

**University of Veterinary Medicine
Doctoral School of Veterinary Science**

Molecular epidemiology of rabies in Hungary

Summary of PhD thesis

Barbara Forró



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University of Veterinary Medicine
Doctoral School of Veterinary Science

Supervisor:

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Dr. Krisztián Bányai.

Institute for Veterinary Medical Research

Centre for Agricultural Research

Hungarian Academy of Sciences

Introduction

The *Lyssavirus* genus includes the zoonotic rabies virus that affects the nervous system by causing infectious brain and spinal cord inflammation/ encephalomyelitis. Rabies virus (RABV) is, the type species of the genus, estimated to cause 59 000 human deaths annually in over 150 countries, most of the cases occurring in Africa and Asia.

The last human case in Hungary was documented in 1994. The urban epidemiological cycle has diminished thanks to the obligatory vaccination of domestic dogs. At present in Hungary the emphasis is on the control of the sylvatic cycle that is primarily maintained by red foxes. Since 1992 rabies vaccine baits have been released into the field for fox immunisation twice annually. Proved by surveillance the oral rabies vaccination in Hungary is so successful that there were no documented RABV cases in 2011 and 2012. However in August 2013 an unexpected outbreak was reported around the city of Kecskemét that lasted for 15 months. Following the outbreak only one or two RABV cases were reported annually. The surveillance of bats rabies (EBLV) in Hungary takes places only with passive monitoring. Seven cases were reported based on examination of 144 samples from 1977 to 2017.

Previously there were no precedents of extensive molecular epidemiology analysis of genetic and epidemic features of RABV and EBLV in Hungary. We aimed at filling this gap, therefore we sequenced RABV (n=61) and EBLV-1 (n=2) genomes, isolated between 1996 and 2018 in Hungary. In addition we aimed to determine the origin of the unexpectedly emerged RABV outbreak in the central region of Hungary during 2013-2014. We also made attempts to analyse the spatial dispersion and the lineage-specific evolution of the RABV strains in Hungary. Our intention was also to find the spreading direction of EBLV-1a and EBLV-1b lineages in Europe based on evolutionary examination of the collected scarce data of EBLV detected in Hungary and of the available European sequences.

Besides that, we sequenced the genome of the first RABV vaccine-derived case in Hungary. We performed genome sequencing from five Lysvulpen oral vaccine samples that had been used between 2012 and 2015 in the Hungarian oral vaccination campaign; thereby we could examine the genetic variability of the vaccine genomes.

Objectives

The aim was to examine the whole genomes of identified Hungarian rabies (RABV and EBLV) samples with molecular epidemiological and evolutionary methods. An objective was to study the RABV and EBLV spatial dispersion inside and between countries with the example of the Hungarian outbreak that recently occurred (2013-2014).

Another goal was to develop a DIVA-TaqMan RT-PCR method for differentiation of Eastern-European wild RABV and vaccine strains. We felt the need for such a method because of the recently identified vaccine-derived RABV case in Hungary.

To get a better understanding of the differences of the variant lineages we examined those proteins that claimed to be responsible mostly for the virus's pathogenicity.

Materials and methods

Origin of the samples

The examined samples of the study were supplied by the National Food Chain Safety Office Veterinary Diagnostic Directorate's (NFCSO VDD) Virology laboratory; all of them were collected via active or passive surveillance.

In this study, we performed whole genome sequencing of 61 RABV originating from red foxes (*Vulpes vulpes*) (n=53), cattle (*Bos taurus*) (n=2), cat (*Felis catus*) (n=1), deer (*Capreolus capreolus*) (n=1) 2, dog (*Canis lupus*) (n=1), goat (*Capra aegagrus hircus*) (n=3); and two EBLV samples originating from serotine bat (*Eptesicus serotinus*). In addition we sequenced five Lysvulpen bait vaccines and one vaccine induced positive RABV sample from red fox and subsequent passages of this isolate (mouse n=1, N2a n=2).

Molecular methods used for rabies samples

All the samples were subjected to RNA isolation, then reverse transcription was performed by random primers to synthesize cDNA. Each RABV and EBLV strains were amplified with partially overlapping primers therefore amplicons were generated covering the whole genomes. Rabies diagnostic primers for the N and G gene were used as positive control during the PCR. For further molecular methods PCR products were isolated from agarose gel.

A DIVA (differentiates infected from vaccinated animals) TaqMan RT-PCR assay was designed to differentiate between vaccine derived and Eastern-European wild-type RABV strains. Primers and probes were designed by the whole genome alignment of Eastern-European (n=28) and vaccine strains (n=7). The degenerate primers amplified 113 nucleotide regions of the polymerase gene. The vaccine probe (FAM) is only able to attach to the vaccine samples, although the conservative probe (HEX) attaches to vaccine and wild RABV strains.

The amplicons belonging to the same isolates were mixed based on light emitting intensity seen in the agarose gel. Then the samples were subjected to whole genome sequencing by using semiconductor sequencing on an Ion Torrent PGM machine. The draft genome's homopolimer and low coverage regions were validated by Sanger sequencing using our designed primers

Bioinformatics methods

The sequencing output were subjected to quality control before processing of the raw reads. The adapter regions were trimmed, the short and useless quality reads were sorted out. Each sample was assembled by reference mapping. The JQ944708 accession number sequence was used for reference mapping of the 31183 RABV sample, then the resulted consensus was used as reference for the rest of the RABV sequences. EF206708 genome from the GenBank was the reference for the vaccine strains. The EBLV reads were mapped to the NC009527 accession number genome. The resulted consensus genomes were manually corrected and annotated. The above-mentioned bioinformatics steps were performed by Geneious 9.1.8 (G9.1.8) software.

For the evolutionary and phylogeographical analyses, the whole genomes were aligned together by the MAFFT algorithm integrated into the G9.1.8 software. The probability of recombination was tested by the RDP4 program. Nucleotide diversity was calculated by DnaSP6. Selective pressure was examined with the Datamonkey webserver algorithms. Phylogeografic trees were generated by the MEGA 6.06. The substitutional models for the phylogeographical analyses were tested by Jmodeltest and MEGA 6.06. BEAST v 1.10.0 software package was used for the Bayesian evolutionary modelling and simulating. BEAUti v 1.10.0 was used to set the substitutional models, the traits (e.g. sampling dates, sampling place), molecular clock, population prior and the sufficient iteration applicable to and used for the sequencing data. For quick and efficient analyses BEAGLE was used. The output log files of the BEAST program were combined by the LogCombiner v1.10.0 and Tracer v 1.6 was used for visualization of the resulted substitutional rates and the verification of the Markov chain convergence by the ESS (Effective Sample Size). The tree-outputs of the BEAST also were combined by the LogCombiner v1.10.0 then the TreeAnnotator summarised the results and generated the Maximum clade credibility (MCC) tree. The time scaled MCC trees were visualized with FigTree v 1.4.3. Spread3 was utilized for rendering the results on the map of Europe. The nucleotide sequences were translated into amino acid sequences with G9.1.8. For the structural modelling of the glycoprotein gene in different lineages the I-Tasser online program and SwissModel software were utilized. The resulted pdb files were visualized by Chimera 1.11.

Results

The molecular epidemiology and evolution of the Hungarian RABV genomes (2006-2018)

The alignment of RABV genomes (2006 – 2018) was 11 799 nucleotide (nt). The RABV genomes' identity varied between 94.36%-100%. A phylogeny tree was created to find the possible lineages. Three possible lineages were detected, the II. and III. cluster's identities (II.: 98.75% – 99.94%; III.: 98.64% – 100%) were more variable than strains in the I. cluster (I.: 99.4 % – 100%). Time scaled tree was generated to estimate the time of the different lineages' separation and to gain information from the epidemiological events. The most recent common ancestor of Hungarian RABV strains, identified since 2006, was separated 170-180 years ago into I., II. and III. clusters. All the RABV samples that belong to the I. lineage were identified within a 100 km distance, during consecutive years. In this sequence group the sample from Nagyhegyes segregated earlier in 1990 (± 7 years) from the rest of the strains in the cluster. The sample from Pusztaradvány was the first sample from the II. cluster to separate around 1970. Interestingly, the rest of the sequences in this group were found ~250 km away in South Hungary in the next two years. Similar tendency was found in the III. cluster, the earliest isolated sample from Pocsaj (2010) grouped with samples from 2016-2017 detected ~100km away. The whole genome sequencing helped shedding light on the fact that III.b subcluster (2013-2014 samples) contains two separate lineages (III.b1 and III.b2), which was not separated during the 2013-2014 outbreak but earlier 2008 (95%HPD, 2007-2010). Substitution rate was estimated for 2006-2018 RABV genome data, which is $1.75E-4$ nucleotide/position/years (95% HPD: $1.14E-4$ – $2.38E-4$). Substitution rate was also estimated for the coding sequences in the genome. The L gene showed the lowest rate $2.17E-4$ nucleotide/position/years). The observed pace difference of evolutionary change raised the possibility of some sort of selective pressure behind the changes. Hence, we examined which codons are under positive selection pressure in RABV CDS. According to our examinations in RABV strains detected between 2006 and 2018, there was no permanent selection pressure. The analysis of CDS regions of RABV genomes showed that the glycoprotein and the phosphoprotein genes are the most variable regions. We found cluster-specific difference in some amino acid positions in the glycoprotein and phosphoprotein genes. However, there were no changes in the phosphoprotein C-terminal domain and the glycoprotein sequences were also conservative in the nAChR and p75 neurotrophin binding positions.

The identified sequences from the 2013-2014 rabies outbreak grouped into the III.b cluster. This sequence data was very homogeneous ($\pi=0.00087$) and covered a very short interval (2 years), therefore posterior density interval (HPD) is less accurate in this estimation. The evolutionary estimations (n=42, using fox samples) gave the same results for the time of the two subclusters' (III.b1, III.b2) separation (2006; 95%HPD: 1998-2010), just like the more complex analysis of the 2006-2018 data (2008; 95%HPD: 2007-2010). The 2013-2014 outbreak spread between the Danube and the Tisza rivers. We wanted to estimate the virus's spatial spread too. According to the result of the analysis the rate of spatial spread was 16.7 km (95% HPD: 6.4-27.6 km/y). Considering the spatial spread rate and the data of the MCC time scaled tree, the results imply that the virus started to spread earlier than the outbreak was noticed.

Phylogeographical analysis of the partial nucleoprotein and glycoprotein genes from European and Hungarian RABV sequences

The analysis of RABV genomes from Hungary (2006-2018) did not give answers to the relations with the RABV strains from neighbouring countries. Therefore, we collected RABV sequences available in the GenBank originating from European countries. We included in the analysis the Hungarian RABV sequences, detected from the 1990s, available in the GenBank (n=6) and the reported case from 1996 sequenced by us. Because of the insignificant number of whole genomes available in GenBank we could only use N and G gene partial RABV sequences. European RABV partial N gene sequences originated from 20 country, were available from 1972 to 2017 interval (n=123, 571 nt). The aligned N gene sequences' identity was between 88.9%-100%. The collected RABV partial G gene (n=198, 672 nt, identity 91.4%-100%) represented 14 countries and included Hungarian (1991-2017) and all available European sequences from the GenBank (1882-2017). According to the results of the RABV partial N gene sequence data analysis, all sequences genetically characterized and identified in our country grouped into two large clades: the Middle European and the Eastern European (Kuzmin's system). The Hungarian RABV strains grouped into the III.b cluster separated around 2000 (95% HPD: 1996-2006) from the close relative Romanian RABV strains. According to the result, it is possible that the 2013-2014 Hungarian outbreak's parental strains were from Romania. We assume at least 5 independent introductions in the Middle European clade and in the Eastern European clade 6 independent introductions seems possible.

Detection of RABV vaccine from red fox

A one and a half years old red fox was shot in Csabasabadi (Békés county) in November 2015. We sequenced the genome of the virus sample from the fox, the mouse passage of it, and two propagations on N2a cell line (n=4). Vaccine bait samples from the Hungarian oral rabies vaccination (ORV) campaign (n=5) were also sequenced. The Csabasabadi samples proved to be identical to the Lysvulpen vaccine used in the campaign. Phylogenetic analysis of the whole genome sequences showed that Lysvulpen vaccine batches used during most recent Hungarian ORV campaigns and the RABV found in the Csabasabadi fox are more closely related to the SAD Bern based vaccine viruses ($\geq 99.9\%$ nt similarity, ≤ 6 nt substitutions) than to the original Lysvulpen vaccine strain (GenBank accession number, EF206708). A DIVA TaqMan RT-PCR assay was designed for the quick analysis of vaccine strains and Eastern-European wild-type RABV strains circulating in Hungary.

Molecular epidemiology of EBLV-1 infections

The first Hungarian EBLV-1 case was detected in 1999, a total of seven cases were documented by 2017. We performed whole genome sequencing on two of the Hungarian samples: the 22540_2011 and 52206_2015. We performed phylogenetic and evolutionary analysis of the Hungarian isolates and all the available EBLV-1 genomes from the GenBank (n=100), these represented 10 countries and were detected between 1968 and 2015. Based on the EBLV-1 phylogenetic reconstruction, the examined genomes grouped into two main lineages, which is EBLV-1a (n=44) and EBLV-1b (n=56). The EBLV-1a grouped into two more clusters, and the EBLV-1b separated into four clusters. Both tested Hungarian bat rabies viruses were included in the EBLV-1a_I subcluster. The nucleotide diversity of the all EBLV-1 genomes were $\pi=0.02968$, the two main cluster's nucleotide diversity were around ~ 0.01 (0.01309 and 0.01798). The EBLV-1b_II group were the most variable subcluster. The analysed EBLV-1 genomes estimated substitutional rate was $3.583E-5$ (95%HPD: $2.55E-5 - 4.61E-5$). We found in this data that the phosphoprotein, matrix protein and the glycoprotein genes' evolution is faster than the polymerase coded by the L CDS, however the difference is insignificant (at most 1.2X). According to the time scaled tree two subclusters of the EBLV-1 were separated around the 1300s. The Hungarian sequences grouped with Slovakian, Polish and Danish isolates and separated from the Slovakian around the 1950s. The phylogenetic and the time scaled tree confirmed that the EBLV-1a group includes Eastern European genomes too; however the EBLV-1b lineages contained only Western European sequences. Our assumption about the possible directions of the EBLV-1a and EBLV-1b

spreading needed to be confirmed by statistical approaches, therefore, we calculated the evolutionary estimation posterior probability and Bayes-factors. Viruses belong to the EBLV-1a cluster mainly spread towards east. The EBLV-1a viruses detected in Hungary were probably introduced from Poland. The EBLV-1b strains spread from Western Europe towards the east. We examined the possible selection pressure on EBLV-1 genes. The 244th amino acid (aa) in the glycoprotein gene and the 141th aa in the phosphoprotein gene of the EBLV-1a genomes are under positive selection pressure. We examined the cluster specific variability of the proteins that play an important role in the pathogenicity with aa alignments of G and P coding regions. We found cluster specific aa in the EBLV-1 glycoprotein sequences in the 19th and 224th positions.

Discussion

The molecular epidemiology of the Hungarian RABV genomes

The 2013-2014 rabies outbreak was detected between the Danube and Tisza rivers. Based on the detected cases the virus spread in a ~10 000 km² area. Since 2008 there was no oral vaccination in this region until the outbreak occurred. The ORV took places only close to the southern and eastern borders in a 50km zone, between 2008 and 2013. Two years before the 2013-2014 outbreak there were no RABV cases diagnosed in Hungary. Then in August 2013 a rabid fox was found in middle of the country, in Kecskemet. By October 2014, 46 additional RABV infections were documented. The analyses of the whole genome sequences and the evolutionary estimations pointed out, the detected RABV samples from the 2013-2014 outbreak groups into two lineages, which were probably separated around 2008. Predecessors of the Central Hungarian rabies epidemic were already split into two genetic branches, and this event could have occurred in areas that were not subjected to surveillance. Based on mathematical models of already known and generally accepted estimations, rabies distributed by red foxes spreads 30-60 km annually. This value is influenced by many factors (relief, weather, population density, vaccination against rabies). In case of less susceptible foxes the spatial distribution is for instance 10-25 km/year according to the literature. Based on the genome sequences of the 2013-2014 outbreak, the origin of the samples and the time of identification, the spatial spread (~17 km/year) was slow, which is supported by the literature. All this can only be interpreted as if the virus had been circulating unnoticed for years in the affected area. To the question, why did the 2013 epidemic broke out in the middle of the country, one of the possible explanations is that the virus could have been incubating and spreading in small mammals (beaver, otter, and muskrat) as these species can easily cross the Tisza. There is also a possibility that the virus

incubated in (badger, marten, raccoon dog, and raccoon), in this case these hosts would have needed longer time to reach the Danube-Tisza area, where the outbreak has happened. The active monitoring only includes red foxes, however the passive surveillance involves all possible host. Thanks to the Hungarian authorities monitoring from 1987 to 2018 we are aware of a rabid raccoon dog (n=1), badger (n=9), and small mustelids (n=59) cases. The peculiarities of small mammalian migration and the fact that bait vaccine primarily attracts foxes (although badgers eat it sometimes), seems to support the possibility that small mammals played a role in spreading rabies, which could have resulted in the occurrence of 2013-2014 rabies outbreak.

Based on the calculation of 2013-2014 outbreak caused by the III.b cluster viruses, it is worth rethinking the dynamics of the Hungarian RABV epidemics. Hungarian rabies cases were detected inside of the actual vaccination zone from 2006 to 2010 and after 2014 thanks to the surveillance. RABV infected wild and domesticated animal cases were detected sporadically many times (i.e. Pusztaradvány, 2008, II.a cluster; Nagyhegyes, 2007, I.b cluster; Pocsaj, 2010, III.a cluster) that showed wider differences on molecular level. In other cases, the genomes of the sporadic isolates were closely related, which can be interpreted as a sign of prolonged latent epidemic (e.g. around Szerencs, 2016-2017, III.c cluster; around Nyírlugos, 2006-2009, I.a cluster). Taken in consideration the results of phylodynamic examinations of the detected cases isolated at similar time around the same foci, we concluded that besides the identified cases, unknown number of undetected rabies cases possibly occurred. However, it is hard to estimate the number of affected individuals as it was represented by a few cases only.

The calculations mentioned above showed that molecular epidemiology and evolutionary examinations based on whole genome sequences can help to provide a more accurate picture of the development and dynamic of an epidemic in a given area. However, we can only gain more information of the origin of the virus in question when we have enough published data from neighbouring countries. We analysed more than a hundred European and 67 Hungarian partial RABV sequences. The Hungarian RABV lineage originated from 1990s was not detectable in Hungary from 2000s. According to the MCC tree based on partial N and G gene sequences detected from 2006-2017, the three Hungarian RABV clusters were separated into further subclusters. We assume that in the recent Hungarian epidemics, we documented partially independent emergence of these RABV clusters. Focusing on 2013-2014 RABV outbreak the N gene sequence analysis of the III.b cluster led us to conclude that the origin of the virus was Romania, but the split in the III.b cluster probably took place in Hungary. For proper evaluation and interpretation of data the

molecular epidemiology methods' standardization would be useful, so whole genes or as far as possible whole genomes would be worth to use for analysis.

Vaccine induced rabies detection from a red fox was another interesting feature of our research, which is the first case in Hungary. The bait vaccines contain live replication-competent attenuated virus strains that may cause vaccine-induced rabies. Laboratory experiments also support this statement. Sporadic infections of wild animals by SAD Bern and SAD B19 vaccines strains were documented in Austria, Germany, Latvia, Romania, Switzerland, Slovenia, after ORV. The Csabaszabadi virus sample originated from fox and Lysvulpen vaccine batches used in the ORV showed 99.9% genome identity, furthermore the tetracycline marker test supported that this infection was caused by a vaccine virus strain used during the previous vaccination campaign. Because of the importance of monitoring such cases, we felt the need for an assay to differentiate between vaccine strain and Eastern-European wild-type RABV strains therefore we designed a DIVA TaqMan RT-PCR method.

The intense rabies elimination program remains successful in Hungary, however the neighbouring countries (Ukraine, Romania, and Serbia) have not been successful yet in rabies elimination. Therefore sylvatic rabies's occasional appearance is to be expected still in Hungary. These cases could be presumed sporadically or epidemically. Nowadays the ORV and the monitoring are organized along EU directives in Hungary. This system is very reliable in detecting RABV cases in a region that is under surveillance. It is important to emphasize that closer cooperation is needed with the neighbouring countries for molecular epidemiology analyses, which is very important for Hungary because it could help to understand the origin of the primary introduction of RABV and the dynamic of the virus spread. To explore rabies cases that are possibly hidden from surveillance, it seems to worth considering the extension of monitoring for other host too.

Molecular epidemiology of EBLV-1 infections

We expected from the examination of the whole EBLV-1 genomes that we gain a more accurate picture about the evolutionary mechanisms and the geographical origins of the Hungarian strains. Two of the seven Hungarian EBLV-1 samples were suitable for whole genome sequencing. Hungarian and European EBLV-1 sequences were examined with Bayes statistical methods. The calculated evolutionary rate of the virus was supported by the published data (EBLV-1a, $3.45E-5$ vs $3.01E-5$; EBLV-1b, $4.04E-5$ vs $4.07E-5$). The spread of two EBLV-1 subtypes (EBLV-1a and EBLV-1b) is supposedly a two-way process; EBLV-1b spreads from North Africa through South Spain into other western countries, while EBLV-1a is distributed from west towards east. Strains of both genetic lineages were found in France and Netherlands. We examined the EBLV-1a and EBLV-1b spatial dispersion in Europe based on the posterior probabilities generated from the results of evolutionary estimations, these results seem to support the literature on both lineages direction of spread. The EBLV-1a strains sequenced by us were possibly introduced to Hungary by bat species migrating between Poland and Hungary. EBLV-1 was in most cases, as well as in Hungary, detected from serotine bats (*Eptesicus serotinus*), which suggests that all the different lineages of EBLV-1 are well adapted to this host. The serotine bat does not migrate, therefore other species likely assist in the distribution, which live in the same habitat as serotine bats and are susceptible to the virus. For instance, Nathusius's pipistrelle (*Pipistrellus nathusii*) presumably plays a role in the spread of EBLV-1, because it is a widespread species with large migration areas.

Currently there are only a few bat rescue organizations in Hungary; hence there is a lack of collection sites with suitable diagnostic background out of the capital city. An organized nationwide active surveillance extended on more bat species would be necessary to fill the gaps in our knowledge.

New scientific results

1. This genetic epidemiology study of rabies in Hungary was the first one. We included in our analyses 79.2% of the detected RABV cases from 2006 to 2017 and we used altogether 61 newly sequenced RABV genomes in our examinations. The examinations based on the RABV genome sequences helped to put a new perspective on the Hungarian RABV epidemics.
2. We concluded from whole genome examinations that three genetically differing variants of RABV clusters were circulating in the last 12 years in Hungary. We identified seven lineages inside the three cluster which suggest at least the same number of independent possible introductions. We assume that the RABV variant known from the 1990s was extinct by the middle of 2000s.
3. The genome analysis pointed out previously misinterpreted results and helped to understand it. For instance, the previous findings suggested falsely that the 2013-2014 outbreak was clonal. We could estimate the rate of virus propagation and the speed of evolutionary changes thanks to the sequence data and background data of geographical information. Based on this analysis we assume that besides of the detected Hungarian cases there was a viral circulation that was unnoticed by the active monitoring.
4. For the first time in Hungary we analysed a vaccine induced rabies case from red fox. We ascertain the genetic similarity with Lysvulpen vaccine strains applied in Hungary by examining the virus genomes identity.
5. We designed a DIVA TaqMan RT-PCR assay to be able to differentiate of vaccine strains and Eastern-European wild-type RABV strains. This method could help quickly identify the vaccine induced cases without DNA sequencing.
6. We also tried to establish the laboratory background of the genomic epidemiology of bats. Despite the scarce case number, we found out that the Hungarian EBLV-1 sequences belong to the EBLV-1a cluster, and based on the evolutionary estimations they were introduced to Hungary probably from Poland by migrating bat species.

Scientific publications

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