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Phylogenetic Analysis of the Rabies Virus N-coding Region in Lithuanian Rabies Isolates

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Abstract

Rabies infection among wild and domestic animals constitutes a well-known problem in Lithuania, but only one dog rabies virus isolate sequence (1992) from Lithuania was used in the European rabies virus phylogenetic analysis. The objective of this work was to determine nucleoprotein (N) gene sequences and genetically characterize the rabies virus isolates in order to learn which virus group (biotype) is circulating in reservoir species in Lithuania. Classical rabies virus isolate nucleoprotein (N) gene sequences from different parts of Lithuania were found to be closely related to each other and demonstrated nucleotide identity from 97.7 to 100% and could be placed in one lineage with 100% bootstrap support. All 12 sequences of raccoon dogs, red foxes, dogs and marten rabies viruses exhibited 97.7 - 99.0% identity to previously published sequences from Eastern parts of Poland, Estonia, Finland, and the North-Eastern part of Russia. Phylogenetic analysis revealed that all Lithuanian strains belong to the North East Europe (NEE) group of rabies virus.

Rabies, nucleoprotein gene, sequencing, group determination

The molecular diversity of classical rabies viruses (Genotype 1, RABV) has been studied at the global level, but there is no current information about the epidemiological status of Lithuanian rabies virus, therefore it is difficult to understand the dynamics of viral dispersion and adaptation. Additionally, only one dog rabies virus isolate sequence (1992) from Lithuania was used in the rabies virus phylogenetic analysis (Bourhy et al. 1999), but this infection among wild and domestic animals in Lithuania constitutes a well known problem: During the last 7 years there were 2 248 reported cases of rabies in the red fox and 2 537 in the raccoon dog populations (Zienius et al. 2007). In contrast, the rabies viruses isolates from Latvia, Estonia, Poland and the European part of Russia (Vanaga et al. 2003; McElhinney et al. 2006; Sadkowska-Todys 2000; Kuzmin et al. 2004) were investigated and analyzed more actively. Consequently, the molecular typing methods are playing an increasingly important role in the understanding of rabies host and geographical distribution in Lithuania.

Hemi-nested RT-PCR (Picard-Meyer et al. 2004) is highly sensitive, up to 300 000 (5.5 log) times more than MIT or RTCIT (Barrat et al. 2006; Franka et al. 2004) and may then be performed to investigate rabies epidemiology, establish species and geographical links. A number of phylogenetic analysis studies have identified the N gene and nucleoprotein as most suitable targets for epidemiological studies of RABV variation (Kissi et al. 1995; Nadin-Davis 1998; Bourhy et al. 1999; Johnson et al. 2002; Botvinkin et al. 2006; Kuzmin et al. 2004). The rabies virus nucleocapsid protein (N) gene was chosen for this analysis because it encodes an internal protein involved in the regulation of transcription and replication and could be an important factor in host adaptation. It presents the virus group-specific core antigen (Schneider et al. 1973) and is the most conserved of the viral components in terms of amino acid sequence similarity within genotypes. Rabies and rabies-related virus isolates that share less than 80% of nucleotide similarity in the N gene (including non-coding regions) and less than 92% of N-amino acid similarity belongs to different genotypes (Bourhy et al. 1993, Kissi et al. 1995). Nucleotide sequence analysis

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permits to understand the transmission of rabies virus from the reservoir host to other hosts, including humans and domestic animals (Nagarajan et al. 2006; David et al. 2004; Kissi et al. 1995).

The objective of this work was to determine nucleoprotein (N) gene sequences and to genetically characterize the rabies virus isolates in order to know which virus group (biotype) is circulating in reservoir species in Lithuania.

Materials and Methods

In total, 30 Lithuanian RABV isolates, collected during the 2005-2006 period, diagnosed as rabies-positive by both the FAT (Dean et al. 1996; OIE 2004) and the MIT (Koprowski 1996; OIE 2004) were used in this study. The isolates were obtained from brain samples received from the National Veterinary Laboratory and came from several Lithuanian districts and from several animal species. The majority of RABV isolates were obtained from wild mammals: red foxes (n = 10), raccoon dogs (n = 8), martens (n = 3), polecats (n = 1) and badgers (n = 1); some from domestic animals: dogs (n = 5) and cats (n = 2). For molecular epidemiological study 12 samples from all the obtained isolates were selected according to the geographical location and animal species and analyzed together with reference to GenBank sequences of the rabies virus isolates from various regions of Eastern, Central, and Northern Europe including the nearest regional Lithuanian neighbours. Total RNA was extracted from infected brain samples using the TRIzol method (Invitrogen, Life Technologies, MD, USA) following the manufacturer's recommendations. RT and PCR were performed according to Amengual et al. (1997) and with primer set N12 (5'-GTAACACCTCTACAATGG-3', nucleotides 57-74) and N8 (5'-AGTTTCTTCAGCCATCTC-3', nucleotides 1585-1568). All nucleotide positions of the primers are numbered according to the PV strains sequence. PCR was carried out in 50 µl volumes containing 5 µl of extracted RNA and the following reagents: 5 µl 10 × PCR buffer (Fermentas), 5 µl $MgCl_2$ (25 mM, Fermentas), 2 µl dNTPs (10 mM, Fermentas), 5 pmol of each outer primers N12 and N8, 0.5 µl (2.5 U) Taq DNA polymerase (Fermentas), 0.25 µl (10 U) RNasin (Promega, Madison, WI, USA), and 0.5 µl (100 U) MMLV reverse transcriptase (Life Technologies). The tubes were then subjected to the following cycle parameters: 42 °C for 30 min, 95 °C for 5 min, and then 35 cycles at 94 °C for 40 s, 56 °C for 40 s, and 72 °C for 1 min.

Nested PCR was carried out with primers N53 (5'-GGATGCCGACAAGATTGTAT-3', corresponding to bases 73-92 of the PV sequence) and N55 (5'- CTAAAGACGCATGTTCAGAG-3', corresponding to bases 491-472 of the PV sequence). For the nested PCR as a template 2 µl of PCR I product and the following reaction mixture were used: 5 µl 10 × PCR buffer (Fermentas), 5 µl $MgCl_2$ (25 mM, Fermentas), 2 µl dNTPs (10 mM, Fermentas), 20 pmol of each nested primers N53 and N55, 0.5 µl (2.5 U) Taq DNA polymerase (Fermentas). The tubes were then subjected to the following cycle parameters: 95 °C for 3 min followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s. A single extension step of 72 °C for 10 min completed the amplification process. The nested PCR resulted a final amplicon of 400 bp.

Prior to sequencing, the PCR products were elctrophoresed on 1.5% agarose gel. Following ethidium bromide staining and desalting in ultrafiltered water, bands of the expected size were excised from the gel, and the DNA was recovered using Nucleospin Extract II kit (Macherey-Nagel GmbH, Germany) following the manufacturer's recommendations. Gel purified PCR products were cycle sequenced using the BigDye™ Terminator Cycle Sequencing kit (v2.0, Applied Biosystems, Foster City, CA, USA) and the ABI310 genetic analyzer (Applied Biosystems). Sequences of both strands of the nucleoprotein N gene products were determined using the same primers as used for the nested PCR amplification. The obtained sequences were assembled by using SeqMan program (Laserge, program package, DNASTAR, Inc., Madison, USA). All sequences reported in this study have been submitted to GenBank, as summarized in Table 1. Clustal W programme from MegAlign Lasergene program package was used for sequence alignment and phylogenetic analyses. For boot strap analyses neighbour-joining (NJ) algorithm from CLC Free Workbench 3.2.3 program package (CLC bios A/S, Denmark) was used. Bootstrap values exceeding 70% were considered significant.

Results

Identification of the rabies viral antigen by FAT and MIT was evaluated in a total of 30 brain samples tested. The RT-PCR with the N12/N8 primer set obtained positive results in 22 rabies samples from 8 red foxes, 6 raccoon dogs, 3 martens, 1 badger, 3 dogs and 1 cat. By using partial region from the amino terminus of the N gene (400 bp), a total of 12 rabies-positive samples representing several Lithuanian regions were sequenced.

Based on newly obtained Lithuanian sequences and the selected rabies virus sequences from Estonia, Russia, Poland and other West European countries available in the GenBank (also summarized in Table 1), a phylogenetic tree was constructed (Fig. 1).

All 12 Lithuanian rabies viruses isolates sequences exhibited 97.7-99.0% identity to

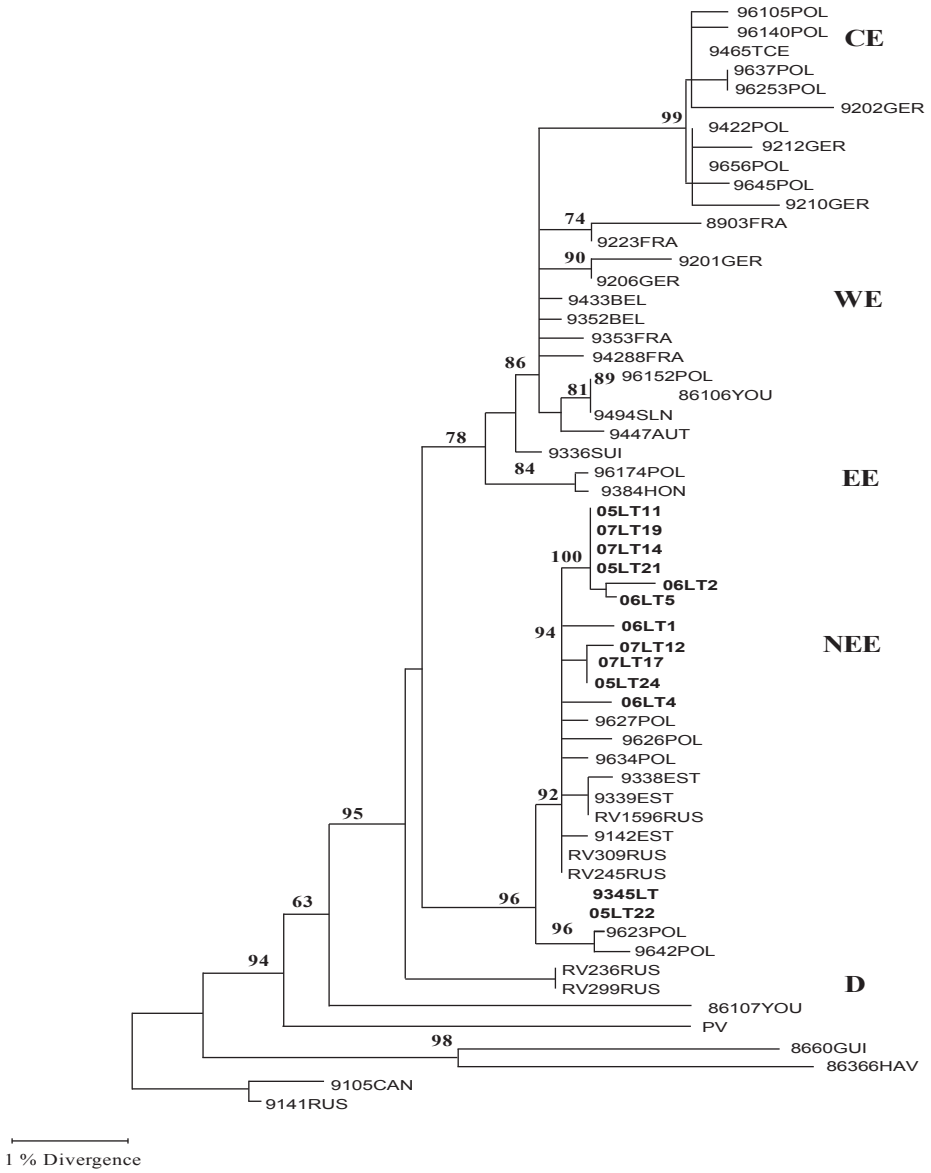

Fig. 1. Phylogenetic tree of classical rabies virus nucleoprotein (N) sequences. The horizontal branches are drawn to scale and the tree is rooted with reference sequences 9141RUS and 9105CAN, representative of Arctic fox lyssaviruses. The numbers at the main nodes indicate the degree of bootstrap support calculated by using neighbour-joining (NJ) algorithm from CLC Free Workbench 3.2.3 program package. The abbreviations for the earlier described European main phylogenetic groups (CE, WE, EE, NEE, D) are given according to Bourhy et al. (1999) and Kuzmin et al (2004). Lithuanian rabies virus isolates are presented in bold.

previously published sequences from Estonia, Finland, Poland and North-Eastern part of Russia. Furthermore, alignment of sequences of different species including the dog (05LT11, 06LT5), red fox (07LT19, 05LT21, 06LT2) and raccoon dog (07LT14) showed that they

Table 1. Summary of classical rabies virus sequences used for phylogenetic analyses of nucleoprotein N gene region of 400 bp. Sequences 22-33 were obtained as part of this study and the other are reference sequences, representing most diverse European rabies virus available in the GenBank. Phylogenetic clusters indicated according to Bourhy et al. (1999): Central Europe (CE), Eastern Europe (EE), Western Europe (WE), North-Eastern Europe (NEE). Group D is representing rabies viruses originated from the centre of the European part of Russia (Kuzmin et al. 2004).

No	Country	No. and abbreviation	Reference / source	Species	Year	Gen Bank accession no.	Phylogenetic cluster
1	Austria	9447AUT	W. Schuller	Red fox	1994	U42708	WE
2	Belgium	9352BEL	F. Cocty	Red fox	1991	U42709	WE
3	Belgium	9433BEL	D. Peharpe	Red fox	1994	U42713	WE
4	Czech Republic	9465TCE	O. Matouch	Red fox	1994	U43010	WE
5	Estonia	9142EST	Kissi et al. (1995)	Raccoon dog	1985	U22476	NEE
6	Estonia	9338EST	K. Kulonen	Red fox	1992	U42707	NEE
7	Estonia	9339EST	K. Kulonen	Raccoon dog	1991	U43432	NEE
8	Former Fed. Rep. Yugoslavia	86107YOU	M. Petrovic	Red fox	1976	U42703	NC
9	Former Fed. Rep. Yugoslavia	86106YOU	Kissi et al. (1995)	Red fox	1972	U22839	WE
10	Finland	9348FIN	K. Kulonen	Raccoon dog	1988	U42716	NEE
11	France	9223FRA	J. Barrat	Red fox	1974	U43433	WE
12	France	8903FRA	Institut Pasteur	Red fox	1989	U42606	WE
13	France	9353FRA	Institut Pasteur	Red fox	1993	U42717	WE
14	France	94288FRA	Institut Pasteur	Red fox	1994	U42992	WE
15	Germany	9202GER	K. Stohr	Red fox	1991	U42701	CE
16	Germany	9201GER	K. Stohr	Red fox	1991	U42994	WE
17	Germany	9206GER	K. Stohr	Red fox	1991	U42996	WE
18	Germany	9210GER	K. Stohr	Red fox	1991	U42997	CE
19	Germany	9212GER	K. Stohr	Red fox	1991	U22475	CE
20	Hungary	9384HON	E. Moskari	Red fox	1993	U42999	EE
21	Lithuania	9345LT	K. Kulonen	Dog	1992	U43002	NEE
22	Lithuania	06LT1	This study	Dog	2006	EU616715	NEE
23	Lithuania	06LT2	This study	Red fox	2006	EU616716	NEE
24	Lithuania	06LT4	This study	Raccoon dog	2006	EU616717	NEE
25	Lithuania	06LT5	This study	Dog	2006	EU616718	NEE
26	Lithuania	05LT11	This study	Dog	2007	EU616719	NEE
27	Lithuania	07LT12	This study	Red fox	2007	EU616720	NEE
28	Lithuania	07LT14	This study	Raccoon dog	2007	EU616721	NEE

No	Country	No. and abbreviation	Reference / source	Species	Year	Gen Bank accession no.	Phylogenetic cluster
29	Lithuania	07LT17	This study	Dog	2007	EU616722	NEE
30	Lithuania	07LT19	This study	Red fox	2007	EU616723	NEE
31	Lithuania	05LT21	This study	Red fox	2006	EU616724	NEE
32	Lithuania	05LT22	This study	Marten	2005	EU616725	NEE
33	Lithuania	05LT24	This study	Raccoon dog	2006	EU616726	NEE
34	Poland	9623POL	D. Seroka	Dog	1991	AF033871	NEE
35	Poland	9642POL	D. Seroka	Fox	1996	AF033879	NEE
36	Poland	9626POL	D. Seroka	Raccoon dog	1986	AF033874	NEE
37	Poland	9627POL	D. Seroka	Fox	1987	AF033875	NEE
38	Poland	9634POL	D. Seroka	Fox	1987	AF033876	NEE
39	Poland	96174POL	J. F. Zmudzinski	Fox	1994	AF033886	EE
40	Poland	96152POL	J. F. Zmudzinski	Fox	1995	AF033884	WE
41	Poland	9645POL	J. F. Zmudzinski	Fox	1993	AF033891	CE
42	Poland	9656POL	J. F. Zmudzinski	Fox	1993	AF033892	CE
43	Poland	9422POL	J. F. Zmudzinski	Raccoon dog	1993	U43004	CE
44	Poland	96253POL	J. F. Zmudzinski	Raccoon dog	1996	AF033903	CE
45	Poland	9637POL	J. F. Zmudzinski	Fox	1996	AF033890	CE
46	Poland	96105POL	J. F. Zmudzinski	Fox	1995	AF033898	CE
47	Poland	96140POL	J. F. Zmudzinski	Raccoon dog	1993	AF033900	CE
48	Russia	RV309RUS	I. Kuzmin et al.	Raccoon dog	2004	AY352504	NEE
49	Russia	RV245RUS	I. Kuzmin et al.	Human	2004	AY352475	NEE
50	Russia	RV1596RUS	I. Kuzmin et al.	Red fox	2004	AY353876	NEE
51	Russia	RV236RUS	I. Kuzmin et al.	Red fox	2004	AY352506	D
52	Russia	RV299RUS	I. Kuzmin et al.	Red fox	2004	AY352479	D
53	Russia	9141RUS	Kissi et al. (1995)	Arctic fox	1990	U22656	NC
54	Switzerland	9336SUI	R. Zanoni	Red fox	1992	U43006	WE
55	Slovenia	9494SLN	P. Hostnik	Red fox	1994	U43009	WE
56	Laboratory strain	PV	Tordo et al. (1986)	Vaccine strain	1986	D42112	NC
57	Canada	9105CAN	Kissi et al. (1995)	Red fox	1990	U22655	NC
58	Guinea	8660GUI	Kissi et al. (1995)	Dog	1986	U22637	NC
59	Burkina Fasso	8636HAV	Kissi et al. (1995)	Dog	1986	U22486	NC

could be placed in one lineage with 100% bootstrap support (Fig.1). Previously published Lithuanian rabies sequence 9345LT also showed very close phylogenetic relationship to newly obtained isolates (97.7-99.0% of identity).

The phylogenetic analysis including a large set of reference sequences revealed that all Lithuanian strains (including the unique Lithuanian sequence 9345LT obtained earlier) belong to North East Europe group of rabies virus described earlier in Bourhy et al. (1999) and could not be subdivided according to geographical location. All new Lithuanian sequences as well as new Russian, Polish or Estonian sequences were well supported from the other group of rabies viruses, which joined isolates from different parts of Europe, with a bootstrap support of 96%. The constructed phylogenetic tree also showed that the other rabies sequences included in this study were placed to West or Central Europe groups with a bootstrap support of 86 and 99%, respectively.

Discussion

Molecular epidemiology based on RT-PCR is an important tool for the classification of animal virus diseases, including rabies virus, and provides a better understanding of epidemiological relationships. This approach has been applied at various geographical levels, from individual countries to worldwide studies (Kissi et al. 1995; Haas 1997; David et al. 2000, De Mattos et al. 2000). It also provides information where a single host dominates the maintenance of rabies virus (Sacramento et al. 1992) or where more than one host are involved in the transmission of the virus (Nadin-Davis et al. 2001; Johnson et al. 2003). The sequence alignment and phylogenetic analysis of Lithuanian rabies virus isolates demonstrates a high degree of similarity between isolates originating from different species. Phylogenetic analysis based on a 400 bp N-coding region sequences demonstrates that all the viruses investigated are genotype 1 viruses and are closely interdependent. The red foxes (*Vulpes vulpes*) remain the principal vector of rabies transmission in Lithuania, but a close relationship (97.7-99.0%) between isolates from raccoon dogs (*Nyctereutes procyonoides*) suggest that the transmission was a recent event with the possibility of direct transmission. Fox rabies viruses from Eastern Europe (EE) tend to group together, as do those from Western Europe (WE) and Central Europe (CE), the latter of which was previously described using monoclonal antibodies (Stohr et al. 1992; Bourhy et al. 1999). The raccoon dog's viruses are also found within a particular geographical area, North-Eastern Europe (NEE), although their precise phylogenetic relationship to the fox rabies strains is uncertain, as indicated by the low bootstrap support for the critical node. NEE group (96% bootstrap support) is now found to cover a wider geographical area, including the eastern parts of Poland, Lithuania, Estonia, Russia and Finland, and includes viruses isolated from both red foxes and raccoon dogs, showing that both species are effective reservoirs for this variant of rabies virus and may represent early cross-species transmission from dog viruses. Raccoon dogs were frequently involved in rabies virus circulation in the NEE region, particularly in Lithuania, where the population of raccoon dogs is the largest, which suggests that the density of susceptible hosts is a major ecological factor in the establishment of rabies virus in a new host species (Bourhy et al. 1999). Phylogenetically, this group was related to other European lineages, and there is no reason to consider it as specific to the raccoon dog (Kuzmin et al. 2004). Our results confirm this suggestion because all Lithuanian rabies strains from raccoon dogs and foxes had 97.7-99.0% nucleotide identity.

By comparing the Lithuanian rabies isolates with those derived from the surrounding countries it became clear that the North-East Europe group is related to RABV found throughout the region. In Poland, the NEE group corresponding to the European fox 3 (F3) phylogenetic subgroup is limited to the eastern side of the Vistula River and to the northern

border of Russia, Byelorussia and Lithuania (Sadkowska-Todys 2000). In Latvia, the direct sequencing of the RT-PCR-amplified products of the rabies virus nucleoprotein encoding region and subsequent sequence analyses resulted in 99.3-100% homology between isolates and 99.0-100% with genotype 1, classical RABV raccoon dog isolate from Estonia in 1995 (Vanaga et al. 2003). The genetically close relationship between the same raccoon dog rabies isolate from Estonia and raccoon dog and fox (99.2% and 98.8% respectively) RABV isolates from northern Lithuania was identified, suggesting that the viruses circulating in foxes and raccoon dogs in this region might have the same origin.

The phylogenetic analysis of the partial N-gene sequences in Lithuanian rabies virus isolates indicates the direct geographical association with the North-East Europe (NEE) group and demonstrates the close relationship among rabies virus strains circulated in the neighbouring countries such as Poland, Latvia, Estonia and Russia.

Fylogenetická analýza litevských izolátů viru vztekliny využívající kódování N-genu

Přestože infekce vztekliny mezi volně žijícími i domestikovanými zvířaty představuje v Litvě dobře známý problém, byl k fylogenetické analýze evropského viru vztekliny sekvenován genom pouze jednoho psího izolátu viru vztekliny (1992) z Litvy. Cílem této práce bylo určit sekvenci nukleoproteinového (N) genu a z hlediska genetiky charakterizovat jednotlivé izoláty viru vztekliny z důvodu rozpoznání skupiny virů (biotypu) cirkulující u rezervoárových živočichů v Litvě. Bylo zjištěno, že sekvence nukleoproteinového (N) genu u izolátů klasického viru vztekliny z různých oblastí Litvy jsou velmi blízce příbuzné. Identita nukleotidů se pohybovala mezi 97,7 až 100 % a tyto izoláty tedy lze umístit do jedné importované linie viru s podložením 100 %. Všechny z 12 sekvencí virů vztekliny u mývala severního, lišky obecné, psa a kuny skalní vykazovalo 97,7-99 % identitu s předchozími publikovanými sekvencemi z východní části Polska, Estonska, Finska a severozápadní části Ruska. Fylogenetická analýza odhalila, že všechny litevské izoláty viru vztekliny patří do severovýchodní skupiny (NEE) viru vztekliny.

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