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DETECTION OF SCHMALLENBERG VIRUS IN CATTLE IMPORTED INTO THE RUSSIAN FEDERATION

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Abstract

Schmallenberg disease, a transmissive vector-borne arbovirus infection affecting cattle, sheep and goats of all age groups, which may lead to significant economic losses. In the Russian Federation, specific antibodies against the causative agent of Schmallenberg disease were first detected in April 2013 and 2014 in the Andreapolsky and Konakovsky districts of the Tver region. In October 2014, specific antibodies were detected in cattle blood in one of the farms of the Novosokolnichesky district of the Pskov region. In this study we provide an overview of the serological and molecular surveillance for Schmallenberg virus (SBV) in the Russian Federation in 2014-2016. Testing of serum samples from cattle born and raised in regions sharing border with previously affected EU countries demonstrated seroconvertion in sentinel animals. The current findings raise concerns with regard to a possible SBV distribution and circulation in Russian cattle. We for the first time report the detection and isolation of SBV in cattle imported into Russia from EU. The virus was isolated on Vero cells and sequenced. The S gene sequence analysis showed 100 % identity of the recovered SBV strain to those isolated in Northern Europe in 2012.

Keywords: Schmallenberg virus (SBV), continuous Vero cell culture, polymerase chain reaction (PCR), phylogenetic analysis, sequencing

For the first time, the disease caused by the Schmallenberg virus was discovered in North Rhine-Westphalia (Germany) and in the northwest of the Netherlands. It is a transmissive arbovirus infection affecting cattle, sheep and goats of all age groups, which is characterized by fever, exhaustion and decrease of milk yield. The distinctive feature of the disease is that the contamination of animals at the stage of pregnancy leads to abortions and stillbirths that entails significant economic losses in case of mass spread of the infection [1].

The causative agent of the ShV belongs to the *Bunyaviridae* family of the *Orthobunyavirus* genus of the *Simbu* serogroup. ShV has the genome consisting of S (short), M (medium) and L (long) segments and is represented by single-stranded RNA [1, 2]. The disease's incubation period lasts from 1 to 4 days, and the viremia – from 1 to 5 days. Upon the experimental challenge of sheep and goats, the clinical signs appear on the 3rd and 5th days [3]. In infected fetuses, the virus is primarily found in the brain. In sick animals, the virus can be isolated from the blood. In molecular studies, the genome of the Schmallenberg virus can be detected in the blood, organs of infected fetuses, in placenta, amniotic fluid, and meconium [4].

The ShV is transmitted in a vector-borne way through blood-sucking insects which are spread throughout the territory of the Russian Federation [5].

The significance of the ShV transmitting agents under agroclimatic conditions of Russia (taking into account the found out biodiversity of biting midges) is covered in the review of A.V. Sprygin et al. [6]. The important role in the animals' contamination is played by the season of a year, depending on which the population of blood-sucking insects varies. The main biological transmitters of the Schmallenberg virus are the biting midges belonging to the *Ceratopogonidae* family of the *Culicoides* genus, which are ones of the smallest blood-sucking dipteran insects of gnats [5]. It was found out that this virus replicates in the salivary glands of female biting midges for 4-16 days depending on the ambient temperature and humidity [3, 7]. Previously, entomological studies showed that ShV has been found out in the pools of biting midges (*Culicoides obsoletus, C. dewulfi, C. pulicaris, C. punc-tatus*), selected in the territories of a number of European countries (Denmark, Norway, Netherlands, Germany, Belgium, Italy and Poland) [8-11]. It is known that ShV is transmitted also by bites of other bloodsucking insects including mosquitoes of the *Culicidae* family [12, 13]. To date, the vertical transmission of this virus through a placenta has been proven, wherein the direct transmission is unlikely. Also, ShV is found in the semen and embryos taken from infected farm animals [14]. The properties of ShV are described in more detail in the review of A.V. Sprygin et al. [7].

The distinctive epidemiological feature of ShV is a high interherd and low intraherd prevalence. The researches conducted in the period from June to September 2016 on sheep in Belgium showed the significant increase in the total (from 25 to 62%) and interherd (from 60 to 96%) seroprevalence in respect of the Schmallenberg virus that indicates the most widespread recirculation of this pathogen since its first appearance in 2011. The ShV circulation has been confirmed by the detection of the virus's RNAs in the pools of *Culicoides obsoletus* biting midges collected around the Antwerp city (Belgium) in August 2016, wherein the minimum morbidity rate of the animals reached 3% [15].

The ShV outbreaks were detected in 2012-2014 in the EU countries (Netherlands, Germany, Belgium, England, France, Italy, Spain, Denmark, Luxembourg, Switzerland, Sweden, Austria, Poland, Finland, Ireland, Norway, Greece, Slovenia, Latvia and other ones) [15, 16]. In the period of 2011-2012 in the territory of Russia, the monitoring of the imported and local animals had been carried out, which has not found the antibodies to ShV in the local farm animals [17]. For the first time in the territory of the Russian Federation, the specific antibodies to ShV were detected in April 2013 and 2014 in the Andreapolsky and Konakovsky districts of the Tver region when investigating the blood serum from cattle in the course of the epizootic monitoring [18, 19]. In October 2014, the specific antibodies to ShV were also found in the blood of cattle in one of the farms in the Novosokolniki district of the Pskov region [20].

The Schmallenberg virus is diagnosed basing on the clinical signs and detection of the viral genome by the PCR method in real-time [21], as well as basing on the virus isolation in insect cell cultures (KC), Syrian hamster kidney (ANC), African green monkey kidney (Vero) or its detection (as the virus kindred to the Akabane disease virus) when intracerebral infection of sucking mice. ShV can be isolated from the blood of infected adult animals and from different tissues of an infected fetus, in particular, from brain biomaterial [22]. For serological diagnostics, the enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence and the virus neutralization test are used [23].

The insufficient knowledge of the causative agent of ShV and unavailability of the precise data about its transmitters in the territory of Russia stimulate the monitoring studies of both the farm animals bred in the territory of the Russian Federation and the animals imported from the EU.

In this publication, for the first time we have summarized the results of the monitoring studies on ShV conducted in Russia from 2014 to 2016. Also, the detection of the PCR-positive animals imported from the EU in 2016 and the discovery in 2015 and 2016 of the seroconversion to Schmallenberg virus in cattle animals born and raised in the regions of Russia, which border on the EU countries where the situation with the Schmallenberg virus is adverse, are described for the first time.

The objective of our work is to accomplish the monitoring molecular-biological investigations of the samples of blood serum and blood from the imported and native cattle animals for the presence of the Schmallenberg virus.

Techniques. We used the samples of stabilized blood and serum from the quarantine animals (heifers) imported from EU countries to Russia, as well as from the local cattle animals. A total of 33,542 stabilized blood samples and 16,749 serum samples of the animals from Voronezh, Vladimir, Kaluga, Bryansk, Ryazan, Rostov, Kirov, Kaliningrad, Leningrad, Ivanovo, Kirov, Tyumen, Rostov, Yekaterinburg, Vologda, Moscow, Pskov, Smolensk, Volgograd, Tver, Lipetsk, Sverdlovsk, Novosibirsk, Tula, Nizhny Novgorod and Kursk regions, Altai Krai, Krasnoyarsk Krai, Karachay-Cherkess Republic, Republic of Bashkortostan, Republic of Crimea, Chuvash Republic, Republic of Dagestan, Republic of Tatarstan, from the Republic of Tajikistan, as well as from the EU countries: France, Germany, Hungary, Austria, Poland, Denmark, Netherlands, Czech Republic, Slovakia and Finland have been investigated. All the samples were taken within the period from 2014 to 2017.

The antibodies to ShV had been detected in these bovine sera using the ID Screen® Capripox Double Antigen Multi-species commercial kit according to the instructions of the manufacturer ("IDvet", France).

To assess the serological status of the cattle animals born and raised in the territory of the Russian Federation (aboriginal cattle) in some regions, the blood serum samples taken from the animals in the spring and autumn periods have been used. The cases when the antibodies were absent in such samples taken in the March-May period, but were detected in the autumn period in the animals which before were seronegative, have been considered as seroconversion.

The total RNA had being isolated from 100 μl of stabilized blood using the QIAamp Viral RNA kit ("Qiagen", Germany).

The PCR in real-time (RT PCR) has been performed according to the protocol described hereinbefore [21].

The isolate of Schmallenberg virus isolated from the cattle animals imported to the Kaliningrad region from Germany had being identified in regards to the S segment using the described primers [24]. The primary nucleotide sequence had being determined with the forward and reverse primers (Applied Biosystems® 3130l Genetic Analyzer, "Applied Biosystems, Inc.", USA). The nucleotide sequences had being equalized using the BioEdit program (https://softfamous.com/bioedit/), the dendrograms have been plotted using the ME-GA 4 software (http://www.megasoftware.net/).

The ShV was isolated from stabilized blood. The blood cells were precipitated by centrifugation for 10-15 minutes at 2,000 rpm, then resuspended with overbuffer saline (OBS, pH 7.2-7.4) and reprecipitated; this procedure was repeated three times. The washed cell fraction was resuspended in sterile OBS having adjusted the volume to the original value. In the obtained samples, the cells were destroyed by ultrasonic vibration (Sonopuls HD 3100, "Bandelin electronic GmbH & Co. KG", Germany) under the amplitude of 16-18 μm (2 times for 30 seconds each with the time interval of 60 seconds). The resulting material was used for the virus isolation in the passaged cell culture of the cells of African

green monkey (Vero). The stages of isolation had being monitored by the real time PCR method.

The cells were grown in 50 cm^3 plastic culturing bottles ("SPL Life Sciences Co., Ltd", Korea) until the formation of a monolayer. After that, the growth medium (manufactured by the FSBI ARRIAH (Federal State Budgetary Institution "All-Russian Research Institute for Animal Health") was removed, the virus-containing material was added and the resulting mixture was incubated for 1 hour at 37°C to adsorb the virus on the monolayer, then the supporting nutrient medium (SNM, manufactured by the FSBI ARRIAH) was added. The infected cell culture had been being examined daily using the microscope for the presence of the characteristic morphological changes. Upon the manifestation of the cytopathic effect (CPE) in 70-80% of the monolayer's area, the virus-containing material was frozen at the temperature of −80°C. The infectious activity of the viral material was determined by microtitring in the Vero cell culture using the conventional method. The virus's titer was calculated by the Reed and Mench method and expressed as lg TCD_{50}/cm^3. The presence of the virus's genome had been confirmed by the real time PCR [21].

Results. In order to monitor the spreading of the Schmallenberg virus, in the period from 2014 to 2016, the monitoring investigations of the bovine blood sera samples taken from the aboriginal animals from the regions of the Russian Federation, as well as of the blood serum samples taken from the animals imported to Russia from the EU countries (a total of 16,749 samples) had been being performed (Table 1, 2). The most part of seropositive animals have been identified in the Kaliningrad, Voronezh and Pskov regions (see Table 2).

1. Prevalence of blood antibodies to Schmallenberg virus in cattle imported into Russia (ELISA test, 2014)

Exporting country	Importing region	Test samples	
		total	seropositive ones, %
France	Voronezh region	522	73.9
Germany	Voronezh region, Kaliningrad region	137	5.8
Czechia	Kursk region	10	0
Sweden	Voronezh region, Tyumen region	160	47.5
Hungary	Kaliningrad region	140	2.8
Austria	Voronezh region	21	0
Poland	Republic of Tatarstan	74	40.5
Denmark	Republic of Tatarstan	408	12.5
Netherlands	Vladimir region	53	0
Slovakia	Kaliningrad region	32	0

2. Prevalence of blood antibodies to Schmallenberg virus in domestic cattle (ELISA test)

Region	Total/positive samples		
	2014	2015	2016
Voronezh region	1345/744	ni	ni
Vladimir region	340/0	ni	ni
Tyumen region	642/263	ni	ni
Tver region	ni	71/0	ni
Kaliningrad region	2273/1488	500/297	431/163
Pskov region	1756/368	645/196	504/50
Nizhny Novgorod Region	1476/107	ni	443/0
Kursk region	296/0	ni	ni
Republic of Tatarstan	1933/63	500/63	ni
The Republic of Dagestan	457/16	ni	ni
Republic of Crimea	ni	621/0	ni
Karachay-Cherkess Republic	959/106	ni	ni

N o t e. ni — not investigated.

Due to the fact of detection of the antibodies to ShV in the local cattle animals, which have been raised in the territory of Russia, in 2015-2016, within a framework of the state monitoring, the paired samples of blood sera taken

from the aboriginal cattle animals of the Kaliningrad and Pskov regions have been investigated (see Table 3).

3. Prevalence of antibodies to Schmallenberg virus in paired blood samples of domestic cattle from two Russian regions (ИФА-тест)

Province	District	Total/positive samples			
		2015		2016	
		spring	autumn	spring	autumn
Kaliningrad	Slavsk	36/0	36/2	36/0	36/7
	Nemansk	36/0	36/1	ni	ni
	Bagrationovsk	36/0	36/7	36/0	36/10
	Nesterov	36/0	36/0	36/0	36/0
	Pravdinsk	36/0	36/0	72/0	72/17
	Krasnoznamensk	ni	ni	36/0	36/9
	Ozyorsk	ni	ni	36/0	36/0
	Krasnogorodsk	32/0	32/2	36/0	36/0
Pskov	Sebezh	55/0	55/2	36/0	36/12
	Palkino	55/0	55/1	ni	ni
	Usvyaty	ni	ni	36/0	36/2
	Kunya	ni	ni	36/0	36/0
	Dedovichi	ni	ni	36/0	36/0

N o t e. ni — not investigated.

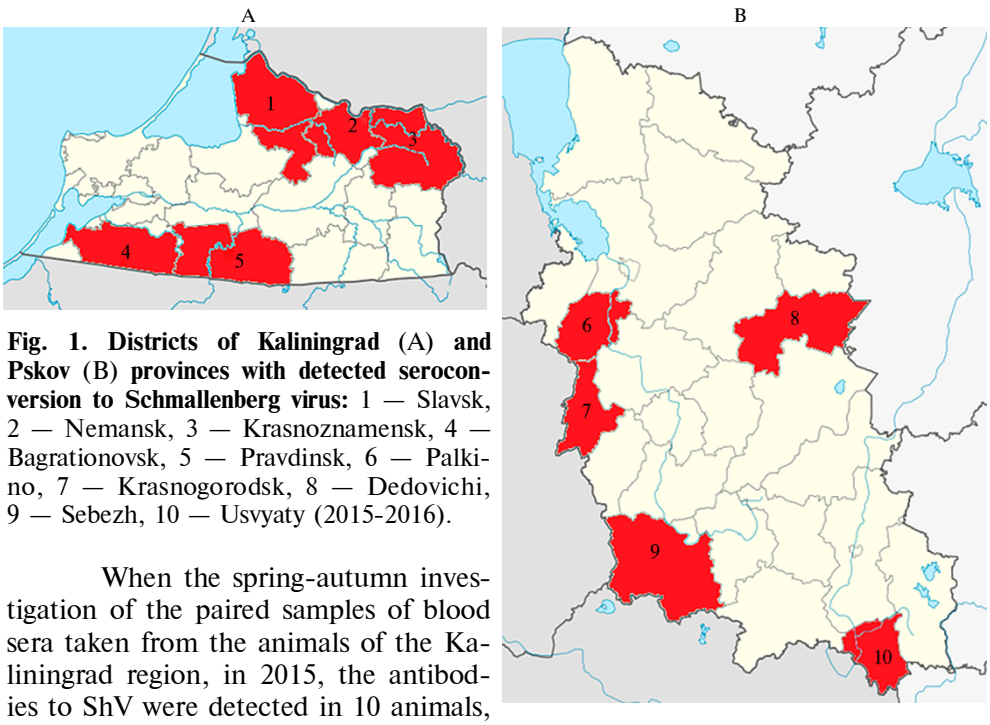

Fig. 1. Districts of Kaliningrad (A) **and Pskov** (B) **provinces with detected seroconversion to Schmallenberg virus:** 1 — Slavsk, 2 — Nemansk, 3 — Krasnoznamensk, 4 — Bagrationovsk, 5 — Pravdinsk, 6 — Palkino, 7 — Krasnogorodsk, 8 — Dedovichi, 9 — Sebezh, 10 — Usvyaty (2015-2016).

When the spring-autumn investigation of the paired samples of blood sera taken from the animals of the Kaliningrad region, in 2015, the antibodies to ShV were detected in 10 animals, in 2016 in 34 ones, and in the Pskov region in 5 and 14 animals, respectively. In each of the regions, the seroconversion was observed in 5 districts (Fig. 1).

When isolating ShV in the Vero cell culture, cytopathic changes were detected at 4th passage 96 hours after the inoculation. The virus's cytopathic effect in the Vero cell culture was initially manifested as the formation of pseudo-syncytium with the fusion of the outer membranes of the cells, later they rounded and merged into conglomerates. Upon the increase of the number of passages, the manifestation of cytopathic effect was found out after 48 hours of culturing (Fig. 2).

The matrasses with the infected cell culture of each passage were frozen, thawed, and the virus's infectious activity was determined in the Vero cell cul-

ture. The titer of infectious activity amounted to 2.83±0.14 lg TCD_{50}/cm^3 at the 5th passage, 3.33±0.00 lg TCD_{50}/cm^3 at the 6th passage, and 3.31±0.07 lg TCD_{50}/cm^3 at the 7th passage.

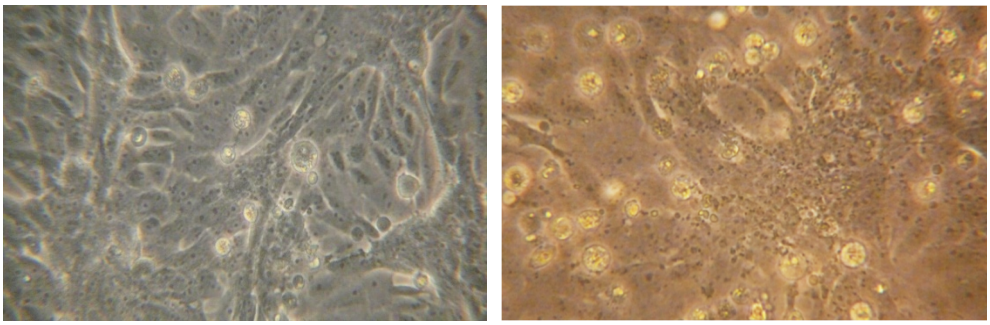

Fig. 2. Monolayers of the 2-day non-infected culture of Vero cells (leftward) **and the cells 48 hours after the infection with the Schmallenberg disease virus** (rightward). Light microscopy (Olympus microscope, Japan).

The 256 bp length fragment of the S segment encoding the nucleocapsid protein was amplified to confirm the isolates' belonging to certain species [24] (Fig. 3). The performed phylogenetic analysis showed that the Kaliningrad/2016 (diagnostic) isolate of ShV has 100% homology with the isolates of ShV found in the Northern Europe countries (see Fig. 3) (the isolate are deposited to the ARRIAH strains collections).

Fig. 3. Phylogenetic position of the Schmallenberg virus Kaliningrad/2016 (diagnostic) isolate. The dendrogram shows the comparison of the nucleotide sequences of the 256 bp fragment of the genome S segment

Also, in the period from 2015 to 2017, the samples of bovine blood were examined for the presence of the Sh. virus's genome. The genome of this pathogen was found in three of 33,542 analyzed samples taken from the animals imported from Germany and Hungary in 2016-2017. Mainly the cattle animals imported from the Netherlands, Germany, Czech Republic, Hungary, Slovakia, France and the animals of local origin were investigated.

Since the detection of the Sh. virus in the territory of a number of EU countries in 2012 [1, 7], in the Russian Federation there has been a problem of the possible bringing in of this pathogen together with the breeding cattle. The course of infection is peculiar with the short duration of the viremia (3-5 days) [7] that significantly reduces the chances of detection of the virus's genome in sick animals during the intravital diagnostics [4]. Because of the absence of mandatory ShV notification in the EU, it is almost impossible to assess the risks of bringing in of this infection into our country due to the wide spread of the virus in the EU countries.

The situation with the spread of ShV is primarily due to the absence of dew attention to the problem from the veterinary and phytosanitary services of European countries despite the scale of the disease spread in these countries and the opinion of the experts of the European Food Safety Authority (EFSA) and the fact that without monitoring and mandatory notification of new cases of ShV it is impossible to assess the situation in a country in regard to this disease. The EU representatives continue to argue that the disease does not cause significant economic damage and does not pose a risk for the livestock sector. That is why the EU countries do not conduct transparent monitoring studies, and in a case of detection of the infection, a country just confines itself to recognizing its territory as endemic [15].

The results of researches of European scientists testify that since the cessation of the mass spread of Sh. virus, it has firmly entrenched itself in the territory of EU [15, 25-27]. In the period from March 3 to 10, 2017, in dairy herds of Ireland, the antibodies to this virus were detected in a total of 256 animals. Also, in the Belgian sheep population an increase in seroprevalence was also noted between June and September 2016 [15].

The Russian Federation is the main trading partner of a number of EU countries in the import of breeding cattle. Moreover, the transit of farm animals to Kazakhstan and other countries gets through the Russian Federation. Since the main transmitters of the Sh. virus are biting midges of the *Culicoides* genus, which are widespread throughout Russia, according to the reviews of the Russian entomofauna and analysis of the situation regarding the possible spread of ShV by its transmitters [6], this may potentially be the factor of the virus spreading in throughout the territory of the Russian Federation.

The serological researches performed in a number of regions of the Russian Federation (see Table 1) have showed that the aboriginal cattle animals are seropositive to the Schmallenber virus, however, according to the official statistics, any signs of congenital malformations in calves have not been found. In order to exclude the error, the analysis of paired samples of blood sera taken from the animals selected in the spring and autumn periods has been performed among the local (aboriginal) cattle animals of the Kaliningrad and Pskov regions. The results have demonstrated the circulation of the virus among the livestock without the manifestation of the clinical signs of the infection (see Table. 3) that testifies about necessity to perform in the listed regions of the entomological investigations of the biomaterial samples in the cases of clinical manifestation of teratogenic effect in pregnant animals. The largest number of seropositive aboriginal animals has been found in the Kaliningrad, Pskov and Voronezh regions in the areas bordering the EU countries. It is possible that the virus circulating in the border countries with the adverse situation is actively brought in to the territory of the Russian Federation by the transmitters or have already entrenched in the said regions. It is important to note that the results of the work of the scientists from the EU countries [15, 26-28] testify that the Schmallenber virus possibly became endemic in Europe.

The antibodies to Sh. virus have also been detected in cattle animals in the Republic of Dagestan (see Table 2). It is likely that the virus entered the territory of the republic from neighboring states (for example, Turkey), which reported the spread of ShV in their territory [27]. Currently numerous data about the spreading of the Sh. virus outside Europe and the Caucasus appear. Thus, in Ethiopia and Mozambique, a mass spread of this pathogen among cattle has been detected [29, 30]. Moreover, the antibodies to ShV have also been detected in cattle animals in China [31].

When investigating the blood sera for the presence of the Sh. virus's ge-

nome, it has been detected in the cattle animals imported from Hungary and Germany to the territory of the Kaliningrad and Tyumen regions. The detection of the antibodies to and the genome of the Schmallenber virus in cattle shows that this pathogen is already circulating in the territory of the Russian Federation and poses a serious threat in case of further spreading.

The S segment of the Kaliningrad/2016 isolate turned out to be 100% homologous to the S segment of the Schmallenber virus isolates found out in Europe (see Fig. 5). The Sh. virus is characterized by high genetic stability despite the fact that its genome is represented by single-stranded RNA. Previously, it was found out [32] that the Sh. virus isolates found in Hungary had 99.3% nucleotide homology. A group of scientists from Turkey also have found out that the Sh. virus was identical by its S segment to the isolates from Germany and Belgium (one nucleotide substitution) [24].

So, as the result of the researches we performed, for the first time since the detection of the Schmallenber virus (ShV) in the EU, it has been reported about detection of the seroconversion of this pathogen in the aboriginal animals in the regions of Russia which border on the EU countries where the situation with ShV is adverse, that testifies about possible bringing in and uncontrolled spreading of the virus in the territory of the Russian Federation. Moreover, the conclusion that the virus could have been brought in earlier both with the imported animals and by the virus transmitters has been confirmed by the fact of detection of the PCR-positive animals imported from the EU. The sequencing of S segment in the viral isolate we isolated showed that it is identical to the ShV isolates circulating in the EU countries. The firm conclusion can be made upon the performance of the relevant entomological studies and the detection of the Schmallenber virus's genome in the virus-transmitting blood-sucking insects. Therefore, we plan the further studying of the potential virus transmitters which support the virus's persistence in the areas where the cases of seroconversion in the animals born and raised in Russia including those obtained from the imported animals have been detected.

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