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Use of real-time polymerase chain reaction for investigation of *Senecavirus* infection occurrence in Russia

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SUMMARY
Senecavirus, previously known as Seneca valley virus, is an emerging virus belonging to *Senecavirus* genus, *Picornaviridae* family, that can cause idiopathic vesicular disease clinically indistinguishable from foot-and-mouth disease, vesicular stomatitis and swine vesicular disease and thereby posing a great threat for pig holdings. Recently, evidence of *Senecavirus* A occurrence in pig herds in such countries as Brazil, the USA, Colombia, China and Thailand has been provided in foreign literature. Accurate diagnosis is crucial for of *Senecavirus* infection control. Results of studying the disease situation with genodiagnostic methods in the Russian Federation are presented in the paper. Primers and probe for real-time RT-PCR described by V. L. Fowler et al. in 2017 were used but the reaction conditions were optimized. Analysis of the method for its sensitivity showed absence of cross-reactivity with other tested viruses. The developed method for virus RNA detection was used to investigate *Senecavirus* occurrence in pig holdings in the Russian Federation. A total of 1,577 samples of biological materials collected form pigs of different ages in 112 holdings located in 37 regions of the country were tested during 2018–2020. *Senecavirus* was detected in one holding located in the Urals Federal Okrug. It was supposed that the infectious agent had entered the said pig holding at the time of putting of the said holding into operation in 2015 and introduction of young breeding animals imported from Canada. This is the first report on *Senecavirus* detection in the Russian Federation. The threat of the pathogen introduction from other countries requires further *Senecavirus* infection investigation and control. The developed method can be used as a potential sensitive method for the said infectious disease diagnosis.

Keywords: *Senecavirus*, real-time reverse transcription-polymerase chain reaction (rt RT-PCR), biomaterials from animals

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Применение полимеразной цепной реакции в режиме реального времени для изучения ситуации по сенекавирусной инфекции в России

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РЕЗЮМЕ
Сенекавирус – новый вирус, принадлежащий к роду *Senecavirus* семейства *Picornaviridae*, ранее называвшийся вирусом долины Сенека, который может вызывать идиопатическую везикулярную болезнь, клинически неотличимую от ящура, везикулярного стоматита и везикулярной болезни свиней, тем самым представляя большую угрозу для свиноводческих хозяйств. В последние годы в зарубежной литературе приводятся сведения о присутствии сенекавируса А в стадах свиней таких стран, как Бразилия, США, Колумбия, Китай и Таиланд. Для обеспечения контроля сенекавирусной инфекции решающее значение имеет точная диагностика. В настоящей работе представлены результаты изучения ситуации по данному заболеванию в Российской

Федерации с использованием методов генодиагностики. В исследовании были использованы праймеры и зонд для полимеразной цепной реакции с обратной транскрипцией в реальном времени, описанные V. L. Fowler et al. в 2017 г., однако условия проведения реакции были оптимизированы. Анализ специфичности метода показал отсутствие перекрестной реактивности с другими тестируемыми вирусами. С помощью разработанного метода обнаружения вирусной РНК было изучено распространение сенекавируса в свиноводческих хозяйствах на территории Российской Федерации. В период с 2018 по 2020 г. было исследовано 1577 образцов биологического материала от свиней разных возрастных групп из 112 хозяйств 37 регионов страны. Сенекавирус был обнаружен в одном из хозяйств Уральского федерального округа. Есть предположение, что на данный свинокомплекс инфекционный агент попал при введении хозяйства в эксплуатацию в 2015 г. и комплектовании племенным молодняком, ввезенным из Канады. Это первое сообщение об обнаружении сенекавируса на территории Российской Федерации. Поскольку существует угроза заноса возбудителя из других стран, возникает необходимость изучения и контроля сенекавирусной инфекции. Разработанный метод может быть использован в качестве потенциального, чувствительного метода для диагностики данного инфекционного заболевания.

Ключевые слова: сенекавирус, полимеразная цепная реакция в реальном времени, биоматериал от животных

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INTRODUCTION

Senecavirus (Seneca valley virus) is a non-enveloped virus containing single-stranded positive-sense RNA that belongs to *Senecavirus* genus in *Picornaviridae* family. *Senecavirus* genome is approximately 7,200 nucleotides in length and is composed of untranslated regions at both genome ends (5'-UTR and 3'-UTR) and large open reading frame (ORF) coding one large polyprotein cleaved into a leader protein (L), 4 structural proteins (VP1–VP4) and 7 non-structural proteins (2A–2C and 3A–3D) [1, 2].

Senecavirus is a relatively new and understudied virus. It was first identified as a contaminant of human retinal cell culture (PER.C6) at the USDA National Veterinary Service Laboratories (NVSL) in 2002 and was used by many researchers as an oncolytic agent for the treatment of human neuroendocrine tumors [3–5]. In 2005, N. J. Knowles and P. Hallenbeck described the whole genome sequence and in 2008, evidence of the *Senecavirus* association with idiopathic swine vesicular disease was obtained in Canada [6, 7].

Interest in *Senecavirus* study has grown significantly in the last few years.

The first *Senecavirus* infection outbreak in a pig holding was reported in Canada in 2007 [7]. Then, clinical symptoms of idiopathic swine vesicular disease caused by *Senecavirus* were found in 6-month-old piglet in the USA in 2012 [8]. Guangdong was the first province in Chine where *Senecavirus* was reported in 2015, then, *Senecavirus* infection was reported in pigs in central and north regions of the country. Currently, at least 14 provinces are affected by the virus, but Guangdong is one of the provinces where the agent detection rate is the highest. The China Animal Health and Epidemiology Centre (CAHEC) reported that only three provinces had not been affected with the *Senecavirus* A [9–11]. Since 2014, numerous cases of *Senecavirus* infection have also been confirmed in such countries as Brazil [1, 3, 12–14], Columbia [15], Thailand [16] and Vietnam [17], that is indicative of the agent wide spread in livestock holdings in different countries for several years.

Clinical manifestations of idiopathic vesicular disease caused by *Senecavirus* are undistinguishable from those of highly dangerous animals diseases caused by other related viruses such as swine vesicular disease virus, vesicular exanthema virus and foot-and-mouth disease virus. Clinical picture in animals is similar in almost all cases. As for adult pigs and weaning piglets, vesicles appear on face, coronary ligaments, lips; aphthae form in the mouth; interdigital lesions, coronary band and feet lesions leading to lameness can occur, fever and sluggishness develop. Ruptured vesicles turn into deep ulcers that heal within 14 days. In addition, muscle weakness, lethargy, excessive salivation, skin hyperemia, diarrhea, neurological manifestations and sudden death (the so-called epidemic transitory neonatal disease) can be observed in piglets of the first week of life. Clinical signs are observed within 3–10 days and then they disappear in survived animals [1, 15, 18–22].

There is a little information on *Senecavirus* transmission routes in the literature. Direct contact appears to be a significant route of the infection transmission. The virus is also excreted in feces and urine. The pathogen can be vertically transmitted, as shown by *Senecavirus* detection in one- and two-day-old piglets [4].

Currently, there are no specific treatment methods and vaccines for the prevention and control of the disease caused by *Senecavirus* A. This could be accounted for the large variety of existing isolates.

Modern laboratory methods developed for *Senecavirus* infection diagnosis include virus isolation in cell culture,

virus neutralization test, competitive enzyme-linked immunosorbent assay (ELISA), conventional reverse transcription polymerase chain reaction (RT-PCR) and real time RT-PCR [18].

The study was aimed at assessment of the *Senecavirus* infection situation in the Russian Federation using genodiagnostic methods.

MATERIALS AND METHODS

Reference strains. CA-01-131395 and MN-88-36695 strains of *Senecavirus* obtained from the Pirbright Institute (United Kingdom) were used as positive control samples to practice the test method and in the test procedure.

Strains of FMD virus (O/Saudi Arabia/08, A/Turkey/06, Asia-1/Shamir 3/89), swine vesicular disease virus (No. 663/73) from the FGBI "ARRIAH" State Collection of Microorganism Strains as well as BHK-21 cell culture were used to examine the test for its specificity.

Pathological materials. Pathological material samples collected from pigs in Russian pig holdings in 2018–2020 were subjected to diagnostic tests.

RNA extraction from 10% biological material suspension was performed using 6 M guanidine thiocyanate and GF/F glass fiber filters [23].

Real-time RT-PCR (rt RT-PCR). Primers and probe described by V. L. Fowler et al. [18] were used. The reaction was performed in 25 µL of the mixture containing 0.5 µL (5 pm) of direct primer and 0.5 µL (5 pm) of reverse primer, 0.5 µL (5 pm) of the probe, 2.5 µL 10× buffer for PCR, 4 µL of 25 mM $MgCl_2$, 0.7 µL of 25 мМ NTPs, 0.2 µL (1 unit) of TaqDNA-polymerase, 0.4 µL (20 Units) of MMLV revertase, 11 µL of nuclease-free water and 5 µL of RNA. Amplification programme included stages of reverse transcription at 60 °C for 30 minutes followed by denaturation at 95 °C for 10 minutes and 50 cycles of PCR itself (denaturation at 95 °C for 15 seconds, annealing and elongation at 60 °C for 1 minute). C1000 Touch™ thermocycler with CFX96 optical reaction module (Bio-Rad, USA) was used to perform all reactions.

Validation. The following method characteristics were determined during its validation: detection limit, specificity, repeatability and reproducibility, amplification efficiency [24, 25].

RESULTS AND DISCUSSION

Primers and the probe described by V. L. Fowler et. al. [18] were used but the reaction conditions were significantly different. Therewith, validation of the method was carried out at the beginning of the work.

RNAs of FMD virus (O/Saudi Arabia/08, A/Turkey/06, Asia-1/Shamir 3/89), swine vesicular disease virus (No. 663/73) as well as BHK-21 cell culture and RNA extracted from tongue epithelium of normal pigs (a total of 9 samples) were used for the rt RT-PCR checking. Only *Senecavirus* RNA-containing samples were tested positive, other samples were tested negative that was indicative of the method specificity (Figure).

Detection limit was determined with serial 10-fold dilutions of CA-01-131395 strain of *Senecavirus*. Initial virus titre was 6 lg $TCID_{50}$/mL. The highest dilution where the virus was detected contained the virus at a titre of 10 $TCID_{50}$/mL.

C*t* values for all dilutions were in the range of 12.2 up to 31.14. Analysis of the data with software programme allowed us to make a standard curve for these RNA dilutions and assess the reaction efficiency that was 92.3%.

The same sample in 10 replicates was tested under the same measuring conditions (one instrument, one operator, i. e., under repeatable conditions) for repeatability test. Closeness between the results of successive measurements of the same sample (*Ct*-value) was recorded.

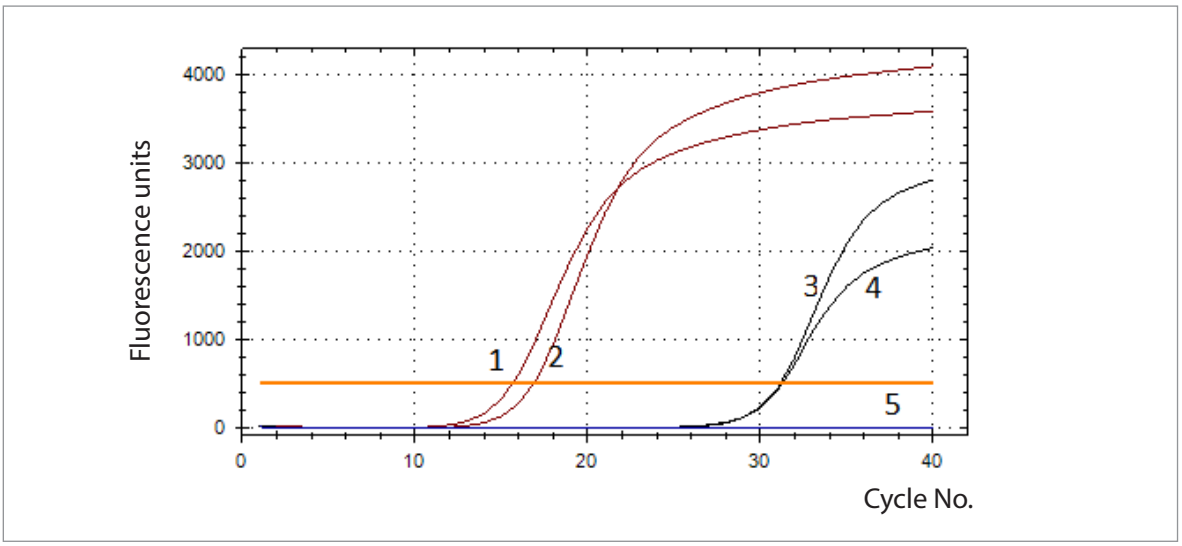

Fig. Detection of Senecavirus with rt RT-PCR
1 – CA-01-131395 reference Senecavirus strain
2 – MN-88-36695 reference Senecavirus strain
3 – field Senecavirus isolate (detected in piglet brain)
4 – field Senecavirus isolate (detected in piglet joints)
5 – Senecavirus-negative samples (foot-and-mouth disease virus, swine vesicular disease virus, BHK-21 cell culture, normal pig epithelium)

Table
Real-time RT-PCR: *Ct*-values for 30 retests and coefficient of variance

Within one run		Using other device		Performed by other operator	
Retest No.	*Ct*	Retest No.	*Ct*	Retest No.	*Ct*
1	12.83	1	12.42	1	12.74
2	12.12	2	12.78	2	12.12
3	12.35	3	12.12	3	12.36
4	12.45	4	12.35	4	13.82
5	12.47	5	12.54	5	12.81
6	12.24	6	12.98	6	12.52
7	12.63	7	13.37	7	12.43
8	12.47	8	12.11	8	12.61
9	12.74	9	12.24	9	13.72
10	12.14	10	12.31	10	12.50
C = 1.86					
C = 2.70					
		C = 3.61			

Coefficient of variance (*C*) was calculated according to the following formula:

$$C = \sigma / X_{mean} \times 100\%,$$

where X – threshold cycle value determined with the rt RT-PCR;

σ – mean squared deviation calculated according to the formula $\sigma = (X_{max} - X_{min}) / K$, where K – coefficient from S. I. Yermolayev's Table; $K = 3.08$ for 10 replicates [24, 25].

Resulting σ = (12.83 – 12.12) / 3.08 = 0.231. Calculated coefficient of variance (*C*) value was $C = 0.231 / 12.444 \times 100\% = 1.86\%$ (Table).

For reproducibility test, also the same sample in 10 replicates was tested under changed testing conditions: 1) by one operator in parallel tests during different time periods (10 days) and 2) by two operators in parallel tests (in 10 retests):

1) $\sigma = (13.37 - 12.11) / 3.74 = 0.337$ (for 20 replicates $K = 3.74$),

$C = 0.337 / 12.483 \times 100\% = 2.70\%$;

2) $\sigma = (13.82 - 12.11) / 3.74 = 0.457$,

$C = 0.457 / 12.643 \times 100\% = 3{,}61\%$.

Estimated coefficient of variance was 2.70% when the test was performed by one operator at different time periods and 3.61% when the test was performed by different operators (Table).

Estimated coefficient of variance values (less than 10%) showed that variation was insignificant and the results were homogeneous.

Thus, the rt RT-PCR characteristics are shown to comply with the requirements for qualitative measuring methods/tests and it can be used for diagnostic tests.

Methodical Guideline for *Senecavirus* detection with real-time reverse transcription polymerase chain reaction were developed based on the method validation results and then used for *Senecavirus* infection diagnosis in Russia.

A total of 1,577 pathological material samples from pigs kept in 112 holdings located in 37 regions of the Russian Federation were tested with the rt RT-PCR for *Senecavirus* genome in 2018–2020. All available biological materials: from embryos to adult pigs, collected from pigs of different age groups were used for testing. Pigs with any clinical manifestations were covered by testing. For example, pigs having the following manifestations were tested: vesicular and mucosal lesions, neurological signs, intestinal pathology, respiratory and reproductive failures, all cases of sudden death as well as biological materials from normal animals. Any animal organs and tissues as well biological fluids were tested. Tissues were collected from pigs with vesicular lesions (aphthae, vesicles, papules, pustules) in FMD outbreak areas.

The Figure shows that a positive fluorescence signal was observed in field samples (brain and joints) from pigs when *Senecavirus* was detected.

No *Senecavirus* was detected in epithelial tissues, parenchymal organ samples, biological samples collected from pigs and all vesicular lesions detected in pigs in 2019 were caused by infection with foot-and-mouth disease (FMD) virus.

However, *Senecavirus* was detected in pigs with neurological signs and arthritis in one holding located in the Chelyabinsk Oblast. It is quite difficult to judge the role of *Senecavirus* A in this pathology, since bacterial pathogens have also been found in this material samples namely *Streptococcus suis* [26]. *Senecavirus* is supposed to be introduced into the pig holding with breeding young pigs imported from Canada in 2015. The virus appeared not to manifest itself clinically.

In conclusion, it should be noted that this is the first report on *Senecavirus* infection in the Russian Federation. Livestock vesicular diseases are clinically, economically and epidemiologically significant. *Senecavirus* can be a new agent potentiating vesicular disease manifestations.

Therefore, *Senecavirus* detection should be included in the differential diagnosis of classical viral vesicular diseases, despite of the fact that *Senecavirus*-caused infectious disease is mild and has no large economic impact. Notwithstanding a single case of *Senecavirus* detection in Russia, there is a threat of its introduction from neighboring countries and hence *Senecavirus* infection investigation and control are required.

CONCLUSIONS

1. Real-time RT-PCR-based method for *Senecavirus* detection was optimized. Absence of cross-reactions with other tested viruses were demonstrated. *Senecavirus* detection limit (titre – 10 $TCID_{50}$/cm) was determined. The main validation characteristics: repeatability (coefficient of variance – 1.86%) and reproducibility (coefficient of variance – 2.70 and 3.61%) were determined. The efficiency of amplification was estimated as 92.3%. The developed method was shown to comply with the criteria for qualitative laboratory test methods and can be used for laboratory diagnosis of *Senecavirus* infection.

2. Methodical Guidelines for *Senecavirus* detection with real-time reverse transcription polymerase chain reaction based on the optimized method were developed. The Methodical Guidelines can be used for *Senecavirus* infection detection in Russian animal holdings.

3. Biological material samples from pigs of different age groups kept in Russian pig holdings were tested for *Senecavirus* with the developed method in 2018–2020. A total of 1,577 samples from pigs kept in 112 holdings located in 37 regions of the Russian Federation were tested. *Senecavirus* was detected in one holding located in the Urals Federal District.

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