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Development of polymerase chain reaction kit for detection of SARS-CoV-2 RNA in biological samples collected from animals

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SUMMARY

Today, global attention is drawn to the same common problem – spread of the novel COVID-19 infection. From the end of December 2019 novel SARS-CoV-2 virus spread over the majority of the countries and on 11 March 2020 the World Health Organization announced pandemic. Global spread of COVID-19 was not limited to the human population and there was a need to test pets and farm animals, who were in contact with the humans. There are more and more reports on SARS-CoV-2 detected in minks, ferrets, dogs, cats, tigers, lions and other animals. Today the key method of COVID-19 diagnosis is polymerase chain reaction, but all currently available test-kits are intended for the virus detection in humans. The paper demonstrates data on the development of the real-time PCR-based method for SARS-CoV-2 RNA detection in the biological samples collected from animals. During the research, an optimal system of primers and a probe were selected, reaction conditions were tested, basic validation specifications (sensitivity, specificity, reproducibility) were set. The validation results demonstrated that the method met all the criteria of the high-quality measurement/test methods and it can be used for diagnostic tests. The test-kit was based of the method intended for SARS-CoV-2 RNA detection in animal biological samples and it was put into the veterinary practice. Animal populations in different regions of the Russian Federation were subjected to the screening tests in order to detect the novel coronavirus genome. No SARS-CoV-2 was reported in herbivorous animals in the Russian Federation. The FGBI "ARRIAH" experts detected only one positive pet animal.

Keywords: novel SARS-CoV-2, real-time polymerase chain reaction, test-kit, animal biological sample

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Разработка тест-системы для обнаружения РНК вируса SARS-CoV-2 в биоматериале от животных методом полимеразной цепной реакции

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РЕЗЮМЕ

Сегодня внимание всего мирового сообщества приковано к одной общей проблеме – распространению новой коронавирусной инфекции (COVID-19). С конца декабря 2019 г. коронавирус SARS-CoV-2 охватил большинство стран мира, и 11 марта 2020 г. Всемирной организацией здравоохранения была объявлена пандемия. Глобальное распространение COVID-19 не ограничилось человеческой популяцией, и возникла необходимость тестирования домашних и сельскохозяйственных животных, находящихся в контакте с человеком. Появляется все больше сообщений о выявлении SARS-CoV-2 у норок, хорьков, собак, кошек, тигров, львов и других животных. Основным методом диагностики COVID-19 на сегодняшний день является полимеразная цепная реакция, однако все существующие в настоящее время тест-системы предназначаются для выявления вируса у людей. В статье представлены данные по разработке метода обнаружения РНК вируса SARS-CoV-2 в биоматериале от животных с помощью полимеразной цепной реакции в реальном времени. В процессе проведенных исследований выбрана оптимальная система праймеров и зонд, отработаны условия реакции, определены основные валидационные характеристики метода (чувствительность, специфичность, воспроизводимость). В результате проведения валидации установлено, что он отвечает требованиям, предъявляемым к качественным методам измерений/испытаний, и может применяться в диагностических исследованиях. На основе разработанного метода создана тест-система для выявления РНК вируса SARS-CoV-2 в биоматериале от животных, которая внедрена в вете-

ринарную практику. Проведены скрининговые исследования популяций животных из различных регионов Российской Федерации с целью выявления генома нового коронавируса. Показано, что среди травоядных животных Российской Федерации вирус SARS-CoV-2 не встречается. Среди домашних животных-компаньонов специалистами ФГБУ «ВНИИЗЖ» был зафиксирован лишь один положительный случай.

Ключевые слова: новый коронавирус SARS-CoV-2, полимеразная цепная реакция в реальном времени, тест-система, биоматериал от животных

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INTRODUCTION

Similar to MERS-CoV and SARS-CoV novel coronavirus SARS-CoV-2 induces severe pneumonia in humans (COVID-19). It is one of the RNA-viruses of family *Coronaviridae*, lineage Beta-CoV B.

The first human cases of pneumonia of unknown origin were reported in Wuhan city, Hubei Province, China [1]. The novel coronavirus was isolated from the respiratory epithelial cells and identified as the disease agent [2]. In 2020, the majority of the countries reported the disease cases in humans, and the World Health Organization (WHO) announced COVID-19 pandemic [3, 4].

In patients COVID-19 is demonstrated with such pronounced clinical signs as fever, dry cough, dyspnea, rhinorrhea, acute tonsillitis, interstitial infiltrate in lungs [5].

Bat coronavirus (*Rhinolophus sinicus*) is considered to be the ancestor of SARS-CoV-2. The virus was transmitted from bats to humans via intermediate host, which was failed to be established [6–10]. Global spread of SARS-CoV-2 is not limited to human population. There are currently lots of reports of the novel coronavirus detected in animals: minks, dogs, cats, tigers and lions. Experimental infection demonstrated that ferrets and cats are highly susceptible to SARS-CoV-2 and they can transmit the virus from the infected animals to the healthy ones through contacts and air [11–17].

Susceptibility of the animals to SARS-CoV-2 could be explained by the fact that the receptor for the virus entry is angiotensin-converting enzyme 2 (ACE2), which is nearly identical and similar in humans and such animal species as mustelids, felines, suids and simian [16, 18, 19]. There is, therefore, a possibility of SARS-CoV-2 reservoir formation in pet population, and, hence, COVID-19 epidemic surveillance should involve diagnostic and monitoring tests of pets.

The recommended method of COVID-19-specific laboratory diagnosis is SARS-CoV-2 RNA detection using polymerase chain reaction (PCR). This tool is ideal for the primary screening owing to its high sensitivity and specificity [20–23].

Currently a sufficient amount of test-kits for SARS-CoV-2 detection in humans have been developed, but they are not intended for the analysis of biological samples from animals [24–27]. Therefore, development of a reliable, sensitive and specific method for COVID-19 diagnosis in animals is a relevant objective.

The research was aimed at the development of a test-kit for SARS-CoV-2 RNA detection in biological samples from animals using real-time polymerase chain reaction.

MATERIALS AND METHODS

Biological samples. The original SARS-CoV-2 isolate recovered from a human was kindly provided by the Smorodintsev Research Institute of Influenza (Ministry of Health of the Russian Federation). Biological samples collected from humans (nasal and oral swabs) and animals (nasal swabs, lungs) were used in the research. The test objects included both domestic animals (pigs, cattle, sheep, goats) and wild animals (boars, elks, deer, Manchurian wapiti, roe deer, yaks, antelopes, European bison, Caucasian turs, red deer, saiga antelopes, ibexes, musk deer, dzerens). For testing food products swabs were collected from the surfaces of meat products and meat preparations as well as from their package.

Reference strains. Specificity of the method was tested using the viruses belonging to *Coronaviridae* family: SARS-CoV-2, transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhoea virus (PEDV), porcine respiratory coronavirus (PRCoV), bovine coronavirus (BCoV), avian infectious bronchitis virus (IBV), as well as heterologous viruses: porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), porcine circovirus type 2 (PCV-2), Aujeszky's disease virus (ADV), infectious bursal disease virus (IBDV), influenza A virus (IAV), infectious bovine rhinotracheitis virus (BHV-1), parainfluenza-3 virus (bPIV-3).

RNA extraction from 10%-suspension of the biological samples was performed using 6 M guanidinium thiocyanate and glass fiber filters GF/F [28]. Nucleic acid was

handled in the environment controlled according to MU 1.3.2569-09 "Organization of laboratories using nucleic acid amplification methods for the work with materials containing microorganisms I–IV pathogenicity groups".

Polymerase chain reaction. The test procedure is described below. Reaction PCR mixture was formulated, which contained 5 µL of 10× Taq-polymerase buffer, 3 mM Mg^{2+}, 0.2 mM dNTPs, 2 units of Taq-DNA polymerase, 5 pmol of each primer, 5 µL of DNA solution and water added to final volume of 50 µL. The reaction was performed using Mastercycler DNA amplification machine (Eppendorf, Germany). The test procedure included reverse transcription at 42 °C for 15 minutes and 35 amplification cycles at the following temperatures: denaturation at 94 °C for 30 seconds, annealing of primers at 55 °C for 30 seconds and elongation at 72 °C for 40 seconds. The test products were analyzed using 0.001% ethidium bromide 2% agararose gel electrophoresis at 50 mA.

Molecular cloning of SARS-CoV-2 N gene was performed using CloneJET PCR Cloning Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Nucleotide sequences. SARS-CoV-2 nucleotide sequences deposited in GenBank database were used in the study. The nucleotide sequences were analyzed using BioEdit software.

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR). Conditions and modes of the reaction are described below. All reactions were performed using C1000 Touch™ thermocycler and CFX96 detection system (Bio-Rad, USA).

Statistical processing of the data was performed using validation analytical techniques [29, 30]. The following performance properties were determined: sensitivity and specificity, precision under the repeatability and reproducibility conditions.

RESULTS AND DISCUSSION

Design of primers. By the start of the works on the development of the test-kit, the WHO had published several procedures on COVID-19 laboratory diagnosis on its website. As a result of their analysis, primers and probe was selected from the extensive list of oligonucleotides recommended for COVID-19 detection. The selected primers and probe were proposed by the Center for Disease Control and Prevention (CDC, USA):

Primer 1 – GACCCCAAAATCAGCGAAAT;

Primer 2 – TCTGGTTACTGCCAGTTGAATCTG;

Probe – ROX ACCCCGCATTACGTTTGGTGGACC BHQ2.

The chosen oligonucleotides are complimentary to SARS-CoV-2 N gene.

Development and validation of the method for SARS-CoV-2 RNA detection using real-time RT-PCR. Reaction mixture composition, temperature and time of the reaction were specified during the optimization procedure.

The reaction was performed with 25 µL of the mixture containing 0.5 µL (5 pm) of direct and indirect primers, 0.5 µL (5 pm) of the probe, 2.5 µL of the 10× PCR buffer, 4 µL of 25 mM $MgCl_2$, 0.7 µL of 25 mM dNTPs, 0.2 µL (1 unit) of Taq-DNA-polymerase, 0.4 µL (20 units) of M-MLV-reverse transcriptase, 1 µL of nuclease-free water and 5 µL of RNA.

The amplification program included reverse transcription phase at 42 °C for 15 minutes followed by denaturation at 95 °C for 5 minutes and 40 cycles of PCR itself (denaturation at 95 °C for 15 seconds, annealing at 55 °C for 15 seconds and elongation at 60 °C for 20 seconds).

Ct ≤ 35 was taken as the reaction threshold value, when the sample was considered positive.

Specificity of the method was tested using several types of viruses of *Coronaviridae* family (SARS-CoV-2, PRCoV, BCoV, TGEV, PEDV, IBV) and other agents of farm animal diseases (PPV, PCV-2, ADV and PRRSV, IBDV, IAV, BHV-1, bPIV-3), as well as RNA/DNA extracted from porcine, bovine and chicken tissues. The positive reaction was observed only with SARS-CoV-2 RNA, thus being indicative of the specificity of the method (Fig. 1).

The sensitivity was assessed by testing serial 10-fold dilutions of SARS-CoV-2 RNA.

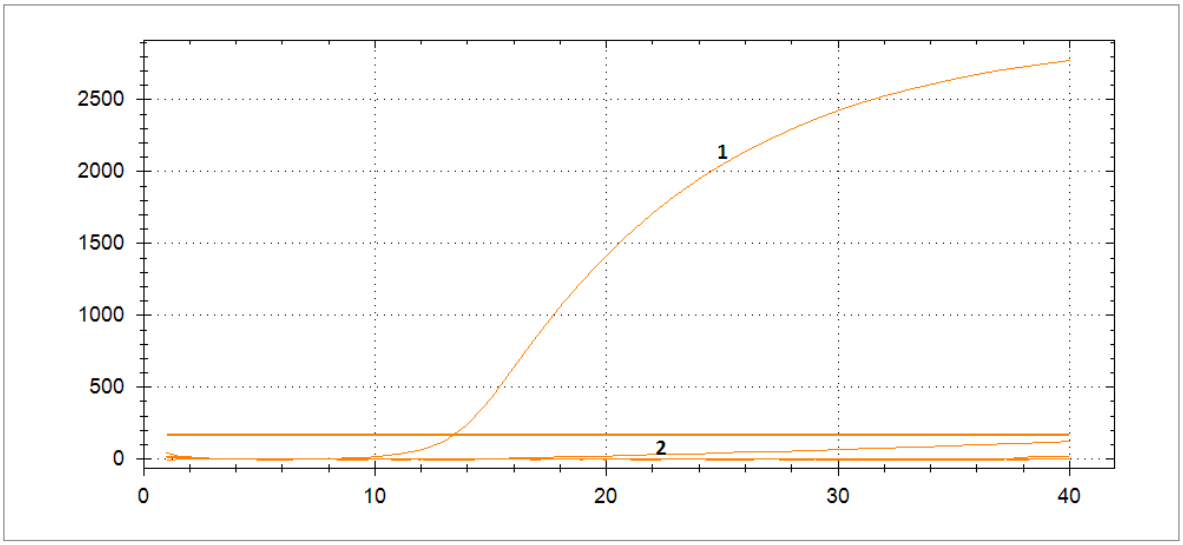

Fig. 1. Detection of SARS-CoV-2 in animal biological samples using real-time PCR:
1 – SARS-CoV-2-containing nasal swab;
2 – SARS-CoV-2-negative sample (TGEV, PEDV, PRCoV, BCoV, IBV, PRRSV, PPV, PCV-2, ADV, IBDV, influenza A virus, BHV-1, bPIV-3, tissue RNA of non-infected pigs, cattle, chickens)

Table 1
Samples used to test sensitivity of the developed test-kit

Sample No.	Description	Status of the sample	Result obtained using the test-kit being validated, *Ct* (interpretation of the result)
1	SARS-CoV-2 RNA sample	positive	14.21 (positive)
2	SARS-CoV-2 RNA sample	positive	14.47 (positive)
3	SARS-CoV-2 RNA sample	positive	16.16 (positive)
4	SARS-CoV-2 RNA sample	positive	18.49 (positive)
5	SARS-CoV-2 RNA sample	positive	21.81 (positive)
6	SARS-CoV-2 RNA sample	positive	19.56 (positive)
7	SARS-CoV-2 RNA sample	positive	23.33 (positive)
8	SARS-CoV-2 RNA sample	positive	24.04 (positive)
9	SARS-CoV-2 RNA sample	positive	22.98 (positive)
10	SARS-CoV-2 RNA sample	positive	23.21 (positive)

The sensitivity was determined as a percentage of positive results obtained in the test-kit being validated to the total number of tests and its was calculated according to the formula:

$$Se = (TP / (TP + FN)) \times 100\%,$$

where TP – true positive result;
FN – false negative result.

All tested SARS-CoV-2 RNA-containing samples demonstrated positive result with the test-kit being validated (Table 1). Therefore, the calculated sensitivity of the validated test-kit amounted to 100%.

Assessment of the test-kit reproducibility. The reproducibility was assessed by testing one positive sample and one negative sample in 10 repeated tests performed under the changed measurement conditions: by the same analyst in parallel tests performed on different days (10 days) and by two different analysts in parallel tests (in 10 repetitions). Precision was assessed by the degree of agreement between the repeated measurements of the same sample. The validated test-kit demonstrated absolute precision, i.e. the positive sample always demonstrated positive result and the negative sample – negative result.

It was therefore established that in its characteristics the real-time RT-PCR is compliant with the requirements to the high quality measurement/test methods and can be used in diagnostic studies.

Following the validation results, methodical guidelines were developed for the detection of SARS-CoV-2 RNA using real-time polymerase chain reaction. These guidelines were further used for COVID-19 diagnosis in animals.

Development of test-kit for SARS-CoV-2 RNA detection using real-time RT-PCR. Production of recombinant plasmid containing SARS-CoV-2 N gene. Any test-kit intended for general use shall meet safety requirements, therefore, a decision was taken to use the recombinant plasmid as positive control.

SARS-CoV-2 N gene region was PCR amplified using coronavirus RNA extracted at the Smorodintsev Research Institute of Influenza (Ministry of Health of the Russian Federation). Primers targeted at the conservative genome region were used for the reaction. Clones containing SARS-CoV-2 N gene region were resulted from the transformation by the ligase mixture of pJET1.2/blunt vector and amplified fragment of the viral gene of the competent *Escherichia coli* JM109 cells. Presence of specific DNA region was checked using nucleotide sequencing.

The recombinant plasmid physical map is shown in Figure 2.

At the next stage, real-time RT-PCR test-kit was developed, which contained specific oligonucleotide primers and fluorescent-labeled oligonucleotide probe. Test conditions were also selected using newly developed diagnosticum contributing to the minimal risk of the test sample contamination and excluding any biased assessments of the results.

For user's convenience the real-time RT-PCR test-kit for detection of SARS-CoV-2 RNA consists of the following components:

No. 1 – RT-PCR mixture;
No. 2 – Taq DNA-polymerase enzyme;
No. 3 – M-MLV reverse transcriptase;
No. 4 – positive control;
No. 5 – negative control.

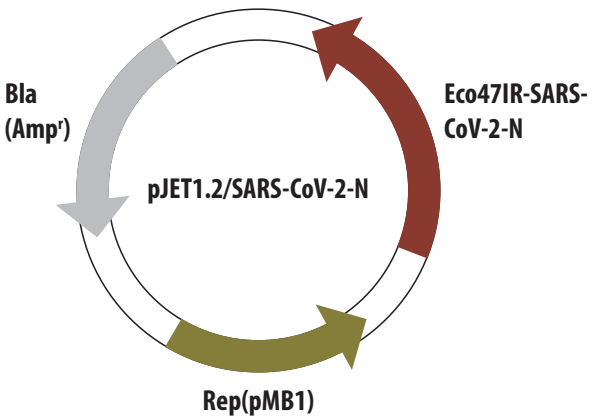

Fig. 2. Physical map of the recombinant plasmid pJET1.2/SARS-CoV-2-N

Table 2
Testing biological samples collected from herbivorous animals for SARS-CoV-2 using "SARS-CoV-2 real-time RT-PCR test-kit"

Animal species	Number of samples tested	Number of positives
pigs	980	0
wild boars	174	0
cattle	220	0
sheep and goats	20	0
wild even-toed ungulates (elks, deer, Manchurian wapiti, roe deer, yaks, antelopes, European bison, Caucasian turs, red deer, saiga antelopes, ibexes, musk deer, dzerens, etc.)	638	0

Fig. 3. "SARS-CoV-2 real-time RT-PCR test-kit" for detection of SARS-CoV-2 RNA in animal biological samples using real-time polymerase chain reaction

Deionized water is used as negative control, and plasmid DNA containing inserted SARS-CoV-2 N gene fragment was used as positive control. Each test-kit comes with the manufacturer's instructions (Fig. 3).

Real-time RT-PCR is performed in one step using programmable amplifier of any model and mixture of PCR reagents and enzymes. RNA can be extracted from the tested samples using any convenient method or commercial test-kit. Negative and positive controls are added to the relative samples.

The results are interpreted according to the presence (or absence) of fluorescence curve intersection with the threshold set at the appropriate level thus corresponding to the presence (or absence) of the cycle threshold value (*Ct*) in the relative column in the table of results. The result is considered positive if the fluorescence accumulation curve for the relative sample has a typical sigmoidal shape and intersects the threshold line.

There are currently lots of reports of SARS-CoV-2 detection in animals (minks, ferrets, cats, dogs, etc.). There is also evidence of the virus detection in herbivorous animals (deer). During the developed method implementation biological samples available in large amounts were used (nasal swabs, esopharyngeal fluids, internal organs, blood) and collected from pigs, cattle, sheep, goats, and wild cloven-hoofed animals. However, no SARS-CoV-2 was detected in these animal species (Table 2). We also failed to detect the viral RNA in the samples of raw meat, meat preparations and in the swabs from their package.

In 2020–2021, the FGBI "ARRIAH" carried out SARS-CoV-2 RNA screening tests of the animal populations in 20 regions of the Russian Federation using the developed test-kit. The published earlier results [11] demonstrated that out of 1,466 tested biological samples from different animal species, only one esopharyngeal swab collected from the domestic cat in Tyumen demonstrated SARS-CoV-2 RNA [11]. The genome of the novel coronavirus was not detected in any of the biological samples collected from the herbivorous animals. Therefore, use of the developed "SARS-CoV-2 real-time RT-PCR test-kit" demonstrated possibility of SARS-CoV-2 detection in pets.

CONCLUSION

The performed studies resulted in the development of the method for SARS-CoV-2 RNA detection using RT-PCR, determination of the validation specifications of the method and preparation of the "SARS-CoV-2 real-time RT-PCR test-kit". The test-kit was used for the analysis of a large number of samples collected from various animal species. The proposed test-kit was put into veterinary practice for the detection of SARS-CoV-2 RNA in animal biological samples within primary and confirmatory diagnosis, for research purposes and monitoring of SARS-CoV-2 spread in animals. The resulted performance specifications of the test-kit comply with the criteria applicable to the high-quality laboratory test methods.

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