FGBI "FEDERAL CENTRE FOR ANIMAL HEALTH" (FGBI "ARRIAH")

FEDERAL SERVICE FOR VETERINARY AND PHYTOSANITARY SURVEILLANCE (ROSSELKHOZNADZOR)

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РУССКО-АНГЛИЙСКИЙ ЖУРНАЛ

SEPTEMBER | СЕНТЯБРЬ №3 [34] 2020

Eradicate rabies in Russia by 2030

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Ветеринария сегодня №3 (34) 2020 научный журнал

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From the Editor-in-Chief

Dear readers, here is a new issue of the journal that is devoted to the World Rabies Day, which is globally celebrated on September 28. This day was set up in 2007 at the initiative of the Global Alliance for Rabies Control (GARC) with the support of the World Health Organization (WHO). The date of the celebration is the anniversary of Louis Pasteur's death (28.09.1895), a microbiologist, one of those who created the rabies vaccine.

The World Rabies Day aims to highlight the problem of the disease spread and raise awareness of the disease consequences.

Rabies is one of the oldest and most dangerous infectious diseases in humans and animals. It affects the central nervous system and is accompanied by paralysis and encephalomyelitis. The disease has an almost absolute death rate. According to the WHO, it is one of the five zoonoses that cause the greatest economic damage and poses a constant threat to the life of humans and animals.

Despite the disease successful eradication in some European countries, the situation in many countries of Eurasia, Africa and America remains complicated. About 70,000 people die of rabies worldwide each year, mostly children from developing countries.

As part of the FAO, WHO and OIE global initiative to eliminate dog-mediated human rabies by 2030, Russia, as a member of these international organizations, will have to take a number of steps in research, information and methodological as well as legislative and practical fields. This area of activity acquired significant support thanks to the decision of the CIS Intergovernmental Council for Cooperation in the Field of Veterinary Medicine dated May 12, 2016, in which the FGBI "ARRIAH" was entrusted to prepare a project for the "Set of joint measures of the CIS countries on the prevention and control of rabies for the period until 2025".

The set of joint actions was developed taking into account the proposals of the veterinary services of the CIS countries, the experience of implementing programs to combat animal rabies in the European Union, the USA and Canada and it is aimed at reducing the spread of disease outbreaks in the CIS countries and minimizing the damage caused by the disease.

According to the WHO, it is one of the five zoonoses that cause the greatest economic damage and poses a constant threat to the life of humans and animals

Only through joint efforts of the international community this dangerous disease can be eradicated. It is important to remember that rabies cannot be cured, but it can be effectively prevented!

Editor-in-Chief
Doctor of Science (Veterinary Medicine)
Artem Ye. Metlin

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Lessons learnt from measures taken to prevent rabies introduction and spread into a long rabies free territory (case study of the Irkutsk Oblast)

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SUMMARY

Rabies is endemic on the considerable part of the Russian Federation, and it is associated with current natural outbreaks of the infection. The highest animal morbidity rates are reported in the central and southern regions of the European part of Russia and in the southern part of Western Siberia. The Irkutsk Oblast is among the few regions of our country, which are rabies free for several decades. The research was aimed at the analysis of factors aiding to the maintenance of the rabies free status of the region. Retrospective study of archive and previously published reports on human and animal rabies cases in the Irkutsk Oblast starting from 1954 was performed. Epidemics of urban rabies ceased in the region in 1970s. Sporadic rabies cases in dogs, reported in 1976 and later, could be imported or could result from the infection from bats. Diagnostic errors were also possible. Rabies is reported in foxes in the Subjects bordering the Irkutsk Oblast: Krasnoyarsk Krai and Republic of Buryatia. It is supposed that in case of the infection introduction the forest-steppe agricultural areas near the Angara River are likely to be affected due to high population of foxes. Relative geographic isolation of the Irkutsk Oblast favors to the long animal rabies freedom of this territory. The vast area of mountain taiga with low fox population serves as an ecological barrier. In 2007–2009 and in 2019, barrier oral vaccination was carried out along the border with the Krasnoyarsk Krai and on the west coast of Baikal Lake. Measures for anti-rabies vaccination of dogs and cats were intensified. Active virological monitoring is performed on a regular basis. The paper demonstrates cartograms of fox and wolf population density along with designation of sites, where oral vaccination of wild carnivores was performed.

Key words: rabies, rabies-free territory, oral barrier vaccination, vaccination of dogs and cats.

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Опыт мероприятий по предупреждению заноса и распространения бешенства на длительно благополучной территории (по материалам Иркутской области)

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РЕЗЮМЕ

Значительная часть территории Российской Федерации эндемична по бешенству, что в настоящее время обусловлено активностью природных очагов инфекции. Наиболее высокие показатели заболеваемости животных отмечаются в центральных и южных регионах европейской части России и на юге Западной Сибири. Иркутская область относится к числу немногих территорий нашей страны, свободных от бешенства на протяжении нескольких десятилетий. Целью исследования был анализ обстоятельств, способствовавших сохранению статуса благополучной по бешенству территории. Проведено ретроспективное изучение архивных и опубликованных ранее данных о регистрации бешенства среди людей и животных в Иркутской области с 1954 г. Эпизоотии городского типа в регионе прекратились в 1970-е годы. Спорадические случаи заболевания собак в 1976 г. и позднее, вероятно, могли быть завозными или возникать в результате заражения от летучих мышей. Не исключено также, что могли иметь место ошибки диагностики. Бешенство среди лисиц регистрируется в соседних с Иркутской областью субъектах – Красноярском крае и Республике Бурятия. Сделано предположение о том, что, в случае заноса инфекции, могут быть поражены сельскохозяйственные районы лесостепного Приангарья с высокой численностью лисицы. Длительному благополучию Иркутской области по бешенству животных способствует относительная географическая изоляция этой части ее территории. Экологическим барьером служат обширные пространства горной тайги с низкой численностью лисицы. В 2007–2009 и 2019 гг. проводилась барьерная оральная вакцинация лисиц на границе с Красноярским краем и на западном побережье Байкала. Усилены мероприятия по вакцинации собак и кошек против бешенства. Систематически проводится активный вирусологический мониторинг. В статье приведены картограммы плотности населения лисицы и волка с обозначением участков проведения оральной вакцинации диких плотоядных.

Ключевые слова: бешенство, свободная от бешенства территория, барьерная оральная вакцинация, вакцинация собак и кошек.

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INTRODUCTION

Rabies is one of the dangerous zoonotic diseases having the highest social and economic significance [1]. The damage posed by this lethal infection is associated with the loss of livestock and hunted animals, major costs of anti-epidemic measures including animal vaccination, diagnostic tests as well as preventive and post-exposure vaccination of humans. Risk of lethal disease cases still

exists even in case of well-organized rabies surveillance. Therefore, preventive measures should be taken in order to avoid the infection introduction into the rabies-free territories. These considerations govern the rabies control strategy and tactics in the regions with various epidemic statuses [2–4].

Rabies is endemic on the major part of the Russian Federation and it is currently associated with active natural outbreaks of the infection. The highest morbidity rates are reported in the central and southern regions of the European part of Russia and in the southern part of Western Siberia. Some Subjects of the Russian Federation, however, remain rabies-free for many years [5–7]. Occasional aggravations of the epidemic situation are typical for rabies, and such aggravations are accompanied by the transformation of the nosoarea boundaries. For example, early in this century the epidemics were for the first time reported in foxes in a number of regions of Eastern Siberia. As a result, for a number of years rabies has been reported in wild and domestic animals in previously free territories (southern part of the Krasnoyarsk Krai, Republics of Khakassia and Buryatia, Zabaikalsky Krai) [8–11]. The Irkutsk Oblast remains the only rabies-free Subject in Eastern Siberia.

The study was aimed at the analysis of the aspects contributing to the maintenance of rabies-free territory against the background of the epidemics affecting the neighboring regions.

MATERIALS AND METHODS

Retrospective study of archive and previously published reports on human and animal rabies cases in the Irkutsk Oblast starting from 1954 was performed [5, 12–15]. Sporadic cases mentioned in the statistical bulletins since 1975 were explored.

Currently taken preventive and surveillance measures are presented based on the data accumulated by the Irkutsk Oblast Veterinary Service. Results of virological monitoring performed in 2007–2019 were reviewed. Brain samples collected from hunted wild animals and rabid suspected, diseased or found dead domestic animals (n = 1002) were tested according to GOST 26075-2013 "Animals. Methods of Laboratory Diagnosis of Rabies".

Data on anti-rabies vaccination of dogs and cats are given according to the official records and reports compiled under Forms 1-Vet and 1-Vet A. The data submitted by the Oblast Animal Disease Control Station in 2018–2019 were used for the estimates of the numbers of the domestic dogs and cats as well as stray animals [16].

The barrier oral vaccination of foxes was carried out by vehicles and four-wheeler ATVs. "Oralrabivac" vaccine (FSE "Shchelkovo biocombinat", Russia) was distributed in 2007–2009; and "Rabistav" vaccine (FSE "Stavropol biofactory") was distributed in 2019 according to the manufacturer's instructions. Dynamics of the fox and wolf population number and density was analyzed basing on the data of winter censuses performed according to the methodical guidelines approved by the Order of the Ministry of Nature of Russia of January 11, 2012 No. 1. The census data were correlated with officially reported hunted volumes of these animal species.

Mapping was performed on the basis of the Natural Earth landscape and geographic map using QGIS 3.2.1 software. Rabies infected territories in the neighboring Subjects of the Russian Federation were mapped according to the data published in 2002–2019 [8, 9, 15].

RESULTS AND DISCUSSION

In the mid-twentieth century, urban rabies epidemics raged in the Irkutsk Oblast as well as in many other regions of Russia. This was evidenced by the morbidity patterns. In 1954–1970, 654 rabies cases were reported in animals (78.7% – dogs, 17.7% – farm animals, 3.8% – cats). No disease cases were reported in wild animals. Over the same period, 29 rabies cases were reported in humans with the maximum number to be reported in 1962. Different publications informed of 36 or 37 human deaths due to rabies [12, 14]. In all cases the source of infection involved domestic animals. Two epidemic peaks were reported: in mid-50s and early 60s. After 1963, the morbidity consistently decreased (see Table). Singular rabies cases were reported in animals after 1970: in 1976 – three cases, in 1998 – one case. In the current century, rabies was also suspected in animals basing on the disease clinical signs and dubious luminescent microscopy results. The diagnosis was not however confirmed by the results of the investigations as well as by the expert and laboratory tests. For example, in 2016 a report on a human being attacked by a wolf in the Ust-Ilymsky Raion was posted in Internet, but the investigation demonstrated that the bites were made by a dog, and laboratory test results did not confirm rabies. In 2007–2019, the regional veterinary laboratory tested for rabies a number of samples collected from 433 foxes, 115 wolves, 208 other wild animal species, 228 dogs and cats, 16 synanthropic rodents and 2 farm animals. The virological monitoring results demonstrated no rabies cases. Therefore, over the past 50 years, no rabies epidemics were observed in the Irkutsk Oblast. Sporadic rabies cases in dogs in 1976 could be imported or resulted from the infection from bats. It is well-known that Irkut lyssiavirus is circulating in the chiropterans in Eastern Siberia [17]. Diagnostic errors were also quite possible. Thus, one rabies case reported in a cat in Irkutsk in 1998 was not confirmed [13].

Since 2002, the rabies situation has dramatically changed in the Subjects of the Russian Federation bordering the Irkutsk Oblast. Since that time the epidemic has been persisting in foxes and involving domestic and farm animals mostly in the insular forest steppes in the south of the Krasnoyarsk Krai and Republic of Khakassia. In 2011–2019, fox rabies outbreaks were reported in the Republic of Buryatia and Zabaikalsky Krai. The outbreaks are occasionally reported in the Republic of Tyva [5, 8–11, 18]. The most geographically close to the Irkutsk Oblast rabies cases were reported in Kansk forest steppe and Angara River valley (Krasnoyarsk Krai) located just 50–60 km from the administrative border [9]. In the Republic of Buryatia, rabies spread along the Selenga River and its tributaries to the north up to Ulan-Ude city (approximately 150 km from the Olkhonsky Raion of the Irkutsk Oblast) [8]. This justified the assessment of possible rabies introduction into the Irkutsk Oblast and implementation of the additional preventive measures.

According to fur production volumes during the rabies epidemics in 1950–1965, the number of fox population in the Irkutsk Oblast was specified by low growth rates varied from 1.4–2.7 ths foxes/year. This once again confirms that there was no epidemic in foxes at that time. Thus, for example, in the Republic of Buryatia the number of fox population drastically reduced after rabies virus introduction in 2017 [19]. Maximal number of wolves (about 400 animals) was hunted in 1950 with progressive four-fold reduction by 1965. Due to a number of social and economic reasons there are no reliable data on fox

Table
History of animal and human rabies morbidity in the Irkutsk Oblast in 1954–1970 (archive data)

Таблица
Динамика заболеваемости животных и людей бешенством в Иркутской области за 1954–1970 гг. (архивные данные)

Category	Year																	
	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971
Animals	173	104	52	79	15	9	19	5	46	71	50	11	1	11	7	0	1	0
Humans*	0	2	0	2	1	1	2	2	9	4	4	1	3	0	0	0	0	0

* Human cases are reported by the date of infection

* Случаи заболевания людей приведены по дате заражения.

and wolf population for the later period (approximately from 1969 to 2000). After 2000, winter census data are available that are indicative of the growth of the population of wolves and mostly of foxes (Fig. 1). The main factor of the current growth of the fox population is apparently the reduction of the hunting pressure. The fox fur is out of demand, and foxes are currently hunted by amateur hunters while hunting hoofed animals. In 2011–2019, on average 489 animals, i.e. about 3% of the recorded number of the population, were hunted annually. As for the wolf, this parameter amounted to 232 animals and 4.8%, respectively.

According to the summaries of the Federal Hunting Reserve Inventory the highest fox population in Eastern Siberia is recorded in the Irkutsk Oblast [20, 21]. The most dense fox population is reported in the Irkutsk-Cheremkhovsk Plain specified by atropogenically transformed forest stepper topography. Mean values of the population density of this animal species are reported in Ekhirit-Bulagant forest steppe and in Taishetsky and Chunsky Raions bordering the Krasnoyarsk Krai. The highest wolf population is observed in the taiga areas (Fig. 2). Comparison of the data on the Irkutsk Oblast and Republic of Buryatia, where rabies has been continuously reported for over three years after it was introduced from Mongolia [19], demonstrates that mean volumes of the wolf population density are similar but mean density of the fox population is higher in the Irkutsk Oblast. The fox population is low in the taiga habitats adjacent to Lake Baikal. The wolf population here is however high due to high number of

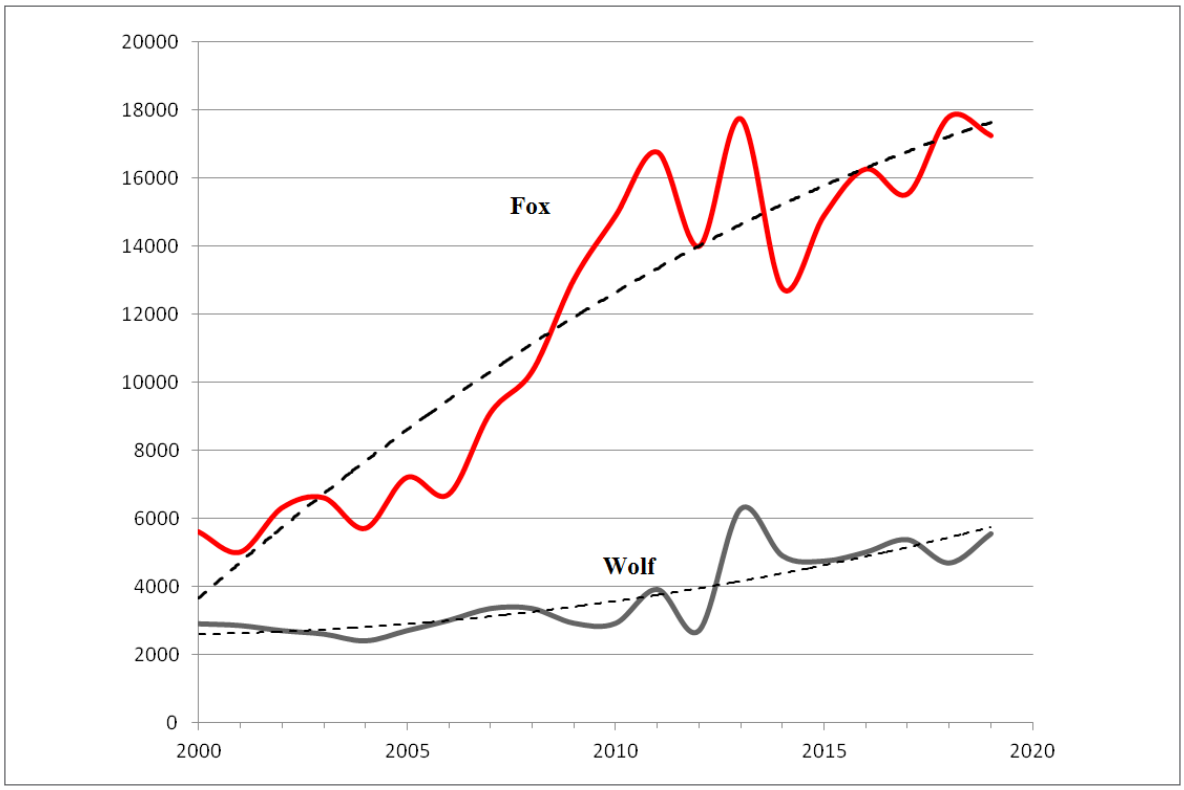

Fig. 1. Dynamics of fox and wolf population numbers in the Irkutsk Oblast by winter census data (number of animals, 2000–2019)

Рис. 1. Динамика численности лисицы и волка в Иркутской области по данным зимних маршрутных учетов (число особей, 2000–2019 гг.)

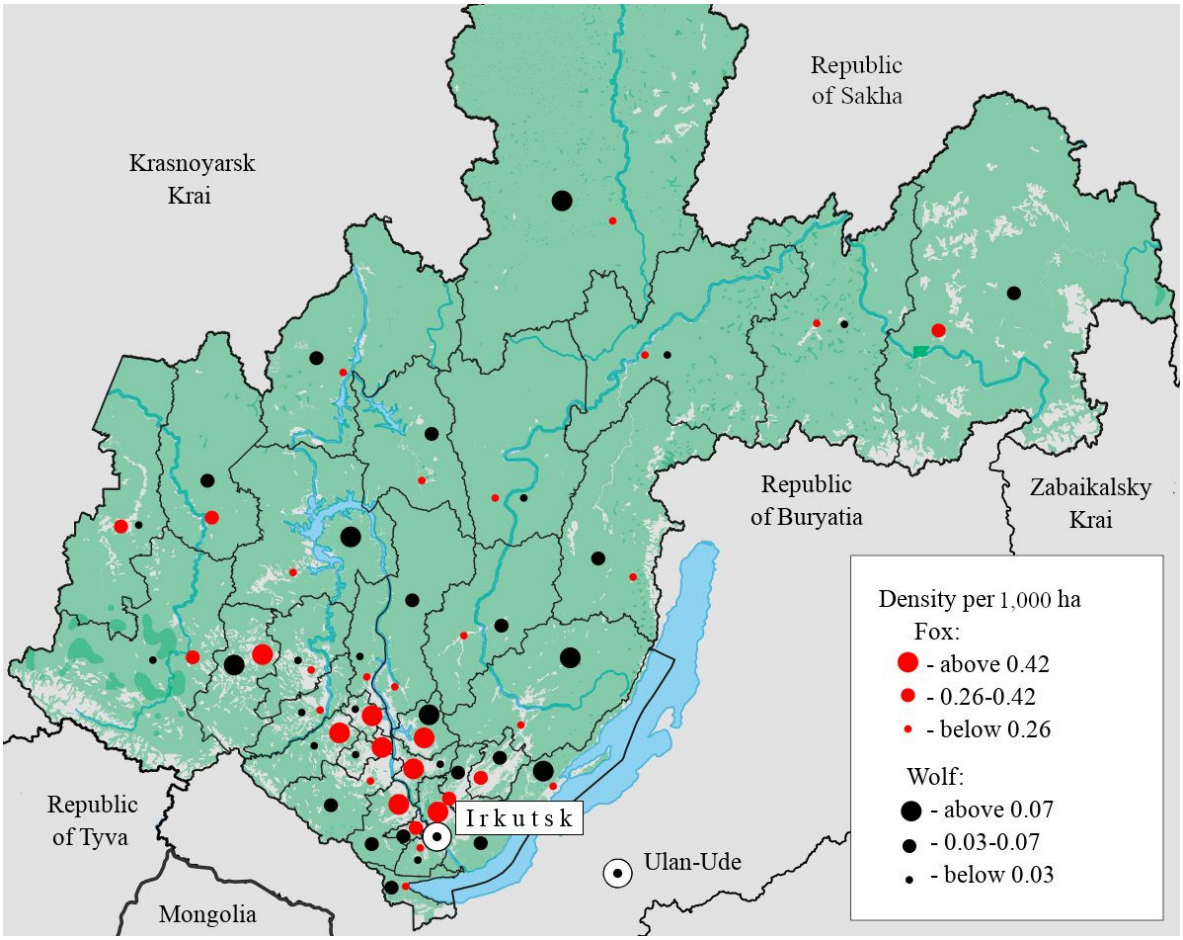

Fig. 2. Density of fox and wolf population in the Irkutsk Oblast by winter census data (average data for 2015–2019 per 1,000 ha of hunting reserves; light colored areas on the map – forest-free territories)

Рис. 2. Плотность населения лисицы и волка в Иркутской области по данным зимних маршрутных учетов (средние данные за 2015–2019 гг. на 1000 га охотничьих угодий; светлые участки на карте – безлесные территории)

wild hoofed animals (Fig. 2). There is low number of foxes on Olkhon Island and the animals mostly feed near the tourist camps and roads. Tazheranskaya steppe provides richer habitat for foxes but their number here is also not high. Pack of wolves (8–11 animals) continuously resides on Olkhon Island. Rare cases of the wolves crossing frozen Lake Baikal from the eastern coast to the western one are known. According to the census the number of foxes and wolves in the Cisbaikalia Natural Park and Baikal-Lena Nature Reserve is estimated within one order of magnitude (wolf – 36–101 animals, fox – 29–116 animals). Therefore, there is a possibility of the rabies virus introduction from the infected regions of the Republic of Buryatia into the specially protected natural areas located along Baikal's coast, but the conditions for further rabies spread in wild animals are minimal here.

Due to the threat of rabies introduction from the Krasnoyarsk Krai, for three years starting from 2007 the barrier oral vaccination was performed in the Nizhneundinsky, Taishetsky, Chunsky and Ust-Ilimsky Raions. The scope of vaccination was increased annually: 2007 – 7.3; 2008 – 37.28; 2009 – 118.0 ths doses. The vaccine was distributed along the 50 km-wide area adjacent to the administrative border. The efforts to form the buffer zone were resumed in 2019: 17 ths doses were used in the Taishetsky Raion along the border with the Krasnoyarsk Krai; three ths doses were distributed in the Olkhonsky Raion (Olkhon Island, southern part of the Irkutsk Oblast) and along the western coast of Lake Baikal (Fig. 3). While selecting the sites for the vaccine distribution the landscape patterns specifying possible habitat and migration of foxes were considered. In the Yenisei part of Siberia and Cisbaikalia, the rabies virus spreads mostly along the river valleys and intermountain forest steppe basins cultivated by humans [8–11]. It is well known that landscape patterns mostly defined the effectiveness of the first oral vaccination campaigns in Central Europe [22, 23]. The Irkutsk Oblast is separated from the Krasnoyarsk Krai, Republic of Tyva and Mongolia by the plexus of Eastern Sayan Mountains with maximal altitudes of over 3.3 ths MASL. Individual ridges with the altitudes of up to 1–2 ths m (Biryusinsky, Gutarsky, Yagi) extend to the north almost up to Taishet. The most probable routes of rabies spread from the west could include territories located northwards – along the Transsiberian railroad between Kansk and Taishet as well as along the Birysa, Chuna and Angara river valleys. In the south, Lake Baikal is an impassable barrier for the rabies virus in summer. However, during 3–4 month after the ice formation singular migrations of wolves from the Republic of Buryatia cannot be excluded. The narrowest part of Lake

Baikal (about 30 km) is in Selenga and Buguldeyka river station. Khamar-Daban and Cisbaikalia mountain ridges are also formidable barriers for foxes, and they protect the Irkutsk Oblast from the south.

Another area of activities involves prevention of the rabies virus introduction with domestic animals. Animal movements by public transport are controlled according to the Order of the Ministry of Agriculture of the Russian Federation of December 14, 2016 No. 635, and thus any possibility of importation of any non-vaccinated animals is nearly excluded. There is, however, a problem of intra-regional transportation by personal transport. For example, over 100 ths people annually come to Lake Baikal in the Olkhonsky Raion, and many of them bring dogs, cats, ferrets and other animals. This further increases the relevance of the vaccination of pets. There are no statistic data on the number of cats and dogs. According to the survey, there is at least one dog and one cat kept on each backyard in the rural communities. Total of 91.8 ths backyards were recorded in 2019, and the scope of the domestic animal vaccination is scheduled according to these data. Starting from 2014, over 80 ths dogs are vaccinated annually. In addition, the number of vaccinated cats has increased significantly (Fig. 4).

After rabies cases ceased to be reported in the Irkutsk Oblast, 3–4 thousand people annually sought medical attention due to injuries caused by animals (160–200 individuals per 100 ths people), and there was a growing trend of this parameter. In different years, from 30% to 77% of such people were vaccinated against rabies [5, 24, 25]. Veterinary monitoring of animals with known owners was performed for 10 days as appropriate and amounted to 75% of total number of cases. However, 25% of cases were due to injuries caused by unknown animals that implies full course of vaccination of the injured individuals. According to the official data, 13 ths stray dogs and cats were recorded in 2019, and Irkutsk accounted for about 10% of them. Of these, 9,855 animals were captured and placed into animal shelters. About 50% of such animals were released after they had been vaccinated, neutered and tagged (microchipping). Monitoring of compliance with animal keeping rules and control of the number of stray dogs and cats still remain intractable social problems.

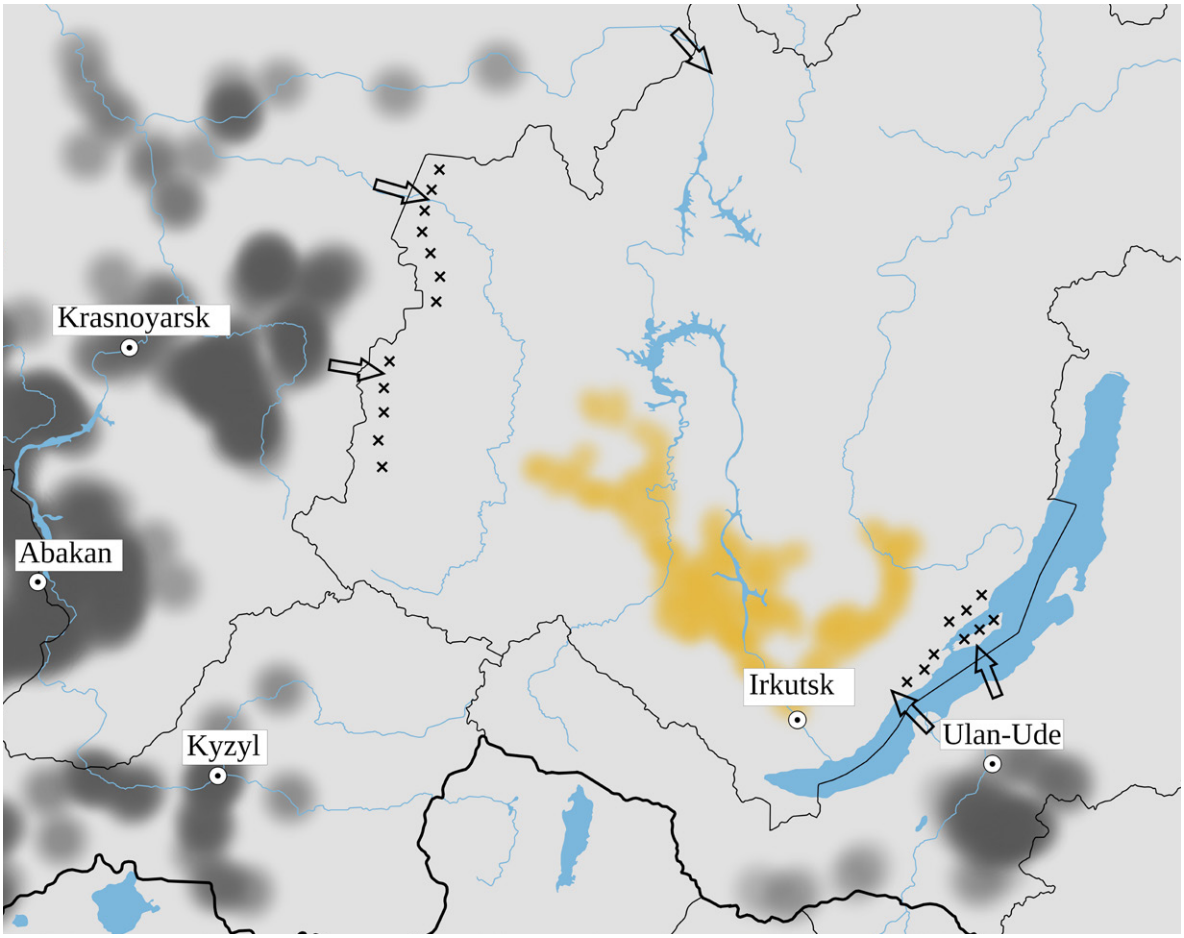

Fig. 3. Estimated probability of rabies introduction and spread in the Irkutsk Oblast and areas of barrier oral vaccination of foxes. Legend: crosses – sites of the vaccine distribution; gray paint – rabies infected neighboring territories; yellow paint – forest-steppe territories optimal for rabies spread in foxes; arrows – most probable routes of rabies introduction with wild animals.

Рис. 3. Оценка возможности заноса и распространения бешенства в Иркутской области и районы проведения барьерной оральной вакцинации лисиц. Легенда: крестики – места раскладки вакцины; серая заливка – неблагополучные по бешенству соседние территории; желтая заливка – лесостепные территории, благоприятные для распространения бешенства среди лисиц; стрелки – пути наиболее вероятного заноса бешенства дикими животными.

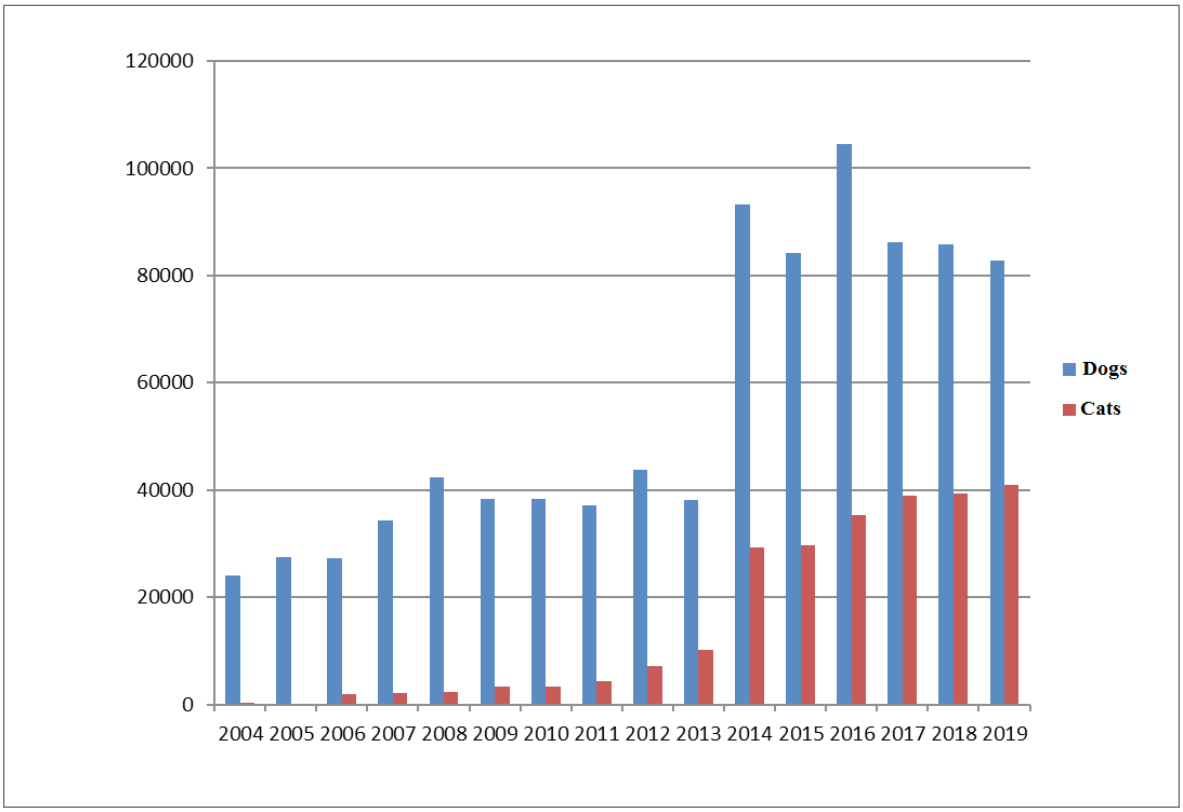

Fig. 4. Numbers of vaccinated dogs and cats in the Irkutsk Oblast (2004–2019)

Рис. 4. Объемы вакцинации собак и кошек против бешенства в Иркутской области (2004–2019 гг.)

CONCLUSION

The probability of rabies introduction into the Irkutsk Oblast has currently increased due to disease presence in the neighboring areas. In case of the infection introduction with the wild animals, the agricultural regions of the forest steppe areas near the Angara are the most likely to be infected due to high fox population. Long disease freedom of the Irkutsk Oblast is favored by relative geographic isolation of this part of its territory. Continuation of the barrier rabies vaccination and preventive measures in the settlements remain of topical significance in the context of Russia's participation in the Zero by 30 strategic plan initiated by the World Health Organization in order to end human deaths from dog-mediated rabies and with regard to the tourism development in the Baikal region.

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Evaluation of rabies control measure effectiveness in the Russian Federation

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SUMMARY
Rabies is a zooanthroponotic disease, causing significant economic damage, resulting from losses due to livestock deaths, costs of preventive measures and diagnostic tests. The disease is transmitted through biting or licking of damaged skin or mucosa. The disease is absolutely fatal and practically all warm-blooded animals are susceptible to it. The paper presents the analysis of statistical data on rabies morbidity and mortality among humans and animals; the assessment of epidemic situation in the Russian Federation, including the target population vaccination coverage and effectiveness evaluation of measures, taken in Russia to prevent rabies spread. The major causes of human mortality are considered. The recommendations on decreasing the disease spread risks are given. It was established that about 60 thousand human deaths and 45 thousand animal deaths from rabies have been reported in the Russian Federation within the past 10 years (from 2010 to 2019). Moreover cases of licking/scratching/biting of humans (397,248 cases in 2019, out of them 10,232 by wild animals) are reported every year. The sources of human infection within the mentioned period were dogs (39%), foxes (18%), cats (14%), raccoon dogs (14%), wolves (4%), polar foxes (4%), ferrets (4%), unknown sources (3%). The analysis of data from veterinary reports showed that the most rabies-infected regions are the Central and Volga Federal Districts. Using the mathematical modeling of the epidemic process the results of preventive measures, taken by the Veterinary Service in case of rabies in the region, were evaluated.

Key words: rabies, analysis, epidemic situation, vaccination, regionalization, anti-epidemic measures.

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УДК 619:616.98:578.824.11:616-036.22(470)

Оценка эффективности противоэпизоотических мероприятий против бешенства, осуществляемых в Российской Федерации

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РЕЗЮМЕ
Бешенство – зооантропонозное заболевание, наносящее значительный экономический ущерб, складывающийся из потерь в результате падежа животных, затрат на проведение профилактических мероприятий и диагностических исследований. Болезнь передается при укусе или ослюнении поврежденных поверхностей кожи, слизистых оболочек. Заболевание характеризуется абсолютной летальностью, ему подвержены практически все теплокровные животные. В работе проведен анализ статистических данных по заболеваемости и смертности людей и животных от бешенства, проведена оценка эпизоотической ситуации в Российской Федерации, включающая охват вакцинацией целевых популяций, а также оценка эффективности мер,

применяемых в России для предотвращения распространения бешенства. Рассмотрены основные причины смертности людей. Даны рекомендации по снижению риска распространения заболевания. Было выявлено, что за последние 10 лет (с 2010 по 2019 г.) на территории Российской Федерации зарегистрировано 60 случаев гибели людей от бешенства и около 45 тысяч случаев падежа животных. Кроме этого, ежегодно фиксируются случаи ослюнений/оцарапываний/покусов людей (397 248 случаев за 2019 г., из них 10 232 – дикими животными). Источниками заражения людей за указанный период являлись: собака (39%), лисица (18%), кошка (14%), енотовидная собака (14%), волк (4%), песец (4%), хорек (4%), неизвестный источник (3%). В результате анализа данных ветеринарных форм отчетности выявили, что самыми неблагополучными по бешенству являются Центральный и Приволжский федеральные округа. В представленной работе с помощью математического моделирования эпизоотического процесса оценены результаты профилактической работы, проводимой ветеринарной службой при возникновении бешенства в регионе.

Ключевые слова: бешенство, анализ, эпизоотическая ситуация, вакцинация, регионализация, противоэпизоотические мероприятия.

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INTRODUCTION

Rabies (or hydrophobia) is one of zoonotic diseases, which occurs in the whole world, except for Antarctica. This is the fatal disease, presenting a significant threat for public health. Notwithstanding the fact, that human cases can be prevented by modern post-exposure prophylaxis tools, control and eradication are possible only in reservoir populations, for example in foxes and domestic dogs [1].

Taking into account the nature of rabies agent reservoir the epidemics can be natural (the disease is spread by wild carnivores) and urban (the virus sources and vectors are stray dogs and cats, the number of which defines the epidemic proportions) [2].

According to the researchers' assessments about 60 thousand people die from rabies and about 29 million people seek for post-exposure medical assistance after contact with a suspected animal [3].

Every year considerable financial resources are dedicated to rabies prevention in animals and reducing the probability of human infections. The vaccination of domestic and wild susceptible animals, post-exposure prophylaxis in humans and control of carnivore movements (regionalization requirements) are carried out in the territory of the Russian Federation.

The purpose of this work was the analysis of statistical data on rabies morbidity and mortality in humans and animals in the Russian Federation in 2010–2019, as well as the evaluation of control measure effectiveness and recommendations on reduction of disease spread risks.

MATERIALS AND METHODS

The statistical data on morbidity and mortality in humans and animals due to rabies virus (Table 1), number of movements of domestic carnivores, number of detected violations in the process of such movements (Data from IS "MERCURY") were used in the study. The population indicators of the epidemic process made it possible to analyze the effect of prevention measures on human morbidity. Data on vaccination of domestic and wild animals allowed to evaluate the reduction of human rabies risks by modeling.

To assess the epidemic situation intensity in the Federal Districts of the Russian Federation the epidemicity index, calculated using the following formula, was used:

$$Ie = t/T,$$

where 'Ie' is the epidemicity index; '*t*' is the number of days (months, years), when the disease was reported; '*T*' is the number of days (months, years) of observation.

Based on the epidemicity index value, the Federal Districts were ranked against their infection level.

To quantitatively assess the effect of vaccination performed on risks of public infection, the modeling of possible number of human infections due to bites of wild and domestic animals was carried out. The modeling was performed using probabilistic approach for two scenarios: 1) preventive vaccination of animals against rabies is not practiced; 2) vaccination is practiced. The probable number of infected humans was modeled using hypergeometric distribution, assessing the probability of '*s*' human infections where data on animal population number (*M*), number of infected animals within it (*D*) and number of reported bites (*n*) are available (Table 1).

The following initial parameters were taken into account: mean potency of the vaccine – 87% [5]; average uptake of vaccine baits by wild animals – 50% [6]. The following assumptions were used: 1) probability of human infection if bitten by an infected animal is 100%; 2) baits were consumed only by foxes [7].

To study the relationship between the vaccination coverage and morbidity level in the Federal Districts in 2010–2019, the correlation analysis using Pearson correlation coefficient (*r*) was performed.

RESULTS AND DISCUSSION

Rabies is endemic to the Russian Federation. In 2010–2019 60 human deaths and 45,219 animals' deaths due to this disease were registered in the country (RF MoA and Rospotrebnadzor data).

Table 1
Statistical data for modeling

Таблица 1
Статистические данные для проведения моделирования

Indicator (for 2019)	Rated value	Source
Number of infected wild animals	587 animals	RF MoA, IAC annual report
Number of infected domestic animals	586 animals	RF MoA, IAC annual report
Number of contacts between wild animals and humans	10,232	Rospotrebnadzor
Number of contacts between domestic carnivores and humans	387,016	Rospotrebnadzor
Number of wild carnivores	753,200 animals	Ministry of Natural Resources, RF Central Hunting Control Institution
Foxes, out of them	496,300 animals	Ministry of Natural Resources, RF Central Hunting Control Institution
Number of domestic carnivores	52,600,000 animals	Evaluated value [4]
Vaccine baits distributed	20,684,436 doses	RF MoA
Domestic carnivores vaccinated	8,548,904 animals	RF MoA

Out of 86 Subjects of the Russian Federation the following regions were permanently infected with rabies in 2010–2018: Republic of Karelia, Kamchatka Krai, Arkhangelsk, Murmansk, Irkutsk, Magadan and Sakhalin Oblasts, Saint Petersburg. In 2019 the following regions became also infected: Republic of Komi, Republic of Chechnya, Kemerovo Oblast and Primorsky Krai.

The infection level in the Federal Districts was ranked based on the epidemicity index (Table 2).

Within the observation period humans got infected in 53.6% of cases after contacts with domestic carnivores (dogs and cats). Herewith the ratio of infections due to dogs was 39% and cats were responsible for 14% of infections. The other rabies infection sources were represented by foxes (18%), raccoon dogs (14%), wolves (4%), polar foxes (4%), ferrets (4%), unknown sources (3%) [9] (Figure 1).

The major reasons for human mortality are: incorrect diagnosis, delayed post-exposure prophylaxis, refusal from vaccination of domestic carnivores [9].

Vaccination

19,842,548 domestic animals, including livestock were vaccinated in the Russian Federation in 2019. Besides 20,684,436 vaccine baits were distributed for wild animals. 8,548,904 cats and dogs out of 52.6 million population were vaccinated, it means 16.25%, which is clearly not enough to develop an effective herd immunity (vaccination is effective if 90% of domestic animals (dogs) and at least 70% of wild carnivores are immunized [10, 11]).

The graphs presenting correlation between morbidity and vaccination coverage in domestic and wild carnivores for the last 10 years by Federal Districts are given below (Fig. 2–4).

The correlation between the vaccination coverage and morbidity was: $r = 0.96$ for domestic carnivores; $r = 0.86$ for wild carnivores; $r = 0.95$ for livestock. Herewith no correlation was established between the number of tests and animal morbidity. Based on the results of correlation analysis, it may be concluded that it is not effective to increase the vaccination coverage, if the current approaches to prevention are maintained, i.e. if the risk populations are not regarded. However this supposed ineffectiveness of the vaccination programme through the increase in urban vaccination coverage among susceptible animals is associated with the practice to vaccinate only registered domestic carnivores (service animals, exhibition animals, animals kept in shelter and so on) alongside a high morbidity of stray and this means non-vaccinated animals. The effectiveness of urban rabies prevention directly depends on the vaccination coverage among target populations [11]. It is recommended to develop measures, aimed at recording and control of urban animals (microchipping, passport system, population control) and increase the vaccination coverage up to 90% [10].

As for wild animals, the supposed ineffectiveness of the vaccination programme through the increase in the number of baits distributed is probably associated with the focus on fox populations, while the number of the other wild carnivore species populations is unknown. Moreover such carnivores are strongly involved into the epidemic process. It is recommended to develop oral vaccination tools for most species of wild carnivores to cover at least 70% of population [10].

Livestock is the dead-end of the rabies epidemic chain. These animals are often vaccinated to comply with the requirements of anti-epidemic measures in the rabies outbreak area, herewith not all livestock facilities stick to biosecurity norms, which creates the risk for the livestock to get infected from wild or stray carnivores. That is why, when planning vaccination of livestock it is recommended to use a risk-oriented approach and pay special attention to the vaccination programme for high risk populations with due consideration of biosecurity requirements and within the set of measures to decrease rabies spread (90% vaccination coverage for urban populations and 70% vaccination coverage for wild carnivores [10, 11]).

Post-Exposure Prophylaxis

A factor, facilitating death rate increase, is a lack of public awareness about potential risks of bites/licking/

Table 2
Ranking of RF Federal Districts based on rabies infection level

Таблица 2
Ранжирование федеральных округов РФ по уровню неблагополучия по бешенству

Federal District	Mean Epidemicity Index (2010–2019)	Rank [8] (risk level*)
Central (CFD)	0.99	0.9–1.0 (catastrophic)
Volga (VFD)	0.978	
North Caucasian (NCFD)	0.88	> 0.9–0.5 (high)
Southern (SFD)	0.86	
Ural (UFD)	0.85	
Siberian (SbFD)	0.77	
Far East (FEFD)	0.35	> 0.5–0.1 (moderate)
Northwestern (NWFD)	0.32	
Baikonur	0.0	> 0.1 (low)

*Risk levels:
catastrophic – the disease was reported in all regions throughout the observation time (practically 100% likelihood of the disease spread to the other Federal Districts);
high – the disease is reported in most regions, the improvement of the situation within the studied period is minimal (high likelihood of the disease spread to the other Federal Districts);
moderate – less than half of the regions are infected (within the observation period the tendency towards improvement was noted; the likelihood of the disease spread to the other Federal Districts is low);
low – the territory is free or sporadic rabies cases are reported (very low likelihood of the disease spread to other regions due to natural or artificial geographical barriers).

*Уровни риска:
катастрофический – заболевание выявлено во всех регионах и за все время наблюдения (практически 100%-я вероятность распространения в другие федеральные округа);
высокий – заболевание наблюдается в большинстве регионов, улучшение ситуации за исследуемый период минимальное (высокая вероятность распространения в другие федеральные округа);
умеренный – меньше половины регионов затронуты заболеванием (в течение срока наблюдения прослеживается тенденция к улучшению эпизоотической ситуации; вероятность распространения в другие регионы мала);
низкий – благополучие территории или спорадические случаи бешенства (очень низкая вероятность распространения в другие регионы ввиду наличия естественных и искусственных географических барьеров).

scratching by both wild and domestic animals and poor patient compliance.

When a victim seeks for a medical help, his/her wound is treated depending on the type of the wound, antirabic immunoglobulin is injected and then COCAV antirabic vaccine is used on 0, 3, 7, 14, 30 and 90 days post exposure. In most cases, this therapy is effective, but if the wound is located close to brain or the wound is vast, the virus high infective dose nullifies all the efforts to prevent the disease clinical signs.

In 2010–2019 60 people died from rabies in the Russian Federation due to the following reasons: failure to seek medical assistance – 67.8%; unauthorized violation of the vaccination scheme – 17.9%; fault of medical staff – 1 person (3.6%); bites of dangerous location, notwithstanding the correct therapy and short incubation period – 2 persons (7.1%); unknown reasons – 1 person (3.6%). 195 persons died from rabies during the previous 20 years [9].

In 2019, 397,248 cases of licking/scratching/biting were reported, out of them 10,232 exposures from wild animals, followed by two human deaths were registered [9].

Due to the fact, that non-carnivorous animals and humans are the end hosts of the infection, we used prevalence values among wild and domestic animals for the risk assessment.

The modeling results (Fig. 5, 6) demonstrate that if the vaccination is not practiced, the most probable number of infected humans can be: 7–8 humans after exposures from wild animals; four humans after exposures from domestic animals. If wild and domestic animals are vaccinated, the probable number of infections is decreased: five humans after exposures from wild animals and three humans after exposures from domestic animals. Herewith the number of infected animals in wild population decreases from 587 to 409 individuals, in domestic population from 586 to 504 individuals.

Hypothetic increase in the vaccine potency to 100% does not significantly influence the situation: the expected number of infected people is not changed.

Some authors describe the 40–60% uptake rate of vaccine baits [6], while the other publications specify 80–90% rates [12]. We took the mean value of a lower uptake rate for our modeling at high risks of spreading. This is associated

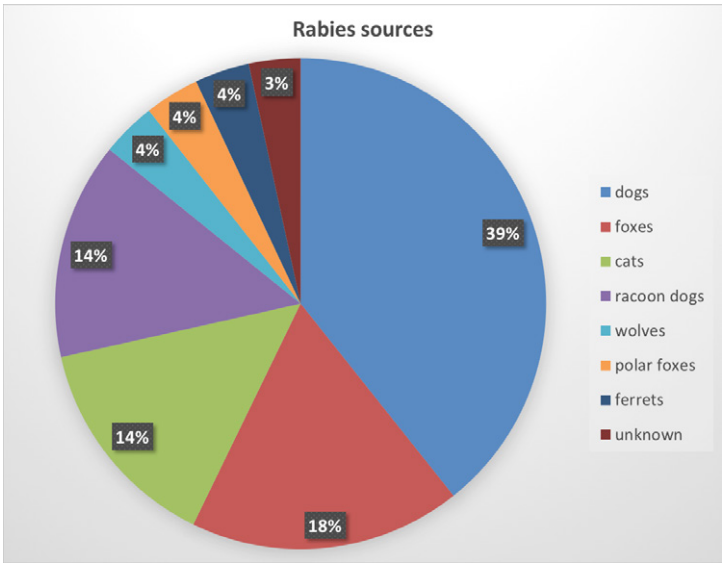

Fig. 1. Human infection sources [9]

Рис. 1. Источники заражения людей бешенством [9]

with the fact that we did not take into account the other species of carnivores, which are not vaccinated in contrast to foxes, but they are involved into the epidemic process (martens, badgers, corsac foxes, etc.). That is why there is a certain proportion of biased data and modeling results, which in turn necessitates the need to increase the volume of output data.

Increased bait uptake rate by wild animals could lead to a greater effect. For example, if bait uptake rate by foxed is 100%, the number of infected people post exposures from wild animals could be decreased to three persons.

The problem of vaccination lies in impossibility to catch and vaccinate all stray animals. It is necessary to immunize domestic carnivores, which have the access to outdoors, and animals, residing in private houses. Today the oral vaccination is envisaged only for foxes due to their specific uptake behavior, needed for effective vaccination [7]. However, other susceptible animals remain intact and are actively involved into the epidemic process.

Average annual human mortality rates due to rabies suggest the lack of public awareness raising events, contributing to understanding of the necessity to take prevention measures.

Animal movement surveillance

In 2010–2019, 10 cases of imported rabies were reported. In most cases, these were tourists, who had contacts with diseased animals in other countries and sought for medical assistance due to clinical signs, manifested at home [9].

279,391 movements of domestic carnivores between regions were registered in 2019. Pursuant to p. 4.11 of Sanitary Rules (SP) 3.1.096-96, Veterinary Rules (VP) 13.3.1103-96 (approved by the State Committee of Public Health Surveillance No. 11 on May 31, 1996 and Ministry of Agriculture and Food No. 23 on June 18, 1996) "Prevention and Control of Infectious Diseases Common for Humans and Animals. 13. Rabies" movements of dogs out of the Oblast (Krai, Republic) are allowed only if they are accompanied with the veterinary certificate, bearing the stamp on rabies vaccination.

Based on the reports of the Rosselkhoznadzor Territorial Administrations eight attempts to move carnivores, accompanied with incorrectly prepared veterinary and sanitary documents were detected and prevented in 2019.

The prevented attempts of illegal movements suggest that the system functions well, but the danger of non-vaccinated domestic carnivores, moved by summer visitors

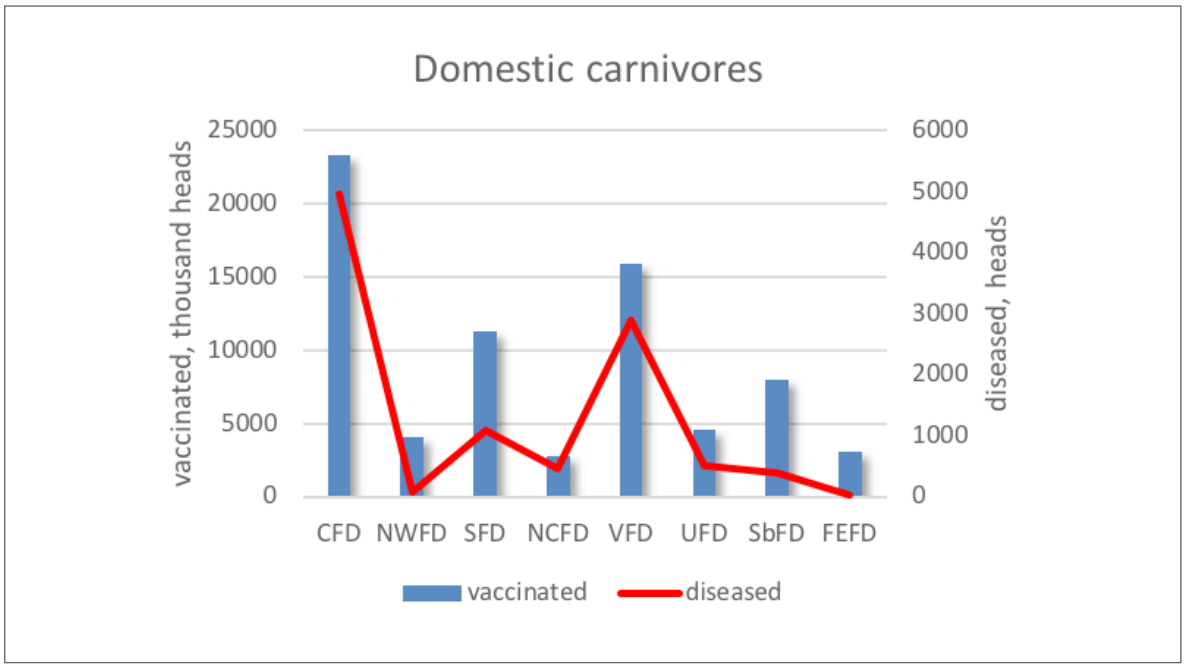

Fig. 2. Correlation between morbidity and vaccination coverage against rabies in domestic carnivores (2010–2019)

Рис. 2. Корреляция заболеваемости с фактическим охватом вакцинацией против бешенства у домашних плотоядных за 2010–2019 гг.

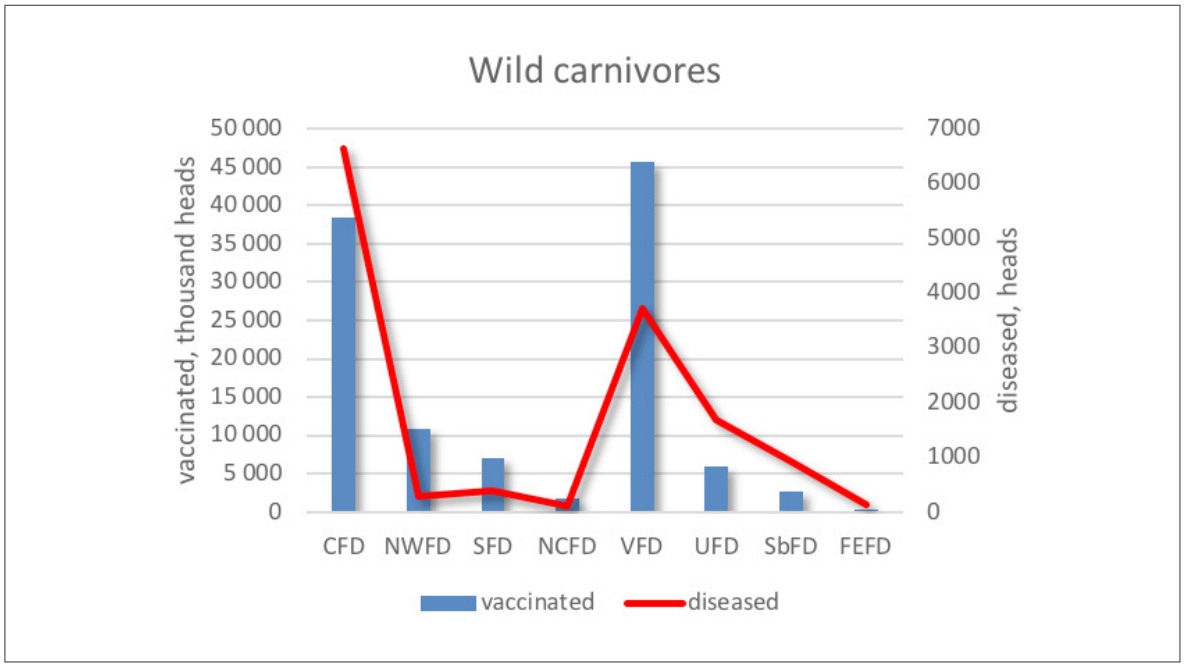

Fig. 3. Correlation between morbidity and amount of distributed baits against rabies in carnivores (2010–2019)

Рис. 3. Корреляция заболеваемости с количеством разложенных приманок против бешенства у диких плотоядных за 2010–2019 гг.

(to the summer houses and back), tourists (for the vacation) and hunters (to hunting areas) should be taken into account.

CONCLUSION

Rabies is a serious threat for human and animal lives. Notwithstanding the efforts made, two persons died and about 400 thousand peoples received antirabic treatment in 2019. These figures suggest the lack of public awareness campaigns and the need to develop and implement additional measures to prevent rabies. Such measures include, except for the stamp on rabies vaccination in the veterinary certificate, if an animal to be moved out of the region, the enhancement of movement

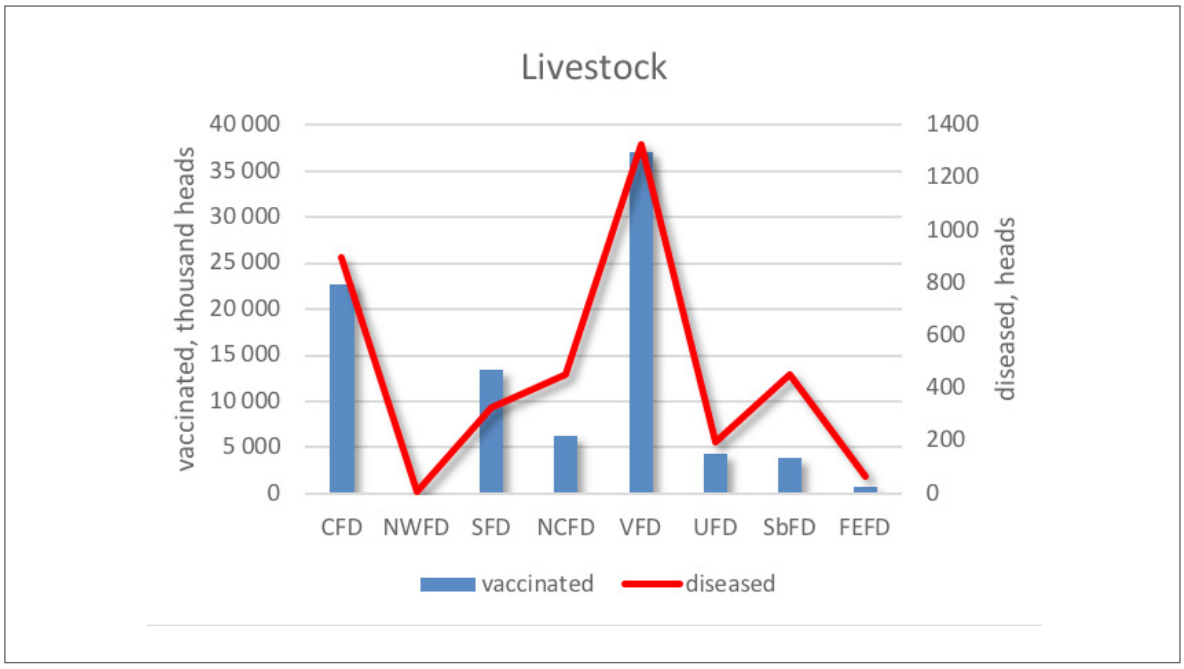

Fig. 4. Correlation between morbidity and vaccination coverage against rabies in livestock (2010–2019)

Рис. 4. Корреляция заболеваемости с фактическим охватом вакцинацией против бешенства у сельскохозяйственных животных за 2010–2019 гг.

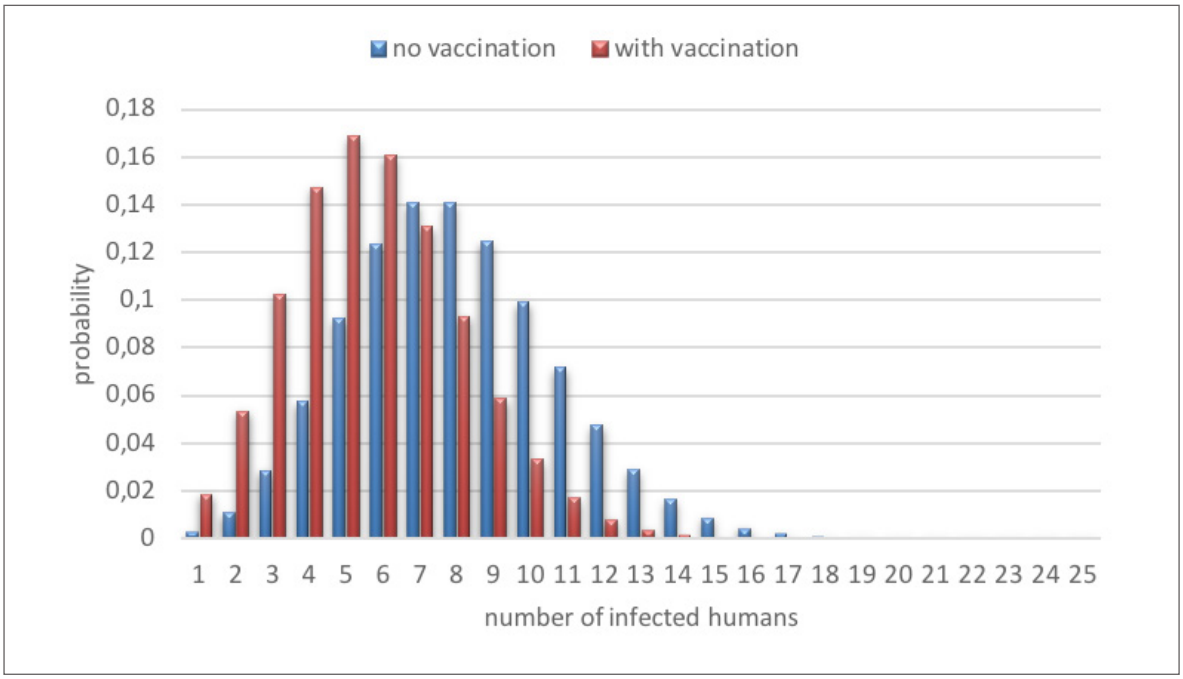

Fig. 5. Distribution of the expected number of infected humans when bitten by wild animals (vaccinated and non-vaccinated)

Рис. 5. Распределение ожидаемого количества инфицированных людей при покусах дикими животными (с вакцинацией и без вакцинации)

control for susceptible non-vaccinated animals within the Oblast (Krai, Republic).

The direct link between vaccination coverage and morbidity among target groups is established, which at first glance, suggests the inadequacy of the programme on vaccination coverage increase. The detailed analysis showed the necessity of a more careful vaccination programme planning, involving control of populations and coverage of a bigger number of animals. The lack of vaccination coverage for the purposes of effective

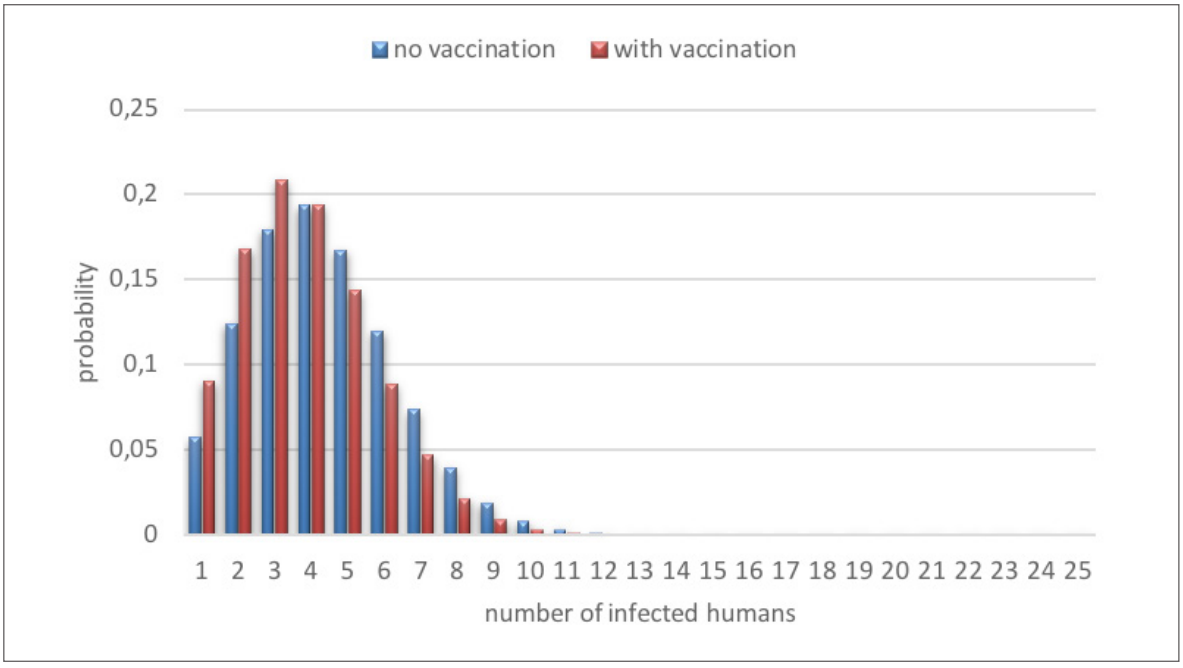

Fig. 6. Distribution of the expected number of infected humans when bitten by domestic animals (vaccinated and non-vaccinated)

Рис. 6. Распределение ожидаемого количества инфицированных людей при покусах домашними животными (с вакцинацией и без вакцинации)

herd immunity and disease restrain was established. It was revealed that out of 52.6 million dogs and cats, only 8,548,904 animals were vaccinated, which is 16.25% of the population. The necessity to improve the methods of wild carnivore vaccination was shown, due to the focus on oral vaccination of foxes.

The data obtained support the relevance of measures, envisaged by the Set of Joint Measures of the CIS Member Countries to prevent and control rabies till 2025. The implementation of the Set will enable to reach rabies freedom in target populations (if 90% of domestic animals (dogs) and 70% of wild carnivores are vaccinated) [10].

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Optimization of cultivation parameters for bovine respiratory syncytial virus strain Vologda/2019

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SUMMARY

There are currently many controversial issues in the study of bovine respiratory syncytial infection. In this regard, it is relevant to study the biological properties of the virus, optimize the methods of its cultivation and select the most technologically advanced methods of designing diagnostic and prevention tools for this disease. The aim of this work was to select sensitive cell systems and to optimize the cultivation parameters in selected cell cultures. The Vologda/2019 strain of the bovine respiratory syncytial infection virus isolated from biological material obtained from a calf with respiratory symptoms was used in the experiment. The strain was adapted to the continuous cell culture derived from bovine turbinate tissue (BT) and deposited in the State collection of microorganism strains at FGBI "ARRIAH". It was established that the continuous cell lines of fetal bovine trachea (FBT) and calf kidney (RBT) are the most sensitive cell systems for the reproduction of the bovine respiratory syncytial virus strain Vologda/2019, the maximum accumulation of the virus was observed in these cell cultures. The cytopathic activity of the virus in the FBT cell culture ranged from 4.78 ± 0.18 to 5.50 ± 0.16 lg $TCID_{50}/cm^3$, and in the RBT cell culture – from 4.00 ± 0.23 to 4.75 ± 0.20 lg $TCID_{50}/cm^3$ at days 4–5 of cultivation. It was determined that in case of multiplicity of inoculation of FBT and RBT cell cultures with the virus at 0.1 lg TCD_{50}/cell and the use of 2% glutamine in the maintenance nutrient medium, as well as 2% horse or cattle blood serum, it is possible to obtain virus material with high cytopathic activity.

Key words: bovine respiratory syncytial virus, Vologda/2019 strain, cultivation, cytopathic activity, virus titer.

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Оптимизация параметров культивирования вируса респираторно-синцитиальной инфекции крупного рогатого скота штамма «Вологда/2019»

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РЕЗЮМЕ

В настоящее время в изучении респираторно-синцитиальной инфекции крупного рогатого скота есть множество спорных вопросов. В связи с этим актуальным является изучение биологических свойств вируса, оптимизация методов его культивирования и подбор наиболее технологичных приемов конструирования средств диагностики и профилактики данного заболевания. Целью настоящей работы являлись выбор чувствительных клеточных систем и оптимизация параметров культивирования в подобранных культурах клеток. В опытах использовали штамм «Вологда/2019» вируса респираторно-синцитиальной инфекции крупного рогатого скота, выделенный из биологического материала, полученного от теленка с признаками респираторной патологии. Штамм адаптирован к перевиваемой культуре клеток слизистой носовых перегородок крупного рогатого скота (ВТ) и депонирован в Государственную коллекцию штаммов микроорганизмов ФГБУ «ВНИИЗЖ». Установлено, что перевиваемые линии клеток трахеи эмбриона крупного рогатого скота (FBT) и почки теленка (RBT) являются наиболее чувствительными клеточными системами для репродукции респираторно-синцитиального вируса крупного рогатого скота штамма «Вологда/2019», в данных культурах клеток отмечалось максимальное накопление вируса. Цитопатическая активность вируса в культуре клеток FBT на 4–5 сут культивирования составила от 4,78 ± 0,18 до 5,50 ± 0,16 lg $ТЦД_{50}$/$см^3$, а в клеточной системе RBT – от 4,00 ± 0,23 до 4,75 ± 0,20 lg $ТЦД_{50}$/$см^3$. Определено, что при множественности заражения культур клеток FBT и RBT вирусом в 0,1 lg $ТЦД_{50}$/кл, использовании в составе поддерживающей питательной среды 2% глютамина, а также 2% сыворотки крови лошади либо крупного рогатого скота удается получить вирусный материал с высокой цитопатической активностью.

Ключевые слова: респираторно-синцитиальный вирус крупного рогатого скота, штамм «Вологда/2019», культивирование, цитопатическая активность, титр вируса.

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INTRODUCTION

Implementation of commercial methods in livestock farming, on the one hand, led to increase in productivity, and on the other hand, caused such serious problems as favorable conditions for massive spread of infectious diseases. Respiratory infections take the leading position among most common bovine diseases [1]. They can emerge both independently (parainfluenza-3, infectious rhinotracheitis, viral diarrhea, adenoviruses, respiratory syncytial infection) and in various associations of viral and bacterial etiology, causing enormous economic losses to livestock industry [2–4].

To date bovine respiratory syncytial virus (BRSV) infection has been registered in many countries across the globe (Japan, Germany, USA, Croatia, Belgium), as well as, since 1975, in Russia [4–6].

BRSV belongs to the family *Pneumoviridae*, genus *Orthopneumovirus*, species *Bovine orthopneumovirus*. All cattle breeds, as well as buffaloes, aurochs, yaks, bison, zebu, etc. are susceptible to the virus. According to the majority of domestic and foreign authors, the most BRSV-susceptible cattle are calves aged 1–12 months. However, the disease is less common in young animals up to 4 weeks of age; this fact may be associated with a more responsible attitude and proper care of newborn calves. There is evidence of outbreak occurrence among adult cattle, which may be associated with the mechanical penetration of the pathogen into the herd, where no measures were applied to prevent BRSV entry. Such cases include purchase of infected livestock, use of non-authorized cattle movement areas for transportation, etc. The incubation period lasts 2–5 days. There are three forms of the disease: subclinical, acute and hyperacute [7].

Diseased or convalescent animals often appear the source of infection. The most likely way of the virus transmission is via respiratory aerosols or from contact, through secretions from eyes, nose and tracheal mucosa. A number of authors have shown that intrauterine infection facilitates circulation of the respiratory syncytial virus in the herd. There is no data on transmission of the BRSV pathogen in cattle with semen; this issue needs further study [6, 8, 9].

At present, many issues related to the course of infection also remain insufficiently studied. It is known that BRSV replicates in respiratory tract cells, however, there are data of some Russian researchers on the virus adaptation to bovine kidney and testicular cell cultures [2, 7, 10].

BRSV diagnosis is made on the basis of epizootological and clinical data, pathological changes and laboratory test results. Laboratory diagnosis includes detection of specific antibodies using enzyme-linked immunosorbent assay (ELISA) or neutralization test; identification of the respiratory syncytial virus genome using real-time reverse transcription polymerase chain reaction (real-time RT-PCR); virus isolation from biological material derived from animals using culture techniques [2, 3, 8]. Blood sera, nasal discharge samples, tracheal and bronchial exudates, lung and bronchi fragments are commonly used as test material [5, 10, 11].

BRSV is often difficult to isolate in cell culture due to its lability and instability in the environment [7, 8]. The result of virus isolation in cell culture and its further identification using PCR or ELISA largely depend on the proper selection, storage, and transportation of biomaterial samples from diseased animals [10]. According to the data of domestic and foreign authors primary trypsinized cultures of bovine embryonic cells (bovine embryonic kidney (BEK) cells, bovine embryonic lung (BEL) cells, etc.) are used for virus isolation [7–9].

Real-time RT-PCR along with retrospective serological methods are the most promising methods for BRSV diagnosis [9]. It should be noted that virological methods play a significant role in the development of diagnostic systems and means of specific disease prevention. It is extremely important to obtain highly potent cultural virus material for production of high-quality test systems, kits, vaccines and specific sera [2, 3, 12].

Thus, the study of the viral biological properties, optimization of cultivation and selection of components are of great relevance for developing diagnostic and prevention tools for this disease.

The aim of this work was to optimize the cultivation parameters for BRSV strain Vologda/2019 in previously selected sensitive cell systems.

MATERIALS AND METHODS

BRSV strain Vologda/2019 isolated from biological material obtained from a calf with respiratory symptoms and adapted to the continuous bovine turbinate tissue (BT) cell culture with the infectivity titer of 4.0 lg $TCID_{50}/cm^3$ was used in the experiment. In 2019 the obtained BRSV strain Vologda/2019 was deposited in the State collection of microorganism strains at FGBI "ARRIAH".

In order to study the cultural properties of the indicated BRSV strain and its adaptation to high-performance cell lines, the following animal cell culture systems were used: bovine calf kidney (RBT), mucous membranes of fetal bovine nasal septum (FBN), fetal bovine trachea (FBT), rhesus monkey kidney (MA-104), goat gonads (YaDK-04) [13].

A 24-hour cell monolayer grown in 25–175 cm^3 plastic flasks was used for BRSV cultivation in continuous cell lines. The initial cell concentration in the cell suspension was 100–300 ths/cm^3. BRSV strain Vologda/2019 was inoculated into the cell culture at a dose of 0.1 $TCID_{50}$/cell. The cultivation time was 6–8 days provided that the monolayer integrity was preserved.

The sensitivity of continuous cell culture to BRSV strain Vologda/2019 was determined by successive passages [12]. For that, the culture inoculation was preceded by virus adsorption in monolayer cells, and no maintenance nutrient medium was added.

The virus was inoculated after removal of growth nutrient medium, then it was allowed to contact with the cell monolayer in CO_2 incubator at 37 °C for 1.5 h and after that maintenance semi synthetic nutrient medium (SSM) supplemented with 2% bovine or horse serum and glutamine was added. The virus material was collected when cytopathic effect (CPE) was demonstrated in 70–80% cell monolayer surface. The obtained virus was stored at minus 80 °C. The virus cytopathic effect was determined by virus microtitration assay [12] in BT or FBT cell cultures. A distinctive feature of this study was that BRSV strain Vologda/2019 was inoculated onto cell monolayer previously grown for 24 hours.

BRSV microtitration for each passage was performed in sterile 96-well flat-bottom microtitration plates at 0.2 cm^3 per well. Dilutions of virus-containing material (VCM) in SSM (10^{-1}–10^{-8}) were prepared in sterile Eppendorf tubes for that pupose [12]. The prepared virus dilutions were transferred with a single-channel mechanical pipette into the wells of a culture plate with a grown monolayer of FBT or BT cell cultures at 0.1 cm^3 per well starting with the highest dilution. The plate was placed in a CO_2-incubator with 5% carbon dioxide at 37 °C for 1.5 h for virus adsorption in the monolayer cells. After virus and cell monolayer contacted, 0.1 cm^3 of SSM supplemented with 2% horse blood serum and glutamine was added. Observation was carried out using Olympus CKX53 inverted microscope (40×–400× magnification) and color phase-contrast sliders of the warm and cold spectrum to increase the image sharpness. The final reading of the virus titration results were performed after 10 days of incubation, provided that the cell monolayer integrity in control wells was preserved [12].

The infectivity level was calculated according to Reed and Muench method and expressed as lg $TCID_{50}/cm^3$ [12].

BRSV antigen titer in inactivated preparations was performed using the 'ELISA kit for antigenic diagnosis of Bovine Respiratory Syncytial Virus (BRSV)' (Bio-X Diagnostics, Belgium) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Five successive passages were performed in RBT, FBN, FBT, MA-104 and YaDK-04 culture systems to study sensitivity of various cell cultures to BRSV strain Vologda/2019.

The virus-induced CPE varied in all cell cultures. At day 3 local changes in individual cells and/or cell groups were observed in RBT cell culture (Fig. 1, 2).

After 4–5 days, local CPE foci were formed in RBT cell culture as a result of BRSV strain Vologda/2019 replication, the main "pattern" of the monolayer became smooth due to cell deformation, and detachment of a large number of cells and their structural elements in suspension was observed (Fig. 3). Figure 4 clearly shows the difference between a supposedly pure and inoculated culture, that is, practically no cell detachment is observed in suspension, there are no changes, the monolayer "pattern" is distinct and individual cells are clearly visible.

At day 7–8 the CPE induced by BRSV strain Vologda/2019 reached 70–80% in RBT cell culture. The monolayer was dispersed, the cells were destroyed, "holes" were formed (Fig. 5). When a similar situation was observed, the virus-containing material was frozen at minus 80 °C and placed for storage. Control test results with no virus inoculation are shown in Figure 6.

Cell changes involving accumulation of destroyed or deformed cells could be observed in FBT cell culture at

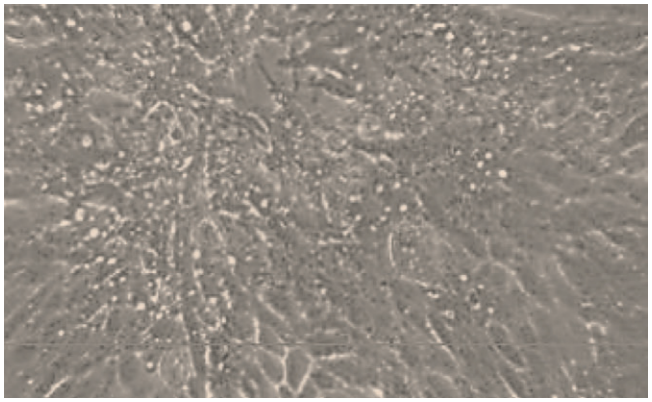

Fig. 1. BRSV-inoculated RBT cell culture (day 3, ×400 magnification)

Рис. 1. Культура клеток RBT, инокулированная вирусом РСИ КРС (3-и сут, увеличение ×400)

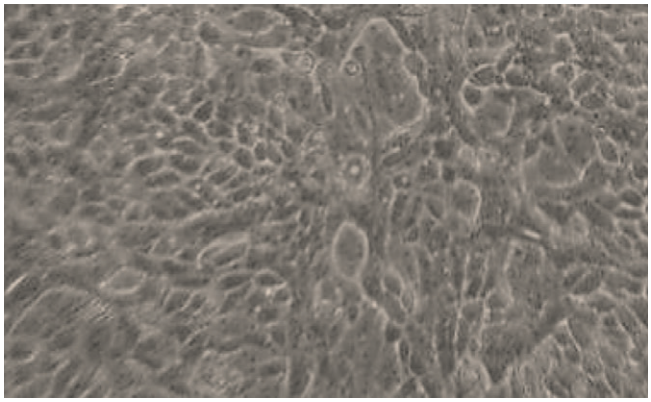

Fig. 2. Non-BRSV-inoculated RBT cell culture (day 3, ×400 magnification)

Рис. 2. Культура клеток RBT без инокуляции вируса РСИ КРС (3-и сут, увеличение ×400)

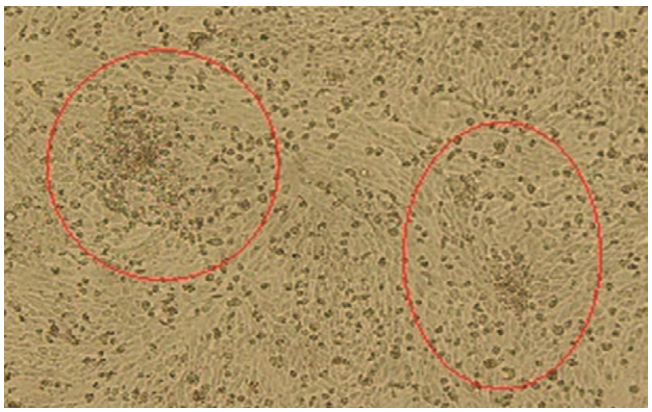

Fig. 3. RBT cell culture inoculated with BRSV (day 5, ×200 magnification)

Рис. 3. Культура клеток RBT, инокулированная вирусом РСИ КРС (5-е сут, увеличение ×200)

Рис. 4. Культура клеток RBT без инокуляции вируса РСИ КРС (5-е сут, увеличение ×200)

Fig. 4. RBT cell culture not inoculated with BRSV (day 5, ×200 magnification)

day 4 of virus cultivation (Fig. 7). Control test results with no virus inoculation are shown in Figure 8.

Further studies showed more pronounced CPE caused by BRSV strain Vologda/2019. By a third passage 80% monolayer destruction and formation of conglomerates and syncytia were observed on day 7 of cultivation in FBT cell culture (Fig. 9). When a similar pattern was observed, the virus containing-material was frozen at minus 80 °C and placed for storage. Control test results with no virus inoculation are shown in Figure 10.

The virus CPE was not observed in MA-104 and FBN cell cultures; the virus titers measured by ELISA confirmed that BRSV strain Vologda/2019 is not replicated in these cell systems (titer decline in percentage equivalence). At passages 4 and 5 no changes were observed in the cell monolayer of MA-104 and FBN cultures.

For YaDK-04 cell culture, the viral CPE was manifested by occasional cell rounding and monolayer thinning. The microtitration test showed that the virus consistently replicates in this cell system, but the levels of virus accumulation differ from those in RBT and FBT cell cultures. This fact indicates the need to optimize the cultivation parameters to achieve the best result.

BRSV strain Vologda/2019 titers were measured for each passage in BT cell culture by microtitration method, and the specificity of the obtained BRSV preparations was confirmed using ELISA (Table 1).

The studies showed that during BRSV strain Vologda/2019 cultivation in FBT cell culture typical CPE was observed on days 4–5 after passages 3–5. By passage 5 the viral CPE level gradually amounted to 5.50 ± 0.16 lg $TCID_{50}/cm^3$. BRSV titer was 1:2 at passage 1 and 1:64 at passage 5 in ELISA. A similar trend was observed during BRSV cultivation in RBT cell culture: on day 4–5 of cultivation (passage 5) the virus titer was 4.75 ± 0.20 lg $TCID_{50}/cm^3$, and it increased to 1:32 in ELISA.

Inoculation of BRSV strain Vologda/2019 in MA-104 and FBN cell systems led to a decrease in its activity to 1.50 ± 0.17 lg $TCID_{50}/cm^3$, and the virus was not detected at passages 4–5. A similar decrease in the virus titer was confirmed by ELISA.

During BRSV strain Vologda/2019 cultivation in YaDK-04 cell culture stable virus activity was noted throughout all 5 passages (2.50 ± 0.17 – 3.33 ± 0.18 lg $TCID_{50}/cm^3$). It is possible that by optimizing the cultivation parameters in YaDK-04 cell system, a higher cytopathic effect of the virus

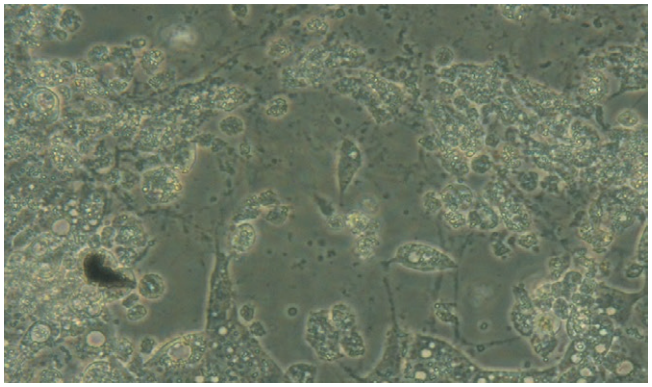

Fig. 5. BRSV-inoculated RBT cell culture (day 7, ×200 magnification)

Рис. 5. Культура клеток RBT, инокулированная вирусом РСИ КРС (7-е сут, увеличение ×200)

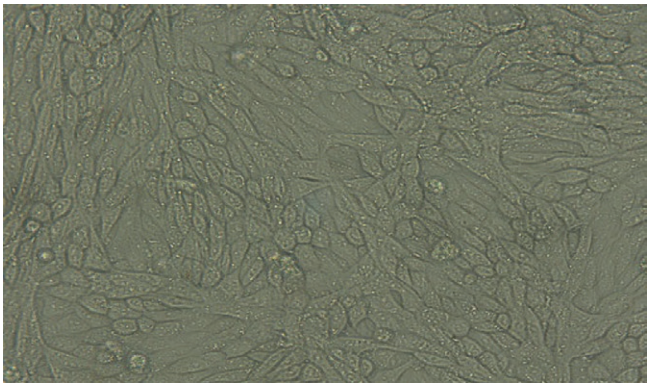

Fig. 6. Non-BRSV-inoculated RBT cell culture (day 7, ×400 magnification)

Рис. 6. Культура клеток RBT без инокуляции вируса РСИ КРС (7-е сут, увеличение ×400)

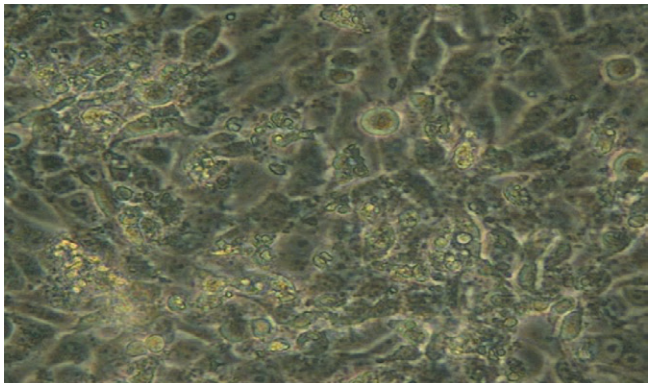

Fig. 7. BRSV-inoculated FBT cell culture (day 4, ×400 magnification)

Рис. 7. Культура клеток FBT, инокулированная вирусом РСИ КРС (4-е сут, увеличение ×400)

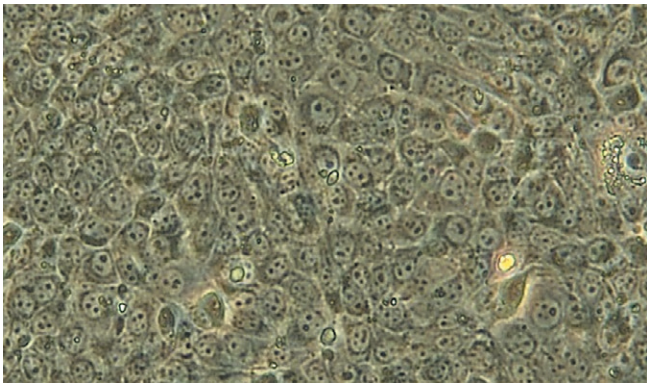

Fig. 8. Non-BRSV-inoculated FBT cell culture (day 4, ×400 magnification)

Рис. 8. Культура клеток FBT без инокуляции вируса РСИ КРС (4-е сут, увеличение ×400)

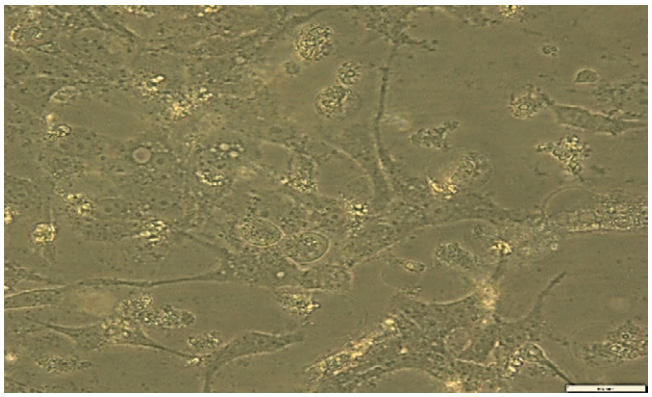

Fig. 9. BRSV-inoculated FBT cell culture (day 7, ×400 magnification)

Рис. 9. Культура клеток FBT, инокулированная вирусом РСИ КРС (7-е сут, увеличение ×400)

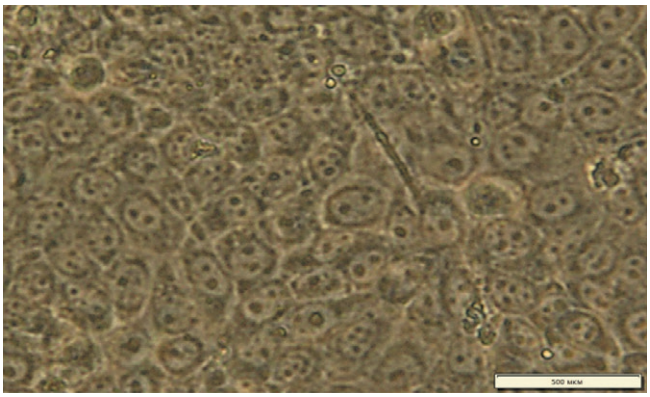

Fig. 10. Non-BRSV-inoculated FBT cell culture (day 7, ×400 magnification)

Рис. 10. Культура клеток FBT без инокуляции вируса РСИ КРС (7-е сут, увеличение ×400)

can be obtained. The virus titer for this viral raw material in ELISA was 1:2 at the 1st passage and 1:4 at the subsequent ones, which is considered a stable result, but it is insufficient for the production of a high-quality virus-containing preparation.

The tests showed that RBT and FBT cell cultures are the most suitable cell systems for BRSV strain Vologda/2019 propagation. MA-104 and FBN cell systems turned out to be unsuitable for BRSV strain Vologda/2019 accumulation and reproduction.

Table 1
Titers of BRSV strain Vologda/2019 in various cell cultures ($n = 3$)

Таблица 1
Титр вируса РСИ КРС штамма «Вологда/2019» в различных культурах клеток ($n = 3$)

Cell culture	Passage No.	Cultivation period, days	Virus titer, lg $TCID_{50}/cm^3$	Virus titer by ELISA, dilution
RBT	1	8–10	3.0 ± 0.25	1:2
	2	8–10	3.63 ± 0.07	1:4
	3	7–10	3.56 ± 0.06	1:4
	4	4–5	4.0 ± 0.23	1:16
	5	4–5	4.75 ± 0.20	1:32
FBT	1	8–10	3.63 ± 0.15	1:2
	2	8–10	4.0 ± 0.22	1:4
	3	7–10	4.33 ± 0.23	1:4
	4	4–5	4.78 ± 0.18	1:32
	5	4–5	5.5 ± 0.16	1:64
MA-104	1	10	1.83 ± 0.16	1:2
	2	10	1.67 ± 0.16	1:2
	3	10	1.50 ± 0.17	1:2
	4	10	n/d	–
	5	10	n/d	–
FBN	1	10	2.23 ± 0.23	1:2
	2	10	1.67 ± 0.10	1:2
	3	10	1.50 ± 0.17	1:2
	4	10	n/d	–
	5	10	n/d	–
YaDK-04	1	9	2.50 ± 0.17	1:2
	2	10	3.25 ± 0.25	1:4
	3	9	3.12 ± 0.18	1:4
	4	8	3.16 ± 0.17	1:4
	5	9	3.33 ± 0.18	1:4

n/d – not detected (не обнаружено);
«–» – negative result (отрицательный результат).

In order to increase the virus titer it was necessary to select the optimal dose for cell culture infection. Highly efficient RBT and FBT cell systems were chosen. To inoculate BRSV strain Vologda/2019 strain into the selected cell cultures, the virus-containing material was diluted to 0.001–0.1 $TCID_{50}$/cell (Fig. 11).

As it is demonstrated in Figure 11, at multiplicity of infection 0.001 $TCID_{50}$/cell, the CPE levels of BRSV strain Vologda/2019 in FBT and RBT cell cultures were 2.50 and 2.75 lg $TCID_{50}/cm^3$, respectively. At multiplicity of infection 0.01 $TCID_{50}$/cell the virus CPE in FBT and RBT cell systems was 4.00 and 3.25 lg $TCID_{50}/cm^3$, respectively. Also, slow virus accumulation was noted in RBT and FBT cell cultures at a given infection dose; the cultivation time to achieve 80% monolayer destruction induced by BRSV was 9 and 12 days, respectively. Given the resistance of cell systems to aging

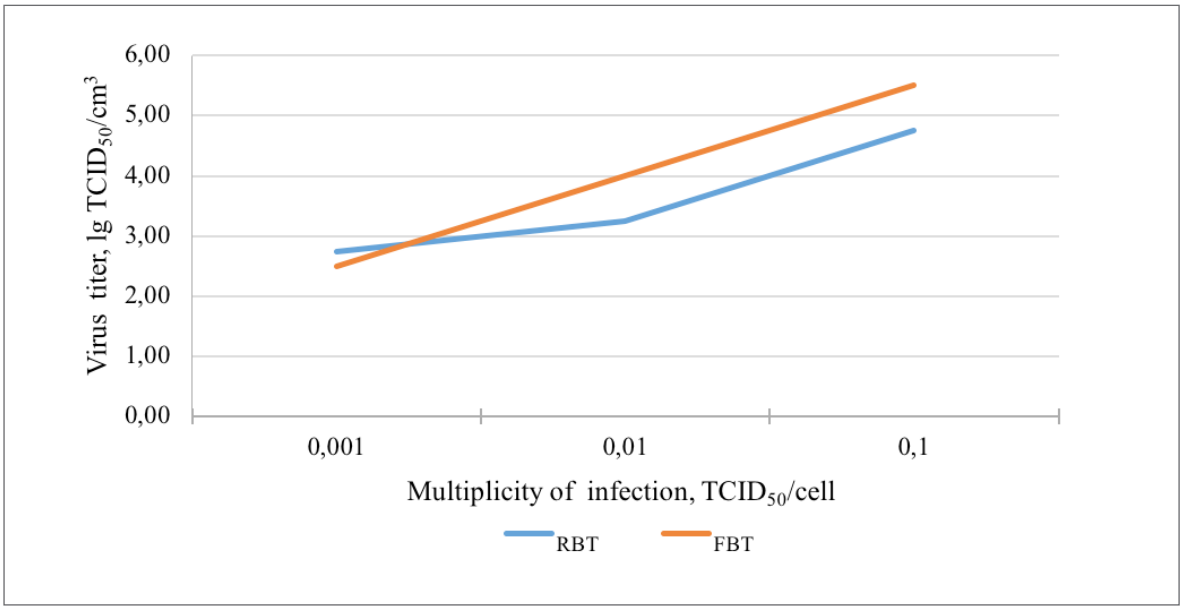

Fig. 11. Effects of inoculation multiplicity on the BRSV strain Vologda/2019 activity (n = 3)

Рис. 11. Влияние множественности заражения на активность вируса РСИ КРС штамма «Вологда/2019» (n = 3)

and death (18 days before the onset of cell degradation for FBT and 16 days for RBT), this result can be considered acceptable. However, the study results showed that with an increase in the multiplicity of infection to 0.1 $TCID_{50}/cm^3$, 80% monolayer destruction and virus accumulation in RBT and FBT cell cultures proceeded faster (4 and 5 days, respectively), while the CPE titer of BRSV strain Vologda/2019 was 4.75 ± 0.16 and 5.50 ± 0.80 lg $TCID_{50}/cm^3$, respectively. Therefore, this level of multiplicity of infection is optimal.

In order to optimize the composition of the semi synthetic nutrient medium used as a maintenance one for inoculation of BRSV strain Vologda/2019, it was decided to assess the effect of supplementation with glutamine at different concentrations of the original tested substance that had been previously shown by T. Yu. Kochish [1] (Table 2).

According to Table 2, the glutamine supplementation of the nutrient medium resulted in gradual increase of BRSV strain Vologda/2019 CPE. When 0.1 and 0.5% glutamine were added to the nutrient medium, the virus titer in RBT cell culture increased from 3.00 ± 0.25 to 3.56 ± 0.06 lg $TCID_{50}/cm^3$, which is a relatively low infectivity level for this virus. When a medium containing 0.1 and 0.5% glutamine was added at passage 3 in FBT cell culture, it was possible to obtain a virus with the infectivity titer 3.63 ± 0.15 and 4.00 ± 0.12 lg $TCID_{50}/cm^3$, respectively. It was found that the most optimal concentration of glutamine in SSM is 2%, where it was possible to achieve the maximal infectivity titer of BRSV by passage 3: 4.75 ± 0.20 lg $TCID_{50}/cm^3$ (RBT) and 5.50 ± 0.08 lg $TCID_{50}/cm^3$ (FBT). A further increase in the concentration of glutamine

Table 2
BRSV strain Vologda/2019 activity in case of glutamine-enriched nutrient medium (*n* = 3)

Таблица 2
Активность вируса РСИ КРС штамма «Вологда/2019» при обогащении питательной среды глютамином (*n* = 3)

Glutamine amount in maintenance medium, %	Virus cytopathic effect, lg $TCID_{50}/cm^3$					
	RBT			FBT		
	Passage 1	Passage 2	Passage 3	Passage 1	Passage 2	Passage 3
0.1	3.00 ± 0.25	3.25 ± 0.25	3.37 ± 0.20	3.12 ± 0.25	3.33 ± 0.22	3.63 ± 0.15
0.5	3.00 ± 0.25	3.37 ± 0.20	3.56 ± 0.06	3.33 ± 0.22	3.63 ± 0.17	4.00 ± 0.12
1	3.56 ± 0.06	3.56 ± 0.17	4.00 ± 0.23	3.63 ± 0.16	4.00 ± 0.22	4.00 ± 0.25
2	4.00 ± 0.23	4.30 ± 0.23	4.75 ± 0.20	4.33 ± 0.23	4.78 ± 0.18	5.50 ± 0.08
3	3.56 ± 0.17	4.00 ± 0.23	3.56 ± 0.06	3.83 ± 0.20	3.83 ± 0.20	3.73 ± 0.12
No-glutamine medium	3.37 ± 0.20	3.00 ± 0.25	3.56 ± 0.06	3.33 ± 0.27	3.63 ± 0.12	4.00 ± 0.17

Table 3
Effect of blood sera concentration in the nutrient medium on BRSV strain Vologda/2019 activity (*n* = 5)

Таблица 3
Влияние концентрации сыворотки крови в питательной среде на активность вируса РСИ КРС штамма «Вологда/2019» (*n* = 5)

Sera concentration in nutrient medium, %	Virus cytopathic effect, lg $TCID_{50}/cm^3$			
	RBT		FBT	
	bovine sera	horse sera	bovine sera	horse sera
0.1	3.25 ± 0.25	3.50 ± 0.15	2.75 ± 0.25	3.63 ± 0.15
0.5	3.37 ± 0.20	3.00 ± 0.25	3.25 ± 0.08	4.00 ± 0.12
1	4.30 ± 0.23	4.00 ± 0.23	3.56 ± 0.19	4.00 ± 0.25
2	4.33 ± 0.12	4.50 ± 0.23	4.75 ± 0.25	5.50 ± 0.16
3	4.00 ± 0.23	3.56 ± 0.17	4.00 ± 0.23	3.73 ± 0.12
Non-sera supplemented medium	3.00 ± 0.25	3.37 ± 0.20	3.50 ± 0.33	4.00 ± 0.17

in the semi synthetic culture medium led to a decrease in the cytopathic effect of the virus in the considered cell systems. When using the medium not supplemented with glutamine in the control samples, the virus titer was at the level from 3.00 ± 0.25 to 4.00 ± 0.17 lg $TCID_{50}/cm^3$.

Based on the data obtained, it can be concluded that 2% glutamine supplementation of the nutrient medium results in increase in the infectivity titer of the virus, and also reduces the time of the virus's maximal accumulation in FBT and RBT cell cultures.

The next stage of the experiment was to study the effect of horse and bovine sera-containing SSM on the virus reproduction.

As it is known, bovine serum is often contaminated with various viral agents, including bovine viral diarrhea virus, which can impact virus accumulation in the cell culture and reduce the cytopathic effect of the virus under study [14]. The use of horse serum could exclude contamination of the viral suspension with this pestivirus and thereby allow obtaining the virus with high biological activity.

Five consecutive passages were performed in RBT and FBT cell cultures to study impact of the tested animal sera concentration on the virus infectivity. The data are presented in Table 3.

Table 3 shows that when SSM was supplemented with 0.1 and 0.5% bovine sera, the virus infectivity levels in RBT cell culture were 3.25 ± 0.25 and 3.37 ± 0.20 lg $TCID_{50}/cm^3$, respectively, and in FBT cell culture – 2.75 ± 0.25 and 3.25 ± 0.08 lg $TCID_{50}/cm^3$, respectively. Gradual increase of bovine serum concentration from 1 to 2% in the nutrient medium resulted in increase in the infectivity titer both in RBT (4.33 ± 0.12 lg $TCID_{50}/cm^3$) and in FBT (4.75 ± 0.25 lg $TCID_{50}/cm^3$). Further increase in the concentration of bovine blood serum caused loss of infectivity in both cultures, which may be associated with the virus lability and its high sensitivity to the nutrient medium components.

When studying the effect of horse serum concentration in a nutrient medium for BRSV strain Vologda/2019 propagation, it was found that the optimal dose was 2%. When this amount of serum was added to the medium, the virus titer reached 4.50 ± 0.23 lg $TCID_{50}/cm^3$ in RBT and 5.50 ± 0.16 lg $TCID_{50}/cm^3$ in FBT, which demonstrated the best result in this study. Addition of a small (0.1%) or large (3%) dose of horse serum to SSM caused decrease in BRSV strain Vologda/2019 infectivity titer.

Thus, we can conclude that the addition of horse or bovine sera has a beneficial effect on replication of BRSV strain Vologda/2019. The optimal concentration of bovine or horse sera in the nutrient medium is 2%. Maximal virus CPE was achieved by adding the indicated amount of serum.

CONCLUSION

The study of sensitivity of continuous cell cultures to BRSV strain Vologda/2019 showed that RBT and FBT cell cultures are effective for obtaining a highly potent virus suspension. These cell culture lines can be used to obtain virus material in order to develop tools for diagnosis and specific prevention of this disease.

The optimal cell culture infection dose is 0.1 $TCID_{50}/cm^3$, thereat the virus titers were 4.75 ± 0.16 lg $TCID_{50}/cm^3$ and 5.50 ± 0.80 lg $TCID_{50}/cm^3$ for RBT and FBT, respectively.

The enrichment of the nutrient medium with 2% glutamine increased the titer of the virus cultivated in these cell systems.

The addition of horse or bovine sera has a positive impact on BRSV strain Vologda/2019 CPE. It has been established that the optimal concentration of serum in the nutrient medium is 2%. BRSV strain Vologda/2019 CPE reached maximal when this amount of serum was added.

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Basic reproduction number for certain infectious porcine diseases: estimation of required level of vaccination or depopulation of susceptible animals

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SUMMARY
Basic reproduction number (R_0) is one of the fundamental quantitative characteristics in epidemiology of infectious human and animal diseases. This parameter reflects the biological properties of the infectious agent, the social and economic aspects of animal husbandry, natural factors associated with the habitat of the animal population invaded by the virus (microorganism), as well as the effectiveness of methods selected for infection control, in particular, the implementation of preventive measures; it also allows foreseeing the number and probability of occurrence of new secondary outbreaks in the area at risk of the disease spread. The paper presents data on the estimation of basic reproduction number (R_0) for a range of infectious porcine diseases. A systematic analysis has been undertaken with respect to the publications available on the estimation of R_0 for various virus isolates of African swine fever, classical swine fever, foot-and-mouth disease, porcine reproductive and respiratory syndrome, Aujeszky's disease, hepatitis E, encephalomyocarditis, porcine circovirus type 2, as well as pleuropneumonia associated with *Actinobacillus pleuropneumoniae*, and diseases caused by pathogenic isolates of *Mycoplasma hyopneumoniae.* Based on the obtained R_0 values, recommendations for the veterinary services are made on preventive vaccination of pigs against the above mentioned diseases in the areas at risk of infection spread. The necessary conditions for wild boar depopulation aimed to prevent new African swine fever outbreaks are identified, namely, the elimination of at least 75% of the wild boar population living in the risk zone within the period of time equal to one infectious period.

Key words: infectious porcine diseases, basic reproduction number (R_0), vaccination, depopulation, wild boars.

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Базовая скорость репродукции для некоторых инфекционных заболеваний свиней: оценка необходимого уровня вакцинации или депопуляции восприимчивого поголовья животных

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РЕЗЮМЕ

Одной из основных количественных характеристик в эпидемиологии/эпизоотологии инфекционных заболеваний человека и животных является базовая скорость репродукции (R_0). Данный показатель отражает как биологические свойства самого инфекционного агента, социально-экономические способы ведения животноводства, природные факторы обитания той популяции животных, в которую внедряется вирус (микроб), так и эффективность выбора методов борьбы с инфекцией, включая проведение профилактических мероприятий, а также позволяет предвидеть количество и вероятность возможного появления новых вторичных очагов инфекции в зоне риска распространения заболевания. В работе представлены данные по оценке базовой скорости репродукции (R_0) для ряда инфекционных болезней свиней. Проведен систематический анализ имеющихся публикаций по оценке этого показателя для различных изолятов вирусов африканской чумы свиней, классической чумы свиней, ящура, репродуктивно-респираторного синдрома свиней, болезни Ауески, гепатита Е, энцефаломиокардита, цирковируса типа 2, а также актинобациллезной плевропневмонии и заболеваний, вызываемых патогенными изолятами *Mycoplasma hyopneumoniae*. На основе полученных количественных значений показателя R_0 даны рекомендации ветеринарным службам по проведению профилактической вакцинации свиней от перечисленных заболеваний в зонах риска распространения инфекции. Определены необходимые условия по депопуляции дикого кабана для предотвращения возникновения новых очагов африканской чумы свиней: уничтожение за время, равное одному инфекционному периоду, не менее 75% обитающей в угрожаемой зоне популяции животных.

Ключевые слова: инфекционные болезни свиней, базовая скорость репродукции (R_0), вакцинация, депопуляция, дикие кабаны.

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INTRODUCTION

Basic reproduction number (R_0) is one of the fundamental quantitative characteristics in epidemiology of infectious human and animal diseases. In population biology, the concept of basic reproduction number is a central one for the "parasite – host" system and is expressed as the average number of offspring that one parasite is able to produce; the number of offspring will depend on the biological characteristics of the parasite [1].

For infectious animal diseases, R_0 is the average number of secondary cases one primary case would "generate/reproduce" in a completely susceptible population during the period of time equal to one infectious period [1, 2].

That is, when one infected individual is introduced into some closed population and has contacts with other animals in it, before the diagnosis is made with subsequent isolation (usually during the infectious period), a certain number of susceptible animals become infected.

Basic reproduction number directly reflects the biological properties of the infectious agent, the social and economic aspects of animal husbandry, natural factors associated with the habitat of the animal population invaded by the virus (microorganism), as well as the effectiveness of methods selected for infection control, in particular, the implementation of preventive measures. A biological agent can invade and persist in the animal population when $R_0 > 1$. In equilibrium, each case in a homogeneously mixing population of susceptible animals produces only one secondary case that later either recovers or dies, i.e. here $R_0 = 1$. When $R_0 < 1$, the epidemic process will die out.

In epizootiology (veterinary epidemiology), the interaction between a population of parasites (biological agents) and their hosts (animals) with a direct infection transmission mechanism is expressed as a simple phenomenological model of the "state and transition" type or the so called "SIR model" (see figure). This model divides the population into several classes (states): S – susceptible animals, I – infectious animals, and R – immune (recovered) or removed animals. That is, when a biological agent is introduced into a population of susceptible animals and they become infected, they transition to the state of infection with subsequent development of post-infection immunity (recovery) or removal from the population (death, emergency slaughter of animals) – the R state.

Based on the "state and transition" model theory developed by A. G. McKendrick and W. O. Kermak in 1927–1933, Head of the Mathematical Division of the World Health Organization N. Bailey suggested a mathematical model of the epidemic process that represents a system of

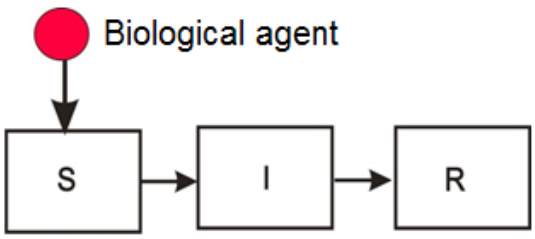

Fig. A simple phenomenological model (SIR model) of the epidemic process

Рис. Простая феноменологическая модель эпизоотического процесса типа SIR

differential equations [1]. The theory of the mathematical model of epidemics starts with considering a homogeneously mixing herd consisting of an animal population of size *N*. Let *S* be the number of susceptible animals, *I* – the number of diseased animals (the sources of infection) and *R* – the number of removed animals in this herd at time *t*, i. e. $S + I + R = N$. It was assumed that the average number of new cases (ΔI) among susceptible animals (*S*) in a certain time interval (Δt) will be proportional to both the number of the sources of infection and the number of susceptibles. If the frequency of contacts between animals within the herd is constant and equals β, then the average number of new cases of the disease during this interval of observation Δt will be $\beta SI\Delta t$, i.e. $\Delta S = -\beta SI\Delta t$.

The transition equation $S - I - R$ can be expressed as a system of differential equations:

$$\frac{\partial S}{\partial t} = -\beta SI$$
$$\frac{\partial I}{\partial t} = \beta SI - \gamma I$$
$$\frac{\partial R}{\partial t} = \gamma I$$

subject to initial condition $(S, I, R) = (S_0, I_0, 0)$ at time $t = 0$.

In this system of equations, the parameter γ (infected animal removal rate) is introduced, i.e. the number of diseased animals removed from the herd during time interval Δt will be $\gamma I\Delta t$.

A classical equation for calculation of R_0 from the above mentioned system of equations will be represented as follows:

$$R_0 = \beta/\gamma.$$

When $R_0 > 1$, infection continues to spread.

MATERIALS AND METHODS

The data of foreign and domestic researchers in the veterinary field are used in the paper. The researchers estimated R_0 by means of experiments in susceptible animals infected with various biological agents using a SIR model of dynamics of observed animal state transitions. To estimate the level of susceptible animal vaccination required to ensure the prevention of highly dangerous animal disease spread (*P*), the following commonly accepted formula was used [3]:

$$P = 1 - 1/R_0.$$

The same formula was used to calculate the required level of wild boar depopulation in the area at risk of African swine fever (ASF) spread.

RESULTS AND DISCUSSION

In the context of an increase in the number of ASF cases in both domestic pigs and wild boars in some European and Asian countries, veterinary specialists carry out extensive research to study the spatiotemporal dynamics of ASF spread [4, 5]. To estimate the dynamics of potential spread of the disease among animals on a farm (a closed population) if even a single animal is infected, as well as between farms, it is necessary to foresee the number and probability of potential new secondary outbreaks of infection in the area at risk of ASF spread. This is a condition for strict control to be implemented with respect to animal and animal product movement/trade at a certain distance from the primary outbreak. One of the possible epidemiological parameters can be the estimated R_0 value showing that, if an ASF outbreak occurs, then new outbreaks can occur in the area of potential risk of infection spread during the period of time equal to one infectious period. Probabilistic quantification of a potential animal disease epidemic, i.e. how many secondary cases can be produced in a herd, if even a single animal becomes diseased (within-herd R_0) or how many secondary outbreaks can occur in the area at risk of infection spread (between-herd R_0) depending on a variety of social, natural and economic factors, is of particular interest for epidemiology. In view of this, the scientific publications available on this topic have been analyzed; the results reported in the publications are presented in Table 1.

The results obtained show that, if even a single ASF case is detected on a farm (within a herd), the number of subsequently affected susceptible animals can vary between 2 and 15 (for serotype II), and the infectious period can last up to 14 days, and this appears to be attributable to the specificity and structure of production [6–9]. In the papers published by Russian researchers [10], basic reproduction number was estimated to range from 4 to 11 within domestic pig populations, and from 2 to 3 – for between-farm spread (for serotype II). In terms of the development of an epidemic, this suggests that, if one animal on a farm is ASFV-infected, it should be expected that subsequently, at the end of one infectious period, at least 11 other animals will become diseased (in some cases – up to 47 animals, as it was determined for serotype I on the island of Malta) [11, 12]. In case of ASFV serotype II spread from the primary outbreak area (a farm) to other farms in the area at risk, from 2 to 17 new outbreaks should be expected to occur during the infectious period [7, 10]. For ASFV serotype IX, the value of R_0 can be about 3 [13].

Due to the lack of effective vaccines for emergency vaccination of animals, one of the ways to control the spread of infection may only be the rapid slaughter (within the incubation period) of all animals in the area at risk of infection spread; besides, quarantine must be strictly observed, and relevant restrictions must be complied with [14]. The necessary condition for preventing the further spread of infection from the primary outbreak area in the area at risk (with regard to the territory of the Russian Federation) is the depopulation of at least $P = 1 - 1/2 = 0.5$ (or 50%) of susceptible animal population in the immediate risk zone [10].

One of the measures taken to eradicate ASF in wild boars is their depopulation (shooting) in the area at risk of infection spread around the primary outbreak of the disease (an animal that has died from ASF or a positive diagnosis established when carrying out diagnostic shooting in the territories inhabited by wild boars). Taking into account the obtained R_0 values for wild boar populations with the maximum R_0 value of up to 4 (3.77) [15, 16], the necessary and sufficient condition for depopulation (the upper confidence limit) to prevent the further spread of the disease will be determined as follows: $P = 1 - 1/4 = 0.75$ (or 75%). This means that, in case a wild boar that has died from ASF is found in some area, at least 75% of the population must be eliminated in the relevant range immediately (within the infectious period of 6 to 15 days). The range of one family usually encompasses a 2 to 5 km radius; but, depending on the natural conditions and landscape, some animals can travel up to 12 to 25 km within several days [17]. For example, if there is a population consisting of 30 boars (about 3–4 families) in the area at risk, then, after even one infected animal has been detected, at least 23 boars must be rapidly (within one

Table 1
The estimated values of within-herd and between-herd basic reproduction number for ASF based on the results of literature data analysis

Таблица 1
Оценочные величины внутристадной и межстадной базовой скорости репродукции для АЧС по результатам проведенного анализа литературных источников

Publication	Genotype	Isolate	Duration of infectious period (days)	Between-herd R_0	Within-herd R_0
S. A. Belyanin et al. (2011)	II		6.8 (5.0–8.6)	–	–
H. C. De Carvalho Ferreira et al. (2013)	I	Malta-78 Netherlands-86	6.8 ± 1.8 4.6 ± 1.4	–	18.0 (6.9–46.9)
J. Pietschmann et al. (2015)	II	Armenia-08	2–9	–	6.1 (0.6–14.5) 5.0 (1.4–10.7)
C. Guinat et al. (2015)	II	Georgia 2007/1	3–14	–	2.8 (1.3–4.8) within a pen 1.4 (0.6–2.4) between pens
V. M. Gulenkin et al. (2011)	II	Russia	5–15	2–3	4–11
M. B. Barongo et al. (2015)	IX	Uganda	–	3.24 (3.21–3.27) 1.63 (1.6–1.72) 1.9 (1.87–1.94)	–
F. I. Korennoy et al. (2017)	I	Ukraine, 1977	7 (within a farm) 19 (between farms)	1.65 (1.42–1.88)	7.46 (5.68–9.21)
C. Guinat et al. (2018)	II	Russia	4.5–8.3	4.4–17.3	–
I. Iglesias et al. (2016)	II	Russia	– (wild boar)	1.58 (1.13–3.77)	–
A. Marcon et al. (2020)	II	Czech Republic Belgium	6 (wild boar)	1.95 1.65	–

incubation period, i.e. 15 days according to the OIE recommendations) eliminated, and appropriate diagnostic tests must be carried out. Only in this case the required and sufficient conditions will be fulfilled to prevent the further spread of ASF in the wild boar population.

Table 2 presents the R_0 values for a number of porcine diseases. These values were obtained by foreign researchers when performing experiments on infection of susceptible animals with a field virus or by means of mathematical modelling using the transmission rate (β) values determined while analyzing the development of natural epidemics [18–21].

The data presented in Table 2 show that for such an infectious disease as classical swine fever the maximum value of R_0 can be about 81.3 (for weaner pigs) [23, 24]. This means that it is necessary to ensure that preventive vaccination conducted in a herd induces protective immunity in 99% of animals [(1 – 1/81.3) × 100%]; therefore, the vaccines used should have high immunogenicity.

To prevent the spread of infection in the area at risk (between the farms), the emergency vaccination (or depopulation) of at least 67% of animals in this area should be carried out [(1 – 1/2.9) × 100%] with $R_0 = 2.9$ [20, 25].

The results obtained by P. L. Eble et al. with respect to FMD show that immunization with a single vaccine dose performed within the study failed to protect pigs in the closed animal population (herd) against the disease transmission. Only as a result of vaccination with a four-fold vaccine dose, the disease might not continue to spread, but might reach equilibrium (with the death or recovery of one animal, $R_0 = 1$) due to the possible development of not fully stable immunity in some animals within the vaccinated animal population [19]. These results indicate that vaccines with high protective activity (immunogenicity) should be created for immunization of pigs, and appropriate vaccination schedules should be developed taking into account the possibility of implementing a DIVA strategy.

The vaccine against Aujeszky's disease used within the study published by M. C. M. De Jong and T. G. Kimman which ensures that at least 90% of animals in the population are protected is able to prevent the further spread of infection within the farm (herd) with $R_0 = 10$ [18].

In order to prevent the transmission of porcine reproductive and respiratory syndrome in a herd, it is necessary to induce protective immunity in at least 91%

Table 2
The estimated values of within-herd and between-herd basic reproduction number for certain porcine diseases based on the results of literature data analysis

Таблица 2
Внутристадная и межстадная оценочные величины базовой скорости репродукции для некоторых болезней свиней, полученные по результатам проведенного анализа данных литературы

Publication	Disease (genome/strain)	Duration of infectious period (days)	Between-herd R_0	Within-herd R_0
A. Stegeman et al. (1999)	classical swine fever	18	2.9	–
E. Weesendorp et al. (2009)	classical swine fever Paderborn strain Brescia strain	–	–	36 17
D. Klinkenberg et al. (2002)	classical swine fever	–	3.39 (between pens)	15.5 (within a pen)
H. Laevens et al. (1999)	classical swine fever	32	–	13.7 81.3 (for weaner pigs)
M. Andraud et al. (2008)	porcine circovirus type 2	35	1.47	5.9 (1.8–10.1)
M. Kluivers et al. (2006)	encephalomyocarditis	–	–	1.4 (within a pen)
H. Maurice et al. (2016)	encephalomyocarditis	–	–	4.2
H. Maurice et al. (2002)	encephalomyocarditis	–	–	1.24 (0.39–4.35)
V. Spyrou et al. (2004)	encephalomyocarditis	–	–	9.87 (a combined estimate for several viruses)
M. C. M. De Jong, T. G. Kimman (1994)	Aujeszky's disease	–	–	10 (non-vaccinated) 0.53 (vaccinated)
P. L. Eble et al. (2008)	foot-and-mouth disease	2.3–6.5	–	40 (non-vaccinated) 11 (single-dose vaccinated) 1 (four-fold-dose vaccinated)
A. G. J. Velthuis et al. (2003)	*Actinobacillus pleuropneumoniae*	from 2 days to several weeks (in case of chronic disease)	10	–
T. Meyns et al. (2004)	*Mycoplasma hyopneumoniae* (virulent isolates)	positive after inoculation 14–32	–	1.47 (0.68–5.38)
E. Pileri (2015)	porcine reproductive and respiratory syndrome, genotype 1	12–14	non-vaccinated 2.78 (2.13–3.43) vaccinated 0.53 (0.19–0.76)	3.53 (2.89–4.18) farm 1 7.11 (3.55–10.68) farm 2
C. Charpin et al. (2012) [22]	porcine reproductive and respiratory syndrome, genotype 1	7–63	–	2.6 (1.8–3.3)
M. Bouwknegt et al. (2008)	hepatitis E	49 (block 1) 13 (block 2)	–	8.8 (4–19) (contact exposure)

of vaccinated animals [(1 – 1/10.68) × 100%] with the maximum R_0 value of 10.68 [18, 26]. The studies conducted show that, if appropriate preventive vaccination has been carried out in the area at risk prior to the outbreak, the disease will not continue to spread between the farms, because the maximum R_0 value is 0.76 [26]. If there are any susceptible animals on the farms, it is necessary to induce protective immunity in at least 71% of animals in the area at risk of infection spread with the maximum R_0 value of 3.43 [(1 – 1/3.43) × 100%].

As for porcine circovirus type 2, in order to prevent the further spread of infection (with the maximum R_0 value of 10.1), it is necessary that immunization should induce protective immunity in 90% of animals [27]. A similar result was found for encephalomyocarditis (with $R_0 = 9.87$) [28–31].

In case of hepatitis E, vaccination-induced protective immunity (for the maximum R_0 value of 19, as determined in the course of experiments on contact infection of pigs) should be about 95% [32].

It was determined that for the virulent isolate of *Mycoplasma hyopneumoniae* [33] the vaccination carried out on a farm should protect at least 81% of animals [(1 – 1/5.38) × 100%] with the upper confidence limit for R_0 being $R_0 = 5.38$. With the said level of pig population protection, the disease should not occur on the farm if infection is introduced into the population.

The data obtained for *Actinobacillus pleuropneumoniae* [21] indicate that, even if there is only one infection outbreak area (a farm), the animals in the area at risk of further spread of the disease should be vaccinated using vaccines with high immunogenicity. Livestock vaccination should cover at least 90% of the total number of farms [(1 – 1/10) × 100%].

CONCLUSION

The above mentioned basic reproduction number (R_0) values for some infectious porcine diseases are indicative of a certain variability in the value of R_0. Apparently, the R_0 values may be dependent on the conditions in which laboratory experiments are carried out, the age group of the animal population selected for experiments, the methods of infection, the virulence of the virus used for infection, etc. Nevertheless, the estimation of R_0 makes a certain scientific contribution to basic applied epidemiology of a number of infectious porcine diseases and is necessary when planning such anti-epidemic activities as preventive immunization or depopulation of susceptible animals aimed to prevent disease outbreaks and the further spread of infection. In particular, with regard to wild boar depopulation in the area at risk of ASF spread, the necessary condition for stopping the further spread of infection is the elimination of at least 75% of the wild boar population living in the risk zone within the period of time equal to one infectious period.

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Development of real-time RT-PCR for N2 subtype avian influenza RNA-virus detection

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SUMMARY
Currently, N2 subtype avian influenza (AI) virus actively circulates in domestic and wild bird populations and is regularly detected in China, other Asian countries and Russia, particularly in combination with H9 hemagglutinin. Therefore, a method for rapid detection of the said infectious agent is urgently required. Data on oligonucleotide primer selection and reverse transcription real-time polymerase chain reaction condition optimization for N2 AI virus detection are presented in the paper. Modified primers and probe proposed by B. Hoffmann in 2006 as well as original primers and probes with the viruses available in the Laboratory working collection and selected during testing were assessed for N2 neuraminidase gene fragment amplification. Optimal concentrations of real-time RT-PCR master mix components and temperature-time mode were determined. Various combinations of primers were tested against ten N2 avian influenza virus isolates that genetically differed from each other in N gene. Nine viruses were isolated from birds in the Russian Federation regions and classified to different genetic groups. The real-time RT-PCR assay was tested for its specificity using AI virus isolates of different neuraminidase subtypes (H5N8, H3N6, H4N6, H5N1, H10N7) as well as samples containing other RNA-viruses: Newcastle disease virus, infectious bronchitis virus and infectious bursal disease virus. As a result of the testing, real-time RT-PCR conditions providing high sensitivity and specificity of the assay were selected and optimized.

Key words: avian influenza virus, real-time RT-PCR, optimization, N2 neuraminidase subtype, sensitivity, specificity.

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Разработка метода ОТ-ПЦР в режиме реального времени для выявления РНК вируса гриппа птиц подтипа N2

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РЕЗЮМЕ

В настоящее время вирус гриппа птиц подтипа N2 активно циркулирует в популяциях домашних и диких птиц, и его регулярно выявляют в Китае, других странах Азии и России, особенно в комбинации с гемагглютинином подтипа H9. Поэтому применение метода для быстрого обнаружения данного инфекционного агента крайне необходимо. В представленной работе приводятся данные по выбору олигонуклеотидных праймеров и оптимизации условий постановки полимеразной цепной реакции с обратной транскрипцией в режиме реального времени для выявления вируса гриппа птиц подтипа N2. Для амплификации фрагмента гена нейраминидазы подтипа N2 были апробированы предложенные в 2016 году B. Hoffmann праймеры и зонд в модификации, а также выбранные в ходе исследования оригинальные праймеры и зонды с вирусами, имеющимися в рабочей коллекции лаборатории. В ходе работы определены оптимальные концентрации компонентов реакционной смеси для проведения полимеразной цепной реакции с обратной транскрипцией в режиме реального времени и температурно-временной режим. Разные комбинации праймеров тестировали на десяти изолятах вируса гриппа птиц подтипа N2, генетически отличающихся друг от друга по гену N. Девять вирусов выделены от птиц из регионов Российской Федерации и относятся к различным генетическим группам. Специфичность метода проверяли методом полимеразной цепной реакции с обратной транскрипцией в режиме реального времени с использованием изолятов вируса гриппа птиц с другим подтипом нейраминидазы (H5N8, H3N6, H4N6, H5N1, H10N7), а также проб, содержащих РНК вирусов ньюкаслской болезни, инфекционного бронхита кур и инфекционной бурсальной болезни. В результате проведенных исследований были подобраны и оптимизированы условия постановки полимеразной цепной реакции с обратной транскрипцией в режиме реального времени, которые обеспечивают высокую чувствительность и специфичность метода.

Ключевые слова: вирус гриппа птиц, ОТ-ПЦР-РВ, оптимизация, подтип нейраминидазы N2, чувствительность, специфичность.

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INTRODUCTION

Avian influenza (AI) is an acute infectious disease caused by the virus of *Orthomyxoviridae* family having segmented negative-sense RNA genome and belonging to *Influenzavirus* A genus [1]. Segmented genome of avian influenza virus allows for its reassortment.

Current classification of avian influenza A viruses is based on antigenic properties of their surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA).

Neuraminidase accounts for 20% of all surface glycoproteins. There may be single NA spikes or local NA clusters surrounded by HA on the virion surface [2]. Neuraminidase plays an important role in AIV infection: it facilitates the virion penetration into respiratory epithelial cells, optimizes HA fusogenic activity, enables release of new virions and prevents their aggregation on the host cell surface.

To date, 16 HA and 9 NA subtypes of the virus in various combinations have been isolated from birds. However, two new HA and NA subtypes of the virus, H17N10 and H18N11, isolated from bats were reported in 2012 and 2013 [3, 4].

In the FGBI "ARRRIAH" Reference Laboratory for Avian Viral Diseases reverse transcription real-time polymerase chain reaction (real-time RT-PCR) with M-gene-targeted primer system is initially used for diagnostic tests aimed at detection of type A avian influenza virus (AIV) in pathological material samples [5–7]. The samples tested positive with M-targeted real-time RT-PCR should be examined for virus H5 and H7 subtypes since the AI viruses of the said subtypes can be highly pathogenic and are notifiable to the OIE. NA subtype should be identified for precise virus characterization.

Neuraminidase inhibition (NI) test is one of the methods used for NA typing [8], but it takes at least two days and requires a panel of high-quality monospecific antisera and control antigens. The test should be carried out under specified conditions since highly toxic reagents have to be used.

Therefore, new highly sensitive, specific and reliable techniques for NA subtype detection and identification are urgently required for prompt diagnosis when it is necessary to identify AIV neuraminidase within a short period of time (within one working day).

Currently, N2 subtype AI virus is quite widespread and actively circulates in domestic and wild bird populations. In 2018, H9N2 AI virus was detected in three commercial poultry establishments in the Primorsky Krai, Russian Federation, as well as on one poultry farm in the Republic of Tajikistan. In 2017–2018, highly pathogenic H5N2 AI outbreaks were reported on one poultry farm in the Kostroma Oblast. In 2019, H9N2 AI virus was detected in the Chelyabinsk Oblast and Zabaikalsky Krai [9, 10].

Hence, development of high sensitive and specific real-time RT-PCR-based assay for N2 subtype AIV genome detection that enables faster results and rapid diagnosis for improvement of preventive measure effectiveness is urgent.

The works were aimed at development of the real-time RT-PCR-based assay with original primer and probe system and optimized parameters for N2 avian influenza virus RNA detection.

MATERIALS AND METHODS

The following N2 AI virus isolates available in the working collection of the FGBI "ARRIAH" Reference

Table 1
Primers and probes used for real-time RT-PCR amplification of N2 AIV N gene fragments

Таблица 1
Праймеры и зонды для амплификации фрагментов гена N подтипа N2 в ОТ-ПЦР-РВ

No.	Primer designation	5′-3′ sequence	Nucleotide base number
1	AIVN2-1316f	GARACYAGAGTRTGGTGGAC	20
2	AIVN2-1319f	ACYAGAGTRTGGTGGACYTC	20
3	AIVN2-1325f	GTRTGGTGGACYTCAAAYAG	20
4	AIVN2-1379-FAM	(FAM) GGAACAGGCTCATGGCCTGATGG (BHQ1)	22
5	AIVN2-1414r	TTTTCTAAAATTGCGAAAGC	20
6	AIVN2-1421r	GGAGTTTTTTTYTAAAATTG	20
7	AIVN2-1432r	AGTAGAAACAAGGAGTTTTT	20
8	AIVN2-1370-FAM	(FAM) GGTACHTATGGAACAGGCT CATGGCCTGATGGRGC (BHQ1)	34
9	AIVN2-1376-FAM	(FAM) TATGGAACAGGCTCATGGCCTGATGG (BHQ1)	26
10	**AIVN2-1367F**	AGTCTGGTGGACYTCAAAYAG	21
11	AIVN2-1488R	AATTGCGAAAGCTTATATAGVCAT	24
12	AIVN2-1444_FAM	(FAM) CCATCAGGCCATGAGCCT (RTQ1)	18
13	**AIVN2-1418r**	GCGAAAGCTTATATAGSCAT	20
14	AIVN2-1428r	TTTTCTAAAATTGCGARAGCTT	22
15	AIVN2-1430r	TTTTTTCTAAAATTGCGARAGC	22
16	AIVN2-1383FAM	(FAM)-CAGGCTCATGGCCTGATGG (RTQ1)	19
17	**AIVN2R-1383FAM**	(FAM) CCATCAGGCCATGAGCCTG (RTQ1)	19

Laboratory for Viral Avian Diseases were used for selection of primers and probes providing high real-time RT-PCR sensitivity and specificity: A/ty/Mass/65 H6N2, A/w.duck/Vladimir/446/09 H4N2, A/bird/Amursky/21/12 H9N2, A/chicken/Kostroma/3175/17 H5N2, A/chicken/Kostroma/2367/18 H5N2, A/chicken/Primorsk/419/18 H9N2, A/chicken/Tadjikistan/2379/18 H9N2, A/chicken/Primorsk/3124/18 H9N2, A/chicken/Chelyabinsk/30/19 H9N2, A/duck/Primorie/2621/2001 H5N2.

Real-time RT-PCR primers were optimized using low virulent H9N2 AIV isolate, A/chicken/Primorsk/419/18, recovered in the Russian Federation (Primorsky Krai) in 2018. The real-time RT-PCR assay was tested for its analytical sensitivity using serial ten-fold dilutions of extracted total RNA of the following isolates in triplicate: A/ty/Mass/65 H6N2, A/bird/Amursky/21/12 H9N2, A/duck/Primorie/2621/2001 H5N2, A/chicken/Tadjikistan/2379/18 H9N2, A/chicken/Primorsk/3124/18 H9N2, A/chicken/Chelyabinsk/30/19 H9N2.

N gene nucleotide sequences of AIV/N2 isolates published in the electronic NCBI GenBank database from 1999 to 2008 (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/) were used for primer selection. Multiple nucleotide sequence alignment was performed with Clustal W tool and BioEdit 7.0 software programme was used for searching for the most conserved regions. The primers and probes were tested for their specificity using on-line Blast resource (NCBI) (http://blast.ncbi.nlm.nih.gov) and AIV isolates of other NA subtypes: (A/w_duck/Altai/1732/2013 H3N6, A/shoveler/Krasnoyarsk/1586/08 H4N6, A/chicken/Adygea/203/06 H5N1, A/mallard/Khabarovsk/12/2014 H10N7) as well as Winterfield 2512 strain of infectious bursal disease virus, H120 strain of infectious bronchitis virus and APMV/wild duck/Rus/Vladimir/44/15 isolate of Newcastle disease virus. Selected primers and probes were synthesized by SINTOL Company (Russia) (Table 1).

All primers and probes except for No. 10–12 were selected in the FGBI "ARRIAH" Reference Laboratory for Viral Avian Diseases. Reverse primer, AIVN2-1418r, and probe (FAM), CCATCAGGCCATGAGCCTG (RTQ1), were modified relevant oligonucleotides described by B. Hoffmann et al. in their publication. Probe and primers No. 10–12 were also recommended by B. Hoffmann et al. [11].

The RNA was extracted from allantoic fluid of SPF chicken embryonated eggs infected with N2 AI virus isolates with 'AmpliPrime RIBO-sorb kit' in accordance with the instruction of its use. Real-time RT-PCR was carried out using deoxynucleoside triphosphates (dNTPs) (Fermentas, cat. No. R0181), GoTaq® Flexi DNA Polymerase, thermostable Taq-DNA-polymerase (Promega, cat. No. M8295) and MMLV reverse transcriptase (SINTOL, cat. No. E-040) with Rotor-Gene Q cycler (Germany).

Table 2
Ct-values for AIV isolates obtained by M gene-targeted real-time RT-PCR

Таблица 2
Значения порогового цикла для изолятов ВГП в ОТ-ПЦР-РВ на ген М

No.	Isolate designation	Original materials (Ct)	Ct-value for dilution					
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	A/bird/Amursky/21/12 H9N2	11.27	14.01	17.08	20.49	23.93	26.86	–
2	A/chicken/Primorsk/419/18 H9N2	11.22	14.98	18.67	22.38	28.46	30.59	36.20
3	A/chicken/Kostroma/2367/18 H5N2	12.47	15.80	19.72	23.48	28.58	30.66	35.31
4	A/w.duck/Vladimir/446/09 H4N2	14.04	18.08	22.07	26.27	30.22	34.35	–
5	A/chicken/Tadjikistan/2379/18 H9N2	12.62	16.49	20.21	23.77	27.14	–	–
6	A/chicken/Chelyabinsk/30/19 H9N2	11.09	15.30	19.06	22.29	26.26	32.21	–
7	A/chicken/Primorsk/3124/18 H9N2	11.08	13.85	17.45	20.86	23.71	27.41	30.45
8	A/duck/Primorie/2621/2001 H5N2	11.27	15.02	18.00	21.50	24.63	28.51	–
9	A/ty/Mass/65 H6N2	14.36	17.37	20.67	24.25	27.28	30.32	–

«–» – negative result (отрицательный результат реакции).

RESULTS AND DISCUSSION

Development of the real-time RT-PCR assay for detection of N2 AI virus RNA included selection of the primers-probe system providing sufficiently high assay sensitivity and specificity. Also, optimal real-time RT-PCR conditions, reaction mix component concentrations and temperature-time mode had to be determined.

Selection of primers and probe. The full-length NA gene nucleotide sequences of N2 AI virus isolates recovered in Eurasian and African countries in 1999–2008 and belonging to different genetic groups that had been published in the electronic NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/) were analyzed to select the primer system for neuraminidase detection.

Initially, 2,822 N gene nucleotide sequences of AIV/N2 isolates were selected for comparative analysis. One hundred and sixty-nine sequences the most different from each other were finally selected for the analysis after step-by-step optimization and removal of the most genetically similar sequences from the selected ones. All sequences contained relatively conserved segments located at both gene ends. The 200-bp region at the N-gene end was considered optimal and selected for the primer incorporation since number of substitutions in the said region was the least in all selected sequences as compared to the other gene segments.

The data previously published by foreign authors were analyzed concurrently with the tests for in-house primer selection. The structure of the primers proposed by B. Hoffmann et al. [11] (No. 10–12, Table 1) was taken into account during in-house primer selection.

As a result, 11 primers and 6 probes were selected and synthesized for further real-time RT-PCR testing to identify the most specific and sensitive system.

One the most suitable primer system (indicated in bold in Table 1) was selected during experimental real-time RT-PCR runs using various primer-probe combinations with N2 AIV isolates and used for further assay optimization aimed at its sensitivity improvement. The reverse primer and fluorescent probe were designed in the FGBI "ARRIAH" Reference Laboratory for Viral Avian Diseases with minimum modifications of the nucleotide sequence proposed by B. Hoffmann et al. Forward primer sequence was identical to that one given in the above said publication [11].

Optimization of real-time RT-PCR conditions. It was required to optimize reaction mix component concentrations and temperature-time mode in order to increase sensitivity, specificity and rate of the real-time RT-PCR assay for N2 subtype avian influenza virus genome detection.

Prepared ten-fold dilutions (10^{-1}–10^{-6}) of extracted total RNAs of N2 AIV isolates were tested with the said real-time RT-PCR targeted for M gene using the primers-probe system recommended by the OIE immediately before tests for optimization of N-targeted real-time RT-PCR conditions [5–7]. The reaction was carried out in accordance with the Methodical Guidelines developed earlier in the FGBI "ARRIAH" [12]. The results of tests of N2 subtype AIV isolates with M-targeted real-time RT-PCR assay (Table 2) were compared to the results obtained by optimized N-targeted real-time RT-PCR assay.

The main task was to optimize reaction mix component concentrations in order to increase sensitivity and specificity of the real-time RT-PCR assay for N2 AIV RNA detection. To do this, N gene-targeted real-time RT-PCR assays using ten-fold dilutions of low virulent H9N2 AIV isolate recovered in the Russian Federation (Primorsky Krai) in 2018 were carried out. A temperature-time mode similar to that one of the M gene-targeted real-time RT-PCR assay was used. Tables 3–6 show cycle threshold (Ct) values for two AIV RNA dilutions (10^{-3} and 10^{-4}) tested in triplicate.

Selection of optimal magnesium chloride concentration for the real-time RT-PCR assay. Mg^{2+} ions as a required reaction mix component are crucial for proper DNA-polymerase functioning. They also have a significant impact on primer hybridization specificity. Optimal Mg^{2+} ion concentration can vary within a fairly wide range

Table 3
Ct-values obtained by real-time RT-PCR during optimal $MgCl_2$ concentration selection

Таблица 3
Значения порогового цикла при подборе оптимальной концентрации $MgCl_2$ в ОТ-ПЦР-РВ

Dilution	$MgCl_2$ amount, µl (concentration 25 mM)							
	1.0	2.0	3.0	4.0	4.5	5.0	5.5	6.0
10^{-3}	–	28.19	27.19	22.74	23.74	23.63	24.13	22.88
10^{-3}	–	27.06	29.33	22.49	23.63	22.58	23.81	23.34
10^{-3}	–	28.14	28.57	22.25	24.08	23.89	23.99	23.22
Mean	–	**27.8**	**28.36**	**22.49**	**23.82**	**23.37**	**23.98**	**23.15**
10^{-4}	–	32.97	31.16	27.03	28.96	27.85	27.55	27.07
10^{-4}	–	32.09	31.12	28.24	28.95	27.03	28.72	27.30
10^{-4}	–	33.16	33.53	28.12	29.76	27.59	29.45	27.34
Mean	–	**32.74**	**31.94**	**27.8**	**29.22**	**27.49**	**28.57**	**27.24**

«–» – negative result (отрицательный результат реакции).

depending on used primers and enzymes [13]. For best results, it is recommended to select Mg^{2+} concentration for the used system of primers and enzymes empirically. The test results are given in Table 3.

Data given in Table 3 show that change in magnesium salt concentration has a significant impact on the amplification process. Specific PCR-product yield was obtained when 2 µl of $MgCl_2$ were added to the reaction mix. Analysis of the obtained results showed that the amplification was effective when 4–6 µl of $MgCl_2$ solution were added. Therewith, Ct-value deviation was minimal, when 5 µl of magnesium chloride solution was used. This amount was considered optimal. It should be noted that increase in magnesium salt concentration could result in decrease in the assay specificity.

Selection of optimal primer concentration for the real-time RT-PCR assay. Optimal concentrations of AIVN2-1367f and AIVN2-1418r primers were selected experimentally. Reaction mixes were prepared using dilutions given in Table 4 and different amounts of the primers, stating from 0.5 µl and up to 2 µl, were added to each of them. Amounts of both primers, forward and reverse, are given in Table 4.

Performed tests show that increase in the primer concentration results in decrease in the assay sensitivity that is clearly demonstrated by data given in Table 4. The least Ct-values were obtained by the assays when 0.5 µl of each of the primers was added to the reaction mix.

Selection of optimal fluorescent probe concentration. Amount of the probe to be added was optimized to increase amplification effectiveness and fluorescence intensity of amplification curves. Test results are given in Table 5. Insignificant changes in real-time RT-PCR assay sensitivity were registered when 0.75 up to 2 µl of the

Table 4
Ct-values obtained by real-time RT-PCR during optimal primer concentration selection

Таблица 4
Значения порогового цикла при подборе оптимальной концентрации праймеров в ОТ-ПЦР-РВ

Dilution	Amount of primers, µl (concentration 10 pmol/µl)			
	0.5	1	1.5	2
10^{-3}	21.03	22.13	22.61	23.34
10^{-3}	21.04	21.98	22.80	23.31
10^{-3}	21.13	22.40	22.09	23.23
Mean	**21.07**	**22.17**	**22.50**	**23.29**
10^{-4}	25.08	26.96	27.44	30.16
10^{-4}	24.69	27.07	28.14	31.07
10^{-4}	24.82	26.58	28.41	28.95
Mean	**24.86**	**26.87**	**28.00**	**30.06**

Table 5
Ct-values obtained by real-time RT-PCR during optimal fluorescent probe concentration selection

Таблица 5
Значения порогового цикла при подборе оптимальной концентрации флуоресцентного зонда в ОТ-ПЦР-РВ

Dilution	Amount of probe, µl (concentration 10 pmol/µl)				
	0.5	0.75	1.0	1.5	2.0
10^{-3}	24.22	22.61	22.71	22.22	22.19
10^{-3}	24.55	23.47	22.65	21.97	22.56
10^{-3}	24.58	22.72	22.96	21.47	21.69
Mean	**24.45**	**22.93**	**22.77**	**21.89**	**22.15**
10^{-4}	29.26	26.20	26.63	25.70	26.88
10^{-4}	28.05	27.61	27.62	25.62	26.32
10^{-4}	27.47	25.91	27.40	25.84	25.98
Mean	**28.26**	**26.57**	**27.22**	**25.72**	**26.39**

probe were added to the reaction mix. Mean Ct-values differed by no more than 1.5 cycles. The most stable results were obtained when 1.5 µl of AIVN2R-1383FAM probe were used. Moreover, changes in the maximum levels of positive sample fluorescence intensity were observed in the reaction graph when the said component in the specified amount was added.

Concentrations of the reaction mix components (magnesium chloride, primers, fluorescent probe) were optimized to increase sensitivity and specificity of the real-time RT-PCR assay for N2 subtype AIV RNA detection. Other components were used in accordance with their manufacturer instructions.

Optimization of the real-time RT-PCR temperature-time parameters. The next step of our works was optimization of temperature-time mode of the PCR itself since temperature parameters of reverse transcription were defined by the enzyme (reverse transcriptase) used. In our case, optimal annealing temperature for the primer system containing selected oligonucleotide primers and fluorescein-labeled probe was determined. The next stages of the PCR – DNA denaturation and synthesis – were carried out in rather narrow temperature range. Denaturation is usually performed at 90–95 °C and DNA chain elongation – at 68–72 °C [13]. Results of primer annealing temperature selection for the real-time RT-PCR assay are shown in Table 6.

According to the data given in Table 6 compatible Ct-values were observed when the primer annealing temperature was 55–60 °C. Generally, this temperature range is suitable for the majority of primer systems used for the real-time RT-PCR assays intended for molecular avian influenza diagnosis. So, it was reasonable to use the same annealing temperatures for N2 subtype AIV identification. Thus, it is possible to carry out real-time RT-PCR assay targeted to different genes (M, H, N) during one thermocycler run and thereby to get the results and to make the diagnosis more quickly. The following temperature and time parameters were used for the optimized real-time RT-PCR: 20 min at 40 °C (reverse transcription); 10 min at 95 °C (polymerase activation); and further 40 PCR cycles comprising DNA denaturation: 10 sec at 95 °C; primer annealing: 35 sec at 55 °C and cDNA elongation: 10 sec at 72 °C.

Comparative sensitivity and specificity of the real-time RT-PCR assay for N2 subtype AIV genome detection. Sensitivity is one of the crucial parameters of real-time RT-PCR assay. Usually analytical sensitivity, i.e. minimum amount of the agent that can be detected by the method in the particular clinical samples, is reported. In this paper comparative sensitivity is reported since two real-time RT-PCR assays: one – for M-gene detection (data are given in Table 2) and the other – for N gene detection (Table 7) have been compared by the said parameter. Serial ten-fold dilutions of total extracted RNAs of 6 AIV isolates were made to test the developed assay for its sensitivity. The real-time RT-PCR assay was carried out in triplicate to obtain more reliable data.

After optimization of component concentrations and temperature and time parameters of the N gene-targeted assay Ct-values for ten-fold AIV isolate dilutions obtained by the said assay were consistent to Ct-values obtained by the M gene-targeted assay. Number of the dilutions tested positive for each AIV isolate was similar for the compared real-time RT-PCR assays.

The assay was tested for its specificity using samples containing N2 and N1, N6, N7, N8 AIV genetic materials

Table 6
Ct-values obtained during optimal primer and probe annealing temperature selection

Таблица 6
Значения порогового цикла при подборе оптимальной температуры отжига праймеров и зонда

Dilution	Annealing temperature, °C		
	60	55	50
10^{-3}	22.04	22.22	21.91
10^{-3}	21.77	21.97	22.82
10^{-3}	21.79	21.47	22.47
Mean value	**21.87**	**21.89**	**22.4**
10^{-4}	25.12	25.70	27.20
10^{-4}	25.39	26.62	27.06
10^{-4}	25.86	25.84	26.58
Mean value	**25.46**	**26.05**	**26.95**

Table 7
Ct-values for AIV isolates obtained by N gene-targeted real-time RT-PCR

Таблица 7
Значения порогового цикла для изолятов ВГП в ОТ-ПЦР-РВ на ген N

Isolate designation	Original materials (Ct)	Ct-value for dilution					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
A/chicken/Tadjikistan/ 2379/18 H9N2	12.19	15.92	19.24	23.16	26.11	–	–
A/chicken/Chelyabinsk/30/19 H9N2	10.30	13.88	17.53	20.75	25.91	29.82	–
A/chicken/Primorsk/3124/18 H9N2	10.62	13.43	17.22	21.05	24.77	29.10	36.73
A/bird/Amursky/21/12 H9N2	10.00	13.57	17.22	21.11	25.54	30.04	–
A/duck/Primorie/2621/2001 H5N2	11.30	14.52	18.31	21.72	25.14	29.25	–
A/ty/Mass/65 H6N2	10.90	14.34	18.54	21.24	26.04	29.70	–

«–» – negative result (отрицательный результат реакции).

as well as samples containing Newcastle disease virus, infectious bronchitis virus and infections bursal disease virus RNAs. All samples containing non-specific pathogens were tested negative with the developed real-time RT-PCR assay that confirmed the said assay specificity.

CONCLUSION

Primer system for N2 subtype AI virus genome detection was selected and the real-time RT-PCR conditions, reaction mix component composition and temperature-time mode, were optimized based on the performed test results. It was demonstrated that the proposed assay was able to detect N2 AIV RNA in biological material samples. High specificity and sensitivity of the real-time RT-PCR assay were proved by successful identification of N2 AIV genetic materials in samples collected from birds and submitted for testing from several regions of the Russian Federation in 2019–2020.

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Bile microbiocenosis in cats suffering from acute cholangiohepatitis

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SUMMARY
Commensal microorganisms are responsible for numerous diseases of animals, including diseases of internal organs (gastroenteritis, pneumonia, nephritis, hepatitis, cholecystitis, etc.). Cholangiohepatitis, one of the most common liver diseases in cats, is often fatal. The focus of the study was the bile of cats, suffering from acute cholangiohepatitis. The bile was sampled using non-lethal method guided by USG. The bile amount, taken from cats by percutaneous puncture of the gall bladder, was 2.6 ± 0.85 cm^3. No complications following the cholecystocentesis were observed in the animals. The microbiocenosis of bile from 51 cats was studied. Acute feline neutrophilic cholangiohepatitis is mostly caused by commensal bacteria. The range of bacterial pathogens includes the isolates of *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Staphylococcus epidermidis*, *Proteus vulgaris*, *Proteus mirabilis*, *Enterobacter cloacae*, *Citrobacter freundii*. The infectious process was caused by two-component associations in 75% of cases, and by three-component associations in 25%. Most common polycomponent bacterial associations included *E. faecalis* + *E. coli* (26.9%), less common – *E. aerogenes* + *E. coli* (15.4%), *P. vulgaris* + *E. coli* (11.5%), *S. aureus* + *E. coli* (11.5%), rarely – *P. aeruginosa* + *E. coli* (7.7%), *S. aureus* + *E. cloacae* (3.9%), *S. aureus* + *E. faecalis* (3.9%), *P. mirabilis* + *E. coli* (3.9%), *S. epidermidis* + *E. coli* (3.9%), *E. coli* + *S. epidermidis* + *E. faecalis* (3.9%), *P. aeruginosa* + *E. coli* + *S. epidermidis* (3.9%), *E. faecalis* + *E. coli* + *C. freundii* (3.9%). The predominant component of the mentioned associations is *E. coli* serovars O101 (28.9%), O41 (2.0%), O141 (15.6%), O26 (13.3%), O138 (13.3%), O15 (6.7%) and O33 (2.2%). It was established that 76.25% of commensal microorganism isolates, recovered from the bile of cats, suffering from feline cholangiohepatitis, were pathogenic for white mice.

Key words: cholangiohepatitis, cats, microbiocenosis, bile, bactobilia.

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Микробиоценоз желчи у кошек при остром холангиогепатите

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РЕЗЮМЕ
Условно-патогенные микроорганизмы являются возбудителями множества заболеваний у животных, в том числе внутренних болезней (гастроэнтерит, пневмония, нефрит, гепатит, холецистит и т. д.). Холангиогепатит – одна из самых распространенных патологий печени у кошек, которая может приводить к летальному исходу. Объектом исследования была желчь кошек, больных острым холангиогепатитом, полученная прижизненно под контролем ультрасонографии. Объем желчи, отобранной у кошек с помощью чрескожной пункции желчного пузыря, составил 2,6 ± 0,85 см^3. Осложнений после

проведения холецистоцентеза у животных не отмечали. Изучен микробиоценоз желчи у 51 кошки. Основной причиной острого нейтрофильного холангиогепатита у кошек являются условно-патогенные бактерии. Спектр бактериальных патогенов представлен изолятами *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Staphylococcus epidermidis*, *Proteus vulgaris*, *Proteus mirabilis*, *Enterobacter cloacae*, *Citrobacter freundii*. В инфекционном процессе принимали участие двухкомпонентные ассоциации в 75% случаев, а трехкомпонентные – в 25% случаев. Из поликомпонентных бактериальных ассоциаций у больных кошек чаще всего встречались *E. faecalis* + *E. coli* (26,9%), реже – *E. aerogenes* + *E. coli* (15,4%), *P. vulgaris* + *E. coli* (11,5%), *S. aureus* + *E. coli* (11,5%), редко – *P. aeruginosa* + *E. coli* (7,7%), *S. aureus* + *E. cloacae* (3,9%), *S. aureus* + *E. faecalis* (3,9%), *P. mirabilis* + *E. coli* (3,9%), *S. epidermidis* + *E. coli* (3,9%), *E. coli* + *S. epidermidis* + *E. faecalis* (3,9%), *P. aeruginosa* + *E. coli* + *S. epidermidis* (3,9%), *E. faecalis* + *E. coli* + *C. freundii* (3,9%). Доминирующим компонентом указанных ассоциаций является *E. coli* сероваров 0101 (28,9%), 041 (20,0%), 0141 (15,6%), 026 (13,3%), 0138 (13,3%), 015 (6,7%) и 033 (2,2%). Установлено, что 76,25% изолятов условно-патогенных микроорганизмов, изолированных из желчи больных холангиогепатитом кошек, были патогенными для белых мышей.

Ключевые слова: холангиогепатит, кошки, микробоценоз, желчь, бактобилия.

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INTRODUCTION

Opportunistic microorganisms are responsible for numerous animal diseases, including diseases of internal organs (gastroenteritis, pneumonia, nephritis, hepatitis, cholecystitis, etc.) [1]. Cholangiohepatitis, one of the most common liver diseases in cats, is often fatal [2, 3]. This condition is characterized by the development of bacterial or immunity-mediated inflammation process in the hepatic parenchyma and bile ducts, by secondary metabolic changes, intoxication and dehydration [2, 4]. According to the data on hepatobiliary disease prevalence in cats, acute (bacterial, neutrophilic) cholangiohepatitis ranks second after hepatic lipidosis [2, 3, 5].

Acute feline neutrophilic cholangiohepatitis is mostly caused by the following opportunistic bacteria: *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus* spp., *Bacteroides* spp., *Streptococcus* spp., *Clostridium* spp. [5]. Development and progressing of feline cholangiohepatitis are conditioned by poor bile flow, because under normal physiological conditions the continuous bile flow and concurrent immunological protection, conferred by the epithelial cells of the bile ducts, maintain the hepatobiliary tract sterile [6]. Bile flow disorder and bile stasis create favourable conditions for backward migration of bacterial pathogens from the small intestine [3, 5, 7]. In the view of the above, biliary dyskinesia in the setting of partial obstruction, followed by ascending biliary infection, is the key factor for the development of acute feline bacterial cholangiohepatitis. Acute inflammation process, edema and thickening of bile duct walls, in turn, also aggravate stasis and facilitate pathological influence [8, 9]. A significant growth in bile pressure inside the hepatic ducts leads to weakening of protective immunological mechanisms, which in turn ensures favourable conditions for growth and development of commensal microorganisms. Further on the inflammation process involves bile ducts and hepatic parenchyma; bacterial translocation into systemic circulation occurs leading to bacteremia [10, 11].

Recently in clinical practice non-lethal evaluation of hepatic tissue and bile sterility has been widely used in case of hepatobiliary tract inflammation in cats [12]. Both bile and hepatic parenchyma can be taken for testing; and the procedure itself is performed under ultrasonographic guidance [13]. However bacteriological testing of bile is more informative, than testing of hepatic parenchyma puncture samples [5]. F. Schiborra et al. analyzed the results, obtained in the process of bacterial microflora study of small pet bile and its cytology [12]. Bile bacterial cultures were isolated in 21.3% cases. Most often *Escherichia coli* and *Enterococcus* spp. were isolated from bile, less often – *Clostridium perfringens*, *Bacteroides* spp. and *Actinomyces* spp., *Lactobacillus* spp., *Lactococcus* spp., *Listeria* spp., *Klebsiella* spp., *Salmonella* spp., *Streptococcus bovis* and *Pseudomonas*. Microbial associations in bile from cats and dogs were detected in 43.8% of cases. Cytology revealed bactobilia in 17.3% of cats. Liver samples taken from ill cats using surgery or laparoscopy more likely showed clean cultures, than samples obtained by percutaneous fine needle biopsy [5].

Thus, study of bacterial associations in the bile of cats, suffering from acute cholangiohepatitis is a vital trend in veterinary medicine of small pets.

In the view of the above, the aim of this study was qualitative and quantitative analysis of bile microbiocenosis of cats, suffering from acute cholangiohepatitis.

MATERIALS AND METHODS

The study was performed from 2015 to 2019 by the Chair for Infectious Diseases, Pathological Anatomy and Judicial Veterinary Medicine under the SEI LPR "Lugansk National Agrarian University" (Lugansk, Lugansk People's Republic). Clinical tests were performed at private veterinary clinics of Donetsk city.

The subject of the testing was the bile of cats, suffering from acute cholangiohepatitis, obtained using non-lethal methods under ultrasonographic guidance. The groups

were formed as the animals were submitted to clinics. The cats were selected for the study based on inclusion and exclusion criteria.

Inclusion criteria: clinical, laboratory and ultrasonographic signs of feline acute cholangiohepatitis.

Exclusion criteria: feline hepatic lipidosis, fatty liver disease, acute hepatitis, aseptic (immunity-mediated) types of cholangitis, oncology in abdominal cavity, positive results for parasites in feces, positive PCR results for infectious peritonitis agents, viral immunodeficiency, viral leukemia and hemotropic mycoplasma.

The acute cholangiohepatitis diagnosis was made in a comprehensive manner, taking into account the history, results of clinical examination, physical examination, morphological and biochemical blood test and ultrasonography [9].

Cholecystocentesis of cats, suffering from acute cholangiohepatitis, was performed under brief multimodal anesthesia. Cats were premedicated using gabapentin orally at the dose of 50 mg/kg; 15 minutes later dexmedetomidine hydrochloride solution was injected intramuscularly (5–10 μg/kg); 20 minutes after that propofol at the dose of 1–2 mg/kg was injected intravenously.

Ultrasound scan of abdominal organs was performed using multi frequency micro-convex probe at the frequency of 6–9 MHz. The optimal site for gall bladder puncture was determined using ultrasonography. The abdominal wall was punctured from the right side. Cholecystocentesis was performed aseptically under ultrasonographic guidance using a syringe (5 cm^3) and a needle 22G (0.7 × 40 mm). Transhepatic access was used. Maximum volume of bile was aspirated into a syringe. The surgical area around the puncture site was treated with 70% ethanol three times. At the end of the procedure the follow-up ultrasonographic scanning of cats' hepatobiliary system was carried out to evaluate the potential damage of the gall bladder.

Bacteriological testing of bile from cholangiohepatitis-affected cats was performed by seeding it on different nutrient media (beef extract broth, beef extract agar, serum broth with glucose and Sabouraud dextrose broth). Following incubation in a thermostat at 38 °C or at room temperature (Sabouraud dextrose broth) for 24–72 hours; colonies of various types were reseeded onto Petri dishes, containing Endo medium, beef extract broth, beef extract agar. Tubes demonstrating no growth of microorganisms were kept in a thermostat at 38 °C for more than 10 days. After studying cultural and morphological properties some typical microorganism colonies were seeded onto beef extract agar, beef extract broth and beef extract semi-liquid agar and incubated at 38 °C for 24 hours. Then tinctorial properties of bacterial cultures using common methods were studied. Microbial mobility was determined by bacterial growth rate in beef extract semi-liquid agar.

All pure bacterial cultures were seeded onto Gissa medium with glucose, maltose, mannose, sucrose, lactose, dulcite and mannitol added. Catalase activity was determined for all bacterial isolates. For this purpose bacterial mass, removed using a loop from the agar surface, was suspended in a slide in one drop of 3% hydrogen peroxide.

Gram-positive cocci were tested for hemolytic and coagulase activity, and growth properties were studied at 45 and 10 °C, pH 9.6, with 40% bovine bile and 6.5% sodium chloride added. To differentiate staphylococci and micrococci oxidation-fermentation test was performed using Hugh and Leifson's medium.

Gram-negative rod-shaped bacteria were additionally tested for fermentation of such hydrocarbons like sorbitol and inositol; lysine decarboxylase, ornithine decarboxylase, β-galactoside and phenylalanine-desaminase activities; ability to synthesize acetylmethylcarbinol, hydrogen sulfide and indole; utilize malonate and sodium citrate.

To suppress *Proteus* culture mobility before testing 96% ethanol was added to bacterial flasks, containing peptone-beef extract agar for 3–5 minutes and then removed. To identify and differentiate Pseudomonas the tested cultures were reseeded onto King's medium in flasks, containing peptone-beef extract agar and kept in a thermostat at 42 °C for 24–48 hours.

The further identification and differentiation of isolated microorganism cultures were performed using conventional methods according to Bergey's Manual of Systematic Bacteriology[1]. *Escherichia* serogroups were determined using "O" *Escherichia coli* Agglutinating Sera.

Bacterial pathogenic properties were studied by biological assay in white mice. Each isolate at the dose of 0.5 cm^3 was injected intraperitoneally to three 14–16 g white mice. The cultures were deemed pathogenic if one or more mice died within 5 days after infection.

All animal experiments were performed in strict compliance with interstate standard on laboratory animal keeping and handling GOST 33216-2014, adopted by Interstate Council on Standardization, Metrology and Certification and pursuant to the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

The final stage of the bacteriological test was focused on species composition and ratios of different pathogenic bacteria species, causing feline acute cholangiohepatitis.

The obtained digital data were processed using personal computer and licensed software MS Excel and Statistica 7.0[2].

RESULTS AND DISCUSSION

The volume of bile, collected from cats by percutaneous puncture of the gall bladder was 2.6 ± 0.85 ml. No complications following the cholecystocentesis were observed in the animals. To study etiological role of bacterial microbiocenosis in the development of acute cholangiohepatitis bacteriological tests were performed. 80 cultures of opportunistic bacteria were isolated from the bile of 51 diseased cats (Table 1).

Out of all isolated microorganisms 59 were Gram-negative and 21 Gram-positive. The most frequent isolates were *E. coli* (45); *E. faecalis* (13) were recovered less often, *S. aureus* (5), *E. aerogenes* (4), *P. aeruginosa* (4), *P. vulgaris* (3), *S. epidermidis* (3), *E. cloacae* (1), *P. mirabilis* (1) and *C. freundii* (1) were isolated seldom. The data obtained are compliant with other studies, published in the past [5, 12].

The results of isolation frequency of opportunistic microorganism monocultures and associations from the bile of acute cholangiohepatitis-affected cats were especially important for practical veterinary medicine.

In the course of the study it was established that 5 species of opportunistic microorganisms were isolated as associations and 25 as monocultures (Table 2).

[1] Bergey's Manual of Systematic Bacteriology. Vol. 1–5. ed. G. M. Garrity. 2nd ed. NY: Springer-Verlag; 2001–2012.

[2] Rebrova O. Yu. Statistical analysis of medical data. Use of STATISTICA software package [Statisticheskij analiz medicinskih dannyh. Primenenie paketa prikladnyh program STATISTICA]. M.: MediaSfera 2002. 312 p. (in Russian)

Table 1
Species composition of bacteria, responsible for feline acute bacterial cholangiohepatitis

Таблица 1
Видовой состав бактерий, вызывающих развитие острого бактериального холангиогепатита у кошек

Microorganism species	Isolates	
	Absolute number	%
Gram-negative		
Citrobacter freundii	1	1.2
Enterobacter aerogenes	4	5.0
Enterobacter cloacae	1	1.2
Escherichia coli	45	56.3
Proteus mirabilis	1	1.2
Proteus vulgaris	3	3.8
Pseudomonas aeruginosa	4	5.0
Gram-positive		
Staphylococcus aureus	5	6.2
Staphylococcus epidermidis	3	3.8
Enterococcus faecalis	13	16.3
Total	80	100.0

As part of associations the most frequent isolates were *E. coli* (43.6%), less often – *E. faecalis* (18.2%), seldom – *S. aureus* (9.1%), *E. aerogenes* (7.2%), *P. vulgaris* (5.5%), *P. aeruginosa* (5.5%), *S. epidermidis* (5.5%), *C. freundii* (1.8%), *E. cloacae* (1.8%) and *P. mirabilis* (1.8%). As monocultures *E. coli* (84.0%) were often recovered, and *E. faecalis* (12.0%) and *P. aeruginosa* (4.0%) were isolated rarely. It should be added that *C. freundii*, *E. aerogenes*, *E. cloacae*, *P. mirabilis*, *P. vulgaris*, *S. aureus* and *S. epidermidis* were not isolated as monocultures from the bile of acute cholangiohepatitis-affected cats.

In qualitative terms 12 variants of bacterial associations were isolated (Table 3). When determining quantitative and qualitative composition of microbiocenoses of cats, suffering from acute cholangiohepatitis, 26 opportunistic microorganism associations were detected (2–3 associates).

The recovery ratio of two-component associations from the bile of acute cholangiohepatitis-affected cats was 75.0% and three-component associations – 25.0%. The most frequent associates of polycomponent opportunistic bacteria associations were *E. coli* + *E. faecalis* (26.9%), less often – *E. coli* + *E. aerogenes* (15.4%), *E. coli* + *P. vulgaris* (11.5%), *E. coli* + *S. aureus* (11.5%), seldom – *E. coli* + *P. aeruginosa* (7.7%), *E. cloacae* + *S. aureus* (3.9%), *E. faecalis* + *S. aureus* (3.9%), *E. coli* + *P. mirabilis* (3.9%), *E. coli* + *S. epidermidis* (3.9%), *E. coli* + *E. faecalis* + *S. epidermidis* (3.9%), *E. coli* + *P. aeruginosa* + *S. epidermidis* (3.9%), *E. coli* + *E. faecalis* + *C. freundii* (3.9%). It should be noted that these were the first obtained data on microbiocenosis quantitative and qualitative composition of the bile from acute cholangiohepatitis-affected cats.

It must be stressed that the most essential component of such associations was *Escherichia*.

The most frequent *Escherichia* serovars isolated from the bile of diseased cats were O101 (28.9%), less often – O41 (20.0%) and O141 (15.6%), seldom – O138 (13.3%), O26 (13.3%), O15 (6.7%) and O33 (2.2%) (Table 4).

Table 2
Isolation frequency of opportunistic microflora monocultures and associations from the bile of cats, suffering from acute cholangiohepatitis

Таблица 2
Частота изоляции монокультур и ассоциаций условно-патогенных микроорганизмов из желчи кошек, больных острым холангиогепатитом

Microorganism species	Isolates recovered			
	As associations		As pure culture	
	number	%	number	%
Citrobacter freundii	1	1.8	0	0
Enterobacter aerogenes	4	7.2	0	0
Enterobacter cloacae	1	1.8	0	0
Escherichia coli	24	43.6	21	84.0
Proteus mirabilis	1	1.8	0	0
Proteus vulgaris	3	5.5	0	0
Pseudomonas aeruginosa	3	5.5	1	4.0
Staphylococcus aureus	5	9.1	0	0
Staphylococcus epidermidis	3	5.5	0	0
Enterococcus faecalis	10	18.2	3	12.0
Total	55	100.0	25	100.0

Pathogenic properties of 80 opportunistic bacteria cultures, isolated from the bile of acute cholangiohepatitis-affected cats, are shown in Table 5.

It was established that out of 80 isolates of opportunistic bacteria, recovered from the bile of diseased cats, 61 isolates were pathogenic for white mice and 19 cultures were non-pathogenic (76.25 and 23.75% correspondingly). It should be noted that all *P. aeruginosa* and *C. freundii* isolates were pathogenic for white mice.

Moreover, 39 out of 45 *E. coli* isolates (86.7%) were pathogenic for white mice, and *S. epidermidis, E. cloacae* and *P. mirabilis* cultures were non-pathogenic. The data obtained indirectly support the hypothesis that non-pathogenic strains can transform to pathogenic ones under certain conditions. We deem that the promising areas of further studies are biological properties of opportunistic bacteria in cats, suffering from acute bacterial cholangiohepatitis, and development of highly effective methods to treat this pathology.

CONCLUSION

The microbiocenosis of bile, collected from acute cholangiohepatitis-affected cats, is represented by the following isolates: *E. coli*, *E. faecalis*, *S. aureus*, *E. aerogenes*, *P. aeruginosa*, *P. vulgaris*, *S. epidermidis*, *E. cloacae*, *P. mirabilis* and *C. freundii*. The prevalence of two-component associations was 75.0%, and of three-component associations – 25.0%. The most frequent biliary bacterial associations were *E. coli* + *E. faecalis* (26,9%), *E. coli* + *E. aerogenes* (15.4%), *E. coli* + *P. vulgaris* (11.5%), *E. coli* + *S. aureus* (11.5%) were detected less often, *E. coli* + *P. aeruginosa* (7.7%), *E. cloacae* + *S. aureus* (3.9%), *E. faecalis* + *S. aureus* (3.9%), *E. coli* + *P. mirabilis* (3.9%), *E. coli* + *S. epidermidis* (3.9%), *E. coli* + *E. faecalis* + *S. epidermidis* (3.9%), *E. coli* + *P. aeruginosa* + S. *epidermidis* (3.9%), *E. coli* + *E. faecalis* + C. *freundii* (3.9%) were detected on rare occasions. In most cases the essential component of these associations was *E. coli* serovars O101 (28.9%), O41 (20.0%), O141 (15.6%), O138 (13.3%), O26 (13.3%), O15 (6.7%) and O33 (2.2%). It was established that 80.0% of opportunistic bacteria isolates, recovered from the bile of cats, suffering from acute cholangiohepatitis, were pathogenic for white mice. Herewith all *P. aeruginosa* and *C. freundii* isolates, and most *E. coli* isolates (86.7%) were pathogenic.

Table 3
Structure of bacterial associations, responsible for feline acute cholangiohepatitis

Таблица 3
Структура ассоциаций бактерий, вызывающих острый холангиогепатит у кошек

Number of microbe associates	Number of associations	Species composition
2	7	*E. coli* + *E. faecalis*
	4	*E. coli* + E. *aerogenes*
	3	*E. coli* + P. *vulgaris*
	3	*E. coli* + S. *aureus*
	1	*E. cloacae* + S. *aureus*
	1	*E. faecalis* + S. *aureus*
	1	*E. coli* + P. *mirabilis*
	1	*E. coli* + S. *epidermidis*
	2	*E. coli* + P. *aeruginosa*
3	1	*E. coli* + *E. faecalis* + S. *epidermidis*
	1	*E. coli* + *P. aeruginosa* + S. *epidermidis*
	1	*E. coli* + *E. faecalis* + C. *freundii*

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Table 4
Serological typification of *E. coli* isolates, recovered from the bile of cats, suffering from acute cholangiohepatitis

Таблица 4
Серологическая типизация изолятов *E. coli*, изолированных из желчи кошек, больных острым бактериальным холангиогепатитом

Serogroups	Number of isolated cultures	
	Absolute number	%
015	3	6.7
026	6	13.3
033	1	2.2
041	9	20.0
0101	13	28.9
0138	6	13.3
0141	7	15.6
Total	45	100.0

Table 5
Pathogenicity of bacterial isolates, recovered from the bile of acute cholangiohepatitis-affected cats, for white mice

Таблица 5
Патогенность для белых мышей изолятов бактерий, выделенных от кошек при остром бактериальном холангиогепатите

Microorganism	Cultures studied	Pathogenic		Non-pathogenic	
		Number of isolates	%	Number of isolates	%
Citrobacter freundii	1	1	1.6	0	0
Enterobacter aerogenes	4	3	4.9	1	5.3
Enterobacter cloacae	1	0	0	1	5.3
Escherichia coli	45	39	63.9	6	31.4
Proteus mirabilis	1	0	0	1	5.3
Proteus vulgaris	3	2	3.3	1	5.3
Pseudomonas aeruginosa	4	4	6.6	0	0
Staphylococcus aureus	5	2	3.3	3	15.8
Staphylococcus epidermidis	3	0	0	3	15.8
Enterococcus faecalis	13	10	16.4	3	15.8
Total	80	61	100.0	19	100.0

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False morel poisoning in a cat

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SUMMARY
The material of this case consisted of a 3.5 kg male Chinchilla cat which brought to Selcuk University Veterinary Faculty Animal Hospital with the complaints of anorexia, stagnation, incoordination, watery diarrhea and severe vomiting. Mushroom intoxications suspected according to the anamnesis. False morel poisoning was diagnosed as a result of physical examination and laboratory test and it was confirmed by presence of ingested spore of morel in fecal flotation examination. As a treatment, 0.9% isotonic NaCl (Polyplex, Polifarma®) solutions 40 ml/kg via IV, maropitant (Cerenia, Zoetis®) 1 mg/kg as an antiemetic and to reduce visceral pain via SC, N-acetylcystein (Nacosel, Haver Farma®) 140 mg/kg via IV, vitamins and amino acid supplementation (Duphalyte, Zoetis®) via IV are administered. For liver health supplementation SAMe and silybin (Denamarin, Nutramax®) 15 mg/kg via PO for 21 days prescribed. On re-examination, the cat was fully recovered. It was concluded that false morel poisoning may cause liver damage, neurologic findings along with the primary gastrointestinal system disorders. Anamnesis, presence of the ingested spore of morel observed during fecal examination is very important in the diagnosis and the prognosis is affected by the magnitude of liver damage.

Key words: cat, false morel, *Gyromitra*, poisoning, intoxication.

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Отравление кота ложными сморчками

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РЕЗЮМЕ
Данный случай произошел с котом породы шиншилла весом 3,5 кг, который был доставлен в лечебницу факультета ветеринарной медицины Университета Сельчук с жалобами на отказ от корма, вялость, нарушение координации, водянистую диарею и сильную рвоту. На основании анамнеза подозревалось отравление грибами. В результате осмотра и лабораторного тестирования было диагностировано отравление ложными сморчками (строчками), впоследствии диагноз подтвердили, обнаружив споры данных ядовитых грибов при флотационном исследовании фекалий. Для лечения вводили внутривенно 40 мл/кг 0,9%-го изотонического раствора хлорида натрия (Polyplex, Polifarma®); в качестве противорвотного и для уменьшения висцеральной боли – подкожно 1 мг/кг маропитанта (Cerenia, Zoetis®); внутривенно 140 мг/кг N-ацетилцистеина (Nacosel, Haver Farma®), а также витамины и аминокислоты (Duphalyte, Zoetis®). Для поддержания функции печени в течение 21 сут, согласно назначению, вводили перорально S-аденозин-L-метионин (SAMe) и силибин (Denamarin, Nutramax®). При повторном обследовании было установлено, что кот полностью выздоровел. Был сделан вывод о том, что отравление ложными сморчками (строчками) наряду с основными нарушениями работы желудочно-кишечного тракта может вызывать поражения печени и неврологические расстройства. Для диагностики очень важен анамнез и выявление проглоченных спор сморчков при флотационном исследовании фекалий, прогноз течения болезни обусловлен степенью поражения печени.

Ключевые слова: кот, ложные сморчки, строчки *Gyromitra*, отравление, интоксикация.

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INTRODUCTION

Mushrooms can be classified as edible or poisonous for both humans and animals. The toxicity of a mushroom depends on its toxin and the dose consumed. Common hepatotoxic mushrooms such as *Amanita ocreata* and *Amanita phalloides* (death cap) contain mainly cyclopeptides (amatoxins) causing acute liver failure both in humans and animals. Mushrooms containing hydrazines (e.g. *Gyromitra* spp., also known as false morel), psilocin and psilocybin (e.g. *Psilocybe* spp., *Panaeolus* spp., *Conocybe* spp. and *Gymnopilus* spp.) and isoxazoles (e.g. *A. pantherine*, *A. muscaria*) are considered neurotoxic. Gastroenterotoxic mushrooms such as *Agaricus* sp. and *Boletus* sp. contain toxins which mainly cause gastrointestinal signs [1]. In general, it is widely known that cats are susceptible to toxicosis from all edible and non-edible mushrooms. However, there is no information to specify the toxic mushroom species for cats or their toxic doses [2].

Morchella, which are the true morels, is a genus of edible sac fungi closely related to the order *Pezizales*. These fungi have a honeycomb appearance due to the network of ridges composing their caps abundance [3]. Like *Morchella*, false morels are also members of the *Pezizales*, but within that group represent the families *Morchellaceae*, *Discinaceae*, and *Helvellaceae*, most often *Gyromitra*. In Turkey, China, North America, India, and Pakistan these fungi are found in abundance. In Turkey, especially in Beyşehir, Konya region, the gathering of *Morchella* occupies an important place economically and touristically [4].

Gyromitra esculenta (*G. esculenta*) is a member of a group of fungi known as false morels. The hat of morel is 5–9 cm long, 5–10 cm in diameter, in colors ranging from red brown to dark brown. The hat is hollow. The stem is 2–3 cm long and 1–3 cm in diameter. In *Morchella* species, the hat is in the form of a petitious hollow, while in the *Gyromitra esculenta* type it is in the form of cerebral folds. When eaten raw it is deadly [5]. False morels were considered to be edible in the past. But according to the studies this has been rejected. Therefore, poisoning cases and deaths still are being reported. Poisonings caused by false morels have been studied and the responsible toxin gyromitrim is identified. The severity of the symptoms depends on the quantity of the ingested substance and the time spent after consumption. In human, the mortality rate approximates to 10% of the patients who have poisoning symptoms. The symptoms of false morel, especially *G. esculenta*, poisoning range from gastroenteritis to death [6, 7]. Gyromitrin's toxic metabolite is monomethylhydrazine (MMH). Monomethylhydrazine binds and inhibits pyridoxal phosphokinase, thereby inhibiting activation of vitamin B6 (as pyridoxal 5-phosphate) from functioning as the key co-factor in the synthesis of GABA. Hydrazines, like MMH, may also form hydrazones and hydrazides that can cause further organ damage. Hydrazones induce lipid peroxidation in the liver causing acute liver injury [8]. The first signs appear after 5–12 h or up to 53 h [9]. Acute liver injury can occur over the next 2 days in most of the cases, also acute kidney injury may occur. A cytolytical hepatitis is sometimes accompanied by a hemolysis. In severe cases of intoxication, neurological disorders, such as nervousness, delirium, coma and convulsions may be seen, as a consequence of MMH [8, 9].

Physical exam findings are often non-specific but may include depression, dehydration, muscle tremors, dry mucous membranes, abdominal distention and confusion. Vomiting and watery diarrhea may be observed [10]. Jaundice is a late finding, typically in more severe cases after the 3 days post-ingestion. Large ingestions may exhibit signs of central nervous system involvement, including nervousness, ataxia and seizures. In human, the mortality rate approximates to 10% of the patients who have poisoning [7].

The treatment must be symptomatic. The hemodynamics, the blood ionic concentration, the azotemia, the creatinemia, and the blood gases must be monitored to compensate for the hydroelectrolytic loss. Acute liver injury and hemolysis can be diagnosed by early serum biochemistry analysis of biological indicators (increase in AST, ALT, bilirubin, triglycerides, LDH) [7].

TEST RESULTS

The material of this case consisted of a 3.5 kg male Chinchilla cat which brought to Selcuk University Veterinary Faculty Animal Hospital with the complaints of anorexia, stagnation, incoordination, watery diarrhea and severe vomiting. In the anamnesis, it was learned that normally, the cat is fed with canned food and occasionally goes out of the house. The owner of the cat did not witness ingestion of the false morel but it was learned that in the area where the house is located (Beyşehir, Konya) false morel can be found in abundance. Approximately 8 h after going out of the house, the cat returned home depressed and with a significant saliva increase observed by the owner.

In the physical examination of the cat, hypersalivation, weakness, abdominal distension and depression were detected. Following the physical examination, blood gases, hemogram, biochemical analysis, fecal flotation examination, abdominal ultrasonography and radiographic examination were performed. In fecal flotation examination plant debris and spores of morels were seen, any other parasite eggs did not observed (Fig. 1).

In the radiographic examination of the abdomen, thickening of the gastric mucosa and abdominal distension were observed (Fig. 2).

With the abdominal ultrasound examination, rugal fold thickening, gastric mucosal edema and abdominal distension (Fig. 3) were confirmed.

Although blood gas and hemogram parameters were within the reference range, anemia and mild leukocytosis were noticed (Table 1).

In serum biochemistry, increased lactate dehydrogenase (LDH), creatine phosphokinase (CPK), aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline

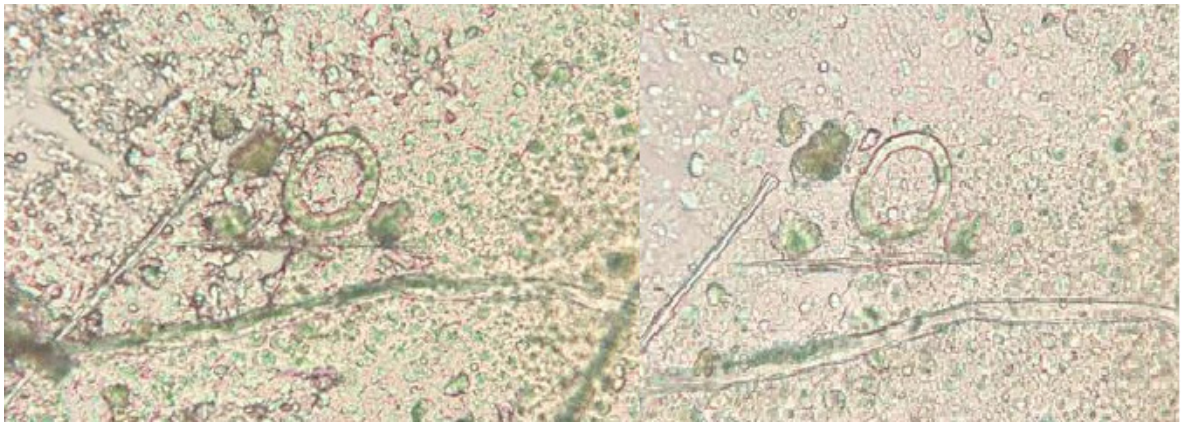

Fig. 1. Image of the spores of morel and plant debris in light microscope (×40 magnification, unstained)

Рис. 1. Изображение спор сморчков и остатков растений при исследовании под световым микроскопом (увеличение ×40, неокрашенные)

phosphatase (ALP) levels, hyperbilirubinemia and hypertriglyceridemia were observed. Other parameters were in the normal reference range (Table 2).

To rule out other diseases cause similar non-specific symptoms, qualitative detection of FeLV antigen/FIV antibody (Asan Easy Test, Asan Pharm) and FIPV antibody (FASTest FIP Ab, Vetlab Supplies) tests were performed and resulted negative.

As a treatment, 0.9% isotonic NaCl (Polyplex, Polifarma®) solutions 40 ml/kg via IV, maropitant (Cerenia, Zoetis®) 1 mg/kg as an antiemetic and to reduce visceral pain via SC, N-acetylcystein (Nacosel, Haver Farma®) 140 mg/kg via IV, vitamins and amino acid supplementation (Duphalyte, Zoetis®) via IV are administered. The cat was discharged from the hospital after admission in good general condition and appetite. For liver health supplementation SAMe and silybin (Denamarin, Nutramax®) 15 mg/kg via PO for 21 days prescribed. After prescribed drug usage, on re-examination, the cat was fully recovered.

DISCUSSION

Mushroom intoxications in animals, and especially in cats, are underreported. In the majority of feline mushroom toxicoses reported, mushrooms were characterised as of unknown origin and were not identified [1]. Cats are potentially susceptible to toxicosis from all edible and non-edible mushrooms; however, there is no information to specify the toxic mushroom species for cats or their toxic doses. In general, mushrooms can cause a variety of non-specific clinical and clinicopathologic signs, which make diagnosis of a mushroom-specific toxicosis difficult [10].

Morels are edible mushrooms, which can be toxic when poorly cooked or eaten raw. Ingestion of raw or poorly cooked morels may cause gastrointestinal disorders (nausea, vomiting, abdominal pain, diarrhea, etc.) with a time to onset that has never been clearly documented. In the previously reported cases, neurological effects appeared after a median of 10 h (12 h in our series) after consumption of *Morchella* sp. Signs and symptoms were very similar to those observed in the present series and included dizziness, tremors, gait or postural instability and gastrointestinal or ocular disorders [11, 12]. In this present report gastrointestinal symptoms were remarkable.

CPK elevation could have been a consequence of muscle damage due to muscle tremors. Increased AST, ALT, ALP levels and hyperbilirubinemia could have been

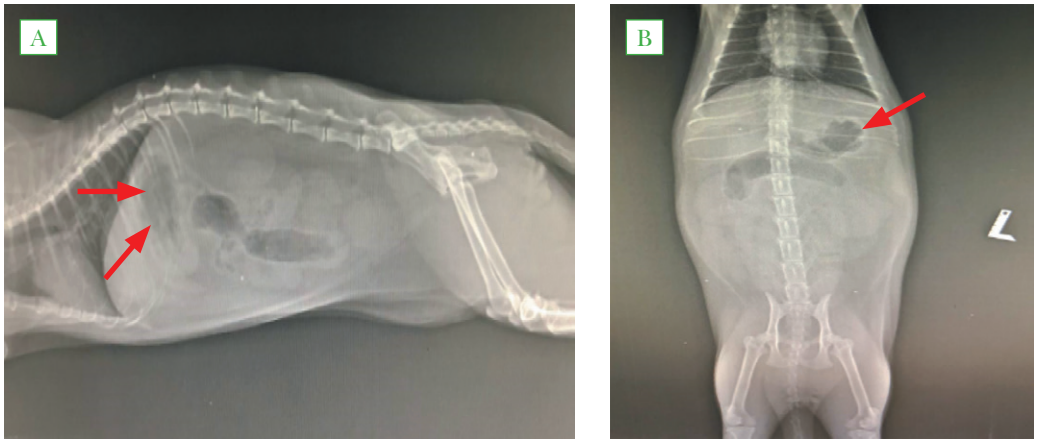

Fig. 2. Radiographic radiopaque (A) latero-lateral and (B) ventrodorsal image of the gastric wall thickening

Рис. 2. Рентгенографическое исследование: А – рентгеноконтрастное в боковой проекции и В – вентродорсальное изображение утолщения стенки желудка

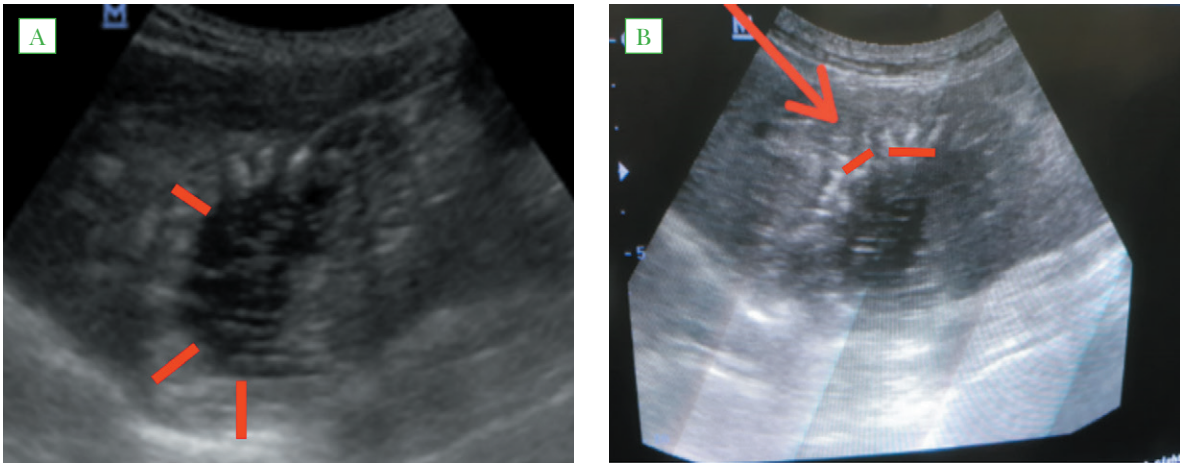

Fig. 3. (A) Rugal fold thickness (5–6 mm) and (B) interrugal thickness (3–4 mm) in the abdominal ultrasonographic images

Рис. 3. Ультразвуковое исследование брюшной полости: А – утолщение складки слизистой желудка (5–6 мм), В – утолщение между складками слизистой желудка (3–4 мм)

the result of hepatotoxicosis or a reactive hepatic consequence of the gastrointestinal inflammation [10]. False morel consumption was thought to be the evidence of the anamnesis by the owner and abundance of the false morel in the area where the house is located, also the presence of the spores of morel in fecal examination. Furthermore, infectious diseases such as FeLV, FIV and FIPV were ruled out grossly through laboratory investigation.

Table 1
Blood gases and hemogram findings

Таблица 1
Газовый состав и данные общего клинического анализа крови (гемограмма)

Parameters	Findings	Reference
pH	7.434	7.35–7.45
K, mmol/L	4.2	3.4–5.6
Na, mmol/L	153	150–165
Cl, mmol/L	119	104–128
Lactate, mmol/L	1.8	0–2
Hct, %	29.8	29–48
Base (Ecf), mmol/L	–9.9	–4÷4
Base (B), mmol/L	–8.8	–4÷4
HCO_3^- (P, st), mmol/L	17.3	19–24
HCO_3^- (P), mmol/L	14.3	19–24
WBC, m/mm³	**21.32 ↑**	5.0–19.0
RBC, m/mm³	**3.50 ↓**	4.0–9.0
MCV, fl	43.3	35.5–55.0
Hct, %	24.4	24.0–45.0
MCHC, g/dl	33.9	28.0–40.0
RDW	9.3	8.0–12.0

pH – hydrogen ion concentration (концентрация ионов водорода), K – potassium (калий), Na – sodium (натрий), Cl – chlorine (хлор), Hct – hematocrit (гематокрит), HCO_3^- – bicarbonate (бикарбонат), WBC – white blood cell (лейкоциты), RBC – red blood cell (эритроциты), MCV – mean corpuscular volume (средний объем эритроцитов), MCHC – mean corpuscular hemoglobin concentrarion (средняя концентрация гемоглобина в эритроцитах), RDW – red cell distribution width (ширина распределения эритроцитов по объему).

Table 2
Serum biochemistry findings

Таблица 2
Показатели биохимического анализа сыворотки крови

Parameters	Findings	Reference
BUN	26.5 mg/dL	14–36
Creatinin	1.0 mg/dL	0.6–2.4
AST	**214 U/L ↑**	10–100
ALT	**184 U/L ↑**	10–100
ALP	**114 U/L ↑**	6–102
Amylase	658 U/L	100–1,200
Glucose	150 mg/dL	64–170
Magnesium	1.7 mg/dL	1.5–2.5
LDH	**780 U/L ↑**	20–500
Total bilirubin	**0.9 mg/dL ↑**	0.1–0.6
Direct bilirubin	**0.5 mg/dL ↑**	0–0.4
Phosphorus	4.4 mg/dL	2.4–8.2
Cholesterol	157 mg/dL	75–220
Albumin	2.9 mg/dL	2.5–3.9
Calcium	7.8 mg/dL	8.2–10.8
Triglycerid	**248 mg/dL ↑**	25–160
Gamma glutamyl transferase	1 U/L	1–10
Protein	6.8 g/dL	5.2–8.8
CPK	**2,162 U/L ↑**	50.00–450.00

CONCLUSION

True morels are widely gathered and considered safe for consumption by amateur mycologists, and true morels can be distinguished from toxic false morels by their appearance (*G. esculenta*, wrinkled cap with brain-like folds). False morel poisoning is characterized by gastrointestinal disorders appearing 6–12 h after ingestion, and in severe cases may be associated with hepatitis, nephritis and neurologic disorders such as tremors, incoordination and seizures [13]. It is generally accepted that the development of neurological signs or symptoms is a dose-dependent effect [14].

Mushroom toxicosis was considered to be the most likely diagnosis. The weak point of this case report is the absence of systematic mycological identification. Prognosis depends on the quantity of the ingested substance, the time spent after consumption and the presence of any concurrent disease of the animal [10]. The Veterinary Poisons Information Service in the United Kingdom has received 867 dog enquiries related with mushroom poisoning, while this number is only 57 in cats [15]. For this reason, it is very important to share mushroom toxicity cases or studies in cats in the scientific world. Finally, it was concluded that anamnesis, presence of the false morel in the area where the animal lives, presence of the ingested spore of the morel in fecal examination are very important in the diagnosis and serum biochemistry analysis in the prognosis as many mushroom poisonings are underrated.

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Immunogenic characteristics of *Avibacterium paragallinarum* (serogroup B) isolates and strains

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SUMMARY
Infectious disease of chickens caused by the bacterium *Avibacterium paragallinarum* remains one of the urgent problems of the poultry industry, as evidenced by numerous reports of recurrent infectious coryza outbreaks in chickens around the world. Bacteriological tests performed in 2014–2019 demonstrated that the disease caused by *Avibacterium paragallinarum* (serogroup B) is endemic in the Russian Federation. The paper presents the results of tests for immunogenic properties of antigens of 13 infectious coryza isolates recovered from the pathological material delivered to the FGBI "ARRIAH" from poultry farms of the Russian Federation and the Republic of Belarus. For this, samples of the vaccine containing formalin-inactivated *Avibacterium paragallinarum* cells and an oil adjuvant were prepared. The poultry was immunized followed by challenge with homologous and heterologous isolates. The degree of manifestation of the disease clinical signs was assessed according to the method proposed by V. E. Soriano. The vaccine sample based on the antigen of the ApB08 isolate induced an insufficient immune response in poultry when infected with the ApB04 and ApB12 isolates. Conversely, a high level of animal protection was demonstrated when infected with the ApB08 isolate. ApB04, ApB08 and ApB12 isolates were comprehensively studied, identified as the most promising for production of vaccines against infectious coryza in chickens, and deposited in the FGBI "ARRIAH" Microorganism Strain Collection under numbers 1116, 5111 and 1818, respectively. Also, a comparative assessment of potency of the experimental vaccine and two commercial products against infectious coryza, including antigens of strains No. 1116, 5111 and 1818, was performed. The experimental vaccine demonstrated maximum protection against infection with *Avibacterium paragallinarum* homologous strains.

Key words: infectious coryza, isolates, strains, vaccine, challenge infection, *Avibacterium paragallinarum*.

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Иммуногенные свойства изолятов и штаммов *Avibacterium paragallinarum* серогруппы В

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РЕЗЮМЕ
Инфекционное заболевание кур, вызываемое бактерией *Avibacterium paragallinarum*, остается одной из актуальных проблем птицеводческой отрасли, о чем говорят многочисленные сообщения о периодических вспышках инфекционного ринита кур в разных странах мира. Проведенные с 2014 по 2019 г.

бактериологические исследования показали, что Российская Федерация эндемична по данному заболеванию, вызываемому *Avibacterium paragallinarum* серогруппы В. Представлены результаты исследования по изучению иммуногенных свойств антигенов 13 изолятов возбудителя инфекционного ринита кур, выделенных из патологического материала, доставленного в ФГБУ «ВНИИЗЖ» с птицефабрик Российской Федерации и Республики Беларусь. Для этого готовили образцы вакцины, содержащей в своем составе инактивированные формалином клетки *Avibacterium paragallinarum* и масляный адъювант. Птиц иммунизировали с последующим контрольным заражением гомологичными и гетерологичными изолятами. Степень проявления клинических признаков заболевания оценивали по методике, предложенной V. E. Soriano. Образец вакцины на основе антигена изолята АрВ08 индуцировал недостаточный иммунный ответ у птиц при инфицировании изолятами АрВ04 и АрВ12. В свою очередь, при заражении изолятом АрВ08 был показан высокий уровень защиты животных. Изоляты АрВ04, АрВ08 и АрВ12 были всесторонне изучены, определены как наиболее перспективные для производства вакцины против инфекционного ринита кур и депонированы в Государственную коллекцию штаммов микроорганизмов ФГБУ «ВНИИЗЖ» под номерами 1116, 5111 и 1818 соответственно. Также была проведена сравнительная оценка иммуногенной активности экспериментальной вакцины против инфекционного ринита кур, включающей антигены штаммов № 1116, 5111 и 1818, с двумя коммерческими препаратами. Экспериментальный препарат показал максимальный процент защиты птиц при заражении гомологичными штаммами *Avibacterium paragallinarum*.

Ключевые слова: инфекционный ринит кур, изоляты, штаммы, вакцина, контрольное заражение, *Avibacterium paragallinarum*.

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INRODUCTION

The agent of infectious coryza in chickens is the representative of the *Pasteurellaceae* family, *Avibacterium paragallinarum* bacteria, known as *Haemophilus paragallinarum*. The disease can occur on its own and it can manifest itself in the form of associations with other infectious diseases [1–4].

Numerous reports of recurrent infectious coryza outbreaks in different countries of the world indicate the urgency of the disease problem. Bacteriological studies carried out at the FGBI "ARRIAH" from 2014 to 2019 showed that the Russian Federation is endemic for infectious coryza caused by *Avibacterium paragallinarum* (serogroup B).

Economic losses in the event of infection can be significant, primarily due to poor growth and decrease in egg production in hens at the productivity peak up to 40% within 2–3 weeks [5, 6]. The disease is characterized by facial swelling and serous-fibrinous inflammation of the infraorbital sinuses, air sacs and the mucous membrane of the upper respiratory tract. With an associative course, the duration and severity of the disease may increase. Improper microclimate in the poultry house can provoke the occurrence of the infection, which subsequently leads to an increase in the mortality of birds up to 20–40% [7].

Diseased birds are the source of the infection. The pathogen can circulate in the flock for a long time, this is especially true for small farms containing birds of different ages. When a disease occurs on such farms, it is very difficult to get rid of or control the pathogen. The pathogen is transmitted aerogenically and with drinking water. The incubation period is from one to ten days with the duration of the disease from 14 to 21 days.

One of the most effective measures to combat infectious coryza in chickens is specific prophylaxis. Vaccination reduces dependence on the constant use of antibacterial drugs, which in turn provides cost savings and also prevents problems associated with antibiotic resistance of microorganisms or antibiotic residues in poultry products. However, the main obstacle in manufacturing an effective inactivated vaccine is the selection of commercial pathogen strains. Herewith, it should be borne in mind that the antigenic structure of *A. paragallinarum* is complex and diverse, and the induced immune response in chickens is serotype-specific, with partial cross-protection between individual serotypes.

Serological typing of *A. paragallinarum* is most often carried out according to two interrelated schemes. The three serogroups of the pathogen – A, B, C, are determined by the agglutination test according to the Page's scheme, and nine serotypes (A1–A4, B-1 and C1–C4) by hemagglutination inhibition assay according to K. Kume's scheme [8, 9].

Currently, a number of foreign drugs for specific prevention of infectious coryza in chickens have been registered in the Russian Federation, which differ in composition, production technology, scheme of use and effectiveness. Most of them provide protection against the most common serotypes of the pathogen, which is their undoubted advantage. The versatility of the drugs is due to the presence of cross-protection between serotypes within serogroup A, as well as between individual serogroup C serotypes. The situation with the choice of a serogroup B strain is much more complicated. According to the scheme proposed by K. Kume, there is only one serotype in serogroup B (B-1), however, as far as immunogenicity is concerned only partial cross-protection is observed between the individual strains/isolates [10–12].

In recent years, outbreaks caused by *A. paragallinarum* serotype B-1 have become more frequent in Europe and Asia, which sometimes has been concurrent with the use of commercial vaccines against infectious coryza [5]. At the same time, the reason for the weak cross-protection

between different strains of serotype B-1 has not yet been established. According to a number of researchers, this serotype is widespread in Argentina, Brazil, China, Ecuador, Egypt, Indonesia, Mexico, Peru, the Philippines, South Africa, Spain, the United States of America, and Zimbabwe [6, 11].

Since different strains of serotype B-1 provide only partial cross-protection with each other, it can be assumed that an effective vaccine can be made from only a few strains isolated in a specific geographic region where this serotype is endemic. In addition, commercial vaccines containing antigens of several variants of serotype B-1 strains may also be effective.

Weak cross-protection between different strains/isolates of serogroup B is possibly associated with their high virulence and the leading role of various pathogenic factors [3, 13].

When testing pathological material from birds delivered in the period from 2014 to 2018 to the FGBI "ARRIAH" from various regions of the Russian Federation and the Republic of Belarus, 13 isolates of the infectious coryza pathogen were recovered using bacteriological methods. Analysis of the pathogen's serological profile showed the prevalence of *A. paragallinarum*, serogroup B circulation [3].

In 2016, at two large poultry farms of the Russian Federation, infected with infectious coryza and using a commercial trivalent emulsion vaccine against infectious coryza, during serological typing of isolates recovered from the material derived from sick poultry, it was established that they belonged to serogroup B. This fact raises doubts about the versatility of the vaccine used in our country. Therefore, researches aimed at finding relevant strains of *A. paragallinarum*, serogroup B, will contribute to the creation of a competitive home-made vaccine.

The purpose of this research was to study the immunogenic properties of isolates and strains of *A. paragallinarum*, serogroup B, recovered in the territory of the Russian Federation and the Republic of Belarus, and the possibility of their use in the development of vaccines.

MATERIALS AND METHODS

Pathogen isolates. We used 13 isolates (ApB01–ApB13) of *A. paragallinarum*, serogroup B, recovered at the FGBI "ARRIAH" from material derived from chickens from poultry farms of the Russian Federation and the Republic of Belarus [3]. The origin of the isolates is shown in Table 1.

Isolate cultivation. Columbia agar and broth (Becton Dickinson and Co., USA) containing the following growth factors: 20 μg/ml nicotinamide adenine dinucleotide (NAD, AppliChem, Germany) and 5% horse blood serum were used as a growth medium for culturing *A. paragallinarum*. Cultivation of the bacteria in an agar medium was carried out at 37 °C for 24 h under conditions of an increased content of carbon dioxide, in a liquid nutrient medium – at 37 °C for 18 h under conditions of a normal atmosphere in an orbital shaker-incubator at 150 rpm.

Antigen preparation. Inactivation of *A. paragallinarum* isolates was performed using formaldehyde solution at 37 °C for 48 h, and the final formalin concentration was 0.2% by volume. Then the cells were centrifuged at 3,000 g for 20 min at 4 °C, the sediment was resuspended in sterile phosphate buffered saline with pH 7.4 to a concentration of 100 units (10^{10} m. c./cm^3) according to the optical turbidity standard. The obtained antigens were stored at a temperature of (2–8) °C [3].

Experimental poultry. Hisex Brown hens 10 weeks of age were used in the testing. The birds were transported from a farm free of infectious coryza. In all the testings in the experimental and control groups there were ten birds each.

Table 1
***A. paragallinarum* isolate origin**

Таблица 1
Происхождение изолятов *A. paragallinarum*

Isolate	The agent isolated from	The year of isolation	Region
ApB01	Infraorbital sinuses	2014	Kostroma Oblast
ApB02	Conjunctival sac	2014	Moscow Oblast
ApB03	Infraorbital sinuses	2015	Moscow Oblast
ApB04	Infraorbital sinuses	2015	Republic of Belarus
ApB05	Infraorbital sinuses	2016	Republic of Tatarstan
ApB06	Lungs	2016	Vladimir Oblast
ApB07	Infraorbital sinuses	2016	Yaroslavl Oblast
ApB08	Infraorbital sinuses	2016	Orenburg Oblast
ApB09	Infraorbital sinuses	2017	Ulyanovsk Oblast
ApB10	Infraorbital sinuses	2017	Moscow Oblast
ApB11	Infraorbital sinuses	2017	Vladimir Oblast
ApB12	Infraorbital sinuses	2018	Republic of Mordovia
ApB13	Infraorbital sinuses	2018	Yaroslavl Oblast

All experiments on the tested poultry were carried out in strict accordance with the interstate standards for laboratory animal management and care, GOST 33216-2014 and GOST 33215-2014, adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of Directive 2010/63/EU of the European Parliament and the Council of the European Union as of 22.09.2010 on the protection of animals used for scientific purposes.

Preparation and use of the vaccine samples. The antigens of 13 *A. paragallinarum* isolates were used to prepare experimental mono- and multistrain vaccine samples. The antigen suspensions were mixed with Montanide ISA 70 VG oil adjuvant (Seppic, France) at a ratio of 30:70 by weight using a Silverson L4RT laboratory homogenizer. The concentration of each antigen in the inoculation dose of 0.5 cm^3 was 5×10^8 m. c. according to the optical turbidity standard. The preparations were injected twice with an interval of 21 days subcutaneously, in the region of the middle third of the neck from the dorsal side in the caudal direction.

Two commercial vaccines registered in the Russian Federation were used as a comparative control.

Determination of the vaccine immunogenic characteristics. The immunogenic characteristics of antigens were determined by the method of vaccination and subsequent challenge of immunized birds with homologous and heterologous isolates. Chickens were infected intranasally with daily broth cultures of isolates at a dose of 0.5 cm^3 containing 10^8 CFU of the pathogen.

Results of the infection. The degree of manifestation of the disease clinical signs was assessed according to the method proposed by V. E. Soriano [14].

The severity of the upper respiratory tract lesions in an infected bird was assessed using a point-based system:

0 – no clinical signs;

1 – weak discharge from the nasal passages and/or slight swelling of the infraorbital sinus area;

2 – moderate discharge from the nasal passages and/or moderate swelling of the infraorbital sinus area;

3 – severe nasal discharge and/or severe swelling of the infraorbital sinuses;

4 – severe nasal discharge and severe swelling of the infraorbital sinuses, rattling.

Clinical signs were monitored daily for each bird. Seven days after infection, the points were calculated for each group, and divided by the total number of the infected birds.

RESULTS AND DISCUSSION

The isolates of *A. paragallinarum* ApB01–ApB13 used in the research were recovered in the period from 2014 to 2018 from birds with respiratory pathology aged 38 to 211 days from farms of Vladimir, Kostroma, Moscow, Orenburg, Ulyanovsk and Yaroslavl oblasts, the Republics of Mordovia and Tatarstan, one isolate was recovered from poultry from the Republic of Belarus.

Isolates ApB04 and ApB08 were recovered during an outbreak of infectious coryza from chickens in 1–2 months following the use of commercial vaccines containing antigens of serogroups A, B and C.

In most cases, clinical signs in the diseased chickens were of the same type and were characterized by swelling of the infraorbital sinuses and conjunctival sacs, sometimes watery discharge from the nasal openings was observed. Some birds demonstrated mouth breathing with rattling due to blockage of the nasal passages. Most of *A. paragallinarum* isolates were recovered from egg layers. The infraorbital sinuses were the main site of pathogen localization. In 24 h of incubation on the agar medium, the culture formed colonies of round and convex shape, with smooth edges and a smooth surface, gray, 0.5–1.0 mm in diameter (S-shape). A characteristic feature of 24-hour cultures was fluorescence of colonies in oblique light, which indicated the presence of a capsule in the bacteria.

In previous researches, we studied the virulent properties of *A. paragallinarum*, serogroup B, isolates for chickens. When the birds were infected with various isolates of *A. paragallinarum*, the duration of the disease periods was observed to be the same. In sick chickens, similar clinical signs manifested by rhinitis, sinusitis and conjunctivitis were observed [1].

When studying the immunogenic properties of the isolates, a trial immunization ($n = 1$) of chickens with a sample of the vaccine with the ApB08 antigen was performed, followed by infection with homologous and heterologous isolates. The reason for choosing this isolate was that, when cultivated on a nutrient medium, it accumulated to high concentrations, while maintaining a stable hemagglutinating activity and a high level of virulence.

The results of infection of birds immunized with a vaccine sample containing the antigen of the ApB08 isolate are presented in Table 2.

The experiment showed that immunization with the vaccine sample containig the antigen of the ApB08 isolate provided protection of birds at a level of at least 80% when infected with homologous and heterologous isolates, except for ApB04 and ApB12. When the birds of the control groups were infected, an incidence of at least 80% was observed with the development of symptoms typical for infectious coryza. The first clinical signs in birds were observed 24–48 h after infection. Clinically, the disease was manifested by watery discharge from the nasal openings and slight one- or two-sided swelling of the infraorbital sinuses. In some cases, clinical signs were limited to the specified above symptoms. In some birds, the exudate gradually became cloudy and acquired a slimy consistency, as a result of which the nasal openings were obstructed, and the bird began to breathe through the mouth. In most of the infected birds, the disease was accompanied by considerable swelling of the infraorbital sinuses and conjunctival sacs, while in the diseased chickens depression, drowsiness and poor feed intake were observed. Sometimes, due to obstruction of the nasolacrimal duct, exudate penetrated into the oral cavity through the palatal cleft. Feathers in the area of the neck and wings were contaminated with the discharged exudate. Some birds developed unilateral or bilateral catarrhal conjunctivitis, subsequently fibrin was observed in the exudate, the eyelids swelled, and the palpebral fissure narrowed. When the infection was localized in the deeper parts of the respiratory tract, in some individuals, breathing was accompanied by rattling. The most severe symptoms of the disease were observed in birds in groups infected with isolates ApB03, ApB04, ApB08, ApB09, ApB12, and ApB13.

To confirm the revealed differences in the protective characteristics of the antigens ApB04, ApB08, and ApB12, an additional experiment on immunization was carried out, followed by challenge with homologous and heterologous isolates (Table 3).

When the immunized poultry were infected with homologous isolates, the percentage of protection was at least 86.7 ± 13.1%, and when infected with heterologous

Table 2
Immunogenic characteristics of ApB08 antigen when infecting chickens with the homologous and heterologous isolates ($n = 1$)

Таблица 2
Иммуногенные свойства антигена ApB08 при заражении кур гомологичным и гетерологичными изолятами ($n = 1$)

Group / Isolate	Vaccine with the antigen of ApB08 isolate		Control	
	% P	S	% D	S
ApB01	100	0	100	1.8
ApB02	100	0	80	1.8
ApB03	80	0.8	100	2.2
ApB04	20	2.8	100	3.0
ApB05	100	0	100	1.6
ApB06	100	0	100	1.8
ApB07	80	0.6	90	1.2
ApB08	100	0	100	2.2
ApB09	80	0.8	100	2.4
ApB10	100	0	100	1.8
ApB11	100	0	100	1.6
ApB12	0	3.0	100	3.2
ApB13	80	0.8	100	2.4

% P – protection rate observed at the infection (процент защиты, наблюдаемый при заражении);
% D – percent of the diseased animals at the infection (процент заболевших животных при заражении);
S – severity of the manifested clinical signs (тяжесть проявления клинических признаков) (по V. E. Soriano [12]).

isolates, it did not exceed 26.7 ± 13.1% ($p \leq 0.05$). As a result of the tests performed, it was found that in terms of immunogenic activity, samples with isolates ApB04, ApB08 and ApB12 differ significantly from each other and therefore, are promising for vaccine production. The isolates were comprehensively studied and deposited in the State Microorganism Strain Collection of the FGBI "ARRIAH" as strains No. 1116, 5111, 1818, respectively.

At the next stage of the research, we carried out comparative tests of an experimental monovalent vaccine produced by the FGBI "ARRIAH", including antigens of strains No. 1116, 5111, 1818, and two commercial polyvalent vaccines (Table 4).

The results of the tests performed demonstrated that both commercial vaccines induce an insufficient immune response in birds when infected with strains No. 1116 and 1818.

In addition to determining the immunogenicity of the tested vaccines, we evaluated the clinical profile of the vaccinated and non-vaccinated birds, which included

Table 3
Immunogenic characteristics of ApB04, ApB08, ApB12 antigens at the control challenge with the homologous and heterologous isolates ($n = 3$)

Таблица 3
Иммуногенные свойства антигенов ApB04, ApB08, ApB12 при контрольном заражении гомологичными и гетерологичными изолятами ($n = 3$)

Group	Isolate antigen	% protection in birds challenged with the isolate		
		ApB04	ApB08	ApB12
Immunized	ApB04	**93.3 ± 13.1**	20.0	26.7 ± 13.1
	ApB08	6.7 ± 13.1	**86.7 ± 13.1**	6.7 ± 13.1
	ApB012	13.3 ± 13.1	20.0	**93.3 ± 13.1**
Naive	–	0	0	6.7 ± 13.1

Table 4
Comparative assessment of potency of the commercial polyvalent vaccines and the experimental monovalent vaccine after infection with *A. paragallinarum* strains No. 1116, 5111, 1818 (*n* = 3)

Таблица 4
Сравнительная оценка иммуногенности коммерческих поливалентных вакцин и экспериментальной моновалентной вакцины после заражения штаммами № 1116, 5111, 1818 *A. paragallinarum* (*n* = 3)

Vaccine	% protection in birds challenged with the strain		
	No. 1116	No. 5111	No. 1818
Commercial vaccine 1	46.6 ± 13.1	**86.6 ± 6.5**	53.3 ± 17.3
Commercial vaccine 2	40.0 ± 11.3	**90.0 ± 11.3**	53.3 ± 13.1
FGBI "ARRIAH" vaccine	**96.6 ± 6.5**	**100**	**90.0 ± 11.3**
Control	0	0	0

determining the severity and duration of the disease when infected with *A. paragallinarum* strains No. 1116, 5111 and 1818 (Fig. 1–3).

As can be seen from Figure 1, after the challenge of vaccinated and non-vaccinated birds with strain No. 1116, the disease incubation period was less than 24 hours. The maximum development of the disease clinical signs was observed in 48 hours after infection. According to a number of researchers [8, 9, 11], in case of intranasal infection of birds, the incubation period is 24–48 h, in case of contact between the diseased and healthy poultry kept in cages – three days, and in case of aerogenic pathogen transmission – up to six days. On the third day, a decrease in the severity of disease symptoms was observed in poultry of the control group and immunized with commercial vaccines, and in the group of birds immunized with the FGBI "ARRIAH" vaccine based on the homologous strain, complete recovery was observed. The duration of the disease in poultry of the control group was seven days, and in chickens immunized with commercial vaccines – five days.

As can be seen in Figure 2, the disease incubation period in birds of the control group was less than 24 hours, and the maximum development of the disease clinical signs was observed 48 hours after infection, with the average point 3.1. The FGBI "ARRIAH" vaccine provided proper protection of birds when challenged with strain No. 5111. Both commercial vaccines also showed a high degree of protection when infected with strain No. 5111, which indicates the presence of cross-immunity between the strains.

As can be seen from Figure 3, the results of infection with strain No. 1818 are quite similar to those for strain No. 5111. The most pronounced disease clinical signs in birds of the control group were observed 48 hours after infection, with an average point of 3.8. In the majority

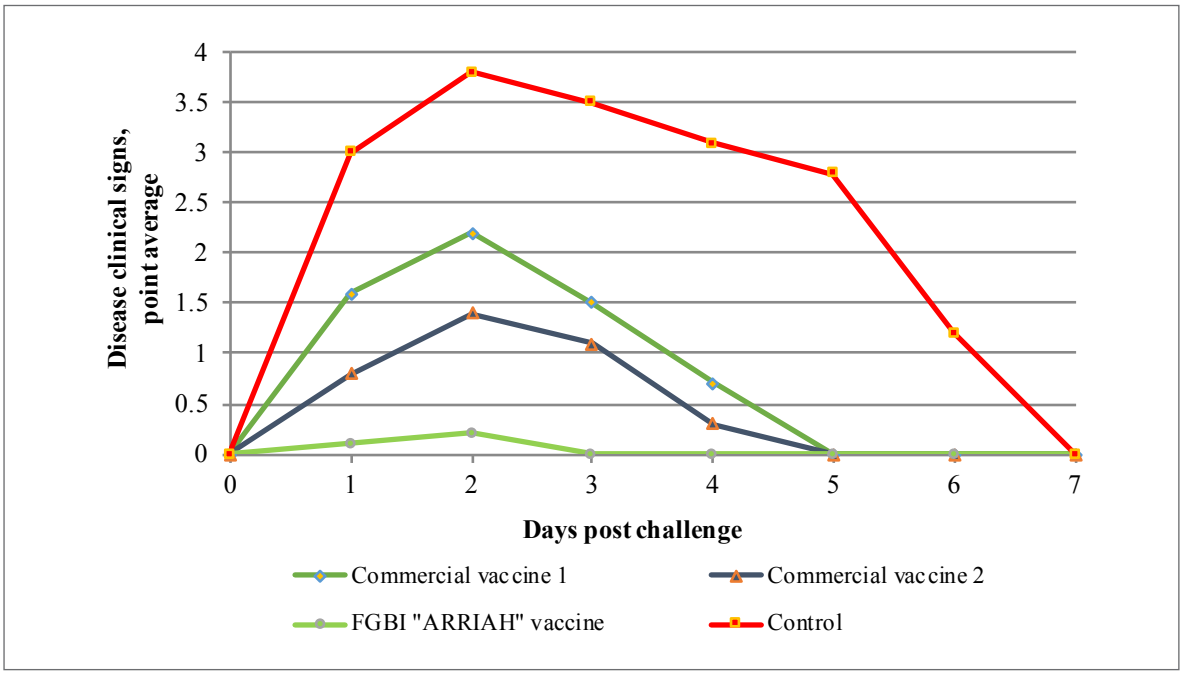

Fig. 1. Clinical profiles of vaccinated and non-vaccinated poultry infected with A. paragallinarum strain No. 1116

Рис. 1. Клинические профили вакцинированных и невакцинированных птиц при заражении штаммом № 1116 A. paragallinarum

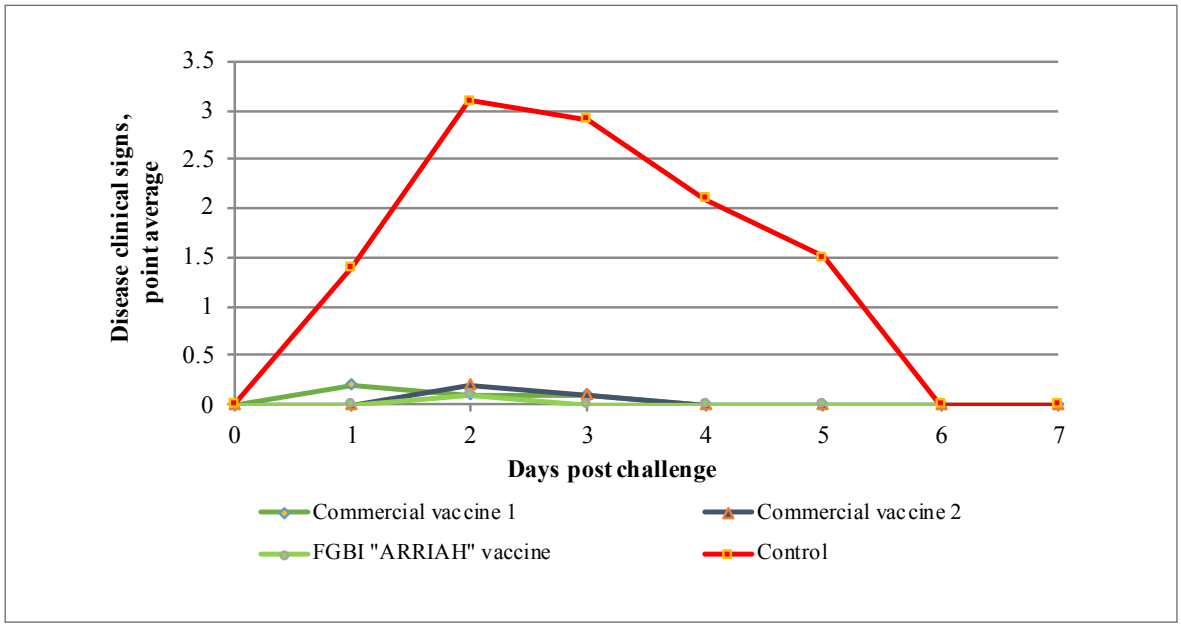

Fig. 2. Clinical profiles of vaccinated and non-vaccinated poultry infected with A. paragallinarum strain No. 5111

Рис. 2. Клинические профили вакцинированных и невакцинированных птиц при заражении штаммом № 5111 A. paragallinarum

of diseased birds, clinical signs were limited to mild, moderate or severe swelling of the infraorbital sinuses and conjunctival sacs; in sick chickens, depression, drowsiness and poor feed intake were observed. On the third day post infection, a decrease in the severity of disease symptoms was observed in birds of the control and experimental groups. The duration of the disease in birds of the control group was seven days, in chickens immunized with the commercial vaccines – four and five days, respectively, and in birds vaccinated with the FGBI "ARRIAH" vaccine – three days. According to a number of researchers [7, 8, 11], the duration of the disease in chickens in natural conditions is usually 2–3 weeks, and in case of an experimental infection – 5–7 days.

Partial cross-protection between the vaccine strain and field isolates of *A. paragallinarum*, serogroup B, does not provide a positive effect of poultry immunization [10–13]. Therefore, as it is like that there is no cross-protective

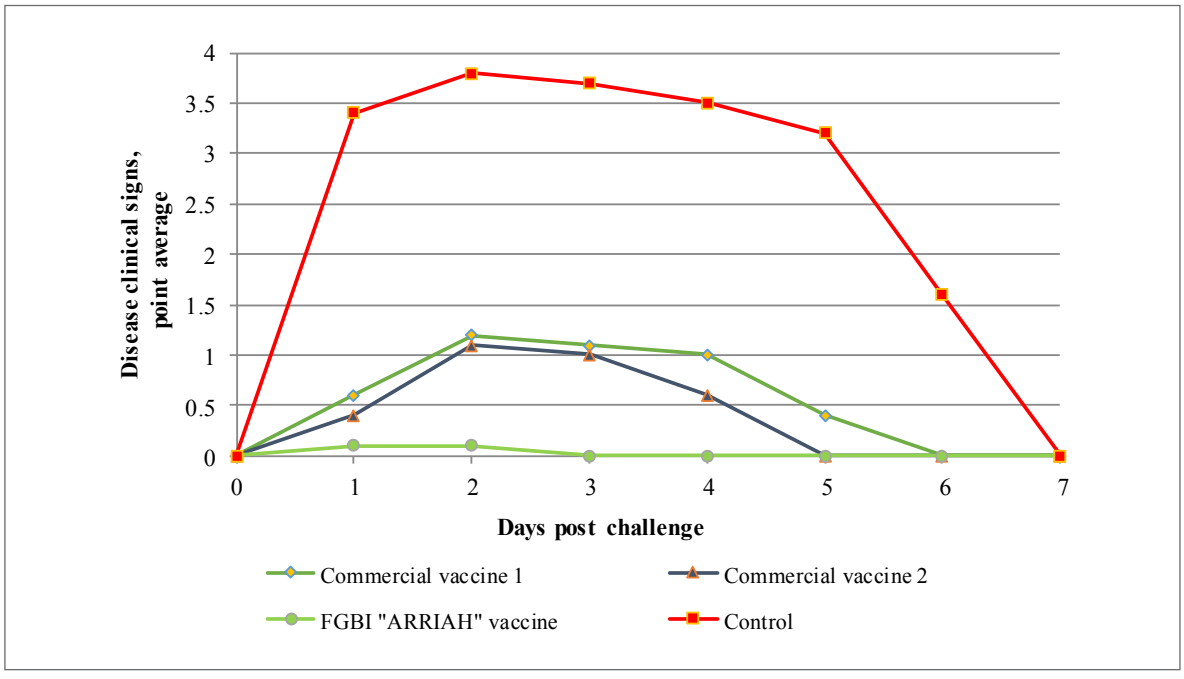

Fig. 3. Clinical profiles of vaccinated and non-vaccinated poultry infected with A. paragallinarum strain No. 1818

Рис. 3. Клинические профили вакцинированных и невакцинированных птиц при заражении штаммом № 1818 A. paragallinarum

mechanism between strains of *A. paragallinarum*, serogroup B, vaccines containing the maximum possible set of pathogen strains relevant to the region of application are most preferred. In addition, the experience of many countries shows that the most effective in this situation is the use of autogenous vaccines, which are quite effective against any circulating serotype of the pathogen [11].

CONCLUSION

The study of the immunogenic characteristics of antigens of 13 isolates of *A. paragallinarum*, serogroup B, recovered from the pathological material from chickens, delivered to the FGBI "ARRIAH" from poultry farms of the Russian Federation and the Republic of Belarus, showed that vaccine samples based on the antigens of the isolates ApB04, ApB08 and ApB12 induced an insufficient immune response in the poultry in case of cross-infection with heterologous isolates. The percentage of protection was 26.7 ± 13.1%. Conversely, during infection with homologous isolates, a high level of protection was demonstrated – 86.7 ± 13.1% ($p \leq 0.05$). Isolates ApB04, ApB08 and ApB12 were comprehensively studied, identified as the most promising for the production of vaccines against infectious coryza of chickens, and deposited in the Microorganism Strain Collection of the FGBI "ARRIAH" under numbers 1116, 5111 and 1818, respectively.

The comparative assessment of the immunogenicity of the experimental vaccine against infectious coryza, containig antigens of strains No. 1116, 5111 and 1818, and two commercial preparations, demonstrated that protection induced in birds immunized with the FGBI "ARRIAH" vaccine based on the homologous strains of *A. paragallinarum* was ≥ 90.0 ± 11.3%, and commercial biological products – 46.6 ± 13.1% and 53.3 ± 17.3%, respectively.

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Mycotoxicological monitoring. Part 3. Feedstuffs from raw grain processing*

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SUMMARY
The paper presents the results of mycotoxicological testing of the production batches of sunflower cake and meal, feedstuffs of soybean and wheat bran processing received from domestic processing establishments and livestock farms from 2009 to 2019. Detection and measurement of the content of fusariotoxins, including T-2 toxin, diacetoxyscirpenol, deoxynivalenol, zearalenone and fumonisins of B group, as well as alternariol, ochratoxin A, citrinin, aflatoxin B_1, sterigmatocystin, cyclopiazonic acid, mycophenolic acid, ergot alkaloids and emodin was carried out by a competitive ELISA in accordance with certified procedure. The summarized results demonstrate the predominant role of alternariol in the contamination of sunflower cake and meal, as well as the frequent occurrence of T-2 toxin, ochratoxin A, citrinin, cyclopiazonic acid, sterigmatocystin, mycophenolic acid and emodin. For the main contaminants, a shift in the medians and 90% percentile towards the lower values of the average and maximum contents was observed, which indicates the possibility of their accumulation beyond the typical range. The summary and results of mycotoxicological study of wheat bran and feedstuffs of soybean processing for a complete list of 14 parameters are presented in this paper for the first time. It was found that the range of mycotoxins that can contaminate soybean meal, cake and full-fat soybean is quite wide, which is consistent with the results of the study of soybean seed mycobiota composition. It was demonstrated that soybean meal can accumulate high concentrations of mycophenolic acid – up to 1,255 μg/kg. As for the wheat bran batches, cases of contamination with diacetoxyscirpenol and the frequent occurrence of T-2 toxin, emodin and ergot alkaloids were detected. The initial monitoring data, systematized and summarized in this paper, are presented in electronic form in the section "Additional materials". The prospects of testing of feedstuffs from processing other oilseeds, as well as from wheat and corn grain processing are discussed.

Key words: sunflower meal/cake, soybean meal/cake, full-fat soybean, wheat bran, mycotoxins, monitoring, enzyme-linked immunosorbent assay.

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Микотоксикологический мониторинг. Сообщение 3. Кормовая продукция от переработки зернового сырья*

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РЕЗЮМЕ

Представлены результаты микотоксикологического обследования производственных партий подсолнечного жмыха и шрота, кормовой продукции от переработки сои и пшеничных отрубей, полученных из перерабатывающих предприятий и животноводческих хозяйств страны за период с 2009 по 2019 г. Детектирование и измерение содержания фузариотоксинов, включающих Т-2 токсин, диацетоксисцирпенол, дезоксиниваленол, зеараленон и фумонизины группы В, а также альтернариола, охратоксина А, цитринина, афлатоксина B_1, стеригматоцистина, циклопиазоновой кислоты, микофеноловой кислоты, эргоалкалоидов и эмодина проведено по аттестованной процедуре с использованием конкурентного иммуноферментного анализа. В ходе обобщения результатов установлена доминирующая роль альтернариола в контаминации подсолнечного жмыха и шрота, а также частая встречаемость Т-2 токсина, охратоксина А, цитринина, циклопиазоновой кислоты, стеригматоцистина, микофеноловой кислоты и эмодина. Для основных контаминантов отмечено смещение медиан и 90%-го процентиля в сторону меньших значений по отношению к средним и максимальным содержаниям, что указывало на возможность случаев их накопления за пределами типичного диапазона. Обобщение и результаты микотоксикологического исследования пшеничных отрубей и кормовой продукции от переработки соевых бобов по полному перечню из 14 показателей приводятся в этой работе впервые. Установлено, что спектр микотоксинов, способных участвовать в контаминации соевого шрота, жмыха и сои полножирной, достаточно широк, что согласуется с результатами изучения состава микобиоты семян этой культуры. В соевом шроте показана возможность накопления высоких концентраций микофеноловой кислоты – до уровня 1255 мкг/кг. В партиях пшеничных отрубей выявлены случаи загрязненности диацетоксисцирпенолом и частая встречаемость Т-2 токсина, эмодина и эргоалкалоидов. Исходные данные мониторинга, систематизированные и обобщенные в данной работе, представлены в электронном виде в разделе «Дополнительные материалы». Обсуждаются перспективы обследования кормовой продукции от переработки семян других масличных культур, а также зерна пшеницы и кукурузы.

Ключевые слова: подсолнечный шрот/жмых, соевый шрот/жмых, соя полножирная, пшеничные отруби, микотоксины, мониторинг, иммуноферментный анализ.

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INTRODUCTION

Feed base improvement is one of the most important tasks in animal farming in the Russian Federation. Animal health, productivity, immunobiological status, the quality and safety of animal products depend heavily on the sanitary state of feedstuffs and the balance of nutrients in them. In domestic compound feeds, macro-components supplementing the grain part are mainly represented by sunflower and soybean cake and meal, to a somewhat lesser extent – by-products of grain and starch processing. A wide network of fat-and-oil establishments and developed grain and starch processing industries completely cover the demand of domestic feed producers for sunflower cake and meal, grain bran, as well as for all types of by-products from complex corn grain processing. Supplies of soybean meal, cake and full-fat extruded soybean traditionally come from the regions specialized mainly in growing this crop (Southern and Far Eastern Federal Districts) and are supported by imported raw materials in order to meet the current market demands.

The first stage of testing of these types of feedstock for mycotoxin contamination was performed at the Laboratory for Mycotoxicology and Feed Hygiene, ARRIVSHE, in 2002–2009 [1, 2]. During this period frequent occurrence of ochratoxin A and citrinin at the level of 190 and 1,020 μg/kg was established in sunflower cake and meal; T-2 toxin, deoxynivalenol, sterigmatocystin, cyclopiazonic acid were less frequent; no zearalenone and diacetoxyscirpenol was detected. In addition, a weak contamination of soybean meal with T-2 toxin was reported. Deoxynivalenol and zearalenone were detected rarely and in small quantities, ochratoxin A and citrinin – in single samples, and fumonisins of B group, aflatoxin B_1, diacetoxiscirpenol, sterigmatocystin and cyclopiazonic acid could not be detected. However, in some imported product batches, the levels of deoxynivalenol exceeded 2,000 μg/kg, and zearalenone – 200 μg/kg. The situation with waste from flour mills received only a limited assessment. Recently, the peculiarities of contamination of sunflower seeds and feedstuffs from their processing have become the subject of special consideration [3–5].

The aim of this work is to sum up the results of testing of production batches of sunflower meal, cake, feedstuffs from soybean processing (meal, oil cake, full-fat soybean) and wheat bran for mycotoxins from 2009 to 2019 and to provide input data to the electronic registration database.

MATERIALS AND METHODS

Pooled samples from the production batches of sunflower meal and cake, soybean meal and cake, full-fat extruded soybean and wheat bran, provided by specialists of the veterinary services, livestock and feed producing establishments, agricultural companies, specialized commercial organizations and owners of backyard farms in 2009–2019 were used for the purpose of the study. As for sunflower cake and meal (121 samples), 107 samples were received from the processing establishments and holdings with documentarily confirmed addresses, located in Belgorod, Volgograd, Voronezh, Kursk, Oryol, Rostov, Saratov, Tambov oblasts, the Krasnodar Krai, the Primorsky Krai, the Republic of Tatarstan; 2 samples were received from the Ukraine, and 12 samples were provided with no data or its reliability was doubtful. Of the 80 samples of soybean meal, cake and processed soybean, 8 were imported, 6 were received from the Far Eastern Federal District (the Amur

oblast, Primorsky Krai). Information about the origin of the rest of the feedstuffs from soybean processing, as well as of 20 samples of feed bran was not available.

The group of mycotoxins to be detected included T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEA), fumonisins of B group (FUM), alternariol (AOH), ochratoxin A (OA), citrinin (CIT), aflatoxin B_1 (AB_1), sterigmatocystin (STG), cyclopiazonic acid (CPA), mycophenolic acid (MPA), ergot alkaloids (EA), and emodin (EMO). Sample preparation was performed in accordance with the official harmonized methodology based on liquid extraction and indirect competitive enzyme immunoassay [6]. Detection limits determined based on 85% antibody binding were as follows: 2 µg/kg (AB_1), 3 µg/kg (EA), 4 µg/kg (T-2, OA, STG), 20 µg/kg (ZEA, AOH, CIT, MPA, EMO) and 50 µg/kg (DAS, DON, FUM, CPA). The following coding pattern was used for filling-in the record form in the database: tested mycotoxins, type of raw material, test year, and the location of the establishment or farm.

Microsoft Excel 2016 and Statistica (Version 6) programmes were used for statistical processing including calculation of percentage of occurrence based on n^+/n ratio and following three values for positive samples – the arithmetical mean, the median and the 90 percentile.

RESULTS AND DISCUSSION

The predominant role in contamination of sunflower meal and cake belonged to AOH produced by fungi of the genus *Alternaria*, with the average values of 306 and 193 µg/kg with the possibility of accumulation up to 1,990 and 953 µg/kg. As for the group of fusariotoxins, only the T-2 detection rate was significant – 21.4 and 37.3% at low concentrations with the ranges of 4–16 and 5–25 µg/kg, only in specific cases it was possible to determine DON and ZEA; DAS and FUM were not detected (Tables 1, 2).

Toxins produced by fungi of other taxa, mainly the genera *Aspergillus* and *Penicillium*, except for AB_1, were detected in both types of products. EA were equally rare in small concentrations; OA, MPA and EMO were found with approximately equal frequency exceeding 50%, while the frequency of contamination with OA was higher than with CIT. For other toxins, these parameters varied by 1.5–2 times in meal and cake. The rows arranged according to the average content values coincided and were as following: CIT, CPA, MPA (75–97 µg/kg) > OA (14 and 16 mg/kg) > STG (7 µg/kg). Medians and threshold concentrations for 90% of the values (90 percentile) for all the contaminants were lower than the average and maximum values, which was indicative of a skewed distribution of the numbers of accumulation cases, i.e. the possibility of their accumulation beyond the typical range for each specific product sample.

Thus, according to the data obtained, sunflower meal and cake are characterized as the ones containing multiple similar contaminants, including AOH, T-2, OA, CIT, CPA, STG, MPA and EMO, and their occurrence frequency varies from 10.4 to 83.8%. This gives every reason to consider

Table 1
Mycotoxins in sunflower meal (summary data for 2009–2019)

Таблица 1
Микотоксины в подсолнечном шроте (обобщенные данные 2009–2019 гг.)

Toxin	Occurrence n^+/n (%)	Content, µg/kg				
		range		average	median	90 percentile
		min	max			
T-2	15/70 (21.4)	4	16	9	9	13
DON	1/70 (1.4)	375	–	–	–	–
DAS	0/34	–	–	–	–	–
ZEA	1/70 (1.4)	66	–	–	–	–
FUM	0/33	–	–	–	–	–
AOH	57/68 (83.8)	19	1,990	306	104	839.2
OA	48/70 (68.6)	4	93	14	10	25.6
CIT	29/67 (43.3)	20	1,020	87	44	106.2
AB_1	0/41	–	–	–	–	–
STG	5/48 (10.4)	4	12	7	6	11.2
CPA	11/58 (19.0)	50	123	77	72	109
MPA	32/61 (52.5)	24	379	75	44	179.1
EA	1/43 (2.3)	11	–	–	–	–
EMO	29/54 (53.7)	15	278	72	52	159.2

n – number of tested samples (число исследованных образцов);
n^+ – number of mycotoxin-containing samples (число образцов, содержащих микотоксин).

Table 2
Mycotoxins in sunflower meal (summary data for 2009–2019)

Таблица 2
Микотоксины в подсолнечном жмыхе (обобщенные данные 2009–2019 гг.)

Toxin	Occurrence n^+/n (%)	Content, µg/kg				
		range		average	median	90 percentile
		min	max			
T-2	19/51 (37.3)	5	25	12	10	18.4
DON	0/51	–	–	–	–	–
DAS	0/23	–	–	–	–	–
ZEA	0/51	–	–	–	–	–
FUM	0/16	–	–	–	–	–
AOH	41/50 (82.0)	20	953	193	79	536
OA	32/51 (62.7)	4	62	16	9.5	36.8
CIT	11/51 (21.6)	20	126	80	79	126
AB_1	0/29	–	–	–	–	–
STG	10/39 (25.6)	4	11	7	5.5	9.2
CPA	21/39	50	142	81	71	120
MPA	9/44 (53.8)	20	334	97	63	222.8
EA	3/33 (9.1)	5	40	19	–	–
EMO	17/30 (56.7)	10	5,000	369.5	59	229.4

n – number of tested samples (число исследованных образцов);
n^+ – number of mycotoxin-containing samples (число образцов, содержащих микотоксин).

both types of the above-mentioned products to be high-risk raw materials. It should be noted that the results of the tests carried out in 2008–2010 for a shorter list of parameters that included T-2, DON, ZEA, FUM, OA and AB_1 in regards to the relevant samples of sunflower meal and cake received from holdings and establishments of the European part of the country, revealed that mycotoxins, except for the missing FUM, were extremely rare (detection frequency of 1.9 to 2.7%) and the meal was much more contaminated with AB_1 in comparison to the cake (28.6%) [7]. It should be admitted that such discrepancies in assessment are very unexpected and difficult to explain.

Soybean meal in the domestic feed production belongs to the main type of raw materials from soybean processing; cake and extruded full-fat soybean are used far less frequently. The summary and results of mycotoxicological testing of these products for a complete list of 14 parameters are presented in this paper for the first time (Fig. 1) for soybean meal (49 samples), the previously established fact that it is less contaminated with mycotoxins compared to the products from sunflower seed processing was fully confirmed [1, 2]. Only T-2, EA and EMO had 10% or higher occurrence frequency, and DAS, OA and CIT were not found, while other toxins were detected less frequently and with average concentrations of tens of µg/kg. Only for MPA in individual samples, concentrations exceeded this threshold and were 337 and 1,255 µg/kg (Fig. 1A). For soybean cake and full-fat soybean (31 samples), the situation was quite similar: the absence of DAS, the presence of T-2 and EMO with the occurrence frequency of more than 10%, and a lower incidence of ZEA and AOH; however, some clear differences were also observed (Fig. 1B). These include not only the increased frequency of contamination with T-2, DON, FUM, EMO and the intensity of accumulation of DON, FUM, but also detection of OA and CIT, although in small quantities close to the method detection limit, and the absence of a number of toxins – AB_1, STG, CPA, MPA and EA.

It can be assumed that the observed shifts in the nature of contamination of cake and full-fat soybean in comparison to meal are associated with different origins of the raw materials, and can also depend on transportation and storage conditions. Similarly noticeable fluctuations in the results are likely to occur for the meal, which also comes from geographically remote areas. Indeed, according to the work of N. Strashilina et al., for 166 samples of meal with non-specified origin, contamination with AB_1 was 100%, all the tested fusariotoxins (T-2, ZEA, DON, and FUM) occurred with a frequency of 20.9 to 28.6%, and OA was detected in 2.2% of the cases [7].

In general, the range of mycotoxins that can contaminate by-products of soybean processing is quite wide and is quite consistent with the results of studies of mycobiota associated with soybean seeds. According to the Slovak University of Agriculture in Nitra, fungi of the genus *Aspergillus* and *Penicillium* were widely present in the

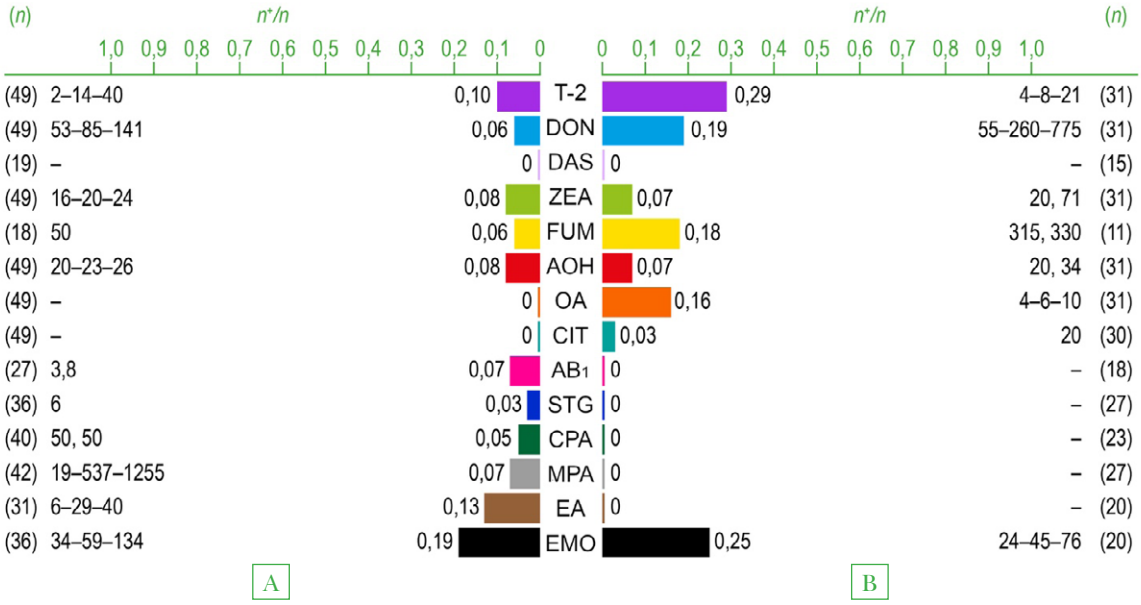

Fig. 1. Mycotoxin occurrence (n^+/n) and content (μg/kg, min – average – max) in soybean meal (A), soybean cake and full-fat soybean (B)

Рис. 1. Встречаемость (n^+/n) и содержание микотоксинов (мкг/кг, мин. – среднее – макс.) в соевом шроте (А), соевом жмыхе и сое полножирной (В)

mycobiota of feed soybean sampled at agricultural establishments of the country and were accompanied by representatives of the genera *Cladosporium*, *Alternaria* и *Fusarium* [8]. The possibility of asymptomatic colonization of soybean with the fungus *Fusarium verticillioides*, capable of FUM biosynthesis, has been recently shown by American researchers [9]. The predominance of the AOH-producing species *Alternaria alternata* and the presence of this toxin is shown in soybeans from Argentina [10, 11]. In soya bran, which is one of the most important components of feed in Brazil, the fungi *Aspergillus* (*A. flavus*, *A. fumigatus*, *A. niger*), *Penicillium* (14.93%), as well as *Fusarium* (3.25%) were frequently detected [12]. Several potentially toxigenic species of the genus *Aspergillus* were identified in soybean seeds and soybean flour imported to Armenia from the USA, Canada, Spain and Greece, with rare occurrence of *Penicillium cyclopium*, *P. lanosum* and *Fusarium moniliforme*, with AB_1 being found in 6 out of 17 samples (7–50 μg/kg),

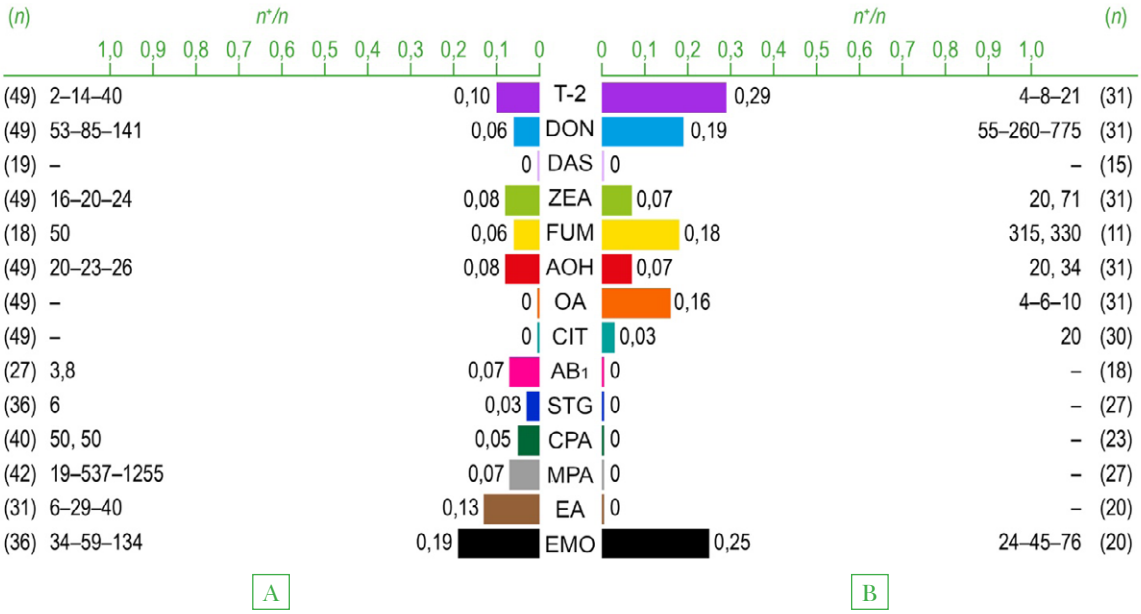

Fig. 2. Mycotoxin occurrence (n^+/n) and content (μg/kg, min – average – max) in wheat bran (A) and wheat grain (B) (cit. for Part 2)

Рис. 2. Встречаемость (n^+/n) и содержание микотоксинов (мкг/кг, мин. – среднее – макс.) в пшеничных отрубях (А) и в зерне пшеницы (В) (цит. по сообщению 2)

and STG (150 μg/kg) and ZEA (2,000 μg/kg) in some single samples [13]. The nature and intensity of mycotoxin contamination of soybean products is undoubtedly conditioned by a combination of factors such as soil, climate and ecology.

Recent decades have been marked by the expansion of the area of soybean cultivation in moderate latitudes, and the relevance of its contamination control remains very high. However, the number of works tackling this issue is small. Recently, the species *Alternaria alternata*, as well as individual representatives of the genera *Cladosporium* and *Fusarium*, have been identified in collection and selection planting soybean seeds in Belarus [14]. Therefore, there is need for mycotoxicological testing of soybean not only in the regions of its traditional cultivation, but also in the territories where lands are actively being exploited – the Belgorod, Rostov oblasts, Altai and Stavropol krais, the Republic of Adygea, and the Republic of Tatarstan. Of undoubted importance is gathering of information regarding other promising types of feedstuffs from oilseed processing, in particular cotton [15] and rapeseed. In 2018, a comprehensive analysis of rapeseed cake sample received from the Krasnodar Krai, revealed only CPA and EMO in small quantities – 50 and 32 μg/kg.

Analysis of 20 samples of wheat bran showed that the frequency of occurrence of T-2, EMO > DON, AOH, OA (Fig. 2A) corresponds to the one typical for wheat grain (Fig. 2B). DAS, ZEA, FUM, AB_1 and CPA were not detected, while OA, CIT, STG, MPA, and EA were identified in single samples. A significantly higher number of T-2, EA and DAS detection can be noticed.

Despite the small number of samples, the data obtained can serve a basis for future research projects aimed at studying the distribution of mycotoxins in fractions separated during the production of wheat flour and cereals. Initial monitoring data with the type of raw material, its origin and the year of sample receipt are provided in electronic form in the section "Additional materials" at: http://doi.org/10.29326/2304-196X-2020-3-34-213-219.

Unfortunately, the situation regarding mycotoxin contamination of distiller's dried grain, obtained by drying and granulating of distillery waste, which is widely used in feed production, is still unclear in our country. However, data from American researchers indicate multiple and intense contamination of the distiller's dried grains with solubles (DDGS) – DON, ZEA and FUM were detected in 70–90% of samples, with their concentrations reaching values of 13,920, 8,107 and 9,042 μg/kg; AB_1 and T-2 at levels up to 89 and 226 μg/kg, respectively, were also often detected [16]. During this period, we did not have an opportunity to continue mycotoxicological testing of gluten feeds, the by-products which are very popular among starch-processing plants. In 2015 and 2016, the laboratory received two samples of corn gluten from the People's Republic of China for testing, one of which demonstrated the presence of fusariotoxins T-2, DON, ZEA, FUM and STG in the amounts of 145, 1,860, 1,080, 1,260 and 11 μg/kg, respectively; the other contained DON and ZEA in the amount of 2,320 and 2,230 μg/kg. The obtained data and preliminary results [2, 7], indicative of the intensive multiple contamination of this product, as well as combined contamination of corn grain (see Part 2) testify to the necessity for mandatory testing of corn gluten for a complete list of parameters. Insufficient attention is paid to other types of products from the complex processing of corn grain, which are widely used in feed production, such as dry corn germ, as well as cake and corn germ meal. However, 16 samples of corn germ cake received in 2009–2010 from holdings in the European part of the country showed that contamination with T-2, DON, ZEA, FUM, OA and AB_1 was quite significant with a frequency of 43.8 to 75% [7].

CONCLUSION

During extensive monitoring, conducted as an annual data collection for the period from 2009 to 2019, multiple contamination of sunflower meal and sunflower oil cake with alternariol, T-2 toxin, ochratoxin A, citrinin, cyclopiazonic acid, sterigmatocystin, mycophenolic acid and emodin was confirmed with the frequency of occurrence from 10.4 to 83.8%, which entitles these products to be regarded as high-risk. For the safe use of raw materials based on soybean, appropriate regional monitoring projects should be considered in the territories of its intensive cultivation and industrial processing. Imported batches of cake and meal should be inspected on a regular basis due to their possible contamination with a wide range of mycotoxins. Cases of diacetoxyscirpenol detection and the frequent occurrence of T-2 toxin, emodin and ergot alkaloids described for wheat bran for the first time show the need for a mandatory incoming control of these products at feed producing establishments.

Additional materials to the paper (records forms with database) can be found at http://doi.org/10.29326/2304-196X-2020-3-34-213-219.

Дополнительные материалы к этой статье (учетные формы с базой данных) можно найти по адресу http://doi.org/10.29326/2304-196X-2020-3-34-213-219.

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Indirect determination of FMDV 146S component concentration in non-inactivated suspension by comparison of graphs of the second derivative for real-time RT-PCR curves

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SUMMARY
During reproduction in biological systems, FMD virus forms four variants of components, three of which do not include RNA of the virus. In the process of industrial production of FMD vaccines, special attention is paid to the number of whole virions, which have the most important biological properties of FMD virus and are the main components that determine the immunogenicity of vaccine preparations. Raw materials for vaccines at various stages of the technological process are tested for concentration of FMDV 146S component. The traditional method of determination is quantitative complement fixation test. In recent years, real-time RT-PCR has been used for indirect determination of FMDV 146S component concentration in a virus-containing suspension. The article presents a new approach to indirect determination of FMDV 146S component concentration in a non-inactivated suspension by comparing the maximum extreme points of the graphs of the second derivative of the fluorescence signal accumulation curves and the number of amplification reaction cycles. The dependence between FMDV 146S component concentration and the maximum extreme points of the graphs of the second derivative of the fluorescence signal accumulation curve is presented in the form of a square function: $C_{146S\ FMDV} = 0.0111(C_p)^2 - 1.0157C_p + 20.446$ with a high accuracy of approximation ($R^2 = 0.993$). The proposed model allows to quantitatively estimate the content of 146S component in virus-containing vaccine raw materials. The presented method allows studying a large number of samples of non-inactivated raw materials for FMD vaccine in 4–5 hours. The main advantage of the proposed method is the capacity to determine the concentration of FMDV 146S component in a suspension with a high level of ballast proteins (more than 7.00 mg/cm^3) and complete viral particles (from 0.01 to 5.00 μg/cm^3).

Key words: FMDV virions, real-time RT-PCR, amplification threshold cycle, graph of second derivative of amplification curve.

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УДК 619:578.835.2:615.371.004.12:616-076

Опосредованное определение концентрации 146S компонента вируса ящура в неинактивированной суспензии при сравнении графиков второй производной для кривых ОТ-ПЦР-РВ

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РЕЗЮМЕ

При репродукции в биологических системах вирус ящура формирует 4 варианта компонентов, три из которых не включают в себя РНК вируса. В процессе промышленного производства противоящурных вакцин особое внимание уделяют количеству цельных вирионов, которые обладают важнейшими биологическими свойствами вируса ящура и являются основными компонентами, определяющими иммуногенность вакцинных препаратов. Сырье для вакцин на различных этапах технологического процесса исследуют с целью определения концентрации 146S компонента вируса ящура. Традиционным методом определения является количественный вариант реакции связывания комплемента. Для опосредованного определения концентрации 146S компонента вируса ящура в вируссодержащей суспензии в последние годы стали использовать полимеразную цепную реакцию с обратной транскрипцией в режиме реального времени. В статье представлен новый подход к опосредованному определению концентрации 146S компонента вируса ящура в неинактивированной суспензии при сравнении максимальных экстремумов графиков второй производной кривых накопления сигнала флуоресценции относительно количества циклов реакции амплификации. Существование зависимости между концентрацией 146S компонента вируса ящура и максимальными экстремумами графиков второй производной кривой накопления флуоресцентного сигнала представлено в виде квадратичной функции $C_{146S\,ВЯ} = 0{,}0111(C_p)^2 - 1{,}0157C_p + 20{,}446$ с высокой достоверностью аппроксимации ($R^2 = 0{,}993$). Предложенная модель позволяет количественно оценивать содержание 146S компонента в вируссодержащем сырье для вакцины. Представленный способ позволяет исследовать большое количество образцов неинактивированного сырья для противоящурной вакцины за 4–5 ч. Основным преимуществом предлагаемого способа является возможность определения концентрации 146S компонента вируса ящура в суспензии, содержащей высокое количество балластного белка (более 7,00 мг/см^3) и количество полных вирусных частиц от 0,01 до 5,00 мкг/см^3.

Ключевые слова: вирионы вируса ящура, ОТ-ПЦР-РВ, пороговый цикл амплификации, график второй производной кривой амплификации.

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INTRODUCTION

In many countries foot-and-mouth disease (FMD) is at the top of the list of animal viral diseases control and prevention measures. FMD is a highly contagious viral acute disease of wild and domestic cloven-hoofed animals and tylopods and is a global problem, which a special attention of international organizations (FAO, OIE) and veterinary services of many countries is paid to [1, 2].

FMD virus (FMDV) genome is represented by a single-stranded positive RNA consisting of approximately 8,500 n. r. (nucleotide residues), surrounded by an icosahedral capsid consisting of 60 copies, each of which is represented by four structural proteins: VP_1 (1D-gene), VP_2 (1B-gene), VP_3 (1C-gene), VP_4 (1A-gene) [3–5].

During reproduction in biological systems FMD virus forms four variants of components: 146S component (whole virion, full particle), consisting of one whole viral RNA molecule and 60 copies of polypeptide, each of which is represented by a complex of proteins VP_1 (1D-gene), VP_2 (1B-gene), VP_3 (1C-gene), VP_4 (1A-gene); 75S particle ("blank" capsid), consisting of 60 copies of polypeptides VP_0 (1AB-gene), VP_1 (1D-gene), VP_3 (1C-gene); 12S particle (capsomer), consisting of structural proteins VP_1 (1D-gene), VP_2 (1B-gene), VP_3 (1C-gene); 3.8S subunit, represented by non-structural protein VP_g. 75S, 12S and 3.8S components do not include FMDV RNA [1, 3].

In the process of industrial production of vaccines, special attention is paid to the number of whole virions, which have the most important biological properties of FMD virus and are the main components that determine the potency of vaccine preparations [2, 3]. Therefore, raw materials for vaccines at different stages of technological process are studied to determine the concentration of 146S component of FMD virus. Traditionally for this purpose quantitative variant of complement fixation test (CFT) is used and the results are evaluated according to methodical recommendations [6]. In recent years, real-time reverse transcription polymerase chain reaction (real-time RT-PCR) has been used for indirect determination of concentration of FMDV 146S component concentration in a non-inactivated virus-containing suspension [1, 7–12]. The presented method is highly sensitive, specific, economical and rapid, and also allows to investigate several dozens of samples of the virus-containing material simultaneously. However, this modification has some disadvantages: if the content of 146S component in the tested sample is less than 0.1 mg/cm^3, the sensitivity of the reaction decreases; if there are a lot of proteins and lipoproteins in the sample, the sorbent particles are sensitized by excess of ballast components that reduces the possibility of virus RNA sorption and decreases the sensitivity of the analysis; where trace amounts of sorbent may end up in the reaction mixture, the background value of the fluorescence may increase and there may be a distortion of the analysis results when determining the a proportionality coefficient between the fluorescence signal and the amount of the whole virus in the sample.

It is therefore an important and challenging task to improve the method for indirect determination of concentration of FMDV 146S component in a non-inactivated suspension on the basis of the method for comparing the maximum extrema of the graphs of the second derivative for real-time amplification reaction curves.

The aim of the research is to develop a method for indirect determination of concentration of FMDV 146S component in the viral suspension by comparing the maximum extrema of the second derivative graphs for the real-time amplification reaction curves.

MATERIALS AND METHODS

Virus. FMD culture virus of Asia-1/Shamir Israel 3/89 strain was used. The virus was propagated in the suspension continuous cell line of baby hamster kidney BHK-21. Non-inactivated FMDV suspensions with ballast proteins contents of more than 7.00 mg/cm^3 were used.

Complement fixation test (CFT). Quantitative CFT was used to determine FMDV 146S component concentration [6].

Determination of 146S component concentration. FMDV 146S component concentration was determined with real-time RT-PCR using values of threshold cycle of amplification (*C*t) according to the above-mentioned requirements [7].

Coating of plates with polyclonal strain-specific antibodies against FMDV. A six-well plate was coated with highly purified strain-specific polyclonal antibodies against FMDV in the volume of 1.5 cm^3 of the suspension with concentration of immunoglobulins G of 5.0 μg/cm^3 at 4 ± 2 °C for 18–20 hours. Open binding sites were blocked with 1% gelatine suspension at 37 ± 1 °C for 30 minutes and the wells were washed with 1/15 M phosphate-buffered saline (PBS) five times.

Strain-specific binding of FMD virus. Samples of suspensions in the volume of 2.4 cm^3 were added to the wells coated with strain-specific FMDV antibodies and incubated at 37 ± 1 °C for 30 minutes. The wells were washed to remove ballast components three times using 1/15 M PBS. The obtained immune complexes were resuspended in 1.0 cm^3 of Eagle's medium (MEM).

Isolation of FMDV virion RNA bound by immune complex. The process of isolation of RNA of 146S FMDV component was based on method by P. Chomczynski [13, 14]. The process resulted in obtaining per 0.2 cm^3 of 12-fold extracts of viral RNA.

Evaluation of purity of eluates of FMDV RNA. The spectral absorption capacity of RNA extracts was measured at wave lengths within the range of 205–325 nm and temperature of 22–25 °C. In the isolated extracts the content of residues of phospholipids, polysaccharides and guanidine isothiocyanate (GITC), carbolic acid, polypeptides and large suspended particles was estimated, determining the optical density (OD) values at 205, 235, 270, 280 and 320 nm, respectively [15]. The RNA eluate was considered free of protein and carbolic acid impurities if the extinction coefficient R_1 (OD_{262}/OD_{280}) was within the range of 1.8–2.2 and was optimally about 2.0. Lower R_1 values indicated the presence of DNA, protein components and carbolic acid residues in the eluate. Higher values of R_1 coefficient indicated the degradation of RNA and the presence of free ribonucleotides. FMDV nucleic acid extract was considered uncontaminated with polysaccharides if the extinction coefficient R_2 (OD_{262}/OD_{235}) was close to 2.000. When 1% RNA is substituted for polysaccharide components, R_2 decreases by 0.002 [16]. Values of R_2 coefficient greater than 2.000 may indicate degradation of RNA molecules. The absence of coarse particle suspension in the eluate is confirmed if OD_{320} is close to zero [15, 16]. If the purity requirements are not met, the stages of serological binding and isolation of FMDV RNA from the source material are repeated.

Real-time RT-PCR for quantitative determination of FMDV 146S particles. Forward-3D-FMDV-primer (5′-ACT-GGT-TTT-ACA-AAC-CTG-TGA-GGT-3′), Reverse-3D-FMDV-primer (5′-GCG-AGT-CCT-GCC-ACG-GAG-TTG-GTT-3′) and 3D-FMDV-ROX/BHQ2-probe (5′-ROX-TCC-TTT-GCA-CGC-CGT-GGG-ACG-3′) were used in the test as oligonucleotides homologous to 3D FMDV gene in 15 pM concentrations per reaction. The concentration of each of the deoxyribonucleoside triphosphates was 0.2 mM. DreamTaq buffer (10×), magnesium chloride and dimethyl sulfoxide in the amounts of 4 mM and 3% of the volume of real-time RT-PCR-mixture, respectively, were used as the basis. MMLV-reverse transcriptase (10 units) and Thermus aquaticus DNA polymerase (1 unit) were used as catalysts for reverse transcription and amplification reaction. The temperature and time parameters of real-time RT-PCR were set according to the above-mentioned requirements [7].

Control during testing of the method. Control during testing of the method. As a positive control, a non-inactivated suspension of culture FMD virus with the content of virions of 1.00 μg/cm^3. A non-inactivated suspension of BHK-21 cells with a concentration of 2.5–3.0 mln/cm^3 was used as negative control.

RESULTS AND DISCUSSION

At the first stage of the study the control panel of ready dilutions of the standard was obtained. A non-inactivated suspension of FMD culture virus of Asia-1/Shamir Israel 3/89 strain with concentrations of 146S component of the virus: 0.01; 0.05; 0.10; 0.20; 0.50; 1.00; 2.00; 2.50; 3.00; 3.50; 4.00; 4.50; 5.00 μg/cm^3 was used as a standard. Then, a viral suspension containing FMD virus was introduced into plates coated with antibodies against FMD virus of a given strain and then viral RNA was isolated from the obtained complexes of "FMDV virion – strain-specific antibodies". As a result, 12-fold viral RNA extracts of each standard dilution were obtained and their purity was assessed by spectral analysis in ultraviolet light. A record of the absorption spectrum of standard dilutions of RNA at wavelengths from 205 to 325 nm is shown in Figure 1.

Based on the results of analysis of control samples in the above-mentioned dilutions demonstrated that $OD_{205\text{-}259}$ and $OD_{263\text{-}325}$ values did not exceed $OD_{260\text{-}262}$, which attests to a high level of purity of the obtained RNA eluates ($n = 3$). The data of spectral study of the standards, shown in Figure 1, showed the absence of marked peaks in the graphs at wavelengths of 205, 235, 270, 280 and 320 nm, which indicated almost complete absence of contamination of RNA extracts with phospholipids, polysaccharides and residues of GITC, carbolic acid, polypeptides and large conglomerates, respectively. The values of R_1 extinction coefficient for the standards are close to the norm of 2.000 (R_1 was 1.995–1.999), which confirmed the absence of DNA and the presence of only trace amounts of protein impurities and carbolic acid residues. Degradation of nucleic acid and presence of free nucleotides in eluates were not observed, as R_1 did not exceed 2.000. The viral RNA extracts of the standard dilutions were not contaminated with

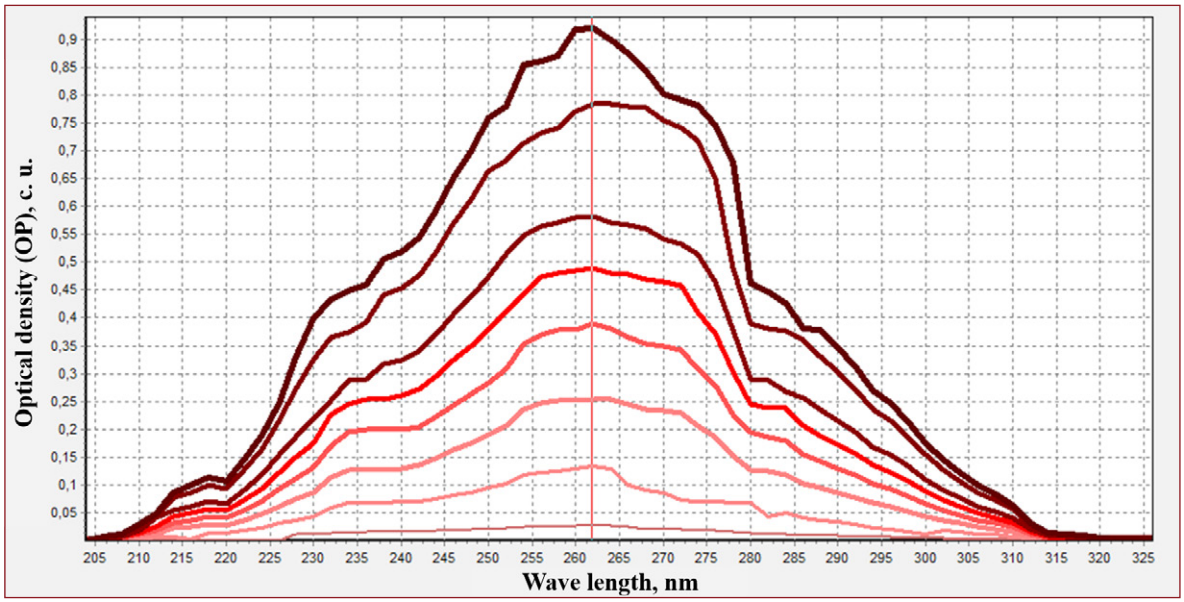

Fig. 1. Spectograms of diluted eluates of FMDV RNA (Asia-1/Shamir Israel 3/89 strain). From bottom to top see graphs for diluted extracts corresponding to the following concentrations of virions: 0.01; 0.10; 0.50; 1.0; 2.0; 3.0; 4.0; 5.0 μg/cm³

Рис. 1. Спектрограммы разведений элюатов РНК вируса ящура штамма Азия-1/Шамир Израиль 3/89. Снизу вверх отражены графики для разведений экстрактов, соответствующих следующим концентрациям вирионов: 0,01; 0,10; 0,50; 1,0; 2,0; 3,0; 4,0; 5,0 мкг/см³

polysaccharides and GITC, as the values of R_2 extinction coefficient were close to the norm of 2.000 and corresponded to 2.000–2.001. Taking into account that when 1% of RNA is substituted for carbohydrates, R_2 value decreases by 0.002 [16], polysaccharide impurities were not detected in the extracts obtained. The degree of RNA destruction in the extracts was not more than 0.5% ((2.001–2.000)/0.002), which is admissible. Thus, FMDV RNA extracts extracted from standard dilutions and used for further studies were characterized by a high purity level.

At the next stage of the work, real-time RT-PCR was carried out according to the above-mentioned recommendations. The analysis was based on the use of 5'-exonuclease activity of Thermus aquaticus of DNA polymerase. In the absence of a target, fluorophore ROX and fluorescence extinguisher BHQ2 in the 3D-FMDV-probe were brought closer due to the maximum use of hydrogen bonds between atoms of H, O and N oligonucleotides. Due to the mechanism of fluorescence-resonance energy transfer the glow is suppressed. Due to the 5'-exonuclease activity of Thermus aquticus of DNA polymerase after annealing the Forward-3D-FMDV-, Reverse-3D-FMDV-primers and 3D-FMDV-ROX/BHQ2-probe the hybridized probe and amplicon were destroyed, their spatial separation was observed, which led to the growth of the detected signal. The increase in the fluorescence level (*Fl*) was proportional to the number of reaction products produced. Monitoring of the signal during 40 cycles (*C*) of real-time PCR allowed to construct kinetic fluorescence curves, which are set by functions of type $Fl = f(C)$.

The obtained data were analyzed with the help of Rotor-Gene FRT-Manager software, which allows to construct graphs of the accumulation of fluorescent signal in real time during the specified number of amplification cycles.

Using the technology of "Maxima" software (or analogue), the graphs of the first and second derivatives for obtained eluates of FMD RNA of each dilution of the standard with known concentrations of 146S component were plotted and average values of maximum extrema (C_p) of the graphs of the second derivative $Fl = f(C_p)$ with projection on the abscissa axis "O-cycles" were calculated.

The value of C_p is an important characteristic of the reaction, which is directly proportional to the number of copies of the original RNA matrix and, consequently, to the concentration of the 146S component of FMD virus, since each such particle contains one molecule of viral RNA [3, 17, 18]. Taking into account that the second derivative of the function $f(C_p)$ ($f''(C_p)$) is continuous in some neighborhoods of the point $C_p = C_{p1}$ and is set at the amplification cycle interval from 0 to 40, there is a certain interval near the point C_p for which in all coordinates on the O-C_p axis the second derivative of the function $f(C_p)$ will be negative. Since $f''(C_p)$ is the first derivative of $f'(C_p)$, it follows from the condition $(f'(C_p)) < 0$ that $f'(C_p)$ on some small interval containing point $C_p = C_{p1}$ will decrease. Taking into account that $f'(C_p) = 0$, on the segment at $C_p < C_{p1}$ the first derivative of the function $f(C_p) > 0$, and at $C_p > C_{p1} - f'(C_p) < 0$. In other words, the first derivative of the function $f(C_p)$ changes the sign from plus to minus when passing through the point $C_p = C_{p1}$, therefore, at the point C_{p1} the function reflecting the process of fluorescence signal accumulation has the maximum extremum [17]. Thus, if the graph of the real-time amplification reaction is represented by the function $Fl = f(C_p)$, $f'(C_p) = 0$ and $f''(C_p) < 0$, then provided that $C_p = C_{p1}$ the obtained function has a maximum at the point with the argument C_{p1}, the value of which is taken into account to establish the dependence between the concentration of FMDV 146S component and the value of C_p.

The advantage of using the second derivative in this case is that when the function of the amplification curve $Fl = f(C_p)$ is multiplied by any multipliers, including the proportionality coefficient α [8, 14], the position of the maxima of the derivatives does not change. The maximum extremum of the second derivative is within the exponential

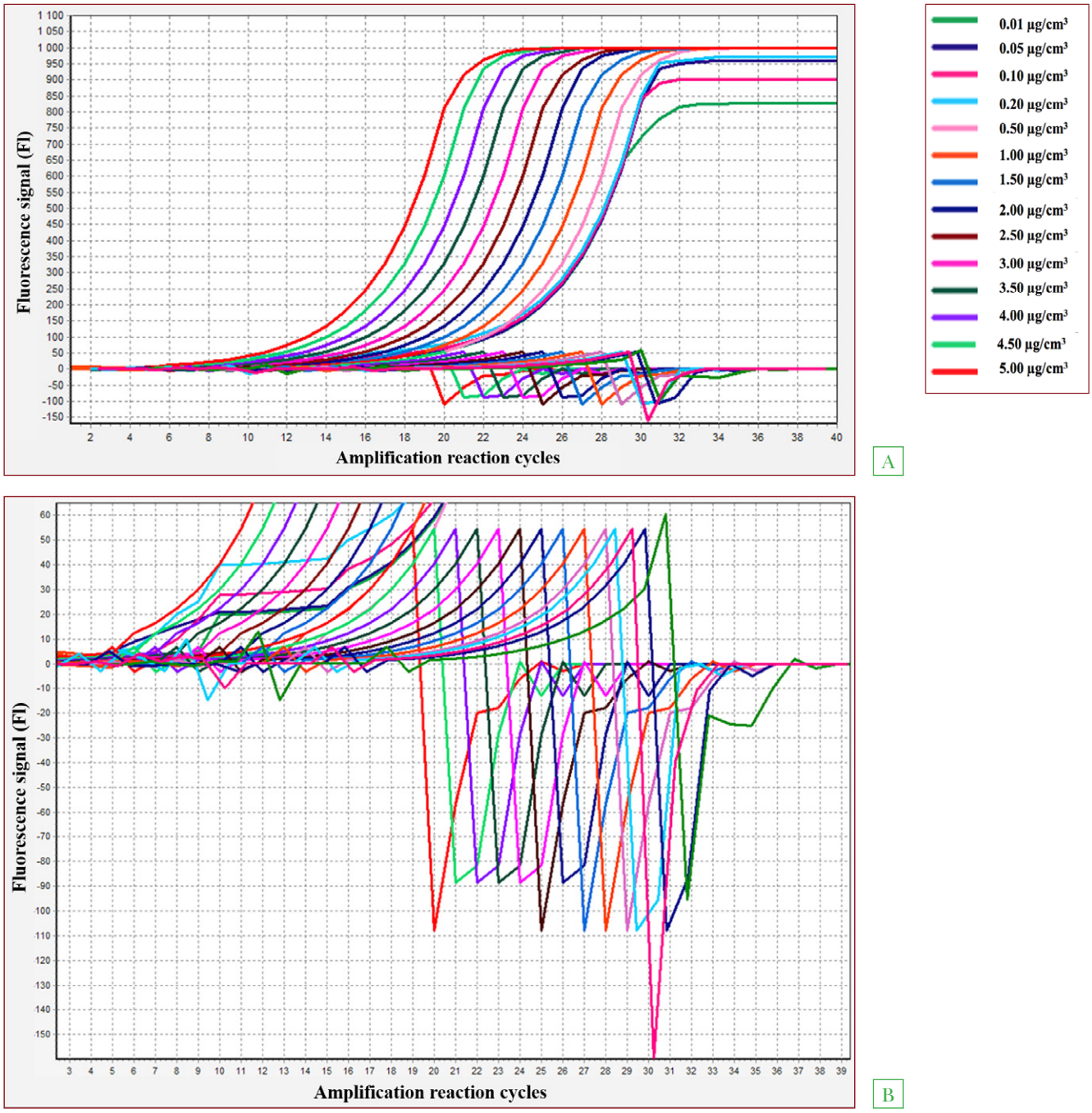

Fig. 2. Mean values of critical points C_p, calculated with the second derivative for real-time amplification reaction graphs during analysis of RNA of standard FMD virus, Asia-1/Shamir Israel 3/89 strain, with the following concentrations of 146S component: 0.01, 0.05, 0.10, 0.20, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00 µg/cm³ (n = 3) (A – graphs of accumulation of fluorescent signal, first and second derivatives; B – graphs of second derivatives for amplification reaction curves)

Рис. 2. Средние значения критических точек C_p, рассчитанные с помощью второй производной для графиков реакции амплификации в реальном времени при анализе РНК стандарта вируса ящура штамма Азия-1/Шамир Израиль 3/89 с концентрациями 146S компонента 0,01; 0,05; 0,10; 0,20; 0,50; 1,00; 1,50; 2,00; 2,50; 3,00; 3,50; 4,00; 4,50; 5,00 мкг/см³ (n = 3) (А – графики накопления флуоресцентного сигнала, первой и второй производных; В – графики второй производной для кривых реакции амплификации)

region of the fluorescence accumulation graph, i.e., in the exponential region, during the analysis of which the efficiency of the amplification reaction does not change [17].

Graphs of the first and second derivatives of the obtained eluates of FMDV RNA of each dilution of the standard with known concentrations of virions of FMD virus are shown in Figure 2.

The results of the experiment on presentation of the system of parallel evaluation of maximum extrema of the graphs of the second derivative for real-time amplification reaction curves (C_p) and the concentration of 146S component of FMDV ($C_{146S\ FMDV}$) in control samples are presented in the table from which it follows that C_p values for all dilutions of the standard of culture FMDV

Table
Relationship between concentration of FMDV 146S component and values of maximum extreme points of real-time amplification reaction curves determined with the second derivative ($n = 3$)

Таблица
Зависимость концентрации 146S компонента вируса ящура и значений максимальных экстремумов кривых реакции амплификации в реальном времени, определенных с помощью второй производной ($n = 3$)

Type of sample	FMDV 146S component concentration, μg/cm³	Determination of FMDV 146S component concentration with real-time RT-PCR							
		according to critical point C_p (crossing point) (proposed method)					according to amplification threshold cycle Ct (threshold cycle) (prototype)		
		C_{p1}	C_{p2}	C_{p3}	C_{pcp}	$C_{virions}$, μg/cm³	Ct_{cp}	$C_{virions}$, μg/cm³	
								per 12× eluate	per 1× eluate
Standard with known concentrations of FMDV 146S component (according to CFT)	0.01	29.85	29.92	29.81	29.86 ± 0.06	0.010 ± 0.003	29.19 ± 0.08	0.058 ± 0.069	0.005 ± 0.005
	0.05	29.82	29.78	29.71	29.77 ± 0.06	0.050 ± 0.010	29.12 ± 0.07	0.304 ± 0.098	0.025 ± 0.009
	0.10	29.59	29.63	29.61	29.61 ± 0.04	0.103 ± 0.020	29.01 ± 0.06	0.660 ± 0.083	0.055 ± 0.038
	0.20	23.34	29.31	29.35	29.33 ± 0.02	0.204 ± 0.030	28.78 ± 0.06	1.440 ± 0.038	0.120 ± 0.050
	0.50	28.53	28.51	28.56	28.53 ± 0.03	0.503 ± 0.041	27.74 ± 0.05	4.985 ± 0.024	0.415 ± 0.050
	1.00	27.21	27.19	27.28	27.23 ± 0.05	1.019 ± 0.032	25.93 ± 0.05	11.160 ± 0.013	0.931 ± 0.052
	1.50	26.02	26.05	26.08	26.05 ± 0.03	1.520 ± 0.031	24.28 ± 0.04	16.741 ± 0.015	1.395 ± 0.048
	2.00	25.03	25.01	25.00	25.01 ± 0.02	1.990 ± 0.030	22.57 ± 0.04	22.560 ± 0.011	1.882 ± 0.051
	2.50	23.94	23.88	23.92	23.91 ± 0.03	2.506 ± 0.028	20.83 ± 0.03	28.503 ± 0.014	2.375 ± 0.039
	3.00	22.99	22.95	23.00	22.98 ± 0.03	2.970 ± 0.027	19.26 ± 0.03	33.840 ± 0.015	2.820 ± 0.045
	3.50	22.01	22.04	21.99	22.01 ± 0.03	3.470 ± 0.034	17.72 ± 0.05	39.060 ± 0.012	3.255 ± 0.044
	4.00	20.99	20.98	21.04	21.00 ± 0.03	4.010 ± 0.030	16.22 ± 0.05	44.160 ± 0.014	3.682 ± 0.038
	4.50	20.10	20.07	20.05	20.07 ± 0.03	4.530 ± 0.031	14.60 ± 0.05	49.684 ± 0.019	4.144 ± 0.061
	5.00	19.29	19.21	19.26	19.25 ± 0.04	5.007 ± 0.033	13.15 ± 0.05	54.601 ± 0.017	4.556 ± 0.055
Negative control	0.00	0.00	0.00	0.00	0.00	0.000	0.00	0.000	0.000

p-criteria: for C_p samples with concentrations of 0.01–0.10 μg/cm³ is less than 0.010, with concentrations of 0.10 –5.00 μg/cm³ – less than 0.005 (for the developed method); for Ct samples with concentrations of 0.01–0.10 μg/cm³ – less than 0.020, with concentrations of 0.10–0.20 μg/cm³ – less than 0.10 μg/cm³, and with concentrations of 0.50–5.00 μg/cm³ – less than 0.005 (for the prototype). To calculate the concentration of 146S particles in 12-fold sample using Ct method (initial method) the following formula was used: $C_{146S} = -3.401(Ct) + 99.333$, to evaluate the content of virions in a one-fold sample the obtained value was divided by 12.

p-критерий: для C_p образцов с концентрациями 0,01–0,10 мкг/см³ составляет менее 0,010, с концентрациями 0,10–5,00 мкг/см³ – менее 0,005 (для разработанного метода); для Ct образцов с концентрациями 0,01–0,10 мкг/см³ – менее 0,020, с концентрациями 0,10–0,20 мкг/см³ – менее 0,10 мкг/см³ и с концентрациями 0,50–5,00 мкг/см³ – менее 0,005 (для прототипа). Для расчета концентрации 146S частиц в 12-кратном образце методом Ct (первоначальный способ) применяли формулу: $C_{146S} = -3,401(Ct) + 99,333$, для расчета содержания вирионов в однократном образце полученное значение делили на 12.

with concentrations of 146S component from 0.01 to 5.00 μg/cm³ are within the range of 29.86 ± 0.06 to 19.25 ± 0.04 respectively. The study of negative control did not reveal fluorescent signal accumulation, which confirmed the absence of FMD virus in the sample. In the presented studies *p*-level of significance is less than 0.010 for control samples of the standard with concentrations of FMDV 146S component from 0.01 to 0.10 μg/cm³ and $p < 0.005$ – from 0.20 to 5.00 μg/cm³.

The dependence of FMDV 146S component concentration and the values of maximum extrema of the graphs of the second derivative for the fluorescence signal accumulation curves is shown in Figure 3 and is presented as a square function $C_{146S\ FMDV} = 0.0111(C_p)^2 - 1.0157C_p + 20.446$ with high accuracy of approximation (R^2 = 99.3%). The model was developed to analyze 12-fold eluates of FMDV RNA obtained by this method. Thus, the existence of dependence between FMDV 146S component concentration in non-inactivated vaccine raw materials and the maximum extreme of the graph of the second derivative for amplification reaction curve in real time was found.

The control samples were studied simultaneously with CFT and classical real-time RT-PCR method with determination of threshold amplification cycle value (Ct) and calculation of 146S component concentration (n = 3) [7]. It should be noted that the preparation of eluates and real-time RT-PCR for the developed method and the prototype differ, in this connection the values of

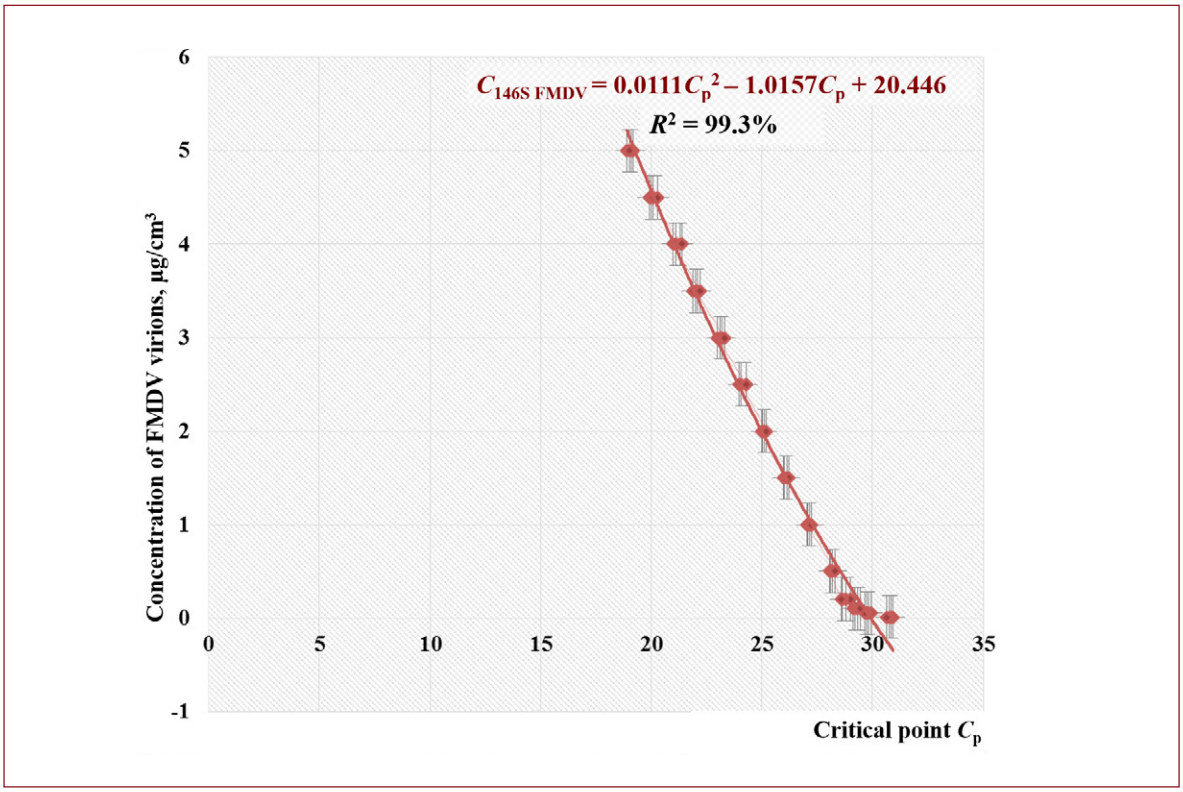

Fig. 3. Relationship between critical point C_p detected with the second derivative for real-time amplification curve and concentration of FMDV 146S component (standard error specified) (n = 3)

Рис. 3. Зависимость критической точки C_p, детектируемой с помощью второй производной для кривой амплификации в режиме реального времени, от концентрации 146S компонента вируса ящура (с указанием стандартной погрешности) (n = 3)

threshold amplification cycles for the obtained graphs of fluorescent signal accumulation of the same sample will be different.

The obtained data are presented in the table, from which it follows that the degree of difference between the results of control analysis by the developed method and the true values of positive standards is 0.00–2.91%. Differences in determination of FMDV 146S component concentration of positive controls with ballast protein content more than 7.00 mg/cm^3 by the initial method in comparison with expected values for samples with 146S component concentrations from 0.01 to 0.10 μg/cm^3 were 45–52%, from 0.01 to 0.10 μg/cm^3 – 17–45%, with full viral particles content from 0.50 to 5.00 μg/cm^3 – 5–17%. In the negative control, FMD virus was not detected with any of the methods presented. Thus, the developed method of indirect determination of FMDV 146S component concentration in the non-inactivated suspension by comparing the maximum extremes of the graphs of the second derivative for the amplification reaction curves in real time allows studying the virus-containing material with concentrations of the 146S component from 0.01 to 5.00 μg/cm^3 with the presence of the ballast protein in the samples more than 7.00 mg/cm^3 within 4–5 hours and with high accuracy.

CONCLUSION

A new approach to indirect determination of FMDV 146S component concentration in a non-inactivated suspension when comparing the maximum extrema of the graphs of the second derivative of the fluorescence signal accumulation curves with the number of amplification reaction cycles is proposed.

The proposed method allows to: 1) increase specificity of sample analysis due to strain-specific binding of FMD virus virions; 2) exclude the possibility of increasing the background fluorescence values due to the use of P. Chomczynski method of sample fraction separation; 3) increase the reliability of the conducted analysis by determining the dependence between the values of FMDV 146S component concentration ($C_{146S\ FMDV}$) and the maximum extrema of the second derivative graphs for amplification reaction curves (C_p).

The existence of dependence between the quantities of 146S particles of FMDV and maximum extrema of the graphs of the second derivative of the fluorescence signal accumulation curve in the form of a square function $C_{146S\ FMDV} = 0.0111(C_p)^2 - 1.0157C_p + 20.446$ with high accuracy of approximation ($R^2 = 0.993$) was determined. The proposed model allows us to quantitatively estimate the content of FMDV virions in non-inactivated raw materials for the vaccine in 4–5 hours.

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Investigation of the healing effects of Afyon Province hot spring waters on experimentally-induced fatty liver in mice

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SUMMARY

Due the increasing number of alcoholic fatty liver disease cases in the world, the development of methods for treating this disease is an urgent task. According to different publications mineral water from hot springs has a beneficial effect on liver cells. In this regard, an investigation was performed with the aim to assess the effectiveness of hot spring water from the Süreyya I spring (Afyonkarahisar province) in treatment of fatty liver disease. 50 one-day-old albino mice with an average body weight of 29.6 g were selected for the experiment. The tests of liver tissue, biochemical and hematological blood tests, as well as blood gas tests performed at this stage, demonstrated deterioration in all parameters. To prove the effectiveness of using hot spring water in the treatment of alcoholic fatty liver disease, two groups of 25 mice each were formed. The animals of the control group were given tap water to drink, and were also bathed (daily) in it for one hour. The mice of the experimental group were given the hot spring water to drink and bathed in it for 15 minutes every day. Histological tests and blood tests were performed on day 1, 7, 14, and 21 of the experiment using five animals randomly selected from each group. On day 21 of the experiment, the animals of the experimental group demonstrated a significant ($p < 0.05$) decrease in the total number of leukocytes, neutrophils, monocytes, as well as in the levels of aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase, low-density lipoproteins, total cholesterol, triglycerides. There was also an increase in erythrocyte, hemoglobin, hematocrit, total protein, albumin and high density lipoprotein levels ($p < 0.05$). The results of histopathological analysis also demonstrated positive dynamics. At the same time, no pronounced positive dynamics was observed in animals of the control group. Moreover, microscopy of liver samples showed an ongoing process of tissue degeneration. The data obtained allow us to conclude that it is advisable to use the hot spring water for the treatment of alcoholic fatty liver disease.

Key words: balneotherapy, biochemistry, hematology, histopathology, fatty liver.

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Исследование лечебного воздействия вод термальных источников провинции Афьон на экспериментально индуцированную жировую дистрофию печени у мышей

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РЕЗЮМЕ

В связи с возрастающим количеством случаев развития алкогольной жировой дистрофии печени в мире актуальной задачей является разработка методов лечения этого заболевания. Из литературных данных известно, что минеральная вода из термальных источников оказывает благотворное влияние на клетки печени. В связи с этим было проведено исследование по оценке эффективности применения термальной воды из источника Süreyya I (провинция Афьонкарахисар) при лечении жировой дистрофии печени. Для эксперимента были отобраны 50 мышей-альбиносов суточного возраста со средней массой тела 29,6 г. Через шесть недель применения этилового спирта у мышей сформировалось ожирение печени. Проведенные на этом этапе исследования тканей печени, биохимический и гематологический анализы крови, а также анализ газового состава демонстрировали ухудшение всех показателей. Для доказательства эффективности применения термальной воды при лечении алкогольной жировой дистрофии печени были сформированы две группы по 25 мышей в каждой. Животных контрольной группы выпаивали водопроводной водой, а также устраивали из нее ежедневные часовые ванны. Мышей опытной группы поили термальной водой, а также купали в ней по 15 мин каждый день. Гистологические исследования и анализы крови проводили на 1, 7, 14 и 21-е сут эксперимента у 5 произвольно выбранных из каждой группы животных. На 21-е сут исследования у животных опытной группы наблюдалось достоверное ($p < 0,05$) снижение общего количества лейкоцитов, нейтрофилов, моноцитов, а также уровней аспартатаминотрансферазы, аланинаминотрансферазы, гамма-глутамилтрансферазы, липопротеинов низкой плотности, концентрации общего холестерина, триглицеридов. Также наблюдалось увеличение уровней эритроцитов, гемоглобина, гематокрита, общего белка, альбумина и липопротеинов высокой плотности ($p < 0,05$). Положительная динамика наблюдалась также по результатам гистопатологического анализа. В то же время у животных контрольной группы ярко выраженной положительной динамики не наблюдалось. Более того, микроскопия проб печени показала продолжающийся процесс дегенерации тканей. Полученные данные позволяют сделать вывод о целесообразности применения термальной воды для лечения алкогольной жировой дистрофии печени.

Ключевые слова: бальнеотерапия, биохимия, гематология, гистопатология, жировая дистрофия печени.

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INTRODUCTION

Fat accumulation exceeding 5% of the total liver mass is called fatty liver disease (FLD) and its incidence is increasing rapidly all over the world [1]. Along with that genetic factors are thought to have impact on the development of the disease, the most important risk factor of alcoholic fatty liver disease (AFLD) is excessive alcohol consumption (> 20 g/day) [2]. Alcohol increases the uptake of fats to liver from the intestine and fatty acid synthesis (lipogenesis) and reduces the digestion of fatty acids (by reducing the beta oxidation of fatty acids), causes fat accumulation in liver cells and ultimately causes AFLD [1, 3]. AFLD can lead to firstly hepatitis and cirrhosis lately [4], and even cancer [5].

During AFLD, hematological, biochemical and histopathological changes are taking place. Alcohol increases the translocation of bacteria from the intestine and leads to increased uptake of bacterial lipopolysaccharides that cause inflammation by activation of Kupffer cells [6]. Therefore, leukocytosis and thrombocytopenia occur frequently in patients with alcoholic steatohepatitis [7]. The most common biochemical finding is elevated transaminases and 2–4 times increase in AST and ALT levels may be seen [8]. The most important histopathological finding of the FDL is the presence of fat vacuoles in hepatocytes in microvesicular or macrovesicular form, or both [9].

It has been reported that hot spring baths contribute significantly to the prevention of hepatitis supporting the incidence of chronic hepatitis by decreasing the portal venous pressure, drinking hot spring water reduces fattening in the liver, stabilizing carbohydrate and lipid metabolism and preventing the progression of pathological process [3].

This study was conducted to determine the efficiency of drinking and bathing applications of Süreyya I hot spring water containing many minerals and compounds that have proven therapeutic efficiency in experimental FLD mice in the treatment of FLD

MATERIALS AND METHODS

Experimental part of this study was made in Experimental Animals Application and Research Center of Afyon Kocatepe University and conducted in accordance with Afyon Kocatepe University Experimental Animals Ethics Committee Instructions (AKUHADYEK) under the report with reference number 42-18 and was supported as Master's Thesis Project by Afyon Kocatepe University Scientific Research Projects Committee (BAPK) under the number 18.SAĞ.BİL.11.

In this research project, 50 Albino mice of the same daily age were used. The animals were kept in plastic cages in a stable environment with equal humidity and heat conditions for 12 hours night and 12 hours day at Afyon Kocatepe University Experimental Animals Application and Research Center. During the study, animals were allowed to receive *ad libitum* rat feed.

Six weeks after alcoholic fatty liver procedure was applied in all animals [10], 50 mice which have same body weight average and constitute the study material were divided into two groups as control group (CG) ($n = 25$) and

Table 1
Weight gain, bouyancy and presence of lesions at the stage of before study, after fatty liver disease formation and after treatment in the animals

Таблица 1
Прибавка в весе, самочувствие и наличие поражений до исследования, после ожирения печени и проведенного лечения животных

Time of indicator measurement by groups		Weight gain or loss (g)	Bouyancy / Clinical lesion formation
BS ($n = 50$)		29.6[b] (25.4–32.5)	Bouyancy and appetency (+++), lesion (–)
AFLF ($n = 44$)		31.6[a] (26.3–32.1)	Bouyancy and appetency (+++), 2 mice dead, 4 mice were operatively exed for examinations, lesion (–)
AT 1st day	CG ($n = 21$)	31.5[a] (26.3–32.0)	Bouyancy and appetency (+++), 1 mouse dead, 5 mice were operatively exed for examinations, lesion (–)
	SG ($n = 22$)	31.70[a] (26.4–32.1)	Bouyancy and appetency (+++), 5 mice were operatively exed for examinations, lesion (–)
AT 7th day	CG ($n = 15$)	31.26[a] (26.3–31.64)	Bouyancy and appetency (+++), 5 mice were operatively exed for examinations, lesion (–)
	SG ($n = 15$)	30.02[b] (24.2–30.4)	Bouyancy and appetency (+++), 1 mouse dead, 5 mice were operatively exed for examinations, lesion (–)
AT 14th day	CG ($n = 9$)	30.64[ab] (25.6–31.0)	Bouyancy and appetency (+++), 1 mouse dead, 5 mice were operatively exed for examinations, lesion (–)
	SG ($n = 10$)	30.12[b] (24.0–30.2)	Bouyancy and appetency (+++), 5 mice were operatively exed for examinations, lesion (–)
AT 21st day	CG ($n = 4$)	30.23[b] (25.6–31.0)	Bouyancy and appetency (+++), 4 mice were operatively exed for examinations, lesion (–)
	SG ($n = 5$)	28.02[c] (23.1–28.9)	Bouyancy and appetency (+++), 5 mice were operatively exed for examinations, lesion (–)

[a–c] The values in the column are statistically significant ($p < 0.05$).
[a–c] Значения в столбце являются статистически значимыми ($p < 0,05$).
BS – before study (до исследования), AFLF – after fatty liver formation (после ожирения печени), AT – after treatment (после лечения), CG – control group (контрольная группа), SG –study group (опытная группа).

study groups (SG) ($n = 25$) for a 21-day treatment period equally and randomly. Normal tap water was added to the CG mice' drinkers, while the hot spring water that was brought from the source each day as fresh added to SG mice' drinkers and they were allowed to reach *ad libitum*. Also, CG mice were bathed in the (35 ± 2) °C tap water as same hour every day, SG mice were bathed in the same temperature with fresh hot spring water for 15 minutes, they were dried with a soft towel after bath, blow dryer was installed gently and then they were put in their cages. In the treatment stage, five animals randomly selected after clinical examination were made on 1, 7, 14 and 21 days after treatment in all of the CG and SG group animals (under xylazine (10 mg/kg) and ketamine (100 mg/kg) anesthesia) and blood and liver tissue samples were taken by intracardiac method for hematological, blood biochemical parameters, blood gases analysis and histopathological examinations [11].

Characteristics of Süreyya I Hot Spring: Süreyya I Hot Spring Water which is volcanic spring and has the property of being the only carbon dioxide water of the region, has been reported as sodium bicarbonate, carbon dioxide, fluoride and silicon thermomineral water class by İstanbul University Çapa Medical Faculty Department of Medical Ecology and Hydroclimatology and mineralization in total has been reported as 4046.8 mg/L by İzmir Community Health Laboratory. Although not specified in this report, it is also accepted in calcium water class since its calcium content is > 150 mg/L.

Clinical Examinations. The body weight (T), mobilization, feed and water consumption, whether they developed lesions of mice were examined and body temperatures, heart frequency (P) and respiratory rate (R) of the animals were measured at the determined measurement times.

Hematological Examinations. Hematological parameters such as erythrocyte (RBC), total leukocytes (WBC), hematocrit (HCT), hemoglobin (HB), mean corpusculer volume (MCV), mean corpusculer hemoglobin concentration (MCHC), lymphocyte (LYM), neutrophil (NEUT), eosinophil (EOS), monocyte (MON) and basophil (BAS) were determined using by commercial test kits and Chemray Brand blood counting device.

Blood Biochemical Examinations. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), urea (UREA), glucose (GLU), triglyceride (TRIG), total cholesterol (TCHOL), high density lipoprotein (HDL), low density lipoprotein (LDL) and magnesium (Mg) levels were measured on the Cobas Integra

400 Plus Roche Brand analyzer (Roche Diagnostics GmbH, Germany).

Blood Gases Analyses. After blood samples were taken to plastic syringes with heparin supplemented as 500 IU liquid heparin for 1 ml of blood prepared previously, pH, partial carbon dioxide pressure (pCO_2), total carbon dioxide concentration (TCO_2), base excess (BE), bicarbonate (HCO_3^-), chlorine (Cl^-), sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) measurements were carried out on portable blood gas analyzer (Edan i15 Veterinary blood gas analyzer) by using commercial cartridges.

Histopathological Examinations. Liver samples were taken under anesthesia of ketamine/xylazine (10 mg/kg/100 mg/kg) [11] one week interval from 5 animals randomly selected from both groups at the determined measurement times. Samples were sent to the relevant laboratories in 10% formol. Histopathological examinations were carried out by experts at Veterinary Control Center Research Institute, Pathology Laboratory in T.R. Ministry of Agriculture and Forestry.

Statistical Analyses. Statistical analyzes of the groups were made according to ANOVA method. Duncan test was used to determine the importance of intragroup differences in the study group. Statistical analyzes were performed using Windows compatible SPSS 18.1 (Inc., Chicago, Il, USA) software. Data were expressed as mean ± standard error and $p < 0.05$ was considered significant.

TEST RESULTS

In this study, sex was not considered a factor in the statistical analysis of the data.

Clinical Findings

Clinical findings of animals are shown in Tables 1 and 2.

When Table 1 is examined, the body weight (bw) averages of the CG mice was 31.5 g (min 26.3, max 32.0) in the weighings performed at the beginning of the treatment phase and the bw averages of the SG mice was 31.7 g (min 26.4, max 32.1) and it was observed that there was no statistical difference in terms of bw ($p > 0.05$). In bw measurements on the 21st day of the study, mean SG animals were found to be statistically significant lower than the CG ($p < 0.05$). When Table 2 is examined, along with there was no statistically significant difference in terms of T in the measured time periods ($p > 0.05$), when compared with the pre-study, it was found that P and R frequencies showed significant ($p < 0.05$) differences in terms of average after fattening formation, and it was found that these averages were significantly higher in CG animals ($p < 0.05$) compared to SG animals during the treatment process.

Hematological Findings

The hematological examination findings of CG and SG animals are shown in Table 3.

When the Table 3 was examined, it was observed that WBC, NEUT, MON, MCV, MCH levels increased significantly after fattening ($p < 0.05$), whereas RBC, HB, HCT, LYM, MCHC and PLT levels were significantly ($p < 0.05$) decreased. In the post-treatment comparisons, WBC, NEUT, MON, MCV and MCH averages in SG animals were lower ($p < 0.05$) and RBC, HB, HCT, LYM and PLT levels were higher ($p < 0.05$).

Blood Biochemical Findings

The averages of blood biochemical analysis findings are shown in Table 4. It was determined that AST, ALT, GGT, UREA, CREA, TCHOL, TRIG and LDL levels increased significantly ($p < 0.05$) after fattening and TP, ALB, HDL, GLU and Mg levels decreased significantly ($p < 0.05$). Although

Table 2
Statistical comparison of body temperature, pulse frequency and respiratory rate

Таблица 2
Статистическое сравнение температуры тела, частоты пульса и частоты дыхания

Time of indicator measurement by groups		Parameters (X ± SD)		
		T (°C)	P (frequency/min)	R (rate/min)
BS ($n = 50$)		37.20 ± 0.14	341.44 ± 45.10^{f}	126.30 ± 20.00^{e}
AFLF ($n = 44$)		37.40 ± 0.18	432.28 ± 65.30^{a}	240.14 ± 35.00^{a}
AT 1st day	CG ($n = 21$)	37.40 ± 0.10	413.04 ± 53.40^{b}	238.28 ± 33.10^{a}
	SG ($n = 22$)	37.30 ± 0.12	392.05 ± 47.10^{c}	221.24 ± 32.00^{b}
AT 7th day	CG ($n = 15$)	37.30 ± 0.00	402.12 ± 45.30^{bc}	229.00 ± 25.00^{ab}
	SG ($n = 15$)	37.10 ± 0.00	376.18 ± 38.20^{d}	178.10 ± 24.10^{c}
AT 14th day	CG ($n = 9$)	37.20 ± 0.20	398.47 ± 32.00^{bc}	180.34 ± 24.00^{c}
	SG ($n = 10$)	37.10 ± 0.10	360.40 ± 28.40^{e}	145.18 ± 20.00^{d}
AT 21st day	CG ($n = 4$)	37.20 ± 0.12	374.10 ± 35.20^{d}	150.30 ± 22.00^{d}
	SG ($n = 5$)	37.00 ± 0.14	337.18 ± 24.30^{f}	130.34 ± 16.10^{e}

$^{a–f}$The values in the column are statistically significant ($p < 0.05$).

$^{a–f}$Значения в столбце являются статистически значимыми ($p < 0,05$).

BS – before study (до исследования), AFLF – after fatty liver formation (после ожирения печени), AT – after treatment (после лечения), CG – control group (контрольная группа), SG –study group (опытная группа).

Table 3
Results of hematology blood tests

Таблица 3
Результаты гематологических исследований крови животных

Time of indicator measurement by groups		Parameters (X ± SD)												
		WBC (10^3/mm^3)	RBC (10^6/mm^3)	HB (g/dl)	HCT (%)	PLT (10^3/mm^3)	MCV (fl)	MCH (pg)	MCHC (g/dl)	LYM (%)	NEUT (%)	EOS (%)	MON (%)	BAS (%)
BS ($n = 50$)		8.30 ± 1.40^{f}	7.93 ± 1.16^{a}	13.42 ± 1.24^{a}	43.23 ± 1.24^{a}	284.90 ± 35.24^{a}	53.48 ± 0.26^{g}	16.48 ± 3.06^{c}	31.18 ± 2.03^{a}	61.42 ± 5.20^{a}	33.30 ± 3.20^{f}	2.10 ± 1.00	3.40 ± 0.20^{d}	NS
AFLF ($n = 44$)		15.40 ± 3.20^{a}	4.48 ± 0.43^{d}	8.27 ± 3.43^{d}	32.48 ± 3.22^{ef}	186.24 ± 32.08^{e}	72.21 ± 5.14^{a}	18.21 ± 6.11^{a}	25.41 ± 3.12^{d}	47.68 ± 6.40^{f}	45.20 ± 4.10^{a}	2.14 ± 0.10	6.30 ± 1.60^{a}	NS
AT 1st day	CG ($n = 21$)	15.28 ± 3.10^{a}	4.62 ± 0.64^{d}	8.26 ± 2.05^{d}	31.98 ± 3.06^{f}	184.38 ± 47.86^{e}	68.36 ± 4.04^{b}	17.87 ± 4.23^{b}	25.56 ± 3.11^{d}	48.26 ± 5.30^{ef}	45.10 ± 4.30^{a}	2.23 ± 0.12	5.20 ± 1.23^{b}	NS
	SG ($n = 22$)	14.06 ± 2.30^{bc}	5.83 ± 0.57^{c}	9.54 ± 2.28^{c}	33.46 ± 2.68^{e}	198.12 ± 55.46^{de}	57.14 ± 4.08	16.15 ± 3.22^{c}	28.51 ± 3.24^{c}	52.18 ± 4.40^{d}	42.20 ± 3.60^{b}	2.16 ± 0.04	4.10 ± 1.22^{c}	NS
AT 7th day	CG ($n = 15$)	14.40 ± 2.34^{b}	5.38 ± 1.10^{c}	8.90 ± 2.11^{d}	32.88 ± 2.27^{e}	186.27 ± 38.22^{e}	61.03 ± 4.28^{c}	16.37 ± 2.11^{c}	27.12 ± 2.23^{c}	49.27 ± 5.10^{e}	44.40 ± 3.00^{a}	2.16 ± 0.20	5.00 ± 1.32^{b}	NS
	SG ($n = 15$)	13.34 ± 2.16^{c}	6.80 ± 1.24^{b}	10.38 ± 2.00^{bc}	37.40 ± 2.06^{c}	214.45 ± 33.26^{c}	55.23 ± 3.32^{e}	15.32 ± 2.08^{d}	27.70 ± 2.03^{c}	55.18 ± 4.34^{c}	40.20 ± 2.30^{c}	2.40 ± 1.40	3.04 ± 1.20^{d}	NS
AT 14th day	CG ($n = 9$)	13.40 ± 2.04^{c}	5.79 ± 1.23^{c}	9.41 ± 2.08^{c}	33.29 ± 2.08^{e}	196.02 ± 27.62^{de}	57.22 ± 3.20^{d}	16.21 ± 1.24^{c}	28.04 ± 1.16^{c}	51.24 ± 4.10^{d}	42.50 ± 2.18^{b}	2.18 ± 0.06	5.69 ± 1.42^{ab}	NS
	SG ($n = 10$)	10.28 ± 1.54^{e}	7.86 ± 1.02^{a}	11.27 ± 1.68^{b}	40.16 ± 1.43^{b}	248.27 ± 28.48^{b}	51.23 ± 2.35^{f}	14.25 ± 1.00^{e}	28.14 ± 0.68^{c}	57.04 ± 3.28^{b}	36.28 ± 2.00^{e}	2.15 ± 0.00	4.03 ± 0.50^{c}	NS
AT 21st day	CG ($n = 4$)	12.04 ± 2.18^{d}	6.23 ± 1.16^{b}	9.87 ± 1.24	35.87 ± 1.66^{d}	201.26 ± 24.32^{d}	57.52 ± 2.22^{d}	15.60 ± 0.55^{d}	27.43 ± 0.55^{c}	54.16 ± 4.24^{c}	38.14 ± 2.10^{d}	2.16 ± 0.00	5.10 ± 0.40^{b}	NS
	SG ($n = 5$)	9.02 ± 1.23^{f}	7.98 ± 1.18^{a}	12.98 ± 1.18^{a}	43.07 ± 1.18^{a}	279.84 ± 21.16^{a}	53.86 ± 1.16^{g}	16.21 ± 0.43^{c}	30.04 ± 0.38^{b}	61.80 ± 2.40^{a}	33.12 ± 1.60^{f}	2.10 ± 0.20	3.28 ± 0.30^{d}	NS

[a-g] The values in the column are statistically significant ($p < 0.05$).

[a-g] Значения в столбце являются статистически значимыми ($p < 0,05$).

BS – before study (до исследования), AFLF – after fatty liver formation (после ожирения печени), AT – after treatment (после лечения),
CG – control group (контрольная группа), SG –study group (опытная группа), NS – non-significant (не значимо).
WBC –white blood cells (лейкоциты), RBC – red blood cells (эритроциты), HB – hemoglobin (гемоглобин),
HCT – hematocrit (гематокрит), PLT – platelets (тромбоциты), MCV – mean corpusculare volume (средний объем эритроцитов),
MCH – mean corpuscular hemoglobin (среднее содержание гемоглобина в эритроците),
MCHC – mean corpuscular hemoglobin concentration (средняя концентрация гемоглобина в эритроците),
LYM – lymphocyte (лимфоциты), NEUT – neutrophils (нейтрофилы), EOS – eosinophils (эозинофилы),
MON – monocyte (моноциты), BAS – basophils (базофилы).

Table 4
Blood biochemical findings of the animals

Таблица 4
Результаты биохимических исследований крови животных

Time of indicator measurement by groups		Parameters (X ± SD)													
		AST (IU/L)	ALT (IU/L)	GGT (IU/L)	TP (g/dl)	ALB (g/dl)	GLU (g/dl)	Mg (mmol/L)	TLPD (mg/dl)	TCHOL (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	TRIG (mg/dl)	CREA (mg/dl)	UREA (mg/dl)
BS ($n = 50$)		94.62 ± 4.24	58.68 ± 6.18^{f}	3.55 ± 0.14^{e}	55.74 ± 6.18^{a}	34.14 ± 0.48^{a}	1.68 ± 0.12^{a}	1.63 ± 0.18^{a}	218.44 ± 23.28^{e}	88.71 ± 8.04^{f}	42.82 ± 6.40^{a}	68.24 ± 17.32^{e}	95.12 ± 15.1^{e}	0.58 ± 0.05^{f}	8.65 ± 3.18^{e}
AFLF ($n = 44$)		248.74 ± 16.14^{a}	165.84 ± 14.23^{a}	8.68 ± 2.13^{a}	36.04 ± 5.32^{de}	22.02 ± 0.54^{g}	0.89 ± 0.24^{e}	0.64 ± 0.04^{d}	459.48 ± 45.26^{a}	176.18 ± 46.12^{a}	23.28 ± 9.23^{e}	136.28 ± 23.44^{a}	207.25 ± 34.12^{a}	3.64 ± 1.05^{a}	15.42 ± 6.27^{a}
AT 1st day	CG ($n = 21$)	243.12 ± 14.27^{a}	167.49 ± 15.16^{a}	8.45 ± 0.54^{a}	35.42 ± 6.04^{e}	23.32 ± 0.48^{f}	0.90 ± 0.20^{e}	0.65 ± 0.16^{d}	458.21 ± 47.14^{a}	174.24 ± 48.13^{a}	23.31 ± 8.65^{e}	135.45 ± 25.16^{a}	206.43 ± 35.21^{a}	3.63 ± 1.12^{a}	15.32 ± 7.12^{a}
	SG ($n = 22$)	242.26 ± 16.23^{a}	162.47 ± 16.32^{a}	8.32 ± 0.47^{a}	37.10 ± 7.23^{d}	24.04 ± 0.46^{ef}	0.91 ± 0.19^{e}	0.68 ± 0.22^{d}	456.03 ± 48.18^{a}	173.45 ± 45.71^{a}	24.12 ± 9.27^{e}	134.12 ± 23.04^{a}	203.18 ± 37.14^{a}	3.62 ± 1.16^{a}	15.04 ± 6.45^{a}
AT 7th day	CG ($n = 15$)	222.18 ± 14.43^{b}	151.48 ± 14.30^{b}	8.18 ± 0.36^{a}	38.18 ± 5.44^{cd}	25.18 ± 0.43^{e}	0.94 ± 0.18^{e}	0.73 ± 0.12^{d}	432.27 ± 35.86^{b}	167.16 ± 38.27^{ab}	25.16 ± 12.21^{e}	130.28 ± 21.25^{ab}	202.28 ± 25.34^{a}	3.54 ± 1.04^{a}	14.03 ± 4.16^{b}
	SG ($n = 15$)	187.74 ± 15.65^{d}	132.61 ± 13.26^{c}	7.44 ± 0.28^{b}	41.32 ± 4.66^{c}	28.15 ± 0.34^{cd}	1.28 ± 0.14^{c}	0.92 ± 0.04^{bc}	345.27 ± 27.21^{d}	122.05 ± 27.34^{d}	35.48 ± 11.51^{c}	112.31 ± 15.14^{c}	178.27 ± 18.23^{b}	2.14 ± 0.64^{c}	11.21 ± 3.09^{d}
AT 14th day	CG ($n = 9$)	204.18 ± 12.32^{c}	141.74 ± 14.25^{bc}	7.69 ± 0.24^{b}	39.23 ± 5.45^{cd}	27.64 ± 0.33^{d}	1.02 ± 0.16^{d}	0.86 ± 0.03^{c}	416.25 ± 31.14^{c}	160.21 ± 28.12^{b}	27.28 ± 10.21^{de}	126.05 ± 14.48^{b}	184.24 ± 19.40^{ab}	2.72 ± 1.02^{b}	12.36 ± 3.07^{c}
	SG ($n = 10$)	161.28 ± 11.43^{f}	106.43 ± 9.46^{e}	5.03 ± 0.12^{d}	47.08 ± 3.46^{b}	31.82 ± 0.35^{b}	1.48 ± 0.13^{b}	1.20 ± 0.04^{b}	276.28 ± 22.21^{e}	106.12 ± 11.22^{e}	40.14 ± 7.22^{b}	83.26 ± 7.41^{d}	130.34 ± 12.22^{d}	0.96 ± 0.27^{e}	9.01 ± 1.16^{e}
AT 21st day	CG ($n = 4$)	183.78 ± 10.44^{e}	129.75 ± 8.55^{d}	6.65 ± 0.11^{c}	42.21 ± 4.33^{c}	29.18 ± 0.26^{c}	1.06 ± 0.09^{d}	0.92 ± 0.03^{bc}	398.00 ± 13.15^{c}	144.21 ± 12.23^{c}	30.16 ± 8.13^{d}	108.07 ± 8.10^{c}	158.21 ± 13.11^{c}	1.48 ± 0.15^{d}	11.27 ± 1.20^{d}
	SG ($n = 5$)	114.76 ± 9.20^{g}	62.48 ± 5.42^{f}	3.88 ± 0.07^{e}	55.19 ± 2.24^{a}	33.18 ± 0.17^{ab}	1.69 ± 0.05^{a}	1.64 ± 0.01^{a}	203.28 ± 11.23^{e}	87.18 ± 5.03^{f}	43.02 ± 5.14^{a}	67.32 ± 6.13^{e}	101.20 ± 9.20^{e}	0.60 ± 0.04^{f}	8.42 ± 0.22^{e}

$^{a-g}$ The values in the column are statistically significant ($p < 0.05$).

$^{a-g}$ Значения в столбце являются статистически значимыми ($p < 0,05$).

BS – before study (до исследования), AFLF – after fatty liver formation (после ожирения печени), AT – after treatment (после лечения),
CG – control group (контрольная группа), SG –study group (опытная группа).
AST – aspartate transaminase (аспартатаминотрансфераза), ALT – alanine aminotransferase (аланинаминотрансфераза),
GGT – gamma-glutamyl transferase (гамма-глутамилтрансфераза), TP – total protein (общий белок), ALB – albumin (альбумин),
GLU – glucose (глюкоза), Mg – magnesium (магний), TLPD – total lipids (общие липиды), TCHOL – total cholesterol (общий холестерин),
HDL – high-density lipoproteins (липопротеины высокой плотности), LDL – low density lipoprotein (липопротеины низкой плотности),
TRIG – triglycerides (триглицериды), CREA – creatinine (креатинин), UREA – мочевина.

Table 5
Blood gases findings of the animals

Таблица 5
Результаты исследования газового состава крови

Time of indicator measurement by groups		Parameters (X ± SD)									
		pH	pCO_2 (mmHg)	HCO_3^- (mmol/L)	BE (mEq/L)	TCO_2 (mmol/L)	LACT (mmol/L)	K^+ (mmol/L)	Na^+ (mmol/L)	Cl^- (mmol/L)	Ca^{2+} (mmol/L)
BS ($n = 50$)		7.35 ± 0.02^{a}	43.18 ± 0.32^{a}	23.54 ± 0.36^{a}	-2.0 ± 0.02^{a}	15.32 ± 0.44^{a}	4.45 ± 0.58^{cd}	8.48 ± 1.05^{a}	157.40 ± 1.42^{e}	113.29 ± 1.35^{h}	10.31 ± 1.33^{a}
AFLF ($n = 44$)		7.24 ± 0.03^{f}	35.64 ± 1.33^{e}	14.86 ± 0.26^{g}	-11.4 ± 0.04^{f}	12.27 ± 2.30^{f}	6.68 ± 1.27^{a}	5.34 ± 1.26^{d}	243.70 ± 12.18^{a}	187.30 ± 9.20^{a}	6.28 ± 1.32^{c}
AT 1st day	CG ($n = 21$)	7.24 ± 0.03^{f}	35.19 ± 1.50^{e}	14.64 ± 0.50^{g}	-11.6 ± 0.04^{f}	12.05 ± 2.22^{f}	6.60 ± 1.23^{a}	5.19 ± 1.27^{d}	234.28 ± 11.20^{a}	183.32 ± 9.22^{a}	6.43 ± 1.04^{c}
	SG ($n = 22$)	7.27 ± 0.02^{e}	33.62 ± 1.34^{e}	14.94 ± 0.38^{f}	-10.6 ± 0.03^{e}	11.61 ± 1.40^{e}	5.88 ± 1.41^{b}	5.12 ± 1.16^{d}	202.27 ± 7.14^{b}	171.40 ± 5.30^{b}	7.28 ± 0.64^{d}
AT 7th day	CG ($n = 15$)	7.26 ± 0.01^{e}	34.19 ± 1.45^{d}	14.13 ± 0.30^{b}	-10.8 ± 0.03^{e}	11.70 ± 1.35^{a}	6.18 ± 1.40^{b}	5.23 ± 1.02^{d}	217.26 ± 5.40^{ab}	166.18 ± 6.23^{c}	7.01 ± 0.55^{b}
	SG ($n = 15$)	7.35 ± 0.02^{cd}	38.65 ± 1.31^{c}	14.85 ± 0.27^{c}	-10.8 ± 0.03^{c}	12.08 ± 0.34^{c}	4.94 ± 0.52^{c}	6.46 ± 0.78^{c}	174.32 ± 4.13^{c}	139.18 ± 3.42^{d}	9.27 ± 0.36^{b}
AT 14th day	CG ($n = 9$)	7.30 ± 0.02^{d}	35.24 ± 1.38^{cd}	20.63 ± 0.20^{e}	-4.2 ± 0.01^{d}	12.62 ± 0.28^{d}	5.86 ± 0.78^{b}	5.88 ± 0.64^{cd}	204.24 ± 4.10^{ab}	145.20 ± 3.36^{e}	8.45 ± 0.43^{c}
	SG ($n = 10$)	7.38 ± 0.02^{b}	41.07 ± 0.33^{b}	23.52 ± 0.22^{b}	-1.2 ± 0.01^{b}	14.70 ± 0.26^{b}	4.67 ± 0.28^{c}	7.38 ± 0.45^{b}	158.22 ± 2.46^{d}	123.28 ± 1.60^{g}	10.12 ± 0.26^{a}
AT 21st day	CG ($n = 4$)	7.32 ± 0.01^{d}	36.19 ± 0.37^{c}	18.02 ± 0.10^{d}	-7.1 ± 0.02^{d}	12.66 ± 0.21^{c}	5.15 ± 0.36^{bc}	6.78 ± 0.36^{c}	179.21 ± 2.76^{c}	134.26 ± 0.43^{f}	9.02 ± 0.17^{b}
	SG ($n = 5$)	7.42 ± 0.01^{a}	43.82 ± 0.03^{a}	27.51 ± 0.12^{a}	-3.1 ± 0.01^{a}	16.01 ± 0.12^{a}	4.12 ± 0.18^{d}	8.68 ± 0.19^{a}	152.27 ± 2.33^{d}	111.28 ± 0.31^{h}	10.89 ± 0.14^{a}

[a-h] The values in the column are statistically significant ($p < 0.05$).

[a-h] Значения в столбце являются статистически значимыми ($p < 0,05$).

BS – before study (до исследования), AFLF – after fatty liver formation (после ожирения печени), AT – after treatment (после лечения), CG – control group (контрольная группа), SG – study group (опытная группа).

pH – hydrogen ion concentration (концентрация ионов водорода), pCO_2 – CO_2 partial pressure (парциальное давление углекислого газа), HCO_3^- – bicarbonate (бикарбонат), BE – base excess (сдвиг буферных оснований), TCO_2 – total CO_2 (общая концентрация углекислого газа), LACT – lactate (лактат), K^+ – potassium (калий), Na^+ – sodium (натрий), Cl^- – chlorine (хлор), Ca^{2+} – calcium (кальций).

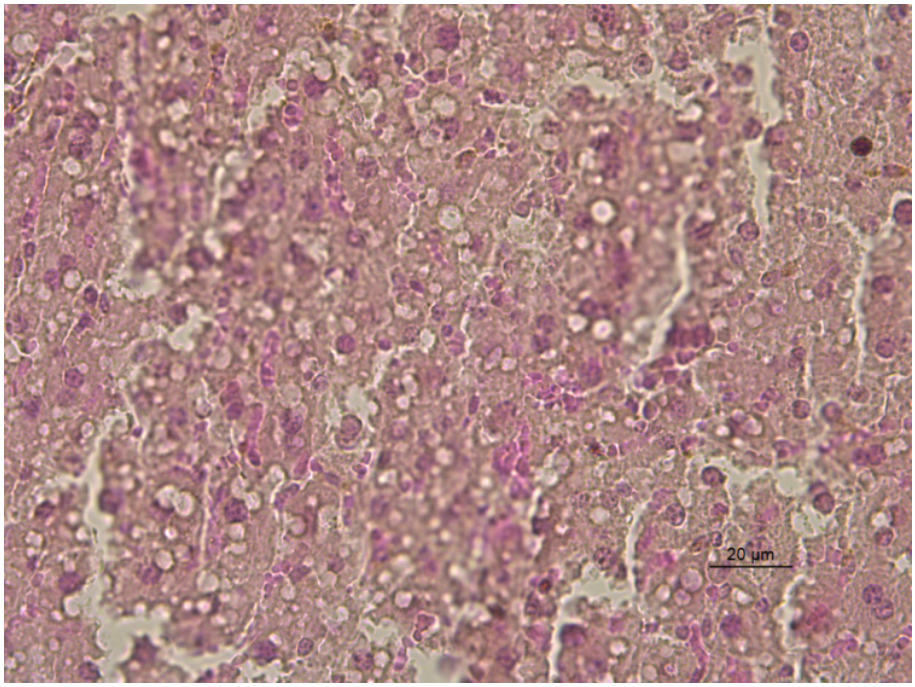

Fig. 1. Formation of fatty liver in mice treated with ethyl alcohol (10×–40×)

Рис. 1. Поражение печени у мышей после применения этилового спирта (увеличение 10×–40×)

TP, ALB, HDL, GLU and Mg levels were increased and AST, ALT, GGT, UREA, CREA, TCHOL, TRIG and LDL levels were decreased in both groups after treatment, in terms of increasing and decreasing parameters, it was observed that the changes in SG animals were statistically significant ($p < 0.05$) than CG animals.

Blood Gases Findings

Statistical comparisons of blood gas analysis results are presented in Table 5 below.

When this table is examined, it was observed that pH, partial CO_2, pCO_2, BE, HCO_3^-, Ca^{2+} and K^+ levels decreased as a result of fatty liver, whereas LACT, sodium Na^+ and Cl^- levels increased, in terms of this increase and decrease in the group comparisons, the highest levels ($p < 0.05$) occurred in SG animals in terms of rate and amount of healing.

Histopathological Findings

Histopathologically, it was found that the fattening was in the form of intrastoplasmic, microvesicular and partly

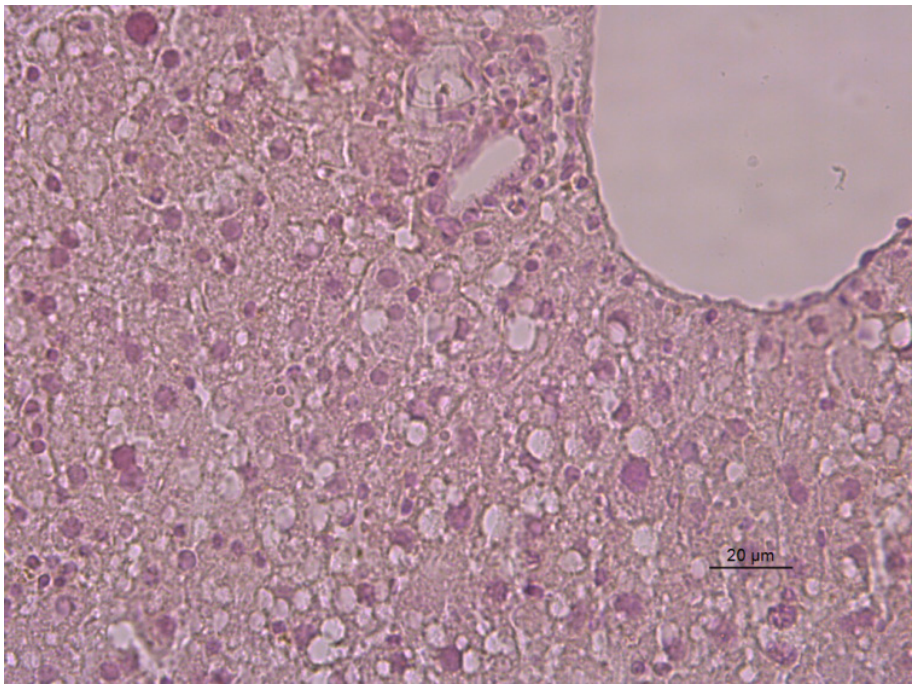

Fig. 2. Ongoing parenchymal degeneration and fattening at the end of day 21 in control animals (10×–40×)

Рис. 2. Паренхиматозная дегенерация печени на 21-е сут у животных контрольной группы (увеличение 10×–40×)

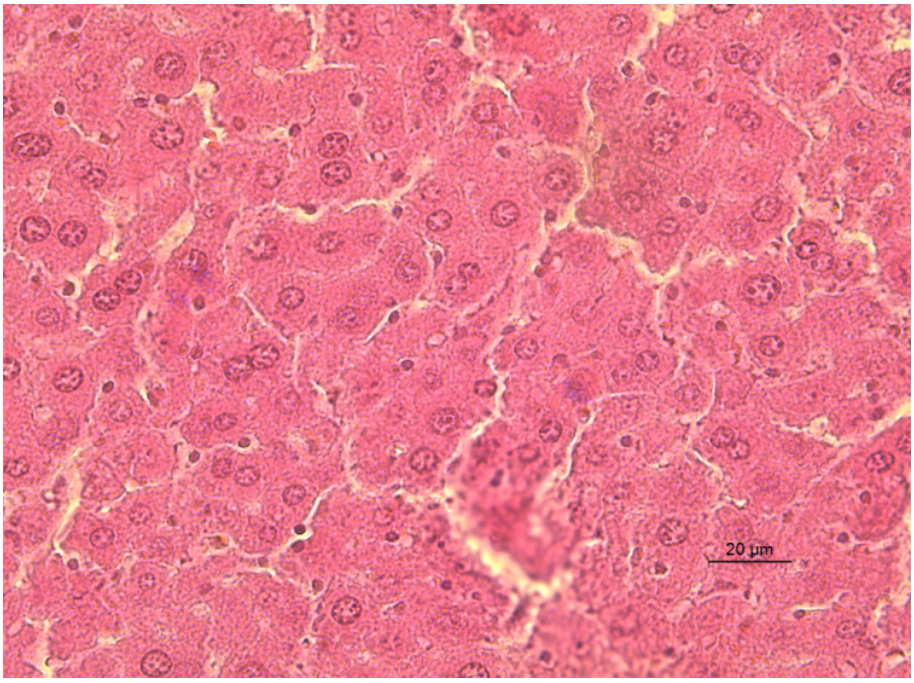

Fig. 3. Normal liver appearance in mice treated with Süreyya I hot spring water at the end of day 21 (10×–40×)

Рис. 3. Внешний вид печени у мышей, получавших воду из термального источника Süreyya I, на 21-е сут исследования (увеличение 10×–40×)

macro-microvescular fattening, and intense parenchymal degeneration and necrosis with severe fattening were partly observed (Fig. 1) after fatty liver formation.

In the comparisons at the end of the 21st day, which is the last day of treatment, it was found that fat formation continued in CG animals treated with tap water (Fig. 2), whereas fat and necrosis cases improved significantly in SG animals treated with hot spring water (Fig. 3). Sections and pictures of each measurement time are archived.

DISCUSSION AND CONCLUSION

It was found that the average bw (31.6 g) after fattening formation was higher compared to the average bw (29.6 g) prior to fattening formation, but the bw average of CG animals (30.23 g) was higher than the average of SG (28.02 g) on the last day of treatment. This finding is consistent with the studies [3] reporting that EtOH causes to weight gain by fastening the liver and that the treatment with hot spring waters leads to weight loss by increasing fat burning and reducing fat intake from the intestines. It was shown that respiratory rate and heart frequencies were statistically significant ($p < 0.05$) in animals whom fatty liver is formed, and these findings were found to be in compliance with the studies indicating that the increased heart rate would be accompanied by increased respiratory frequency [10].

It was observed that WBC, LYM numbers which were initially high, were decreased in the SG animals which drink Süreyya I water and are daily bathed with this water. These findings were found to be compatible with the studies [12] indicating that hot spring waters had an immunosuppressive effect and that T-lymphocytes in blood decreased significantly in hyperthermal baths, and that hyperthermal waters provoked ACTH hormone level and cortisol production and caused T-lymphocytopenia and eosinopenia.

Chronic alcohol consumption has been reported to lead to elevated MCV levels with leukocytosis and thrombocytopenia [13], and leucocytosis and thrombocytopenia have been tended to normal levels upon discontinuation of alcohol consumption [14]. The fact that the lower MCV, WBC and higher PLT levels were obtained in SG animals which drank Süreyya I hot spring water compared to CG and initial measurements fattening, and the that this case reached the most significant level in the last week of treatment support these researches' declarations. Increased MCV has been reported to be important biomarkers of chronic alcohol dependence [15], and high MCV levels accompanied by clinical inflammation syndrome resulting in Mg deficiency in leukocyte and macrophage activation and excessive production of free radicals [16]. Since the Süreyya I hot spring water used in our study is rich in Mg, it is thought that Mg may contribute to the normalization of leukocytosis and high MCV levels by showing anti-inflammatory effect.

In our study, in animals with fatty liver, it was found that TRIG, LDL, TCHOL levels were significantly increased, HDL cholesterol levels were decreased, but in the treatment of animals treated with hot spring water, however, in the SG animals given hot spring water with the beginning of the treatment period, a reverse course was formed in these parameters and the improvement in the lipid profile gradually increased and the best results were obtained in the animals in the SG and in the last week. These findings are consistent with the findings of the researchers [17] who reported that acute or chronic natural use of mineral waters had significant regulatory effects on serum lipid profile. As a matter of fact that, C. L. Hsu et al. [18] reported that rich mineral waters decreased TCHOL levels by increasing fecal cholesterol and bile acid excretion in feces.

The spectacular effects of rich mineral waters on obesity have been revealed by the stimulation mechanism of

mitochondrial genesis and the identification of components controlling energy release from fats. Mg and Ca are the main components that play a role in reducing fat [19]. Süreyya I hot spring is rich in Mg and Ca ions. Mg reduces lipid accumulation due to high cholesterol intake [20]. Y. Kishimoto et al. indicated that Mg uptake also inhibits intestinal fat absorption and may improve postprandial hyperlipidemia in healthy individuals [21]. M. Kimura et al. showed that mineral water containing 600 and 1,000 ppm Mg could reduce cholesterol levels by 18% and 15%, respectively [22]. It was reported that Mg-rich water decreases lipid peroxidation by increasing hepatic low-density lipoprotein receptor and cholesterol-7a-hydroxylase (CYP7A1) gene activation that play a role in cholesterol catabolism, so leads to decrease in TCHOL and LDL levels [23]. However, there are studies that report that Mg-rich mineral waters treat fatty liver by inhibiting cholesterol and fatty acid synthesis by increasing the AMP-activated protein kinase enzyme level [24].

Calcium in the diet prevents adipocyte lipid accumulation and weight gain, increases lipolysis and thus significantly accelerates weight loss. Moreover, it has recently been shown that calcitriols released in response to low calcium diets stimulate Ca flow in human adipocytes and thus support adiposity [25]. In spite of all these positive effects, it has been reported that Mg and Ca do not have an effect alone in reducing fat and other elements contribute to it [26]. HCO_3^- is the leading element of these elements, and water with rich HCO_3^- has been reported to have a reducing effect on total and LDL cholesterol [17]. In our study, Süreyya I hot spring water used for treatment purposes is included in the bicarbonate hot spring water class and has a high concentration of HCO_3^- besides high Mg and Ca levels. As a matter of fact, in the measurements we made, the group with the highest increase in blood HCO_3^- levels was determined to be the SG group and also the lowest levels of TCHOL, LDL and TRIG were obtained in this group. It has also been reported that Cl^- containing bicarbonate water stimulates bile acid excretion and reduces TRIG concentration in the intestine [27].

In our study, it was found that the measured levels of AST, ALT, GGT, UREA and CREA were found to be high in the measurements following the fattening and TP, ALB, GLU, HDL levels were low. With the commencement of the treatment period, in SG animals given Süreyya I hot spring water, a continuous positive improvement was observed in these parameters until the last week of the study when compared with CG animals. Similar findings were found in a study C. Pereira et al. reporting that high levels of AST, ALT, GGT, UREA, CREA decreased in mice with metabolic syndrome, whereas TP, ALB, HDL levels increased [16]. Plasma AST and ALT are considered important markers in the detection of liver damage [28]. I. Chen et al. argued that treatment with high mineral waters reduced lipid accumulation in the liver by increasing daily fecal lipid and bile acid output [29]. Also, it was reported that rich mineral waters reduced lipid peroxidation and related hepatic malondialdehyde (MDA) content in the livers and prevent hepatic damage, boron and Mg contained in it increased antioxidant capacity against oxidative stress and decreased high ALT and AST levels [18].

It was reported that ALB, TP, Mg and K levels were significantly decreased in patients with AFLD similar to our findings, whereas GGT and bilirubin levels were elevated and these findings were important markers of alcohol-related severe fat [30]. In our study, high levels of GGT and low levels of TP, ALB, Mg and K were found to support the findings of these researchers. At the end of the treatment period, the fact that the most important healings related to these parameters are detected in SG animals proves the effectiveness of treatment with hot spring water. As a matter of fact, it has been reported that hot spring water and baths normalize the intake of minerals and proteins from the intestines by reducing portal venous pressure [9].

Overdose and excessive consumption of alcohol cause ketoacidosis associated with metabolic acidosis [27]. In this case, a decrease in blood pH, pCO_2 and HCO_3^- levels and an increase in LACT levels are detected [31]. In our study, similar findings were found in animals with fatty liver formed. However, it was observed that after starting treatment with Süreyya I hot spring water in SG animals, a reversal of these parameters started and withdrawn to physiological limits towards to end of the treatment, metabolic acidosis due to lactic acidosis improved when compared to CG. L. Xu et al. reported that LACT levels decreased significantly after 21-day balneotherapy, along with there was an increase in TCO_2 levels, this increase was not statistically significant.

In our present study, in histopathological sections of liver tissue samples taken after the formation of fatty liver intrastoplasmic, microvesicular and partly macro-microvesicular fat was observed, and intense parenchymal degeneration and necrosis foci with severe fattening were partly observed. These data are consistent with the studies that report that up to 90% of chronic alcohol users will experience steatosis in the centrilobular or perivenular area [33]. In the histopathological comparisons at the end of the 21st day, it was found that although fattening in liver and necrosis healed in SG animals treated with hot spring water, fattening were still continued in CG animals treated with tap water. This finding has been detected for the first time and constitutes a reference.

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Peer-review of monograph "Viral Diseases of Sturgeons and Salmon" L. P. Buchatsky, Yu. P. Rud, N. N. Matvienko.

K.: DIA, 2020. 240 p. ISBN 978-617-7785-10-0

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Рецензия на монографию «Вирусные болезни осетров и лососей» Л. П. Бучацкий, Ю. П. Рудь, Н. Н. Матвиенко.

Киев: ДИА, 2020. 240 с. ISBN 978-617-7785-10-0

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Для цитирования: Ярыгина Е. И. Рецензия на монографию «Вирусные болезни осетров и лососей» Л. П. Бучацкий, Ю. П. Рудь, Н. Н. Матвиенко. Киев: ДИА, 2020. 240 с. ISBN 978-617-7785-10-0. *Ветеринария сегодня.* 2020; 3 (34): 239. DOI: 10.29326/2304-196X-2020-3-34-239.

The information presented in the book "Viral Diseases of Sturgeons and Salmon" meets the requirements for this type of publications and contains current data on the main viral diseases of sturgeons and salmon.

Eighteen chapters of the book are devoted to different viral diseases of fish and two chapters – to methods of laboratory diagnosis, prevention and treatment of sturgeon and salmon viral infections.

The authors were the first to present in detail the world history of studying of sturgeon and salmon viral diseases, and to describe biological characteristics of viruses causing fish diseases both in natural water bodies and in specialized fish farms of Ukraine. The book also gives information on genomes and presents schemes of virus virions structure of different families that cause diseases of aquaculture animals.

The book is well illustrated, and color photographs make it easy to determine the degree of pathological and histological changes in fish as a result of the development of epizootic process caused by a particular representative of the kingdom of *Vira*.

The etiological role of the described pathogens was proved by the results of polymerase chain reaction and philogenetic analysis of nucleotide sequences of viruses isolated from infected fish, including in Ukraine.

The authors presented a scheme of diagnosis of viral diseases of fish, which reflects the biological characteristics of aquaculture animals.

The list of fish cell lines is of great interest, as well as diagnostic test systems for the identification of viral diseases of sturgeons and salmon in the laboratory of fish farming biotechnologies of the Institute of Fisheries of the National Academy of Agrarian Sciences of Ukraine.

In conclusion, the authors provided current data on methods of prevention and treatment of viral diseases of fish. The information about DNA-vaccines and fish vaccination schedules is of great interest for the reader.

The advantage of the book are the references at the end of each chapter, which contain scientific publications both of the authors of this publication, and other researchers, including foreign ones, which indicates the objectivity of the information presented in the monograph.

The book "Viral Diseases of Sturgeons and Salmon" will allow students of biological and veterinary profiles, students of advanced training courses, as well as specialists working in the field of ichthyopathology, to better understand the problems and get acquainted with modern methods of laboratory diagnosis of sturgeon and salmon viral diseases.

The book "Viral Diseases of Sturgeons and Salmon" (by L. P. Buchatsky, Yu. P. Rud, and N. N. Matvienko) should be recommended for specialists of the state veterinary service and veterinary surveillance bodies, as well as for professional education – for students of high schools and various advanced training courses.

World Organisation for Animal Health

Holding of World Rabies Day in the Russian Federation

Budimir Plavsic
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The international health organizations of the Tripartite Alliance – the World Organisation for Animal Health (OIE), the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) – will carry out an awareness-raising campaign in many countries on the occasion of World Rabies Day celebrated by the international community on September 28.

It is planned to organize events in Russia in partnership with national health authorities and scientific institutes in order to strengthen multilateral cooperation for Russia to reach zero human deaths from dog-mediated rabies by 2030, and total eradication in following years.

According to the World Health Organization, rabies kills approximately 60 thousand people worldwide every year. Although human rabies infections are 100% deadly, this disease is 100% preventable. All veterinarians promote and implement vaccination of dogs and cats, sometimes other endangered species in endemic areas, as proven measure to prevent the transmission of virus from wild reservoirs (e.g. red fox) to humans. For total eradication of rabies from some territory it is needed to carry out oral rabies vaccination of wild animals. However, it is needed to go step by step, from adoption of clear, well designed strategy, development of modern legislation and collaboration with several stakeholders. Such coordination on eradication on rabies is efficient too for prevention and control of other zoonotic diseases – including those with pandemic potential.

Since 2015, the WHO, OIE, FAO, and the Global Alliance for Rabies Control (GARC) with Member Countries have been implementing the Global Strategic Plan to end human deaths from dog-mediated rabies by 2030. The plan is based on the One Health approach recognizing the interconnections between human, animal, and environmental health. This partnership, so-called "United Against Rabies collaboration" leverages existing infrastructure, measures and expertise of human, veterinary and wildlife health institutions in a coordinated way to empower, engage and enable countries to save human lives from this preventable disease. As animal bites cause almost all human cases, we can prevent rabies deaths by increasing awareness and knowledge, vaccinating animals to prevent the disease at its source and administering life-saving treatment after people have been bitten by rabid dogs or other animals.

All countries should develop and implement strategic framework to eliminate rabies – based on following pillars:

1. *Preparedness and communication*. Preparedness includes surveillance, detection, control, and eradication activities, based on implementation of dog population management and vaccination programme in domestic animals, supplemented with oral rabies vaccination of wild animals. In addition, communication to public, communities and the authorities, recognising that all players share the responsibility to limit the spread of infection and to eradicate the disease, is of crucial interest.

2. *Surveillance and detection*. Early warning of rabies cases and the ability to closely track the spread of the disease is critical for rapid deployment of resources to contain the spread of the virus. An effective surveillance

and detection system will save lives by enforcing the veterinary authorities to activate the response plans to prevent further cases, carry out control mechanisms including vaccination.

3. *Control and eradication*. The most effective way to protect the country is to keep rabies beyond the borders of the country by mass vaccination of dogs as the major vector for human infection. Mathematical models and practical experiences from some countries show that sustained vaccination coverage of at least 70% of dog populations (including stray dogs) is sufficient to stop the transmission of rabies between dogs, and from dogs to humans. Also, the implementation of large-scale and sustained oral vaccination in the relevant areas of national interest may become the starting point for the gradual elimination of rabies across the country. However, if the neighbouring countries are not implementing similar measures the disease can be easily re-introduced. Therefore, control, progressive elimination, and eradication of rabies at regional level is a more realistic outcome if all the countries in the region have the same approach.

4. *Disease freedom*. In contrast to the previous pillars, this one is focused on changing the strategy from vaccination of companion animals and wildlife to non-vaccination policy with well-organized surveillance and early reporting system. However, this phase is based on continuous awareness maintenance.

OIE COMMUNICATION CAMPAIGN

The OIE encourages joining the social media campaign "Rabies Ends Here" which calls on the dog owners caring about their pets to get them vaccinated against rabies regularly. For this purpose, the World Organisation for Animal Health has developed new communication tools that are available at *https://trello.com/b/66Bqv1ld/world-rabies-day-toolkit*.

FURTHER INFORMATION

OIE Rabies Portal:
https://www.oie.int/en/animal-health-in-the-world/rabies-portal

OIE technical disease card for rabies:
https://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/RABIES_FINAL.pdf

Dog population control programme:
https://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_aw_stray_dog.htm

OIE Collaborating Centres and Reference Laboratories for rabies in Europe:
https://rr-europe.oie.int/en/the-oie-reference-centres-reference-laboratories-and-collaborating-centres/collaborating-centres/?page-nb=1&1828=1920

https://rr-europe.oie.int/en/the-oie-reference-centres-reference-laboratories-and-collaborating-centres/reference-laboratories/?page-nb=1&1830=1966

The list of the countries with a self-declared rabies-free status published by the OIE:
https://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Self-declarations/Archives/ENG_archive_2000_Dec_2019.pdf

Zero by 30. The Global Strategic Plan to end human deaths from dog-mediated rabies by 2030:
https://www.oie.int/en/animal-health-in-the-world/rabies-portal/action-plan

WHO fact sheet on rabies:
https://www.who.int/news-room/fact-sheets/detail/rabies

WHO, FAO and OIE unite in the fight against rabies:
https://www.oie.int/fileadmin/Home/eng/Media_Center/docs/pdf/FAO_OIE_WHO_Rabiesfactsheet.pdf

Food and Agriculture Organization
of the United Nations (FAO)

PRESS RELEASE

Online training helps veterinarians in lumpy skin disease preparedness

9 July 2020,
Budapest, Hungary

To ensure preparedness, early detection, and response against the incursion of any transboundary animal disease, it is paramount to train the first responders – the veterinarians, and as many as possible. With this in mind, FAO offers an electronic training kit in the form of a four-week online course on lumpy skin disease (LSD) preparedness.

Lumpy skin disease is a vector-borne disease in cattle that has been spreading gradually over the past years from Africa, through the Middle East and Turkey, and into the Balkans and Russia. More recently, the disease has made a big jump into Asia, to countries with the highest cattle numbers worldwide, such as Bangladesh, China, and India, and threatening Central Asia and other Asian countries. Never in history has lumpy skin disease such a wide geographic distribution.

The disease comes with high economic costs, considerable trade disruptions, and serious impacts on local livelihoods. Moreover, the disease is completely new to some countries, meaning that farmers and veterinary services have never seen or fought it before.

FAO has already developed in the past standardized training materials (presentations, guidelines, etc.), which can be easily translated and adapted to countries specifics, and rolled out quickly to reach most national veterinarians through the training-of-trainer (or cascade) approach. This was already implemented in North Macedonia (2017) and Belarus, Moldova and Ukraine (2018).

"Recently, we've realized that, even with continuous trainings, we cannot cover everyone; so to meet the

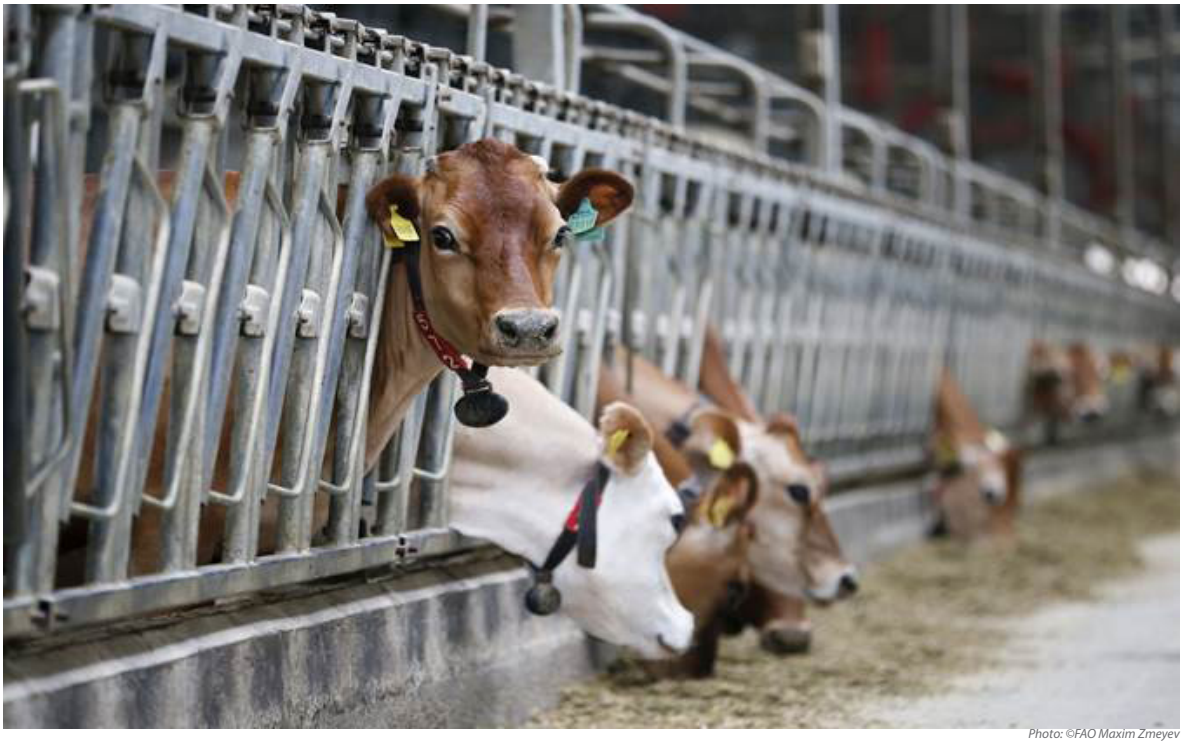

Photo: ©FAO Maxim Zmeyev

rising demand of both infected and at-risk countries for lumpy skin disease training, we have switched to online," said Daniel Beltran-Alcrudo, FAO animal health officer. He pointed out the "obvious advantages" of online trainings – a format that is easy, requiring only a stable internet connection, and cost-effective to scale up, reaching people in remote locations who can then learn at their own pace. Online training has now proven more relevant than ever in these times of COVID-19-related restrictions to travelling and face-to-face meetings.

The current pilot course has been co-organized by FAO and the European Commission for the Control of Foot-and-Mouth Disease (EuFMD), under the umbrella of the Global Framework for the Progressive Control of Transboundary Animal Diseases (GF-TADs). The course materials have been developed jointly by a team of experts from FAO and the Friedrich-Loeffler-Institute in Germany.

The main goal of the pilot is to elicit experts' feedback to further improve the course. Participants include invited representatives of veterinary services from 44 countries, all the way from Western Europe to East Asia. There are also international agencies, and vaccine manufacturers, as well as lumpy skin disease experts, enrolled in the course for a total of 282 participants.

A Russian version of the course is planned for the second half of the year.

ABOUT THE COURSE

The 10-hour tutored course has six training modules that cover a range of topics, such as the overview of the disease, clinical and pathological diagnosis, sampling and laboratory diagnosis, epidemiology and outbreak investigation, surveillance, and control and eradication. The course starts with an introductory webinar on the training and the trainers through short technical presentation. Trainers are experts in either diagnostics, epidemiology, or the control of disease. Each participant can access the training material, discussion forum, recordings of the webinars, and a list of further resources.

Each week during the four-week period, a specific topic will be in focus for the participants and trainers to interact on in the discussion forum. Interactions are further facilitated by the trainers' questions.

The course ends with a final course assessment and a closing webinar covering topics that proved to be difficult or have sparked the most discussion in the forum. Finally, through the feedback section, participants can rate the course content and provide suggestions for further improvement.

LINKS

Vets from the Balkans and Eastern Europe discuss disease risk communication and outbreak management:
http://www.fao.org/europe/news/detail-news/en/c/1271970/?utm_source=press%20release&utm_medium=email&utm_campaign=fao

Predicting the areas at risk for lumpy skin disease (LSD) spread within Eurasia:
http://www.fao.org/ag/againfo/programmes/en/empres/news_080219.html?utm_source=press%20release&utm_medium=email&utm_campaign=fao

Train of trainers program for field veterinarians on LSD in Moldova (TCP/RER/3605):
http://www.fao.org/ag/againfo/programmes/en/empres/news_080219.html?utm_source=press%20release&utm_medium=email&utm_campaign=fao

Cascade training to prepare field veterinarians in Eastern Europe against lumpy skin disease (LSD):
http://www.fao.org/ag/againfo/programmes/en/empres/news_260618b.html?utm_source=press%20release&utm_medium=email&utm_campaign=fao

RELATED PUBLICATIONS

Lumpy skin disease – A field manual for veterinarians:
http://www.fao.org/documents/card/en/c/1fcf63b0-80e9-4f8e-825f-10ea6e998479/

Sustainable prevention, control and elimination of Lumpy Skin Disease – Eastern Europe and the Balkans:
http://www.fao.org/3/a-i7827e.pdf

Spatial analysis of lumpy skin disease (LSD) in Eurasia – Predicting areas at risk for further spread within the region:
https://doi.org/10.1111/tbed.13090

Editable leaflets to allow quick awareness against priority transboundary animal diseases:
http://www.fao.org/index.php?id=94206&utm_source=press%20release&utm_medium=email&utm_campaign=fao

In memory of Vitaly Alexandrovich Sergeev

On June 14, 2020, the honored Russian scientist, Doctor of Science (Biology), Professor Vitaly Sergeev passed away.

Vitaly Alexandrovich Sergeev was born in the village of Bueraki, Sengileyevsky Raion, Ulyanovsk Oblast. He graduated with honors from Moscow Veterinary Academy (1945–1949), where he began his scientific activities while still a student. His scientific work at that time was awarded with the prize of the Ministry of Higher and Secondary Special Education of the USSR and a Certificate of Honor.

From 1949 to 1956 V. A. Sergeev worked in the field of microbiology at the All-Union Institute of Experimental Veterinary Medicine (VIEV, Moscow). From 1956 V. A. Sergeev started working in virology. From 1960, he headed laboratories at the following institutes: the All-Russian Scientific Research Institute of Veterinary Virology and Microbiology (1960–1979), the Institute of Medico-Biological Problems, Ministry of Health of the USSR (1980–1981), the VIEV (1982–1989), the Institute of Veterinary Medicine in Kiev (1990–1993). From 1994 V. A. Sergeev started to work as a scientific advisor at the Scientific Production Association NARVAC (Moscow).

Professor V. A. Sergeev was a major scientist in the field of general and veterinary virology and specific prevention of animal viral diseases, his name is widely known to national and foreign experts. He made a significant contribution to the theory and practice of a new scientific direction – biotechnology of manufacturing antiviral drugs, and was deservedly recognized in this field of science.

He proposed a number of original and valuable solutions, protected by 35 inventor's certificates, which were awarded with four gold, three silver and one bronze medals of the VDNKh of the USSR. Professor V. A. Sergeev was the author of more than 350 scientific papers. The results of the author's long-term research on cell and virus cultivation, on virus structure and biology are presented in five monographs: "Reproduction of animal viruses in tissue culture", "Reproduction and cultivation of animal viruses", "Structure and biology of animal viruses", "Cell culture in veterinary medicine and biotechnology", "Viral vaccines". Two monographs published in 1976 and 1983 were presented at international book fairs and were highly praised.

Under the guidance of professor V. A. Sergeev, 60 candidate and doctoral theses were completed, and a scientific school which trains highly qualified specialists in the field of general and veterinary virology was created. V. A. Sergeev was awarded the medals "For the victory over Germany in the Great Patriotic War of 1941–1945" and "For labor valor". A characteristic feature of his scientific activity was its high scientific, methodological and organizational

level, purposefulness, close connection with practice, implementation of research results in the national economy.

Vitaly A. Sergeev will remain forever in our memory as a great scientist in the field of biotechnology, the development of specific prevention tools and methods for animal disease diagnosis. He was a shining example of a scientist with inexhaustible energy, grand ideas, an advocate of professional achievements based on extensive knowledge combined with unique analytical abilities, a strategist in the field of theory and practice of infectious animal disease prevention, a respected mentor, a role model of mutual assistance and understanding among colleagues and co-workers.

Eternal memory to Vitaly Alexandrovich Sergeev, a talented scientist, a respected colleague, a bright and kind person!

Administration,
FGBI "ARRIAH" staff members

GENERAL REQUIREMENTS FOR SUBMITTED PAPERS

- Papers in two languages – Russian and English – that comprise results of own scientific studies, being up to 6–8 pages (up to 10 pages for reviews) but at least 5 pages (single-spaced, size 12) are accepted for publication. Optimal paper size: **3,000–6,000 words.**
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STRUCTURE OF PRESENTED PAPER

1. **UDC**
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4. **Summary** (brief and accurate outline of the paper containing data and conclusions of described works): 200–250 words, not more than 2,000 characters.
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7. **For citing**
8. **Conflict of interest**
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10. **Introduction**
11. **Materials and methods**
12. **Results and Discussion**
13. **Conclusion**
14. **References** (Vancouver style – sequentially numbered list of references in the order of their citing (appearing in the text).
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The paper should be presented as follows: Microsoft Word, font Times New Roman, font size – 12, line spacing – 1, margins – 2 cm, first line indent – 1 cm, text alignment – justified text.

Figures, tables, diagrams, graphs, etc. shall be numerated, have indicated sources and fit to page printing. Table name should be given under the table; figure/graph name – under the figure/graph.

Paper originals and copies are not returned. Authors shall guarantee that submitted materials have not been published before. Compliance with all above-said requirements of the editorial board is essential for paper acceptance for publication in Veterinary Science Today journal.

PLEASE CONTACT OUR EDITOR OFFICE FOR DETAILED CONDITIONS OF PUBLICATION

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FGBI "FEDERAL CENTRE FOR ANIMAL HEALTH"
(FGBI "ARRIAH")
ФГБУ «ФЕДЕРАЛЬНЫЙ ЦЕНТР ОХРАНЫ ЗДОРОВЬЯ ЖИВОТНЫХ»

OIE REGIONAL REFERENCE LABORATORY FOR FOOT AND MOUTH DISEASE
РЕГИОНАЛЬНАЯ РЕФЕРЕНТНАЯ ЛАБОРАТОРИЯ МЭБ ПО ЯЩУРУ

OIE REFERENCE LABORATORY FOR HIGHLY PATHOGENIC AVIAN INFLUENZA AND LOW PATHOGENIC AVIAN INFLUENZA (POULTRY) AND NEWCASTLE DISEASE
РЕФЕРЕНТНАЯ ЛАБОРАТОРИЯ МЭБ ПО ВЫСОКОПАТОГЕННОМУ И НИЗКОПАТОГЕННОМУ ГРИППУ ПТИЦ И НЬЮКАСЛСКОЙ БОЛЕЗНИ

The researchers of the Federal Centre for Animal Health (FGBI "ARRIAH") have developed a novel anti-rabies vaccine. The product is intended for vaccination of farm animals as well as for vaccination of cats and dogs.

- The vaccine induces strong and long-lasting immune response against rabies in 21 days post single administration. Post the immunization with the developed vaccine, the level of the virus neutralizing antibodies is two-three-fold higher as compared to the levels induced by the sorbate anti-rabies vaccine.
- ARRIAH-RABIVAC vaccine induces stable immunity in animals that lasts for over a year.
- The vaccine is low-reactogenic, i.e. it does not cause any side effects. No systemic or local reactions have been reported in target animals post the vaccine administration.
- ARRIAH-RABIVAC vaccine is recommended for rabies prevention in farm animals. The scientists emphasize that meat and milk from the vaccinated animals can be used without any restrictions.
- The recommended administration dose of the new vaccine is lower as compared to the sorbate products.

ARRIAH-RABIVAC
INACTIVATED EMULSION CULTURE RABIES VACCINE

Intended use:

The vaccine is intended for rabies prevention and emergency anti-rabies immunization of cattle and small ruminants, pigs, horses, camels, dogs and cats.

Composition:

Dosage form – emulsion for injections. The vaccine is formulated from the inactivated rabies virus strain ARRIAH (similar to strain Schelkovo-51), cultivated in BHK-21 cell suspension, inactivated with aminoethyl ethyleneimin and emulsified with oil adjuvant.

Biological properties:

The vaccine induces immune response against rabies virus in 21 days post single administration. The immunity duration is 12 months.

Usage:

Do not vaccinate suspected rabid animals.

Preventive vaccination of animals against rabies shall be performed once; the animals shall be revaccinated in a year. Cattle and small ruminants, horses and pigs shall be immunized at the age of 3 months or older, dogs and cats – at the age of 2 months or older.

Animals shall be emergently vaccinated not later than 48 hours after their possible exposure. The vaccine shall be aseptically administered twice with a 7-day interval at the doses specified below.

Thoroughly shake the vaccine bottles before use. Treat the injection site with 70% ethanol.

All target animal species (cattle, small ruminants, horses, camels, pigs, dogs, cats) shall be vaccinated intramuscularly at the following doses:

– horses, cattle, camels, pigs – 2 cm^3;

– sheep and goats, large and medium-sized adult dogs – 1 cm^3;

– cats, puppies of all dog breeds at the age of 2 months or older, small-sized adult dogs – 0.5 cm^3.

Product description:

The vaccine is filled in the appropriate glass or plastic bottles at 1, 4, 10, 20, 50, 100 and 200 cm^3. The bottles are stoppered with rubber stoppers and sealed with aluminum caps.

Shelf-life and storage conditions:

The vaccine shelf-life is 24 months from the production date provided the storage and transportation conditions are met. Do not use the expired vaccine. The vaccine shall be used within 10 hours after opening the bottle. The vaccine shall be stored and transported in a dry dark place at 2–8 °C within the shelf-life. The vaccine may be transported in the manufacturer's package at not above 20 °C for maximum 5 days. Do not freeze the vaccine.

Rabies is an acute infectious disease caused by the virus of the genus Lyssavirus in the family Rhabdoviridae. The disease results in rabies-specific encephalitis (inflammation of the brain) in animals and humans. The disease is transmitted with the saliva of the diseased animal through a bite or contact with damaged skin or mucosa.

To buy FGBI "ARRIAH" manufactured veterinary products please contact +7(4922)52-99-24, +7(4922)26-15-25.