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GENOME VARIATIONS OF *PUUMALA* VIRUS STRAINS CIRCULATING IN NIZHNEKAMSKY AND TUKAEVSKY DISTRICTS OF THE REPUBLIC OF TATARSTAN

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Abstract

Puumala virus (PUUV), which causes hemorrhagic fever with renal syndrome (HFRS), is endemic in Russia, including the Republic of Tatarstan (RT). Although hundreds of HFRS cases are recorded in RT annually, little is known about the genetic diversity of PUUV in Tatarstan. For this study, partial PUUV sequences of the S and M genome segments were obtained from bank vole populations in Tukayevsky and Nizhnekamsky districts. The genetic diversity among newly identified PUUV isolates was in the range of 0.0–3.3% for both S and M genome segments, with these isolates displaying from 5.1% to 7.8% of nucleotide sequence differences from the strains previously identified in Russia. All the recovered PUUV genome sequences phylogenetically clustered with the known PUUV strains of the previously defined Russian (RUS) genetic lineage and clearly differed from other strains circulating in the RT. It was found that the genome composition of PUUV/Nizhnekamsky/MG_134/2015 strain is likely to be a result of the intra-lineage recombination or genetic reassortment of two distinct PUUV strains.

Keywords: *Puumala* virus (PUUV) genome, *Hantaviruses*, *Bunyaviridae*, hemorrhagic fever with renal syndrome (HFRS), S segment, M segment, recombination, reassortment

Introduction

Hemorrhagic fever with renal syndrome (HFRS) is endemic in Eurasia, including the European part of Russia where the Republic of Tatarstan (RT) is located. In most cases, *Puumala* virus (PUUV) causes a mild form of a disease, which is often referred to as *nephropathia epidemica* (NE) in Europe [1, 2]. When the PUUV infection progresses in humans, it triggers an acute onset with flu-like symptoms. Later, patients display symptoms of impaired renal function, and disturbed hemodynamics develops in many cases [1, 3, 4]. Most of the patients fully recover with no post-morbid complications [1]. Of all human infectious diseases occurring in the territory of Russia, 47.3% are zoonotic infections. HFRS cases represent 90% of zoonotic infections, with the mortality rate of 0.1–0.4% [5]. In the European part of Russia, 83.3% of HFRS cases are registered in the Volga Federal District (VFD) [5, 6]. Among the most affected regions in the VFD, the RT follows Udmurtia, Republic of Bashkortostan and Mordovia [7]. Although several investigations have been conducted to map the distribution and genetic diversity of PUUV in Tatarstan, there is still more to be done to fully describe variations of the PUUV strains circulating in the RT.

PUUV is a leading cause of HRFS in the RT. This zoonotic agent belongs to the genus *Hantavirus*, family *Bunyaviridae* [8]. In nature, PUUV is known to cause lifelong asymptomatic infection in its main natural host, the bank vole (*Myodes glareolus*) [9]. The PUUV virus is transmitted to humans by inhalation of aerosolized contaminated urine and feces [10].

The RNA genome of PUUV is tri-segmented of the negative polarity [11]. The three segments are arranged according to their size and code for specific proteins: the small (S) segment codes for the nucleocapsid protein (N protein); the medium (M) segment codes for the surface glycoproteins G1 and G2 (Gn and Gc, produced by cleavage of the glycoprotein precursor, GPC); the large (L) codes for the RNA-dependent RNA-polymerase (RdRp) [10, 12].

Currently, eight PUUV genetic lineages have been discovered to circulate in their reservoir rodent populations and infect humans worldwide [10]. The latest PUUV genetic lineage has been detected in Latvia (LAT) and found to be co-circulating with the Russian (RUS) PUUV genetic lineage [13]. Generally, the genetic diversity of the virus lineages is structured geographically [10, 17]. Depending on the location, the genetic diversity of the local virus strains usually varies between 0 and 10%, reaching 15% in larger areas.

The goal of the current study was to look into the genetic variations of the PUUV strains circulating in bank vole populations of two RT districts, Tukayevsky and Nizhnekamsky, both situated in the east-central part of the RT. The partial nucleotide sequences of the PUUV S and M genome segments (566 and 1014 nucleotides, respectively) from the bank voles captured in 2015 were determined. These sequences were compared to the known PUUV strains from the Russian Federation (RF) regions Samara, Udmurtia and Republic of Bashkortostan as well as few other selected PUUV strains that belong to different genetic lineages. The S segment of RT PUUV strains detected in the course of our previous work was also included in the comparison [7].

1. Materials and Methods

Frozen rodent lung tissue samples were obtained from the Center for Hygiene and Epidemiology in the Republic of Tatarstan (Kazan, Russia). Data on the location of the trapping sites are given in Fig. 1. Total RNA was extracted from lung tissues using Trizol Reagent (Invitrogen, USA) following the manufacturer's protocol. The concentrations of RNA were measured using a NanoDrop 2000 UV-vis Spectrophotometer (Thermo Fisher Scientific, USA) as specified by the manufacturer.

For cDNA synthesis, RevertAid Reverse Transcriptase (Thermo Fisher Scientific, USA) was used. The PCR amplification of the partial S and M segment sequences (723- and 1132-bp fragments, respectively) was performed as described in our previous report using the same techniques as for the M segment [14]. The resulting amplicons were separated with 1.0% gel electrophoresis. The sequencing was carried out using ABI PRISM 310 and Big Dye Terminator 3.1 Sequencing Kit (ABI, USA). The primers used for RT-PCR and sequencing are summarized in Table 1.

Fig. 1. The location of rodent trapping sites

Table 1

Primers used for RT-PCR amplifications and sequencing

Genome segments	Primer designations	Primer sequences, 5' → 3'
S	PuuV-For*	CTGCAAGCCAGGCAACAAACAGTGTCAGCA
	PuuV-Rev*	TCTGCCACATGATTTTTGTCAAGCACATC
M	F1452PUUVM	TCTTTAATCCCAGGAGTTGC
	R2582PUUVM	AAATTGTCCCTATTAAACACAC

*Primers were previously described in [14].

The DNASTAR software (Lasergene package) [15] and Mega v7.0 [16] were used for sequences comparison and for phylogenetic analysis by the maximum parsimony method. For comparison, strains PUUV/Kazan/MG_003/2014, PUUV/Kazan/MG_004/2014, PUUV/Kazan/MG_032/2014, and PUUV/Kazan/MG_037/2014 (our previous data described in [7]), known PUUV strains from Samara, Udmurtia, and the Republic of Bashkortostan, and other PUUV strains from some genetic lineages were used. The sequence accession numbers for the partial S segment tree are as follows: AB433843 (Samara_49/CG/2005), Z84204 (Puu/Kazan), Z21497 (Udmurtia/894Cg/91), M32750 (CG1820), AB297665 (DTK/Ufa-97), AF442613 (CG17/Bashkiria-2001), JN657230 (PUUV/Jelgava/ Mg136/2008), JN657229 (PUUV/Madona/Mg99/2008), JQ319168 (PUUV/Konnevesi/Mg_O22B/2005), JN831950 (PUUV/Pieksamaki/human_kidney/2008), HE801633 (Sotkamo 2009), AF367071 (CRF366_Omsk), KP292966 (Kuchuk170/ Mg/2007), AJ238790 (Gomselga), AJ238789 (Kolodozero), GQ339485 (Mangelbo/Mg1/05), GQ339487 (Munga/Mg16/05), AY526219 (Umea/hu), KT247596 (PUUV/Jura/Mg2/2010), KT247595 (PUUV/Orleans/Mg29/2010), and KJ994776 (Mu/07/1219_Lower Saxony) strains. The accession numbers of the partial M segment tree are as follows: Z84205 (Puu/Kazan), AB433850 (Samara_49/CG/2005), L08754 (K27_Bashkiria), AB297666 (DTK/Ufa-97), M29979 (CG1820), JQ319174 (PUUV/Konnevesi/Mg_M78B/2005), JN831948 (PUUV/Pieksamaki/human_lung/2008), HE801634 (Sotkamo 2009), U14136 (Vranica), KT247603 (PUUV/Ardennes/Mg156/2011), KT247599 (PUUV/Jura/Mg214/2010), KT247601 (PUUV/Orleans/Mg29/2010), and

KJ994777 (Mu/07/1219_Astrup) strains. The corresponding *Tula* virus sequence, accession number EU439951 and NC_005228 for the partial S and M segments, respectively, was used as an outgroup.

2. Results and Discussion

Overall, 14 and 10 bank voles were trapped in 2015 in the forests located in Nizhnekamsky and Tukayevsky districts, respectively. The PUUV RNA was detected in five and three bank voles captured at sites S1, S2, S3, S4, and S5, all located in the east-central part of the RT. Thus, the infection rate in the corresponding populations of bank voles was 33.3%. The PUUV identity was confirmed by sequencing all the detected eight strains and comparing them to the known selected PUUV strains retrieved from the NCBI GenBank database. The number and designations of the sequenced samples are shown in Table 2.

Table 2

The number of nucleotide sequences obtained per site and designation of samples

Specific district and pool designation	Trapping sites	Sequences obtained	Sample designations
Nizhnekamsky (NIZ pool)	S1	1	PUUV/Nizhnekamsky/MG_134/2015
	S2	1	PUUV/Nizhnekamsky/MG_137/2015
	S3	1	PUUV/Nizhnekamsky/MG_139/2015
	S4	2	PUUV/Nizhnekamsky/MG_154/2015* PUUV/Nizhnekamsky/MG_158/2015
Tukaevsky (TUK pool)	S5	3	PUUV/Tukaevsky/MG_260/2015 PUUV/Tukaevsky/MG_262/2015 PUUV/Tukaevsky/MG_265/2015

* The M segment sequence was not retrieved from this sample.

The comparative analysis of the partial S segment sequences (566 bp long, nucleotide positions from 240 to 805) revealed that the virus strains obtained from the bank voles within the sites S4 and S5 were shown by low divergence (0.4% and 0.0%, respectively). The sequence divergence between the PUUV strains from different sites (S1, S2, S3, and S4) detected in Nizhnekamsky district was higher, ranging from 0.4% to 2.5%. The sequences of the PUUV strains from Nizhnekamsky and Tukaevsky districts differed by 2.2–3.3%. The low divergence observed between the RT PUUV strains suggested that these strains belong to the same genetic lineage. These results agree with the observations made in Belgium, in which the low divergence was detected in PUUV nucleotide sequences obtained within the same locality [17]. The comparison of the sequences obtained demonstrated 5.1–7.8% of divergence from the RUS genetic lineage (Kazan, Udmurtia, and Samara strains). In addition, the PUUV sequences obtained in the current study displayed 15.1–20.6% of nucleotide differences from the strains belonging to the FIN, LAT, CE, and other lineages (Table 3). Previously, the data obtained in northern Finland and Central Europe showed the PUUV genetic diversity between strains belonging to the same lineage to be 0.2–4.9%, while the between-lineage diversity was observed to be 15.3–16.6% [11, 12]. Therefore, one can conclude that all partial PUUV S segments detected in the current study are closely related to the RUS genetic lineage.

Table 3

The partial PUUV S segment nucleotide diversity between the samples investigated and the strains from GenBank

Strain	Kazan	Udmurtia	Samara	Baskiria-2001	DTK_Ufa-97	Kazan_003	Kazan_004	Kazan_032	Kazan_037	Sotkamo 2009	Konnevesi	Jelgava	Orleans	Umea
134	5.4	6.2	5.8	5.6	5.8	7.9	5.0	15.9	7.5	16.1	15.2	15.1	18.8	16.0
137	6.5	7.4	5.9	6.8	7.0	9.0	6.0	15.9	8.6	16.1	15.2	15.1	20.3	17.2
139	6.9	7.8	6.5	7.2	7.4	9.2	6.2	15.4	8.8	15.6	15.2	15.8	19.6	15.8
154	5.5	7.4	5.9	6.8	7.0	9.0	6.0	15.9	8.6	16.1	15.2	15.1	20.3	17.2
158	5.1	7.0	5.9	6.4	6.6	8.6	5.6	16.3	8.2	16.5	15.2	15.3	20.6	17.2
260 262 265	5.8	7.0	6.1	6.3	6.5	8.7	5.8	16.8	8.3	16.7	15.9	17.0	19.0	16.4

The analysis of the partial S segment nucleotide sequence isolated from eight bank voles revealed 25 point mutations. The most frequent ones were T-C substitutions (36%). Twenty-two nucleotide substitutions were in the third position of the corresponding codons and did not lead to changes in the deduced amino acid sequences. This may be a result of the strong negative selection operating at the N protein level [7]. In addition, the strains PUUV/Tukaevsky/MG_260/2015, PUUV/Tukaevsky/MG_262/2015, and PUUV/Tukaevsky/MG_265/2015 had one nucleotide substitution found in the third position of the codon (A/G756T) that led to the amino acid substitution of glutamic acid for aspartic acid (Glu238Asp) in the deduced N protein sequences. Two remaining nucleotide point mutations found in the first position of codons, C454T and C463T, were silent.

The comparison of the deduced amino acid sequences showed that the sequences of all five strains from Nizhnekamsky district were identical to the known Samara strain (Fig. 2). The sequences of the strains from Tukaevsky district had a specific amino acid marker (Asp) in position 238, which was previously found in the Kazan and Udmurtia strains. These findings suggest that the three strains are more closely related to the Kazan and Udmurtia strains than strains circulating in Nizhnekamsky district. Thus, we found two variants of the N protein amino acid sequences in the investigated strains.

The nucleotide sequence comparisons of the partial M segment (1014 bp long, nucleotide positions from 1499 to 2512) of the PUUV strains investigated demonstrated low nucleotide sequence divergence (from 0.1% to 0.3%) among the samples obtained from the site S5. Compared to the S segment, the M segment sequences showed a similar level of nucleotide diversity, with the low values of 0.0% to 2.5% among the strains isolated from bank voles captured at sites S1, S2, S3, and S4 in Nizhnekamsky district. The sequence divergence between the strains from Nizhnekamsky and Tukaevsky districts varied from 2.1% to 2.4%. Likewise, the sequences obtained showed 6.8–7.6% of nucleotide differences when compared to the Kazan and Samara PUUV strains of the RUS genetic lineage (Table 4).

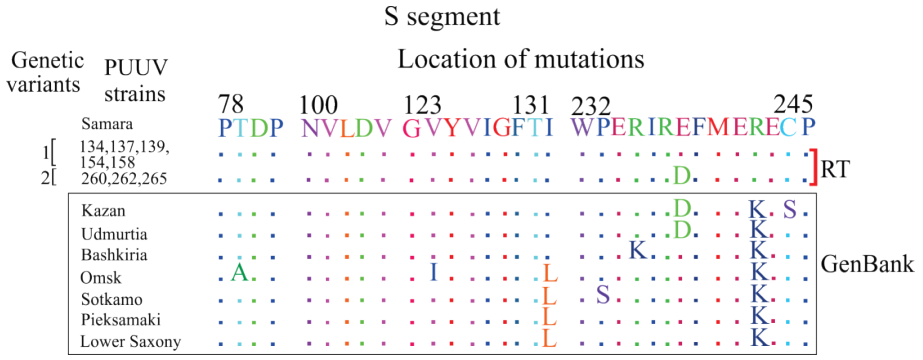

Fig. 2. Amino acid sequence alignment of the PUUV partial N protein between the PUUV strains investigated and the selected sequences from GenBank

Table 4

The partial PUUV M segment nucleotide diversity between the samples investigated and the strains from GenBank

Strain	Kazan	Samara	K27_Baskiria	DTK_Ufa-97	Sotkamo 2009	Konnevesi	Orleans	Jura	Ardennes	Astrup	Vranica
134	6.8	7.4	15.5	15.5	18.9	18.9	24.3	23.2	21.1	24.1	24.9
137	7.4	7.4	14.9	14.9	16.4	17.6	24.3	23.0	20.1	23.7	23.4
139	7.6	7.4	15.0	15.0	16.8	17.5	24.8	24.8	20.5	24.3	24.5
158	7.4	7.4	14.9	14.9	16.4	17.6	24.3	23.0	20.1	23.7	23.4
260 262 265	6.8	7.1	14.8	14.8	17.7	18.3	24.5	23.4	19.7	23.7	24.0

The PUUV sequences obtained displayed a higher divergence from the PUUV sequences of the FIN, LAT, CE and other lineages, with the difference varying from 16.4% to 24.8%. This data showed that the M segment nucleotide sequences of the PUUV strains obtained in the current study are closely related to the sequences of the PUUV strains of the RUS genetic lineage. The results are consistent with our previous observations and with the earlier results described by others, specifically, observations made in Central Europe where the divergence for the M segment reached 18.7–19.9% [7, 11]. Therefore, we postulate that the sequences of the M segment belong to the RUS genetic lineage circulating in the east-central part of the RT.

The sequence analysis of the partial M-segment coding region (1014 bp long) revealed 58 point mutations that were observed consistently in the investigated strains when compared to the Samara and Kazan strains. The most common mutations were transition T-C substitutions (25.8%). As expected, 48 mutations occupied the third position of the codon and were silent. In addition to these mutations, the strains PUUV/Nizhnekamsky/MG_137/2015, PUUV/Nizhnekamsky/MG_139/2015, and PUUV/Nizhnekamsky/MG_158/2015 carried a specific mutation C2415T located in the second position of the codon (GTC), which led to the substitution of alanine for valine (Ala792Val) (Fig. 3). This amino acid substitution was found to be similar

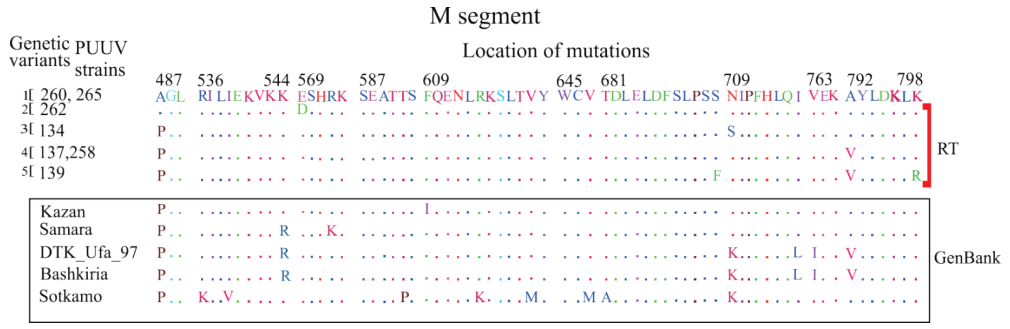

Fig. 3. Amino acid sequence alignment of the PUUV partial glycoprotein precursor between the PUUV strains investigated and selected sequences from GenBank

to the substitution observed in the strains DTK_Ufa-97 and K27_Baskiria at the same location. However, the latter mutation was generated by a different codon (GTA) and does not represent an evidence for direct genetic relation between these strains.

Additionally, the sequence PUUV/Nizhnekamsky/MG_134/2015 differed from the remaining three sequences by having a unique nucleotide substitution positioned in the second position of codon (A2169G); this mutation led to the substitution of the amino acid an asparagine for a serine (Asn670Ser). Furthermore, the deduced protein sequence of PUUV/Tukaevsky/MG_262/2015 contained one amino acid, which did not match the corresponding sequences of the remaining two strains from the TUK pool.

The phylogenetic trees inferred from the PUUV partial S segment sequences and the partial M segment demonstrated similar topologies (Fig. 4 and Fig. 5, respectively). The strains identified in the current study formed two subclades, the NIZ and the TUK, corresponding to the geographic locations of the bank vole trapping sites in the RT districts. These strains clustered with the PUUV strains previously detected in the VFD, which belong to the RUS genetic lineage and formed a separate branch together with the previously identified PUUV strains PUUV/Kazan/MG_003/2014, PUUV/Kazan/MG_004/2014, PUUV/Kazan/MG_032/2014, and PUUV/Kazan/MG_037/2014.

It is worth mentioning that the S-segment nucleotide sequence of the strain PUUV/Nizhnekamsky/MG_134/2015 appropriately clustered with the NIZ subclade sequences while the M segment sequence of this strain fell into the TUK subclade. It is suggested that, at least in some cases, the PUUV genome may be comprised of several components that have different origins. Such a phenomenon is usually attributed to either whole genome segment reassortment or RNA recombination. Previously, the existence of PUUV genetic reassortant was detected, which included segments from the N-SCA and FIN genetic lineages [8]. In other studies, it was revealed that some reassortant genomes consisted of the segments derived from several genetic variants of the FIN lineage [9]. In the current study, the genome of the strain PUUV/Nizhnekamsky/MG_134/2015 possibly contained parts of the S and M segments that originated from the genetically distinct PUUV strains belonging to the same genetic lineage, thus, supporting the hypothesis about possible recombination or reassortment.

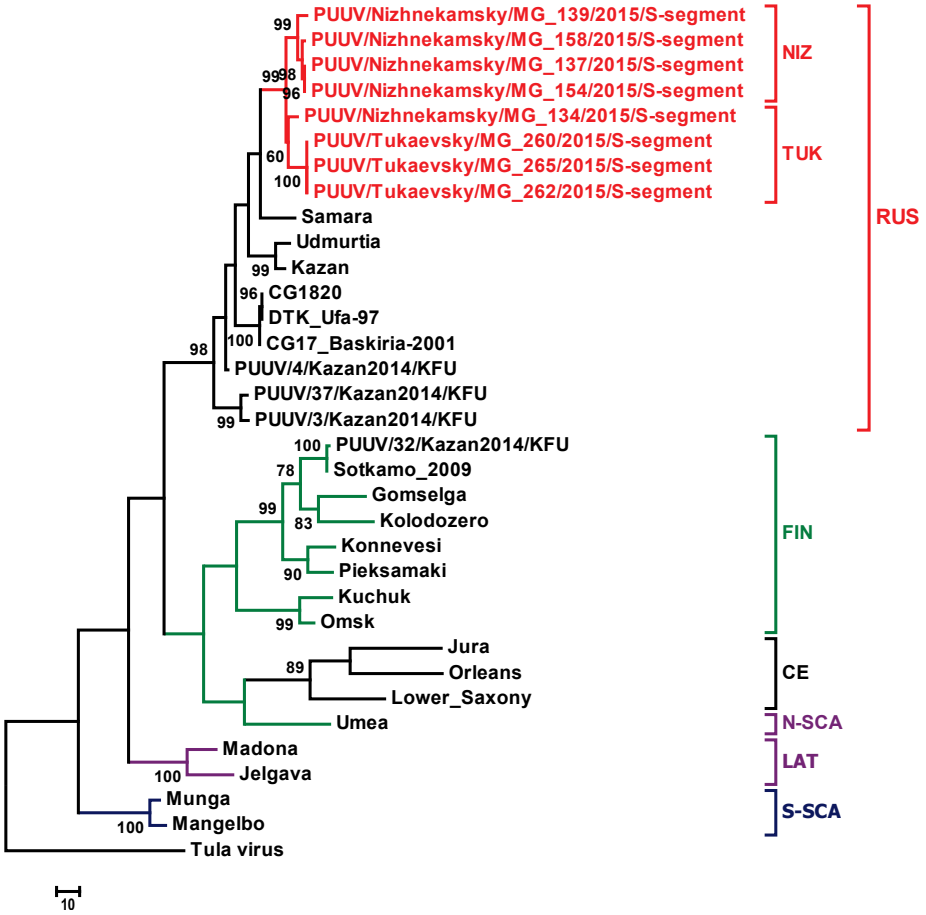

Fig. 4. Phylogenetic tree based on the partial S segment sequences of the PUUV strains from the RT (shown in red) and the PUUV strains Samara, Kazan, DTK_Ufa-97, CG1820, CG17_Baskiria-2001 and our previously described strains (PUUV/3/Kazan2014/KFU, PUUV/4/Kazan2014/KFU, PUUV/32/Kazan2014/KFU and PUUV/37/Kazan2014/KFU) from the RUS genetic lineage. A few selected strains from FIN, CE, N-SCA, LAT, and S-SCA genetic lineages were also included in the study. The corresponding sequence of the *Tula* virus (TULV) was used as an outgroup. The bootstrap values of the maximum parsimony analysis are given above the branches and only values higher than 60% are shown

The S and M segment nucleotide sequences divergence between the PUUV/Nizhnekamsky/MG_134/2015 and other strains isolated in the current study reached 2.2–2.5% and 2.2–2.5%, respectively. As mentioned earlier, all the observed nucleotide substitutions in the S segment of the strain PUUV/Nizhnekamsky/MG_134/2015 were silent and its deduced amino acid sequence was identical to both sequences of the other strains from Nizhnekamsky district and the Samara strain. Unlike the S segment, the deduced amino acid sequences of the M segment of the strain PUUV/Nizhnekamsky/MG_134/2015 differed from all the strains investigated. However, all the PUUV strains identified shared one common amino acid substitution, where alanine was substituted for proline (A487P) within the most variable region of the N protein ORF with respect to the sequences of the M segment for all

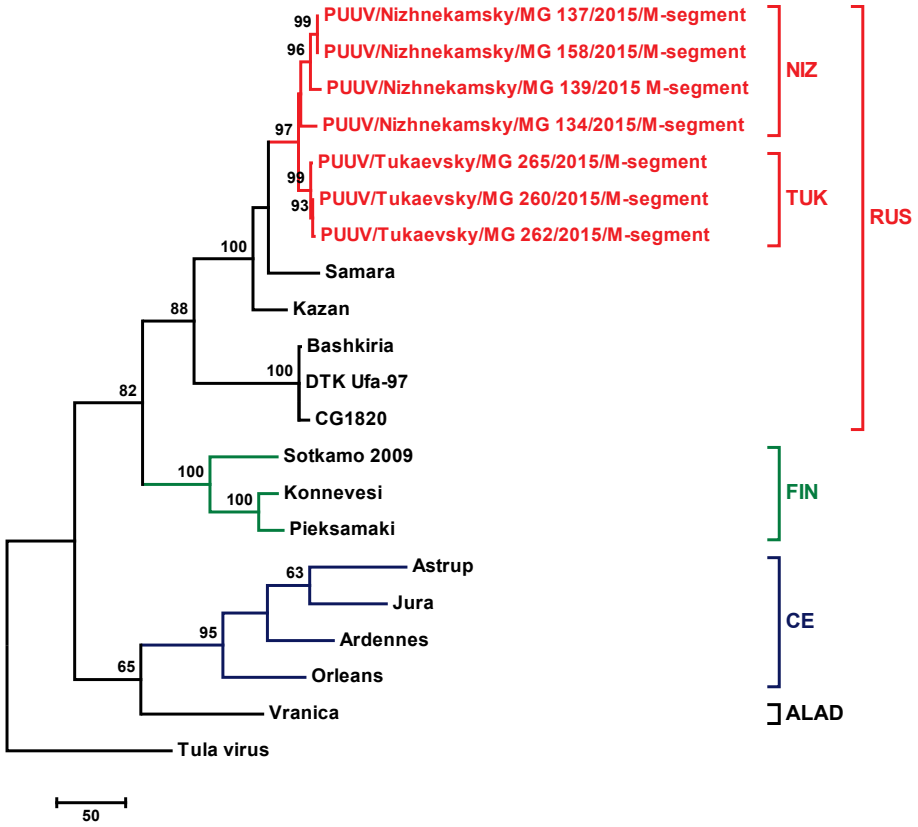

Fig. 5. Phylogenetic tree based on the partial M segment sequences of the PUUV strains from RT (shown in red) and the PUUV strains Samara, Kazan, DTK_Ufa-97, CG1820, K27_Baskiria from the RUS genetic lineage, and a few selected strains from the FIN, CE, and ALAD genetic lineages. The corresponding sequence of TULV was used as an outgroup. The bootstrap values of the maximum parsimony analysis are given above the branches and only values higher than 60% are shown

the PUUV strains from the NIZ pool (Fig. 3). On the other hand, the sequences of the strains PUUV/Nizhnekamsky/MG_137/2015, PUUV/ Nizhnekamsky/MG_139/2015, and PUUV/Nizhnekamsky/MG_158/2015 differed from those of the strains from the TUK pools, Samara and Kazan strains, and one strain from the NIZ pool in that they had a unique nucleotide substitution located in the second position of the codon C2415T. This mutation led to the amino acid change from valine to alanine (V792A) (Fig. 3).

Furthermore, we found two variants of the deduced N protein amino acid sequence among the eight PUUV strains and five variants of the M segment among the seven strains isolated from the bank vole population in the east-central part of the RT. Considering these data, the observed mutations in the strain PUUV/Nizhnekamsky/MG_134/2015 are of great interest. Whether these mutations are a result of the recombination or reassortment remains to be investigated.

In the previous study [14], we analyzed the partial S segment sequences of the PUUV strains isolated from the HFRS patients in the RT (Kazan) and the Republic

of Mordovia (Saransk). The comparison of the PUUV isolates (the RUS genetic lineage) from the patients and the corresponding partial S segment sequences of the current NIZ and TUK pools revealed a higher genetic divergence ranging from 4.1% to 12.7% (data not shown). This suggests that the PUUV strains mentioned above are not closely related; they are likely to belong to different genetic variants of the PUUV RUS lineage. The RT is situated around the confluence of two large rivers, Volga and Kama, and is divided traditionally into the Pre-Volga, Pre-Kama, and Trans-Kama areas. According to the information obtained from the patients in our previous study, the infections occurred in the RT regions around Kazan, i.e., in the Pre-Kama area. The PUUV strains described in the current investigation were isolated in the Trans-Kama area and seem to diverge significantly from the PUUV isolates detected in humans in the Pre-Kama.

It is reasonable to assume that the Kama River acted as a natural barrier for bank vole migrations. Consequently, the rodent populations currently occupying opposite banks of the river formed as a result of different migration routes and the corresponding PUUV strains circulating in these populations were of different origins. To confirm this suggestion, further investigations will need to focus on the search for PUUV strains circulating in bank vole populations in the Pre-Kama and Trans-Kama areas and their genome characterization.

Conclusions

Our data revealed that the PUUV strains detected in the bank vole populations in the Nizhnekamsky and Tukaevsky districts of the RT belong to the same genetic lineage, the RUS lineage. Based on the partial S and M sequences, the analysis of genetic diversity showed geographical clustering of the PUUV genetic variants. The PUUV strains obtained were not closely related to the strains previously isolated from HFRS patients in Pre-Kama area. Interestingly, one PUUV isolate, PUUV/Nizhnekamsky/MG_134/2015, displayed the S segment sequence that clustered with the Tukaevsky subclade while the M segment sequence fell into the Nizhnekamsky subclade, indicating a possible recombinant or reassortant origin of this strain.

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Вариабельность генома штаммов вируса *Puumala*, циркулирующих в Нижнекамском и Тукаевском районах Республики Татарстан

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Аннотация

Вирус *Puumala* (PUUV), является основным возбудителем геморрагической лихорадки с почечным синдромом (ГЛПС) как в целом в России, так и в Республике Татарстан (РТ). В РТ ежегодно регистрируется несколько сотен случаев заболевания ГЛПС, но на данный момент о генетическом разнообразии PUUV в республике известно мало. Для штаммов PUUV, выявленных в популяциях рыжей полёвки в Тукаевском и Нижнекамском районах, получены последовательности участков S- и M-сегментов генома. Дивергенция нуклеотидных последовательностей S- и M-сегментов между выявленными штаммами составила 0.0–3.3%, а по сравнению со штаммами, ранее идентифицированными в России, – 5.1–7.8%. На филогенетическом дереве все выявленные штаммы находятся в кладе генетической линии RUS отдельно от других штаммов PUUV, циркулирующих в РТ. Геном штамма PUUV/Nizhnekamsky/MG_134/2015, вероятно, является результатом внутрилинейной рекомбинации или реассортации между двумя штаммами PUUV.

Ключевые слова: геном вируса *Puumala* (PUUV), *Hantaviruses*, *Bunyaviridae*, геморрагическая лихорадка с почечным синдромом (ГЛПС), S- сегмент, M-сегмент, рекомбинация, реассортация

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