proteolysis. Valine has less diverse functions within a body [4, 25].

According to several foreign works, to provide early diagnosis of bovine subclinical mastitis, milk amino acid composition should be assayed by high-performance liquid chromatography [26]. Moreover, indicator values associated with the most common staphylococcal mastitis are mentioned there [27]. However, since these data are rarely reported, these veterinary studies are important and promising.

Thus, amino acid levels in milk of cows infected with bovine immunodeficiency virus or co-infected with bovine leukemia and immunodeficiency viruses was 5-20 times lower than in healthy cows. Essential and conditionally essential amino acids (i.e., methionine, glutamine, histidine and glycine) showed more significantly decreased weight percentages. Infected cows demonstrated substantially lower levels of tyrosine, leucine and serine than healthy ones. Less significant decrease was mentioned in arginine, lysine, proline and asparagine levels whereas tyrosine became a limitative factor as a result of phenylalanine deficiency. Excessive relative concentrations of threonine and serine were detected. Disproportional weight percentage of leucine-isoleucine and valine was seen in milk of healthy and infected cows. The storage even got this worse. BIV- and BLV-BIV-infected cow milk demonstrated an evident alteration of amino acid composition when stored in a refrigerator. Several essential amino acids (i.e., arginine, histidine and methionine) were not detected in refrigerated milk of infected cows on the day 6 of storage. Tyrosine weight percentage increased on the third day of the storage followed by further decrease. Although weight percentage of phenylalanine, leucine, isoleucine, valine, serine and glycine increased on the day 6 of the storage, proline and threonine demonstrated reduced weight percentages. Imbalanced and instable amino acid composition of milk collected in BLV-BIV- and BIV-infected cows indicates uncontrolled inner processes. Infected animals should be timely removed from a flock, and bovine health monitoring system should be improved.

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CHARACTERIZATION OF MESENCHYMAL STEM CELLS ISOLATED FROM FELINE AND CANINE ADIPOSE TISSUE

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Abstract

Multipotent mesenchymal stem cells (MMSCs) are a promising tool of regenerative medicine for treatment of various small animal diseases. MMSCs have a high proliferative activity, multipotent properties, low immunogenicity, as well as the ability to migrate to the damaged tissue and promote its healing and regeneration. Currently, the methods of regenerative medicine are actively developing to solve problems that are difficult to cope with alternative treatments. However, data on the use of these cells in the clinic are ahead of the study of the properties of these cells in culture. This paper is our first report on isolation cells with phenotype similar to mammalian multipotent mesenchymal stem cells from feline and canine adipose tissue. The aim of the presented research was to study the cells properties with a phenotype similar to MMSC isolated from feline and canine adipose tissue (AT) in vitro. Isolation of cells was achieved by mechanical and enzymatic treatments of the AT. For enzymatic dissociation, the tissues were treated with a 0.01 % collagenase type II solution based on DMEM-LG (PanEco, Russia) with low glucose (1 g/l) at 37 °C for 60 min. The comparative analysis of properties of the derived cellular populations is carried out. Cells isolated from feline and canine adipose tissue had similar morphological characteristics and were represented by two cellular types: small round cells and larger narrow spindle like fibroblast. They had a strong adhesion to cultural plastic and high colonies formation ability, 88.3±0.10 % for feline MMSCs and 88.0±0.15 % for canine MMSCs. The generation time of feline MMSCs was 34.6±0.02 h, while in canine MMSCs it was 50.0±0.01 h. Mitotic index of feline and canine MMSCs was 3.4 ‰ and 2.7 ‰, respectively. The ability of the MMSCs to induced osteo-, chondro- and adipogenic differentiation in vitro was demonstrated using StemPro® Osteogenesis Differentiation Kit, StemPro® Chondrogenesis Differentiation Kit и StemPro® Adipogenesis Differentiation Kit (Gibco, USA), respectively. Adipogenic differentiation accompanied by the appearance of rounded cells with lipid vesicles in the cytoplasm that were identified with the specific dye Oil red O. Specific staining of feline and canine MMSCs for endogenous alkaline phosphatase was positive on day 14 of culture in the induction medium. MMSCs stained by von Kossa revealed extracellular matrix formation on day 21 after induction. Alcian blue staining of cells cultured in chondrogenic medium for 21 days visualized formation of round structures with isogenic groups similar to the lacunae of hyaline cartilage. Thus, it was shown that cells isolated from feline and canine adipose tissue exhibit in culture the properties of MMSC. The derived cell cultures were propagated and deposited to Kovalenko VIEV Specialized Collection of somatic cell cultures of farm and commercial animals.

Keywords: multipotent mesenchymal stem cells, adipose tissue, culture, induced differentiation in vitro, feline, canine

Development of methods of stem cell derivation from animal tissues without a health damage, as well as their culture and storage provided wide use of these cells in various scientific branches. As laboratory models, the cell cultures used in cell biology, genetics, toxicology, virology, medicine and biotechnologies for a long time. In terms of veterinary virology, they are applicable to viral reproduction studies, di-

agnostic testings and production of various antiviral products. To this end, diploid cell cultures derived from animal organs/tissues and their fetuses are often used. These cell cultures have several disadvantages (including lack of standardization and short-term culture (up to 50 cytogenerations)). Continuous immortalized (immortal) cell lines are the most promising in this regard. However, their application is restricted with loss of tissue specificity resulted from long-term culture that leads to viral attenuation. Moreover, continuous cells isolated from farm animal tissues are typically contaminated with viruses. In light of this, multipotent mesenchymal stem cells (MMSCs) derived from animals can be considered as a new cell model that has several advantages over diploid and continuous cell cultures.

Mammalian MMSCs are promising tool to solve many veterinary, medical and biotechnological issues. Human MMSCs were detected in bone marrow (BM), adipose tissue (AT), skeletal muscles, placenta, umbilical blood and other tissues [1]. MMSCs have unique properties. They can maintain genome stability during self-renewal in vitro (> 50 cytogenerations) for a long time, and form bone, chondral and adipose cells in vitro during induced differentiation [2]. Previously, we isolated MMSCs from equine umbilical blood [3], as well as from bovine BM and AT [4]. MMSCs extracted from equine umbilical blood are suitable for equine infectious anemia virus study [3]. Small domestic animal MMSC cultures should be obtained, in particular, due to their promising application to manufacturing biotechnology of carnivore antiviral products (including vaccines to combat canine, mink, feline and fox parvoviral enteritis, distemper and, of course, rabies).

Nowadays, a great focus is placed on human MMSC biology in vitro studies. At the same time, the cells are regarded as a biomedical cell product (Federal Law no. 180-FZ 'On Biomedical Cell Products' dated 23.06.2016). It is generally believed that, in terms of cell therapy, MMSCs act as immunosuppressors [5]. According to reported data, similar studies involve small domestic animals, i. e., cats [6-9] and dogs [10, 11]. MMSCs represent promising cell material to treat osseous and chondral disorders (including osteoarthritis and herniated disks in cats and dogs) [12-14]. In this regard, we need a great number of cells obtained with their conversion to culture. The optimum culture conditions to provide efficient increase in cell mass are of primary concern. It was noted that current practical MMSC application, unfortunately, anticipates their studies in culture. In this view, these cell properties should be assessed beyond tissue/organ they were derived from. Preclinical studies of biomedical cell products are conducted in laboratory animals (i.e., mice or rats). In turn, this often complicates interpretation of a study findings. In this light, cats and dogs can be more suitable models, and their MMSC isolation can solve an issue related to pre-clinical study control. Isolation and study of MMSC properties in culture as per appropriate human cell standards enable development of large banks and national collections of certified stem cell cultures.

In the paper we derived canine and feline adipose cells demonstrating all the primary features of multipotent mesenchymal stem cells. Also, we obtained their cultures. Chemically induced cells differentiate into adipocytes, chondrocytes, and osteocytes. It was found that feline MMSC cultures have higher proliferative activity.

Our purpose was to isolate cells with a phenotype similar to multipotent mesenchymal stem cells from feline and canine adipose tissue in vitro, and to study their properties in culture.

Techniques. AT was collected in 8-month-cats (n = 3) and 1-year-dogs (n = 3) experienced ovariectomy as per the Declaration of Helsinki (World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects, 1964-2013) in the veterinary clinic of Kovalenko All-Russian Research Institute of Experimental Veterinary. These samples

were shipped to a laboratory for < 30 min. Cells were isolated as per the previously described method (16). Finished inner AT sample (2-4 g) was carefully washed with PBS (PanEco, Russia) without Ca^{2+} and Mg^{2+} ions. Then, it was pulverized and subjected to enzymatic treatment with 0.01% collagenase type II solution based on DMEM LG (PanEco, Russia) with low glucose (1 g/l) at 37 °C for 60 min. Collagenase action was neutralized with equal volume of DMEM LG medium with 10% fetal bovine serum (FBS), and centrifuged at 1000 g for 10 min. Cells were washed twice in DMEM LG medium with antibiotics (final streptomycin and penicillin concentrations are 50 μg/ml and 50 U/ml, respectively). Further, it was precipitated with centrifugation at 800 g for 10 min.

After final precipitation we added DMEM LG to cell precipitate. It was resuspended carefully and filtered through cell grids (SPL Life Sciences Co., Ltd., Korea) on a step-by-step basis. At the first stage stromal vascular fraction (SVF) cells were collected with 70 μm grids. To select stem cells, we filtered SVF through 10 μm filters.

Counted in a Goryaev chamber (1.2×10^6), cells were placed in a culture flask with 25 cm^2 growth surface area. DMEM LG with 10% FBS (HyClone, Perbio Scientific, Belgium) and antibiotics (final streptomycin and penicillin concentrations are 50 μg/ml and 50 U/ml, respectively) was a primary MMSC growth culture medium. In 24 h the medium was replaced by a fresh one; adhesive cells were caused to grow in a CO_2-incubator (5% CO_2, 37 °C).

Duration of tested MMSCs' cell cycle was assessed in virtue of doubling time. G_0-pool was not considered. Growth rate of cellular populations was evaluated in virtue of cell number alteration until a monolayer was formed. Mean doubling time was calculated as per the formula as follows: $t_d = t/\log_2 (N_t/N_0)$, where t_d — doubling time, t — a period between an initial and a final cell countings, N_0 and N_t — initial and final cell count, respectively [17]. Mitotic index of each cellular population was calculated during the logarithmic phase. It is expressed as a ratio of generation number and total cell count multiplied by 1000 (‰).

Native and Romanowsky-Giemsa-stained cell morphology was evaluated visually (Axio Observer D.1 inverted phase-contrast microscope, Carl Zeiss, Germany, magnification ×100, ×200, ×630; AxioVision Rel. 4.8 software, Carl Zeiss, Germany).

Clone formation efficiency was assessed during cell plating (1.5×10^3) in culture flasks (25 cm^2). It was expressed as ratio of total cell count and number of clones formed on day 10 of culture.

Feline and canine ability to inductive differentiation into adipocytes, chondrocytes, and osteocytes in vitro was studied with StemPro® Osteogenesis Differentiation Kit, StemPro® Chondrogenesis Differentiation Kit and StemPro® Adipogenesis Differentiation Kit (Gibco, USA). In this regard, MMSCs were plated in 12-well plates (SPL Life Sciences Co., Ltd., Korea) (1×10^5 cells/well) at 2-3 passages. When cells reached 70-80% of a monolayer, we removed working medium and added induction media as per the manufacturer's guidelines. Induction media were replaced every 4 days for 21 days. Feline and canine MMSC differentiation was evaluated on day 14 and day 21. Cells were fixed with ice-cold methanol (–20 °C) for 10 min. Then, they were stained with specific colorants. All the colorants were produced by Sigma-Aldrich (USA). Cell alkaline phosphatase (AP) was assayed with Alkaline Phosphatase Kit (Sigma-Aldrich, USA). To detect phosphates and carbonates associated with the osteogenic differentiation of MMSCs, cells were stained by von Kossa method according to the reagent manufacturer's guidelines. During silvering fixed cells were treated with 2% aqueous

$AgNO_3$ solution for 10-15 min. Then, they were placed under direct lamp light (60 W) for 1 hour. Cells were washed with distilled water and treated with 2.5% aqueous sodium thiosulphate solution for 1 min. They were washed with tap water and put under a microscope (Axio Observer D.1, Carl Zeiss, Germany, magnification ×100, ×200, ×630). To assess chondrogenic MMSC differentiation, we used Alcian blue stain (LabPoint, Russia) staining mucopolysaccharides produced by chondral cells in extracellular matrix. Adipogenic differentiation efficiency was evaluated with Oil Red O staining to detect lipid inclusions in cell cytoplasm. Nuclei were counterstained with hematoxylin.

To perform statistical processing, we calculated arithmetic mean (*M*) and its standard error (±SEM). Difference significance was evaluated with Student's *t*-test ($p < 0.05$).

Results. MMSCs derived from canine and feline AT demonstrated strong adhesive properties. In 24 h after the isolation they were detected adherent to a culture flask bottom. This meets one of the minimum obligatory criteria for culture mammalian MMSCs [2]. Cell number increased on day 4 of culture. Cells derived from feline and canine AT had similar morphological properties (Fig. 1, A, B). Final cellular populations represented two following cell types: small round cells (⌀=10±0.2 μm, arrowed in the Figure) and larger (⌀=20±0.5 μm) narrow spindle ones with fibroblast-like morphological properties. A single cytogeneration took 34.6±0.02 h and 50±0.01 h in three feline and canine MMSC cultures, respectively. Feline and canine MMSC mitotic indices were 3.4‰ and 2.7‰, respectively (see the Table). Feline MMSCs formed a monolayer on day 12 (Fig. 1, C) of culture when 1×10^4 cells/cm^2 were plated; canine MMSCs formed a monolayer on day 15 under the same conditions (Fig. 1, D). According to the tabulated data, MMSCs derived from canine and feline AT showed high clone formation efficiency. Also, this verifies their affiliation with MMSCs.

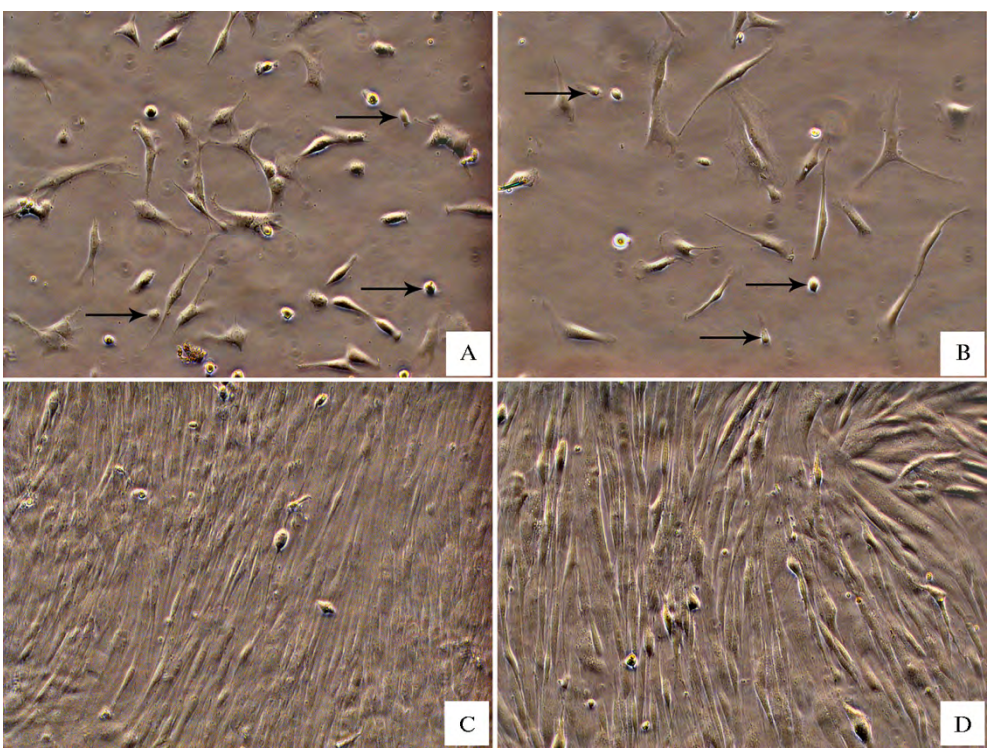

Fig. 1. Multipotent mesenchymal stem cell (MMSC) phenotype cells derived from small domestic animal adipose tissue on day 4 after isolation (A — feline MMSCs, B — canine MMSCs)**; on day 12 (mon-**

olayer) after isolation (C — feline MMSCs) **and on day 15 (monolayer) after isolation** (D — canine MMSCs). Small round cells are arrowed. Native preparation, phase-contrast microscopy (microscope Axio Observer D.1, Carl Zeiss, Germany; magnification ×200).

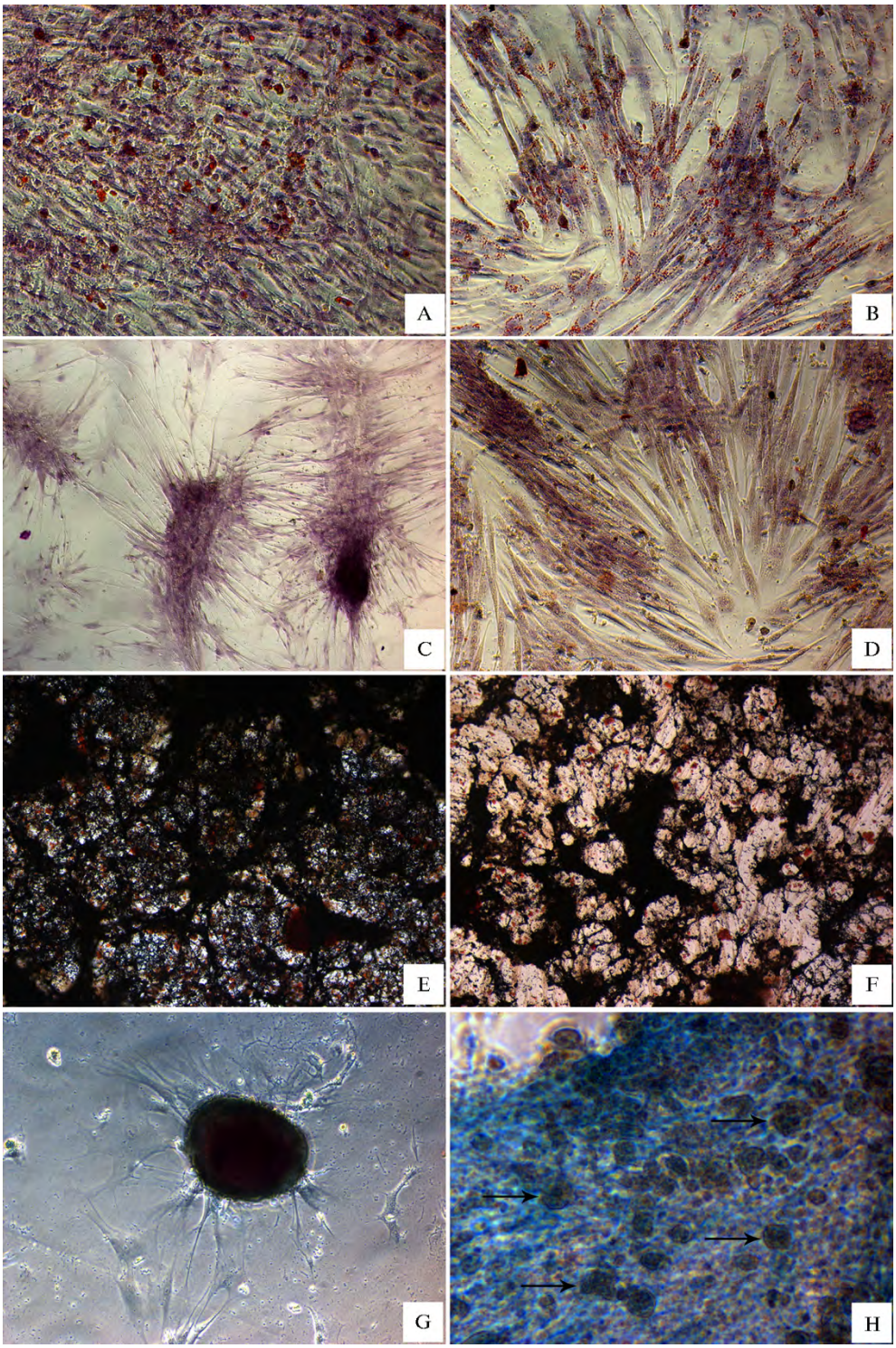

Fig. 2. Ability of induced multipotent mesenchymal stem cells (MMSCs) **derived from small domestic animals' adipose tissue to form adipose, osseous and chondral tissues:** A, B — feline and canine MMSCs, respectively, on day 21 of culture (Oil Red O staining, adipogenic medium); C, D — feline and canine MMSCs on day 14 of culture (alkaline phosphatase staining), E, F — feline and canine MMSCs on day

21 (von Kossa silvering) (osteogenic medium); G, H — feline and canine MMSCs on day 21 (Alcian blue staining, chondrogenic medium). Isogenic groups whose morphological properties are similar to hyaline cartilage lacunae are arrowed. Phase-contrast microscopy (microscope Axio Observer D.1, Carl Zeiss, Germany), magnification - ×200 (A, B, D, G), ×100 (C, E, F) and ×630 (H)..

Properties of multipotent mesenchymal stem cell (MMSC) **culture in vitro derived from feline and canine adipose tissue** (M±SEM, n = 3)

Cell culture	Sample	Metaphases/cell count	MI, ‰	Cytogeneration time, h	Clone formation efficiency, %
Feline MMSCs	1	34/1000	3.4	36.0±0.02	89.0±0.22
	2	33/1000	3.3	33.0±0.04	89.0±0.01
	3	36/1000	3.6	35.0±0.04	87.0±0.07
	Average	34.3/1000	3.4	34.6±0.02	88.3±0.10
Canine MMSCs	1	28/1000	2.8	50.0±0.01	88.0±0.40
	2	24/1000	2.4	52.0±0.02	87.0±0.01
	3	30/1000	3.0	48.0±0.06	89.0±0.05
	Mean	27.3/1000	2.7	50.0±0.01	88.0±0.15

Note. MI is mitotic index. Means (M) and their error (±SEM) of three independent tests are presented

Previously it was shown that MMSCs derived from feline [18-21] and canine [22, 23] AT are stained with fluorochrome-labeled antibodies (AB) to CD29 (β-1 integrin), CD44 (hyaluronic acid receptor), CD90 (Thy-1), CD105 (endoglin) and CD166 (ALCAM) antigens (AG), and are not stained with AB to AG markers of hematopoietic cells CD34 (sialomucin), CD45 (LCA, leukocyte common AG) and CD73 (5′-terminal nucleotidase)). In this case, we evaluated the cell affiliation with MMSCs according to their functional properties (i.e., in vitro induced adipose, osseous and chondral cell formation). Cells derived from feline and canine AT demonstrated morphological changes even in 14 days after induction of medium containing adipogenic growth factors. Adipocyte differentiation was accompanied by formation of round cells with lipid vesicles in cytoplasm which were detected with a specific Oil Red O staining (Fig. 2, A, B).

It is a known fact that a direct relationship is seen between AP cell level and osteogenic differentiation [16]. Feline and canine MMSC staining for specific activity of endogenous AP demonstrated positive results even on day 14 of culture in the induction medium (Fig. 2, C, D). In 21 days after the induction (von Kossa silvering) we detected extracellular matrix in vitro as indicated by black coloration of phosphates and carbonates (Fig. 2, E, F). This evidences an ability of isolated MMSCs (i.e., both types) to osteogenic differentiation in vitro.

Round structures stained with Alcian blue were observed after 21-day cell culture in the chondrogenic medium (Fig. 2, G). Mature chondroblasts start to produce chondral intercellular matrix containing mucopolysaccharides [24] that turn blue with the stain. Although cells could be hardly differentiated morphologically in preparations, isogenic groups whose morphological properties are similar to hyaline cartilage lacunae were visualized (Fig. 2, 3). Thus, MMSCs derived from feline and canine inner AT can form cells similar to osseous, adipose and chondral ones after induced differentiation in vitro. Our findings comply with other reported data [25-27]. Owing to maintained functional abilities in culture, we can assume that feline and canine MMSCs can be a promising material to treat various osseous and chondral disorders in small domestic animals. Our results improve our knowledge concerning cell behavior in culture. So, we can take a step closer to further understanding of processes occurring in progenitor cells in vitro.

Therefore, we obtained multipotent mesenchymal stem cell (MMSC) cultures isolated from feline and canine adipose tissue (AT). These isolated cells exhibit all the primary MMSC properties such as strong adhesion to plastic surface, high clone-forming ability (88.3±0.10% for feline MMSCs vs. 88.0±0.15%

for canine MMSCs), as well as differentiation into adipocytes, chondrocytes, and osteocytes after chemical induction in vitro. Comparative analysis of feline and canine MMSCs revealed insignificant differences. Despite similar morphological properties of cell cultures, a single MMSC cytogeneration took 34.6±0.02 h and 50.0±0.01 h in cats and dogs, respectively; mitotic index is 3.4‰ and 2.7‰, respectively, indicating higher proliferative activity of feline MMSC cultures. The derived cell cultures were propagated and deposited to Kovalenko VIEV Specialized Collection of continuous somatic cell cultures of farm and commercial animals.

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