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Genetic heterogeneity of Russian, Estonian and Finnish field rabies viruses

A. E. Metlin[1,2,3], **S. Rybakov**[2], **K. Gruzdev**[2], **E. Neuvonen**[1], **A. Huovilainen**[1]

[1] Finnish Food Safety Authority, EVIRA, Helsinki, Finland
[2] FGI Federal Centre for Animal Health, Vladimir, Russia
[3] Faculty of Veterinary Medicine, Department of Basic Veterinary Sciences, University of Helsinki, Helsinki, Finland

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Summary

Thirty-five field rabies virus strains were collected in recent years in different regions of the Russian Federation in order to characterize their genetic heterogeneity and to study their molecular epidemiology. In addition to the Russian viruses, seven archive samples from Estonia and Finland and two Russian vaccine strains were also included in the study. The viruses collected were subjected to two different reverse transcription-polymerase chain reaction tests, the amplicons were sequenced and the sequences were analysed phylogenetically. Among the field viruses studied, two main phylogenetic groups were found and designated as Pan-Eurasian and Caucasian according to their geographic origin. The Pan-Eurasian group, comprising some reference viruses from Europe, was further divided into four subgroups. All of the vaccine strains were clearly different from the field strains. No recombination between the field and vaccine virus strains was observed. The data obtained here show the critical role of geographical isolation and limitation for the genetic clustering and evolution of the rabies virus and also help in predicting its distribution from rabies-affected areas to rabies-free areas.

Introduction

The rabies virus belongs to the genus *Lyssavirus* within the family *Rhabdoviridae* within the order *Mononegavirales* [37]. The genus *Lyssavirus* is further divided into seven genotypes. Genotype 1 is known to be the most widespread and comprises the classical rabies viruses including the majority of field, laboratory and vaccine strains. Rabies-related viruses isolated in Africa belong to genotypes 2, 3 and 4, with the prototypes Lagos bat virus, Mokola virus and Duvenhage virus, respectively. Viruses isolated in bats in Europe represent genotypes 5 and 6 (EBLV-1 and EBLV-2). The Australian bat Lyssavirus (ABLV) represents the seventh genotype [9]. Four more bat viruses isolated in the former USSR have yet to be included in the existing or new genotypes [1, 4].

The rabies virus genome is a non-segmented negative-strand RNA molecule of about 12000 nucleotides (nt), which encodes five major viral proteins. With molecular methods, the genetic variability of rabies viruses has become much easier to

The study was carried out at the Finnish Food Safety Authority, EVIRA, Animal Diseases and Food Safety Research, Virology Unit, Helsinki, Finland.

Correspondence: Artem E. Metlin, Finnish Food Safety Authority, EVIRA, Mustialankatu 3, 00790 Helsinki, Finland
e-mail: artem.metlin@inbox.ru

study. The reverse transcription-polymerase chain reaction test (RT-PCR), subsequent nucleotide sequencing and phylogenetic analysis are important tools for molecular epidemiological studies. Several genome regions have been used for these purposes. Although the G gene and G-L intergenic region (pseudo-gene, ψ-region) are far more variable than the N gene [14], both of these regions have been used for the genetic characterization of rabies viruses [5, 11, 16, 20, 27].

Rabies is endemic in the Russian Federation: 5253 animal rabies cases were reported in Russia in 2005, a 40% increase in cases compared with 2004. In Estonia, 266 animal-rabies cases were reported in 2005 [30]. Finland has been a rabies-free country since 1991, and only one imported case, in a horse from Estonia, was reported in 2003 [25]. Since the last outbreak in 1988–1989, the spread of rabies has been prevented by vaccinating cats and dogs, and by oral immunization of wild carnivores covering the Finnish–Russian southeast border region [25, 28, 33]. This oral-vaccination campaign was initially organized annually from 1989 until 2003 and has since been conducted twice a year. Since 2003, the campaign has also covered the Russian side of the border, the territory of Leningrad region and the Republic of Karelia.

Several phylogenetic and molecular-epidemiological studies on rabies have been conducted over the past 10 years. Bourhy et al. [5] classified rabies viruses of European origin into four main groups: the NEE-group (North-East Europe), the EE-group (East Europe), the WE-group (Western Europe) and the CE-group (Central Europe). Kuzmin et al. [20] recently studied a wide range of rabies viruses isolated in the territory of the former Soviet Union and classified them into five groups (A, B, C, D and E). The data show that viruses with the same geographical origin often group together in phylogenetic analysis. Mansfield et al. [22] have demonstrated the existence of two groups within the general Arctic group: arctic 1 and 2, the latter with two subgroups and a separate Arctic-like group. Studying isolates from countries in the Middle and Near East has demonstrated the existence of a few closely related groups that are different from the viruses of European and African origin. No host-dependent relationships were found in this study [8].

Recent monoclonal antibody (mAb) characterization of rabies viruses from Russia, Finland and Estonia revealed the existence of five antigenic groups [23]. Most of them had a similar mAb reaction pattern to the European genotype 1 rabies viruses, but two field viruses originating in the Tver region had an identical reaction pattern with vaccine strains from the SAD group. This led the authors to suspect that those strains could be reverted vaccine strains because several types of the oral anti-rabies live-attenuated vaccines have been applied in the Tver region.

The main goal of the present study is to evaluate the phylogeny and molecular epidemiology of rabies viruses isolated in different regions of Russia, Finland and Estonia and to discuss the cross-border spread of the rabies virus in the Northern European and Caucasian parts of Russia. Furthermore, we wanted to look for correlations between geography and natural barriers and to compare the wild-type rabies viruses with the vaccine strains used for the oral anti-rabies immunization of wild carnivores in Russia and Finland. All of this work was carried out within the framework of the Finnish-Russian collaboration programme to prevent the spread of rabies to Finland and also to rabies-free areas in Russia.

Materials and methods

Field isolates and vaccine strains

Brain samples of 35 rabid animals were collected in 14 administrative regions, including northwestern, western, southern, Caucasian, central and Siberian parts of the Russian Federation (Table 1). Four Finnish isolates were collected during the outbreak in 1988–1989 [28] and three Estonian isolates in 1989–1990 [17]. They were isolated in murine neuroblastoma (MNA) cells and stored at −70 °C. Two Russian vaccine strains were included in this study (RV-97 and "Sheep"): the RV-97 strain is now in use for the production of the live-attenuated oral anti-rabies vaccine SINRAB (FGI "Federal Centre for Animal Health", Vladimir, Russia) and is known to be a derivate of the RB-71 strain. The "Sheep" strain is the parent strain for the RB-71 strain and was used for producing brain anti-rabies vaccines in the past.

Diagnostic and molecular techniques

The fluorescent antibody test (FAT) and cell culture inoculation test were performed using rabies antinucleocapsid con-

Table 1. Information on isolates/strains collected for the present study

Code of isolate or strain	Origin	Animal species	Phylo-group	Reference	GeneBank accession number	
					N-gene fragment	G-pseudogene fragment
1	2	3	4	5	6	7
3502f	Russia, Altai	red fox	C	Kuzmin et al. [20]	AY352455	–
40_2005	Russia,	lynx	Eurasian	this study	DQ462431	DQ468336
41_2005	Bashkiria	cattle			DQ462432	DQ468337
48_2005		cat			DQ462433	DQ468338
51_2005		dog			DQ462434	DQ468339
69_2005		red fox			DQ462436	DQ468341
23_2002	Russia,	red fox			AY705417	AY702678
33_2002	Belgorod	dog			AY705419	AY702680
41_2002		pig			AY705420	AY702681
28_2005	Russia, Bryansk	dog			DQ462428	DQ468333
33_2005		red fox	North-European		DQ462429	DQ468334
RV262		red fox	D	Kuzmin et al.	AY352457	–
857r	Russia, Chabarovsk	raccoon dog	B	[20]	AY352458	–
248c	Russia, Chita	steppe fox			AY352460	–
10_2005	Russia,	red fox	Eurasian	this study	DQ462422	DQ468327
12_2005	Krasnodar	cattle	Caucasian		DQ462423	DQ468328
18_2005		dog			DQ462424	DQ468329
21_2005		dog			DQ462426	DQ468331
25_2005		red fox	Eurasian		DQ462427	DQ468332
1305f		red fox	C	Kuzmin et al. [20]	AY352461	–
RVHK	Russia, Krasnoyarsk	human	A		AY352462	–
Leningrad_	Russia,	red fox	North-European	this study	DQ462437	DQ468342
1990	Leningrad				DQ462430	DQ468335
4_2005	Russia, Moscow	red fox	Central-Russian		DQ462435	DQ468340
6_2005		red fox			AY705416	AY702677
20_2002	Russia,	red fox	Eurasian		AY705422	AY702683
623_2003	N. Novgorod	dog			AY705425	AY702686
78_2002		cat				
647_2003	Russia, Novosibirsk	red fox			AY705423	AY702684
3687f	Russia, Omsk	red fox	C	Kuzmin et al.	AY352470	–
3605f		red fox		[20]	AY352467	–
18_2002	Russia, Penza	red fox	Eurasian	this study	AY705412	EF095204
3_2002	Russia, Pskov	raccoon dog	North-European	this study	AY705418	AY702679
7_2002		red fox			AY705424	AY702685
RV1596		red fox	E	Kuzmin et al.	AY352474	–
RV245		human		[20]	AY352475	–
188_2002	Russia, Ryazan	cattle	Central-Russian	this study	EF095203	AY702673
RV234	Russia, Tula	dog	D	Kuzmin et al.	AY352476	–
765w	Russia, Tuva	wolf	C	[20]	AY352483	–
3561d		dog			AY352481	–

(*continued*)

Table 1 (*continued*)

Code of isolate or strain	Origin	Animal species	Phylo-group	Reference	GeneBank accession number	
					N-gene fragment	G-pseudogene fragment
1	2	3	4	5	6	7
RV250		rodent	A		AY352480	–
84_2002	Russia, Tver	wolf	Central-Russian	this study	AY705426	AY702687
85_2002		raccoon dog			AY705427	AY702688
88_2002		raccoon dog			AY705428	AY702689
96_2002		red fox			AY705431	AY738335
189_2002	Russia,	wolf	Central-Russian	this study	AY705413	AY702674
190_2002	Vladimir	raccoon dog			AY705414	AY702675
51_2001		red fox			AY705421	AY702682
2_2005		red fox			DQ462425	DQ468330
2070f	Russia, Volgograd	red fox	C	Kuzmin et al. [20]	AY352484	–
1_2002	Russia, Voronezh*	red fox	Eurasian	this study	AY705407	AY702668
120_1989	Estonia	red fox	North-European	this study	AY705410	AY702671
124_1989		red fox			AY705411	AY702672
EST_1990		red fox			AY705429	EF095205
9342EST		raccoon dog	E	Bourhy et al. [5]	U43432	–
113_1988	Finland	raccoon dog	North-European	this study	AY705409	AY702670
109_1988		red fox			AY705408	AY702669
RV1511_1989		cattle		Metlin et al.	DQ269940*	EF095206
1904_2003		horse		[24, 25]*	AY705415*	AY702676
9445FRA	France	red fox	WE	Bourhy et al.	U42700	–
8663FRA		red fox		[5]	U42605	–
8903FRA		red fox			U42606	–
9213ALL	Germany	red fox	CE		U42702	–
RV308	Georgia	human	UG	Kuzmin et al. [20]	AY352497	–
V686	Iran	cow	1a	Nadin-Davis	AY854581	–
V703		sheep	1	et al. [26]	AY854583	–
408s	Kazakhstan	sheep	C	Kuzmin et al.	AY352490	–
409f		red fox		[20]	AY352489	–
86111YOU	Yugoslavia	red fox	WE	Bourhy et al. [5]	U42706	–
2054	USA, Colorado	big brown bat	out-group	Smith et al. [38]	AF394888	–
RV-97	Vaccine strains			this study	AY705430	AY702690
'Sheep'					AY705406	AY702667
Nishigahara				Ito et al. [12]	AB044824	
RC_HL					AB009663	
PV				Tordo et al. [36]	M13215	
SAD B19				Conzelmann et al. [7]	M31046	

* For N-gene partial sequences only.

jugate (BioRad, France) and the monoclonal FITC-conjugate Centocor (Centocor Inc, Malvern. PA, USA) according to protocols recommended by the World Organization for Animal Health (OIE) [29]. Purification of total RNA, cDNA synthesis, RT-PCR and sequencing of the PCR products were carried out in principle as described earlier [24]. Two primer pairs were designed: N-forward 3′-ACTCTAATGACAACT CACAARATGTG-5′ and N-reverse 5′-AGTGAATGAGAT TGAATACATGACC-3′ amplify an N-gene segment of 384 base pairs (nt 626–1010), G-forward 3′-GGGAAGGTCA-TATCTTCATGGG-5′ and G-reverse 5′-ATGAAA GCACC GTTGGTCACTG-3′ amplify 280 nucleotides (4835–5115) covering the end of the G gene and part of the G-L intergenic region. The numbering of the nucleotides is according to the SAD B19 sequence [7]. The primer sequences were further cut out. The CLUSTALW [35] program was used for sequence analysis and multiple-sequence alignment.

Phylogenetic analyses

Phylogenetic analyses were conducted using the MEGA Version 3.1 program [18, 19]. Minimum Evolution phylogenetic trees were constructed and bootstrapped with 1000 replicates. Neighbour-Joining phylogenetic trees were also built using the Kimura-2 parameter. In addition to the field strains, six rabies vaccine strains were included in the phylogenetic analysis. The sequences obtained in this study were compared with a wide variety of sequences published in the Gene Bank database, and then representatives of the genogroups created were used to construct the final phylogenetic trees. The rabies virus isolated from a bat in Colorado was used as an out-group. All of the sequences used in this study are listed in Table 1 and put in alphabetical order according to their geographical origin.

Results

All brain samples were studied using FAT and were found to be rabies virus positive. Viruses originating from Finland and Estonia stored as cell culture suspensions were re-isolated in MNA cells. The positive diagnosis was further supported by RT-PCR and nucleotide sequencing.

Two types of tests were applied for analysing phylogenetic relationships: the Minimal Evolution and Neighbour-Joining methods. Because both methods produced the same clustering of viruses, only the Minimum Evolution tree is shown and discussed below (Fig. 1). Trees based on both of the genome regions produced a similar grouping. Only the tree based on the partial N-gene sequences is shown.

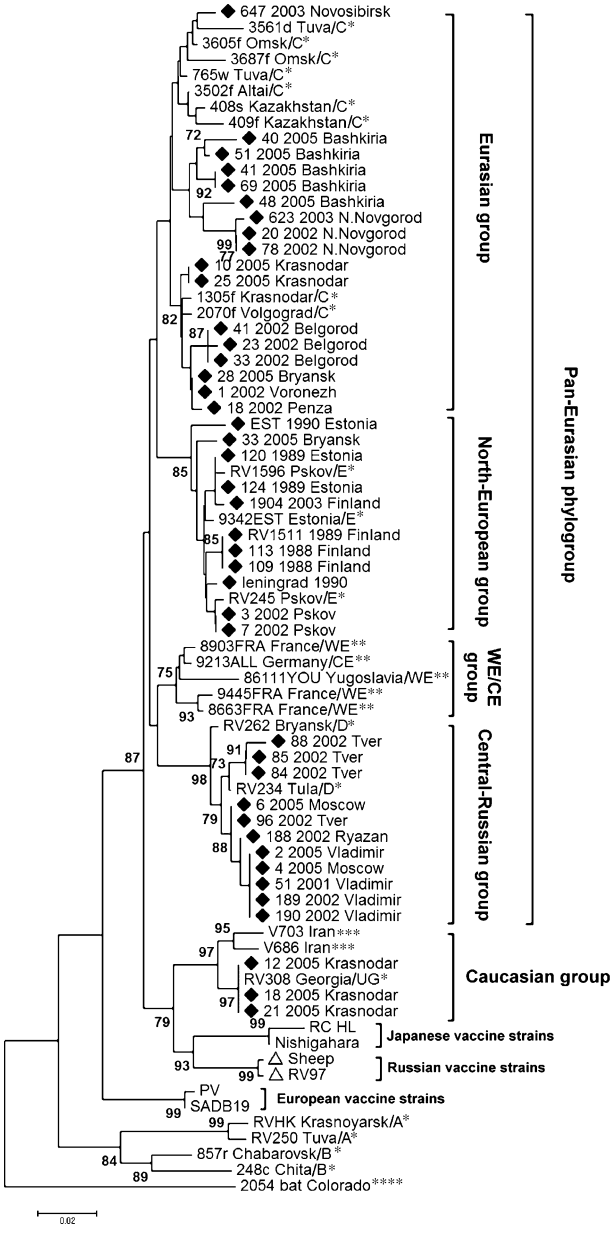

Fig. 1. Minimum Evolution phylogenetic tree of 69 field rabies viruses and 6 vaccine strains according to a partial fragment of the N-gene. Russian vaccine strains are marked by △, and field viruses from Russia, Estonia and Finland obtained or this study are marked by ◆. Referent viruses marked as follows: *Kuzmin et al. [20]; **Bourhy et al. [5]; ***Nadin-Davis et al. [26]; ****Smith et al. [38]. The strain names and geographical origins are given for all field viruses. Numbers indicate the bootstrap value from 1000 replicates, only values ≥70% are shown

Fig. 2. Geographical distribution of rabies viruses groups revealed in this study. *N-E* North-European Group (includes archival viruses from Finland, Estonia and Leningrad region), *Eurasian* Eurasian group, *C-R* Central-Russian group, *Cau* Caucasian group (circled). Lakes, rivers, gulfs, seas and oceans are light grey. *Arm* Armenia; *Azr* Azerbaijan; *Czh* Czech Republic; *Est* Estonia; *Geo* Georgia; *Lth* Lithuania; *Lva* Latvia; *Mld* Moldova; *Rom* Romania; *Svk* Slovak Republic; *Uzb* Uzbekistan

Two main phylogenetic groups, Pan-Eurasian and Caucasian were found among the field viruses. The geographical location of the phylogenetic groups is shown on the map (Fig. 2). According to the N-gene tree, the Pan-Eurasian group was further divided into four subgroups: Eurasian, North-European, Central-Russian, and a fourth group including some European viruses classified by Bourhy et al. [5] as members of the Western Europe (WE) and Central Europe (CE) groups. According to the partial N-gene sequence analysis, the nucleotide differences within these groups were a maximum of about 2 and 4–7% between subgroups.

The Eurasian group comprises viruses from European (Belgorod, Voronezh, Penza, Krasnodar), Central (N. Novgorod), Asian (Bashkiria) and Siberan (Novosibirsk) parts of Russia. Several viruses (1305f – Krasnodar; 2070 – Volgograd; 3502f – Altai; 3687f and 3605f – Omsk; 765w and 3561d – Tuva; 408s and 409f – Kazakhstan), whose sequences were published before by Kuzmin et al. [20], also fell into this group.

The North European group comprises viruses from Finland and Estonia, as well as from the Pskov, Bryansk and Leningrad regions of Russia. Archival viruses from Finland, Estonia and the Leningrad region (N-E) group are also indicated on the map. The Finland and Leningrad region are nevertheless officially rabies-free areas at present. The RV1596 and RV245 viruses from Pskov [20] and the 9342EST virus from Estonia [5] are members of this group.

Viruses from the geographically close (Moscow, Vladimir, Tver, and Ryazan) regions constitute a separate Central-Russian group. Comparison of partial N-gene sequences has shown that the RV234 from Tula and RV262 from Bryansk [20] viruses also belong to this group. The nucleotide sequences of the fox rabies virus 96_2002 from Tver and the SAD B19 strain, which have a similar MAb pattern [23], differ by 7.2% in the N-gene fragment and by about 14% in the G-L intergenic region.

Viruses isolated in the southern part of the Krasnodar region near the Georgian border and the Black Sea coast (12_2005, 18_2005, 21_2005) make up a separate Caucasian group. They are closely related to each other and also to the RV308 virus from Georgia, which remained ungrouped in earlier studies [20]. The RV703 and RV686 viruses from Iran (1 and 1a genetic groups, respectively, by Nadin-Davis et al. [26]) were also included in the Caucasian phylogenic group. Viruses from the Caucasian group differ from the viruses which form the Pan-Eurasian group according to N-gene sequences by 6–7% and according to G-L intergenic region sequences by about 9%.

All of the vaccine strains studied clearly differed from the field viruses. The Russian vaccine strains ''Sheep'' and RV-97 were grouped together with the RC-HL and Nishigahara vaccine strains of Japanese origin. No recombining of the field and vaccine virus strains was observed.

Discussion

Rabies is still a serious veterinary and public health problem in Russia and in the Baltic countries. Phylogeny and molecular epidemiological studies often help to locate the origin of a virus. They also make

it possible to develop effective rabies control programmes and to prevent the introduction of the rabies infection into rabies-free areas in the future.

In this study, two main phylogenetic groups were established among the field viruses studied: Pan-Eurasian and Caucasian. The general Pan-Eurasian group comprises four subgroups: the Eurasian, North-European and Central-Russian subgroups, which correspond to the phylogenetic groups C, D and E published by Kuzmin et al. [20], respectively, and the WE/CE group of Bourhy et al. [5]. Several nomenclatures for the grouping of rabies viruses inside genotype 1 were offered by different research groups [5, 20]. The geographical clustering is becoming clearer and more evident because of the fact that more material is accumulating all the time, in this report too.

The Eurasian subgroup comprises viruses with a wide geographical origin (Fig. 2): from Siberia (Novosibirsk) to the western (Belgorod) and southwestern (Krasnodar) regions of the Russian Federation. Viruses from Omsk, Altai (Russia) and Kazakhstan, previously classified by Kuzmin et al. [20] as members of group C, also belong to this group. This group seems to be the most widespread in Russia.

Viruses from the North-European group have been positive for the P-41 mAb [23, 24], indicating the Arctic antigenic group. The North-European group includes viruses from Finland and Estonia, plus the Pskov, Bryansk and Leningrad regions. It is obvious that viruses circulating in Estonia and the Pskov region of Russia are very similar.

Previous antigenic and genetic studies have confirmed that the isolates responsible for the Finnish outbreak (1988–89) were related but not identical to the viruses isolated in the Baltic region [5, 15, 17, 23], suggesting that rabies had perhaps gradually moved from the Russian/Estonian territories after prolonged circulation among local hosts before being detected. In this study, the causative agents (strains 113_1988, 109_1988 and RV1511, Fig. 1) belong to the North-European group, which corresponds to group E by Kuzmin et al. [20] and to group NEE by Bourhy et al. [5]. Several explanations, such as animal-to-animal transfer, human-mediated transfer, for instance by railways, or the movement of infected animals across the frozen sea have been suggested [15]. The way in which the virus was introduced to the rabies-free area is still unclear, but here we report a close phylogenetic relationship among viruses isolated in Finland in the period 1988–89 and 2003, in Estonia in 1989–90, in the Leningrad region (Russia) in 1990 and in the Pskov region (Russia) in 2002. Two ways of transmission seem to be more probable: animal-to-animal gradual terrestrial movement via/from the Leningrad region or across the frozen surface of the Gulf of Finland directly from Estonian territory. According to Nyberg et al. [28], the last explanation seems to be more probable because most cases were found on the Western side of the Kymi river and one case was found on an island on the southern rim of the coastal ice [28]. It is possible that the virus was introduced in both ways. Rabies could still be reintroduced to Finland by imported animals, as in 2003 [25] but due to the annual oral vaccination campaigns on both sides of the southeastern Finnish-Russian border, the development of mass sylvatic epidemic in southeastern Finland seems improbable.

The Central-Russian group comprises viruses mostly from the Central part of Russia and seems to overlap with viruses classified earlier as group D [20]. Viruses from these regions clearly differ even from those isolated from bordering regions like the N. Novgorod region. The Oka and Volga rivers (Fig. 2) can be regarded as factors limiting virus circulation by separating Moscow and Vladimir and parts of the Ryazan and Tver regions from the other regions. Rivers significantly limit the intensive movement of wild carnivores and allow the independent evolution of viruses in separated regions. During the last century, some barriers, such as the Vistula River in Poland, were able to limit the spread [5] of rabies from Eastern to Western and Southern Europe. Of course, this barrier is not absolute, and animals are able to cross rivers in the wintertime, when the water is frozen, or go over bridges or swim, but major rivers remain a considerable limiting factor for the development of rabies epidemics. This fact must be taken into account when anti-rabies programmes, especially oral-vaccination campaigns are developed.

Besides rivers, mountain ranges were also capable of strongly limiting the circulation of the rabies virus. In our study, viruses from the northwestern part of the Krasnodar region (10_2005 and 25_2005) clearly differed from viruses isolated in the area situated on the other side of the Caucasian Mountains (12_2005, 18_2005 and 21_2005), near the Georgian border. This area is separated from the other territory of the Krasnodar region by the Caucasian Mountain Range. According to the partial N-gene sequence, the V703 and V686 viruses from Iran [26] and the RV308 virus from Georgia [20] belong to this phylogroup. The Ural Mountains could also play a role in the separation of viral circulation, but in order to verify this, further studies must be conducted.

All of the field viruses studied here clearly differed from the vaccine strains, and also from the 96_2002 strain that was isolated in the Tver region, with a mAb reaction pattern similar to some vaccine strains of the SAD group [23]. The oral vaccination of wildlife with various live-attenuated vaccines has been conducted in that region, and the recombination between field and vaccine strains would have been theoretically possible. Although the recombination rate within the genus *Lyssavirus* is low, there are some data suggesting that it might occur [6]. Russian, Estonian and Finnish field rabies viruses and Russian vaccine virus strains were analysed phylogenetically on the basis of two distant genome regions. Basically, the grouping of viruses was similar in both regions, which provides no evidence for the idea of the recombining of field viruses and vaccine strains.

The pathogenicity of rabies viruses seems to depend, at least in part, on the presence of an arginine or a lysine residue at position 333 of the mature viral glycoprotein [34]. Although our study does not cover this genome region, it is clear on the basis of sequence data obtained that none of the strains isolated from sick animals were attenuated vaccine strains. This indicates that the vaccines used in the region were stable and did not revert back to their native pathogenic form.

A specific host for phylogenetic groups could not be perfectly defined in this study because the virus members of the phylogroups determined were found in both wild and domestic/farm animals. However, viruses from the North-European group are mostly hosted by red foxes and raccoon dogs [5, 15, 28], which transmit them to domestic and farm animals. The Caucasian phylogenetic group can also be associated with dogs, but further studies need to be conducted to support this statement.

It is necessary to continue investigations on the genetic characteristics of the field and vaccine strains of the rabies virus in order to determine their similarities and differences. These data can be used when planning the use of vaccines in different geographical regions. The wide genetic heterogeneity of the field rabies virus strains verified in this study indicates that there should still be a permanent alertness to the potential spread of rabies and that international rabies control programmes must be established in rabies-affected areas.

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