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Molecular and genetic characteristics of the multicomponent flavi-like Kindia tick virus (Flaviviridae) found in ixodid ticks on the territory of the Republic of Guinea

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Introduction. Ixodid ticks are vectors for pathogens of many infectious diseases. Recently, during the study of *Rhipicephalus geigyi* ticks collected from livestock in the Republic of Guinea, a new multicomponent flavi-like RNA virus, called Kindia tick virus (KITV), was discovered with an unusual mechanism for the implementation of genetic information.
The **aim** of the work is to detect and study the genetic diversity of KITV in ixodid ticks collected in the territory of the Kindia province of the Republic of Guinea.
Materials and methods. In 2021, 324 specimens of ticks of the species *Amblyomma variegatum*, *Rh. geigyi*, *Rh. annulatus*, *Rh. decoloratus*, *Rh. senegalensis* were collected from cattle. The detection of viral RNA was carried out in individual samples of ticks by RT-PCR, followed by the determination of the nucleotide sequence and phylogenetic analysis.
Results and discussion. KITV detection rates in ticks of the species *Rh. geigyi* was 12.2%, *Rh. annulatus* – 4.4%, *Rh. decoloratus* – 3.3%. However, the KITV genetic material has not been identified in Am. variegatum ticks, which are one of the dominant species in West Africa. For all virus isolates, a partial nucleotide sequences of each of the four viral segments (GenBank, OK345271–OK345306) were determined. The phylogenetic analysis showed a high level of identity (98.5–99.8%) for each of the four segments of the viral genome with those previously found in the Republic of Guinea. The obtained KITV isolates are most genetically close to Mogiana tick virus, which was previously detected in South America in *Rh. microplus* ticks and significantly differed from other multicomponent viruses circulating in Europe and Asia, including the Russian Federation.
Conclusion. KITV genetic material was found in three species of ixodid ticks collected from livestock in a number of prefectures of the Republic of Guinea. The infection rate in ticks was 3.3–12.2%. The continuation of research in this direction remains relevant.

Keywords: *multicomponent flavi-like viruses; Kindia tick virus; KITV; ixodid ticks; Republic of Guinea*

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НАУЧНАЯ СТАТЬЯ
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Молекулярно-генетическая характеристика многокомпонентного флавиподобного вируса Kindia tick virus (Flaviviridae), обнаруженного в иксодовых клещах на территории Гвинейской Республики

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Введение. Иксодовые клещи – переносчики возбудителей многих инфекционных болезней. Недавно при исследовании клещей *Rhipicephalus geigyi*, собранных с домашнего скота в Гвинейской Республике, обнаружен новый многокомпонентный флавиподобный РНК-содержащий вирус, получивший название Kindia tick virus (KITV), с необычным механизмом реализации генетической информации.
Цель работы – обнаружение и исследование генетического разнообразия KITV в иксодовых клещах, собранных на территории провинции Киндиа Гвинейской Республики.
Материал и методы. В 2021 г. с крупного рогатого скота собрано 324 экземпляра клещей видов *Amblyomma variegatum*, *Rh. geigyi*, *Rh. annulatus*, *Rh. decoloratus*, *Rh. senegalensis*. Детекция вирусной РНК проводилась в индивидуальных образцах клещей методом ОТ-ПЦР с последующим определением нуклеотидной последовательности и проведением филогенетического анализа.
Результаты и обсуждение. Инфицированность KITV клещей вида *Rh. geigyi* составила 12,2%, *Rh. annulatus* – 4,4%, *Rh. decoloratus* – 3,3%. Однако генетический материал KITV в клещах *Am. variegatum*, являющихся одним из доминирующих видов в Западной Африке, выявлен не был. Для всех изолятов вируса определена частичная нуклеотидная последовательность каждого из четырёх вирусных сегментов (GenBank, OK345271–OK345306), филогенетический анализ которых показал высокий уровень их тождественности (98,5–99,8%) по каждому из четырёх сегментов вирусного генома с ранее обнаруженными в Гвинейской Республике. Полученные изоляты KITV наиболее генетически близки к Mogiana tick virus, который ранее был обнаружен в Южной Америке в клещах *Rh. microplus*, и существенно отличаются от других многокомпонентных вирусов, циркулирующих в странах Европы и Азии, в том числе и в Российской Федерации.
Заключение. Генетический материал KITV обнаружен в трёх видах иксодовых клещей, собранных с домашнего скота ряда префектур Гвинейской Республики. Уровень инфицированности клещей составил 3,3–12,2%. Актуальным остаётся продолжение исследований в данном направлении.

Ключевые слова: *многокомпонентные флавиподобные вирусы; Kindia tick virus; KITV; иксодовые клещи; Гвинейская Республика*

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Introduction

Outbreaks in the past few decades give a new perspective on emerging and re-emerging infectious viral diseases. Quintessential examples of emergency situations representing the danger of such infections can be seen in avian influenza H_5N_1 and Zika fever epidemics as well as in the ongoing pandemic of novel coronavirus infection COVID-19 caused by the SARS-CoV-2 virus. These infectious diseases owe their emergence to the secondary or cross-species transmission of viral agents from their natural hosts to humans [1].

At the same time, some researchers have found that various viruses representing different families build the virus-host relationship, following certain patterns. Representatives of the family Flaviviridae demonstrate multiple examples of such interactions [2–4]. For example, dengue, Zika or yellow fever viruses are transmitted by mosquitoes, the tick-borne encephalitis virus is transmitted by ticks, while the hepatitis C virus is not transmitted by arthropod vectors.

Recently, new flavi-like viruses have been discovered. One of them is the Jingmen tick virus (JMTV) that was first isolated from *Rhipicephalus microplus* ticks collected in Hubei province in China and was named after the area where it was found – the Jingmen Region [5]. Almost at the same time, a similar multicomponent flavivirus, the Guaico Culex virus (GCXV), was detected in mosquitoes of the genus *Culex* in Peru, Panama, and and Trinidad in the Caribbean Sea in the Caribbean Sea [5, 6].

In addition to ectoparasites, genetic material of the JMTV virus (RC27) was also detected in plasma of primates – red colobus monkeys (the genus *Piliocolobus* (Rochebrune, 1877)) living in Uganda as well as in sera collected from cattle and rodents [5–8]. JMTV is believed to be responsible for the outbreaks of febrile illness in China and Kosovo [7, 9, 10]. The above findings led to conclusion that the virus was able to cross the species barrier and effectively replicate both in ectoparasites and in warm-blooded animals.

The later studies of different species of arthropods and vertebrates discovered other multicomponent flavi-like viruses having the genome structure and genetic information transmission mechanism similar to JMTV. For example, the Mogiana tick virus (MGTV) was isolated from salivary glands of the *Rh. microplus* tick in Brazil [11]. Later, the circulation of the previously unknown Alongshan (ALSV) virus was discovered in Northeast China. Shortly after, this pathogen was isolated from *Ixodes ricinus* ticks (Linnaeus, 1758) in the southeast of Finland and in some regions of Russia [12, 13]. Multicomponent flavi-like viruses were also found in Turkey in *Rh. sanguineus sensu lato* (Latreille, 1806), *R. turanicus* (Pomerantsev, 1936), *R. bursa* (Canestrini & Fanzago, 1878), *Hyalomma marginatum* (Koch, 1844), *Haemaphysalis inermis* (Birula, 1895), *Ha. parva* (Neumann, 1897) ticks [14]. The similar multicomponent virus was detected in blood of Crimean-Congo hemorrhagic fever (CCHF) patients in Kosovo and in the south of Russia [15].

The genome structure in all flavi-like viruses is characterized by segmentation and existence of genes encoding two major non-structural viral proteins, which are related to proteins of representatives of the family Flaviviridae (NS3 and NS5); however, multicomponent viruses have genomes with 4 (typical of most of the viruses isolated from ticks, bats, monkeys, and humans) or 5 (as in viruses isolated from mosquitoes) segments, which are flanked at 5′ and 3′ ends by non-translated regions, and are polyadenylated (**Fig. 1**). Segment 1 encodes the non-structural NS5-like protein having homology with the NS5 protein (RNA-dependent RNA polymerase) of classical representatives of the family Flaviviridae. Segment 3 encodes the non-structural protein having high homology with the flavivirus NS3 protein. The NS3 protein, along with NS5, plays the central role in replication of the virus and is one of the best-studied non-structural proteins. The NS3 N-terminal domain has protease activity, which is essential for

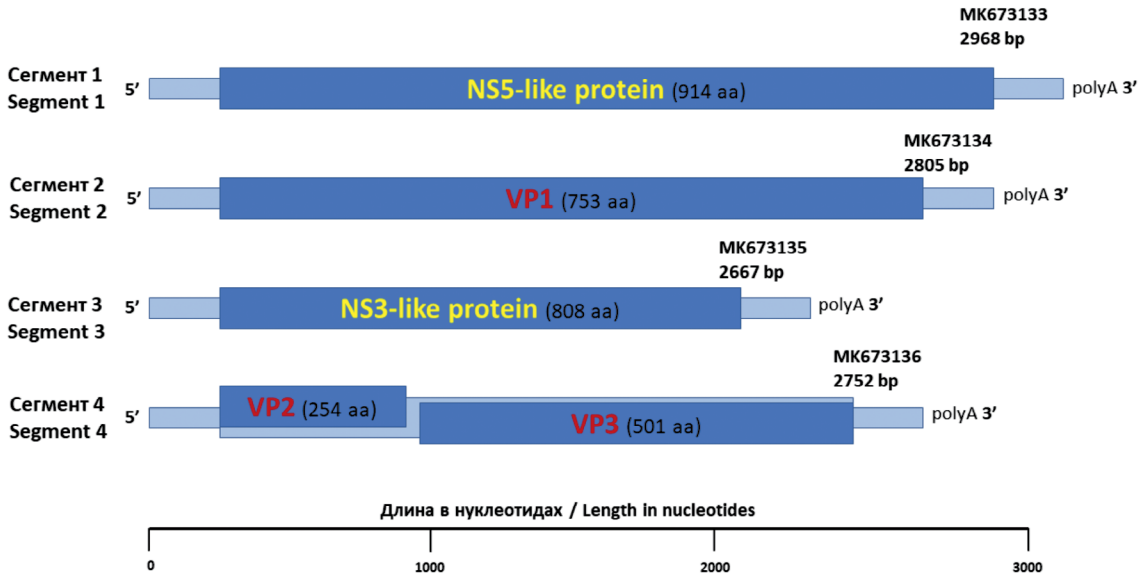

Fig. 1. Scheme of the structure of the genome of multicomponent flavi-like viruses on the example of KITV.
Рис. 1. Схема строения генома многокомпонентных флавиподобных вирусов на примере KITV.

polyprotein processing, while the C-terminal domain performs the function of helicase and is involved in viral RNA capping and synthesis. The structure of the NS3 protein has been thoroughly studied in most of the non-segmented flaviviruses, and their analysis shows high homology not only in the structure, but also in mechanisms of ATP hydrolysis, RNA recognition and unwinding [16]. Structural proteins VP1, VP2, and VP3 are encoded in the second and fourth fragments and do not have any known homologs both among flaviviruses and among other identified viruses. Segment 2 encodes putative glycoprotein VP1. Proteins VP2 (putative capsid protein) and VP3 (putative viral membrane protein) are encoded in segment 4 and have partially overlapping reading frames [14]. Some researchers have conducted a few serological studies based on VP1 and VP2 structural proteins to analyze the circulation of specific antibodies in sera from sheep and cattle, which are feeders of arthropod vectors [17], or from patients with tick bite history [12].

Today, the pathogenicity of multicomponent flavi-like viruses has been proven for livestock animals and humans. However, the available information is fragmentary and limited. It is quite possible that their role in infectious pathology can be more significant than it is commonly believed. The evidence that multicomponent flavi-like viruses are well represented and are detected in Asia, Europe, Africa, and America necessitates further studies of this group of pathogens.

There are grounds to believe that tick-borne flaviviruses originated in Africa from the Kadam-like virus (KADV) approximately 28.5 thousand years ago. Subsequently, they spread in Europe, Asia, and America. There is a strong possibility that African multicomponent flaviviruses such as the Kindia tick virus (KITV) are the most likely candidates for predecessors of modern representatives of this group of pathogens [18].

The multicomponent flavi-like virus tentatively named the Kindia tick virus was first isolated in Guinea during the metagenomic studies; it was isolated from suspensions of *Rh. geigyi* ticks collected from cattle in the Kindia Region [15, 19]. It was found that KITV had the highest homology with MGTV isolated from *Rh. microplus* ticks in South America.

The difference between KITV nucleotide/amino acid sequences and sequences of other representatives of segmented flavi-like viruses was 7–28/3.1–21.8% (for segment 1); 7.7–20.6/3.4–20.3% (for segment 2); 2.3–29.3/1.3–20.8% (for segment 3), and 0.4–22/0.2–15% (for segment 4).

The **purpose** of this study was the further search and molecular-genetic characterization of multicomponent flavi-like viruses in ixodid ticks collected in Guinea.

Materials and methods

The tests were conducted at the laboratory of the Russian-Guinean Center for Epidemiology and Prevention of Infectious Diseases, which is located at the site of the Research Institute of Applied Biology in Kindia, the Republic of Guinea [20].

Ticks were collected from April to May in 2021 during the examination of adult free-grazing livestock animals in Coyah, Dubreka, Forecariah, Kindia, Telimele prefectures in the Kindia Region (**Fig. 2**). The collected and pre-treated material was tested using the polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) with reagent kits manufactured in Russia to detect pathogen markers: AmliSens CCHFV-FL for CCHF virus RNA; AmpliSens TBEV, B. burgdorferi sl, A. phagocytophilum, E. chaffeensis / E. muris-FL for cDNA of the tick-borne encephalitis virus, Borrelia burgdorferi sl, Ehrlichia chaffeensis / E. muris and DNA Anaplasma phagocytophilum; AmpliSens Coxiella burnetii-FL for DNA Coxiella burnetii, the AmpliSens Rickettsia spp. SFG-FL reagent kit

for DNA of the Rickettsia of the tick-borne spotted fever Rickettsia spp. group (Central Research Institute of Epidemiology, Russia) as well as the Gene Francisella tularensis-RGF for Francisella tularensis DNA (Microbe Russian Research Anti-Plague Institute, Russia). Samples without the above pathogens were selected for the further studies.

A total of 324 individual samples of ticks belonging to species *Am. variegatum*, *Rh. geigyi*, *Rh. annulatus*, *Rh. decoloratus,* and *Rh. Senegalensis* were collected, prepared, and analyzed for the study. Ectoparasites were washed twice with 70% ethanol to remove external impurities and microflora; they had been stored at –20 °C before they were used in the further tests. Tick species were identified by morphological characteristics in accordance with the identification guide [21] using a binocular with magnification ×56 and subsequent taxonomic verification of the nucleotide sequence of the ribosomal RNA gene fragment.

The analyzed samples were prepared using the TissueLyser II laboratory homogenizer (QIAGEN, Germany) in 500 µl of sterile phosphate buffered saline. Viral RNA was extracted from 100 µl of tick suspension using phenol/chloroform extraction and the ExtractRNA™ Reagent kit (Evrogen, Russia). cDNA was produced by the reverse transcription reaction using the REVERTA-L commercial kit (AmpliSens, Russia) in accordance with the manufacturer's instructions.

The tested samples were screened for the presence of KITV genetic material using PCR and the pair of KITV1_bF/KITV1_bR primers complementary to segment 1 (the NS5-like gene, segment 1) (**Table 1**). Amplicons partially overlapping all 4 segments of the KITV genome were obtained for positive samples. The amplification reaction was performed in 25 µl of reaction mixture containing 12.5 µl of 2X PCR master mix (BioLabMix, Russia), 0.2 µM of each primer and 1.5 µl of cDNA. The primers used in the study and the expected amplicon length are shown in **Table 1**. RT-PCR was performed in the T100 thermocycler (BioRad, United States) following the amplification protocol: activation of polymerase at 95 °C for 5 min; then 38 successive cycles: denaturation at 95 °C for 15 sec, annealing of primers at 57 °C for 20 sec, elongation at 72 °C for 45 sec, final elongation at 72 °C for 5 min. The reaction products were analyzed using electrophoresis in 2% agarose gel containing ethidium bromide at concentration of 2 µg/ml.

Nucleotide sequences of the PCR fragments were determined with the help of the ABI PRISM® BigDye™ Terminator v3.1 regent kit (ThermoFisher Scientific, United States) and then analyzed using the ABI PRISM 3130 se-

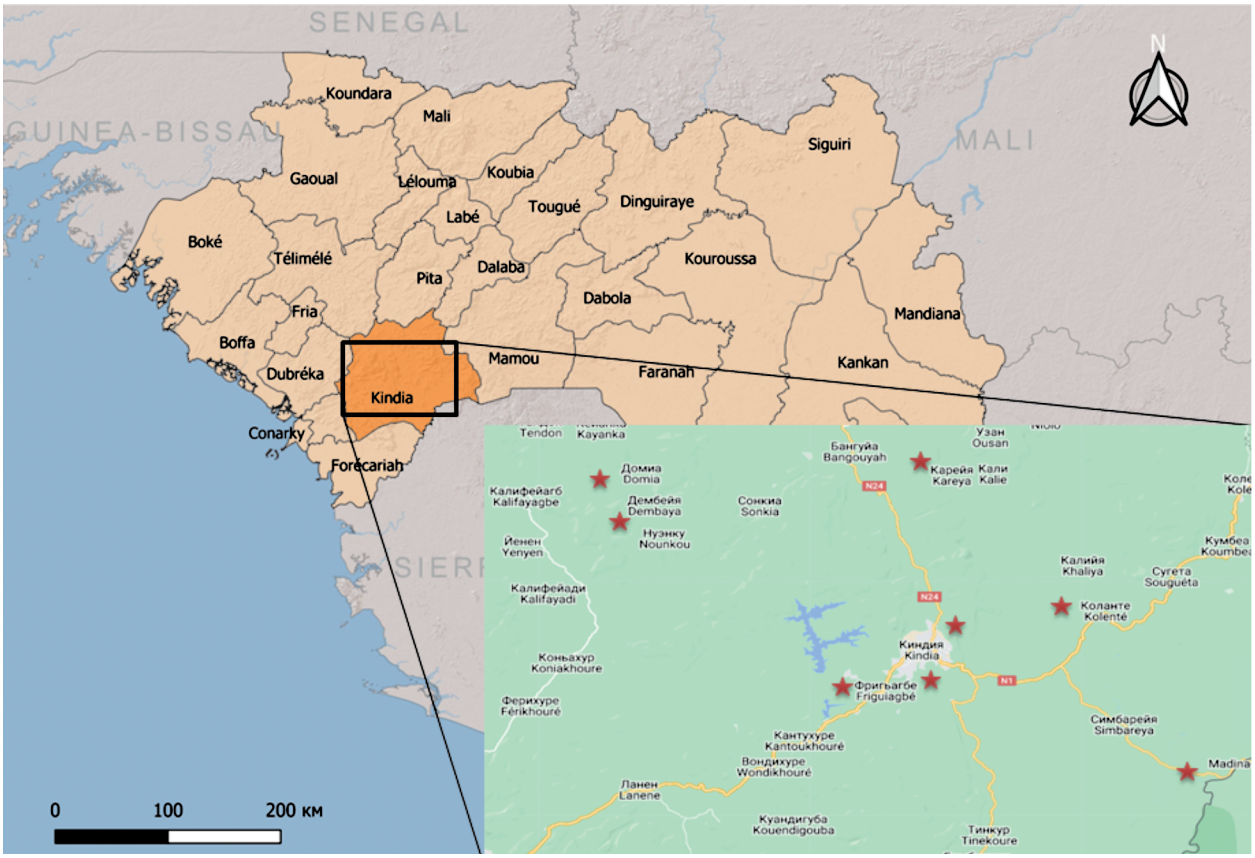

Fig. 2. Territories of Kindia Prefecture (Republic of Guinea) where samples were collected for research. Tick collection sites are marked with asterisks.

Рис. 2. Территории префектуры Киндиа (Гвинейская Республика), где были собраны пробы для исследований. Места сбора клещей обозначены звездочками.

Table 1. Oligonucleotide primer used in the work
Таблица 1. Последовательности олигонуклеотидных праймеров, используемых в работе

Target gene Ген-мишень	Primer Праймер	Primer sequence Структура праймера	T annealing, °C Температура отжига, °C	Amplicon length, bp Длина ампликона, п.н.
Internal transcribed spacer 2	Boophits 2F	GCCGTCGACTCGTTTTGA	57	825
	Boophits 2R	TCCGAACAGTTGCGTGATAAA		
KITV Segment 1 Сегмент 1	KITV1_aF	TGAAGAACGTCAAGCCCTGG	58	602
	KITV1_aR	GCTGACCCACGAACCTGTTT		
	KITV1_bF	AAAGAAGGGCTCTGAGGGC	58	607
	KITV1_bR	CTTATACAGGCCCTGTCCCG		
	KITV1_cF	GAAGTGCGGATGGAGCGTAG	58	619
	KITV1_cR	ACCTGTGGGAGCAGAAGGAT		
KITV Segment 2 Сегмент 2	KITV2_aF	AACTTTGGGAGTGACCAGGG	58	617
	KITV2_aR	GATAAGGCCGTCAGAGCGAG		
	KITV2_bF	CAGGGACGAGACATTGCCAA	58	555
	KITV2_bR	CCGTGGAGTAGTGGACCGTA		
KITV Segment 3 Сегмент 3	KITV3_aF	AATTGGAGAGGCAGAGGGGA	58	609
	KITV3_aR	GACCTTGTTGGACCAGGTCA		
	KITV3_bF	GGCAACTCATGACCTGGTCC		
	KITV3_bR	AGGACCACTGTGGCGTAGT	58	558
KITV Segment 4 Сегмент 4	KITV4_aF	CCCTACCAGGCCTGATACGA		
	KITV4_aR	TAGTAGCGGGCCAGGTTGTA	58	615
	KITV4_bF	GCGGAGAGAGAGAAAACGCA		
	KITV4_bR	ACAGGTTCACGAACACAGCC	58	617

Table 2. Detection of KITV RNA among different species of ticks by RT-PCR
Таблица 2. Выявление РНК KITV среди разных видов иксодовых клещей методом ОТ-ПЦР

Species of ticks Вид клеща	Number of samples (of which positive) Количество экземпляров (из них положительных)	Prevalence of KITV Встречаемость KITV
Am. variegatum	197 (0)	0% (95% CI 0–1.9)
Rh. geigyi	49 (6)	12.2% (95% CI 5.7–24.2)
Rh. annulatus	45 (2)	4.4% (95% CI 1.2–14.8)
Rh. decoloratus	30 (1)	3.3% (95% CI 0.7–16.1)
Rh. senegalensis	3 (0)	0% (95% CI 0–46.2)
Всего Total	324 (9)	2.8% (95% CI 1.5–5.1)

quencer (Applied Biosystems, United States). The obtained chromatograms were analyzed using the SeqMan program (DNAstar, United States).

The phylogenetic analysis was conducted using Mega X software, the maximum likelihood method and the Tamura 1992 (T92) three-parameter evolutionary model [22]. Statistical reliability of nodes of the phylogenetic tree was assessed with the bootstrap method for 1000 repetitions.

The partial nucleotide sequences of segments of KITV isolates detected in this study were deposited to GenBank database with accession numbers OK345271–OK345279 – for segment 1, OK345280 – OK345288 – for segment 2, OK345289–OK345297 – for segment 3, OK345298–OK345306 – for segment 4.

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).

Results and discussion

During the study, KITV RNA was detected in 9 samples. Partial nucleotide sequences of each segment were identified for all isolates: segment 1 – 1609 base pairs (bp), segment 2 – 980 bp, segment 3 – 1114 bp and segment 4 – 1116 bp. It was found that differences in nucleotide and amino acid sequences of KITV compared to other representatives of segmented flavi-like viruses were as follows: for segment 1 – from 7 to 28% and from 3.1 to 21.8%; for segment 2 – from 7.7 to 20.6% and from 3.4 to 20.3%; for segment 3 – from 2.3 to 29.3% and from 1.3 to 20.8% and for segment 4 – from 0.4 to 22% and from 0.2 to 15%, respectively (**Table 2**).

The homology of nucleotide sequences isolated in 2021 for segment 1 was 98.5–100%; for segment 2 – 99.6–100%; for segments 3 and 4 – 99.5–100%. When compared with the KITV/2017/1 isolate obtained in 2017, the homology level was 98.7–99.2%; compared to other multicomponent flavi-like viruses, it ranged from 72 to 90% for fragment 1; from 60 to 90% – for fragment 2; from 73 to 90% – for fragment 3 and from 61 to 90% – for fragment 4.

Fig. 3 shows that all the sequences isolated in 2021 are clustered into one group together with the KITV/2017/1 isolate [18]. It is noteworthy that the phylogenetic trees of the detected isolates of KITV and other multicomponent viruses, which were constructed for each segment, have almost identical topology. In 2021, KITV was found in three species of ixodid ticks, but the sequences were quite con-

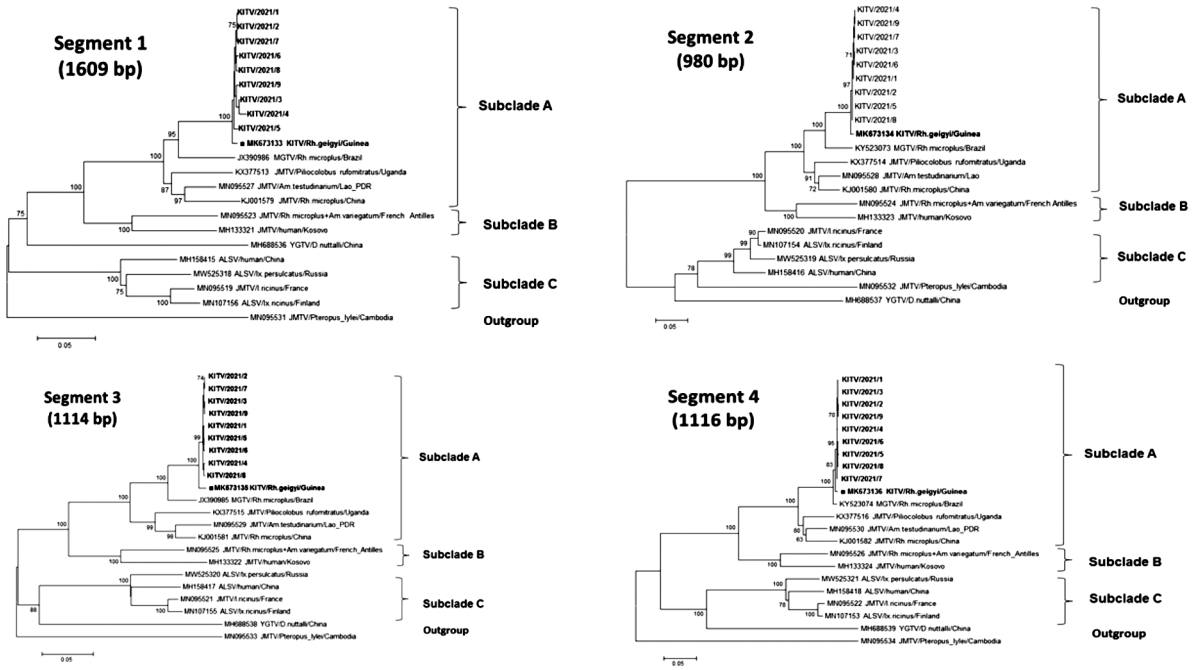

Fig. 3. Phylogenetic analysis of KITV isolates from individual ixodid ticks for all four genome segments.
Рис. 3. Филогенетический анализ изолятов KITV из индивидуальных иксодовых клещей для всех четырёх сегментов генома.

served, and no significant differences associated with the taxonomic difference among ectoparasites were found.

Some researchers have reported that multicomponent flavi-like viruses are subdivided into at least three subclades [23]. The analysis of each of the four genome segments shows that the KITV virus and its identified isolates are explicitly clustered into subclade A that also includes MGTV (Brazil), JMTV (China, Asia) and JMTV/primate/Uganda variant (Africa). Subclade B includes JMTV isolates from the Antilles and JMTV/human/Kosovo variants (MH133313–MH133316) isolated from CCHF patients. Subclade C is comprised of JMTV isolates from France (MN095527–MN095530) and the Alongshan virus detected in domestic animals and humans in China and during later years in Europe and Russia.

It can be assumed that redistribution/recombination events are typical of viruses with a multicomponent genome, contributing to their genetic variation. However, the available data suggest that the JMTV genome is exceptionally stable among vertebrates and invertebrates, thus leading to the conclusion that the virus has been well adapted to both hosts. Nucleotide sequences of different segments of representatives of this group demonstrate a high level of homology among geographically distanced viruses circulating in Europe, Asia, Africa, and South America. The genome analysis does not provide the possibility to identify apparent mutations, which could capture the adaptation of the virus to the vertebrate or invertebrate host. The genome conservation may be associated with the long-range spread of the same virus. The clear picture of such wide scale spread of the virus can be obtained through studies of the role played by migratory birds, rodents and, possibly, bats.

Conclusion

The study has found that the Kindia tick virus is the first segmented flavi-like virus detected in West Africa. The KITV genetic material was obtained from *Rh. geigyi*, *Rh. annulatus* and *Rh. decoloratus* ixodid ticks collected from domestic livestock; the infection rate was 3.3–12.2%. The phylogenetic analysis of four fragments KITV showed a high level of homology (98.5–100%) with the previously detected isolates isolated in Guinea in 2017 and made it possible to cluster them into subclade A of JMTV-like viruses.

Thus, the obtained results demonstrate the importance of further studies of the KITV circulation in West Africa, identification of species of reservoirs/vectors and assessment of the pathogenicity for humans and animals.

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