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Development and validation of highly sensitive multiplex real-time RT-PCR assay for detection of classical swine fever virus genome

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ABSTRACT

Classical swine fever (CSF) remains a challenge for pig farming industry all over the world despite the measures taken. The last CSF case in the Russian Federation was reported in 2020, however, the threat of the disease emerging still persists. A set of anti-epidemic measures including mainly preventive vaccination and annual diagnostic monitoring using molecular-genetic and serological methods is required for CSF virus introduction prevention and rapid eradication of potential disease outbreaks. Therefore, a real-time reverse transcription-polymerase chain reaction using an internal control sample has been developed. Therefore, a real-time reverse transcription-polymerase chain reaction using an internal control sample has been developed. Modified primers (locked nucleic acids containing conformationally blocked nucleosides) providing a higher affinity to the DNA matrix and physicochemical stability and a FAM-labeled TaqMan probe were selected for 5'-untranslated region of the genome. The following validation parameters were defined: accuracy, repeatability, reproducibility, specificity and sensitivity. For comparative analysis of the developed assay sensitivity, swabs, samples of organs and tissues collected from pigs experimentally infected with an epizootic strain of the classical swine fever virus (spleen, kidney, liver, blood, lymph nodes, rectal and oral smears), animal-contaminated feed and virus-containing material with known virus titres were also tested in parallel with coded test systems No. x1 and x2. The developed assay was shown to have 100% diagnostic sensitivity and detection limit of 0.23 lg $CCID_{50}/cm^3$. Therewith, the results of analysis of test systems No. x1, x2 based on above parameters were lower that could give rise to false positive real-time RT-PCR results and incorrect diagnosis. Thus, described assay can be used for extensive monitoring of classical swine fever in the Russian Federation.

Keywords: classical swine fever, real-time RT-PCR, internal control sample, diagnostic sensitivity, analytical sensitivity

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Разработка и валидация высокочувствительного метода мультиплексной ОТ-ПЦР-РВ для обнаружения генома вируса классической чумы свиней

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РЕЗЮМЕ

Проблема классической чумы свиней в свиноводстве по-прежнему остается актуальной во всем в мире, несмотря на принимаемые меры. Последний случай данного заболевания в Российской Федерации регистрировали в 2020 г., однако сохраняется угроза эмерджентного возникновения болезни.

Для предотвращения заноса вируса классической чумы свиней и быстрой ликвидации потенциально возможных вспышек необходимо проведение комплекса противоэпизоотических мероприятий, преимущественно включающих вакцинопрофилактику и ежегодный диагностический мониторинг на основе молекулярно-генетических и серологических исследований. В связи с этим разработан метод полимеразной цепной реакции с обратной транскрипцией в режиме реального времени с использованием внутреннего контрольного образца. Праймеры в модификации Locked Nucleic Acid (конформационно блокированных нуклеозидов), обеспечивающие более высокий уровень аффинности к ДНК-матрице и физико-химической стабильности, и FAM-меченый TaqMan-зонд были подобраны к 5′-нетранслируемой области генома. Также определены валидационные показатели: правильность, сходимость, воспроизводимость, специфичность и чувствительность. С целью сравнительного анализа чувствительности параллельно тестировались зашифрованными тест-системами № х1, х2 образцы смывов, органов и тканей, полученных от свиней, экспериментально зараженных эпизоотическим штаммом вируса классической чумы свиней (селезенка, почка, печень, кровь, лимфатические узлы, ректальные и оральные мазки), корма, контаминированного животными, и вируссодержащего материала с известными титрами. Показаны 100%-я диагностическая чувствительность и предел детекции в 0,23 lg ККИД$_{50}$/см^3 разработанного метода. При этом показатели тест-систем № х1 и х2 были ниже, что может приводить к ложноотрицательным результатам полимеразной цепной реакции с обратной транскрипцией в режиме реального времени (ОТ-ПЦР-РВ) и влиять на недостоверную постановку диагноза. Таким образом, представленный метод может использоваться при проведении широкомасштабного мониторинга классической чумы свиней в Российской Федерации.

Ключевые слова: классическая чума свиней, ОТ-ПЦР-РВ, внутренний контрольный образец, диагностическая чувствительность, аналитическая чувствительность

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INTRODUCTION

Classical swine fever (CSF, *Pestis suum*) is one of the major viral diseases having a significant impact on pig farming and wild boar hunting [1].

The CSF etiological agent is a *Pestivirus C* with a positive-sense, single-stranded RNA genome of 12.3 kb in length of the genus *Pestivirus*, family *Flaviviridae* [2]. RNA molecule contains 2 nontranslated regions (5′-NTR and 3′-NTR), as well as one open reading frame coding for 13 proteins (4 structural and 9 nonstructural proteins) [3].

According to the recommendations of the World Organization for Animal Health (WOAH) classical swine fever is subject to notification [4]. And despite the fact that CSF was eradicated in the European countries and in Russia, where the last outbreak in domestic pigs was reported in 2019 and the last outbreak in wild boars was reported in 2020, the threat of this transboundary disease introduction still persists, that requires systematic disease monitoring [5]. Due to the lack of reliable data on the number of samples tested within passive serological monitoring, it is difficult to reliably prove absence of virulent CSFV circulation in wild boar population in Russia [6].

Currently, the polymerase chain reaction (PCR) assay is widely used as one of the most rapid, specific and sensitive molecular biological methods for the pathogen genetic material detection [7]. However, the classical PCR assay with electrophoretic detection in agarose gel is a time- and labour-consuming method posing a high risk of cross-contamination [8]. The real-time multiplex reverse transcription polymerase chain reaction (real-time RT-PCR) with an internal control sample (ICS) minimizing unreliable results is the most suitable for CSF diagnosis, including screening and monitoring [9].

TaqMan probes enabling real-time hybridization-fluorescence detection of PCR products and being the most practical and reliable ones for pestivirus infection diagnosis are used in some assays [10]. Most real-time RT-PCR-based test systems described earlier for the CSFV genome detection amplify a fragment of 5′-nontranslated region (5′-NTR) and demonstrate sufficient sensitivity and specificity [11]. Modification of oligonucleotides in locked nucleic acids (LNA) increases primer affinity to the target DNA and provides for physicochemical stability [12]

Exogenous ICS allows for avoiding false negative results due to errors both at the stage of sample preparation (nucleic acid extraction) and at the stage of the target fragment amplification [13].

The study was aimed at development and validation of highly sensitive, specific and reproducible multiplex real-time RT-PCR assay enabling diagnosis of all CSFV subgenotypes circulating in the Russian Federation territory (1.1, 1.2, 2.1, 2.3) in the period from 1982 to 2020 [5, 14]. Such assay should have characteristics meeting all the requirements for a real-time PCR-based test system and be widely applicable in monitoring for the infection diagnosis [15].

MATERIALS AND METHODS

Viruses and bacteria. The following CSFV strains were used for the assay development: reference Shimen strain, 684 strain, 719 strain (subgenotype 1.1), vaccine SK strain (subgenotype 1.2), CSF Amur 19-10/WB-12555 strain and CSF Tigrovoe 16/WB-634 (subgenotype 2.1), 275 strain (subgenotype 2.2), 368, 870, 843 strains (subgenotype 2.3) and CSFV 589, 924, 925, 929, 917, 918, 920, 926, 927, 930 strains with unidentified virus genotype isolated in the period of 1982–2020.

The following heterologous porcine disease agents were used for determination of the assay analytical specificity: vaccine VK-DEP strain of Aujeszky's disease virus, Irkutsky 2007 strain of American porcine reproductive and respiratory syndrome virus (PRRSV), reference Mozambique-78 strain of African swine fever virus (ASFV) genotype V; Chelyabinsk 2021 isolate of bovine viral diarrhea virus (BVDV) genotype II, field isolate of swine erysipelas agent (*Erysipelothrix rhusiopathiae*). The biological pathogens were obtained in the form of freeze-dried material from the State Microorganism Strain Collection and working microorganism collection of the Federal Centre for Animal Health.

Animals. Piglets at the age of 2–2.5 months weighing 10–15 kg and obtained from CSF-free farms located in the Vladimir Oblast were used for primary cell culture preparation. The piglets were euthanized and testicular explants were collected in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes.

Cultivation. CSFV was propagated in the initially trypsinized swine testicle (ST) cell culture cultivated in Eagle-MEM medium prepared according to the Federal Centre for Animal Health procedure and supplemented with 10% bovine fetal serum and 50 μg/cm^3 of gentamicin sulphate [16]. The virus reproduction was identified with real-time RT-PCR according with the methodical guidelines[1].

Internal control sample (ICS). MS2, RNA-containing bacteriophage belonging to *Leviviridae* family and pathogenic for *Escherichia coli*, was selected as an ICS [17].

Designing of primers and probes. cDNA nucleotide sequences of different CSFV subgenotypes imported from the GenBank database were aligned and subjected to comparative molecular-genetic analysis using Bioedit v7.2.5 and NCBI: Nucleotide BLAST software. Conserved segments of CSFV genome served as a criterion for selection of optimal primers. Primers and probe for amplification and hybridization of ICS fragment were selected based on the literature data [18]. Synthesis of oligonucleotides was performed at the Syntol company (Russia).

Extraction of nucleic acids. CSFV RNA was extracted from the virus-containing ST cell culture suspension, genomes of heterologous viruses and bacteria were extracted from freeze-dried materials with nucleosorption method using RIBO-sorb reagent kit for DNA/RNA extraction from biological materials (Central Research Institute of Epidemiology, Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing, Russia) in accordance with the manufacturer's instructuctions[2].

Real-time RT-PCR procedure. PCR master mix produced by the Eurogene (Russia) contained the following components: OneTube RT-PCR TaqMan kit consisting of OneTube RT-PCRmix, TM-MMLV revertase, nuclease-free water. All PCR stages (reverse transcription, amplification and fluorescence-hybridization detection) were performed in automatic Rotor-Gene Q thermocycler using provided software (QIAGEN, Germany).

Positive control sample (PCS). Vaccine SK strain of CSFV (virus titer – 3.5 lg $CCID_{50}$/cm^3) in the form of freeze-dried material dissolved in 4.0 cm^3 of saline solution and thermally inactivated by heating for 60 minutes at +60 °C was used as a positive control. Tests for inactivation completeness were carried out by three blind passages in ST cell culture according to the methodical guidelines[1].

Negative control sample (NCS). Nuclease-free water produced by the Eurogene (Russia) was used as a negative control.

Validation. Validation parameters were determined in accordance with the recommended guidelines for publication of the quantitative real-time PCR development outcomes (MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments) [19]. To determine accuracy, repeatability and reproducibility CSFV 719 strain-positive sample was analyzed in 6 repeated reactions, in 3 parallel tests performed by the same operator within one day and in 3 parallel tests performed by two operators within 3 days. Analytical sensitivity (detection limit) was estimated using 10-fold dilutions of CSFV 719, 684, Shimen strain with known titer. Detection limit was expressed as minimal virus titer (lg $CCID_{50}$/cm^3) detected by the assay under validation. Amplification efficiency was calculated according to the formula:

$$E = (10^{1/slope} - 1) \times 100\%,$$

where slope – the slope value of the linear region of the curve constructed using the Ct values plotted on a logarithmic scale according to cDNA matrix concentration.

The real-time RT-PCR assay was tested for its analytical specificity using known CSFV RNA-negative samples containing extracted genomes of heterologous viruses and bacteria as well as using CSFV strains of different subgenotypes. To tests the assay for its diagnostic sensitivity a panel of 27 true positive samples (spleen, liver, kidney, blood, lymph nodes, muscle, oral and rectal swabs) collected from the pigs experimentally infected with epizootic CSFV strain at different stages of the infection process as well as contaminated feed samples was prepared. The assay was tested for its diagnostic specificity by examination of 27 known negative samples of complete mixed feed for pigs, 10% spleen, liver, lymph node suspensions, porcine meat products, whole blood, oral and rectal swabs that were free from CSF and were prepared at the Federal Centre for Animal Health Reference Laboratory for ASF for official animal disease monitoring implementation and for commercial tests of samples from pig holdings located in the European part of the Russian Federation. The assay

[1] Kolbin I. S., Vlasova N. N., Igolkin A. S., Elsukova A. A., Gavrilova V. L., Puzankova O. S. Methodical guidelines for isolation of classical swine fever virus with real-time polymerase chain reaction with fluorescent hybridization probe for the product detection in primary cell cultures (porcine spleen, porcine bone marrow, porcine kidney, lamb testicle, swine testicle) approved by the Federal Centre for Animal Health on 14 September 2021, No. 42-21. Vladimir: Federal Centre for Animal Health, 2021. 56 p. (in Russ.)

[2] Instruction on use of RIBO-sorb reagent kit for DNA/RNA extraction from clinical materials: approved by the Order No. 1337-Pr/09 of the Federal Service for Surveillance in Health Care of 20 February 2009. https://www.amplisens.ru/upload/iblock/259/RIBO-sorb.pdf (in Russ.)

was tested for its diagnostic and analytical sensitivity in comparison with coded Russian test-systems No. x1, x2 by parallel testing of the samples. The coded test systems were used according to their manufacturers' instructions. The selected test systems are the most commonly used in Russia for the CSF diagnosis and contain ICS similar to that one used in the tested assay.

The data were statistically processed with Microsoft Excel. GraphPad Prism programme was used for graph plotting.

RESULTS AND DISCUSSION

Oligonucleotide design. Alignment and comparative assessment of nucleotides sequences of CSFV strains of different subgenotypes showed that, as expected, 5'-NTR was the most conservative genome region. Forward and reverse LNA-containing primers amplifying 120 nucleotides fragment were selected for this region (Fig. 1).

TaqMan probes for the target fragment of CSFV genome and for ICS fragment were labelled by fluorophores: 6-FAM (6-carboxyfluorescein) and Cy5.5 (cyanine-5.5), respectively.

Optimization of real-time RT-PCR conditions. Thermal-temporal profile and quantitative composition of the PCR mix were determined during the optimization.

The PCR mix for one reaction contained 1X OneTube RT-PCRmix, 0.4 pmol of forward primer and 0.4 pmol of reverse primer, 0.2 pmol of TaqMan-probe specific for the CSFV fragment; 0.1 pmol of forward primer and 0.1 pmol of reverse primer and TaqMan probe specific for ICS fragment, 1X TM-MMLV revertase, 10.0 µL of RNA matrix. Nuclease-free water was added to make the final reaction mix volume of 25.0 µL.

The PCR procedure included reverse transcription at 50 °C for 25 minutes and double-cycling amplification: general denaturation to inactivate revertase and activate DNA polymerase with a "hot-start" at 95 °C for 10 minutes, the first 10 cycles without fluorescence detection (denaturation at 95 °C for 10 seconds, primer annealing at 60 °C for 40 seconds, elongation at 72 °C for 10 seconds), then 35 cycles with fluorescence detection on the Green and Crimson channels (denaturation at 95 °C for 10 seconds, primer annealing at 55 °C for 40 seconds, elongation at 72 °C for 10 seconds).

ICS selection and optimization. MS2 bacteriophage is used as ICS for diagnosis of diseases caused by RNA viruses (hepatitis C, acquired human immunodeficiency syndrome, etc.). The phage of the *Leviviridae* family is a small icosahedral virion, pathogenic for *Escherichia coli*. The genome is made of single-stranded RNA of 3,569 nucleotides in length [20]. MS2 was chosen as ICS owing to its RNA genome and safety for humans, animals, and plants [21].

Intact ST cell culture, 10% spleen suspension samples collected from pigs infected with epizootic CSFV strain and from CSF-free pigs, culture CSFV 719 strain and NCS with different MS2 bacteriophage contents in the sample were tested to determine sufficient ICS concentration for adding to RNA extraction system (Table 1).

As a result, the optimal ICS amount was 3.2×10^3 PFU of MS2 bacteriophage per 100 µL of the sample. MS2 suspension with recommended titer was tested by storing at a temperature of +4 °C, and was found to have a stable threshold amplification cycle (Ct) with changes of ± 2 Ct when it was used for real-time RT-PCR after six-months storage. Ct value remained stable during five freezing (–20 °C) / thawing (room temperature) cycles.

Assessment of accuracy, repeatability and reproducibility. The developed assay under validation was shown to have 100% accuracy, repeatability and reproducibility during tests since known CSFV 719 strain-positive sample was found positive during 6 repeats in 3 parallel tests performed by one operator within one day and 3 parallel tests performed by two operators during three days (Table 2).

However, according to the graph (Fig. 2), Ct value increased by 3.09 ± 0.81 on day 3 of testing of the same sample as compared to that one on day 2 of testing by two operators that could be accounted for multiple freezing/thawing of the virus-containing material.

Assessment of analytical sensitivity. Mean minimum CSFV titers detectable by the real-time RT-PCR assay in 0.23 lg $CCID_{50}/cm^3$ were determined for CSFV 719, 684 and Shimen strains.

According to Table 3, comparative analysis of parallel tests of the virus strains using different real-time RT-PCR-based test systems showed that for CSFV 684 strain the detection limit of the developed assay was 1.2 lg higher than detection limits of test systems No. x1 and x2, and for reference Shimen strain the detection limit of the developed assay was 2.0 lg higher than that one of the test system No. x2. CSFV 719 strain-containing sample with a titer of 7.5 lg $CCID_{50}/cm^3$ was tested with the developed assay with positive results, while inconclusive results were obtained when the same sample was subjected to parallel tests using test systems No. x1 and x2.

A graph of correlation was plotted based on obtained Ct values ($n = 3$) and 10-fold dilutions of CSFV 719 strain (Fig. 3).

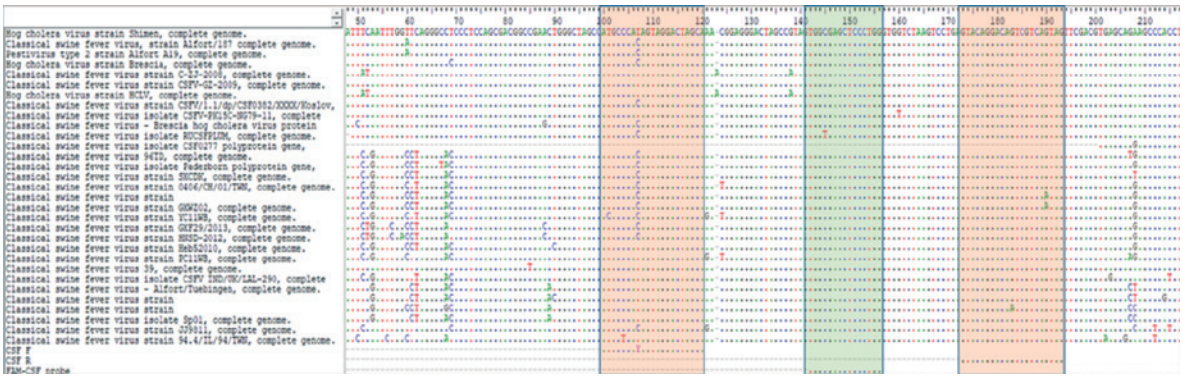

Fig. 1. Alignment of CSFV genome 5'-untranslated region (5'-NTR) sequences obtained from the GenBank (forward and reverse primer annealing sites are given in orange, TaqMan probe hybridization region is given in green)

Table 1
Results of internal control sample (ICS) titration with real-time RT-PCR using samples of different types

Sample	ICS titer (PFU/reaction)	Ct/Green	Ct/Crimson
NCS	10^5	–	20.22
Intact ST cell culture		–	26.02
10% spleen suspension from CSF-free pig		–	–
10% spleen suspension from CSFV infected pig		14.28	–
CSFV 719 strain-containing suspension		6.15	–
NCS	3.2×10^5	–	19.85
Intact ST cell culture		–	25.67
10% spleen suspension from CSF-free pig		–	22.80
10% spleen suspension from CSFV infected pig		13.62	23.22
CSFV 719 strain-containing suspension		5.66	–
NCS	10^6	–	14.11
Intact ST cell culture		–	15.93
10% spleen suspension from CSF-free pig		–	17.90
10% spleen suspension from CSFV infected pig		15.12	14.73
CSFV 719 strain-containing suspension		5.95	26.07
NCS	3.2×10^6	–	11.70
Intact ST cell culture		–	13.21
10% spleen suspension from CSF-free pig		–	15.21
10% spleen suspension from CSFV infected pig		12.10	10.48
CSFV 719 strain-containing suspension		5.56	24.25
NCS	10^7	–	12.34
Intact ST cell culture		–	17.82
10% spleen suspension from CSF-free pig		–	11.93
10% spleen suspension from CSFV infected pig		14.43	–
CSFV 719 strain-containing suspension		8.37	–

"–" – negative result; PFU – plaque-forming unit; Ct/Green – cycle threshold value for CSFV genome detection; Ct/Crimson – cycle threshold value for ICS detection.

The high statistical parameters calculated using correlation analysis were as follows: reaction efficiency E = 105%, adequacy coefficient $R^2 = 0.9928$ and significance criterion p value < 0.0001, that were indicative of the prospects of this real-time RT-PCR assay use for further development of quantitative PCR with reference samples.

Assessment of analytical specificity. Samples containing heterologous virus and bacteria genomes (ASFV, Aujeszky's disease virus, PRRSV, erysipelas agent and BVDV) were tested with the real-time RT-PCR assay with negative results and samples containing CSFV strains were tested CSFV RNA-positive with the real-time RT-PCR assay, so analytical specificity of the real-time RT-PCR assay was 100% (Table 4).

ICS fragment amplification was found to be inhibited at high Ct value on the Green channel (CSFV genome) that could be accounted for consumption of the reaction mix components (deoxyribonucleotide triphosphates, DNA polymerase, etc.) for the target PCR product synthesis with a large number of the matrix copies [20]. This should be taken into account when interpreting the results.

Determination of diagnostic sensitivity and specificity. All 27 true positive samples containing CSFV or collected from the pigs experimentally infected with epizootic CSFV strain as well as samples of the feed contaminated by infected animals were tested positive for CSFV genome with the real-time RT-PCR assay. Similarly, all 27 true negative samples were tested negative for CSFV RNA with the real-tine RT-PCR assay (Fig. 4).

Thus, the tests showed that diagnostic sensitivity and specificity of the assay were as high as possible and equaled to 100%. At the same time, the real-time RT-PCR-based test system No. x2 did not detect a positive blood sample collected from pigs experimentally infected with epizootic CSFV strain, as well as a positive feed sample collected in the animal facility where the pigs experimentally

Table 2
Developed real-time RT-PCR assay accuracy, repeatability and reproducibility values

Accuracy			
Sample	Ct/Green	(mean value ± SD)	Ct/Crimson
CSFV 719 strain	5.31	5.32 ± 0.148	–
CSFV 719 strain	5.22		27.29
CSFV 719 strain	5.30		26.01
CSFV 719 strain	5.52		27.27
CSFV 719 strain	5.33		26.67
CSFV 719 strain	5.28		29.37
Repeatability			
Procedure	Ct/Green	(mean value ± SD)	Ct/Crimson
First measurement	5.38	5.64 ± 0.237	28.63
First measurement	5.74		26.85
First measurement	5.71		22.68
First measurement	5.61		23.78
First measurement	5.59		23.87
First measurement	5.86		24.41
Second measurement	5.63	5.44 ± 0.493	23.14
Second measurement	5.37		23.95
Second measurement	5.02		23.62
Second measurement	5.11		23.11
Second measurement	5.96		23.01
Second measurement	5.56		23.70
Third measurement	5.59	5.60 ± 0.095	28.84
Third measurement	5.60		30.30
Third measurement	5.58		28.72
Third measurement	5.74		30.26
Third measurement	5.57		28.06
Third measurement	5.57		28.87

Reproducibility (Ct/Green)					
Day 1		Day 2		Day 3	
first operator	second operator	first operator	second operator	first operator	second operator
5.52	5.07	5.67	6.51	7.83	8.95
5.27	5.10	5.61	4.70	8.01	9.19
5.46	5.82	5.65	4.55	6.71	9.17
5.42	5.98	5.64	5.14	8.28	8.98
5.38	5.33	5.73	5.44	8.02	9.30
5.46	5.06	5.78	5.49	9.21	9.34
5.4183 ± 0.125	5.3933 ± 0.591	5.68 ± 0.0917	5.305 ± 1.019	8.01 ± 1.166	9.155 ± 0.233

"–" – negative result; Ct/Green – cycle threshold value for CSFV fragment; Ct/Crimson – cycle threshold value for ICS fragment.

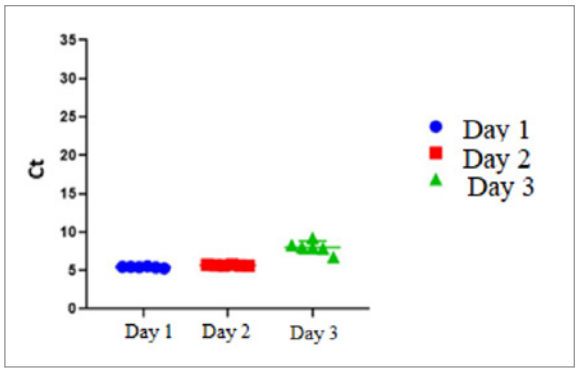

Fig. 2. Distribution of Ct values for known positive sample in different days of testing when the real-time RT-PCR assay was assessed for its reproducibility

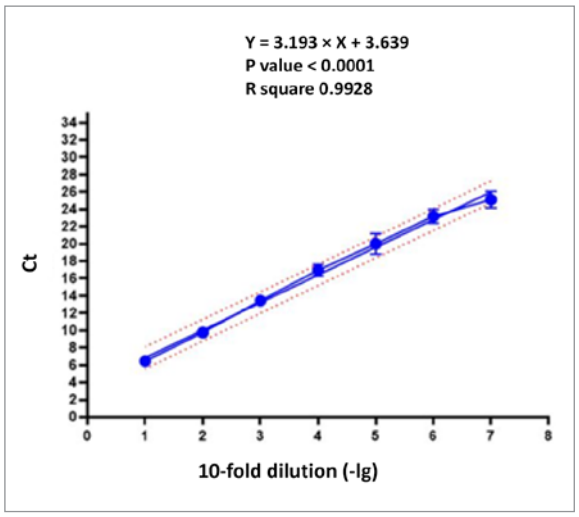

Fig. 3. Graph of linear correlation of CSFV 719 strain dilutions with Ct-values

infected with the same strain were kept, test-system No. x1 also did not detect the said positive blood sample that was indicative of their lower diagnostic sensitivity (92.6% for test system No. x2 and 96.3% for test system No. x1) than that one of the developed assay under validation.

Analysis and interpretation of the results. The recommended parameters of PCR assay for the Green and Crimson channels were identical: dynamic baseline setting, slope correction, emission reduction by 10%, linear scale, and threshold value of 0.05. The results were interpreted based on the presence or absence the standard curve intersection with the set threshold line that corresponds to Ct value presence or absence in relative line of the results table (Fig. 5).

Also, 2-fold dilution of the RNA extracted from CSFV 719 strain suspension with final titer of 7.5 lg $CCID_{50}/cm^3$ was tested to establish the PCR assay parameters and to determine maximum Ct value at which the sample could be interpreted as "positive" (Table 5).

The obtained data showed that the maximum Ct value was 27.83 that was equivalent to CSFV 719 strain titer of 0.23 lg $CCID_{50}/cm^3$.

The result was considered reliable when the correct results were obtained for positive and negative controls.

The sample was considered positive for CSFV genome when Ct value on Green channel did not exceed 28. In this

Table 3
Real-time RT-PCR results for 10-fold dilutions of various epizootic CSFV strains

Virus dilution	Shimen strain (titer – 4.0 lg $CCID_{50}/cm^3$)	719 strain (titer – 7.5 lg $CCID_{50}/cm^3$)	684 strain (titer – 6.2 lg $CCID_{50}/cm^3$)
	Ct/Green	Ct/Green	Ct/Green
Non-diluted	11.88 (pos)	5.41 (pos)	6.79 (pos)
$1:10^1$	16.18 (pos)	9.75 (pos)	9.89 (pos)
$1:10^2$	20.52 (pos)	12.53 (pos)	13.69 (pos)
$1:10^3$	22.64 (pos)	16.68 (pos)	16.97 (pos)
$1:10^4$	27.06 (pos)	18.79 (pos)	20.24 (pos)
$1:10^5$	–	20.60 (pos)	23.45 (pos)
$1:10^6$	–	21.57 (pos)	26.30 (pos)
$1:10^7$	–	24.71 (pos)	–
$1:10^8$	–	–	–
Test system No. x1			
Ct/Yellow (result)			
Non-diluted	12.89 (pos)	6.44 (pos)	8.27 (pos)
$1:10^1$	16.77 (pos)	8.33 (pos)	12.89 (pos)
$1:10^2$	18.67 (pos)	13.56 (pos)	15.08 (pos)
$1:10^3$	21.96 (pos)	17.67 (pos)	17.36 (pos)
$1:10^4$	25.78 (pos)	18.82 (pos)	22.85 (pos)
$1:10^5$	–	24.01 (pos)	26.57 (inconcl)
$1:10^6$	–	22.79 (pos)	–
$1:10^7$	–	28.19 (inconcl)	–
$1:10^8$	–	–	–
Test system No. x2			
Ct/Yellow (result)			
Non-diluted	19.07 (pos)	10.60 (pos)	9.37 (pos)
$1:10^1$	22.42 (pos)	13.34 (pos)	12.69 (pos)
$1:10^2$	25.28 (pos)	16.46 (pos)	16.13 (pos)
$1:10^3$	–	17.67 (pos)	19.19 (pos)
$1:10^4$	–	20.55 (pos)	22.22 (pos)
$1:10^5$	–	22.55 (pos)	24.68 (pos)
$1:10^6$	–	25.44 (pos)	–
$1:10^7$	–	28.02 (inconcl.)	–
$1:10^8$	–	–	–

"–" – negative result; pos – positive result; inconcl – inconclusive result;
Ct/Green – cycle threshold value for CSFV fragment; Ct/Yellow – cycle threshold value for CSFV fragment obtained when real-time PCR-based test systems No. x1 and x2 were used in accordance with their manufactures' instructions.

Table 4
Assessment of analytical specificity of the real-time RT-PCR assay when the assay was used for CSFV genome detection (*n* = 2)

Sample	Ct/Green	Ct/Crimson	Result of test for CSFV genome
Field *Erysipelothrix rhusiopathiae* isolate	–	19.08	negative
PRRSV Irkutsky 2007 strain	–	13.03	negative
Aujeszky's disease virus VK-DEP strain	–	13.73	negative
ASFV Mozambique-78 strain	–	15.39	negative
BVDV Chelyabinsk 2021 strain	–	15.31	negative
Reference CSFV Shimen strain	18.64	15.39	positive
CSFV Amur 19-10/WB-12555 strain	12.19	16.88	positive
CSFV Tigrovoe 16/WB-634 strain	17.26	18.02	positive
CSFV 275 strain	11.27	–	positive
CSFV 719 strain	6.56	–	positive
CSFV 843 strain	6.27	–	positive
CSFV 917 strain	6.24	–	positive
CSFV 918 strain	21.8	16.7	positive
CSFV 920 strain	21.16	14.88	positive
CSFV 926 strain	14.12	16.07	positive
CSFV 927 strain	13.61	17.91	positive
CSFV 930 strain	27.25	14.61	positive
CSFV 368 strain	10.01	19.04	positive
CSFV 589 strain	11.81	19.81	positive
CSFV 684 strain	6.79	17.15	positive
CSFV 870 strain	9.64	18.45	positive
CSFV 924 strain	13.26	18.89	positive
CSFV 925 strain	13.93	17.04	positive
CSFV 929 strain	15.28	19.12	positive
NCS	–	15.17	negative
PCS (vaccine SK strain of CSFV)	19.57	18.76	positive

"–" – negative result; Ct/Green – cycle threshold value for CSFV fragment; Ct/Crimson – cycle threshold value for ICS fragment.

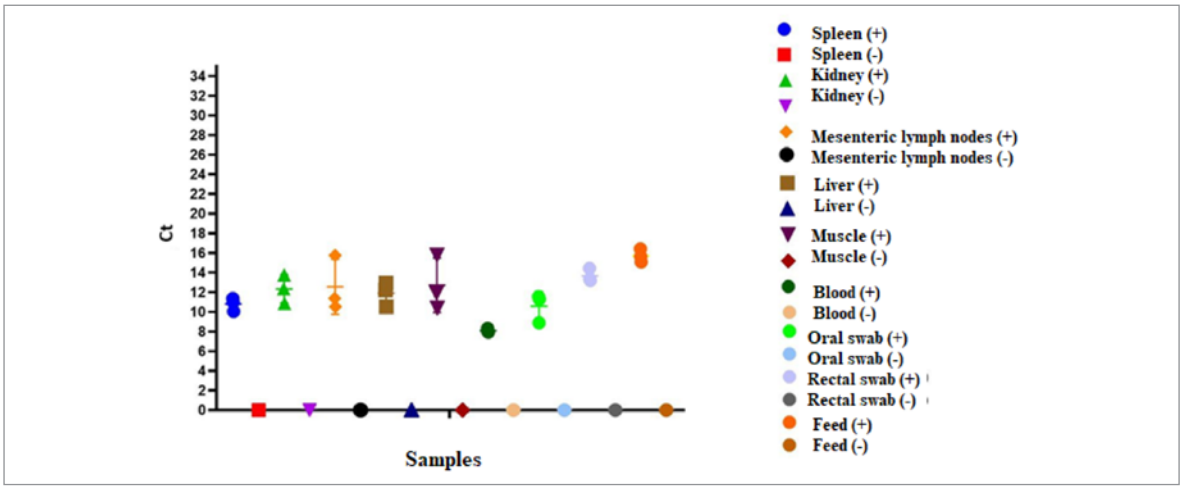

Fig. 4. Kinetics of Ct values for the panel of samples selected for determination of diagnostic sensitivity and specificity (samples – 10% suspensions of the indicated organs; "+" – true positive sample; "–" – true negative sample; l/n – lymph nodes; porcine raw meats and products were used as negative muscle samples)

Table 5
Real-time RT-PCR results for two-fold dilutions of CSFV RNA

CSFV RNA dilution	Ct/Green
non-diluted	6.04
$1:2^{1}$	6.86
$1:2^{2}$	7.55
$1:2^{3}$	8.75
$1:2^{4}$	12.02
$1:2^{5}$	11.75
$1:2^{6}$	12.06
$1:2^{7}$	12.90
$1:2^{8}$	13.42
$1:2^{9}$	14.61
$1:2^{10}$	15.61
$1:2^{11}$	16.32
$1:2^{12}$	17.19
$1:2^{13}$	18.21
$1:2^{14}$	19.27
$1:2^{15}$	20.14
$1:2^{16}$	21.17
$1:2^{17}$	22.08
$1:2^{18}$	22.98
$1:2^{19}$	24.06
$1:2^{20}$	24.74
$1:2^{21}$	24.22
$1:2^{22}$	26.13
$1:2^{23}$	26.18
$1:2^{24}$	27.83
$1:^{22}5$	27.01
$1:2^{26}$	–
$1:2^{27}$	–
$1:2^{28}$	–
$1:2^{29}$	–
$1:2^{30}$	–

CSFV RNA was extracted from CSFV 719 strain-containing suspension; titer 7.5 lg $CCID_{50}/cm^3$, "–" – negative result.

case, the result was valid regardless of the values on Crimson channel.

A result for the presence of the CSFV genome was interpreted as negative when there was no Ct value on Green channel, but Ct value on Crimson channel did not exceed 31.

If Ct value on Green channel exceeded 28, but on Crimson channel was less than 31 the PCR result was considered inconclusive.

When there were no Ct values on Green and Crimson channels, as well as when Ct value on Crimson channel exceeded 31, the PCR result was considered invalid.

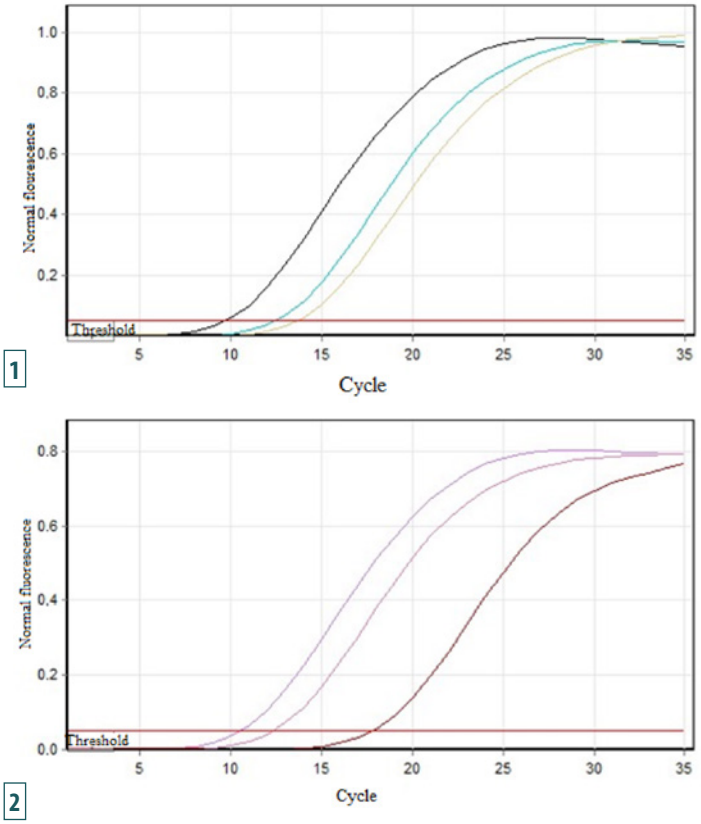

Fig. 5. Fluorescence curves: 1 – for Green channel (CSFV fragment); 2 – for Crimson channel (ICS fragment)

In case of inconclusive or invalid results the sample was to be retested starting from the RNA extraction stage in order to confirm absence or presence of CSFV genome in the sample.

CONCLUSION

The developed assay has high validation characteristics: 100% accuracy, repeatability, reproducibility, analytical specificity, diagnostic sensitivity and specificity, detection limit of 0.23 lg $CCID_{50}/cm^3$. Such characteristics make the developed real-time PCR-RV assay competitive on the domestic market of diagnostic tools and suitable for use for large-scale monitoring of CSF situation in Russia.

REFERENCES

1. Malik Y. S., Bhat S., Kumar O. R. V., Yadav A. K., Sircar S., Ansari M. I., et al. Classical swine fever virus biology, clinicopathology, diagnosis, vaccines and a meta-analysis of prevalence: A review from the Indian perspective. *Pathogens*. 2020; 9 (6):500. https://doi.org/10.3390/pathogens9060500

2. Stark R., Meyers G., Rümenapf T., Thiel H. J. Processing of pestivirus polyprotein: cleavage site between autoprotease and nucleocapsid protein of classical swine fever virus. *Journal of Virology*. 1993; 67 (12): 7088–7095. https://doi.org/10.1128/jvi.67.12.7088-7095.1993

3. Blome S., Staubach C., Henke J., Carlson J., Beer M. Classical swine fever – an updated review. *Viruses*. 2017; 9 (4):86. https://doi.org/10.3390/v9040086

4. Edwards S., Fukusho A., Lefèvre P.-C., Lipowski A., Pejsak Z., Roehe P., Westergaard J. Classical swine fever: the global situation. *Veterinary Microbiology*. 2000; 73 (2–3):

103–119. https://doi.org/10.1016/s0378-1135(00)00138-3

5. Oganesyan A. S., Shevtsov A. A., Shcherbakov A. V., Korennoy F. I., Karaulov A. K. Classical swine fever: a retrospective analysis of the epizootic situation in the Russian Federation (2007–2021) and forecast for 2022. *Veterinary Science Today*. 2022; 11 (3): 229–238. https://doi.org/10.29326/2304-196X-2022-11-3-229-238

6. Shevtsov A. Classical swine fever: prospects of eradication. *Animal Husbandry of Russia*. 2021; (10): 27–30. https://www.elibrary.ru/jfxalo (in Russ.)

7. Zhu H., Zhang H., Xu Y., Laššáková S., Korabečná M., Neužil P. PCR past, present and future. *BioTechniques*. 2020; 69 (4): 317–325. https://doi.org/10.2144/btn-2020-0057

8. Hirschhorn J. W., Avery A., Schandl C. A. Managing a PCR contamination event in a molecular pathology laboratory. *In: Clinical Applications of Nucleic Acid Amplification. Methods in Molecular Biology. Eds. M. B. Myers, C. A. Schandl.* 2023; 2621: 15–26. https://doi.org/10.1007/978-1-0716-2950-5_2

9. Canal C. W., Hotzel I., de Almeida L. L., Roehe P. M., Masuda A. Differentiation of classical swine fever virus from ruminant pestiviruses by reverse transcription and polymerase chain reaction (RT-PCR). *Veterinary Microbiology*. 1996; 48 (3–4): 373–379. https://doi.org/10.1016/0378-1135(95)00156-5

10. McGoldrick A., Lowings J. P., Ibata G., Sands J. J., Belak S., Paton D. J. A novel approach to the detection of classical swine fever virus by RT-PCR with a fluorogenic probe (TaqMan). *Journal of Virological Methods*. 1998; 72 (2): 125–135. https://doi.org/10.1016/s0166-0934(97)00208-5

11. Hoffmann B., Beer M., Schelp C., Schirrmeier H., Depner K. Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. *Journal of Virological Methods*. 2005; 130 (1–2): 36–44. https://doi.org/10.1016/j.jviromet.2005.05.030

12. Latorra D., Arar K., Hurley J. M. Design considerations and effects of LNA in PCR primers. *Molecular and Cellular Probes*. 2003; 17 (5): 253–259. https://doi.org/10.1016/S0890-8508(03)00062-8

13. Monpoeho S., Coste-Burel M., Costa-Mattioli M., Besse B., Chomel J., Billaudel S., Ferre V. Application of a real-time polymerase chain reaction with internal positive control for detection and quantification of enterovirus in cerebrospinal fluid. *European Journal of Clinical Microbiology and Infectious Diseases*. 2002; 21 (7): 532–536. https://doi.org/10.1007/s10096-002-0766-5

14. Gavrilova V. L., Puzankova O. S., Latyshev O. Ye., Igolkin A. S., Manin B. L., Zhbanova T. V., Yuzhakov A. G. Studying cultural and molecular characteristics of classical swine fever virus isolates recovered in 2016. *Proceedings of the Federal Centre for Animal Health*. 2017; 15: 82–101. https://www.elibrary.ru/xrdrrb (in Russ.)

15. Gilep A., Bashko N., Usanov S., Kamyshnikov V. Import-substituting domestic diagnostic tools. *Laboratory Diagnostics. Eastern Europe*. 2014; (4): 28–45. https://elibrary.ru/taxtgh (in Russ.)

16. Kolbin I. S., Igolkin A. S., Gavrilova V. L., Puzankova O. S., Aronova Ye. V., Yelsukova A. A., Vlasova N. N. Determination of reproductive properties of virulent and vaccine classical swine fever virus strains in primary and continuous cell cultures. *Veterinary Science Today*. 2022; 11 (2): 149–155. https://doi.org/10.29326/2304-196X-2022-11-2-149-155

17. Dreier J., Störmer M., Kleesiek K. Use of bacteriophage MS2 as an internal control in viral reverse transcription-PCR assays. *Journal of Clinical Microbiology*. 2005; 43 (9): 4551–4557. https://doi.org/10.1128/jcm.43.9.4551-4557.2005

18. O'Connell K. P., Bucher J. R., Anderson P. E., Cao C. J., Khan A. S., Gostomski M. V., Valdes J. J. Real-time fluorogenic reverse transcription-PCR assays for detection of bacteriophage MS2. *Applied and Environmental Microbiology*. 2006; 72 (1): 478–483. https://doi.org/10.1128/aem.72.1.478-483.2006

19. Bustin S. A., Benes V., Garson J. A., Hellemans J., Huggett J., Kubista M., et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*. 2009; 55 (4): 611–622. https://doi.org/10.1373/clinchem.2008.112797

20. Buckwalter S. P., Sloan L. M., Cunningham S. A., Espy M. J., Uhl J. R., Jones M. F., et al. Inhibition controls for qualitative real-time PCR assays: Are they necessary for all specimen matrices? *Journal of Clinical Microbiology*. 2014; 52 (6): 2139–2143. https://doi.org/10.1128/jcm.03389-13

21. Zambenedetti M. R., Pavoni D. P., Dallabona A. C., Dominguez A. C., Poersch C. de D., Fragoso S. P., Krieger M. A. Internal control for real-time polymerase chain reaction based on MS2 bacteriophage for RNA viruses diagnostics. *Memórias do Instituto Oswaldo Cruz*. 2017; 112 (5): 339–347. https://doi.org/10.1590/0074-02760160380

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