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Spatio-temporal clustering of African swine fever virus (Asfarviridae: *Asfivirus*) circulating in the Kaliningrad region based on three genome markers

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Abstract

Introduction. The rapid spread of African swine fever in the Kaliningrad region makes it necessary to use the methods of molecular epidemiology to determine the dynamics and direction of ASF spread in this region of Russia.

The aim of the study was to determine single nucleotide polymorphisms within molecular markers *K145R*, *O174L* and *MGF 505-5R* of ASFVs isolated in Kaliningrad region and to study the circulating of the pathogen in European countries by subgenotyping and spatio-temporal clustering analysis.

Materials and methods. Blood samples from living domestic pigs and organs from dead domestic pigs and wild boars, collected in the Kaliningrad region between 2017 and 2022 were used. Virus isolation was carried out in porcine bone-marrow primary cell culture. Amplicons of genome markers were amplified by PCR with electrophoretic detection and subsequent extraction of fragments from agarose gel. Sequencing was performed using the Sanger method.

Results. The circulation of two genetic clusters of ASFV isolates on the territory of the Kaliningrad has been established: epidemic (K145R-III, MGF 505-5R-II, O174L-I – 94.3% of the studied isolates) and sporadic (K145R-II, MGF 505-5R-II, O174L-I – 5.7%).

Conclusion. The broaden molecular genetic surveillance of ASFV isolates based on sequencing of genome markers is necessary in the countries of the Eurasian continent to perform a more detailed analysis of ASF spread between countries and within regions.

Keywords: *African swine fever; genome markers of spread; spatio-temporal analysis; genetic variants, Kaliningrad region*

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ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
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Пространственно-временной кластерный анализ циркуляции вируса африканской чумы свиней (Asfarviridae: *Asfivirus*) в Калининградской области на основе трех генетических маркеров

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Резюме

Введение. Стремительное распространение африканской чумы свиней (АЧС) в Калининградской области обусловливает необходимость использования методов молекулярной эпизоотологии для определения динамики и вектора распространения инфекции в данном субъекте России.

Цель работы – установить характер однонуклеотидного полиморфизма генов *K145R, O174L, MGF 505-5R* у изолятов вируса АЧС, выделенных в Калининградской области, и изучить циркуляцию возбудителя в странах Восточной Европы методом субгенотипирования и пространственно-временного кластерного анализа.

Материалы и методы. В качестве образцов биологического материала использовали пробы крови от живых и органов от павших домашних свиней и диких кабанов, отобранные в Калининградской области в 2017–2022 гг. Выделение вируса АЧС и идентификацию изолятов проводили в первичной культуре клеток костного мозга свиньи. Подготовку ампликонов целевых маркерных областей генома выполняли методом ПЦР с электрофоретической детекций и последующей экстракцией фрагментов из агарозного геля. Секвенирование осуществляли по методу Сэнгера.

Результаты. Установлена циркуляция на территории субъекта-эксклава Российской Федерации вируса АЧС, принадлежащего двум генетическим кластерам: эпизоотическому (K145R-III, MGF 505-5R-II, O174L-I – 94,3% от изученных изолятов) и спорадическому (K145R-II, MGF 505-5R-II, O174L-I – 5,7%).

Заключение. Необходимо совершенствование молекулярно-эпизоотологического мониторинга генетических вариантов вируса АЧС в странах евро-азиатского континента на основе маркерных фрагментов генома внутри генотипа II, что позволит проводить наиболее детальный анализ распространения АЧС.

Ключевые слова: *африканская чума свиней; генетические маркеры распространения; пространственно-временной анализ; генетические варианты; Калининградская область*

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Этическое утверждение. Авторы подтверждают соблюдение институциональных и национальных стандартов по использованию лабораторных животных в соответствии с Consensus author guidelines for animal use (IAVES 23 July 2010). Протокол исследования одобрен Комиссией по биоэтике ФГБУ «ВНИИЗЖ» (протокол № Б-3/2021 от 24.05.2021).

Introduction

The large-scale transboundary spread of African swine fever virus (ASFV) across Eurasia requires new approaches to study virus circulation. For this purpose, molecular epizoological clustering and multigenic analysis of newly isolated isolates have been recommended [1]. Due to the considerable length of the pathogen genome (189 kbp), it is possible to use various markers of spread in a spatio-temporal study. Most markers are identified by detecting single nucleotide polymorphisms (SNPs) or differences in the number of tandem repeats (TRS) observed in comparative analysis of full genome sequences of ASFV isolates from biological material from infected animals (domestic pigs or wild boars) [2].

The target for ASFV genotyping is the 475 bp nucleotide sequence of the C-terminal region of the *B646L* gene, which has traditionally been used to identify 24 genotypes [3, 4]. ASF outbreaks in Europe and Asia are caused by genotype II, except for the enzootic situation on the island of Sardinia (Italy), where genotype I has been registered

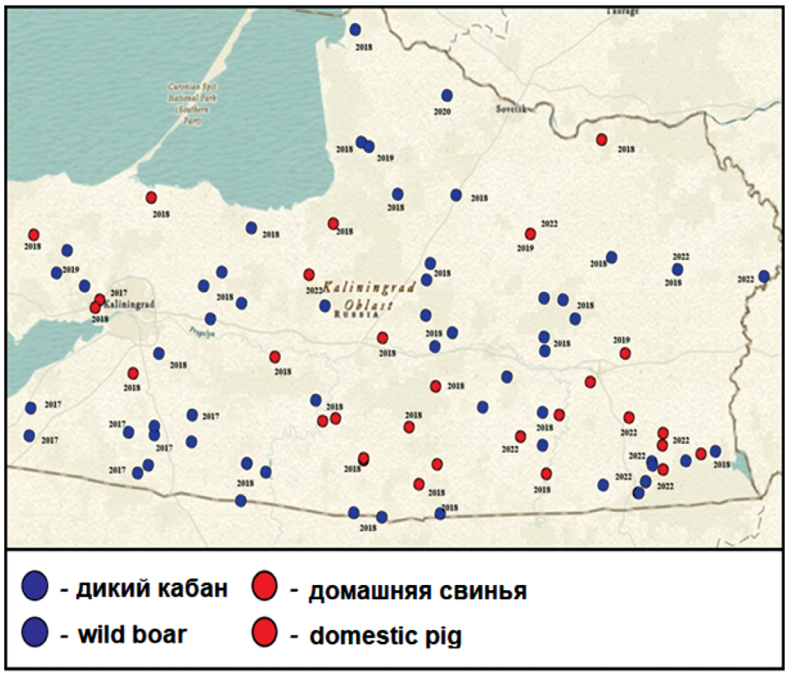

Fig. 1. Spread of ASF in the Kaliningrad region (2017–2022).

Рис. 1. Распространение АЧС на территории Калининградской области (2017–2022 гг.).

since 1978, and the People's Republic of China, where an epizootic outbreak in domestic pigs caused by genotype I was established in 2021 [5, 6]. In 2023, a recombinant variant between genotypes I and II with 20 recombination sites was described for the first time in China [7].

Due to the predominant circulation of ASFV virus genotype II in Eurasia and the detection of various genetic variants, Gallardo C. et al., 2023 recommended a method that allows the identification of 24 clusters (subgenotypes, genetic groups) of distribution based on various markers [1]. Mazloum A. et al, 2023 proposed six major (locus *B646L*, central variable region (CVR) of gene *B602L*, intergenic regions *I73R/I329L* and *MGF 505-9R/10R*, *K145R* and *O174L* genes) and seven alternative genomic markers (intergenic regions *A179L/A137R* and *C315R/C147L*, loci *I267L*, *MGF 505-5R*, *MGF 110-7L*, *MGF 505-9R*, *MGF 360-10L*) to differentiate isolates belonging to genotype II [2].

Cluster genetic analysis is a relatively new and exploratory approach for the study of ASF in the Russian Federation. In this regard, the most interesting and representative object of study is the epizootic in the territory of the Kaliningrad Region.

Kaliningrad region is an exclave of Russia, geographically located in Eastern Europe. It borders with Poland in the south and Lithuania in the north and east, which have been recognized as affected by ASFV since 2014 [8, 9]. The first ASFV cases in the Kaliningrad region were observed in the wild boar population in early November 2017. During November–December 2017, 6 epizootic outbreaks among wild boar (Bagrationovsky district) and 1 among domestic pigs in the personal subsidiary farm (PSF) of a citizen living in the rural settlement of Sosnovka (Polessky district) were recorded in the region. In the winter-spring period of 2018, 6 wild boar carcasses diagnosed with ASFV were found on the territory of the subject in the areas bordering Poland. From June to September 2018, the infection spread widely among domestic pigs in household farms and several pig farms in the Kaliningrad region, affecting 7 previously safe districts (Gvardeysky (Suvorovo settlement), Pravdinsky (Prudy, Novobiyskoye, Bely Yar, Izvilino, Krasnoye, Sergeevka, Belkino), Chernyakhovsky (Penki, Glushkovo) and others). From November 2018 to January 2020, single cases of the disease were registered [10]. At the end of July 2022, after 2.5 years of absence of outbreaks, an epizootic outbreak of ASFV was registered in PSFs of the Nesterovsky district. A total of 16 cases were notified for 2022. During the entire epizootic period in the territory of Kaliningrad region 87 cases of ASFV (56 in wild boar and 31 in domestic pigs) were officially confirmed, as seen in **Figure 1**[1].

Earlier studies analyzing 9 full genome sequences of isolates isolated in 2017–2019 in the Kaliningrad region showed clonal development of ASFV outbreaks [11]. Thus, an additional mutation in the K145R gene occurred at the beginning of the epizootic in this region. Prolonged circulation of the genetic variant of the virus with a unique SNP among susceptible animals (wild boars) formed a divergent spatio-temporal ASFV cluster (pool) in eastern Europe.

The enzootic situation of ASFV in the Kaliningrad region of Russia and the discussion about the origin of the pathogen in Eastern European countries make it neces-

[1]Rosselkhoznadzor. Epidemic ASF situation in the Russian Federation, 2007–2024. Available at: https://fsvps.gov.ru/wp-content/uploads/2024/02/karta-vspyshek-za-2007-2024-na-05.02.png (In Russian).

sary to use molecular epizoological methods in outbreak investigations. In this regard, the aim of the study was to determine the distribution of genetic variants *K145R*, *MGF 505-5R*, *O174L* in a statistically significant number of ASFV virus isolates circulating in the territory of the Kaliningrad region and to assess the epizootic situation on the basis of molecular clustering.

Materials and methods

ASFV virus isolates. Biological material for virus isolation was organ samples (tubular bones, spleen, lymph nodes) from fallen domestic and wild pigs, as well as blood from live domestic pigs sampled in the Kaliningrad region of the Russian Federation in 2017–2022 and tested positive by polymerase chain reaction. Virus isolation was performed in primary culture of pig bone marrow cells (BMC) obtained from donors on a contractual basis [12]. After identification of 26 isolates, they were accumulated in BMC cell culture at a titer of at least 6.5 lg $HAdU_{50}/cm^3$.

DNA extraction and amplification of target fragments. Total DNA extraction was performed from virus-containing culture material using the DNA-Sorb B reagent kit (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia). Amplification of nucleotide regions of *K145R*, *MGF 505-5R*, and *O174L* genes was performed by PCR with electrophoretic detection according to the previously developed protocol [13]. Fragments were isolated from agarose gel slides using GeneJET Gel Extraction Kit (ThermoFisher Scientific, USA).

Sequencing. The reaction was performed using Big Dye Terminator Kit version 1.1 or 3.1 (Applied Biosystems, USA) on an ABI Prism 3130/3130xl automated sequencer (Applied Biosystems, USA) according to the manufacturer's instructions.

Alignment and phylogenetic analysis. Multiple alignment of nucleotide sequences and comparative analysis with other isolates retrieved from GenBank (**Appendix**) were performed using the CLUSTAL W algorithm in the Bioedit v7.2.5 program. The sequences of target genes in 9 ASF virus isolates from the Kaliningrad region, published earlier [12], were included in analysis. The phylogeny of isolates was studied using the Mega X software according to the recommended model by the Neighbor–Joining method with 1000 initial Bootstrap iterations [14].

Graphical display of molecular epizootic maps was performed in ArcGIS program.

Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with Consensus author guidelines for animal use (IAVES 23 July 2010). The research protocol was approved by the Bioethics Commission of the Federal State Budgetary Institution «ARRIAH» (protocol b/n of30.04.2020).

Results

K145R. A fragment of the *K145R* gene (501 bp in length) was obtained for all 26 isolates by Sanger sequencing. All identified isolates from Kaliningrad region (35) had point mutations (substitutions) in this fragment and differed from the reference strain *Georgia 2007/1* (NC_044959.2), as seen in **Figure 2** *a*.

According to Figure 2 *a*, based on the nucleotide alignment of the partial sequence of the *K145R* gene, all ASFV isolates under study can be divided into three genetic variants: *K145R-I*, similar to *Georgia 2007/1*; *K145R-II* with one C > A substitution at position 434; *K145R-III* with two substitutions (C > T at position 291 and C > A at position 434). It was found that the majority, 33 out of 35 (94.3%) isolates in the study region belonged to the genetic variant *K145R-III*, which is exclusive for this region. At the same time, 2 (5.7%) isolates of *ASFV/Kaliningrad/DP2017/15355* and *ASFV/Kaliningrad/DP2022/9201* belonged to *K145R-II*, a variant previously unregistered throughout Russia and characteristic of Eastern Europe (Poland, Lithuania, Ukraine, Germany). Spatio-temporal analysis of ASFV spread based on the *K145R* marker is shown in **Figure 3** *a*.

O174L. Analysis of the 673 bp sequences obtained by sequencing of the *O174L* gene fragment showed that all (100%) isolates from the Kaliningrad region lacked a 14-nucleotide insertion at position 50–63 of the *O174L* gene and was identical to the reference sequence of strain Georgia 2007/1 (Figure 2 *b*). The absolute enzootic nature of the *O174L-I* variant of ASF virus in the Kaliningrad region is shown in Figure 3 *b*.

MGF 505-5R. Sequence analysis of a 641 bp fragment in all samples studied showed that 100% of the isolates studied in this study belonged to the Eastern European variant *MGF 505-5R-II*, which was characterized by a nonsynonymous G > A substitution at position 988 of the gene when compared with the reference strain II of genotype *Georgia 2007/1* (Figure 2 *c*). The geographical distribution of the *MGF 505-5R* genetic groups of ASFV in Europe is presented in Figure 3 *c*.

Phylogenetic relatedness of ASFV isolates from Kaliningrad region of the Russian Federation, Poland, Lithuania, Germany, Romania and Ukraine is presented on the rooted tree made complexly on the basis of concatenated sequence of 3 markers: *K145R*, *MGF 505-5R* and *O174L* (**Figure 4**).

As shown in Figure 4, two groups within genotype II were identified. Group 1 is completely homologous to strain *Georgia 2007/1*. Group 2 includes 3 subgroups: group 2 proper (corresponding to genetic variants *K145R-I*, *O174L-II*); 2.1 (*K145R-II*, *MGF 505-5R-II*, *O174L-I* or *O174L-II*) and 2.2 (*K145R-III*, *MGF 505-5R-II*, *O174L-I*). Kaliningrad region is predominantly characterized by group 2.2 (94.3%). In Form 76, group 2.1 (5.7%) was registered.

Epizoological data with the geographical location of the sampling site, name of the isolate, date of the outbreak, source of the infectious agent, marker genetic group are presented in **Table** and the **Appendix.**

Discussion

Reference diagnostic tests for ASF in FGBI ARRIAH (Vladimir) provided the study with isolates from biological material from domestic and wild pigs, select-

Fig. 2. Multiple nucleotide alignment of genome marker fragments *K145R* (*a*), *O174L* (*b*) and *MGF 505-5R* (*c*) of ASFV isolates obtained by Sanger sequencing.

The figures show an example of identified SNPs or TRSs and their position in the open reading frame.

Рис. 2. Множественное нуклеотидное выравнивание маркерных фрагментов генов *K145R* (*а*), *O174L* (*б*) и *MGF 505-5R* (*в*) изолятов вируса АЧС, полученных при секвенировании по методу Сэнгера.

На рисунках показан пример выявленных ОНП или TRS и их позиции в открытой рамке считывания.

ed from 35 (40.2%) out of 87 ASFV foci notified in the territory of Kaliningrad region (sequences are published in GenBank, accession numbers are presented in the **Appendix**). The large statistical sample size allows us to use the results of point (local) Sanger sequencing of marker regions of the genome to analyze virus circulation and the formation of spatio-temporal genetic clusters in the exclave region.

Since 2016, the genotype II ASFV circulating in the countries of eastern Europe (Poland, Lithuania, Ukraine, etc.), as well as in the Kaliningrad region of the Russian Federation, has significant nucleotide heterogeneity in comparative analysis with the original strain *Georgia 2007/1* [1]. Point mutations in open reading frames and intergenic regions (substitutions/insertions/deletions) in ASFV insignificantly affect culture and immunobiological properties [15–17]. At the same time, the applied significance of genetic changes in the molecular epizoology of ASFV is quite high and is constantly being updated [18]. A comprehensive analysis of such loci as *K145R*, *MGF 505-5R*, *O174L*, *MGF 110-7L*, *IGR I73R/I329L* is necessary to study ASFV clusters in Europe.

Previously presented data show that the formation of the genetic cluster K145R-II, MGF 505-5R-II, MGF 110-7L-II began in 2016 in Ukraine and Poland [19, 20]. The spatio-temporal array of this cluster is observed from 2016 to 2020 in Poland, from July 2017 to March 2022 in Lithuania. In parallel, an O174L-II variant with an insertion in the gene of the same name was registered in Poland in 2016, which always corresponds to MGF 505-5R-II [21, 22]. At the same time, O174L-II is combined with K145R-II in some cases (2016–2020) and with K145R-I in others (2018–2019). However, O174L-II has not been reported in Lithuania, Ukraine and the Kaliningrad region [1, 11, 20]. 19 ASF outbreaks in Romania (2019) correspond to the O174L-II, K145R-I group,

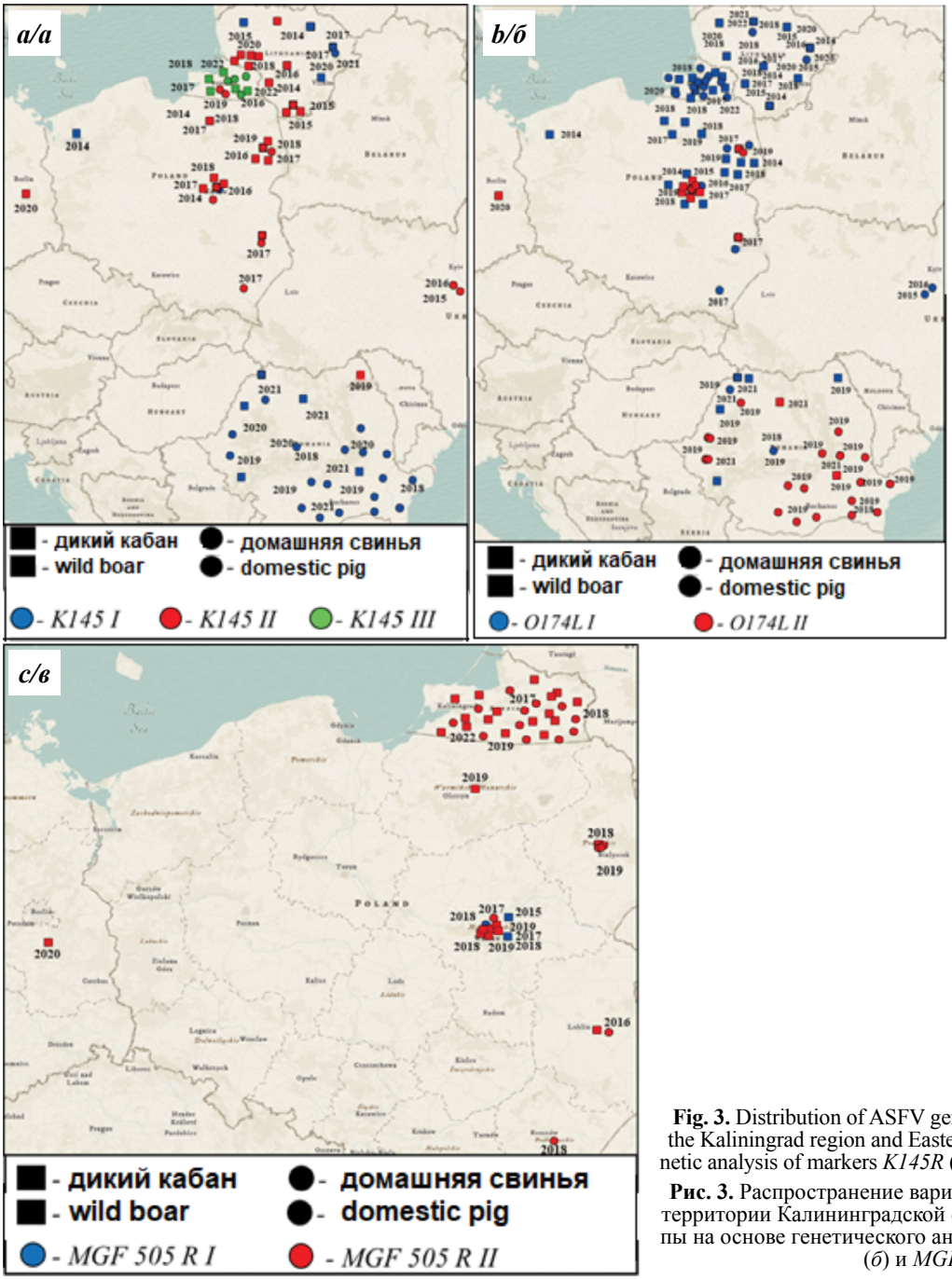

Fig. 3. Distribution of ASFV genotype II variants in the territory of the Kaliningrad region and Eastern European countries based on genetic analysis of markers *K145R* (*a*), *O174L* (*b*) and *MGF 505-5R* (*c*).

Рис. 3. Распространение вариантов вируса АЧС генотипа II на территории Калининградской области и стран Восточной Европы на основе генетического анализа маркеров *K145R* (*а*), *O174L* (*б*) и *MGF 505-5R* (*в*).

and only one isolate 6 km from the Ukrainian border was categorized as K145R-II and O174L-I [1]. Further, similar changes in the genome (O174L-II, K145R-II) were observed in the ASFV isolate isolated from wild boar in Germany (2020) [2].

The MGF 505-5R-II variant is characteristic of isolates with K145R-II or K145R-III, and has never been found in ASFV with K145R-I. On the contrary, isolates with MGF 505-5R-I paralleled the K145R-I gene variant [21]. The emergence and circulation of virus belonging to variants K145R-I, O174L-II in Poland and Romania in 2018–2019 may be due to point repair of the K145R gene, which tends to occur in ASFV due to the presence of Pol X DNA polymerase, or recombination [7, 23]. For a deeper study of the molecular evolution of ASFV in Romania, full genomic sequencing of isolates with analysis of the *MGF 505-5R* gene is necessary.

It should be noted that the synonymous G to A substitution at position 60 of the *MGF 110 7L* gene has always been combined with the above-described ONP in the *MGF 505-5R* region [1, 11, 19, 20]. In this regard, it is economically feasible to investigate one of these loci of choice [2].

In the Kaliningrad region of Russia, a unique cluster of K145R-III, MGF 505-5R-II, O174L-I of ASFV was formed between 2017 and 2022. The substitution at po-

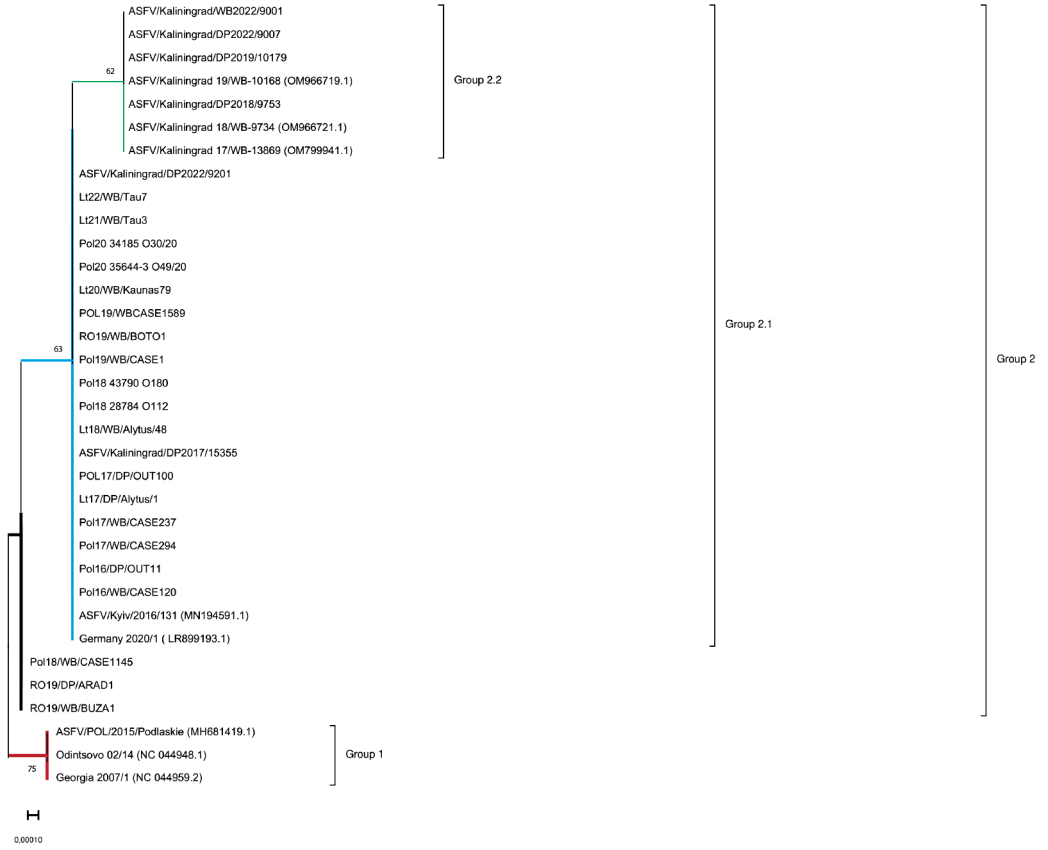

Fig. 4. Neighbor-joining dendrogram showing phylogenomic of ASFV isolates from eastern Europe based on clustering analysis.

Рис. 4. Дендрограмма, построенная методом присоединения соседей (Neighbor-joining) и отображающая филогенетическое родство изолятов вируса АЧС из Восточной Европы на основе кластерного анализа.

sition 434 of the K145R gene corresponded to both K145R-III and K145R-II variants. However, K145R-III had an additional substitution at position 291 of the original gene, hence it was a daughter gene variant of the maternal K145R-II gene.

In the conducted study it was found that the isolate *ASFV/Kaliningrad/DP2017/15355*, isolated for the first time from biomaterial from domestic pigs in the study region (December 2017, Polessky district, Sosnovka village) was classified as K145R-II, despite the fact that the first ASFV outbreak in the region was caused by a virus with K145R-III (*ASFV/Kaliningrad 17/WB-13869* isolate obtained from biomaterial from a fallen wild boar found in November 2017 in Bagrationovsky district) [11]. Interestingly, the first cases of ASFV in Lithuania caused by the virus with K145R-II were registered in July 2017, while Poland and Ukraine were recognized as affected by ASFV in 2017 [24].

In 2018–2019, 65 ASFV outbreaks were registered on the territory of the Kaliningrad region, and all isolates belonged to genetic group K145R-III, MGF 505-5R-II and O174L-I. At the same time, the K145R-II variant did not spread due to the successful elimination of the 2017 outbreak in the private farm of a citizen of Polessky district, which limited the transition of ASFV into the wild fauna. Thus, the time of formation of the genetic group unique for the region is the fall of 2017, and the original host is the European wild boar *Sus scrofa*. At the same time, the possibility of ASFV entry into the Kaliningrad region in 2017 from other regions of Russia, where variants different in markers such as *K145R*, *MGF 505-5R* and *MGF 110-7L* from the *Georgia 2007/1* strain have never been registered before, is unlikely [25, 26].

After a prolonged absence of virus with K145R-II (2018–2021) in the Kaliningrad region, an isolate of *ASFV/Kaliningrad/DP2022/9201* was isolated in 2022 at a large pig breeding farm in the village of Pokrovskoye, Chernyakhovsky district, belonging to this variant. However, K145R-II has never been detected in infected wild boars in the region, which are one of the characteristic factors in the spread of ASFV, including the 2022 isolates [27–31]. However, the disease was registered during the second half of 2022 in Lithuania/Poland/Ukraine, and the genetic sequence of the pathogen in individual countries (Lithuania) corresponded to K145R-II and O174L-I[2] [1, 32].

The most important marker of ASFV spread is also the intergenic region of *IGR I73R/I329L*. Thus, 2 variants of IGR-I (2 insertions of 10-nucleotide tandem repeats identical to strain *Georgia 2007/1*) and IGR-II (3 insertions of tandem repeats) are found in Poland, the

[2]Rosselkhoznadzor. Number of ASF outbreaks in European countries 2012–2024. Available at: https://fsvps.gov.ru/files/kolichestvo-vspyshek-achs-v-stranah-evropy-2012-2023-gg-na-05-09-2023/?ysclid=lsox29ofky735423172 (in Russian)

Table. Molecular and epizootological data on ASFVs isolated in the Kaliningrad region from 2017 to 2022
Таблица. Молекулярно-эпизоотологические данные об изолятах вируса АЧС, выделенных в Калининградской области 2017–2022 гг.

№	Isolate Наименование изолята	Place of collection Место отбора образцов	Date of outbreak registration Дата регистрации вспышки	Source of isolation Источник возбудителя инфекции	*K145R*	*MGF 505-5R*	*O174L*
1	ASFV/Kaliningrad/DP2017/15355	Private household plot, Sosnovka village, Polessky district ЛПХ, п.Сосновка, Полесский район	18.11.2017	Domestic pig Домашняя свинья	II	II	I
2	ASFV/Kaliningrad/WB2018/9737	Hunting farm, Gvardeysky district Охотничье хозяйство, Гвардейский район	03–04.07.2018	Wild boar Дикий кабан	III	II	I
3	ASFV/Kaliningrad/WB2018/9767	Forest area, Ilyichevo village, Polessky district Лесной массив, п. Ильичево, Полесский район	07–08.07.2018	Wild boar Дикий кабан	III	II	I
4	ASFV/Kaliningrad/DP2018/9716	Private household plot, Pravdinsky district, Sergeevka village ЛПХ, Правдинский район, п. Сергеевка	22.06.2018	Domestic pig Домашняя свинья	III	II	I
5	ASFV/Kaliningrad/DP2018/12537	Peasant farm, Nemansky district, Zhilino village, st. Dorozhnaya, 1 КФХ, Неманский район, п. Жилино, ул. Дорожная, 1	06–10.07.2018	Domestic pig Домашняя свинья	III	II	I
6	ASFV/Kaliningrad/WB2018/12513	Hunting farm, Chernyakhovsky district Охотничье хозяйство, Черняховский район	18.07.2018	Wild boar Дикий кабан	III	II	I
7	ASFV/Kaliningrad/DP2018/9729	Private household plot, Pravdinsky district, Belkino village ЛПХ, Правдинский район, п. Белкино	22.06.2018	Domestic pig Домашняя свинья	III	II	I
8	ASFV/Kaliningrad/WB2018/9732	Zhavoronkovo village, Chernyakhovsky district п. Жаворонково, Черняховский район	25.06.2018	Wild boar Дикий кабан	III	II	I
9	ASFV/Kaliningrad/DP2018/9728	Private household plot, Chernyakhovsky district, Penki village ЛПХ, Черняховский район, п. Пеньки	22.06.2018	Domestic pig Домашняя свинья	III	II	I
10	ASFV/Kaliningrad/WB2017/16199	Forest, Novoselovo settlement, Bagrationovsky district Лесной массив, п. Новоселово, Багратионовский район	27.11.2017	Wild boar Дикий кабан	III	II	I
11	ASFV/Kaliningrad/WB2018/12518	Forest, Krasnopolye village, Gusevsky district Лесной массив, п. Краснополье, Гусевский район	30.07–07.08.2018	Wild boar Дикий кабан	III	II	I
12	ASFV/Kaliningrad/DP2018/9723	Private household plot, Pravdinsky district, Bely Yar village ЛПХ, Правдинский район, п. Белый Яр	22.06.2018	Domestic pig Домашняя свинья	III	II	I
13	ASFV/Kaliningrad/WB2018/14814	Hunting farm, Guryevsky district, Kurgany village Охотничье хозяйство, Гурьевский район, п. Курганы	24.08–03.09.2018	Wild boar Дикий кабан	III	II	I
14	ASFV/Kaliningrad/DP2018/6809	Private household plot, Pravdinsky district, Novo-Biiskoye village ЛПХ, Правдинский район, п. Ново-Бийское	08.06.2018	Domestic pig Домашняя свинья	III	II	I

For continuation of the table, see page 249

№	Isolate Наименование изолята	Place of collection Место отбора образцов	Date of outbreak registration Дата регистрации вспышки	Source of isolation Источник возбудителя инфекции	*K145R*	*MGF 505-5R*	*O174L*
15	ASFV/Kaliningrad/DP2018/9753	Pig farm of IV compartment, Pravdinsky district, Novo-Biiskoye village Свинокомплекс IV компартмента, Правдинский район, п. Ново-Бийское	10.07.2018	Domestic pig Домашняя свинья	III	II	I
16	ASFV/Kaliningrad/DP2018/9724	Private household plot, Pravdinsky district, Prudy village ЛПХ, Правдинский район, п. Пруды	22.06.2018	Domestic pig Домашняя свинья	III	II	I
17	ASFV/Kaliningrad/DP2018/9741	Breeding farm, Slavsky district Племенной завод, Славский район	10.07.2018	Domestic pig Домашняя свинья	III	II	I
18	ASFV/Kaliningrad/DP2018/9727	Private household plot, Pravdinsky district, Krasnoe village ЛПХ, Правдинский район, п. Красное	22.06.2018	Domestic pig Домашняя свинья	III	II	I
19	ASFV/Kaliningrad/DP2018/12528	Pig farm of compartment III, Kaliningrad, town. Them. A. Kosmodemyanenko Свинокомплекс III компартмента, г. Калининград, пгт. им. А. Космодемьяненко	06–10.07.2018	Domestic pig Домашняя свинья	III	II	I
20	ASFV/Kaliningrad/DP2018/14812	Private household plot, Gusevsky district, Mayakovskoye village ЛПХ, Гусевский район, п. Маяковское	21–23.08.2018	Domestic pig Домашняя свинья	III	II	I
21	ASFV/Kaliningrad/DP2019/10179	Pig farm of compartment IV, Gusevsky district, Tamanskoye village Свинокомплекс IV компартмента, Гусевский район, п. Таманское	23.08.2019	Domestic pig Домашняя свинья	III	II	I
22	ASFV/Kaliningrad/WB2019/10169	Hunting farm, Zelenogradsky district Охотничье хозяйство, Зеленоградский район	18.05.2019	Wild boar Дикий кабан	III	II	I
23	ASFV/Kaliningrad/WB2022/8997	Hunting farm, Nesterovsky district Охотничье хозяйство, Нестеровский район	27.07.2022	Wild boar Дикий кабан	III	II	I
24	ASFV/Kaliningrad/WB2022/9001	Forest area near the village of Tokarevka, Nesterovsky district Лесной массив вблизи п. Токаревка, Нестеровский район	01.08.2022	Wild boar Дикий кабан	III	II	I
25	ASFV/Kaliningrad/DP2022/9007	Private household plot, Nesterovsky district, Kalinino village ЛПХ, Нестеровский район, п. Калинино	04.08.2022	Domestic pig Домашняя свинья	III	II	I
26	ASFV/Kaliningrad/DP2022/9201	Breeding farm, Chernyakhovsky district, Pokrovskoye village Племенная ферма, Черняховский район, п. Покровское	15.08.2022	Domestic pig Домашняя свинья	II	II	I
27	ASFV/Kaliningrad 17/WB-13869	Forest, Krasnoarmeyskoye settlement, Bagrationovsky district Лесной массив, п. Красноармейское, Багратионовский район	07.11.2019	Wild boar Дикий кабан	III	II	I

For continuation of the table, see page 250

№	Isolate Наименование изолята	Place of collection Место отбора образцов	Date of outbreak registration Дата регистрации вспышки	Source of isolation Источник возбудителя инфекции	*K145R*	*MGF 505-5R*	*O174L*
28	ASFV_Kaliningrad_18_WB-12523	Hunting farm, Slavsky district Охотничье хозяйство, Славский район	07.08.2018	Wild boar Дикий кабан	III	II	I
29	ASFV_Kaliningrad_18_WB-12524	Forest, Shirokoe village, Pravdinsky district Лесной массив, п. Широкое, Правдинский район	30.07.2018	Wild boar Дикий кабан	III	II	I
30	ASFV_Kaliningrad_18_WB-9735	Forest area, Pravdinsky district Лесной массив, Правдинский район	03.07.2018	Wild boar Дикий кабан	III	II	I
31	ASFV_Kaliningrad_18_WB-9763	Forest, Osinovka village, Gvardeysky district Лесной массив, п. Осиновка, Гвардейский район	07.07.2018	Wild boar Дикий кабан	III	II	I
32	ASFV_Kaliningrad_18_WB-9766	Hunting farm, Polessky district Охотничье хозяйство, Полесский район	08.07.2018	Wild boar Дикий кабан	III	II	I
33	ASFV_Kaliningrad_19_WB-10168	Forest, Zapovednoe village, Slavsky district Лесной массив, п. Заповедное, Славский район	13.05.2019	Wild boar Дикий кабан	III	II	I
34	ASFV_Kaliningrad_18_WB-9734	Forest area, Kamenskoye village, Chernyakhovsky district Лесной массив, п. Каменское, Черняховский район	25.06.2018	Wild boar Дикий кабан	III	II	I
35	ASFV_Kaliningrad_18_WB-12516	Forest area, p. Shuvalovo, Chernyakhovsky district Лесной массив, п. Шувалово, Черняховский район	13.05.2018	Wild boar Дикий кабан	III	II	I

Note. The analysis used sequences of 26 isolates described for the first time in the current study and data on 9 ASFV isolates published previously and retrieved from GenBank. Isolates with the genetic variant *K145R*-II are highlighted in red, isolates with *K145R*-III are highlighted in green.

Примечание. В анализе использованы последовательности 26 изолятов, описанных впервые в текущем исследовании, и данные о 9 изолятах вируса АЧС, опубликованные ранее и импортированные из GenBank. Красным цветом выделены изоляты с генетическим вариантом *K145R*-II, зеленым цветом – с *K145R*-III.

Kaliningrad region of the Russian Federation, and Lithuania [1, 11]. In 7 isolates from Warmińsko-Mazurskie Voivodeship (eastern subject of Poland bordering the Kaliningrad region) isolated in 2019–2020, 5 insertions of tandem repeats in the intergenic region (IGR-IV) were detected. All 7 samples with IGR-IV belonged to genetic variants K145R-II, MGF 505-5R-II, and O174L-I [21]. Subsequent analysis of the *IGR I73R/I329L* locus in isolates from Kaliningrad region is relevant to confirm the absence of IGR-IV.

Analysis of sequencing data objectively proves the pathways of molecular evolution of ASF pathogen on the Eurasian continent. The trend of epizootic development was manifested by the shift of outbreaks from the south-east (Georgia, Armenia, Azerbaijan, central Russia) to the west (Ukraine, Belarus, Poland, Lithuania, Latvia, Estonia, Germany, Kaliningrad region) in the period from 2007 to 2017 [18]. The scheme of territorial origin and circulation of ASFV in the Kaliningrad region, proposed on the basis of spatio-temporal and phylogenetic studies, is presented in **Figure 5**. Molecular epizoological clustering based on local sequencing data is also confirmed by the results of the full-genome phylogenetic analysis of Eurasian isolates performed by Y. Zhang et al. [18].

Conclusion

ASFV cases in the Kaliningrad region of the Russian Federation were registered during 2017–2022. Molecular genetic analysis of 35 virus isolates from biological material from domestic and wild pigs demonstrated the circulation in the territory of the exclave region of the virus belonging to two genetic clusters: epizootic (K145R-III, MGF 505-5R-II, O174L-I – 94.3% of the isolates studied) and sporadic (K145R-II, MGF 505-5R-II, O174L-I – 5.7%). At the same time, the second cluster, widely spread in Poland and Lithuania, was first described in the Kaliningrad region only in December 2017 – the first ASFV outbreak in domestic pigs in PSF (isolate *ASFV/Kaliningrad/DP2017/15355*), and in August 2022 – epizootic outbreak at a breeding pig farm (*ASFV/Kaliningrad/DP2022/9201*). The absence of a 14-nucleotide insertion in the O174L gene in ASFV isolates isolated among susceptible animals of the Kaliningrad region was demonstrated.

The results of the spatio-temporal analysis demonstrate the unlikely possibility of ASFV pathogen entry into the Kaliningrad region in 2017 from the central subjects of Russia, as genetic heterogeneity of virus isolates according to the three studied markers is observed.

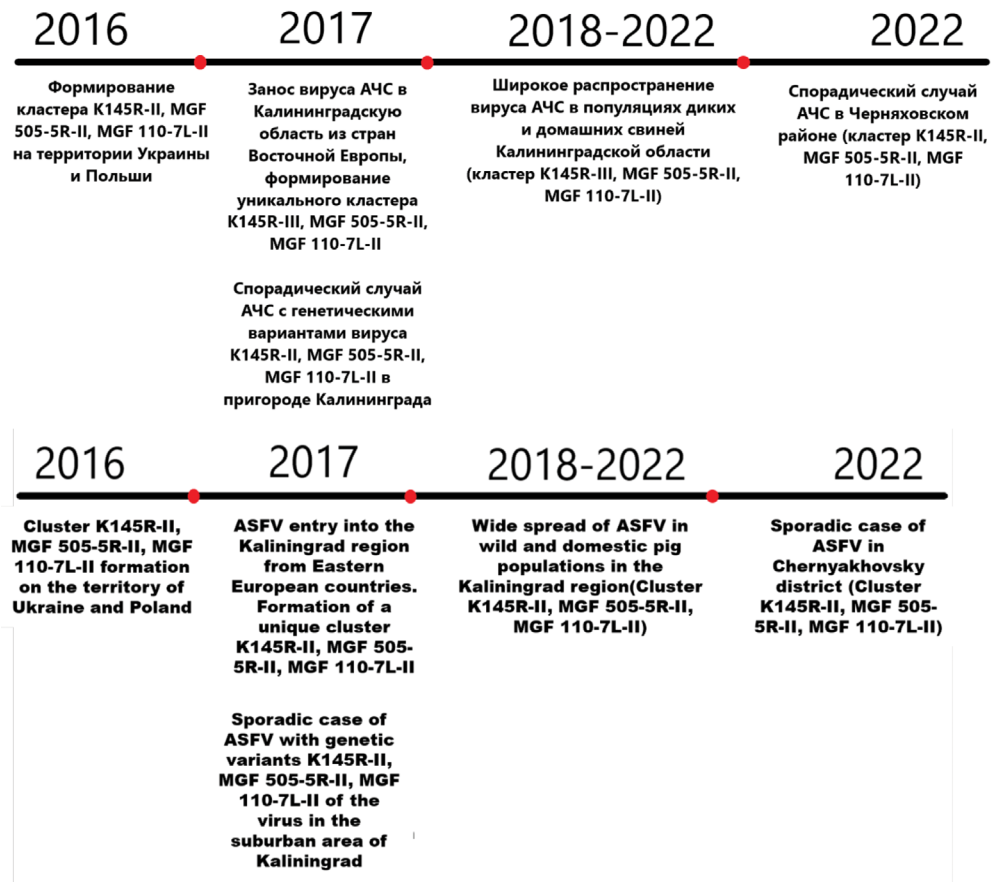

Fig. 5. The pattern of territorial origin and circulation of ASFV in Kaliningrad region based on analyses of published and original data.

Рис. 5. Схема территориального происхождения и циркуляции вируса АЧС в Калининградской области на основе анализа опубликованных и полученных данных.

Further monitoring of ASFV subgenotypes in Eurasia is a very relevant direction in molecular epizoology, which may become a basic method and have applied value in ASFV outbreak investigation.

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