Virology Research Unit
Finnish Food Safety Authority Evira
Helsinki, Finland
and
Department of Veterinary Biosciences
Faculty of Veterinary Medicine
University of Helsinki

EUROPEAN BAT LYSSAVIRUS TYPE 2 IN FINLAND

Surveillance, evolutionary analysis,
and prevention with vaccination

Tiina Nokireki

ACADEMIC DISSERTATION
To be presented, with the permission of the Faculty of Veterinary Medicine
of the University of Helsinki, for public examination in lecture room XII,
University main building, Unioninkatu 34,
Helsinki on 24th November 2017, at 12 noon.

Helsinki 2017

Supervised by

Professor Liisa Sihvonen, DVM, PhD
Department of Veterinary Biosciences
Faculty of Veterinary Medicine
University of Helsinki, Finland
and
Finnish Food Safety Authority Evira
Helsinki, Finland

Miia Jakava-Viljanen, DVM, PhD
Virology Research Unit
Finnish Food Safety Authority Evira
Helsinki, Finland

Reviewed by

Docent Tytti Vuorinen
Department of Virology
University of Turku, Finland

Professor in Virology Espen Rimstad
Department of Food Safety and Infection Biology
Faculty of Veterinary Medicine
Norwegian University of Life Sciences, Norway

Opponent

Docent Ruska Rimhanen-Finne, DVM, PhD
National Institute for Health and Welfare
Helsinki, Finland

ISBN 978-952-225-161-9 (pbk)
ISBN 978-952-225-162-6 (PDF)

Unigrafia
Helsinki 2017

To Axel and Iida

"Is not an event in fact more significant and noteworthy the greater the number of fortuities necessary to bring it about?"
(Milan Kundera)

ABSTRACT

European bat lyssavirus type 2 (EBLV-2) was first isolated in Finland from a Daubenton's bat (*Myotis daubentonii*) in 2009. Rabies in bats was already suspected in 1985, when a Swiss biologist died in Finland of lyssavirus infection, later identified as EBLV-2 infection. However, the origin of the infection could not be confirmed at that time. In 1986, 183 bats were analyzed for lyssaviruses in active surveillance, and passive rabies surveillance was ongoing, but lyssavirus was not detected in bats before 2009. In 2010–2011, during another active surveillance study, samples from 774 bats were analyzed for EBLV viral RNA. In addition, sera from 423 bats were analyzed for the presence of lyssavirus antibodies. Antibodies were detected in 2010 and 2011 from two locations and from one location, respectively. All seropositive bats were Daubenton's bats. All locations where seropositive bats were detected were in close proximity to where the EBLV-2-positive Daubenton's bat was found in 2009. No EBLV viral RNA was detected in any of these bats. In 2016, EBLV-2 was detected from a diseased Daubenton's bat for the second time from a location about 100 kilometers from where the Daubenton's bat was found in 2009. These data provide proof that EBLV-2 is endemic in the Finnish Daubenton's bat population.

In phylogenetic analysis, the Finnish EBLV-2 strains formed a monophyletic group separate from other bat-type lyssaviruses with significant support. EBLV-2 shared the most recent common ancestry with Bokeloh bat lyssavirus (BBLV) and Khujand virus (KHUV). EBLV-2 showed limited diversity compared to rabies virus (RABV) and appears to be well adapted to its host bat species. The slow tempo of viral evolution was evident in the estimations of divergence times for EBLV-2: the current diversity was estimated to have built up during the last 2000 years. In a phylogenetic tree of partial N gene sequences, the Finnish EBLV-2 strains clustered with strains from Central Europe, supporting the hypothesis that EBLV-2 circulating in Finland might have a Central European origin. The Finnish EBLV-2 strains and a Swiss strain (1993) were estimated to have diverged from other EBLV-2 strains during the last 1000 years, and the Finnish strains (1985 and 2009) appear to have evolved from a common ancestor during the last 200 years.

Rabies vaccine is used to protect against rabies virus before or after potential exposure. Since all the currently available vaccines are based on RABV, the vaccines are also used to protect against other lyssaviruses, and additionally against EBLV-2 infection based on cross-protection. We assessed the level of protection afforded by two commercial rabies vaccines, one for humans and one for animals, against intracerebral challenge in mice with EBLV-2 isolated from a bat in 2009. We compared this with protection using the same mouse model against challenge

with RABV isolated from a Finnish raccoon dog in 1989. When challenged with RABV, all the vaccinated mice survived. When challenged with EBLV-2, 75% to 80% of the vaccinated mice survived. All vaccinated mice developed sufficient to high virus-neutralizing antibody (VNA) titers against RABV, ranging from 0.5 to 128 IU/ml. RABV-based vaccines also appear to offer good cross-protection against EBLV-2 circulating in the Finnish bat population.

To investigate the factors influencing the response to rabies vaccination, we assessed the success of vaccination measured from the antibody response in dogs (n = 10 071) and cats (n = 722) sampled during 2009–2013. We examined the factors influencing the response to vaccination when animals failed to reach a rabies antibody titer of ≥0.5 IU/ml. Of the dog and cat samples, 10.7% (95% confidence interval CI 10.1–11.3) and 3.5% (95% CI 2.3–5.0), respectively, had a vaccination antibody titer <0.5 IU/ml. In dogs, vaccination with two commercial vaccines (odds ratio OR ranging from 2.5 to 13.6), vaccination over six months previously (OR from 4.2 to 4.5), and vaccination of dogs >60 cm or larger (OR from 2.3 to 3.2) resulted in a higher risk of failing to reach an antibody level of at least 0.5 IU/ml. In dogs up to a year old, these risks were higher than in older dogs. In cats, the type of vaccine did not appear to play a role.

ACKNOWLEDGEMENTS

This study was conducted at the Finnish Food Safety Authority Evira, in the Research Department in the Virology Research unit. This work was financially supported by the Ministry of Agriculture and Forestry (grant MMM 2682/311/2009) and the Finnish Food Safety Authority Evira. I thank Director General Matti Aho, the head of Evira, Professor Pia Mäkelä, Head of the Research Department, and Professor Tuija Gadd, Head of Virology Research unit. I also thank the former Director General, Jaana Husu-Kallio, former Head of the Research Department, Professor Tuula Honkanen-Buzalski, and former head of Virology Research unit, Liisa Kaartinen.

I am most grateful to my supervising professor, Liisa Sihvonen, for her constant support, encouragement, and her belief in me during this work, and for the opportunity to work in veterinary virology. I am grateful to my supervisor, Miia Jakava-Viljanen, for taking me into this project and for her valuable support.

I would like to acknowledge the reviewers of my thesis, Docent Tytti Vuorinen and Professor in Virology Espen Rimstad, for their valuable comments, which greatly improved this thesis. Roy Siddall is thanked for language editing.

I warmly thank my co-authors and collaborators (in order of appearance): Anita Huovilainen, Thomas Lilley, Eeva-Maria Kyheröinen, Christine Ek-Kommonen, Liisa Sihvonen, Miia Jakava-Viljanen, Tarja Sironen, Olli Vapalahti, Anna-Maija Virtala, Teemu Smura, Veera Karkamo, and Tuija Gadd, for their valuable contribution to the original publications.

I am grateful to the many people who have provided samples. Thomas Lilley, Eeva-Maria Kyheröinen, Veronika Laine, and Riitta Räisänen are thanked for providing bat samples, and other bat workers for their assistance in the field.

Special thanks to colleagues at Virology Research Unit for helping in the diagnostic work for rabies and providing essential peer support. Thanks to Laura London and Ulla Rikula for commenting on this thesis. The excellent technical staff of our unit are acknowledged for their contributions to this work. Special thanks to my trusted partner in the laboratory, Tiina Peltonen and also to Arja Engblom, Merja Hautala, Mervi Holopainen, Marika Karlsson, Essi Kuitunen, Sari Kiuru and Laura Sneitz. Raili Heinonen, Sanna Karumaa, Marika Karlsson, Ulla-Maija Kokkonen and Karoliina Alm are acknowledged for their work with our laboratory animals. I am grateful for the provision of EBLV-2 virus strain RV 628 and positive control sera from the European Virus Archive. Daniel Horton is thanked for providing mFAVN SOP.

I warmly thank my family and loved ones for all their support and love during all these years. Mom and Dad have always emphasized the importance of education

and have offered both financial and timely support. Thanks to my sister Sari and her family, to Matti and to my friends, especially Anu Näreaho and Jutta Talarmo, for their help, support and friendship. My children, Axel and Iida, are the most valuable and precious achievement of mine and the true meaning of life. I am truly proud of you. Love you.

Espoo, September 2017 *Tiina Nokireki*

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Nokireki T, Huovilainen A, Lilley T, Kyheröinen EM, Ek-Kommonen C, Sihvonen L, Jakava-Viljanen M. Bat rabies surveillance in Finland. BMC Vet Res. 2013 Sep 8;9:174. doi: 10.1186/1746-6148-9-174.

II Jakava-Viljanen M, Nokireki T, Sironen T, Vapalahti O, Sihvonen L, Huovilainen A. Evolutionary trends of European bat lyssavirus type 2 including genetic characterization of Finnish strains of human and bat origin 24 years apart. Arch Virol 2015; 160:1489–1498. DOI10.1007/s00705-015-2424-0.

III Nokireki T, Jakava-Viljanen M, Virtala A, Sihvonen L. Efficacy of rabies vaccines in dogs and cats and protection in mouse model against European bat lyssavirus type 2. Acta Vet Scan 2017;59:64. DOI 10.1186/s13028-017-0332-x.

IV Nokireki T, Sironen T, Smura T, Karkamo V, Sihvonen L, Gadd T. Second case of European bat lyssavirus type 2 detected in a Daubenton's bat in Finland. Acta Vet Scan 2017;59:62. DOI 10.1186/s13028-017-0331-y.

The publications are referred to in the text by their Roman numerals.

The author's contribution to the publications included in this thesis was as follows:

I Participated in the design of the study, set up the rabies serology, analyzed the samples and data and drafted the manuscript.
II Participated in the design of the study, isolated the virus and was responsible for the virus growth, participated in the phylogenetic and molecular clock analysis and drafted the manuscript.
III Participated in the study design, conducted the challenge experiment, performed the serological and virological analysis, participated in the statistical analysis and drafted the manuscript.
IV Was responsible for antigen detection, virus isolation, RT-PCR analysis, diagnostic sequencing and drafting the manuscript.

ABBREVIATIONS

AHVLA	Animal Health and Veterinary Laboratories Agency
AD	anno Domini
ABLV	Australian bat lyssavirus
ARAV	Aravan virus
BHK-21	Baby hamster kidney cell line
BSL-3	Bio safety level 3 laboratory
BBLV	Bokeloh Bat Lyssavirus
CI	confidence interval
DUVV	Duvenhage virus
ELISA	enzyme-linked immunosorbent assay
EBLV-1	European bat lyssavirus type 1
EBLV-2	European bat lyssavirus type 2
EFSA	European Food Safety Authority
EU	European Union
Evira	Finnish Food Safety Authority
FAT	fluorescent antibody test
FAVN	fluorescent antibody virus neutralization
FAO	Food and Agriculture Organization
GBLV	Gannoruwa bat lyssavirus
HKY	Hasegawa-Kishino-Yano model
IKOV	Ikoma lyssavirus
IU	international units
IRKV	Irkut virus
KHUV	Khujand virus
LBV	Lagos bat virus
LLEBV	Lleida bat lyssavirus
MEM	Minimum Essential Medium
MLD_{50}	minimum lethal dose
MNA	mouse neuroblastoma cell line
MOKV	Mokola virus
NMRI	Naval Medical Research Institute
RIG	rabies immune globulins
RABV	rabies virus
RIDT	rapid immunodiagnostic test
RTCIT	rabies tissue culture infectious test
RFFIT	rapid fluorescent focus inhibition test
SHIBV	Shimoni bat virus
VNA	virus-neutralizing antibody
WCBV	West Caucasian bat virus
WHO	World Health Organization

INTRODUCTION

Viruses from the genus *Lyssavirus* can cause a disease called rabies. Rabies is an acute encephalomyelitis that can affect all warm-blooded vertebrates, predominantly mammals. It has the highest case fatality rate of conventional infectious diseases, approaching 100%. Rabies caused by rabies virus (RABV) is responsible for about 55 000 to 75 000 human deaths and 1.74 million disability-adjusted life years every year, mostly in Africa and Asia (Knobel et al. 2012). RABV is transmitted between mammals via bites, the domestic dog (*Canis famialiaris*) being the main source of rabies infections in humans. The true reservoir of lyssaviruses is thought to be bats (*Chiroptera*) (Kuzmin and Tordo 2012). In a small number of cases, rabies in humans and other mammals is caused by a lyssavirus other than RABV and is contracted from bats. Disease caused by other lyssaviruses resembles rabies caused by RABV and can therefore also be called rabies. Data on virus–host interaction is mostly based on knowledge of RABV, but appears to apply in many aspects to other lyssaviruses as well.

The *Lyssavirus* genus includes 14 recognized species: classical rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus type 1 (EBLV-1), European bat lyssavirus type 2 (EBLV-2), Australian bat lyssavirus (ABLV), Irkut virus (IRKV), West Caucasian bat virus (WCBV), Khujand virus (KHUV), Aravan virus (ARAV), Shimoni bat virus (SHIBV), Bokeloh bat lyssavirus (BBLV), and Ikoma lyssavirus (IKOV) (Anonymous 2017a). Novel lyssaviruses include Lleida bat lyssavirus (LLEBV), which was found in a bent-winged bat (*Miniopterus schreibersii*) in Spain in 2011 (Arechiga-Ceballos et al. 2013), and Gannoruwa bat lyssavirus (GBLV), which was isolated from a Greater Indian Fruit Bat in Sri Lanka in 2015 (Gunawardena et al. 2016). Lyssaviruses can be divided into phylogroups (Badrane et al. 2001). Phylogroup I comprises the classical rabies virus (RABV) and the majority of bat lyssaviruses (DUVV, EBLV-1, EBLV-2, ABLV, IRKV, KHUV, ARAV, BBLV, GBLV), whereas MOKV, LBV and SHIBV form phylogroup II (Badrane et al. 2001, Kuzmin et al. 2010, Gunawardena et al. 2016). WCBV, IKOV, and LLEBV may be representatives of possible new phylogroups (Kuzmin et al. 2005, Marston et al. 2012, Arechiga-Ceballos et al. 2013).

In the Americas, bats are infected with rabies virus RABV, but outside the Americas, other lyssavirus species are present in bat populations. EBLV-2 is known to associate with two closely related *Myotis* bat species, Daubenton's (*Myotis daubentonii*) and pond bats (*M. dascyneme*). EBLV-2 has caused two human deaths: in 1985 in Finland and in 2002 in the UK (Lumio et al. 1986, Roine et al. 1988, Fooks et al. 2003).

Rabies caused by RABV is preventable by pre- or post-exposure vaccination and the use of immunoglobulins in humans and by pre- and post-exposure vaccination of animals. However, all the currently available vaccines are based on RABV, and while they offer partial cross-protection against other lyssaviruses in phylogroup I, protection against lyssaviruses outside phylogroup I is considered poor.

2 REVIEW OF THE LITERATURE

2.1 History of rabies research

Rabies is one of the oldest known diseases. Democritus, Aristotle, Hippocrates, and Celsus already described the clinical features of rabies and understood that it can be transmitted through the bite of a rabid animal. Cordamus assumed that a "poison" was present in a rabid dog's saliva. Celsus wrote in the first century anno Domini (AD): "The patient is tortured at the same time by thirst and by invincible repulsion towards water". A variety of literature in the period from 800–700 BC contained passages describing this disease. In Homer's Iliad, Hector is compared to a rabid dog. Fracastotoro (1478–1553) proposed a theory of contagion. Morgagni (1735–1789) concluded that rabies was not transmitted by blood but via the nerves. Pathogenesis studies in the 1700s demonstrated that the infective agent of rabies was transmitted via saliva and could be transmitted between species. Louis Pasteur (1822–1895) started his work on rabies in 1880. By 1884, he and his colleagues had developed the first rabies vaccine, which was administered to a person in 1885. Over the next decades, hundreds of thousands of people were treated with this vaccine at the Institut Pasteur in Paris. Neuroparalytic complications of early vaccines were noted, and in 1911, Sir David Semple reported on a method for preparing an inactivated vaccine, the Semple vaccine, which was widely used for several decades (Jackson 2013a, Kuzmin and Tordo 2012).

A diagnostic breakthrough was achieved in 1903, when Adolchi Negri observed eosinophilic cytoplasmic inclusion bodies in neurons in the brains of rabid dogs (Negri bodies). Remlicher discovered in 1903 that the etiological agent of rabies was ultrafilterable, and rabies virus was isolated for the first time. In the 1950s and the 1960s, electron microscopic studies were performed on Negri bodies. Furthermore, in the 1950s, Goldwasser and Kissling used indirect fluorescent antibody staining (FAT) in order to demonstrate rabies virus antigen in impression smears of the brain of rabid animals. This technique played an important role in the first modern experimental pathogenesis studies, which demonstrated that rabies virus is highly neurotropic and that the virus spreads to the central nervous system in the axoplasm of peripheral nerves. In 1983, the first full-length rabies virus glycoprotein gene was cloned (Jackson 2013a, Kuzmin and Tordo 2012).

2.2 Lyssaviruses within the family *Rhabdoviridae*

Rabies is caused by RNA viruses belonging to the genus *Lyssavirus* within the family *Rhabdoviridae* in the order *Mononegavirales*. The name of the genus originates from Greek mythology. Lyssa was a goddess or spirit of rage, fury, raging madness, and frenzy. The type virus of this genus is rabies virus RABV (Dietzgen and Kuzmin 2012).

Genetically, lyssaviruses form a very tight monophyletic cluster within the Rhabdoviridae. Species demarcation is based on antigenic cross-reactivity using polyclonal antisera and monoclonal antibodies, on genetic characteristics, topology and consistency of phylogenetic trees and ecological characteristics. The Lyssavirus genus includes fourteen species that have been accepted by the International Committee on Taxonomy of Viruses: classical rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus type 1 (EBLV-1), European bat lyssavirus type 2 (EBLV-2), Australian bat lyssavirus (ABLV), Irkut virus (IRKV), West Caucasian bat virus (WCBV), Khujand virus (KHUV), Aravan virus (ARAV), Shimoni bat virus (SHIBV), Bokeloh bat lyssavirus (BBLV) and Ikoma lyssavirus (IKOV) (Anonymous 2017a). There are two novel tentative lyssaviruses, Lleida bat lyssavirus (LLEBV) (Arechiga-Ceballos et al. 2013) and Gannoruwa bat lyssavirus (GBLV) (Gunawardena et al. 2016). The timeline of different lyssavirus discoveries is presented in Figure 1, and the phylogenetic relationship of different lyssaviruses in Figure 2.

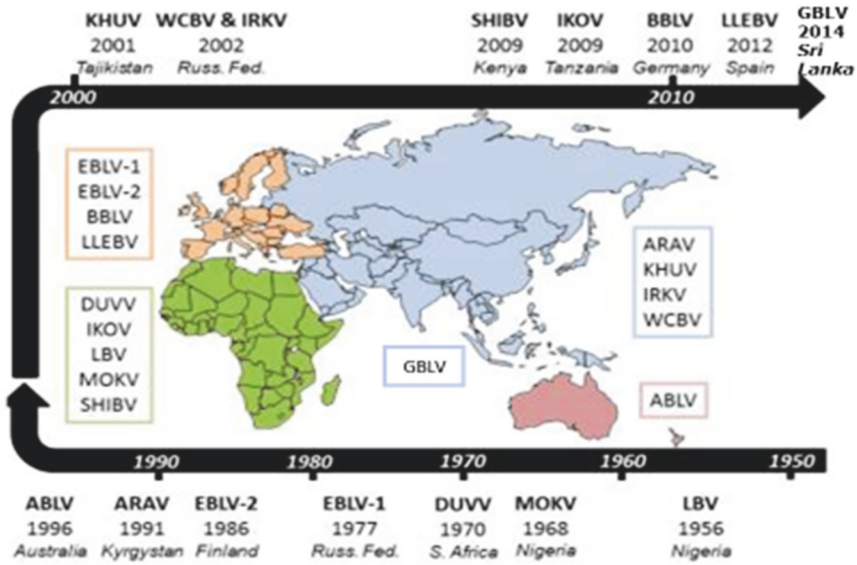

Figure 1. A Lyssavirus timeline. Regions where different lyssaviruses are found are colored and dates for initial isolations are shown. Modified by Banyard et al. 2014

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4147683/

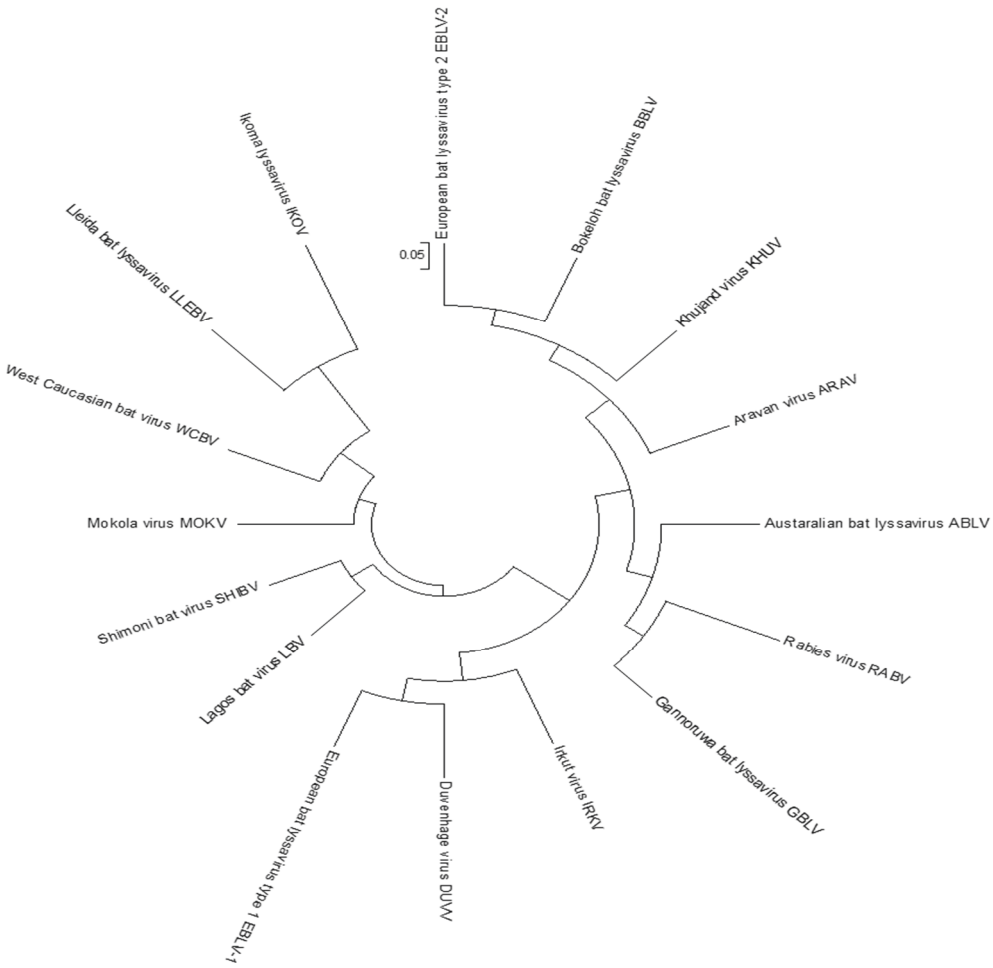

Figure 2. A phylogenetic analysis of the lyssaviruses. The analysis is based on 405 nucleotides of the nucleoprotein gene. Sequences were aligned by Clustal W in the program Mega 6.0.

Based on phylogenetic reconstructions and serological cross-reactivity, members of the genus can be divided into phylogroups. Phylogroup I comprises the classical rabies virus (RABV) and the bat lyssaviruses DUVV, EBLV-1, EBLV-2, ABLV, IRKV, KHUV, ARAV, BBLV and GBLV, whereas MOKV, LBV, and SHIBV form phylogroup II (Badrane et al. 2001, Kuzmin et al. 2010, Gunawardena et al. 2016). WCBV, IKOV, and LLEBV may be representatives of a possible new phylogroup (Kuzmin et al. 2005, Marston et al. 2012, Arechiga-Ceballos et al. 2013).

Phylogenetic analyses and the virus–host relationship suggest that all 16 currently known species of lyssaviruses probably originated in bats. With the exception of Mokola virus and Ikoma lyssavirus, all other lyssavirus species have been isolated from bats. Most lyssaviruses have a limited geographical distribution and are also limited to a certain host species. RABV has a worldwide distribution and has adapted to several different hosts (Table 1).

T**able 1.** Viruses currently included in the genus Lyssavirus

Recognized and proposed lyssavirus species	Natural host	Year and place of first isolation	Geographical range	Known human cases (n)	Phylo-group	Remarks
Rabies virus (RABV)	Terrestrial mammals Bats of multiple species	1903 Turkey	Worldwide New World	Yes (55000/y) Yes (100/y)	I	Causes the vast majority of human cases in the world.
European bat lyssavirus type 1 (EBLV-1)	*Insectivorous bats* (predominantly *Eptesicus serotinus)*	1954 Germany	Europe	Yes (1)	I	Spillover infections to wild and companion animals have been documented.
Lagos bat virus (LBV)	Several fruit and insectivorous bat species	1956 Nigeria	Sub-Saharan Africa	No	II	Spill-over infections to wild and companion animals have been documented. Constitutes several lineages.
Mokola virus (MOKV)	Unknown	1968 Nigeria	Sub-Saharan Africa	Yes (1)	II	Isolations from wild and domestic mammals.
Duvenhage virus (DUVV)	Insectivorous bats	1970 South Africa	Sub-Saharan Africa	Yes (3)	I	
European bat lyssavirus type 2 (EBLV-2)	*Insectivorous bats (Myotis daubentonii, M. dasycneme)*	1985 Finland	Europe	Yes (2)	I	
Aravan virus (ARAV)	*Insectivorous bat Myotis blythii*	1991 Kyrgystan	Central Asia	No	I	Known by a single isolate.
Australian bat lyssavirus (ABLV)	Pteropodid bats of several genera and insectivorous bats	1996 Australia	Australia	Yes (3)	I	Spillover infections to horses have been documented.
Khujand virus (KHUV)	*Insectivorous bat Myotis mystacinus*	2001 Tajikistan	Central Asia	No	I	Known by a single isolate.
Irkut virus (IRKV)	*Insectivorous bat Murina leucogaster*	2002 Russian federation	Eastern Asia	Yes (1)	I	Known by three isolates.
West Caucasuian bat virus (WCBV)	*Insectivorous bat from genus Miniopterus*	2002 Russian Federation	South-eastern Europe	No	III/IV?	Known by a single isolate.
Shimoni bat virus (SHIBV)	*Insectivorous bat Hipposideros commersoni*	2009 Kenya	Kenya	No	II	Known by a single isolate.
Ikoma lyssavirus	*Unknown*	2009 Tanzania	Tanzania	No	III/IV?	Known by a single isolate from *Civettictis civetta.*
Bokeloh bat lyssavirus (BBLV)	*Insectivorous bat Myotis nattereri*	2010 Germany	Germany, France	No	I	
Lleida bat lyssavirus (LLEBV)	*Insectivorous bat Miniopterus schreibersii*	2012 Spain	Spain	No	III/IV?	Known by a single isolate.
Gannoruwa bet lyssavirus (GBLV)	*Pteropus spp*	2014 Sri Lanka	Sri Lanka	No	I	Known by four isolates.

2.3 Structure of the virion, genome, and proteins

Lyssaviruses have large enveloped virions, 100–430 nm in length and 45–100 nm in diameter, with a bullet-shape or cone-shaped morphology. They are composed of an infectious nucleocapsid enveloped by a host-derived lipid membrane containing glycoprotein spikes.

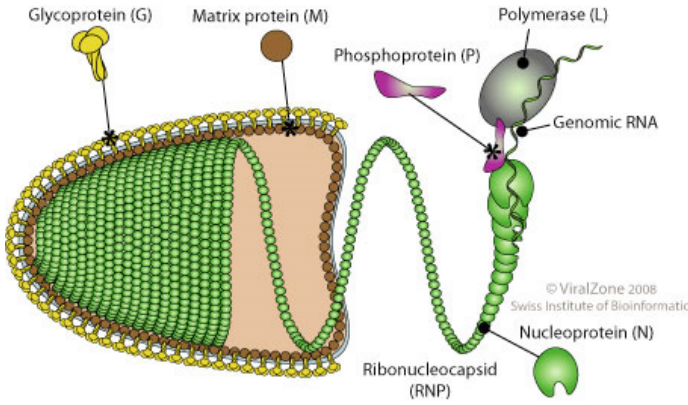

Figure 3. Structure of a lyssavirus.
http://viralzone.expasy.org/all_by_species/22.html
Reprinted with permission from ViralZone, Swiss Institute for Bioinformatics

The negative-sense, single-stranded lyssavirus genome is about 12 kb in length and it encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA polymerase (L), in the order 3′-N-P-M-G-L-5′. Each gene is flanked by intergenic regions with a high degree of divergence (Marston et al. 2007). The variation within genomes could be related to polymerase errors during replication (Assemberg et al. 2010).

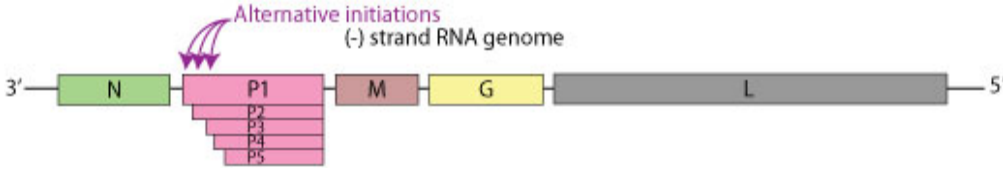

Figure 4. Genetic organization of lyssaviruses.
http://viralzone.expasy.org/all_by_species/22.html
Reprinted with permission from ViralZone, Swiss Institute for Bioinformatics

2.4 Virus–host interactions

2.4.1 Pathogenesis

Lyssaviruses are highly neurotropic. The lyssavirus replicates in limited numbers in the peripheral tissue at the site of inoculation. It spreads along peripheral nerves and the spinal cord to the central nervous system, where it disseminates and replicates, causing acute encephalomyelitis. From the central nervous system, it spreads along nerves to various organs, including the salivary glands. It has been shown that the lyssavirus remains at or near the entry site for most of the long incubation time. The incubation time in humans is usually between 20 to 90 days. There is uncertainty over what happens during the incubation period. The infection of muscle fibers may be a critical pathogenetic step for the virus to gain access to the peripheral nervous system, but studies also suggest that rabies virus is capable of direct entry into peripheral nerves (Jackson and Fu 2013)

The virus glycoproteins enable the virus to bind to neurospecific receptors and to gain entry into cells. At least three receptors have been proposed: nicotinic acetylcholine receptor, neural cell adhesion molecule receptor, and low-affinity p75 neurotrophin receptor. The virus spreads from the peripheral entry site to the central nervous system by retrograde fast axonal transport within motor and perhaps also sensory axons. This occurs at a rate of 50 to 100 mm per day. Infection also occurs early in motor neurons in the spinal cord and primary sensory neurons in dorsal root ganglia. Once central nervous neurons are infected, there is rapid dissemination of the virus along neuroanatomical pathways. Centrifugal spread to peripheral sites is essential for transmission of the virus to its natural hosts, and salivary gland infection enables the transmission of infectious oral fluids. The lyssavirus can also be detected in the retina and cornea, hair follicles, tongue, adrenal glands, heart, liver, and pancreas (Jackson and Fu 2013).

2.4.2 Clinical signs

Bats are the natural reservoir of lyssaviruses. The detection of seropositive bats without clinical signs suggests that bats can be infected and clear the infection. Lyssavirus infection can also cause clinical disease in bats. Furthermore, a long incubation period associated with infection has been recorded (Banyard et al. 2014). When clinical disease is present, bats are often weak and unable to fly. They show abnormal behavior, uncoordinated movements, spasms, and paralysis. Infected bats are grounded, agitated, and aggressive, and they may make repeated attempts to bite. There are no observations that bats can survive infection if the clinical disease develops (Banyard et al. 2011).

The initial clinical signs of rabies in animals are often non-specific and may include lethargy, poor appetite, and diarrhea or vomiting. The clinical condition

progressively deteriorates. Changes in behavior are one of the first signs. An animal may become more reclusive or attention-seeking, and it might unpredictably and intermittently attack humans, other animals, or any object it comes into contact with. A wild animal might move slowly or act tame. There may be irritation or parasthesis at the site of initial entry of the virus. Saliva production increases and the ability to swallow decreases. Pupil size may be unequal, and there might be facial and tongue paresis and phonation changes. In the end stage, animals become paralytic, comatose, and moribund. The infection leads to death within ten days after the appearance of clinical signs (Hanlon 2012).

Non-specific prodromal symptoms in humans include fever, chills, malaise, fatigue, insomnia, anorexia, headache, anxiety, and irritability for up to 10 days prior to the occurrence of neurological symptoms. About 80% of patients develop an encephalitic form of rabies, also known as a furious form, and the rest develop a paralytic form. In the encephalitic form, people suffer from generalized arousal or hyperexcitability separated from lucid periods. Intermittent episodes of confusion, hallucination, agitation, and aggressive behavior may occur. There might be symptoms of autonomic dysfunction. From 50% to 80% develop hydrophobia, a characteristic symptom of rabies. Encephalitic rabies leads to flaccid paralysis, coma, and multiple organ failure. Death occurs within 14 days of the onset of clinical symptoms. In paralytic or dump rabies, flaccid muscle weakness develops early in the course of the disease (Jackson 2012b). There are some differences in the clinical manifestations of dog- and bat-acquired RABV rabies cases. Encephalopathy, hydrophobia, and aerophobia have been reported to be more common in dog-acquired rabies. Bat-acquired cases were more often misdiagnosed and lacked a bite history. Abnormal cranial nerve, motor, and sensory examinations, tremor, myoclonus, local sensory symptoms, symptoms at the exposure site, and local symptoms in the absence of a bite or scratch were more common in patients with bat-acquired rabies (Udow et al. 2013).

The clinical disease of the Swiss biologist who died in Finland in 1985 of EBLV-2 infection lasted for 23 days, and was a combination of the paralytic and furious forms of rabies. Severe ascending destruction of the brain was observed. An unusual progression from isolated brainstem death to cortical brain death occurred (Roine et al. 1988). The second case of human rabies due to EBLV-2 from the UK in 2002 presented with rabies that had mixed components of furious and paralytic forms, which was strikingly similar to the EBLV-2 case from Finland (Nathwani et al. 2003).

2.4.3 Pathological changes

Despite the severe clinical neurological signs, the neuropathological findings are usually quite mild. Rabies results from neuronal dysfunction rather than structural

damage. The macroscopic examination is unremarkable or it shows a spectrum of mild and non-specific changes such as edema, congestion of blood vessels, and petechiae. Microscopic findings include mononuclear inflammatory cell infiltration, microglial activation and neuronophagia. The presence of Negri body viral inclusions in the cytoplasm is a unique and diagnostic feature of infection with rabies, but it is only present in about 40% of cases. There are marked abnormalities in dendrites and axons, vacuolation in neurons, and axonal swelling. These morphological changes may be sufficient to explain the clinical disease (Rossiter and Jackson 2013). In the EBLV-2 human case of 1985, the patient's brain showed severe lytic changes (Roine et al. 1988).

2.4.4 Immunology

Infection induces the expression of innate immunity, inflammatory cells in the central nervous system, and apoptosis. The latter two correlate with the enhancement of permeability of the blood–brain barrier and the attenuation of the lyssavirus (Lafon 2013).

Lyssavirus causes an innate immune response, which involves the release of type I interferons, inflammatory cytokines and chemokines, the activation of complement, and the attraction of macrophages, neutrophils, and NK cells to eliminate the virus locally at the entry site and set up a specific immune response of B and T cells extraneurally. The innate immune response is triggered within hours after the entry of the virus and it is not pathogen specific. After its entry into nerves, lyssavirus elicits an innate immune response in the infected neurons, which are protected from destruction by infiltrating T cells and by mechanisms limiting the inflammation of neuronal tissue. Down-regulation of immune responsiveness in the periphery facilitates the propagation of the virus in the nervous system (Lafon 2013).

An adaptive immune response is tailored against a specific microbe and it takes several days to develop. The triggering of the adaptive immune response takes place in lymphoid organs such as the lymph nodes or spleen. The CD4 (T helper) and CD8 (cytotoxic T) lymphocytes recognize foreign antigens. The CD4 cells limit the proliferation of pathogens, provide help for antibody production by B cells and CD 8 cells kill infected cells via cytotoxicity. A strong B cell response is mounted in the spleen. The entry of B cells into the infected neural system and the secretion of antibodies contribute to the clearance of the virus from the nervous system (Lafon 2013). The type of immune response where antibodies are found in body fluids is called a humoral immune response, while the type where specialized cytotoxic cells destroy virus infected cells is called a cell-mediated immune response (Tizard 2013). The primary correlate of protection is the presence of neutralizing

antibodies. Cell-mediated immunity is important and acts in synergy with the humoral response (Moore et al. 2006).

In rabies, clearance of the infection by T cells is inefficient and is inactivated by the virus: the cells undergoing death are the leucocytes and not infected neurons. During the course of encephalitic lyssavirus infection, B cells are almost undetectable in the brain (Lafon 2013). In the majority of human rabies cases, there is no detectable antibody response until some days after the development of clinical disease (Johnson et al. 2010). The limited immune response of the host could be due to a number of factors. The virus primarily replicates in the nervous system, which is an immunoprivileged location, and the induction of inflammation in the central nervous system is tightly regulated in order to avoid neuronal deficit of the host. It is also suggested that the infectious dose during a bite is too small to trigger an immune response, enabling the virus to infect nerves. The lack of antibodies could be a result of immunosuppression induced by the virus (Johnson et al. 2010). In mice, there is an increase in inflammatory cytokines and chemokines in response to EBLV-2 infection, which leads to a dramatic increase in T cell infiltration and provides evidence for a robust immune response to lyssavirus infection that may not commonly occur in RABV infection (Mansfield et al. 2008).

2.5 Vaccines

2.5.1 Rabies vaccines

Rabies vaccines are defined as a standardized formulation containing defined amounts of immunogens, which are either inactivated (killed), live-attenuated, or biotechnology derived. Rabies vaccines are formulated for their specific purpose and for application by either the injectable or oral route, and they are produced in eggs or in cell culture. Vaccination is highly effective at preventing disease when administered before or shortly after exposure to rabies virus. Since Pasteur's first rabies vaccine in 1885 until about the mid-twentieth century, rabies vaccines were based on virus grown in inoculated animals. Over the past 35 years, several safer and more potent vaccines have been developed by growth in cell cultures: human diploid cell vaccine, purified chick embryo cell vaccine, purified Vero cell vaccine, and purified duck embryo vaccine (Anonymous 2016).

All currently available vaccines are based on RABV. Studies have been conducted to investigate the cross-neutralization of sera against different lyssaviruses and cross-protection challenge. Consequently, they offer variable cross-protection against other lyssaviruses (Horton et al. 2010, Brookes et al. 2005a, Malerzyk et al. 2009, 2014, Hanlon et al. 2005, Liu et al. 2013). Protection is inversely related to the genetic distance of the lyssavirus in comparison to RABV (Brookes

et al. 2005, Malerczyk et al. 2009, Fekadu et al. 1988). However, there are no official recommendations by WHO on whether there would be a need to modify the current procedure for the pre- and post-exposure treatment of people or other mammals who are exposed to other lyssaviruses than RABV (Anonymous 2013, Anonymous 2016).

Moreover, a question has been raised about whether there would be need or means to develop a pan-lyssavirus vaccine. More cross-reactive vaccine formulations may be necessary in areas where a threat to the human population comes from lyssaviruses other than RABV. Recent advances in the antigenic characterization of different lyssaviruses will aid in future cross-reactive vaccine design. Studies have concentrated on the development of recombinant viruses or glycoproteins as potential novel vaccines, or swapping domains within the G protein between different lyssaviruses to produce a cross-phylogroup antibody response. However, there is a potential for the development of novel pan-lyssavirus vaccines from inactivated preparations of viruses from each phylogroup (Evans et al. 2012).

2.5.2 Response to vaccination

Factors associated with the success of rabies vaccination and antibody detection depend on the vaccine, the receiver of the vaccine, the person vaccinating, sampling, and the methods used to evaluate the success of vaccination (Figure 5).

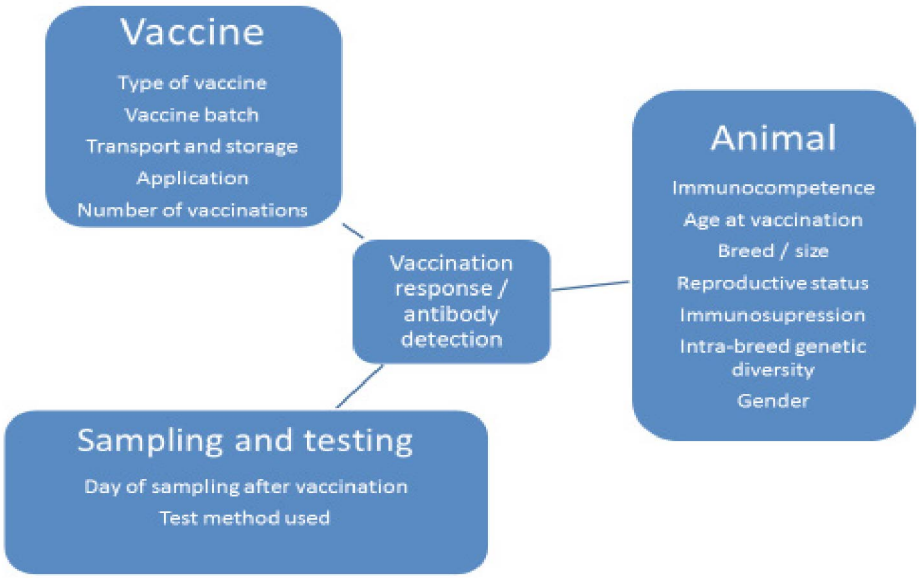

Figure 5. Factors associated with the success of rabies vaccination and antibody detection.

Because laboratory methods to measure the humoral response are easier to perform than those measuring the cellular response, VNA tests are used to verify that an immune response has occurred (Moore et al. 2006). According to WHO recommendations, 0.5 IU/ml is a sufficient antibody level, considered to represent a level of immunity in humans that correlates with the ability to protect against rabies infection (Anonymous 2013). The same threshold titer is applied in dogs, cats, and ferrets to confirm a satisfactory response to vaccination before international transportation.

Rabies vaccines differ in how they induce the antibody level of 0.5 IU/ml in dogs (Berntsson et al. 2011, Mansfield et al. 2004, Minke et al. 2008, Kennedy et al. 2007). This may be due to differences in the immunogenicity of the vaccines or the potency of the vaccine batches. The antibody level peaks at slightly different times after vaccination with different vaccines, and the level starts to decline afterwards (Mansfield et al. 2004). In post-vaccination serological studies, the percentage of dogs with inadequate titers has been 3.1–8.1% (Sihvonen et al. 1995, Mansfield et al. 2004, Berndtsson et al. 2011), and for cats 2.85% (Mansfield et al. 2004). As many as 53% (95% CI 41% to 65%) of imported rescue dogs from Eastern Europe were found to have inadequate titers after rabies vaccination (Klevar et al. 2015).

Some individuals respond to vaccination earlier and produce high titers, while others respond later and produce lower titers (Moore et al. 2006). There are reports that vaccinated animals without detectable antibodies survive challenge, indicating that other immune mechanisms protect against rabies infection. On the other hand, the presence of detectable neutralizing antibodies to rabies at the time of challenge does not indicate protection for all the animals (Aubert 1992, Hooper et al. 1998). An active immune response and antibody response induced by rabies vaccines is measurable 7–10 days after vaccination. Responses in humans generally persist for two to three years, and even up to twenty years after vaccination (Moore et al. 2006). The time between vaccination and sampling is a significant factor when estimating the immune response of an animal. In addition, the laboratory tests used to measure the immune response vary and the results are not always comparable.

On the population level for rabies, the critical, theoretical vaccination coverage required to control the disease needs to be about 20% to 40% of the dog population. In practice, the achievement of this goal is hampered by factors responsible for a decline in the proportion of the vaccinated population over time. Empirically, consecutive annual vaccinations that achieve 70% vaccination coverage have proven effective in eliminating dog rabies (Knobel et al 2013).

2.5.3 Vaccination failures

There are many reasons why vaccination might not lead to protective immunity. The vaccine might be ineffective if it contains the wrong strain of antigen or the

amount of antigen is insufficient. There can be a fault in the manufacturing process, the cold chain during transport, or in the storage of the vaccine (Tizard 2013). Incorrect administration can also cause vaccination failure.

Even animals given an adequate dose of an effective vaccine may fail to be protected. The vaccine might be given too late if the animal is vaccinated during the incubation period, or the animal may fail to mount an immune response. Heavily parasitized or malnourished animals might be immunosuppressed, and some virus infections induce immunosuppression. Stress, pregnancy, extremes of cold or heat, fatigue, and malnutrition reduce the normal immune response. The most common reason for failing to respond to vaccination is the presence of maternal antibodies (Tizard 2013). Maternal antibodies may interfere with immune responses, particularly in puppies eight weeks of age or younger vaccinated with modified live rabies vaccine under field conditions (Aghomo et al. 1990). However, at least under experimental conditions, maternal antibodies and the immune function may not limit the immune response to inactivated vaccines that stimulate both B cell and T cell responses (Siegrist 2012). World Health Organization (WHO) guidelines recommend that all dogs, including puppies less than three months of age, are vaccinated during mass vaccination campaigns (Anonymous 2013).

2.6 Epidemiology

2.6.1 Epidemiology of bat-related lyssaviruses other than RABV

Those lyssaviruses for which an adequate number of isolates exist are bat-specific virus variants that are maintained in particular bat species within a geographically limited area. In Europe, EBLV-1, EBLV-2, BBLV, and LLBLV have been detected in bats. During 1977–2016, 1179 rabies cases (7.1% of all bats tested) were reported in 11 of the 45 known indigenous bat species in 20 European countries, most cases being EBLV-1. The majority of cases were detected in the Netherlands, Germany, Denmark, Poland, France, and Spain (WHO Rabies Bulletin Europe).

The first isolation of EBLV-2 from a bat took place in 1987 from a pond bat in the Netherlands (Nieuwenhuijs 1987). Elsewhere in Europe, EBLV-2 has sporadically been isolated from the Daubenton's bat in Switzerland (Megali et al. 2010), the UK (Whitby et al. 2000, Harris et al. 2007), Germany (Freuling et al. 2008), and Finland (Jakava-Viljanen et al. 2010). In 2013, EBLV-2 was found from two Danish Daubenton's bats using a molecular diagnostic strategy (Rasmussen et al. 2014) and in Norway in a Daubenton's bat (Moldal et al. 2017).

In the UK, the first EBLV-2 positive bat was reported in 1996, and EBLV-2 has been isolated from 15 Daubenton's bats in the UK since 1996 (Johnson et al. 2016). In 2003, a seroprevalence of 0.05% to 3.8% in Daubenton's bats was

recorded. However, the virus was not detected from the 218 swabs tested (Brookes et al. 2005a). A study from 2003 to 2006 revealed a seroprevalence of 1.0% to 4.1% (Harris et al. 2009). Live EBLV-2 was first isolated in Scotland in 2009. A single oral swab sample from a Daubenton's bat was positive out of ca. 900 tested (reviewed in Schatz et al. 2013). These data suggest that EBLV-2 circulates at low levels in the UK and Scotland.

In Switzerland, 837 bats were examined between 1976 and 2009 and three samples were found to be positive for EBLV-2. During active surveillance in 2009, one oral swab out of 237 was positive for EBLV-2 and three Daubenton's bats were seropositive (Megali et al. 2010). EBLV-2 was first isolated in Germany in 2007. Between 1997 and 2007, a total of 1800 bats were tested in Germany, including 45 Daubenton's bats and one pond bat (Freuling et al. 2008). In the Netherlands, 5 pond bats were found to be positive for EBLV-2 out of 129 pond bats analyzed, resulting in a prevalence of 3.9%. No new cases have been detected since 1997 (Freuling et al. 2008, Van der Poel et al. 2005).

Virus-neutralizing antibodies have been found in many bat species throughout Europe. Due to cross-reactivity between different lyssaviruses, seropositivity cannot be linked to a specific lyssavirus (Schatz et al. 2013). Fourteen Daubenton's bats sampled in 2008–2013 in Sweden had antibodies against lyssavirus when using EBLV-1 or EBLV-2 as an antigen (Hammarin et al. 2016). Seropositive Daubenton's bats have also been found in the UK, Switzerland (Schatz et al. 2013), Finland (Nokireki et al. 2013), and Latvia (Dobrostana et al. 2017).

No spillover of EBLV-2 to other animals has been detected. In experimental infections, EBLV-2 has been shown to cause fatal disease in foxes (Cliquet et al. 2009), sheep (Brookes et al. 2007), and mice (Banyard et al. 2014, Healy et al. 2013). In Europe, spillover of EBLV-1 has been seen in natural infections of stone marten (*Martes foina*) (Müller et al. 2004), sheep (Tjørnehøj et al. 2006), and domestic cats (Tjørnehøj et al. 2004). Spillovers of Lagos bat and Mokola viruses have been recorded in cats, dogs, and wild carnivores and Australian bat lyssavirus in horses (Markotter et al 2006, Sabeta et al 2007. Shinwari et al 2014).

2.6.2 Epidemiology of rabies virus RABV

In the new world, bats are infected with RABV, and RABV has not been detected in bats outside the Americas. A rabies virus variant from hematophagous bats causes between 100 000 and 500 000 cattle deaths per year in Latin America, and spillover to cats, foxes, and other mammals has occurred (Barrett 2011).

Among domestic animals, dogs are the most important animal reservoirs for spillover cases of RABV to humans, whereas within wildlife species, foxes, raccoons, and skunks constitute the greatest threat to spillover to both humans and domestic

animals. The rabies situation in wildlife is complicated due to the presence of different terrestrial and bat vector species.

In Europe, the red fox (*Vulpes vulpes*) has been the main reservoir of RABV. The raccoon dog (*Nyctereutes procyonoides*) was introduced into Europe in the 1920s, and could also act as a reservoir, since it played a significant role in the epidemiology of rabies in Eastern and Central Europe. In the USA, terrestrial species may be limited in their distribution, with raccoons (*Procyon lotor*) being present across much of the eastern United States, the gray fox (*Urocyon cinereoargenteus*) in Arizona and Texas, and both red and arctic (*Alopex lagopus*) foxes in Alaska and parts of Canada. In Asia and Africa, dog-mediated urban rabies is epidemiologically the most important form of rabies (Hanlon 2013)

Host switching has been proposed to be the evolutionary mechanism of lyssaviruses. Lyssaviruses have evolved through spillover from bats to terrestrial mammals. Such transmission to a novel host can alter the genome to enable efficient replication and maintenance within the recipient species. Host switching can lead to the evolution of a novel human pathogen. In most cases, spillover infections are dead-end infections. However, further transmission within new species may occur if productive infection and viral excretion take place (Badrane and Tordo 2001).

Two case reports provide evidence for the maintenance of virus in a terrestrial species following presumed transmission from a bat species. In 1993, a small outbreak of rabies cases occurred in foxes on Prince Edward Island, Canada. The genetic detection of a bat RABV variant within this fox population was confirmed, and from the detection of low levels of virus in salivary gland material, it was assumed that some degree of intraspecific transmission among the foxes had occurred (Daoust et al. 1996). There was a second case in Arizona, USA, in 2001 of an infection of a number of skunks with a bat RABV variant. This virus was phylogenetically most similar to that known to be present in populations of *Eptesicus* and *Myotis* spp. from the same region (Leslie et al. 2006).

2.6.3 Human rabies of bat origin

In the 16th century, it was reported that many soldiers died as a result of hematophagous bat bites in Darien, located south of what is now known as Panama. However, the link between hematophagous bat bites and rabies was not established until the beginning of the 20th century. Presently, it is well known that insectivorous, frugivorous, and hematophagous bats in the Americas act as wildlife reservoirs for the rabies virus and can transmit the disease to humans (Johnson et al. 2010). A rabies virus variant in hematophagous bats in Latin America causes a significant number of human deaths, estimated to be around 100 each year. In the USA, around 1 to 4 human deaths occur each year following bites by insectivorous bats (Barrett 2011).

A small number of rabies cases in humans result from other lyssaviruses present in bats. Although numerous human contacts with European bat species have been reported, primarily from handling sick or injured animals (Brass 1994), only a few EBLV-induced human casualties have been conclusively demonstrated: in Voroshilovgrad, Ukraine (1977), in Belgorod, Russia (1985), in Finland (1985), and in the UK in Scotland (2002) (Botvinkin et al. 2003, Anonymous 1986, Lumio et al. 1986, Roine et al. 1988, Fooks et al. 2003). The Russian case was genetically typed as being EBLV-1a, while the Ukrainian case was assumed to be EBLV-1 based on antigenic profiling (Botvinkin et al. 2003, Selimov et al. 1989). The virus variants responsible for human rabies cases in the UK and Finland were typed as being EBLV-2a and b, respectively (Fooks et al. 2003). Subsequently, two other human rabies cases following a bat bite have been described in the Ukraine (Botvinkin et al. 2003). However, these cases were clinically diagnosed and on the basis of anamnesis, and no virological or pathological results are available.

A human case due to a bat contact was reported from Africa in 1970, which led to the isolation of Duvenhage virus. Two additional human cases, from 2006 and 2007, have been reported. One confirmed lethal Mokola virus infection was documented from Nigeria in 1969 (reviewed in Johnson et al. 2010). In Queensland, Australia, ABLV has caused three human cases of rabies, two in the late 1990s and one in 2013 (Samaratunga et al. 1998, Hanna et al. 2000, Francis et al. 2014). In 2007, a fatal human case of a bat lyssavirus infection occurred in Primorye Territory (the Russian Far East). The nucleotide sequence was closest to the bat lyssavirus species Irkut virus (IRKV), with 95% identity. IRKV had been isolated earlier from a dead greater tube-nosed bat (*Murina leucogaster*) in Irkutsk (Leonova et al. 2009).

2.7 Control and prevention

2.7.1 Human rabies

Rabies caused by RABV in humans is preventable by the promotion of behavior that prevents exposure to rabid animals, by eliminating rabies from dog populations, and by the administration of pre-exposure vaccination or emergency post-exposure treatment if such exposure should occur. Appropriate prophylaxis given within a short time after exposure prevents the occurrence of rabies. After possible exposure, the wound, abrasion, and splashes are first cleansed with water and soap for 15 minutes and then disinfected. After cleaning, medical advice should be sought immediately (Anonymous 2013).

The control of rabies in humans needs to include rabies control in the reservoir species. Programs to increase awareness among the general public of the benefits of responsible dog ownership, basic care of suspected rabid bites, and avoiding

animal exposure is promoted through campaigns and child education. People are educated to avoid contact with possibly infected animals, including bats, and use exclusion housing or netting to prevent bats from entering the house. Bites and scratches can be avoided by the use of appropriate protective clothes and a proper handling technique.

Rabies biologics for humans include vaccines and rabies immune globulins (RIG). The former are used for both pre- and post-exposure prophylaxis, and the latter only during post-exposure prophylaxis. The administration of RIG is intended to provide an immediate supply of rabies-neutralizing antibodies until the production of active immunity in response to vaccine administration. This immunity only persists for a short time, but the idea is to intervene before the virus reaches the nervous system (Rupprecht and Slate 2012). Pre-exposure prophylaxis does not eliminate the need for post-exposure prophylaxis after possible exposure, but it simplifies the post-exposure treatment so that two doses of vaccine separated by three days are sufficient.

Pre-exposure prophylaxis vaccination is recommended for anyone who is at continual, frequent, or increased risk of exposure to the rabies virus as a result of their residence or occupation, such as laboratory workers, veterinarians, and animal handlers. Travelers in high-risk areas should be vaccinated after a risk assessment. Children living in or visiting rabies-affected areas are at particular risk and should be given pre-exposure prophylaxis. One dose is given on each of days 0, 7, and 21 or 28. Day 0 is the date of administration of the first dose of vaccine. Periodic booster doses of rabies vaccine are not necessary for people living in or travelling to high-risk areas who have received a complete primary series of pre- or post-exposure prophylaxis. Only people whose occupation puts them at continual or frequent risk of exposure should receive periodic booster injections. If available, monitoring of rabies virus neutralizing antibody of personnel at risk is preferred to routine boosters. For people potentially at high risk of laboratory exposure to high concentrations of live rabies virus, a neutralizing antibody titration should be performed every 6 months. If the titer falls below 0.5 IU/ml of serum, one booster dose of vaccine should be given (Anonymous 2013).

Factors that should be taken into consideration in deciding to initiate post-exposure prophylaxis include the epidemiological likelihood that the implicated animal was rabid, the severity of exposure, the clinical features of the animal, its vaccination status, and its availability for observation and laboratory testing. All exposures determined to represent a risk of rabies require post-exposure prophylaxis. Rabies immunoglobulin is administered only once, preferably at the same time or as soon as possible after the initiation of post-exposure vaccination. Vaccination regimens follow WHO's recommended protocols and typically consist of multiple injections over a period of four weeks. In Finland, vaccination

recommendations are given by the National Institute for Health and Welfare (Anonymous 2017b)

2.7.2 Dog-mediated rabies

The control of rabies in domestic dogs has important implications not just for the health of the dogs but also for public health. Each year, an estimated 7 million people are bitten by suspected rabid dogs. The most cost-effective way to eliminate human rabies is to eliminate dog rabies. In the 19th century, muzzling and dog movement restriction were used to control dog rabies. By the early 1900s, animal rabies vaccines had been developed. During the second half of the 20th century, dog rabies was eliminated through mass vaccination from Western Europe. Livestock deaths due to canine rabies are still poorly quantified, but are probably an important loss in impoverished livestock-dependent communities (Hampson et al. 2015).

Dog population management aims to stabilize or reduce the population size or density, adjust the population structure, improve the overall health, or alter the behavior of the dog population. Intervention includes culling and sterilization programs, sometimes combined with rabies vaccines. It is important to note that dog rabies can be very effectively controlled by vaccination programs alone (Knobel et al. 2013). Mass culling of dogs should not be an element of a rabies control strategy: it is ineffective and can be counterproductive to vaccination programs.

Intensive information and education campaigns are needed to strengthen community support and volunteer engagement in order to increase dog vaccination and responsible pet ownership. Surveillance and diagnostic facilities need to be strengthened to include rapid diagnostic measures (Anonymous 2013).

2.7.3 Sylvatic wildlife rabies

Depopulation of foxes only results in a transient lull in the prevalence of rabies. Oral rabies vaccination of foxes and raccoon dogs has reduced rabies cases in Europe, and several countries are now free of rabies in wildlife (other than bats). Only concerted actions across borders will lead to the successful control of rabies (Anonymous 2015).

2.7.4 Bat-related rabies

Vaccination of free-living bats is not feasible and there is therefore no effective way to eradicate lyssaviruses from bats. Culling of bats is not appropriate considering their valuable role in the environment, but hematophagous bats have been culled in Latin America by the administration of anticoagulants. In Europe, bats are under legal protection and conservation (Council Directive 92/43/EEC on the

conservation of natural habitats and of wild fauna and flora 94/43/EEC and the Agreement on the conservation of populations of European bats). The spillover of lyssaviruses from bats to other mammals and humans can be managed. The impact of hematophagous bat-related rabies on cattle can be minimized by vaccinating the cattle (Barrett 2011).

2.7.5 Surveillance of rabies

Surveillance involves the systematic continuous collection, analysis, and interpretation of data in order to demonstrate the absence of disease or to identify its presence and distribution based on the diagnostic confirmation of suspected and probable cases in humans and animals (Anonymous 2013).

Several recommendations by international organizations have been made on rabies surveillance (EFSA, WHO, OIE, EU). Passive surveillance implies the detection of rabies in suspected cases without actively searching for a disease. To determine the real-time spatial and temporal distribution of rabies by specific virus variants, passive surveillance is inadequate. Active rabies surveillance is targeted and typically initiated by the investigator with a designed sampling scheme.

2.7.6 Legislation concerning rabies surveillance and control

In Finland, rabies in humans is a notifiable communicable disease according to the Ministry of Social Affairs and Health: Communicable Diseases Decree 146/2017. Rabies in animals is a notifiable disease according to the Act on Animal Diseases (441/2013). The measures for the control of rabies are provided in Decision No. 724/2014 of the Veterinary and Food Department, including the investigation of all suspected cases by the veterinary authorities, notification procedures, and mandatory vaccination of hunting dogs and dogs used by officials. Dogs, cats, and ferrets entering Finland shall be vaccinated against rabies in accordance with Regulation No. 576/2013 of the European Parliament and of the Council.

2.8 Bats associated with lyssaviruses

Bats are the second most diverse group of mammals on Earth, with more than 1150 species. One in five mammal species is a bat, and they are found on every major land mass except for the Polar Regions and a few islands. Bats are reservoirs of many potentially infectious agents, such as Ebola virus, Nipah virus, SARS-like coronavirus, and lyssaviruses (Barrett 2011).

About 70% of bat species are insectivores, while most of the rest are frugivores. A few hematophagous bat species feed on blood from animals. Lyssaviruses have

been detected in many of the bat species that have been surveyed and should be assumed to be present in all bat species globally. Many countries have conducted few or no surveys on bats, and data are very limited for African and Eurasian bats, as well as Indian and South-East Asian bats. For some lyssavirus species, only one or a few isolates are available (Barrett 2011). In Europe, circa 45 bat species are present and lyssaviruses have been identified from at least eleven different bat species.

2.8.1 Bats in Finland

In Finland, thirteen species of bats have been recorded. All of these, apart from the northern bat (*Eptesicus nilssonii*), are at the northernmost limit of their range. Most of the species are restricted to southern and central Finland. Only five bat species are common, and the rest are either very rare or only a few individuals have ever been seen (Table 2). The distribution ranges of bats in Finland are still poorly known due to the limited number of bat studies carried out in the country.

Daubenton's bats are small, weighing about 5 to 15 g, and insectivorous. They are widespread and common in Finland. Daubenton's bats hibernate from October till March or April. They are facultative seasonal migrants, covering middle-range distances of 100–150 km between summer and winter roosts, and the longest recorded distance covered is 257 km (Hutterer et al. 2005). However, Daubenton's bats may show strong roost fidelity during the breeding season and between breeding seasons. Several studies have demonstrated that females in particular change their roost fairly often, every few days (Lucan et al. 2010). Fidelity is also apparent for larger areas with good feeding habitats and several roosting sites (Kapfer et al. 2008). Daubenton's bats prefer to roost in abandoned woodpecker cavities and bird or bat boxes. The majority of the animals hunt over water or in the vicinity of waterways, but they also hunt in forests, parks, or meadows. They usually forage close to the roost, within a distance of one kilometer (Parsons et al. 2003). Daubenton's bats are partially opportunistic trawling bats; insects are usually caught directly from the water surface.

Table 2. Status and distribution of the bat species recorded in Finland, modified from Kyheröinen et al. 2010. Status categories for red-list species are based on Liukko et al. 2010.

Species in Latin	Common name in English	Distribution in Finland	Status
Eptesicus nilssonii	The northern bat	Widespread in southern Lapland	Common
Eptesicus serotinus	Serotine bat	Southern Finland	Only a few records (first record in 2008)
Myotis brandtii	Brandt's bat	Widespread in southern and central Finland	Common
Myotis dasycneme	Pond bat	Eastern Finland	Only a few records in Finland (first record 2002)
Myotis daubentonii	Daubenton's bat	Widespread in southern and central Finland	Common
Myotis mystacinus	Whiskered bat	Widespread in southern and central Finland	Common
Myotis nattereri	Natterer's bat	Southern Finland	Rare, endangered
Nyctalus noctula	Common noctule	Southern Finland	Rare, migrant
Pipistrellus nathusii	Nathusius pipistrelle	Southern Finland	Restricted, migrant
Pipistrellus pipistrellus	Common pipistrelle	Southern Finland	Rare migrant (first record 2001)
Pipistrellus pygmaeus	Soprano pipistrelle	Southern Finland	Rare, migrant (first record 2007)
Plecotus auritus	Common long-eared bat	Southern and Central Finland	Common, widespread
Vespertilio murinus	Parti-colored bat	Southern Finland	Rare, migrant

2.9 Laboratory diagnosis of lyssaviruses

Because the signs of rabies may vary, laboratory diagnosis of the suspected animal is essential. Reliable diagnosis of rabies in animals might save human lives. A positive result leads to a public health investigation and post-exposure treatment of people, as well as to control measures to prevent the further spread of rabies within the animal population. A negative result may rule out the need for post-exposure treatment of humans and the management of domestic exposed animals (Hanlon and Nadin-Davis 2012).

Rabies diagnosis in animals is post-mortem diagnosis. The virus, viral genome or antigen can be detected in a brain sample. Serology can be used to detect antibodies due to rabies vaccination. Seroconversion due to natural infection only happens in the late gestation of the disease with advanced clinical signs, if at all, and is not

therefore a useful tool for demonstrating natural infection in animals other than bats. For bats, saliva swabs can be used to detect lyssaviruses or viral genome from live bats. Ante-mortem diagnosis is possible during the clinical human disease. The best samples are skin biopsy from the nape of the neck, saliva, serum, or cerebrospinal fluid (Anonymous 1996, 2015).

2.9.1 Detection of the virus

The direct fluorescent antibody test (FAT) is a rapid, sensitive, and specific method for diagnosing rabies and it is the gold standard method. The test is based on the microscopic examination of impressions or smears of brain tissue after incubation with anti-rabies globulin or antibodies conjugated with fluorescein isothiocyanate. Immunoperoxidase methods can be used as an alternative to FAT. An ELISA that detects rabies antigen is a variation of the immunochemical test. A rapid immunodiagnostic test (RIDT) can be used under field conditions and in developing countries with limited diagnostic resources.

Detection of the replicating virus after inoculation of a tissue suspension in cell cultures (RTCIT) or in laboratory animals shows the infectivity of the virus. Various molecular diagnostic tests, e.g. the detection of viral RNA by reverse transcription PCR (RT-PCR), PCR-ELISA, *in situ* hybridization, and real-time PCR, are used as rapid and sensitive additional techniques for rabies diagnosis. They are useful for confirmatory diagnosis as a first step in virus typing followed by sequencing (Anonymous 1996, 2015).

2.9.2 Detecting rabies antibodies

The main application of serology for rabies is to determine the response to vaccination in domestic animals prior to international travel, in wildlife populations following oral immunization, and for people after vaccination. For bats, seropositivity can be used as a tool to detect natural or experimental infection with lyssaviruses.

As neutralizing antibodies are considered a key component of the adaptive immune response against rabies virus, the gold standard tests are virus neutralization (VN) tests: the fluorescent antibody virus neutralization (FAVN) test and the rapid fluorescent focus inhibition test (RFFIT). Indirect ELISAs have been developed that do not require high-containment facilities and produce rapid results (Anonymous 1996, 2015).

2.10 Potency tests of vaccines

Varying requirements relating to quality, safety, and efficacy apply for manufacturers to obtain authorization or a license for a vaccine. The potency of inactivated virus vaccines is established and controlled using tests formulated by the European Pharmacopoeia. For live vaccines that are prepared for oral vaccination of animals, safety and efficacy in the target animals and safety in non-target species must be demonstrated (Anonymous 1996, 2015).

3 AIMS OF THE STUDY

This study was conducted to investigate the epidemiology of the EBLV-2 in Finland, to characterize the EBLV-2 viruses, and to assess the efficacy of vaccination against EBLV-2.

The specific aims of this study were as follows:

1. To study the epidemiology of EBLVs in Finnish bat populations (I) and to gain a better understanding of the public health risk that EBLV-infected bats pose (I, III, IV).

2. To describe the phylogenetic analysis of Finnish EBLV-2 strains isolated from a human in 1985 and from diseased bats in 2009 and 2016 (II, IV).

3. To calculate the tempo of viral evolution and divergence times for EBLV-2 viruses by molecular-clock estimation (II).

4. To assess the efficacy of animal and human vaccines against challenge with EBLV-2 isolated from a bat in Finland in comparison to challenge with RABV isolated from a raccoon dog (III).

5. To assess the factors influencing the success of rabies vaccination of the dog and cat population in Finland (III).

4 MATERIALS AND METHODS

4.1 Active and passive surveillance of bats (I, IV)

Dead bats or bats euthanized after showing clinical signs of rabies have been submitted to Evira for autopsy and laboratory analysis by veterinarians, members of the public, animal shelters, and bat biologists. The number of bats tested during 1985–2016 was 331, and details are presented in Table 5. Active surveillance took place in 2010–2011. Bats of seven bat species (Table 6) were caught from 71 sampling sites. Capture was carried out using a combination of harp traps and mist nets. Daubenton's bat roosts were primarily sampled. Samples were also collected from an old mill at a single site. Most of the samples were taken in southwest Finland, from Hamina in the east to Turku in the west.

Saliva samples were collected from 459 and 315 bats in 2010 and 2011, respectively. The specimens were collected from the oral cavity and oropharynx using dry nylon fiber- tipped oral swabs (Copan) and transported in a Copan Universal Transport Medium (UTM-RT) System (Copan Diagnostics Inc.). They were kept frozen until laboratory analysis.

Blood samples were collected from a total of 423 bats of six different species (Table 6). Blood samples were collected from 275 and 148 bats in 2010 and 2011, respectively. Sera were collected into a single 75-µl capillary tube from the interfemoral vein after lancing with a 27-gauge needle. The capillary tubes were centrifuged. Serum for rabies antibody screening was stored in Eppendorf tubes, which were kept frozen at -20 °C until laboratory analysis.

4.2 Laboratory analysis (I, II, III, IV)

4.2.1 Antigen detection (I, II, III, IV)

Rabies laboratory diagnosis was performed on brain samples according to the OIE Manual of Standards for Diagnostic Tests and Vaccines, using a standard fluorescent antibody test (FAT). Smears prepared from a sample of brain tissue were fixed in high-grade cold acetone, air dried, and then stained with specific conjugate. Two conjugates were used: rabies conjugate anti-nucleocapsid (Bio-Rad, USA) and FITC anti-rabies monoclonal globulin (Fujirebio Diagnostics Inc., USA), which were diluted according to the manufacturers' instructions. FAT slides were then examined for specific fluorescence using a fluorescence microscope.

4.2.2 Virus isolation (II, III, IV)

Virus isolation was performed in mouse neuroblast cell culture, according to a procedure described in the OIE manual. In addition, the bat brain suspension of the 2009 isolate was inoculated into suckling mice. Eight newborn mice (ScaNmri) at 2 days old were intracerebrally infected with 20 µl of the suspension in the Biosafety Level 3 (BSL-3) laboratory. When the mice started to develop signs of encephalitis, they were euthanized and the brains were collected and examined using a fluorescence antibody test (FAT) for the presence of lyssavirus. The human virus isolate of 1985 (Lumio et al. 1986, Roine et al. 1988) used in this study was the first archived newborn mouse passage, and had been kept at -70 °C since 1986.

4.2.3 Nucleic acid detection (I, II, IV)

The RNA for PCR studies was extracted from the samples with a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was tested for reaction EBLV-1 and EBLV-2 (I) with the OneStep real-time reverse transcription-polymerase chain test (OneStep RT-PCR kit, Qiagen, Hilden, Germany). The reaction volume was 50 µl and the temperature profile of cDNA synthesis and amplification was: 50 °C for 30 min, 95 °C for 15 min, and 45 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 20 s. The sequences for the primer (JW12, N165-146) and the probe (LysGT5 and LysGT6) were published by Wakeley et al. (2005).

Primers for whole genome sequencing (II) were designed with the program PCR Suite (van Baren 2004) and modified afterwards, if needed. The OneStep RT-PCR kit (Qiagen, Germany, Hilden) was used to amplify 34 overlapping fragments. The following thermal profile was used: a single cycle of reverse transcription for 30 min at 50 °C, 15 min at 94 °C for reverse transcriptase inactivation and DNA polymerase activation, followed by 30 amplification cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C.

For the bat sample of 2016 (IV), the OneStep RT-PCR kit (Qiagen, Germany, Hilden) was used to amplify two fragments. The reaction volume was 25 µl and the temperature profile of cDNA synthesis and amplification was 30 min at 50 °C, 15 min at 94 °C for reverse transcriptase inactivation and DNA polymerase activation, followed by 30 amplification cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C. Primers (N127, N66CS, N512 and N8m) were published by Davis et al. (2005).

All the primers used are presented in Table 3.

Table 3. Primers and probes used in the RT-PCR and sequencing. Primer and probe positions by Wakeley et al. (2005) are given relative to the Pasteur virus genome (accession no. M13215). The position numbering by Jakava-Viljanen et al. (2015) is according to the EBLV-2 isolate RV1333 (EF157977). Sequence positions by Davis et al. (2005) are numbered relative to the rabies virus PV genome.

References: 1. Wakeley et al. (2005), 2. Jakava-Viljanen et al. (2015), 3. Davis et al. (2005).

Primer	Sequence 5'-3'	Position	Reference
N165-146	gca ggg tay ttr tac tca ta	165–146	1
JW12	atg taa cac cyc tac aat g	55–73	1
1f	acg ctt aac gac aaa acc ag	1–20	2
1r	tag ctc tcc caa tcg tca gg	326–345	2
2f	cgc tag gtt gga tcc tga tg	256–275	2
2r	ggc gca cat ctt gtg agt ag	636–655	2
3f	cca acg tag ctg aca gaa tgg	558–578	2
3r	aca tct cgt gag gtg cac ag	1066–1085	2
4f	cgg gag tta cat ggg tca ag	1015–1034	2
4r	gtc tgg cct gat gat tcg ag	1354–1373	2
5f	cag gat cat ggt caa tgg	1291–1308	2
5r	tcc caa cac cct caa ggt ag	1812–1831	2
6f	aag aag aag gaa gcg atg agg	1745–1765	2
6r	tgc gct att tct gct tca ac	2116–2135	2
7f	acc tgc gct gga atg gtc	2070–2087	2
7r	ggg agc cat agg tca tca tc	2591–2610	2
8f	agt gag agg ttg cag gga tg	2530–2549	2
8r	act ctg ccc att gaa aca cc	2869–2888	2
9f	ttc cag agg gaa tga act gg	2826–2845	2
9r	ggt gtt cag tcg ggt gtt tc	3245–3264	2
10f	ctt tta tga gca ata gaa caa aac c	3186–3210	2
10r	atg tcg gat cac ctg cag tc	3688–3704	2
11f	aac tac cac gtt caa gag aaa gc	3619–3641	2
11r	ttt gcc tca tcg tga ttt tg	4115–4134	2
12f	tga aac tgt gtg gaa tct ctg g	4059–4080	2
12r	atg ctg ttg aag cat tgc ag	4518–4537	2
13f	tcc tca tca caa tgg agt ctt c	4441–4462	2
13r	ccc act ttg gga agt gac ag	4830–4849	2
14f	aaa gag agc caa acc caa cc	4787–4805	2
14r	att gca tcc tct ccc act tg	5154–5171	2
15f	acc ggt aca cag ggt ctt gc	5076–5095	2
15r	gca tct atc tcc ggt tcg ac	5458–5477	2
16f	aga tga ttg atc ccc tgg ag	5414–5433	2
16r	gag gca ctt tcg act tct gg	5747–5766	2
17f	cgc aca atc cat gat gtc tc	5697–5716	2
17r	gaa tca gga ggg agt tga acc	6173–6193	2

18f	tct cag agt gcc aac tgt ctg	6106–6126	2
18r	gtt cct tca agc tgg ctc ac	6415–6434	2
19f	tta gtg cag agg gct gaa gg	6340–6359	2
19r	tat ggg atc aaa ggg tgg tc	6709–6728	2
20f	ctg gct aaa cgg atc ctc ag	6634–6653	2
20r	aag aat tcc ctg ggg ttg ac	6964–6983	2
21f	ccg tcc cca gtg aga aag tc	6917–6936	2
21r	gac ctt gtc ccg tga ctc tg	7209–7228	2
22f	ttg gcg aac tac atc tta ccc	7129–7149	2
22r	tga gtc cct ctt ggg tca ac	7641–7660	2
23f	agc aca ggg aga caa cca ag	7590–7609	2
23r	gtg aaa tac cgc ctg gac tg	7979–7998	2
24f	gtc gca cag cat tca caa tc	7924–7943	2
24r	agc aga atg gtt gga ctt gc	8332–8351	2
25f	ccg gac ttg ggt gat aga ag	8254–8273	2
25r	aaa ttg ccg tcg aat tgt tc	8567–8586	2
26f	gct cat cct tcc tcg gaa tac	8513–8533	2
26r	gat ttg agt ccc tgg caa tg	9021–9040	2
27f	cca acg tcc atg ttg tca ag	8966–8985	2
27r	aga cat ccg gga aca tga ag	9417–9436	2
28f	caa gtg cat ccg acc gat ag	9369–9388	2
28r	cag atc gaa gtg agg gtt cc	9831–9850	2
29f	tgt tga ggc tag aca atc atc c	9788–9809	2
29r	taa ggt gtc ttc ccc gtg ac	10151–10167	2
30f	atc cga ctc agg cag ttg ag	10105–10124	2
30r	gag gcc atg agg tca ttc ac	10606–10625	2
31f	tgg aat ctc cag aac tgt gc	10539–10558	2
31r	tgg cct tgt agt ctg ggt tc	10923–10942	2
32f	ctc tcg atc aat ggt cca ctc	10867–10887	2
32r	tta gcc aag gtc cct ctt tg	11287–11306	2
33f	tga agt cga gtc att cct agt cc	11238–11260	2
33r	gct act acc ggc aag tcg ag	11672–11691	2
34f	aag caa gtc ata cga gga ag	11533–11552	2
34r	acg ctt aac aaa aaa aac ata g	11909–11930	2
N127	atg taa cac ctc tac aat gg	55–74	3
N66CS	gtg ctc car ttt gcr cac a	578–596	3
N512	gtg gcw gay aga atg gaa ca	493–512	3
N8m	cag tct cyt cng cca tct c	1567–1586	3
Probes			
LysGT5	aac arg gtt gtt tty aag gtc cat aa	80–105	1
LysGT6	aca raa ttg tct tca arg tcc ata atc ag	81–109	1

Table 4. Lyssavirus strains used in the phylogenetic studies.

Species	Abbreviation	Country	Host	Year	Accession number	Reference
European bat lyssavirus 2	EBLV-2	Finland	Homo sapiens	1985	JX129233	II
European bat lyssavirus 2	EBLV-2	Finland	Myotis daubentonii	2009	JX129232	II
European bat lyssavirus 2	EBLV-2	Finland	Myotis daubentonii	2016	MF326269	IV
European bat lyssavirus 2	EBLV-2	Netherlands	Myotis dasycneme	1987	U89480	Amengual B. J. Gen Virol 78 (Pt 9), 2319-2328 (1997)
European bat lyssavirus 2	EBLV-2	Netherlands	Myotis dasycneme	1989	AY062089	Johnson N. Arch Virol 147 (11), 2111-2123 (2002)
European bat lyssavirus 2	EBLV-2	Netherlands	Myotis dasycneme	1986	EU293114	Delmas O. PLoS ONE 3 (4), E2057 (2008)
European bat lyssavirus 2	EBLV-2	Netherlands	Myotis dasycneme	1993	AY863404	Davis P. J Virol 79 (16), 10487-10497 (2005)
European bat lyssavirus 2	EBLV-2	Switzerland	Myotis daubentonii	1992	AY212117	Johnson N. Vet Rec 152 (13), 383-387 (2003)
European bat lyssavirus 2	EBLV-2	Switzerland	Myotis daubentonii	1993	U89479	Amengual B. J Ge. Virol 78 (Pt 9), 2319-2328 (1997)
European bat lyssavirus 2	EBLV-2	Switzerland	Myotis daubentonii	1993	AY212118	Johnson N. Vet Rec 152 (13), 383-387 (2003)
European bat lyssavirus 2	EBLV-2	Switzerland	NA	2002	AY863408	Davis, P. J Virol 79 (16), 10487-10497 (2005)
European bat lyssavirus 2	EBLV-3	Switzerland	Myotis daubentonii	2002	KF831571	Deubelbeiss AN. Unpublished
European bat lyssavirus 2	EBLV-2	Germany	Myotis daubentonii	2006	JQ796805	McElhinney L. Zoonoses Public Health 60 (1), 35-45 (2013)
European bat lyssavirus 2	EBLV-2	Germany	Myotis daubentonii	2007	GU227648	Freuling C. Vet Microbiol 131 (1-2), 26-34 (2008)
European bat lyssavirus 2	EBLV-3	Germany	Myotis daubentonii	2013	KF826149	Schatz J. PLoS Negl Trop Dis 8 (5), E2835 (2014)
European bat lyssavirus 2	EBLV-2	United Kingdom	Myotis daubentonii	1996	U89478	Amengual B. J Gen Virol 78 (Pt 9), 2319-2328 (1997)
European bat lyssavirus 2	EBLV-2	United Kingdom	Homo sapiens	2002	EF157977	Marston DA. J Gen Virol 88 (PT 4), 1302-1314 (2007)
European bat lyssavirus 2	EBLV-2	United Kingdom	Myotis daubentonii	2002	AY212120	Johnson N. Vet Rec 152 (13), 383-387 (2003)
European bat lyssavirus 2	EBLV-2	United Kingdom	Myotis daubentonii	2003	JQ796808	McElhinney L. Zoonoses Public Health 60 (1), 35-45 (2013)
European bat lyssavirus 2	EBLV-2	United Kingdom	Myotis daubentonii	2004	JQ796807	McElhinney L. Zoonoses Public Health 60 (1), 35-45 (2013)
European bat lyssavirus 2	EBLV-2	United Kingdom	Myotis daubentonii	2004	KF155004	Marston DA. BMC Genomics 14 (1), 444 (2013)
European bat lyssavirus 2	EBLV-2	United Kingdom	Myotis daubentonii	2006	JQ796809	McElhinney L. Zoonoses Public Health 60 (1), 35-45 (2013)
European bat lyssavirus 2	EBLV-2	United Kingdom	Myotis daubentonii	2007	JQ796810	McElhinney L. Zoonoses Public Health 60 (1), 35-45 (2013)
European bat lyssavirus 2	EBLV-2	United Kingdom	Myotis daubentonii	2008	JQ796811	McElhinney L. Zoonoses Public Health 60 (1), 35-45 (2013)

European bat lyssavirus 2	EBLV-2	United Kingdom	Myotis daubentonii	2008	JQ796812	McElhinney L. Zoonoses Public Health 60 (1), 35-45 (2013)
European bat lyssavirus 2	EBLV-2	United Kingdom	Myotis daubentonii	2009	JQ796806	McElhinney L. Zoonoses Public Health 60 (1), 35-45 (2013)
European bat lyssavirus 2	EBLV-2	Norway	Myotis daubentonii	2015	KX644899	Moldal T. BMC Vet Res. 2017;13:216
Khujand virus	KHUV	C Asia Tajikistan	Myotis mystacinus	2001	EF614261	Kuzmin IV. Virus Res 97 (2), 65-79 (2003)
Bokeloh bat lyssavirus	BBLV	Germany	Myotis nattereri	2010	JF311903	Freuling C.M., Emerging Infect. Dis. 17 (8), 1519-1522 (2011)
Bokeloh bat lyssavirus	BBLV	Germany	Myotis nattereri	2012	KF245925	Freuling C. Virus Res 177 (2), 201-204 (2013)
Aravan virus	ARAV	C Asia Kyrgystan	Myotis blythi	1991	EF614259	Kuzmin IV. Virus Res 97 (2), 65-79 (2003)
Australian bat lyssavirus	ABLV	Australia	Homo sapiens	1986	AF418014	Warrilow D. Virology 297 (1), 109-119 (2002)
Australian bat lyssavirus	ABLV	Australia	Pteropus sp.	1996	NC003243	Gould AR. Virus Res 89 (1), 1-28 (2002)
Australian bat lyssavirus	ABLV	Australia	Pteropus scapulatus	2014	KT868956	O'Dea M. Unpublished
Rabies virus	RABV	S America FR Guiana	Dog ex a bat	1990	EU293113	Delmas O. PLoS ONE 3 (4), E2057 (2008)
Rabies virus	RABV	S America Brazil	Artibeus lituratus	1998	AB519641	Mochizuki NJ. Vet Med Sci 73 (6), 759-766 (2011)
Irkut virus	IRKV	E Siberia	Murina leucogaster	2002	FJ905105	Kuzmin IV. Virus Res 111 (1), 28-43 (2005)
Irkut virus	IRKV	China	Murina leucogaster	2012	JX442979	Liu Y. PLoS Negl Trop Dis 7 (3), E2097 (2013)
Duvenhage virus	DUVV	S Africa	Homo sapiens	1971	EU293119	Delmas O. PLoS ONE 3 (4), E2057 (2008)
European bat lyssavirus 1	EBLV-1	France	Epticus serotinus	2003	EU293109	Delmas O. PLoS ONE 3 (4), E2057 (2008)
European bat lyssavirus 1	EBLV 1	Germany	Epticus serotinus	1968	EF157976	Marsto D. J Gen Virol 88 (PT 4), 1302-1314 (2007)
West Caucasian bat virus	WCBV	Russia Caucasian region	Miniopterus schreibersi	2002	EF614258	Kuzmin IV. Virus Res 111 (1), 28-43 (2005)
Lleida bat lyssavirus	LLBV	Spain	Miniopterus schreibersi	2012	JX402204	Ceballos NA. Emerging Infect Dis 19 (5), 793-795 (2013)
Lagos bat virus	LBV	W Africa Nigeria	Eidolon helvum	1956	EU293110	Delmas O. PLoS ONE 3 (4), E2057 (2008)
Shimoni bat virus	SHIBV	Africa Kenya	Hipposideros commersoni	2009	GU170201	Kuzmin IV. Virus Res 149 (2), 197-210 (2010)
Ikoma virus	IKOV	Tanzania	Civettictis civetta	2009	JX193798	Marston D. J. Virol. 86 (18), 10242-10243 (2012)
Mokola virus	MOKV	Nigeria	Shrew	1968	NC006429	Le Mercier P. J Gen Virol 78 (Pt 7), 1571-1576 (1997)
Mokola virus	MOKV	Zimbabwe	Feline	1993	KF155007	Marston D. BMC Genomics 14 (1), 444 (2013)
Gannowuwa bat lyssavirus	GBLV	Sri Lanka	Pteropus giganteus	2015	KU244269	Gunawardena PS. Emerging Infect Dis 22 (8), 1456-1459 (2016)

After agarose gel electrophoresis, the bands were cut from the gel and DNA was extracted with the Qiaquick Gel Extraction Kit (Qiagen, Germany, Hilden). PCR products were sequenced using an ABI 3100 Avant Genetic Analyzer (Applied Biosystems) with the primers used in the PCR and a Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems). Before sequencing, the reaction products were purified using the DyeEx 2.0 Spin Kit (Qiagen, Germany, Hilden). The sequences were analyzed with Sequencing Analysis software version 6.0 (Applied Biosystems).

The newly obtained genome sequences (GenBank accession numbers JX129232, JX129233 and MF326269) were aligned with other published lyssavirus bat-type isolates extracted from GenBank to evaluate the genetic diversity of EBLV-2 strains and their relationship with other bat-associated genotypes. Amino acid sequences were deduced using the "translate" function of the program MEGA v.5.1 (Tamura et al. 2011). Phylogenetic trees of full-length genomes and partial N coding sequences were calculated using the maximum likelihood approach in the program MEGA with 1000 bootstrap replicates. The pairwise sequence identities were calculated using CLUSTAL W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), with default settings. The lyssavirus strains used in these phylogenetic analyses are presented in Table 4.

For evolutionary time-scale analysis (II), the program BEAST (Drummond et al. 2007) was used to estimate the substitution rate of EBLV-2 and time of divergence from the phylogenetically closest lyssavirus species. The data set used for estimating the rate of evolution was based on the partial N coding sequences (400 bp) of EBLV-2 strains isolated from the years 1986 to 2012. The Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution was used (Hasegawa et al. 1985), and both strict and relaxed lognormal clock models. Convergence of parameters was assessed using TRACER (Drummond et al. 2007), and each run was continued until the effective sampling size of all parameters was greater than 200.

4.2.4 Serological analysis (I, III)

Serological testing for EBLV virus neutralizing antibodies from bat samples was performed using a fluorescent antibody virus neutralization test (Cliquet et al. 1998) according to modification (mFAVN) by the UK Animal Health and Veterinary Laboratories Agency (AHVLA). The volume of sera used was 30 µl. The EBLV-2 antigen, strain RV 628, was provided by the European Virus Archive supported by the European Community. Two positive control sera, both provided by the European Virus Archive, were used in each test. Samples needed to be pooled to obtain the minimum serum volume required for the test. In total, 167 pools were tested. Samples from animals of the same species, gender, and site were pooled. Samples were analyzed in duplicate and serially diluted using a 3-fold series. A titer of 1:27 was chosen to distinguish positive from negative samples.

The serum samples of the vaccinated mice were analyzed using the rapid fluorescent focus inhibition test RFFIT (OIE). Serial dilutions of test sera were mixed with the challenge virus preparation and BHK-21 cells. Samples were fixed and stained with specific conjugate (FITC Anti-Rabies Monoclonal Globulin, Fujirebio Diagnostics). Residual virus was detected using a standard fluorescence microscope. The serum neutralization end-point titer was defined as the dilution factor of the highest serum dilution at which 50% of the observed microscopic fields contained one or more infected cells.

The antibody responses of dogs and cats were determined using the FAVN test (Cliquet et al. 1998). This test involves the neutralization of a constant amount of rabies virus CVS-11 strain adapted to cell culture before inoculating cells susceptible to rabies virus (BHK-21). The serum titer is the dilution at which 100% of the virus is neutralized in 50% of the wells. This titer is expressed in IU/ml by comparing it with the neutralizing dilution of OIE serum of dog origin under the same test conditions. Dogs and cats with titers of ≥0.5 IU/ml had passed the test and dogs with titers of <0.5 IU/ml had failed the test.

4.3 Vaccination challenge of mice with RABV and EBLV-2 after vaccination with a human vaccine and an animal vaccine (III)

Three- to four-week-old NMRI mice (Harlan, NL; n = 20 per challenge virus and n = 5 vaccine controls) were vaccinated intra-peritoneally with 0.1 ml of vaccine diluted 1:10 in physiological saline solution with a 16-mm needle. With the human vaccine (Rabies-Imovax®; Sanofi-Pasteur MSD, France, batch G1391-4), the mice were vaccinated either once or twice with a two-week interval. With the animal vaccine (Rabisin® vet; Merial, France, batch L374051), the mice were vaccinated once. Vaccines were purchased from Helsinki University Pharmacy.

The potency test protocol was modified from the European Pharmacopoeia protocol: the minimum lethal dose (MLD50) for the intra-cranial challenge was determined and the 50 MLD_{50} was used, and mice were challenged intra-cranially with 30 µl of 10% virus suspension according to the procedure described by WHO (Anonymous 1996) 28 days after vaccination. The suspension was prepared with MEM and later with physiological saline. The mice were anesthetized by inhalation anesthesia using isoflurane and were given 0.05–0.1 mg/kg buprenorphine hydrochloride subcutaneously at the time of intra-cranial challenge. The back titration of 5 mice per group was set up with 50 MLD_{50}, 5 MLD_{50} and 0.5 MLD_{50} of each virus. Five mice per vaccine that were not challenged served as vaccine controls. The mice were monitored twice per day for any clinical signs of rabies, and to minimize suffering they were killed when signs of rabies infection were

obvious (weight loss, behavioral changes, neurological signs, and paralysis) or when the observation period of six weeks had ended. Serum was collected from the vaccinated mice prior to the challenge, approximately three weeks after the vaccination. At the end of the trial, the brains and sera of the mice were collected either at the time they were euthanized due to rabies signs or when they were euthanized after the monitoring period.

4.4 Post-vaccination analysis of dog and cat samples (III)

This was a case-control study with a duration of five years. During 2009–2013, serum samples from dogs (n = 10 071) and cats (n = 722) were sent to the Finnish Food Safety Authority Evira for post-vaccination efficacy tests. Of these samples, 1055 dogs that did not pass the test and had submission data available comprised the case group for dogs. An approximately similar number of dogs with submission data that passed the test were randomly assigned to the control group (n = 1626). In cats, only 25 failed the test (cases) and a much larger number of cats that passed were randomly assigned to the control group (n = 241). Submission forms for these samples were evaluated. Three (3) inactivated rabies vaccines were used for dogs and cats in Finland during 2009–2013: Wistar-G52 strain vaccine, BHK-21cell vaccine with Pasteur RIV strain, and Flury LEP strain vaccine.

4.4.1 Statistical analysis (III)

The 95% confidence intervals (CI) for valid percentages (excluding missing values) were calculated with Jeffrey's method using EpiTools (Sergeant 2015). Statistical analyses were performed using the statistical software SPSS 22.0 (IBM SPSS Statistical Package version 22, USA). The outcome variable was failure to reach the required antibody level (0 for antibody level ≥0.5 IU/ml, denoting the ability to reach the required antibody level, and 1 for <0.5 IU/ml, denoting failure). Independent variables collated in the dataset were the vaccine used, age at vaccination, gender, the number of vaccinations, and the time from vaccination to sampling, and for dogs, the breed and height of the dog as measured at the withers. Dogs were categorized into five different breed size groups based on their height (<30 cm, 30–45 cm, 46–60 cm, >60 cm, and unknown).

Animals were divided into two age groups: up to one year and over one year old. Based on the time interval between vaccination and sampling, three groups were created: sampling less than three months after vaccination, three to six months after vaccination, and more than six months after vaccination. First, Fisher's exact test and crude logistic regression analyses were performed to examine the pairwise associations between the outcome and each independent variable separately.

Variables with $p \leq 0.2$ were included in the multivariable logistic regression analysis, with separate models for cats and dogs, and variables with Wald's $p < 0.05$ were included in the final model. Correlations between independent variables were calculated with Spearman's test and no significant correlations were found. A causal diagram was used to assess potential confounders; their impacts on the other variables in the model were verified, but none needed to be included in the models. Pairwise interactions were assessed. Since in dogs a significant interaction was found between the vaccine used and the age of the dog, two separate models were created: one for dogs up to one year old and another for older dogs. Additionally, for younger dogs, the time interval between vaccination and sampling was categorized into two groups only: up to or more than six months. The goodness of fit of the final model was assessed with the Omnibus test, Nagelkerke's R2, and the Hosmer and Lemeshow test, and by calculating the area under the curve (AUC).

4.5 Ethical approval and permissions (I, III)

The National Animal Experiment Board of the County Administrative Board of Southern Finland approved the sampling of bats, including blood and saliva sampling (permit numbers: Lilley ESLH-YM-2007-01055, Kyheröinen ESLH-2009-04958/Ym-23), which followed Finnish legislation, namely the Finnish Act on the Use of Animals for Experimental Purposes (62/2006). Bats were captured and handled under permits from the following regional environmental centers (Centre for Economic Development, Transport and the Environment): Southeast region, KASELY/379/20/07.01/2010; Southern Finland, LUO 459/UUS-2009-L-388-254 and UUDELY/475/07.01/2011; Southwest region, LOS-2007-L-182-254; and Eastern region, POSELY/501/07.01/2010 and ESAELY/557/07.01/2010.

The National Animal Experiment Board of the County Administrative Board of Southern Finland approved the diagnostic mouse inoculation test (permission number ESLH-2008-06899/Ym-23).

The vaccination challenge was carried out with the permission of the National Animal Experiment Board of the County Administrative Board of Southern Finland (permission number ESAVI-2010-06673/Ym-23 and ESAVI/955/04.10.07/2013), which followed the Finnish Act on the Use of Animals for Experimental Purposes (62/2006).

5 RESULTS

5.1 Passive and active surveillance of the bats (I, IV)

In the passive surveillance of 331 samples during 1985–2016 (Table 5), two positive Daubenton's bat were detected by FAT, RTCIT (Fig. 6), and by RT-PCR and subsequent sequencing: one in 2009 and the other in 2016. The 2016 Daubenton's bat was a cachexic adult female, weighing 7 g. Only 15 Daubenton's bats were submitted for laboratory analysis, and two were positive for EBLV-2, which would indicate that 13% (95% Cl 3.7–38%) of all passive surveillance Daubenton's bat were positive for EBLV-2 in Finland.

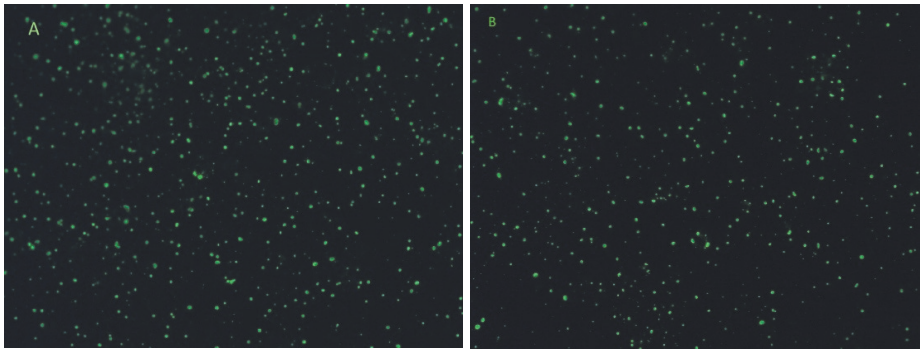

Figure 6. RTCIT image of the 2016 Daubenton's bat (A) and positive control (B)

Table 5. Number of bats tested for rabies in the passive surveillance in Finland 1985–2016.

Year	Unknown/ N.A.	*Eptesicus nilssonii*	*Myotis daubentonii*	*Myotis mystacinus*	*Myotis brandtii*	*Plecotus auritus*	In total	Number of positive samples
2016		9	2	4	3	1	19	**1**
2015	4	13	3	6			26	0
2014	2	11		1			14	0
2013	3	7		3		1	14	0
2012	8	16	1	6		1	32	0
2011	3	6		4			13	0
2010		6	1	1			8	0
2009	4	13	3	2		2	24	**1**
2008							0	0
2007	1	2					3	0
2006		1					1	0
2005			1				1	0
2004		1		2		1	4	0
2003			1				1	0
2002		3					3	0
2001	1						1	0
2000	2						2	0
1999		2					2	0
1998		9					9	0
1997	1						1	0
1996	5						5	0
1995	5						5	0
1994	2						2	0
1993	1	1					2	0
1992	3	6	1	3		1	14	0
1991	5						5	0
1990	4	3					7	0
1989	3	3					6	0
1988	13						13	0
1987	35						35	0
1986	3	33	2	1	8	14	61	0
1985	1						1	0
In total	109	145	15	33	11	21	331	**2**

Figure 7. The EBLV-2-in'fected Daubenton's bat appearing in the day. (Photo: Riitta Räisänen)

Antibodies were detected from Daubenton's bats from two sampling sites in 2010, in the city of Turku (60°27′05″N, 022°16′00″E) and in Nauvo (60°11′35″N, 21°54′25″E), and from one sampling site in 2011 in the city of Naantali (60°28′05″N, 22°01′35″E). No EBLV RNA was detected in the active surveillance in any of the oropharyngeal swabs analyzed (Table 6).

The seropositive samples were from 3 to 9 (due to the pooling of samples) male Daubenton's bats. In the pool collected in 2010 from Turku, all individuals were adults and caught while flying. In the pool collected in Nauvo, all bats were adults and they were caught in roosts. In the seropositive pool collected in 2011 from Naantali, one individual was an adult and two were juveniles, and they were caught while flying. Altogether, 268 samples from Daubenton's bat were analyzed, resulting in a seroprevalence of 1.12% to 3.36%. A 95% confidence interval for the true prevalence of 0.2–4% was calculated, as described by Rogan and Gladen (1978). All the positive sites were from the same geographical area (Fig. 8).

Table 6. Number of bats tested for cross-neutralizing lyssavirus antibodies and viral EBLV-1 and -2 per species, and number of positive bats during active sampling in 2010 and 2011.

Species	Number of bats (number of positive bats) tested for lyssavirus antibodies	Number of bats (number of positive bats) tested for EBLV-1 and -2 RNA
M. daubentonii	268 (3–9*)	399 (0)
M. brandtii	71 (0)	129 (0)
P. auritus	38 (0)	98 (0)
E. nilssonii	29 (0)	108 (0)
M. mystacinus	16 (0)	36 (0)
M. nattereri	1 (0)	1 (0)
P. nathusii	0	3 (0)
Total	423 (3–9*)	(0) * Pooled samples

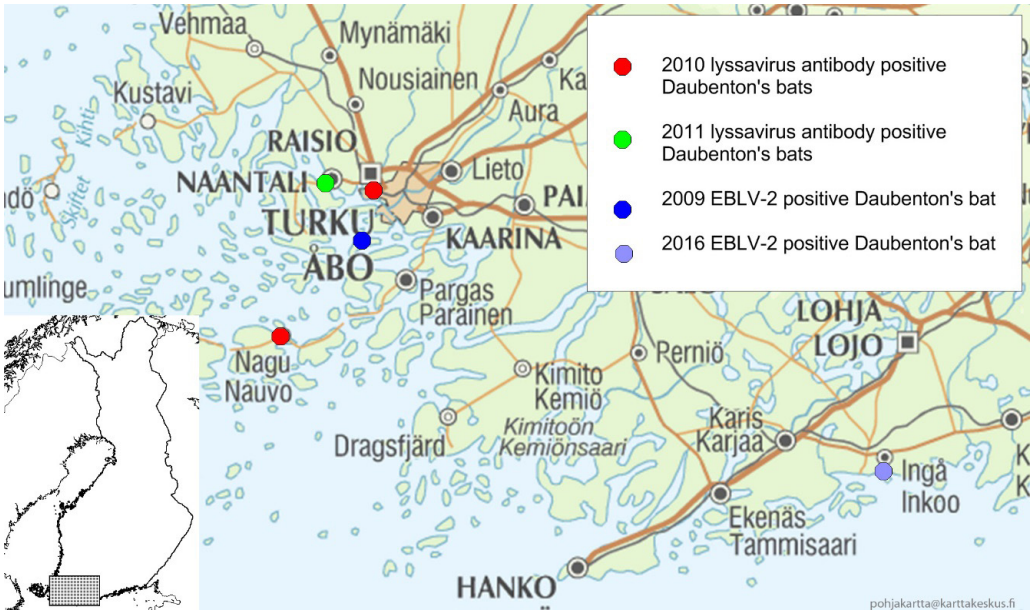

Figure 8. Geographical locations of the lyssavirus antibody-positive Daubenton's bats sample collection sites and the locations where two EBLV-2-positive Daubenton's bats were found. Map: Pia Vilen.

5.2 Virus isolation of the EBLV-2 strains 2009 and 2016 (II, IV)

Inoculation of the bat brain samples of 2009 and 2016 into MNA cells revealed the presence of virus in a limited number of cells after 3 days of incubation. In addition to the brain sample, several organs were tested for the 2016 Daubenton's bat. The MNA cell culture was positive for the 2016 isolate from the brain, spinal cord, and salivary glands (Table 7).

Table 7. Results of lyssavirus detection from different organ and swab samples from the 2016 Daubenton's bat. (FAT = fluorescent antibody test, RTCIT = rabies tissue culture infectious test, NA = not applicable)

	Brain	Spinal cord	Salivary gland	Mouth swab	Anal swab	Tongue	Intestines	Liver	Eye	Lung	Trachea	Bladder	Tonsils
FAT	+	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
RTCIT	+	+	+	-	-	-	-	-	-	-	-	-	-
RT-PCR	+	+	+	-	-	-	-	-	-	-	-	-	-

The brain suspension of the 2009 bat isolate was inoculated into suckling mice. The mice began to develop signs of encephalitis on day 12 p.i. By day 17 p.i., all 8 inoculated mice were euthanized. The presence of the virus in all the brain samples was confirmed by FAT.

5.3 Genetic characterization and evolutionary time-scale analysis of the EBLV-2 strains from a human case in 1985 and from a bat in 2009 (II)

The complete lengths of the EBLV-2 1985 (GenBank accession number JX129233) and 2009 (GenBank accession number JX129232) genomes were 11 928 and 11 927 nucleotides, respectively. Twenty nucleotides at the 5' end and 22 nucleotides at the 3' end of the genomes were primer derived and were excluded from all the phylogenetic analyses.

The general genome organization was typical for lyssaviruses, consisting of five structural genes, N, P, M, G, and L, and non-coding regions between them and at both ends of the genome. The lengths of the genes and the number of deduced amino acids (in parentheses) were 1356 (451 aa), 894 (297 aa), 609 (202 aa), 1575 (524 aa), and 6384 (2127 aa), respectively. The long untranslated region between the G and L genes was the only area that varied in length between the two Finnish EBLV-2 strains, being 510 bp in FI-85 and 509 bp in FI-09. The corresponding

lengths for other completely sequenced EBLV-2 strains were 511 bp for EU293114 and 512 bp for KF155004 and EF157977.

The nucleotide identity between EBLV-2 1985 and 2009 strains was 99.6%. There were four amino acid differences between the two Finnish strains: N gene ah 106 (N><S), G gene aa 158 (V><A), L gene aa 154 (R><K), and aa 1656 (G><D). The N, P, M, G, and L genes of the FI-85 and FI-09 strains shared 98–99%, 98%, 99–100%, 97–99%, and 99% amino acid identity, respectively, with the previously published EBLV-2 strains. The non-coding regions were found to be highly divergent, yet rather similar for all EBLV-2 strains.

A phylogenetic tree was calculated based on the two novel EBLV-2 genomes and full-length bat-related lyssavirus genomes obtained from GenBank (Fig. 9). The analysis revealed that all five EBLV-2 strains are monophyletic. The phylogenetic tree also demonstrated that EBLV-2 strains share the most recent common ancestry with BBLV and KHUV, and more distant ancestry with ARAV, RABV and ABLV. A second major cluster of lyssaviruses consists of IRKV, DUVV, and EBLV-1. The clearly most divergent group is formed by WCBV. IKOV, LBV, SHIV, and MOKV.

In order to include more EBLV-2 strains, we calculated phylogenetic trees (Fig. 10) based on partial N gene sequences. EBLV-2 strains were also monophyletic in this region. Some discrepancies were observed between the two trees, especially in the composition of the major clusters that were not reproduced in the phylogeny based on partial N-gene sequences.

The substitution rate of European bat lyssavirus type 2 was estimated using the two Finnish strains isolated 24 years apart as a calibrator. The overall rate was 7.67×10^{-5} substitutions per site per year. The current diversity of EBLV-2 was estimated to have appeared during the last 2000 years. However, EBLV-2 and other phylogroup I viruses were estimated to have already diverged from other lyssaviruses at about 8000 years ago (Fig. 11). The divergence of Finnish EBLV-2 strains and a strain from Switzerland from other EBLV-2 strains has occurred during the last 1000 years. The two Finnish strains have evolved from a common ancestor during the last 200 years.

The EBLV-2 isolate from Daubenton's bat in 2016 (GenBank accession number MF326269) was 98% identical with the two other EBLV-2 isolates of 1985 and 2009. This 2016 isolate and a new strain from a Daubenton's bat from Norway are included in Figure 12.

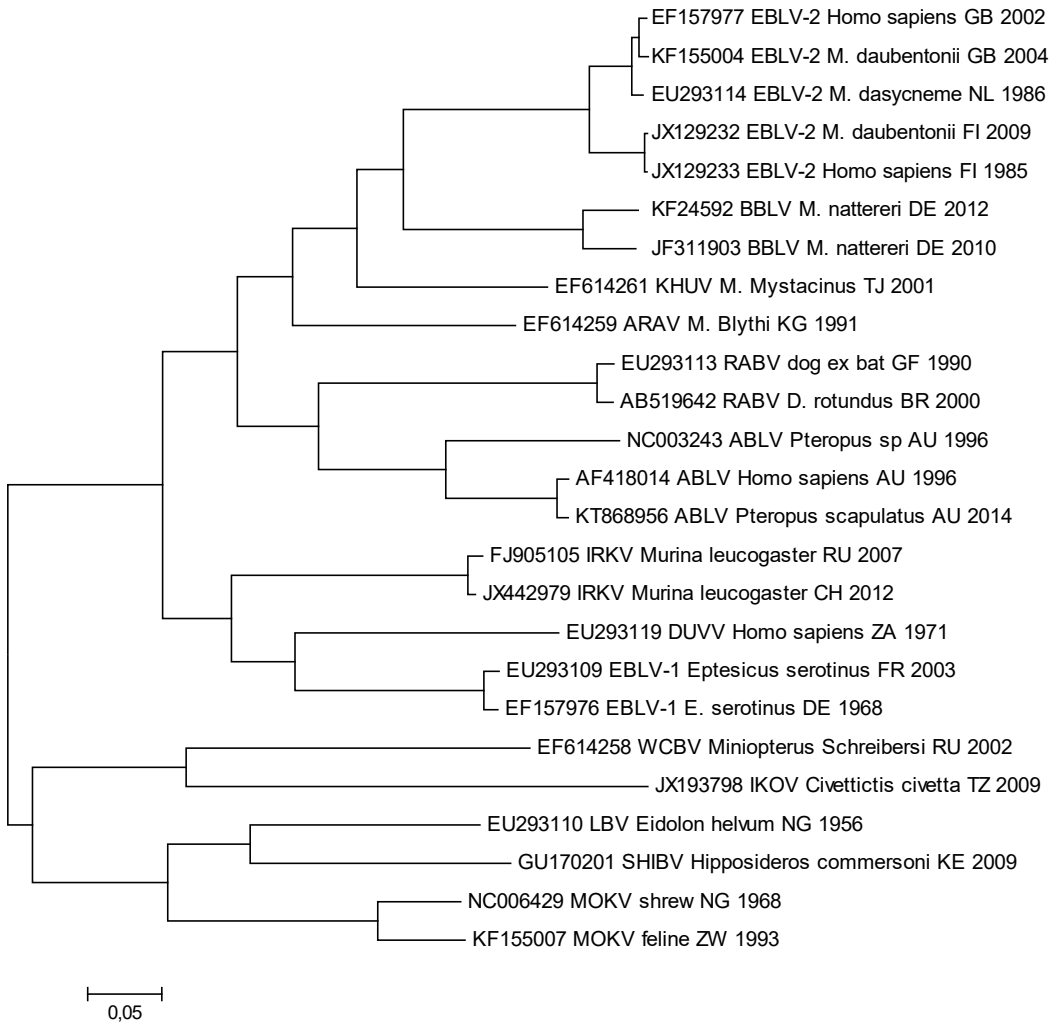

Figure 9. Phylogenetic tree of bat-related lyssaviruses based on whole genome sequences available.

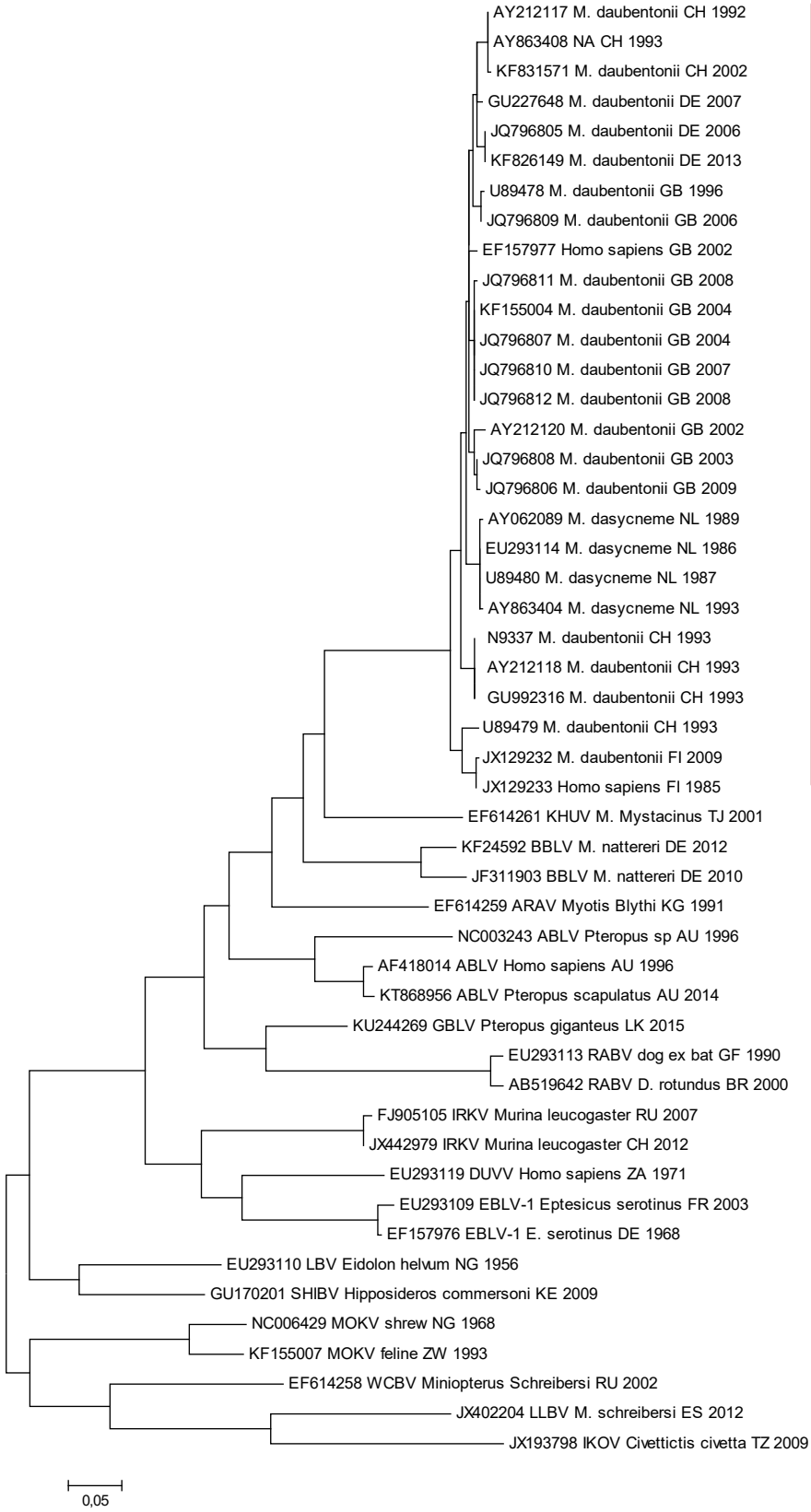

Figure 10. Phylogenetic tree of bat-related lyssaviruses based on partial N-gene sequences.

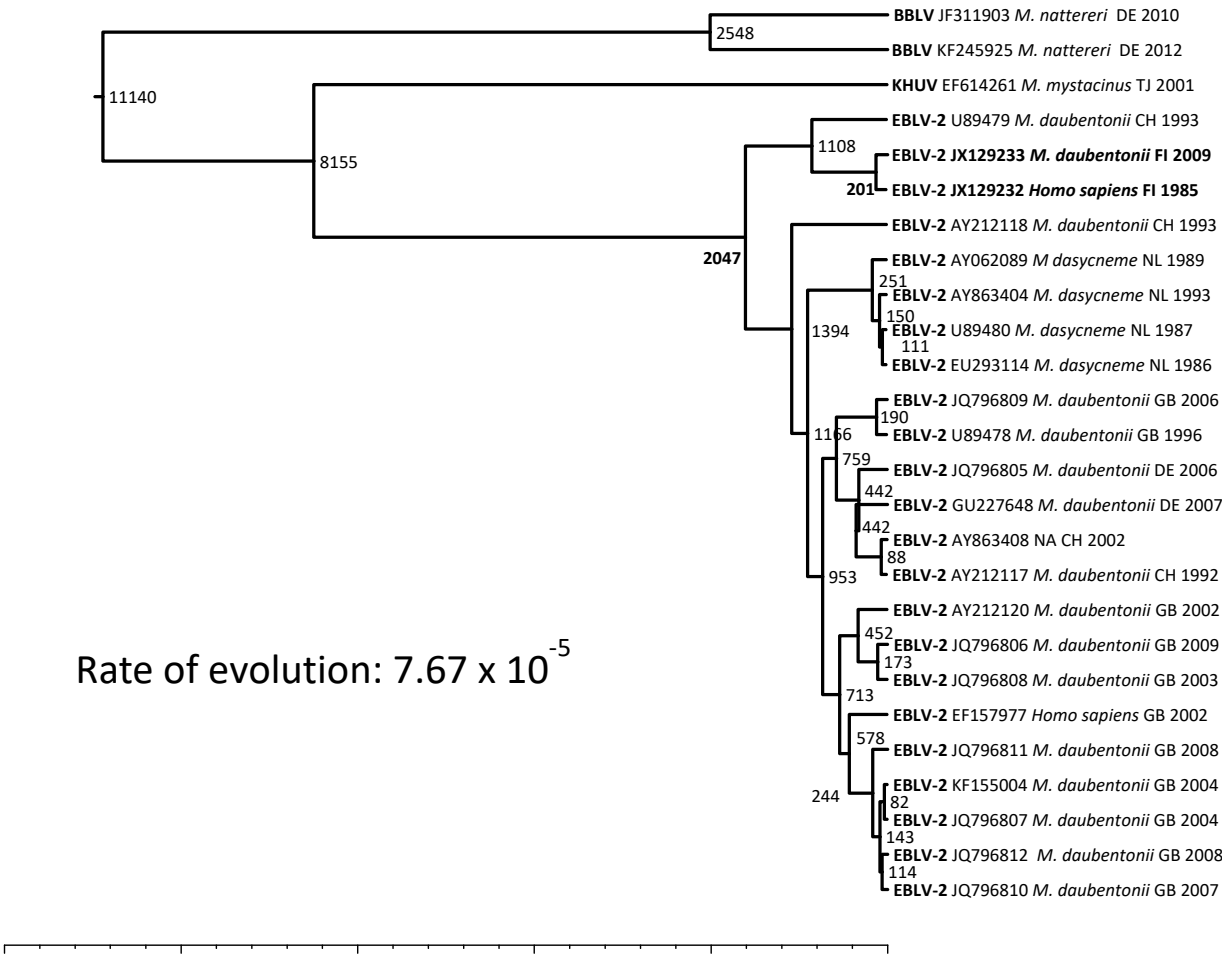

Figure 11. Phylogenetic tree of EBLV-2 based on partial N-gene (400 nt) sequences with a molecular-clock estimate. The program BEAST was used to estimate the substitution rate of EBLV-2 and time of divergence from the phylogenetically closest lyssavirus species. The HKY model of nucleotide substitution was used, and both strict and relaxed lognormal clock models. Convergence of parameters was assessed using TRACER, and each run was continued until the effective sampling size of all parameters was greater than 200.

Figure 12. Phylogenetic tree of EBLV-2 lyssaviruses based on partial N-gene sequences.

5.4 Challenge of mice with RABV and EBLV-2 after vaccination with a human vaccine and an animal vaccine (III)

When challenged with RABV isolated from a Finnish raccoon dog in 1989, all of the vaccinated mice survived. When challenged with EBLV-2 isolated from a Daubenton's bat in 2009, 75% to 80% of the vaccinated mice survived (Table 8). All vaccinated mice developed sufficient to high VNA titers against RABV ranging from 0.5 to 128 IU/ml. Mice that succumbed after challenge with EBLV-2 virus had a VNA titer of 2–64 IU/ml against RABV (individual results not shown).

From the group of 20 mice vaccinated with Rabisin, 11 died or were euthanized on the day of intra-cranial challenge with RABV and EBLV-2, while 12 and 8 mice vaccinated with SAG2 oral live vaccine died or were euthanized at the intra-cranial challenge with RABV and EBLV-2, respectively, and the experiment was discontinued. The experiment was repeated after modification with the vaccine Rabisin.

Table 8. Rabies vaccination protection following an intra-cranial challenge in mice

		Survival after challenge with	
	Total number of mice	**RABV 1989**	**EBLV-2 2009**
		n (%)	n (%)
Rabies Imovax, vaccinated twice	20	20 (100)	16 (80)
P-value[1)]		0.000	0.002
Rabies Imovax, vaccinated once	20	ND	15 (75)
P-value[1)]			0.005
Virus control 50 MLD_{50}	5	0 (0)	0 (0)
Virus control 5 MLD_{50}	5	2 (40)	2 (40)
Virus control 0.5 MLD_{50}	5	4 (80)	4 (80)
Rabisin, vaccinated once	20	20 (100)	16 (80)
P-value[1)]			0.002
Virus control 50 MLD_{50}	5	0 (0)	0 (0)
Virus control 5 MLD_{50}	5	2 (40)	2 (40)
Virus control 0.5 MLD_{50}	5	3 (60)	4 (80)

[1)]P-value derived using Fisher's exact test for the number of vaccinated and challenged mice that survived relative to the total and compared with the 50 MLD_{50} virus control mice

5.5 Results of the serological and statistical analysis of vaccinated dogs and cats (III)

Of the 10 071 dog samples analyzed during 2009–2013, 1073 (10.7%; 95% confidence interval CI 10.1–11.3) had a rabies antibody titer of <0.5 IU/ml. Of the 722 cats analyzed, only 25 (3.5%; 95% CI 2.3–5.0) had a rabies antibody titer of <0.5 IU/ml.

Younger dogs were associated with lower antibody titers ($p < 0.05$). Since there was a significant interaction between the variables dog age and the vaccine used, two multivariable logistic regression models for dogs were constructed: one for dogs up to one year old and another for older dogs. For younger dogs, vaccine C (Flury LEP vaccine) was used significantly more often than for older dogs ($p < 0.05$). For both age groups, dogs vaccinated with vaccine C only and those vaccinated with vaccine B (Pasteur RIV vaccine) only had a significantly higher risk of failing to reach an antibody level of 0.5 IU/ml in comparison to dogs vaccinated with vaccine A (Wistar-G52 vaccine) only. Additionally, for both age groups, vaccination with vaccine C only had the highest risk of failing to reach an antibody level of 0.5 IU/ml. In younger dogs, these risks were higher than in older dogs. In younger dogs, if the time between vaccination and sampling was over three months, the risk of failing to reach an antibody level of 0.5 IU/ml was significantly higher than if the time span was shorter. In older dogs, the risk was higher if the time was over six months. For older dogs (over one year) of a larger size (>30 cm), the risk of failing to reach an antibody level of 0.5 IU/ml was greater. In younger dogs, those over 60 cm had a higher risk compared to smaller dogs.

In cats, we observed no statistically significant differences between the vaccines used. However, there was a similar tendency towards a higher risk of failing to reach an antibody level of 0.5 IU/ml for vaccination with vaccine C only compared to vaccination with vaccine A only. Cats that were vaccinated at the age of up to one year old had a significantly higher risk of failing to reach an antibody level of 0.5 IU/ml than cats vaccinated at an older age. Similarly to dogs, cats that were sampled for testing three to six months or over six months after vaccination had a significantly higher risk of failing to reach an antibody level of 0.5 IU/ml than cats that had been sampled less than three months after vaccination.

6 DISCUSSION

6.1 Epidemiology of bat lyssavirus in Finland

EBLV-2 has been isolated from Finnish Daubenton's bats twice, in 2009 and in 2016. Both bats showed neurological signs before death. The locations are only circa 100 kilometers apart. Furthermore, antibodies against lyssavirus have been detected in Daubenton's bats from the same geographical area. These data provide a strong indication that EBLV-2 is endemic in the Finnish Daubenton's bat population. Due to the small number of samples from passive and active surveillance from other areas in Finland, the presence of lyssaviruses in other parts of the country remains unclear.

Passive surveillance of bat rabies might be a sufficient surveillance method for obtaining information on the incidence of bat lyssavirus, but it does not provide information on the prevalence of lyssavirus infection in a certain population. Active sampling of oral swabs has rarely resulted in positive findings, and our findings are consistent with this, since we did not find a viral genome from swab samples tested in active surveillance. Moreover, active sampling requires permission from the competent authority due to the protection and conservation of bats, and is relatively expensive and labour-intensive. More samples from sick or dead bats should be sent for laboratory analysis. Daubenton's bat samples are particularly difficult to obtain, probably because they do not usually roost in buildings, reducing the likelihood of people finding grounded bats. This leads to lower passive sampling of this species in comparison to other bat species. There is a significant correlation between the number of bats examined and the number of positive bats. Harris et al. (2007) suggested that seven or more dead or symptomatic Daubenton's bats should be examined yearly in order to find at least one EBLV-2-positive sample. In Finland, 2 out of 15 Daubenton's bats sampled in passive surveillance during 1985–2016 were positive for EBLV-2.

Serological testing can be used as an indicator of past exposure to lyssavirus in bats. Seropositive Daubenton's bats have been recorded in Finland and also in the UK, Switzerland, Sweden and Latvia. Results from our surveillance indicate that the lyssavirus seroprevalence in the Finnish bat population is low, even though all seropositive sampling sites exhibited high Daubenton's bat densities and consisted of individuals of the same large population (Laine et al. 2013). The seroprevalence of Daubenton's bats has also been low in other countries. The results of serosurveillance are not fully comparable because of differences in the methods used, as well as in the threshold values. In addition, the different lyssavirus-induced antibodies cross-react to some extend in laboratory analysis.

In an experimental infection, Daubenton's bats were infected with EBLV-2, but none of the bats seroconverted (Johnson et al. 2008). Seroconversion appears to depend not just on the lyssavirus species infecting the bat, but also on the viral dose, bat species infected and route of exposure (Franka et al. 2008). Seroprevalence probably underestimates the true prevalence of lyssavirus infections in the bat population.

Of the 13 bat species recorded in Finland, Daubenton's bats, pond bats *(Myotis dasycneme)*, serotine bats (*E. serotinus*), Natterer's bats (*M. nattereri*), whiskered bats (*M. mystacinus*), common pipistrelle (*P. pipistrellus*), Nathusius' pipistrelle (*P. nathusii)* and common long-eared bat (*Plecotus auritus*) have been shown to be infected with lyssaviruses. *Myotis dasycneme*, the bat species associated with EBLV-2 infections in the Netherlands, and *M. nattereri,* the bat species associated with Bokeloh bat lyssavirus infections in Germany and France, are rare in Finland. Only a few observations of the species *E. serotinus*, the main reservoir host for EBLV-1 infections, have so far been recorded in Finland. The first observation of *E. serotinus* was made in 2008. The most common bat species in Finland is the northern bat (*Eptesicus nilssonii*), which has not been associated with lyssavirus infections. The common bent-wing bat, or Schreibers' bat (*Miniopterus schreibersii*), the greater tube-nosed bat (*Murina leucogaster*), the lesser mouse-eared bat (*Myotis blythii*), Commerson's leaf-nosed bat (*Hipposideros commersoni*), and *Pteropus* spp., associated with several lyssavirus species infections, have never been recorded in Finland. Furthermore, the presence of different bat species in separate parts of the country is not fully known. It can be speculated that climate change might influence the geographical distribution of different bat species and therefore also the distribution of lyssaviruses (McIntyre et al. 2017).

Nevertheless, we consider the health risk to the general public as negligible when people have no contact with bats through work or hobbies. However, new lyssaviruses have recently been discovered, and it is possible that there may still be undetected bat lyssaviruses in many parts of the world. Resident bat species in Finland could also be infected with other lyssaviruses than EBLV-2. Proper handling of bats in order to avoid bites and subsequent exposure, and protecting houses from bats entering them are the most effective preventive measures against bat-induced lyssavirus infections. Education about bats and possible zoonotic diseases they might have, without inducing unnecessary fear, is very important to enable the peaceful co-existence of humans, domestic animals, and bats. In case exposure does occur, people should be aware of the need to seek medical advice for themselves and veterinary advice for their domestic animals. Health care workers and veterinarians also need to be informed about the necessary actions and legislation in place in order to implement proper measures after possible exposure to lyssaviruses. People who come into contact with bats due to their work or hobby,

should be vaccinated against rabies, even though current rabies vaccines induce only partial cross-protection against other lyssaviruses than RABV.

6.2 Phylogenetic analysis of Finnish EBLV-2 isolates

EBLV-2 has been isolated in Finland from a diseased bat researcher in 1985 and from Daubenton's bats in 2009 and 2016. All Finnish EBLV-2 isolates are very closely related and share high nucleotide identity. The overall organization and length of the regions of fully characterized EBLV-2 isolates of 1985 and 2009 were similar to the other three EBLV-2 isolates fully characterized. Comparison with other EBLV-2 sequences demonstrates a high degree of homology (Marston et al. 2007, Delmas et al. 2007, Marston et al. 2013).

EBLV-2 isolates cluster according to the host species, but also according to the geographical place of isolation. These are correlated, since EBLV-2 has been isolated from Daubenton's bats in the UK, Germany, Switzerland, Finland, Denmark, and Norway and from the pond bat (*M. dasycneme*) in the Netherlands. The closest relatives to EBLV-2 are Bokeloh bat lyssavirus isolate from *Myotis nattereri* and Khujand virus isolate from *Myotis mystacinus*. Thus, the host species of these lyssavirus species are also related. EBLV-2 additionally shared ancestry with Aravan virus and with a lineage consisting of rabies virus RABV and Australian bat lyssavirus. Another major cluster of lyssaviruses consists of IRKV, DUVV, and EBLV-1, suggesting that EBLV-1 and EBLV-2 diverged a long time ago. The tree topology was somewhat different in the phylogeny based on either the complete coding sequences or the partial N sequences.

In earlier studies, it has been suggested that the Finnish strain from 1985 and a strain from Switzerland 1993 form lineage EBLV-2b, whereas the rest of the EBLV-2 isolates from the UK and the Netherlands form lineage EBLV-2a (Amengual et al. 1997, McElhinney et al. 2013, Megali et al. 2010, Davis et al. 2005). In our study, there was a division into subgroups when full genomes were compared (Fig. 9). Unfortunately, the full genome of the Swiss strain was not available for analysis, and only strains from the UK and Germany were available. When more strains were compared using N-gene sequences, we could not confirm the suggested subgroup division (Figs 10 and 12).

It has been shown that the genetic structure of Daubenton's bats is relatively homogeneous in western parts of Europe. Daubenton's bats migrate between the UK and the mainland of Europe. This supports the clustering of EBLV-2 according to the geographical place of isolation from the western parts of Europe (Smith et al. 2011). This could be the explanation for the proposed lineage 2a. As Finnish Daubenton's bats do not migrate to Central Europe, the finding that the Finnish EBLV-2 is closely related to the EBLV-2 strain from Switzerland cannot be explained

by migration. Interpretation of the molecular epidemiology of these strains is further complicated by the history of the Swiss bat biologist who died of EBLV-2 infection in Finland in 1985. He was bitten 51 days before the onset of clinical signs by a Daubenton's bat, which showed signs of clinical rabies. Unfortunately, the bat was freed before the patient developed symptoms and it was not therefore available for laboratory tests (Roine et al. 1988). It can be speculated whether the freed bat was not originally from Finland (Lumio et al. 1986, Roine et al. 1988).

The current linage of EBLV-2 arose circa 2000 years ago. The most recent common ancestor (TMRCA) occurred circa 8000 years ago. The Finnish EBLV-2 strains and a Swiss strain have evolved from a common ancestor during the last 1000 years (Figure 12). Tao et al. (2013) estimated that the TMRCA of all lyssaviruses was approximately 5030 years. The West Caucasian bat virus divided first, and phylogroups I and II then divided about 4000 years ago. The overall rate of evolution appears somewhat slower for the bat-type lyssaviruses than for RABV. This is in line with bats being considered the true reservoir of lyssaviruses. The overall evolution rate for EBLV-2 in our study was 7.67×10^{-5} substitutions per site per year (95% HPD interval). For RABV, it has been suggested to be $1.56–1.78 \times 10^{-4}$ (Kuzmina et al. 2013) and 1×10^{-3} to 5.5×10^{-4} (Bourhy et al. 1992, Hughes 2008, Badrane and Tordo 2001, Hanada 2004). Timescale estimation performed on a limited set of recently sampled sequences cannot provide realistic inferences for viruses evolving under the constraints of purifying selection. Evolutionary rates are increased towards the present because of the transient mutations yet to be removed by purifying selection. Therefore, timescale estimations based on substitution rates are useful for the time frame encompassed by the sampling period. They cannot be easily extrapolated for longer periods of time (Rupprecht et al. 2017).

Streicker et al. (2012) demonstrated that the local host environment determines lyssavirus evolutionary rates. Hibernation, migration, and the coloniality of the bats might influence viral evolution (Streicker et al. 2012, Mollentze et al. 2014). Virus evolution in lineages in bat species from tropical or subtropical climates and in bats that remain active year-round was faster in comparison to bats in the temperate climate zone and to those bats that hibernate. This could be a major factor in a cold climate region. Lyssaviruses associated with closely related bat species or sub-species often had dissimilar evolutionary rates. Increased information on the complete genome sequences of lyssaviruses is fundamental to understanding their epidemiology and evolution. It is important to monitor and characterize lyssaviruses circulating in human and animal populations. For a detailed analysis of evolutionary history, complete genomes should be used. The limitation of our study was the number of whole genome sequences available for comparison. Streicker et al. (2016) demonstrated that with adequate data on both the virus and the host and with accurate models, it would be possible to predict the timing and location of the emergence of disease from animals to humans (Streicker et al. 2016).

Some bat species have an extensive geographical distribution, while others appear to have a very restricted geographical distribution. Also, some lyssaviruses are able to infect a wide range of bat species, while others are only found in certain species. The underlying reasons for virus-host restrictions and the differences in habitation tendencies are still unknown. For many lyssavirus species, only a few isolates or just a single isolate are available, which makes it difficult to study host and viral properties.

6.3 Rabies vaccination and protection against EBLV-2

VNAs are the main method of protection during rabies infection, and the role of cell-mediated and innate immunity is not as well understood, even though both play an important role. Measuring the VNA titer is the most common way to assess the success of rabies vaccination. Challenge by using vaccinated laboratory animals is another way of studying the potency of a rabies vaccine.

The immune response elicited by RABV-based rabies vaccines has been shown to be capable of cross-protection against other lyssaviruses in phylogroup I, but not for those that belong to phylogroup II or potential new phylogroups (Liu 2013, Hanlon 2005, Malerzyk 2009 and 2014, Fekadu 1988). Even though EBLV-2 and RABV both belong to phylogroup I, the protection induced by rabies vaccines has been limited in experimental virus challenge studies in mice, even with production of VNAs (Brookes et al. 2005, Fekadu 1988). Separate studies have drawn different conclusions on cross-protection, but information is still limited and factors such as the virus strain, type of cell culture used for vaccine production, and the use of adjuvants of the vaccine influence the vaccination response.

Possible exposure to other lyssaviruses than RABV has raised concern over whether RABV-based vaccines offer sufficient protection. Studies have suggested that either higher serum VNA titers (Brookes et al. 2005) or a higher dose of rabies immunoglobulins is required to neutralize other lyssaviruses than RABV (Liu et al. 2013). The development of novel vaccines that would stimulate a pan-lyssavirus neutralizing immune response is of importance to those at occupational risk of infection with lyssaviruses divergent from RABV (Evans et al. 2012). Considering that vaccine development is time consuming, laborious, and expensive, pan-lyssavirus vaccines are not expected in the near future and protection against lyssaviruses other than phylogroup I relies on the prevention of exposure and thorough wound care.

There is marked individual variation in the comparative neutralization patterns of human sera against different lyssaviruses (Brookes et al. 2005, Horton et al. 2010), and this is probably also true for animals. In our study, all mice developed a sufficient VNA titer against RABV after vaccination, but mice that had VNA

titers of 2–64 IU/ml, which is higher than the accepted threshold, succumbed after challenge with EBLV-2 virus. All vaccinated mice survived the challenge with RABV. This indicates that the RABV antibody level does not clearly correlate with protection against EBLV-2. Therefore, it is still unclear what RABV antibody level would be indicative of protection.

When we first started our potency test, the mice vaccinated with animal vaccines suffered from an anaphylactic reaction with a fatal outcome during and immediately after challenge with the virus suspension. They started breathing heavily, stopped moving, their extremities turned blue, and despite our efforts to support their critical functions, died or were euthanized after a few minutes. This was not seen in mice vaccinated with human vaccine or when the virus suspension was prepared in physiological saline instead of MEM. Therefore, we started using physiological saline in the virus suspension and we did not encounter any further problems of this kind. This unforeseen reaction was probably due to the purification process during manufacture. In general, veterinary vaccines often are less purified than human vaccines. This is reflected in the prices of the vaccines; the human product has on average a ten-fold higher price than animal parental rabies vaccines.

6.4 Factors influencing the success of rabies vaccination of the dog and cat population in Finland

The difference between the proportions of dogs (around 11%) and cats (around 3%) failing to reach the level of 0.5 IU/ml is an interesting finding. This might be caused by differences in genetic variation within these two species. Most dogs in our study material were pedigree dogs, whereas the majority of cats were barn cats. Moreover, cats are more homogeneous in size and there is much greater variability in size between dogs of different breeds. The key genetic factor of immune responsiveness is situated within the major histocompatibility complex (MHC). It has been suspected that the cat has a more limited diversity in immune response genes than dogs, but recent research has revealed variation in the MHC in cats, as is found in dogs (Day 2007). In our study, the different commercial vaccines induced the antibody level of 0.5 IU/ml differently in dogs. This has also been shown in other earlier studies (Berntsson et al. 2011, Mansfield et al. 2004, Minke et al. 2008, Kennedy et al. 2007). This difference between the vaccines was not statistically significant in cats, perhaps due to the type two error of too small sample sizes, since there were so few (n = 25) failures to reach 0.5 IU/ml in cats. Clearly, veterinarians should be aware of the differences between vaccines in their ability to induce antibody production, especially when they are vaccinating dogs with a higher risk of failing to reach the required antibody level.

Several factors influence the outcome of an animal's antibody level after vaccination, namely the vaccine used, the vaccination procedure, and the animal receiving vaccination. Sihvonen et al. (1995) demonstrated that a single vaccination of dogs with rabies vaccine induced moderate but short-term seroconversion in 96.9% of dogs, but in 17% of dogs the antibody titer did not last for a whole year. Based on our studies, it is advisable that dogs needing a sufficient antibody test result due to travel or those that will be going to rabies endemic areas should be vaccinated twice with rabies vaccine and then regularly boosted according to the manufacturer's instructions, and the choice of vaccine needs to be taken into consideration.

The time between vaccination and sampling was a significant risk factor for both dogs and cats failing to reach the antibody level of 0.5 IU/ml. The antibody level peaks at slightly different times after vaccination depending on the vaccine, and the level starts to decline afterwards (Mansfield et al 2004). Larger dogs had a greater risk of failing to reach the required antibody level. Increasing the dose would not probably be a solution, since it has been shown that if there is a sufficient antigen to create a response, larger doses will not increase antibody production. Kennedy et al. (2007) suggested that larger breeds might have deeper sub-cutaneous fat, which could reduce the level of the antibody response. The breed might also be a factor, not just the size of the dog, since even though most failures to reach the anticipated antibody level were in larger breeds, some smaller breeds had significant test failure rates (Kennedy et al. 2005). The higher risk of failing to reach 0.5 IU/ml in dogs and cats aged under one year old could be due to the administration of vaccines before the animal has reached full immunocompetency or due to maternal antibodies (Mansfield et al. 2004, Berndtsson et al. 2011).

In rabies-free countries, the rabies vaccination coverage of cats and dogs is likely to be smaller than in rabies-endemic countries. Furthermore, the legislation does not usually include mandatory vaccinations in rabies-free countries. For example, in Finland, we estimated based on the number of vaccine doses (other vaccines than rabies vaccines) sold that about 10–20% of cat population of circa 700 000 has been vaccinated. The vaccination coverage for rabies vaccines is probably even lower than this. The dog population in Finland has been estimated to be around 650 000 (Finnish Kennel Club 2014). The rabies vaccination coverage is higher for dogs than cats, estimated at around 40–65% based on the number of rabies vaccines sold in Finland (personal communication Martina Reims). Cats, especially in the countryside, often freely go outside and are therefore able to encounter wildlife. They are consequently at greater risk of exposure to rabid animals and especially to bats than dogs. Natural spillover of EBLV-2 to other mammals than humans has not been demonstrated. Even though EBLV-2 infection of dogs and cats through bat contact is very unlikely to occur due to the low prevalence of lyssavirus in the bat population and the small likelihood of such an encounter, dogs

and cats are probably susceptible to EBLV-2 infection. Dogs and cats vaccinated against rabies are highly likely to be protected against EBLV-2 infection as well. However, it has to be remembered that protection against succumbing to rabies and measuring antibody titers in order to see that vaccination has succeeded are two different aspects.

7 CONCLUSIONS

1. EBLV-2 circulates in bats in Finland, even though the seroprevalence is low. Our results indicate that passive surveillance of dead or sick bats is a relevant means to study the occurrence of lyssavirus infection, but the number of bats submitted for laboratory analysis should be higher and from a wider geographical area in order to obtain reliable information on the lyssavirus situation in the country.

2. In Finland, the health risk to people who have no contact with bats through work or hobbies is considered negligible.

3. The three EBLV-2 strains isolated in Finland are highly similar to each other but also with other EBLV-2 strains characterized.

4. The results from the mouse model indicate that dogs and cats vaccinated with RABV-based rabies vaccines are cross-protected against EBLV-2.

5. The mouse model also indicated that people vaccinated with RABV-based vaccines with an antibody titer ≥0.5 IU/ml are most probably protected against EBLV-2. However, measurement of the RABV antibody titer only provides a partial indication of protection against EBLV-2.

6. Dogs, and especially cats, seldom fail to reach the antibody titer of 0.5 IU/ml after rabies vaccination, but several risk factors for dogs and some for cats failing to reach this level were identified.

8 REFERENCES

Aghomo HO, Oduye OO, Rupprecht CE. The serological response of young dogs to the Flury LEP strain of rabies virus vaccine. Veterinary Research Communications 1990;14: 415. doi:10.1007/BF00343220.

Amengual B, Whitby JE, King A, Cobo JS, Bourhy H. Evolution of European bat lyssaviruses. J Gen Virol. 1997;78:2319-2328.

Anonymous. Bat rabies in the Union of Soviet Socialist Republics. Rabies Bull Eur: 1986;4:12-14.

Anonymous. International Committee on Taxonomy of Viruses (ICTV) 2017a. [http://ictvonline.org/organization.asp] Accessed 10.3.2017.

Anonymous. Vesikauhurokote. 2017b. https://www.thl.fi/en/web/rokottaminen/rokotteet/vesikauhurokote] Accessed 15.9.2017.

Anonymous. Rabies (infection with rabies virus). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2016 [http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/]

Anonymous. World Health Organization Expert consultation on rabies. 2nd report 2013.

Anonymous. World Health Organization. Laboratory techniques in rabies. Edited by Meslin F, Kaplan M, Koprowski H. 1996.

Arechiga-Ceballos AN, Vázquez Morón S, Berciano JM, Nicolás O, Aznar López C, Juste J, Rodríguez Nevado C, Aguilar Setién A, Echevarría JE. Novel lyssavirus in Spain. Emerg Infect Dis 2013;19: 793–795.

Assenberg R, Delmas O, Morin B, Graham SC, De Lamballerie X, Laubert C,

Coutard B, Grimes JM, Neyts J, Owens RJ, Brandt BW, Gorbalenya A et al. Genomics and structure/function studies of Rhabdoviridae proteins involved in replication and transcription. Antiviral Res. 2010;87:149–161.

Aubert MF. Practical significance of rabies antibodies in cats and dogs. Rev Sci Tech. 1992; Sep;11(3):735-60.

Badrane H, Bahloul C, Perrin P, Tordo N. Evidence of two Lyssavirus phylogroups with distinct pathogenicity and immunogenicity. J Virol 2001;75:3268-3276.

Badrane H, Tordo N. Host switching in Lyssavirus history from the Chiroptera to the Carnivora orders. J Virol. 2001;75(17):8096-104.

Banyard AC, Hayman D, Johnson N, McElhinney L, Fooks AR. Bats and lyssaviruses. Adv Virus Res. 2011;79:239-89.

Banyard AC, Healy DM, Brookes SM, Voller K, Hicks DJ, Núñez A, Fooks AR. Lyssavirus infection: 'low dose, multiple exposure' in the mouse model. Virus Res. 2014; 6;181:35-42.

Barrett J. Lyssaviruses in Investigating the role of bats in emerging zoonoses. FAO animal production and health manual 2011. Edited by Newman S, Field H, Epstein J, de Jong C.

Berndtsson LT, Nyman A-K, Rivera E, Klingeborn B. Factors associated with the success of rabies vaccination of dogs in Sweden. Acta Vet Scand. 2011;53:22.

Botvinkin AD, Poleschuk EM, Kuzmin IV, Borisova TI, Gazaryan SV, Yager P, Rupprecht CE. Novel lyssaviruses isolated from bats in Russia. Emerg Infect Dis 2003;9:1623-1625.

Bourhy H, Kissi B, Lafon M, Sacramento D, Tordo N. Antigenic and molecular characterization of bat rabies virus in Europe. J Clin Microbiol. 1992;30(9):2419-26.

Brass DA. Rabies in bats. Natural History and Public Health Implications.1994. Ridgefield CN, Livia Press.

Brookes SM, Parsons G, Johnson N, McElhinney LM, Fooks AR. Rabies human diploid cell vaccine elicits cross-neutralising and cross-protecting immune responses against European and Australian bat lyssaviruses. Vaccine. 2005:23(32):4101-9.

Brookes SM, Aegerter JN, Smith GC, Healy DM, Jolliffe TA, Swift SM, Mackie IJ, Pritchard JS, Racey PA, Moore NP, Fooks AR. European bat lyssavirus in Scottish bats. Emerg Infect Dis 2005, 4:572-578.

Brookes SM, Klopfleisch R, Müller T, Healy DM, Teifke JP, Lange E, Kliemt J, Johnson N, Johnson L, Kaden V, Vos A, Fooks AR. Susceptibility of sheep to European bat lyssavirus type-1 and -2 infection: a clinical pathogenesis study. Vet Microbiol. 2007;15;125(3-4):210-23.

Cliquet F, Picard-Meyer E, Barrat J, Brookes SM, Healy DM, Wasniewski M, Litaize E, Biarnais M, Johnson L, Fooks AR. Experimental infection of foxes with European Bat Lyssaviruses type-1 and 2. BMC Vet Res. 2009;19:5:19.

Cliquet F, Aubert M, Sagne L: Development of a fluorescent antibody virus neutralization test (FAVN test) for the quantitation of rabies-neutralising antibody. J Immunol Methods 1998;212:79-87.

Daoust PY, Wandeler AI, Casey GA. Cluster of rabies cases of probable bat origin among red foxes in Prince Edward Island, Canada. J. Wildl. Dis. 1996;32:403–406.

Davis PL, Holmes EC, Larrous F, Van der Poel WH, Tjørnehøj K, Alonso WJ, Bourhy H. Phylogeography, population dynamics and molecular evolution of European bat lyssaviruses. J Virol 2005;79:10487-10497.

Day MJ. Immune system development in the dog and cat. J Comp Pathol. 2007;137 Suppl 1:S10-5.

Delmas O, Holmes EC, Talbi C, Larrous F, Dacheux L, Bouchier C, Bourhy H. Genomic diversity and evolution of lyssaviruses. PLoS One. 2008;3:e2057. doi: 10.1371/journal.pone.0002057.

Dietzgen RG, Kuzmin IV Taxonomy of Rhabdoviruses in Rhabdoviruses, Molecular taxonomy, evolution, genetics, ecology, host-vector interactions, cytopathology and control. Caister Academic Press 2012. ISBN: 978-1-908230-11-9.

Dobrostana M, Cvetkova S, Muizniece Z, Petersons G, Rodze I, Saulite V. Presence of anti-rabies antibodies among bats in Latvia. Proceedings of Rabies Serology meeting 14.-15. June 2017.

Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 2007;8:7:214.

Evans JS1, Horton DL, Easton AJ, Fooks AR, Banyard AC. Rabies virus vaccines: is there a need for a pan-lyssavirus vaccine? Vaccine. 2012;14;30(52):7447-54. doi: 10.1016/j.vaccine.2012.10.015.

Fekadu M, Shaddock JH, Sanderlin DW, Smith JS. Efficacy of rabies vaccines against Duvenhage virus isolated from European house bats (Eptesicus serotinus), classic rabies and rabies-related viruses. Vaccine 1988;6:533-9.

Francis JR, Nourse C, Vaska VL, et al. Australian Bat Lyssavirus in a child: the first reported case. Pediatrics 2014;133: e1063-e1067. doi: 10.1542/peds.2013-1782.

Freuling C, Grossmann E, Conraths FJ, Schameitat A, Kliemt J, Auer E, Greiser-Wilke I, Müller T. First isolation of EBLV-2 in Germany. Vet Microbiol 2008, 131:26–34.

Fooks AR, McElhinney LM, Pounder DJ, Finnegan CJ, Mansfield K, Johnson N, Brookes SM, Parsons G, White K, McIntyre PG, Nathwani D. Case report: isolation of a European bat lyssavirus type 2a from a fatal human case of rabies encephalitis. J Med Virol 2003;71:281-28.

Franka R, Johnson N, Müller T, Vos A, Neubert L, Freuling C, Rupprecht CE, Fooks AR. Susceptibility of North American big brown bats (*Eptesicus fuscus*) to infection with European bat lyssavirus type 1. J Gen Virol 2008, 89:1998-2010.

Gunawardena PS, Marston DA, Ellis RJ, Wise EL, Karawita AC, Breed AC, McElhinney LM, Johnson N, Banyard AC, Fooks AR. Lyssavirus in Indian Flying Foxes, Sri Lanka. Emerg Infect Dis. 2016;22(8):1456-9.

Hammarin AL, Berndtsson LT, Falk K, Nedinge M, Olsson G, Lundkvist Å. Lyssavirus-reactive antibodies in Swedish bats. Infect Ecol Epidemiol. 2016;6:31262. doi: 10.3402/iee.v6.31262.

Hampson K Coudeville L, Lembo T, Sambo M, Kieffer A, Attlan M, Barrat J, Blanton JD, Briggs DJ, Cleaveland S, Costa P, Freuling CM, Hiby E, Knopf L, Leanes F, Meslin FX, Metlin A, Miranda ME, Müller T, Nel LH, Recuenco S, Rupprecht CE, Schumacher C, Taylor L, Vigilato MA, Zinsstag J, Dushoff J; Global Alliance for Rabies Control Partners for Rabies Prevention. Estimating the global burden of endemic canine rabies. PLoS Negl Trop Dis, 2015;9(4):e0003709. doi:10.1371/journal.pntd.0003709.

Hanada K, Suzuki Y, Gojobori T. A large variation in the rates of synonymous substitution for RNA viruses and its relationship to a diversity of viral infection and transmission modes. Mol Biol Evol 2004;21:1074-1080.

Hanlon C, Kuzmin IV, Blanton JD, Weldon WC, Manangan JS, Rupprecht CE. Efficacy of rabies biologics against new lyssaviruses from Eurasia. Virus Res. 2005;111(1):44-54.

Hanlon C, Nadin-Davis S. Laboratory diagnosis of rabies in Rabies Scientific basis of the disease and its management. Edited by Jackson A. Elsevier 2013. ISBN 978-0-12-396547-9.

Hanlon C. Rabies in terrestrial animals in Rabies Scientific basis of the disease and its management. Edited by Jackson A. Elsevier 2013. ISBN 978-0-12-396547-9.

Hanna JN, Carney IK, Smith GA, et al. Australian bat lyssavirus infection: a second human case, with a long incubation period. Med J Aust 2000;172:597-599.

Harris SL, Mansfield K, Marston DA, Johnson N, Pajamo K, O'Brien N, Black C, McElhinney LM, Fooks AR. Isolation of European bat lyssavirus type 2 from a Daubenton's bat (*Myotis daubentonii*) in Shropshire. Vet Rec 2007;161:384–386.

Harris SL, Aegerter JN, Brookes SM, McElhinney LM, Jones G, Smith GC, Fooks AR. Targeted surveillance for European bat lyssaviruses in English bats (2003-06). J Wildl Dis 2009;45(4):1030-1041.

Hasegawa M, Kishino H, Yano T. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 1985;22:160-174.

Healy DM, Brookes SM, Banyard AC, Núñez A, Cosby SL, Fooks AR. Pathobiology of rabies virus and the European bat lyssaviruses in experimentally infected mice. Virus Res. 2013;172(1-2):46-53.

Horton DL, Voller K, Haxton B, Johnson N, Leech S, Goddard T, Wilson C, McElhinney LM, Fooks AR. European bat lyssavirus type 2 in a Daubenton's bat in Scotland. Vet Rec 2009;165:383–384.

Horton DL, McElhinney LM, Marston DA, Wood JL, Russell CA, Lewis N, Kuzmin IV, Fouchier RA, Osterhaus AD, Fooks AR, Smith DJ. Quantifying antigenic relationships among the lyssaviruses. J Virol. 2010;84:11841-8.

Hooper DC, Morimoto K, Bette M, Weihe E, Koprowski H, Dietzschold B. Collaboration of antibody and inflammation in clearance of rabies virus from the central nervous system. J Virol. 1998;72(5):3711-9.

Hughes GJ. A reassessment of the emergence time of European bat lyssavirus type 1. Infect. Gen.Evol 2008;8:820-824. doi: 10.1016/j.meegid.2008.08.003.

Hutterer R, Ivanova T, Meyer-Cords, Rodrigues L. Bat Migrations in Europe. A Review of Banding data and Literature. Naturshcutz und Biologische Vielfalt Heft 28. Federal Agency for Nature Conservation. Bonn, Germany. 2005. 162 p.

Jackson A. History of rabies research in Rabies Scientific basis of the disease and its management. Edited by Jackson A. Elsevier 2013. ISBN 978-0-12-396547-9.

Jackson A. Human disease in Rabies Scientific basis of the disease and its management. Edited by Jackson A. Elsevier 2013. ISBN 978-0-12-396547-9.

Jackson A, Fu Z. Pathogenesis in Rabies Scientific basis of the disease and its management. Edited by Jackson A. Elsevier 2013. ISBN 978-0-12-396547-9.

Jakava-Viljanen M, Lilley T, Kyheröinen E-M, Huovilainen A. First encounter of European bat lyssavirus type 2 (EBLV-2) in a bat in Finland. Epid Inf 2010;138:1581-1585.

Jakava-Viljanen M, Nokireki T, Sironen T, Vapalahti O, Sihvonen L, Huovilainen A. Evolutionary trends of European bat lyssavirus type 2 including genetic characterization of Finnish strains of human and bat origin 24 years apart. Arch Virol. 2015;160:1489-98.

Johnson N, Vos A, Freuling C, Tordo N, Fooks AR, Müller T. Human rabies due to lyssavirus infection of bat origin. Vet Microbiol. 2010;142:151-9.

Johnson N, Vos A, Neubert L, Freuling C, Mansfield KL, Kaipf I, Denzinger A, Hicks D, Núñez A, Franka R, Rupprecht CE, Müller T, Fooks AR. Experimental study of European bat lyssavirus type-2 infection in Daubenton's bats (*Myotis daubentonii*). J Gen Virol 2008;89:2662-2672.

Johnson N, Goddard TM, Goharriz H, Wise E, Jennings D, Selden D, Marston DA, Banyard AC, McElhinney LM, Fooks AR. Two EBLV-2 infected Daubenton's bats detected in the north of England. Vet Rec 2016;179:311-312.

Kapfer G, Rigot T, Holsbeek L, Aron S. Roost and hunting site fidelity of female and juvenile Daubenton's bat (*Myotis daubentonii*) (Kuhl, 1817) (Chiroptera: Vespertilionidae). Mamm Biol 2008, 73: 267-275.

Kennedy LJ, Barnes A, Happ GM, Quinnell RJ, Bennett D, Angles JM, Day MJ, Carmichael N, Innes JF, Isherwood D, Carter SD, Thomson W, Ollier WE. Extensive interbreed, but minimal intrabreed, variation of DLA class II alleles and haplotypes in dogs. Tissue Antigens. 2002;59:194-204.

Kennedy LJ, Lunt M, Barnes A, McElhinney LM, Fooks AR, Ollier, WER. Do dogs vary in their response to rabies vaccination? Proceedings of the 48th Annual Congress of the British Small Animal Veterinary Association 2005:550.

Kennedy LJ, Lunt M, Barnes A, McElhinney L, Fooks AR, Baxter DN, Ollier WER. Factors influencing the antibody response of dogs vaccinated against rabies. Vaccine 2007;25:8500-8507.

Klevar S, Høgåsen HR, Davidson RK, Hamnes IS, Treiberg Berndtsson L, Lund A. Cross-border transport of rescue dogs may spread rabies in Europe. Vet Rec. 2015;176:672.

Knobel D, Lembo T, Morters M, Townsend S, Cleaveland S, Hmapson K. Dog rabies and its control in Rabies Scientific basis of the disease and its management. Edited by Jackson A. Elsevier 2013. ISBN 978-0-12-396547-9.

Kuzmin IV, Mayer AE, Niezgoda M, Markotter W, Agwanda B, Breiman RF, Rupprecht CE. Shimoni bat virus, a new representative of the Lyssavirus genus. Virus Res 2010;149:197-210.

Kuzmin IV, Tordo N. Genus Lyssavirus in Rhabdoviruses Molecular taxonomy, evolution, genomics, ecology, host-vector interactions, cytopathology and control. Edited by Dietzgen R, Kuzmin I. Caister Academic Press 2012.

Kuzmin IV, Hughes GJ, Botvinkin AD, Orciari LA, Rupprecht CE. Phylogenetic relationships of Irkut and West Caucasian bat viruses within the Lyssavirus genus and suggested quantitative criteria based on the N gene sequence for lyssavirus genotype definition. Virus Res 2005;111:28-43.

Kuzmina NA, Kuzmin I, Ellison JA, Taylor ST, Bergman DL, Dew B, Rupprecht CE. A reassessment of the evolutionary timescale of bat rabies viruses based upon glycoprotein gene sequences. Virus Genes 2013;47:305-310.

Lafon M. Immunology in Rabies Scientific basis of the disease and its management. Edited by Jackson A. Elsevier 2013. ISBN 978-0-12-396547-9.

Laine VN, Lilley TM, Norrdahl K, Primmer CR. Population genetics of Daubenton's bat (*Myotis daubentonii*) in the Archipelago Sea, southwest Finland. Ann Zool Fenn 2013; 5:303-315. doi: 10.5735/085.050.0505.

Leonova GN, Belikov SI, Kondratov IG, Krylova NV, Pavlenko EV, Romanova EV, Chentsova IV, Petukhova SA. A fatal case of bat lyssavirus infection in Primorye Territory of the Russian Far East. WHO Rabies Bull. Eur. 2009;33:5–8.

Leslie MJ, Messenger S, Rohde RE, Smith J, Cheshier R, Hanlon C, Rupprecht CE. Bat-associated rabies virus in skunks. Emerg Infect Dis 2006;12:1274–1277.

Liu Y, Chen Q, Zhang F, Zhang S, Li N, Lian H, Wang Y, Zhang J, Hu R. Evaluation of rabies biologics against Irkut virus isolated in China. J Clin Microbiol. 2013 51(11):3499-504. doi: 10.1128/JCM.01565-13.

Liukko U, Henttonen H, Hanski IK, Kauhala K, Kojola I, Kyheröinen E: **Nisäkkäät.** In Rassi P, Hyvärinen E, Juslén A, Mannerkoski I (eds). The 2010 Red Data List of Finnish Species. Ympäristöministeriö & Suomen ympäristökeskus, Helsinki, 2010, 311-319.

Lučan RK, Radil J. Variability of foraging and roosting activities in adult females of Daubenton's bat (Myotis daubentonii) in different seasons. Biologia 2010;65:1072-1080.

Lumio J, Hillbom M, Roine R, Ketonen L, Haltia M, Valle M, Neuvonen E, Lähdevirta J. Human rabies of bat origin in Europe. Lancet 1986;15:378.

Malerczyk C, Selhorst T, Tordo N, Moore S, Müller T. Antibodies induced by vaccination with purified chick embryo cell culture vaccine (PCECV) cross-neutralize non-classical bat lyssavirus strains. Vaccine. 2009;27:5320-5.

Malerczyk C, Freuling C, Gniel D, Giesen A, Selhorst T, Müller T. Cross-neutralization of antibodies induced by vaccination with Purified Chick Embryo Cell Vaccine (PCECV) against different Lyssavirus species. Hum Vaccin Immunother. 2014;10(10):2799-804

Mansfield KL, Burr PD, Snodgrass DR, Sayers R, Fooks AR: Factors affecting the serological response of dogs and cats to rabies vaccination. Vet Rec 2004, 154:423-426.

Mansfield KL, Johnson N, Nunez A, Hicks D, Jackson AC, Fooks AR. Up-regulation of chemokine gene transcripts and T-cell infiltration into the central nervous system and dorsal root ganglia are characteristics of experimental European bat lyssavirus type 2 infection of mice. J Neurovirol 2008;14:218-28. doi: 10.1080/13550280802008297.

Markotter W, Randles J, Rupprecht CE, Sabeta CT, Taylor PJ, Wandeler AI, Nel LH. Lagos bat virus, South Africa. Emerg Infect Dis. 2006 Mar;12(3):504-6.

Marston DA, McElhinney LM, Johnson N, Müller T, Conzelmann KK, Tordo N, Fooks AR. Comparative analysis of the full genome sequence of European bat lyssavirus type 1 and type 2 with other lyssaviruses and evidence for a conserved transcription termination and polyadenylation motif in the G-L 3' non-translated region. J Gen Virol. 2007 Apr;88(Pt 4):1302-14.

Marston DA, Horton DL, Ngeleja C, Hampson K, McElhinney LM, Banyard AC, Haydon D, Cleaveland S, Rupprecht CE, Bigambo M, Fooks AR, Lembo T. Ikoma lyssavirus, highly divergent novel lyssavirus in an African civet. Emerg Infect Dis 2012;18:664-667.

Marston DA, McElhinney LM, Ellis RJ, Horton DL, Wise EL, Leech SL, David D, de Lamballerie X, Fooks AR. Next generation sequencing of viral RNA genomes. BMC Genomics 2013;14:444.

McElhinney LM, Marston DA, Leech S, Freuling CM, van der Poel WH, Echevarria J, Vázquez-Moron S, Horton DL, Müller T, Fooks AR. Molecular epidemiology of bat lyssaviruses in Europe. Zoonoses Public Health. 2013;60:35-45.

McIntyre K, Setzkorn C, Hepworth P, Morand S, Morse A, Baylis M. Systematic assessment of the climate sensitivity of important human and domestic animals pethogens in Europe. Sci Rep. 2017;7:7134.

Megali A, Yannic G, Zahno ML, Brügger D, Bertoni G, Christe P, Zanoni R. Surveillance for European bat lyssavirus in Swiss bats. Arch Virol 2010;155:1655-1662.

Minke JM, Bouvet J, Cliquet F, Wasniewski M, Guiot AL, Lemaitre L, Cariou C, Cozette V, Vergne L, Guigal PM. Comparison of antibody responses after vaccination with two inactivated rabies vaccines. Vet Microbiol 2008;133:283-286.

Moldal T, Vikøren T, Cliquet F, Marston DA, van der Kooij J, Madslien K, Ørpetveit I. First detection of European bat lyssavirus type 2 (EBLV-2) in Norway. BMC Vet Res. 2017;13:216.

Mollentze N, Biek R, Streicker DG. The role of viral evolution in rabies host shifts and emergence. Curr Opin Virol 2014;8:68-72.

Moore SM, Wilkerson MJ, Davis RD, Wyatt CR, Briggs DJ. Detection of cellular immunity to rabies antigens in human vaccinees. J Clin Immunol. 2006;26(6):533-45.

Müller T, Cox J, Schafer R, Johnson N, McElhinney LM, Geue JL, Tjornehoj K, Fooks AR. Spill-over of European Bat Lyssavirus type 1 into a stone marten (Martes foina) in Germany. J Vet Med B Infect Dis Vet Public Health 2004;51: 49–54.

Nadin-Davis SA, Real LA. Molecular phylogenetics of the lyssaviruses--insights from a coalescent approach. Adv Virus Res 2011;79:203-38.

Nathwani D, McIntyre PG, White K, Shearer AJ, Reynolds N, Walker D, Orange GV, Fooks AR. Fatal human rabies caused by European bat Lyssavirus type 2a infection in Scotland. Clin Infect Dis. 2003;37:598-601.

Nieuwenhuijs J. WHO Rabies Bulletin Europe 1987;4:8. (http://www.who-rabies-bulletin.org/).

Nokireki T, Huovilainen A, Lilley T, Kyheröinen EM, Ek-Kommonen C, Sihvonen L, Jakava-Viljanen M. Bat rabies surveillance in Finland. BMC Vet Res. 2013 Sep 8;9:174. doi: 10.1186/1746-6148-9-174.

Parsons K, Jones G. Dispersion and habitat use by Myotis daubentonii and Myotis nattereri during the swarming season: implications for conservation. Anim Conserv 2003;6:283–290.

Rasmussen TB, Chrlél M, Baagøe, Fjederholt E, Kooi EA, Belsham GJ, Bøtner A. Dtection of European bat lyssavirus type 2 in Danish Daubenton's bats using a molecular diagnostic strategy. In Proceedings of 8th Annual EPIZONE Meeting, 23.-25.9.2014, Copenhagen, Denmark.

Rogan W, Gladen B: Estimating prevalence from the results of a screening test. Am J Epidemiol 1978;107:71-76.

Roine RO, Hillbom M, Valle M, Haltia M, Ketonen L, Neuvonen E, Lumio J, Lähdevirta J. Fatal encephalitis caused by a bat-borne rabies-related virus. Clinical findings. Brain 1988;111:1505-1516.

Rupprecht C, Slate D. Rabies prevention and control: advances and challenges in Rhabdoviruses, Molecular taxonomy, evolution, genetics, ecology, host-vector interactions, cytopathology and control. Caister Academic Press 2012. ISBN: 978-1-908230-11-9.

Rupprecht C, Kuzmin I, Meslin F. Lyssaviruses and rabies: current conundrums, concerns, contradictions and controversies [version 1; referees: 2 approved]. F1000Research 2017, 6(F1000 Faculty Rev):184 (doi: 10.12688/f1000research.10416.1) Accessed 24 June 2017.

Rossiter J, Jackson A. Pathology in Rabies Scientific basis of the disease and its management. Edited by Jackson A. Elsevier 2013. ISBN 978-0-12-396547-9.

Sabeta CT, Markotter W, Mohale DK, Shumba W, Wandeler AI, Nel LH. Mokola virus in domestic mammals, South Africa. Emerg Infect Dis. 2007 Sep;13(9):1371-3. doi: 10.3201/eid1309.070466.

Samaratunga H, Searle JW, Hudson N. Non-rabies Lyssavirus human encephalitis from fruit bats: Australian bat Lyssavirus (pteropid Lyssavirus) infection. Neuropathol Appl Neurobiol 1998;24: 331-335.

Schatz J, Fooks AR, McElhinney L, Horton D, Echevarria J, Vázquez-Moron S, Kooi EA, Rasmussen TB, Müller T, Freuling CM. Bat Rabies Surveillance in Europe. Zoonoses Public Health 2013;60:22-34.

Selimov MA, Tatarov AG, Botvinkin AD, Klueva EV, Kulikova LG, Khismatullina NA. Rabies-related Yuli virus; identification with a panel of monoclonal antibodies. Acta Virol 1989;33:542-546.

Sergeant, ESG, 2015. Epitools epidemiological calculators. AusVet Animal Health Services and Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease. [http://epitools.ausvet.com.au/content.php?page=home]

Shinwari MW, Annand EJ, Driver L, Warrilow D, Harrower B, Allcock RJ, Pukallus D, Harper J, Bingham J, Kung N, Diallo IS. Australian bat lyssavirus infection in two horses. Vet Microbiol. 2014 Oct 10;173(3-4):224-31.

Siegrist C-A. Vaccine immunology. In Vaccines. 6th edn. Eds Plotkin S., Orenstein W., Offit P. USA: Saunders 2012:16–32.

Sihvonen L, Kulonen K, Neuvonen E, Pekkanen K. Rabies Antibody in Vaccinated Dogs. Acta Vet Scand 1995;36:87-91.

Smith GC, Aegerter JN, Allnutt TR, MacNicoll AD, Learmount J, Hutson AM, Atterby H. Bat population genetics and Lyssavirus presence in Great Britain. Epidemiol Infect 2011;10:1463-1469.

Streicker DG, Lemey P, Velasco-Villa A, Rupprecht CE. Rates of viral evolution are linked to host geography in bat rabies. PLoS Pathog 2012;8(5):e1002720. doi: 10.1371/journal.ppat.1002720.

Streicker DG, Winternitz JC, Satterfield DA, Condori-Condori RE, Broos A, Tello C, Recuenco S, Velasco-Villa A, Altizer S, Valderrama W. Host-pathogen evolutionary signatures reveal dynamics and future invasions of vampire bat rabies. Proc Natl Acad Sci U S A. 2016;27;113(39):10926-31.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 2011;28:2731-2739.

Tao X, Guo Z, Li H, Han N, Tang Q, Liang G. Investigation of the evolutionary history of the lyssaviruses. Virol Sin. 2013;28:186-189.

Tizard IR. Veterinary Immunology. 9th Edition 2013. Saunders. ISBN: 978-1-4557-0362-3.

Tjørnehøj K, Rønsholt L, Fooks AR. Antibodies to EBLV-1 in a domestic cat in Denmark. Vet Rec. 2004;30;155(18):571-2

Tjørnehøj K, Fooks AR, Agerholm JS, Rønsholt L. Natural and experimental infection of sheep with European bat lyssavirus type-1 of Danish bat origin. J Comp Path 2006;134: 190–201.

Udow SJ1, Marrie RA, Jackson AC. Clinical features of dog- and bat-acquired rabies in humans. Clin Infect Dis. 2013;57(5):689-96.

Van der Poel WHM, Van der Heide R, Verstraten E, Takumi K, Lina PHC, Kramps JA. European bat lyssaviruses, the Netherlands. Emerg Infect Dis 2005;11:1854–1859.

van Baren MJ, Heutink P. The PCR Suite. M.J Bioinformatics 2004;20:591-593.

Wakeley PR, Johnson N, McElhinney LM, Marston D, Sawyer J, Fooks AR. Development of a Real-Time, TaqMan Reverse Transcription-PCR Assay for Detection and Differentiation of Lyssavirus Genotypes 1, 5, and 6. J Clin Microbiol 2005;43: 2786–2792.

Whitby JE, Heaton PR, Black EM, Wooldridge M, McElhinney LM, Johnstone P. First isolation of a rabies-related virus from a Daubenton's bat in the United Kingdom. Vet Rec. 2000;147:385-388.

World Health Organization. Rabies Bulletin Europe (http://www.who-rabiesbulletin.org/). Accessed in March 2017.