

## Open Access

Berl Münch Tierärztl Wochenschr 125,  
249–254 (2012)  
DOI 10.2376/0005-9366-125-249

© 2012 Schlütersche  
Verlagsgesellschaft mbH & Co. KG  
ISSN 0005-9366

Korrespondenzadresse:  
ilojkic@veinst.hr

Eingegangen: 28.09.2011  
Angenommen: 15.11.2011

Online first: 07.05.2012

[http://vetline.de/zeitschriften/bmtw/  
open\\_access.htm](http://vetline.de/zeitschriften/bmtw/open_access.htm)

### Summary

### Zusammenfassung

U.S. Copyright Clearance Center  
Code Statement:  
0005-9366/2012/12505-249 \$ 15.00/0

## Short communication

Department of Virology, Croatian Veterinary Institute, Zagreb, Croatia<sup>1</sup>  
Institute of Epidemiology, WHO Collaborating Centre for Rabies Surveillance and  
Research, Friedrich-Loeffler-Institute, Federal Research Institute of Animal Health,  
Wusterhausen, Germany<sup>2</sup>

## Diversity of currently circulating rabies virus strains in Croatia

### *Diversität der derzeit zirkulierenden Tollwutviren in Kroatien*

Ivana Lojkić<sup>1</sup>, Željko Čač<sup>1</sup>, Tomislav Bedeković<sup>1</sup>, Nina Lemo<sup>1</sup>, Mate Brstilo<sup>1</sup>,  
Thomas Müller<sup>2</sup>, Conrad M. Freuling<sup>2</sup>

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 Gehirnpriproben 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                | Zagrebacka             | 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-Podravska  | 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).

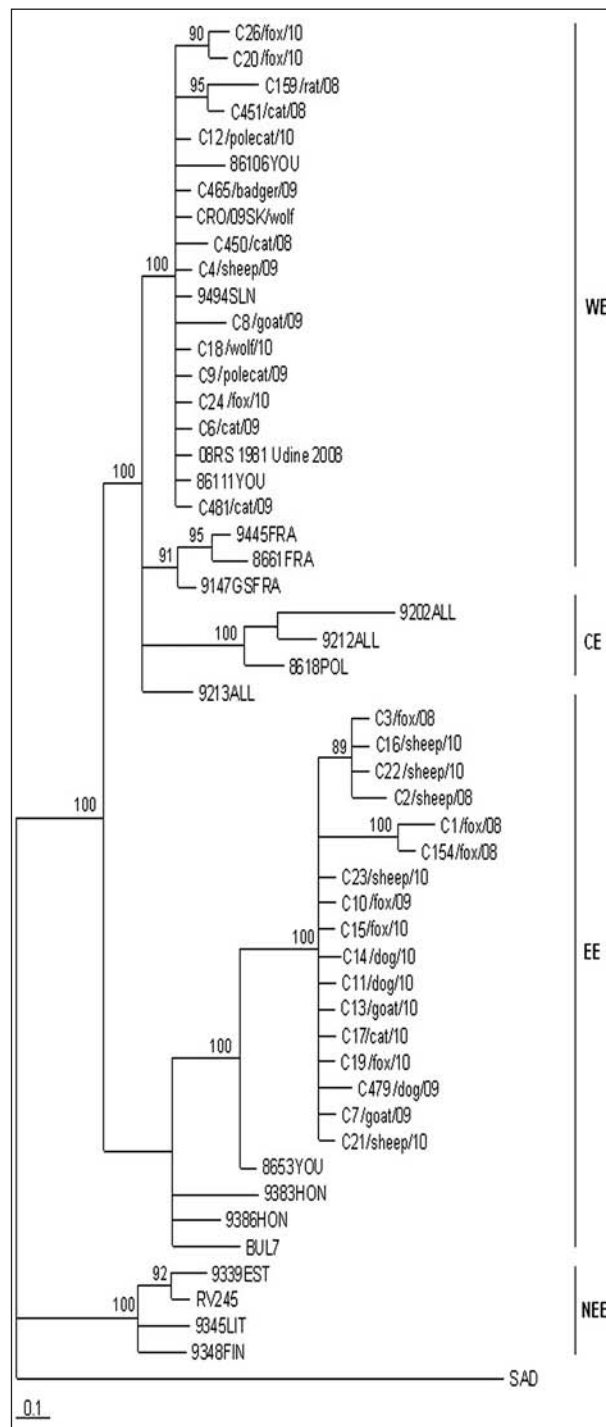
The most common nomenclature for phylogenetic lineages of RABVs in Europe are geographical-associated groups designated the North-Eastern Europe (NEE) group, the Eastern Europe (EE) group, the Western Europe (WE) group and Central Europe (CE) group (Bourhy et al., 1999). However, this nomenclature may 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    | 54 | 90 | 117 | 228 | 302      |
|----------------|----|----|-----|-----|----------|
| aa position    | 18 | 30 | 39  | 76  | 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   | .   | <b>G</b> |
| C2/sheep/08    | A  | T  | G   | G   | <b>G</b> |
| C3/fox/08      | A  | T  | A   | G   | <b>G</b> |
| C154/fox/08    | .  | T  | A   | .   | <b>G</b> |
| C7/goat/09     | .  | T  | A   | G   | <b>G</b> |
| C479/dog/09    | .  | T  | A   | G   | <b>G</b> |
| C10/fox/09     | .  | T  | A   | G   | <b>G</b> |
| C11/dog/10     | .  | T  | A   | G   | <b>G</b> |
| C13/goat/10    | .  | T  | A   | G   | <b>G</b> |
| C14/dog/10     | .  | T  | A   | G   | <b>G</b> |
| C15/fox/10     | .  | T  | A   | G   | <b>G</b> |
| C16/sheep/10   | A  | T  | A   | G   | <b>G</b> |
| C17/cat/10     | .  | T  | A   | G   | <b>G</b> |
| C19/fox/10     | .  | T  | A   | G   | <b>G</b> |
| C21/sheep/10   | .  | T  | A   | G   | <b>G</b> |
| C22/sheep/10   | A  | T  | A   | G   | <b>G</b> |
| C23/sheep/10   | .  | T  | A   | G   | <b>G</b> |

## 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.

## Acknowledgement

This research was supported by grant No. 048-0481186-1183 from the Ministry of Science, Education and Sports, Republic of Croatia and by the German Federal Ministry for Education and Research (BMBF, grant 01K11016A), Germany.

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

## References

- Aubert MF, Cliquet F, Smak JA, Brochier B, Schon J, Kappeler A (2004): Rabies in France, The Netherlands, Belgium, Luxembourg and Switzerland. In: King AA, Fooks AR, Aubert M, Wandeler AI (eds.): Historical perspective of rabies in Europe and the Mediterranean Basin. OIE (World Organisation for Animal Health), Paris, 129–145.
- Borčić B (1978): Rabies is spreading again in Croatia! Lijec Vjesn 100: 563–564 (in Croatian).
- Bourhy H, Kissi B, Audry L, Smreczak M, Sadkowska-Todys M, Kulonen K, Tordo N, Zmudzinski JF, Holmes EC (1999): Ecology and evolution of rabies virus in Europe. J Gen Virol 80: 2545–2557.

- Čač Ž, Brstilo M, Rajić A (1994):** The Rabies in Croatia 1986–1992. *Vet Stanica* 25: 159–166 (in Croatian).
- Čač Ž (1996):** Sylvatic Rabies in Croatia: Appearance, spreading and current situation. *Infekt Glasnik* 16: 1–4 (in Croatian).
- De Benedicts P, Battisti C, de Dacheux L, Marciano S, Ormelli S, Salomoni A, Caenazzo S, Lepelletier A, Bourhy H, Capua I, Cattoli G (2011):** Lyssavirus detection and typing using pyrosequencing. *J Clin Microbiol* 49: 1932–1938.
- Dean DJ, Abelseth MK, Athanasiu P (1996):** The fluorescence antibody test In: Meslin FX, Kaplan MM, Koprowski H (eds.) *Laboratory techniques in rabies*. World Health Organization, Geneva, 88–93.
- Dietzgen RG, Calisher CH, Kurath G, Kuzmin IV, Rodriguez LL, Stone DM, Tesh RB, Tordo N, Walker PJ, Wetzel T, Whitfield AE (2011):** Family *Rhabdoviridae*. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds.), *Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses*. Elsevier, San-Diego (in press).
- Freuling CM, Beer M, Conraths FJ, Finke S, Hoffmann B, Keller B, Kliemt J, Mettenleiter TC, Muhlbach E, Teifke JP, Wohlsein P, Müller T (2011):** Novel Lyssavirus in Natterer's Bat, Germany. *Emerg Infect Dis* 17: 1519–1522.
- Geue L, Schares S, Schnick C, Kliemt J, Beckert A, Freuling C, Conraths FJ, Hoffmann B, Zanoni R, Marston D, McElhinney L, Johnson N, Fooks AR, Tordo N, Müller T (2008):** Genetic characterisation of attenuated SAD rabies virus strains used for oral vaccination of wildlife. *Vaccine* 26: 3227–3235.
- Heaton PR, Johnstone P, McElhinney LM, Cowley R, O'Sullivan E, Whitby JE (1997):** Heminested PCR assay for detection of six genotypes of rabies and rabies-related viruses. *J Clin Microbiol* 35: 2762–2766.
- Huelsenbeck JP, Ronquist F (2001):** MrBayes: Bayesian inference of phylogeny. *Bioinformatics* 17: 754–755.
- Johnson N, Dicker A, Mork T, Marston DA, Fooks AR, Tryland M, Müller T (2007a):** Phylogenetic comparison of Rabies viruses from an outbreak on the Svalbard Islands. *Vector Borne Zoonotic Dis* 7: 457–60.
- Johnson N, Fooks AR, Valtchovski R, Müller T (2007b):** Evidence for trans-border movement of rabies by wildlife reservoirs between countries in the Balkan Peninsula. *Vet Microbiol* 120: 71–76.
- Kissi B, Tordo N, Bourhy H (1995):** Genetic Polymorphism in the Rabies Virus Nucleoprotein Gene. *Virology* 209: 526–537.
- Kodrnja E (1970):** Rabies movement and eradication in Croatia 1946–1969. *Vet Arhiv* 40: 303–313 (in Croatian).
- Kuzmin IV, Botvinkin AD, McElhinney LM, Smith JS, Orciari LA, Hughes GJ, Fooks AR, Rupprecht CE (2004):** Molecular epidemiology of terrestrial rabies in the former Soviet Union. *J Wild Dis* 40: 617–663.
- Kuzmin IV, Mayer AE, Niezgodna M, Markotter W, Agwanda B, Breiman RF, Rupprecht CE (2010):** Shimoni bat virus, a new representative of the Lyssavirus genus. *Virus Res* 149: 197–210.
- Larget B, Simon DL (1999):** Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Mol Biol Evol* 16: 750–759.
- McElhinney LM, Marston DA, Freuling CM, Cragg W, Stankov S, Lalosević D, Lalosević V, Müller T, Fooks AR (2011):** Molecular diversity and evolutionary history of rabies virus strains circulating in the Balkans. *J Gen Virol* 92: 2171–2180.
- McElhinney LM, Marston D, Johnson N, Black C, Matouch O, Lalosevic D, Must, K, Smerczak M, Zmudzinski JF, Botvinkin AD, Aylan O, Vanek E, Cliquet F, Müller T, Fooks AR (2006):** Molecular epidemiology of rabies viruses in Europe. *Dev Biol (Basel)* 125: 17–28.
- McElhinney LM, Marston D, Stankov S, Tu C, Black C, Johnson N, Jiang Y, Tordo N, Müller T, Fooks AR (2008):** Molecular epidemiology of Lyssaviruses in Eurasia. In: Dodet B, Fooks AR, Müller T, Tordo N (eds.), *Towards the elimination of rabies in Eurasia*. Karger, Basel, 125–131.
- Metlin AE, Rybakov S, Gruzdev K, Neuvonen E, Huovilainen A (2007):** Genetic heterogeneity of Russian, Estonian and Finnish field rabies viruses. *Arch Virol* 152: 1251–1258.
- Petrović M (1976):** Review on the situation of rabies in Yugoslavia. *Med Pregl* 29: 219–225.
- Rihtarič D, Hostnik P, Grom J, Toplak I (2011):** Molecular epidemiology of the rabies virus in Slovenia 1994–2010. *Vet Microbiol* 152: 181–186.
- Rupprecht CE, Barrett J, Briggs D, Cliquet F, Fooks AR, Lumlerdacha B, Meslin FX, Müller T, Nel LH, Schneider C, Tordo N, Wandeler AI (2008):** Can rabies be eradicated? *Dev Biol (Basel)* 131: 95–121.
- Russell CA, Real LA, Smith DL (2006):** Spatial Control of Rabies on Heterogeneous Landscapes. *PLoS ONE* 1(1): e27. doi: 10.1371/journal.pone.0000027.
- Schneider LG, Uhlmann W (1979):** Rabies in individual countries. *Rabies Bull Eur* 3: 10–13.
- Slavica A, Severin K, Čač Ž, Cvetnić Ž, Lojkić M, Deždek D, Konjević D, Pavlak M, Budinščak Z (2010):** A model of the spatial spread of sylvatic rabies on Croatian territory over the period of thirty years. *Vet Stanica* 41: 199–210 (abstract in English).
- Steck F, Wandeler A (1980):** The epidemiology of fox rabies in Europe. *Epidemiological review* 2: 71–96.
- Tamura K, Dudley J, Nei M, Kumar S (2007):** MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Mol Biol Evol* 24: 1596–1599.
- Turcitu M, Barboi G, Vuta V, Mihai I, Boncea D, Dumitrescu F, Codreanu M, Johnson N, Fooks A, Muller T, Freuling C (2010):** Molecular epidemiology of rabies virus in Romania provides evidence for a high degree of heterogeneity and virus diversity. *Virus Res* 150: 28–33.
- World Health Organisation (2005):** Expert Consultation on Rabies, First report. *World Health Organ Tech Rep Ser* 931: 1–121.

**Address for correspondence:**

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