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«ВЕТЕРИНАРИЯ СЕГОДНЯ»
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Serological monitoring of avian influenza and Newcastle disease in the Russian Federation in 2019

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SUMMARY
More than 30,000 samples of blood serum from domestic, wild and synanthropic birds from 50 regions of the Russian Federation were submitted to the FGBI "ARRIAH" (Vladimir) Reference Laboratory for Avian Viral Diseases to be tested for avian influenza and Newcastle disease within the framework of monitoring activities conducted by the Rosselkhoznadzor in 2019. As a result of the laboratory diagnosis, antibodies to type A influenza virus were detected in vaccinated chickens from two poultry farms in the Perm and Primorsky Krais (A/N9). The detected antibodies were specific to the haemagglutinin subtype of the vaccine antigen. As for the backyards in the RF Subjects, where scheduled vaccination against avian influenza A/H5 is carried out, a low level of immunity was seen in the Rostov and Astrakhan Oblasts (35 and 44%, respectively) while a high level of immunity was observed in the Republic of Altai, Krasnodar Krai, the Chechen Republic and the Primorsky Krai (69, 78, 80 and 88%, respectively). High seroprevalence of Newcastle disease virus in adult poultry in indoor holdings was associated with mass vaccination against the disease. In broiler chickens, post-vaccination antibodies were observed, on average, in 42% of the studied blood serum samples. Antibodies to the Newcastle disease virus were detected in 39% of samples from backyard chickens. Seroprevalence in wild and synanthropic birds was high. The obtained results suggest that the risk of introduction and spread of avian influenza and Newcastle disease in industrial poultry farms and in backyards remains.

Key words: avian influenza, Newcastle disease, epizootology, monitoring, poultry, wild birds.

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Серологический мониторинг гриппа птиц и ньюкаслской болезни в Российской Федерации в 2019 году

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РЕЗЮМЕ
В рамках мониторинговых мероприятий, проводимых Россельхознадзором, в референтную лабораторию вирусных болезней птиц ФГБУ «ВНИИЗЖ» (г. Владимир) в течение 2019 года было доставлено более 30 000 проб сыворотки крови от домашних, диких и синантропных птиц из 50 регионов

Российской Федерации для исследования на грипп птиц и ньюкаслскую болезнь. В результате лабораторной диагностики антитела к вирусу гриппа типа А были выявлены у вакцинированных кур из двух птицеводческих предприятий Пермского и Приморского краев (A/H9). Выявленные антитела были специфичны вакцинному антигену по подтипу гемагглютинина. В личных подсобных хозяйствах граждан субъектов Российской Федерации, где проводится плановая вакцинопрофилактика гриппа птиц A/H5, установлен низкий уровень иммунитета в Ростовской и Астраханской областях (35 и 44% соответственно) и высокий уровень – в Республике Алтай, Краснодарском крае, Чеченской Республике и Приморском крае (69, 78, 80 и 88% соответственно). Высокая серопревалентность ньюкаслской болезни была установлена для взрослой птицы в промышленных хозяйствах закрытого типа, что связано с массовой вакцинацией против данного заболевания. У цыплят-бройлеров отмечали наличие поствакцинальных антител в среднем в 42% исследованных проб сыворотки крови. В 39% проб от кур из личных подсобных хозяйств были обнаружены антитела к вирусу ньюкаслской болезни. Серопревалентность у диких и синантропных птиц была высокой. Полученные результаты свидетельствуют о сохранении риска заноса и распространения гриппа птиц и ньюкаслской болезни в промышленных птицеводческих хозяйствах и личных подсобных хозяйствах граждан.

Ключевые слова: грипп птиц, ньюкаслская болезнь, эпизоотология, мониторинг, домашняя птица, дикая птица.

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INTRODUCTION

Highly pathogenic avian influenza (HPAI) and Newcastle disease (ND) are the World Organisation for Animal Health (OIE) listed diseases that are subject to mandatory notification when detected in the territory of the country.

In poultry, these diseases can cause damage to vital organs and the subsequent death of all the unvaccinated flock, thus, leading to large economic losses to poultry farms [1–3].

Newcastle disease has become widespread in many parts of the world [4–7]. In 2019, Newcastle disease outbreaks notified to the OIE occurred in the United States, Mexico, Israel, Kazakhstan, and Russia. According to the immediate notifications made by the Veterinary Services of the Russian Federation, ND outbreaks were reported in 9 regions of the country (18 affected areas) in 2019. Newcastle disease epidemics caused by Newcastle disease virus (NDV) subgenotype VII(L) were large-scale in terms of their geographical distribution (Krasnodar Krai, Chechen Republic, Stavropol Krai, Primorsky Krai, Saratov oblast, Zabaykalsky Krai, Altai Krai, Omsk and Kursk oblasts) and long-term (from January to December 2019).

Recently, there has been a steady decline in the number of countries reporting HPAI outbreaks. According to the OIE, in 2018 and 2019, a total of 33 and 20 countries, respectively, reported on HPAI outbreaks. HPAI was mainly caused by a highly virulent HPAI virus of the H5 subtype with various neuraminidases, with H5N1 and H5N8 subtypes being the most prevalent [8–11].

According to the immediate notifications received from the Veterinary Services of the Subjects of the Russian Federation, in 2018, HPAI virus of the H5 subtype was detected in poultry flocks in 15 regions (more than 80 cases), in 2019 – only in the Rostov oblast (2 cases), which was reported to the OIE.

However, since the beginning of 2020, the opposite trend demonstrating the increased HPAI activity has been observed. In January 2020, the OIE received reports on HPAI outbreaks from 12 countries (China, Germany, Romania, Slovakia, Ukraine, Czech Republic, Hungary, Poland, Taiwan, Saudi Arabia, Israel and Vietnam). Since a number of countries has common borders with the Russian Federation, and Russia is involved in trade relations with other countries, there is a real risk of disease introduction into the territory of the Russian Federation.

The necessity of monitoring studies on avian influenza (AI) and Newcastle disease is determined by the risk of introduction of new virus strains into the country, the risk of pathogen introduction into commercial poultry farms, the emergence of epidemics that lead to great economic losses [8, 9, 12, 13].

This paper presents the results of the serological monitoring of Newcastle disease and avian influenza in poultry, synanthropic and wild birds conducted in the Russian Federation in 2019 within the framework of the Rosselkhoznadzor diagnostic and preventive measures for highly dangerous animal diseases, aimed at protecting the territory of the Russian Federation against the introduction of animal diseases from abroad and against their further spread.

MATERIALS AND METHODS

Biological material (serum samples) was provided for the study by the Rosselkhoznadzor Territorial Administrations.

The study was carried out using commercial kits for the detection of antibodies to Newcastle disease and avian influenza viruses, produced by the FGBI "ARRIAH", according to the manufacturer's instructions and diagnostic materials (avian influenza virus antigens of subtypes H5, H7 and H9 and homologous sera) produced by GD (the Netherlands) and IZSVe (Italy) according to the standard method [14]. Enzyme-linked immunosorbent assay (ELISA) Kits were used to test sera from chicken and turkey for avian influenza and from chicken for ND; haemagglutination inhibition (HI) kits were used to test poultry sera (from chicken, turkey, duck, goose, quail and pheasant) as well as sera of wild and synanthropic birds. The received

serum samples were inactivated by heating at 56 °C for 30 min. HI test results were considered positive if the serum titer was ≥ 4.0 $\log_2$ for avian influenza and ≥ 3.0 $\log_2$ for Newcastle disease.

RESULTS AND DISCUSSION

As part of the State epidemic monitoring (Rosselkhoznadzor Order No. 1519 of 28.12.2018 "On laboratory tests within the Rosselkhoznadzor activities for ensuring compliance with the World Trade Organization (WTO) Sanitary and phytosanitary (SPS) Agreement requirements upon Russia accession to the WTO for 2020"), in 2019, the poultry sera (28,262 samples) received from 49 regions of the Russian Federation were tested for the presence of antibodies to the Newcastle disease virus by HI test and ELISA. The total of 20,374 samples of poultry serum (from chicken, turkey, quail and goose) were delivered from 139 commercial poultry farms located in 41 regions of the Russian Federation (Fig. 1).

1,315 serum samples from young chicken in commercial and parent flocks (up to 100 days old) were tested, and Newcastle disease virus specific antibodies were detected in 936 samples. The percentage of seropositive poultry in the commercial and parent flocks was minimal – 48% – in the Central (CFD) and maximum – 98% – in the Southern (SFD) Federal Districts. Up to 60% of positive samples were found in poultry from the Siberian (SiFD) and the Far Eastern (FEFD) Federal Districts. In the Privolzhsky (PFD), the Ural (UFD), and the Northwestern (NWFD) Districts the number of positive samples ranged from 87 to 92%.

The study of 13,389 serum samples collected from adult poultry in all the Federal Districts of the Russian Federation revealed 11,749 positive results. The lowest number of positive samples (49%) was observed in the Northwestern Federal District. In other districts, the number of positive samples ranged from 79% in the Privolzhsky Federal District (PFD) to 100% in the Ural Federal District (UFD).

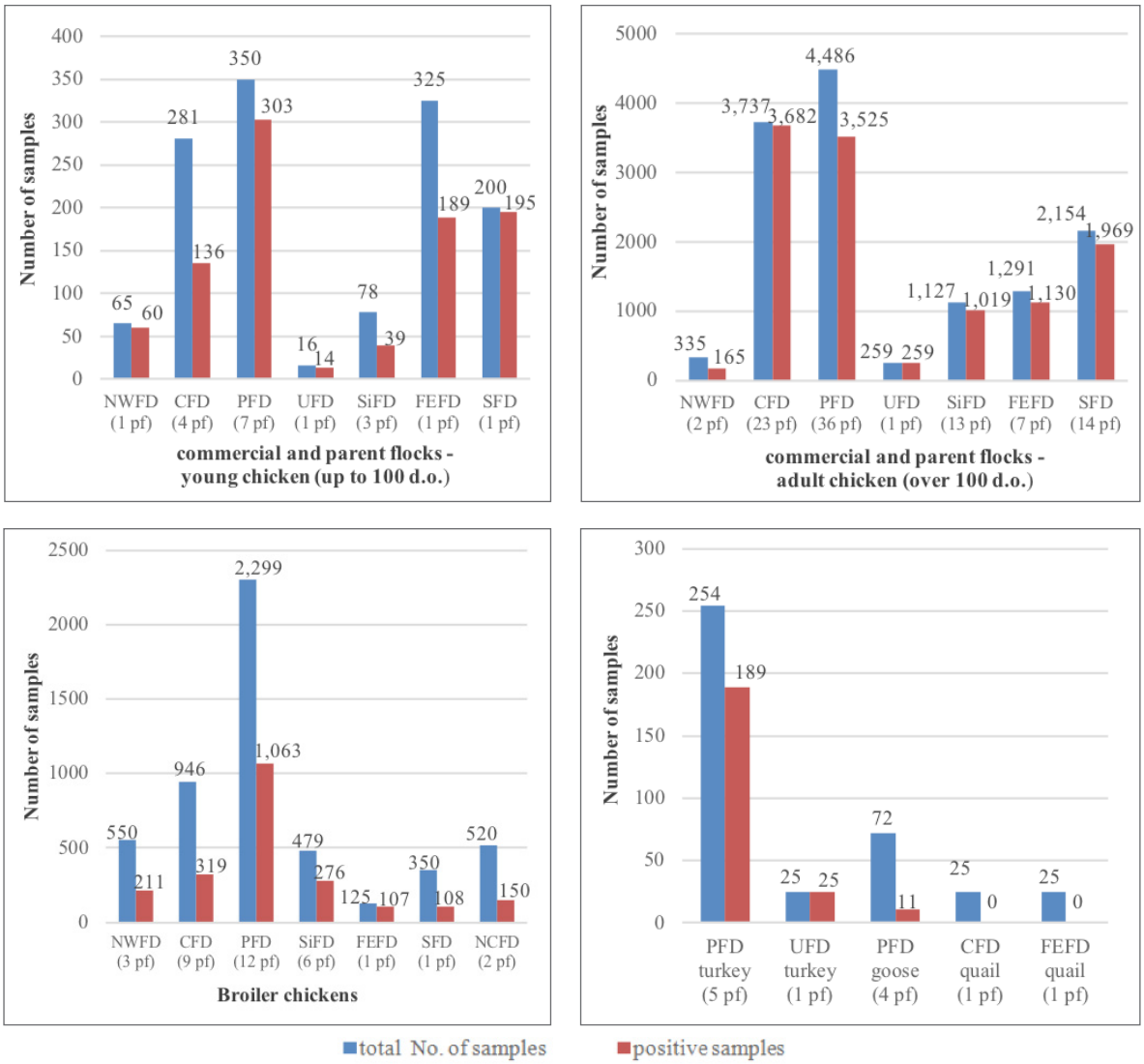

Fig. 1. Detection of antibodies to NDV in poultry sera, submitted from Russian industrial poultry farms, using HI test and ELISA.
The number of poultry farms from which samples were delivered is shown in parentheses.

Рис. 1. Результаты исследований сывороток крови птиц из промышленных птицеводческих предприятий РФ в РТГА и ИФА на наличие антител к вирусу ньюкаслской болезни.
В скобках указано количество птицеводческих хозяйств, из которых доставляли пробы.

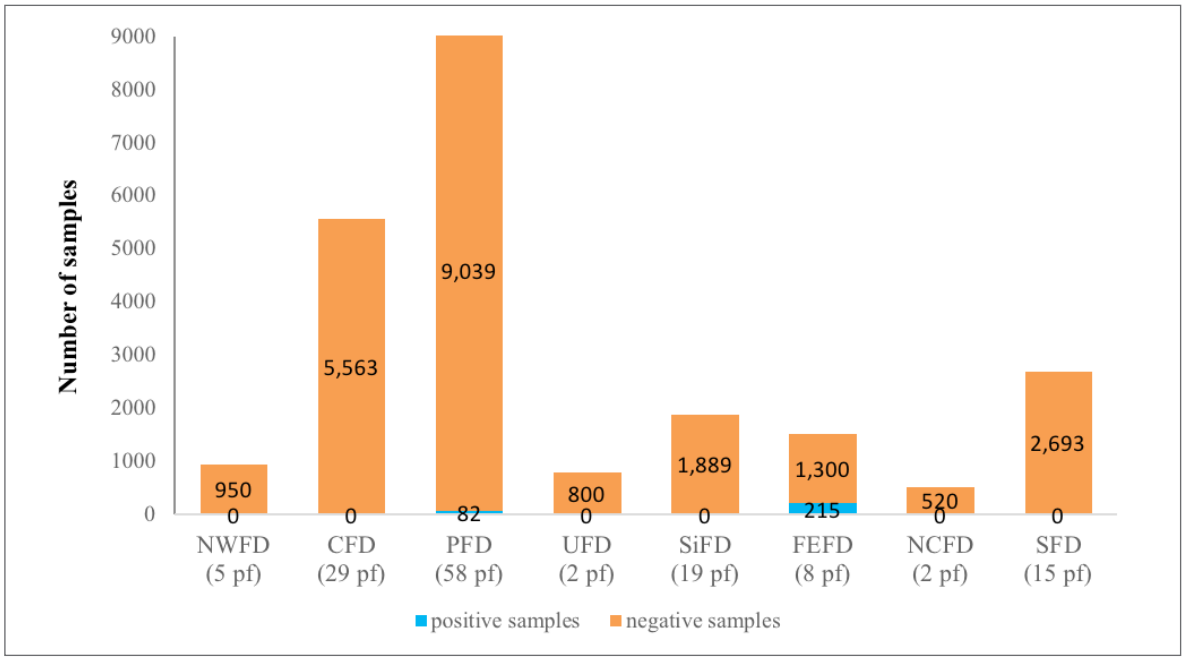

Fig. 2. Detection of antibodies to AI virus in poultry sera, submitted from Russian industrial poultry farms, using ELISA and HI test in 2019.
The number of poultry farms from which samples were delivered is shown in parentheses.

Рис. 2. Выявление антител к вирусу гриппа птиц в сыворотках крови птиц из промышленных птицеводческих хозяйств РФ в ИФА и РТГА в 2019 г.
В скобках указано количество птицеводческих хозяйств, из которых доставляли пробы.

The study of 5,269 serum samples collected from broiler chicken in 7 Federal Districts of the Russian Federation revealed the presence of Newcastle disease virus specific antibodies in 2,234 samples. The lowest number of positive samples was found on the farms of the North Caucasus Federal District (NCFD) (29%), and the highest number – in the Far Eastern Federal District (86%), in the remaining districts the number of positive samples ranged from 31% to 58%. A relatively low average percentage of positive samples in broiler chicken can be explained by the fact that different farms used different vaccination schedules, and that the level of post-vaccination antibodies was not always high enough by the time of blood sampling (at 30–45 days of age).

In the study of serum samples collected from turkey in the PFD and UFD, antibodies to the Newcastle disease virus were detected in 74% and 100% of samples from vaccinated birds, respectively. No antibodies to the Newcastle disease virus were found in the quail sera. When studying goose sera, specific antibodies were detected in birds from two regions – the Republic of Mari El and the Republic of Tatarstan.

According to the information available in the accompanying documents, the antibodies detected in serum samples collected from poultry on commercial poultry farms were induced by the Newcastle disease virus vaccine strains contained in live or inactivated vaccines. On some of the farms vaccination did not result in a satisfactory antibody response in broiler chickens.

The main goal of serological monitoring studies on influenza type A in poultry is the control of the infection in unvaccinated flocks, as well as the control of post-vaccination immunity in vaccinated flocks.

In 2019, 22,754 poultry serum samples (from chicken, turkey, duck, goose, quail) were delivered from 138 commercial poultry farms, located in 41 regions and 8 Federal Districts of the Russian Federation, to be tested for avian influenza (Fig. 2).

Antibodies to the avian influenza virus were found in chicken from one of the farms in the Perm Krai (subtype H9) and in chicken from two poultry farms in the Primorsky Krai (also subtype H9). Vaccination against avian influenza was carried out on all the commercial farms. The detected antibodies were specific to the vaccine antigen by the hemagglutinin subtype. No avian influenza virus antibodies were detected in other poultry species.

In 2019, 8,699 poultry serum samples (from chicken, turkey, goose, duck, quail, pheasant) received from backyards and small scale farms located in 29 regions of the Russian Federation were tested for avian influenza (Tables 1 and 2).

Avian influenza virus specific antibodies were detected only in the samples collected from chicken vaccinated against influenza; samples from other poultry species showed negative results (Tables 1 and 2).

According to the accompanying documents, vaccination against avian influenza was carried out using inactivated vaccines containing the antigen of the homologous influenza virus A/H5. The number of positive results in vaccinated flocks in various regions ranged from 35% to 80%. Low levels of post-vaccination antibodies were observed in the Rostov and Astrakhan oblasts (35 and 44%, respectively), and high levels (69, 78, 80, 88%) – in the Republic of Altai, Krasnodar Krai, Chechen Republic and Primorsky Krai, respectively.

In 2019, 7,888 poultry serum samples received from backyards and small scale farms located in 27 regions in 7 Federal Districts of the Russian Federation were tested for antibodies to the Newcastle disease virus (Tables 1 and 2).

Table 1
Detection of antibodies to ND and AI viruses in poultry sera, submitted from backyards and small scale farms in the Russian Federation, using ELISA and HI test in 2019

Таблица 1
Выявление антител к вирусам ньюкаслской болезни и гриппа птиц в сыворотках крови домашних птиц из ЛПХ и КФХ РФ в ИФА и РТГА в 2019 г.

Federal district	Subject of the Russian Federation	Number of tested samples			
		ND		AI	
		total	positive	total	positive
NWFD	Murmansk oblast	n/t	n/t	10	0
CFD	Vladimir oblast	444	151	397	0
	Bryansk oblast	55	24	55	0
	Kursk oblast*	70	30	70	0
	Oryol oblast	191	7	148	1
	Smolensk oblast	76	32	76	0
PFD	Republic of Tatarstan	287	85	389	0
	Republic Of Mari El	n/t	n/t	75	0
	Republic of Mordovia	36	0	36	0
	Samara oblast	50	50	50	0
UFD	Tyumen oblast	325	12	325	0
SiFD	Omsk oblast*	1	1	1	0
	Altai Republic	100	0	139	69
	Republic of Tyva	155	46	155	0
	Irkutsk oblast	31	0	31	0
FEFD	Zabaikalsky Krai*	362	50	362	0
	Amur oblast	475	1	575	0
	Khabarovsk Krai	350	41	350	0
	Primorsky Krai*	300	300	600	264
SFD	Astrakhan oblast	200	39	400	106
	Volgograd oblast	300	144	300	0
	Krasnodar Krai*	100	100	100	78
	Republic of Adygeya	173	140	173	0
	Rostov oblast	1,914	459	2,092	245
NCFD	Republic of Ingushetia	400	265	400	0
	Republic of Dagestan	200	88	200	0
	Stavropol Krai*	510	233	520	3
	Karachay-Cherkess Republic	200	128	200	0
	Chechen Republic*	583	339	470	374

n/t – not tested (пробы не исследовали);
* Regions, where ND outbreaks were reported in 2019 (according to the data received from the Veterinary Services).
* Регионы, в которых, по данным ветеринарных служб в 2019 г., были зарегистрированы вспышки ньюкаслской болезни.

Newcastle disease virus-specific antibodies were detected in three poultry species: chicken, turkey and goose. Antibodies to the Newcastle disease virus were detected in 39% of all the chicken serum samples tested in the Russian Federation. No specific antibodies were detected in unvaccinated chicken in three regions – the Republic of Mordovia, the Irkutsk oblast, and the Republic of Altai. According to the accompanying documents, chicken for backyards were purchased mainly from commercial poultry farms, where they had been vaccinated against Newcastle disease, however, after that they were not vaccinated anymore. The majority of small scale farms carried out re-vaccination of poultry against Newcastle disease. According to the accompanying documents, antibodies to the Newcastle disease virus were detected in 65% of the tested samples taken from vaccinated chicken. The low percentage of positive results may be indicative of insufficient effectiveness of the preventive vaccination against Newcastle disease carried out on small scale farms and in backyards in the studied regions.

In vaccinated turkeys, the antibodies were found in 100% of the tested samples received from the Samara oblast and in one sample from a small scale farm in the Amur oblast. No antibodies were found in serum samples from non-vaccinated turkeys received from the Rostov oblast and the Zabaikalsky Krai. Goose serum samples (133 samples) were delivered from 7 regions and 6 Federal Districts. Antibodies to the Newcastle disease virus were found in 3 samples from the Republic of Tatarstan.

Many bird species are natural reservoirs and carriers of infectious diseases, therefore, constant monitoring of epidemic situation in wild birds shall be one of the components in the system for the prediction, control and prevention of diseases, including such highly dangerous viral diseases as avian influenza and Newcastle disease.

Table 3 presents the HI test results for sera from wild and synanthropic birds collected in 2019.

No antibodies to avian influenza virus of H5 and H7 subtypes were found in the study of 236 serum samples collected from wild and synanthropic birds in 6 Subjects of the Russian Federation.

Antibodies to the Newcastle disease virus were detected in 153 out of 255 samples received from 4 Subjects of the Russian Federation, in 123 samples from synanthropic birds (crows and pigeons) and in 30 samples from wild birds. In most cases, positive samples from pigeons were collected near large poultry farms, which poses a risk of disease introduction into the poultry farms.

Figure 3 shows the location of the RF regions where antibodies to avian influenza and Newcastle disease viruses were detected in poultry and wild birds.

Of the 50 regions represented in the monitoring studies, Newcastle disease and avian influenza viruses specific antibodies were not detected in two oblasts (the Murmansk and the Arkhangelsk oblast).

It should be noted that the serological study conducted in the Russian Federation in 2019 did not show the presence of antibodies to avian influenza virus in unvaccinated poultry, while the studies conducted in 2017–2018 showed the presence of post-infectious antibodies to influenza A/H5 virus in serum samples from birds in the Altai Krai, the Rostov and Kaliningrad oblasts, and to influenza A/H9 virus in serum samples from birds in backyards and on two poultry farms in the Primorsky Krai. Analysis of the serological monitoring data collected over the past three

years showed a continuous unstable situation regarding Newcastle disease in the Russian Federation, because, despite the high seropositivity of poultry on commercial poultry farms, inadequate level of protection on small scale farms due to the absence of mass vaccination poses a constant threat of the disease occurrence.

CONCLUSION

Thus, the results of the serological studies on Newcastle disease conducted in the framework of the State epidemic monitoring in 2019 showed a high level of positive results in birds on commercial poultry farms, associated with mass vaccination against this disease, and insufficient protection of poultry against Newcastle disease virus on small scale farms, which is confirmed by the reporting of primary disease outbreaks, caused by the virulent virus strain, specifically in poultry on small scale farms. Seroprevalence of Newcastle disease in wild and synanthropic birds, which are the most likely natural reservoir of Newcastle disease virus strains with different pathogenicity, was high in some regions of the Russian Federation.

The results of the monitoring studies on avian influenza showed, in general, the absence of avian influenza virus specific antibodies in the studied unvaccinated poultry on commercial poultry farms and in backyards and a low level of post-vaccinal immunity to avian influenza on small scale farms in certain regions of the Russian Federation.

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Table 2

Detection of antibodies to ND and AI viruses in sera collected from different poultry species in backyards and small scale farms in the Russian Federation in 2019

Таблица 2

Выявление антител к вирусу ньюкаслской болезни и гриппа птиц в сыворотках крови разных видов птиц из ЛПХ и КФХ РФ в 2019 г.

Bird species	Number of tested samples			
	ND		AI	
	total	positive	total	positive
chicken	7,020	2,711	7,572	1,140
turkey	580	51	711	0
duck	155	0	186	0
goose	133	3	212	0
quail and pheasant	n/t	n/t	18	0
Total	7,888	2,765	8,699	1,140

n/t – not tested (пробы не исследовали).

Table 3

Detection of antibodies to ND virus and AI viruses in sera from wild and synanthropic birds using HI test

Таблица 3

Результаты исследования в РТГА проб сыворотки крови диких и синантропных птиц на наличие антител к вирусам ньюкаслской болезни и гриппа птиц

Subject of the Russian Federation	Bird species	Number of tested samples / number of positive samples		
		ND	HPAI H5	HPAI H7
Yaroslavl oblast	field and forest birds (capercaillie, black grouse, woodcock)	10/10 (100%)*	10/0	10/0
	waterfowl (wild ducks and geese)	27/20 (74%)	27/0	27/0
	synanthropic birds	12/12 (100%)	12/0	12/0
Samara oblast	synanthropic birds (pigeons)	30/0	30/0	30/0
Republic of Mordovia	synanthropic birds (crows, pigeons)	112/88 (79%)	112/0	112/0
	field and forest birds (lark, starling, rook)	10/0	10/0	10/0
Republic of Tatarstan	synanthropic birds (pigeons)	14/12 (86%)	n/t	n/t
Krasnoyarsk Krai	synanthropic birds (pigeons)	30/11 (37%)	25/0	25/0
Republic of Tyva	semiaquatic birds (great crested grebe, dabchick, herring gull, kite)	10/0	10/0	10/0
Total		255/153 (60%)	236/0	236/0

n/t – not tested (не исследовали);
* Positive sample percentage of the total number of samples tested.
* В скобках указан процент положительных от общего количества исследованных проб.

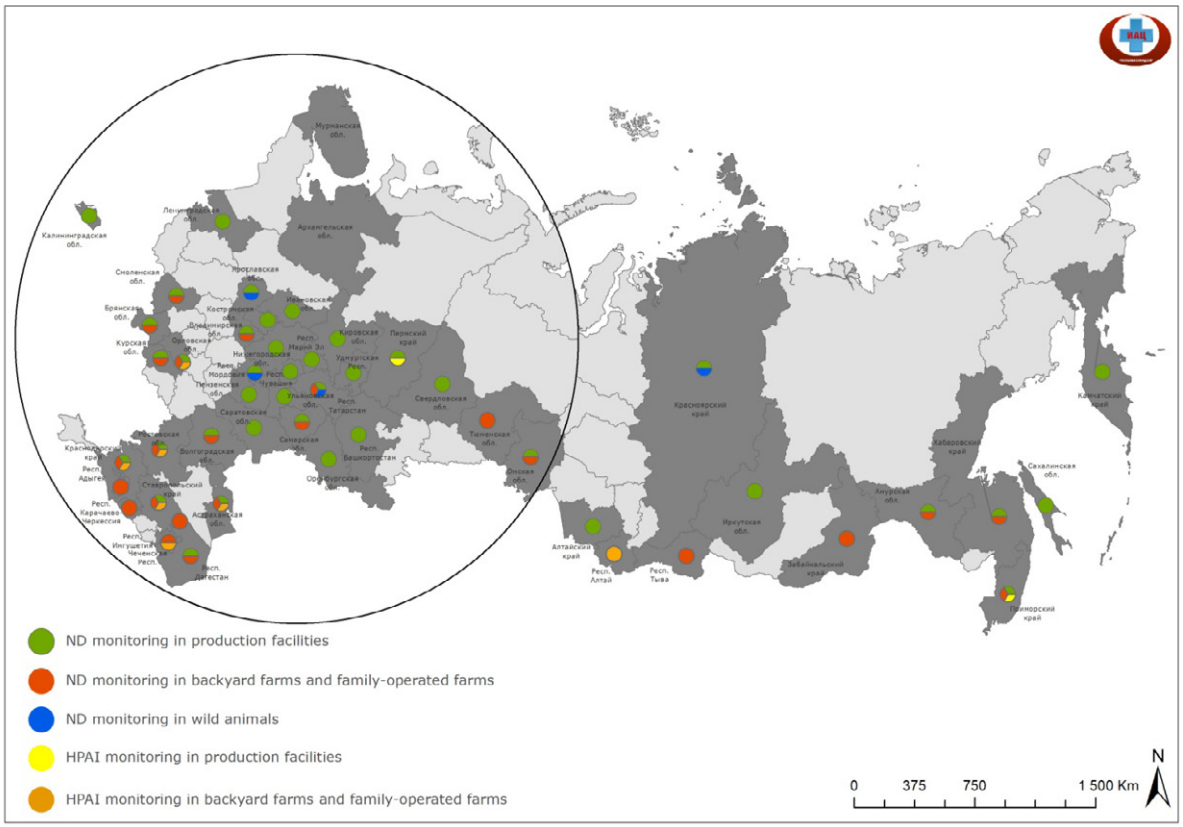

Fig. 3. RF Regions in which monitoring studies revealed antibodies to ND and AI viruses in 2019 (highlighted regions – the ones from which samples were delivered)

Рис. 3. Регионы Российской Федерации, на территории которых в ходе мониторинговых исследований у птиц было выявлено наличие антител к вирусам гриппа птиц и ньюкаслской болезни в 2019 г. (выделены регионы, из которых доставлены пробы)

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Results of scientific expedition to natural biotopes of the Republic of Tyva in 2019 with the purpose of infectious disease monitoring in wild bird populations

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SUMMARY

The results of the scientific expedition to Tere Khol and Uvs Nuur Lakes in the Republic of Tyva with the purpose of active monitoring of highly dangerous diseases in wild migratory waterfowl and epidemic analysis of these biotope water areas are presented in the paper. The Uvs Nuur Lake is a kind of an indicator for avian influenza introduction to the Russian Federation, because this is the resting and nesting area for many migratory wild birds during the period of mass migrations from Central and South-East Asian countries. In the process of active monitoring the complete autopsy of bird carcasses with description of organs and systems and sampling for laboratory diagnostics were performed. Droppings (pooled samples), parts of internal organs from dead and shot birds, blood (if possible) served as biological and pathological material for testing. While sampling, species were identified using an ornithological guide. The autopsy of dead waterfowl and birds shot for diagnostic purposes demonstrated a high worm burden of nematodes and cestodes. Two samples from European herring gulls were positive for avian influenza type A virus genome and subtype H13N6 was identified in one of them. Avian paramyxovirus serotype 1 (APMV-1), the agent of Newcastle disease, was found in one sample from gulls. The lakes of the Republic of Tyva are the most significant sites for sampling of biological material from wild birds, as the primary detection of highly pathogenic avian influenza virus in this territory is a serious signal of potential further virus spread and a precursor to a probable epizooty. Notwithstanding the absence of AIV very virulent isolate detections in wild bird populations the middle term prognosis for 2020 can be designated as cautious, as the avian influenza epidemic situation is deteriorating globally, especially in the European countries, and the threat of the virus introduction to the Russian territory with migratory birds still exists.

Key words: avian influenza, wild migratory bird, lake, epizooty, invasion, parasitic disease.

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Результаты научной экспедиции в природные биотопы Республики Тыва в 2019 году для проведения мониторинга инфекционных болезней в популяциях диких птиц

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РЕЗЮМЕ

Представлены результаты научной экспедиции на озёра Тере-Холь и Убсу-Нур Республики Тыва с целью активного мониторинга особо опасных болезней среди диких перелетных водоплавающих птиц и проведения эпизоотологического исследования акватории выбранных биотопов. Озеро Убсу-Нур является своеобразным индикатором заноса вируса гриппа птиц на территорию Российской Федерации, так как оно служит местом стоянок и гнездований многих диких перелетных птиц в период массовых миграций из стран Центральной и Юго-Восточной Азии. При проведении активного мониторинга проводилось полное вскрытие трупов птиц с описанием органов и систем и отбором проб для лабораторной диагностики. В качестве биологического и патологического материала отбирали пробы помета (пуловые пробы), кусочки внутренних органов от мертвых и отстрелянных птиц, кровь (при возможности). При отборе образцов для исследований проводили идентификацию вида с использованием орнитологического определителя. При патологоанатомическом исследовании мертвых или убитых с диагностической целью водоплавающих птиц выявлена высокая степень глистной инвазии нематодами и цестодами. В двух пробах от чаек серебристых был выявлен геном вируса гриппа птиц типа А и в одной из них идентифицирован подтип H13N6. В одной пробе от чаек выявлен парамиксовирус 1-го серотипа (APMV-1) – возбудитель ньюкаслской болезни. Озёра Республики Тыва являются важнейшими точками пробоотбора биоматериала от диких птиц, поскольку первичное обнаружение вируса высокопатогенного гриппа на данных территориях является серьезным сигналом о возможности дальнейшего распространения возбудителя и предвестником возможной эпизоотии. Несмотря на отсутствие случаев обнаружения высоковирулентных изолятов вируса гриппа в дикой популяции, среднесрочный прогноз на 2020 г. можно охарактеризовать как «осторожный», поскольку наблюдается ухудшение эпизоотической ситуации по гриппу в мире, особенно в европейских странах, и сохраняется угроза заноса вируса на территорию России с перелетными птицами.

Ключевые слова: грипп птиц, дикая перелетная птица, озеро, эпизоотия, инвазия, паразитоз.

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INTRODUCTION

Natural ecosystems, where anthropogenic factor does not influence biotopes, are extremely interesting for veterinary, medical and biological sciences, as they provide the possibility to study patterns of infectious and epizootic process development and peculiarities of infectious agent ecology in its habitat, where natural disease foci can occur and persist[1]. Ecosystems of the Republic of Tyva, in particular the Tere Khol and Uvs Nuur Lakes, were deliberately selected for studies of the avian influenza virus ecology. Researchers of the FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH") undertake scientific expeditions to the lakes of the Republic of Tyva every year; the Uvs Nuur Lake is some kind of an indicator for avian influenza introduction to the Russian Federation, because this is the resting and nesting areas for many migratory wild birds during the period of mass migrations from Central and South-East Asian countries. For example in 2006 mass deaths of wild migratory birds (great crested grebe/*Podiceps cristatus* was the prevailing species) were reported in the lake area due to infection with H5N1 highly pathogenic influenza. In June 2009 and 2010 dead birds of various species were found at the lake (great-crested grebe/*Podiceps cristatus*, bean goose/*Anser fabalis*, Eurasian spoonbill/*Platalea leucorodia*, Pallas's gull/*Larus ichthyaetus*, gadwall/*Anas strepera*, great cormorant/*Phalacrocorax carbo*, Eurasian coot/*Fulica atra*, great egret/*Egretta alba*, common pochard/*Aythya ferina*), died due to the infection with H5N1. H5N1 virus was isolated from dead great-crested grebes also in 2015. Since 2011 diagnostically relevant titres of specific antibodies to H5, H7 and H9 virus subtypes have been detected in sera from shot birds, which suggests avian influenza virus circulation in migratory waterfowl. The influenza A/H5N8 virus was isolated from dead and shot birds at the Uvs Nuur Lake in May 2016; this subtype is responsible for the epizooty in agricultural poultry in Russia (late 2016 – January, 2019). That is why annual comprehensive monitoring in waterfowl populations is an actual and important task of practical veterinary medicine.

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MATERIALS AND METHODS

2019 expedition to the Republic of Tyva was arranged within FAO/IAEA research program (Contract No. 22555/R0). In order to prevent shooting of the Red Book and remnant species for the purposes of active monitoring, the official game manager was included into the expedition team. Complete autopsy of bird carcasses followed by description of organs and systems and sample collection for laboratory diagnostics was performed. Droppings (pooled samples), parts of internal organs from dead and shot birds, blood (if possible) served as biological and pathological material for testing. While sampling, species were identified using an ornithological guide [1, 2]. Samples were transported to the laboratory in sealed water-proof insulated containers with cool packs in compliance with current standards [3]. For the purposes of the epizootological study of the lake areas, conventional epizootological techniques and photo- and video equipment were used.

To prepare suspension, samples of internal organ parts and droppings were homogenized and pH 7.4 sterile saline solution was added. For further testing 1 ml of the

Fig. 1. Tere Khol Lake area, 2019

Рис. 1. Акватория оз. Тере-Холь, 2019 г.

prepared suspension was transferred into a tube and centrifuged at low rpm to clarify.

Total RNA was extracted using RIBO-sorb test kit (Cat. No. K2-1-Et-100) in accordance with the manufacturer's guidelines.

Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) was performed in one run using OneStep RT-PCR Kit (Qiagen, cat. No. 210212), 25 mM magnesium chloride solution (Promega, in kit with Cat. No. M8296) and primer kits specific for genes M, H, N. 25 µl mixture was made from 7.5 µl of de-ionized water; 5 µl 5× RT-PCR buffer; 2.75 µl of 25 mM of мМ magnesium chloride solution, 1 µl of 10 mM dNTP solution, 1 µl of each direct and reverse primer solutions (10 picomol/µl); 0.75 µl of fluorescent probe solution (10 picomol/µl); 1 µl of reverse transcriptase and polymerase enzyme solution; 5 µl of total RNA solution. Reverse transcription was performed for 30 min at 50 °C. For amplification the following temperature and time parameters were used: 95 °C – 10 min (polymerase activation), then 40 cycles, each consisting of 3 stages (95 °C – 10 s, 55 °C – 35 s, 72 °C – 10 s).

RESULTS AND DISCUSSION

The fresh water Tere Khol Lake is located 1,300 m above sea level in the south-east of the Republic of Tyva in the Tere Khol tectonic hollow, not far from the Mongolian border. Lack of anthropogenic factor and feed abundance in the lake area attract wild waterfowl, notwithstanding the poor offshore vegetation. The lake is extremely marshy and water has a specific swamp smell (Fig. 1). During examination no wild bird carcasses were found.

The Russian part of the Uvs Nuur Lake is located in the southern part of the Republic of Tyva at the border with Mongolia (Fig. 2). The lake is situated in the tectonic hollow – Great Lakes Depression, in an endorheic basin, is highly saline and its bitterly-salty water is unfit for human consumption. Every year the lake area diminishes due to drying up. The shores in the Russian part (10 km long) are marshy and hard to access. The climate in the basin is sharply continental with great fluctuations in daily and annual temperatures. While examining the lake shore three dead European herring gulls (*Larus argentatus*) were found.

Fig. 2. Uvs Nuur Lake area (Mongolian border), 2019

Рис. 2. Акватория оз. Убсу-Нур (граница с Монголией), 2019 г.

Table
Number of samples taken in natural biotopes

Таблица
Количество отобранных проб в природных биотопах

Species	Tere Khol Lake	Uvs Nuur Lake
Order *Charadriiformes*		
Black-headed gull (*Larus ridibundus*)	3	–
European herring gull (*Larus argentatus*)	–	8
Common tern (*Sterna hirundo*)	2	–
Wader (*Limicolae*)	1	–
Common snipe (*Gallinago gallinago*)	1	–
Order *Anseriformes*		
Garganey (*Anas querquedula*)	2	–
Common pochard (*Aythya ferina*)	1	1
Order *Gruiformes*		
Eurasian coot (*Fulica atra*)	3	–
Order *Podicipediformes*		
Great-crested grebe (*Podiceps cristatus*)	1	–
Order *Suliformes*		
Cormorant (*Phalacrocorax*)	–	2
Droppings (pooled samples)	28	19
Aquatic organisms	18	–

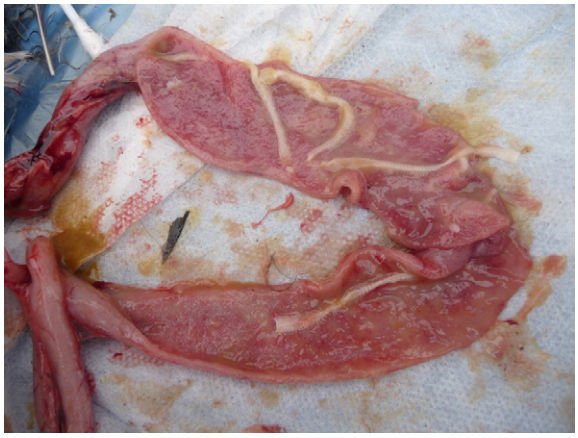

Fig. 3. Cestodes in intestines (drepanidotaeniosis)

Рис. 3. Цестоды в кишечнике (дрепанидотениоз)

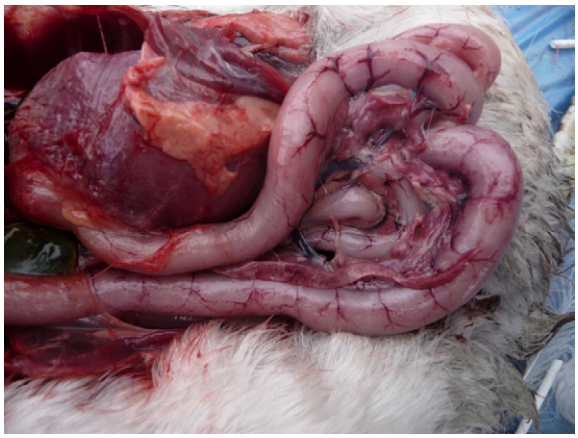

Fig. 4. Distention and vascular congestion of intestinal loops

Рис. 4. Вздутие и полнокровие сосудов петель кишечника

The following bird species were met during the examination of the lakes' areas: cranes (*Grus*), waders (*Limicolae*), gulls (*Laridae*), mallard (*Anas platyrhynchos*), common pochard (*Aythya ferina*), garganey (*Spatula querquedula*), Eurasian coot (*Fulica atra*), great-crested grebe (*Podiceps cristatus*), ruddy shelduck (*Tadorna ferruginea*), perching birds (*Passeriformes*).

Within the active monitoring in 2019 90 samples of biological materials were taken from wild birds: parts of intestines, kidneys, spleen, sometimes brain and droppings (see Table).

The autopsy of dead and killed for diagnostic purposes birds showed single and combined invasions (cestodes and nematodes) in 50% cases. When adult cestodes were detected in small intestines (Fig. 3), distention and vascular congestion of intestinal loops, catarrh of mucosal membrane, which was congested and edemic (Fig. 4), gallbladder enlargement with discharge of bile into the stomach lumen and pancreatitis (pancreas distention, hyperemia, edema and softness) were observed. White cestodes were 12–20 cm long, the width of their proglottids were bigger than their length (Fig. 5). Heavy 0.5–5 cm nematode burden was revealed in cormorants, gulls and ducks (Fig. 6–8). Moreover, the proventriculus mucosa was hyperemic and extremely edemic with petechial hemorrhages. The gizzard cuticle was boggy and easily detachable; hyperemia and petechia were observed under the cuticle. The tapeworm infestation was assessed as heavy. Such a heavy tapeworm burden in wild bird populations can be explained by the fact that optimal conditions for the propagation of intermediate hosts for avian parasites, in particular of crustaceans, like cyclops, daphnia, gammarids as well as mollusks and other aquatic invertebrates, exist in the concerned ecosystems of the Republic of Tyva. Intermediate hosts for cestodes are cyclops, in organisms of which larvae (cysticercoids) develop; gammarids are intermediate hosts for many tapeworms, responsible for tetramerosis, streptacarosis and other diseases in bids[2].

During the necropsy of dead gulls, found on the Uvs Nuur Lake shore, diffuse hemorrhages were observed under the head skin and in the oviduct (Fig. 9–10), inflammation of pancreas and small intestines. Avian influenza type A genome was detected in two samples. Subtype H13N6 was identified in one of them. Sample taken from the third gull revealed the presence of paramoxyvirus serotype 1 (APMV-1), causing Newcastle disease.

[2] Kotelnikov G. A. Tapeworm tests of animals and environment [Gel'mintologicheskie issledovaniya zhivotnyh i okruzhayushchej sredy]: guiding book. M: Kolos; 1983. 208 p.

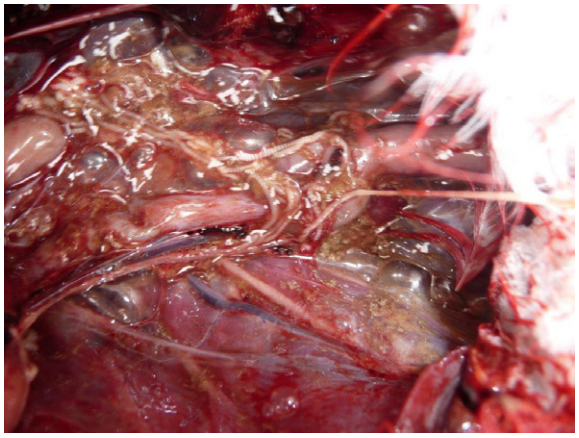

Fig. 5. Cestodes in abdominal cavity (European herring gull)

Рис. 5. Цестоды в брюшной полости (чайка серебристая)

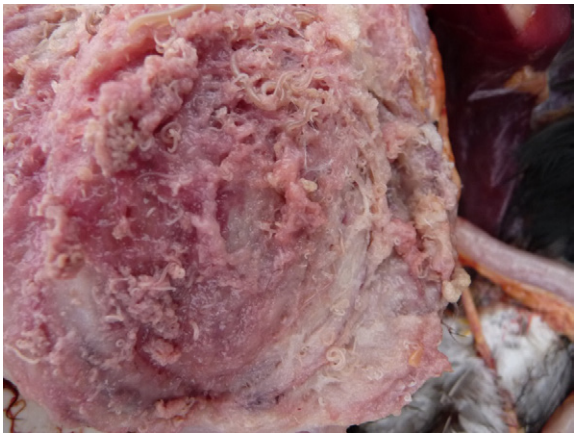

Fig. 6. Nematodes in proventriculus (European herring gull)

Рис. 6. Нематоды в железистом желудке птиц (чайка серебристая)

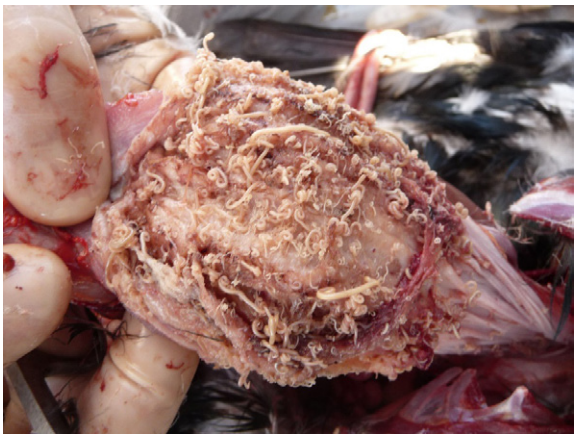

Fig. 7. Nematodes in gizzard (cormorant)

Рис. 7. Нематоды в мышечном желудке птиц (баклан)

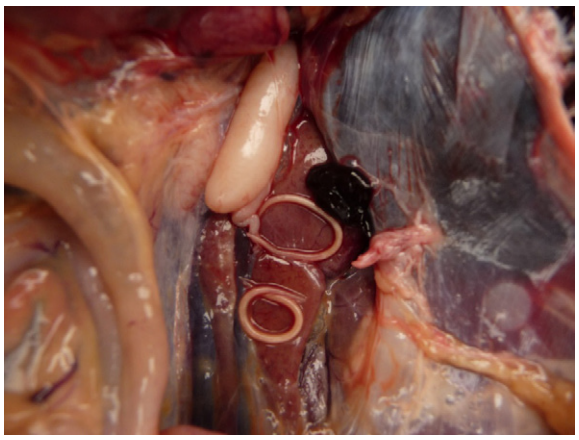

Fig. 8. Nematodes in abdominal cavity (great-crested grebe)

Рис. 8. Нематоды в брюшной полости птиц (чомга)

Notwithstanding the PCR positive results, we failed to isolate these pathogens using chicken embryos and cell cultures.

No avian influenza type A genetic material was found in the samples of aquatic organisms (gammarids), taken from the shores. However, there are reports of avian influenza virus accumulation in aquatic organisms, in particular in mussels and daphnia, inhabiting natural lakes and rivers. For example, A. Pathak et al. studied the survival rate of low pathogenic (H9N2) avian influenza virus in bamboo shrimps (*Atyopsis moluccensis*) and found that this species is able to accumulate the influenza agent and support its circulation in the biotope [4].

Notwithstanding the fact that no highly avian influenza virus virulent isolates were detected in wild fauna, the middle term forecast for 2020 can be characterized as "cautious", because the risk of avian influenza virus introduction into the Russian territory together with migratory birds still remains. The further spread of H5Nx avian influenza viruses, potential epizootic emergence and severity of the epizootic process will depend on direct and indirect contacts between wild virus-carrier birds and poultry.

CONCLUSIONS

1. Heavy cestode and nematode burden of wild migratory bird populations in the Republic of Tyva was established.

2. Major post-mortem lesions due to inflammations in organs and tissues are associated with parasitic diseases in studied birds.

3. Detection of avian influenza virus subtype H13N6 and Newcastle disease agent in gulls suggest circulation

Fig. 9. Hemorrhages under head skin

Рис. 9. Кровоизлияния под кожей головы

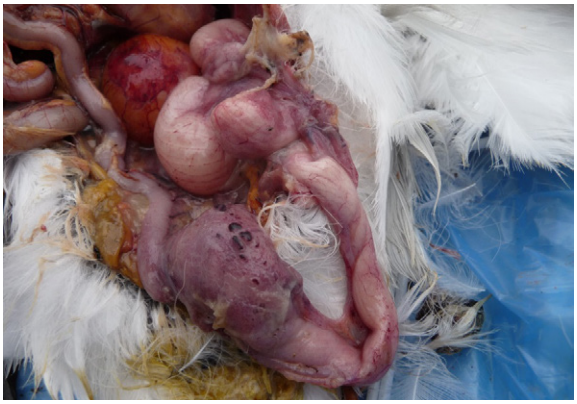

Fig. 10. Hemorrhages in oviduct

Рис. 10. Кровоизлияния в яйцеводе

of these viruses in the wild bird populations and this virus carriage state is a common case.

4. Lack of mass deaths in wild birds and negative results of laboratory tests for highly pathogenic avian influenza enables to predict a low risk of the agent introduction from the Central and South-East Asian countries through the migration routes, located across the Republic of Tyva in 2017–2019 [5–7].

5. The middle term prognosis for 2020 can be characterized as "cautious", because the avian influenza epidemic situation is deteriorating globally, especially in the European countries, and the threat of the virus introduction to the Russian territory with migratory birds still exists.

6. The lakes of the Republic of Tyva are the most important sites for sampling of biological material from wild birds, because the primary detection of highly pathogenic avian influenza virus in this territory is an essential signal of the agent further spread and a precursor of a potential epizooty.

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Avian mycoplasmosis monitoring in the Russian Federation in 2019

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SUMMARY
Under the conditions of intensive poultry farming and high concentration of poultry in a limited area mycoplasmoses cause significant economic damage to commercial poultry farming. Of greatest interest are respiratory mycoplasmosis and infectious synovitis, the causative agents of which are *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, respectively. Considering that both diseases are included in the OIE list of notifiable diseases, it is necessary to perform constant control for their spread. The paper presents an analysis of serological test results for antibodies to mycoplasmosis agents in 2019. Six respiratory mycoplasmosis positive samples – and 129 infectious synovitis positive samples were detected when testing 2,401 chicken serum samples by the enzyme-linked immunosorbent assay (ELISA). The results of monitoring tests of samples received from 31 poultry farms (nine RF Subjects) indicate a decrease in the number of *Mycoplasma gallisepticum*-infected stocks and stable *Mycoplasma synoviae* situation. The respiratory mycoplasmosis epidemic situation on indoor poultry farms might have improved due to obtaining the poultry for commercial parent stocks from mycoplasmosis-free sources, better sanitary and hygienic conditions, elimination of the disease provoking factors, and the use of the disease-specific means of prevention in the parent broiler stocks. The infectious synovitis situation remains tense despite the fact that the number of *Mycoplasma synoviae*-infected farms decreased in comparison with the monitoring results for 2015–2018. Most seropositive stocks were detected on the layer farms (50.0% – in 2019). It seems appropriate to increase the amount of tests to be performed and the number of farms covered by testing in 2020 to more fully present the disease spread situation in commercial poultry farming in Russia.

Key words: serological monitoring, respiratory mycoplasmosis (*Mycoplasma gallisepticum*), infectious synovitis (*Mycoplasma synoviae*), enzyme-linked immunosorbent assay, specific antibodies.

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Мониторинг микоплазмозов птиц в Российской Федерации в 2019 году

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РЕЗЮМЕ
Микоплазмозы в условиях интенсивного ведения птицеводства и высокой концентрации поголовья птицы на ограниченной территории наносят промышленному птицеводству значительный экономический ущерб. Наибольший интерес представляют респираторный микоплазмоз и инфекционный синовит птиц, возбудителями которых являются *Mycoplasma gallisepticum* и *Mycoplasma synoviae* соответственно. Поскольку оба заболевания включены в список нотифицируемых болезней Всемирной организации здравоохранения животных (МЭБ), необходим постоянный контроль за их распространением. В статье представлен анализ результатов серологических исследований на предмет выявления антител к возбудителям микоплазмозов птиц за 2019 г. При тестировании 2401 пробы сывороток крови кур методом иммуноферментного анализа было выявлено 6 проб, поло-

жительных на респираторный микоплазмоз птиц, и 129 проб, положительных на инфекционный синовит. Результаты мониторинговых исследований проб, поступивших из 31 птицеводческого хозяйства 9 субъектов Российской Федерации, свидетельствуют о снижении количества инфицированных *Mycoplasma gallisepticum* стад и стабильной ситуации по *Mycoplasma synoviae*. Улучшение эпизоотической ситуации по респираторному микоплазмозу на птицефабриках закрытого типа может быть связано с комплектованием родительских промышленных стад из благополучных по данному заболеванию источников, изменением санитарно-гигиенических условий, ликвидацией факторов, провоцирующих заболевание, а также с применением средств специфической профилактики в родительских стадах бройлерного производства. Ситуация по распространению инфекционного синовита остается напряженной, несмотря на уменьшение количества хозяйств, инфицированных *Mycoplasma synoviae*, по сравнению с результатами мониторинга 2015–2018 гг. Наибольшее количество серопозитивных стад выявлено в хозяйствах яичного направления продуктивности, их удельный вес в 2019 г. составил 50,0%. Для более полного отражения ситуации по распространению данных инфекций в промышленном птицеводстве России представляется целесообразным в 2020 г. увеличение объема исследований и количества обследованных хозяйств.

Ключевые слова: серологический мониторинг, респираторный микоплазмоз (*Mycoplasma gallisepticum*), инфекционный синовит (*Mycoplasma synoviae*), иммуноферментный анализ, специфические антитела.

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INTRODUCTION

Currently, a significant and urgent problem in poultry farming is infectious diseases of mycoplasma etiology. *Mycoplasma gallisepticum*, which causes respiratory mycoplasmosis and *Mycoplasma synoviae*, which causes infectious synovitis of birds, are of most interest for veterinary medicine [1–3].

Clinically, diseases are manifested by respiratory disorders (shortness of breath, cough, sinusitis, wheezing), conjunctivitis, tendonitis, loss of appetite, weight loss and egg production decrease (Fig. 1–4) [4, 5]. Mycoplasmosis is usually complicated by a secondary viral and bacterial infection. The most common causative agents of such infections are viruses of infectious bronchitis (IBV), infectious laryngotracheitis (ILTV), avian pneumovirus (APV), *Escherichia coli*, *Ornithobacterium rhinotracheale* (ORT), etc. [2, 6].

Fig. 1. Eggshell apex abnormalities associated with infectious synovitis

Рис. 1. Синдром стекловидной вершины яйца при инфекционном синовите

In countries with developed industrial poultry farming, mycoplasmosis in poultry causes significant economic damage due to emergency slaughter, decreased egg yield (up to 30%) and meat (up to 16%) productivity, as well as culling young and adult birds [7–9].

Due to the high potential for wide mycoplasmosis spread with breeding material (hatching eggs and day-old chickens) during export-import operations, the diseases are classified as economically significant [10, 11].

Most methods for determining the level of antibodies to *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in chicken serum are based on enzyme-linked immunosorbent assay (ELISA). The advantages of the method are its high sensitivity, specificity, the ability to conduct large-scale studies, quick results, small amount of samples tested and the ability to automate almost all stages of the reaction, including result recording and processing.

For many years, the FGBI "ARRIAH" has been performing mycoplasmosis monitoring, because these diseases (respiratory mycoplasmosis, infectious synovitis) are the OIE listed diseases and require constant monitoring of their spread.

The main goal of this research was to analyze the results of serological monitoring of breeding poultry farms in the Russian Federation for avian mycoplasmosis in 2019.

MATERIALS AND METHODS

Diagnostic kits. "Single serum dilution ELISA kit for detection of antibodies to *Mycoplasma gallisepticum*" and "Single serum dilution ELISA kit for detection of antibodies to *Mycoplasma synoviae*" (manufacturer – FGBI "ARRIAH").

Solutions and reagents. To prepare the solutions, double distilled water with pH of 6.0 and electrical conductivity of 0.5 S/cm was used. For ELISA we used: buffer solution (conc.) – for dilution of control and test sera and anti-species conjugate; buffer solution (conc.) for inter-stage

washes to remove unbound components; anti-chicken IgG immunoperoxidase conjugate; substrate – ABTS solution (2,2-azino-di(3-ethylbenzothiazoline-6-sulfonic acid); stop solution – 5% sodium dodecyl sulfate solution (solution for stopping the reaction).

Equipment: Freezer Indesit DF 4180 W (Russia); Bosch refrigerator (Germany); Indesit 4180 W refrigerator (Russia); drying cabinet LP-309 (Russia); air thermostat TV-20 PZ-"K" (Russia); basic pH-meter PB-11 "Sartorius" (Germany); mechanical dozers of variable volume "Biohit" (Finland), "Socorex" (Switzerland); automatic reader "ELx800" (USA); VCT computer (Russia); psychrometric hygrometer "VIT-1" (Russia); combined device "Testo 608-H1" (China); digital thermometer (logger) "Testo-174T" (Germany); stopwatch SDSpr-1-2-000 (Russia).

Materials for testing: Blood serum of chickens of different age groups received from poultry farms of the Russian Federation. Along with the sera from breeding and parent stocks, blood serum from commercial poultry was studied to objectively assess the mycoplasmosis situation.

Statistical data analysis. Statistical data processing was performed using the computer program "Statistica for Windows" (USA, Release 4.3; Inc., 1993). Result recording, data processing, storage and analysis were carried out using the SINKO-IFA computer program developed at the FGBI "ARRIAH".

RESULTS AND DISCUSSION

When monitoring mycoplasma infections of poultry, the main task was to determine diagnostically significant titers of antibodies to the causative agents of respiratory mycoplasmosis and infectious synovitis, while the parent and grandparent chicken stocks (breeding stock) were the key objects of monitoring.

In 2019, as part of monitoring studies, 2,041 ELISA tests were performed.

Serum samples came from 31 poultry farms from 9 Subjects of the Russian Federation. According to the results of laboratory studies, specific antibodies to avian mycoplasmosis were detected: 6 *Mycoplasma gallisepticum* positive samples (0.58%) and 129 *Mycoplasma synoviae* positive samples (12.64%).

Determination of serological status of farms for respiratory mycoplasmosis

Blood serum samples to be tested for respiratory mycoplasmosis were obtained from 12 layer poultry farms. Samples from two poultry farms were seropositive, which is 16.7% of the total. As for broiler production, test material came from 19 poultry farms. Samples from one poultry establishment were seropositive, which is 5.26% of the total.

The distribution of titers of antibodies to *Mycoplasma gallisepticum* in the tested samples depending on the age of the poultry is shown in Table 1.

An inconsiderable presence of antibodies to *Mycoplasma gallisepticum* in layer poultry was observed when testing sera collected from poultry over 300 days of age (3.3%), which indicates the circulation of the disease causative agent in the stock.

A low level of antibodies to the respiratory mycoplasmosis agent in broiler poultry of less than 40 days of age indicates the "purity" of parental stocks. Starting from day 130 of age, the number of seropositive poultry increased inconsiderably. It should be noted that the improvement in the respiratory mycoplasmosis epidemic

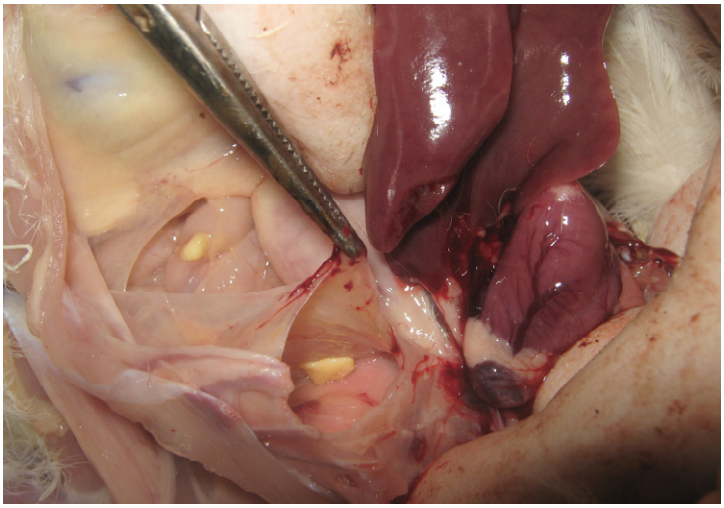

Fig. 2. Airsacculitis – pathognomonic characteristic of respiratory mycoplasmosis

Рис. 2. Аэросаккулит – патогномоничный признак респираторного микоплазмоза

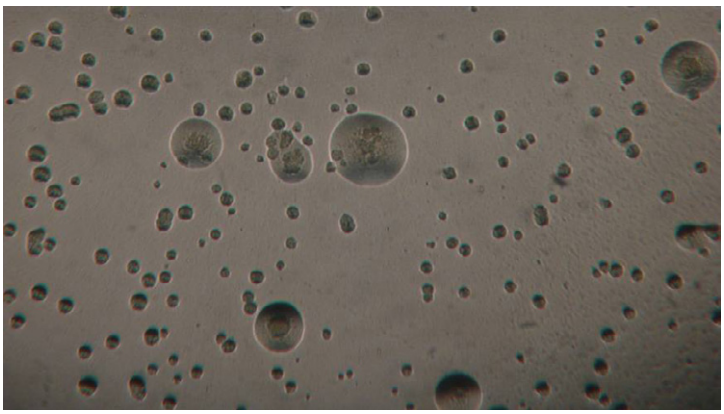

Fig. 3. Mycoplasma gallisepticum colonies in dense medium

Рис. 3. Колонии Mycoplasma gallisepticum на плотной среде

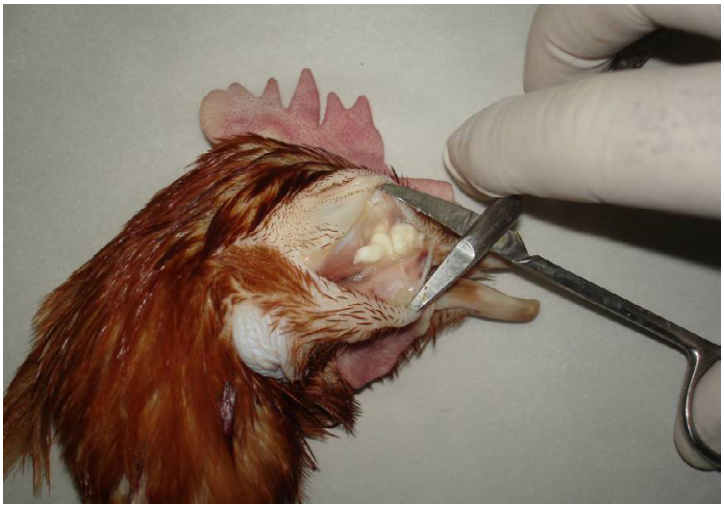

Fig. 4. Catarrhal and fibrous sinusitis associated with respiratory mycoplasmosis

Рис. 4. Катарально-фибринозный синусит при респираторном микоплазмозе

Table1
Level of antibodies to *Mycoplasma gallisepticum* distributed by age groups on layer and broiler poultry farms in the Russian Federation

Таблица 1
Уровень антител к *Mycoplasma gallisepticum* по возрастным группам на птицефабриках РФ яичного и бройлерного направления

Age groups, days	Layer farms		Broiler farms	
	Number of positive/ tested samples	ELISA titer	Number of positive/ tested samples	ELISA titer
11–40	n/t	n/t	2/25 (8%)	1,305 ± 14
131–200	0/10 (0%)	0	3/25 (12%)	1,135 ± 20
201–300	0/10 (0%)	0	n/t	n/t
Older than 300 days of age	1/30 (3.3%)	2,370 ± 25	n/t	n/t

n/t – not tested (не исследовали).

Table 2
Level of antibodies to *Mycoplasma synoviae* distributed by age groups on layer and broiler poultry farms in the Russian Federation

Таблица 2
Уровень антител к *Mycoplasma synoviae* по возрастным группам на птицефабриках РФ яичного и бройлерного направления

Age groups, days	Layer farms		Broiler farms	
	Number of positive/ tested samples	ELISA titer	Number of positive/ tested samples	ELISA titer
1–10	n/t	n/t	4/25 (16%)	1,289 ± 109
11–40	n/t	n/t	11/50 (22%)	1,822 ± 306
41–60	8/25 (32%)	2,416 ± 102	n/t	n/t
61–130	n/t	n/t	n/t	n/t
131–200	11/55 (20%)	1,288 ± 198	n/t	n/t
201–300	5/10 (50%)	1,796 ± 240	n/t	n/t
Older than 300 days of age	90/225 (40%)	2,381 ± 306	n/t	n/t

n/t – not tested (не исследовали).

situation on indoor poultry farms can be associated with obtaining poultry for commercial parent stocks from mycoplasmosis-free sources.

Decrease in the number of positive poultry and the level of antibodies with increasing age indicates improvement in the sanitary and hygienic conditions at the sites and elimination of the disease provoking factors. Thus, according to the results of recent monitoring (2012–2017), the number of adult (more than 300 days of age) poultry with positive reaction was significantly higher and reached 40%.

Determination of serological status of farms for infectious synovitis

Blood serum samples to be tested for infectious synovitis were submitted from 12 layer poultry farms. Samples from six poultry farms were seropositive, which is 50% of the total. Seropositive samples were also detected on one of 17 broiler farms, which is 5.9% of the total.

The distribution of titers of *Mycoplasma synoviae* antibodies in the tested samples depending on the age of the poultry is shown in Table 2.

The presence of specific antibodies to *Mycoplasma synoviae* in layer poultry at the age of 41–60 days (32%) is indicative of infectious synovitis circulation in the stock, which was probably transmitted transovarially from the chicken parent stock. An increase in antibody titers after transferring replacement stock to the commercial or parent stocks (130 days of age) also indicates the exposure of the poultry to the field infection agent. Given the retrospective analysis data, it should be emphasized that the infectious synovitis situation, unlike respiratory mycoplasmosis, continues to be tense, although it tends to improve. Therefore, in 2018 the proportion of infected households was 57.1%, in 2017 – 66.7%, in 2016 – 78.6%, and in 2015 – 90.32%.

The data specified in the Table 2 demonstrate that the situation on infectious synovitis in the commercial broiler farming is similar. It should be noted that the level of specific antibodies to the infectious synovitis agent prior to transferring to the productive stock is diagnostically insignificant, however, an increase in titers is observed with the age of the poultry, which may be associated with the activation of latent infection.

CONCLUSION

According to the results of the tests performed, a trend toward noticeable decrease in the number of *Mycoplasma gallisepticum* infected stocks was observed. Seroprevalence of the respiratory mycoplasmosis agent in poultry on layer farms in 2019 was 16.7%, which is 21.3% lower than in 2018. The stabilization of the epidemic situation may be associated with delivering poultry from the disease-free sources, with improvement of sanitary conditions on farms, elimination of stress factors, and use of adequate antibiotic therapy. It is well known that specific prophylaxis carried out in the broiler parent stocks reduces transovarial transmission of the agent, so its use could also improve the epidemic situation. Despite an inconsiderable improvement in the epidemic situation comparing with the monitoring results of 2015–2018 (as there is decrease in the number of *Mycoplasma synoviae* infected farms) and given the similarity of monitoring performed on farms each year, the infectious synovitis situation remains tense. The largest number of seropositive stocks was found on the layer farms (50.0%) in 2019. However, poultry farms maintain high production rates due to the asymptomatic course of infection, which is mainly associated with the prevention of technological and feed stress, which delay clinical disease manifestations.

Considering the fact that respiratory mycoplasmosis and infectious synovitis are included in the OIE list of notifiable diseases due to their cross-boundary distribution with the breeding material, it seems appropriate to increase the amount of tests and the number of farms covered by testing in 2020 to more fully reflect the disease spread situation in commercial poultry farming in Russia.

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Studies on development of early immunity against type O FMD in naturally susceptible animals

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SUMMARY
FMD risk in the Russian Federation dictates the need for enhanced measures aiming to prevent the introduction of FMD virus and comprising systematic monitoring research and mass vaccination of susceptible animals in the buffer zone. Research into the development of vaccines for early protection confirm that their use induces the formation of virus-neutralizing antibodies in naturally susceptible animals in the outbreak area, which protects from FMD infection, limits its spread and contains it within the primary outbreak. Taking into account the high speed of the infection spread, such a control measure as using FMD vaccines which induce early protection should be adopted immediately after the occurrence of the outbreak. The article presents the results of the research into the formation of humoral immunity in naturally susceptible animals triggered by administration of inactivated emulsion FMD vaccines capable of ensuring early protection against type O FMD. Culture FMD virus of O/Primorsky/2012, O/Saudi Arabia/08 and O/Mongolia/2017 strains was used for vaccine production. Immunogenic activity of vaccines was tested in cattle, pigs, and sheep. It was found that monovalent emulsion FMD vaccine based on O/Mongolia/2017 strain induced the formation of virus-neutralizing antibodies in the quantity necessary to protect against the homologous strain in seven days after a single administration in the dose of 2 cm^3. Vaccines based on O/Saudi Arabia/08 and O/Primorsky/2012 FMDV strains can protect animals from infection with heterologous O/Mongolia/2017 strain at early stages if a double dose is administered. Vaccines based on the above-mentioned strains induce early immunity formation (seven days after vaccination) against type O FMD. We suggest using the given products in the zones of a higher risk of the virus introduction.

Key words: type O FMD, inactivated monovalent emulsion vaccine, early protection, virus neutralization test.

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УДК 619:616.98:578.835.2:616-085.371:616-076

Изучение формирования раннего иммунитета у естественно восприимчивых животных против ящура типа О

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РЕЗЮМЕ
Риск возникновения вспышек ящура на территории Российской Федерации диктует необходимость усиления противоящурных мероприятий, направленных на предупреждение заноса вируса в страну и включающих проведение систематических мониторинговых исследований, а также осуществление

поголовной вакцинации восприимчивых животных в буферной зоне. Исследования по разработке вакцин для ранней защиты подтверждают, что при их использовании происходит выработка вируснейтрализующих антител у естественно восприимчивых животных в зоне вспышки, что служит защитой от заражения ящуром, приводит к сдерживанию инфекции и ее купированию в первичном очаге. Учитывая высокую скорость распространения инфекции, такая мера контроля, как применение вакцин против ящура, обеспечивающих раннюю защиту, должна применяться сразу после вспышки. Представлены результаты исследований по формированию гуморального иммунитета у естественно восприимчивых животных на введение инактивированных эмульсионных противоящурных вакцин, способных обеспечить раннюю защиту против ящура типа О. Для изготовления вакцин использовали культуральный вирус ящура штаммов О/Приморский/2012, О/Саудовская Аравия/08 и О/Монголия/2017. Иммуногенную активность вакцин проверяли на крупном рогатом скоте, свиньях и овцах. Выявлено, что моновалентная эмульсионная противоящурная вакцина из штамма О/Монголия/2017 через 7 сут после однократного введения в дозе 2 см^3 индуцировала выработку вируснейтрализующих антител в количестве, достаточном для защиты от заражения гомологичным штаммом. Вакцины из штаммов вируса ящура О/Саудовская Аравия/08 и О/Приморский/2012 при введении двойной дозы способны защитить животных на ранних сроках от заражения гетерологичным штаммом вируса ящура О/Монголия/2017. Вакцинные препараты из указанных штаммов вызывают формирование иммунитета в ранние сроки (на 7-е сут после вакцинации) против ящура типа О. Данные препараты предлагается использовать в зонах повышенного риска заноса вируса.

Ключевые слова: ящур типа О, инактивированная моновалентная эмульсионная вакцина, ранняя защита, реакция нейтрализации.

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INTRODUCTION

Foot-and-mouth disease is a viral disease of wild and domesticated cloven-hoofed animals which is economically important due to a large number of naturally susceptible species, significant impact on animal productivity and rapid spread within and between neighbouring geographical regions. Foot-and-mouth disease outbreaks result in high economic losses incurred by huge costs of sanitary measures aimed at eradication of the disease [1–3].

Currently, despite the awareness of cattle owners and trained veterinary staff, FMD outbreaks may remain unnoticed on farms in many countries until the disease spreads beyond the primary outbreak. This situation is due to a lack of identification of FMD clinical signs, delayed or unavailable information on diseased animals, and illegal movement of livestock or contaminated animal products [4, 5].

Between September 2017 and March 2018, about 30 outbreaks of type O FMD in cattle, sheep and goats were registered in several Mongolian aimags bordering Russia in the north and China in the south. The risk of outbreaks in the Russian Federation requires enhanced FMD control measures aimed at preventing the introduction of the virus into the country, including systematic monitoring studies as well as general vaccination of susceptible animals in the buffer zone [4, 6, 7]. Given the high speed of infection spread, FMD vaccines providing early protection of animals should be used immediately after the outbreak has occurred.

Vaccines have been shown to be highly effective and undeniably useful in controlling infectious diseases over many decades. Mono- and polyvalent culture inactivated vaccines are widely used for specific FMD prevention [3]. Studies on the development of vaccines for early protection confirm that their use induces the formation of virus-neutralizing antibodies in naturally susceptible animals in the outbreak area, which protects against FMD infection, limits the infection spread, and contains it within the primary outbreak [1, 2, 7].

The aim was to study humoral and protective immunity during the formation of early protection in naturally susceptible animals after administration of the inactivated emulsion FMD vaccine based on O/Mongolia/2017 strain against the homologous strain as well as vaccines based on O/Saudi Arabia/08 and O/Primorsky/2012 strains against the heterologous strain O/Mongolia/2017.

MATERIALS AND METHODS

Virus. To study humoral immunity, we used O/Mongolia/2017 homologous strain and O/Primorsky/2012 and O/Saudi Arabia/08 heterologous strains of FMD cultural virus.

For challenging of naturally susceptible animals we used aphthous FMD virus of O/Mongolia/2017 strain adapted to these animals.

Cell lines. Monolayer continuous cell lines from the Siberian mountain goat kidney (SMGK30) and Syrian baby hamster kidney (BHK21) were used for propagation of the virus. Primary monolayer cell culture of pig kidney (PK) was used to evaluate antibody titer and antigen innocuity after inactivation.

Animals. In the studies, we used: 1) 26 Holstein-Friesian cows weighing 250–300 kg; 2) 16 pigs of different breeds weighing 30–40 kg; 3) 4 sheep of Romanov breed weighing 20–30 kg.

All animal experiments were conducted in strict accordance with the Interstate Standard for the keeping and

care of laboratory animals (GOST 33216-2014), adopted by the Interstate Council for Standardization, Metrology and Certification, as well as the requirements of Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2012 on the protection of animals used for scientific purposes.

Inactivation of the virus, purification and concentration of the antigen. FMD virus was inactivated with aminoethylethyleneimine solution (AEEI). Polyhexamethylene guanidine (PGMG) solution was used to purify the antigen from ballast proteins, including non-structural proteins. Concentration was performed by ultrafiltration in a tangential flow.

Determination of component composition of inactivated suspension. The number of immunogenic components in the inactivated suspension was estimated in accordance with Methodical recommendations for determining the concentration of 146S, 75S, and 12S components of vaccine strains of FMD culture virus in complement fixation test (CFT) [8].

Selection of adjuvant and antigen-adjuvant ratio. Montanide ISA 206 VG was chosen as an adjuvant for the production of monovalent emulsion vaccines against FMD because research literature showed that it induced the formation of antibodies on days 4–7 after immunization [5, 9]. The ratio of antigen to adjuvant was 50:50.

Vaccines. Three samples were produced to study the immunogenic activity of emulsion vaccines against FMD, which were administered in a single dose of 2 cm^3 intramuscularly, and in a double dose – intramuscularly in two sites per 2 cm^3.

The first vaccine was based on O/Mongolia/2017 FMD virus strain. A double dose (4 cm^3) contained 24.22 μg of 146S component and a single dose (2 cm^3) – 12.11 μg.

The second vaccine was based on O/Saudi Arabia/08 FMD strain with 146S component in a dose of 4 cm^3 – 30.1 μg.

The third vaccine was based on O/Primorsky/2012 strain containing 29.2 μg of 146S particles in a double dose (4 cm^3).

Challenging. Seven days after vaccination, animals were challenged with adapted FMD virus of O/Mongolia/2017 strain. Challenging was carried out to the mucosa of the tongue at a dose of 10^4 ID_{50}/0.20 cm^3 (two sites per 0.10 ± 0.05 cm^3).

Blood sampling. Blood sampling was carried out on days 4 and 7 after vaccination.

Determination of VNA titer. The quantity of virus-neutralizing antibodies (VNA) in serum samples was determined by neutralization reaction using a monolayer of PK cell culture according to the generally accepted method [10].

Evaluation of viral antigen innocuity. Innocuity of the inactivated virus was controlled by inoculation into the monolayer PK cell culture.

Evaluation of vaccine immunogenicity. Vaccine immunogenicity was studied in cattle and pigs. The level of humoral immunity in neutralization reaction was determined in sheep. The blood sera obtained on days 4 and 7 after immunization were analyzed by neutralization reaction. Then, cattle and pigs were challenged with O/Mongolia/2017 control strain of FMD virus, 7 days later all animals were euthanized and pathological examination was performed. Animals that had no lesions on their limbs were considered to be protected against FMD. Primary ulcers were not considered.

Statistical data processing. All measurements were made in triplicate. The obtained results were statistically processed to determine arithmetic mean values and the degree of reliability of statistical difference between mean values by Student-Fisher difference method, as well as the determination coefficient [11]. Diagrams were made in Microsoft Excel 2010.

RESULTS AND DISCUSSION

At the first stage of the studies pigs were immunized with monovalent emulsion FMD vaccines based on O/Mongolia/2017, O/Primorsky/2012 and O/Saudi Arabia/08 strains. Subsequent assessment of the formation of early immunity against homologous and heterologous strains was carried out. The results of the study are shown in Figure 1.

Figure 1 shows that in animals immunized with the vaccine based on O/Saudi Arabia/08 strain, antibody titers against the homologous strain increased by 1.3 from day 4 to 7 after vaccination. Titers of antibodies against O/Primorsky/2012 and O/Mongolia/2017 heterologous FMDV strains day 4 after immunization were 2.1 and 1.9 times lower compared to the homologous strain and amounted to 1.95 ± 0.10 and 2.05 ± 0.17 log_2, respectively. On day 7 after the inoculation, VNA titers against O/Primorsky/2012 and O/Mongolia/2017 heterologous strains were 2.5 and 2.1 times lower than those against the homologous strain and corresponded to the values of 2.10 ± 0.10 and 2.35 ± 0.10 log_2, respectively.

In pigs vaccinated with the vaccine based on O/Primorsky/2012 strain, the number of antibodies against the homologous strain on day 4 after immunization was 3.35 ± 0.17 log_2, against O/Saudi Arabia/08 and O/Mongolia/2017 heterologous strains – 2.30 ± 0.22 and 3.25 ± 0.18 log_2. Translating log_2 into natural numbers, it can be noted that on day 4 titers of antibodies against O/Saudi Arabia/08 and O/Mongolia/2017 heterologous strains were 2.1 and 1.1 times lower, respectively, compared to the homologous strain. The difference between the level of humoral immunity against the homologous FMD virus O/Primorsky/2012 and the heterologous strain O/Mongolia/2017 is insignificant. On day 7 after immunization the number of antibodies against the homologous strain was 4.20 ± 0.29 log_2, which is 1.8 times higher than on day 4 after vaccination. Antibody levels on day 7 after vaccination against O/Saudi Arabia/08 and O/Mongolia/2017 heterologous strains were 2.4 and 1.5 times lower respectively.

In pigs immunized with the vaccine based on O/Mongolia/2017 strain on days 4 and 7 after the inoculation, antibodies titers against the homologous strain were 3.00 ± 0.36 and 3.90 ± 0.30 log_2, respectively. On day 7, the number of antibodies against the homologous strain increased by 1.9. The content of antibodies against O/Saudi Arabia/08 and O/Primorsky/2012 heterologous strains on day 4 after vaccination in serum was 1.50 ± 0.18 and 1.75 ± 0.18 log_2, which is 2.8 and 2.4 times lower than against the homologous strain. On day 7 after immunization, the VNA titers against O/Saudi Arabia/08 and O/Primorsky/2012 heterologous strains were 3.7 and 1.6 times lower than those for the homologous strain and amounted to 2.00 ± 0.17 and 3.25 ± 0.20 log_2, respectively.

As it follows from Figure 1, the level of humoral immunity in pigs vaccinated with monovalent early protection vaccines against FMD increases by day 7 post-vaccination.

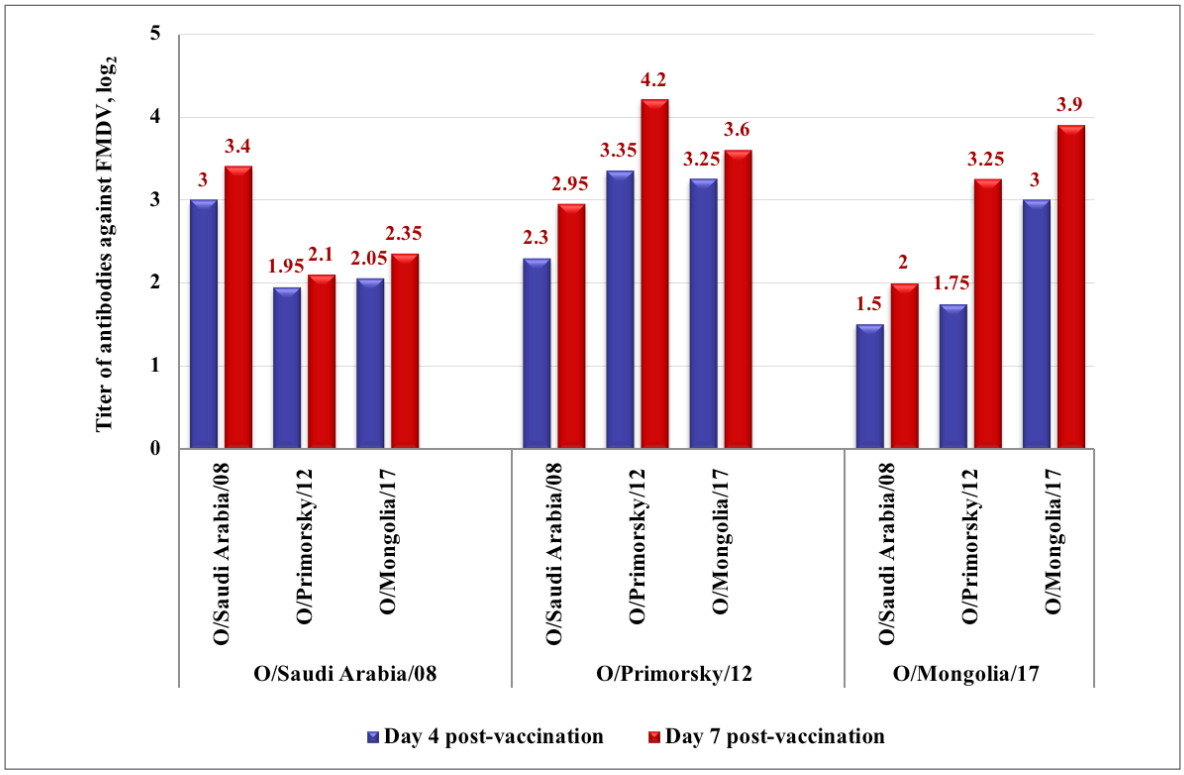

Fig. 1. Dynamics of VNA formation in pigs after use of vaccines based on O/Saudi Arabia/08, O/Primorsky/2012 and O/Mongolia/2017 strains in a double dose (4 cm³) against homologous and heterologous FMDV strains, day 4 and 7 post-vaccination

Рис. 1. Динамика образования ВНА у свиней после применения вакцин из штаммов О/Саудовская Аравия/08, О/Приморский/2012 и О/Монголия/2017 в двойной дозе (4 см³) против гомологичных и гетерологичных штаммов вируса ящура на 4-е и 7-е сут после вакцинации

The products manufactured have the best immunogenic activity mainly in relation to homologous strains. In further experiments, pigs were infected with O/Mongolia/2017 strain 7 days after vaccination to assess their protective immunity. One non-vaccinated animal was used for control. The results of the challenging are presented in Table 1.

Table 1 shows that animals immunized with vaccines based on O/Primorsky/2012 and O/Mongolia/2017 strains did not manifest FMD signs after challenging. The emulsion FMD vaccine based on O/Saudi Arabia/08 strain 7 days after administration and subsequent challenging did not protect 1 out of 5 pigs. VNA titre in the diseased animal was 2.00 $\log_2$. The control animal also contracted FMD.

The next stage of the work was devoted to comparative analysis of formation of early immunity in cattle after administration of a single and double dose of monovalent emulsion FMD vaccines. Antibody levels in blood of immunized animals against homologous and heterologous strains of FMD virus were estimated. The results of the study are shown in Figure 2.

The data presented in Figure 2 demonstrates that when the cattle was administered the monovalent emulsion FMD vaccine based on O/Mongolia/2017 strain at a dose of 2 cm³ on the 4th day the antibody titers against the homologous strain amounted to 2.83 ± 0.33 $\log_2$, and with immunization at a dose of 4 cm³ – to 3.46 ± 0.24 $\log_2$. When the vaccine was administered at a dose of 2 cm³ on day 4 post-vaccination, the content of antibodies against the homologous strain was 1.6 times lower compared to the case when a double dose was inoculated.

On day 7 post-vaccination with a single dose, the level of VNA against O/Mongolia/2017 strain was 4.67 ± 0.23 $\log_2$, and with a double dose – 5.00 ± 0.21 $\log_2$, which is an insignificant difference. After the administration of the vaccine, the VNA against the homologous strain on day 7 increased by 3.6 times compared to the data obtained on day 4 post-vaccination. When a double dose of the vaccine was used from the 4th to 7th days after immunization, there was a 2.9-fold increase in the titre of antibodies against O/Mongolia/2017 strain.

When the vaccine based on O/Mongolia/2017 strain was administered at a dose of 2 cm³ on the 4th day after immunization of cattle, the level of VNA against heterologous strains O/Saudi Arabia/08 and O/Primorsky/2012 was 2.21 ± 0.41 and 2.46 ± 0.29 $\log_2$, respectively. The number of antibodies against the homologous strain was 2.83 ± 0.24 $\log_2$, which is 1.5 and 1.3 times higher than against the heterologous strains O/Saudi Arabia/08 and O/Primorsky/2012, respectively.

It was found that on day 4 after the administration of a double dose of monovalent emulsion vaccine based on O/Mongolia/2017 strain, the level of VNA against the homologous strain was 3.46 ± 0.19 $\log_2$, against heterologous strains O/Saudi Arabia/08 and O/Primorsky/2012 – 3.21 ± 0.33 and 3.21 ± 0.22 $\log_2$, respectively. In other words, the values of antibody after administration of this vaccine at a dose of 4 cm³ against the homologous strain were 1.2 times higher than against the heterologous strain.

On day 7 after the administration of a single dose of the vaccine based on O/Mongolia/2017 strain, the level

Table 1
Study of humoral and protective immunity in pigs vaccinated with a double dose of inactivated emulsion vaccines against FMD

Таблица 1
Исследование гуморального и протективного иммунитета свиней, вакцинированных двойной дозой инактивированных эмульсионных вакцин против ящура

Vaccine strain	Titers of antibodies against FMDV, $\log_2$ ($M \pm m$)						Results of challenging with O/Mongolia/17 strain day 7 post-vaccination (challenged/ protected)
	Day 4 post-vaccination			Day 7 post-vaccination			
	O/Saudi Arabia/08	O/Primorsky/12	O/Mongolia/17	O/Saudi Arabia/08	O/Primorsky/12	O/Mongolia/17	
O/Saudi Arabia/08	3.00 ± 0.38	1.95 ± 0.10	2.05 ± 0.17	3.40 ± 0.36	2.10 ± 0.10	2.35 ± 0.35	5/4
O/Primorsky/12	2.30 ± 0.22	3.35 ± 0.17	3.25 ± 0.18	2.95 ± 0.15	4.20 ± 0.29	3.60 ± 0.27	5/5
O/Mongolia/17	1.50 ± 0.18	1.75 ± 0.18	3.00 ± 0.36	2.00 ± 0.33	3.25 ± 0.26	3.90 ± 0.30	5/5
Control							1/0

of VNA against the homologous strain was 4.67 ± 0.23 $\log_2$, and against the heterologous strains O/Saudi Arabia/08 and O/Primorsky/2012 corresponded to the values of 3.58 ± 0.12 and 3.92 ± 0.19 $\log_2$. Thus, on day 7 after immunization, the number of antibodies against the homologous strain exceeded the VNA content against the heterologous strains O/Saudi Arabia/08 and O/Primorsky/2012 by 2.1 and 1.7 times, respectively.

After the administration of the vaccine at a dose of 4 cm^3 on day 7, the titer of antibodies against homologous strain O/Mongolia/2017 was 5.00 ± 0.22 $\log_2$, against heterologous strains O/Saudi Arabia/08 and O/Primorsky/2012 – 4.46 ± 0.33 and 4.33 ± 0.22 $\log_2$, respectively. That is, on the 7th day after the administration of a double dose of the vaccine based on O/Mongolia/2017 strain, the level of antibodies against heterologous strains practically did not

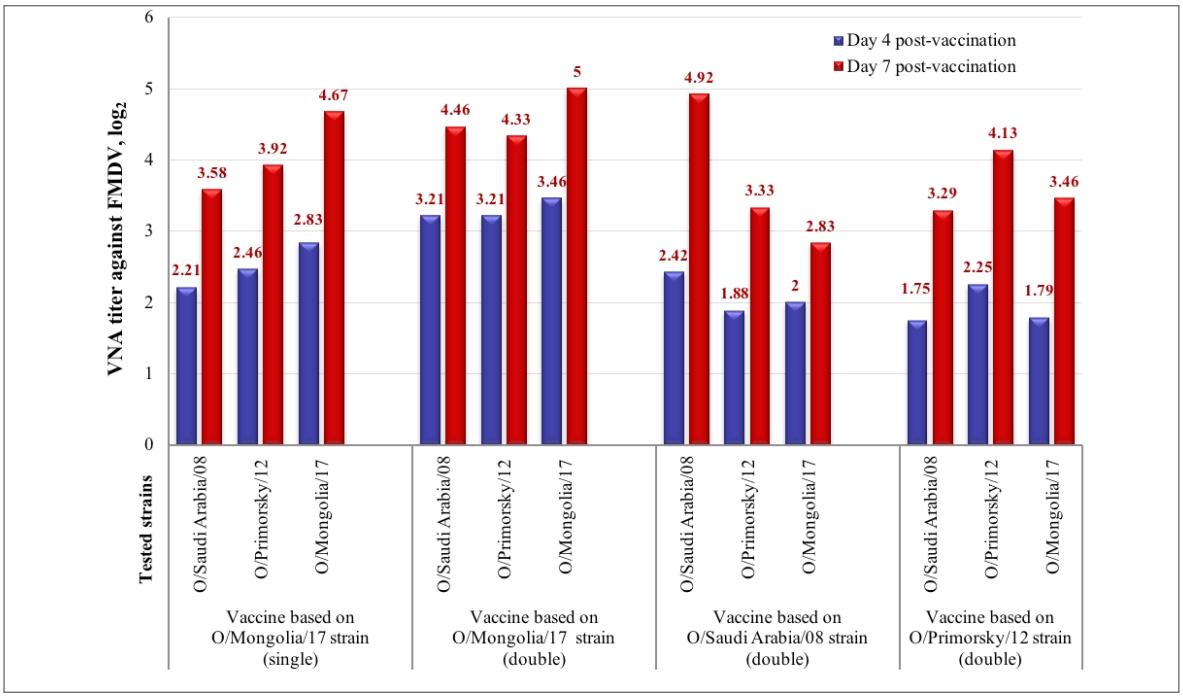

Fig. 2. Dynamics of VNA formation in cattle after administration of a single or double dose of the vaccine based on O/Mongolia/2017 strain, double dose of vaccines based on O/Saudi Arabia/08 and O/Primorsky/2012 strains against homologous and heterologous FMDV strains, day 4 and 7 post-vaccination

Рис. 2. Динамика образования ВНА у КРС после применения одной и двойной дозы вакцины из штамма О/Монголия/2017, двойной дозы вакцин из штаммов О/Саудовская Аравия/08 и О/Приморский/2012 против гомологичного и гетерологичных штаммов вируса ящура на 4-е и 7-е сут после вакцинации

differ from the VNA titre against the homologous strain. It should be noted that the immunization of cattle using a double dose on day 7 if compared to the administration of a single dose of the vaccine allowed increasing the number of antibodies against strains O/Saudi Arabia/08 and O/Primorsky/2012 by 1.8 and 1.3 times, respectively.

From the data presented in Figure 2, it follows that on day 4 and 7 after the inoculation, antibody levels were 2.42 ± 0.14 and 4.92 ± 0.15, respectively, in cattle vaccinated with a double dose of emulsion FMD vaccine based on O/Saudi Arabia/08 strain. The values of antibody titers on days 4 and 7 after immunization increased by 5.7.

On day 4 after the vaccination, the antibody titer values against heterologous strains O/Primorsky/2012 and O/Mongolia/2017 were 1.88 ± 0.15 and 2.00 ± 0.35 $\log_2$, respectively. The difference between antibody titers against homologous and heterologous strains was 1.5 and 1.3, respectively.

On day 7 after the vaccination with the same vaccine, VNA against heterologous strains O/Primorsky/2012 and O/Mongolia/2017 corresponded to the values of 3.33 ± 0.19 and 2.83 ± 0.12 $\log_2$, which is 3.0 and 4.3 times lower than the antibody titer against the homologous strain.

In animals vaccinated with monovalent emulsion vaccine based on O/Primorsky/2012 strain at a dose of 4 cm^3, the antibody titers against homologous strain on days 4 and 7 after immunization were 2.25 ± 0.14 and 4.13 ± 0.11 $\log_2$, respectively. The activity of cattle's humoral immunity against the homologous strain increased by 3.7 on days 4 to 7 after immunization.

On day 4 after the vaccination, VNA against the heterologous strains O/Saudi Arabia/08 and O/Mongolia/2017 was 1.75 ± 0.13 and 1.79 ± 0.10 $\log_2$, respectively, which is 1.4 times lower than against the homologous strain. On day 7 after immunization, antibody titers against heterologous strains corresponded to the values of 3.29 ± 0.12 and 3.46 ± 0.10 $\log_2$. Thus, the number of antibodies against heterologous strains O/Saudi Arabia/08 and O/Mongolia/2017 decreased by 1.8 and 1.6 times compared to the data for the homologous strain.

The results of the study of protective immunity on day 7 post-vaccination in cattle vaccinated with monovalent FMD vaccines are presented in Table 2. Two non-vaccinated animals were used as controls.

According to Table 2, after the administration of the monovalent emulsion vaccine based on O/Mongolia/2017 strain at a dose of 2 cm^3 we observed the formation of VNA in titers sufficient to protect against direct challenging with the homologous strain. The results of challenging with O/Mongolia/2017 strain 7 days after vaccination showed that all six animals immunized with a double dose of vaccine based on O/Primorsky/2012 and O/Mongolia/2017 strains had no FMD signs. The emulsion FMD vaccine based on O/Saudi Arabia/08 strain 7 days after administration did not provide protection against challenging of one of the six animals. The diseased animal on day 7 after the vaccination had VNA titre equal to 2.75 $\log_2$. FMD signs were also observed in two control animals.

At the next stage of the work the formation of early immunity in sheep after immunization with monovalent emulsion FMD vaccine based on O/Mongolia/2017 strain in a dose of 4 cm^3 was studied. Estimation of the sheep's immune status was difficult due to the lack of a virus adapted to this type of animal, for this reason, no challenging was carried out. The results of the determination of VNA titre on day 4 after immunization of the animals are presented in Table 3.

Table 3 shows that on day 4 after immunization the level of antibodies against the homologous strain was

Table 2
Study of humoral and protective immunity in cattle vaccinated with monovalent emulsion vaccines against FMD

Таблица 2
Исследование гуморального и протективного иммунитета у КРС, привитых моновалентными эмульсионными вакцинами против ящура

Vaccine strain, dose	Titers of antibodies against FMDV, $\log_2$ ($M \pm m$)						Results of challenging with O/Mongolia/17 strain day 7 post-vaccination (challenged/protected)
	Day 4 post-vaccination			Day 7 post-vaccination			
	O/Saudi Arabia/08	O/Primorsky/12	O/Mongolia/17	O/Saudi Arabia/08	O/Primorsky/12	O/Mongolia/17	
O/Mongolia/17 2 cm^3	2.21 ± 0.41	2.46 ± 0.29	2.83 ± 0.33	3.58 ± 0.12	3.92 ± 0.19	4.67 ± 0.23	6/6
O/Mongolia/17 4 cm^3	3.21 ± 0.33	3.21 ± 0.22	3.46 ± 0.24	4.46 ± 0.12	4.33 ± 0.11	5.00 ± 0.21	6/6
O/Saudi Arabia/08 4 cm^3	2.42 ± 0.14	1.88 ± 0.15	2.00 ± 0.22	4.92 ± 0.15	3.33 ± 0.19	2.83 ± 0.12	6/5
O/Primorsky/12 4 cm^3	1.75 ± 0.13	2.25 ± 0.14	1.79 ± 0.10	3.29 ± 0.12	4.13 ± 0.11	3.46 ± 0.10	6/6
Control 1							1/0
Control 2							1/0

Table 3
Study of humoral and protective immunity in sheep vaccinated with inactivated emulsion vaccine against FMD based on O/Mongolia/2017 strain

Таблица 3
Исследование гуморального иммунитета у овец, привитых противоящурной инактивированной эмульсионной вакциной из штамма O/Монголия/2017

Vaccine strain	Dose	No. of an animal	VNA titers day 4 post-vaccination (log_2) against FMDV strains		
			O/Saudi Arabia/08	O/Primorsky/12	O/Mongolia/17
O/Mongolia/17	4 cm^3	1	2.50	3.00	3.75
		2	3.25	2.75	3.75
		3	3.00	3.50	3.50
		4	3.00	3.75	4.00
		M ± *m*	2.94 ± 0.38	3.25 ± 0.38	3.75 ± 0.14

3.75 ± 0.14 log_2. Titers of VNA against the heterologous strains O/Saudi Arabia/08 and O/Primorsky/2012 corresponded to the values of 2.94 ± 0.38 and 3.25 ± 0.38 log_2, respectively. Thus, on the 4th day after the administration of the vaccine, sheep formed early immunity, the activity of which against the heterologous strains O/Saudi Arabia/08 and O/Primorsky/2012 was 1.8 and 1.4 times lower compared to the homologous strain. In other words, the use of FMD inactivated emulsion vaccine based on O/Mongolia/2017 strain contributes to the formation of early immunity in sheep after administration of the vaccine.

CONCLUSION

Studies on the formation of early immunity in naturally susceptible animals against FMD type O indicate that FMD emulsion vaccine based on O/Mongolia/2017 strain on day 7 after the administration of a double dose to pigs induced the formation of VNA in the amount of 3.90 ± 0.30 log_2. On day 7 after immunization of cattle with this vaccine using a dose of 2 cm^3 VNA titer against homologous strain corresponded to the value of 4.67 ± 0.23 log_2, a dose of 4 cm^3 – 5.00 ± 0.21 log_2. Results of challenging of cattle and pigs with O/Mongolia/2017 strain 7 days after immunization showed that all animals vaccinated with homologous virus had no generalized FMD signs.

When a double dose of FMD emulsion vaccine based on O/Primorsky/2012 strain was administered, on day 7 after immunization, the VNA titer in pigs against the heterologous strain O/Mongolia/2017 was 3.60 ± 0.27 log_2 and in cattle – 3.46 ± 0.10 log_2. According to the results of the challenging, all animals vaccinated with FMD vaccine based on O/Primorsky/2012 strain were protected against O/Mongolia/2017 FMDV strain.

FMD inactivated emulsion vaccine based on O/Saudi Arabia/08 strain 7 days after administration at a dose of 4 cm^3 contributed to the formation of VNA against O/Mongolia/2017 strain in pigs in the amount of 2.35 ± 0.35 log_2, in cattle – 2.83 ± 0.12 log_2. At the same time, after challenging with O/Mongolia/2017 FMDV strain, immunization with this vaccine using a double dose did not protect one out of five gilts and one of six cows. In diseased animals on day 7 post-vaccination, the VNA titers were 2.75 log_2 (cattle) and 2.00 log_2 (pig).

On day 4 after vaccination of sheep with FMD inactivated emulsion vaccine based on O/Mongolia/2017 strain at a dose of 4 cm^3 VNA titer against the homologous strain was 3.75 ± 0.14 log_2, which attested to the fact that this preparation could form early immunity. The level of humoral immunity in sheep vaccinated with this vaccine against strains O/Saudi Arabia/08 and O/Primorsky/2012 was 1.8 and 1.4 times lower compared to the homologous strain. Studies conducted in cattle suggest that FMD inactivated emulsion vaccine based on O/Mongolia/2017 strain can provide protective immunity in sheep as well.

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Bovine mycoplasmosis occurrence on livestock farms in the Russian Federation for 2015–2018

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SUMMARY

Mycoplasmosis control remains urgent in view of wide spread of bovine mycoplasmoses in the countries with intensive animal farming and trade relations between the Russian Federation and foreign partners including import of pedigree livestock and stud bull semen. Results of testing 1,186 biomaterial samples (blood, sera, nasal swabs, milk, preputial swabs, vaginal swabs, aborted and stillborn fetuses) collected from animals that demonstrated clinical signs of respiratory and reproductive disorders in 34 different regions of the Russian Federation for 2015–2018 are presented in the paper. The samples were tested with real-time polymerase chain reaction (qPCR) for genomes of the following mycoplasmosis agents: *Mycoplasma bovis*, *Mycoplasma bovigenitalium*, *Mycoplasma dispar*. As a result, *M. bovis* genome was detected in 10.1% of the samples, *M. bovigenitalium* genome was detected in 8.6% of the samples and M. *dispar* genome was detected in 37.15% of the samples. Also, 927 semen samples submitted from Russian and foreign breeding farms were tested with PCR. Test results showed presence of *M. bovis* and *M. bovigenitalium* genomes in semen samples collected from native bull population. Presented data support Russian scientists' conclusions on wide mycoplasmoses occurrence in cattle in the Russian Federation territory and risk of the disease agent introduction through semen import. All of these highlight the need for control of semen products as a source for mycoplasmosis spread as well as insufficiency of single testing of semen for granting the disease-free status to the breeding farm for genetic material marketing.

Key words: *Mycoplasma bovis*, *Mycoplasma bovigenitalium*, *Mycoplasma dispar*, polymerase chain reaction, spread, cattle, biomaterials, semen.

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Распространение микоплазмозов крупного рогатого скота на животноводческих фермах в Российской Федерации в период с 2015 по 2018 год

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РЕЗЮМЕ

Учитывая широкое распространение микоплазмозов крупного рогатого скота в странах с развитым животноводством и торговые связи Российской Федерации с зарубежными партнерами, в том числе импорт племенного скота и спермы от быков-производителей, проблема контроля микоплазмозов не теряет своей актуальности. В работе представлены результаты исследования 1186 проб биоматериала (кровь, сыворотка крови, назальные смывы, молоко, смывы с препуции и вагинальные смывы, абортированные и мертворожденные плоды), полученных от животных с клиническими признаками респираторной и/или репродуктивной патологии из 34 различных регионов Российской Федерации в период с 2015 по 2018 г. Указанные образцы были исследованы на наличие геномов таких возбудителей микоплазмозов, как *Mycoplasma bovis*, *Mycoplasma bovigenitalium*, *Mycoplasma dispar*, методом полимеразной цепной реакции в реальном времени. В результате проведенных исследований геном *M. bovis* был обнаружен в 10,1% проб, геном *M. bovigenitalium* выявлен в 8,6% проб, а геном *M. dispar* регистрировали в 37,15% проб. Также с помощью ПЦР-исследования было протестировано 927 образцов семенной жидкости, поступивших из отечественных и иностранных племенных хозяйств. Полученные результаты показали наличие геномов *M. bovis* и *M. bovigenitalium* в образцах спермы от местного поголовья быков. Представленные данные подтверждают выводы отечественных ученых о широком распространении микоплазмозов среди крупного рогатого скота на территории Российской Федерации и угрозе заноса возбудителей заболевания с ввозимой спермой. Все это указывает на необходимость контроля спермопродукции, как источника распространения микоплазмозов, а также на недостаточность однократного исследования семени для присвоения племенному хозяйству статуса благополучия для реализации генетического материала.

Ключевые слова: *Mycoplasma bovis*, *Mycoplasma bovigenitalium*, *Mycoplasma dispar*, полимеразная цепная реакция, распространение, крупный рогатый скот, биоматериал, сперма.

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INTRODUCTION

Mycoplasma mycoides subsp. *mycoides* SC (Mmm SC), *Mycoplasma bovis* (*M. bovis*), *Mycoplasma bovigenitalium* (*M. bovigenitalium*) and *Mycoplasma dispar* (*M. dispar*) play a significant role in bovine mycoplasmoses development. High disease incidence has a significant impact on animal health situation in animal industry and results in substantial economic losses in meat and dairy industries [1, 2].

M. bovis is the second most common bovine mycoplasmosis agent after Mmm SC; it is one of the major pathogens causing numerous diseases: inflammation of the respiratory tract, arthritis, keratoconjunctivitis, mastitis, etc. [2, 3]. *M. bovis*-associated mastitis in cattle and buffaloes has been already recognized as a serious problem worldwide [4, 5], and the infection caused by the said pathogen is of steadily growing importance due to increase in the said disease outbreaks in the major dairy producing countries [6–8]. Thus, *M. bovis* was detected in animals in the south-east of France [9] and in Czech Republic [10]. According to available reports, *M. bovis* prevalence in the north of Greece was 8.2% [11], in Poland in cattle population – 76.6% [12]. *M. bovis* prevalence in cattle in South America was slightly lower – 6.2% [13].

M. bovis prevalence heterogeneity in different countries can be accounted for various densities of susceptible animals [9]. Sporadic nature of *Mycoplasma*-associated mastitis in France is accounted for small herd sizes as well as effective management practice.

As the agent can be transmitted through infected milk, animal handling, veterinary and zootechnical procedures [14], it is particularly important that the animals without any clinical signs of the disease could be a source of the infection. The risk of mycoplasmosis outbreaks increases when new animals are introduced into the herd [15].

Likelihood of *Mycoplasma* infection in cattle raised under semi-intensive farming systems is 4.6 times higher than in free-ranging animals [16]. This is due to the fact that the risk of the pathogen transmission via direct contact between animals increases when the animals are reared under semi-intensive systems [17].

M. bovis was isolated in 2.2% of tested vaginal samples taken from cows in Egypt whereas *M. bovigenitalium* detection rate was 13.3% [18]. *M. bovigenitalium* was detected using similar tests in Brazil (9.29%) [19] and Japan (7.4%) [20].

M. bovigenitalium-associated genital infections in cows are characterized by granular vaginitis, vulvovaginitis with mucous and purulent vaginal discharges that could result in infertility and occasionally necrotic endometritis [21].

Economic losses due to *M. bovigenitalium* infection are attributed to infertility and poor reproductive performance of animals [21, 22].

Many researchers believe that *M. dispar* is responsible for bovine respiratory diseases that are widespread and characterized by upper respiratory tract mucosa inflammation and lung lesions. Though microorganisms of the said *Mycoplasma* species cause mild pneumonic lesions increased *M. dispar* occurrence confirms their role in bovine respiratory disease pathogenesis. Under unfavorable conditions mycoplasmas by themselves or in combination with other infectious agents can cause serious respiratory diseases resulting in economic losses in large animal farming holdings with high animal density [23, 24].

The study was aimed at analysis of *M. bovis*, *M. bovigenitalium* and *M. dispar* prevalence in different Subjects of the Russian Federation and tests of native and imported stud-bull semen samples for genomes of the above-said mycoplasmas.

MATERIALS AND METHODS

The following samples were used for tests: blood, sera, nasal swabs, milk, preputial swabs, vaginal swabs, aborted and stillborn fetuses. The samples were collected from animals with clinical signs of respiratory and reproductive disorders in 34 different regions of the Russian Federation for 2015–2018. Tests of 115 biomaterial samples were carried out in 2015; 337 biomaterial samples were tested in 2016; 373 biomaterial samples were tested in 2017 and 361 biomaterial samples were tested in 2018.

Additionally, stud bull semen samples (483 semen straws) obtained from breeding holdings located in different regions of the Russian Federation were tested in the FGBI "ARRIAH" (Vladimir).

Furthermore, 444 semen samples collected from stud bulls in different Russian and foreign breeding centres were tested in the Unit for Gene Diagnosis of Infectious Animal Diseases of the FGBI "VGNKI" (Moscow).

Seminal fluid was periodically collected from four stud bulls with impaired reproductive performance to test for *M. bovis* and *M. bovigenitalium* shedding with semen.

Samples were preliminary processed in accordance with the requirements of the Methodical Guidelines 1.3.2569-09 "Operation procedures for the laboratories using nucleic acid amplification techniques for tests of the materials containing Pathogenicity Group I–IV microorganisms". In the FGBI "ARRIAH" the agent DNA was extracted with AllPrep DNA/RNA Mini Kit (Qiagen, Germany); in the FGBI "VGNKI" the agent DNA was extracted with RIBO-prep kit (AmpliSens, Russia) in accordance with the relevant instruction for use.

PCR assays of the biological materials were performed in the FGBI "ARRIAH" with real-time polymerase chain reaction (qPCR) using own commercial kits for *M. bovis*, *M. bovigenitalium* and *M. dispar* detection in accordance with their instructions for use.

Stud bull semen straw samples were tested in the FGBI "VGNKI" with qPCR in accordance with the methods developed earlier [25].

TEST RESULTS

Tests of the biomaterials submitted from different regions of the Russian Federation

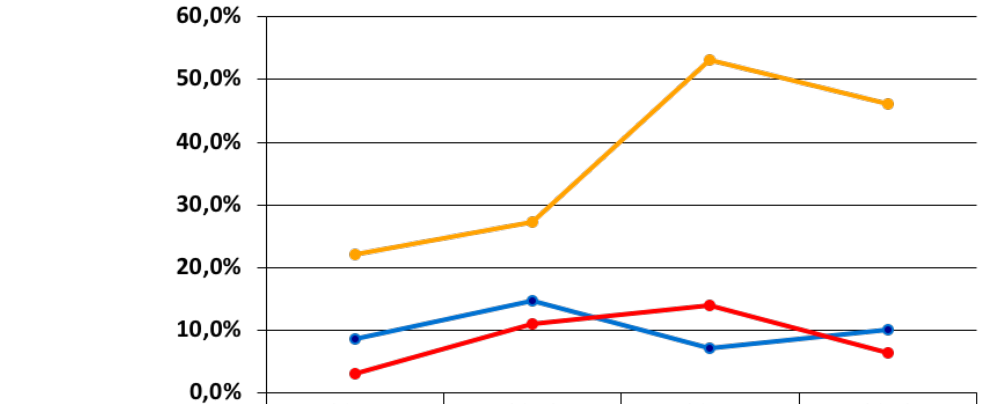
Figure 1 shows results of qPCR tests of 1,186 biomaterial samples collected from cattle in 34 Subjects of the Russian Federation for 2015–2018.

Figure 1 shows that average detection rate of *M. bovis*, *M. bovigenitalium*, *M. dispar* genome was 10.1%, 8.6% and 37.15%, respectively, for the whole test period.

It should be noted that *M. dispar* was detected more often than *M. bovis* and *M. bovigenitalium* based on the analysis of *Mycoplasma*-positive samples. Average percentage of *M. dispar* genome-positive samples out of all samples that had been PCR-positive for *Mycoplasma* for 4 years was 58.75%, whereas for *M. bovis* and *M. bovigenitalium* it was 32.50% and 8.75%, respectively.

Tests of semen samples

A total of 241 semen samples collected from native donor stud bulls and 242 semen samples collected from imported donor stud bulls were tested in the FGBI "ARRIAH" for assessment of seminal fluid quality. Bovine *Mycoplasma*



	2015	2016	2017	2018
M. bovis	8,5%	14,6%	7,2%	10,1%
M. bovigenitalium	3,0%	11,0%	14,0%	6,4%
M. dispar	22,1%	27,2%	53,2%	46,1%

Fig. 1. Results of tests of biological materials for mycoplasma genomes (2015–2018)

Рис. 1. Результаты выявления генома микоплазм в пробах биологического материала в 2015–2018 гг.

Table 1
Results of tests of 483 semen product samples carried out in the FGBI "ARRIAH"

Таблица 1
Результаты исследований 483 образцов спермопродукции, проведенных в ФГБУ «ВНИИЗЖ»

Pathogen	Number of positive semen samples			
	native		imported	
	number of samples	%	number of samples	%
M. bovis	28	11.6	6	2.5
M. bovigenitalium	70	29.0	10	4.1
Total	98	40.6	16	6.6

genomes were detected in 114 semen samples (23.6%) out of 483 semen samples submitted from 13 Subjects of the Russian Federation in 2015–2018.

Data on tests of seminal fluid for *Mycoplasma* contamination are presented in Table 1.

Data given in Table 1 show that in Russian semen samples *M. bovigenitalium* genome was detected most frequently (29%), while *M. bovis* genome was detected only in 11.6% of the samples.

M. bovigenitalium and *M. bovis* genomes were detected in 10 (4.1%) and 6 (2.5%) semen samples, respectively, out of 242 imported semen samples.

Test results for 2015–2018 were analyzed to determine detection rates of *M. bovis* and *M. bovigenitalium* in seminal fluids (Fig. 2).

The analysis revealed that detection rate of *M. bovis* (2.1%) and *M. bovigenitalium* (6.3%) genomes was the lowest in 2015. However, it had increased up to 6 and 15.5%, respectively, by 2018 (Fig. 2). *M. bovis* genome detection rate was maximum in 2016: 9.8% of *M. bovis*-positive samples out of total number of tested samples. The highest number of *M. bovigenitalium* genome-containing samples was detected in 2017: 25% of *M. bovigenitalium*-positive samples out of total number of tested samples.

M. bovis and *M. bovigenitalium* genomes were detected with qPCR in 187 samples (42.1%) out of 444 semen samples obtained from Russian and foreign breeding centres that were tested in the FGBI "VGNKI".

No *M. bovis* genome was detected in semen samples submitted from Russian breeding holdings, whereas *M. bovigenitalium* DNA was detected in 60.7% of tested samples (Table 2).

M. bovigenitalium and *M. bovis* genomes were detected in 22.3 and 3%, respectively, of tested semen straws obtained from breeding holdings located in the UK, USA and Netherlands. Therewith, *M. bovis* DNA was detected only in semen straws submitted from US breeding centres.

Test of stud bulls for Mycoplasma shedding

For tests for *Mycoplasma* shedding with semen, seminal fluid samples were collected from four stud bulls with impaired reproductive performance for 2015–2018. Collected samples were tested for *M. bovis* and *M. bovigenitalium* genomes with qPCR. Test results are given in Table 3.

The results given in Table 3 show that only *M. bovigenitalium* genome was detected in semen collected from the tested bulls. It should be noted that *M. bovigenitalium* genome was detected within the period of 2015–2017 while

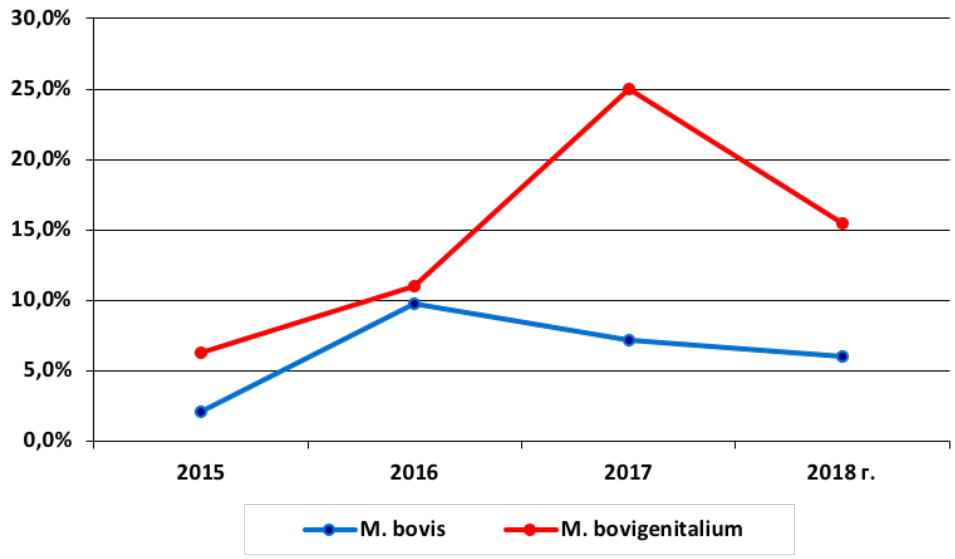


Fig. 2. M. bovis and M. bovigenitalium detection in semen product samples in 2015–2018

Рис. 2. Выявление геномов M. bovis и M. bovigenitalium в пробах спермопродукции в 2015–2018 гг.

Table 2
Results of tests of 444 semen product samples carried out in the FGBI "VGNKI"

Таблица 2
Результаты исследований 444 образцов спермопродукции, проведенных на базе ФГБУ «ВГНКИ»

Pathogen	Number of positive semen samples			
	native		imported	
	number of samples	%	number of samples	%
M. bovigenitalium	128	60.7	52	22.3
M. bovis	0	0	7	3.0
Total	128	60.7	59	25.3

samples collected from the same stud bulls in 2018 were tested negative.

The frequency of *Mycoplasma* genome detection in stud bull semen can be associated with many external and internal factors: introduction of infected animals in the herd, regular antimicrobial treatment, etc. *Mycoplasma* infections have remained understudied so far and require further investigations in the field of their diagnosis and clinical infection in animals.

DISCUSSION

Mycoplasmoses are one of the most common infectious diseases in cattle. About 100,000 new cases of clinical *Mycoplasma* infections are reported annually in cattle in Germany and the USA [26].

M. dispar genome was the most often detected in biomaterials from cattle with clinical signs of respiratory disorders (Fig. 1). It is important to note that while *M. bovigenitalium* is a genital pathogen in some cases its genome has been detected in respiratory organ samples and in nasal swabs. Analysis of detection dynamics shows an upward trend for detection rate of *M. dispar* genome in tested samples (Fig. 1), whereas detection rate of *M. bovis* and *M. bovigenitalium* remains approximately at the same level; this highlights the need for systematic monitoring of the said pathogens.

The fact that mycoplasmas can contaminate stud bull semen is of great concern. Use of uncertified semen can significantly undermine *Mycoplasma* freedom of the whole populations in breeding and/or artificial insemination centres. Results of the test carried out in the FGBI "VGNKI" showed that *M. bovis* genome was detected in 3% of stud bull semen samples from foreign breeding centres and was not detected in stud bull semen samples from Russian breeding holdings. *M. bovigenitalium* DNA was detected in 60.7% of stud bull semen samples from Russian breeding holdings and in 22.3% of stud bull semen samples from foreign breeding centres (Table 2, Fig. 2).

During the tests carried out in the FGBI "ARRIAH", *M. bovis* and *M. bovigenitalium* genomes were detected in 11.6 and 29.0% of semen samples collected from native stud bulls and in 2.5% and 4.1% of imported semen samples, respectively (Table 1).

Notably, there is a difference between results of semen straw tests performed by the FGBI "VGNKI" and FGBI "ARRIAH". This can be accounted for different origin of samples selected for tests. Samples from large-scale animal holdings including those being both production and breeding centres were submitted to the laboratory of the FGBI "ARRIAH". The samples were not separated during the said tests.

Semen purchased directly in the breeding centres was tested in the FGBI "VGNKI". Also, differences in control of stud bull health in Russian breeding centres and foreign breeding centres importing semen straws to the Russian Federation should be considered.

Mycoplasmosis is a factor-associated infectious disease triggered by stress, animal overcrowding, wet conditions, increased air humidity, inappropriate diet, etc.

Antibiotic treatment carried out in the holdings for *Mycoplasma* control contributes to the herd health improvement. However, it is important to understand that this measure alone is not sufficient for the complete recovery of the animals due to long-term *Mycoplasma* persistence in animal body and periodical *Mycoplasma* shedding by the animals. This reveal the need for systematic monitoring of the said infections (Table 3).

Presented data support results of the tests performed by Russian scientists that are indicative of wide mycoplasmoses occurrence in cattle in the Russian Federation and risk of the *Mycoplasma* agent introduction through imported semen [27, 28].

The above-said test results highlight the need for control of semen products (especially imported ones) as a source of *Mycoplasma* spread as well as insufficiency of single testing of semen for granting the disease-free status to the breeding holding for genetic material marketing.

CONCLUSION

Test results are indicative of bovine mycoplasmoses occurrence in the holdings located in different regions of the Russian Federation for 2015–2018. Identification of *M. bovis* and *M. bovigenitalium* genomes in semen from Russian and foreign breeding centres reveals high risk of the further spread of pathogenic mycoplasmas in the absence of systematic surveillance aimed at their spread prevention.

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Table 3
Results of periodic tests of bull semen for *M. bovis* and *M. bovigenitalium* genomes

Таблица 3
Результаты периодических исследований спермы быков на наличие геномов *M. bovis* и *M. bovigenitalium*

No.	Animal No.	Sampling date	Test results	
			M. bovis	*M. bovigenitalium*
1	1	25.07.15	no genome was detected	no genome was detected
2		15.05.16	no genome was detected	no genome was detected
3		05.09.17	no genome was detected	**genome was detected**
4		10.12.17	no genome was detected	no genome was detected
5		27.10.18	no genome was detected	no genome was detected
6	2	14.03.15	no genome was detected	**genome was detected**
7		18.10.15	no genome was detected	**genome was detected**
8		14.03.16	no genome was detected	no genome was detected
9	3	10.04.15	no genome was detected	no genome was detected
10		03.12.15	no genome was detected	no genome was detected
11		21.01.16	no genome was detected	no genome was detected
12		20.10.17	no genome was detected	no genome was detected
13		27.10.18	no genome was detected	no genome was detected
14	4	17.04.15	no genome was detected	**genome was detected**
15		16.07.15	no genome was detected	**genome was detected**
16		02.12.15	no genome was detected	**genome was detected**
17		28.04.16	no genome was detected	no genome was detected
18		07.09.16	no genome was detected	**genome was detected**
19		14.02.17	no genome was detected	no genome was detected
20		13.08.17	no genome was detected	no genome was detected
21		16.12.17	no genome was detected	no genome was detected
22		07.02.18	no genome was detected	no genome was detected
23		26.05.18	no genome was detected	no genome was detected
24		28.09.18	no genome was detected	no genome was detected
25		20.12.18	no genome was detected	no genome was detected

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Development and use of indirect liquid-phase ELISA test system for detection of PRRS virus antigen during in-process control of raw materials intended for vaccine production

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SUMMARY
Porcine reproductive and respiratory syndrome (PRRS) being endemic and reported in the most countries in the world remains one of the most challenging diseases in pig industry. The main disease control measures include preventive vaccination and animal movement control within and outside the country as well as diagnostic testing of pigs in the population. Live and inactivated vaccines are used for specific prevention of porcine reproductive and respiratory syndrome. Complete and irreversible infectious agent inactivation with maximum epitope preservation and protective immunity in immunized animals are the main requirements for inactivated vaccines. Therefore, continuous improvement of methods for vaccine quality control at various vaccine production stages is of current importance. Results of development of the test system based on indirect liquid-phase enzyme-linked immunosorbent assay (ELISA) for PRRS virus antigen detection and activity testing in infectious and inactivated virus-containing cell cultures at intermediate stages of the vaccine production process are described in the paper. The test-system development process included purified and concentrated virus antigen as well as hyperimmune rabbit sera preparation. Specificity of purified and concentrated virus antigen was confirmed with real-time polymerase chain reaction. The developed test-system was shown to detect the virus antigen at initial infectivity titre of 4.87–7.21 lg $TCID_{50}/cm^3$ corresponding to ELISA titre (dilution) of 1:4 up to 1:64. Methodical Guidelines for detection of porcine reproductive and respiratory syndrome virus antigen with indirect liquid-phase enzyme-linked immunosorbent assay (ELISA) (2019) were developed based on the work results, commissioned and approved by the FGBI "ARRIAH" Scientific Board.

Key words: porcine reproductive and respiratory syndrome, enzyme-linked immunosorbent assay, antigen.

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Разработка и применение тест-системы на основе непрямого жидкофазного блокирующего варианта ИФА для определения антигена вируса РРСС при технологическом контроле вакцинного сырья

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РЕЗЮМЕ

Одной из наиболее актуальных проблем свиноводства остается репродуктивно-респираторный синдром свиней, который является эндемичным заболеванием и регистрируется в большинстве стран мира. К основным мерам борьбы с заболеванием относятся профилактическая вакцинация, контроль за передвижением животных как внутри страны, так и за ее пределами, а также постоянное проведение диагностических исследований в популяции свиней. В качестве средств специфической профилактики репродуктивно-респираторного синдрома свиней применяются живые и инактивированные вакцины. Основным требованием, предъявляемым к инактивированным препаратам, является полная и необратимая инактивация инфекционного агента при максимальной сохранности антигенной детерминанты и иммунная защита привитых животных. Поэтому актуальной задачей является постоянное совершенствование методов контроля качества вакцин на различных технологических стадиях их производства. В статье представлены результаты разработки тест-системы на основе непрямого жидкофазного блокирующего иммуноферментного анализа для выявления и определения активности антигена вируса репродуктивно-респираторного синдрома свиней в инфекционных и инактивированных вируссодержащих препаратах клеточных культур на промежуточных этапах производства вакцинных биопрепаратов. Разработка тест-системы включала получение очищенного и концентрированного препарата антигена вируса, а также специфических гипериммунных сывороток крови кроликов. Специфичность очищенного и концентрированного антигена вируса подтверждали с помощью полимеразной цепной реакции в режиме реального времени. Показано, что разработанная тест-система позволяет выявлять антиген вируса с исходным титром инфекционной активности в диапазоне от 4,87 до 7,21 lg $ТЦД_{50}/см^3$, соответствующий значению титра (разведения) при постановке иммуноферментного анализа от 1:4 до 1:64. В результате проведенной работы были разработаны, прошли комиссионные испытания и утверждены ученым советом ФГБУ «ВНИИЗЖ» «Методические рекомендации по выявлению антигена вируса репродуктивно-респираторного синдрома свиней в непрямом жидкофазном блокирующем варианте иммуноферментного анализа» (2019 г.).

Ключевые слова: репродуктивно-респираторный синдром свиней, иммуноферментный анализ, антиген.

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INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) has been and remains a significant challenge for the majority of the countries where pig faming is well-developed including Russia. PRRS is currently endemic and almost all countries in the world including Russia, USA, China as well as West European countries suffer great economic losses [1, 2].

Analysis of anti-PRRS measures taken worldwide shows that the main measures include preventive vaccination, animal movement control within and outside the country as well as permanent diagnostic testing of pigs in the population [3]. Preventive vaccination covers more and more pigs every year. Therefore, continuous improvement of the vaccines and methods for control at various vaccine production stages is of current importance.

It is known that inactivated antiviral vaccine production is a complex technological process and complete and irreversible infectious agent inactivation with maximum epitope preservation and protective immunity in vaccinated animals are the main requirements for inactivated vaccines.

Qualitative and quantitative characterization of the virus antigen is a fundamental prerequisite for produced anti-PRRS vaccine effectiveness. If the virus infectivity can be controlled at the stage of the vaccine raw material scaling up with titration in susceptible cell culture, then, after inactivation, quantification of PRRS antigen, a parameter characterizing the ability to induce immune response, is difficult.

The study was aimed at development of the method for determination of PRRS virus antigen activity both in cultural virus-containing materials intended for the vaccine production and in the semi-finished vaccine.

MATERIALS AND METHODS

Antigen. Aminoethylethyleneimine-inactivated KPR-96 strain of European genotype PRRS virus (FGBI "ARRIAH" Collection of Microorganism Strains) grown in Marc-145 continuous cell culture (rhesus macaque kidney), a trophovariant of MA-104 cell culture, (infectivity titre – 4.87–7.21 lg $TCID_{50}/cm^3$) was used.

Animals. The following laboratory animals were used for tests: seronegative Chinchilla rabbits (live weight – 2–2.5 kg) and clinically healthy piglets at the age of 2 months (live weight – 20–25 kg) obtained from the PRRS-free holdings.

All tests in the animals were carried out in strict compliance with the interstate standards on laboratory animal keeping and handling: GOST 33216-2014 and GOST 33215-2014, adopted by the Interstate Council on Standardization, Metrology and Certification as well as in accordance with the requirements laid down in Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on protection of the animals used for scientific purposes.

Specific hyperimmune sera. Specific hyperimmune rabbit sera prepared by thrice immunization of rabbits with preliminary purified and concentrated emulsified antigen supplemented with oil adjuvant were used.

Anti-species conjugate. Horseradish peroxidase-conjugated anti-rabbit IgG (H+L) immunoglobulins (Sigma, USA) were used as anti-species conjugate.

Tested preparations: cultural virus and antigen specimens prepared during the vaccine raw material scaling-up, exhibiting different infectivity levels and collected at different production stages. Suspension of normal uninfected cell culture equivalent to the tested specimen as well as antigens of Aujezsky's disease virus (ADV) and porcine parvovirus (PPV) were used as negative controls. Cultural virus-containing preparation with known infectivity titre was used as positive control.

Methods. Common methods for PRRSV cultivation, infectivity titre determination, antigen preparation through the virus inactivation and low speed-centrifugation concentration; microneutralization assay (MNA), Bradford protein assay; real-time polymerase chain reaction (qPCR) were used.

Enzyme-linked immunosorbent assay (ELISA) was carried out in accordance with the Methodical Guidelines for detection of porcine reproductive and respiratory syndrome virus antigen with indirect liquid-phase enzyme-linked immunosorbent assay developed in the FGBI "ARRIAH" [4].

Porcine Reproductive and Respiratory Syndrome Virus Antibody Test Kit (IDEXX, USA) was used for testing porcine sera for specific antibodies against PRRS virus, the test results were read in accordance with the test-kit manufacturer instruction.

Statistical processing of the results. Standard methods for processing of the selected variables as well as elements of correlation-regression analysis were used. All calculations and graphical plottings were performed using Microsoft Office Excel applications.

RESULTS AND DISCUSSION

Preparation of active and specific antigen is one of the important stages of the ELISA test-kit development. The virus-containing cultural fluid was purified from ballast proteins and cell fragments with low-speed centrifugation using Beckman Coulter J-26 XP centrifuge (USA) equipped with JA-14 rotor at 4,500 rpm for 40 min.

Resulting clarified supernatant was centrifuged with Beckman Coulter J-26 XP centrifuge (USA) with JA-18 rotor at 17,000 rpm for 2.5 hours and then the supernatant was removed and pellet was resuspended with 10 ml of TNE-buffer.

For the further PRRS virus antigen purification and concentration the resuspended pellet was centrifuged in 30% sucrose-layer gradient with high-speed Beckman Coulter Optima L-80 XP (USA) centrifuge with SW-28 rotor at 27,000 rpm for 2.5 hours. The resulting pellet was resuspended with TNE-buffer at the ratio of 100:1 of the initial volume.

The specificity of the purified and concentrated PRRS virus antigen was confirmed with qPCR. C_t-values of all antigen samples tested with qPCR were less 35 (C_t) and within the range of 17–23 (C_t), that was indicative of high virus levels in the preparations.

Protein concentration in the PRRS virus antigen preparations measured with Bradford protein assay using bovine serum albumin standard solutions at wavelength of 595 nm was 0.5–1.0 mg/ml. The purified and concentrated antigen was used for preparation of hyperimmune sera required for ELISA test- system development.

The test-system development comprised selection of optimal antigen dilution, specific hyperimmune serum and anti-species conjugate. The test results were read with Sunrise spectrophotometer (Tecan, Austria) at wavelength of 405 nm. The test-system development included selection of suitable buffer systems and blocking solutions, determination of the component adsorption time and temperature.

Optimal antigen dilution was determined for each batch with serial-dilution ELISA. The last antigen dilution that gave the following optical density (OD) values was considered to be working dilution: control positive serum OD_S – within the range of 1.0–1.5 AU (when tested sera were diluted at 1:100) and negative serum OD_S – not higher than 0.25 AU. The data are given in Figure 1.

Based on the obtained data, PRRS virus antigen dilutions giving control positive serum OD_S of 1.0–1.2 (at 1:100 dilution of the tested sera) that were equal to the antigen dilutions of 1:50 – 1:100 (P/N 8.8–8.75) were used as working PRRS virus antigen dilutions.

As a result, the Methodical Guidelines for detection of porcine reproductive and respiratory syndrome virus antigen with indirect liquid-phase enzyme-linked immunosorbent assay (2019) were developed, commissioned and approved by the FGBI "ARRIAH" Scientific Board [4].

Testing of the developed test-system for its sensitivity and specificity was the next stage of the study. Tests of the virus-containing specimens before and after their inactivation as well as virus antigen at different stages of its purification and concentration with the proposed method were carried out. Test results are given in Table 1.

The data given in Table 1 show direct correlation between infectivity titres and ELISA dilutions. For example, infectivity titre of the 10×-concentrate of the virus was 7.21 ± 0.11 lg $TCID_{50}/cm^3$, and its ELISA titre was 1:32 – 1:64 both before and after inactivation; infectivity titre of the 3×-concentrate of the virus was 6.15 ± 0.14 lg $TCID_{50}/cm^3$, its ELISA titre was 1:16 – 1:32 both before and after inactivation. While supernatants with infectivity titres of 1.15 ± 0.09 and 1.35 ± 0.12 lg $TCID_{50}/cm^3$ demonstrated negative ELISA values – < 1:2.

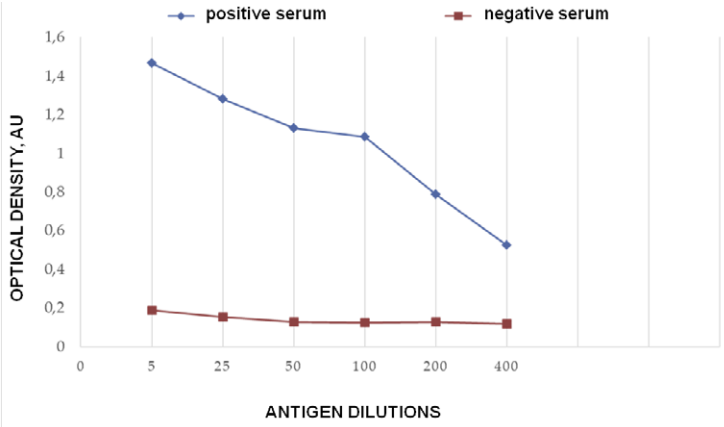

Fig. 1. Specific and normal rabbit serum activity, 1:100 dilution

Рис. 1. Активность специфической и нормальной сывороток крови кролика в разведении 1:100

Table 1
Comparative testing of PRRS virus for its infectivity and antigenicity with titration and ELISA

Таблица 1
Сравнительная оценка инфекционной и антигенной активности вируса РРСС, определенной методом титрования и с помощью ИФА

Type of sample	Number of samples	Mean titre, lg $TCID_{50}/cm^3$	Mean ELISA titre* (dilutions)
Virus-containing suspension (before inactivation)	25	4.87 ± 0.12	1:4
Virus-containing suspension (before inactivation)	75	5.67 ± 0.19	1:8 – 1:16
Antigen-containing suspension (inactivated)	75	not tested	1:8
Virus-containing suspension (×10 concentrate of PRRSV antigen) (before inactivation)	10	7.21 ± 0.11	1:32 – 1:64
Antigen-containing suspension (×10 concentrate of PRRSV antigen) (inactivated)	10	not tested	1:32 – 1:64
Supernatant (before inactivation)	10	1.15 ± 0.09	< 1:2
Virus-containing suspension (×3 concentrate of PRRSV antigen) (before inactivation)	15	6.15 ± 0.14	1:16 – 1:32
Supernatant (before inactivation)	15	1.35 ± 0.12	< 1:2
Antigen-containing suspension (×3 concentrate of PRRSV antigen) (inactivated)	15	not tested	1:16 – 1:32
Supernatant (inactivated)	15	not tested	< 1:2
Negative control (cultural fluid of normal non-PRRSV-infected cell culture)	5	0	< 1:2
Negative control (PPV antigen)	5	0	< 1:2
Negative control (AD virus antigen)	5	0	< 1:2

* ELISA test result in titres: > 1:2 – positive result; < 1:2 – negative result.

* Результат ИФА в титрах: > 1:2 – положительно; < 1:2 – отрицательно.

Correlation between developed indirect liquid-phase ELISA-derived results and virus infectivity titre (T, $TCID_{50}$) calculated for the tested virus-containing cultural fluid specimens was determined based on results of numerous tests. For ELISA the following was taken as a value indicating the relative amount of reacting antigen: $D = K^-/S$, where K^- and S – negative control and tested sample optical density, respectively; D – estimated independent parameter and lgT – estimated dependent parameter.

Obtained results are presented in the form of graph in Figure 2.

Figure 2 shows the following regression model for tested parameters: lgT″ = 1.808(D) + 0.769, where lgT″ – expected titre for the specified D value; the coefficient of model validity is as follows: $R^2 = 0.891$.

Obtained data are indicative of apparent correlation between relative amount of ELISA-reactive antigen (D) and logarithmic titres of the virus (lgT) expressed as correlation coefficient (R = 0.944). Plotted model of correlation between specified parameters was as follows: lgT″ = 1.808(D) + 0.769 and covered at least 89.1% of empirical points. Within the $2 \le D \le 3.15$ range the model allows infectivity titre of the sample to be predicted with statistical uncertainty (regression error) of $m \le 0.18$ lg.

Tests shows that developed sensitive and specific competitive liquid-phase ELISA can be used for in-process laboratory control carried out at the stages of PRRS virus antigen preparation for determination of semi-finished vaccine activity.

During the laboratory experiment specific antibody levels were determined in piglets immunized intramuscularly at a dose of 2 cm^3 with tested vaccine specimens prepared based on the virus antigen with different infectivity levels determined with titration and developed ELISA test-system. Piglet sera were tested in accordance with the instruction for use of Porcine Reproductive and Respiratory Syndrome Virus Antibody Test Kit (IDEXX, USA). The results are given in Table 2.

The animals were PRRS virus-seronegative before vaccination. Twenty-eight days after vaccination specific antibodies were detected only in 30% of the piglets immunized with vaccine specimen 1 (ELISA virus antigen activity – 1:4)

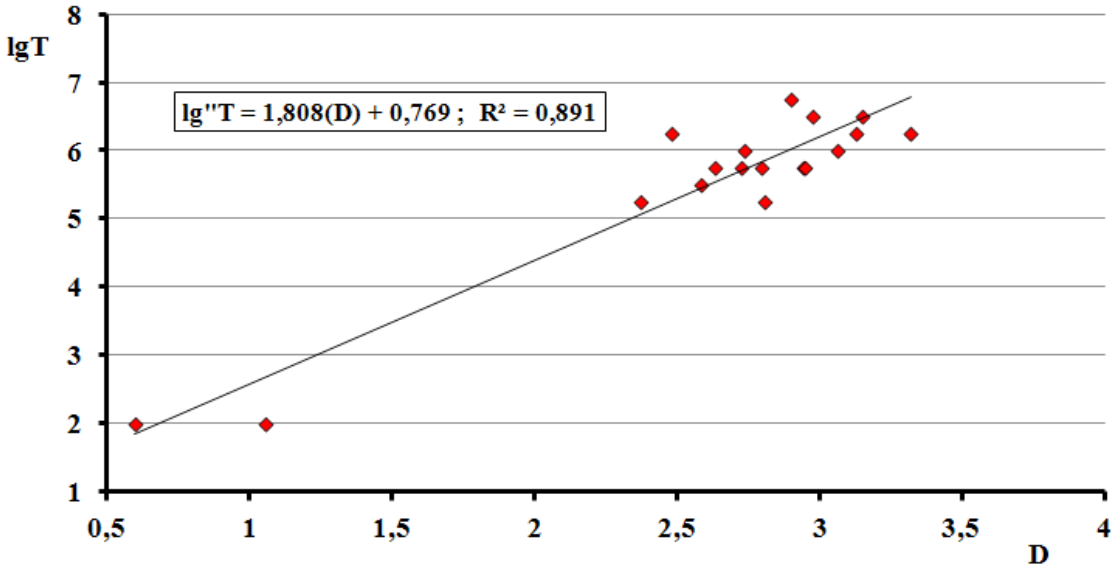

Fig. 2. Logarithmic titres of the virus infectivity (lgT, ◆) determined for tested cultural fluid samples corresponding to relative amounts of ELISA-reacting antigen (D)

Рис. 2. Логарифмические оценки титра инфекционной активности вируса (lgT, ◆), установленные в тестируемых образцах культуральной жидкости соответственно показателям относительного количества реагирующего антигена (D) в ИФА

whereas vaccine specimens 2 and 3 (ELISA virus antigen activity – 1:8 and higher) induced pronounced immune response in the piglets detectable both with ELISA and MNA.

CONCLUSION

ELISA test-system to quantitate porcine reproductive and respiratory syndrome virus antigen in tested specimens of semi-finished anti-PRRS vaccine during in-process laboratory control was developed based on test results. Specific ELISA components were prepared, cut-off values were determined, correlation between relative amount of ELISA-reactive antigen and logarithmic titre of the virus expressed as the following correlation coefficient: R = 0.944 was established. Inactivated PRRSV antigen with ELISA activity of at least 1:8 – 1:16 induces specific immunity that is evidenced by presence of diagnostic levels of specific anti-PRRSV antibodies in animals.

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Table 2
Tests of inactivated emulsion anti-PRRS vaccine samples demonstrating different antigen activity for their antigenic properties

Таблица 2
Изучение антигенных свойств вакцины против РРСС инактивированной эмульгированной с различной активностью антигена

Inactivated emulsion vaccine against PRRS	PRRS virus/antigen activity		Vaccine antigenicity (mean antibody level in the group of piglets 28 days after vaccination	
	before inactivation (lg $TCID_{50}/cm^3$)	after inactivation (ELISA)	ELISA (s/p)	MNA (log_2)
Specimen No. 1	5.0	1:4	10/3* 0.31 ± 0.06	< 2.0
Specimen No. 2	5.75	1:8	10/8* 0.58 ± 0.1	2.74 ± 0.12
Specimen No. 3	6.25	1:16	10/10* 1.1 ± 0.1	3.57 ± 0.11

* Number of tested/positive samples.
ELISA result: $s/p < 0.4$ – no specific antibodies; $s/p \geq 0.4$ – presence of specific antibodies.
MNA results: < 2.0 – no microneutralization antibodies; ≥ 2.0 – presence of specific antibodies.

* Количество исследованных/положительных проб.
Значение ИФА: $s/p < 0,4$ – специфические антитела отсутствуют; $s/p \geq 0,4$ – наличие специфических антител.
Значение РМН: < 2,0 – специфические антитела отсутствуют; ≥ 2,0 – наличие специфических антител.

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Nidoviruses associated with aquatic animals

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SUMMARY

Nidoviruses comprise the most complex grouping among other viruses with respect to their multiplicity, phylogeny, systematics, species identification, genetic relationships within a taxon, progressive variability. Like other single-stranded RNA viruses, nidoviruses have a relatively high ability to mutate and recombine, which allows them to quickly adapt to new hosts and new ecological niches. Although most of the known representatives of nidoviruses are associated with terrestrial hosts, more and more data has recently appeared on nidoviruses recovered from aquatic organisms. This review is the analysis of current data on the representatives of the order *Nidovirales* associated with aquatic animals. They are all included in the eight families based on the current classification of viruses. The most studied among them are members of the families *Coronaviridae*, *Tobaniviridae* and *Roniviridae*. Representatives of the other families of aquatic animal nidoviruses were identified using metagenomic deep sequencing (metagenomics), but their effect on the host organism has not yet been adequately studied. Data on the distribution of nidoviruses among aquatic animals in different global aquatic systems are presented, clinical signs of the disease are described, a brief description of nidoviruses and their genomes is given. Nidoviruses of aquatic animals as the earliest members of the animal kingdom are supposed to have played a possible role in the evolution of terrestrial animal nidoviruses. Therefore, aquatic animal nidoviruses could play a significant role in the formation of new natural reservoirs unknown to science, as well as in their interspecies transfer between marine, freshwater and terrestrial hosts.

Key words: nidoviruses, coronaviruses, tobaniviruses, roniviruses, spread, pathogenesis.

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Нидовирусы, ассоциированные с водными животными

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РЕЗЮМЕ

Нидовирусы в отношении их многочисленности, филогенеза, систематики, видовой идентификации, генетических связей внутри таксона, прогрессивной изменчивости являются наиболее сложной группировкой среди прочих вирусов. Как и другие вирусы с односпиральной РНК, нидовирусы обладают сравнительно высокой способностью к мутациям и рекомбинациям, что позволяет им быстро адаптироваться к новым хозяевам и новым экологическим нишам. Хотя бóльшая часть известных представителей нидовирусов ассоциирована с наземными хозяевами, в последнее время появляется все больше сведений о нидовирусах, изолированных из водных организмов. В обзоре анализируется современная информация о представителях отряда *Nidovirales*, ассоциированных с водными животными. Согласно современной классификации вирусов все они входят в состав восьми семейств. Наиболее изученными среди них являются члены семейств *Coronaviridae*, *Tobaniviridae* и *Roniviridae*. Представители остальных семейств нидовирусов водных животных были выявлены методом углубленного секвенирования (метагеномики), но их влияние на организм хозяев пока изучено недостаточно. Приведены данные по распространению нидовирусов среди водных животных в различных водных системах мира, описаны клинические признаки заболевания, дана краткая характеристика нидовирусов и их геномов. Предполагается возможная роль нидовирусов водных животных, как наиболее древних представителей животного мира, в эволюции нидовирусов наземных животных. Поэтому нидовирусы водных животных могут иметь большое значение для установления новых, неизвестных науке природных резервуаров, межвидового их переноса между морскими, пресноводными и наземными хозяевами.

Ключевые слова: нидовирусы, коронавирусы, тобанивирусы, ронивирусы, распространение, патогенез.

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Nidoviruses (*Nidovirales*) are the order of enveloped viruses with a single-segment linear single-stranded positive-sense (+)RNA. They comprise the most complex grouping among other viruses as regards their multiplicity, phylogeny, systematics, species identification, genetic relationships within a taxon, progressive variability. As obligate parasites, nidoviruses form parasitic systems with reservoir hosts belonging to all relevant veterinary and medical categories – food-producing and small domestic animals, humans, synanthropes, rodents, bats, wild animals, birds, fish.

The spectrum of pathogenicity and epidemiological significance of nidoviruses varies from fairly balanced relationships of mutual tolerance within the parasite-host system, when viruses remain "orphan" and do not cause specific pathology, to severe, fatal, nosologically determined epidemic infections such as porcine transmissible gastroenteritis (TGEV), feline infectious peritonitis (FIPV), bovine winter dysentery, avian infectious bronchitis, severe acute respiratory syndrome and Middle East respiratory syndrome passing through a series of intermediate phenomena at the clinical and epidemic levels. Speaking of the latter, the probability of the development of factorial, conditionally dependent pathology in the form of pneumoenteritis resulting from stressful effects on the host organism with a decrease in its resistance, provoking a parasitic system imbalance that turns the host carrier into an active source of infection, needs serious attention [1, 2].

Although most of the known nidoviruses are associated with terrestrial hosts, more and more information has recently appeared on nidoviruses isolated from aquatic organisms. Some aquatic animal nidoviruses have been recently identified by metagenomic deep sequencing, but their effect on the host organism has not yet been sufficiently

Table
Nidoviruses associated with aquatic organisms

Таблица
Нидовирусы, ассоциированные с водными организмами

Family	Genus	Type species	Host
Coronaviridae	*Alphacoronavirus*	*Harbor seal coronavirus* (HSCoV)	Seal
	Gammacoronavirus	1. *Beluga whale coronavirus* (SW1) 2. *Bottlenose dolphin coronavirus* (BdCoV)	Beluga whale Dolphin
	Alphaletovirus	*Microhyla alphaletovirus 1* (MLeV)	Microhyla*
Tobaniviridae	*Bafinivirus*	1. *White bream virus* (WBV) 2. *Fathead minnow nidovirus* (FHMNV)	White bream Minnow
	Oncotshavirus	1. *Chinook salmon nidovirus* (CSBV) 2. *Crucian carp nidovirus (CCNV)*	Chinook salmon Crucian carp
Roniviridae	*Okavirus*	1. *Gill-associated virus* (GAV) 2. *Yellow head virus* (YHV) 3. *Palaemon nidovirus* (PAN)	Shrimp Shrimp Swimming crab
Euroniviridae	*Charybnivirus*	1. *Charybnivirus* (CharNV) 2. *Decronivirus* (DecNV)	Crab Shrimp
	Paguronivirus	*Paguronivirus* (PagRV)	Pagurus crayfish
Mononiviridae	*Alphamononivirus*	*Planidovirus 1* (PSCNV)	Planaria
Mesoniviridae	*Alphamesonivirus*	*Alphamesonivirus 1* (NDiV)	Mosquitoes*
Abyssoviridae	*Alphaabyssovirus*	*Aplysia abyssovirus 1* (AAbV)	Mollusk
Medioniviridae	*Bolenivirus*	*Botrylloides leachi virus*	Tunicates

* Animals living in the aquatic environment only at the larval stage.

* Животные, обитающие в водной среде лишь на личиночной стадии.

Fig. 1. Pacific seals (Phoca vitulina richardsii) on the Central California coast (https://specials-images.forbesimg.com/imageserve/1170425445/960x0.jpg?fit=scale)

Рис. 1. Тихоокеанские тюлени (Phoca vitulina richardsii) на побережье Центральной Калифорнии

studied. In this regard nidoviruses of aquatic animals, as the earliest members of the animal kingdom could play a significant role in the formation of new natural reservoirs unknown to science, as well as in their interspecies transfer between marine, freshwater and terrestrial hosts.

According to the current classification of viruses (International Committee on Taxonomy of Viruses (ICTV), 2019), all novidoviruses associated with aquatic organisms belong to eight families (See the Table). The most studied among them are members of the families *Coronaviridae*, *Tobaniviridae* and *Roniviridae*. The representatives of other families of aquatic animal nidoviruses have not been adequately studied.

Harbor seal coronavirus was first reported in the United States in 1987 in three common seals (*Phoca vitulina*) housed at the Seaquarium in Florida [3]. One animal exhibited leukocytosis, accompanied by dehydration and hyperchloremia, and two seals died without showing any clinical signs. The pathological examination of all three animals revealed extensive bronchoalveolar hemorrhages with severe diffuse pulmonary congestion. The spleen, visceral and peripheral lymph nodes were characterized by lymphoid depletion. Using immunofluorescence staining method and antisera against various coronaviruses, it was shown that positive results were observed only using antisera against alpha-coronaviruses (TGEV, FIPV, CCoV – canine coronavirus enteritis) and negative results were obtained when the antisera against bovine beta-coronavirus (BCoV) were used. Based on that, it was decided to assign this virus (HSCoV) to the genus *Alphacoronavirus*. A short nucleotide sequence of harbor seal coronavirus has been published in GenBank (NCBI, Acc. No. FJ766501) (https://www.ncbi.nlm.nih.gov/nuccore/FJ766501.1/).

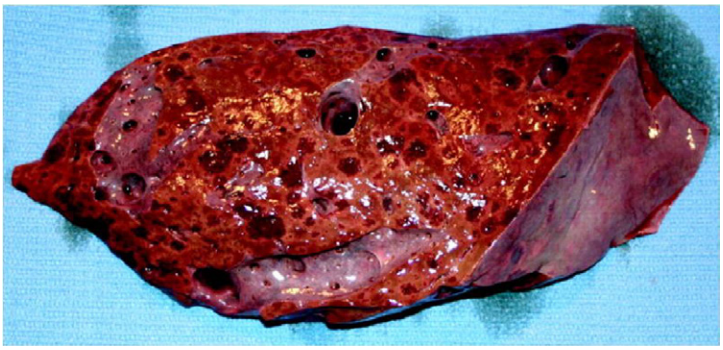
In June 2000 twenty-one individuals in a herd of resting Pacific seals (*Phoca vitulina richardsii*) were found dead on the central coast of California (Fig. 1) [4].

Three different viruses were isolated from pathological material obtained from the dead seals. Coronavirus was identified in five animals using polymerase chain reaction (PCR). The biopsy of the dead seals revealed pulmonary stagnation and extensive hemorrhages.

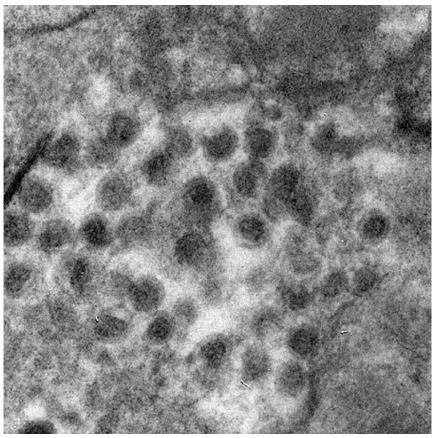
Coronavirus of beluga whale (*Delphinapterus leucas*) belonging to the family *Monodontidae*, order *Cetacea* and found in all coastal waters of the Arctic seas, including the White, Bering, Okhotsk, and occasionally Baltic seas, was first identified in the USA in 2008 and was named SW1 [5]. A captive-born male beluga whale died from acute liver failure after suffering an acute generalized pulmonary disease. Histological examination revealed numerous red-yellow hepatic necroses (Fig. 2).

Electronic microscopic examination of liver tissue revealed a great number of round viral particles 60–80 nm in diameter in infected tissues. Attempts to isolate the pathogen in continuous cell lines failed. The viral genome was approximately 31,700 nucleotides and encoded nonstructural (ORF1 and ORF1b) and structural (ORF2, ORF3, ORF4 and ORF11) proteins. The SW1 nucleotide sequence was deposited in GenBank (NCBI, Acc. No. EU111742). The deduced amino acid sequences showed no similarity to proteins from known coronaviruses in 2008. At present its close relationship with Bottlenose dolphin coronavirus has been established [6].

Coronavirus of bottlenose dolphin (*Tursiops truncatus*), inhabiting temperate and warm waters of the World Ocean, as well as the Mediterranean, Baltic and Black Seas, was discovered by Chinese researchers in 2014 [6]. The



A



B

Fig. 2. A. Necrosis areas in the liver of the beluga whale affected by SW1 coronavirus.
B. The aggregates of spherical viral particles in infected tissues [5] (https://jvi.asm.org/content/jvi/82/10/5084/F1.large.jpg)

Рис. 2. А. Некрозы в печени белухи, пораженной коронавирусом SW1. В. Скопления сферических вирусных частиц в инфицированных тканях [5]

virus was named BdCoV. Complete genome sequencing showed that BdCoV is closely related to Beluga whale coronavirus (SW1) and they both belong to gammacoronaviruses.

The BdCoV genome is 32,000 nucleotides and in 2014 it was the largest among all known coronaviruses. The large size of its genome is a result of multiple unique open reading frames (NS5a, NS5b, NS5c, NS6, NS7, NS8, NS9, NS10) located between the M and N genes. The major difference between the two abovementioned coronaviruses was observed in the proteins encoded by their spike (S) genes [6].

Microhyla alphaletovirus 1 (MLeV) was detected by metagenomic analysis of the common intracellular RNA pool of the ornamented pygmy frog *Microhyla fissipes* [7]. This frog is up to 3–4 cm in size and is widespread in many countries of Southeast Asia. The virus genome contains 22,304 nucleotides, the nucleotide sequence is available in GenBank (Acc. No. GECV01031551). In the process of frog metamorphosis the number of viral transcripts decreases by 7–14 times; they are not detected at all in adult frogs [7].

Carp fish bafiniviruses are classified in a separate genus (*Bafinivirus*), which is included in the family *Tobaniviridae*. The genus name is made up of the first two letters of the English words '**ba**cilla', '**fi**sh' and '**ni**dovirus' and is due to the fact that the virus has a bacilliform shape (Fig. 3).

The fish bafinivirus was first discovered in grass carp (*Ctenopharyngodon idella*) by German ichthyopathologists in 1987 [8]. Grass carp has been introduced to many countries and now ranks first in the world in terms of commercial rearing. The virus was reported in apparently healthy grass carp from Hungary during cross-border veterinary control. The virus isolate multiplied in CAR (continuous) cells (goldfish [*Carassius auratus*] fin cells), CLC cells (common carp [*Cyprinus carpio*] leukocytes) and FHM cells (fathead minnow [*Pimephales promelas*] caudal stem cells) at 15–25 °C. The infected cells fused, then lysed. Electron micrographs of affected cells revealed that the virus particles were bacilliform in shape measuring 170–220 nm in length and 50–55 nm in diameter. Staining with an orange acridine solution showed that the virus contains a single-stranded RNA. The virus is inactivated by chloroform, acid solutions (pH 3) and at a temperature of 56 °C.

A year later, a similar virus was reported in Japan during an outbreak of acute infection of common carp (*Cyprinus carpio*). Affected individuals demonstrated erythema on the abdomen and hepatic and renal necrosis. The virus is transmitted to carp fry via the water at a temperature of 20 °C. The bafinivirus was described in color carp (*C. carpio haematopterus*) in the same country, during the study of the disease named 'ana-aki-bio' [9]. Developed lesions were observed in visceral organs, the virus particles were identified in hematopoietic tissue and spleen. Infected epithelioma papillosum cyprin (EPC) cells displayed karyopyknosis and intracytoplasmic vacuolization.

The bafinivirus of white bream, or silver bream (*Blicca bjoerkna* L.), a fish species which is widespread in the basins of the Baltic, Black and Caspian seas, in the European and Caucasian countries, was first detected in Germany during studies of fish health in the natural environment [10]. The virus is named WBV in the English-language sources. The virus does not induce visible pathological changes in the white bream body, its effects are manifested only in continuous fish culture cells.

WBV particles are bacilliform in shape (130–160 nm in length and 37–45 nm in diameter). The virion is covered with a lipid envelope with surface projections (20–25 nm in length). The polyadenylated WBV RNA has five genes encoding open reading frame: ORF1a, ORF1b, ORF2, ORF3 and ORF4. The ORF1a/1b gene encodes pp1a and pp1ab polyproteins containing proteinase, polymerase and other replication enzymes common to all nidoviruses, and ORF2, ORF3, and ORF4 are the surface projection glycoprotein (S), membrane protein (M), and nucleocapsid (N) protein, respectively [11].

Fat-minnow bafinivirus (*Pimephales promelas*) was discovered in the USA in 1997 [12]. Minnows (*Phoxinus*) are the genus of small (not larger than 20 cm) freshwater fish in the family *Cyprinidae*. The virus is named *Fathead minnow virus* (FHMNV). It replicates in the EPC, FHM and RTG (rainbow trout gonads) cells at 15–25 °C. Syncytium is produced in FHMNV-infected cells.

The disease of the fathead minnow becomes evident by behavioral changes characterized by fish initially swimming erratically in circles before becoming listless either at the tank bottom or with their head orientated upward at the water surface. The course of the disease is consistent with the temperature. In 19 °C water mortalities accumulate rapidly from 3 days post-challenge, but 17 °C water delays mortality onset to 13 days post-challenge. Hemorrhages are observed on the skin as well as in the liver,

kidneys and spleen of infected individuals. Hemorrhages in the muscles may also be evident and the kidneys in many fish may become visible due to musculature edema and darkening of the trunk kidney. FHMNV is highly host-specific; channel catfish, goldfish and golden shiners are not FHMNV susceptible [12].

FHMNV virions are bacilliform in shape (130–185 nm in length and 31–47 nm in diameter). The complete nucleotide sequence of the virus has been established [11]. The virus single-stranded RNA contains 27,000 nucleotides and is similar to white bream coronavirus. Phylogenetic analysis using the conserved region in the gene helicase motif of FHMNV showed that it is most closely related to WBV. A comparison of the gene products of helicase (pp1ab domain), S, M, N, and ORF1ab with the WBV gene products shows different levels of homology ranging from 15 (protein S) to 70% (helicase) [13].

Chinook salmon (*Oncorhynchus tshawytscha*) **nidovirus** was discovered in Canada in 2014 [14]. The virus is named CSBV. It replicates and induces cytopathic effects in RTG-2 and EPC cells at the temperatures of 15, 20 and 25 °C. Viral particles are rod-shaped, 45 nm in diameter and 120–130 nm in length. The genome of the virus contains 27,004 nucleotides and the organization of genes corresponds to that of nidoviruses. The complete nucleotide sequence is available in the GenBank database (NCBI) (Acc. No. KJ681496). Based on the amino acid composition, this virus was found to be related to WBV and FHMNV [15].

Another salmonid virus was detected by Canadian researchers in Atlantic salmon (*Salmo salar*) [15]. The virus is named ASBV. Analysis of the complete nucleotide sequence of its genome revealed that it is 99% identical to Chinook salmon coronavirus (CSBV). However, it has a large deletion in the pp1a replicase polyprotein gene. Genome sequence analysis also revealed the alleged sixth protein, which may be the envelope protein [15, 16]. A broad spectrum of sensibility of continuous cell cultures to this virus may indicate a wide range of its hosts in the natural environment.

Crucian carp or goldfish (*Carassius auratus*) **nidovirus** was first described by Chinese researchers in 2019 [17]. The virus is named CCNV (*Crucian carp nidovirus*). The CCNV genome contains 25,971 nucleotides, it has five open reading frames encoding 1ab polyprotein (pp1ab), peplomer glycoprotein (S), membrane protein (M) and nucleocapsid protein (N). By the organization of the genome, this virus is related to Chinook salmon nidovirus.

Shrimp okavirus. Shrimp nidoviruses belong to the genus *Okavirus* in the family *Roniviridae*. The family name combines two words that designate the form of the virion (**ro**d-shaped) and the taxonomic name of the order (***Ni****dovirales*). The name of the genus *Okavirus* comes from the name of the shrimp lymphoid organ (in English 'oka'), in which the virus is most commonly detected. Okaviruses comprise a complex group consisting of six genotypes. Genotype 1 is the YHV shrimp okavirus that causes a disease called "yellow head" affecting tiger shrimp (*Penaeus monodon*); genotype 2 includes shrimp gill-associated okavirus (GAV); viruses of genotypes 3–6 are low pathogenic and do not cause symptoms or signs of the disease.

Yellow head disease (YHD) was first reported in Thailand in giant tiger shrimp. The disease onset is announced by a rapid abnormal increase in feed consumption followed several days later by an equally rapid cessation in feeding and swelling of the digestive glands. A day later

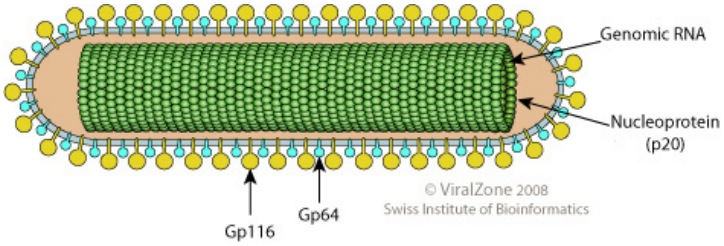


Fig. 3. Schematic structure of bafiniviruses (https://viralzone.expasy.org/resources/Roniviridae_virion.jpg)

Рис. 3. Схема строения бафинивирусов

infected individuals begin to swim erratically and restlessly at the water surface [18]. YHV can affect shrimp and other species, as well as some krill species. To date the virus has been detected in Southeast Asia, Australia, America, and East Africa [19, 20]. YHV leads to 90–100% shrimp mortality within 3–5 days after the onset of the first symptoms. It was commonly observed that diseased shrimps had bleached or yellowed gills and pale-yellow hepatopancreas (paired structures in the small intestine of invertebrates functioning as both the pancreas and liver). One more day later the shrimp cephalothorax becomes intensely yellow. Then the number of affected individuals increases sharply, leading to almost complete population losses within three days after the onset of symptoms. The YHV virus can remain viable in water at 25–28 °C for about 4 days after the infection resolves.

Shrimp larvae weighing 5–15 g are the most susceptible to GAV ovirus [21, 22]. Adult shrimps are also susceptible to the virus. Coronavirus infection occurs when shrimps scavenge on dead individuals or directly via contaminated water and infected nets or tools. The disease can be both acute and chronic, the pathogen is transmitted both horizontally and vertically. Shrimp mortality can be significant in case of acute-stage infection, and the virus can invade all tissues of mesodermal and ectodermal origin, most often – the lymphoid organ. Basophilic cytoplasmic inclusion bodies can be observed in necrotic cells.

Shrimp okavirus virions have an outer envelope and are rod-shaped with rounded ends measuring 40–60 × 150–200 nm. The envelope is covered with spikes (peplomeres) protruding about 11 nm above the surface of the virion. Nucleocapsids are 20–30 nm in diameter and appear as a coiled filament with a 5–7 nm periodicity. Long filamentous nucleocapsid precursors (approximately 15 nm in diameter and 80–450 nm in length) occur in the cytoplasm of infected cells, where they acquire envelopes by budding on the membranes of the endoplasmic reticulum. Newly formed mature virions often appear in the form of congregations, as a result of which paracrystalline arrays may be visualized.

Shrimp okaviruses contain one linear segment of a single-stranded positive-sense RNA, the length of which varies from 26,235 nucleotides for the GAV virus to 26,662 nucleotides for YHV. The YHV okavirus ORF4 is significantly shorter as compared to that of other known genotypes, and cannot always be expressed. The GAV genome complete sequence and YHV genome partial sequences are available [21].

Along with the *Okavirus* genus, a new genus only represented by the nidovirus isolated from the Chinese mitten crab *Eriocheir sinensis* is supposed to be assigned to

the family *Roniviridae* [23]. This crab is a dangerous invasive species that has spread from the Yellow Sea to many European and North American countries. It is found in the Karelian reservoirs and the Volga River. The crab nidovirus (EsRNV, *Eriocheir sinensis ronivirus*) causes the so called "sighs disease" because affected crabs make sounds similar to sighs at night. EsRNV virions are rod-shaped, 16–18 nm in diameter and 15–20 nm, occasionally up to 400 nm, in length. Experimental infection of crabs resulted in 100% mortality within 13–17 days. The viral particles are found in the connective tissues of many organs, including gills, hepatopancreas, heart, intestines, and ovaries [23].

The genomes of the members of the family *Euroniviridae* were studied using bioinformatic methods, but the biological properties of these nidoviruses are not yet well known.

Planaria nidovirus was discovered at the end of 2018 in the Mediterranean planaria *Schmidtea mediterranea* [24]. This is a type of flatworm that lives in fresh water on the islands of the Mediterranean Sea, in Spain and Tunisia, and is particularly useful for studying regeneration processes. Planaria nidovirus was detected by metagenomic analysis of a common pool of intracellular RNA. The virus was named *Planarian secretory cell nidovirus* (PSCNV). It is propagated in planarian secretory cells. The electron microscope examination demonstrated presence of spherical, slightly elongated virus particles, associated with the endoplasmic reticulum membranes, 90–150 nm in diameter, in the cytoplasm of affected cells. The PSCNV genome contains 40,671 nucleotides [24]. It is currently the largest genome among all known RNA viruses.

The main difference between PSCNV and other nidoviruses is the presence of an unusually large reading frame ORF1b in its genome. It is suggested that PSCNV diverged early from nidoviruses and acquired additional genes typical of large DNA viruses, namely genes encoding ankyrin and fibronectin. Newly acquired genes may significantly affect the virus-new host interaction [24].

Mosquito nidovirus was detected in Vietnam by metagenomic analysis from a pool of blood-sucking mosquitoes of the genus *Culex* [25]. The virus was named Nam Dinh (NDiV) due to the name of the area where mosquitoes were collected for research. It does not cause pathological changes in mosquitoes. The virus replicates in the continuous mosquito cell line C6/36 derived from the *Aedes albopictus* members. Virions are spherical, 60–80 nm in diameter. The virus genome contains 20,192 nucleotides and has 5 open reading frames. Based on its genomic structure this virus was assigned to a separate family *Mesoniviridae*, which is in transition from "large" to "small nidoviruses" (*Arteriviridae*).

Nidovirus of aplysia, or sea hare (*Aplysia*), one of the largest herbivorous mollusks reaching 1 m in length and living in warm and subtropical seas, was simultaneously described by two independent research groups [7, 26]. Like the Planaria virus, the Aplizia nidovirus was also detected in the gastropod mollusk *Aplysia californica* by metagenomic analysis of the common pool of intracellular RNA. The BLAST bioinformatics analysis showed that based on its genome structure the virus has properties that are typical for nidoviruses. The virus is named AAbV. The genome contains 35,906 nucleotides, which indicates its belonging to the "large" nidoviruses. The largest amount of virus RNA are found in neurons, as well as it is detected in the gills, salivary glands and muscles. Similar nidoviruses have been described in tunicates (*Botrylloides leachi*), sea snails (*Turritella* sp.) and other aquatic organisms [27, 28].

CONCLUSION

The analysis of the literature available on this topic reveals that the application of the genomic identification principles based on metagenomics gives the possibility of detecting an unpredictable number of new viruses, as it follows from how nidoviruses spread among aquatic animals. The study of the global virosphere significantly expands information on potential reservoirs of emerging pathogens [29]. It is generally recognized that their overwhelming majority are of zoogenic origin [30]. The interest has recently increased significantly in this group of viruses due to the outbreak of severe acute coronavirus infection COVID-19 [31]. Like other single-stranded RNA viruses, nidoviruses have a relatively high ability to mutate and recombine, which allows them to quickly adapt to new hosts and new ecological niches [2, 13, 32]. For instance, SARS-CoV-1-like coronaviruses have been isolated from humans, Himalayan palm civets and raccoon dogs [33]. Phylogenetic analysis of the genomes of various animal nidoviruses suggests that many of them can be descendants of aquatic animal nidoviruses.

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Studies of clinical symptoms of panleukopenia in cats in the Donetsk People's Republic

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SUMMARY

Feline panleukopenia is a highly contagious viral disease of the members of the family *Felidae* caused by a DNA-virus of the family *Parvoviridae*. After infection, the virus is detected in saliva, nasal discharge, urine and feces; it is transmitted through excrements, water, food, and, according to some reports, by blood-sucking insects. The disease is characterized by gastrointestinal tract, central nervous system, hematopoietic tissue lesions. The paper describes the clinical signs of panleukopenia in cats in the territory of the Donetsk People's Republic. Based on the records of a state-financed veterinary hospital and a charitable animal shelter located in the town of Yasinovataya, as well as those of private veterinary clinics and a veterinary office located in Donetsk, a classification of the clinical signs of the disease in pet and stray cats is suggested with the signs grouped according to their occurrence rate and by age groups. The paper provides evidence of feline panleukopenia occurrence in the territory of the Donetsk People's Republic. In particular, the period from 2015 to 2018 was characterized by an increase in panleukopenia morbidity in stray animals; pet cats were also involved in the epidemic process. It was found that most of the clinical symptoms of panleukopenia were reported in cats of all age categories irrespective of their sex. Specific symptoms characteristic for different age groups were reported. It was found that panleukopenia morbidity rates for stray kittens aged 0–12 months were 1.8 times higher than those for pet kittens of the same age group. The disease was reported in adult pet cats 2.4 times less frequently than in stray cats. It is shown that the number of the disease cases in female cats is 1.5 times higher than in male ones. It is concluded that the violation of quarantine rules and insufficient vaccination coverage in cats are risk factors for the disease outbreak occurrence precipitating the multiple recurrent outbreaks of the disease.

Key words: panleukopenia, cats, clinical symptoms, morbidity.

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Изучение клинических симптомов при панлейкопении у кошек на территории Донецкой Народной Республики

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РЕЗЮМЕ

Панлейкопения кошек – высококонтагиозное вирусное заболевание представителей семейства кошачьих, возбудителем которого является ДНК-содержащий вирус из семейства *Parvoviridae*. После заражения вирус обнаруживается в слюне, в отделяемом из носа, моче и фекалиях, распространяется через экскременты, с водой и пищей, а также, по некоторым данным, через кровососущих насекомых. Болезнь сопровождается поражениями органов желудочно-кишечного тракта, центральной нервной системы, кроветворной ткани. В статье рассмотрены клинические признаки у кошек при заболевании панлейкопенией на территории Донецкой Народной Республики. На основании отчетных документов государственной больницы ветеринарной медицины и благотворительного питомника города Ясиноватая, а также частных ветеринарных клиник и ветеринарного кабинета города Донецка предложена классификация клинических признаков заболевания среди домашних и бродячих кошек, сгруппированных по частоте встречаемости и возрастным группам. Показано, что территория Донецкой Народной Республики является неблагополучной по заболеваемости панлейкопенией среди кошек. Так, за период с 2015 по 2018 г. ситуация характеризовалась ростом заболеваемости бродячих животных с вовлечением в эпизоотический про-

цесс домашних кошек. Установлено, что большинство клинических симптомов панлейкопении регистрируются у всех возрастных категорий кошачьих вне зависимости от половой принадлежности. Отмечены специфические симптомы, характерные для разных возрастных категорий. Установлено, что показатели заболеваемости панлейкопенией бродячих котят возрастной группы 0–12 месяцев в 1,8 раза выше по отношению к домашним котятам той же возрастной категории. У взрослых домашних кошек заболевание регистрировали в 2,4 раза реже по сравнению с бездомными кошками. Показано, что количество случаев заболевания среди самок в 1,5 раза выше по сравнению с заболеванием среди самцов. Сделан вывод, что нарушение правил карантина и недостаточный охват кошек вакцинацией являются факторами риска возникновения очагов заболевания и приводят к многократным повторным вспышкам болезни.

Ключевые слова: ветеринария, панлейкопения, кошки, клинические симптомы, заболеваемость.

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INTRODUCTION

Feline panleukopenia continues to be a topical issue in feline infectious disease pathology in spite of the fact that it can be relatively effectively prevented by vaccination [1–5]. Feline panleukopenia (FPL) is a highly contagious infectious disease of the members of the family *Felidae* caused by a DNA-virus of the family *Parvoviridae*.

Just a few decades have passed since the disease was first reported; the results of the studies published with regard to this topic indicate that panleukopenia morbidity and mortality show persistent upward trends [1, 6–8]. In particular, K. Van Brussel et al. [8] described the outbreaks of feline panleukopenia between 2014 and 2018 in Australia, New Zealand and the United Arab Emirates (UAE) where the disease had not been reported for several decades. The authors hold the view that each of these outbreaks was caused by a distinct feline panleukopenia virus (*Felis parvovirus*, FPV) with two viral lineages present in eastern Australia. The viruses from the UAE outbreak formed a lineage of unknown origin. The authors explain the new reported cases of the disease by the fact that feline panleukopenia viruses circulating in the feral cat population may occasionally infect pet cats [6, 8, 9].

The examples found and analyzed by the authors allow to identify the following pattern: the violation of quarantine rules and insufficient vaccination coverage in cats are common factors in all the disease outbreaks precipitating the multiple recurrent outbreaks of the disease [6, 8, 10].

According to the literature data, animal shelters are conducive environments for the disease outbreak occurrence or recurrence because of a large number of susceptible animals living together in a confined area [1, 4]. It should be emphasized that such factors as young age, immunological naivety or immunosuppression, close contact and co-morbidities (the presence of endo- and ectoparasites) exarcebate the disease progression [2, 7]. Issues related to the spread, prevention and treatment of panleukopenia [1, 2, 4, 5, 9, 11, 12], as well as the studies and detailing of the clinical signs that can most accurately characterize this pathology [7, 11, 13–15] still remain relevant for veterinary practitioners.

In view of the above, the aim of this paper was the detailed analysis of the clinical signs of panleukopenia in pet and stray cats in the territory of the Donetsk People's Republic.

MATERIALS AND METHODS

To achieve the aim of the paper, commonly available research methods were applied when studying the documentary records of veterinary clinics and analyzing the findings. The following types of veterinary records were used: a veterinary case log, an infectious disease registration log, animal patients' records.

The monitoring studies were conducted at the facilities of the Yasinovataya municipal state-financed veterinary hospital, the charitable animal shelter of the LLC "RPA "Yasinovataya machine-building plant", the private veterinary office "Invet", as well as of the veterinary clinics ("Bagira", "Aybolit", "Dingo") of the city of Donetsk.

The cases covered by the study included the diseased cats that had received ambulatory care in the period from 2015 to 2018.

The findings were statistically processed and presented in the form of a diagram and tables.

RESULTS AND DISCUSSION

During the reporting period, 1,216 cats with the signs of panleukopenia were reported. The diagnosis was made based on the case history, clinical signs and confirmed by laboratory tests of blood (a complete blood count and a biochemical analysis). For blood tests, commercial immunochromatography assay (ICA) test kits were used in 60% of cases.

The reported clinical signs of panleukopenia in cats were analyzed and grouped according to their occurrence rate and by the age of animals. The obtained results are presented in Table 1.

Data in Table 1 show that cats of all ages can be infected by panleukopenia virus, but young kittens (the 1–6 month age group) are the most susceptible: the body stops resisting when colostral antibody levels decline to the threshold value, and the kittens develop the pronounced clinical signs of the disease.

Primary symptoms in this category of animals include the following: apathy, hyperthermia, ataxy, crouched posture, exicosis, thirst (however, animals refuse to drink); dry, cyanotic oral mucosa; edematous laryngeal mucosa; cachexia; dull dishevelled coat; dry and atonic skin.

Besides, non-specific symptoms (48.8%) were reported in most kittens. The following gastrointestinal symptoms (11.8%) were recorded: vomiting (foamy vomit with

Table 1
Clinical signs of panleukopenia in cats

Таблица 1
Клинические признаки при заболевании панлейкопенией кошек

Symptoms	Age group				
	1–6 months	6–12 months	1–6 years	6–12 years	over 12 years
Primary	142	93	72	28	21
%	35.0	38.0	23.7	15.2	27.3
GIT	48	54	37	51	16
%	11.8	22.0	12.2	27.7	20.8
CNS	18	3	–	1	8
%	4.4	1.2	0.0	0.5	10.4
Reproductive system	–	–	38	17	4
%	0.0	0.0	12.5	9.2	5.2
Non-specific	198	95	157	87	28
%	48.8	38.8	51.6	47.4	36.3
Total number of animals (100%)	406	245	304	184	77

GIT – gastrointestinal tract (желудочно-кишечный тракт);
CNS – central nervous system (центральная нервная система).

admixed bile and blood), after abdominal palpation – pain and vomiting, diarrhea (voluminous, watery, often with admixed blood and fibrin, foamy). Palpation revealed non-mobile, thickened, painful (resembling a rubber tube), fluid- and gas-dilated intestinal loops; a succussion splash was heard on auscultation. The disease was also characterized by other symptoms such as rhinitis, optic nerve atrophy, retinal dysplasia, seizures, paresis, paralysis of limbs and internal organ sphincters, head tremor (cerebellar hypoplasia), often followed by death, and these may be indicative of intrauterine infection of kittens from the cats that had not been vaccinated against panleukopenia or from the virus carrier cats.

In animals aged between 6 and 12 months primary and non-specific symptoms were mainly reported (38.0 and 38.8% respectively), whereas central nervous system symptoms were reported only in 3 cases (1.2%), and this may be indicative of the fact that the animals had not been intrauterinally infected, but had contracted the disease independently. The signs of central nervous system disorders can also be regarded not as stand-alone ones, but as the symptoms of intoxication.

The following symptoms were reported in pregnant queens (the 1–12 year age group): during the first stage of gestation – fetal death and resorption, return to estrus; during the second stage – abortion or passage of mummified fetuses; during the third stage – congenital malformations (the replication and spread of the virus in the lymphoid tissues, eyes and nervous system of the fetus resulting in hydrocephalus, ophthalmic abnormalities and, first and foremost, in cerebellar hypoplasia). As for the male cats of this age group, unproductive copulations were reported.

Along with primary and non-specific symptoms of panleukopenia, such symptoms as moist rales (pulmonary edema), optic nerve atrophy were often reported in cats aged over 12 years. The last-mentioned symptom is ambiguous, since it is associated with age-related changes rather than with the disease. Gastrointestinal symptoms (20.8%) also require further differential diagnosis taking into account age-related changes. The symptoms of reproductive disorders (5.2%) mostly manifest themselves in the form of spontaneous abortions and return to estrus.

The figure shows the percentages of reported clinical symptoms of feline panleukopenia by age groups.

Panleukopenia morbidity in pet and stray cats was also analyzed in terms of age and sex. The results are presented in Table 2.

According to the data presented in Table 2, the number of panleukopenia cases in stray kittens aged 0–12 months is 1.8 times higher than in pet kittens of the same age group. Panleukopenia cases in adult stray cats of the 1–12 year age group were reported 2.4 times more frequently than in pet cats of the same age group. This is due to the fact that pet cats, unlike stray ones, are kept under supervision and any changes in the animal's behavior can prompt the owner to contact the veterinarian. The number of diseased kittens is 2.1 times higher than that of adult cats, and the number of diseased stray cats is 1.9 times higher than that of pet cats. Morbidity rates for female cats are 1.5 times higher than for male cats.

As a result of the study, it was found that most of the clinical symptoms of panleukopenia were reported in cats of all age groups irrespective of their sex. However, attention should be paid to the fact that specific symptoms characteristic for certain age groups were reported.

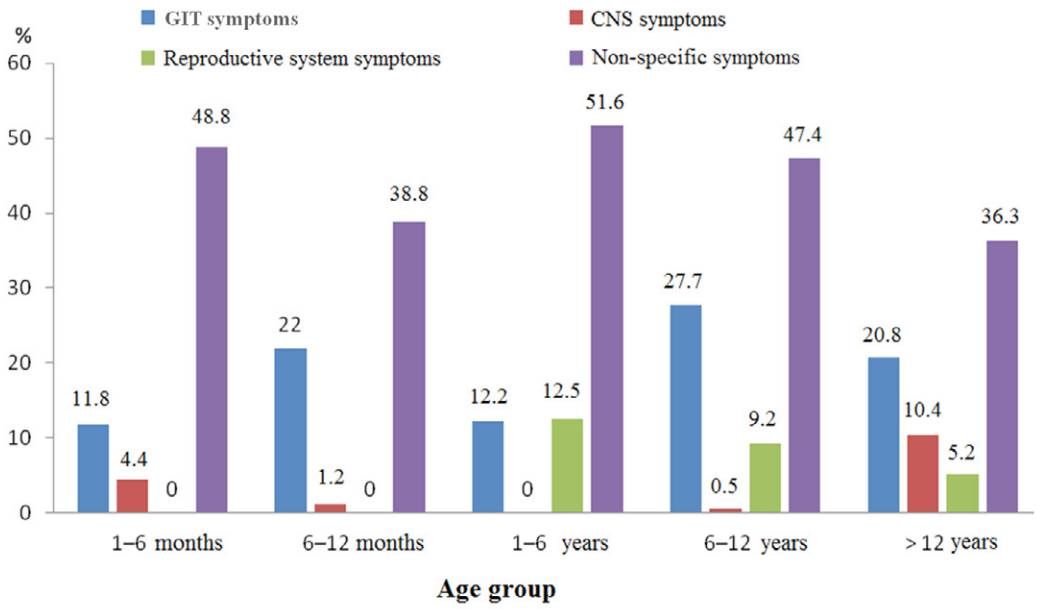

Fig. Reported symptoms of feline panleukopenia by age groups, %

Рис. Регистрируемые симптомы при панлейкопении кошек по возрастным категориям в процентах

Besides, it should be noted that the period from 2015 to 2018 was characterized by an increase in panleukopenia morbidity in stray animals; pet cats were also involved in the epidemic process.

The analysis of data from the studies reveals the following pattern: an increase in morbidity occurs early in the year because of the feline mating season; the next peak occurs in spring and is associated with the loss of colostral immunity in young animals; another one that occurs in autumn and winter period results from the dispersal of young animals seizing new territories and related conflicts. The reduction of the rates is observed during the offspring raising period when adult cat movements are limited and young animals have not yet become independent.

Summing up the intermediate results, it can be stated that the following measures still remain relevant: activities to raise awareness with regard to observance of veterinary and sanitary, as well as animal hygiene measures; balanced feeding, appropriate care and maintenance of cats in homes and in animal shelters; mandatory quarantine of all the animals entering catteries and shelters; obligatory disinfection (of animal facilities, tools, equipment and supplies), disinsection and deratization activities. Spaying and neutering of stray and pet cats contribute to the improvement of the epidemic situation.

We see the prospects for further research on this issue in more detailed studies and improvement of diagnostic methods, as well as in refinement of existing treatment regimens for this pathology. In our view, systemic disease prevention measures help reduce morbidity in pet and stray cats, and this is directly related to the annual vaccination of the entire animal population.

CONCLUSION

Feline panleukopenia persists in the territory of the Donetsk People's Republic. The period from 2015 to 2018 was characterized by an increase in panleukopenia morbidity in stray animals; pet cats were also involved in the

Table 2
Panleukopenia morbidity rates in cats by age and sex

Таблица 2
Характеристика заболеваемости кошек панлейкопенией по возрастным и половым признакам

Cat age/sex	Stray cats		Pet cats		Total number of cases
	animals	%	animals	%	animals
Kittens aged 0–12 months	526	64.3	292	35.7	818
Adult cats aged 1–12 years	282	70.9	116	29.1	398
of which – female	144	60.5	94	39.5	238
– male	51	31.9	109	68.1	160
Total	808	–	408	–	1,216

epidemic process. Notably, most of the clinical symptoms of panleukopenia were reported in cats of all age groups irrespective of their sex. Specific symptoms characteristic for different age groups were also reported.

Panleukopenia morbidity rates for stray kittens aged 0–12 months were 1.8 times higher than those for pet kittens of the same age group. The disease was reported 2.4 times less frequently in adult pet cats than in stray cats. The number of the disease cases in female cats is 1.5 times higher than in male ones.

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Agr-typing of methicillin-susceptible *Staphylococcus aureus* (MSSA) isolated from non-human primates

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SUMMARY

Staphylococcus aureus (*S. aureus*) is a microorganism that causes a great number of diseases in humans and animals, including sepsis, pneumonia, food toxico-infections, wound abscess, etc. Numerous studies on genotyping *S. aureus* strains isolated from humans, food and mastitis in cattle and small ruminants have been carried out. The lack of information on the genotyping of methicillin-susceptible *S. aureus* detected in monkeys served as a stimulus to conduct a similar research, since staphylococcal infections in the primates are widespread. The present study is devoted to molecular genetic testing of *S. aureus* isolated from different biological samples taken from monkeys and is based on typing of *agr* polymorphic locus which acts as a regulator of pathogenic gene expression. As a result of PCR analysis of 301 *S. aureus* isolates it was established that most of *S. aureus* belonged to *agr* IV (55%), and *agr* I (34%) was the second most group. Data resulting from the study differ from the results of other researchers published in literary sources, who performed typing of *Staphylococcus* isolated from people with *agr* I prevailing. In conducting the study, neither distinct correlation between microbial isolation source and *agr* complex groups, nor relationship between the diseases and *S. aureus* group specificity were detected. Prevalence ratio of each *agr* group is nearly similar in *S. aureus* isolated from rhesus macaques and crab-eating macaques. But in hamadryas baboons and green monkeys II and III groups of *agr* complex were not detected.

Key words: monkeys, *Staphylococcus aureus*, *agr* complex, *agr* groups and alleles.

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Agr-типирование метициллин-чувствительных *Staphylococcus aureus* (MSSA), выделенных у низших приматов

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РЕЗЮМЕ

Staphylococcus aureus (*S. aureus*) – микроорганизм, вызывающий большое количество заболеваний у человека и животных, включая сепсис, пневмонии, пищевые токсикоинфекции, нагноение ран и другие. По генотипированию штаммов *S. aureus*, выделенных у людей, из пищевых продуктов и при маститах у крупного и мелкого рогатого скота, проведено много исследований. Отсутствие данных по типированию метициллин-чувствительных *S. aureus*, обнаруженных у обезьян, побудило провести аналогичное исследование, поскольку инфекции стафилококковой природы у приматов широко распространены. Настоящая работа посвящена молекулярно-генетическому исследованию *S. aureus*, изолированных из разных биологических образцов от обезьян, на основе типирования полиморфного локуса *agr*, являющегося регулятором экспрессии генов патогенности. В результате исследования методом полимеразной цепной реакции 301 изолята *S. aureus* установлено, что большинство *S. aureus* относилось к группе *agr* IV (55%), на втором месте по распространенности оказался *agr* I (34%). Полученные в ходе исследования данные отличаются от опубликованных в литературных источниках результатов других исследователей, которые проводили типирование стафилококков, выделенных от людей, у которых превалирует *agr* I. При проведении работы не

выявлено четкой корреляции между источником выделения микроба и группами комплекса *agr*, а также не отмечена связь между заболеваниями и принадлежностью *S. aureus* к определенной группе. Соотношения распространенности каждой группы *agr* примерно одинаковы у *S. aureus*, изолированных у макаков-резусов и макаков яванских, но у павианов гамадрилов и мартышек зеленых II и III группы комплекса *agr* не обнаружены.

Ключевые слова: обезьяны, *Staphylococcus aureus*, комплекс *agr*, группы и аллели *agr*.

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INTRODUCTION

Infections caused by *Staphylococcus aureus* (*S. aureus)* play a significant role in veterinary science and medicine. Although *S. aureus* is a commensal microbe first of all, it can also cause a wide spectrum of diseases in humans and animals, which can significantly vary in their severity (skin infections, meningitis, endocarditis, osteomyelitis, bacteremia, toxic shock syndrome, food poisoning, mastitis, abscess, pneumonia, etc.). Being a zoonotic pathogen, it is responsible for infectious diseases characterized by septicemia and sepsis [1]. *S. aureus* has developed a complex regulatory network for control of virulence factors' production in order to survive and adapt to different ecological niches. One of the main functions of this interrelated network is the perception of different environmental signals and reaction by changing the production of virulence factors essential for survival in host, including cell-surface adhesins, extracellular enzymes and toxins. Accessory gene regulator (*agr*) system is considered to be the most investigated. It represents a gene cluster which regulates the expression of different housekeeping genes, various virulence factors and adhesive proteins recognizing the macromolecules of host cells and is also involved in quorum sensing. *Agr* locus is characterized by the polymorphism of its autoinducing peptide (AIP). Four main *agr* groups (I, II, III, IV) can be distinguished according to their variable regions upon which *agr*-typing is based [2–4]. It is established by the molecular genetic testing that *agr* groups are spread in different geographic zones, but at the same time detection of the prevailing groups may vary in each region [3].

There is little information available in literature on investigation of *S. aureus* isolated from animals. S. Monecke et al. provide data on *S. aureus* research in wild and exotic animal species, being kept in captivity [5]. In some cases, the molecular typing proved that human strains were transmitted to these animals. Many studies are devoted to the genetic peculiarities of *S. aureus* recovered in bovine mastitis [6–11]. Molecular genetic testing of *S. aureus* isolated from monkeys is performed at Adler monkey farm relatively recently [12–14]. No studies on *agr*-typing of *S. aureus* isolated from monkeys have been carried out abroad. Thus, given the important role of *agr* genes, this study was aimed at detection and identification of *agr* groups of *S. aureus* isolates recovered from different biological samples taken from monkeys.

MATERIALS AND METHODS

Recovery and identification of bacterial isolates. S. aureus isolates (301 isolates) recovered from different monkey species kept at Adler monkey farm are included in the study. *S. aureus* isolates were recovered from feces ($n = 62$) and nasal mucosa ($n = 32$) of live animals and from different organs of dead animals: lungs ($n = 101$), liver ($n = 36$), spleen ($n = 26$), kidneys ($n = 17$), lymph nodes ($n = 27$). For the purpose of the study, lungs were taken from monkeys died of pneumonia, and other organs – from monkeys with intestinal diseases.

Bacteriological and biochemical testing using standard methods were carried out for staphylococci isolation, as previously described [13, 14]. Identification was performed based on morphological, tinctorial and biochemical properties. Species identification was performed using commercial tests systems "Multimicrotests for biochemical identification of staphylococci (MMT C)" (NPO "Immunotex", Russia).

S. aureus strain DNA extraction. Total staphylococci DNA extraction was performed from bacterial suspensions prepared from day-old agar *S. aureus* cultures and suspended with 100 µl of NaCl solution using DNA-sorb-B reagent kit (InterLabService Ltd., Russia) according to the manufacturer's instruction.

S. aureus mecA gene detection with PCR. Polymerase chain reaction with hybridization-fluorescence detection was carried out using commercial AmpliSens® MRSA-screen-titre-FRT test system (InterLabService Ltd., Russia) in Rotor-Gene fluorescence-detecting thermocycler (USA) according to the attached instruction.

Agr complex allele detection and typing. Agr-typing of specific groups was performed using primers described in the scientific publications [4], and designed by the Evrogen (Russia) (Table 1).

Ready-to-use ScreenMix-HS amplification mixes (Evrogen, Russia) were used for multiplex PCR; final volume – 25 µl for the reaction. Amplification was performed in "Tercyc" thermocycler (Company DNA-Technology LLC, Russia) according to the following programme: predenaturation – at 95 °C for 5 min; denaturation – at 95 °C for 10 sec, annealing – at 50 °C for 10 sec, prolongation – at 72 °C for 20 sec (32 cycles) and final elongation – at 72 °C for 5 min.

Gel-electrophoresis. Amplification products were visualized with 1.2% agarose gel electrophoresis stained with ethidium bromide solution at voltage gradient of 90 V. Amplicon sizes were determined using 100–1200 base pair DNA marker (Evrogen, Russia).

Table 1
***Agr* locus genes and primers used in the analysis**

Таблица 1
Гены локуса *agr* и праймеры, использованные в исследовании

Gene/locus	5′–3′ Sequence	Scale, μmol	Amplicon (base pairs)
agr loci (*agrB*)	F: ATGCACATGGTGCACATGC	0.6	
agr I	R: GTCACAAGTACTATAAGCTGCGAT	0.4	441
agr II	R: TAT TAC TAA TTG AAA AGT GGC CAT AGC	0.4	575
agr III	R: GTAATGTAATAGCTTGTATAATAATACCCAG	0.4	323
agr IV	R: CGATAATGCCGTAATACCCG	0.4	659

RESULTS AND DISCUSSION

S. aureus isolates (301) were identified using phenotypic and biochemical tests. All tested cultures demonstrated hemoltical and lecithinase activity, fermented mannitol under anaerobic conditions, coagulated rabbit plasma. The results obtained completely matched (100%) the results of PCR with hybridization-fluorescence detection, which confirmed *Staphylococcus* type and showed the lack of mecA gene isolates in the genome, i.e. all the cultures were methicillin-susceptible (MSSA).

As a result of *agr*-typing, most of MSSA were attributed to *agr* group IV (55%). *Agr* I (34%) was the second frequent type. Detection frequency of other groups was far below (Table 2).

As Table 2 shows, *agr* complex allele IV dominated almost in all MSSA isolates recovered from the organs of dead monkeys, fecal and nasal cultures. Frequency of its detection varied from 41% in *S. aureus* isolated from kidneys to 61% in *S. aureus* isolated from the lymph nodes. However, no allele III was detected in 27 *S. aureus* isolates recovered from the lymph nodes.

Agr IV group also prevailed in MSSA isolated from monkeys of the following species: macaque (rhesus macaque, crab-eating macaque) and hamadryas baboons (Table 3). No *agr* complex groups II and III were detected in *S. aureus* recovered from hamadryas baboons and green monkeys.

As it is known, *S. aureus* is a commensal microorganism of mammal microbiota, but at the same time it expresses different pathogenicity factors becoming the cause of various hospital-acquired and community-acquired infections. Secretion of various surface cell proteins, toxins and adhesins is regulated by *agr* locus. Method of *S. aureus* classification on the basis of *agr*-typing was firstly used by P. Dufour et al. [2], who divided isolates of this microbe into four groups. This division is based on *agrC* gene encoding the autoinducing peptide receptor and *agrD* gene encoding the autoinducing peptide [2, 4]. *S. aureus* strains recovered from humans are characterized by four *agr* system allele groups connected with the genetic background and presence of the pathogenicity factors [15]. However, the relative distribution of *agr* groups in *S. aureus* isolates recovered from monkeys is still unknown.

Numerous studies have established that different *agr* groups may be associated with certain virulence factors and diseases caused by *S. aureus* [1, 15, 16]. It was determined that *S. aureus* isolates recovered from humans, belonging to *agr* I represent a group consisting of hospital-acquired and community-acquired isolates,

Table 2
***S. aureus* isolates belonging to *agr* groups according to the isolation source**

Таблица 2
Принадлежность изолятов *S. aureus* к *agr*-группам в зависимости от источника выделения

Source of isolation	Number of isolates according to *agr* groups, *n* (%)				Total
	agr I	*agr* II	*agr* III	*agr* IV	
Lung	35 (35%)	5 (5%)	3 (3%)	58 (57%)	101
Liver	12 (33%)	3 (8%)	2 (6%)	19 (53%)	36
Spleen	8 (31%)	1 (4%)	2 (8%)	15 (57%)	26
Kidney	8 (47%)	1 (6%)	1 (6%)	7 (41%)	17
Lymph node	11 (35%)	2 (4%)	–	14 (61%)	27
Nasal swab	13 (41%)	2 (6%)	1 (3%)	16 (50%)	32
Feces	16 (26%)	8 (13%)	3 (5%)	35 (56%)	62
Total	103 (34%)	22 (7%)	12 (4%)	164 (55%)	301

Table 3
***Agr* group distribution in *S. aureus* isolates obtained from different monkey species**

Таблица 3
Распространение *agr*-групп у изолятов *S. aureus*, полученных от различных видов обезьян

Monkey species	Number of isolates according to *agr* groups				Total
	agr I	*agr* II	*agr* III	*agr* IV	
Rhesus macaque	39	7	3	64	113
Crab-eating macaque	32	11	7	55	105
Southern pig-tailed macaque	3	2	1	4	10
Green monkey	4	–	–	5	9
Anubis baboon	5	1	1	8	15
Hamadryas baboons	15	–	–	25	40
Bear macaque	2	1	–	–	3
Capuchin monkey	1	–	–	2	3
Patas monkey	2	–	–	1	3
Total	103	22	12	164	301

rare sporadic strains; *agr* II and *agr* III groups are mainly hospital-acquired epidemiologic clones; *agr* IV – is a rare group, resembling *agr* I [17]. Staphylococci belonging to *agr* III group are associated with invasive diseases, such as bacteremia [3]. *S. aureus* comprising *agr* I groups are capable to penetrate into epithelial cells and cause mastitis in cows and sheep [9].

Studies of foreign and domestic researches demonstrate that allele I is prevailing among *S. aureus* isolates recovered from humans in different geographic regions [4, 5, 16]. In some research works aimed at investigation of *S. aureus* isolated in cow mastitis *agr* I was also dominating [3, 5, 7, 11], but in other studies *agr* II and *agr* III took the first place [8]. *Agr* IV group in mastitis of cattle and small ruminants was found in very rare cases or was not found at all [5, 7, 8, 11]. It is described in works of L. M. De Almeida et al. [9] that alleles I and II were detected in *S. aureus* in sheep milk and sheep mastitis. Data on distribution of *agr* groups in *S. aureus* isolated at pig farms were also various. Indeed, in such cases some authors [18] report on detection of only *agr* I in *S. aureus*, but the others – only of *agr* IV group [1]. It is probable that the spread of the given alleles of gene regulating complex in *S. aureus* isolated from animals, is connected with the geographical region, as well as in humans.

In this investigation, most of MSSA isolates recovered from monkeys were attributed to *agr* IV group (55%), and *agr* I (34%) was the second most group. *Agr* II alleles were detected in 7% and *agr* III – in 4% of tested isolates. *Agr* IV group dominated in staphylococci isolated from monkey lungs affected with pneumonia and in those isolated from the organs affected by intestinal infections. According to the research results it is established that the isolates colonizing monkey nasal mucosa and recovered from kidneys equally belong to *agr* I and *agr* IV. Allele IV of the tested complex is 20–30% more likely to be found in *S. aureus* detected in other organs and fecal samples. It is also mentioned in the study that the prevalence ratio of each *S. aureus agr* group is nearly similar, in case with rhesus macaques and crab-eating macaques. But no alleles II and III were detected among all the tested hamadryas baboons and green monkeys. Data obtained in the course of the work differ from those concerning the distribution of *agr* complex alleles in *S. aureus* strains isolated from humans, where *S. aureus agr* I groups are prevailing [2–4, 15].

The recent study suggests further molecular-genetic examination with *spa*- and *coa*-typing of isolates in order to understand the epidemiology of *S. aureus* in monkeys at monkey farm and compare the obtained results with the molecular characteristics of epidemiological clones recovered from humans.

CONCLUSIONS

1. *Agr* IV group dominated in *S. aureus* isolates recovered from monkeys.

2. No correlation between the source of microbe isolation and *S. aureus* distribution to a certain *agr* group was detected.

3. No *agr* complex alleles II and III were detected in *S. aureus* isolates recovered from hamadryas baboons and green monkeys.

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Studies of biofilms and phenotypic characteristics of *Candida* fungi

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SUMMARY

Yeast-like fungi of the *Candida* genus are causative agents of the infectious lesions of the mucous membrane of the gastrointestinal, respiratory, urogenital tracts and skin of mammals, sepsis, and disseminated infection in birds. The search and testing of multilevel algorithms for biofilm identification when exposed to chemotherapeutic and disinfectant drugs for blocking the synthesis or destruction of the intercellular matrix in the development of superficial, deep and systemic candidiasis of animals are relevant for developing and improving diagnostic and antiepidemic measures. It was established that the formation of biofilm heterogeneous structure comprises multiple stages implementing the processes of intercellular communication due to the synthesis of a polymer matrix composites. Optical microscopy revealed a three-dimensional structure of biofilms in the form of a dense network consisting of yeast cells, hyphal and pseudohyphalic forms surrounded by an intercellular polymer matrix. *Candida* spp. pathogenicity factors contribute to infection of susceptible species due to adhesion, invasion, secretion of hydrolases, and dimorphism. Formation of mono-species or poly-species biofilms of microorganisms, including *Candida* spp., causes the development of superficial, deep and systemic candidiasis. Detection of a large amount of yeast and micellar phases in *C. albicans* and *C. africana* isolates was a differential sign of a significant degree of colonization of the mucous membranes of the larynx, pharynx, and tonsils in case of localized and systemic lesions in pigs. The results of studies of the biofilm heterogeneous structure and phenotypic signs of yeast-like fungi can be used in a comparative study of biological characteristics and the identification of common patterns and differential signs of microorganisms, optimization of mycological diagnostics, and also in the development of antimycotic drugs.

Key words: microscopic fungi, *Candida* spp., biofilms, optical density, microscopy, phenotypic characters.

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Исследование биопленок и фенотипических признаков грибов рода *Candida*

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РЕЗЮМЕ

Дрожжеподобные грибы рода *Candida* – возбудители инфекционной патологии слизистой оболочки желудочно-кишечного, дыхательного, урогенитального трактов и кожи млекопитающих, сепсиса и диссеминированной инфекции птиц. Для разработки и совершенствования диагностических и противоэпизоотических мероприятий актуальность представляют изыскание и апробация многоуровневых алгоритмов индикации биопленок при воздействии химиотерапевтических и дезинфицирующих препаратов для блокировки синтеза или разрушения межклеточного матрикса при развитии поверхностных, глубоких и системных кандидозов животных. Установлено, что формирование гетерогенной структуры биопленок представляет собой множество этапов, реализующих процессы межклеточной коммуникации за счет синтеза полимерного матрикса. При оптической микроскопии выявлялась трехмерная структура биопленок в виде плотной сети, состоящей из дрожжевых клеток, гифальных и псевдогифальных форм, окруженных межклеточным полимерным матриксом. При инфицировании восприимчивых видов этиологическая значимость факторов патогенности *Candida* spp.

реализуется за счет адгезии, инвазии, секреции гидролаз, диморфизма. Формирование моновидовых или поливидовых биопленок микроорганизмов, в том числе и *Candida* spp., обусловливают развитие поверхностных, глубоких и системных кандидозов. Индикация в большом количестве дрожжевой и мицеллярной фаз у изолятов *C. albicans* и *C. africana* являлась дифференциальным признаком значительной степени колонизации слизистых оболочек гортани, глотки и миндалин при локальных и системных патологиях свиней. Результаты исследований гетерогенной структуры биопленок и фенотипических признаков дрожжеподобных грибов могут быть использованы при сравнительном изучении биологических свойств и выявлении общих закономерностей и дифференциальных признаков микроорганизмов, оптимизации схемы микологической диагностики, а также при разработке антимикотических препаратов.

Ключевые слова: микроскопические грибы, *Candida* spp., биопленки, оптическая плотность, микроскопия, фенотипические признаки.

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INTRODUCTION

Of the numerous representatives of the genus *Candida*, 20 species were recognized as pathogenic for humans, of which, primarily, *C. albicans*, *C. tropicalis*, *C. krusei*, *C. kefyr*, *C. glabrata*, *C. guilliermondii*, *C. parapsilosis* [1, 2]. The etiological structure of candidiasis in farm animals is represented by *C. albicans* and *C. tropicalis* species recovered from the calves' pancreas (48.07%) and rennet (75.0%), piglets' stomach (88.26%), as well as the oral cavity, esophagus and crop of young birds with pathological legions [3–5]. Clinical signs of the disease are observed in the early postnatal period (up to 2 months of age) in particular, curd-like patches were detected in the stratified squamous epithelium of the digestive system (oral cavity, esophagus, paunch, reticulum, omasom) of calves and lambs [3]. In case of mastitis in cows, *C. krusei* ($n = 14$) and *C. parapsilosis* ($n = 6$) were more often detected in milk samples, *C. lipolytica*, *C. lusitaniae*, *C. neoformans* – less often [6]. Visceral candidiasis of piglets, which occupies the 4th place in the nosological profile of infectious lesions, reaches 13.36% occurrence frequency [4]. 13 (68.42%) *C. albicans* and 6 (31.57%) *C. africana* microorganism cultures were recovered from tissues and organs of pigs with clinical signs of candidiasis and identified [7]. In case of candidiasis in silver-black foxes, dogs and cats, both minor lesions of the skin and epidermic derivatives, as well as intense lesions in the form of ulcers, hyperkeratosis and local alopecia are reported [8]. Chickens and poults younger than one month of age are the most susceptible to this disease. Subacute or chronic candidiasis occurs in birds in the form of epidemic outbreaks. As a rule, it is characterized by formation of intense patches of yellow-gray color, tightly attached to the mucous membrane of the esophagus, crop, and when removed, ulcerative foci are exposed [9, 10].

In case of chronic infectious lesions of farm animals, direct correlations were established between the morphological and densitometric characteristics of biofilms and the effect of virulence factors of microorganisms, including yeast-like fungi [11–14]. *Candida* spp. virulence factors contribute to infection of susceptible species due to synthesis of polymeric substances, transcriptional control of adhesion, invasion, and secretion of toxins [15–20]. Heteromorphic population growth promotes the interaction between microorganisms of various taxonomic groups, which leads to virulence and protection of microbial biofilms from the immune response, as well as from the effects of chemotherapeutic drugs and disinfectants [21–24]. *Candida* spp. mRNA transport is inactivated when the so-called She3 adapter is removed, the hyphae become specifically defective, which is accompanied by atypical growth and decrease in the ability to damage the monolayer of epithelial cells due to a decrease in the production of phospholipase B [25, 26]. The search and testing of multilevel algorithms for biofilm identification when exposed to chemotherapeutic and disinfectant drugs for blocking the synthesis or destruction of the intercellular matrix in the development of superficial, deep and systemic candidiasis of animals are relevant for developing and improving diagnostic and anti-epidemic measures.

The research is aimed at studying the morphometric and densitometric characteristics of biofilms and phenotypic characteristics of reference strains and isolates of *Candida* spp. yeast-like fungi.

MATERIALS AND METHODS

Strains. Biofilms and phenotypic characteristics were tested using reference strains (ATCC): *Candida albicans* ATCC 14053, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750, *C. glabrata* ATCC 66032 [27]. The isolates we recovered were also used in the experiments: *C. albicans* and *C. africana* – from pig lymph nodes in case of localized or systemic lesions; *C. albicans* and *C. tropicalis* from vaginal mucus of dogs in the presence of clinical signs of candidiasis, as well as *C. humilis* from compound feed for cattle [7, 25].

Nutrient media. The microorganisms were cultured for 24 hours at 37, 42, 45 °C using liquid and dense nutrient media: cardiac broth (HiMedia, India), Sabouraud (bioMerieux, France), HiCrome Candida Agar (HiMedia, India). The presence of chlamydospores was taken into account when inoculating a 24-hour culture of microorganisms from Sabouraud's culture medium on the rice agar (API-System R.A.T., France) and cultivated for 24 hours at 25 °C.

To assess the formation of germ tubes, the microorganisms were cultured in 1.0 ml of meat-peptone broth (MPB) with addition of cattle blood serum (FSUE NPO Microgen, Russia) at 37 °C for 5 hours.

Phenotypic characteristics. The morphological, cultural, and biochemical properties of microorganisms were studied by conventional methods using differential diagnostic media and test systems [1, 2].

For recording the enzymatic features, *Candida* spp. day-old culture (optical density OD = 0.5 at a wavelength of 620 nm) was added to the wells of the HiCandida Identification Kit test system (HiMedia, India) and cultured for 48 hours at 22.5 °C.

Morphometric parameters of biofilms. Microorganisms were cultured for 48 hours at 37 °C on coverslips placed in Petri dishes with 20 ml of MPB and 5 ml of suspension of 18-hour cultures of microorganisms at a concentration of 105 CFU/ml. The preparations were fixed with a mixture of alcohol and ether (1:1) for 10 min; they were stained with a 0.5% methylene blue solution [12, 21].

The preparations were tested using optical microscopy BIOMED MS-1 Stereo (Russia).

Densitometric characteristics. When cultured in a 96-well plate (Medpolymer, Russia), the crystal violet binding (HiMedia, India) reading was performed using an Immunochem-2100 photometric analyzer (HTI, USA). The test samples (optical density OD 580, wavelength 580 nm) were added to the wells of the plates and cultured at 37 °C for 48 hours. Then, the liquid was removed, the wells were washed three times with 200 µl of phosphate-buffered solution (pH 7.3). Fixation was performed using 150 µl of 96% ethanol for 15 min. Then the plate wells were dried for 20 min at 37 °C and a 0.5% solution of crystalline violet was added, and again placed in a thermostat at 37 °C. In 5 min, the contents of the wells were removed, washed three times with 200 µl of phosphate-buffered saline (pH 7.2) and dried. The dye was eluted with 200 µl of 96% ethanol for 30 min [16, 28, 29].

The data obtained during the experiment were processed by the method of statistical analysis using Student's t-test, the results were considered reliable at $p \leq 0.05$.

RESULTS AND DISCUSSION

Candida spp. – gram-positive oval-based budding microorganisms. The yeast phase of the tested species was represented by relatively large round or oval budding cells of 2.0–6.0 µm in diameter, the micellar phase – by groups of round small cells (blastospores) and hyphae.

The microorganisms *C. albicans* and *C. humilis* had round, subspherical, elliptical cell shapes 2.0–3.0 × 3.0–5.0 µm in size.

The *C. parapsilosis* and *C. tropicalis* cells of 1.7–2.0 × 3.0–4.0 µm in size had an elliptical elongated shape; mycelium with groups of round small cells – blastospores – was also detected (Fig. 1).

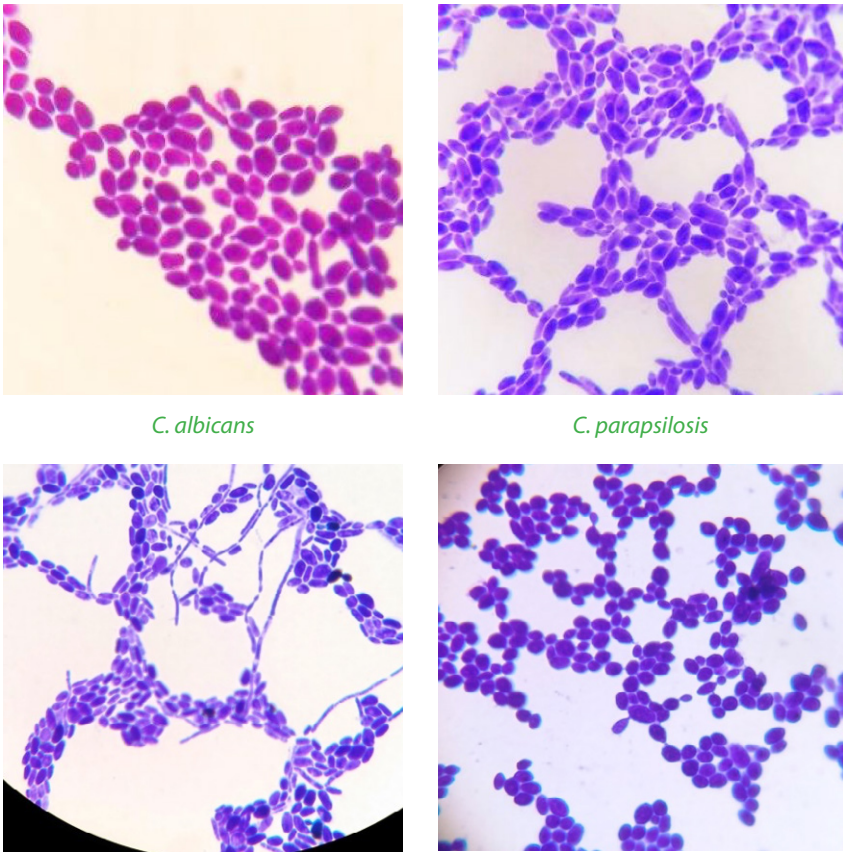

Fig. 1. Morphology of yeast-like fungi.
Growth at 25 °C for 24 h in Sabouraud medium. Methylene blue staining, oc. 10, ob. 100, immersion.

Рис. 1. Морфология дрожжеподобных грибов.
Рост при 25 °С 24 ч на среде Сабуро. Окрашивание метиленовым синим, ок. 10, об. 100, иммерсия.

Detection of a large number of yeast and micellar phases in *C. albicans* and *C. africana* isolates recovered from pig lymph nodes was a differential sign of a significant degree of colonization of the larynx, pharynx, and tonsils mucous membranes in case of localized and systemic lesions.

The population immobilization of the mature three-dimensional biofilm architectonics, in accordance with the cultivation conditions, was accompanied by co-aggregation between yeast and micellar forms combined by an exocellular matrix, the presence of long branched hyphal forms forming dense pseudomycelial structures. In their central part microcolonies had a more pronounced matrix, therefore, yeast forms and mycelium were not detected. In the peripheral part of biofilms, as a rule, the exocellular matrix gradually thinned, individual yeast cells and micellar forms were detected (Fig. 2).

When evaluating densitometric caracteristics of the microorganism culture, it was found that after 48 hours of *C. albicans* (ATCC 14053) and *C. africana* cultivation recovered from the lymph nodes of piglets, the absolute optical density values (OD_s) were in the range from 0.423 ± 0.11 to 0.510 ± 0.19, and the intensity of biofilm formation (*ID*) was ≥ 0.3–0.4.

C. albicans and *C. tropicalis* microorganisms (ATCC 750) isolated from dog vaginal mucus discharge, as well as *C. glabrata* (ATCC 66032), *C. parapsilosis* (ATCC 22019) demonstrated OD_s values ranging from 0.331 ± 0.10 to 0.350 ± 0.08, biofilm formation intensity *ID* ≥ 0.2–0.3.

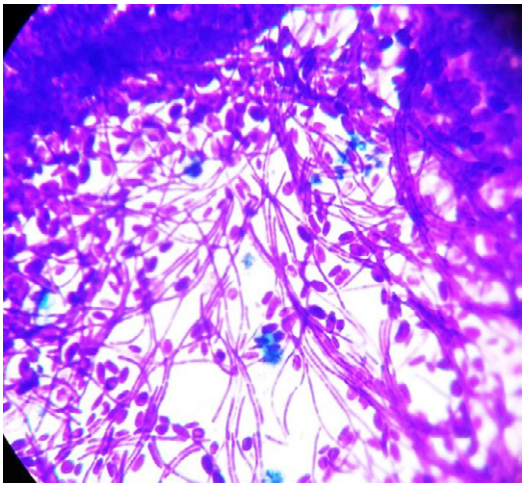

Fig. 2. Morphology of yeast-like fungi C. tropicalis. Growth at 37 °C for 48 h in Sabouraud medium. Methylene blue staining, oc. 10, ob. 100, immersion.

Рис. 2. Морфология дрожжеподобных грибов C. tropicalis. Рост при 37 °C 48 ч на среде Сабуро. Окрашивание метиленовым синим, ок. 10, об. 100, иммерсия.

C. humilis isolated from cattle feed demonstrated OD_s in the range from 0.208 ± 0.06 to 0.288 ± 0.11, and the intensity of biofilm formation *ID* ≥ 0.1–0.2 (Table 1).

Table 1
Densitometric parameters of *Candida* spp. biofilm formation

Таблица 1
Денситометрические показатели формирования биопленок *Candida* spp.

Microorganism species	Optical density ($n = 3$)		
	OD_s*	Δ (OD_s – OD_c)**	Intensity (*ID*)***
Reference strains			
C. albicans, ATCC 14053	0.423 ± 0.11	0.325 ± 0.20	≥ 0.3–0.4
C. tropicalis, ATCC 750	0.342 ± 0.13	0.244 ± 0.22	≥ 0.2–0.3
C. parapsilosis, ATCC 22019	0.350 ± 0.08	0.252 ± 0.17	≥ 0.2–0.3
C. glabrata, ATCC 66032	0.288 ± 0.11	0.130 ± 0.20	≥ 0.1–0.2
Isolates			
C. albicans (piglets' lympnode)	0.482 ± 0.09	0.384 ± 0.18	≥ 0.3–0.4
C. africana (piglets' lympnode)	0.510 ± 0.19	0.412 ± 0.28	≥ 0.3–0.4
C. albicans (dogs' vaginal mucus discharge)	0.398 ± 0.16	0.300 ± 0.25	≥ 0.2–0.3
C. tropicalis (dogs' vaginal mucus discharge)	0.331 ± 0.10	0.213 ± 0.19	≥ 0.2–0.3
C. humilis (compound feed)	0.208 ± 0,06	0.110 ± 0,15	≥ 0.1–0.2

* OD_s – optical density of the sample (оптическая плотность образца);
** OD_c – optical density of the control sample (оптическая плотность контроля);
*** *ID* – intensity: the difference between the optical density of the test sample (ODs) and control sample (ODc)
(интенсивность: разность оптической плотности исследуемого образца (OD_s) и контроля (OD_c).

Table 2
Phenotypic characteristics of *Candida* spp.

Таблица 2
Фенотипические признаки *Candida* spp.

Microorganism species	Distinctive features in 48 hours of cultivation			
	Sabouraud medium			HiCrome Candida Agar
	37 °C	42 °C	45 °C	
Reference strains				
C. albicans, ATCC 14053	+	+	+	Green colour of the colony
C. tropicalis, ATCC 750	+	+	+	Blue colour of the colony
C. parapsilosis, ATCC 22019	+	+	–	Pale-pink colour of the colony
C. glabrata, ATCC 66032	+	+	+	Cream colour of the colony
Isolates				
C. albicans (piglets' lympnode)	+	+	+	Green colour of the colony
C. albicans (dogs' vaginal mucus discharge)	+	+	+	Green colour of the colony
C. africana (piglets' lympnode)	+	+	–	Green colour of the colony
C. tropicalis (dogs' vaginal mucus discharge)	+	+	+	Blue colour of the colony
C. humilis (compound feed)	+	+	+	Lilac colour of the colony

«+» – species characteristic growth is observed (присутствие характерного для вида роста);
«–» – species characteristic growth is not observed (отсутствие характерного для вида роста).

The reference strains and isolates of yeast-like fungi *Candida* spp., regardless of the recovery source, demonstrated growth characteristic of the species, which is quite informative in terms of sediment, film and the degree of medium turbidity. Taking into account the microorganism temperature tolerance, the distinctive feature of *C. africana* and *C. parapsilosis* species was the absence of growth on Sabouraud medium at 45 °C (Table 2).

HiCrome Candida Agar contains chloramphenicol, which inhibits the growth of concomitant microorganisms. The presence of chromogenic substrates makes it possible to differentiate *Candida* spp. colonies, which differed in size, shape, color and consistency. At the same time, the *C. albicans* and *C. africana* species formed similar green colonies on this medium.

A diagnostic test for the presence of germ tubes, the precursors of true hyphae, makes it possible to differentiate fungi in 5 hours of microorganism cultivation in MPB at 37 °C with the addition of cattle blood serum. The tested types of microorganisms were able to grow in the presence of cycloheximide, no urease activity was observed. *C. africana*, unlike other species, fermented sucrose and raffinose and did not ferment maltose, *C. humilis* and *C. glabrata* species did not ferment galactose and xylose, *C. parapsilosis* did not ferment trehalose (Table 3).

In general, the formation of a heterogeneous structure of *Candida* spp. fungi reference strain and isolate biofilms represents many stages of intercellular communication processes due to the synthesis of a polymer matrix. The results of studying biofilm heterogeneous structure as well as phenotypic characteristics and virulence factors of the microorganisms expand the boundaries of general and special mycology, and the applied aspects – the identification of common patterns and differential characteristics of saprophytes, potentially pathogenic and pathogenic microorganisms – have potential for optimization of the infectious lesion diagnostics and the development of antimycotic preparations.

CONCLUSION

When studying the morphometric parameters of *Candida* spp. fungi reference strain and isolate biofilms the three-dimensional structure of biofilms was detected in the form of a dense network consisting of yeast cells, hyphal and pseudohyphal forms surrounded by an intercellular polymer matrix. Detection of a large number of yeast and micellar phases in *C. albicans* and *C. africana* isolates was a differential characteristics of a significant degree of colonization of the larynx, pharynx, and tonsils mucous membranes in case of localized and systemic lesions of pigs.

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Table 3
Differential diagnostic characteristics of *Candida* spp.

Таблица 3
Дифференциально-диагностические признаки *Candida* spp.

Species	Carbonhydrates											
	Urease	Melibiosis	Lactose	Maltose	Suchrose	Galactose	Cellobiose	Inositol	Xylose	Dulcite	Raffinose	Trehalose
Reference strains												
C. albicans, ATCC 14053	–	–	–	+	+	+	–	–	+	–	–	+
C. tropicalis, ATCC 750	–	–	–	+	+	+	+	–	+	–	–	+
C. parapsilosis, ATCC 22019	–	–	–	+	+	–	–	–	+	–	–	–
C. glabrata, ATCC 66032	–	–	–	+	–	–	–	–	–	–	–	+
Isolates												
C. albicans (piglets' lympnode)	–	–	–	+	+	+	–	–	+	–	–	+
C. albicans (dogs' vaginal mucus discharge)	–	–	–	+	+	+	–	–	+	–	–	+
C. africana (piglets' lympnode)	–	–	–	–	+	+	–	–	+	–	+	+
C. tropicalis (dogs' vaginal mucus discharge)	–	–	–	+	+	+	+	–	+	–	–	+
C. humilis (compound feed)	–	–	–	+	–	+	–	–	+	–	–	+

«+» – positive test result (положительный тест);
«–» – negative test result (отрицательный тест).

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Mycotoxilogical monitoring. Part 2. Wheat, barley, oat and maize grain*

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SUMMARY

Results of mycotoxicological survey of representative samples of feed and procured wheat, barley, oat and maize grain batches are demonstrated. The samples were submitted by the Veterinary Service officials, livestock farmers and feed mill operators, agricultural producers, specialized commercial business operators and farm owners in seven Federal Districts of the Russian Federation in 2009-2019. Similar amounts of wheat and barley grain samples were received from the Central, Volga, Ural and Siberian Federal Districts. The amount of wheat samples delivered from the Southern Federal District prevailed over the number of barley samples, and the maize samples were mostly delivered from the regions of the Central Federal District. *Fusarium* toxins including T-2 toxin, diacetoxyscirpenol, deoxynivalenol, zearalenone and fumonisins of group B as well as alternariol, ochratoxin A, citrinin, aflatoxin B1, sterigmatocystin, cyclopiazonic acid, mycophenolic acid, ergot alkaloids and emodin were detected and measured according to the validated competitive ELISA procedure. Generalization of the results demonstrated domination of *Fusarium* toxins and active involvement of alternariol in the contamination of all types of feed grains as well as high occurrence of emodin in ear cereals and increased occurrence of T-2 toxin and ochratoxin A in barley. Shift of medians and 90%-percentile of the basic contaminants to lower values as compared to mean and maximal ones was reported thus being indicative of their possible accumulation at the levels outside the typical range. The highest levels of T-2 toxin, deoxynivalenol and ochratoxin A as well as 90%-percentile values exceeded the acceptable levels. The maize grains demonstrated the whole complex of the tested *Fusarium* toxins with the prevalence of T-2 toxin, deoxynivalenol, zearalenone and fumonisins; and the maximal amounts of these mycotoxins by several times exceeded the accepted regulatory levels. Diacetoxyscirpenol, aflatoxin B1, sterigmatocystin, cyclopiazonic acid and ergot alkaloids are classified as rare feed grain contaminants. High prevalence of alternariol and emodin known as "diarrhea factor" as well as maize grain contamination with mycophenolic acid (mycotoxin having an immunosuppressive effect) are for the first time reported in this paper. These data support the need of their introduction in the group of regulated substances significant for public health. Original monitoring data systematized and summarized in the paper are given in electronic format in section Additional materials.

Key words: wheat, barley, oat and maize grain, mycotoxins, monitoring, enzyme-linked immunosorbent assay (ELISA).

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Микотоксикологический мониторинг. Сообщение 2. Зерно пшеницы, ячменя, овса, кукурузы*

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РЕЗЮМЕ

Представлены результаты микотоксикологического обследования средних образцов от партий фуражного и заготовляемого зерна пшеницы, ячменя, овса и кукурузы, предоставленных специалистами ветеринарных служб, животноводческих и комбикормовых предприятий, компаний-

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сельхозпроизводителей, специализированных коммерческих организаций и владельцами крестьянских фермерских хозяйств из 7 федеральных округов Российской Федерации в период с 2009 по 2019 г. Сравнимые по объему выборки зерна пшеницы и ячменя были получены из Центрального, Приволжского, Уральского и Сибирского федеральных округов. Из Южного федерального округа число образцов пшеницы было больше, чем ячменя, а зерно кукурузы поступало в основном из субъектов Центрального федерального округа. 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 проводили по аттестованной процедуре с использованием конкурентного иммуноферментного анализа. В ходе обобщения результатов установлена доминирующая роль фузариотоксинов и активное участие альтернариола в контаминации всех видов зернофуража, а также частая встречаемость эмодина в зерне колосовых культур и повышенная распространенность Т-2 токсина и охратоксина А в зерне ячменя. 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 их накопления за пределами типичного диапазона. Наибольшие уровни Т-2 токсина, дезоксиниваленола и охратоксина А, а также показатели 90%-го процентиля превышали допустимые нормы содержания. В зерне кукурузы комплекс анализированных фузариотоксинов представлен полностью с наибольшей встречаемостью Т-2 токсина, дезоксиниваленола, зеараленона и фумонизинов, и максимальные количества этих микотоксинов в несколько раз превышали принятые уровни нормирования. Диацетоксисцирпенол, афлатоксин B_1, стеригматоцистин, циклопиазоновая кислота и эргоалкалоиды отнесены к редким контаминантам кормового зерна. Факт обширной распространенности в зерне альтернариола и эмодина, известного как «диарейный фактор», а также контаминации зерна кукурузы микофеноловой кислотой – микотоксином с иммунодепрессивным действием, приведены в данной работе впервые. Эта информация подтверждает необходимость их введения в группу нормируемых санитарно-значимых показателей. Исходные данные мониторинга, систематизированные и обобщенные в данной работе, представлены в электронном виде в разделе «Дополнительные материалы».

Ключевые слова: зерно пшеницы, ячменя, овса, кукурузы, микотоксины, мониторинг, иммуноферментный анализ.

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INTRODUCTION

Grain contamination with toxic metabolites of microscopic fungi (mycotoxins) has been and remains the focus of attention of the global agricultural science being the key issue of the veterinary welfare [1, 2]. In our country the hygiene of the feed grain is of practical importance for all livestock sectors not only due to significant scope of its use in the compound feed but also due to still growing interest to hydroponic fodder and new technologies of its preservation under high humidity conditions. The feed rations of the majority of food-producing animals and poultry are based on grain ingredients – wheat, barley and maize grain with oat grain being of traditional interest for horse breeding sector.

For many years, the wide spread of *Fusarium* spp. contamination of grain, often growing into long-lasting epiphytoties, has been gaining increased attention due to the risk of toxicoses in animals [3]. In 1995–2005, the targeted governmental program was implemented in order to assess the consequences of the grain contamination with toxigenic *Fusarium* fungi and to forecast the development of the situation in the cultivation areas [4]. That was when the data on *Fusarium* toxins' spread pattern were obtained for the first time [5, 6]. Hereafter, regional surveys were initiated as for other mycotoxins significant for public health [7]. Enforcement of the Technical Regulation of the Customs Union on Safety of Grain (TR CU 015/2011) prescribing their admissible levels in feed was an important outcome of these efforts [8].

According to the global scientific data the list of mycotoxins capable of inducing chronic intoxication and highly dangerous remote effects in animals is far from being exhausted, and improvement of the system of grain safety control remains highly relevant. An increasing number of countries realize the need for regular monitoring researches and continuous operation of specialized structures for coordination of actions and rapid expert evaluation of the received data. In our country, the long-run objective of the establishment of the national information resource aimed at the mitigation of mycotoxicosis risk in farm animals involves systematization of analytical investigations and data reporting under the unified approach, which combines their overall assessment and accessibility for updating and use by the experts.

The work was aimed at compilation of the results of the survey of mycotoxin contamination of feed and procured wheat, barley, oat and maize grain delivered from seven Federal Districts of the Russian Federation in 2009–2019 with the source data being reported to the electronic database.

MATERIALS AND METHODS

The test objects included representative samples of feed and procured grain batches submitted by the Veterinary Service officials, livestock farmers and feed mill operators, agricultural producers, specialized commercial business operators and small-scale farm owners in 2009–2019. Harvesting areas were documented and officially confirmed for 623 wheat, oat, barley and maize samples, 30 samples were delivered without data on their origin, and four samples were collected from the imported consignments (Table 1). Twenty-seven oat grain samples were submitted for testing mostly from the Tula, Moscow Oblasts and Krasnodar Krai as well as from the Bryansk, Kursk, Tyumen Oblasts and Sakha (Yakutia) Republic.

The tested mycotoxins included T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEA) and fumonisins of group B (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). The samples were prepared using standardized procedure based on liquid-liquid extraction and indirect competitive ELISA [9]. 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 record form in the database: type of grain (WH, BAR, OA, MAI), region (FD and REGION), reporting year (1–11), tested mycotoxins. Microsoft Excel 2016 and Statistica (Version 6) software were used for statistical data processing including calculation of percentage of occurrence based on n^+/n ratio and the following three values for positive samples – the arithmetical mean, the median and the 90% percentile.

RESULTS AND DISCUSSION

The data in Table 1 demonstrate that the coverage of the grain sampling areas was the highest in the Central and Volga Federal Districts (FD). In the Southern, Ural and Siberian FD this parameter was significantly lower and as for the Northwestern and Far Eastern FD the samples were delivered only from two regions of each. The total numbers of tested samples of wheat, barley and maize were quite comparable, but their regional distribution was uneven. Comparable sizes of wheat and barley grain samples were delivered only from the Central, Volga, Ural and Siberian FD, but samples delivered from the Southern FD considerably varied in size. Maize grain was mostly submitted from the regions of the Central FD.

All tested mycotoxins except from DAS and AB_1 were detected in wheat grain (Table 2). T-2 and DON as well as AOH and EMO were the most frequently detected ones. The occurrence rate of other mycotoxins amounted to 1.0%–4.9%. The quantities of toxins generally varied within two orders of magnitude; only in case of ZEA the variation range did not exceed one order of magnitude. Shift of medians to lower values as compared to mean ones was reported for all contaminants being indicative of unified dissymmetric distribution of contents where half of the data was less than other ones. The highest quantities exceeded the threshold concentrations calculated for 90% of values (90% percentile) being the most pronounced in case of T-2, DON, AOH and EMO that suggested their possible abnormal accumulation against the typical contamination. Moreover, in case of DON the 90% percentile value several times exceeded its admissible level of 1,000 μg/kg [8]. It should be emphasized that against the relatively rare occurrence both maximum levels of OA and CIT detected in grain and their threshold concentrations calculated for 90% of values were significantly higher than the recommended standards [8, 10].

In barley grain the highest occurrence rates were reported for T-2 and EMO, moderate ones – for DON, AOH and OA, the lowest ones – for ZEA, CIT, STG and MPA. DAS, FUM, AB_1 and CPA were absent (Table 3). Two- and even more-fold median displacement from the average values to the lower ones was reported for all contaminants excluding AOH, and the excess of the highest values over the threshold concentrations calculated for 90% of the values (90% percentile) was reported for all but EA. With high prevalence of T-2 contamination the 90% percentile value was consistent with the admissible levels and the highest amount exceeded it in over six times. As for less prevalent DON and OA, the comparison of the both parameters indicated real possibility of the excess contamination of this type of grain [8]. Higher T-2 occurrence in barley grain as

Table 1
Areas, from which representative samples of wheat, barley and maize grain were delivered (according to documented and official data, 2009–2019)

Таблица 1
Территории, с которых направлены средние образцы зерна пшеницы, ячменя, кукурузы (согласно документальным и ответственным подтверждениям, 2009–2019 гг.)

Federal District (Region) of the Russian Federation	Number of samples		
	wheat	barley	maize
Central (Belgorod, Bryansk, Voronezh, Kaluga, Kursk, Lipetsk, Moscow, Oryol, Ryazan, Tambov, Tula, Yaroslavl Oblasts)	65	58	190
Volga (Kirov, Nizhny Novgorod, Orenburg, Penza, Samara, Saratov, Ulyanovsk Oblasts, Republic of Bashkortostan, Republic of Mordovia, Republic of Tatarstan)	41	29	2
Southern (Volgograd, Rostov Oblasts, Krasnodar Krai, Stavropol Krai)	75	14	9
Urals (Kurgan, Sverdlovsk, Tyumen, Chelyabinsk Oblasts)	17	12	–
Siberian (Irkutsk, Novosibirsk, Omsk Oblasts, Altai Krai, Krasnoyarsk Krai)	44	26	–
Northwestern (Vologda, Pskov Oblasts)	1	1	–
Far Eastern (Amur Oblast, Primorsky Krai)	2	1	2
Origin is not specified	14	6	10
Imported grain	–	1	3
Total	259	148	216

Table 2
Mycotoxins in wheat grain (summary data, 2009–2019)

Таблица 2
Микотоксины в зерне пшеницы (обобщенные данные 2009–2019 гг.)

Toxin	Occurrence n^+/n (%)	Level, μg/kg				
		range		mean	median	90% percentile
		min	max			
T-2	83/259 (32.0)	2	225	26	12	48
DON	62/259 (23.9)	40	7,920	1,092	550	3,155
DAS	0/74	–	–	–	–	–
ZEA	11/259 (4.2)	10	215	68	40	205
FUM	6/165 (3.6)	75	1,990	478	150	1,200
AOH	52/240 (21.7)	11	675	60	32	116
OA	12/247 (4.9)	4	270	52	8	236
CIT	8/163 (4.9)	24	1,000	349	128	853
AB_1	0/101	–	–	–	–	–
STG	3/125 (2.4)	4	250	90	–	–
CPA	1/132 (1.0)	63	–	–	–	–
MPA	4/151 (2.6)	63	1,255	602	545	1,179
EA	4/121 (3.3)	6	144	43	10	105
EMO	56/126 (44.4)	5	706	118	60.5	301

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

compared to wheat grain as well as concordance of DON and ZEA occurrence parameters support the previously reported data [11]. More pronounced OA concentration in barley grain (20.4%) as compared to wheat grain (4.9%) was reported for the first time.

DAS, ZEA, FUM, AB_1 and CPA were not detected in 27 tested oat grain samples; OA, CIT, STG, MPA and EA were detected in singular samples; other mycotoxins were ranged as T-2 > EMO > DON > AOH according to their occurrence rate (see the Figure). Maximum concentrations of T-2 and DON detected in oat grain exceeded the current standards [8].

In spite of low amount of samples and irregular testing of this type of grain during the reported years, the obtained data can be useful for the complex research project targeted at the examination of oat contamination with toxicogenic fungi in the regions practicing intensive cultivation of this grain, especially in the northern part of the Non-Black-Earth region, Volga FD and other territories [12, 13]. Moreover, these data are of indisputable value for the horse breeding sector, where this grain is widely used due to its high nutrition and content of biologically active substances having a stimulating effect of the behavioral responses of horses. Previous pilot study of several samples submitted by the equestrian centers and horse breeding farms also demonstrated high T-2 contamination, moderate DON and AOH contamination and low ZEA contamination [12].

In maize grain the group of contaminants was more diverse and only STG was not included in it. The complex of the tested *Fusarium* toxins was represented in full with high prevalence of T-2, DON and FUM and lower prevalence of ZEA and DAS (Table 4). The highest levels of T-2, DON, FUM and ZEA several times exceeded their regulatory standards. The data on the dominating role of these mycotoxins were consistent with the previous conclusion [14] and supported the relevance of their introduction into the list of regulated substances in the maize grain supplied for use in feed [8]. Among other tested metabolites the significant contribution of AOH and MPA should be noted with their occurrence rate 13.3% and 8.6% and maximum levels 295 μg/kg and 397 μg/kg, respectively. OA, CIT and EMO mycotoxins were rare to detect and their levels amounted to maximum 50 μg/kg and AB_1, CPA and EA were detected only sporadically.

Results of tests of wheat, barley, oat and maize grain contaminants relevant for public health carried out in 2009–2019 were for the first time consolidated and analyzed in detail in this paper. Dominant role of *Fusarium* toxins along with potential high-level accumulation as well as evidence of co-contamination with all components of the complex were confirmed for maize grain. Significant EMO occurrence in ear cereals was reported that is likely to be the basic route of its introduction into the compound feeds (see Part 1). The role of this secondary metabolite for plants is not quite clear for now and has been actively

Table 3
Mycotoxins in barley grain (summary data, 2009–2019)

Таблица 3
Микотоксины в зерне ячменя (обобщенные данные 2009–2019 гг.)

Toxin	Occurrence n^+/n (%)	Level, μg/kg				
		range		mean	median	90% percentile
		min	max			
T-2	105/148 (70.9)	4	660	48	25	100
DON	35/148 (23.6)	50	7,060	654	130	2,380
DAS	0/60	–	–	–	–	–
ZEA	2/148 (1.4)	100	1,250	675	–	–
FUM	0/67	–	–	–	–	–
AOH	29/137 (21.2)	5	397	85	71	141
OA	29/142 (20.4)	4	250	23	5	71
CIT	5/137 (3.6)	30	1,120	300	146	734
AB_1	0/69	–	–	–	–	–
STG	2/107 (1.9)	10	40	25	–	–
CPA	0/92	–	–	–	–	–
MPA	1/106 (1.0)	334	–	–	–	–
EA	4/94 (4.3)	5	164	49	14	120
EMO	68/94 (72.3)	13	1,400	205	101	495

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

discussed in the scientific publications [15]. Genotoxic AOH was classified as significant for public health and relevant for all types of feed grain as well as highly active immunosuppressive MPA that is relevant for maize grain.

To see the source data of the monitoring with the regions and sampling date specified please follow the link http://doi.org/10.29326/2304-196X-2020-2-33-139-145. In section Additional materials. The option of the database updating as well as its accessibility allow for any selection of data processing *inter alia* by type of grain, occurrence of contamination with an individual mycotoxin or co-contamination with mycotoxins as well as by region or territory. Over the recent years, DON and ZEA co-contamination has been frequently occurring in wheat [11], the same as T-2 and DAS co-contamination – in maize grain [14]. The data received as for individual territories allow for unique possibility to forecast risk of contamination in the specific area with due consideration of the soil-climatic and cultivation factors, which are able to influence the mycotoxicological situation due to their effect on the microscopic fungus growth conditions, competitive interactions and toxin production [16–18].

CONCLUSION

During the large-scale monitoring involving annual data collection in 2009–2019 general characteristics and peculiarities of mycotoxin contamination of wheat, barley, oat and maize grain were determined. The findings supported the relevance of the regular assessment of the grain contamination with *Fusarium* toxins and ochratoxin A. The list of public health-significant substances regulated in grain is recommended to be extended by inclusion of emodin (anthraquinone toxin known as "diarrhea factor") as well as alternariol, citrinin and mycophenolic acid having especially hazardous forms of toxicity and negative remote effects. The obtained data can be relevant

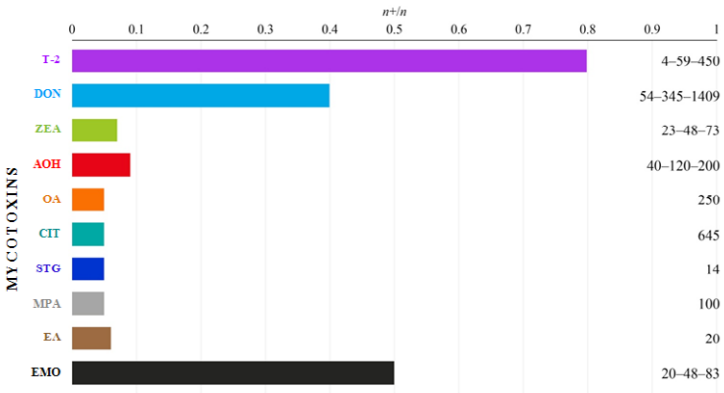

Fig. Mycotoxin occurrence (n^+/n) and content (μg/kg, min – mean - max) in oat grain (summarized data)

Рис. Встречаемость (n^+/n) и содержание микотоксинов (мкг/кг, мин. – среднее – макс.) в зерне овса (обобщенные данные)

Table 4
Mycotoxins in maize grain (summarized data, 2009–2019)

Таблица 4
Микотоксины в зерне кукурузы (обобщенные данные 2009–2019 гг.)

Toxin	Occurrence n^+/n (%)	Level, μg/kg				
		range		mean	median	90% percentile
		min	max			
T-2	203/216 (94.0)	2	1,040	151	120	310
DON	154/216 (71.3)	50	6,590	740	382.5	1,963
DAS	10/59 (17.0)	50	250	122	92.5	205
ZEA	50/216 (23.2)	20	4,455	345	55.5	674
FUM	167/216 (77.3)	50	15,800	1,334	500	3,152
AOH	21/158 (13.3)	20	295	71	57	140
OA	4/164 (2.4)	5	16	10	–	–
CIT	3/141 (2.1)	20	40	27	–	–
AB_1	1/113 (1.0)	16	–	–	–	–
STG	0/112	–	–	–	–	–
CPA	1/75 (1.3)	126	–	–	–	–
MPA	8/93 (8.6)	25	397	158	100	345
EA	1/67 (1.5)	6	–	–	–	–
EMO	2/74 (2.7)	25	45	35	–	–

n – number of tested samples (число исследованных образцов);

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

for the assessment of the general situation in the agricultural production, verification of the criteria of the mycotoxin regulation in grain and for the improvement of the system of grain product control.

Additional materials to the paper (records forms with database) can be found at: http://doi.org/10.29326/2304-196X-2020-2-33-139-145.

Дополнительные материалы к этой статье (учетные формы с базой данных) можно найти по адресу: http://doi.org/10.29326/2304-196X-2020-2-33-139-145.

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Рецензия на монографию В. В. Макарова, Н. Я. Махамата, А. М. Гулюкина, М. И. Гулюкина «Сибирская язва: современное представление и мировое распространение».

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The monograph is devoted to anthrax, one of the major epizootological and epidemiological challenges.

The monograph publication is important due to almost total lack of the data on epizootic peculiarities of the global nosoarea of anthrax in animals suitable for ecological-epizootological modeling and prediction.

Analysis of one hundred fifty-year veterinary and medical efforts for anthrax control in Russia and in the world described in Introduction showed obvious and impressive progress in solving this problem. Therewith, the monograph authors note some outstanding issues of the further anthrax control strategy, prediction, modeling, agent ecology associated with apparent sapronotic nature of the infection.

"Modern knowledge of anthrax" section, one of the important monograph sections, contains general information on the infection, infection agent and its two alternative forms (vegetative form – in the diseased animal organism and spore form – outside the animal organism) determining infectious and epizootic processes, respectively. First outstanding achievements in specific disease prevention as well as ecological aspects of anthrax infection, routes of human and animal infection, landscape and climate factor effects on the spore persistence in soil being a covert source of the infection, susceptibility of animals to the disease are described in the section. The authors draw attention to epizootological and epidemiological significance of the disease in food-producing grazing herbivores – large and small ruminants.

The section also contains data on various anthrax forms in humans that are associated with infection routes: cutaneous form (local form) caused by the agent contact to

the skin, as well as inhalation and enteric forms (systemic forms). The disease incidence in humans was shown to depend on the level of exposure to the infection in each country taking into account data on susceptible animal population and epizootic, epidemic and ecological co-factors.

The materials provided in the second section "Veterinary epidemiology of anthrax. Current situation" are of great interest. Considering the importance of this issue the monograph authors analyzed human cases and anthrax outbreaks in animals occurred in many countries in the world for the last 40–50 years as well as assessed current situation in the Russian Federation and in the world based on recent national publications with particular emphasis to anthrax emergence and consequences of mass anthrax morbidity in animals in the Yamalo-Nenets Autonomous Okrug in 2016. Also, information on possibilities and cases of anthrax agent use for terrorist purposes is given in the section.

Veterinary epidemiological pattern for global anthrax spread at the current stage (2007–2017) based on the authors' investigation results is provided in the third section of the monograph. The investigation was carried out as a systematic review in accordance with general evidence-based medicine requirements, epizootological analysis and evidence-based epizootology principles and methods using the most complete international animal health information databases (ProMED and WAHIS) with total coverage of reports on anthrax in humans and animals and common methods of quantitative, graphical epidemiology, biometrics and statistical data processing.

Comparative evaluation of the intensity of epizootic and epidemiological processes in anthrax-affected countries in Africa (16 countries), Asia and Middle East (14 countries), Europe (18 countries), in Australia, Canada, Argentine, Colombia, Haiti, Peru and Uruguay based on performed investigations are presented.

Multi-year and annual dynamics of anthrax outbreaks in cattle in the Republic of Chad for 2010–2015 is given.

Susceptibility of animals and incidence in humans as an indicator of unfavorable epizootic situation, as well as risk factors, seasonality and focality as major features of anthrax epizootic process were characterized for the infected countries.

There are a lot of interesting perfect illustrations of anthrax history, global infection nosoarea in 21st century, infection cycle, mortality rates in animals of different species in the world in 2007–2017, cutaneous anthrax in humans, anthrax incidence, mortality, lethality in humans and different contacts with infected materials and other data in the monograph.

In epilogue containing the final interpretation of given materials, the authors provide a clear characterization of anthrax as a typical naturally-focal non-transmissible sapronosis for which soil plays a role of reservoir and source of the infection for mammals naturally contacting with it.

References include selected publications, mainly original publications on anthrax veterinary epidemiology and nosogeography.

The authors have shown the importance of the problem that is an undoubted advantage of the monograph "Anthrax: Modern Knowledge and Global Occurrence".

The monograph is of great interest to wide range of readers, experts in infectious pathology and epizootology. It will be useful for students, postgraduates, lecturers, participants of advanced training courses organized at veterinary higher educational institutions, veterinarians and medical specialists.

In memory of Alexander Nikolayevich Burdov

Alexander N. Burdov, Doctor of Science (Veterinary Medicine), RSFSR Honored Scientist, participant of the Great Patriotic War, Director of the All-Union Foot-and-Mouth Disease Research Institute (nowaday FGBI "ARRIAH") in 1981–1992 (11 years), passed away in March 2020 at the age of 93 after a long battle with a disease.

Alexander N. Burdov was born on September 15, 1926 in Vyazovka village, Mainsky Raion, Ulyanovsk Oblast. In 1945, while being a military cadet, he was sent to the Far East, where he participated in military operations against Japan. After demobilization in 1946, he entered the Kiev Veterinary Institute. After graduation in 1951, the USSR MoA assigned Alexander N. Burdov to work in the Kazakh SSR as a head of a veterinary health unit, Senior Veterinarian of the MTS, head of the Raion veterinary laboratory.

In 1959–1962, Alexander N. Burdov studied in the full-time postgraduate school of the All-Union Institute of Experimental Veterinary Medicine, and as soon as he graduated from it, he was employed as a Chief Veterinarian in the Chief Veterinary Administration of the USSR MoA. In 1963, he defended a Candidate's thesis.

In April 1964, Alexander N. Burdov was promoted to the position of the Director of the USSR MoA Research Agricultural Institute (Kazakh SSR), where under his leadership the team of employees implemented and put into the veterinary practice a number of developments being of significant national and economic importance. In 1971, Alexander N. Burdov defended the Doctor's dissertation in the AURIVVM (Pokrov). In 1972, he was appointed as the Head of Department in the Chief Administration of the Research and Production Institutions of the USSR MoA. In 1978, Alexander N. Burdov was assigned to work as the Director of the USSR MoA Veterinary Laboratory in the People's Republic of Congo, and later in 1981 he was appointed as the Director of the All-Union Foot-and-Mouth Disease Research Institute, which was headed by him until 1992.

Alexander N. Burdov was a highly qualified expert in highly dangerous animal disease virology and epidemiology. He published 150 research papers, received 6 patents for inventions. Monograph "Foot-and-Mouth Disease" published under Alexander N. Burdov's edition (1990) summed up the best domestic and foreign practices on the disease control.

Lots of activities on the production of new biological products, means and methods for animal protection against quarantine and poor studied diseases were carried-out under his scientific supervision. The contribution of Alexander N. Burdov to the development of the method of the large-scale cell and FMDV cultivation is especially significant. The domestic technology of culture FMD vaccine production was developed on the basis of

this method. The same is applied to the radically new production technologies of the recombinant DNA-based and chemically synthesized vaccines.

Twelve Candidates of Science were trained under the supervision of Alexander N. Burdov actively participated in scientific and public activities; he was the chairman of the Specialized Thesis Council of the AUFMDRI, member of the Specialized Doctor Dissertation Council of the AURIVVM, member of the Scientific and Technical Committee of the USSR Agroindustry Committee, Head of the FMD Coordination Centre of CMEA Member-States.

In 1990, Alexander N. Burdov was awarded the honorary title "RSFSR Honored Scientist" for the advancement of Veterinary Science and training of highly professional specialists.

Alexander N. Burdov was awarded the Order of the October Revolution, Order of The Patriotic War 2nd Class, Order of Friendship of Peoples, two Orders of the Red Banner of Labour and many medals.

In 1992, Alexander N. Burdov retired.

Alexander Nikolayevich was a man of great scientific learning and work capacity. He always took professional and creative initiative and was strictly committed to his principles. All these have earned him a well-deserved renown, respect and wide popularity both in our country and abroad. He will be remembered by his colleagues and friends as this kind of person.

Administration,
Trade Union Committee,
FGBI "ARRIAH" staff members

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

OIE Regional Reference Laboratory for Foot and Mouth Disease
Региональная референтная лаборатория МЭБ по ящуру

The 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 main activities of the Federal State Financed Institution "Federal Centre for Animal Health" (FGBI "ARRIAH") in the field of avian disease control include development and implementation of highly effective products for treatment and prevention of avian diseases, diagnostic test systems and methods in veterinary practice.

The FGBI "Federal Center for Animal Health" (FGBI "ARRIAH")
Manufactures vaccines against the following diseases:
Infectious bursal disease, Newcastle disease, infectious bronchitis, Marek's disease, egg drop syndrome 76, infectious laryngotracheitis, infectious encephalomyelitis, reovirus tenosynovitis, hydropericarditis, mycoplasmosis, fowl pox, duck hepatitis, avian metapneumovirus, etc.
Offers the following diagnostic testing including:

• Determination of antibodies by ELISA using test systems of domestic and foreign production.

• Identification of bacterial and viral genomes using the polymerase chain reaction (PCR):

– avian infectious bronchitis;
– infectious bursal disease;
– infectious encephalomyelitis;
– infectious laryngotracheitis;
– egg drop syndrom-76;
– Marek's disease;
– Newcastle disease;
– reoviral tenosynovitis;
– adenovirus infection;
– metapneumovirus infection;
– avian influenza;
– avian anaemia;
– duck hepatitis;
– enteritis of geese;
– fowl pox;
– *Mycoplasma gallisepticum*;
– *Mycoplasma synoviae*;
– *Mycoplasma meleagridis*.

• Identification of genomes of viruses of infectious bursal disease, avian infectious bronchitis, Newcastle disease, encephalomyelitis, reovirus, etc. using sequence analysis.

• Bacteriological tests for hemophilia, ornithobacteriosis, salmonellosis, pasteurellosis, etc.

To meet the needs of domestic veterinary laboratories for avian influenza diagnostic tools, the Rosselkhoznadzor subordinate FGBI "ARRIAH" has launched production of a test kit for detecting antibodies against avian influenza virus H9 subtype using hemagglutination inhibition test. The kit was successfully tested and demonstrated high specificity and sensitivity.

The test kit was developed in the Reference Laboratory for Avian Viral Diseases and is based on the current strain of the A/H9 virus, antigenically related to isolates identified in the territory of the Russian Federation. This kit is used to determine the level of antibodies to avian influenza virus H9subtype in the sera of both domestic and wild birds.

This test kit can be used for detection of post-infection antibodies in accordance with the "Veterinary rules for laboratory diagnosis of avian influenza A (as of April 03, 2006 No. 105)" and assessment of the efficacy of means for low-pathogenic avian influenza specific prophylaxis.

The leaflet to the kit for detecting antibodies to the avian influenza virus H9 subtype using the hemagglutination inhibition test is available on the FGBI "ARRIAH" website in the section "Products and services".

To purchase veterinary preparations and diagnostic kits produced at FGBI "ARRIAH", please contact us:
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