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Korrespondenzadresse:
ilojkic@veinst.hr

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Summary

Zusammenfassung

Short communication

Department of Virology, Croatian Veterinary Institute, Zagreb, Croatia¹
Institute of Epidemiology, WHO Collaborating Centre for Rabies Surveillance and
Research, Friedrich-Loeffler-Institute, Federal Research Institute of Animal Health,
Wusterhausen, Germany²

Diversity of currently circulating rabies virus strains in Croatia

Diversität der derzeit zirkulierenden Tollwutviren in Kroatien

Ivana Lojkić¹, Željko Čač¹, Tomislav Bedeković¹, Nina Lemo¹, Mate Brstilo¹,
Thomas Müller², Conrad M. Freuling²

Sylvatic rabies has been present in Croatia for more than three decades, with the red fox (*Vulpes vulpes*) as the main reservoir. The present epidemic of sylvatic rabies in Croatia started already in 1977 and in the past ten years the disease has become enzootic in the entire country and thus represents a considerable veterinary and public health threat.

A genetic characterization and phylogenetic analysis of rabies virus isolates (RABV) from Croatia was performed using panel of 32 selected rabies-positive brain samples from domestic and wild animals collected between 2008 and 2010. Based on the comparison of 367-nucleotide sequences of a conserved region of the nucleoprotein (N) gene (nucleotides 75–441), the phylogenetic analysis revealed a low genetic diversity of currently circulating RABV strains in Croatia. 18 RABV isolates mainly originating from Eastern Croatia clustered with the formerly established Eastern European (EE) lineage, and the rest (14) were identical with the West European (WE) group. Both phylogenetic groups seem to coincide in central regions on both sides along the Save River. A high sequence identity in the N gene of the RABV isolates from neighbouring countries was found.

Keywords: fox rabies, rabies, Croatia, molecular epidemiology

Die sylvatische Tollwut mit dem Rotfuchs (*Vulpes vulpes*) als Reservoir trat in Kroatien erstmals Jahr 1977 auf. Im vergangenen Jahrzehnt hat sich die Epidemie über das ganze Land ausgebreitet und stellt eine große Herausforderung für das öffentliche Veterinär- und Gesundheitswesen dar.

Um die Diversität der Tollwutviren (Rabies-Virus, RABV) in Kroatien zu untersuchen, wurden 32 Tollwut-positive Gehirnproben von Haus- und Wildtieren, die zwischen 2008 und 2010 getestet wurden, weiter charakterisiert. Basierend auf dem Vergleich der Nukleotid-Sequenzen einer konservierten Region des Nukleoprotein (N)-Gens (Position 75–441) ergab die phylogenetische Analyse eine geringe genetische Vielfalt der gegenwärtig zirkulierenden RABV-Varianten in Kroatien. 18 RABV-Isolate, die vorwiegend aus östlichen Regionen Kroatiens stammen, waren der osteuropäischen Linie (Eastern European, EE) zuzuordnen, während 14 dem westeuropäischen (Western European, WE) Typ entsprachen. Interessanterweise gibt es eine Überlappung der Verbreitungsgebiete in den zentralen Regionen Kroatiens, wo beide Varianten auf beiden Seiten entlang des Flusses Save gemeinsam vorkommen. Insgesamt zeigte sich auch eine hohe Sequenzidentität mit Isolaten aus benachbarten Ländern.

Schlüsselwörter: Fuchstollwut, Tollwut, Kroatien, Typisierung

Introduction

Rabies is caused by an infection with a single-stranded, negative-sense RNA lyssavirus species within the family *Rhabdoviridae* of the order *Mononegavirales* (Kuzmin, et al., 2010; Dietzgen, et al., 2011; Freuling, et al., 2011). The prototypic classical rabies virus (RABV) has its reservoirs in species of the order *Carnivora*, and also in bats in the Americas (World Health Organisation, 2005).

In Europe, sylvatic rabies with the red fox (*Vulpes vulpes*) as reservoir is considered to have emerged from the former Russian-Polish border and the region of Gdansk in Northern Poland (Steck and Wandeler, 1980). Within

two decades, i. e. 1950s–1970s the disease spread south-westwards across Europe reaching its western-most periphery in France in 1968 (Aubert et al., 2004).

The first evidence for the arrival of the fox-mediated rabies front in Croatia dates back to 1977 when a rabid fox was hunted near the village Bole in northern part of the country close to the Hungarian border. The rabies epidemic was presumably introduced by infected foxes from neighbouring Vojvodina and Hungary, since cases of fox rabies had already been reported earlier in those regions (Petrović, 1976; Schneider and Uhlmann, 1979). At the end of 1989, sylvatic rabies affected all continental territories of Croatia except the Dalmatian towns of Biograd and Dubrovnik, where rabies was detected in

TABLE 1: RABVs used in this study, their epidemiological information and GenBank accession numbers

Country	County	Isolate	Year isolated	Hostspecies	Accession	Reference
Croatia	Zagrebicka	C1/fox/08	2008	red fox	JF683622	This study
	Sisacko-Moslavacka	C2/sheep/08	2008	sheep	JF683635	This study
	Sisacko-Moslavacka	C3/fox/08	2008	red fox	JF683642	This study
	Varazdinska	C154/fox/08	2008	red fox	JF683629	This study
	Krapinsko-Zagorska	C159/rat/08	2008	rat	JF683630	This study
	Bjelovarsko-Bilogorska	C450/cat/08	2008	cat	JF683644	This study
	Karlovacka	C451/cat/08	2008	cat	JF683645	This study
	Karlovacka	C4/sheep/09	2009	sheep	JF683643	This study
	Karlovacka	C6/cat/09	2009	cat	JF683649	This study
	Pozesko-Slavonska	C7/goat/09	2009	goat	JF683650	This study
	Splitsko-Dalmatinska	C8/goat/09	2009	goat	JF683651	This study
	Splitsko-Dalmatinska	C9/polecat/09	2009	polecat	JF683652	This study
	Koprivnicko-Krizevacka	C10/fox/09	2009	red fox	JF683623	This study
	Primorsko-Goranska	C465/badger/09	2009	badger	JF683646	This study
	Vukovarsko-Srijemska	C479/dog/09	2009	dog	JF683647	This study
	Sisacko-Moslavacka	CRO_09SK/wolf	2009	wolf	GU134624	This study
	Sibensko-Kninska	C481/cat/09	2009	cat	JF683648	This study
	Brodsko-Posavska	C11/dog/10	2010	dog	JF683624	This study
	Varazdinska	C12/polecat/10	2010	polecat	JF683625	This study
	Viroviticko-Podravsk	C13/goat/10	2010	goat	JF683626	This study
	Pozesko-Slavonska	C14/dog/10	2010	dog	JF683627	This study
	Pozesko-Slavonska	C15/fox/10	2010	red fox	JF683628	This study
	Koprivnicko-Krizevacka	C16/sheep/10	2010	sheep	JF683631	This study
	Sisacko-Moslavacka	C17/cat/10	2010	cat	JF683632	This study
	Sibensko-Kninska	C18/wolf/10	2010	wolf	JF683633	This study
	Sisacko-Moslavacka	C19/fox/10	2010	red fox	JF683634	This study
	Sisacko-Moslavacka	C20/fox/10	2010	red fox	JF683636	This study
	Pozesko-Slavonska	C21/sheep/10	2010	sheep	JF683637	This study
	Koprivnicko-Krizevacka	C22/sheep/10	2010	sheep	JF683638	This study
	Pozesko-Slavonska	C23/sheep/10	2010	sheep	JF683639	This study
	Karlovacka	C24/fox/10	2010	red fox	JF683640	This study
	Sisacko-Moslavacka	C26/fox/10	2010	red fox	JF683641	This study
Bosnia and Herzegovina		8653 YOU	1986	wolf	U42704	Bourhy et al. (1999)
		86111 YOU	1986	red fox	U42706	Bourhy et al. (1999)
Bulgaria		BUL 7	2003	red fox	DQ300294	Johnson et al. (2007b)
Estonia		9339EST	1991	raccoon dog	U42707	Bourhy et al. (1999)
France		8661 FRA		hedgehog	U43434	Bourhy et al. (1999)
		9147 GSFRA		red fox	U22474	Kissi et al. (1995)
		9445 FRA		red fox	U42700	Bourhy et al. (1999)
Finland		9348 FIN		raccoon dog	U42716	Bourhy et al. (1999)
Germany		9202 ALL		red fox	U22475	Bourhy et al. (1999)
		9213 ALL		red fox	U42702	Bourhy et al. (1999)
		9212 ALL	1991	red fox	U22475	Kissi et al. (1995)
Hungary		9383 HON		red fox	U42998	Bourhy et al. (1999)
		9386 HON		red fox	U43000	Bourhy et al. (1999)
Italy		08RS 1981 Udine 2008		red fox	FJ424484	De Benedictis et al. (2008)
Lithuania		9345 LIT		dog	U43002	Bourhy et al. (1999)
Poland		8618 POL		raccoon dog	U22840	Kissi et al. (1995)
Russia		RV245		human	AY352475	Kuzmin et al. (2004)
Serbia		86106 YOU	1972	red fox	U22839	Kissi et al. (1995)
Slovenia		9494 SLN		red fox	U43008	Bourhy et al. (1999)
		SAD B19		vaccine	EF206709	Geue et al. (2008)

the mid of 1990s. Only islands in the Adriatic Sea have remained rabies free (Čač et al., 1994; Čač, 1996).

Between 1977 and 2010, a total of 108 190 domestic and wild animals were submitted for routine rabies testing in Croatia. Of the total submissions, 16 723 (15.5%) were positive (unpublished). Foxes represented the highest number of submissions (> 60%) followed by dogs and cats (unpublished). The last human rabies case was reported in 1964 (Kodrnja, 1970; Borčić, 1978). The fact that no further human rabies cases were recorded is mainly attributed to forceful implementation of a variety of measures such as quarantine, registration and mandatory vaccination of dogs, capture and destruction of stray dogs and cats, preventive vaccination of persons in high-risk occupational groups, strict application of post exposure prophylaxis, education campaigns and close cooperation between veterinary and public health institutions. Nevertheless, rabies incidence in Croatia has been increasing since 1988 reaching peaks of 1042 (17.9%) and 1061 (19.6%) rabies cases in 1999 and 2008, respectively, representing an increasing public and animal health problem.

Since the first field trials conducted in Switzerland in 1978 oral rabies vaccination (ORV) of foxes has become the method of choice for fox rabies control in Europe (Rupprecht et al., 2008). Due to vigorous ORV programmes terrestrial rabies virtually disappeared in large parts of Europe and today most Western European countries have achieved a rabies free status. Although ORV field trials had been conducted on the Istrian peninsula along common borders with Slovenia as early as 1991, a nationwide ORV programme was implemented in 2011 with financial help of the European Union.

European RABVs are part of the cosmopolitan lineage (Kissi et al., 1995), and several molecular studies increased the understanding of the diversity of RABV in Europe (Bourhy et al., 1999; Kuzmin et al., 2004; McElhinney et al., 2006; Metlin et al., 2007; Johnson et al., 2007a; McElhinney et al., 2008).

The most common nomenclature for phylogenetic lineages of RABVs in Europe are geographical-associated groups designated the North-Eastern Europe (NEE) group, the Eastern Europe (EE) group, the Western Europe (WE) group and Central Europe (CE) group (Bourhy et al., 1999). However, this nomenclature may not be adequate anymore (McElhinney et al., 2011), since RABV has now been eliminated from most of Western and Central Europe, and for example RABVs of the NEE group were found in Rumania (Turcitu et al., 2010).

Only recently, the degree of heterogeneity and virus diversity of RABVs from the Balkans including Bulgaria, Romania, Slovenia, and the former Republic of Yugoslavia (FRY) was elucidated (Johnson et al., 2007b; Turcitu et al., 2010; Rihtarič et al., 2011; McElhinney et al., 2011). However, no such comprehensive analysis has been performed for RABVs from Croatia. Therefore, the objective of the present study was to characterize a panel of selected RABVs from Croatia collected between 2008 and 2010, and thus complete the phylogenetic picture of RABV strains circulating on the Balkan peninsula.

Material and Methods

Of 17 628 animals submitted for rabies routine diagnostic to the Laboratory for Rabies and General Virology,

Croatian Veterinary Institute in Zagreb, Croatia, between 2008 and 2010, 2497 were positive with the direct fluorescent antibody test (DFA) (Dean et al., 1996). Whenever possible rabies positive brain specimens were archived and stored at -20°C . A panel of rabies positive brain samples ($N = 32$) from the entire country for the years 2008–2010 was selected for this study according to

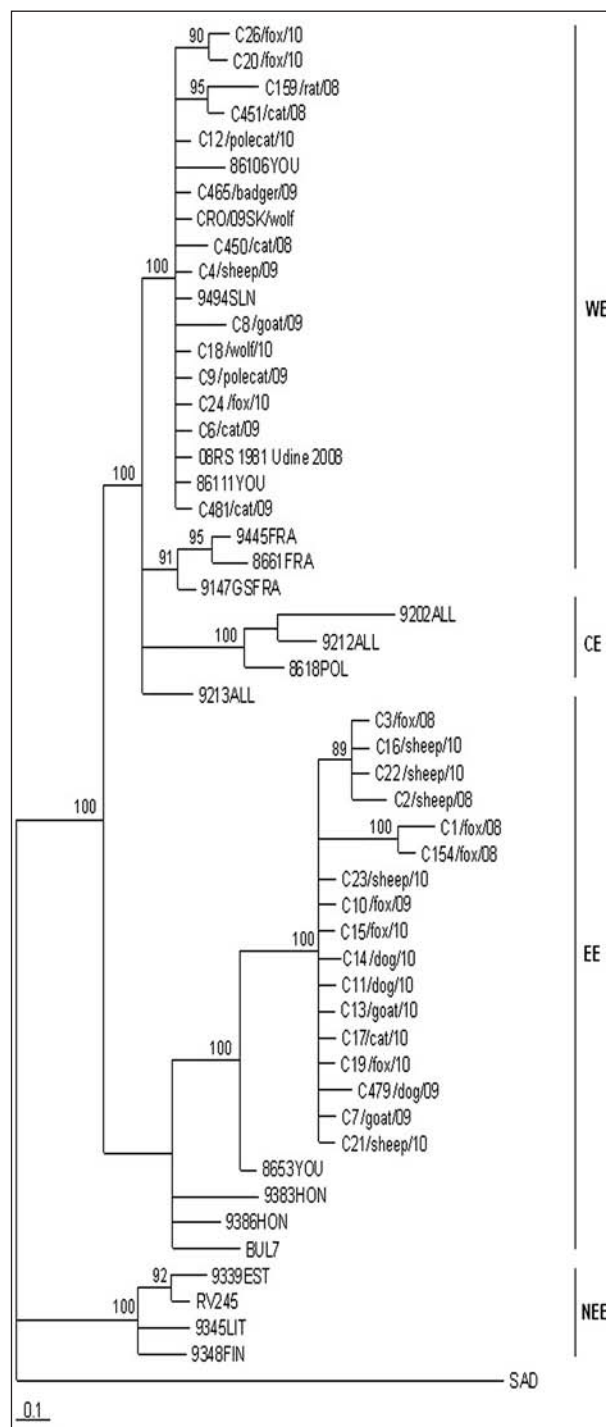


FIGURE 1: Phylogenetic analysis based on a 367 bp region of the RABV N gene comparing a panel of Croatian sequences with those from various regions of Eastern, Western, Central and Northern Europe. Formerly established phylogroups by Bourhy et al. (1999) are indicated. Posterior probability values (%) are shown for all nodes.

availability of the original archived brain material, geographical and species origin (Tab. 1).

For RNA extractions brain samples were initially homogenized in Dulbecco Minimum Essential Medium (DMEM) to gain 10% brain suspensions. RNA was extracted from supernatant of centrifuged suspensions using the RNEasy Mini Kit (Qiagen, Germany) and eluted in 50 µl final volume according to the manufacturer's instructions. For partial sequencing a one step reverse-transcription polymerase chain reaction (RT-PCR) was performed with 5 µl RNA and 2 pmol of pan-lyssavirus primer JW12 (5'-ATG-TAACACCYCTACAATG-3') and JW6DPL (5'-CAAT-TCGCACACATTTGTG-3') essentially as described (Heaton et al., 1997) using Superscript III with Platinum Taq polymerase (Invitrogen, USA) following the manufacturer's instructions in a 50 µl final volume. Amplification of the 606 bp fragment of the N gene was conducted on 2720 Thermal Cycler (Applied Biosystems, USA). Amplified products were visualized by agarose gel electrophoresis (1%) with ethidium bromide, and subsequently purified using Exosap (USB, Germany). Purified PCR products were sequenced in both directions by MacroGen Inc. (Korea) using the Big Dye sequencing kit (Applied Biosystems, USA). Nucleotide sequences generated in this study have been submitted

to GenBank and were assigned the following accession numbers: JF683622-JF683652.

To infer the genetic relationship of Croatian RABV, the panel of the 32 RABV N-gene sequences was compared to available published sequences from GenBank (Tab. 1) using the online BLAST program. Forward and reverse sequences for each isolate were aligned and from 517 bp nucleotide consensus sequences obtained a 367 bp part (nucleotides 75–441 of the challenge virus standard (CVS) reference strain; GenBank accession no. D42112) was used for further analysis. Sequence identities of nucleotides, as well as those of amino acids, were analyzed using the ClustalX implemented in Mega4 software (Tamura et al., 2007). Phylogenetic tree was calculated using MrBayes v3.0b3 (Huelsenbeck and Ronquist, 2001). In Bayesian Inference (BI) analysis (Larget and Simon, 1999) four incrementally heated Markov Chains were run for 1 000 000 generations (ngen = 1 000 000), sampling every 100 generations (samplefreq = 100), where 1250 samples were discarded (burnin = 1250). A consensus tree was constructed from the tree output file produced in the BI analysis using TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>) with the vaccine virus strain SAD B19 as outgroup.

TABLE 2: Positions of base substitutions characteristic for Croatian RABVs of EE lineage based on 367-nt fragment of N, starting from the translation initiation codon. The second base mutation of the codon 101 resulting in amino acid substitution (Asn101 to Ser101) is marked bold. The EE isolates are shaded with grey colour

nt position aa position	54 18	90 30	117 39	228 76	302 101
C450/cat/08	G	C	C	A	C
C451/cat/08
C159/rat/08	.	.	T	.	.
C4/sheep/09
C6/cat/09
C8/goat/09
C9/polecat/09
C465/badger/09
CRO/09SK/wolf
C481/cat/09
C12/polecat/10
C18/wolf/10
C20/fox/10
C24/fox/10
C26/fox/10
C1/fox/08	A	T	A	.	G
C2/sheep/08	A	T	G	G	G
C3/fox/08	A	T	A	G	G
C154/fox/08	.	T	A	.	G
C7/goat/09	.	T	A	G	G
C479/dog/09	.	T	A	G	G
C10/fox/09	.	T	A	G	G
C11/dog/10	.	T	A	G	G
C13/goat/10	.	T	A	G	G
C14/dog/10	.	T	A	G	G
C15/fox/10	.	T	A	G	G
C16/sheep/10	A	T	A	G	G
C17/cat/10	.	T	A	G	G
C19/fox/10	.	T	A	G	G
C21/sheep/10	.	T	A	G	G
C22/sheep/10	A	T	A	G	G
C23/sheep/10	.	T	A	G	G

Results

The 32 RABV isolates selected and characterized in this study comprise samples from foxes (N = 9), dogs (N = 3), cats (N = 5), wolves (N = 2), sheep and goats (N = 9), polecats (N = 2) and one each from a rat and a badger (Tab. 1). The samples originated from 15 out of 21 counties of mainland Croatia. For all samples, the amplicon obtained was the correct size (~600 bp).

Phylogenetic analysis, using a 367 bp region of the RABV nucleoprotein gene, places the Croatian sequences (N = 32) within two distinct lineages within the cosmopolitan RABV cluster (Fig. 1). 18 isolates mainly originating from Eastern Croatia clustered with the formerly established Eastern Europe (EE) lineage (Bourhy et al., 1999), whereas 14 were clustered with the Western Europe (WE) group (Fig. 1). Both phylogenetic groups seem to coincide in Central Croatian regions on both sides along the Save River (Fig. 2). Within these groups there is 2% average divergence between isolate sequences. Nucleotide and amino acid sequence identity from the EE and WE group ranged between 96.7% and 100%. Within the WE group most Croatian isolates (N = 14) were identical and also shared 100% sequence identity with recent isolates from Slovenia and Italy. No host specific pattern was observed considering the species involved (Fig. 1; Tab. 1).

Sequence analysis of the 32 RABV isolates revealed five variable positions within the aligned 367 bp fragment. Four silent nucleotide substitutions were characteristic exclusively for RABV isolates of the EE lineage (Tab. 2). A single nucleotide substitution resulted in an amino acid (aa) substitution from Asparagine to Serine (Asn101 to Ser101) and was characteristic exclusively for Croatian RABV isolates of the EE lineage. All analysed WE rabies viruses, including the Croatian RABVs had Alanine (GCC) at that position.

Discussion

This is the first time that a larger panel of RABV isolates collected in Croatia was characterized using partial N gene sequencing. For all samples RT-PCR corroborated previous FAT results. The phylogenetic analysis of partial N gene (367 nt) sequences of 32 representative positive samples collected from all mainland territories between 2008 and 2010 (Fig. 2) and subsequent comparison with GenBank sequences revealed the Croatian sequences belonging to the cosmopolitan lineage of rabies viruses that have been reported throughout the world (Kissi et al., 1995).

Specifically, RABVs from Croatia are either of the WE or EE lineage that is associated with the fox epidemic in Europe. The analysis of RABVs from a range of species indicates that the fox is the sole reservoir and vector of the disease in Croatia.

No RABV of the NEE and the CE lineages were found, supporting other studies that especially NEE may not have established in the Western Balkans (McElhinney et al., 2011), including Croatia.

EE strains are most prevalent in Eastern Croatia, while WE are exclusively found in the southern and western region. In central regions along the Save River both lineages are present (Fig. 2). The fact that there is an average of 2% genetic divergence between RABV sequences indicates a low genetic diversity of currently circulating rabies virus strains in Croatia. This is in accordance with recent findings from neighbouring regions in Italy, Slovenia, Montenegro and Bosnia and Herzegovina, where WE and EE strains were predominantly found, respectively (De Benedictis et al., 2011; Rihtarič et al., 2011; McElhinney et al., 2011). Although a limited panel was used in this study, the results from neighbouring countries indicate that the panel is representative for the circulating RABVs in Croatia. However, the existence of other variants cannot be excluded.

A Slovenian isolate collected in 2002 close to the Croatian border that shared the Serine at position 101, characteristic exclusively for Croatian RABV isolates of the EE lineage (Tab. 2), suggests that this variant has been circulating for more than eight years in the region, and that trans-border movement of infected animals occurs as previously described (Johnson et al., 2007b).

Interestingly, a higher degree of heterogeneity and virus diversity was found in Romania, Serbia, and Bulgaria with six, four and three RABV variants circulating, respectively (Turcitu et al., 2010; Johnson et al., 2007b; McElhinney et al., 2011).

One reason for the comparatively low genetic diversity of currently circulating RABV strains in the Western Balkans, including Croatia, could be that natural barriers such as rivers or high mountain ranges could have reduced the spread of distinct RABVs as shown before (Bourhy et al., 1999; Johnson et al., 2007b; McElhinney et al., 2011). In fact, rabies reached Croatia from the Northeast and it was calculated that rivers considerably reduced the speed of the epidemic in Croatia (Slavica et al., 2010). Given the distribution of the lineages in Croatia (Fig. 2) it is likely that the EE lineage was the first that eventually reached the Save River. WE on the other hand may have spread along the coast line and recently also reached this central area. The occurrence of both lineages on both sides of the river Save suggests, that it represents such natural barrier, but infected animals may

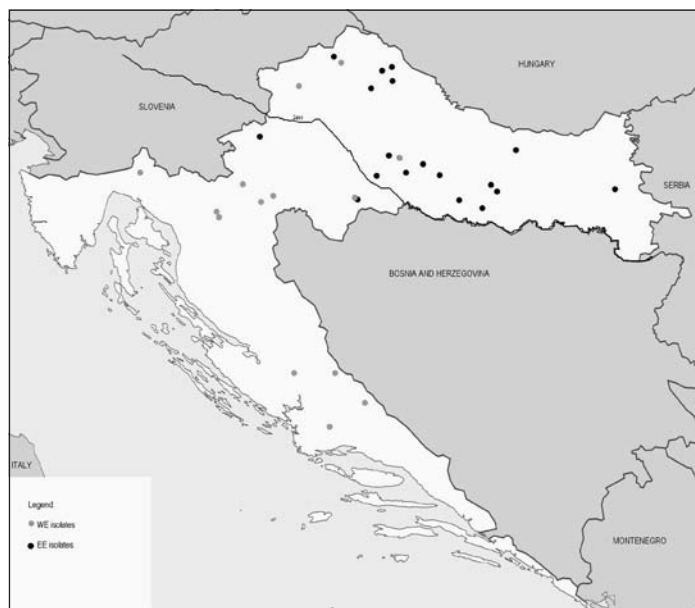


FIGURE 2: Map of Croatia showing the origin of samples and the occurrence of the two distinct lineages of RABV strains. The Save river is indicated. Grey and black dots represent virus strains belonging to WE and EE group of RABV, respectively.

cross using bridges etc. as seen before (Johnson et al. 2007b). Therefore, ORV programmes could make use of the Save when only limited resources are available that would not allow for covering the entire country. Such approach was well confirmed in the study of Russel et al. (2006).

Molecular epidemiological studies have helped to understand the spatio-temporal dynamics of RABV in Europe, and as rabies will eventually be eliminated in this region, they also provide a historical snapshot of RABV diversity.

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Conflict of interest: The authors declare that no competing interests exist.

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Address for correspondence:

Ivana Lojkić
Department of Virology
Croatian Veterinary Institute
Savska cesta 143
10000 Zagreb
Croatia
ilojkic@veinst.hr