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Development of the test kit for detection of SARS-CoV-2 antibodies in sera of susceptible animals

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SUMMARY

The novel coronavirus infection COVID-19, caused by the SARS-CoV-2, has triggered a pandemic, and has also been reported in animal populations – in farm minks, dogs and felines: domestic cats, lions and tigers. The susceptibility of some animal species to the SARS-CoV-2 has been proven by experimental infection. Serological methods are effectively used to detect the infection in animals. Currently, methods such as neutralization test, immunofluorescence assay and enzyme-linked immunoassay are used to detect antibodies to coronaviruses. Thanks to these studies, a test kit was developed based on an indirect enzyme-linked immunoassay to detect the SARS-CoV-2 antibodies in sera of susceptible animals. The use of a purified concentrated inactivated virus as an antigen allows the detection of antibodies to various SARS-CoV-2 immunodominant proteins (S and N). The reaction conditions were optimized, and a positive-negative threshold was established by testing of 154 negative sera from animals of six species (ferrets, minks, foxes, Arctic foxes, cats and dogs). The method reproducibility analysis showed that the average value of the variation coefficient did not exceed 7%, which is an acceptable value. The specificity and sensitivity of the neutralization test, when testing 30 sera from ferrets was 100 and 92.6%, respectively. The high diagnostic sensitivity and specificity shown by testing of 50 serum samples from minks, foxes, cats and dogs with different immune status, allow us to recommend the developed test kit for screening and monitoring tests and post-vaccination immunity control.

Keywords: COVID-19, SARS-CoV-2, antibodies, enzyme-linked immunoassay.

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Разработка тест-системы для выявления антител к вирусу SARS-CoV-2 в сыворотках крови восприимчивых животных

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РЕЗЮМЕ

Новая коронавирусная инфекция (COVID-19), вызываемая вирусом SARS-CoV-2, стала причиной пандемии, а также была зарегистрирована в популяциях животных – у норок фермерских хозяйств, собак и представителей кошачьих: домашних кошек, львов и тигров. Доказана чувствительность некоторых видов животных к вирусу SARS-CoV-2 при экспериментальном заражении. Для выявления случаев инфицирования животных эффективно применяются серологические методы. В настоящее время для обнаружения антител к коронавирусам используют такие методы, как реакция нейтрализации, иммунофлуоресцентный метод и иммуноферментный анализ. В результате проведенных исследований была разработана тест-система на основе непрямого варианта иммуноферментного анализа для выявления антител к вирусу SARS-CoV-2 в сыворотках крови восприимчивых животных. Использование

в качестве антигена очищенного концентрированного инактивированного вируса позволяет выявлять антитела к различным иммунодоминантным белкам (S и N) SARS-CoV-2. Оптимизированы условия постановки реакции, установлен позитивно-негативный порог при исследовании 154 негативных сывороток крови от животных шести видов (хорьков, норок, лис, песцов, кошек и собак). При определении воспроизводимости метода среднее значение коэффициента вариации не превышало 7%, что является допустимым значением. Специфичность и чувствительность относительно реакции нейтрализации при исследовании 30 сывороток крови от хорьков составила 100 и 92,6% соответственно. Высокая диагностическая чувствительность и специфичность, показанные при исследовании 50 сывороток крови от норок, лис, кошек и собак с разным иммунным статусом, позволяют рекомендовать разработанную тест-систему для проведения скрининговых исследований и контроля поствакцинального иммунитета.

Ключевые слова: COVID-19, SARS-CoV-2, антитела, иммуноферментный анализ.

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INTRODUCTION

Coronaviruses are one of the major pathogens of mammals (including humans), amphibians and birds [1]. The novel coronavirus SARS-CoV-2 (Severe acute respiratory syndrome-related coronavirus 2) is the causative agent of COVID-19, a 2019 coronavirus disease. The SARS-CoV-2 belongs to *Nidovirales* order, *Orthocoronavirinae* subfamily, *Betacoronavirus* genus (International Committee on Taxonomy of Viruses, ICTV). Coronaviruses are enveloped RNA viruses with single-stranded unsegmented RNA [2]. The main structural proteins are nucleoprotein (N), spike protein (S), envelope protein (E), and matrix protein (M). The S-protein, consisting of two subunits S1 and S2, forms a trimer on the viral membrane. The S1 subunit contains a receptor-binding domain (RBD), which is responsible for binding of the host cell to the receptors, and the S2 subunit facilitates fusion between the viral membranes and the host cell [2–4]. The S protein is the most variable in the representatives of different genera of *Coronaviridae* family and is responsible for their transmissivity and adaptation abilities. The S2 protein is more conservative than S1 [4].

The susceptibility to the SARS-CoV-2 infection of different species is currently understudied. There are a number of reports about the SARS-CoV-2 experimental infections in ferrets, cats, dogs and pigs [5–7]. The SARS-CoV-2 was found to replicate effectively in cats and ferrets. Dogs were less susceptible to the infection, and ducks and chickens were not susceptible at all [8]. B. S. Pickering et al. showed that pigs are sensitive to nasal infection with a large dose of the SARS-CoV-2 [7]. In natural environments (in zoos), the SARS-CoV-2 genome was detected in cats, dogs, tigers, and lions, showing signs of respiratory disease. In the spring of 2020, the SARS-CoV-2 infection in minks and their deaths were reported on farms in the Netherlands [9].

The peculiarity of this viral disease is the development of antibodies to the components of viral particles, the detection of which confirms the past disease or asymptomatic carrier state and indicates the presence of immunity. The duration and strength of immunity to the SARS-CoV-2 in different species is not sufficiently studied.

Currently, various methods are used to detect antibodies to animal coronaviruses: neutralization test, enzyme-linked immunoassay, immunofluorescence assay [5, 6, 8–11]. In 2020, IDvet (France) released the ID Screen® SARS-CoV-2 Double Antigen Multi-species ELISA multi-enzyme immunoassay kit for the detection of antibodies to the SARS-CoV-2 nucleoprotein in serum, plasma and whole blood of different species. There are a number of reports on the development of other non-commercial ELISA kits for the detection of antibodies to the S1 protein receptor-binding domain (RBD) in animal blood [5, 10, 11].

Due to the COVID-19 wide spread of in the world and the infection risk for humans and animals, the goal was to develop a domestic ELISA test kit for detection of antibodies against SARS-CoV-2 antigen in sera of susceptible animals of various species to control post-vaccination immunity and conduct screening tests.

MATERIALS AND METHODS

Antigen. As an antigen in the enzyme-linked immunoassay (ELISA), the SARS-CoV-2 "variant B" strain, inactivated with β-propiolactone and cultured in Vero cells was used. Purification and concentration of the inactivated virus-containing culture liquid included low-speed centrifugation and ultracentrifugation through a 30% sucrose layer. The resulting 100-fold concentrated product was used for ELISA. The presence of the virus was confirmed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Sera. The SARS-CoV-2-free serum samples from animals (ferrets, minks, foxes, cats, dogs, and other susceptible animals) were used as negative controls), positive controls were the SARS-CoV-2-specific sera of animals (ferrets, minks, foxes, cats, dogs, and other susceptible animals) with a titer of at least 1:800.

Indirect ELISA. Antigen working dilution in a carbonate-bicarbonate buffer (pH 9.6) was added to the wells of a 96-well plastic plate (Nunc-Immuno Plates, Denmark), incubated overnight at 4 °C, blocked with a 1% skimmed milk powder solution (Carl Roth GmbH, Germany) in a tris-HCl buffer solution (0.02 M tris-HCl, 0.15 M NaCl, 0.1% twin-20)

(TBR-T), pH 7.4, for 1 hour at room temperature and then washed three times using TBR-T. The serum samples were titrated by two-fold serial dilutions for testing, starting from 1:50 dilution and using 1% skimmed milk solution in TBR-T. When testing single-diluted sera, 1:100 dilution was used as a working solution. The test and control sera were added into the plate wells in a volume of 100 µl and incubated for 45 minutes at 37 °C. The bound antibodies were detected by adding 100 µl of working dilution of protein A from *Staphylococcus aureus* / horseradish peroxidase conjugate (Sigma, USA) diluted by dilution buffer to each well of the plate. The plates were incubated for 45 minutes at 37 °C. After each stage, the wells were washed 3–4 times with a TBR-T buffer solution. To visualize the resulting complex, 100 µl of ABTS substrate solution [2,2-azino-di-(3-ethyl benzoaminosulfonate)] was added to each well and incubated for 10–15 minutes at room temperature. The reaction was stopped by adding 100 µl of a stop solution (1% sodium dodecyl sulfate) to each well. The reaction was read by spectrophotometric method at a wavelength of 405 nm. The highest serum dilution that gave an optical density (OD) of 2 or greater standard deviations above the negative control was deemed the antibody titer. S/N value (where S is the OD of the test sample, N is the OD of negative control) was determined by a single serum dilution method for each sample. To determine the positive-negative threshold, 154 sera from clinically healthy animals of different species were tested. The antibody titer, S/N value and standard deviation were calculated, the sum of the mean and two standard deviations determined the upper limit of the negative values, and the sum of the mean and three standard deviations determined the lower limit of the positive values.

Electrophoresis and immunoblotting. Protein electrophoresis was performed in 10% SDA-PAGE for 1 hour at a constant voltage of 200 V. Separated viral proteins were transferred to a nitrocellulose membrane with a pore size of 0.45 microns for 1 hour at a voltage of 100 V using Mini Trans-Blot Electrophoretic transfer cell, BioRad, USA according to the manufacturer's instructions. The membrane was incubated for 1 hour in a 1% milk powder solution in TBR-T buffer, at pH 7.4. Then they were treated with normal and the SARS-CoV-2 positive animal sera diluted 1:100 with TBR-T buffer for 1 hour while being stirred on a shaker. After soaking in a Protein A-horseradish Peroxidase Conjugate (Sigma, USA) for 1 hour, the membrane was stained with a substrate mixture including 4-chloro-1-naphthol and 0.04% hydrogen peroxide. Each step was finished by a 3–4 washings of the membrane with TBR-T buffer.

Statistical data processing. For statistical data processing, we used the program Statistica 10.0 (Stat Soft. Inc., USA).

RESULTS AND DISCUSSION

After purification and concentration of the virus-containing culture fluid, the presence of the virus in the resulting preparation was determined by SDS-PAGE electrophoresis of viral proteins and immunoblotting using specific sera (Fig. 1, 2). The polypeptide position after electrophoresis corresponded to the data published by other authors for SARS-CoV-2 [2, 4].

The testing of sera from animals vaccinated against coronavirus infection (COVID-19) by immunoblotting showed that antibodies were developed against the main immunodominant proteins: spike protein S1- and S2-subunits and N-protein, this is confirmed by the data of other researchers [10, 11].

The resulting inactivated virus material was used as an antigen in indirect ELISA to detect antibodies to SARS-CoV-2.

When developing the ELISA test kit, the reaction conditions were optimized: the antigen working dilution (1:300) and immunoperoxidase conjugate working dilution (1:10000) was determined. Analyzing 30 optical density values of tested control sera, diluted 1:100, their acceptable values were established: not higher than 0.2 for a negative control; not lower than 0.4 for a positive control.

To obtain an objective assessment of the immune response, it was necessary to establish a positive-negative threshold. 154 sera from clinically healthy animals of different species (ferrets, minks, foxes, Arctic foxes, cats and dogs),

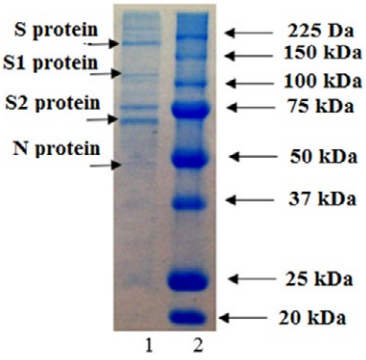

Fig. 1. SARS-CoV-2 antigen. SDS-PAGE electrophoresis, Coomassie Brilliant Blue G-250 staining:
1 – purified SARS-CoV-2 antigen protein fractions;
2 – molecular weight marker (BioRad, USA)

Рис. 1. Антиген SARS-CoV-2. Электрофорез в 10%-м ДСН-ПААГ, окрашивание Coomassie Brilliant Blue G-250:
1 – белковые фракции очищенного антигена SARS-CoV-2;
2 – маркер молекулярных весов (BioRad, США)

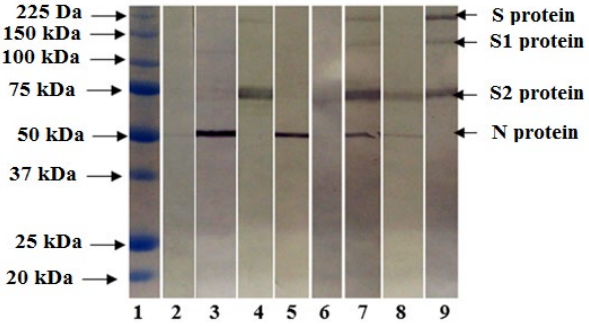

Fig. 2. SARS-CoV-2 antigen protein immunoblotting using normal and coronavirus-specific animal sera:
1 – molecular weight marker (BioRad, USA);
2, 6 – purified SARS-CoV-2 antigen protein fractions using normal ferret and cat sera;
3, 4, 5, 7, 8, 9 – purified SARS-CoV-2 antigen protein fractions using coronavirus-specific sera of ferret (3), mink (4), fox (5), cat (7, 8), dog (9)

Рис. 2. Иммуноблоттинг белков антигена SARS-CoV-2 с нормальной и специфическими к коронавирусу сыворотками крови животных:
1 – маркер молекулярных весов (BioRad, США);
2, 6 – белковые фракции очищенного антигена SARS-CoV-2 с нормальной сывороткой крови хорька и кошки;
3, 4, 5, 7, 8, 9 – белковые фракции очищенного антигена SARS-CoV-2 со специфической к коронавирусу сывороткой крови хорька (3), норки (4), лисы (5), кошки (7, 8), собаки (9)

Table 1
Results of the method reproducibility study

Таблица 1
Результаты исследования воспроизводимости метода

Serum type	Operator / day of run	Indicator			
		Mean S/N value	Standard deviation (σ)	Coefficient of variation (CV%)	Mean value CV%
Repeatability (by 6 tests)					
Weakly positive	1/1	4.44	0.205	4.62	3.70
	2/1	4.01	0.125	3.12	
	1/2	3.76	0.144	3.82	
	2/2	4.05	0.129	3.17	
Strongly positive	1/1	6.00	0.174	2.91	2.43
	2/1	6.04	0.144	2.39	
	1/2	6.05	0.285	4.71	
	2/2	6.25	0.170	2.72	
Reproducibility (by 24 tests)					
Weakly positive	2 operators / 2 days	4.07	0.280	6.99	5.12
Strongly positive	2 operators / 2 days	6.08	0.197	3.24	

not vaccinated against COVID-19, were examined in triplicate on different days. As a result, the established mean S/N value of negative sera was 1.333, and the standard deviation was 0.3769. Based on the statistical analysis, the result was considered negative if S/N was ≤ 2.1; positive if S/N was ≥ 2.5; intermediate values were considered inconclusive. When the serial dilution method was used, the antibody titer less than or equal to 1:100 was considered negative, greater than or equal to 1:200 value was considered positive.

The reproducibility of the method was evaluated by the coefficient of variation within one run and between the runs, doing retests of 2 positive serum samples from susceptible animals (ferrets) with different concentrations of antibodies (low and high). The coefficient of variation of S/N values within one run was evaluated by 6 tests conducted by one operator, the coefficient of variation between the runs – by 24 tests conducted on 4 different plates by two operators on different days (Table 1).

The results obtained by repeated ELISAs showed that the mean value of the coefficient of variation did not exceed 7%. Thus, the use of the developed test kit for the detection of the SARS-CoV-2 antibodies by indirect ELISA gave reproducible results.

To confirm the specificity of the developed method, sera specific for α-coronavirus (porcine transmissible gastroenteritis virus), β-coronavirus (bovine coronavirus), γ-coronavirus (infectious bronchitis virus) and pestivirus (bovine diarrhea virus) were used. It was shown (Table 2) that the activity of the SARS-CoV-2 antigen against heterologous sera did not exceed the background level (reaction to non-immune serum).

In order to assess the relative specificity and sensitivity of the test kit, the results obtained using the developed ELISA and neutralization test were compared using 2 × 2 contingency table. Table 3 shows the results of the testing by two assays of 30 serum samples taken from ferrets 28 days after immunization with pilot vaccines against coronavirus infection (COVID-19) of carnivorous animals (produced by the FGBI "ARRIAH"). The data obtained indicate that 3 samples were negative and 25 samples were positive in both assays. Two samples, positive in neutralization test, when showed negative results when tested by ELISA.

The ELISA specificity and sensitivity compared to these parameters of the neutralization test used for testing of postvaccinal sera was 100 and 92.6%, respectively.

Table 2
Results of SARS-CoV-2 antigen antibody detection in homologous and heterologous animal sera by ELISA

Таблица 2
Результаты выявления антител к антигену SARS-CoV-2 в гомологичных и гетерологичных сыворотках крови животных методом ИФА

No.	Sample	Antibody titer*	S/N	Qualitative assessment of the result
1	Porcine transmissible gastroenteritis virus specific serum	50	0.88	negative
2	Bovine coronavirus specific serum	50	0.78	negative
3	Bovine diarrhea virus specific serum	100	0.99	negative
4	Infectious bronchitis virus specific serum	50	0.57	negative
5	Negative control serum	100	1.00	negative
6	SARS-CoV-2 positive control serum	800	5.70	positive

* Antibody titer is the reciprocal value of the serum dilution
(Титр антител – величина, обратная разведению сыворотки).

In order to determine the diagnostic sensitivity and specificity, 50 serum samples from different species (minks – 14, foxes – 10, cats – 14 and dogs – 12) were tested before and after (after 4–6 weeks) vaccination against COVID-19 with pilot vaccines produced by the FGBI "'ARRIAH". The results of the studies are presented in Table 4. Antibodies to SARS-CoV-2 were detected in the sera of all vaccinated animals; before immunization, all serum samples were negative in ELISA.

The results of antibody detection using the developed test kit were compared with the results, obtained by the commercial IDvet kit (France). For this purpose, 44 sera from different species were tested using the FGBI "ARRIAH" test kit and the IDvet commercial kit for the detection of antibodies to the SARS-CoV-2 nucleoprotein in accordance with the manufacturer's instructions.

Antibodies to the SARS-CoV-2 were detected in all serum samples of vaccinated animals, tested using the developed test kit (Table 5).

When testing by the IDvet kit, the SARS-CoV-2-specific antibodies were detected only in the sera of vaccinated ferrets, foxes, and minks. No specific antibodies were found in the blood of vaccinated dogs and cats. Since the IDvet kit is designed to detect antibodies only to the SARS-CoV-2 nucleoprotein, it can be assumed that the level of antibodies to protein N in the sera of cats and dogs is very low, which is also confirmed by immunoblotting of these sera (Fig. 2). In serum samples from non-vaccinated pigs, virus-specific antibodies were not detected by both ELISA test kits.

Table 3
Analysis of ELISA relative sensitivity and specificity for SARS-CoV-2 antibody detection in animal sera

Таблица 3
Оценка относительной чувствительности и специфичности ИФА для выявления антител к вирусу SARS-CoV-2 в сыворотках крови животных

Neutralization test*	ELISA		
	Positive	Negative	Total
Positive samples	25/a	2/c	27/a+c
Negative samples	0/d	3/b	3/d+b
Total samples	25	5	n = 30

* Sera were tested by neutralization test at the "N. F. Gamaleya NRCEM"of the Ministry of Health of the Russian Federation (Moscow) / Исследование сывороток крови в реакции нейтрализации выполнено в ФГБУ «НИЦЭМ им. Н. Ф. Гамалеи» Минздрава России (г. Москва);
a – true-positive results (истинно положительные результаты);
b – true-negative results (истинно отрицательные результаты);
c – false-negative results (ложноотрицательные результаты);
d – false-positive results (ложноположительные результаты).

Table 4
Results of SARS-CoV-2 antibody detection in animal sera before and after vaccination against coronavirus infection (COVID-19) by ELISA

Таблица 4
Результаты выявления антител к вирусу SARS-CoV-2 в сыворотках крови животных до и после вакцинации против коронавирусной инфекции (COVID-19) животных в ИФА

No.	Serum samples	Antibody titer*	S/N**	Number of samples / positives
1	Minks before vaccination	64 ± 9	1.45 ± 0.11	7/0
2	Minks after vaccination	829 ± 216	6.03 ± 0.89	7/7
3	Foxes before vaccination	50 ± 0	1.45 ± 0.11	5/0
4	Foxes after vaccination	400 ± 0	3.61 ± 0.65	5/5
5	Cats before vaccination	50 ± 0	1.31 ± 0.08	7/0
6	Cats after vaccination	543 ± 95	5.03 ± 0.47	7/7
7	Dogs before vaccination	83 ± 11	1.69 ± 0.13	6/0
8	Dogs after vaccination	667 ± 84	4.33 ± 0.40	6/6

* Average value of the antibody titer ± standard error of the mean, where the antibody titer is the reciprocal value of the serum dilution (Среднее значение титра антител ± стандартная ошибка среднего, где титр антител – величина, обратная разведению сыворотки);
** S/N value ± standard error of the mean (значение S/N ± стандартная ошибка среднего).

CONCLUSION

The developed indirect ELISA test kit for detection of antibodies to the SARS-CoV-2 showed high specificity (100%), sensitivity (92.6%) and reproducibility and can be used for screening tests for the SARS-CoV-2 in different susceptible species and control of the immunity after vaccination against coronavirus infection (COVID-19) of animals.

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Table 5
Results of SARS-CoV-2 antibody detection in animal sera by ELISA

Таблица 5
Результаты выявления антител к вирусу SARS-CoV-2 в сыворотках крови животных в ИФА

No.	Serum samples	ELISA (FGBI "ARRIAH")		ELISA (IDvet)	
		Antibody titer*	Number of samples / positives	S/P (%)**	Number of samples / positives
1	Ferrets after vaccination	1,600 ± 0	5/5	707 ± 66	5/5
2	Minks before vaccination	50 ± 0	2/0	47 ± 9	2/0
3	Minks after vaccination	1,025 ± 225	8/8	529 ± 58	8/8
4	Foxes after vaccination	300 ± 58	4/4	208 ± 62	4/4
5	Non-vaccinated pigs	50 ± 0	4/0	3.1 ± 1.8	4/0
6	Non-vaccinated Arctic foxes	50 ± 0	2/0	2 ± 0	2/0
7	Cats before vaccination	50 ± 0	3/0	1 ± 0	3/0
8	Cats after vaccination	560 ± 264	5/5	0.80 ± 0.01	5/0
9	Dogs before vaccination	50 ± 0	4/0	8.3 ± 6.6	4/0
10	Dogs after vaccination	700 ± 205	6/6	2.40 ± 1.09	6/0

Serum samples from vaccinated animals were taken 4-5 weeks after immunization using experimental vaccines against coronavirus infection (COVID-19) produced by the FGBI "ARRIAH" (Пробы сыворотки крови от вакцинированных животных отобраны через 4–5 недель после иммунизации экспериментальными вакцинными препаратами против коронавирусной инфекции (COVID-19) производства ФГБУ «ВНИИЗЖ»);
* the average value of the antibody titer ± the standard error of the mean, where the antibody titer is the reciprocal value of the serum dilution (среднее значение титра антител ± стандартная ошибка среднего, где титр антител – величина, обратная разведению сыворотки);
** mean S/P ± standard error of the mean (среднее значение S/P ± стандартная ошибка среднего).
Interpretation of the results obtained by IDvet kit (Интерпретация результатов в наборе IDvet): S/P ≤ 50% – negative result (результат отрицательный), S/P ≥ 60% – positive result (результат положительный), 50% < S/P < 60% – inconclusive result (результат сомнительный).

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