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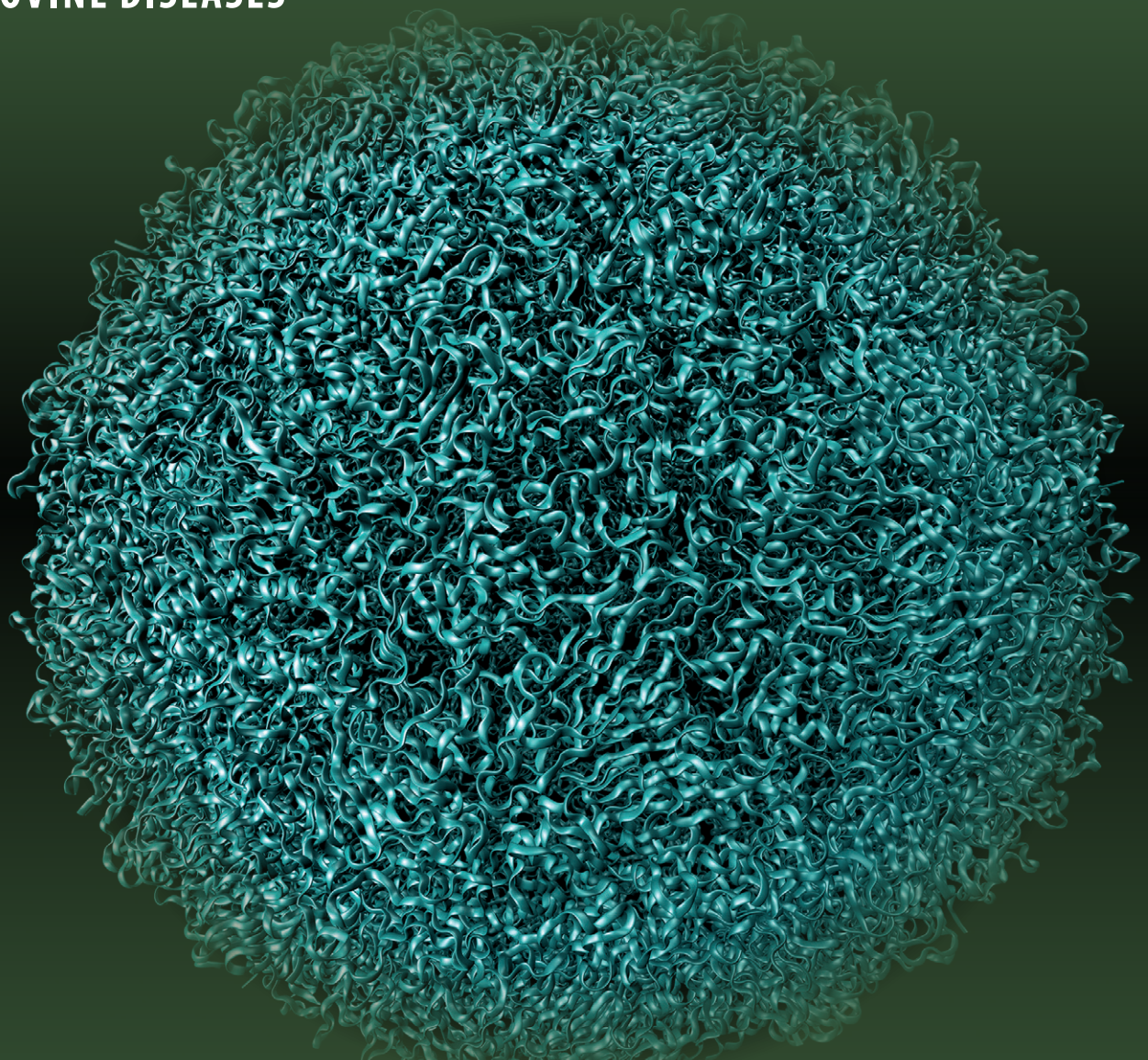

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Bovine nebovirus infection (review)

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ABSTRACT

Animal husbandry is one of the main agricultural industries in most countries over the world as well as in the Russian Federation, and its profitability is determined by three main factors: the animal genetic potential, complete diet and freedom from infectious, invasive and mass non-infectious diseases. One of the most significant and difficult tasks is to generate and rear healthy young cattle. Digestive disorders clinically manifested by diarrhea resulting in apparent dehydration, toxemia, enophthalmos, membrane pathology, immunodeficiency and metabolic disorders are prevalent among neonatal calf diseases in early postnatal period. Massive diarrhea in neonatal calves is characterized by significant polymorphism, involving a wide range of various factors including genetic, physiological, sanitary and hygienic as well as infectious factors. Infectious agents are the main causes of massive gastroenteritis in neonatal calves. In most cases viruses serve as triggers for gastrointestinal pathology development and bacteria play the secondary role. For a long time, rotaviruses, coronaviruses and pestiviruses have been believed to play the main role in etiology of massive neonatal calf diarrhea. In recent years, a number of new and understudied viruses, including kobuvirus, nebovirus, norovirus, torovirus and astrovirus, have been detected in fecal samples from diarrheic calves and their role in diarrhea development has not been definitively determined. Their role as primary pathogens, coinfection agents or commensals remains unclear. Recently these animal pathogens have widely spread in different countries of the world. At the end of the XX century – beginning of the XXI century, large numbers of cattle were imported to the Russian Federation, including cattle from the nebovirus-infected countries. Data on nebovirus infection (occurrence, pathogen characteristics, disease clinical signs and epizootological features) are given in the paper.

Keywords: review, dairy and meat cattle, neonatal calves, yaks, diarrhea, neboviruses, genetic heterogeneity, recombinations

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Небовирусная инфекция крупного рогатого скота (обзор литературы)

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РЕЗЮМЕ

Животноводство является одной из основных отраслей сельского хозяйства в большинстве стран мира, в том числе и в Российской Федерации, рентабельность которой обуславливают три основных фактора: генетический потенциал животных, полноценное кормление и благополучие по инфекционным, инвазионным и массовым незаразным болезням. Получение и выращивание здорового молодняка крупного рогатого скота является одной из наиболее важных и трудных задач. В структуре заболеваний новорожденных телят в ранний постнатальный период превалирующее место занимают нарушения функции пищеварительной системы, клинически проявляющиеся диареей, обуславливающей развитие выраженной дегидратации, токсемии, энофтальмии, мембранопатологии, иммунодефицитов и нарушений обмена веществ. Массовые диареи новорожденных телят отличаются значительным полиморфизмом, включающим широкий спектр различных факторов, в том числе генетических, физиологических, санитарно-гигиенических и инфекционных. Ведущей причиной массовых гастроэнтеритов новорожденных телят являются инфекционные агенты. В большинстве случаев вирусы служат пусковым механизмом в развитии патологии желудочно-кишечного тракта, а бактерии играют вторичную роль. Долгое время считалось, что первостепенное значение в этиологии массовых диарей новорожденных телят имеют ротавирусы, коронавирусы и пестивирусы (возбудители вирусной диареи –

болезни слизистых). В последние годы в пробах фекалий больных диареей телят был обнаружен ряд новых и малоизученных вирусов, в том числе кобувирус, небовирус, норовирус, торовирус и астровирус, роль которых в развитии диареи окончательно не определена. Остается невыясненной их роль в качестве первичных патогенов, агентов коинфекции или комменсалов. В последнее время произошло широкое распространение данных возбудителей болезней животных в различных странах мира. В конце XX – начале XXI века в Российскую Федерацию было импортировано большое количество крупного рогатого скота, в том числе из стран, неблагополучных по небовирусной инфекции. В статье приведены сведения о небовирусной инфекции крупного рогатого скота (распространение, характеристика возбудителя, клинические признаки заболевания, эпизоотологические особенности болезни).

Ключевые слова: обзор, крупный рогатый скот молочных и мясных пород, новорожденные телята, яки, диарея, небовирусы, генетическая гетерогенность, рекомбинации

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For a long time, rotaviruses, coronaviruses, parvoviruses, enteroviruses and pestiviruses, one of which is bovine viral diarrhea – mucosal disease agent have been believed to play the main role in etiology of massive diarrhea in neonatal calves [1, 2, 3, 4, 5, 6, 7, 8, 9, 10]. Inactivated vaccines were developed in the Russian Federation for prevention of rotavirus, coronavirus infections and bovine viral diarrhea-mucosal disease caused by genotype I virus [1, 2, 3, 4]. To date, bovine viral diarrhea-mucosal disease agents belonging to more than 15 subgenotypes of all 3 genotypes have been reported to circulate in the country [3]. Recently, some new and understudied viruses including kobuviruses, toroviruses, neboviruses, noroviruses and astroviruses were detected in fecal samples from diarrheic calves [5, 6, 11, 12, 13, 14, 15] which role in the disease development has not been yet clearly defined. Such agent diversity makes difficult identification of etiological factors responsible for gastrointestinal pathologies in neonatal calves and leads to the choice of insufficiently effective specific preventive tools and great economic losses. This aspect is very important for the holdings involved in high-yielding animal breeding.

In 1976, RNA virus was detected in fecal samples collected from diarrheic neonatal calves kept in the settlement located near Newbury in the South England and was named *Newbury agent 1*. Electronic microscopy showed that the said virus virions were small (36.6 nm in diameter) non-enveloped icosahedral (E = 3) particles consisting of 90 dimers of main structural protein VP1 (58–62 kDa). There were calicivirus-characteristic 32 cup-shaped depressions on the external capsid surface. Isolated agent was classified to *Caliciviridae* family. The virion molecular mass was 15 MDa, sedimentation coefficient was 170–187 S, buoyant density in CsCl is 1.34 g/cm^3. As all other caliciviruses, the isolated agent was stable in the environment, resistant to acid, heating and to chloroform agent. Calves were found to be infected with nebovirus by fecal-oral route or by contact. However, the isolated virus differed from *Caliciviridae* family representatives known at that time [6, 16, 17, 18, 19].

New system for calicivirus classification based on results of molecular biological tests of the viruses was proposed in the beginning of XXI century [20]. *Caliciviridae* family comprised four genera: vesiviruses, sapoviruses, noroviruses, and lagoviruses. *Newbury 1* virus was classified to *Norovirus* genus. Therewith, molecular biological test results showed that *Newbury 1/76UK* virus as some viruses identical to it isolated from feces of diarrheic neonatal calves differed from all known noroviruses. In 1980, the calicivirus related to *Newbury 1/76UK* virus was isolated from fecal samples collected from diarrheic calves in the holding located in Nebraska (Ohio, USA). However phylogenetic analysis of the nucleotide sequences showed that this agent named after the place of sampling (*Nebraska/80/US*), differed from all known viruses and, as a result, the isolated virus was classified to a new genus – *Nebovirus* genus [18, 21].

Nebovirus is a non-enveloped virus with icosahedral geometries, and T = 3, T = 1 symmetry and is 35 nm in diameter. Cattle is the main host of the virus. This pathogen can cause necrotic hepatitis resulting in fatal hemorrhages. Nebovirus replicates in intestinal epithelial cell cytoplasm. Neboviruses, as all other caliciviruses, are stable, highly resistant to physical and chemical environmental factors and retain infectivity at pH 2.7 for 3 hours at room temperature. The viruses are resistant to ether, chloroform, guanidine, sodium deoxycholate, bile acids. The agent remains infectious at 60 °C for 30 minutes [6, 19, 22, 23].

In 2010, *Newbury 1/76/UK* and *Nebraska/80/US* viruses were classified to new *Nebovirus* genus of *Caliciviridae* family. Then, isolated nebovirus strains were classified based on the results of VP1 nucleotide sequence phylogenetic analysis [14, 21, 23]. Nebovirus genome is a single-stranded RNA with molecular weight of 2.6–2.8 MDa, 7.4 kbp in length and is organized into two major ORFs encoding nonstructural polypeptide with the major structural capsid protein (VP1) gene in the frame with non-structural polypeptide.

All isolated neboviruses have closely related genome structures but genetically and antigenically diverse. Nebovirus genome is liable to mutations that resulting in antigenic drift and recombinations as well as emergence of new antigenically altered agent variants [14, 24, 25, 26, 27, 28]. Mutations occur in the genome segments responsible for calicivirus binding to receptors of intestinal mucosal epithelial cells [1]. The neboviruses are believed to evolve through recombination [23, 24, 27, 29, 30]. Nebovirus replication and assembly take place in cytoplasm and

the viral particles are released from the cell by lysis. All known caliciviruses have similar replication cycles: they interact with many cell attachment factors (glycans) and with co-receptors (proteins) for adsorption and penetration, use cell membranes for formation of replication complexes [1, 16, 17, 29]. Attempted cultivation of nebovirus in MDBK and PB cells was unsuccessful [6, 21].

Pathomorphological changes and clinical signs caused by nebovirus infection are similar to that ones caused by rotavirus, coronavirus and kobuvirus infections, as well as by bovine viral diarrhea – mucous disease virus that makes difficult clinical and postmortem diagnosis [11, 31].

Neboviruses replicate in intestinal villi epithelium as well as in the immune system cells. The virus replication is characterized with intestinal villus expanding and blunting, epithelial cell detachment, crypt epithelial hyperplasia, cytoplasm vacuolization, infiltration of affected cells. The most pronounced changes are recorded in the mucous membrane of the proximal intestine (duodenum, jejunum and ileum), where inflammatory processes accompanied by intestinal villus atrophy and intestinal gland hypertrophy are found [9, 29, 31, 32]. Necrosis of small intestine villi epithelium was reported. Decreased enzymatic activity in cells and secondary disaccharide deficiency development resulting in diarrhea were reported [9, 32].

Nebovirus infection has the following epizootological characteristics: long-term agent shedding by diseased animals and virus carrier animals, high contagiousness and virus persistence in the environment. Infected (diseased and convalescent) animals are the nebovirus reservoir and source. The virus-contaminated feed and water can be nebovirus transmission factors.

Incubation period in the nebovirus-infected neonatal calves is considered to be 12–48 hours, and the disease lasts for 2–30 days. Duodenum and jejunum mucosal lesions were detected in gnotobiotic calves 12 hours after experimental infection with *Newbury 1/76UK*. Nebovirus was detected in enterocytes located on villi sides [18]. Anorexia, diarrhea, enophthalmos, dehydration and metabolism disorders were reported in neonatal calves with the disease caused by nebovirus infection kept in animal holdings. Duodenum and jejunum mucosal inflammation was observed in necropsied dead animals. No difference in clinical signs was observed in colostrum-deprived calves experimentally infected with nebovirus *Newbury 1/76UK* and *Nebraska 80/US* strains. No difference in postmortem lesions was also observed in necropsied dead calves [9, 16, 18, 21, 26, 33, 34].

Chinese researchers investigated the causes of diarrhea outbreaks in newborn yak calves kept on the Qinghai-Tibetan Plateau. For this purpose, 354 fecal samples were taken from newborn animals on 55 farms. Nebovirus RNA was detected with polymerase chain reaction in 22% of the samples. Phylogenetic analysis of 78 virus isolates showed that 69 of them were closely related to *Nebraska*-like strains, and 9 isolates circulating on 6 farms in 2 administrative districts were representatives of a new nebovirus genotype [13].

In 2012, mass gastrointestinal disorder was reported in neonatal calves on the farm located in the town of Kirklareli (East Thrace, Turkey) where 250 cows and 200 calves were kept. About 60% of calves became diseased and 30% of them died. Lesions characteristic for rotavirus and coronavirus infections were detected in necropsied dead animals. Rotavirus, coronavirus and *Cryptosporidium* were detected in fecal samples collected from diseased calves. Calicivirus demonstrating 65% homology to *Nebraska* nebovirus was detected in 3 fecal samples when the samples were tested with molecular methods. Isolated pathogen was named as *Kirklareli virus* after the sampling place. It was classified to *Nebovirus* genus based on phylogenetic analysis data. Detailed analysis of the obtained data has suggested that *Kirklareli virus* may be the ancestor of *Nebovirus* genus [21].

Guo Z. et al. detected norovirus and nebovirus in fecal samples collected from gastroenteritis-affected neonatal calves on one Chinese dairy farm that was indicative of co-circulation of these pathogens causing mixed infections [14].

Rotavirus was detected in 73.2% of fecal samples, coronavirus was detected in 36.6% of fecal samples, bovine viral diarrhea virus was detected in 31.7% of fecal samples and nebovirus was detected in 41.8% of fecal samples during tests of the fecal samples collected from neonatal calves kept on 13 commercial dairy farms located in 5 Chinese provinces for etiology of mass gastrointestinal diseases. Two-three pathogens were detected in many samples and all 4 viruses were detected in some samples. Phylogenetic analysis data showed that all tested nebovirus isolates belonged to *Nebraska* group (*Nebraska-like strains*), 14 out of them belonged to lineage 1 and 4 out of them belonged to lineage 3. Also recombinations in nebovirus VP1 were detected [32]. The data obtained by Chinese researchers indicate that nebovirus is widespread in meat and dairy cattle herds as well as yak herds in the country [13, 14, 26, 27, 32].

According to numerous publications, nebovirus was isolated from fecal samples taken from diarrheic neonatal calves in England [16, 18, 19, 23], Brazil [12], Hungary [15], Germany [34], France [30], Italy [24], Sweden [35], Iran [36], China [13, 14, 26, 27, 32], USA [33, 37], Tunisia [28], Turkey [21, 38, 39], South Korea [40] and other countries. Some researchers detected the virus in fecal samples collected from both diseased and clinically healthy calves kept on the same farms. It is suggested that the biological materials have been collected at different periods of the pathological process (incubation period or convalescence stage). This could also explain the different level (4.8–41.8%) of the detected nebovirus prevalence.

Characteristics of neboviruses detected in the fecal samples from neonatal calves and circulating in various countries are given in the table below.

CONCLUSION

Nebovirus infection of neonatal calves is registered in many countries of the world having close economic ties with the Russian Federation. A large number of dairy and meat cattle were imported into Russia from Germany, the USA, France, Hungary and some other countries where nebovirus infection was diagnosed. All this is indicative of high probability of importation of animals infected with various infectious disease pathogens that is confirmed by the presence of pestiviruses (bovine viral diarrhea – mucosal disease agents) in biological material samples taken from aborted fetuses and dead neonatal calves born from imported heifers. Evidence of recombinant nebovirus strain circulation was found. In some cases, other pathogens including norovirus and astrovirus, were detected together with nebovirus in pathological material samples taken from the same animals.

Table
Characteristics of the neboviruses circulating in the countries

Countries	Nebovirus genotype	Publication number
England	*Newbury* 1/76/*UK; Nebraska80/US*	16, 18, 19, 23
USA	*Nebraska80/US*	33, 37
Germany	*Newbury* 1/76/*UK*	34
France	*Nebraska80/US; Dijon A216/06/FR*	30
China	*Newbury 1-like strains; Nebraska-like strains; Dijon A216-like strains*	13, 14, 26, 27, 32
Brazil	*Newbury* 1/76/*UK*	12
Turkey	*Newbury 1-like strains; Nebraska-like strains; Kirklareli virus*	21, 38, 39
Italy	*Newbury* 1/76/*UK; Nebraska80/US*	24
Hungary	*Newbury* 1/76/*UK*	15
Iran	*Newbury* 1/76/*UK; Nebraska80/US*	36
Tunisia	*Nebraska80/US; Dijon A216-like strains*	28
South Korea	*Newbury 1/76/UK; Nebraska-like strains*	40
Sweden	*Newbury 1-like strains; Nebraska-like strains*	35

Pathomorphological changes caused by nebovirus infection are similar to that ones caused by rotavirus, coronavirus, norovirus, torovirus infections, as well as of bovine viral diarrhea – mucous disease. Diarrhea in neonatal calves is generally caused by mixed infections. All this makes much more difficult clinical and postmortem diagnosis of detected gastrointestinal disorders as well as specific prevention of viral diarrheas.

Presented data on bovine nebovirus infection and its wide spread in many countries of the world show that further studies, particularly on breeding farms, monitoring tests of pathological material samples from neonatal calves with clinical signs of gastrointestinal diseases, development of tools and methods for this infection control and prevention are required.

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Avian colibacillosis – current aspects

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ABSTRACT

Colibacillosis is a bacterial disease of humans, animals and birds caused by *Escherichia coli*, pathogenic gram-negative bacillus. Despite its secondary nature, colibacillosis widely affects poultry farms and causes significant economic losses. The disease spread is closely associated with antibiotic resistance problem because a diseased carrier bird may be a reservoir of antibiotic-resistant *Escherichia coli* strains. In addition, genes of virulence and resistance have been proven to be transferred from avian *Escherichia* strains to extra intestinal pathogenic strains that are dangerous to humans. Colibacillosis is transmitted aerogenically, alimentally, rarely transovarially, with droppings, mucus, feed, water, handling tools and operating personnel. Birds are most susceptible at the age of 1–14 days and at the onset of laying period. The disease may present as acute, subacute and chronic forms and is most often manifested by catarrhal hemorrhagic enteritis with profuse foamy diarrhea, respiratory tract lesions, fibrinous peritonitis and polyserositis, as well as a significant decrease in weight gains, stunting, egg laying decrease or complete cessation. Colibacillosis is diagnosed comprehensively taking into account the epizootic situation, findings of clinical examination and postmortem examination of dead or emergency-slaughtered poultry as well as laboratory test and bioassay results. Bacteriological, serological and molecular genetic methods are used for the disease diagnosis. Colibacillosis prevention includes improvement of poultry keeping practice (control of feed and water quality, disinfection, pest control, microclimate control) as well as timely complex vaccination of all poultry. The disease shall be treated taking into account primary etiological factors and bacteria sensitivity to antimicrobials.

Keywords: review, colibacillosis, poultry farming, epizootiology, *Escherichia coli*

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Колибактериоз птиц – актуальные вопросы

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РЕЗЮМЕ

Колибактериоз – бактериальная болезнь человека, животных и птиц, вызываемая патогенной грамотрицательной палочкой *Escherichia coli*. Несмотря на то что болезнь носит секундарный характер, колибактериоз затрагивает птицеводческие хозяйства повсеместно, нанося значительный экономический ущерб. Распространение болезни тесно связано с проблемой антибиотикорезистентности, поскольку больная птица-носитель может являться резервуаром штаммов *Escherichia coli*, устойчивых к действию антибактериальных средств. Кроме того, доказана возможность передачи генов вирулентности и резистентности от птичьих штаммов эшерихий внекишечным патогенным штаммам, опасным для человека. Колибактериоз передается аэрогенно, алиментарно, реже – трансовариально через помет, слизь, корма, воду, предметы обихода и обслуживающий персонал. Наиболее восприимчивы птицы в возрасте 1–14 сут и в период начала яйцекладки. Болезнь протекает в острой, подострой и хронической форме и чаще всего сопровождается катарально-геморрагическим энтеритом с профузной пенистой диареей, поражением респираторного тракта, фибринозным перитонитом и полисерозитом, а также значительным снижением привесов, отставанием в росте, снижением или полным прекращением яйценоскости. Диагноз «колибактериоз» ставится комплексно с учетом эпизоотической ситуации, данных клинического осмотра и патолого-анатомического вскрытия павшей или вынужденно убитой птицы, а также результатов лабораторных исследований и постановки биопробы. Для диагностики заболевания применяют бактериологические, серологические и молекулярно-генетические методы. Профилактика колибактериоза достигается путем улучшения условий содержания птицы

(контроль качества корма и воды, дезинфекция, дератизация, контроль параметров микроклимата), а также своевременной комплексной вакцинацией всего поголовья. Лечебные мероприятия должны планироваться, основываясь на первичных этиологических факторах и чувствительности бактерий к противомикробным средствам.

Ключевые слова: обзор, колибактериоз, птицеводство, эпизоотология, *Escherichia coli*

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INTRODUCTION

Colibacillosis (escherichiosis) is a zooanthroponotic bacterial septicaemic disease of poultry and wild birds, affecting mainly gastrointestinal and respiratory tracts, causing high mortality and serious economic losses [1].

It is generally believed throughout the world that avian colibacillosis occurs as a secondary disease when the immunity is suppressed and overall body resistance is decreased. This is most often associated with shortages in poultry keeping and feeding practices, serious poultry management shortcomings, as well as the presence of viral (avian influenza, Newcastle disease, acute infectious bronchitis, infectious bursal disease, etc.) and bacterial (pasteurellosis, *Haemophilus* infection, staphylococcosis, mycoplasmosis, etc.) diseases [2, 3, 4]. *Escherichia coli* infections cause losses due to embryonated egg and chick deaths, poor development of convalescent poultry, reduced egg laying and weight gain, culling of carcasses, spread of pathogenic and antibiotic-resistant strains among adult poultry [1, 2, 3]. "Colibacillosis" diagnosis is the most common in veterinary reporting since the disease is not a "quarantine disease", and *E. coli* are found in the vast majority of outbreaks and have no negative impact on the holding reputation [4].

Colibacillosis poses a serious threat both to birds and humans. The uncontrolled use of chemotherapeutics in poultry and animal farming industry resulted in formation of a reservoir of antibiotic-resistant *E. coli* strains capable of being transmitted to humans through processed products produced by commercial agricultural establishments [5, 6]. Whole genome sequencing has shown that human extra-intestinal pathogenic *E. coli* (ExPEC) is genetically similar to avian pathogenic *E. coli* strains. Virulence and antibiotic resistance genes are proven to be horizontally transferred. Moreover, avian ExPEC-specific ColV (colicin V) plasmid have been detected in human ExPEC isolates that suggests a possible zoonotic transmission of pathogenic *Escherichia* from poultry to humans [7, 8].

In February 2017, the World Health Organization published a list of 12 species of bacteria resistant to antimicrobials and posing the most threat to human health. *E. coli* was classified to category 1 comprising bacteria with critical antibiotic resistance together with other *Enterobacteriaceae* family members, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, resistant to carbapenems and producing extended-spectrum beta-lactamases [9].

The scientific novelty of the presented review is in description of modern methods for *E. coli* detection and identification, new trends in colibacillosis treatment and prevention.

The work was aimed at summarizing scientific literature data on avian colibacillosis and highlighting the most important aspects of its investigation.

AGENT CHARACTERIZATION

Avian colibacillosis is caused by *E. coli* (avian pathogenic *Escherichia coli*, APEC), the most widespread member of *Enterobacteriaceae* family that was named after T. Escherich, German pediatrician, who isolated the said pathogen from intestinal contents in children for the first time in 1885 [10]. Straight or slightly curved Gram-negative rods that are motile due to flagella and peritrichial cilia and occur singly less often in pairs in smears (Fig. 1).

On dense media *E. coli* bacteria form smooth round colonies of medium size (1.5–2.5 mm) (Fig. 2). Furthermore, they actively ferment glucose, lactose, mannitol, arabinose, galactose with acid and gas production; they typically do not ferment sucrose and dulcite; produce indole, do not produce hydrogen sulfide; reduce nitrates

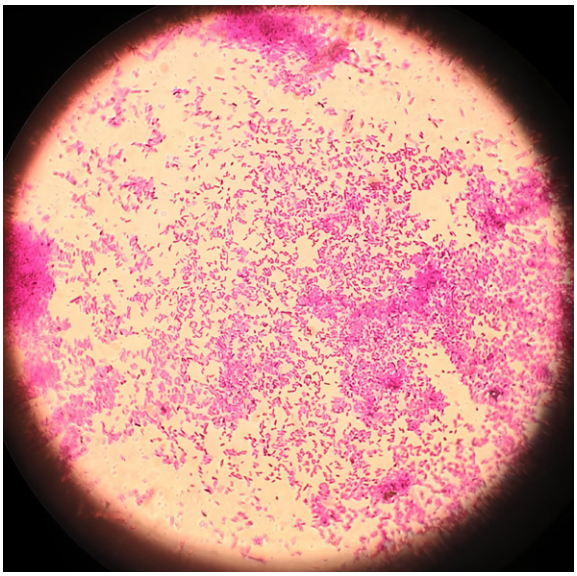

Fig. 1. Morphology of Gram-stained E. coli (magnification 40×)

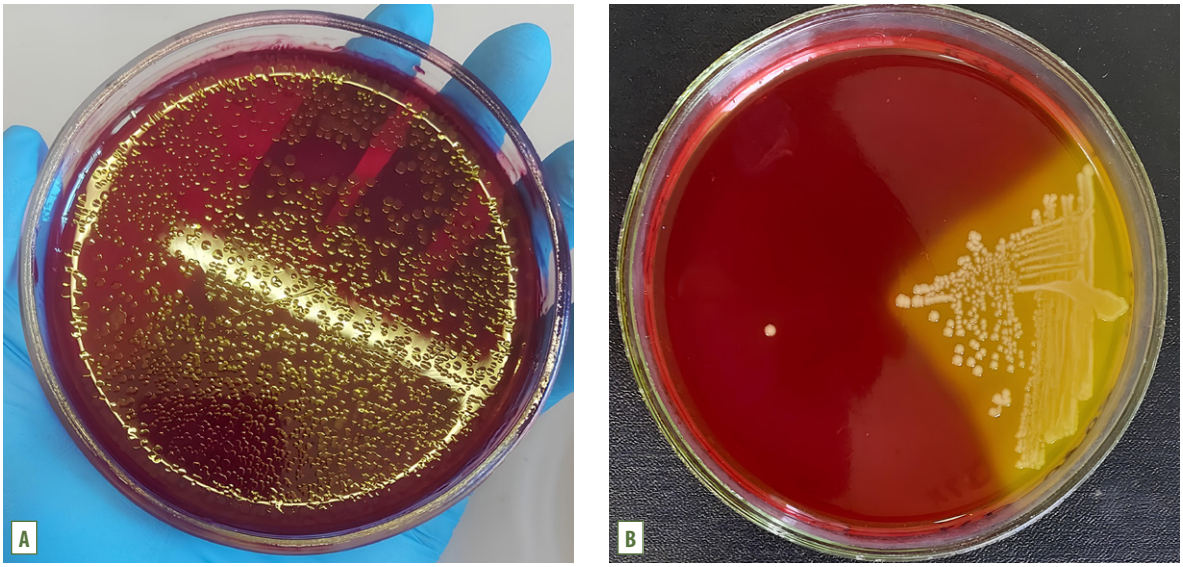

Fig. 2. E. coli growth on dense nutrient media:
A – E. coli colonies with characteristic metallic sheen on Endo agar; B – E. coli colonies on XLD-agar

to nitrites, do not dilute gelatin, do not grow on a citrate-containing medium, do not digest urea; demonstrate positive methyl red test reaction and negative Voges–Proskauer test reaction (Fig. 3) [11, 12].

E. coli bacteria are rather persistent in the environment. The pathogen is decontaminated by heating to 60 °C for 15 minutes and decontaminated instantly by boiling at 100 °C. The bacteria are able to survive in the environment (water, soil, household items) for several months, in food – for more than 30 days. In addition, *E. coli* are highly susceptible to most disinfectants (formaldehyde, chlorine preparations, sodium hydroxide, etc.) and antibiotics (tetracyclines, aminoglycosides, rifampicin, etc.), however, they are able to acquire resistance to antimicrobial drugs through R-plasmids. Horizontal transfer of resistance plasmids promotes the spread of resistance within the bacterial population [10, 13, 14, 15].

All poultry, ornamental, exotic and wild bird species are susceptible to the disease.

Colibacillosis is transmitted alimentary, aerogenically and transovarially. The infection transmission factors include feed, bedding, handling tools, rodents, synanthropic birds, operating personnel.

Chicks at the age of 1–14 days are the most susceptible and can be affected with acute disease in the form of sepsis. Poultry during the laying period are also susceptible. Convalescent adult birds become pathogen-carriers and often serve as a reservoir of pathogenic *E. coli* strains [1, 2].

Incubation period varies from several hours to 6 days.

The disease occurs in acute (sepsis), subacute and chronic forms and characterized by hyporexia or anorexia, depression, significantly reduced weight gain, foamy yellow-green diarrhea, and when respiratory tract is affected – by rales, sneezing and profuse nasal discharges. The following postmortem lesions are found in necropsied birds: petechiae and ecchymoses on mucous membranes and internal organs, catarrhal hemorrhagic enteritis, pancreatitis, splenitis, fibrinous peritonitis and polyserositis; cecum is often swollen (Fig. 4) [1, 16, 17].

CURRENT ASPECTS OF DIAGNOSIS

Bacteriological methods. Samples are inoculated on dehydrated Endo's or Levin's medium and incubated at temperature of 37–38°C for 24 hours for the bacteria isolation and differentiation. Then the most characteristic *E. coli* colonies are selected and smears are prepared, Gram-stained and examined under microscope. When

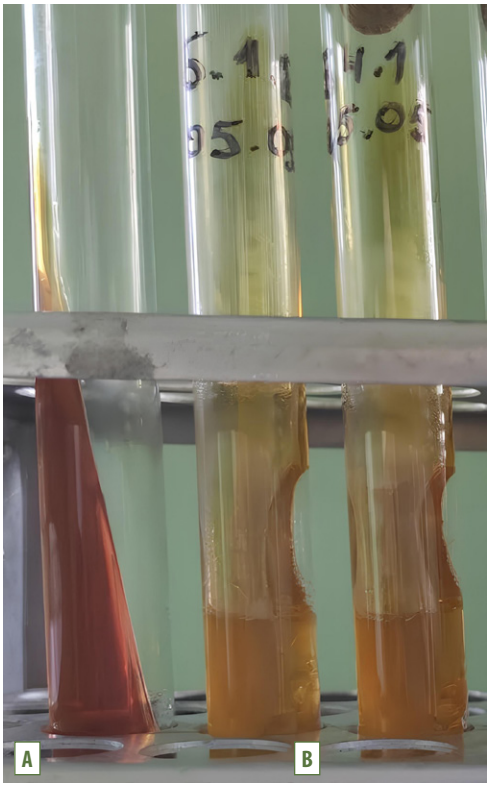

Fig. 3. Biochemical properties of E. coli on triple sugar iron agar: A – control sample containing medium not inoculated with the culture; B – test samples with E. coli: the medium slant turns yellow due to lactose fermentation, and glucose and sucrose fermentation with acid and gas formation discolors the medium column with butt splitting

Gram-negative rounded rods are detected in the smears, the colonies are re-seeded on Olkelnitsky's triple sugar agar, Gissa's, Simmons media, Kligler agar for testing them for biochemical properties. Paper indicator discs are used for the same purpose [17, 18]. The diagnosis is made based on *E. coli* isolation from cardiac blood, bone marrow, liver, spleen and pericardium tissues. Isolation of *E. coli* from intestine is considered diagnostically insignificant [19].

Serological methods. *E. coli* has 900 known serotypes. They are differentiated by examination of bacteria antigenic properties.

Somatic O-antigen is a lipopolysaccharide-protein complex of outer membrane of bacterial wall and defines the serological group of the bacteria; O-antigen-lacking strains form rough colonies of R-form and most often are non-virulent. To date, 175 O serogroups have been identified. Prevailing serogroups are O1, O2 and O78 but they account for only 15 up 60% of isolates depending on testing [20].

Motile *Escherichia* have flagellar H-antigen consisting of the flagellin protein and are thermolabile. Totally, there are about 70 H-antigen variants.

Some bacteria possess capsular mucopolysaccride thermostable K-antigen (Vi-antigen) located outside the somatic antigen. This allows bacteria to block agglutination reaction to specific O-serum. About 100 K-antigen variants and 3 classes among them: A, B and L. Antigens of class A are thermostable and antigens of class B and L are thermolabile.

Also, 17 types of fimbrial F-antigens required for bacteria adhesion have been described. F-antigens are classified into mannose-sensitive and mannose-resistant depending on whether agglutination is inhibited in the presence of mannose or not.

There are several diarrheagenic groups within known *E. coli* serotypes: entertoxicogenic (ETEC); enteroinvasive (EIEC); enteropathogenic (EPEC); enterohemorrhagic (EHEC); enteroaggregative (EA) и diffusely

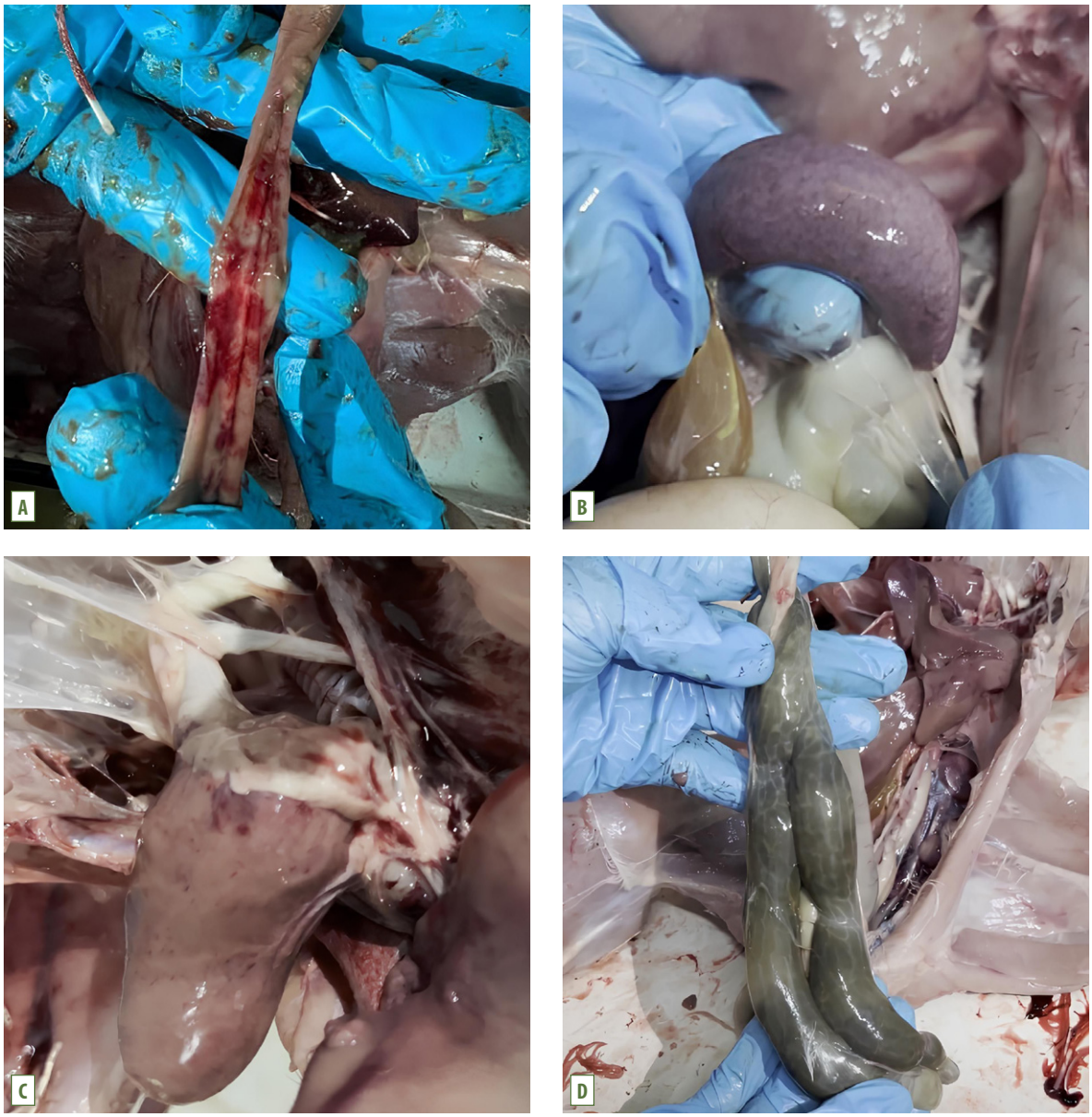

Fig. 4. Postmortem lesions detected in turkey poults infected with E. coli culture:
A – multiple hemorrhages in intestine mucosa; B – splenitis; C – hemorrhages in epicardium; D – cecum swelling

adherent (DAEC) *E. coli*. Colibacilloses are classified based on different pathogenicity factors possessed by the agent [11, 16, 18, 21].

Slide or tube agglutination test with *E. coli*-specific sera is performed for serological *Escherichia* differentiation. At first, suspension is prepared by adding 0.85% sodium chloride solution to concentration of 2–3 billion microbial cells/cm^3, and then it is heat treated – heated in a water bath at 100 °C for 1 hour and autoclaved at 1 atm for 2 hours to destroy the surface K-antigen [16]. Serotyping remains the most frequently used diagnostic method in laboratories, but it only allows the identification of a limited number of APEC strains. Therefore, this method cannot be used as an effective diagnostic tool, particularly since serotype does not reflect the virulence trait [20].

Molecular-genetic methods. Tests by polymerase chain reaction (PCR) are used for rapid pathogen detection in clinical samples. DNA from tracheal and intestinal mucosa scrapings, parenchymal organ and red bone marrow pieces are used for the PCR. Control sample and negative control are concurrently tested with PCR [22]. Colibacillosis is diagnosed when at least one of the determinants of pathogenicity is detected in samples: P-fimbrial protein (*papC*), temperature-sensitive hemagglutinin (*tsh*), iron-binding protein (*fyuA/irp 2*), aerobactin (*iutA/iucD*), increased serum survival protein (*iss*), colicin V plasmid (*cva/cvi*), enteroaggregative *E. coli* heat-stable toxin (*astA2*), vacuolating autotransporter toxin (*vat*) [8, 23]. Some other virulence genes involved in the development of avian colibacillosis have also been identified: genes encoding adhesins (*F1, P* and *Stg fimbriae, curli* and *EA/I*), host immunity protection factors (*OmpA*, lipopolysaccharide and K1), iron acquisition systems (*Iro* proteins, yersiniabactin and iron acquisition *Sit* locus), autotransporters (*AatA*), phosphate transport system, sugar metabolism and *IbeA* protein.

Numerous studies have demonstrated that these virulence factors (VFs) are rarely all present in the same isolate, showing that APEC strains constitute a large heterogeneous group. Different isolates may harbor different associations of virulence factors, each one is able to induce avian colibacillosis [20].

Today, double digest selective label (DDSL) technique is one of the most accurate and highly specific methods of genotyping. It allows pathogen DNA to be detected in samples as well as the infection pathways to be tracked, close related strains and mutations to be identified. The accuracy of this method determined based on discrimination index, exceeds the accuracy of pulsed-field gel electrophoresis – gold standard for genotyping [24].

CURRENT ASPECTS OF THERAPY

Colibacillosis therapy includes use of antibacterial agents. Mass monitoring carried out in 2015–2020 has shown that *E. coli* strains are currently susceptible to almost all classes of antibiotics, except for carbapenems [8]. Preliminary testing of *Escherichia* for susceptibility to antibiotics followed by selection of appropriate drug is an important therapy stage [23]. The Committee for Veterinary Medicinal Products of the European Medicines Agency actively revises guidelines for use of antibiotics in animal farming industry including dosing regimen optimization to prevent expansion of the resistome (a set of antibiotic resistance genes) among animals and, as a result, in humans [25].

Antimicrobial peptides, phytobiotics, probiotics, bacteriophages are alternatives widely used for bacterial disease therapy. According modern studies, they are found to be highly effective for both *in vitro* and *in vivo* colibacillosis control [26, 27, 28].

Antimicrobial peptides (AMPs) are a promising natural alternative to traditional antibiotics. AMPs are characterized by rapid and highly selective antimicrobial effect, as well as a low drug resistance tendency and easy synthesizability. Moreover, they demonstrate immunomodulatory and anti-endotoxin activity, inhibit proinflammatory reactions, stimulate chemotaxis and differentiation of immune cells [29, 30, 31]. Kathayat D. et al. during their study performed in 2021 found that AMPs derived from *Lactobacillus rhamnosus* probiotic bacteria were active against resistant *E. coli* serotypes isolated from birds, as well as reduced the number of *Enterobacteriaceae* in chicken caeca, with minimal impact on intestinal microbiota [32].

Inhibition of *Escherichia* quorum sensing (QS), a phenomenon underlying the bacteria social behavior, ability to regulate gene expression in response to changes in the microorganism population density, has become a new strategy for avian colibacillosis control. This regulation mechanism serves to enhance bacteria survivability. Quorum sensing inhibitors (QSI) are targeted to bacteria signaling circuit disruption and population virulence reducing. The use of QSI for avian colibacillosis treatment is described. Some studies showed that QSI significantly reduced the mortality of chickens caused by the infection. Moreover, some inhibitors (QSI-8 and QSI-10) have beneficial effects on the intestinal microbiota through increasing the number of *Butyricicoccus* spp. and *Lactobacillus* spp. [7, 33, 34, 35].

The use of bacteriophages for avian colibacillosis treatment has been widely described. Their principle of action is a highly specific effect on bacterial cells causing their lysis. Some studies demonstrated successful application of lytic bacteriophages for inhibition of pathogenic *E. coli* strain development. At the same time, many authors note a decrease in the effectiveness of biologicals *in vivo* [36, 37, 38, 39]. Sørensen P. E. et al. indicate the prospects of investigations related to the use of bacteriophages in poultry farming, however, they emphasize the problem of bacteriophage-resistant mutant APEC strain emergence. Bacteria are able to develop phage resistance through various mechanisms: spontaneous mutations, acquisition of restriction-modification (R-M) systems, adaptive immunity associated with clustered regularly interspaced short palindromic repeats (CRISPRs) [40].

CURRENT ASPECTS OF PREVENTION

Nonspecific prevention of avian colibacillosis relies on good management practice including appropriate veterinary and sanitary measures: use of all-in-all-out management practice and sanitary breaks before restocking, good egg acceptance and incubation hygiene practice, timely disinfection and pest control, use of pathogenic *Escherichia*-free feedstuffs and feedstuffs protected from rodents and wild birds, microclimate maintenance [23, 41, 42].

Current trend in poultry farming industry is introduction of alternative feed additives having immunostimulating

effect. Such additives include: prebiotics, probiotics, synbiotics, essential oils. Lactic acid bacilli are known to synthesize bacteriocins and bacteriocin-like factors that are antagonists of putrefactive, pathogenic and opportunistic microflora due to their ability to produce lactabiotics, lactacins having antimicrobial activity, as well as hydrogen peroxide, lysozyme, interleukins, interferons, etc. [23, 26, 43].

The effect of biologicals inoculated *in ovo* is being actively studied. To date, it has been found that the injection of probiotics and phage cocktails into the amniotic fluid prevents colibacillosis at the stage of chick hatching, but is ineffective for mass long-term prevention on large poultry farms [44, 45].

Specific prevention includes timely vaccination of poultry contributing to disease freedom maintaining.

There are live and inactivated vaccines, monovaccines against colibacillosis, as well as combined vaccines against several diseases. Live vaccines in poultry farming industry are most often administered to group of poultry with drinking water, by coarse and fine spraying. Whereas, inactivated vaccines are administered to each bird parenterally: intramuscularly, subcutaneously in the middle third of the neck, cutaneously, intranasally, intraocularly or cloacally [16, 23, 24, 41, 42, 43, 46].

Studies conducted in Japan in 2017 [47] showed high effectiveness of live attenuated vaccine against colibacillosis caused by *E. coli* serovar O78. Attenuated *E. coli* strain was constructed in 2012 by the allelic exchange procedure based on the mutant AESN1331strain containing a deletion in the C-reactive protein (*crp*) gene, lacking virulence-associated genes (*iss, tsh, cvaA* and *papC*) and susceptible to many antimicrobials, except for nalidixic acid [48].

Non-cellular vaccines based on bacterial outer membrane vesicles (OMVs) – proteolipid nanostructures secreted by Gram-negative bacteria and enriched with various immunoactive molecules (cell wall components, membrane proteins, cytoplasmic proteins and bacterial nucleic acids) are described. Such vaccines confer strong cross-immunity against several *E. coli* serogroups through enhancement of nonspecific serum immune factors, specific antibody response and spleen and peripheral blood lymphocyte proliferation. Studies carried out by R. Hu et al. in 2020 demonstrated the effective use of OMVs vaccines based on *E. coli* O1, O2 and O78 serogroups, that reduced bacterial load and stimulated proinflammatory cytokine production [49, 50].

Non-live vaccines based on bacterial ghosts (BGs) – cell envelopes devoid of genetic and cytoplasmic components and produced by controlled expression of lysis E gene of PhiX 174 bacteriophage are currently of scientific and practical interest. Bacterial ghosts are proven to possess adjuvant properties and also show tropism to host antigen-presenting cells, allowing the induction of humoral and cellular immune responses (in particular, they maintain high levels of IgY, IgA and IFN-γ, as well as increase the production of proinflammatory IL-6, IL-1β and TNFSF15 cytokines). At the same time, the bacterial ghosts do not have any endotoxicity and exhibit antigenic properties of living bacteria. Antigenic epitopes are transferred in inner or outer membrane proteins, as well as in flagella, fimbria or periplasm. Bacterial ghost-based vaccines advantages include simplicity of production method, safety, long shelf life without need for a cold chain, possible needle-free administration and universality [51, 52].

CONCLUSIONS

Russian and foreign scientific literature review has allowed us to make the following conclusions.

1. Currently, colibacillosis is of great concern for poultry industry for many reasons. Firstly, colibacillosis causes severe economic losses on farms, affects poultry performance and compromises poultry welfare. Secondly, convalescent carrier birds are able to form reservoirs of resistant *E. coli* strains. Thirdly, APEC strains are genetically similar to with extra-intestinal pathogenic human *E. coli* strains, that makes transfer of virulence and antibiotic resistance genes between them possible.

2. Bacteriological, serological and molecular genetic test methods are used for *E. coli* detection and identification. Bacteriological methods enable coliform isolation from pathological materials but cannot be used for pathogenicity determinant identification and serotyping. Serological diagnostic methods using *E. coli*-specific sera are used for serotyping. The said methods are considered less informative for avian colibacillosis diagnosis since they do not allow for bacteria virulence determination. Molecular genetic diagnostic methods are more specific and informative since they allow for detection of bacteria in pathological materials as well as identification of pathogenicity determinants, determination of the genetic relationship between isolates and the infection pathways.

3. Antibiotic resistance is a problem attracting attention of researchers and medical and veterinary practitioners around the world. Therefore, search for safe and environment-friendly alternatives to antibiotics has become of current importance for treatment of bacterial diseases (including colibacillosis). The successful use of antimicrobial peptides, quorum sensing inhibitors and bacteriophages for avian colibacillosis treatment is described. It has been experimentally proven that the above-mentioned agents are capable of suppressing the pathogenic *Escherichia* reproduction in poultry, demonstrate immunomodulatory activity and at the same time do not have a negative effect on intestinal microbiocenosis. The examination of biosafe alternatives is a promising area for further studies, and, nevertheless, antibiotics remain the first choice drugs for the treatment of bacterial diseases.

4. Widespread refuse to use in-feed antibiotics as a non-specific prevention and growth stimulation tools has resulted in the large-scale use of feed additives: prebiotics, probiotics, synbiotics, phytobiotics, essential oils, etc. Numerous studies have shown positive effect of these additives on poultry performance, intestinal microbiota, non-specific immunity response and of intestinal barrier function maintenance.

5. Wide serological *Escherichia* diversity makes difficult specific avian colibacillosis prevention. Attenuated vaccines based on mutant APEC strains lacking virulence genes but retaining susceptibility to antimicrobials are being actively developed. Moreover, synthetic vaccines based on outer membrane vesicles and ghost cells are of great scientific interest. To date, such vaccines have been found to have specific effect as well as to stimulate cellular and humoral immunity.

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Global avian influenza situation (2019–2022). Host range expansion as evidence of high pathogenicity avian influenza virus evolution

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ABSTRACT

High pathogenicity avian influenza has a significant negative impact on poultry farming, international trade and health of wild bird populations, therefore the infection requires the utmost attention of the entire international community. The article investigates the evolutionary and epidemic processes observed in recent years in many countries of the world where avian influenza outbreaks occur; describes the ways of the infection spread; the prevalence of the virus types for the last several years, as well as the expansion of the host range, including among representatives of the *Mammalia* class. The change in the ratio between the virus types starting from 2020, when H5N8 subtype was responsible for the overwhelming number of the disease outbreaks reported, until 2022, when an obvious predominance of H5N1 subtype was detected is demonstrated. A noticeable expansion of the disease-affected areas in Central and South America, the influence of migration, anthropogenic and other factors on influenza spread are highlighted. The conditions facilitating the occurrence of the infection outbreaks affecting mammals, wild animals and livestock, zoo and companion animals are described. Cases of mammals' infection on the North American and Eurasian continents in zoos, nature parks, backyards and fur farms, as a rule, coincide in time with the infection outbreaks in waterfowl populations. The WAHIS data were analyzed and the high ability of the virus to spillover from birds to mammals, such as martens (minks, otters, ferrets, badgers), cats (domestic cats, cougars, leopards, lynxes), pinnipeds (common seals, grey seals), bears (brown, grizzly, American black), bottlenose dolphins, skunks, foxes, opossums, raccoons was demonstrated. Changes in the habitats of both migratory birds and mammals, including due to some human economic activities, add an ecological and urban component to the complex task of the control and prevention of the epidemic, also posing a potential threat to humans.

Keywords: review, avian influenza, mammals, disease situation, expansion of the host range, atypical hosts

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Эпизоотическая ситуация в мире по гриппу птиц (2019–2022 гг.). Расширение спектра хозяев как проявление эволюции вируса высокопатогенного гриппа птиц

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РЕЗЮМЕ

Высокопатогенный грипп птиц оказывает значительное негативное влияние на птицеводство, международную торговлю и здоровье популяций диких птиц, вследствие чего данная инфекция требует самого пристального внимания всего международного сообщества. В статье рассмотрены эволюционные и эпизоотические процессы, наблюдаемые в последние годы при возникновении вспышек гриппа птиц во многих странах мира,

описаны пути распространения инфекции, превалентность типов вируса в течение нескольких лет, а также вопросы расширения спектра восприимчивых животных, в том числе среди представителей класса *Mammalia*. Показано изменение соотношения типов вируса с 2020 г., когда подавляющее количество обнаруженных вспышек заболевания приходилось на вирус гриппа подтипа H5N8, до 2022 г., когда было выявлено явное преобладание подтипа H5N1. Отмечено заметное расширение ареала заболевания в Центральной и Южной Америке, влияние миграционных, антропогенных и иных факторов на распространение гриппа. Указаны предпосылки к формированию очагов инфекции с участием млекопитающих: как диких, так и сельскохозяйственных, зоопарковых и животных-компаньонов. Случаи инфицирования млекопитающих на Северо-Американском континенте и в Евразии на территориях зоопарков, природных парков, в домохозяйствах и на зверофермах, как правило, совпадают по времени со вспышками инфекции в популяции водоплавающих птиц. Проведен анализ данных WAHIS и показана высокая способность вируса передаваться от птиц в популяцию млекопитающих, таких как куньи (норки, выдры, хорьки, барсуки), кошачьи (домашние кошки, пумы, леопарды, рыси), ластоногие (обыкновенные тюлени, длинномордые тюлени), медведи (бурые, гризли, американские черные), афалины, скунсы, лисы, опоссумы, еноты. Изменение ареалов обитания как перелетных птиц, так и млекопитающих, в том числе вследствие особенностей хозяйственной деятельности человека, добавляет эколого-урбанистическую составляющую к сложному вопросу борьбы с распространением и предотвращением возникновения эпизоотии, представляющей угрозу в том числе и для человека.

Ключевые слова: обзор, грипп птиц, млекопитающие, эпизоотическая ситуация, расширение спектра хозяев, нетипичные хозяева

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INTRODUCTION

High pathogenicity avian influenza (HPAI) has a significant negative impact on poultry farming, international trade and health of wild bird populations, therefore the infection requires the utmost attention of the entire international community.

This disease is caused by viruses divided into multiple subtypes, the genetic characteristics of which can change rapidly over time [1, 2].

Avian influenza viruses (AIVs) are divided into 16 subtypes by hemagglutinin (H1–H16) and into 9 subtypes by neuraminidase (N1–N9) [3]. In addition, new AIV type A subtypes – H17N10 and H18N11 isolated from bats in Guatemala have been identified [4, 5].

Wild birds remain the major reservoir of AI in wild nature, while the virus persistence in the population does not significantly affect the general condition of the hosts [6, 7]. However, the introductions of the virus into unadapted groups of poultry result in severe epidemics with huge economic losses. First of all, this refers to the AIVs of subtypes H5, H7 and H9 [8, 9, 10].

Such a reservoir poses a certain risk, which should be taken into account when animal health measures are planned and implemented [7, 11].

The AI virus is very stable in the environment, can survive for a long time at low temperatures and easily spreads between farms, inside ecosystems, including with fomites (contaminated handling items, equipment). The virus can cross the host-range barrier and infect, although less frequently, animals such as rats, mice, weasels, ferrets, pigs, cats, tigers, dogs and horses. There are known cases of AIV isolation from various mammalian species, including humans [3, 12, 13].

The aim of the study was to analyze the avian influenza evolutionary and epidemic processes in the world in 2019–2022, and these results will provide a more objective picture and will serve the basis for AI monitoring expansion.

MATERIALS AND METHODS

The study was carried out in the Information and Analysis Centre of the Veterinary Surveillance Department at the ARRIAH (Vladimir). The data on AI cases (for example, date, place) were collected from WAHID/WAHIS database of the World Organization for Animal Health (WOAH). Several scientific publications by foreign and domestic authors were also used.

RESULTS AND DISCUSSION

The disease spread. An infected bird sheds the AIV with droppings and through the respiratory tract. The infection is transmitted through direct contact with droppings, through feed and water. The disease has a pronounced seasonality and transboundary nature. Influenza viruses easily spread with migrating birds, creating natural reservoirs of the infection in nesting sites [14, 15, 16].

Poultry is particularly susceptible to the infection. Due to crowding, breed features, technology of intensive poultry farming AI can quickly cause epidemic in the population. In addition, the genetic variability of the

Table 1
Various subtypes of avian influenza virus reported in the world in 2019–2022

Year	Countries	Isolated AIV subtypes
2019	Bhutan, Vietnam, Ghana, Egypt, India, Indonesia, China, Nepal	H5N1
	Egypt, Taiwan	H5N2
	Taiwan	H5N5
	Vietnam, Cambodia, China	H5N6
	Bulgaria, Egypt, Iran, Israel, Kuwait, Namibia, Nigeria, Pakistan, Poland, South Africa	H5N8
	Mexico	H7N3
	China	H7N9
	Bangladesh, Bulgaria, Denmark, Russia	H5 (or not typed)
2020	United Kingdom, Vietnam, Egypt, India, Italy, China, Laos, Nigeria, Netherlands, Senegal	H5N1
	Taiwan	H5N2
	Germany	H5N3
	Belgium, United Kingdom, Germany, Denmark, Italy, Netherlands, Russia, Slovenia, Taiwan, Sweden	H5N5
	Vietnam, China, Philippines	H5N6
	Bulgaria, United Kingdom, Hungary, Germany, Denmark, Egypt, Spain, Italy, Ireland, Iraq, Iran, Israel, Kuwait, Lithuania, Netherlands, Norway, Poland, Russia, Romania, Saudi Arabia, Slovakia, Slovenia, Ukraine, France, Croatia, Czech Republic, Sweden, South Africa, South Korea, Japan	H5N8
	Palestine	H5N9
	Mexico, USA	H7N3
	Australia	H7N7
	Belgium, Germany, Kazakhstan, Ukraine	H5 (or not typed)
2021	Austria, Belgium, Benin, Bosnia and Herzegovina, Hong Kong, United Kingdom, Hungary, Vietnam, Germany, Denmark, Israel, Ireland, Spain, Italy, India, Cambodia, Canada, Latvia, Luxembourg, Mauritania, Mali, Niger, Nigeria, Netherlands, Norway, Poland, Portugal, Russia, Romania, Senegal, Serbia, Slovakia, Slovenia, Taiwan, Togo, Faroe Islands, Finland, France, Croatia, Czech Republic, Sweden, Estonia, South Africa, South Korea, Japan	H5N1
	Serbia, Taiwan	H5N2
	United Kingdom, Germany, Denmark, Ireland, Netherlands	H5N3
	Germany, Netherlands, Sweden, Switzerland	H5N4
	Hungary, Germany, Iran, Romania, Russia, Taiwan, Sweden	H5N5
	Austria, Belgium, Vietnam, Denmark, China, Czech Republic	H5N6
	Austria, Algeria, Afghanistan, United Kingdom, Hungary, Vietnam, Germany, Hong Kong, Denmark, India, Iran, Iraq, Spain, Israel, Ireland, Italy, China, Latvia, Lithuania, Netherlands, Norway, Poland, Romania, Serbia, Slovakia, Ukraine, Finland, France, Croatia, Czech Republic, Sweden, Estonia, South Korea, Japan	H5N8
	Mexico	H7N3
	Lithuania	H7N7
	Austria, Belgium, Ghana, Germany, Kazakhstan, Laos, Lesotho, Pakistan, Romania, Russia, Ukraine, Sweden, South Africa	H5 (or not typed)
2022	Albania, Austria, Algeria, Belgium, Hungary, United Kingdom, Vietnam, Germany, Greece, Greenland, Gabon, Guinea, Hong Kong, Honduras, Denmark, Israel, India, Italy, Iceland, Ireland, Spain, Cameroon, Canada, Cyprus, Colombia, Lithuania, Latvia, Luxembourg, Macedonia, Mali, Mexico, Moldova, Nepal, Niger, Namibia, Nigeria, Netherlands, Norway, Portugal, Poland, Romania, Russia, Serbia, Slovakia, Slovenia, USA, Taiwan, Togo, Faroe Islands, Philippines, Finland, France, Croatia, Czech Republic, Montenegro, Chile, Sweden, Switzerland, Ecuador, South Africa, South Korea, Japan	H5N1
	Germany, Poland, Taiwan, South Africa, Japan	H5N2
	USA	H5N4
	Norway, Finland	H5N5
	Albania, Iraq, Israel, Philippines	H5N8
	Mexico	H7N3
	Austria, Belgium, Canada, Kazakhstan, Peru, Japan	H5 (or not typed)

virus contributes to the wide spread of the infection and the emergence of the variants capable of spillover.

Changes in the spectrum of isolated AIVs occur every year. And if in 2020 the overwhelming number of reported outbreaks accounted for AIV H5N8 subtype, then in 2022 there was a clear predominance of H5N1 subtype (Table 1, Fig. 1). In addition, the list of countries that have reported HPAI outbreaks is expanding.

During 2022, new AI outbreaks were reported by Mali, Iceland, Reunion (France). For the first time during

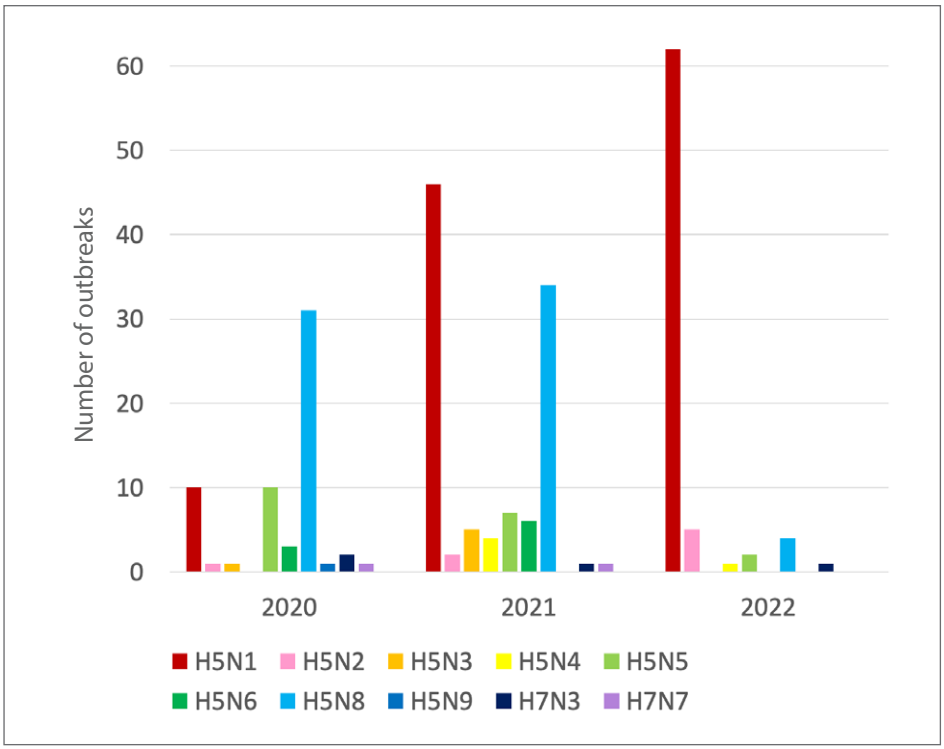

Fig. 1. Prevalence of influenza virus subtypes by year

the observation period (up to January 2023 inclusive), HPAI outbreaks were reported by Colombia, Ecuador, Peru, Venezuela, Panama, Chile and Bolivia [3].

Thus, currently the disease distribution range is expanding in Central and South Americas.

The influence of migration factors on HPAI spread. To date, 14 global migration routes of migratory birds are recognized, 8 of which are more or less related to the Russian territory [17].

Two migration routes seem to be the most relevant on the Eurasian continent for AI possible distribution: Central Asian (the localization of H9 subtype is Pakistan) and East Asian (the localization of H5 subtype is Southeast Asia), since they cross the endemic areas [6].

Overcoming considerable distances, migratory birds are able to introduce AIV to new territories, resting, feeding and nesting sites. By contacting the local fauna, migratory birds spread the virus in new territories and into a new population, maintaining the pathogen circulation and evolving new outbreaks [18].

It should be noted that migration routes are not always clearly defined, however, molecular genetic tests of fecal or cloacal swabs from migratory waterfowl make it possible not only to identify AIV, but also to determine the genetic lineage of the recovered isolate [19].

Synanthropic birds are a kind of the vector of virus transmission from a wild reservoir to susceptible poultry and mammals in the anthropogenic environment. The combination of the described factors has recently led to the occurrence of sporadic cases in animals, not typically affected by this virus.

Potential factors of mammals' infection with HPAI virus. Contact with infected poultry and wild waterfowl, in particular feeding on infected bird meat, is one of the ways of mammals' infection, which creates conditions for AIV spillover [20].

In the wild, the risk of mammals' infection with influenza A viruses is primarily associated with their dietary patterns: hunting or scavenging birds. The role of wild mammals feeding on waterfowl and their carcasses is understudied in influenza epizootics. Contact with synanthropic waterfowl in urban environments can also cause infection of mammalian animals, in particular cats and dogs [21, 22].

Cases of infection with AIV H5N1 in domestic dogs have been published in literature. One of the first cases was reported in 2004 in Thailand and is presumably associated with feeding a dog with infected dead ducks from AI infected areas [23]. In 2009 in Egypt, AIV H5N1 isolates were recovered from nasal swabs collected from donkeys that had been in contact with infected poultry [24]. Studies show that foxes, martens and civets are susceptible to infection with the AIV subtype H5N1 [3, 25, 26, 27].

The susceptibility of Felidae family members to HPAI. *Felidae* family members show a rather high susceptibility to infection with AIV H5N1 subtype [21, 28, 29, 30]. In 2003 and 2004, tigers (*Panthera tigris*) and leopards (*Panthera pardus*) died in Thai zoos due to infection with AIV H5N1 subtype. As a result of the disease outbreak that occurred in the Sriracha Tiger Zoo in October 2004, 147 tigers died or were euthanized [31]. Researchers attribute the infection of big cats to a previous HPAI outbreak in poultry [32, 33, 34].

Testing of isolates recovered from tigers showed that H5N1 is more pathogenic for cats than other AIV subtypes, in addition, changes in the hemagglutinin of these isolates can contribute to an increase in the virus infectivity for mammalian hosts [32, 33].

First domestic cat infections (*Felis catus*) with AIV H5N1 were reported in Thailand in 2004 and coincided in time with outbreaks among poultry [34, 35].

In 2004 HPAI H5N1 outbreak at the Phnom Tamao Wildlife Rescue Centre in Cambodia caused the infection

of wild cats of 5 species: lions (*Panthera leo*), Asian golden cats (*Catopuma temminckii*), clouded leopard (*Neofelis nebulosa*), tigers (*Panthera tigris*) and leopards (*Panthera pardus*) [36].

Fatal cases of domestic cat infection with high pathogenicity H5N1 were reported in Iraq in early 2006; it is noted that the infection of animals occurred during the disease outbreak among poultry [37].

One of the first cases of infection with high pathogenicity H5N1of domestic cats in Europe was reported during an influenza outbreak among wild birds on the German island of Rugen in the Baltic Sea in February 2006, where 3 stray cats were found dead [38]. Around the same period, AIV H5N1 isolates were derived from 3 cats kept in a pet shelter in Graz (Austria) after contact with an infected swan in the same shelter. No clinical signs of the disease were found in cats [39]. In 2006, an AIV isolate was recovered from the internal organs of a dead cat in the Republic of Dagestan (Russia) [40].

In early 2013, a case of infection with HPAI virus H5N1 of a 4-month-old Bengal tiger cub in the Jiangsu Province zoo (China) with a lethal outcome was reported [41].

Specialists of the Erasmus University Medical Center (the Netherlands) conducted an experimental infection of European short-haired cats with H5N1 using various methods. Experimental infections with H5N1 virus isolated from a fatal human case confirmed that cats can develop severe clinical signs after intratracheal inoculation. The experiment also confirm that the virus can also be transmitted horizontally from cat to cat [34]. These findings are remarkable, as clinical disease resulting from infection with influenza viruses had not been noticed in cats before [42, 43, 44].

More recent studies have demonstrated that domestic cats can become infected via several routes and shed the virus in aerosols and with faeces, which can facilitate horizontal transmission route [45, 46].

In 2010–2012 in China, serum samples and nasal swabs collected from hundreds of stray cats living in close proximity to poultry farms or poultry markets were tested. As a result, it was determined that some of the animals were infected with AIV H5N1 [47, 48]. In December 2016, HPAI virus was detected in cats in South Korea. Genetic analyses indicated that the feline isolates were similar to AI H5N6 viruses isolated in chicken farms nearby [49].

Isolation of the HPAI virus from mammals in recent years. In 2015, the WOAH was notified about the detection of H5N1 in tigers in the Nanning Zoo (Guangxi, China) [3].

The first documented case of avian influenza (H5N1) infection with characteristic clinical signs in a lion also occurred in Ezhou zoo, Hubei province, China in 2016 [50].

Sporadic cases of AIV infection in marine mammals were reported by the UK Health Security Agency. According to the Agency, AIV H5N8 was isolated from a gray seal (*Halichoerus grypus*) in 2017, and H3N8 from a gray seal and two common seals (*Phoca vitulina*) in 2020 [51].

In November 2020, carcasses of 4 common seals, one gray seal and one red fox were found in Surrey county (England), which were submitted to the laboratory for diagnostic testing. Histopathology of the organ tissues of the fox and one of the seals showed lesions indicative of an acute systemic viral infection. Using virological and molecular biological test methods it was established that animals were infected with AIV H5N8. Two seal carcasses were decomposed so they were disposed of without diagnostics. Since tests for concurrent conditions were not conducted, other factors may have influenced the severity of the disease. There were no previously confirmed cases of highly pathogenic H5N8 infection in foxes [3, 52].

A phylogenetic analysis of H5N1 isolates recovered from 3 red foxes in December 2021 to February 2022 conducted by the Netherlands researchers showed that they were related to HPAI H5N1 clade 2.3.4.4b viruses that are found in wild birds. This suggests that the virus was not transmitted between the foxes [53]. Nevertheless, surveillance in mammals should be expanded to closely monitor the emergence of zoonotic mutations for pandemic preparedness.

The data on the reported AI cases in atypical hosts (mammals) given in Table 2 in section Supplementary files at https://doi.org/10.29326/2304-196X-2023-12-4-293-302, show that recently the AIV has acquired the ability to spillover from birds to mammals, such as mustelids (minks, otters, ferrets, badgers), felines (domestic cats, cougars,

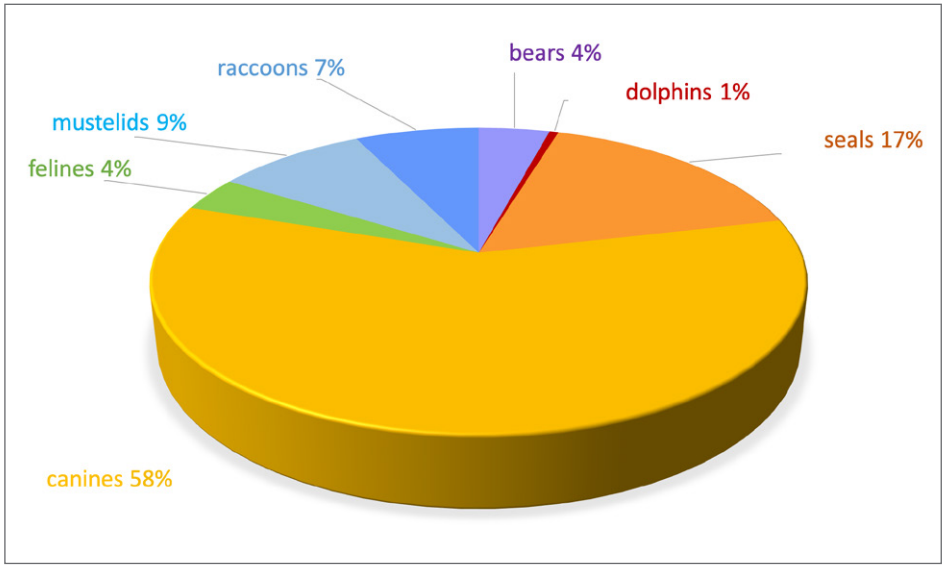

Fig. 2. Ratio of influenza virus infected animals (by families)

leopards, lynxes), pinnipeds (common seals, long-muzzled seals), bears (brown, grizzlies, American black), skunks, bottlenose dolphins, foxes, possums, raccoons (Fig. 2). Cases of infection were reported on the North American and Eurasian continents in zoos, nature parks and households.

As a rule, cases of HPAI infections among mammals coincide in time with outbreaks in waterfowl. The number of reported cases among atypical hosts increased significantly from sporadic cases in 2021 to more than a hundred in 2022. The largest number of such cases was reported in the USA and Canada. France, the United Kingdom, the Netherlands, Denmark and other countries reported AIV infections in mammals (Fig. 3).

Monitoring of the AIV spread in waterfowl, especially beyond the outbreaks, is one of the ways to obtain important epizootological information.

AI transmission from birds to mammals can play an important role in the evolution of new strains of mammalian viruses [57, 58]. Expansion of HPAI distribution area in recent years, an increase in the number of reports about the AIV infection in mammals, as shown in Table 2, a significant increase in the variety of species suffering from pronounced clinical symptoms, suggests a growing zoonotic potential of high pathogenicity H5N1. However, researchers believe that in order to cause a serious pandemic in the human population, the interspecies transmission of influenza viruses is not enough [59].

In the modern urbanization conditions, the number of contacts between migratory and synanthropic birds, domestic animals and humans increases many times, which, in turn, creates provocative conditions both for the virus transmission to atypical hosts and for the occurrence of mutations that carry anthropozoonotic risks (Fig. 4).

Therefore, the WHO Scientific Advisory Group for the Origins of Novel Pathogens (SAGO) is analysing emerging and re-emerging highly dangerous infections, including AI.

CONCLUSION

The comprehensive monitoring of both domestic and wild animals, if there is a possibility of the wild animal contacts with poultry and wild birds (especially waterfowl) is feasible as there is a need for timely measures in order to prevent or reduce the risk of the virus circulation in atypical hosts. The emergence of such a natural reservoir, which includes both typical hosts and mammals, can create conditions for the AIV circulation scheme, which is difficult to monitor.

Currently HPAI affects a wide range of birds and mammals. The disease also attacks rare, endangered species, which may lead to irreparable loss of species diversity.

The change (reduction) of habitats of both migratory birds and mammals due to the expansion of human economic activity, especially on the Eurasian continent, adds an ecological and urban component to AI control challenge and makes control and prevention more complex.

Current complex HPAI situation requires the development and implementation of improved measures, taking into account the latest trends in the disease epizootology.

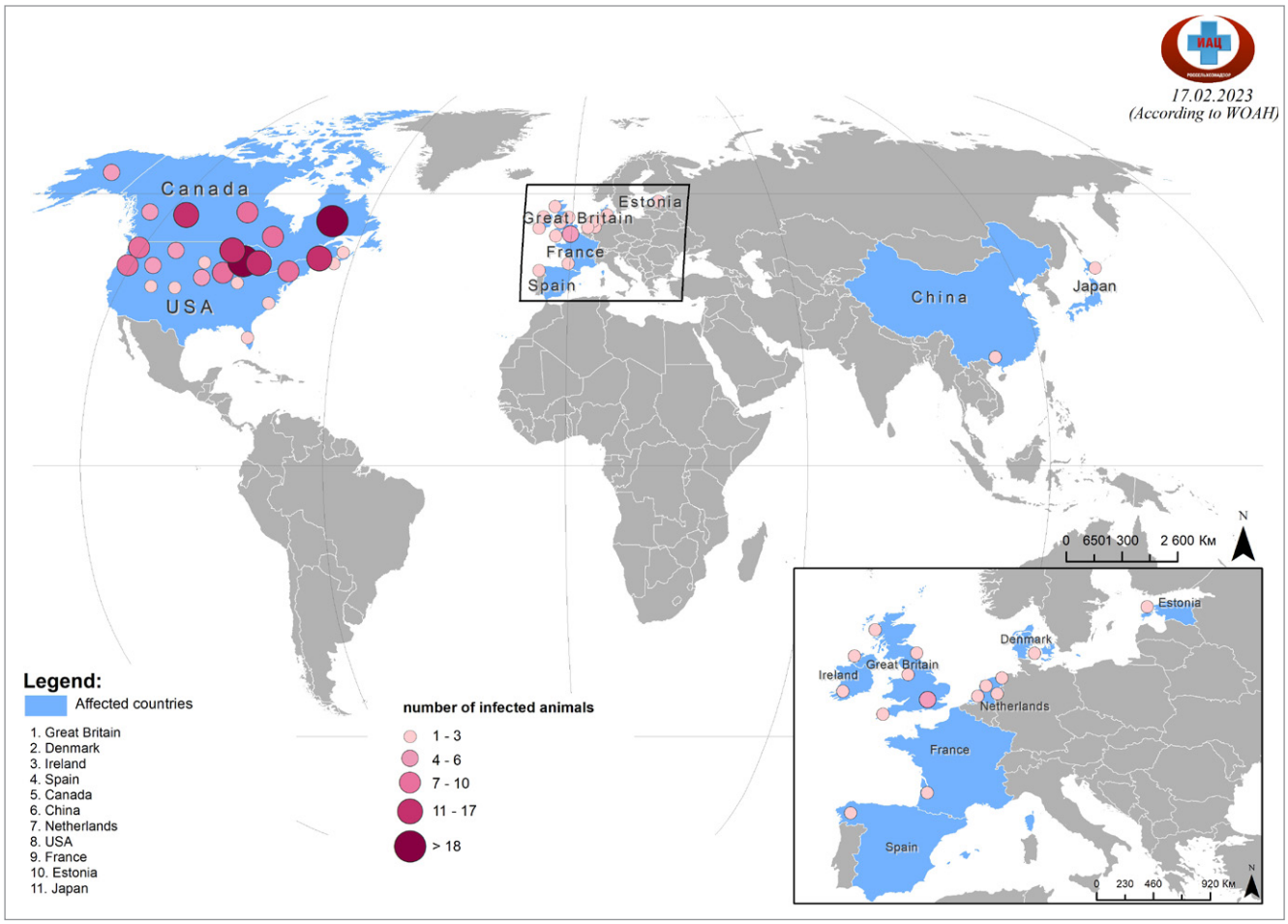

Fig. 3. Occurrence of HPAI infections in mammalians (according to the WOAH data)

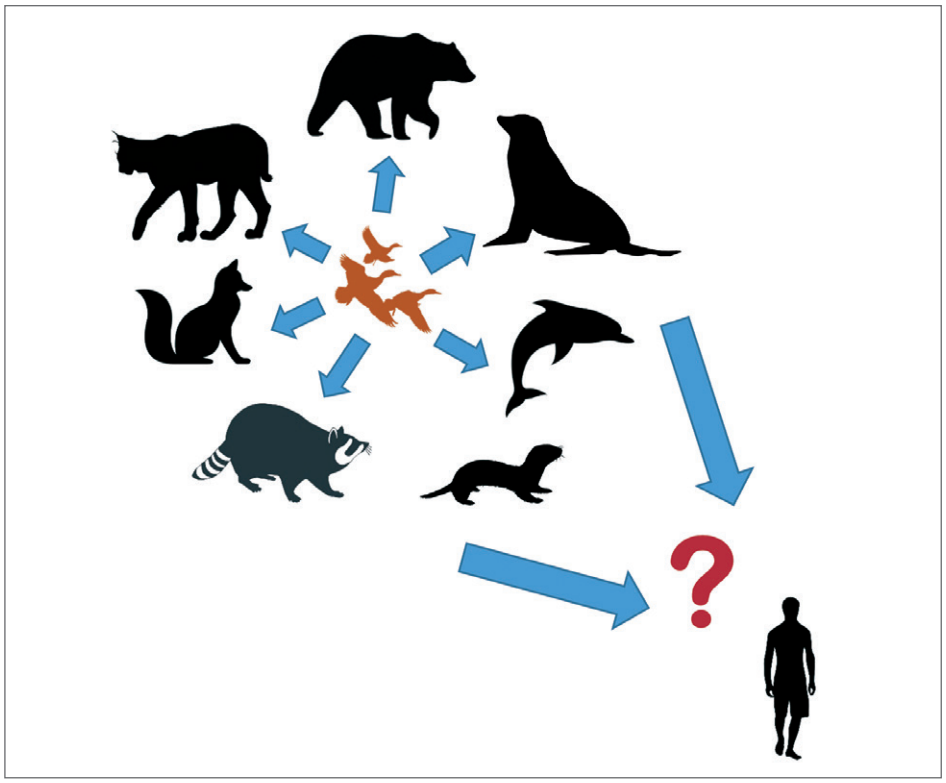

Fig. 4. AIV spillover risks

Currently, there is no sufficient evidence of human infection after contact with infected mammals. The AI virus is able to affect a wide range of avian species and mammals, and thus presents a potential risk to humans.

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Feline panleukopenia (review)

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ABSTRACT

Feline panleukopenia, a disease globally known since the beginning of the last century and originally attributed to canine distemper, has expanded its range of hosts since its discovery as a result of successful infections (both natural and experimental) in mustelids, raccoons and even primates. Evolutionarily, the disease pathogen gave rise to a new infectious agent – canine parvovirus, which, despite its DNA structure, demonstrates a relatively high mutation rate and the emergence of new variants. The disease is in most cases fatal to newborn kittens and causes severe manifestations in adult cats, severely affecting the vital systems of the body. The prognosis is often (up to 50%) unfavorable, while the animal's age plays a key role. Current preventive measures can ensure protection, however, vaccines are used in the absence of adequate testing on cats and dogs (for ethical reasons) and have a number of limitations in use. The persistence of the infectious agent in the environment and the growing number of stray animals allow the infectious agent to circulate unhindered in these populations, threatening the health of domestic cats and endangered felines in nature reserves and zoos. Easing of legislation for leading research centers, regulation of the number of stray animals, adequate prevention measures for target groups in animal shelters, nurseries and zoos can contribute to a significant reduction in the circulation in susceptible populations of pathogens not only of this disease, but also of the majority of other dangerous infections, such as rabies, feline rhinotracheitis, canine distemper and others.

Keywords: review, feline panleukopenia, parvoviruses, *Felidae*

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РЕЗЮМЕ

Панлейкопения кошек – болезнь, известная в мире с начала прошлого века и первоначально отнесенная к чуме плотоядных, – с момента обнаружения расширила круг своих хозяев в результате успешных заражений (как в естественных условиях, так и искусственно) куньих, енотовых и даже приматов. Эволюционно возбудитель заболевания дал начало новому инфекционному агенту – парвовирусу собак, который, несмотря на ДНК-архитектуру, демонстрирует сравнительно высокую скорость мутаций и появление новых вариантов. Болезнь в большинстве случаев смертельна для новорожденных котят и вызывает сильные страдания взрослых кошек, тяжело поражая жизненно важные системы организма. Исход часто (до 50%) неблагоприятный, причем возраст кошки играет одну из ключевых ролей. Существующие меры профилактики способны защитить животных, однако вакцинные препараты используются при отсутствии адекватных испытаний на кошках и собаках (по этическим соображениям) и имеют ряд ограничений в применении. Устойчивость инфекционного агента в окружающей среде и растущее число безнадзорных животных позволяют беспрепятственно циркулировать возбудителю инфекции в данных популяциях, угрожая благополучию домашних кошек, а также вымирающих кошачьих в заповедниках и зоопарках. Послабления в законодательстве для ведущих исследовательских центров, регуляция численности безнадзорных животных, адекватная профилактика среди целевых групп в приютах, питомниках и зоопарках могут способствовать значительному снижению циркуляции в подверженных риску популяциях возбудителей не только данной болезни, но и большинства других опасных инфекций, например бешенства, ринотрахеита кошек, чумы плотоядных и других.

Ключевые слова: обзор, панлейкопения кошек, парвовирусы, кошачьи

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INTRODUCTION

Feline panleukopenia (FPL), also known as feline distemper, is a highly contagious disease of cats (*Carnivora*, *Felidae*) characterized by high mortality in newborn kittens (> 90%) with an acute course and neurological disorders such as ataxia and blindness. Older kittens develop panleukopenia, neutropenia due to bone marrow and lymphatic tissue infection, and diarrhea due to enterocyte disruption. The clinical form of the disease is most often diagnosed in animals aged 2–5 months, and subclinical or mild disease forms prevail in older cats [1, 2]. Panleukopenia in cats is caused by such subspecies of *Protoparvovirus carnivoran 1* as FPV (90–95% of cases) and some strains of canine parvovirus (< 10% of cases) [3].

Feline panleukopenia virus (FPV) can also infect raccoons (*Procyonidae*) and mustelids (*Mustelidae*). Feline panleukopenia has been known as a separate nosological unit since the 1920s, but *Canine parvovirus* (CPV) occurred as an infectious agent only in the late 1970s [2, 4]. Interestingly, FPV mutates slowly as a result of random genetic drift, while CPV demonstrates a rate of genomic substitutions similar to that of RNA viruses, with about 10^{-4} substitutions per site per year [5].

The disease is widespread on all continents and in most countries. Vaccines against FPL are usually developed based on avirulent virus strains with replication capacity (attenuated live vaccines) [6].

This review deals with the disease that causes high mortality in domestic cats (*Felis catus*), thus posing a great interest with regard to improving quality of life for companion animals taking into account significant economic costs on pet keeping and treatment, as well as pedigree breeding and preservation of endangered feline populations. In addition, the spillover of the pathogen to wild susceptible animals and some fur animal species makes these populations vulnerable and poses a threat of the pathogen's entry to fur farms or establishment of virus reservoirs in wild animals.

The FPL agent is a non-enveloped virus containing a single-stranded DNA (ssDNA) and an icosahedral capsid [7, 8]. It belongs to the genus *Protoparvovirus*, one of the eleven genera of vertebrate viruses in the subfamily *Parvovirinae*, the family *Parvoviridae*. The FPV and CPV jointly with associated variants found in various carnivorous species such as minks and raccoons make up the species *Protoparvovirus carnivoran 1* [9].

The virus genome consists of 5.1 kbp and contains 2 open reading frames: non-structural (NS) and structural (VP) proteins. The NS gene encodes the NS1 and NS2 proteins involved in DNA replication, capsid assembly and intracellular transport, the VP gene encodes the capsid proteins VP1 and VP2. The virus capsid consists of 60 molecules of viral proteins, approximately 10% VP1 and 90% VP2, the latter of which allows the virus to bind to the transferrin receptor (TfR) of the host cell [10, 11]. Moreover, changes in the species-specific binding of capsid proteins to the host receptor determine susceptibility to FPV or CPV [12]. This phenomenon is reflected in the adaptation of the capsid protein to the receptors of other hosts, which ensured effective interspecies distribution, as can be seen from the example of infection of cats with new strains of canine parvovirus type 2 (CPV-2) [13]. The evidence suggests that the original type 2 (CPV-2) came from FPV infecting only cats in 1978, and gave rise to the antigenic variant CPV-2a through 5–6 non-synonymous mutations in the VP gene, which led to a change in the amino acid composition of VP2 in 1979–1981 [14].

The range of hosts includes domestic and wild cats, raccoons, minks, foxes [13, 15, 16]. Domestic dogs are not susceptible to the FPL agent, since the virus cannot bind with the host's TfR target cells. However, there are studies showing that during experimental infection of dogs, FPV replicated in lymphoid tissues (thymus, bone marrow), but this was not enough for successful infection *in vivo* [17, 18]. At the end of the XX century, the viral DNA was isolated from the faeces and formalin-fixed small intestines of captive cheetahs (*Acinonyx jubatus*) and free-living African wildcat (*Felis lybica*) and honey badger (*Mellivora capensis*) [15]. In 2008 FPV was recovered from 500 macaques demonstrating signs of hemorrhagic enteritis in the Chinese Experimental Animal Centre. The virus was identified morphologically, genetically, and the result of bioassay performed in felines was positive [19]. In 2022 a 9-month-old leopard (*Panthera pardus*) was diagnosed with panleukopenia in the Wildlife Rescue Center in India. This endangered feline species had been brought from a Transit Animal Treatment Center, which was, in fact, a shelter for providing care and treatment to wild animals. The genome sequence of the isolated virus was 99.14% similar to the sequence of nucleotides recovered from the raccoon virus [20]. An interesting case of FPV isolation from a banded linsang (*Prionodon linsang*) in Thailand was described by N. Inthong et al. in 2019. Amino acid analysis of VP2 protein revealed a high level of homology (over 98%) with FPV. The isolate was closely related to the FPV strains from Japan, South Korea and China [21].

The pathogen is transmitted through direct contact with the secretions of infected animals, including faeces, blood, urine. In addition, there is a vertical transmission pathway, while infection can result in abortion, mummification and stillbirth [22].

Parvoviruses are extremely stable in the environment, and indirect transmission probably plays an important role in spreading and maintaining the agent circulation in a population, especially in wild carnivores. Transmission between domestic and wild carnivores is assumed to be easy, and fomites may be factors in long-distance transmission. Apparently, this causes high mortality in intact populations [23].

In populations where parvoviruses are constantly circulating, new cases occur mainly among young animals that get infected due to decrease in colostral antibody titers, and the infection dynamics in seasonal breeders may strongly depend on stock replacement, often resulting in cyclical incidence [23].

After FPV entry into the cell via clathrin-mediated endocytosis, endosomes with a virion fuse with the nuclear membrane. After the virus is released from the capsid in the nucleus, it uses the DNA polymerase of the host cell for replication [24, 25]. Since the virus can replicate only in actively dividing cells (occurring in the S-phase of mitosis), it possesses tropism to lymphoid tissue, bone marrow, epithelium of intestinal crypts and other actively dividing cells of newborn kittens. FPV can replicate in Purkinje cells of the cerebellum of kittens under 10 days of age [26]. The tissue tropism determines the clinical signs and pathological-anatomical picture.

The incubation period lasts 4–5 days, and the clinical course of the disease can rapidly develop causing death. The gate of infection are the palatopharyngeal tonsils, after which viremia develops rapidly. The primary pathological site of virus replication is located in the intestinal crypts due to the high mitotic activity of the latter, causing severe enteritis and diarrhea. The virus also targets lymphoid tissue, which leads to pancytopenia (less than 4,000 cells/μL). In the later stages of the disease it can be observed that a white blood cells count returned to normal. In some cases, icterus may also be noted [22].

The disease clinical signs demonstrate the nervous system pathology (depression, ataxia, anorexia), fever, the digestive tract pathology (vomiting, diarrhea), hypersalivation [19, 27, 28, 29]. The development of the clinical picture directly depends on the age of the animal. Thus, the virus replicates in multiple tissues and often causes cerebellar hypoplasia, and, consequently, nervous disorders in newborn animals. The virus replication is limited to lymphoid cells and small intestine cells, causing temporary panleukopenia and diarrhea in older animals [30]. Interestingly, newborn animals show no signs of diarrhea, probably due to a lower rate of reproduction of intestinal epithelial cells at the beginning of life, but infection of fetuses and newborns usually leads to death or irreversible disabling damage to organ systems [31].

Necropsy performed in kittens and adult cats showed the following common pathological and anatomical picture: local small- and large-focal enteritis with spot and/or petechial hemorrhages in the serous membrane. Lesions are most pronounced in the jejunum and ileum. Thickening of the intestinal walls accompanied by edema and hemorrhagic lymphadenitis of the mesenteric lymph nodes are often found [32, 33]. Histological lesions in the small intestine include multi-focal necrosis and loss of crypt architecture. The effects of secondary bacterial infection are also observed. FPV is known to be teratogenic in case of intrauterine infections. In the last stages of pregnancy, the virus targets mitotically active brain and eye tissues. This leads to cerebellar hypoplasia, hydrocephalus, and retinal dysplasia [34, 35].

The DNA molecule of *Protoparvovirus carnivoran 1* persists for a long time in the tissues of convalescent animals, leaving behind a molecular trace [16]. The virus can remain latent in peripheral blood monocytes, as evidenced by the successful FPV cultivation from monocytes of healthy cats with high titers of virus neutralizing antibodies [36, 37, 38]. The antibody-mediated immune response viewed as a production of virus neutralizing antibodies is prevalent in FPV-induced infection. Colostral immunity plays an important role in the protection of newborns. The infection mainly occurs in young animals aged 2–4 months. Cell-mediated immunity also plays an important role in the recovery [31].

The treatment of animals with panleukopenia primarily includes transfusion therapy with electrolyte replenishment. Since the disrupted intestinal crypt architecture contributes to bacteremia, and intensification of neutropenia aggravates this process and often leads to sepsis, antibacterial therapy with broad-spectrum drugs (especially against gram-negative and anaerobic bacteria) is required. An easily digestible diet is preferred, feeding should not be suspended. It is important to understand that many cats with panleukopenia, especially those that come from shelters, also have a parasitic infection, and therefore coprolarvoscopy, coproovoscopy and appropriate treatment with anthelmintics is an important factor, since intestinal parasitosis is a common concomitant disease [10, 22, 39].

As previously reported, FPV is highly resistant to environmental factors, as well as to many detergents [39, 40, 41]. In shelters, staff may act as mechanical virus carriers and, therefore, pose a hazard to unvaccinated cats [42]. Diseased carnivores shed the pathogen in high titers (up to 10^9 $TCID_{50}$ per gram of faeces), and the virus quickly accumulates in nurseries and shelters, since the latter are characterized by initial populations of animals with an unknown vaccination history and frequent personnel turnover. Due to the high contagiousness of FPV, susceptible animals can get infected even after thorough disinfection of premises [40]. There is an acute problem of high feline mortality due to FPL in shelters in Europe, the USA and Australia [10, 43, 44]. Therefore, it is recommended that only kittens and cats subjected to successful vaccination are placed in such an environment [40].

Live and inactivated vaccines against FPL inducing stable immunity levels in animals have been developed. Live vaccines generally induce rapid development of protective antibodies in immunocompetent cats [45]. However, even a single dose of an inactivated vaccine against FPV can confer a sufficient antibody-mediated response in previously uninfected cats within a short period of time [46]. Despite this, there are some restrictions on the use of live attenuated vaccines: 1) the vaccine shall not be used in pregnant females due to the risk of virus penetration to the fetus and subsequent dysfunction of the developing cerebellum; 2) the vaccine shall never be administered to kittens under 4 weeks of age for the same reason: to avoid damage to the cerebellum, which is still developing in newborn kittens. At the moment, there are no studies available aimed at finding out what vaccine type or manufacturer are more effective. Due to the high resistance of the virus in the environment and the wide spread

of the disease in the world, all cats are at risk of infection. Animals that are mostly kept in households can become infected via fomites. Therefore, vaccination is recommended for all cats lacking adequate immunity [47].

As a rule, the colostral antibody titers in kittens decrease to a threshold level by 12 weeks of age, so the first vaccination is carried out at the age of 8–9 weeks, and followed by revaccination in 3–4 weeks. The FPL vaccination strategy should be based on the preliminary determination of maternal antibody titers, since their high concentration in animal blood can lead to the neutralization of the virus vaccine strains included in live vaccines [6, 47, 48]. In this regard, the advantage of inactivated vaccines should be emphasized, since the immune response induced post administration is not affected by the level of colostral antibodies.

Recent amendments in the Russian legislation [49], having an ethical background, may significantly limit tests of preventive and therapeutic medicinal products in target animals. This state of affairs forces us to receive incomplete and not always reliable information about the effectiveness of such products, which means that it puts animal populations that are in need of protection from infection, in a vulnerable position.

Forecast. FPV can cause a serious and potentially fatal disease in cats; 30–50% of diseased animals die despite intensive treatment [50, 51].

According to F. Porporato et al. [52] and F. Ferri et al. [53], either FLV immunocompetent cats or cats without signs of depression and with a higher body weight during admission to the clinic demonstrated a high survival rate. There is a high probability of an unfavorable outcome on day 3 of hospitalization or later even with adequate leukopenia treatment provided.

CONCLUSION

Feline panleukopenia, known for more than a hundred years, still remains a serious feline concern that is not receiving enough attention in the world. Strict ethical standards for companion animals may limit the adequate implementation of preventive measures. The high degree of the pathogen's resistance in the environment poses a threat of its entry into animal husbandry and zoos, as well as the establishment of wildlife reservoirs. Veterinary services in most world countries need to pay closer attention to this feline infection in the era of significant costs to ensure safe coexistence of companion animals and humans.

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Retrospective analysis of enzootic bovine leucosis spread in Republic of Dagestan considering natural and climatic conditions

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ABSTRACT

Retrospective analysis of enzootic bovine leucosis (EBL) data received by the Republic of Dagestan Veterinary Laboratory and Veterinary Department of the Republic of Dagestan was made. From 1988 to 2022, the Republican veterinary laboratories serologically tested 3,205,118 animal sera for the antibodies to bovine leukaemia virus (BLV) antigen, and 76,133 (2.4%) of them were positive. High BLV infection levels were detected in 1988 (32.2%), 1989 (21.3%), 1991 (23.3%), 1993 (23.0%), 2005 (24.2%), 2010 (23.0%), and the lowest ones were reported in the recent years: 2020 – 1.0%, 2021 – 1.0%, 2022 – 0.5%. In 2022, diagnostic testing of 875,312 serum samples was carried out, which included 476,493 sera collected from bovines in high-altitude and mountainous areas of Dagestan. In the plain areas, 255,312 bovine animals were tested for leucosis, and 122,967 animals were tested in the sub-mountain areas. The animal infection with BLV in these natural and climatic conditions was reported as follows: high-altitude and mountainous areas – 0.5% (2,313 animals), plain areas – 0.8% (1,925 animals), sub-mountain areas – 0.1% (109 animals). Additional 20,540 serum samples were tested in the laboratories at the transhumance pasture veterinary units, and 170 BLV seropositive animals (0.83%) were detected. No EBL was diagnosed in the laboratories of the Derbent, Kochubeysk, Ulankholsk, Bakressk veterinary units, but other four laboratories detected high level of BLV seropositive animals (Kizlyarsk – 14.6%, Babayurt – 3.6%, Tarumovsk – 3.0%, Kyzylyurt – 1.06%). Thus, EBL is widespread in animals, especially in the plain areas of the Republic of Dagestan.

Keywords: enzootic bovine leucosis (EBL), epizootic map, natural and climatic zones, dynamics of EBL spread, Republic of Dagestan

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Ретроспективный анализ распространения лейкоза крупного рогатого скота в Республике Дагестан с учетом природно-климатических условий

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РЕЗЮМЕ

Проведен ретроспективный анализ эпизоотологических данных по лейкозу крупного рогатого скота ГБУ РД «Республиканская ветеринарная лаборатория» и Комитета по ветеринарии Республики Дагестан. С 1988 по 2022 г. в ветеринарных лабораториях республики серологическим методом были проведены исследования 3 205 118 проб крови животных с целью выявления антител к антигену вируса лейкоза крупного рогатого скота, из них 76 133 (2,4%) дали положительный результат. Высокий уровень инфицированности животных вирусом лейкоза крупного рогатого скота выявлен в 1988 (32,2%), 1989 (21,3%), 1991 (23,3%), 1993 (23,0%), 2005 (24,2%), 2010 (23,0%) годах, а наименьший установлен в последние годы: в 2020 г. – 1,0%, в 2021 г. – 1,0%, в 2022 г. – 0,5%. В 2022 г. проведены диагностические исследования 875 312 проб сыворотки крови, из которых 476 493 были отобраны от крупного рогатого скота из высокогорных и горных районов Дагестана. В равнинной части республики на лейкоз было исследовано 255 312 гол. крупного рогатого скота, в предгорных районах – 122 967 гол. Инфицированность животных вирусом лейкоза крупного рогатого скота в данных природно-климатических зонах составила: в высокогорных и горных – 0,5% (2313 гол.), в равнинной зоне – 0,8% (1925 гол.), в предгорной – 0,1% (109 гол.). Еще 20 540 проб сыворотки крови были исследованы в лабораториях ветеринарных станций отгонного животноводства, в результате выявили 170 (0,83%) животных, сероположи-

тельных к вирусу лейкоза крупного рогатого скота. В лабораториях Дербентской, Кочубейской, Уланхольской, Бакресской ветеринарных станций лейкоз у крупного рогатого скота не диагностирован, а в других четырех выявлен высокий уровень серопозитивности животных к ВЛКРС (Кизлярская – 14,6%, Бабаюртовская – 3,6%, Тарумовская – 3,0%, Кизилюртовская – 1,06%). Таким образом, лейкоз крупного рогатого скота имеет повсеместное распространение среди животных, особенно на территории равнинной зоны Республики Дагестан.

Ключевые слова: лейкоз крупного рогатого скота, эпизоотическая карта, природно-климатические зоны, динамика распространения вируса лейкоза крупного рогатого скота, Республика Дагестан

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INTRODUCTION

Enzootic bovine leucosis (EBL) is a disease widely spread in many countries of the world and in the regions of the Russian Federation [1, 2, 3, 4]. The disease is caused by bovine leukemia virus (BLV) and specified by long subclinical course. EBL is mostly chronic and seldom acute [5, 6, 7, 8]. Definite time period (3–5 years and more) is necessary for the disease to transform from the subclinical to hematological or clinical phase. This is entirely dependent on the immunological and physiological status of the animal. Reasons or negative factors affecting the immune status of the BLV-infected animal can include concomitant diseases (brucellosis, tuberculosis, etc.), poor feeding, stress (veterinary manipulations, insect bites, injuries, etc.). Effects of radiological elements as well as natural and climatic conditions (temperature, humidity, rarified air, etc.) cannot also be excluded [9, 10, 11, 12, 13, 14].

In 2020, in the Republic of Dagestan, there were over million bovines and the number of cows amounted to 481 thousand of them; in 2021 – 951 thousand animals including 462 thousand cows. In 2022, the veterinary laboratories serologically tested 875,312 bovine blood sera that amounted to 92% of the total bovine population in the Republic. When 5–6-month old calves born from the cows not subjected to EBL testing are included, the coverage of the serologically tested population can reach 100% for the Republic.

The study was aimed at the retrospective analysis of EBL epizootic situation taking into account natural and climatic zones in the Republic of Dagestan.

Based on the aim the following tasks were identified:

1. Retrospective analysis of EBL-situation over the recent 35 years.

2. Examination of BLV spread in animals in different natural and climatic zones of the Republic.

3. Compilation of EBL epizootic map for the municipal raions and okrugs in the Republic.

MATERIALS AND METHODS

In order to conduct EBL epizootological monitoring in municipal raions and okrugs, official statistical data of the GBI RD "Republican Veterinary Laboratory" and the Republic of Dagestan Veterinary Committee were used. EBL situation in the Republic over the past 35 years was studied in the form of a retrospective analysis, taking into account the effect of natural and climatic conditions on EBL spread in animals. The epizootic map of EBL spread in the Republic was compiled taking into account the territorial boundaries of raions and municipal okrugs.

Serological and hematological tests of animal blood for EBL were carried out according to the "Guidelines for bovine leukosis diagnosis" [15], and the BLV spread situation was studied according to the "Guidelines for epizootological survey of bovine leukosis" [16].

RESULTS AND DISCUSSION

In 1988–2022, serological tests of 3,205,118 animal blood samples were performed to detect antibodies to the BLV antigen, and 76,133 (2.4%) test results were positive. A high proportion of BLV infection was detected in animals in 1988 (32.2%), 1989 (21.3%), 1991 (23.3%), 1993 (23.0%) and 2005 (24.2%), 2010 (23.0%). The lowest level of infection was established in the recent years: in 2020 – 1.01%, in 2021 – 1.0%, in 2022 – 0.5% (Table 1). In our opinion, the low level of animal infection with BLV is associated with a high coverage of bovines with diagnostic tests, and the highest percentage of seropositive farm animals is associated with the sampling of animals located on the public farms in the plain zone. At the same time, hematological tests were conducted in veterinary laboratories among the seropositive animals for the detection of persistent leukocytosis. A total of 40,057 samples were hematologically tested. As a result, 5,612 (14.0%) animals demonstrated development of persistent leukocytosis. Over the past 35 years, the largest number of bovine blood samples were subjected to hematological tests in 1988 (9,451), 1989 (9,127), 1990 (4,657) and 2019 (6,070). The proportion of hematologically diseased animals in these years amounted to 9.4% (888), 11.3% (1,031), 11.0% (512), 24.4% (1,482), respectively. In 2006, 2007, 2008 and 2022, hematological tests of animal blood were not conducted in the Republic, and the number of samples tested in other years was insignificant and did not reflect the real situation on EBL incidence. Nevertheless,

Table 1
Retrospective analysis of EBL spread in the Republic of Dagestan

Year	IDA tested			Hematological tests		
	total tested, animals	IDA-positive	seropositive, %	total tested, animals	persistent leukocytosis	%
1988	9,248	2,977	32.2	9,451	888	9.4
1989	31,823	6,783	21.3	9,127	1,031	11.3
1990	18,592	3,678	19.8	4,657	512	11.0
1991	8,613	2,006	23.3	1,277	168	13.2
1992	8,777	1,161	13.2	755	26	3.4
1993	5,157	1,186	23.0	1,039	21	2.0
1994	11,413	1,538	13.5	401	16	4.0
1995	9,575	1,219	12.7	733	8	1.1
1996	6,773	979	14.5	145	–	0
1997	6,041	462	7.6	18	–	0
1998	5,162	384	7.4	128	–	0
1999	4,112	151	3.7	72	–	0
2000	2,553	48	1.9	511	–	0
2001	2,300	68	3.0	49	–	0
2002	2,610	197	7.5	60	13	21.7
2003	2,133	23	1.1	20	–	0
2004	3,287	60	1.8	72	–	0
2005	3,127	758	24.2	286	53	18.5
2006	2,658	335	12.6	–	–	–
2007	–	–	–	–	–	–
2008	20,007	581	2.9	–	–	–
2009	10,109	1,822	18.0	281	103	36.7
2010	9,328	2,148	23.0	451	193	42.8
2011	7,417	1,214	16.4	136	61	44.9
2012	5,977	172	2.9	81	22	27.2
2013	7,210	1,220	16.9	447	135	30.2
2014	5,504	295	5.4	233	39	16.7
2015	7,310	1,016	13.9	79	14	17.7
2016	10,842	1,433	13.2	296	86	29.1
2017	7,466	577	7.7	188	45	23.9
2018	223,293	8,998	4.0	1,202	292	24.3
2019	625,970	15,578	2.5	6,070	1,482	24.4
2020	524,930	5,361	1.0	1,265	251	19.8
2021	720,489	7,188	1.0	527	153	29.0
2022	875,312	4,517	0.5	–	–	–
Total	**3,205,118**	**76,133**	**2.4**	**40,057**	**5,612**	**14.0**

IDA – immunodiffusion assay.

high percentage of the leukemia diseased animals of the number of hematologically tested ones was noted in 2009 (36.7%), 2010 (42.8%), 2011 (44.9%), 2013 (30.2%), 2016 (29.1%) and 2021 (29.0%).

In 2007, EBL diagnostic tests of animal blood were not carried out, and the number of serologically tested animals did not exceed 32 thousand bovines/year until 2018. Large-scale serological EBL tests were started in 2019. Then 625,970 animal serum samples were tested, in 2020 – 524,930, in 2021 – 720,489, in 2022 – 875,312, of which 15,578 (2.5%), 5,361 (1.0%), 7,188 (1.0%), 4,517 (0.5%) samples were seropositive, respectively.

Table 2
Epizootic analysis of EBL spread in 2022 considering the natural and climatic zones of the Republic of Dagestan

Raions and municipal okrugs	Total IDA-tested, animals	IDA-positive, animals	Seropositive animals, %
Plain area			
Babayurtovsky	17,944	91	0.51
Kizilyurtovsky	15,589	2	0.013
Kizlyarsky	24,022	721	3.0
Tarumovsky	29,956	334	1.1
Khasavyurtovsky	56,744	52	0.09
Karabudakhkentsky	15,414	75	0.5
Kumtorkalinsky	6,188	308	5.0
Magaramkentsky	25,693	0	–
Nogaysky	18,163	3	0.02
Kayakentsky	10,534	18	0.17
Derbentsky	12,479	5	0.04
Kaspiysk city	576	0	–
Makhachkala city	15,397	260	1.7
Khasavyurt city	5,222	3	0.06
Derbent city	571	0	–
Dagestanskiye Ogni town	507	0	–
Kizlyar town	313	53	16.9
Total	**255,312**	**1,925**	**0.8**
Sub-mountain area			
Kazbekovsky	18,306	0	–
Kaytagsky	8,559	0	–
Sergokalinsky	7,468	50	0.67
Tabasaransky	20,743	1	0.005
Khivsky	8,790	0	–
Buynaksky	29,327	24	0.08
Novolaksky	10,165	34	0.33
Suleyman-Stalsky	19,609	0	–
Total	**122,967**	**109**	**0.1**
High-altitude and mountainous areas			
Agulsky	7,967	0	–
Akushinsky	58,933	65	0.1
Akhtynsky	10,706	0	–
Kurakhsky	11,492	0	–
Gergebilsky	21,018	107	0.51
Gunibsky	33,855	762	2.3
Kulinsky	25,131	4	0.02
Laksky	25,471	37	0.15
Levashinsky	24,612	2	0.008
Rutulsky	14,673	13	0.09
Untsukulsky	15,817	114	0.7
Khunzakhsky	21,669	20	0.09
Shamilsky	30,780	263	0.9
Botlikhsky	36,994	208	0.6
Gumbetovsky	18,513	0	–
Dakhadayevsky	20,241	96	0.5
Tlyaratinsky	13,892	76	0.5
Charodinsky	20,014	385	1.9
Tsumadinsky	17,247	72	0.4
Tsuntinsky (Bezhtinsky uchastok)	14,680	0	–
Akhvakhsky	22,264	89	0.4
Dokuzparinsky	10,524	0	–
Total	**476,493**	**2,313**	**0.5**

IDA – immunodiffusion assay.

In view of the above, it can be concluded that the number of diagnostic tests is annually increasing, and animal BLV infection rates are decreasing.

In 2022, EBL diagnostic tests were carried out on 875,312 serum samples, of which 476,493 were collected from cattle in the high-altitude and mountainous regions of Dagestan. 255,312 bovines were tested for EBL in the plain part of the Republic and 122,967 animals in the sub-mountain areas (Table 2). BLV seropositivity of cattle in the natural and climatic conditions of the Republic was at the following level: in the plain areas – 0.8% (1,925 animals), in high-altitude and mountainous areas – 0.5% (2,313 animals), in the sub-mountain areas – 0.1% (109 animals). A high degree of BLV infection in animals is mainly reported in the areas located on the plain, as well as in mountainous areas where transhumance farms are located in the flat areas. The highest epizootic BLV spread was reported in the following raions and municipal okrugs: Kizlyar town (16.9%), Kumtorkalinsky (5.0%), Kizlyarsky (3.0%), Gunibsky (2.3%), Charodinsky (1.9%) raions, Makhachkala city (1.7%), Tarumovsky Raion (1.1%). In other raions and municipal okrugs, the animal BLV seropositivity level did not exceed 1.0%. In 2022, the Republican veterinary laboratories did not detect BLV antibodies by diagnostic tools in the blood of animals in 11 raions (Magaramkentsky, Kazbekovsky, Kaytagsky, Khivsky, Suleyman-Stalsky, Agulsky, Akhtynsky, Kurakhsky, Gumbetovsky, Tsuntinsky (Bezhtinsk uchastok), Dokuzparinsky) and in 3 municipal okrugs (Kaspiysk, Derbent, Dagestanskiye Ogni).

Analyzing the data in Table 2, it can be noted that the lowest BLV spread (0.1%) in animals was reported in the areas that are located in the sub-mountain areas

Table 3
EBL monitoring performed by the transhumance pasture veterinary units in the Republic of Dagestan in 2022

Transhumance veterinary stations	Total tested, animals	IDA-positive, animals	Seropositive animals, %
Tarumovsk	4,310	129	3.0
Kizlyar	82	12	14.6
Bakres	3,515	0	–
Kizilyurt	1,228	13	1.06
Babayurt	446	16	3.6
Kochubeysk	5,888	0	–
Ulankholsk	3,892	0	–
Derbent	1,179	0	–
Total	**20,540**	**170**	**0.83**

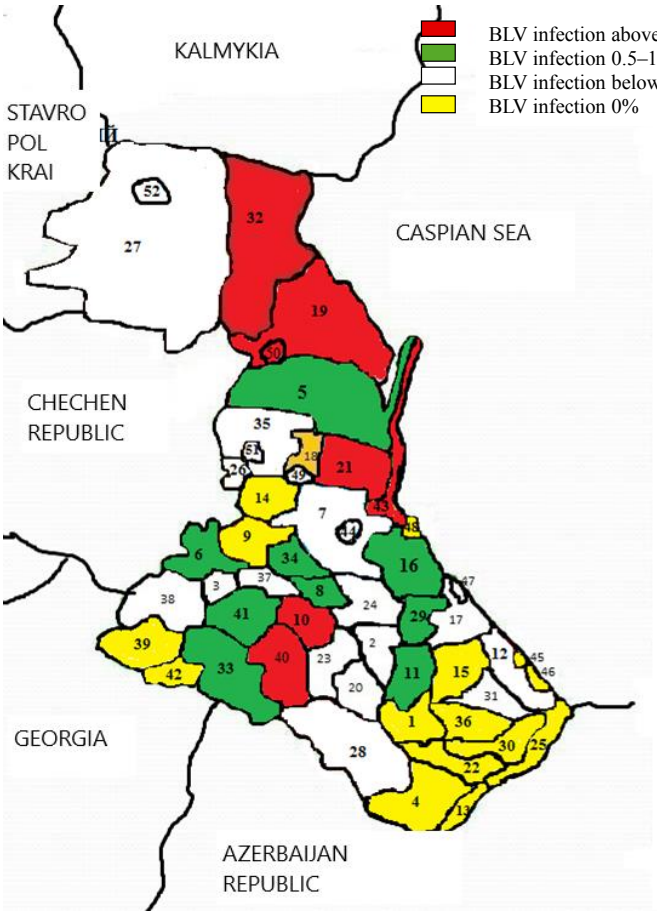
IDA – immunodiffusion assay.

of the Republic. This is due to the fact that the farm animals in these areas have limited contact with the infected animals located in the plain areas of the Republic. However, in the mountainous and high-altitude areas of the Republic, many livestock farmers graze their cattle and small ruminants on mountain meadows in summer, and in the transhumance areas on the lowland plaining in winter, where, in our opinion, contact with infected animals may occur.

Thus, the main reasons for BLV spread in the mountainous areas of the Republic include the following: joint grazing of indigenous animals and animals from the mountainous areas in the plain areas, veterinary manipulations (tagging, blood collection, obstetrics, etc.) at the transhumance veterinary stations, etc. As noted earlier [9], the high level of EBL spread in the plain areas of the Republic is associated with the historical importation of infected animals from the disease-infected farms in the north-western and central regions, as well as from the Baltic Republics and Ukraine during the Soviet period. An important factor in the spread of the infection in the plain areas of the Republic of Dagestan also involves high concentration and intensification of animal reproduction on public farms [17, 18, 19].

In the Republic, in the places of transhumant management of cattle and small ruminants, the transhumance veterinary stations are located in the plain areas, where diagnostic tests of animal blood for various diseases are carried out, including EBL. In 2022, specialists of these veterinary stations serologically tested 20,540 animal blood samples for EBL, of which 170 (0.83%) showed positive results (Table 3). In the laboratories of four out of the eight veterinary stations (Derbent, Kochubeysk, Ulankholsk, Bakres), EBL was not diagnosed by serological method, and in the other four a high level of animal BLV seropositivity was detected (Kizlyar – 14.6%, Babayurt – 3.6%, Tarumovsk – 3.0%, Kizilyurt – 1.06%).

Comparative analysis of EBL epizootological data in municipal okrugs, raions and related transhumance areas



Municipal raions

1. Agulsky
2. Akushinsky
3. Akhvakhsky
4. Akhtynsky
5. Babayurtovsky
6. Botlikhsky
7. Buynaksky
8. Gergebilsky
9. Gumbetovsky
10. Gunibsky
11. Dakhadayevsky
12. Derbentsky
13. Dokuzparinsky
14. Kazbekovsky
15. Kaytagsky
16. Karabudakhkentsky
17. Kayakentsky
18. Kizilyurtovsky
19. Kizlyarsky
20. Kulinsky
21. Kumtorkalinsky
22. Kurakhsky
23. Laksky
24. Levashinsky
25. Magaramkentsky
26. Novolaksky
27. Nogaysky
28. Rutulsky
29. Sergokalinsky
30. Suleyman-Stalsky
31. Tabasaransky
32. Tarumovsky
33. Tlyaratinsky
34. Untsukulsky
35. Khasavyurtovsky
36. Khivsky
37. Khunzakhsky
38. Tsumadinsky
39. Tsuntinsky
40. Charodinsky
41. Shamilsky
42. Bezhtinsky uchastok

Municipal okrugs

43. Makhachkala
44. Buynaksk
45. Dagestanskiye Ogni
46. Derbent
47. Izberbash
48. Kaspiysk
49. Kizilyurt
50. Kizlyar
51. Khasavyurt
52. Yuzhno-Sukhokumsk

Fig. Epizootic map of EBL spread in the Republic of Dagestan in 2022

(Kizlyarsky Raion – 3.0%, Kizlyar Municipal Okrug – 16.9%, Kizlyar Transhumance Veterinary Station – 14.6%, etc.) shows the interdependence (relatedness) of BLV spread indicators in animals. This is due to the fact that the BLV-infected farm animals in the plain area come into contact with healthy cattle in the transhumance areas.

An epizootic map of EBL spread in municipal raions and okrugs of the Republic was compiled on the basis of the official statistical data for 2022, received by the GBI RD "Republican Veterinary Laboratory" (Fig.).

As one can see, EBL is widespread in the middle and northern parts of the Republic, where a large number of public (family-operated) farms are concentrated in the plain areas. The southern part of Dagestan remains EBL-free, with the exception of several raions (Dakhadayevsky, etc.). There are transhumance farms located in the flat parts in the mountainous and high-altitude areas marked in red and green on the map, which explains BLV spread in these climatic zones.

Thus, EBL is reported in all natural and climatic zones of the Republic of Dagestan, but it has received a high degree of spread on the lowland plains.

CONCLUSIONS

As a result of the work carried out to study the EBL epizootic situation in 1988–2022, the following conclusions can be made.

1. The number of serological tests of animal blood samples is annually increasing (from 9,248 in 1988 to 875,312 in 2022), while the proportion of BLV-infected farm animals is decreasing (from 32.2% in 1988 to 0.5% in 2022).

2. The spread of infection is reported in all natural and climatic zones of the Republic, but the highest level is observed in the plain area and in transhumance zones located on lowland plains.

3. The epizootic map compiled on the basis of the official statistical data for 2022 shows high degree of EBL spread in many municipal raions and okrugs of the Republic of Dagestan.

In summing up, a general conclusion can be made: in early 2023 the Republic of Dagestan remained an EBL-infected region of the Russian Federation.

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Testing and identification of bovine viral diarrhea virus isolates recovered in Russia between 2019 and 2022

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ABSTRACT

Studying the agents of bovine viral diarrhea (BVD) is an important task given the high probability of new isolate introduction into the Russian Federation, as well as the need to take into account the genotype and subgenotype of the virus circulating in a herd when developing vaccines and diagnostic kits for the infection. During the work performed, 6 BVD virus isolates were recovered and identified. The recovery of these isolates in the lamb testicle cell subculture revealed that Bashkiria/2019, Kirov/2020 and Samara/2020 isolates belong to non-cytopathic bovine viral diarrhea virus biotypes, Chelyabinsk/2021 isolate demonstrated the characteristic cytopathic effect in the monolayer and was classified as a cytopathic variant of the virus, the adaptation of Belgorod/2021 and Udmurtiya/2020 isolates to this cell system was not possible. The study also identified the species of the recovered isolates. Based on the analysis of the nucleotide sequence of genome 5′-untranslated region (5′-UTR) fragment, these isolates were classified as belonging to three genotypes of the virus. The phylogenetic analysis showed that Chelyabinsk/2021 and Udmurtiya/2020 isolates belong to genotype 2 and demonstrate, respectively, 98% and 99% homology with reference 890 strain of BVD virus. The recovered Bashkiria/2019, Samara/2020, Kirov/2020 isolates were classified as belonging to subtypes 1i, 1f and 1b of genotype 1, and Belgorod/2021 isolate represents genotype 3 of the virus. The findings from the study confirm the presence of all three genotypes of bovine viral diarrhea virus in the Russian Federation and reiterate the need for the development of specific prevention and diagnosis tools for the disease.

Keywords: bovine viral diarrhea, pestiviruses, genotype 2, isolate, polymerase chain reaction, sequencing

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Изучение и идентификация изолятов вируса вирусной диареи крупного рогатого скота, выделенных на территории России с 2019 по 2022 г.

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РЕЗЮМЕ

Изучение возбудителей вирусной диареи крупного рогатого скота является важной задачей в связи с высокой вероятностью заноса новых изолятов на территорию Российской Федерации, а также с необходимостью учитывать генотиповую и субгенотиповую принадлежность циркулирующего в стаде вируса при разработке вакцин и средств диагностики инфекции. В ходе проделанной работы было получено и идентифицировано 6 изолятов возбудителя вирусной диареи крупного рогатого скота. При выделении данных изолятов в субкультуре клеток тестикул ягненка установили, что изоляты Bashkiriya/2019, Kirov/2020 и Samara/2020 относятся к нецитопатогенным биотипам вируса вирусной диареи крупного рогатого скота, изолят Chelyabinsk/2021 проявлял характерное цитопатическое действие в монослое и был отнесен к цитопатогенному варианту вируса, а изоляты Belgorod/2021 и Udmurtiya/2020 не удалось адаптировать к данной клеточной системе. Также при проведении исследования была определена видовая принадлежность полученных изолятов. При анализе нуклеотидной последовательности фрагмента 5′-нетранслируемой области (5′-UTR) генома данные изоляты отнесены к трем генотипам вируса. Филогенетический анализ показал, что изоляты Chelyabinsk/2021 и Udmurtiya/2020 принадлежат к генотипу 2 и имеют соответственно

98%-ю и 99%-ю гомологию с референтным штаммом 890 возбудителя вирусной диареи крупного рогатого скота. Выделенные изоляты Bashkiria/2019, Samara/2020, Kirov/2020 были отнесены к субтипам 1i, 1f и 1b генотипа 1, а изолят Belgorod/2021 является представителем генотипа 3 вируса. Данные исследования подтверждают присутствие всех трех генотипов вируса вирусной диареи крупного рогатого скота на территории Российской Федерации и необходимость разработки средств специфической профилактики и диагностики против данного заболевания.

Ключевые слова: вирусная диарея крупного рогатого скота, пестивирусы, генотип 2, изолят, полимеразная цепная реакция, секвенирование

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INTRODUCTION

Bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus*, family *Flaviviridae,* and is represented by different genotypes: genotype 1 (pestivirus A, *Pestivirus bovis*, BVDV-1), genotype 2 (pestivirus B, *Pestivirus tauri*, BVDV-2), genotype 3 (pestivirus H, HoBi-like pestivirus, *Pestivirus brazilense*, BVDV-3), as well as by two phenotypes – cytopathic (CP) and non-cytopathic (NCP). At the same time, the genotypes are divided into subtypes: 21 subtypes of genotype 1 (1a–1u), 6 subtypes of genotype 2 (2a–2f) and 4 subtypes of genotype 3 are known [1, 2].

BVDV infection has a wide variety of clinical manifestations and leads to significant economic losses in meat and dairy farming all over the world. The infection is accompanied by reproductive disorders, respiratory diseases, immune system malfunction, erosive-ulcerous inflammation of gastrointestinal mucosa, chronic diseases with predisposition to the development of secondary bacterial and other viral infection. A distinctive feature of the virus is its ability to cross the placental barrier and, depending on gestation period in a cow, to infect a fetus and thus to cause persistent infection [3]. As a result, immunotolerant calves are born that act as a continual source of the pathogen for non-immune animals.

The key feature of BVDV is its genotypic and phenotypic diversity that underlies the polymorphism of the clinical presentations of the disease, as well as virulence and antigenicity variation of the virus. BVDV genome is a single-stranded, positive-sense RNA with a length of around 12.3 thousand nucleotides. It has one open reading frame (ORF) of about 4,000 codons in length that encodes 12 structural and non-structural proteins (Npro-C-Erns-E1-E2-p7-NS2/NS3-NS4A-NS4B-NS5A-NS5B), flanked at 5′- and 3′-ends by untranslated regions (5′-UTR and 3′-UTR), p80-125, and basic protein gp53 responsible for virus neutralizing antibody (VNA) induction [4].

The specific feature of the virus, which is its antigenic variability and differences in virulence and reproductive properties, may be associated with genomic reorganizations, mutations or recombinations.

The distribution of the virus types and subtypes has regional specificities and depends on animal husbandry practices, stocking density, livestock performance, new animal introduction frequency and other factors. Genotype 1 BVDV is spread globally, with its outbreaks being most commonly reported in the countries of Europe. The largest number of subtypes (as many as 21) was detected in cattle in Italy and China. The isolation of genotype 2 pestivirus from cattle was reported in the USA, Canada, Brazil, Uruguay, Germany, Slovakia, Italy, South Korea, Japan, Mongolia and Russia [5].

Mucosal disease, one of the forms of diverse BVD clinical presentation, develops following persistent infection and is accompanied by animal death at the age between 6 months and 2 years. The disease occurs when an animal persistently infected with an NCP virus is superinfected with a homologous CP variant of the virus [6]. Outbreaks may also be caused by an NCP virus of genotype 2 not accompanied by a CP virus [6, 7].

The isolates of genotype 2 BVDV are less common than genotype 1 BVDV isolates. They cause acute and hyperacute disease characterized by high mortality, thrombocytopenia and hemorrhages [8, 9, 10, 11]. Studying the agents of BVD is very important given the high probability of new isolate introduction into the Russian Federation. Besides, the genotype and subgenotype of the virus circulating in a herd should be taken into account when developing specific prevention and diagnosis tools for the infection [6, 7].

Specific immunity plays an important role in combating BVD. Due to its wide spread, a long latency and virus shedding period, the high number and density of animals on farms, the infection eradication is difficult and even impossible to achieve without vaccine prevention. Immunization is intended to shield animals from viremia and spread of the virus, to prevent the infection of target reproductive and lymphatic system cells in order to protect a fetus against the infection and immune suppression development. A few decades ago, most vaccines contained genotype 1 BVDV strains. But, taking into account

the antigenic variability of BVDV, the development and production of both attenuated and inactivated vaccines based on the strains of two virus genotypes were initiated. There are more than 180 licensed vaccines against BVD in the USA [9]. In Russia, there are no registered immunological products containing genotype 2 BVDV.

The aim of the study is to test the material isolated from cattle with respiratory and reproductive disorders, collected on farms of the Russian Federation in 2019–2022, as well as to perform the phylogenetic characterization of the recovered isolates.

MATERIALS AND METHODS

Biological material samples were submitted to the Reference Laboratory for Bovine Diseases for testing for the presence of BVDV RNA. Before isolation, sample preparation was carried out under laboratory conditions. Stabilized blood, nasal swabs and/or a 5–10% suspension of pathological material were used for the tests. To prepare the suspension, a sample of the material was homogenized into a paste using a sterile porcelain mortar and a pestle. Then a 10% suspension was prepared by adding nuclease-free water to the mortar and mixing it with the homogenate. The total RNA was extracted from 0.1 mL of the tested biological material using a "RIBO-sorb" test kit (Central Research Institute for Epidemiology of the Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor), Russia), or a "Viral RNA kit" (QIAGEN, Germany), or equivalent in accordance with the manufacturer's instructions.

The detection of genotype 2 BVDV genome was carried out using the following oligonucleotide primers: BD1 5′-GTAGTCGTCAGTGGTTCG-3′ (positions 188–205 nt, NADL strain), BD4 5′-GCCATGTACAGCAGAGAT-3′ (positions 383–366 nt, NADL strain), BD2 5′-CGACACTCCATTAGTTGAGG-3′ (positions 204–223 nt, 890 strain), BD3 5′-GTCCATAACGCCACGAATAG-3′ (positions 320–301 nt, 890 strain), flanking the conservative virus genome region with a length of 261 base pairs, and a method for the virus detection and strain differentiation based on genome 5′-UTR region [12] in a programmable Rotor-Gene™ 6000 thermal cycler (Corbett Research Pty Ltd., Australia).

The sequencing of the samples was carried out using an automated ABI Prism® 3100 sequencer (Applied Biosystems, the USA) in accordance with the manufacturer's instructions.

The nucleotide sequences of the synthesized fragments were analyzed using sequence alignment methods with BVDV-1, BVDV-2 and BVDV-3 sequences received from the GenBank international database and the genetic database of the Federal Centre for Animal Health.

The resulting nucleotide sequences were analyzed using BioEdit 7.0 software package. The phylogenetic tree was constructed by maximum likelihood method using Mega 11 software. Branch topology was confirmed by bootstrap analysis.

Table 1
PCR tests of pathological material samples, 2019–2022

Region	Number of tested farms	Number of samples	PCR test results	
			positive	negative
Kursk Oblast	1	6	0	6
Ivanovo Oblast	1	10	0	10
Republic of Bashkortostan	**1**	**11**	**5**	**6**
Krasnodar Krai	2	10	0	10
Tambov Oblast	1	33	0	33
Belgorod Oblast	**2**	**24**	**3**	**21**
Stavropol Krai	1	9	0	9
Republic of Mordovia	1	17	0	17
Mari El Republic	1	2	0	2
Novosibirsk Oblast	1	10	0	10
Kirov Oblast	**3**	**40**	**5**	**35**
Udmurt Republic	**1**	**12**	**3**	**9**
Kamchatka Krai	1	4	0	4
Vologda Oblast	1	7	0	7
Kostroma Oblast	1	10	0	10
Chelyabinsk Oblast	**1**	**6**	**1**	**5**
Krasnoyarsk Krai	1	10	0	10
Samara Oblast	**1**	**40**	**1**	**39**
Rostov Oblast	1	2	0	2
Total	23	263	18	245

Table 2
Results of RT-PCR tests of culture fluid samples collected during cultivation of BVDV isolates in lamb testicle cell culture

BVDV isolate	Ct value		Presence of CPE
	passage 2	passage 5	
Bashkiria/2019	25.75	21.93	–
Kirov/2020	26.56	19.63	–
Samara/2020	25.64	18.25	–
Belgorod/2021	30.86	33.22	–
Udmurtiya/2020	29.59	34.51	–
Chelyabinsk/2021	25.26	17.02	+

The samples that tested BVDV RNA positive with polymerase chain reaction (PCR) were used for the virus isolation in cell culture. To exclude contamination with a NCP BVDV, the virus isolation was carried out in the subcultured lamb testicle (LT) cell lines. The infected monolayer was cultivated at a temperature of (37 ± 1) °C. Serum-free semi-synthetic nutrient medium prepared in accordance with the Federal Centre for Animal Health formula, supplemented with a 10% Baytril solution was used as a maintenance medium. The identification of the isolates was carried out after 5 serial passages in LT cell culture by testing the culture fluid with reverse transcription polymerase chain reaction (RT-PCR).

RESULTS AND DISCUSSION

Between 2019 and 2022, 263 samples (nasal swabs, lip erosion scrapings, pathological material samples) submitted to the laboratory from 23 farms located in 19 Subjects of the Russian Federation were subjected to PCR testing.

All the samples were tested for BVDV genome with RT-PCR. Eighteen samples, i.e. 6.8% of the total number of the tested samples, tested positive.

Nasal discharge samples collected from a 1-month-old calf with respiratory dysfunction on a farm located in the Chelyabinsk Oblast tested positive. BVDV genome was also detected in fetal bovine serum samples (the Belgorod Oblast), intestinal mucosa samples from calves with clinical manifestations characteristic of BVD with mucosal inflammation (the Republic of Bashkortostan, the Samara and Kirov Oblasts), samples from the aborted fetus of a first-calf cow recently imported to Russia (the Udmurt Republic). The diversity of the clinical picture of the disease observed in the animals confirms the data on the polymorphism of its clinical presentations.

To recover BVDV isolates, serial passages in LT cell subculture were performed. The virus reproduction was assessed based on characteristic cytopathic effect (CPE), as well as by RT-PCR tests of the culture fluid, on the basis of the fact that the highest cycle threshold (Ct) values correspond to the minimum BVDV accumulation level. In total, 6 BVDV isolates were recovered and subsequently used in the work (Table 2).

Bashkiria/2019, Kirov/2020 and Samara/2020 isolates demonstrated no CPE after 5 serial passages in LT cell culture; however, a significant decrease in Ct values was indicative of the positive dynamics of the virus activity.

The analysis of Ct values for Belgorod/2021 and Udmurtiya/2020 isolates allowed to conclude that the use of LT cell culture for the virus accumulation is ineffective, that is why a search for more sensitive cell systems is required.

During Chelyabinsk/2021 isolate recovery, no apparent changes were observed in the cell monolayer at passages 1 and 2. At passage 3, the detachment of individual cells with a changed morphology from the monolayer surface was observed. CPE was also characterized by the rounding of cells, which gradually formed separate aggregations. Low Ct values based on RT-PCR test results for Chelyabinsk/2021 isolate by passage 5 were indicative of the virus accumulation in LT cell culture [13].

The identification of the species of the recovered isolates was carried out by PCR product sequencing. The nucleotide sequences of 5′-UTR region fragments were identified and compared with the sequences available in the GenBank international database and the genetic database of the Federal Centre for Animal Health. The constructed phylogenetic tree is presented in the figure.

As the dendrogram shows, the recovered virus isolates belong to different BVDV genotypes, the genotypes demonstrate a 70% homology.

Among 6 tested isolates, 2 isolates were classified as belonging to genotype 2. Chelyabinsk/2021 isolate genome sequencing revealed its 98% identity with reference 890 strain of genotype 2 BVDV (sequence U18059.1). This strain was first isolated during the infection outbreak in the USA in the 1990s [7]. Udmurtiya/2020 isolate was 99% homologous to reference 890 strain of genotype 2 BVDV (sequence FJ795044.1) recovered from a fetal bovine serum sample [14].

Genotype 1 BVDV is widely spread in Russia and all over the world. Based on the test results, Kirov/2020 isolate was found to be identical to reference Osloss strain of subtype 1b BVDV; Samara/2020 isolate was found to be 98% homologous to subtype 1f BVDV isolate. Bashkiria/2019 isolate demonstrated a 98% identity with 23-13 strain classified as subtype 1i BVDV (sequence FJ493484.1) [15].

The genetic analysis of Belgorod/2021 isolate revealed its close relationship with 2 isolates of genotype 3 BVDV (sequences JN967748.1 and JN967731.1) and FBS 37 strain (sequence MK017821.1) previously isolated from fetal bovine serum samples [16].

Thus, the findings from the study confirm reports that genotype 1 BVDV prevails in Russia and is spread not only in the central part of the country, but also in the Volga Federal District (subtypes 1b, 1f and 1i).

Over the past 15 years, BVD outbreaks caused by genotype 2 BVDV were reported in some regions of the Russian Federation. Respiratory disorders caused by the virus of the said genotype were detected in calves in the Ural region and Siberia [1, 4]. Koteneva S. V. et al. [17] detected genotype 2 BVDV in internal organ samples from an aborted fetus and a deadborn calf of a local breed (the Novosibirsk and Tyumen Oblasts). Three BVDV-2 subtypes (2a, 2b and 2c) were detected in imported and domestic animals in Siberia. Based on the previously conducted studies, it was found that subtype 2a was recognized as one of the major etiological agents responsible for reproductive disorders in cows [17, 18, 19, 20]. This study revealed the presence of genotype 2 BVDV in the European part of Russia (the Udmurt Republic and the Chelyabinsk Oblast areas located in Europe). According to the international classification (ICTV) of pestiviruses, both isolates (Udmurtiya/2020 and Chelyabinsk/2021) represent subtype 2a BVDV.

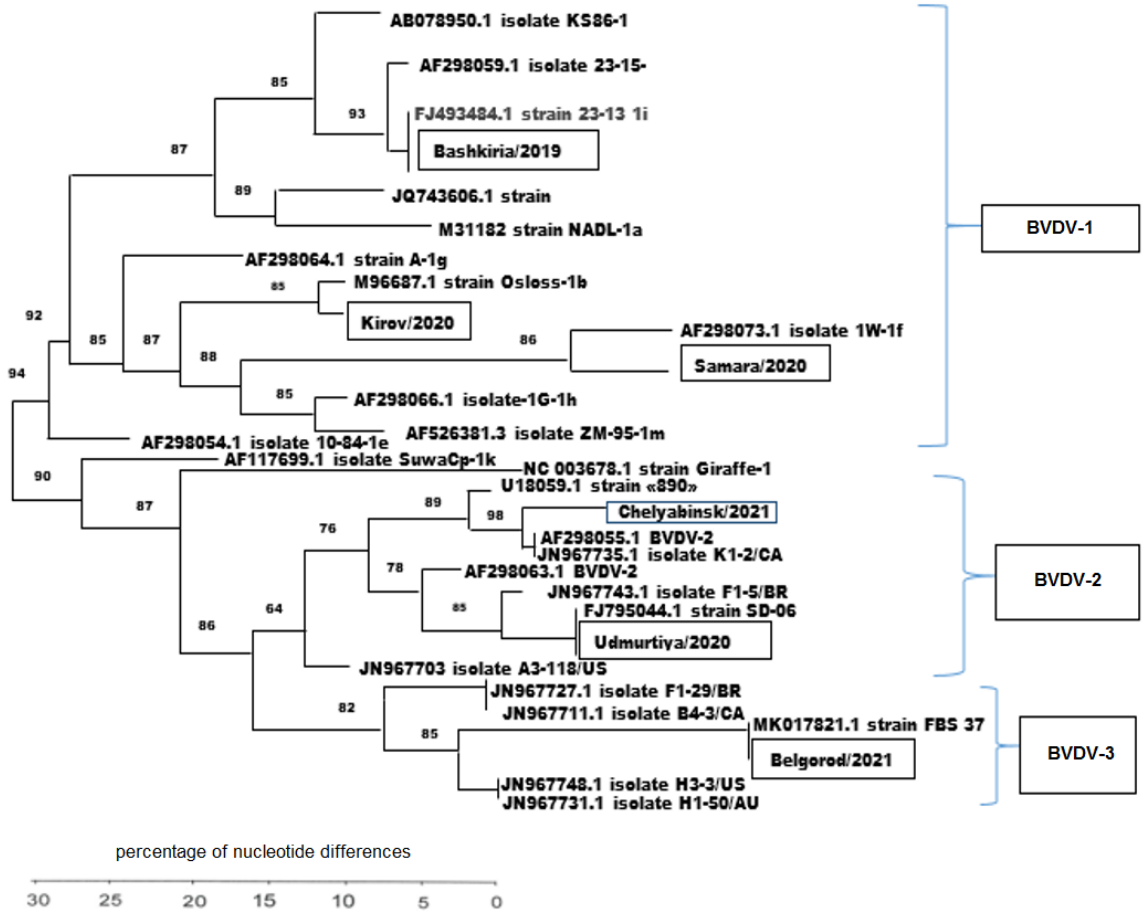

Fig. Dendrogram constructed based on BVDV genome 5'-untranslated (5'-UTR) region fragment sequencing results. The recovered isolates are shown in squares

Genotype 3 BVDV was previously detected on livestock farms in Russia [1, 3, 21, 22]. This study reports the detection of genotype 3 BVDV in the Central Federal District of the country.

CONCLUSION

RT-PCR tests of the biological material samples submitted from livestock farms to the Reference Laboratory for Bovine Diseases detected BVDV genome in 6.1% of the samples. During the work performed, 6 BVDV isolates were recovered in the cell system and identified. The isolates were classified as belonging to different BVDV biotypes: Bashkiria/2019, Kirov/2020 and Samara/2020 isolates were classified as belonging to non-cytopathic phenotypes, Chelyabinsk/2021 isolate demonstrated the characteristic cytopathic effect in LT cell culture monolayer and was classified as a cytopathic variant of the virus, the adaptation of Belgorod/2021 and Udmurtiya/2020 isolates to this cell system was not possible.

Besides, the genetic identification of BVDV was carried out based on conservative 5'-UTR region. The phylogenetic analysis showed that Chelyabinsk/2021 and Udmurtiya/2020 isolates belong to genotype 2 and are, respectively, 98% and 99% homologous to reference 890 strain of subtype 2a BVDV. The recovered Bashkiria/2019, Samara/2020 and Kirov/2020 isolates were classified as belonging to subtypes 1i, 1f and 1b, respectively. Belgorod/2021 isolate represents genotype 3 BVDV.

The findings from the study confirm the presence of three genotypes of BVD virus in Russia.

The recovered Chelyabinsk/2021 isolate will be used for further studies of the cultural properties of genotype 2 BVD virus.

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Evaluating wound-healing effect of silicon-zinc-boron-containing glycerohydrogel and its effect on mammary glands of high producing dairy cows

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ABSTRACT

Cow's milk quality, which may deteriorate due to inflammatory processes in the mammary glands, remains one of the important problems of dairy farming and requires effective, safe and affordable therapeutic agents. Nanocomposite silicon-zinc-boron-containing glycerohydrogel (Si-Zn-B-gel) may become a very good solution to the issue. The paper demonstrates wound-healing effect of the glycerohydrogel and confirms its effectiveness for teat hyperkeratosis treatment in dairy cows. Results of a rat-burn model – based experiment suggest that Si-Zn-B-gel is a promising wound healing agent for topical use. Thus, on Day 9 complete re-epithelialization of the burn surface was observed, with fibrous structures prevailing in the granulation tissue of the dermal layer, on Day 19 a mature scar was formed with a longitudinal alignment of collagen fibers. The production tests conducted in high producing dairy cows have demonstrated good therapeutic effect of the Si-Zn-B gel for teat-end hyperkeratosis and confirmed its long-term effect, which helps to longer maintain the results achieved during treatment. After a 7-day treatment physiological structure of up to 27.8% teats improved, on Day 14 of the experiment, no severe hyperkeratotic lesions were observed and the number of teats that correspond to the physiological norm was 72.2%. Analysis of the data collected shows that the Si-Zn-B-gel is effective for teat-end hyperkeratosis treatment, thus, it prevents mastitis in animals and improves the milk quality.

Keywords: silicon-zinc-boron-containing glycerohydrogel, 10% methyluracyl ointment, wound healing effect, long-term effect, high producing dairy cows, mammary glands, mastitis, teat-end hyperkeratosis, milk quality

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Оценка ранозаживляющего эффекта кремнийцинкборсодержащего глицерогидрогеля и влияние его на молочную железу высокопродуктивных коров

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РЕЗЮМЕ

Качество получаемого коровьего молока, снижающееся прежде всего за счет наличия у животных воспалительных процессов в молочной железе, остается одной из важных проблем молочного животноводства, что требует разработки высокоэффективных, безопасных и доступных терапевтических средств. Большим потенциалом для решения данного вопроса обладает нанокомпозитный кремнийцинкборсодержащий глицерогидрогель (Si-Zn-B-гель). В работе представлены данные, подтверждающие наличие ранозаживляющей активности глицерогидрогеля и эффективность его использования в схемах лечения гиперкератоза сосков молочной железы коров. Результаты исследования, проведенного на экспериментальной модели термического ожога кожи крыс, свидетельствуют о том, что Si-Zn-B-гель является перспективным ранозаживляющим средством для местного применения. Так, на 9-е сут была зафиксирована полная эпителизация ожоговой поверхности, при этом в грануляционной ткани дермального слоя кожи преобладали волокнистые структуры, к 19-м сут формировался зрелый рубец с продольно ориентированными коллагеновыми волокнами. Проведенные производственные исследования на высокопродуктивных коровах показали терапевтическую эффективность применения Si-Zn-B-геля в лечении гиперкератоза сосков у коров и наличие пролонгированного действия, что увеличивает сроки результативности проведенной терапии. Через 7 дней лечения установлено увеличение количества сосков с физиологической структурой до 27,8%, на 14-е сут после опытного периода зафиксировали отсутствие поражений сосков тяжелой формой гиперкератоза, при этом количество сосков, соответствующих физиологической норме, составило 72,2%. Анализ данных, полученных в результате исследований, показывает, что применение Si-Zn-B-геля позволяет производить эффективное лечение гиперкератоза сосков молочной железы, тем самым обеспечивать профилактику мастита у животных и повышать качество получаемого молока.

Ключевые слова: кремнийцинкборсодержащий глицерогидрогель, 10%-я метилурациловая мазь, ранозаживляющая активность, пролонгированное действие, высокопродуктивные коровы, молочная железа, мастит, гиперкератоз сосков, качество молока

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INTRODUCTION

Production of high quality and competitive dairy products is a top priority for the Russian Federation. The solution to the task is a matter of special importance for the national economy, when health promotion improves the quality of life and maximizes competitive advantages of producers and regions and ensures food security [1, 2, 3, 4]. Today, our country is one of the world's largest producers of milk and dairy products. It is now ranked number four in the world, at the same time quality improvement remains one of the primary tasks of the domestic livestock industry [3, 5]. The mammary gland of high producing dairy cows becomes overactive during lactation, which may result in such diseases as mastitis that affects milk production, safety and quality [1, 3, 6, 7, 8].

Teat-end hyperkeratosis is a risk factor for clinical mastitis in cows as it disrupts the teat canal barrier function, thus, significantly increasing the risk of penetration of opportunistic and pathogenic microflora into the mammary gland resulting in its damage and inflammation [5, 9, 10, 11, 12, 13, 14]. The current regimens implemented worldwide to treat and prevent diseases of mammary glands in animals offer a wide variety of medicinal products containing cosmetic, antiseptic, probiotic and emollient components, however, the agricultural holdings with milk yield more than 8000 kg per cow, see no stable or long-lasting positive effect of these therapeutic measures [7, 11, 15]. Therefore, modern practical science shall search for new, more effective approaches to treat teat-end hyperkeratosis and prevent mastitis in cows [7, 11]. Nanocomposite silicon-zinc-boron-containing glycerohydrogel (Si-Zn-B-gel), developed by I. Ya. Postovsky Institute of Organic Synthesis, Ural Branch of Russian Academy of Sciences [16], may become a very good solution to the issue.

The gel chemical formula is:

$$3Si(C_3H_7O_3)_4 \times ZnC_3H_6O_3 \times 2HB(C_3H_6O_3)_2 \times 15C_3H_8O_3 \times 70H_2O.$$

At the molecular level, the developed substance contains silicon, zinc and boron atoms, has a gel-like consistency and pronounced wound-healing, regenerating, antibacterial and fungicidal effect [16, 17]. Consequently, it is an urgent task to study its therapeutic effectiveness for treatment of teat-end hyperkeratosis in cows and to confirm its prolonged effect.

The goal of the experiment is to study effectiveness of the Si–Zn-B-gel for treatment of teat-end hyperkeratosis in high producing dairy cows.

The following tasks need to be addressed to achieve this goal.

1. Evaluate specific wound-healing effect of Si-Zn-B-gel in comparison with 10% methyluracyl ointment using a rat thermal burn model.

2. Study clinical efficacy of the developed glycerohydrogel in comparison with 10% methyluracyl ointment in experimental groups of cows with teat-end hyperkeratosis.

MATERIALS AND METHODS

The experiment involved white mongrel rats at the age of 10 weeks; high producing black-and-white cows at the age

of 4–6 lactation with an average annual milk yield per cow of more than 8000 kg.

Preclinical experiments were carried out using an experimental rat thermal burn model to evaluate the specific wound-healing effect of the Si-Zn-B-gel in comparison with 10% methyluracyl ointment. II–IIIa degree thermal burns were caused by heated metal devices (at a temperature of 98–100 °C) applied directly to the skin of an individual for 40 seconds [18]. The treatment course was assessed at the final stage of the wound healing and was characterized by primary intention resulting from the use of Si-Zn-B-gel (19 days). During this period, healing was reported in three groups of rats: control group – a burn without treatment; Experimental Group 1 – a burn treated with 10% methyluracyl ointment; Experimental Group 2 – a burn treated with Si-Zn-B-gel. The number of animals in each group was 10.

All tests in animals were carried out in strict compliance with intergovernmental standards on laboratory animal keeping and handling adopted by the Intergovernmental Council for Standardization, Metrology and Certification as well as in accordance with Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes.

Histomorphological tests were carried out to confirm the wound healing effect. Pieces of burn-affected skin from experimental rats were used as the biomaterial. At the first stage, the material was fixed in a 10% buffered formalin. The material was then dehydrated and soaked in the embedding medium. Paraffin medium "Histomix" (LLC "BioVitrum", Russia) was used for subsequent three-time soaking and embedding the material. Next, rotary microtome Leica RM 2255 (Leica Microsystems, Germany) was used to prepare 6 µm-thin sections of paraffin-embedded specimen. The micro-preparations were stained with hematoxylin and eosin and using Weigert – Van Gieson stain method. Histological changes were photographed on a "Micros" microscope (Austria).

Field trials. The clinical efficacy of glycerohydrogel was assessed on a farm of the Sysertsky Raion of the Sverdlovsk Oblast. For this purpose, two groups of cows (9 animals in each) were formed. All the animals were diagnosed with teat-end hyperkeratosis of different levels. The experimental animals were treated with Si-Zn-B-gel applied to the teats 2 times a day for 7 days. The control group was treated with 10% methyluracil ointment following the same scheme.

Conventional methods were used to *clinically test* the teat-ends [19]. The level of teat-end hyperkeratosis was assessed using an upgraded diagnostic scale, represented by a panel of 18 photographs [9]. The mammary gland was tested for clinical mastitis by palpation and strip-cup test, subclinical mastitis was diagnosed using rapid test Keno™test (CID LINES, Belgium).

Ultrasound scanning of the mammary glands was performed using the veterinary ultrasound scanner Ecoson 900V (West Medica Produktions- und Handels-GmbH, Austria). Two types of multi-frequency transducers (convex and linear) were used to test mammary gland parenchyma of cows. The teat cistern was examined with a 7.5 MHz linear scanner. Scanning in segmental and frontal planes was used. Teat-end condition was assessed using a plastic teat dip cup with a water buffer (water 38 °C) and a multi-frequency probe attached to it. The resulting echograms were processed using software provided by an ultrasound scanner [5, 20].

The milk was centrifuged in order to remove blood, if detected. To do this, a pooled sample of the residual milk (40–50 mL) was put into a plastic container, then heated up to 20–22 °C; after that 5–7 mL of milk was poured into the test tube and centrifuged for 10 minutes at 1000 rpm. A red ring sediment at the bottom of the tube suggested there was blood in the tested milk. The milk quality was tested using "Lactan 1–4 M" analyzer (Sibagropribor, Russia).

RESULTS AND DISCUSSION

The preclinical trials carried out to assess the specific wound healing effect on the experimental rat-burn model revealed that the percentage of incomplete healing associated with the application of Si-Zn-B-gel and 10% methyluracyl ointment was 0.12 and 3%, respectively. Thus, it demonstrates good prospects for the gel use. Following application of the soft dosage forms, in addition to the accelerated healing of the damaged skin, tissue crusts were formed on Day 3 of treatment. Complete crust detachment was observed on Day 9 and hair regrowth together with primary intention was observed on the new tissue at the end of the experimental application of Si-Zn-B-gel (Table 1).

Additionally, histomorphological tests were performed to study the wound healing effect, the test results are given in Figures 1–5. Necrotic epidermis with lymphocytic infiltration was found in the control group (that received no treatment) on Day 9 post the thermal burn. Granulation tissue (represented by functionally active fibroblasts and forming sinusoidal capillaries) was formed in the underlying dermis. The tissue was diffusely infiltrated with lymphoid elements (Fig. 1).

Weigert and Van Gieson stain method demonstrated that the scar area had collagen fibers of different maturity and different diameter, while the elastic fibers were identical (Fig. 2*a*). On Day 19 of the experiment, re-epithelization of the largest part of the skin defect was observed. The dermal scar was represented by functionally active

Table 1
Dynamics of rat skin regeneration in a thermal burn

Group of animals	Day 2	Day 9	Day 19	% incomplete healing
Control	40 × 20 mm (800 mm^2)	35 × 18 mm (630 mm^2)	20 × 10 mm (200 mm^2)	25
Experimental 1	40 × 20 mm (800 mm^2)	25 × 12 mm (300 mm^2)	7 × 3 mm (21 mm^2)	3
Experimental 2	40 × 20 mm (800 mm^2)	13 × 6 mm (78 mm^2)	3 × 0.3 mm (0.9 mm^2)	0.12

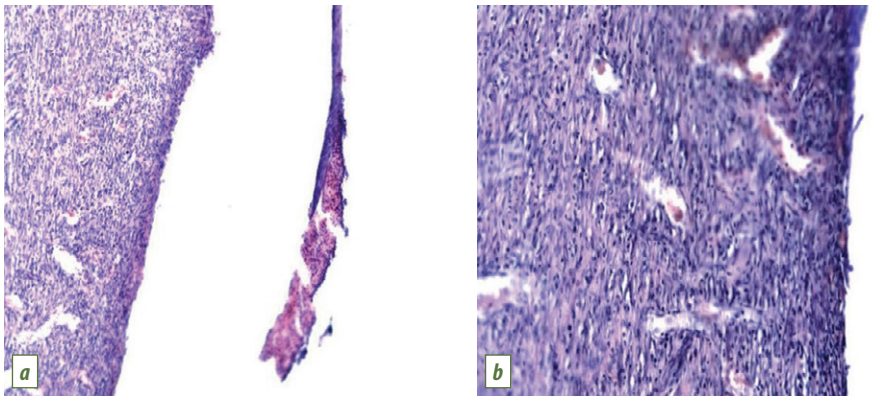

Fig. 1. Epidermis on Day 9 of the experiment (without treatment):
a) thermal burn on rat skin; stained with hematoxylin and eosin, magnification 100×;
b) granulation tissue formed after the burn;
stained with hematoxylin and eosin, magnification 200×

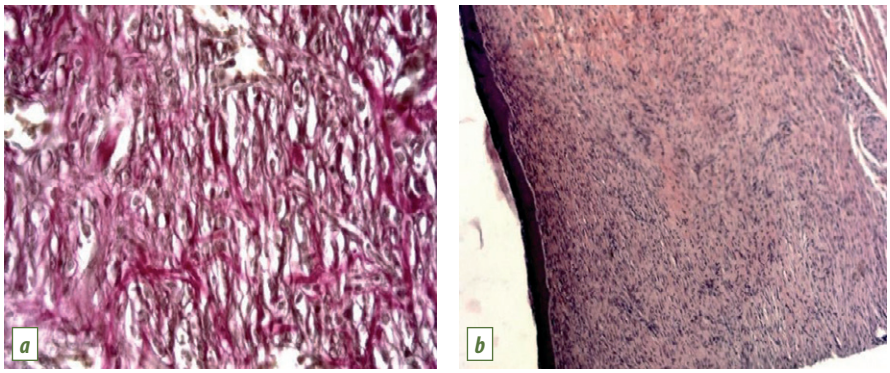

Fig. 2. Control group (without treatment):
a) collagen fibers formed in granulation tissue on Day 9 of the experiment;
Weigert and Van Gieson stain, magnification 400×;
b) forming dermal scar in the projection of the thermal burn on Day 19 of the experiment;
stained with hematoxylin and eosin, magnification 100×

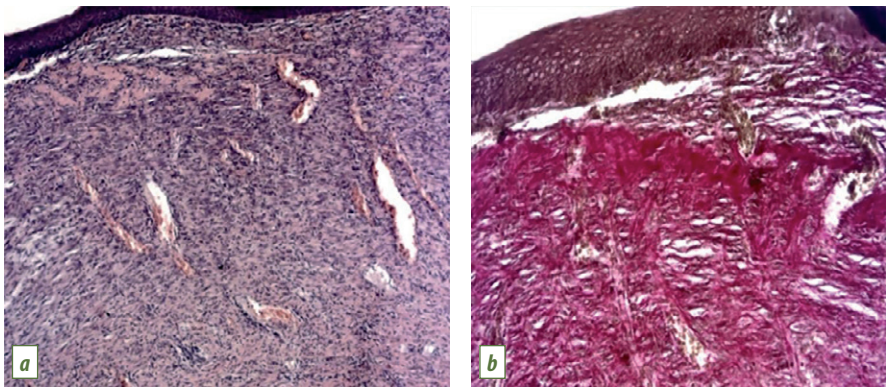

Fig. 3. Experimental Group 1 (10% methyluracyl ointment used for treatment) on Day 9 of the experiment:
a) a scar forming in the dermal layer in the projection of a thermal burn;
stained with hematoxylin and eosin, magnification of 100×;
b) scar of the dermal layer in the projection of a thermal burn;
Weigert and Van Gieson staining, magnification 100×

fibroblasts formed by randomly oriented collagen fibers. There were sporadic vessels, lymphoid infiltration was minimal (Fig. 2*b*).

On Day 9 of the experiment Experimental Group 1, where methyluracil was applied topically, demonstrated complete re-epithalization of the defect. The dermal scar is represented mainly by fibroblast cells formed by collagen fibers and sinusoidal vessels. Lymphoid infiltration was minimal (Fig. 3*a*). Weigert and Van Gieson stain method demonstrated that the scar area was represented by collagen fibers of different maturity degree and different diameter. Elastic fibers were sporadic (Fig. 3*b*).

On Day 19 after exposure, the group that was treated with 10% methyluracyl ointment demonstrated complete re-epithelization of the defect, acanthosis foci were reported with simultaneous formation of keratinous cysts. A dermal scar was formed with collagen fibers. Angiomatosis was sporadic, vessels – with collapsed walls. Lymphoid elements in the area of the scar were sporadic (Fig. 4*a*). Weigert and Van Gieson stain method demonstrated that the scar area in the dermal layer had mature dense collagen fibers similar in diameter (Fig. 4*b*).

On Day 9 of the experiment, Experimental Group 2, that received Si-Zn-B-gel treatment, demonstrated complete re-epithelization of the defect in the burn area. Granulation tissue was formed in the dermal layer from functionally active fibroblasts, sinusoidal capillaries, infiltrated with lymphoid elements (Fig. 5*a*). On Day 19 of the experiment, a mature scar was formed with a longitudinal alignment of collagen fibers; complete re-epithelization of the defect was observed with a fibrous scar in the dermal layer (Fig. 5*b*).

Thus, on Day 9 of the experiment, the control group (received no treatment) demonstrated longer burn healing with signs of exudative inflammation. On Day 19, the exudation phase was followed by the proliferation phase characterized by regeneration and replacement of the damaged tissues due to the influx of fibrinogen molecules to the defect region resulting in fibrin formation. At the same time, functionally active fibroblasts and mature randomly oriented collagen fibers form scar tissue, which becomes a dermal scar on Day 19 of the experiment.

In Experimental Group 1, where 10% methyluracyl ointment was topically applied, on Day 9 of the experiment complete re-epithelialization and dermal scar formation were observed, represented mainly by granulation tissue infiltrated by lymphocytes and granulocytes. By Day 19, the epidermis in the defect area got thicker; the scar tissue was formed mainly with collagen fibers and granulation tissue.

In Experimental Group 2, where Si-Zn-B-gel was used, on Day 9 of the experiment, the burn wounds fully re-epithelized, with fibrous structures prevailing in the granulation tissue of dermis, and on Day 19 a mature scar with longitudinally oriented collagen fibers was formed. The results of burn treatment with 10% methyluracyl ointment (Fig. 3*a*) and Si-Zn-B-gel (Fig. 5*a*) compared on Day 9, demonstrate that the developed hydrogel effectively triggers granulation process resulting in formation of young connective tissue, which stimulates the healing process; whereas, application of methyluracil immediately leads to scarring.

Experiments conducted in high producing dairy cows to study effectiveness of Si-Zn-B-gel for teat-end hyperkeratosis treatment showed a decrease in the number of udder quarters with pronounced changes in the teat ends.

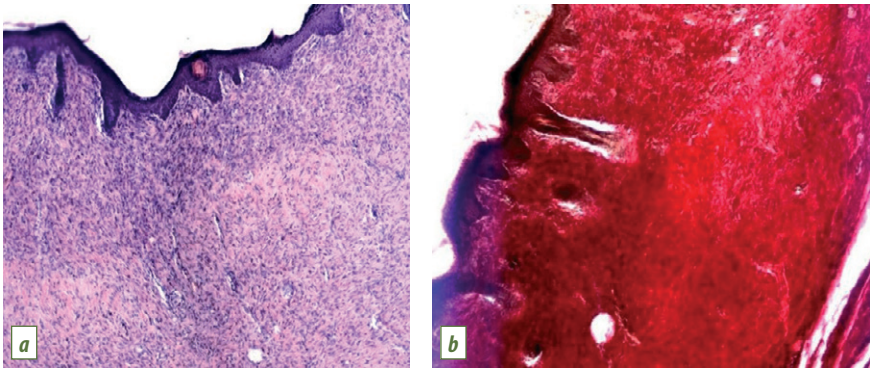

Fig. 4. Experimental group 1 (10% methyluracyl ointment used for treatment) on Day 19 of the experiment:
a) scar of the dermal layer in the projection of the thermal burn;
staining with hematoxylin and eosin, magnification of 100×;
b) scar of the dermal layer; Weigert and Van Gieson staining, magnification 100×

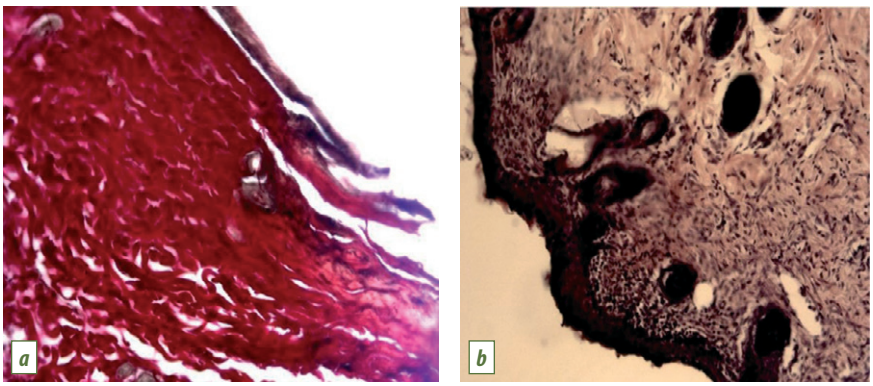

Fig. 5. Experimental Group 2 (treatment with Si-Zn-B-gel):
a) burn area on Day 9 of the experiment; staining with hematoxylin and eosin, magnification 100×;
b) fibrous scar in the dermal layer on Day 19 of the experiment; Weigert and Van Gieson staining, magnification of 200×

At the end of the experimental period (which took 7 days), the group of animals who received the hydrogel demonstrated regeneration of the teat-end epidermis, the number of teat-ends with a physiologically normal structure increased to 27.8%. On Day 14 after application of Si-Zn-B-gel, no severe hyperkeratosis-associated lesions of teat-ends were reported, at the same time the number of physiologically normal teat-ends grew up to 72.2%. On Day 21 after the end of the experiment, a diagnostic test for hyperkeratosis revealed that teat-end condition remained at the achieved level with a tendency to improve (Fig. 6*a*).

The experiment results showed that the number of teat-ends with radial cracks decreased by 2.7 times in the control group treated with 10% methyluracyl ointment, and physiologically normal teat-ends accounted for 38.9%. On Day 14 after the treatment period with 10% methyluracyl ointment, no complications in animals were detected following and the number of physiologically normal teat-ends accounted for 84.1%. The teat-end examination done after the treatment course (on Day 21 after the end of the treatment) reflected a deterioration, which is most likely caused by the negative factors of milking process and animal husbandry. Thus, the number of healthy teat-ends decreased by 15.5%, and the number of teat-ends with hyperkeratosis increased by 2 times – up to 31.4% (Fig. 6*b*). While the experimental group of animals kept under the same conditions showed improvement of the teat-ends condition, so, the number of teat-ends with hyperkeratosis decreased to 13.9%, and the number of physiologically normal teat-ends accounted for 86.1%. These changes may suggest Si-Zn-B-gel has a more a prolonged effect and is more effective in comparison with 10% methyluracyl ointment.

Diameter of teat-end callosities measured during the experiment suggests that both medicines gradually reduce the callosity size. In the experimental and control groups the mean callosity diameter decreased by 1.6 times. Therefore, at the beginning and at the end of the experiment, the callosity size in the experimental group was 7.8 and 4.8 mm, respectively, in the control group at the beginning of the experiment the mean diameter was 7.9 mm, by the end of the experiment it decreased to 4.9 mm.

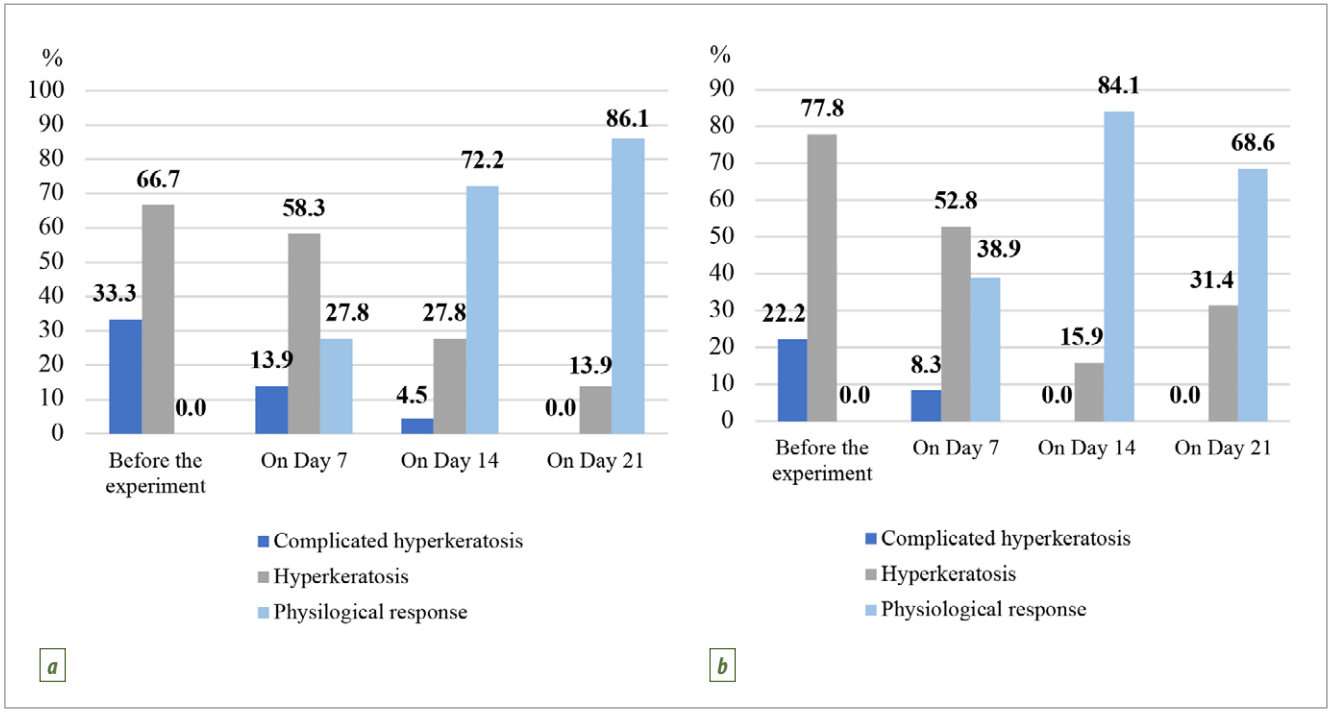

Fig. 6. Results of the cows' teats examination during the treatment process:
a) experimental group – use of Si-Zn-B-gel; b) control group – use of 10% methyluracyl ointment

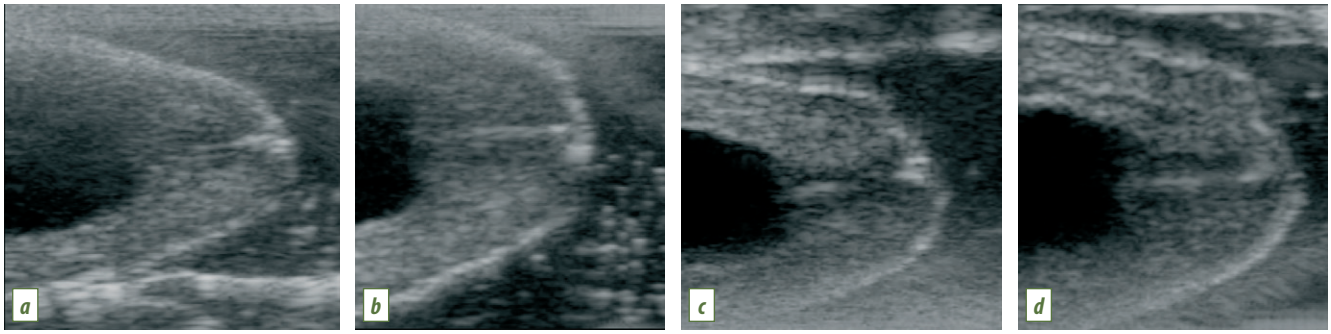

Fig. 7. Ultrasound examination of the teat canal: a) before the use of Si-Zn-B-gel; b) after the use of Si-Zn-B-gel; c) on Day 7 after the use ; d) on Day 14 after the use

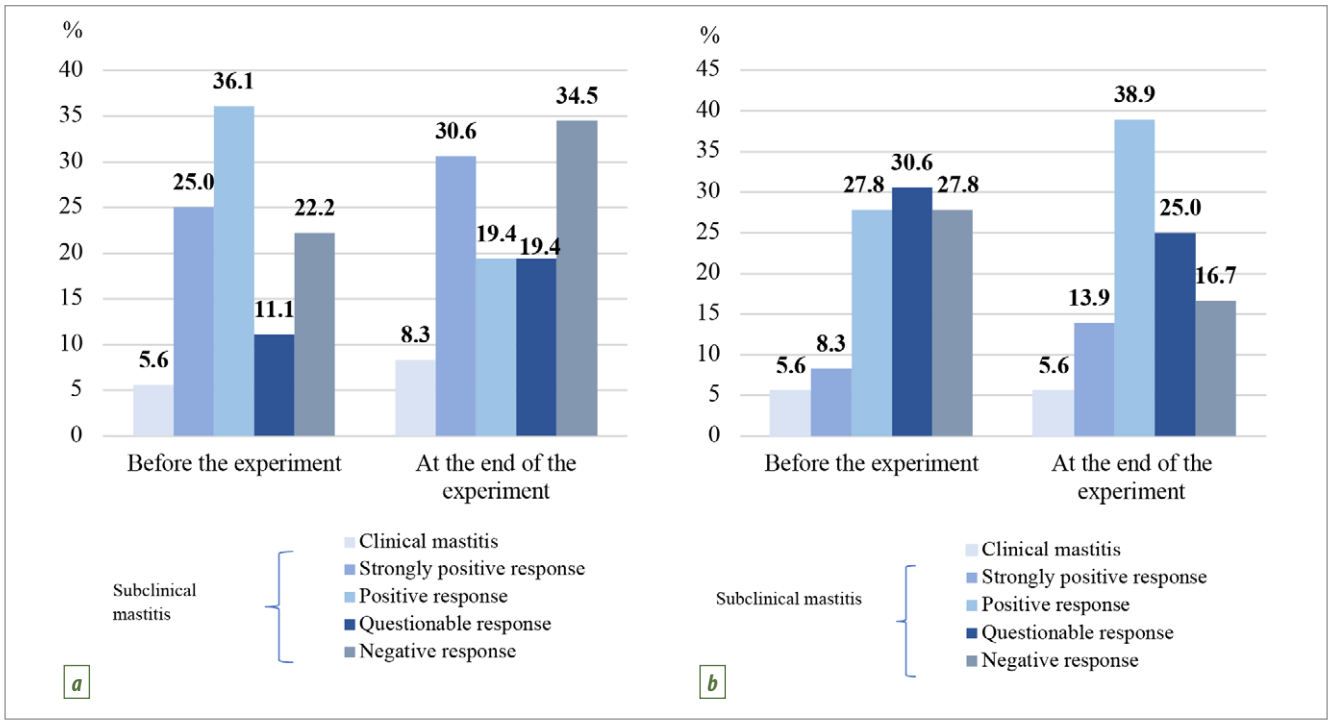

Fig. 8. Mastitis incidence rate during the treatment:
a) experimental group – use of Si-Zn-B-gel; b) control group – use of 10% methyluracyl ointment

Ultrasound examination detected changes in the teat canals associated with a decrease in the volume of the affected tissue around the external teat orifice, as it can be seen in hyperechoic tissue in the picture; with the final recovery on Day 14 after the therapy (Fig. 7).

The condition of the mammary gland was assessed by examining each quarter of the udder for clinical and subclinical mastitis. As a result, latent inflammation (strongly positive and positive response) was reported in 61.1% of quarters. At the end of the experimental period, the number of quarters with a questionable and negative response to subclinical mastitis increased by 8.3 and 12.6%, respectively. There was a 16.7% decrease in positive response to latent mastitis, at the same time, there was a slight (2.7%) increase in clinical mastitis, which most likely results from faulty milking techniques and had nothing to do with the effectiveness of the developed glycerohydrogel (Fig. 8*a*). In the control group, the proportion of diagnosed clinical mastitis before and after the therapy was 5.6%, however, there was a decrease in the number of udder quarters with questionable and negative response to subclinical mastitis by 5.6 and 11.1%, respectively (Fig. 8*b*).

At the beginning of the experiment, 33% of milk samples from the animals of the experimental group had a weakly positive reaction to blood impurities, whereas all milk samples from the control group gave a negative result. At the end of the experimental period, milk samples from the cows of the experimental group showed a negative result, one positive result was detected in the control group.

Estimating solids-not-fat, protein, fat and density in milk from animals of both groups revealed no significant differences between the groups (Table 2).

CONCLUSION

Evaluation of the specific wound-healing effect of the two medicines showed that by the end of the experiment, the group of rats that received Si-Zn-B-gel to treat thermal burn had a mature scar with longitudinally oriented collagen fibers, and the group of animals treated with 10% methyluracyl ointment (applied topically) demonstrated thickening of the epidermis and the scar tissue included collagen fibers and foci of granulation tissue. The overall duration of the treatment in both experimental groups was the same (the same number of days); however, the use of Si-Zn-B-gel resulted in improved morphostructural indicators, which offers great potential for the product application.

The data obtained for cows treated with Si-Zn-B-gel confirm its therapeutic efficacy. Thus, there was an increase in the number of udder quarters not affected

Table 2
Milk quality assessment in experimental and control groups

Group of animals	Fat, %	Protein, %	Solids-not-fat, %	Density,°A
Control	4.68 ± 3.10	3.02 ± 0.49	7.90 ± 1.23	26.25 ± 6.03
Experimental group	3.95 ± 3.14	3.05 ± 0.40	7.99 ± 0.83	27.16 ± 5.03

$P \leq 0.05$.

by the inflammatory process; an increase in the number of teats without hyperkeratosis; the teat-end callosity diameter decreased. The newly developed product had a positive effect on the tissues of the teat-ends of cows, as confirmed by the ultrasound examination, which showed a decrease in the volume of the affected tissue around the external teat orifice.

A comparative assessment of the Si-Zn-B-gel and 10% methyluracyl ointment revealed the prolonged action of the former resulting from its longer therapeutic effect. Thus, there was a decrease in the diagnosed teat-end hyperkeratosis in the experimental group during the whole observation period, however, in the control group, first, there was a decrease in the number of animals with teat-end hyperkeratosis, and then followed by an increase. Similar data were received when diagnosing latent inflammatory diseases in the bovine mammary glands. The results obtained show effectiveness of the developed Si-Zn-B-gel due to its pronounced wound-healing, regenerating, antibacterial, fungicidal activity and prolonged action, which makes it possible to recommend it as a medicine to treat teat-end diseases in lactating cows.

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Biological properties of foot-and-mouth disease virus A 2205/G-IV strain

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ABSTRACT

According to the World Organisation for Animal Health, foot-and-mouth disease (FMD) is regularly reported in domestic and wild cloven-hoofed animals in Africa. G-I, G-IV, G-VI, G-VII, ASIA/Iran-05 genetic lineages of serotype A FMD virus are considered to be the most widespread on the African continent. Given the close economic and trade relations maintained by the Russian Federation with the countries of North Africa, of particular interest for us is studying the FMD virus of serotype A G-IV genetic lineage, which has been responsible for the infection outbreaks in the naturally susceptible animal population of the said region every year since 2012, and there is a risk of introduction of this virus genotype into the Russian Federation. Therefore, the issues of FMD introduction risk assessment and timely diagnosis are relevant for the Veterinary Service of Russia. FMD virus A 2205/G-IV strain tested for its biological and antigenic properties in cell cultures and naturally susceptible animals (cattle and pigs) was adapted for its reproduction in initially trypsinized porcine kidney (PK) cell culture, continuous monolayer cell cultures (IB-RS-2, PSGK-30, YaDK-04, BHK-21) by five serial passages. The virus was considered to be adapted when 90–95% cytopathic effect developed within 14–19 hours after the cell culture infection. The virus adapted to the cell cultures was tested for its infectivity with microtitration in IB-RS-2 cell culture. The virus strain tested for vaccine matching with microneutralization test (MNT) demonstrated significant difference from production A/Turkey/06, A_{22} No. 550/Azerbaijan/64, A_{22}/Iraq/64, A/Iran/97, A No. 2155/Zabaikalsky/2013, A No. 2166/Krasnodarsky/2013, A No. 2269/ARRIAH/2015 strains of FMD virus.

Keywords: foot-and-mouth disease virus, genotype, cell culture, vaccine matching, Africa

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Биологические свойства штамма A 2205/G-IV вируса ящура

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РЕЗЮМЕ

По данным Всемирной организации здравоохранения животных (ВОЗЖ), в странах Африки регулярно регистрируют ящур среди домашних и диких парнокопытных животных. Наиболее распространенными на Африканском континенте считаются генетические линии G-I, G-IV, G-VI, G-VII, ASIA/Iran-05 вируса ящура серотипа А. Поскольку Российская Федерация поддерживает тесные торгово-экономические отношения со странами Северной Африки, для нас особый интерес представляет изучение вируса ящура серотипа А генетической линии G-IV, который начиная с 2012 г. ежегодно является причиной вспышек инфекции в популяции естественно восприимчивых животных данного региона, при этом существует риск заноса вируса данного генотипа на территорию Российской Федерации. В связи с этим вопросы оценки риска заноса и своевременной диагностики ящура являются актуальными для ветеринарной службы России. В ходе исследований по изучению биологических и антигенных свойств штамма А 2205/G-IV вируса ящура в культурах клеток и организме естественно восприимчивых животных (крупный рогатый скот и свиньи) вирус адаптировали к репродукции в первично трипсинизированной культуре клеток свиной почки (СП), перевиваемых монослойных культурах клеток (IB-RS-2, ПСГК-30, ЯДК-04, ВНК-21) в течение пяти последовательных пассажей. При наступлении 90–95%-го цитопатического действия в течение 14–19 ч после инфицирования культуры клеток вирус ящура считали адаптированным. Инфекционную активность адаптированного к культурам клеток вируса изучали титрованием микрометодом в культуре клеток IB-RS-2. Оценка антигенного соответствия в реакции микронейтрализации показала значительное отличие изучаемого штамма от производственных вакцинных штаммов А/Турция/06, A_{22} № 550/Азербайджан/64, A_{22}/Ирак/64, А/Иран/97, А № 2155/Забайкальский/2013, А № 2166/Краснодарский/2013, А № 2269/ВНИИЗЖ/2015 вируса ящура.

Ключевые слова: вирус ящура, генотип, культура клеток, антигенное соответствие штаммов, Африка

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INTRODUCTION

Foot-and-mouth disease (*Aphthae epizooticae)* is an acute highly contagious infectious disease of cloven-hoofed animals, which is caused by an epitheliothropic RNA virus of the family *Picornaviridae*, genus *Aphthovirus*. The disease is characterized by fever, hypersalivation, aphthous lesions of oral mucosa, muzzle, interdigital cleft and coronary band skin, reduced milk and meat performance of livestock [1, 2, 3]. The disease was first described in the mid-XVI century, and, to this day, it continues to adversely impact the development of global trade and economy, as well as food security of the countries, since it is associated with enormous losses in the animal husbandry sector of agriculture [4, 5]. According to the current classification of the World Organisation for Animal Health (WOAH), foot-and-mouth disease belongs to the group of transboundary infections [6].

FMD virus has 7 serotypes significantly different from one another: A, O, C (not reported since 2004), SAT-1 (South Africa Territories-1), SAT-2, SAT-3, Asia-1 [7, 8]. The genetic diversity of field isolates and lack of cross-resistance to different FMDV serotypes in animals contribute to its wide spread all over the world. However, strict compliance with restrictions aimed at excluding the entry of livestock susceptible to foot-and-mouth disease from FMD infected regions into the countries free from the infection, as well as the application of state-of-the-art diagnosis and prevention tools help to prevent the occurrence of new FMD outbreaks. However, wild cloven-hoofed animals that are also susceptible to the virus can migrate at long distances and continuously maintain its persistence in a herd [9, 10, 11, 12]. Wild cloven-hoofed animals sharing the same territory with livestock act as a source of infection, and this is one of the causes of numerous new FMD outbreaks [13, 14, 15].

No country, not even the one with a highly effective animal disease prevention and control system in place, is shielded from FMD virus introduction, and FMD occurrence and further spread may cause a huge economic damage for agriculture [16]. The WOAH data on FMD infected countries of the world show that the virus is present in the susceptible animal population of three continents, with the highest number of FMD endemic countries being reported in Africa [17, 18, 19, 20]. Given the close trade and economic partnership maintained by the Russian Federation with the countries of this geographical region, studying the FMD virus circulating in Africa continues to be an urgent matter.

The aim of the study is to investigate the biological, antigenic and reproduction properties of foot-and-mouth disease virus A 2205/G-IV strain.

MATERIALS AND METHODS

FMDV A 2205/G-IV isolate (AFRICA topotype of G-IV genetic lineage) was provided to the FGBI "ARRIAH" by the World Reference Laboratory for Foot-and-Mouth Disease (Pirbright, Great Britain) for research purposes. The virus had been isolated from the pathological material collected from cattle in the Arab Republic of Egypt during FMD outbreaks in February 2018. During the study, the virus was adapted for its reproduction in initially trypsinized and continuous monolayer cell cultures.

Cell culture. The following cell lines were used for FMD virus A 2205/G-IV strain adaptation to cell cultures: PK (initially trypsinized porcine kidney cell culture), IB-RS-2 (continuous porcine kidney cell culture), PSGK-30 (continuous cell line of porcine origin), BHK-21 (continuous newborn Syrian hamster cell culture) and YaDK-04 (continuous domestic goat gonad cell culture). The cell cultures were tested for their susceptibility to FMDV A 2205/G-IV strain by serial passages in 25 cm^3 plastic culture flasks with a completely formed monolayer supplemented with maintenance nutrient medium. The infected cell monolayer was incubated at a temperature of (37.0 ± 0.2) °C until the development of apparent cytopathic effect (CPE). The time required for the development of 90–95% CPE reduced gradually with each subsequent passage.

Tests of the strain for biological activity in cell culture. The virus adapted to the cell cultures was tested for its biological activity by microtitration in 96-well culture plates. For this purpose, 4-fold serial dilutions of the virus were prepared in duplicate using sterile Eagle's medium (pH 7.6) containing kanamycin, an antibiotic, at a concentration of 20 IU/cm^3. Freshly prepared IB-RS-2 cell suspension with a concentration of (0.8–1.0) × 10^6 cells/cm^3 demonstrating no signs of contamination with foreign microorganisms was used as a biological activity indicator. The plates containing the reaction components were covered and placed into a carbon dioxide (CO_2) incubator with CO_2 concentration of 5% at a temperature of (37.0 ± 0.2) °C for 48 hours. The test results were recorded based on the specific CPE caused by the virus in the cell culture using an inverted microscope. The virus biological activity titre was calculated according to the Karber method and expressed as lg TCID/cm^3.

Animals. Six 8–10-month-old Russian Black Pied calves with a weight of 260–295 kg and six 4-month-old Large White gilts with a weight of 35–40 kg originating from infectious disease free farms of the Vladimir Oblast were used for FMDV A 2205/G-IV strain adaptation and infectivity titre determination in naturally susceptible animals.

Animal experiments conducted as part of the FMDV strain tests for its biological properties were carried out in compliance with interstate standard GOST 33215-2014 "Guidelines for accommodation and care of animals" adopted by the Interstate Council for Standardization, Metrology and Certification (Protocol No. 73-P of 22 December 2014).

FMD virus adaptation in cattle. For the virus adaptation, the virus culture suspension was administered to the animals intradermalingually in 4 sites at a volume of 0.1 cm^3. At passage 2, a 10% virus suspension prepared from passage 1 aphthous material was administered. Every 12 hours, the animals were examined for FMD clinical manifestations based on the presence of aphthous lesions.

Tests of FMD virus for infectivity in cattle. The infectivity titre of the studied FMD virus strain was determined according to the Henderson method. For this purpose, 10-fold dilutions of a 10% aphthous suspension of passage 2 FMD virus were prepared using phosphate buffer solution (PBS). The prepared virus dilutions were administered intradermalingually to two calves. The titration results were recorded after 24 hours based on the presence of aphthae at the virus-containing material inoculation site. The virus infectivity titre in cattle was expressed as lg ID_{50}/0.1 cm^3.

FMD virus adaptation in pigs was carried out by infecting 4-month-old piglets (2 piglets per passage) with the virus culture suspension administered intradermally in the coronary band. For passage 2, a 10% suspension prepared from passage 1 aphthous material with PBS was used. FMD clinical signs (aphthous lesions) were recorded every 12 hours.

Tests of FMD virus for infectivity in pigs were carried out by administration of the virus suspension (10-fold dilutions of a 10% suspension of passage 2 aphthae with PBS) to two gilts intradermally in the coronary band according to the Graves and Cunliffe method. The virus titration results in pigs were recorded after 24 hours based on the presence of aphthae at the virus-containing material dilution inoculation site. The virus infectivity titre in pigs was expressed as lg ID_{50}/0.1 cm^3.

Microneutralization tests of the isolate for antigenic properties. For determination of antigenic relationship (r_1 value) between A 2205/G-IV strain and production strains of serotype A FMD virus with microneutralization test (MNT), reference bovine sera from animals immunized with monovalent vaccines based on the following FMD virus strains were used: A/Turkey/06, A_{22} No. 550/Azerbaijan/64, A_{22}/Iraq/64, A/Iran/97, A No. 2155/Zabaikalsky/2013, A No. 2166/Krasnodarsky/2013, A No. 2269/ARRIAH/2015. The tests were carried out in accordance with "Methodical guidelines for determination of antigenic relationship between field isolates and production strains of foot-and-mouth disease virus using cross microneutralization test"[1], the test results were interpreted according to M. Rweyemamu [21].

Reference serum titres against 100 $TCID_{50}$ of homologous and heterologous viruses were determined with MNT by the serum cross-titration with five doses of the virus, calculated using linear regression equation and expressed as lg. Antigenic relationship coefficient (r_1 value) was calculated as the antilog of difference between serum titre (lg) against the heterologous virus and serum titre (lg) against the homologous virus.

The test results were interpreted as follows: $r_1 \geq 0.3$ suggests that there is an antigenic relationship between the field isolate and the production strain and that the vaccine based on the production strain will confer protection against the field virus; $r_1 < 0.3$ indicates that the field isolate is different from the production strain and that the vaccine based on this strain will not confer protection against the field virus.

RESULTS AND DISCUSSION

FMDV A 2205/G-IV strain adaptation to cell cultures. FMD virus A 2205/G-IV strain was adapted by five serial passages in IB-RS-2, PSGK-30, BHK-21, YaDK-04 and PK cell cultures. Test results are presented in Table 1.

Table 1
Results of FMDV A 2205/G-IV strain adaptation in cell cultures ($n = 3$)

Cell culture	Passage No.	CPE development period, hours	Virus titre, lg $TCID_{50}$/cm^3
IB-RS-2	1	18	5.50 ± 0.13
	2	15	6.73 ± 0.22
	3	15	7.03 ± 0.07
	4	11	7.60 ± 0.07
	5	7	6.59 ± 0.30
PSGK-30	1	20	6.80 ± 0.12
	2	15	7.25 ± 0.15
	3	15	6.81 ± 0.23
	4	9	7.70 ± 0.24
	5	9	7.28 ± 0.16
BHK-21	1	22	7.28 ± 0.24
	2	23	7.03 ± 0.25
	3	20	7.13 ± 0.37
	4	15	7.25 ± 0.25
	5	11	7.22 ± 0.22
YaDK-04	1	12	6.64 ± 0.11
	2	11	7.01 ± 0.40
	3	10	6.69 ± 0.02
	4	10	7.60 ± 0.07
	5	11	7.27 ± 0.37
PK	1	10	7.28 ± 0.17
	2	7	6.88 ± 0.12
	3	10	6.85 ± 0.03
	4	9	7.00 ± 0.05
	5	7	6.94 ± 0.80

[1] MU 76-12 Methodical guidelines for determination of antigenic relationship between field isolates and production strains of foot-and-mouth disease virus using cross microneutralization test: approved by the Rosselkhoznadzor on 13.09.2017. Vladimir: FGBI "ARRIAH"; 2017. 24 p.

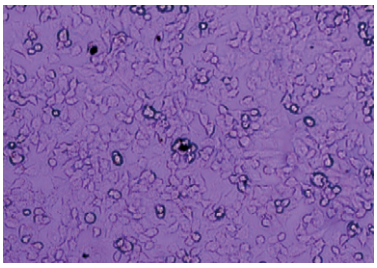

Fig. 1. Negative control BHK-21 cell culture 72 hours after the start of cultivation (200× magnification)

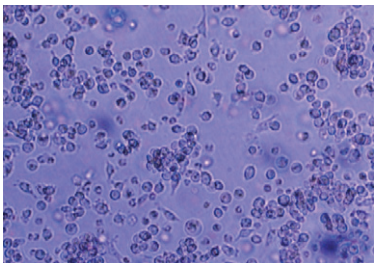

Fig. 2. FMDV A 2205/G-IV strain CPE in BHK-21 cell culture 20 hours after inoculation (200× magnification)

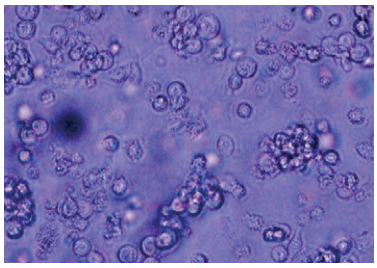

Fig. 3. Degenerative changes in BHK-21 cells as a result of FMDV A 2205/G-IV strain replication (400× magnification)

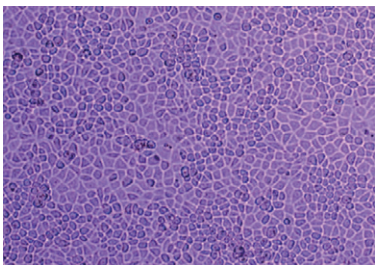

Fig. 4. Negative control IB-RS-2 cell culture 72 hours after the start of cultivation (200× magnification)

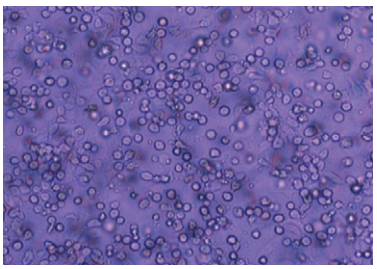

Fig. 5. FMDV A 2205/G-IV strain CPE in IB-RS-2 cell culture 15 hours after inoculation (200× magnification)

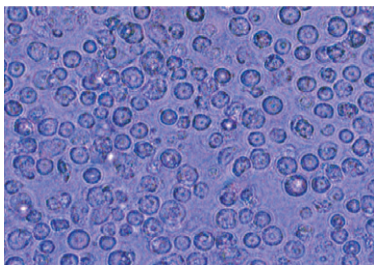

Fig. 6. Degenerative changes in IB-RS-2 cells as a result of FMDV A 2205/G-IV strain replication (400× magnification)

The negative control cell culture remained unchanged during 72 hours of observation (Fig. 1, 4). The formation of groups of rounding vacuolated cells (Fig. 2, 5) subsequently demonstrating degenerative changes of nuclei such as karyolysis and karyorrhexis (Fig. 3, 6) was interpreted as specific CPE characteristic of FMD virus. Then the cells became wrinkled and decreased in size, and that eventually led to the monolayer destruction. The above changes in the cells were indicative of the active replication of the virus.

The findings allow to conclude that FMD virus was successfully adapted to PSGK-30 and BHK-21 cell cultures. The most stable virus infectivity titre (between 7.03 ± 0.25 and 7.28 ± 0.24 lg $TCID_{50}/cm^3$) was recorded during the virus adaptation in BHK-21 cell culture. The highest infectivity of FMD virus A 2205/G-IV strain was 7.70 ± 0.24 lg $TCID_{50}/cm^3$ at passage 4 in PSGK-30 cell culture monolayer. The virus infectivity titre in IB-RS-2 cell culture increased gradually with every passage: from 5.50 ± 0.13 lg $TCID_{50}/cm^3$ at passage 1 to 7.60 ± 0.07 lg $TCID_{50}/cm^3$ at passage 4, but then began to decrease at passage 5. By contrast, the virus titre decreased during serial passages in PK cell culture. In YaDK-04 cell culture, CPE was steadily observed on average 11 hours after the virus inoculation. The infectivity titre increased in an undulating manner from 6.64 ± 0.11 lg $TCID_{50}/cm^3$ at passage 1 to 7.27 ± 0.37 lg $TCID_{50}/cm^3$ at passage 5.

FMDV A 2205/G-IV strain adaptation to naturally susceptible animals. To adapt FMD virus A 2205/G-IV strain to naturally susceptible animals, the virus containing material with activity of 6.81 ± 0.23 lg $TCID_{50}/cm^3$ prepared by passage 3 in PSGK-30 cell culture was used.

During the experiment, the animals were daily observed, first and foremost for their general health state, for lameness (piglets) and salivation (calves), for the severity and number of aphthous lesions at the virus suspension inoculation sites, their body temperature was measured.

At passage 1, apparent and well formed primary aphthae characteristic of foot-and-mouth-disease were found in the oral cavity (cattle) and on the coronary bands (pigs) 28 hours after the inoculation. The virus adaptation in naturally susceptible animals was considered to be successful based on the development of pronounced FMD clinical signs. The resulting aphthous material was used to prepare a 10% suspension for the virus infectivity tests in cattle and pigs, as well as in initially trypsinized PK cell culture. The results of the virus titration in animals and in PK cell culture are shown in Table 2.

Table 2
Results of infectivity titre determination for 10% aphthous suspension during FMDV A 2205/G-IV strain adaptation

Description of material	FMD virus infectivity titre in biological systems		
	in cattle, lg $ID_{50}/0.1\ cm^3$ ($n = 1$)	in pigs, lg $ID_{50}/0.1\ cm^3$ ($n = 1$)	in PK cell culture, lg $TCID_{50}/0.1\ cm^3$ ($n = 3, p < 0.01$)
10% aphthous suspension passage 1 in cattle	4.00	–	4.67 ± 0.30
10% aphthous suspension passage 1 in pigs	–	3.25	4.33 ± 0.17
10% aphthous suspension passage 2 in cattle	5.50	–	6.00 ± 0.14
10% aphthous suspension passage 2 in pigs	–	5.00	5.92 ± 0.22

The infectivity titres of 10% aphthous suspensions of the virus prepared using the material collected from cattle and pigs were found to be as follows: at passage 1 in animals – 4.00 and 3.25 lg ID_{50}/0.1 cm^3; at passage 2 – 5.50 and 5.00 lg ID_{50}/0.1 cm^3, respectively. In initially trypsinized PK cell culture, the infectivity titres of 10% aphthous suspensions of the virus prepared using the aphthae collected from cattle and pigs were found to be as follows: at passage 1 – 4.67 ± 0.30 and 4.33 ± 0.17 lg $TCID_{50}$/0.1 cm^3; at passage 2 – 6.00 ± 0.14 and 5.92 ± 0.22 lg $TCID_{50}$/0.1 cm^3, respectively, and this is indicative of FMDV A 2205/G-IV strain adaptation to naturally susceptible animals.

Microneutralization tests of the strain for antigenic properties. High variability of FMDV within one serotype leads to the emergence of new isolates that may differ from the previously recovered strains of the virus as regards their virulence, immunogenicity and antigenic properties. Among FMDV serotypes, serotype A FMD virus demonstrates the most pronounced antigenic variability, and this may result in strain-specific diagnosis problems. In view of this, the determination of antigenic relationship between the newly recovered FMDV isolates and the characterized and production strains of heterologous genotypes is of particular interest when studying FMD virus. The results of FMDV A 2205/G-IV strain tests for antigenic relationship are presented in Table 3.

FMD virus A 2205/G-IV strain was found to be antigenically different from production FMD virus A/Turkey/06, A_{22} No. 550/Azerbaijan/64, A_{22}/Iraq/64, A/Iran/97, A No. 2155/Zabaikalsky/2013, A No. 2166/Krasnodarsky/2013, A No. 2269/ARRIAH/2015 strains and not related to them. The findings are consistent with the data of the World Reference Laboratory for Foot-and-Mouth Disease (Pirbright, Great Britain) [22].

Table 3
Antigenic relationship (r_1) between A 2205/G-IV strain and production strains of serotype A FMD virus ($n = 3$) in MNT

FMD virus strains (genotypes)	r_1 value
A_{22} No. 550/Azerbaijan/64 (A/ASIA/Iraq-64)	0.22
A_{22}/Iraq/64 (A/ASIA/Iraq-64)	0.15
A/Iran/97 (A/ASIA/Iran-97)	0.21
A/Turkey/06 (A/ASIA/Iran-05)	0.08
A No. 2155/Zabaikalsky/2013 (A/ASIA/Sea-97)	0.06
A No. 2166/Krasnodarsky/2013 (A/ASIA/Iran-05SIS-10)	0.19
A No. 2269/ARRIAH/2015 (A/ASIA/G-VII)	0.25

CONCLUSION

The results of FMDV A 2205/G-IV strain tests for its biological and infectious properties are indicative of its high stability in cattle and pigs, and this may pose a significant risk in case of genotype A/AFRICA/G-IV FMD virus introduction into FMD free countries.

The tests of FMD virus A 2205/G-IV strain for its antigenic properties revealed its significant difference from production serotype A FMDV strains included in the vaccines used for the preventive immunization of naturally susceptible animals in the Russian Federation and neighbouring countries (r_1 values ranged from 0.06 to 0.25). The test results show that emergency response measures to be implemented in case of occurrence of foot-and-mouth disease caused by the virus of A/AFRICA/G-IV genetic lineage require the development and production of diagnostica and vaccines based on FMDV strains that are homologous or closely related to genotype A/AFRICA/G-IV FMD virus.

To ensure the economic stability and food security, as well as FMD freedom of the Russian Federation, to minimize the economic damage in case of possible FMD outbreak occurrence, we consider it appropriate to continuously monitor the global FMD situation in order to assess the risk of the disease agent introduction into Russia, to develop tools for timely FMD diagnosis based on heterogeneous field isolates of FMD virus that are not typical for our geographical region.

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Dynamics of blackleg epizootic process in the Republic of Kazakhstan

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ABSTRACT

In 2012–2021, 2,030 outbreaks of acute infectious animal diseases were registered in the Republic of Kazakhstan. Among all the diseases blackleg accounted for 20.7% (421 outbreaks), which suggests that the nosological unit is of high epizootological significance among other infectious animal diseases in the country. Analysis of the blackleg outbreaks registered over the recent 10 years demonstrates a significant annual growth in the number of outbreaks (from 19 to 81 outbreaks) in the Republic of Kazakhstan and the disease persistence in the territory. Within the mentioned period, the average number of blackleg-infected animals per one outbreak ranged between 1 and 3 animals, which proves that blackleg is a non-contagious disease. The research indicates that blackleg is a seasonal disease in the Republic of Kazakhstan with an incidence rise in autumn. The data analysis for 2012–2022 did not reveal any regular blackleg epizooties. The epizootological zoning made it possible to conclude that the blackleg situation in 6 oblasts (which account for 42.8% of the total territory) was rather tense; in 5 oblasts (35.7% of the country's territory) the epizootic situation was less tense and the remaining 3 (21.5%) oblasts are disease-free. Therefore, blackleg zoning in the Republic makes it possible to use a differential approach to planning preventive veterinary and control measures, depending on the intensity of the epizootic situation. The research results will help to improve the system of blackleg surveillance, to predict the disease spread in animals and can be used to develop anti-epizootic measures.

Keywords: blackleg, outbreak, intensity of the epizootic situation

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Динамика эпизоотического процесса эмфизематозного карбункула животных на территории Республики Казахстан

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РЕЗЮМЕ

В 2012–2021 гг. на территории Республики Казахстан было зарегистрировано 2030 очагов острых инфекционных болезней животных, доля эмфизематозного карбункула животных из их числа составила 20,7% (421 очаг), что указывает на важное эпизоотологическое значение данной нозоединицы в инфекционной патологии животных в стране. Результаты анализа количества зарегистрированных очагов эмкара за 10 лет свидетельствуют о ежегодном (от 19 до 81 очага) значительном распространении заболевания на территории Республики Казахстан и о его стационарности. За этот период показатель

очаговости по эмкару составил в среднем от 1 до 3 животных на один очаг, что свидетельствует о неконтагиозности эмкарной инфекции. Результаты исследований указывают на то, что эмкар в Республике Казахстан имеет сезонный характер с подъемом заболеваемости в осенние месяцы года, анализ данных за 2012–2022 гг. периодичности эпизоотий не выявил. Проведенное эпизоотологическое зонирование позволило установить, что в 6 областях, площадь которых составляет 42,8% площади всей территории республики, наблюдался высокий уровень напряженности эпизоотической ситуации по эмкару; в 5 областях, занимающих 35,7% территории страны, отмечена низкая степень напряженности эпизоотической ситуации; остальные 3 (21,5%) являются благополучными по заболеванию. Таким образом, зонирование территории республики по эмкару дает возможность дифференцированно планировать профилактические ветеринарные мероприятия и меры борьбы с ним по отдельным территориям (зонам) в зависимости от напряженности эпизоотической ситуации. Полученные результаты исследований позволят усовершенствовать систему эпизоотологического надзора за эмкарной инфекцией, прогнозировать возможное территориальное расширение распространения заболеваемости животных и могут быть использованы при разработке противоэпизоотических мероприятий.

Ключевые слова: эмкар, эпизоотический очаг, напряженность эпизоотической ситуации

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INTRODUCTION

Blackleg (*Gangraena emphysematosa*) is an acute, non-contagious toxic infection of cattle, characterized by rapidly increasing crepitate swellings in muscles and lameness. Cattle, including buffaloes, are susceptible to blackleg. In sheep, blackleg causative agent is of no particular importance; it is more often isolated during malignant edema. Cattle of improved breeds, purebreds and particularly meat cattle (that have more muscle mass and are thick-fleshed) are more susceptible to blackleg. Animals brought to the infected area from other farms, or imported animals often get infected. Cattle of any age can get diseased, but young animals aged from 3 months to 3–4 years are the most susceptible [1, 2, 3, 4, 5, 6, 7].

Blackleg is caused by an anaerobic bacterium *Clostridium chauvoei*. It is a straight or slightly curved rod with rounded ends, observed either single or in pairs, less often in short chains; gram-positive in young cultures. The pathogen spores are very stable: they remain viable in soil for several years, in rotting muscles and manure for up to 6 months and at the bottom of water-bodies for over 10 years. Under appropriate conditions, the bacteria can survive and multiply in soil. Many researchers have studied the blackleg pathogen properties [8, 9, 10, 11, 12]. The blackleg agent synthesizes and releases an exotoxin. Hemotoxic and necrotizing components were found in the toxin. The ability of the agent to produce aggressins is another diagnostic criterion [13, 14, 15, 16].

Blackleg is seen in livestock all over the world, regardless of the geographical location and soil and climatic conditions. It causes great economic damage to the infected farms resulting from the livestock death and the cost of anti-epizootic measures [17, 18, 19, 20, 21]. In the CIS countries, the disease has been registered in all regions [22, 23, 24].

Blackleg has been known to herdsmen since ancient times. Kazakhs have long been able to distinguish this disease from anthrax and named it "karasan" (black thigh, black rump).

In Kazakhstan blackleg is an issue of top priority among other animal infectious diseases. Many regions of the Republic, regardless of their geographical location and soil and climatic conditions, are permanently infected by the disease [25, 26, 27]. If detected late or the required measures are taken with delays, the disease can cause serious damage to the livestock industry of the Republic resulting from the animal death and the cost of anti-epizootic measures. Regardless of the scheduled vaccinations and measures taken to prevent, detect and eliminate the outbreaks, the disease remains a serious concern in the infected areas that needs further investigation.

Due to the urgency of the problem, the purpose of this research is to implement monitoring activities and to draw a blackleg map of the Republic of Kazakhstan so that to depict current epizootic situation based on the epizootic tension.

The novelty of the research consists in studying common patterns and epizootic trends of the disease spread and in obtaining new epizootological data that will be used to draw an epizootic map of the Republic of Kazakhstan that will depict blackleg-infected areas, thus, helping to efficiently plan and implement measures to control the disease.

The purpose of the work is to assess the epizootic situation on blackleg in 2012–2021 and to zone the territory

of the Republic of Kazakhstan following analysis of the epizootic tension.

MATERIALS AND METHODS

For the purposes of this research, we used the blackleg diagnostic methods officially stipulated by GOST 26503-85. For the purposes of epizootological monitoring, we used the methods described in relevant guidelines [28, 29].

Tension of the epizootic situation on blackleg was calculated using the formula:

$$W = n / N \times t / T,$$

where W – the value of the epizootic tension; n – the number of blackleg outbreaks in 2012–2021; N – the total number of outbreaks of acute infectious diseases in 2012–2021; t – the time when the disease was registered; T – the observation time (years).

In order to study the blackleg-related epizootic process and ensure control over the disease, we collected and analyzed statistics and official reports from the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan and the Republican Veterinary Laboratory. We used the results obtained by the veterinary laboratories and project executors during serological and bacteriological tests, during epizootological and immunological monitoring and blackleg control activities. We used the materials collected after clinical and epizootic examination of blackleg outbreaks in the districts and oblasts.

For the zoning purposes, we used retrospective data on blackleg outbreaks for some years in oblasts. The data were analyzed and an epizootic map was drawn to depict the oblasts with varying risk of infection.

RESULTS AND DISCUSSION

In order to assess the blackleg situation in the Republic of Kazakhstan, we analyzed epizootic data for 10 years.

Table 1 shows the number of outbreaks of acute infectious animal diseases and blackleg registered in the Republic of Kazakhstan in 2012–2021.

During the analyzed period 2,030 outbreaks of acute infectious animal diseases were registered in the Republic, 421 of them were blackleg outbreaks, that is, the disease share in the total number of outbreaks of acute infectious diseases was 20.7%. Outbreaks of rabies and blackleg were most often recorded in 2012–2021. Next in descending order are pasteurellosis, avian influenza, bovine viral diarrhea and rhinotracheitis, etc. In some years, in some oblasts, the share of blackleg in the total number of infectious diseases ranged from 68.8% (2021, Aktobe Oblast) to 86.2% (2019, West Kazakhstan Oblast). These data demonstrate epizootological importance of blackleg among other animal pathogens in the Republic of Kazakhstan.

The number of registered outbreaks over a certain period can also add to the country's epizootic landscape. Thus, the average annual number of blackleg outbreaks per oblast in the Republic can be calculated by dividing the total number of blackleg outbreaks over a ten-year period by the number of territorial units of Kazakhstan: 421 / 14 = 30.

Therefore, the oblasts, where the number of blackleg outbreaks is above 30, can be regarded as territories with a high blackleg prevalence: West Kazakhstan Oblast – 176 outbreaks, East Kazakhstan Oblast – 83, Zhambyl Oblast – 52, Almaty Oblast – 36, Aktobe Oblast – 33, and below 30 – with medium and low prevalence: Pavlodar

Table 1
Number of outbreaks of acute infectious animal diseases and blackleg in 2012–2021

Oblast	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	For the whole period
West Kazakhstan	14/7	45/15	64/23	57/31	23/8	10/3	31/10	29/25	61/47	14/7	348/176
East Kazakhstan	47/5	28/0	32/8	55/5	18/3	24/12	29/13	25/13	38/12	24/12	320/83
Zhambyl	57/4	32/3	21/4	29/2	25/4	34/15	15/7	24/10	8/1	30/2	275/52
Almaty	22/0	18/0	10/0	14/1	15/1	24/14	13/5	20/7	8/4	11/4	155/36
Aktobe	8/2	15/0	8/1	10/2	11/1	9/2	12/3	9/3	15/8	16/11	113/33
Pavlodar	0/0	4/0	6/1	14/3	6/2	3/1	2/0	6/2	26/5	14/3	81/17
Kostanay	7/3	18/0	2/0	17/1	10/2	4/0	7/0	44/1	19//1	17/1	145/9
Karaganda	2/0	10/1	8/2	19/0	8/0	7/0	11/1	5/1	15/1	8/2	93/8
Atyrau	4/0	6/0	9/0	9/0	16/0	7/0	14/1	6/1	7/1	0/0	78/3
Akmola	9/1	15/0	12/0	23/0	2/0	1/0	7/0	4/0	25/1	23/1	121/3
North Kazakhstan	1/0	8/0	3/0	3/0	2/0	2/0	2/0	2/0	56/0	47/1	126/1
Kyzylorda	0/0	0/0	0/0	6/0	0/0	0/0	0/0	0/0	4/0	0/0	10/0
Mangystau	4/0	4/0	4/0	3/0	0/0	0/0	15/0	1/0	6/0	0/0	37/0
Turkistan	5/0	9/0	15/0	18/0	13/0	17/0	5/0	8/0	38/0	0/0	128/0
Total	180/22	212/19	194/39	277/45	149/21	142/47	163/40	183/63	326/81	204/44	2,030/421

* In the numerator – total number of outbreaks of acute infectious diseases; in the denominator – number of blackleg outbreaks.

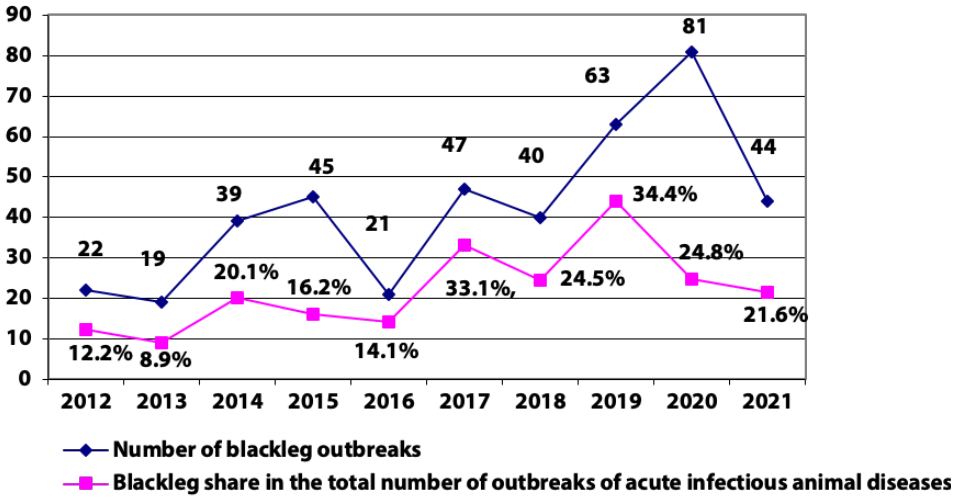

Fig. 1. Registration of blackleg outbreaks and its share in the total number of acute infectious animal diseases in the Republic of Kazakhstan (2012–2021)

Oblast – 17, Kostanay Oblast – 9, Karaganda Oblast – 8, Atyrau Oblast – 3, Akmola Oblast – 3, North Kazakhstan Oblast – 1. The remaining three oblasts (Kyzylorda, Mangystau, Turkistan) are considered free from this infection.

Figure 1 shows dynamics of blackleg registration in the Republic of Kazakhstan and its share in the total number of acute infectious animal diseases in 2012–2021.

As you can see, the curve reflecting the share of blackleg in the total number of infectious animal diseases has been running almost parallel to the curve showing the annual number of the disease outbreaks all these years. The only exception is 2020, when the largest number of blackleg outbreaks (81) was recorded over a 10-year period, and its share in the total number of acute infectious animal diseases decreased by almost

Table 2
Indicators of the epizootic process in case of blackleg in the Republic of Kazakhstan for 2012–2021

Oblast	Indicators of the epizootic process						
	n	N	Blackleg share	t	T	Epizooty index	W
West Kazakhstan	176	348	50.5	10	10	1.0	0.51
East Kazakhstan	83	320	25.9	9	10	0.9	0.23
Aktobe	33	113	29.2	9	10	0.9	0.26
Zhambyl	52	275	18.9	10	10	1.0	0.19
Almaty	36	155	23.2	7	10	0.7	0.16
Pavlodar	17	81	20.9	7	10	0.7	0.15
Kostanay	9	145	6.2	6	10	0.6	0.04
Karaganda	8	93	8.6	6	10	0.6	0.05
Atyrau	3	78	3.8	3	10	0.3	0.01
Akmola	3	121	2.5	3	10	0.3	0.01
North Kazakhstan	1	126	0.8	1	10	0,1	0.001
Kyzylorda	0	10	0	0	10	0	0
Mangystau	0	37	0	0	10	0	0
Turkistan	0	128	0	0	10	0	0
Total	421	2030	20.7	7.1	10	0.71	0.15

n – number of blackleg outbreaks; N – total number of outbreaks of acute infectious animal diseases in 2012–2021;
blackleg share – blackleg share in the total number of outbreaks of acute infectious animal diseases;
t – number of years when the disease was reported; T – time of observation; W – intensity of epizootic situation.

one and a half times (24.8%) compared to the previous year (34.4%).

Thus, the number of outbreaks registered over 10 years (2012–2021) was analyzed and the analysis showed there had been a significant annual increase in blackleg outbreaks in the Republic of Kazakhstan (from 19 to 81) and the infection was persistent.

To characterize the epizootic process, we apply an outbreak criterion, i.e. the average number of animals that have got diseased within one infected settlement. In 2012–2021, based on the criterion, when the average number of diseased animals in one outbreak in the Republic of Kazakhstan ranged between 1 to 3 animals per outbreak, we confirmed that blackleg is not a contagious disease. These data are consistent with the data of other researchers [30].

We earlier studied blackleg seasons in the Republic of Kazakhstan within 2016–2020. It was found that the maximum number of registered outbreaks was observed in November. Sixty nine outbreaks were recorded in November, which accounted for 27.4% of the total number (252) of registered blackleg outbreaks. This indicator was, in descending order: 25.4% in October, 11.9% in September, 10.3% in August, 7.9% in July, 4.7% in June, 2.7% in March, 2.4% in December; 1.9% were reported both in January and in February; and 1.6% in April and May of the total number of registered blackleg outbreaks over a 5-year period [31].

These data suggest seasonal manifestation of blackleg in the Republic of Kazakhstan (August, September, October and November), which makes it possible for researchers and veterinarians to specify the causes and factors of this pattern and adjust the ongoing preventive and anti-epizootic measures.

During epizootological observation, it is important to establish the frequency of epizooties – ups and downs repeated at certain intervals, usually lasting for several years. The regular time intervals are especially typical for epizooties of such infectious diseases that, due to the high contagiousness of their causative agents, affect most susceptible animals, as well as for spontaneously developing epizooties when effective anti-epizootic measures are not carried out. The blackleg process monitoring in the Republic of Kazakhstan in 2012–2021 did not help to determine the frequency of epizooties.

Based on the major criteria of the blackleg epizootic process over the past 10 years, the Republic of Kazakhstan was zoned according to the epizootic tension.

The tension of the epizootic situation is a comparative feature of particular territories, which implies intensive disease manifestation assessed according to some epizootological indicators.

Table 2 shows the main indicators of blackleg epizootic process in the Republic of Kazakhstan for 2012–2021, that are used to assess epizootic tension.

It was established that the mean tension indicator for the blackleg situation in the Republic of Kazakhstan in 2012–2021, was 0.15. Based on this, oblasts with the indicators of 0.15 and above were classified as territories with an increased epizootic tension, and below 0.15 – as territories with a low epizootic tension. Oblasts with W = 0 (Kyzylorda, Mangystau, Turkistan) were free from the disease during the period.

Based on the results obtained, a map of the Republic of Kazakhstan was drawn to depict zones according to the tension of the epizootic situation on blackleg in 2012–2021.

Figure 2 shows that during these years, 6 oblasts (i.e. West Kazakhstan, East Kazakhstan, Aktobe, Zhambyl, Almaty, Pavlodar) accounting for 42.8% of the Republic territory demonstrated an increased epizootic tension; 5 oblasts (Kostanay, Karaganda, Atyrau, Akmola, North

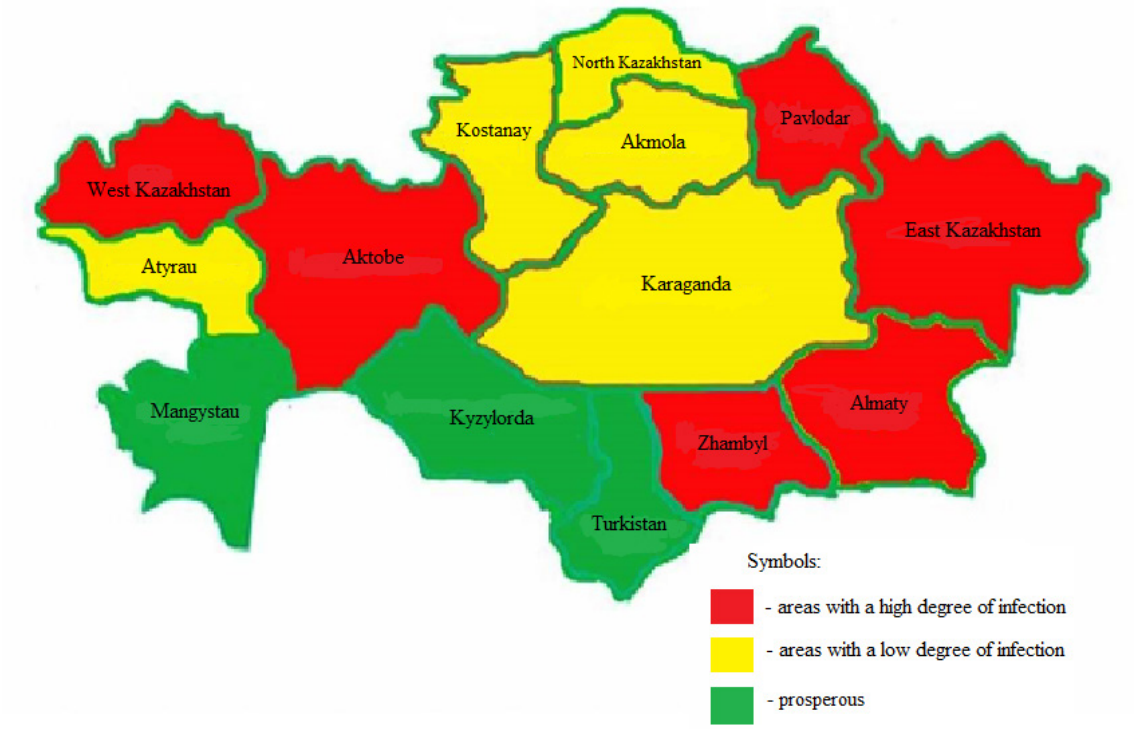

Fig. 2. Zoning the Republic of Kazakhstan according to the intensity of the blackleg epizootic situation in 2012–2021

Kazakhstan) accounting for 35.7% of the country's territory, demonstrated lower degree of epizootic tension.

Thus, 78.5% of the Republic of Kazakhstan turned out to be blackleg – infected from 2012 to 2021. The remaining three oblasts (21.5%): Kyzylorda, Mangystau, Turkistan – were free from the disease.

The epizootic zoning provides data that can be used to take a differentiated approach to planning preventive veterinary measures and measures to control blackleg in particular territories (zones), depending on the epizootic tension.

In the oblasts with an increased epizootic tension, preventive measures shall be applied on all the farms for all the susceptible farm animals of all the age groups.

In the oblasts with a low epizootic tension, preventive vaccination of susceptible livestock in the disease-infected settlements shall be provided. In the territories where the disease has not been registered during the entire research, general veterinary and sanitary measures shall be taken.

The obtained results make it possible to improve the system of blackleg surveillance, to predict the disease spread in animals and these results can be used to develop anti-epizootic measures.

CONCLUSION

The research results show that the ultimate goal has been achieved: the epizootic situation in 2012–2021 was assessed and the Republic of Kazakhstan was zoned according to the epizootic tension.

It was found, that 2,030 outbreaks of acute infectious animal diseases were registered in the Republic of Kazakhstan from 2012 to 2021 (10 years), 421 out of them were blackleg outbreaks, that is, the share of this disease in the total number of acute infectious diseases was 20.7%, which suggests epizootological significance of blackleg in the infectious pathology of animals.

The analysis of the registered outbreaks during this period indicates that every year blackleg easily spreads in the Republic of Kazakhstan (from 19 to 81 outbreaks) and remains persistent.

In order to characterize the blackleg epizootic process, we calculated the average number of blackleg-infected animals per one outbreak (outbreak criterion) as ranging between 1 and 3 animals. It proves that blackleg is a non-contagious disease.

The research indicates that blackleg in the Republic of Kazakhstan is seasonal (August, September, October and November) and the frequency of epizooties was not established during observation of the epizootic processes in 2012–2021.

Based on the main indicators of the epizootic process over the past 10 years, territory of the Republic of Kazakhstan was zoned according to the tension of the epizootic situation on blackleg in 2012–2021. The zoning makes it possible to use a differential approach to planning anti-epizootic measures, depending on the tension of the epizootic situation.

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Goat-derived cell line (*Capra hircus*) TCh generated by karyological and morphological transformation of YaDK-04 CCL during subcultivation with lanthanide-treated bovine serum

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ABSTRACT

Ability of the continuous cell lines to evolve enables generation of new transformed cell cultures with unlimited life potential and different from the original prototypes in the process of sequential cultivation. There are practically no universal mechanisms and methods for new cell line generation. But it was noted that cell immortalization is associated with chromosomal rearrangements (chromatid morphology) and changes in the number of chromosomes. The paper presents the results of the generation of a new *Testis Capra hircus* (TCh) cell line, suitable for effective replication of dermatotropic and other types of animal viruses, in order to scale up viral material used for the manufacture of the means for animal disease specific prevention and diagnosis. The monolayer TCh cell line was transformed from the continuous YaDK-04 cell line as a result of more than 50 passages in the growth medium supplemented with 10% of lanthanide-treated bovine serum. Use of the bovine serum purified and supplemented with lanthanides during the cultivation of the continuous cell line YaDK-04 led to significant chromosomal rearrangements and contributed to the formation of a stable and productive new TCh cell line, which differed in cytomorphological and karyological characteristics and had unlimited potential for passaging without changing the cell karyotype and morphology. The novel continuous cell line proved to be suitable for effective reproduction of such disease pathogens as lumpy skin disease, sheep pox, peste des petits ruminants agents. These are mainly viruses of dermatotropic origin.

Keywords: continuous cell line, proliferative activity, immortalization, cytopathic effect, infectious activity

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Получение клеточной линии козьего происхождения (*Capra hircus*) TCh как результат кариологической и морфологической трансформации ПЛК ЯДК-04 при субкультивировании с применением сыворотки крупного рогатого скота, обработанной лантаноидами

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РЕЗЮМЕ

Способность перевиваемых линий клеток к эволюции дает возможность создавать в процессе последовательного культивирования новые трансформированные клеточные культуры, обладающие неограниченным жизненным потенциалом и отличающиеся от исходных прототипов. Универсальных

механизмов и способов получения новых клеточных линий практически не существует. Но было замечено, что иммортализация клеток связана с хромосомными перестройками (морфология хроматид) и изменением количества хромосом. Представлены результаты получения новой клеточной линии тестикул козленка *Testis Capra hircus* (TCh), пригодной для эффективной репродукции дерматотропных и других видов вирусов животных, с целью наработки вирусного материала, применяемого для изготовления средств специфической профилактики и диагностики заболеваний животных. Монослойная линия клеток TCh трансформировалась из перевиваемой линии клеток ЯДК-04 в результате проведения более 50 пассажей культивирования в ростовой среде с добавлением 10% сыворотки крови крупного рогатого скота, обработанной лантаноидами. Применение сыворотки крови крупного рогатого скота, очищенной и обогащенной лантаноидами, при культивировании постоянной линии клеток ЯДК-04 привело к значительным хромосомным перестройкам и способствовало формированию стабильной и продуктивной новой клеточной линии TCh, которая отличалась по цитоморфологическим и кариологическим признакам и обладала неограниченным потенциалом к пассированию без изменения кариотипа и морфологии клеток. Новая перевиваемая линия клеток оказалась пригодной для эффективной репродукции возбудителей таких болезней, как заразный узелковый дерматит, оспа овец, чума мелких жвачных животных. В основном это вирусы дерматотропного происхождения.

Ключевые слова: перевиваемая (постоянная) линия клеток, пролиферативная активность, иммортализация, цитопатическое действие, инфекционная активность

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INTRODUCTION

The mechanisms of formation of continuous cell lines (CCL) have not been fully established. But due to quite frequent appearance of infinitely dividing (immortal) CCL, it can be assumed that the mechanism of formation of the continuous cell lines has some patterns, manifested in changes in the cell karyology and morphology during *in vitro* cultivation. One of such patterns involves the increase in proliferative activity – a sign of immortality (immortalization), which occurs by passage 50 after trypsinization of tissues or organs [1, 2, 3]. Changes occurring at the chromosomal and genetic levels of the cells under the effect of various physico-chemical and biological factors are likely to accumulate [4, 5, 6]. The mechanism of stable heterochromatin methylation in metacentric sections of chromosomes is formed and thereby the ability to unlimited division of the main population of cell lines develops under stable cultivation conditions [3, 7, 8, 9].

In veterinary virology, three lines of goat-derived cell lines (*Capra hircus*) obtained from goat gonads are known: CG-91 [10], YaDK-04 [11], TCh [12]. In fact, these lines are derivatives of the single trypsinization, which was carried out in the late 1980s. They were formed under different cultivation conditions and effect of additional chemical factors. Whereas the first two lines have low proliferative activity (split ratio 1:2, 1:4), instability of cultivation and a near-diploid chromosome complement), the third line (TCh), obtained using lanthanide treated bovine serum, has significant chromosomal rearrangements and surpasses its predecessors in performance and stability. This report provides an example of the evolution of the goat gonad cell line and describes the proposed mechanism for the formation of a stable and productive CCL suitable for the effective reproduction of dermatotropic and other animal viruses.

The work was aimed at the study of the karyological and cultural transformation of the TCh cell line obtained by subcultivation of the continuous YaDK-04 cell line, as well as at the assessment of the degree of sensitivity of the new cell subline to the viruses – agents of the animal diseases.

MATERIALS AND METHODS

The cells were cultured according to the generally accepted technique in glass and plastic flasks using classical media MEM, DMEM, DMEM/F-12 supplemented with 10% of the lanthanide treated bovine serum.

Phenotyping of the cell lines was carried out using Olympus CX41 phase contrast microscope (Japan) and ML-2B fluorescent microscope (Russia).

The cell cultures were identified by the karyological method of metaphase spread preparation according to P. S. Moorhead technique [13].

Viral material. Lumpy skin disease virus strain LSD Cattle/Dagestan/2015 with infectivity 5.0 lg $TCID_{50}/cm^3$, production sheep pox virus strain "ARRIAH" with infectivity 5.5 lg $TCID_{50}/cm^3$ and production peste des petits ruminants virus (PPRV) strain "ARRIAH" with infectivity 5.0 lg $TCID_{50}/cm^3$ were used in the study [14, 15, 16, 17].

Cell culture. Cultivation of the viruses was carried out in the cell cultures of various origin, which were obtained from the Cell Cultivation Unit of the Federal Centre for Animal Health.

The infectivity of the obtained viral material was determined by titration using YaDK-04 cell line.

RESULTS AND DISCUSSION

There are no any patterns in the formation of the continuous cell lines of goat origin (*Capra hircus*). Use of a variety of nutrient media and sera, duration of passaging do not allow identification of any significant factor that

would affect the karyological and genetic transformation of the cell lines. The first two variants described below have a unique origin, however they turned out to be similar in characteristics.

It is well known that the effectiveness of cell cultivation is associated with the quality of the nutrient medium, the growth properties of which are largely dependent on the quality of animal serum in its composition. Lanthanide treated bovine serum was used in this study [18]. The use of lanthanides in serum at the stage of its production leads to flocculation of latent microorganisms and endotoxins, which settle and can be removed by separation and ultrafiltration. The lanthanides themselves remain in the medium and participate in the biochemical processes of cultivation. The duration of passaging under homogeneous conditions can lead to certain changes observed in the further study of the cell cytomorphology.

Characteristics of the continuous goat gonad cell line CG-91. The only continuous goat gonad cell line (*Capra hircus*) CG-91, which has no analogues in other countries, was obtained at the Federal Centre for Animal Health in 1990 (RF Patent No. 2061753) [10]. This CCL is quite difficult to cultivate as the split ratio is no more than 1:2–1:4 and the modal class is 59 chromosomes. The CCL subcultivation resulted in almost immediate elimination of the small metacentric Y-chromosome, and the culture was formed as a pseudodiploid.

The CCL is derived (established) from the organs spontaneously, as a result of the long passaging with a variety of nutrient media, as well as with supplementation with animal sera and using some other technological approaches associated with cultivation temperature, subculture methods, duration of cultivation, use of conditioned media, etc. [19, 20, 21]. In this particular case, the CG-91 cell line was stabilized as continuous when cultured in DMEM. As an additive, 10% of different types of bovine sera, including fetal one, were used. Morphological features of CG-91 CCL lie in the fact that spindle-shaped cells predominate in the cell population along with a small number of epithelial and fibroblast-like cells. When the culture is reseeded, the spindle-shaped cells undergo a fibroblast-like transformation under the effect of the dispersing solution or begin taking a spherical shape, while up to 20% of the total number of cells die (Fig. 1). To mitigate the process of trypsinization, 0.5% glucose solution is added to the dispersing solution. The cells survived after the reseeding repair during sedimentation and adhesion to the substrate and form a confluent monolayer in 3–4 days.

CG-91 cell line was found to be sensitive to type A, O, C, Asia-1 foot-and-mouth disease virus, as well as to African horse sickness causative agent.

Characteristics of continuous goat gonad subline YaDK-04. The continuous cell subline YaDK-04 was derived from CG-91 CCL by targeted selection aimed at the increase of the cell biomass and their sensitivity to animal viruses [11]. The selection was carried out using limiting dilution of the cell suspension when reseeded using Eagle's medium supplemented with 0.25% lactalbumin hydrolysate.

Using the limiting dilution method during the cell line reseeding, the subline performance was increased at passage 36 and amounted to 100 million cells from 300 cm^2 culture vial. The morphology of the cells and monolayer remained the same and spindle-shaped cells

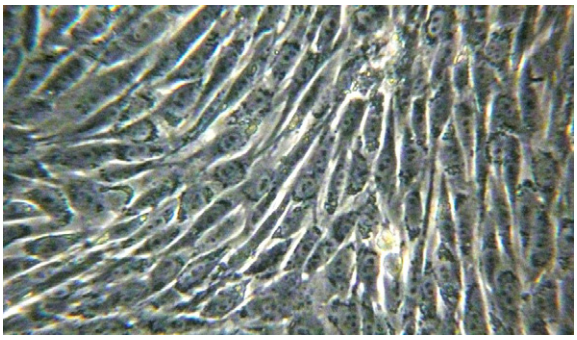

Fig. 1. YaDK-04 cell line morphology

prevailed (Fig. 1). The karyotype became more variable and amounted to 57–60 chromosomes (Fig. 2, 3). The predominant population is the modal class of 59 chromosomes (58%).

As a result of the selection, the list of the viruses efficiently reproduced on this subline was extended. Thus, Aujeszky's disease virus titer reached 8.00–8.75 lg $TCID_{50}/cm^3$, sheep pox virus titer – 5.5–6.0 lg $TCID_{50}/cm^3$, pneumovirus titer – 5.0 lg $TCID_{50}/cm^3$.

Both described variants of goat gonad cells are practically diploid cultures with minimal transformations in karyotype and morphology, the split ratio of which does not exceed 1:4. These CCLs periodically demonstrated suppressed growth activity, therefore, change of growth

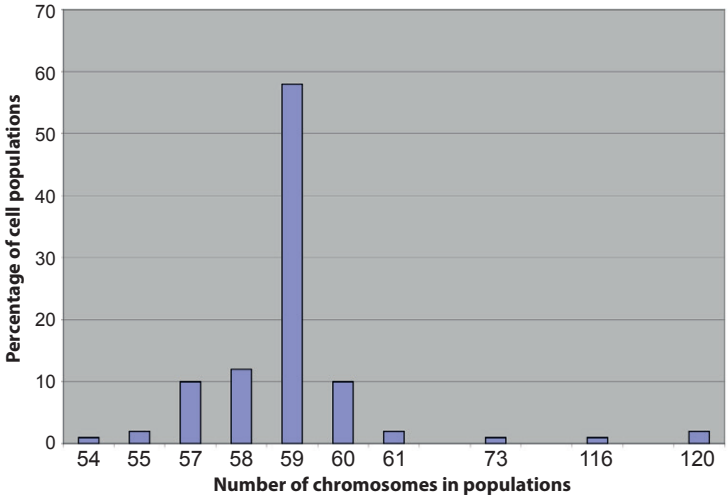

Fig. 2. YaDK-04 cell line karyogram

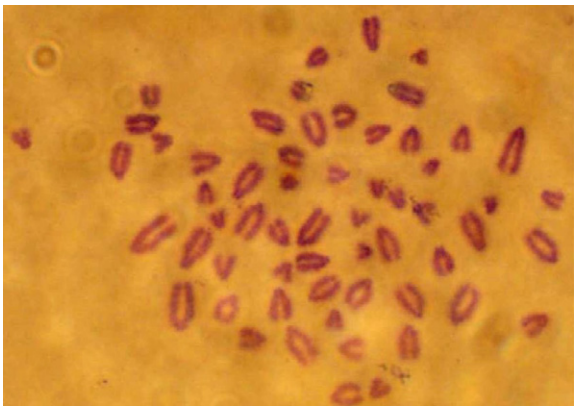

Fig. 3. Metaphase spread of YaDK-04 cell line – 59 chromosomes

ingredients and recovery from the cryobank were required. Propagation of these variants for the production of the specific vaccine products required large material and labor costs.

Production of new continuous TCh cell line. When analyzing YaDK-04 karyogram, we noted sufficient chromosome variability in cell populations – from 54 to 120. This fact suggests that culture selection is possible to isolate cells different from the previous clones. A monolayer goat gonad CCL (YaDK-04) was used as a starting material for this purpose, which was characterized by variable split ratio stability ranging from 1:2 to 1:4.

Use of lanthanide treated bovine serum in the culture technology resulted in the signs of stabilization of the continuous cell line proliferation. Long-term serial cell culture passaging was performed with 72–96 hours cycle and without cryo stage. The activities aimed at the adjustment of the lanthanide treated bovine serum properties was carried out in parallel. Using the tested cell culture, information was obtained about the serum toxicity, its adhesive properties and growth activity. This allowed the serum manufacturers to eliminate latent viruses and endotoxins thus significantly improving the quality of the formed YaDK-04 monolayer and increasing the cell performance [18, 21, 22].

The long-term serial passaging of YaDK-04 without cryo stage was carried out under standard conditions with 10% of the lanthanide treated bovine serum. By passage 44, the cell morphology began to change: the epithelial-like cells prevailed and they became dense by the end of the logarithmic growth phase (Fig. 4). During passages 44 and 55, the karyological examination was carried out, whose results demonstrated significant rearrangements in the karyotype (Fig. 5, 6). Populations with a hyperploid set of chromosomes and 2–4 metacentric elements predominated (Fig. 6, 7). The split ratio increased to 1:6 or higher and maintained by passage 70.

Cultivation for over 50 passages without cryo stage, split ratio increase, morphological and karyological changes indicated production of a new stable lamb testicle subline, which was named TCh (*Testis Capra hircus*). This CCL consisted of predominant epithelial-like cells; spindle-shaped and fibroblast-like cells in smaller numbers concentrated on the substrate amongst the main population.

After the obtained subline was subjected to over 70 serial passages (within 18 months), no signs of degeneration were observed and the CCL was characterized by stable cultural parameters.

Long-term cultivation with lanthanide treated blood serum resulted in a significant transformation of the karyotype. For karyological analysis of the TCh cell subline, chromosomal preparations were prepared using P. S. Moorhead method [13], 100 metaphase spreads

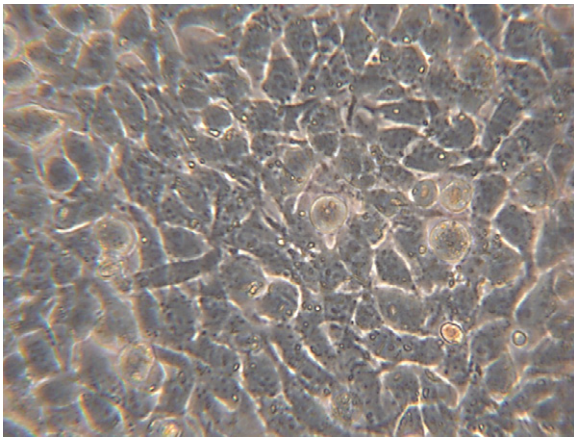

Fig. 4. Morphology of TCh CCL (passage 70) 48 hours

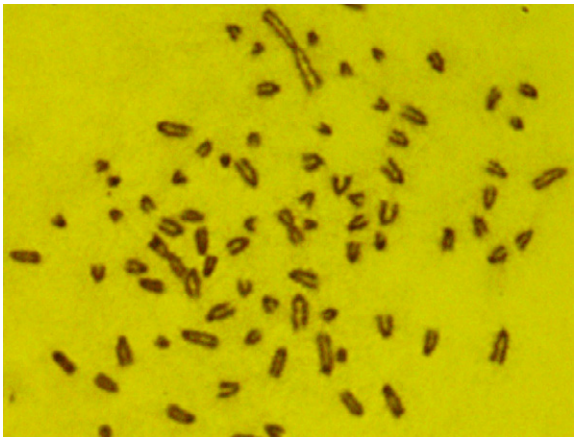

Fig. 6. TCh-2 CCL metaphase spread (passage 70)

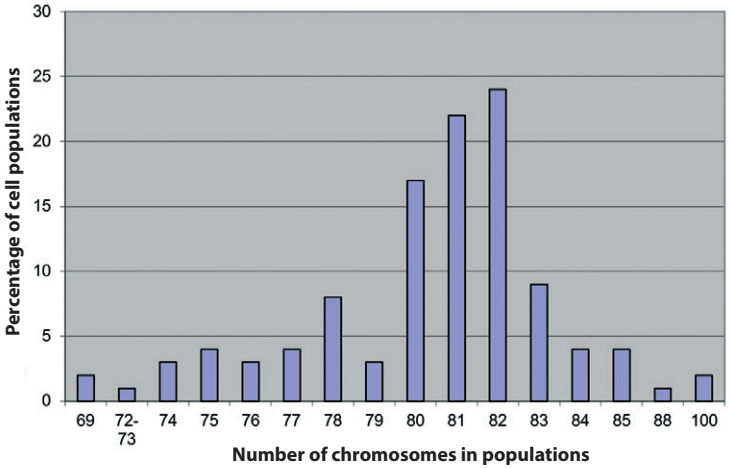

Fig. 5. TCh CCL karyogram (passage 70)

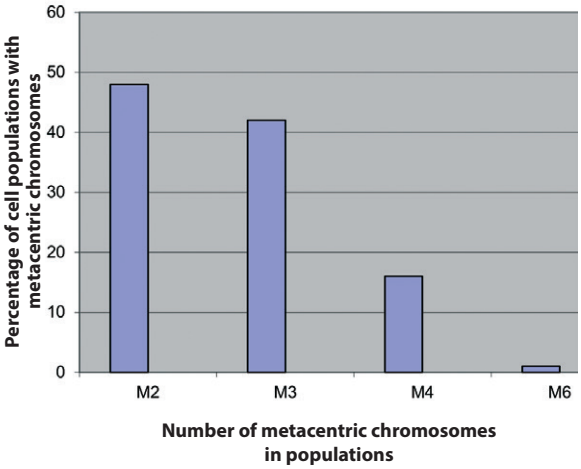

Fig. 7. Percentage of cell populations with different number of metacentric chromosomes in TCh CCL

were photographed, the chromosomes were counted and a karyogram was compiled. According to the analysis results, it was found that the TCh cell subline formed a karyologically heterogeneous population of cells, mainly hyperploid ones. The modal class of the cells in 50 serial passages was 117 chromosomes – 14%, near-tetraploid population – 70%, near-diploid population – 29%. Karyotype variability was 57–122 chromosomes. During passage 66, a new population with predominant cells of the modal class of 82 chromosomes appeared – 24%. The subline stabilized at this karyological level. This population had unlimited potential for passaging without a significant karyotype changes.

By means of karyological analysis, modal class indication and appearance of marker chromosomes, the culture was identified as lamb testicle cells (*Capra hircus* L.).

Characteristics of continuous TCh cell line. The proliferative activity of the resulting cell population was high, which indicates the suitability of the subline for cultivation on an industrial scale. Biotechnological properties of the CCL differed significantly from similar properties of the original cell lines. Possibility of cultivation in roller bottles made it possible to significantly increase the efficiency of the manufacture of the specific antiviral drugs. The potential of the culture turned out to be such that the population recovered from the "foam" to the complete monolayer in case any cells remained after the primary cell subcultivation.

In addition to hyperploidy (modal class – 82 chromosomes), a reliable indicator of the new cell population included the emergence of 2 to 4 metacentric chromosomes (Fig. 6, 7).

The selection with lanthanide treated bovine serum resulted in CCL transformation, and the morphological status of TCh subline cells differed significantly from YaDK-04 and CG-91. The permanent morphology of the TCh CCL was observed during 40 passages of continuous cultivation without cryo stage.

When the TCh CCL was used as a control during the studies of the cytopathic effect (CPE) of various viruses without changing the medium, morphological changes along with the signs of cell and monolayer aging were reported: cytoplasm granulation increased, the intercellular space thickened, vacuoles appeared, small amounts of cellular detritus were localized on the monolayer. All these trophic changes differed from the specific cell degeneration caused by exposure to viruses.

Methods and conditions of TCh cell subline cultivation. TCh monolayer is cultivated in the nutrient medium in two ways: on stationary horizontal surfaces (in culture flasks) and in roller bottles.

Monolayer cultivation in stationary conditions at (37 ± 0.5) °C is the most popular method for the manufacture of culture vaccines and virological studies. TCh cell line is grown in culture flasks with a growth area of 300, 175, 75, 25 cm^2. If necessary, the cell line is used in a microneutralization tests in the plates of various size.

Serial passaging starts with defrosting of the 5 cm^3 ampoule containing cells at the concentration of 5–7 million/mL. In case of the produced TCh subline, there is no need to change the medium every 24 hours. After 72 hours, the cell culture forms a complete monolayer, which can be subcultivated at 1:6. For serial passaging, the semisynthetic nutrient medium + 199 (or DMEM/F-12) is used at 1:3 and 10% of the lanthanide treated bovine serum.

Table 1
Biotechnological specifications of TCh CCL

Growth area of the culture flask, cm^2	Surface type	Split ratio	Monolayer formation, hours	Monolayer characteristics
300	Glass, plastic	1:4	48	Dense, with cell layering
300	Glass, plastic	1:6	72	Dense, with cell layering
300	Glass, plastic	1:8	72–96	Dense, with cell layering
850	Glass, plastic	1:6.6	72	Dense
850	Glass, plastic	1:8.5	72	Dense
1,700	Corrugated plastic	1:12	72–96	Dense

During the serial passaging with the cycle of 72–96 hours, a dense monolayer is formed with partial layering with epithelial-like and spherical cells.

Active proliferation of TCh CCL occurred at pH ranging from 7.3 to 6.8. Acidification of the growth nutrient medium below 6.8 is a sign of the depletion of the stock of ingredients and the need for reseeding or changing the medium.

Roller cultivation of the TCh subline cells is carried out in stationary conditions. Using the same conditions and components, the cells are transferred from the stationary flasks with the growth area of 300 cm^2 into roller bottles with the growth surface of 850 and 1,700 cm^2. The split ratio in this case can reach 1:6.6–1:12 (Table 1). The optimal split ratio for the complete and stable monolayer is 1:6. The culture grown by the roller method is especially in demand for PPRV cultivation.

Reproduction of animal viruses on TCh CCL. The main advantage of the new TCh cell line production involved stability of its cultivation. The TCh cell line began to be actively used for large-scale cultivation of the substrate for the manufacture of vaccine products against such diseases as LSD, sheep and goat pox and PPR. Each of the agents of the above-mentioned infectious animal diseases made its own specific CPE in the TCh cell culture.

During lumpy skin disease virus (LSDV) reproduction in the TCh cell culture, detachment and degeneration of a part of the cell monolayer occurred in the terminal stage. The degenerated cells assembled into aggregates, but part of the monolayer remained on the substrate. There were also spherical, not completely destroyed cells in the suspension (Fig. 8).

The cytopathic effect of the sheep pox virus differed from the CPE of the LSDV and it was manifested by the detachment of the major part of the spherical cells that assembled into aggregates. Degenerative changes were observed in the cells remaining on the substrate: they became spindle-shaped and vacuolated (Fig. 9).

The PPRV totally affected the TCh CCL that was manifested by the degeneration of the individual cells and the whole monolayer to an amorphous state (Fig. 10). In most cases, the monolayer destruction reached 100%.

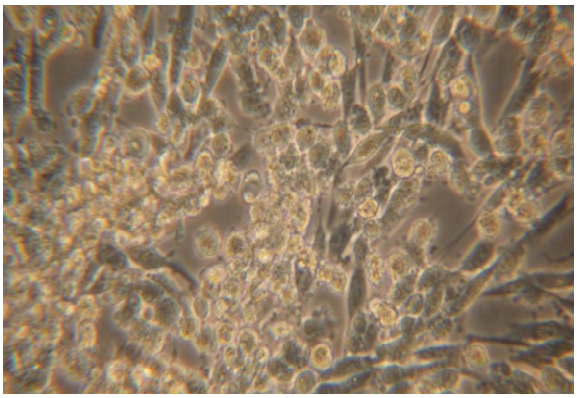

Fig. 8. TCh CCL monolayer cells post infection with LSDV

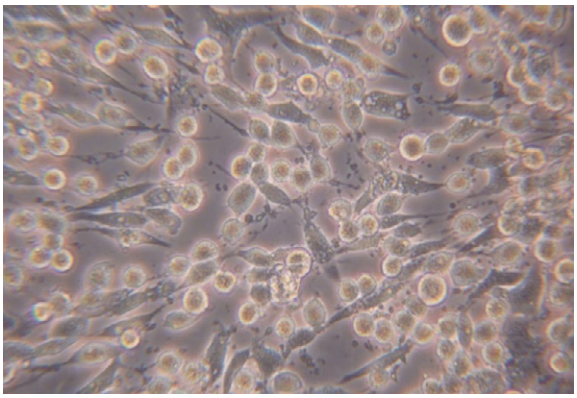

Fig. 9. TCh CCL monolayer cells post infection with sheep pox virus

Comparative analysis of LSDV reproduction in various cell cultures. The following cell lines were used in the comparative analysis: TCh, FBN (continuous fetal bovine nasal septum cell culture), MDBK (continuous bovine kidney cell culture), Taurus-2 (continuous calf kidney cell culture), SIRC (continuous rabbit corneal cell line), PO (continuous sheep kidney cell culture), PT (calf kidney cell subculture) and TYa (lamb testicle cell subculture).

The infectivity of the obtained viral material was determined by titration in 96-well culture plates using YaDK-04 cell suspension. Titration results were recorded according to the virus cytopathic effect during 96–120 hours. The virus titer was calculated by Reed & Muench method and expressed in lg $TCID_{50}/cm^3$.

The data in Table 2 demonstrate that the maximum accumulation of the LSDV was recorded at the level of passage 7 in the homologous continuous TCh cell culture (5.48 ± 0.16 lg $TCID_{50}/cm^3$) and TYa cell subculture (5.17 ± 0.15 lg $TCID_{50}/cm^3$). In FBN and SIRC cell cultures, the infectivity titer amounted to 4.00 ± 0.16 and 4.00 ± 0.12 lg $TCID_{50}/cm^3$, respectively. MDBK, Taurus-2 cell cultures, PO and PT turned out to be insensitive to the LSDV [23, 24].

In order to optimize the parameters of the LSDV cultivation in TCh and TYa cell cultures, the effect of cultivation period on the virus reproduction was studied.

The data in Table 3 demonstrate that the level of LSDV accumulation in TYa and TCh cell cultures after 72 and 96 hours of cultivation did not differ significantly and ranged from 5.00 ± 0.00 to 5.08 ± 0.18 and from 5.25 ± 0.17 to 5.33 ± 0.14 lg $TCID_{50}/cm^3$, respectively, but significantly exceeded the values recorded after 24,

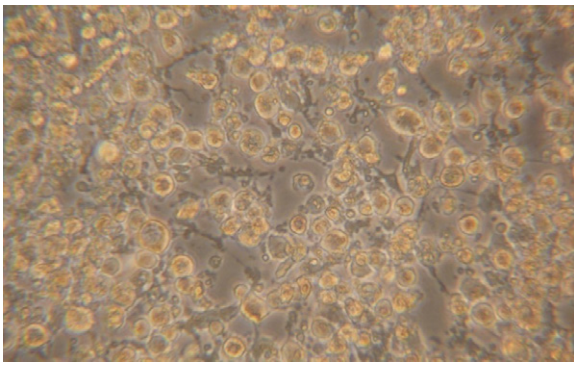

Fig. 10. TCh CCL monolayer cells post infection with PPRV

Table 2
LSDV replication in various cell cultures ($n = 3$)

Cell culture	Virus infectivity, lg $TCID_{50}/cm^3$						
	Number of passages						
	1	2	3	4	5	6	7
TCh	3.33 ± 0.00	4.16 ± 0.16	4.25 ± 0.18	5.25 ± 0.17	5.30 ± 0.14	5.47 ± 0.16	5.48 ± 0.16
TYa	3.02 ± 0.14	4.20 ± 0.16	4.17 ± 0.25	4.50 ± 0.15	5.17 ± 0.14	5.14 ± 0.15	5.17 ± 0.15
FBN	3.01 ± 0.12	3.35 ± 0.15	3.30 ± 0.16	4.17 ± 0.15	4.25 ± 0.16	4.12 ± 0.18	4.00 ± 0.16
SIRC	3.01 ± 0.14	3.15 ± 0.17	3.25 ± 0.12	4.01 ± 0.12	4.15 ± 0.13	4.00 ± 0.15	4.00 ± 0.12
PT	3.02 ± 0.12	2.20 ± 0.25	2.15 ± 0.17	1.35 ± 0.17	n/d	n/d	n/d
MDBK	1.25 ± 0.16	1.15 ± 0.13	n/d	n/d	n/d	n/d	n/d
Taurus-2	1.27 ± 0.13	1.12 ± 0.14	n/d	n/d	n/d	n/d	n/d
PO	3.05 ± 0.15	2.17 ± 0.15	1.35 ± 0.17	n/d	n/d	n/d	n/d

n/d – not detected.

48 and 120 hours of cultivation. The data obtained show that the optimal period of the virus cultivation should be taken as 72–96 hours.

Thus, it was experimentally proved that, despite the fact that the degree of the LSDV accumulation in the TCh cell culture does not statistically differ from that in the TYa subculture, the TCh is a more promising cultivation system for industrial purposes, since this cell line is the most stable and combines such properties as high proliferative activity, sensitivity to LSDV and homology of its origin.

Comparative analysis of sheep pox virus reproduction in various cell cultures. The following cell lines were used in the comparative analysis: TCh, PO, PS (continuous saiga kidney cell culture), PB (ram kidney subculture) and TB (ram testicle subculture) [22, 24, 25, 26]. Three serial passages were performed.

The infectivity of the obtained viral material was determined by titration in 96-well culture plates using YaDK-04 cell suspension. Titration results were recorded according to the cytopathic effect of the virus during 72–120 hours. The virus titer was calculated by Reed & Muench method and expressed in lg $TCID_{50}/cm^3$.

The data in Table 4 demonstrate that the maximum accumulation of the sheep pox virus was observed in TCh, PB and TB cell cultures. By passage 3 the virus titer amounted to 5.50 ± 0.18, 5.50 ± 0.25 and 5.50 ± 0.25 lg $TCID_{50}/cm^3$, respectively. In PO and PS cell cultures, the level of the virus infectivity was low and by passage 3 it amounted to 3.25 ± 0.12 and 3.25 ± 0.25 lg $TCID_{50}/cm^3$, respectively.

Thus, the resulted data indicate that the combination of high proliferative activity, sensitivity to the virus and homology of the origin makes TCh CCL indispensable for the manufacture of the products for sheep pox specific prevention.

Comparative analysis of the PPRV reproduction in various cell cultures The following cell lines were used in the screening process: TCh, PO, PS, TK (goat testicle subculture) and SPEV (continuous pig embryo kidney cell culture). Five serial passages were performed for each cell culture.

The virus was harvested at 80–90% destruction of the cell monolayer. The virus-containing material of each passage was titrated in penicillin vials in YaDK-04 cell culture by the serial 10-fold dilutions. The virus titer was calculated by Ashmarin modified Kärber method and expressed in lg $TCID_{50}/cm^3$.

Already at the first passages, the virus CPE was reported for all cell cultures, and it was most clearly expressed in the TCh cell line (Fig. 10). Cytopathic manifestations of the PPRV in the TCh cell culture consisted in the fact that almost all cells were deadhered on day 3 of cultivation, their membranes and cytoplasm lost their native structure, and partial aggregation of cells occurred.

It was found that at all passage levels the highest virus accumulation was observed in the TCh cell line (virus activity ranged from 5.25 ± 0.00 to 5.33 ± 0.18 lg $TCID_{50}/cm^3$). The virus titer in TK and SPEV cell cultures was significantly lower and amounted to 3.50 ± 0.00 and 4.33 ± 0.18 lg $TCID_{50}/cm^3$ by passage 5, respectively. At passage 1, the virus infectivity was quite high in PO and PS cell cultures (4.58 ± 0.14 and 5.00 ± 0.18 lg $TCID_{50}/cm^3$, respectively), but later a stable and consistent decrease in the level of the virus accumulation was observed. So,

Table 3
Effect of LSDV cultivation period in various lamb testis cell cultures and TCh ($n = 3$)

Cultivation period, hours	Virus titer, lg $TCID_{50}/cm^3$	
	TYa cell culture	TCh cell culture
24	2.66 ± 0.14	2.83 ± 0.22
48	3.78 ± 0.00	3.75 ± 0.17
72	5.08 ± 0.18	5.25 ± 0.17
96	5.00 ± 0.00	5.33 ± 0.14
120	4.75 ± 0.08	5.00 ± 0.00

Table 4
Sheep pox virus accumulation in various cell cultures ($n = 3$)

Cell culture	Virus infectivity, lg $TCID_{50}/cm^3$		
	Number of passages		
	1	2	3
TCh	5.00 ± 0.25	5.48 ± 0.16	5.50 ± 0.18
PO	4.20 ± 0.16	4.50 ± 0.25	3.25 ± 0.12
PS	2.50 ± 0.25	3.08 ± 0.18	3.25 ± 0.25
PB	5.00 ± 0.25	5.14 ± 0.15	5.50 ± 0.25
TB	5.00 ± 0.25	5.17 ± 0.14	5.50 ± 0.25

Table 5
Dynamics of PPRV accumulation in various cell cultures ($n = 3$)

Cell culture	Virus titer, lg $TCID_{50}/cm^3$	
	Passage 1	Passage 5
TCh	5.25 ± 0.00	5.33 ± 0.18
TK	4.20 ± 0.16	3.50 ± 0.00
SPEV	4.15 ± 0.13	4.33 ± 0.18
PO	4.58 ± 0.14	2.08 ± 0.14
PS	5.00 ± 0.18	3.33 ± 0.18

by passage 5, the virus titer in PO cell culture amounted to 2.08 ± 0.14 lg $TCID_{50}/cm^3$, in PS cell culture – to 3.33 ± 0.18 lg $TCID_{50}/cm^3$ (Table 5).

Thus, the study results indicate that the optimal cell line for the PPRV reproduction is the TCh cell culture [12]. The virus titer obtained in this culture was consistently high during five serial passages and ranged from 5.25 ± 0.00 to 5.33 ± 0.18 lg $TCID_{50}/cm^3$, thus indicating the possibility of using this cell culture for the production of the viral raw material for large-scale manufacture of the vaccines based on the tested virus.

CONCLUSION

Long-term use of lanthanide treated bovine blood serum during cultivation of YaDK-04 CCL resulted in the formation of a new TCh cell line, which significantly differed in cytomorphological and karyological features from the original one. We assume that lanthanides, bearing high electrical charge cations, affect

the formation of chromosomal variability and instability of the nucleosomes, especially at the distal ends of acrocentric chromosomes. Two processes occur: emergence and stable reduplication of the hyperploid cell population, as well as accumulation of acrocentrics along with the formation of stable metacentric chromosomes. These rearrangements are correlated with the increase in the proliferative activity of the line and stability of cultivation during long-term passaging.

An important result of the production of the new cell line is the fact that sensitivity to dermatotropic and other viruses did not change. And high productivity of the cell populations enabled cost-effective production of culture vaccines.

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Effect of mesenchymal stem cells on animal semen during storage

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ABSTRACT

Mesenchymal stem cells (MSCs) have been known to mankind since the mid-20th century. The comprehensive study revealed their high biologically active potential. Capacity of forming several types of body tissues was demonstrated. The stem cells, like any other cells, exert their effect on surrounding cells and tissues by secreting extracellular vesicles. The extracellular vesicles of the stem cells possess biological activity of parent cells. Taking into account the regenerative potential of the mesenchymal stem cells, they are currently used in medicine, and also in veterinary medicine for treatment of various injuries of the companion animals. Effect of the mesenchymal stem cells on boar and rat sperm cells during 12-hour storage was studied. The study results demonstrated that during 12 hours of coincubation, the porcine MSCs contributed to the survival of the boar sperm cells and maintenance of their motility at 60–80% (depending on the solvent) as compared to the controls. Such a significant effect was not however observed during coincubation of the rat sperm cells with rat MSCs. But it should be noted that before the 3rd hour of coincubation, the experimental sperm motility was higher than that of the control. By hour 5 of the observation, this difference was leveled. The rat and boar sperm cells are likely to have different physiological characteristics, which were reflected in the results obtained. Therefore, possibility of using the MSCs for the storage and cryopreservation of the semen of some animals was demonstrated, but this requires further research.

Keywords: mesenchymal stem cells (MSCs), pig, boar, rat, sperm cells

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Влияние мезенхимных стволовых клеток на сперму животных при хранении

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РЕЗЮМЕ

Мезенхимные стволовые клетки известны человечеству с середины XX в. В ходе проведенного всестороннего изучения выявлен их высокий биологически активный потенциал. Показана возможность образовывать несколько видов тканей организма. Стволовые клетки, как и любые другие, оказывают свое воздействие на окружающие клетки и ткани путем выделения внеклеточных везикул. Внеклеточные везикулы стволовых клеток обладают биологической активностью родительских клеток. Принимая во внимание регенеративный потенциал мезенхимных стволовых клеток, в настоящее время их применяют в медицине, а также в ветеринарии для лечения различных травм животных-компаньонов. Были проведены исследования по изучению влияния мезенхимных стволовых клеток на сперматозоиды хряка и крысы при хранении в течение 12 ч. Согласно полученным результатам, мезенхимные стволовые клетки свиньи способствуют выживанию сперматозоидов хряка и сохранению их подвижности на 60–80% (в зависимости от растворителя) по сравнению с контролем в течение 12 ч соинкубирования. Однако такого значительного эффекта не наблюдали при соинкубировании сперматозоидов крыс с мезенхимными стволовыми клетками крысы. Но необходимо отметить, что до 3-го ч соинкубирования подвижность сперматозоидов в опыте была выше, чем в контроле. К 5-му ч наблюдения эта разница нивелировалась. Вероятно, сперматозоиды крысы и хряка имеют различные физиологические особенности, которые отразились на полученных результатах. Таким образом, показана возможность использования мезенхимных стволовых клеток для хранения и, возможно, криоконсервации спермы некоторых животных, но для этого требуется проведение дальнейших исследований.

Ключевые слова: мезенхимные стволовые клетки, свинья, хряк, крыса, сперматозоиды

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INTRODUCTION

Among the various practical problems of animal husbandry in agriculture, one of the most urgent ones involves the increase of the efficiency of herd reproduction by maximizing the use of high-value breeders. This is of great importance for ensuring further progress in animal husbandry. An important area for improving reproduction is the use of artificial insemination. Artificial insemination is a highly effective method of improving the pedigree and performance properties of animals by using high-value breeders. One of the determining factors of artificial insemination effectiveness is the improvement of methods for storing animal semen in a chilled or deep-frozen state. However, when the semen is being technologically processed, diluted with synthetic media and stored chilled or deep-frozen, the spermatozoa are significantly damaged structurally and physiologically. This results in the failure of the plasma membranes' permeability and release of a number of enzymes and other cell metabolic components from the spermatozoa thus leading to the considerable loss of the semen fertility [1]. The causes for the decrease in the fertility of chilled and frozen boar semen have not yet been definitively clarified. One of them is activation of lipid peroxidation processes. When the spermatozoa are cool-stored, superoxide radicals being the precursors of highly active oxygen radicals can accumulate in them. Their formation leads to the damage of the sperm DNA, proteins, lipids of the cytoplasmic membrane, etc. In this regard, researches are being conducted to improve the protective properties of synthetic diluents intended for semen of a specific animal species by introducing various natural or synthetic antioxidants, as well as other biologically active substances into their composition [2].

Development of technologies for *in vitro* storage of the semen opens up new opportunities for its use in animal reproductive medicine. This is especially relevant for artificial insemination in pig farming [3]. The use of this method allows to collect the semen sample at any convenient time in advance, as well as, if necessary, to carry out safe transportation. The main goal in the development of the semen storage technologies is to increase the duration of storage without significant loss of fertility [4].

The preservation of the rat semen is also of great importance in biology. Laboratory rats have been used in biomedical research for over 170 years. For many reasons, rats are preferred over mice in studies of physiology, neurobiology, pharmacology and behavior. The rat is the main animal model for the pharmaceutical industry due to its similarities with the humans in terms of drug binding and toxicological profiles, and almost every new medicinal product is tested in these animals [5]. This is due to the fact that rats and humans have similar genomic regions, so that breast cancer in rats, for example, is sensitive to hormones and has a greater similarity to human's in the disease stages as compared to the one in mice. Assisted reproductive technologies and genome-editing technology have facilitated the creation of the genetically modified rats worldwide. Genome-editing technology has increased the value of laboratory rats as important models for human diseases and medicinal product development in physiology and toxicology. However, not all newly bred rat breeds can reproduce naturally. Such animals are often bred through *in vitro* fertilization. Therefore, the long-term *in vitro* preservation of the rat sperm vitality is relevant [6].

Stem cells and their derivatives can act as biologically active substances that protect spermatozoa from damage. Discovery of stem cells and study of their properties expanded not only knowledge in cell biology, but also the possibilities of medicine [7, 8]. It has been shown that mesenchymal stem cells (MSCs) of an adult organism are not carcinogenic, non-immunogenic and can be used to stimulate regeneration of damaged tissues. The simplest and least traumatic way to obtain MSCs is to derive them from the adipose tissue [9, 10]. MSCs mediate their therapeutic effect by releasing biologically active molecules into the environment that are "packed" into microvesicles (MVs). The MVs, in their turn, are membrane vesicles, whose attachment or internalization to target cells results in a wide range of their epigenetic and phenotypic changes. Such regulation of physiological and pathological processes demonstrates promising non-cellular therapeutic possibilities of the MVs. Intercellular communication through vesicle secretion is currently considered a common phenomenon in all mammalian cells [11, 12].

A number of studies demonstrated that MSCs of all animals have similar biological activity, as a result of which they have found their application in veterinary medicine [13, 14, 15]. Currently, MSCs are successfully used to treat various pathological conditions in animals [16, 17].

Studies of the possible use of MSCs and their derivatives in reproductive veterinary technologies were started relatively recently. The studies have found that supplementation of the cryomedia with adipose tissue-derived MSCs increases the percentage of vital and motile canine spermatozoa after cryopreservation as compared with freezing without MSCs [18]. According to the published data,

a number of researchers have obtained good results when adding air-conditioned medium from MSCs of aminotic origin to the cryomedium. Herewith, they demonstrated that this supplement enhanced sperm motility and vitality, membrane integrity and mitochondrial activity of canine spermatozoa after defrosting. This effect was most likely mediated by natural MVs contained in large amount in the conditioned media during cell cultivation [19].

In view of the above, the effect of adipose tissue-derived MSCs on animal spermatozoa was studied. In this paper we present the results of storing boar and rat semen with the MSCs of these animals.

MATERIALS AND METHODS

Production of adipose tissue-derived MSC culture and its analysis. In rats (n = 3), subcutaneous adipose tissue was collected after decapitation in order to avoid the effect of an anesthetic on the course and results of the experiment. The incision site on the animal's abdomen was shaved, the skin was treated with alcohol. The skin incision was performed with sterile surgical instruments along the midline of the abdomen. Then subcutaneous fat was collected into a sterile container with saline solution (PanEco, Russia) supplemented with 1% of penicillin-streptomycin (PanEco, Russia).

A fragment of the subcutaneous adipose tissue was collected from pigs (n = 3) into a sterile container under general anesthesia in the veterinary operating room.

The adipose tissue samples were transported at max 15 °C for 2–4 hours after their collection. The cells were isolated in sterile conditions of the cell laboratory according to the previously described standard procedure [20]. The stem cell-containing stromal vascular fraction was isolated as follows: the adipose tissue was mechanically crushed with sterile scissors into fragments of about 1 mm^3 and incubated in the crab collagenase solution (BioloT, Russia) at a final concentration of 0.2% for 1 hour at 37 °C while rocking on the shaker. The resulted cell suspension was precipitated by centrifugation at 1,500 rpm for 10 minutes. The supernatant was removed. Then the deposited cells were washed: the precipitate was resuspended three times in 0.9% NaCl solution (PanEco, Russia) and precipitated by centrifugation at 1,500 rpm for 10 minutes. The obtained cells were cultured in α-MEM medium (PanEco, Russia) containing 10% blood fetal bovine serum – FBS (PanEco, Russia), 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (PanEco, Russia). After 24 hours, the nutrient medium was replaced with a fresh growth medium. At the same time, cells that were not attached to the plastic culture flask were removed. The growth medium was subsequently changed once every 3 days. As soon as the monolayer reached 80% density, the MSCs were reseeded. The cells were reseeded by trypsinization using 0.25% trypsin-EDTA solution (PanEco, Russia). Cells of passages 4–5 were further used. This is due to the fact that the MSCs of passage 3 are already evaluated as homogeneous and stable populations, and the cells of passage 8 are aging cells beginning to show signs of genetic instability and decrease in differentiation potential.

To perform flow cytofluorometry, the MSCs were removed from the plastic culture flask by trypsinization. They were further washed with Dulbecco sodium phosphate buffer solution – DPBS (PanEco, Russia) by centrifugation for 5 minutes at 1,500 rpm and fixed with 4% formalin solution for 20 minutes at room temperature. After that, they were washed with DPBS, resuspended and the aliquots were stained with antibodies (AT) according to the manufacturer's instructions: monoclonal PE/Cyanine7 anti-mouse Thy-1.1 AT (Biolegend, USA); monoclonal PE anti-mouse/rat CD29 AT (Biolegend, USA); monoclonal PerCP/Cyanine5.5 anti-mouse CD73 AT (Biolegend, USA); CD34 Alexa Fluor 647 monoclonal mouse AT (Santa Cruz Biotechnology, USA). The cells were analyzed for the presence of the stem cell membrane markers using flow cytofluorimeter BD FACSAria™ III (BD Biosciences, USA). The flow cytometry result is expressed as a percentage of the total number of cells in the sample (at least 100 ths cells/aliquot).

MSCs differentiation. To study the differentiation capacities of the obtained cell cultures, the passage 4 cells were seeded on 12-well plates at 30 ths cells/well and incubated in the growth medium until the monolayer was formed. In order to induce the differentiation, the cell cultures were subsequently incubated with special media. Differentiation was carried out in three directions: osteogenic, adipogenic and chondrogenic. The differentiation results were recorded using an inverted AxioObserver Z1 microscope (Carl Zeiss, Germany).

For osteogenic differentiation, α-MEM medium was used, which was supplemented with 10% FBS, 100 nM dexamethasone (Sigma, USA), 0.5 µM ascorbic acid 2-phosphate (Sigma, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (PanEco, Russia). α-MEM medium supplemented with 10% FBS, 0.5 µM ascorbic acid 2-phosphate, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine was used as a control medium. For osteogenic differentiation, 0.2 µM of β-glycerophosphate solution (Sigma, USA) was added to the medium as well as to the control medium from day 10 of incubation. The media were changed every three days.

Von Kossa staining technique was used to determine mineralization, which is a sign of osteogenic differentiation. This reaction is based on the binding of silver ions to phosphate groups. The resulted compound undergoes photochemical degradation with the release of silver ions, staining the mineral deposits gray-brown. For this purpose, the nutrient medium was removed from the wells of the plate before staining. The cells were washed with 0.9% NaCl solution and fixed with 4% formalin solution for 30 minutes at room temperature. Then the wells were threefold thoroughly washed with a sufficient amount of distilled water and filled with a 2% silver nitrate and distilled water solution. The plates were incubated in the dark for 10 minutes. Then they were washed with distilled water and incubated in bright light for 1 hour.

To induce adipogenic differentiation, DMEM High glucose medium (PanEco, Russia) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 1 µM dexamethasone, 100 µM indomethacin (Sigma, USA), 500 µM 3-isobutyl-1-methylxanthine (IBMX, Sigma, USA) and 10 ug/mL insulin (Sigma, USA) was used. From day 10, the medium was replaced with a maintenance one free from dexamethasone, indomethacin and IBMX. At all stages DMEM High glucose supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine was used as a control medium. The media were changed every three days.

Qualitative staining of lipid inclusions with Sudan-3 stain (Sigma, USA) was used for adipogenic differentiation. For this purpose, the nutrient medium was removed from the cell culture and fixed with a 4% formalin solution for 30 minutes at room temperature. Before staining, the wells were threefold thoroughly washed with a sufficient amount of distilled water for 5 minutes.

When staining the cells with Sudan-3, their nuclei were additionally stained with hematoxylin and eosin. Sudan-3 stain was prepared by dissolving 0.02 g of powder in 10 mL of 70% ethanol. The mixture was incubated for 2 hours at 58 °C. The solution was subsequently filtered and the samples were stained at room temperature for 15–30 minutes. Then they were washed with 0.9% NaCl solution and stained with hematoxylin and eosin. Hematoxylin and eosin solutions were prepared and the cell cultures were stained according to the standard procedure.

For chondrogenic differentiation, 9×10^5 of passage 3–6 cells were taken and the same number was taken for control. They were washed from the nutrient medium and precipitated. MSCs for differentiation were resuspended with 90 µL of the chondrogenic differentiation medium, the same number of cells were resuspended in 90 µL of the control medium. The experimental and control suspensions were applied by drops at 10 µL for each of the 3 wells of a 24-well plate. MSCs were incubated at 37 °C for 2 hours for cell adhesion, after which 500 µL of the appropriate medium was added to each well. *α*-MEM supplemented with 10% FBS, 0.5 µM ascorbic acid 2-phosphate (Sigma, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine was used as a control medium. DMEM-High glucose medium supplemented with 10% FBS, 0.5 µM ascorbic acid 2-phosphate, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 1× ITS (Sigma, USA), 100 µM/L sodium pyruvate (PanEco, Russia), 1 µM dexamethasone (Sigma, USA), 0.5 µM ascorbic acid 2-phosphate (Sigma, USA), 10 ng/mL TGF-*β*1 (Sigma, USA) was used as a chondrogenic differentiation medium. The media were changed every three days.

For chondrogenic differentiation identification, the staining with Alcian Blue stain (Sigma, USA) was performed for the detection of acidic mucopolysaccharides being the marker of chondrogenation. For this purpose, the cell cultures were fixed in 95% ethanol and then in 70% ethanol for 5 minutes in each. After that, the cell cultures were threefold washed with distilled water for 30 seconds and stained with Alcian Blue solution for 1 hour. Then they were washed under running water for 2 minutes. Distilled water was poured over the samples and the samples were examined through the inverted visible light microscope. The Alcian Blue stain solution was prepared by dissolving 1 g of the powder in 100 mL of 0.1 M HCl.

Obtaining and analysis of animal sperm. The epididymides were derived from healthy male Wistar rats ($n = 5$) after decapitation, and from boars ($n = 3$) – immediately after slaughter. The epididymides were aseptically excised and transferred in a sterile Petri dish. The semen derived from one excised epididymis was washed with heated citrate buffer (Diaem, Russia), from the other – with DPBS in the ratio of 1:2. In case the semen was rich in spermatozoa, it was diluted 10-fold. All studies of the collected samples were carried out under the light microscope on slides heated to 37 °C. The samples meeting the standards were selected for further experiments [21].

To determine the content of pathological forms of spermatozoa, 0.05% eosin stain solution (PanEco, Russia) was added to a drop of semen on the slide in the ratio of 1:2. The semen and stain mixture was left for 3–5 minutes, after which three smears were made and visualized using the immersion microscopy; 100–200 cells were counted, identifying pathological forms and determining their percentage. The number of live (unstained) and dead (stained) spermatozoa was simultaneously calculated. Normally, the vital cells in the sample should amount to at least 58%. When assessing motility, the attention was paid to morphological defects such as droplets or curved tails. The number of sperm cells with abnormal morphology should not exceed 10%.

To determine the motility, the semen was diluted 20-fold with a heated citrate buffer/DPBS solution using semi-automatic dispensers. The number of forward-moving spermatozoa was calculated among 2 hundred counted spermatozoa, and percentage of progressive motility was determined. Ejaculates with the motility below 70% and high content of agglutinated cell groups were rejected. The calculation was performed in at least four different fields. All results were presented as a percentage of the total number of spermatozoa.

The integrity of the acrosomes in semen was determined by staining the samples with Coomassie G250 stain (Sigma, USA). The semen samples were placed on slides and incubated in a freshly prepared stain solution (0.22% Coomassie Blue G250, 50% methanol, 10% glacial acetic acid, 40% water) for 2 minutes. After that, the slides were thoroughly washed with distilled water to remove excess staining and examined under the light microscope.

The MSCs and spermatozoa were coincubated in CO_2 incubator at 37 °C for 12 hours in the ratio of 1 million rat/pig MSCs per 5 million rat/pig spermatozoa in 1 mL of citrate buffer/DPBS. The control semen samples were incubated at the concentration of 5 million germ cells per 1 mL of citrate buffer/DPBS without MSCs. The number of live and dead spermatozoa, their motility, as well as integrity of the acrosomes were determined in 3, 5 and 12 hours after the start of the experiment.

Compliance with ethical standards. The permission of the local Ethics Committee of Kazan Federal University (No. 1 of 23 February 2015 for research on the subject Genetic and cell therapy in regenerative veterinary medicine) was obtained to carry out procedures for the collection of adipose tissue.

Processing the results. Statistical processing of the obtained data was carried out by primary statistical analysis tools using Excel 2016 software. The results are presented as the arithmetic mean of the sample ± standard deviation. Secondary statistical data processing was performed using the nonparametric Mann – Whitney U-test. The differences were considered significant at a significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

The cells isolated from the animal adipose tissue adhered to the plastic culture flask and had a fibroblast-like morphology (Fig. 1).

The isolated cells expressed MSC markers: Thy-1, CD29, CD73 and did not express the hematopoietic stem cell marker CD34 (Table 1).

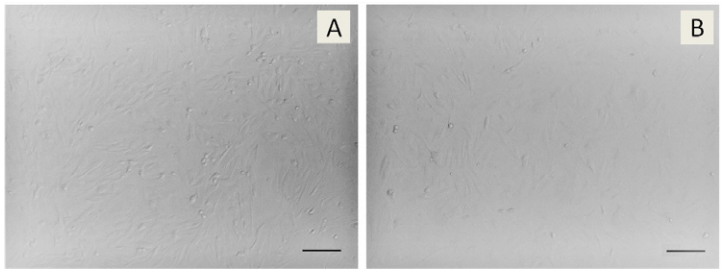

Fig. 1. Primary MSC culture: A – pigs; B – rats (100× magnification)

The results of differentiation of isolated rat and pig cells in 3 directions are shown in Figure 2.

Thus, the affiliation of isolated and cultured pig and rat cells to the MSC pool is confirmed by the typical morphology at *in vitro* growth, presence of specific MSC markers and lack of markers of other stem cells, as well as their differentiation in 3 directions.

At the next stage, 23 ± 1 and 160 ± 3 million spermatozoa/mL were obtained from rat and boar epididymides, respectively. Results of coincubation of rat/boar spermatozoa with rat/pig MSCs are shown in Figure 3.

Table 1
Number of animal MSCs ($n = 3$) in the isolated cell population demonstrating typical markers of MSC, %

Marker / Animal	Thy-1	CD29	CD73	CD34
Rat	95 ± 3	97 ± 1	95 ± 6	0
Pig	98 ± 2	94 ± 3	92 ± 4	0

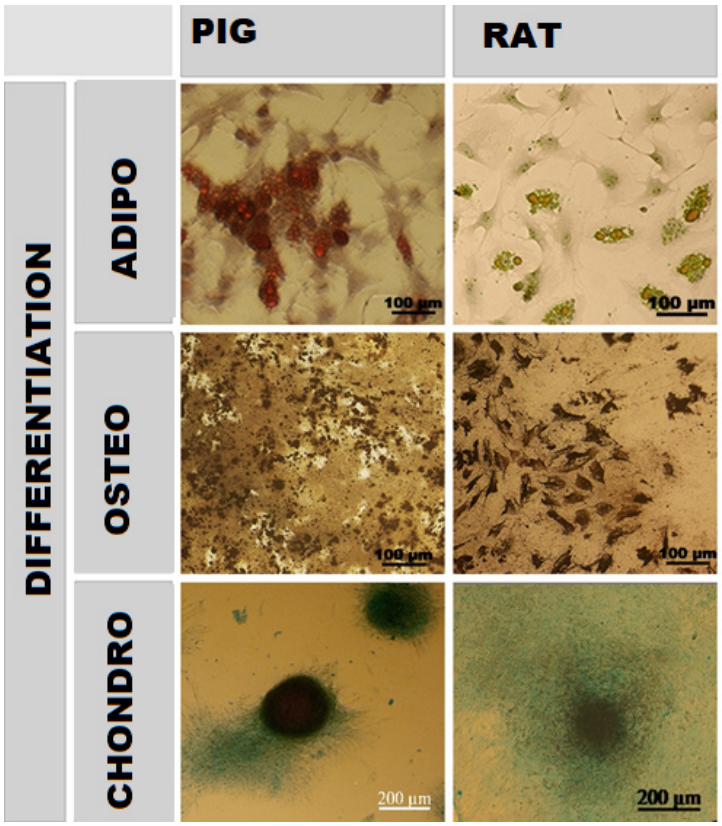

Fig. 2. Differentiation of rat and porcine MSCs of adipogenic origin (magnification is indicated in the pictures)

The vitality and progressive motility of spermatozoa are considered to be the key factors that can affect the rate of both artificial and natural insemination. The semen evaluation based on these parameters is still the gold standard for *in vitro* and *in vivo* fertility prediction [22]. According to the data obtained, the rat spermatozoa are more sensitive to storage in citrate buffer and DPBS as compared to boar spermatozoa. At the same time, they practically lose their motility after 3 hours of storage in DPBS and citrate buffer solutions, both with and without MSCs. Rat spermatozoa retained the same vitality for 5 hours, regardless of what solution they were in, while boar spermatozoa better retained their vitality and motility in solutions with MSCs. Herewith, the minimal loss of motility and the maximal number of live boar spermatozoa were recorded when stored in DPBS with MSCs as compared to citrate buffer with MSCs. But there is a significant positive effect of the presence of MSCs in the storage medium as compared to the solutions without MSCs: by hour 12 of the experiment, 18% of vital boar spermatozoa were in the citrate buffer, and when MSCs were added to the citrate buffer, the vitality amounted to 88%. When stored in DPBS, by hour 12, there were 6% of vital spermatozoa in the samples, and 88% in the medium with MSCs.

The data obtained on the change in the integrity of acrosomes throughout the whole experiment indicated that they remained practically intact in rat spermatozoa by hour 12 of incubation. Moreover, introduction of MSCs into the incubation medium had a positive effect on their integrity. In boars, breakdown of acrosomes occurred already by hour 3 of incubation in both control and experimental samples. Herewith, adding MSCs to the solution also had a protective effect on preserving the integrity of boar spermatozoa (Table 2).

It is known that in order to preserve *in vitro* motility and vitality of boar spermatozoa, the researchers recommend adding various substances to the diluent, for example, cresacin biostimulator. It has a positive effect on the motility of germ cells and belongs to organic chemical compounds [23]. However, addition of MSCs may have a positive effect not only on the spermatozoa themselves, but also on sows during insemination, since, according to the published data, the addition of the MSC conditioned medium to stallion semen samples not only did not worsen the semen parameters during 2 hours of incubation, but also mitigated early inflammatory endometrial reactions in mares during artificial insemination with these samples. The researchers associate the obtained effect with MVs extracted by the MSCs in the culture medium [24].

Our studies have shown absence of both pronounced positive and negative effects of MSCs on the rat semen as compared with the boar spermatozoa. This can be explained by the fact that, according to the published data [25], there are differences in the content of organic compounds, in particular proteins and their compounds, in the cell membrane of rat and boar spermatozoa (Table 3), as a result of which the germ cells of these animals react differently to signals from the outside.

For example, concentrations of total cholesterol of phospholipids, desmosterol, and phosphatidylethanolamine in the cytoplasmic membrane of rat and boar caudal spermatozoa are almost the same. But the content

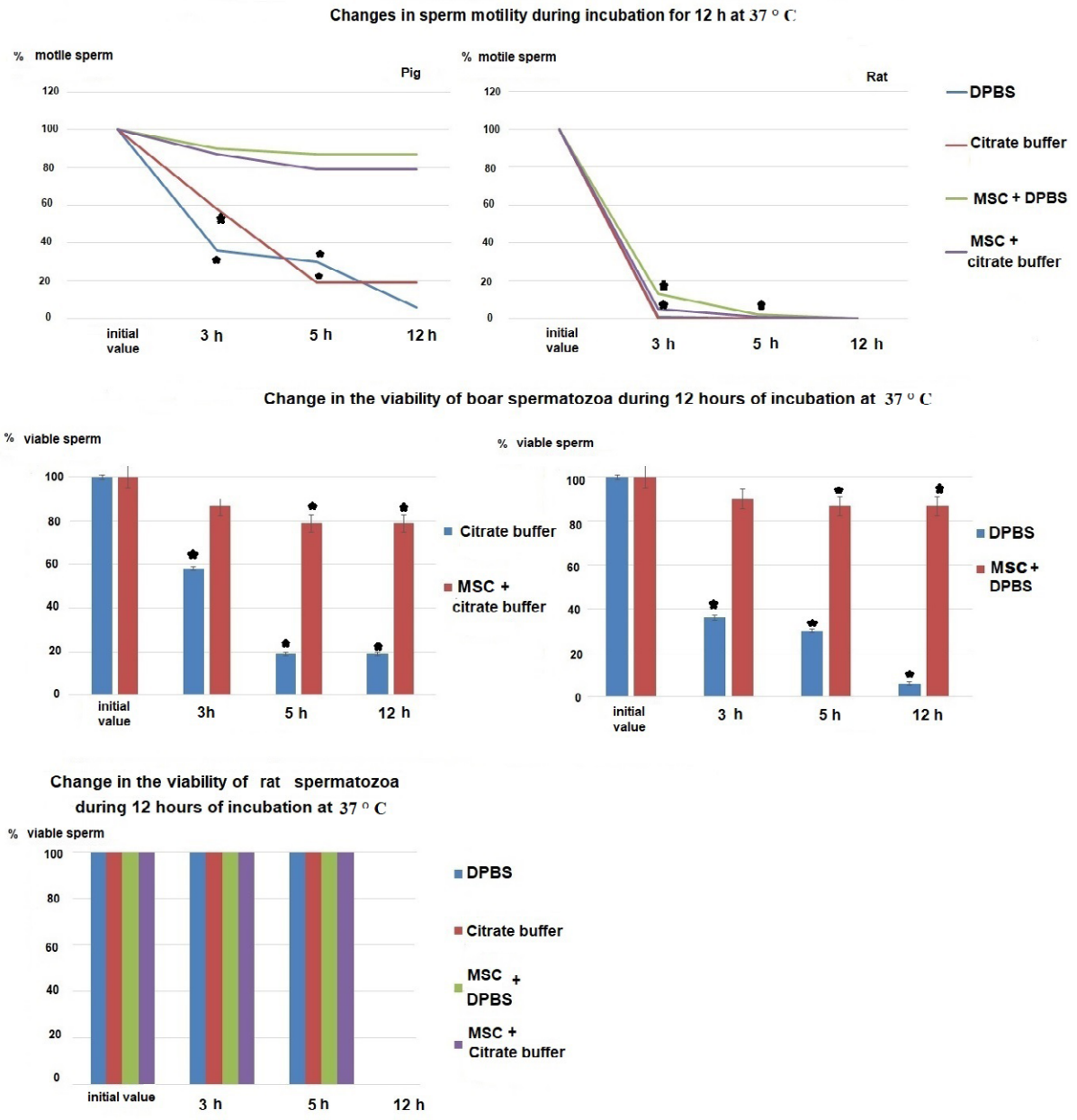

Fig. 3. Measuring vitality and motility of the boar and rat sperm cells (p ≤ 0.05 as compared to initial group value)*

of sphingomyelin and phosphatidylserine varies significantly. The amount of sphingomyelin in the cytoplasmic membrane of rat caudal spermatozoa is nearly two-times lower than in boar spermatozoa. It is well known that sphingomyelin has an effect on spermatozoa motility and integrity of their membranes. There is evidence of positive correlation between the content of this phospholipid in the cell membrane of human spermatozoa and their motility [26]. Rat spermatozoa contain less sphingomyelin as compared to boar spermatozoa. That is probably why they lose motility in the control sample already by hour 3 of incubation. But even under these conditions, rat spermatozoa retain greater motility with MSCs (Fig. 2) as compared to the control group. Different content of proteins responsible for the motion activity of spermatozoa in the membrane of boar and rat male germ cells, and, as a result, different performance, are most likely due to different length of the genital tract in females of these animals.

Phospholipid phosphatidylserine concentrations also differ significantly in boar and rat spermatozoa. It is in charge of the activity of spermatozoa. This phospholipid is normally located on the inner side of the cell membrane, and when transferred to the outer side as a result of various processes in the cell, it is an early marker of apoptosis [27]. In their article, P. Agrawal et al. [25] determined concentration of phosphatidylserine in the whole membrane of spermatozoa. Therefore, it is impractical to draw any conclusions from these data regarding the work carried out.

It is also known that rat spermatozoa are extremely sensitive to centrifugation, pipetting and cooling conditions due to their long tail, shape, head size and membrane composition [28]. Lack of any effect on rat spermatozoa during the experiment may be explained by the small number of MVs isolated from the MSCs added to the storage medium. Mokarizadeh A. et al. [29] in their studies demonstrated the data on cryopreservation of rat spermatozoa with MVs collected during the cultivation

Table 2
Changes in rat and boar acrosome integrity during the experiment, %

	Diluent		Original	3 hours	5 hours	12 hours
Rat	Citrate buffer	control	0	0	0	0.71
		adding MSCs	0	0	0	0.33*
	DPBS	control	0	0	0.31	2.00
		adding MSCs	0	0	0	0.33*
Boar	Citrate buffer	control	0	0.33	0.67	1.20
		adding MSCs	0	0.33	0.71	1.10
	DPBS	control	0	0.67	0.82	0.97
		adding MSCs	0	0.33*	0.51*	0.67*

* $p \leq 0.05$ as compared to the group control value at the given time point.

Table 3
Cytoplasmic membrane composition of rat and boar caudal sperm cells (according to P. Agrawal, 1988), μg

Substance		Rat	Boar
Phospholipid protein		0.63	1.47
Total cholesterol of phospholipids		0.18	0.17
Desmosterol		0.32	0.32
Phospholipids	phosphatidylserine	10	3
	phosphatidylethanolamine	31	28
	sphingomyelin	30	62
	diphosphate diglycerol	1	–
	other	8	7

of adipose tissue-derived MSCs. The researchers noted that addition of 25 μg of MVs based on total protein does not have any significant effect on spermatozoa as compared to the control without MVs. At the same time, cryopreservation of rat spermatozoa with 50 and 100 μg of MVs based on total protein significantly increases the number of vital and motile spermatozoa after defrosting. Unfortunately, the task of this work was not to isolate and determine the effect of MSCs derivatives on the state of rat spermatozoa, so we cannot reliably confirm this assumption.

CONCLUSION

In the last few decades, the majority of the research has been focused on the methods aimed to improve the efficiency of the semen storage/cryopreservation, which is considered one of the main problems in reproductive biotechnology. The applied approaches are based on the protection of spermatozoa from the harmful effects of storage and cryopreservation procedures, including use of various extenders, cryoprotectors, antioxidants and nutritional components. Moreover, some of these studies have focused on repair damaged sperm during the freeze-thaw process [30]. Repair of the spermatozoa damaged during storage is considered extremely important for improving their vitality and fertility [31]. It is known that stem cells excrete paracrine factors that enhance cellular protection and trigger anti-apoptotic and antioxidant mechanisms [32]. And stem cell derivatives, MVs, have effects similar to their parent cells on the surrounding tissues and cells [33]. The use of the stem cells and their derivatives (MVs) is likely to protect semen from the negative effects of storage/cryopreservation, such as oxidative stress, apoptosis, DNA damage and loss of mitochondrial activity. When analyzing the results obtained, it is impossible to unambiguously recommend the use of MSCs in the storage of spermatozoa of all animals. It is necessary to clarify the features of the interaction between animal spermatozoa and MSCs, mechanism of such effects and features of artificial insemination of animals with such semen. This will allow filling in serious gaps in knowledge and technologies for long-term storage of germ cells. This research area is of great importance for animal science and biotechnology as for preservation of germ cells of various species of rare and endangered animals. Along with scientific progress related to the discovery of sperm fertility markers, there is an urgent need to improve vitality and fertility of spermatozoa after storage (thawing) by improving storage methods (cryopreservation) in order to obtain high-performance farm animals to ensure global food security.

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Provision of veterinary services in livestock holdings in the Russian Federation

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ABSTRACT

Being the primary link in the disease notification system, specialists of the veterinary service in livestock holdings ensure continuous monitoring and control of animal health. This analysis includes assessment of the actual availability of veterinary services in the absolute majority of animal holdings in 85 Subjects of the Russian Federation. In total, the study covered 6,226,368 holdings for major livestock species, such as cattle and small ruminants, pigs, poultry, horses and fur animals. Small-scale holdings have been shown to account for the largest proportion (99.7%) of the total number of livestock farms, while the proportion of holdings where animal health control is daily organized has varied from 0.03% in fur farms to 3% in poultry farms. The significant role of animal owners in small-scale holdings within the implementation of epizootological surveillance has been determined. It was revealed that the main populations of pigs, poultry and fur animals are concentrated in large-scale livestock farms. The study results indicate a relatively favorable situation in pig and poultry holdings, where only single cases of lack of veterinary service were reported. On the contrary there are multiple cases of lack of veterinary care in the farms for rearing cattle, small ruminants, fur animals. The paper highlights the mechanisms for the implementation of tasks assigned to the state veterinary service in terms of the organization of planned preventive and diagnostic veterinary measures in large pig breeding establishments. Proposals were made to introduce a legally fixed obligation for animal owners in large-scale livestock holdings to establish and maintain the production veterinary service, as well as a proposal to establish divisions of the state veterinary service in large-scale livestock establishments.

Keywords: veterinary service, livestock holdings, animal health control, biosafety, epizootological surveillance

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Организация ветеринарного обслуживания животноводческих хозяйств в Российской Федерации

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РЕЗЮМЕ

Специалисты ветеринарной службы животноводческих хозяйств обеспечивают постоянное наблюдение и контроль за здоровьем животных, являясь первичным звеном в системе нотификации болезней. В рамках настоящей аналитической работы проведена оценка фактического наличия ветеринарного обслуживания в абсолютном большинстве хозяйств по содержанию животных 85 субъектов Российской Федерации. В общей сложности исследованием было охвачено 6 226 368 хозяйств по содержанию основных видов сельскохозяйственных животных, таких как крупный и мелкий рогатый скот, свиньи, птица, лошади, а также пушные звери. Показано, что мелкие хозяйства составляют основную долю (99,7%) от общего числа животноводческих хозяйств, при этом удельный вес хозяйств, где организован ежедневный ветеринарный контроль за здоровьем животных, варьирует от 0,03% в пушном звероводстве до 3% в птицеводческих хозяйствах. Определена важная роль владельцев животных мелких хозяйств в осуществлении эпизоотологического надзора. Выявили, что основная популяция свиней, птицы и пушных зверей сконцентрирована в животноводческих хозяйствах категории «крупные». Результаты исследования свидетельствуют об относительно благоприятной обстановке в хозяйствах по содержанию свиней и птицы, где зафиксированы лишь единичные случаи отсутствия ветеринарного обслуживания. В хозяйствах по содержанию крупного и мелкого рогатого скота, пушных животных, напротив, отмечаются множественные случаи отсутствия ветеринарного обслуживания. В работе освещается вопрос механизмов реализации возложенных на государственную ветеринарную службу задач, связанных с организацией плановых профилактических и диагностических ветеринарных мероприятий

на крупных свиноводческих предприятиях. Высказаны предложения о введении законодательно закрепленной обязанности для владельцев животных крупных животноводческих хозяйств создавать и содержать производственную ветеринарную службу, а также предложение о создании подразделений государственной ветеринарной службы на крупных животноводческих предприятиях.

Ключевые слова: ветеринарная служба, животноводческие хозяйства, ветеринарный контроль, биологическая безопасность, эпизоотологический надзор

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INTRODUCTION

It has become especially noticeable in recent decades that in the process of evolution of the Veterinary Service's objectives the emphasis has shifted to production of global public goods, that is, the formation of an environment favorable for human and animal life, economy and national development [1, 2].

The global political situation in recent years indicates strengthening of anti-Russian policy in some foreign countries. Particular concern is induced by the evidence of military biological activity of biolaboratories located in neighboring states, which does not exclude the possibility of natural and artificial animal infection outbreaks on the territory of our country as a result of intentional or unintentional actions of concerned parties.

In this regard, increased attention should be addressed to ensuring biological and food safety. In particular, the latter cannot be fully implemented without maintaining stable indicators of animal disease freedom and livestock productivity, which is extremely problematic without taking into account the data on the prevalence of infectious animal diseases (primarily highly dangerous and economically significant ones) in the Russian Federation, as well as neighbouring states and trading partner countries.

The implementation of measures for prevention and eradication of infectious animal diseases is the main task of the veterinary medicine in the Russian Federation [3]. At the same time, early detection and timely measures in response to infection outbreaks in susceptible animal population play the major role in disease prevention.

In this case, it is difficult to overestimate the role of livestock holding veterinary service (in-house veterinary service), whose specialists ensure constant animal health monitoring and control, being the primary link in the disease notification system.

The Veterinary Service controls the product manufacture, as the veterinary measures are inextricably linked with the livestock production, especially in commercial livestock facilities.

In this regard, the aim of this study was to investigate the peculiarities of animal health control measures to be implemented in livestock holdings of the Russian Federation.

MATERIALS AND METHODS

The theoretical basis of the study was the analysis of the regulatory and legislative framework governing the organization of livestock farms' veterinary services in the Russian Federation.

The practical basis for the analysis of the situation on the implementation of veterinary services in the livestock farms of the country was the official information posted by the executive authorities of the Subjects of the Russian Federation in the veterinary field in the FGIS "VetIS" (Assol.Express component) as part of the annual data collection by the Federal Service for Veterinary and Phytosanitary Surveillance on the functional status and activities of the authorized veterinary executive authorities of the Subjects of the Russian Federation and their subordinate institutions.

The assessment of the actual availability of qualified veterinary services in the absolute majority of livestock holdings in 85 Subjects of the Russian Federation was carried out. In total, the study covered 6,226,368 farms for keeping major livestock species, such as cattle and small ruminants, pigs, poultry, horses and fur animals.

For this study, farms with the following livestock population were classified as "small-scale": pig farms and farms for keeping up to 1,000 cattle, farms for keeping up to 500 cattle and horses, small-scale fur farms where the number of females in the main herd does not exceed 100–200 animals, and outdoor poultry holdings (poultry farms). Farms where the number of animals exceeded these values were categorized as "large-scale", and poultry farms were considered indoor establishments [4, 5, 6, 7].

The processing of quantitative (numerical) data was carried out using Microsoft Office Excel software.

Such methods of data analysis as generalization and formalization of information, comparative analysis, descriptive statistics, expert opinions were used in the study.

RESULTS AND DISCUSSION

Emergency situations, regardless of their nature, require an immediate professional response in order to reduce the socio-economic consequences. In relation to animal diseases, the veterinary service shall be able to detect and respond promptly to emerging epizootic

situations, and perform early detection, since any delays lead to the spread of the disease to vast areas, which makes infection control more difficult and expensive, and in certain cases almost impossible. The effectiveness of early detection of the disease directly depends on the ability of the veterinary service to conduct epizootological surveillance (ES). A number of domestic and foreign researchers and expert international organizations (WOAH, FAO, WHO) propose definitions of epizootological/epidemiological surveillance, which can be summarized in terms of essence and content as follows: ES is the continuous collection of zoosanitary (that is, related to animal health) information, its analysis and prompt communication to interested parties in order to take appropriate measures and ensure intervention during the epizootic process [8, 9, 10, 11, 12, 13].

Veterinary specialists of livestock farms are the primary and integral part of the ES, and primarily passive ES. The advantage of passive ES consists in the fact that it has a relatively high sensitivity with a relatively low specificity, since it does not require any targeted actions on the part of the veterinary service for a specific infectious disease, but is implemented by means of general constant monitoring of animal health by veterinary specialists directly in the susceptible animal population [14]. It is also implemented through the initiative and the obligation of animal owners to immediately notify veterinary specialists of all cases of sudden death or mass morbidity of animals and their unusual behavior [3].

The aim of passive ES is to identify all changes in animal health with their subsequent identification and differentiation. At the same time, the probability of detecting animals with health and behavior disorders (lethargy, anorexia, constrained postures, visible clinical signs) increases with intensification of this surveillance. The effectiveness of surveillance is directly dependent on the qualifications of the person carrying it out, and to a lesser extent it will be affected by an increase in the duration or frequency of observation of animals and the technical equipment of the specialist.

An analysis of the situation with veterinary services provided in farms in our country has shown that the absolute majority of farms under study belong to the category of "small-scale" holdings whose livestock population includes several animals (Fig. 1). It is such holdings (backyards, family-operated farms) that make up the main share (99.7%) of the total number of livestock farms.

Small-scale holdings. Due to the significant predominance of small-scale farms, it is quite problematic for veterinary specialists to organize daily animal health control and examination of animals in such sites. As a rule, a significant number of such farms have a low biosecurity level, which indicates the importance of ES implementation aimed at population health control of the animals contained in them.

The study results show that the proportion of small-scale holdings where animal health control is daily organized varies from 0.03% in fur industry to 3% in poultry farms (Fig. 2).

Veterinary services in small-scale holdings are generally provided by specialists of authorities and organizations included in the system of the State Veterinary Service of the Russian Federation (hereinafter referred to as specialists of the State Veterinary Service), not on a daily basis, but following the animal owners' requests to the State Veterinary Service institutions or during implementation of mandatory veterinary measures (planned and emergency) as specified by the Russian Federation legislation.

Direct visual monitoring of the animals' condition is carried out by their owners or handling personnel. That basically means that the most important role in the implementation of passive ES is assigned/delegated to the animal owners. Despite the fact that the animal owners have the legal obligation to immediately notify the specialists of the State Veterinary Service about all cases of sudden death or mass mortality in animals and their unusual behavior, the effectiveness and objectivity of the ES primary stage is directly dependent on the owners' competence in terms of animal health and normal behavior [3].

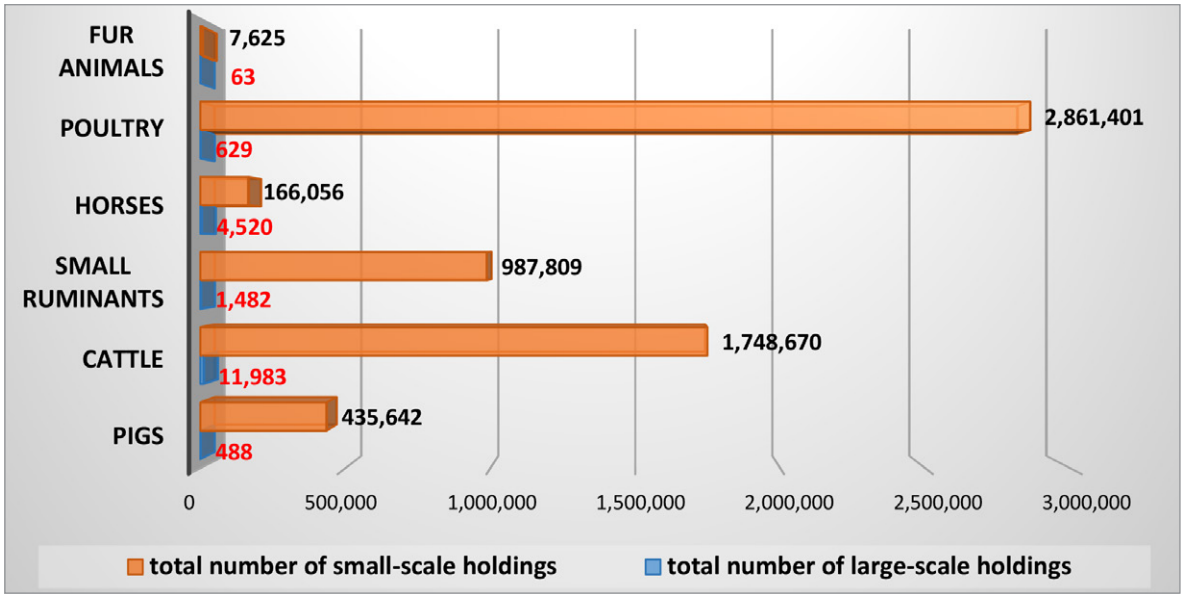

Fig. 1. Distribution of large-scale and small-scale livestock holdings

It is an obvious fact that livestock animals and products thereof can be, under certain circumstances, a source of pathogens and other biological factors posing hazard to animal and human health [15]. Intentional or unintentional actions of the animal owners in the form of non-compliance or formalized implementation of the Russian Federation veterinary legislation, including incompliance with the rules for keeping animals, hiding animal disease cases, lack of awareness of the threats of a particular animal disease, ignoring animal disease signs, etc. can contribute to the occurrence of such circumstances. Therefore, in order to reduce the likelihood of dangerous biological factors and increase the objectivity of passive ES, the institutions of the State Veterinary Service and national livestock associations need to pay increased attention to raising awareness of animal owners in the veterinary field, involving them in active participation in the implementation of effective animal health surveillance. Taking into account the fact that today there are new ways of communication that are more familiar to citizens and are regularly used by them, the most popular of which are social networks, the generally accepted approaches to awareness-raising activities (leaflets, posters, meetings, etc.) can be supplemented with training seminars, including online (webinars), by participation in groups and communities in social networks, as well as by sending information notices and newsletters. Besides, for the above purposes, we consider it possible and feasible to introduce, especially for potential animal owners and support personnel at the initial stage of their work, the so-called veterinary minimum – a set of minimum necessary knowledge that allows them to ensure their own biological safety and the safety of surrounding people and animals, as well as compliance with the veterinary legislation of the Russian Federation. It is advisable to introduce a duty for animal owners and handling personnel with work experience to periodically improve their skills and update existing knowledge, since current legislation, including that in the veterinary field, regularly undergoes significant amendments. Such an initiative can be implemented both at the federal and regional levels. Similar practices exist in the field of handling weapons, as well as nature management and hunting [16, 17, 18].

Large-scale holdings. Despite the fact that small-scale farms numerically prevail over large ones, most pig, poultry and fur animal populations are concentrated in large-scale holdings accounting for ten times higher than the livestock in small-scale farms (Fig. 3).

On the contrary, the population of small ruminants and horses is mostly concentrated in small-scale farms, and as for cattle, with the development of industrial animal husbandry the prevail of small-scale farms is actually mitigating by now.

Large-scale holdings are also characterized by such a concentration of production, that results in a high livestock density in a relatively small area, strengthening and expanding functional ties with other farms and standardization of veterinary services, which, on the one hand, predisposes to improving the quality of services (planning, supply and implementation of preventive/antiepizootic treatment plans, research), and on the other hand, it increases the globalization level of threats associated with both veterinary services and the introduction and spread of infections, especially in the pre-epizootic period.

In addition, large-scale holdings are more involved in the process of providing the population with food and raw materials for its production, that is, ensuring food safety and food independence of our country. In this regard, there should be no doubt about the need for daily veterinary control of animals, which consists in the activities of a veterinary specialist for the primary registration

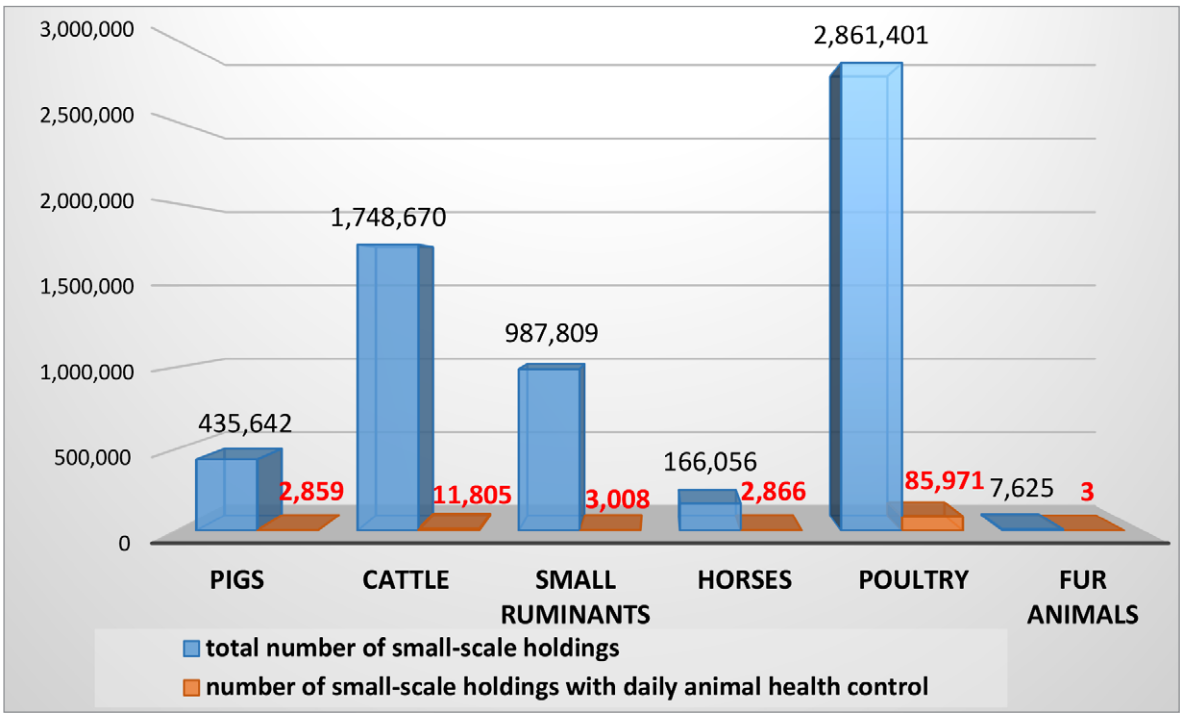

Fig. 2. Provision of veterinary services in small-scale livestock holdings

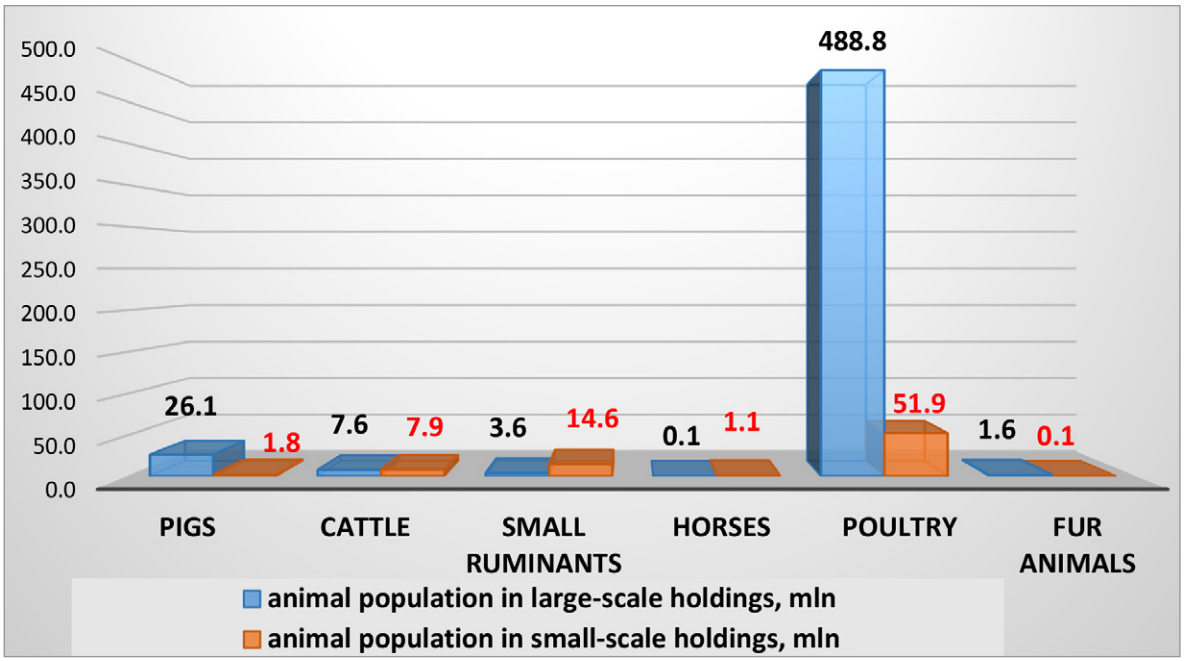

Fig. 3. Distribution of livestock population in large-scale and small-scale holdings

of diseases and deaths of animals, as well as in conducting diagnostic tests, preventive, therapeutic, veterinary and sanitary measures and meat inspection carried out by veterinary specialists directly in livestock farms, and also in training staff on the effective assessment of changes in the animals' condition. Otherwise, lack of daily veterinary control in livestock holdings may lead to an inappropriate assessment of the animal condition by the handling personnel, delaying the diagnosis date, including intentional actions due to the financial dependence of personnel on productivity indicators, which, in turn, leads to the activation (spread) of the epizootic process and the formation of infection outbreaks. That is, the pathogen invasion into a susceptible animal population in large-scale farms is expected to result in the "epizootic scenario" in most cases.

The study results showed that the situation with the veterinary services at large-scale livestock establishments is quite heterogeneous.

As the data presented in Figure 4 show, a relatively favorable situation is observed in pig and poultry farms, where lack of veterinary service provision is only reported in single cases. On the contrary, an extremely unfavorable situation is detected in the holdings for other livestock species. In particular, the situation with large-scale cattle and small ruminant holdings where only 30 and 14%, respectively, are subjected to daily animal health monitoring is of certain concern. The situation is complicated by the fact that livestock industry is an important element in providing the country's population with irreplaceable food, as well as with meat and dairy source materials for food production.

It is also worth focusing on veterinary service provision in pig holdings. Only specialists of the State Veterinary Service are responsible for implementing measures to prevent porcine diseases in farms pursuant to the current legislation of the Russian Federation. First of all, this includes such measures as vaccination against classical swine fever, Aujeszky's disease, anthrax and brucellosis, as well as conducting routine allergic tests for tuberculosis and animal sampling in order to prove the absence of FMD and ASF virus circulation in a certain area. That is, it is legally established that persons who are not specialists of the State Veterinary Service are not entitled to carry out these activities. It is unequivocal that the above-mentioned preventive, diagnostic and other veterinary measures should be preceded by the veterinary examination of animals for compliance with the standard health requirements (for example, a clinical examination of animals before vaccination), which is impossible if there is lack of specialists of the State Veterinary Service directly in the site where animals are kept. The specialists of the State Veterinary Service generally implement these measures almost simultaneously in all pig farms located in the area under their responsibility in accordance with the Plan of diagnostic tests, veterinary-preventive and anti-epizootic measures in holdings of all forms of ownership in the Subject of the Russian Federation. At the same time, the holdings are classified based on the animal health status (Compartments I–IV) [19].

At the same time, the provisions of the "Rules for establishing the animal health status of pig facilities, as well as organizations engaged in pig slaughter, processing and storage of porcine products" (hereinafter referred to as the Rules) specify a requirement that a higher level of the compartment can be assigned if this farm was not technologically connected with the lower compartment, including links via visits of veterinary specialists. The farms applying for Compartment II–IV status should not be visited by veterinary specialists or officials of competent control (surveillance) authorities who were in contact with domestic or wild pigs during the previous two weeks and participated in anti-epizootic measures aimed at eradication of porcine infectious diseases [19]. In our opinion, taking into account these Rules and the fact that over the past few years there has been a decrease in the staff/

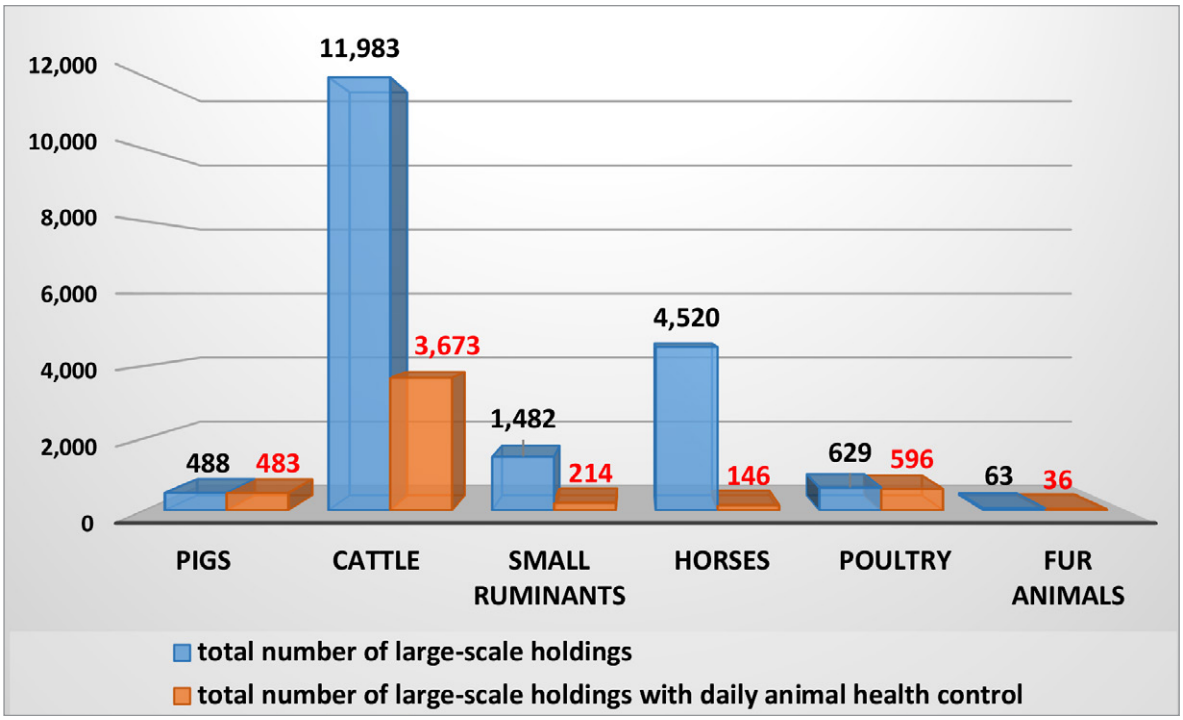

Fig. 4. Provision of veterinary services in large-scale holdings

actual number of veterinary specialists of the State Veterinary Service, especially in therapeutic and preventive institutions at the raion level (animal disease control stations, veterinary clinics, etc.), it is becoming increasingly difficult for the State Veterinary Service to organize legally required and mandatory measures aimed at preventing porcine infectious diseases and ensuring animal disease (including highly dangerous disease) freedom in the area under their responsibility.

In terms of assessing the probability of iatrogenic agent entry into pig breeding establishments, it can be unequivocally concluded that the provisions of these Rules are feasible and necessary for reducing the risks indicated above. Ignoring the Rules and non-compliance with its requirements is an obstacle to improving the biosecurity and safety of a pig breeding establishment, and consequently, obtaining a higher animal health status by the farm. However, the mechanism for implementing veterinary rules with regard to animal diseases taking into account the limited staff of the State Veterinary Service in the area under responsibility should be quite flexible when implemented in practice. This depends both on the biosecurity system of establishments and on the level of guarantees provided by the establishment's veterinary service to government officials. Therefore, the organization of effective interaction between public and private veterinary services is becoming increasingly important in current conditions.

The studies conducted under the auspices of the World Organization for Animal Health on the possibilities of public-private partnership to establish effective veterinary services and animal health systems in more than 100 countries around the world, it was shown that the interaction of the public and private veterinary sector is a means of optimizing animal health systems. Interactions between public and private veterinary services were typologically categorized into transactional, joint and transformational, but in all countries the animal health control function of the State Veterinary Service was preserved [20].

Summarizing examples of interaction between the public and private veterinary sectors and the organization of surveillance in Europe [21] and the Middle East [20], we note a general trend: with the consolidation of farms, as well as transition to commercial livestock husbandry and establishment of megaholdings, these establishments begin to play a leading role in ensuring animal disease freedom in the area of their location helping to improve animal health status of small-scale farms. However, the issue of the effectiveness of epizootological surveillance for animal population in such holdings by the official service is debatable and ambiguous due to the differences in legislation and the agricultural policy of the countries.

In recent years, there has been a tendency in the Russian Federation to switch to industrial production of agricultural products at large-scale establishments. A small traditional farm (a backyard) is becoming less significant in egg, meat and milk production and in providing employment for the population. The major part of the livestock population is concentrated in large-scale farms. Turning the focus of veterinary surveillance to large-scale farms and the transformation of interaction between public and private veterinary services is a natural process caused by global trends in the development of agriculture [1, 2]. Considering the increasing role of large-scale holdings in the Russian Federation, as well as the need to maintain control by the State Veterinary Service, the constant presence of specialists of the State Veterinary Service at establishments today seems to be the most reliable solution to the problem of improving the effectiveness of the state policy implementation in the veterinary field.

CONCLUSION

The unfavourable situation evolved with the organization of veterinary services in large-scale livestock holdings. In particular, the situation in farms for keeping cattle and small ruminants, horses and fur-bearing animals is of concern, since the vast majority of farms do not arrange animal health control on a daily basis.

The current situation is possibly caused by a legal gap, since the animal owners of large-scale livestock establishments are not obligated to organize animal health control on a daily basis according to the Russian Federation legislation. A possible way out of this situation may be the legal obligation for animal owners of large-scale livestock holdings to establish and maintain an in-house veterinary service, that is, to hire veterinary specialists carrying out work on a permanent basis with the restriction on simultaneous servicing of other farms, the staff number of which can be determined based on the production capacity and specifics of the livestock establishment, and also taking into account the provisions of the "Methodological guidelines on labour standardization of veterinary specialists" [22].

In order to arrange optimal conditions for implementing preventive measures in pig rearing holdings, it is proposed to establish the State Veterinary Service divisions at large-scale livestock establishments. It is assumed that the specified division should be included in the system of the Subject's State Veterinary Service institutions, it will be independent in its activities from the livestock establishment owner and financed within the government subsidies for the state task only for these purposes. For many years such State Veterinary Service divisions established in facilities for slaughter of livestock (poultry), as well as for processing and storage of livestock products have shown their high importance and efficiency [23].

In our view, the presence of a specialist of the State Veterinary Service at a livestock establishment on a permanent basis will contribute to improving the effectiveness of planned preventive veterinary measures, the objectivity of assessing changes in animal condition by farm personnel, increasing the level of awareness of the State Veterinary Service as regards the epizootic situation in the territory under its responsibility and possible threats of the situation's deterioration, which will ultimately be reflected in the achievement and maintenance of disease freedom in the Subjects of the Russian Federation, as well as in ensuring the veterinary safety of animal products.

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Andrey I. Dudnikov (on the 95th anniversary of his birth)

December 12, 2023 marked the 95th anniversary of the birth of Andrey Ivanovich Dudnikov, Honored Veterinarian of the Russian Federation, holder of three Orders of the Red Banner of Labor, laureate of the State Prize of the Russian Federation in the field of science and technology, Doctor of Science (Veterinary Medicine), Professor, first Head of the Laboratory for Inactivated Vaccines in the All-Union Foot-and-Mouth Disease Research Institute (currently FGBI "ARRIAH", Vladimir).

Andrey I. Dudnikov was born in the village of Kryuk, Novooskolsky Raion of the Belgorod Oblast on December 12, 1928. In 1952, he graduated with honors from the Kharkov Veterinary Institute. For four years he worked as the Head of the Transport Veterinary Station at the Novy Oskol railway station in the Belgorod Oblast.

From 1956 to 1964, he worked as a Junior Researcher at the Laboratory of Virology at the Ukrainian Research Institute of Experimental Veterinary Medicine (Kharkov), where he defended his Candidate of Science thesis in 1964, and then was invited for Senior Researcher position in the All-Union Foot-and-Mouth Disease Research Institute.

Since foot-and-mouth disease as a highly dangerous infection posed a great threat to the country due to the lack of specific disease prevention tools, the Laboratory for Inactivated Vaccines was established at the All-Union Foot-and-Mouth Disease Research Institute in 1966 and headed by Andrey I. Dudnikov.

The Laboratory staff activities were aimed at the development of safe and highly effective inactivated vaccines against foot-and-mouth disease (FMD). Andrey I. Dudnikov led and was directly involved in the improvement of technology for adsorbed lapinized virus-based vaccine production and its putting into practice at national 10 biological factories. Anti-FMD emulsion vaccine using domestic mineral and synthetic oils and emulsifiers was developed and put into production. Commercial production of mono- and polyvalent adsorbed and emulsion anti-FMD vaccines as well as universal vaccines for rapid protection of animals from FMD based on large-scale virus cultivation in BHK-21 cell suspension was commenced under Andrey I. Dudnikov leadership.

National strategy for foot-and-mouth disease eradication through the use of developed immunobiologicals was launched in the country and facilitated by mass application of safe and effective vaccines. By the early 1990s, foot-and-mouth disease had been eradicated and epizootic virus strain circulation had been terminated in the country.

Professor Andrei I. Dudnikov created his own scientific school, three Doctor of Science theses and more than twenty Candidate of Science theses were successfully defended under his scientific supervision. He published more than 500 scientific papers. The priority of Andrei I. Dudnikov's research is protected by 38 USSR inventor's certificates and 12 Russian Federation patents for the inventions. He was an excellent teacher, highly committed and hardworking expert, knowledgeable in vaccinology, being a real "farther" for his subordinates and early-carrier researchers and that's why he was deeply respected and cherished by his apprentices and colleagues. Andrey I. Dudnikov had been the Deputy Director for Research at the Institute till 1990.

In 2009, Andrey I. Dudnikov passed away. His adherents worthily continue to put his ideas into practice: new methods for preparation of epizootic virus isolates for vaccine production and virus inactivation; improved methods for virus purification and concentration, virus immunogenic components quantification within vaccine formulation. Since its early days until today, the Laboratory has been a unique fundamental pillar for research and experiments at the FGBI "ARRIAH".

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