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Surveillance and pathogenicity of lyssaviruses

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For my family

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1.Abbreviations

A	Adenine
AA	Amino acid
ABLV	Australian bat lyssavirus
ARAV	Aravan virus
BBLV	Bokeloh bat lyssavirus
C	Cytosine
CDC	Centers for Disease Control and Prevention
CNS	Central nervous system
DNA	Deoxyribonucleic acid
dRIT	Direct rapid immunohistochemical test
DUVV	Duvenhage virus
EBLV-1	European bat lyssavirus 1
EBLV-2	European bat lyssavirus 2
f.p.	Footpad
FAO	Food and Agriculture Organization of the United Nations
FAT	Fluorescence antibody test
FITC	Fluorophore conjugated
FLI	Friedrich-Loeffler-Institut
G	Guanine
GARC	Global Alliance for Rabies Control
GBLV	Gannoruwa bat lyssavirus
Gln	Glutamine
His	Histidine
i.m.	Intramuscular
i.n.	Intranasal
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
IGR	Intergenic region
IKOV	Ikoma virus
indels	Insertions and deletions

IRKV	Irkut virus
kb	Kilobase
KHUV	Khujand virus
LBV	Lagos bat virus
LFD	Lateral flow device
LLEBV	Lleida bat lyssavirus
MIT	Mouse inoculation test
MOKV	Mokola virus
mRNA	Messenger ribonucleic acid
N	Any base
nAchR	Nicotinic acetylcholine receptor
NASBA	Nucleic acid sequence based amplification
NCAM	Neuronal cell adhesion molecule
nm	Nanometre
nt	Nucleotide
OIE	World Organization for Animal Health
p75NTR	Low affinity nerve growth factor
PEP	Post-exposure prophylaxis
RABV	Rabies virus
RNA	Ribonucleic acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
RTCIT	Rabies tissue culture infection test
RT-LAMP	Reverse transcription loop mediated isothermal amplification
RT-PCR	Reverse transcription polymerase chain reaction
s.c.	Subcutaneous
SHIBV	Shimoni bat virus
STAT	Signal transducer and activator of transcription
T	Thymine
TIS	Transcription initiation signal
TTS	Transcription termination signal
UTR	Untranslated region

W	Adenine or Thymine
WCBV	West Caucasian bat virus
WHO	World Health Organization
Y	Cytosine or Thymine

2.Introduction

Rabies, caused by lyssaviruses, is a zoonotic disease responsible for an estimated 59000 human deaths per year and is classified as a neglected zoonotic disease by the World Health Organization (WHO). Since rabies is almost always fatal once clinical signs develop, preventive vaccination of risk groups or prompt application of post exposure prophylaxis is of utmost importance. In most of Europe, rabies has been eliminated in terrestrial animals, but is still present in bats. In order to understand bat rabies epidemiology, dynamics and possible emergence, bat rabies surveillance is performed in parts of Europe including Germany and five bat lyssaviruses have been detected so far. European bat lyssavirus 1 (EBLV-1) was identified as the main cause of bat rabies in Europe. For this lyssavirus, spill-over infections in other mammals including humans have been recorded.

Rabies in terrestrial mammals is caused by rabies virus (RABV). Dogs are the main source for human infection, with dog-mediated human rabies being endemic especially in Asia and Africa. Unfortunately, rabies surveillance, as the basis for control and elimination, is hampered for various reasons including challenges in rabies diagnosis. For this reason alternative test methods for rabies diagnosis in the form of point of care tests, were developed, e.g. lateral flow devices (LFDs).

This thesis focuses on aspects of lyssavirus surveillance and pathogenicity in order to clarify further the potential impact of bat lyssaviruses on human health in Europe. As for the latter, the pathogenicity of EBLV-1 isolates with certain genetic variations was assessed. Also, previously established enhanced passive bat rabies surveillance in Germany was continued. Furthermore, to facilitate and strengthen surveillance efforts particularly in the developing world, commercially available LFDs for rabies diagnosis were evaluated.

3.Literature review

3.1. History of rabies with focus on Europe

The over 4200 years old Eshnunna code contains the first known record of a disease resembling rabies (Baer et al., 1996). In the next 1500 years there is hardly any mention of rabies, followed by a period (ca. 5th BC to 5th AC) with many records, describing the symptoms, transmission, preventive measures, as well as suggesting cures (Neville, 2004). Progress concerning rabies was only made in the 19th century with first pathogenesis studies (Jackson, 2013) and subsequent development of the first rabies vaccine by Louis Pasteur. Pasteur discovered that through repeated inoculation of desiccated nerve tissue from rabid animals, rabies infection could be prevented. The first person who successfully received the vaccine was a boy named Joseph Meister, who had been severely bitten two days earlier by a rabid dog (Suzor and Pasteur, 1887). This so called nerve tissue vaccine was further enhanced to reduce unwanted side effects and used in many countries for several decades (Jackson, 2013).

A vaccine for dogs was developed in the early 1920´s (Baer, 1975a). Previously, the only control measures for elimination of dog rabies were veterinary control measures, e.g. dog movement restriction, muzzling and elimination of stray dogs, leading to successful elimination in some countries, i.e. Prussia, Denmark, Norway and Sweden (Tarantola, 2017). Mass vaccination of dogs resulted in elimination of dog-mediated rabies in most of Europe by the mid of the last century. Great progress was also made in the Americas, resulting in the disappearance of dog-mediated rabies in many countries (Vigilato et al., 2013, Velasco-Villa et al., 2017).

Parallel to the control of the disease in dogs, terrestrial rabies in wildlife, i.e. foxes, came into focus as a source of rabies infections. In Europe, fox mediated rabies emerged in the 1940´s and spread across most of the continent within a few decades, representing new challenges in rabies control (Müller and Freuling, 2011). These were met in the 1970´s with the development of orally applicable vaccines and corresponding baits, and the implementation of oral rabies vaccination, which led to the elimination of terrestrial rabies in Western and Central Europe (Müller et al., 2012).

Although the association of rabies with dogs has been known for millennia, it was only much later recognized that bats also transmit rabies. In 1930´s an outbreak of rabies occurred and vampire bats were identified as the source of infection (Baer, 1975b, Pawan, 1936).

In Europe, the first bat rabies case was discovered in 1954 in Hamburg, Germany, when a boy was bitten by a bat, which subsequently tested positive for rabies. The child received post exposure treatment and survived (Mohr, 1957). Between 1954 and 1989 a total of 4705 bats were tested for rabies in Europe of which 379 were positive for rabies (Kappeler, 1989). Serotyping of bat rabies isolates in Europe showed that they did not belong to the known classical rabies virus (RABV), but were similar to Duvenhage virus (Fekadu et al., 1988a, Schneider et al., 1985). In 1988, a distinction between Duvenhage virus and European lyssaviruses was made (Dietzschold et al., 1988), followed by their separation into two serotypes/biotypes, EBL1 and EBL2 (now called EBLV-1 and EBLV-2) (King et al., 1990, Hirose et al., 1990, Bourhy et al., 1992). The discovery of other bat lyssaviruses in Europe followed, i.e. West Caucasian bat lyssavirus in 2002, Bokeloh bat lyssavirus in 2010 and Lleida bat lyssavirus in 2013 (Freuling et al., 2011, Arechiga Ceballos et al., 2013, Botvinkin et al., 2003).

3.2. Lyssaviruses

The word lyssa is of ancient Greek origin and translates as madness (Baer, 1975b). It is suggested that its root is either lysis (loosing – loss of rational faculties), lykos (wolf) or lud (violent) (Jackson, 2013, Neville, 2004). A lyssavirus infection results in rabies, a zoonotic disease, which is almost always fatal once clinical signs develop (Taylor and Nel, 2015).

3.2.1. Virus Taxonomy

The term “lyssaviruses” comprises all viruses of the Genus *Lyssavirus*, which belongs to the family *Rhabdoviridae* of the order *Mononegavirales*. Originally, lyssaviruses were separated into serotypes or genotypes. Following taxonomic guidelines, lyssaviruses were then classified into species according to species demarcation criteria that include homology of the viral genomes (King et al., 2012). Currently, the genus *Lyssavirus* comprises 16 species, of which 14 are officially recognized by the International Committee on Taxonomy of Viruses

(ICTV) (King et al., 2012). Two lyssaviruses, i.e. Lleida bat lyssavirus (LLEBV) and Gannoruwa bat lyssavirus (GBLV), although approved by the executive committee still await ratification from the ICTV (Table 1) (International Committee on Taxonomy of Viruses 2017). Furthermore, two isolations of a novel lyssavirus were made from Japanese house bats (*Pipistrellus abramus*) in Taiwan in November 2016 and April 2017, although this virus does not have an assigned name yet (Shu-min, 2016, Chatterjee, 2017).

Lyssaviruses can be further divided into two phylogroups, based on their phylogenetic, immunologic and pathogenic properties (Badrane et al., 2001). Phylogroup 1 comprises most lyssavirus species, i.e. RABV, EBLV-1, EBLV-2, BBLV, ABLV, GBLV, IRKV, ARAV, KHUV and DUVV, whereas phylogroup 2 includes three African lyssavirus species, i.e. LBV, MOKV and SHIBV (Table 1) (Kuzmin et al., 2005, Kuzmin et al., 2010, Badrane et al., 2001). IKOV, WCBV and LLEBV do not belong to either phylogroup and it has not been determined yet if they group together in a third phylogroup, or if a fourth phylogroup comprising IKOV and LLEBV should be established (Table 1) (Arechiga Ceballos et al., 2013, Banyard et al., 2014a). This separation into different phylogroups is very important, as there is no cross neutralization between viruses of the different groups. Therefore, commercial rabies vaccines, which are based on RABV, elicit protection against lyssaviruses of phylogroup 1, but do not reliably protect against lyssaviruses of the other phylogroups (Fekadu et al., 1988b, Badrane et al., 2001, Malerczyk et al., 2014, Brookes et al., 2005, Malerczyk et al., 2009).

Phylogroup	Virus name	Species name	Abbreviation	Reservoir	Distribution
1	Rabies virus	*Rabies lyssavirus*	RABV	*Chiroptera* , *Carnivora*	worldwide except Australia and Antarctica
	European bat lyssavirus 1	*European bat 1 lyssavirus*	EBLV-1	*Chiroptera*	Europe
	European bat lyssavirus 2	*European bat 2 lyssavirus*	EBLV-2	*Chiroptera*	Europe
	Bokeloh bat lyssavirus	*Bokeloh bat lyssavirus*	BBLV	*Chiroptera*	Europe
	Australian bat lyssavirus	*Australian bat lyssavirus*	ABLV	*Chiroptera*	Australia
	Irkut virus	*Irkut lyssavirus*	IRKV	*Chiroptera**	Asia
	Aravan virus	*Aravan lyssavirus*	ARAV	*Chiroptera**	Asia
	Khujand virus	*Khujand lyssavirus*	KHUV	*Chiroptera**	Asia
	Gannoruwa bat lyssavirus	-	GBLV	*Chiroptera*	Asia
	Duvenhage virus	*Duvenhage lyssavirus*	DUVV	*Chiroptera*	Africa
2	Lagos bat virus	*Lagos bat lyssavirus*	LBV	*Chiroptera*	Africa
	Mokola virus	*Mokola Lyssavirus*	MOKV	Shrews (*Crocidura* sp.), small rodent (*Lopyhromys sikapusi*)*	Africa
	Shimoni bat virus	*Shimoni bat lyssavirus*	SHIBV	*Chiroptera**	Africa
3-4	West Caucasian bat virus	*West Caucasian bat lyssavirus*	WCBV	*Chiroptera**	Europe
	Lleida bat lyssavirus	-	LLEBV	*Chiroptera**	Europe
	Ikoma virus	*Ikoma Lyssavirus*	IKOV	African civet (*Civettictis civetta*)*	Africa

Table1: Members of the genus lyssavirus (modified from Gunawardena et al., 2016, Banyard et al., 2014a), including phylogroup, reservoir and distribution. GBLV and LLEBV have no assigned species name, since they have not yet been approved by the ICTV. Asterisks indicate that the reservoir has not been confirmed, due to the limited number of isolations of the respective lyssaviruses.

3.2.2. Virus structure and viral genome

As a member of the *Rhabdoviridae*, lyssavirus virions are rod- or bullet-shaped (*rhabdos* – rod) and measure between 100-250nm in length with a diameter of roughly 80 nm (Matsumoto, 1962, Davies et al., 1963).

The ribonucleoprotein (RNP) comprises the viral genomic ribonucleic acid (RNA) encapsidated by the nucleoprotein, while the viral RNA-dependent RNA-polymerase and the phosphoprotein are associated with the RNP, forming together the core structure of the virion (Davis et al., 2015). The RNP is surrounded by a host derived membrane with the matrix protein forming a bridge between the virus membrane and the core (Mebatsion et al., 1999). The surface of the virus particle is covered by glycoprotein homotrimers, the only surface protein of the virus (Gaudin et al., 1992).

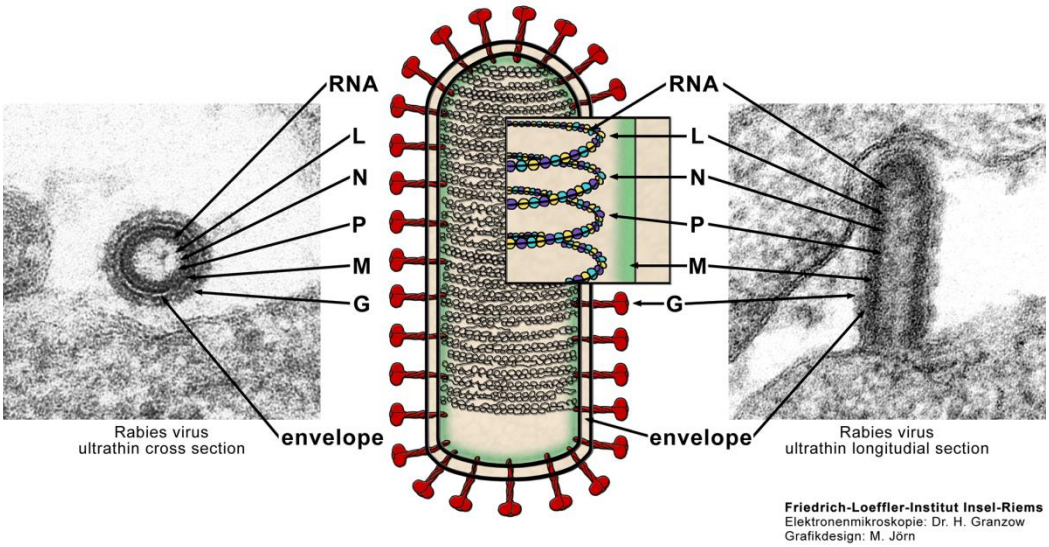

Figure 1: Structure of the lyssavirus type species RABV depicted as schematic illustration and electron microscopic pictures. G: glycoprotein; L: RNA-polymerase; M: matrix protein; N: nucleoprotein; P: phosphoprotein. (© Friedrich-Loeffler-Institut Insel Riems, Dr. H. Granzow, M. Jörn)

3.2.2.1. Viral RNA

The lyssavirus genome consists of a ca. 12 kilobases (kb) long single stranded negative sense RNA (Marston et al., 2007). It carries the genes for the five virus proteins in a strictly conserved order (3´ – nucleoprotein gene – phosphoprotein gene – matrix protein gene – glycoprotein gene – RNA-polymerase gene – 5´) (Tordo et al., 1986a). The genes are separated by intergenic regions. At the termini of the genomic RNA noncoding leader and trailer sequences are present (Wunner and Conzelmann, 2013). The length of the intergenic regions (IGR) are 2 nucleotides (nts) between the nucleoprotein gene and the phosphoprotein gene, 5nts between the phosphoprotein gene and the matrix protein gene as well as between the matrix protein gene and the glycoprotein gene and 19nts to 24nts between the glycoprotein gene and the RNA-polymerase gene. Exceptions are the IGRs of MOKV and WBCV between the matrix protein gene and the glycoprotein gene, and between the glycoprotein gene and the RNA-polymerase gene, which are significantly longer (Marston et al., 2007). The lengths of the IGRs influence downstream transcription (Finke et al., 2000). Each gene starts with a transcription initiation signal (TIS) (5´-AACAYYNCT-3´; A: Adenine, C: Cytosine, T: Thymine, Y: C or T, N: any base) and ends with a transcription termination polyadenylation signal (TTS) (5´-WGAAAAAAA-3´; W: A or T, G: Guanine) respectively (Marston et al., 2007). The length of the untranslated regions (UTRs), comprising the regions between the TIS and the start codon for translation and between the stop codon and the TTS, vary. The 3`-UTRs of the virus genes are shorter (20-30nts) compared to the 5´-UTRs (100-500nts) (Wunner and Conzelmann, 2013). The 5`-UTR of the glycoprotein gene, also called pseudogene, is particularly long and believed to be the remnant of a former 6th gene (Tordo et al., 1986b).

3.2.2.2. Viral proteins

The nucleoprotein is the most conserved lyssavirus protein. The high conservation of the amino acid (AA) sequence is most likely due to its function in encapsidation of the viral RNA, for which interaction sites need to be conserved. Through the formation of the RNP, the nucleoprotein protects the viral genome from host ribonucleases (RNases) (Albertini et al., 2011). Serotyping of lyssavirus isolates and subsequent division in serotypes/biotypes was

routinely performed with monoclonal anti-nucleoprotein antibodies (Schneider, 1982, Dietzschold et al., 1988, King and Crick, 1988, Bourhy et al., 1992, Hirose et al., 1990). Since demarcation of lyssaviruses switched to genotypes and species based on nucleotide sequences, the corresponding nucleoprotein gene is now preferably used for sequence comparisons (Nadin-Davis and Real, 2011). Furthermore, due to the conserved AA sequence of nucleoprotein and its abundance in host cells as the first transcribed protein, anti-nucleoprotein antibodies are commonly used in lyssavirus antigen detection assays (OIE, 2012). The phosphoprotein is, in contrast to the nucleoprotein, the least conserved of the lyssavirus proteins (Marston 2007). The phosphoprotein is dimeric and has multiple functions in RNA replication, virus assembly and as an interferon antagonist. As a non-catalytic cofactor of the viral polymerase, the phosphoprotein stabilizes the former and places the phosphoprotein – RNA-polymerase complex on the viral RNA (Wunner and Conzelmann, 2013). Furthermore, the phosphoprotein prevents the aggregation and nonspecific binding of newly synthesized nucleoprotein to cellular RNA, by acting as a chaperone (Liu et al., 2004, Yang et al., 1998) . The phosphoprotein is also a major interferon (IFN) antagonist, by interfering with gene expression of IFN, and STAT protein signalling caused by IFN (Rieder and Conzelmann, 2011). The matrix protein plays an important role in virus assembly and budding. It is responsible for the bullet shaped structure of the virion, by condensing the RNP into its helical structure. Attachment of the core structure to glycoprotein in the host membrane is also mediated by the matrix protein, therefore the matrix protein is essential for budding of virus particles (Mebatsion et al., 1999). Furthermore, the matrix protein inhibits transcription and promotes RNA replication, regulating the balance between the two processes (Finke et al., 2003). On the host side, the matrix protein can inhibit RNA translation and induces apoptosis (Gholami et al., 2008, Komarova et al., 2007, Kassis et al., 2004).

The glycoprotein is probably the most extensively analysed protein since it is an important pathogenicity determinant as well as an immunogen (Wunner and Conzelmann, 2013). It is a transmembrane protein and consists of a carboxyl-terminal domain (endodomain), a transmembrane domain and an ectodomain as well as an amino-terminal 19AA signal peptide, which is cleaved of and acts as a membrane insertion signal during protein maturation (Anilionis et al., 1981). As the only viral surface protein glycoprotein enables attachment of virus particles to host cell membranes by binding to specific host cell

receptors. After endocytosis of the virus particle, the glycoprotein mediates pH dependent fusion of virus and endosomal membrane (Gaudin et al., 1993). Furthermore, it facilitates retrograde axonal transport (Mazarakis et al., 2001). The glycoprotein is glycosylated at one or more sites in the ectodomain, with one glycosylation site known to be conserved in at least seven lyssavirus species (Badrane et al., 2001). Glycosylation is essential for the intracellular transport of glycoprotein and important for its antigenicity (Shakineshleman et al., 1992). Similar to matrix protein, glycoprotein can also induce apoptosis of the host cell (Prehaud et al., 2003, Faber et al., 2002, Morimoto et al., 1999).

The RNA-dependent RNA-polymerase is by far the largest of the lyssavirus proteins. It forms the enzymatic component of the polymerase complex and is essential for transcription and replication (Wunner and Conzelmann, 2013). No efficient in vitro system exists for RABV messenger RNA (mRNA) synthesis, but the RABV RNA-polymerase displays great sequence similarity to the RNA-polymerase of another member of the *Rhabdoviridae*, Vesicular stomatitis virus (VSV) (Villarreal and Holland, 1974, Kawai, 1977, Tordo et al., 1988). Therefore, it is presumed that the functions of the two RNA-polymerases are similar and most of the functions of RABV RNA-polymerase were determined by studying VSV RNA-polymerase. The RNA-polymerase initiates transcription of the virus genome, is responsible for nucleotide polymerization and has furthermore important enzymatic functions in mRNA capping, methylation and 3´-polyadenylation. RNA-polymerase also displays protein kinase activity and is responsible for phosphorylation of phosphoprotein in transcriptional activation (Banerjee, 1987). Recently the catalytic activity of the RNA-polymerase in mRNA capping was shown for rabies virus (Ogino et al., 2016).

3.2.3. Replication cycle

Lyssavirus infection starts with glycoprotein mediated attachment of the virus to the host cell. Various host cell receptors are known to play a role in virus attachment. These include the nicotinic acetylcholine receptor (nAchR), the neuronal cell adhesion molecule (NCAM) and the low affinity nerve growth factor (p75NTR), although none of these receptors were proven to be essential for virus infection (Lentz et al., 1982, Reagan and Wunner, 1985, Thoulouze et al., 1998, Tuffereau et al., 1998, Jackson and Park, 1999). Following attachment

the virus particle is engulfed into lysosomes and the core structure of the virus particle is released by fusion of the virus envelope with the endosomal membrane. This fusion process is triggered by a low pH within the endosome (Superti et al., 1984). Transcription and replication probably takes place within inclusion bodies, in neuronal cells termed Negri bodies, in the cytoplasm of the host cell (Lahaye et al., 2009). RNA transcription is initiated by the polymerase at the 3´-end of the genome, starting at the TIS and terminated at the TTS where the polymerase stutters to and fro for polyadenylation (Barr et al., 1997). Afterwards it dissociates from the template RNA and re-associates at the next TIS. Re-association of the polymerase does not always occur, its likelihood depending on the length of the IGR (Finke et al., 2000). Therefore, the lyssavirus genes downstream of the viral genome are less frequently transcribed, resulting in a negative transcription gradient (Banerjee, 1987). High levels of matrix protein modulate the switch from RNA transcription to replication (Finke et al., 2003). In the replication mode, full-length positive-sense RNA antigenomes are produced by the polymerase, which then act as templates for new full-length negative-sense RNA production (Banerjee, 1987). The RNP, associated with phosphoprotein and RNA-polymerase form new core structures of the virions, which are then tightly condensed into their helical structures by matrix protein (Mebatsion et al., 1999). Budding of the virus particles is facilitated by the interaction of matrix protein with glycoprotein, which is inserted into the host cell membrane following maturation in the endoplasmic reticulum (Schnell et al., 2010).

3.3. Rabies disease

The Latin word rabies is probably derived from the old Sanskrit word *rabhas* (“to do violence”), whereas the German word “Tollwut” originated from Indo-Germanic *Dhvar* (to damage) and middle German wuot (rage) (Baer, 1975b). Rabies is responsible for an estimated 59000 human deaths per year and is classified by the WHO as a neglected zoonotic disease (Hampson et al., 2015, WHO, 2013b). In 2015, the United Nations drafted an agenda with the goal to end neglected tropical diseases by 2030 (United Nations, 2016). Three month later a joint collaboration of the WHO, the OIE, the Food and Agriculture Organization of the United Nations (FAO) and the Global Alliance for Rabies Control (GARC)

developed a global framework for the elimination of dog-mediated human rabies by 2030 (WHO & OIE, 2016).

3.3.1. Pathogenesis

Lyssaviruses are transmitted through the inoculation of virus containing saliva via bites or scratches of the infected animals (Fekadu et al., 1982). It is believed that prior to neuroinvasion lyssaviruses replicate in the muscle at the site of inoculation. This is supported by the ability of lyssaviruses to replicate in denerved muscle tissue in vivo and to bind to nAchRs at the postsynaptic membranes (Lafon, 2005, Charlton et al., 1997, Charlton and Casey, 1981, Murphy et al., 1973a). This replication step is not mandatory and immediate infection of neurons following inoculation can occur (Shankar et al., 1991, Coulon et al., 1989). Neuroinvasion starts with the infection of primary motor neurons at the presynaptic membranes via NCAM, p75NTR or other unknown receptors (Lafon, 2005). Once inside the neuron, virus particles reach the neuronal cell body via retrograde axonal transport along the microtubular motor network, where replication takes place (Ceccaldi et al., 1989, Bijlenga and Heaney, 1978). Afterwards transportation and budding from another synapse occur, resulting in the distribution of the virus in the central nervous system (CNS) (Ugolini, 2011). At the same time the virus undergoes a centrifugal spread along neurons to the peripheral organs (Jackson et al., 1999, Murphy et al., 1973b). In the salivary glands, budding of virus particles from mucogenic acinar cells occurs, followed by shedding of the virus with the saliva (Dierks et al., 1969, Fekadu et al., 1982). Lyssavirus infection results in encephalomyelitis, although only little histopathological changes are observed (Yan et al., 2001, Murphy, 1977).

3.3.2. Clinical picture

For human rabies, disease progression can be divided into five stages according to symptoms, i.e. the incubation period, the prodrome, the acute neurological phase, coma and death (Hemachudha et al., 2002). The incubation periods following exposure are usually between one and two months. Very long incubation periods of up to several years following

a bite have also been observed, rendering the connection of bite history to subsequent clinical symptoms difficult (Hemachudha et al., 2002, Smith et al., 1991, Rupprecht et al., 2002). The incubation period ends with the prodromal phase, where first unspecific clinical symptoms appear, e.g. fever or local symptoms like burning, numbness, tingling or itching (Hemachudha, 1994). The acute neurological phase can manifest either as furious (encephalitic) rabies or as paralytic rabies (Hemachudha et al., 2002). The three symptoms characteristic for furious rabies are fluctuating consciousness, phobic spasms and signs of autonomic dysfunctions including piloerection and hypersalivation (Hemachudha, 1994). Roughly a third of the rabies patients develop paralytic rabies, which usually starts with weakness in the bitten extremity, progressing to other parts of the body (Hemachudha, 1994). Here, phobic spasms are observed in roughly half of the patients. The patients then lapse into a coma and subsequently die, in most cases due to circulatory insufficiency, manifesting as arrhythmia or hypotension (Hemachudha, 1994). The average survival time, after the onset of clinical symptoms, is two weeks and shorter for patients with furious rabies than for patients with paralytic rabies (Hemachudha et al., 2002, Mitrabhakdi et al., 2005).

Similar to humans the initial clinical signs in animals are often unspecific and include lethargy, diarrhoea and vomiting. This is followed by a rapid deterioration, where behavioural changes in the form of tameness up to aggressiveness, parasthesiasis and autonomic dysfunctions, e.g. hypersalivation, can occur. Other neurologic dysfunctions include tremors, seizures, paresis and paralysis. Death is caused most likely by severe autonomic dysfunctions (Hanlon, 2013).

3.3.3. Pathogenicity

Although rabies disease almost always results in death once clinical signs develop, not every lyssavirus infection leads to the development of clinical signs (Taylor and Nel, 2015). Especially bats seem to be able to clear the virus before it reaches the CNS and develop neutralizing antibodies (Kuzmin and Rupprecht, 2015). The pathogenicity of lyssavirus isolates depends on various factors, including the inoculation dose, inoculation route, as well as the animal species, age and immune status (Banyard et al., 2014b, Coulon et al., 1994,

Jackson and Fu, 2013). Furthermore, pathogenicity depends on the lyssavirus species and the respective isolate (Kgaladi et al., 2013, Badrane et al., 2001, Cunha et al., 2010, Perrin et al., 1996). Differences in the pathogenicity of isolates belonging to one lyssaviruses species were found for LBV and RABV, and are most likely linked to genetic differences of the respective isolates (Kgaladi et al., 2013, Cunha et al., 2010, Markotter et al., 2009a). Sites in the genome or the respective protein that have an influence on the properties of a virus, so called pathogenicity determining sites, have been identified for RABV with the help of reverse genetics (Virojanapirom et al., 2016, Tuffereau et al., 1989, Rieder et al., 2011). They include not only amino acid exchanges in the respective proteins but also nucleotide exchanges in UTRs and IGRs (Finke et al., 2000, Conzelmann et al., 1990, Virojanapirom et al., 2016). Unfortunately, not many reverse genetic systems exist for other lyssaviruses, and knowledge about pathogenicity determinants in field virus isolates is poor (Orbanz and Finke, 2010, Nolden et al., 2016).

3.3.4. Vaccination and post exposure prophylaxis

Vaccination against rabies is the foremost tool for preventing, controlling and eliminating the disease in terrestrial mammals and humans. In human medicine parenteral vaccination against rabies is recommended for certain risk groups, e.g. laboratory workers, veterinarians, animal handlers and bat conservationists (WHO, 2013a). Post-exposure prophylaxis (PEP) after contact with a rabid or rabies suspected animal includes local wound treatment, vaccination and administration of rabies immunoglobulin. The extent of PEP, especially concerning immunoglobulin administration, should be assessed for every case and depends on several factors (WHO, 2013b), including the epidemiological situation, the clinical features and vaccination status of the animal, the severity of the exposure, and the vaccination history of the patient (WHO, 2013a). There is no treatment proven to prevent death once clinical signs develop and reports of patients surviving rabies are very rare (Rupprecht et al., 2017).

Two vaccination strategies for terrestrial mammals exist, which are parenteral vaccination with inactivated or live recombinant vectored RABV vaccines and oral vaccination with live attenuated RABV vaccines (WHO, 2013a). Parenteral vaccination is performed for domestic

animals and is the main tool for the control and elimination of dog rabies (Fooks et al., 2014). Oral vaccination, performed in terrestrial wildlife populations, can successfully control and eliminate rabies, as exemplified by the elimination of fox rabies in most of Europe (Freuling et al., 2013a). Due to the residual pathogenicity of oral vaccines, sporadic vaccine induced rabies cases have occurred (Vuta et al., 2016, Robardet et al., 2016, Hostnik et al., 2014, Müller et al., 2009, Fehiner-Gardiner et al., 2008).

3.4. Rabies epidemiology

3.4.1. Terrestrial rabies

Terrestrial rabies, i.e. RABV in non-bat reservoirs, is endemic on all continents, except Australia and Antarctica and has been eliminated in Western and Central Europe (Müller et al., 2015, Sparkes et al., 2016, Nel and Markotter, 2007). The last rabies case in terrestrial wildlife in Germany was reported in 2006, and in 2008 Germany self-declared a “rabies free” status according to the standards by the World Organization for Animal Health (OIE), which is defined as freedom of rabies caused by RABV (OIE, 2013).

The causative agent of terrestrial rabies is RABV and although all mammals are potentially susceptible to rabies, not all can serve as a reservoir for RABV. Next to the host species being highly susceptible, virus evolutionary factors, e.g. efficient and balanced replication and excretion and modification of host behaviour, as well as certain ecological factors, i.e. host density, social structure and population size must be met for independent transmission cycles to occur within a species (Mollentze et al., 2014).

Known terrestrial reservoirs of RABV belong to the order *Carnivora* and include dogs (*Canis lupus familiaris*), red foxes (*Vulpes vulpes*), grey foxes (*Urocyon cinereoargenteus*), arctic foxes (*Vulpes lagopus*), bat-eared foxes (*Otocyon megalotis*), raccoon dogs (*Nyctereutes procyonoides*), raccoons (*Procyon lotor*), skunks (*Mephitidae*), Indian mongoose (*Herpestes auropunctatus*), jackals (*Canis aureus*, *C. adustus*, *C. mesomelas*) and Chinese ferret badgers (*Melogale moschata*) (WHO, 2013a). The most important reservoir for human infection is the dog, as 99% of human rabies cases are transmitted by rabid dogs (WHO, 2013a). Although elimination of dog rabies is feasible and was successful in Europe, Japan, parts of

Asia, North and most of South America, it still poses a problem in many parts of Asia and Africa (Coleman et al., 2004, WHO, 2013a). Asia has the highest rabies burden with India alone accounting for over 35 % of human rabies deaths (Hampson et al., 2015, Banyard et al., 2013). This is followed by Africa where ca. 36.4% of human rabies deaths occur. Asia and Africa also account for 95% of the estimated 3.7 million disability-adjusted life years, a measure for the years annually lost to rabies due to disease or premature death (Hampson et al., 2015).

3.4.2. Bat rabies

With ~1300 known species (Racey, 2015), bats (*Chiroptera*) are the second largest mammalian order after rodents (Burland and Wilmer, 2001). Bat speciation is performed either using morphological features or through phylogenetic analyses of host genes, though the first is challenging when dealing with cryptic species (Burland and Wilmer, 2001). With a quarter of all bat species threatened, many are protected, including all 53 bat species present in Europe (Racey, 2015, Lina, 2016, UNEP/EUROBATS, 1994). Although bats have certain characteristics which are presumed to make them special reservoirs for viruses, i.e. capability of flight, high roost densities, torpor and hibernation, long life spans and echolocation (Calisher et al., 2006), there is no clear indication that bats harbour a greater number of zoonotic viruses compared to other animal groups (Olival et al., 2015).

Interestingly, the first virus discovered in bats was RABV and most lyssaviruses were found in bats or are known to have bats as their reservoir, which is why these animals are believed to be the ancestral hosts of all lyssaviruses (Banyard et al., 2011, Rupprecht et al., 2017). Within bats lyssaviruses display a restricted geographical distribution. This results in the conundrum that RABV is the only lyssavirus present in bats in the new world, while in the old world RABV has only been found in terrestrial mammals and other lyssavirus species are present in bats (Banyard et al., 2014a, Rupprecht et al., 2017). RABV is also responsible for most spill-over infections of rabies from bats into terrestrial mammals and humans, and is the only lyssavirus where sustained spill-overs from bats into other mammals are known to have occurred (Badrane and Tordo, 2001, Kuzmin et al., 2012, Velasco-Villa et al., 2005, Daoust et

al., 1996, Leslie et al., 2006). Spill-over infections of other bat lyssaviruses are rare and have only been described sporadically (Johnson et al., 2010).

3.4.2.1. Bat rabies in Europe

Rabies is a notifiable disease in almost all European countries and monitored in the scope of surveillance programs, but less than half of these include bat rabies surveillance (Müller et al., 2016). Two forms of rabies surveillance exist: (i) Passive surveillance comprises the sampling of sick, rabies suspected or dead bats and bats which had contact to humans or domestic animals via bites or scratches. (ii) Active surveillance on the other hand describes the monitoring of lyssaviruses in free living bats, either through the detection of antibodies against lyssaviruses in serum samples or the detection of lyssavirus RNA or antigen in saliva samples (Med Vet Net Working Group, 2005). Despite great efforts, detection of virus RNA in oral swabs from European bats was rare and interpretation as well as comparison of serological results from different studies proves challenging due to cross neutralization of phlyogroup 1 lyssaviruses as well as the non-standardized serological test procedures (Freuling et al., 2009a, Schatz et al., 2014a). Thus, it was recommended to focus on passive bat rabies surveillance (Schatz et al., 2013b, Schatz et al., 2014a).

A total of ca. 1123 bat rabies cases in Europe have been reported to the WHO Rabies Bulletin Europe in the scope of passive bat rabies surveillance between 1977 and 2015 (Anonymous, 2017). In most cases EBLV-1 was identified as the causative agent (Schatz et al., 2013a). Two bat species serve as reservoir for this lyssavirus, i.e. the Serotine bat (*Eptesicus serotinus*) and the Isabelline serotine bat (*Eptesicus isabellinus*) (Vazquez-Moron et al., 2008, Montano-Hirose et al., 1990). Furthermore, EBLV-1 can be divided into two genetic distinct sublineages. While EBLV-1a is present in an area between France, the Netherlands and Russia, EBLV-1b is present rather in western parts of Europe between Spain and Poland (Amengual et al., 1997, Davis et al., 2005). A third genetic sublineage comprising isolates from the Isabelline serotine bat from the Iberian Peninsula has been proposed (Vazquez-Moron et al., 2011). Furthermore, additional variation in the genome, in the form of insertions and deletions (indels) in the UTRs of EBLV-1 isolates were found (Freuling et al., 2012, Johnson et al., 2007). Single EBLV-1 infections of other bat species were recorded for

the common pipistrelle bat (*Pipistrellus pipistrellus*), the common noctule bat (*Nyctalus noctula*), the Nathusius' pipistrelle bat (*Pipistrellus nathusii*), the brown long-eared bat (*Plecotus auritus*) and the Barbastelle bat (*Barbastella barbastellus*) (Müller et al., 2007, Schatz et al., 2014a) as well as for a Natterer's bat (*Myotis nattereri*), a common bent-winged bat (*Miniopterus schreibersii*), a mouse-eared bat (*Myotis myotis*) and a greater horseshoed bat (*Rhinolophus ferrumequinum*) (Serra-Cobo et al., 2002). Additionally, EBLV-1 infections of Egyptian fruit bats (*Rousettus agypticatus*) in a colony from a Danish zoo have been reported (Rønsholt et al., 1998). Spill-over infections in terrestrial mammals, including two human rabies cases caused by EBLV-1, also occurred. These comprised EBLV-1 infections in sheep in Denmark, cats in France and a stone marten in Germany (Müller et al., 2004, Dacheux et al., 2009, Tjornehoj et al., 2006). The first presumed spill-over of EBLV-1 to a human occurred in 1977 in the Ukraine, where a small girl was bitten by a bat of unknown species (Fooks et al., 2003a). The second human rabies case caused by EBLV-1 occurred in Russia in 1985, when a girl was bitten by a bat and died approximately one month later after developing rabies like symptoms (Selimov et al., 1989).

The second lyssavirus present in Europe is EBLV-2 with 21 confirmed cases in bats until 2012 (Schatz et al., 2013a). The majority of EBLV-2 cases were isolated from Daubenton's bats (*Myotis daubentonii*). In the Netherlands, EBLV-2 was isolated from five Pond bats (*Myotis dasycneme*), the only bat species from which EBLV-2 was isolated there (Schatz et al., 2013a, van der Poel et al., 2005). Two genetic lineages, EBLV-2a and EBLV-2b, exist for EBLV-2, although the phylogenetic support is weaker compared to EBLV-1. Due to limited data no correlation of the lineages with certain bat species can be established (Amengual et al., 1997, McElhinney et al., 2013). Two human rabies cases caused by EBLV-2 have been recorded 1985 in Finland (Lumio et al., 1986) and 2002 in Scotland (Fooks et al., 2003b).

Since the discovery of BBLV in 2010 in Germany, two additional cases were detected in 2012 in Hemilly, France and Bavaria, Germany (Picard-Meyer et al., 2013, Freuling et al., 2013b, Freuling et al., 2011). All cases were found in Natterer's bats (*Myotis nattereri*), which is presumed to be the reservoir for BBLV (Banyard et al., 2014a).

Single isolations of two additional lyssavirus species were detected in Common bent-winged bats (*Miniopterus schreibersii*) in Europe. WCBV was isolated in 2002 in the European Caucasus and LLEBV in 2012 in Spain (Botvinkin et al., 2003, Arechiga Ceballos et al., 2013,

Kuzmin et al., 2005). These lyssaviruses do not belong to phylogroup 1 (Banyard et al., 2014a).

3.4.2.2. Passive bat rabies surveillance in Germany

In Germany passive bat rabies surveillance can be divided further into routine surveillance and enhanced passive bat rabies surveillance. Routine surveillance is performed by the regional veterinary laboratories of the sixteen federal states and was initiated in 1982 as beforehand bats were tested only sporadically for rabies (Müller et al., 2007). A presumed bat mediated human rabies case in Denmark further sparked surveillance efforts, as before bat rabies surveillance was only thought to be of interest for scientific reasons (Seidler et al., 1987). In the scope of routine surveillance only bats with symptoms suggestive of rabies or where human contact in form of bites and scratches has occurred are examined (Müller et al., 2007). Approximately 2076 bats were tested for bat rabies during routine surveillance until 2015 (Anonymous) with 272 detected rabies cases (Anonymous, 2017). The majority of cases were caused by EBLV-1, but one case of EBLV-2 as well as the first case of BBLV in 2010 were also detected during routine surveillance (Schatz et al., 2014a).

In 1998, an additional enhanced passive bat rabies surveillance study was initiated, where dead found bats from bat collections, not suspected of rabies, were included (Schatz et al., 2014a). Until June 2013 a total of 5478 bats were investigated, with the most frequently submitted bat species being the common pipistrelle bat and the common noctule bat. Fifty-six bat rabies cases were found during enhanced passive bat rabies surveillance, and in 52 cases EBLV-1 and in three cases EBLV-2 were identified as the causative agents (Schatz et al, 2014). The resulting prevalence of bat rabies during enhanced passive bat rabies surveillance was much lower compared to routine surveillance and more likely represents the true prevalence of bat rabies in Germany (Schatz et al, 2014).

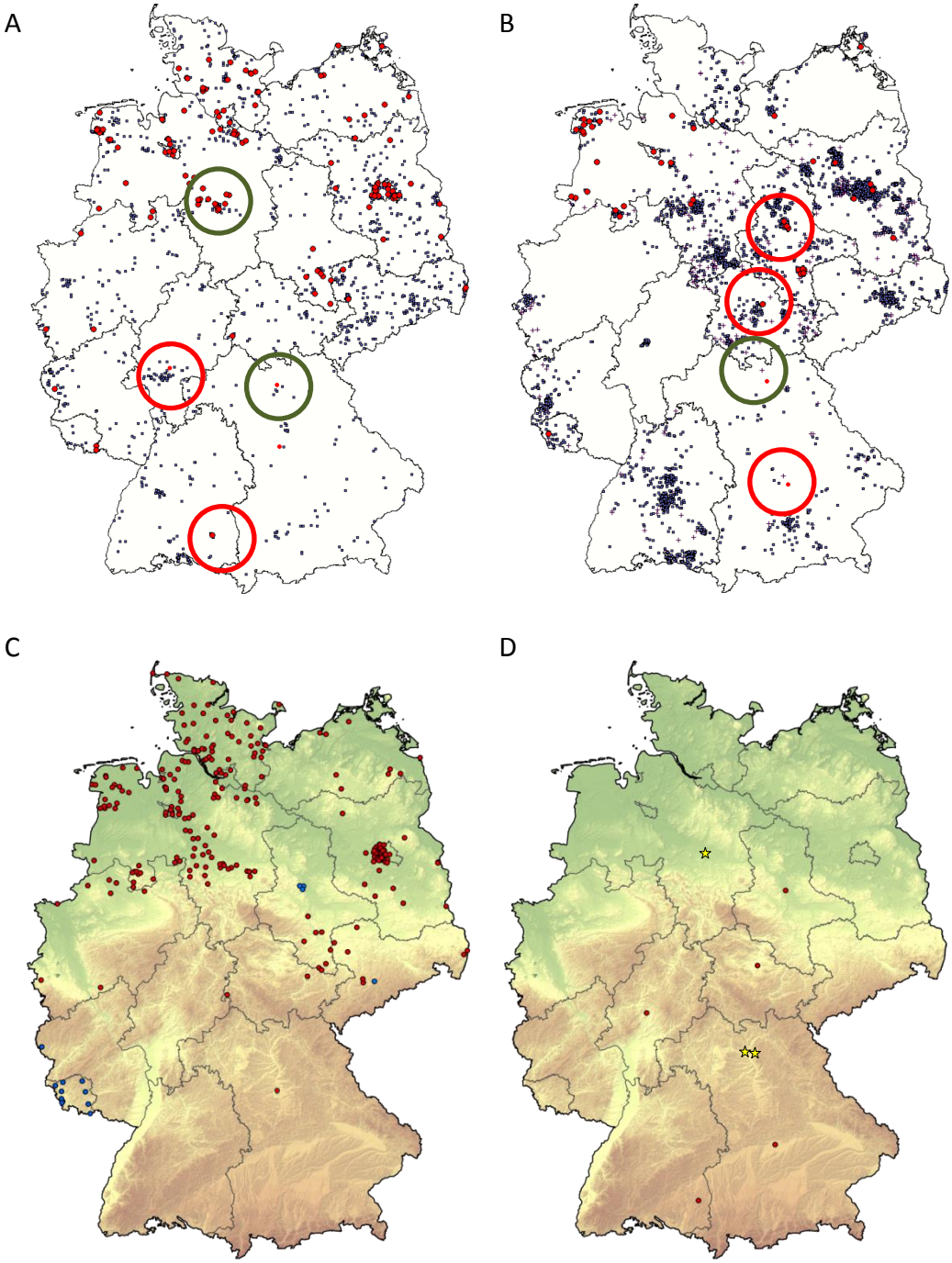

Figure 2: Surveillance efforts and geographical distribution of reported bat rabies cases in Germany until 2014. A) Bats sampled during routine surveillance which tested negative (blue dots) and positive (red dots) for bat rabies. B) Bats sampled during enhanced passive surveillance which tested negative (blue dots) and positive (red dots) for bat rabies. A) + B) Red and green circles mark cases of EBLV-2 and BBLV respectively. C) Bat rabies cases caused by EBLV-1a (red dots) and EBLV-1b (blue dots). D) Bat rabies cases caused by EBLV-2 (red dots) and BBLV (stars). (© Friedrich-Loeffler-Institut Insel Riems)

3.5. Rabies diagnosis

Rabies diagnosis in animals is preferably made post mortem from brain material and ideally different brain regions, i.e. brain stem, Ammon´s horn, cortex, cerebellum and thalamus, are tested (Müller, 2017, OIE, 2012). Ante mortem diagnosis should only be performed in human suspect rabies cases, and comprises the testing of preferentially saliva and head skin biopsies at different time points (Crepin et al., 1998, WHO, 2013a). Since virus shedding in saliva is intermittent and skin biopsies not always positive, these tests can only confirm but not exclude rabies (WHO, 2013a). Due to late seroconversion, serology is rarely used for ante-mortem diagnosis but mostly for epidemiological studies and the assessment of seroconversion following vaccination (OIE, 2012).

3.5.1. Standard diagnostic tests

The gold standard for rabies diagnosis is the fluorescence antibody test (FAT), due to accuracy and speed, which is recommended by the WHO and OIE (OIE, 2012). Virus antigen is detected in brain smears fixed on slides, by staining them with fluorophore-conjugated (FITC) anti-rabies antibodies. The slides are evaluated under a fluorescence microscope, which emits ultraviolet light, causing the fluorophore to emit a light signal. This can be seen as green fluorescence (Dean et al., 1996, Goldwasser and Kissling, 1958). If the FAT result is inconclusive or in cases of human exposure further tests are recommended (OIE, 2012). The rabies tissue culture infection test (RTCIT) has replaced the mouse inoculation test (MIT), which is no longer recommended due to ethical reasons (Müller et al., 2016). In the RTCIT virus is isolated in mouse neuroblastoma cell culture from brain suspensions, thus only viable virus can be detected (Webster and Casey, 1996, Rudd and Trimarchi, 1989). Although not yet recommended by the WHO as a diagnostic test, reverse transcription polymerase chain reaction (RT-PCR) is increasingly used in rabies diagnosis, e.g. for the verification of FAT results (Müller, 2017, WHO, 2013a, Dacheux et al., 2010, Fooks et al., 2009). Next to a high sensitivity, RT-PCR has the additional advantage that identification of the lyssavirus species in the scope of multiplex RT-PCR or sequencing of PCR products is possible (Woldehiwet, 2005).

3.5.2. Challenges in rabies diagnosis

Sample acquisition and transport to the laboratory are the first steps for diagnosing rabies and already pose difficulties. To obtain brain samples, the skull needs to be opened, which should be performed by well-trained technicians, with precautionary measures taken to avoid accidental exposure or cross contamination. To circumvent these challenges, alternative sampling methods were developed where brain material is obtained by insertion of a straw or syringe either via the occipital foramen or following trepanation of the eye socket (Montano-Hirose et al., 1991, Barrat and Halek, 1986). Immediately after removal, sample material should ideally be frozen and in this condition transported to a laboratory (OIE, 2012). In order to preserve sample material for testing without freezing, storage in Formalin or 50% Glycerol is possible, although these preservation techniques present some disadvantages. Since Formalin inactivates the virus, virus isolation is no longer possible and FAT as well as PCR display decreased sensitivity. Although Glycerol does not inactivate the virus, it only protects the sample from putrefaction and virus inactivation due to high temperatures still occurs and it is recommended by the OIE to store Glycerol samples in a refrigerator (OIE, 2012, Barrat, 1996). The implementation of standard diagnostic tests for rabies is also challenging for developing countries. The FAT for example needs expensive FITC anti-rabies antibodies and a fluorescence microscope for evaluation. Furthermore, evaluation should only be performed by trained staff (Duong et al., 2016, Woldehiwet, 2005, Banyard et al., 2013). Similar to FAT, RTCIT also requires a fluorescence microscope and trained staff, as well as an established cell culture system and adequate biosafety (Duong et al., 2016, Webster and Casey, 1996). Expensive equipment is also needed for RT-PCR and without appropriate standardization and quality control false positive as well as false negative results can occur (Duong et al., 2016, OIE, 2012, Notomi et al., 2000).

These problems are especially apparent in developing countries in Asia and Africa, where dog rabies still poses a major human health threat (Fooks et al., 2009, Banyard et al., 2013). Coupled with lack of awareness of the general public, weak capacity for field investigation of rabies cases, poor infrastructure, poor veterinary and health capacity as well as their insufficient cooperation, and inadequate reporting systems, the result is inadequate rabies surveillance (Townsend et al., 2013, Banyard et al., 2013). In fact, insufficient surveillance leads to an underestimation of the situation, therefore attracting little attention from policy

makers, resulting in a cycle of neglect (Cleaveland et al., 2014). Furthermore, surveillance is crucial for canine rabies elimination programs, since it provides data on the progress and cost effectiveness, enabling sustainable implementation (Townsend et al., 2013).

3.5.3. Alternative diagnostic tests

To overcome some of the challenges in rabies diagnosis, alternative test methods have been developed. The direct rapid immunohistochemical test (dRIT) was developed by the Centers for Disease Control and Prevention (CDC) as an alternative to FAT (Lembo et al., 2006). In dRIT rabies antigen is detected with biotinylated anti-N antibodies. Biotin catalyses the formation of a red precipitate which can be observed using a conventional light microscope (Coetzer et al., 2014a). The evaluation of the test with a light microscope is the biggest advantage of dRIT compared to FAT. Other advantages are easier interpretation of test results and reduced costs compared to FAT (Fooks et al., 2009, Coetzer et al., 2014a). The dRIT was evaluated in several studies and showed sensitivities between 83% and 100% depending on the antibodies used and was able to detect other lyssaviruses apart from RABV, i.e. MOKV, LBV and DUVV (Coetzer et al., 2014a, Coetzer et al., 2014b, Lembo et al., 2006, Madhusudana et al., 2012).

Novel approaches for the rapid amplification and detection of lyssavirus RNA are reverse transcription loop mediated isothermal amplification (RT-LAMP) and nucleic acid sequence based amplification (NASBA), where amplification occurs at a constant temperature, eliminating the need for a high precision thermal cycler (Notomi et al., 2000, Compton, 1991). NASBA allows direct isothermal amplification of RNA (Compton, 1991). Gene amplification occurs at a relatively low temperature of 40°C, which is advantageous for the in situ detection of viral RNA, due to less cell damage compared to RT-PCR (Sugiyama et al., 2003). RNA amplification requires three enzymes, which are the T7 RNA polymerase, the avian myeloblastosis virus reverse transcriptase and RNase H (Guatelli et al., 1990). Two studies assessed NASBA techniques for the detection of RABV RNA, with mixed results (Wacharapluesadee et al., 2011, Wacharapluesadee and Hemachudha, 2001). In rabies diagnosis, NASBA was used for the ante mortem diagnosis of rabies in humans (Wacharapluesadee and Hemachudha, 2010). Isothermal amplification of deoxyribonucleic

acid (DNA) during RT-LAMP requires a higher temperature of 65°C compared to NASBA. As, similar to RT-PCR, direct amplification of virus RNA with LAMP is not possible, a reverse transcription step has to be included. RT-LAMP displays very high specificities, which can have a negative effect on the sensitivities of the assays (Ito et al., 2014). Indeed two RT-LAMP assays developed for rabies diagnosis displayed reduced sensitivity due to sequence variation of the RABV isolates (Saitou et al., 2010, Boldbaatar et al., 2009). For the development of a RT-LAMP assay to detect RABV isolates of two African lineages, two primer sets, 12 primers in total, were needed (Hayman et al., 2011). Therefore, the development of a RT-LAMP able to detect a broad spectrum of RABV isolates is challenging.

Another test, developed as a point of care test for the detection of antigen is the lateral flow device (LFD), also called lateral flow assay, rapid immunodiagnostic test or rapid immunochromatographic strip test (O'Farrell, 2013, Kang et al., 2007, Mak et al., 2016) . The test principle is based on fluid migration along a membrane. A sample is added to the sample area of a test strip from where it migrates along the strip, the antigen in the sample reacting with conjugated and fixed antibodies, resulting in the development of lines visible to the naked eye (O'Farrell, 2013). Since the test allows a one-step analysis, it is easy to perform. Furthermore, the test can be stored at room temperature and does not need any additional equipment and chemicals. Therefore, it has potential for field use. Coupled with being rapid and cost effective it is applied in many areas, e.g. environmental science, human and animal health (Ngom et al., 2010, Posthuma-Trumpie et al., 2009). LFDs require small sample volumes. These result subsequently in small quantities of analyte, which can lower the sensitivity of the test. On the other hand, small sample volumes can be advantageous, when not much material is available, as long as sensitivities are adequate. Due to test components and manufacturing processes the tests can display variable sensitivity and test reproducibility (O'Farrell, 2013). For rabies diagnosis one commercially available LFD, produced by Bionote, has been evaluated in several studies reporting sensitivities between 88% and 100% (Table 3).

No. of samples & Sample material	No. of rabies positive samples	Lyssavirus species	Run time	Sensitivity	Specificity	Reference
44 brain, 7 saliva	29	RABV	10 min	91,7%	100,0%	(Kang et al., 2007)
25 brain	21	RABV, LBV, MOKV, DUVV	5 min	100,0%	100,0%	(Markotter et al., 2009b)
177 brain	78	RABV EBLV-1, EBLV-2, ABLV	5-10 min	>88,3%	100,0%	(Servat et al., 2012)
110 brain	20	RABV	5 min	95,0%	98,5%	(Yang et al., 2012)
115 brain	85	RABV	5-10 min	96,5%	100,0%	(Reta et al., 2013)
80 brain	32	RABV	5-10 min	96,9%	100,0%	(Voehl and Saturday, 2014)
34 brain	24	RABV	5-10 min	91,7%	100,0%	(Sharma P, 2015)
11 brain	6	RABV	5 min	85,7%	100,0%	(Ahmad and Singh, 2016)
48 brain	34	RABV	5-10 min	100,0%	100,0%	(Lechenne et al., 2016)
73 brain	43	RABV	5-10 min	95,3%	93,3%	(Lechenne et al., 2016)

Table 3: Summary of studies evaluating Bionote LFD and respective results.

4.Objectives

Assessment of EBLV-1 pathogenicity:

Five lyssavirus species circulate in Europe, with EBLV-1 being responsible for most bat rabies cases, including spill-over infections in other mammals including humans. The pathogenicity of EBLV-1 has so far only been assessed in studies using single isolates as representatives. Since these studies were all designed differently no comparison can be made between the different isolates used. As there are genetic differences between EBLV-1 isolates and it is known for other lyssaviruses that genetic variation can influence the pathogenicity, one aim of this thesis was to assess the pathogenic properties of EBLV-1 isolates with genetic differences using the same experimental design.

Enhanced passive bat rabies surveillance in Germany:

In Germany three bat lyssavirus species have been detected, which present a human health threat. To better understand the epidemiology and dynamics of bat rabies in Germany, enhanced passive bat rabies surveillance was performed until 2014. This surveillance was continued to keep up to date with the situation also with respect of the discovery of novel lyssaviruses in Germany in 2010 and Spain in 2013.

LFDs as a tool for rabies surveillance in developing countries:

Terrestrial rabies caused by RABV, especially dog-mediated rabies, is responsible for most human rabies cases. Dog-mediated human rabies is still prevalent in many regions but foremost in developing countries in Asia and Africa. To establish efficient rabies control measures, adequate rabies surveillance is required, which is difficult due to the nature of standard diagnostic tests for rabies. To circumvent this problem other test methods were developed, including LFDs. So far only one commercially available LFD has been evaluated, although several products are on the market, without anything known regarding their performance. Therefore, the performance of several commercially available LFDs was assessed.

5.Results

5.1. Comparative analysis of European bat lyssavirus 1 isolates in the mouse model

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The manuscript is presented in its published form and has therefore its own reference section. References and abbreviations from the manuscript are not included in the relevant sections at the beginning and the end of this document. The corresponding supplement material has been added directly following the reference section of this publication.

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RESEARCH ARTICLE

Comparative analysis of European bat lyssavirus 1 pathogenicity in the mouse model

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Abstract

European bat lyssavirus 1 is responsible for most bat rabies cases in Europe. Although EBLV-1 isolates display a high degree of sequence identity, different sublineages exist. In individual isolates various insertions and deletions have been identified, with unknown impact on viral replication and pathogenicity. In order to assess whether different genetic features of EBLV-1 isolates correlate with phenotypic changes, different EBLV-1 variants were compared for pathogenicity in the mouse model. Groups of three mice were infected intracranially (i.c.) with 10^2 TCID50/ml and groups of six mice were infected intramuscularly (i.m.) with 10^5 TCID50/ml and 10^2 TCID50/ml as well as intranasally (i.n.) with 10^2 TCID50/ml. Significant differences in survival following i.m. inoculation with low doses as well as i.n. inoculation were observed. Also, striking variations in incubation periods following i.c. inoculation and i.m. inoculation with high doses were seen. Hereby, the clinical picture differed between general symptoms, spasms and aggressiveness depending on the inoculation route. Immunohistochemistry of mouse brains showed that the virus distribution in the brain depended on the inoculation route. In conclusion, different EBLV-1 isolates differ in pathogenicity indicating variation which is not reflected in studies of single isolates.

Author summary

European bat lyssavirus 1 (EBLV-1) is one of fourteen officially recognized lyssavirus species causing rabies, a zoonosis resulting inevitably in death once clinical signs appear. EBLV-1 is responsible for most bat rabies cases detected in Europe, and spill-over infections in humans highlight its zoonotic potential. In our study, we compared eight genetically diverse EBLV-1 isolates in the mouse model using various routes of inoculation. Although EBLV-1 isolates displayed very high sequence conservation, significant differences in pathogenicity, i.e. in incubation periods and mouse survival, were observed. Furthermore, depending on the inoculation route the clinical picture as well as the virus

antigen distribution within the brain varied. Thus, transfer of results obtained with single isolates to the whole lyssavirus species can be misleading, and results indicating reduced pathogenicity obtained with single EBLV-1 isolates in previous studies have to be carefully interpreted.

Introduction

Rabies is an acute, progressive and incurable viral encephalitis, caused by negative strand RNA viruses of the Lyssavirus genus belonging to the order Mononegavirales, family Rhabdoviridae, which is transmitted by bites of infected mammals. Taxonomically, the etiological agents are classified into 14 officially recognized and two yet unassigned lyssavirus species [1–3]. Intriguingly, for the great majority of lyssaviruses bats (*Chiroptera*) are the reservoir leading to the assumption that bats are the true ancestral host of all lyssaviruses [4]. Hence, rabies is the most significant viral zoonosis associated with bats as almost all bat lyssaviruses have caused fatal spillovers into humans and terrestrial mammals [5–7].

Bat rabies in Europe was initially discovered in 1954 [8]. Subsequent virus characterization using monoclonal antibodies showed that the viruses isolated from bats at the time were distinct from classical rabies virus (RABV) and were assigned as European bat lyssaviruses types 1 and 2 (EBLV-1 and -2) [9, 10]. In recent years, novel lyssavirus species have been detected in European bats, namely West Caucasian bat lyssavirus (WCBV) [11], Bokeloh bat lyssavirus (BBLV) [12] and Lleida bat lyssavirus (LLBV, [3]).

European bat lyssavirus 1 (EBLV-1) is the most common of the five lyssavirus species circulating in European bats and responsible for the majority of all recorded bat rabies cases in Europe [13]. The reservoir hosts of EBLV-1 are the serotine bat (*Eptesicus serotinus*) and the Isabelline serotine bat (*Eptesicus isabellinus*) [14], but occasional cases were also found in other bat species, e.g. the brown long-eared bat (*Plecotus auritus*), the common pipistrelle (*Pipistrellus pipistrellus*), and the Nathusius' pipistrelle (*Pipistrellus nathusii*), [15] as well as in sheep [16], cats [17] and a stone marten [18]. The zoonotic potential of EBLV-1 is demonstrated by the fact that at least two confirmed human cases occurred in Russia and the Ukraine [19, 20].

The nucleoprotein (N)-gene is the most conserved gene across all lyssaviruses [21] and frequently used for phylogenic analyses since its diversity allows a good separation between lyssavirus species [22]. Based on partial N-gene sequences, EBLV-1 can be divided into two distinct sublineages EBLV-1a and EBLV-1b, the first predominantly found in Central and Eastern Europe, the second in southwest Europe [23, 24]. Recently, a third sublineage of EBLV-1 comprising isolates from the Isabelline bat on the Iberian peninsula has been proposed [14]. Genetically, EBLV-1 isolates show a very high nucleotide identity above 99% in EBLV-1a and 98% in EBLV-1b, respectively. The overall heterogeneity at nucleotide level is less than 3.3% [23].

Thus far, full genome sequences have never been assessed for genomic differences although evidence for those was found in the form of insertions and deletions (indels) in areas usually not sequenced for phylogenetic analysis. For example, a six nucleotide insertion was identified in the 3′untranslated region (UTR) of EBLV-1b isolates [25, 26] and a single nucleotide insertion in EBLV-1a isolates in the same area. Furthermore, a 35 nucleotide deletion was found in the G-L intergenic region of one EBLV-1a isolate [26]. The potential impact of these genomic differences as well as the influence of the overall genetic diversity within EBLV-1 sublineages on the pathogenicity remains elusive, since so far only single representative EBLV-1 isolates were used in pathogenicity studies. Those were usually comparative studies of different lyssavirus species or studies aimed at efficacy testing of antibodies and vaccines [27–33]. However,

differences in pathogenicity within other lyssavirus species had been observed, i.e. for Lagos bat virus (LBV) and Rabies virus (RABV) [34–36]. Against this background and the conundrum of reduced pathogenicity in experimental animal studies on one side and human casualties on the other, this study aimed at analyzing different EBLV-1 isolates representing all three sublineages to assess variability in pathogenicity.

Materials and methods

Ethics statement

All in vivo work was performed according to European guidelines on animal welfare and care according to the Federation of European Laboratory Animal Science Associations (FELASA). The characterization of lyssaviruses in the mouse model was reviewed and approved by the review board of the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei M-V (LALLF, Document-ID: AZ LALLF.M-V/TSD/7221.3–2.1-002/11).

Viruses

Ten lyssavirus isolates originating from the archive of the Friedrich-Loeffler-Institute (FLI) Riems were included in the study. Two viruses belonged to RABV and eight isolates to EBLV-1, consisting of five EBLV-1a isolates, two EBLV-1b isolates and a single EBLV-1 isolate of the proposed third sublineage, here termed EBLV-1c. Five isolates have already been described previously including EBLV-1 isolates with insertions and deletions [25, 26], a distant EBLV-1a isolate and an EBLV-1a isolate responsible for a human rabies case in Russia [19]. Properties of selected isolates are detailed in Table 1. Cell lines used in this study for viral propagation, titration, replication kinetics and serology were obtained from the Collection of Cell Lines in Veterinary Medicine (CCLV) established at FLI, Riems, Germany.

Table 1. Isolates and viruses used in the study including details of their respective characteristics, year of isolation, host and origin.

Lab ID	Name	Viral species	Characteristics	Year	Host	Origin	Accession numbers
13454	13454_EBLV-1a_ref	EBLV-1a	Isolate used for the infection of foxes and ferrets [27, 33] available as a recombinant virus [37],	2000	*Eptesicus serotinus*	Germany	LT839615
5782	5782_EBLV-1a_del	EBLV-1a	35nt deletion in G-L region [26]	2001	unknown	Germany	LT839611
5776	5776_EBLV-1a_ins	EBLV-1a	1nt (A) insertion in N-P region [26]	2001	unknown	Germany	LT839614
976	976_EBLV-1a_dist	EBLV-1a		1992	*Pipistrellus nathusii*	Germany	LT839610
13027	13027_EBLV-1a_Yuli	EBLV-1a	human rabies case (Yuli)[19]	1982	human	Russia	LT839613
20174	20174_EBLV-1b	EBLV-1b	-	2008	*Eptesicus serotinus*	Germany	LT839609
5006	5006_EBLV-1b_ins	EBLV-1b	6nt (AAAAGA) insertion in N-P region, as described before [25]	2000	*Eptesicus serotinus*	Germany	LT839612
13424	13424_EBLV-1c	EBLV-1c	-	1989	unknown	Spain	LT839608
35009	35009_RABV_CVS	RABV	fixed RABV strain, challenge virus standard (CVS), batch 1, ANSES Nancy, France	1996	-	-	LT839616
5989	5989_RABV_dog_azerb	RABV	RABV field strain, used in experimental studies [38, 39]	2002	dog	Azerbaijan	LN879480

A, Adenine;

G, Guanine;

Brain samples of mice inoculated with isolates highlighted in grey were subjected to IHC.

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Viral propagation and replication kinetics

Prior to mouse inoculation virus stocks were produced for all lyssaviruses. Viral propagation, titration and replication kinetics of the lyssavirus isolates were conducted on mouse neuroblastoma cells (Na 42/13, CCLV-RIE 0229). For virus propagation cells were infected at a multiplicity of infection (MOI) of 0.001, incubated at 37˚C and 5% CO_2 for at least for 72 hours. When 100% of the monolayer was infected supernatant virus was harvested. Depending on the isolates an additional passaging was required. After harvesting infectious virus titres were determined by endpoint titration, calculated using the Spearman-Karber method [40] and expressed as tissue culture infective dose 50 (TCID50).

Replication kinetics were determined by one step and two step growth curves. For each isolate Na 42/13 cells were infected at MOIs of 0.01 and 3, and subsequently incubated at 37˚C and 5% CO2 for 96 hours. Supernatant virus titres were determined at 0, 16, 24, 48, 72 and 96 hours post infection by endpoint titration. For each isolate two biological as well as two technical replicates were done.

Mouse inoculation and sampling

Three to four week old female Balb/c mice (Charles River, Germany) were inoculated with the selected isolates (Table 1) using three different inoculation routes and two different viral doses. While groups for intramuscular (i.m.) and intranasal (i.n.) inoculation consisted of six animals, three animals were used in positive (intracranial, i.c.) as well as in negative (mock infected) control groups. Groups were housed in individual cages and mice had access to water and food ad libitum.

For each isolate two groups of mice were inoculated i.m. into the right or left gluteal muscle using high (10^5 TCID50/30μl) and low (10^2 TCID50/30μl) viral doses. Because viral propagations of isolates 5989_RABV_dog_azerb and 20174_EBLV-1b did not yield viral titres of 10^5 TCID50/30μl, undiluted supernatant with titres of 10^4 TCID50/30μl for both isolates were used as a high dose for i.m. inoculation. Additionally, one group of mice was i.n. inoculated with 5 μl of viral suspensions (10^2 TCID50/10μl) in each nostril using a pipette. Positive and negative controls were inoculated i.c. either using 10^2 TCID50/30μl of viral suspension or 30μl of cell culture medium.

All mice were marked with earclips for identification and monitored daily for 45 days post infection (dpi). Weight and clinical scores, ranging from zero up to four, were recorded daily (see S1 Table). With onset of clinical signs mice were examined twice daily. At a clinical score of three or when the weight loss exceeded 20% mice were anaesthetized using Isoflurane and euthanized through cervical dislocation. All remaining animals were euthanized 45 days after inoculation.

Upon euthanasia, brain samples were taken from all mice. From animals inoculated with six representative isolates (Table 1) that died during the observation period half of the brain was fixed in 4% paraformaldehyde (PFA) for additional immune-histochemical analysis. Furthermore, blood was collected by heart puncture in 600μl tubes (BD Microtainer, SST Tubes), allowed to settle for at least 30 min and centrifuged for 3 min at 10,000×g. Afterwards, the serum was transferred to 1.5 ml tubes and stored at -70˚C until serological testing.

Detection of viral antigen

Brain samples were tested for the presence of lyssaviral antigen using the fluorescence antibody test (FAT) as described elsewhere [41]. In brief, brain smears were heat-fixed on slides followed by staining with a FITC-conjugated polyclonal antibody (SIFIN, Berlin, Germany) for 30 minutes. Slides were examined under a fluorescence microscope and considered positive if

green fluorescence was present. Defined positive and negative controls were included in every test run.

Furthermore, paraffin embedded brain samples of selected animals (see above, Table 1) were subject to histochemical analysis as described before [42, 43]. Briefly, after fixation in 4% PFA and embedding in paraffin wax (FFPE), samples were cut in 3μm thick paramedian sections and dewaxed, followed by immunohistochemistry (IHC) using an anti-nucleoprotein (N) polyclonal rabbit serum N161-5 [37]. The amount of viral antigen in the complete paramedian cross sections as well as in different brain regions i.e. the medulla, the cerebellum, the cortex and the olfactory bulb was semi-quantitatively analyzed using a four plus scoring system.

Serological assays

Sera were tested for the presence of virus neutralizing antibodies (VNAs) in a modified rapid fluorescence focus inhibition test (RFFIT) [44, 45] using a homologous RABV and EBLV-1 isolate as test virus and BHK21-BSR/5 (CCLV-RIE 0194/260) and BHK21-C13 (CCLV-RIE 017971113) cells, respectively. The WHO international standard immunoglobulin (2nd human rabies immunoglobulin preparation, National Institute for Standards and Control, Potters Bar, UK) adjusted to 0.5 and 1.5 international units (IU) for RABV and EBLV-1, respectively, was used as positive control [45]. A naive bovine serum was used as negative control. The potential immune response to infection was assessed qualitatively and sera were considered positive if neutralizing activity was equal or above the respective positive controls.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA) with p-values < 0.05 considered significant. Replication kinetics were analyzed by calculating the area under the curve (AUC) followed by statistical analysis using an ordinary one-way ANOVA combined with Tukey′s multiple comparison test. To infer statistical differences in survival rates the Mantel-Cox test (log-rank test) was used, while incubation periods were evaluated using the same statistical analysis as for the replication kinetics. For statistical analyses of results obtained in IHC data were stratified in respect to (i) inoculation route and (ii) the different isolates following i.m. inoculation. To this end, the Kruskal-Wallis test was applied and adjusted p-values for direct comparison of two groups were obtained using Dunn′s multiple comparison test.

Full genome sequencing

Total RNA was extracted from 2 ml cell culture supernatant using TriFast (VWR Peqlab, Erlangen, Germany) together with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and DNase (Qiagen) treatment as recommended by the supplier. The RNA was further concentrated using Agencourt RNAclean XP beads (Beckman Coulter) and used as input for the preparation of cDNA sequencing libraries as described elsewhere [46]. Sequencing was carried out on an Illumina MiSeq instrument using the MiSeq reagent kit, version 3 (Illumina, San Diego, USA) in 2x300 bp paired end mode. A combination of reference based mapping along appropriate references and de-novo assembly as implemented in the 454 software suite (version 3.0, Roche) was used to generate EBLV-1 and CVS full-genomes. These sequences were annotated in Geneious [47], version 10, http://www.geneious.com] and submitted to the European Nucleotide Archive under study number PRJEB20390 (Table 1). For sequence comparison and phylogenetic analysis, 7 full-length EBLV-1 reference sequences were aligned with sequences obtained in this study for a total number of 15 sequences, using the MAFFT plugin in Geneious. A maximum-likelihood tree was calculated from this alignment using the optimal

substitution model GTR+G and 1000 bootstrap replicates as incorporated in MEGA7 [48]. The protein coding regions were translated in amino acid sequences and screened for amino acid exchanges in known pathogenicity determining sites.

Results

Replication kinetics

All viruses grew to maximum titres ranging between $10^{6.5}$ and 10^{9} TCID50/ml with the highest titres at different time points observed for 35009_RABV_CVS for MOI 0.01 while 5006_EBLV-1b_ins had the lowest titres for MOI 3 (S1 Fig). However, the differences observed were below the level of significance.

Incubation periods

After i.c. inoculation first clinical signs within the groups appeared between five and eight dpi, while the mean incubation periods varied between five and ten days. Significant differences were observed between isolate 5776_EBLV-1a_ins with a mean incubation period 10 dpi and isolates 5782_EBLV-1a_del and 20174_EBLV-1b with mean incubation periods of 5 and 6 days respectively (p-values: 0.0067 & 0.0439) (Fig 1a).

Groups of mice inoculated i.m. with high doses of the lyssavirus isolates started to show clinical sings between 5 and 10 dpi. Mean incubation periods varied between the groups from 6 to 13 dpi with significant differences between EBLV-1 isolates (p-value: 0.0003). 5006_EBLV-1b_ins had significant longer incubation periods compared to all other EBLV-1 isolates (p-values: 0.0004–0.0433) with the exception of 5776_EBLV-1a_(ins) (p-value: 0.4443). For the latter isolate, a significant difference in the mean incubation period could be observed compared to 13027_EBLV-1a_Yuli (p-value: 0.0483, Fig 1b). Following i.m. inoculation with low doses only mice inoculated with isolates 5989_RABV_dog_azerb and 5782_EBLV-1a_del developed clinical signs after an average of 17 and 9 dpi, respectively (Fig 1c). Mean incubation periods after i.n. inoculation ranged between 8 and 10 dpi (p-value >0.05, Fig 1d).

Clinical sings

The clinical picture of mice inoculated i.c. usually included general signs like weight loss, ruffled fur, a hunched back and slowed movements. In rare occasions spasms, aggressiveness or increased activity was observed.

Following i.m. inoculation the clinical signs usually started in the inoculated limb with either spasms in mice inoculated with EBLV-1 resulting in hypermetria or a wobbly gait, or paralysis in mice inoculated with RABV and eventually included the second hind limb. At this point, ruffled fur, a hunched back or trembling were commonly observed in RABV infected animals, but only sporadically in mice inoculated with EBLV-1. Aggressive behavior was rarely observed and restricted to 5006_EBLV-1b_ins and 976_EBLV-1a_dist infected mice.

Intranasal inoculation of EBLV-1 isolates resulted in clinical signs like tameness, aggressiveness, circular movement and occasionally automutilation. Notably, of the two mice which developed clinical signs following i.n. inoculation with 5989_RABV_dog-azerb one developed tremor whereas the other did not show any clinical signs, except for progressive weight loss, eventually leading to euthanasia.

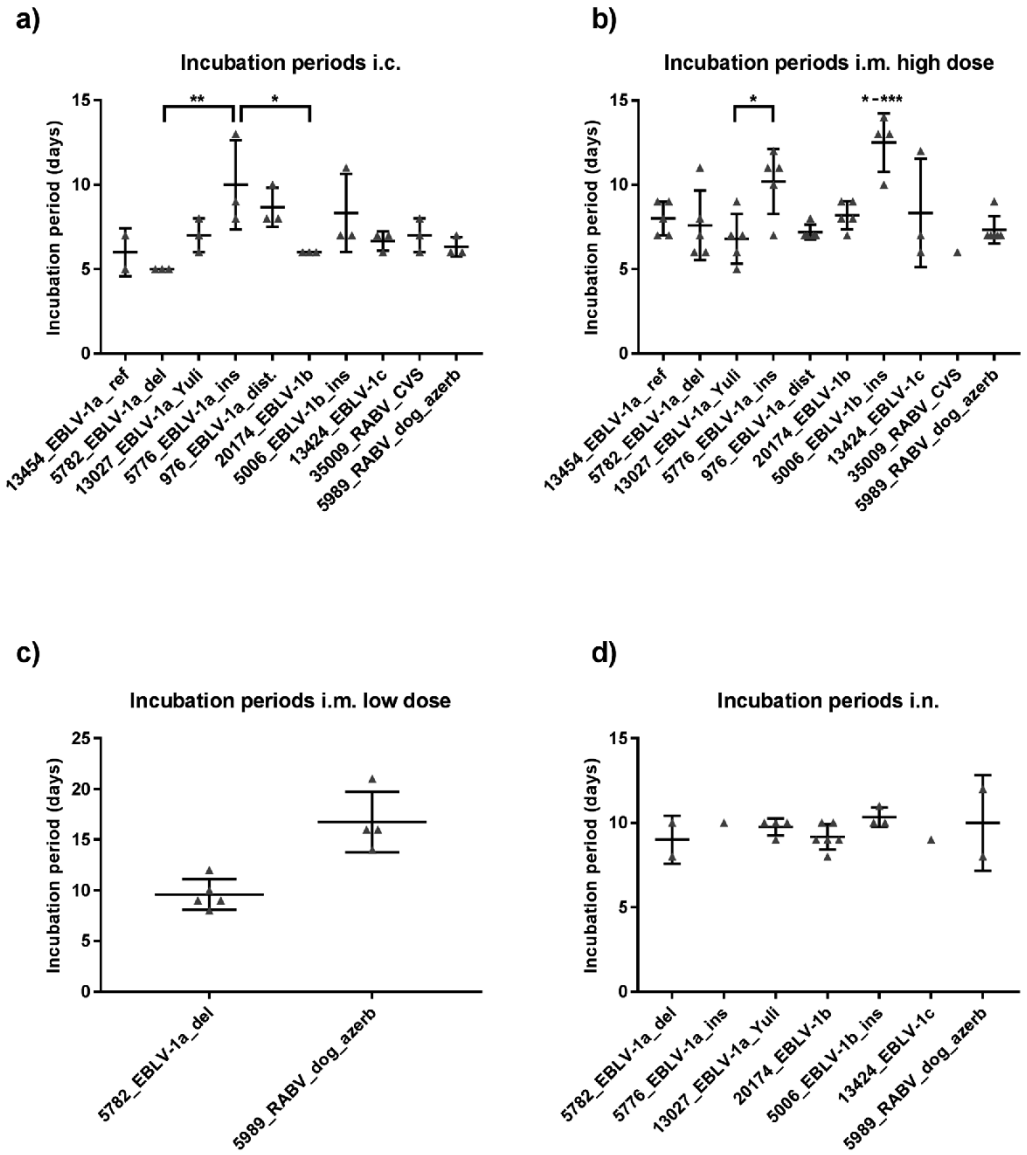

Fig 1. Incubation periods following i.c., i.m., and i.n. inoculation. Individual incubation periods of mice following a) i.c. inoculation, b) i.m. inoculation with high doses, c) i.m. inoculation with low doses and d) i.n. inoculation of the isolates (grey triangles) with n = 3 for i.c. and n = 6 for i.m. and i.n. inoculated mice. Mean and standard deviation (SD) of the isolates are indicated by the horizontal bars and whiskers, respectively. P-values, obtained using an ordinary one-way ANOVA combined with Tukey's multiple comparison test, are indicated by asterisks, with * indicating p-values ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001.

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Pathogenicity and survival

All mice inoculated i.c. with the isolates developed clinical signs and died, except for one mouse inoculated with 13454_EBLV-1a_ref (S2 Fig).

Survival among i.m. high dose infected EBLV-1 groups varied between 50% for 13424_EBLV-1c and 17% for all other EBLV-1 isolates except 5006_EBLV-1b_ins (33%) (p-values > 0.05). After RABV infection 83% (35009_RABV_CVS) and 0% (5989_RABV_dog azerb) of mice survived, respectively (p-value: 0.004). Only isolates 5989_RABV_dog_azerb and 5782_EBLV-1a_del were pathogenic following i.m. inoculation with a low dose, resulting in a significant difference in survival between the RABV isolates (p-value: 0.02) as well as between 5782_EBLV-1a_del and the other EBLV-1 isolates (p-value < 0.0001, Fig 2a–2c).

I.n. inoculation resulted in significant differences in survival within the EBLV-1a isolates and within the EBLV-1b + 1c isolates (p-value < 0.03). Compared to isolates 13454_EBLV-1a_ref and 976_EBLV-1a_dist, isolate 13027_EBLV-1a_Yuli displayed a significant lower survival (p-value: 0.0191). No mice survived following inoculation with isolate 20174_EBLV-1b which resulted in a significant difference in survival compared to isolates 5006_EBLV-1b_ins and 13424_EBLV-1c (p-value: 0.0055 & 0.0061) as well as compared to isolate 13454_EBLV-1a_ref, 976_EBLV-1a_dist (p-values: 0.0008) and isolates 5776_EBLV-1a_ins and 5782_EBLV-1a_del (p-values: 0.0024 & 0.028). Survival following inoculation with the RABV isolates was similar in both groups (p-value>0.05) (Fig 2d)

All mock infected mice did not show clinical sings and survived until the end of the observation period.

Antigen detection

All mice which were euthanized or died during the experimental stage were positive while all animals that were killed at the end of the observation period were negative using FAT. With

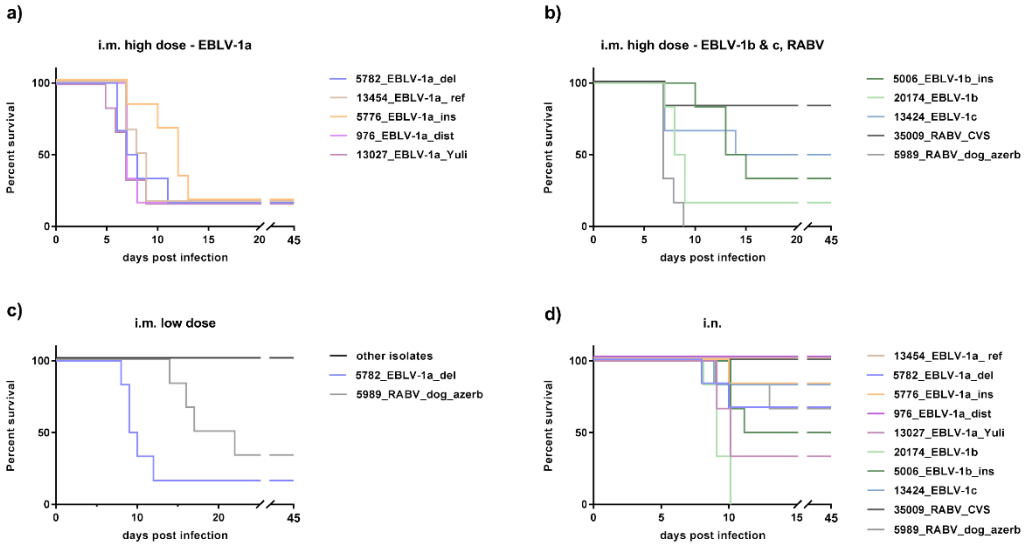

Fig 2. Kaplan-Meyer survival plots following i.m. infection with high doses (a, b), low doses (c) and i.n. infection (d). Six Balb/c mice were inoculated per group.

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IHC, the amount of antigen in the brain varied depending on the inoculation route, with a lower antigen content in the paramedian cross sections and the olfactory bulb following i.m. inoculation compared to i.c. (p-values: 0.0379 & <0.0001) and i.n. inoculation (p-values: 0.0003 & <0.0001; Fig 3a and 3b). No significant difference could be observed upon comparison of the isolates following intramuscular inoculation (p-values: >0.58).

Serology

VNAs were detected both in survivors as well as in animals that succumbed to infection after i.c and i.m inoculation. Following i.m. high dose inoculation seroconversion of the mice varied between 17% and 100%. In groups inoculated with isolates 5006_EBLV-1b_ins and 35009_RABV_CVS all mice which succumbed to disease did not seroconvert whereas all survivors did. Overall seroconversion was higher following i.m. inoculation with high doses (58% for EBLV-1 and 92% for RABV) compared to low doses (19% for EBLV-1 and 33% for RABV) (S3 Fig). Following 5989_RABV_dog_azerb i.m. low dose infection only mice which succumbed to disease seroconverted whereas the opposite was true for isolate 5782_EBLV-1a_del, where only survivors seroconverted. None of the animals inoculated i.n. as well as the mock infected control group developed VNAs.

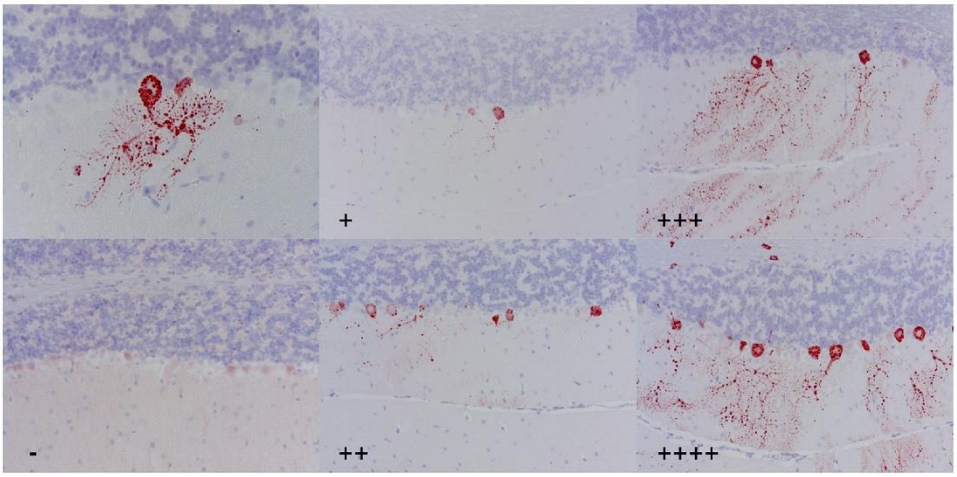
Comparison of nucleotide and amino acid sequences

Sequence analysis of the EBLV-1 full genome sequences revealed nucleotide identities within the lineages above 98.8% for EBLV-1a and above 97.4% for both EBLV-1b and EBLV-1c. Also, the heterogeneity between the groups was below 5%, as visualized in the branching pattern of the phylogenetic tree (Fig 4). For isolate 5006_EBLV-1b_ins an additional single nucleotide insertion (nt) in the G-gene UTR (position 3308) was discovered. In total the number of single nucleotide polymorphisms was 567. At amino acid (aa) level a total of 71 aa exchanges were found among the EBLV-1 isolates. Of those, 28 resulted in a change of the respective aa property (S2 Table). Differences in the aa sequences of the EBLV-1 isolates could be observed in two known pathogenicity determining sites (S3 Table). One aa exchange was in the phosphoprotein of isolate 5006_EBLV-1b_ins at position 176 where Serine was exchanged with Proline (S176P). Furthermore in the glycoprotein at position 503 of the EBLV-1a isolates a Glycine was present, whereas the EBLV-1b and 1c isolate had a Serine at this position. All isolates had a glycosylation site in the glycoprotein at position 319, while both RABV isolates had additional sites at position 37 and isolate 35009_RABV_CVS at position 204.

Discussion

Pathogenicity studies are essential e.g. to characterize individual viruses and to understand virus-host interactions. The latter studies are preferentially performed in the respective reservoir host. Unfortunately, most lyssaviruses including EBLV-1 have their reservoir in bats, with evident challenges in performing studies in those bat species. Although initial studies were performed with EBLV-2 in Daubenton's bats [49] and with EBLV-1 in the Serotine bat [50], the protected status of these animals, as well as their challenging husbandry and handling precludes using these species for comparative analyses. As an alternative, infection of mice was established as a model to study lyssavirus pathogenesis.

Most pathogenicity studies were performed using RABV [51], demonstrating differences in virus characteristics depending on the isolates used [35, 36, 51]. Comparative analyses of different lyssavirus isolates within one species were also published for LBV, where distinct differences in pathogenicity between isolates were also recorded [34]. Although some studies included EBLV-1, only single isolates were used as representatives. In those studies, various



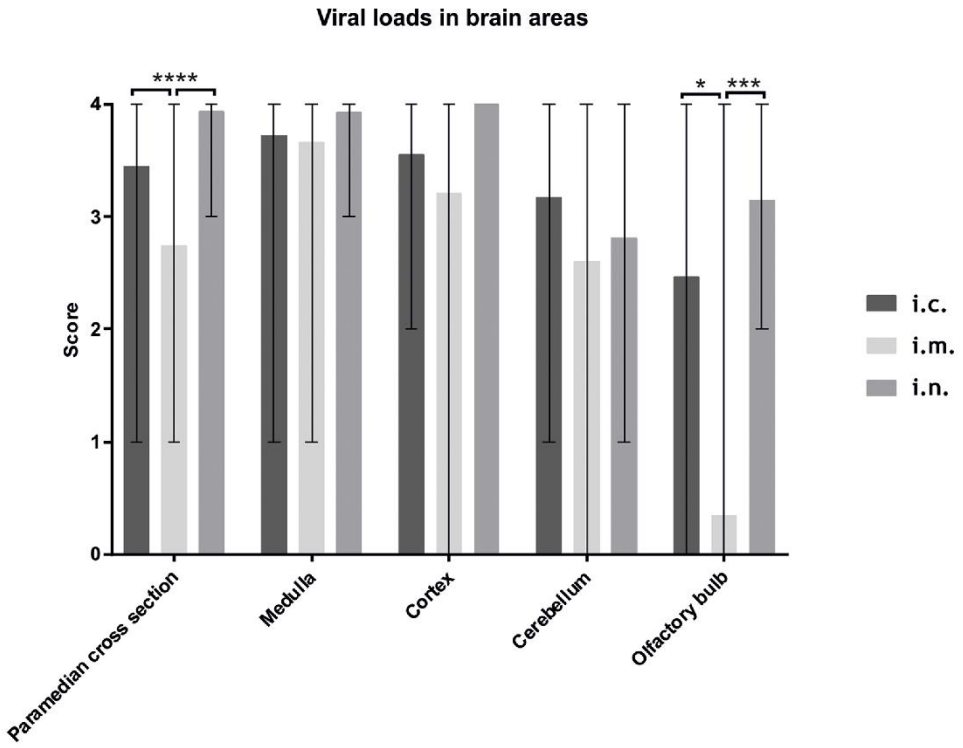


Fig 3. IHC score for the amount of lyssavirus antigen and antigen distribution in the different brain regions. a) IHC pictures of the cerebellum depicting a lyssavirus antigen positive Purkinje cell (upper right) and the score ranging from negative (-) up to four plus (++++). b) Distribution of viral antigen in the paramedian cross sections, the medulla, the cortex, the cerebellum and the olfactory bulb with respect to the inoculation route. Whiskers indicate the range of the data sets. P-values below 0.5 are indicated by asterisk, * indicating p-values ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001.

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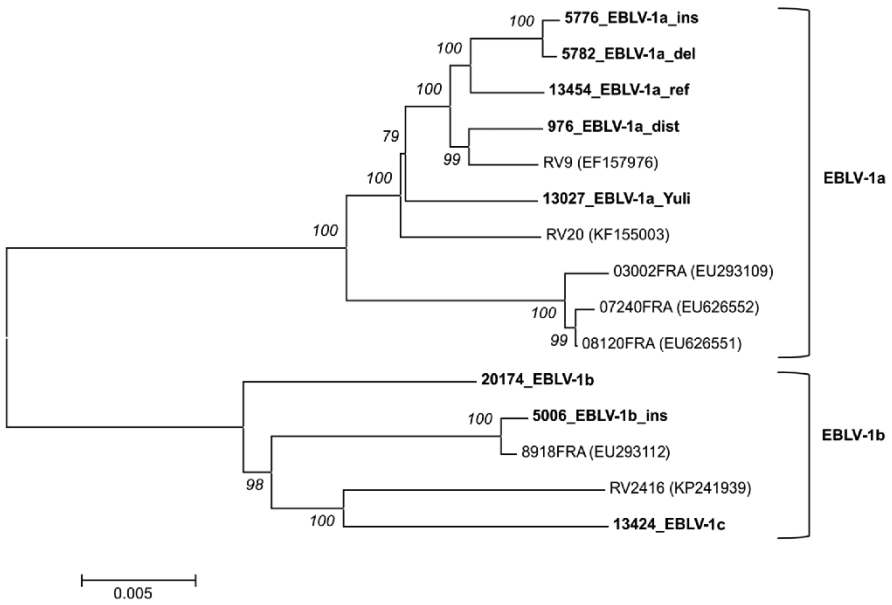

Fig 4. Phylogenetic relationship of EBLV-1 isolates inferred from all currently available full-length genome sequences. The observed phylogenetic grouping is in accordance with the classification into the distinct sublineages EBLV-1a and EBLV-1b. For EBLV-1c currently only one full-length genome sequence is available. Sequences obtained in this study are highlighted in bold type and bootstrap support values are indicated in italics.

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mouse breeds, application routes, cells for virus propagation and viral doses were used. Furthermore, titres were usually expressed as MICLD50/MLD50 (S4 Table), thus preventing direct comparison. Therefore, in this study the pathogenicity of eight EBLV-1 isolates was compared under identical conditions. All known sublineages were included as well as isolates containing insertions and deletions attempting to represent the diversity of this lyssavirus species.

We observed significant differences in the pathogenicity between the EBLV-1 isolates, with isolate 5782_EBLV-1_del displaying a higher pathogenicity following i.m. inoculation with a low dose compared to all other EBLV-1 isolates. This is remarkable, considering that the nucleotide sequence is 99.6% identical with isolate 5776_EBLV-1a_ins which was not pathogenic after i.m. low dose application. Overall, there is a high nucleotide identity among the EBLV-1 isolates and the only distinctive feature of 5782_EBLV-1a_del on nucleotide level is the 35nt deletion in the pseudogene region as described before [26]. On protein level no aa exchange in isolate 5782_EBLV-1a_del could be observed which would explain this increase in pathogenicity compared to the other EBLV-1 isolates (S3 and S4 Tables).

While deletions or insertions in the pseudogene region of fixed RABV strains did not change their pathogenicity after intracranial inoculation [52, 53], experimental studies using chimeric viruses revealed that the pseudogene contributes to neuroinvasiveness after peripheral infection [54]. Another possible reason for this difference could be an aa exchange in a so far unknown pathogenicity determining site of this EBLV-1 isolate. In order to identify and verify responsible differences further studies using reverse-genetics are warranted.

Following i.n. inoculation, interestingly, the majority of EBLV-1 isolates displayed a higher pathogenicity compared to i.m. low-dose inoculation, although the same viral dose was used.

Within i.n. inoculated mice significant differences were observed, with survival rates of the isolates varying between 0% and 100% (Fig 2d). However, there was no correlation with the pathogenicity following i.m. inoculation, as here most EBLV-1 isolates were similar pathogenic. Isolate 5782_EBLV-1a_del which was highly pathogenic following i.m. inoculation with a low dose, was less pathogenic following i.n. inoculation compared to isolate 20174_EBLV-1b. Even isolates 20174_EBLV-1b and 5006_EBLV-1b_ins with a high identity on nucleotide level of 97.8% had a difference in mortality of 50%. It is unclear why certain viruses are pathogenic via i.n. inoculation while others seem to be apathogenic.

Previous studies investigating intranasal or aerosol infection used the fixed RABV strain CVS and EBLV-2 with different results regarding pathogenicity [55–58]. But although these results seemed to sometimes contradict each other, it has to be noted that the studies were all designed differently. In fact, one study showed that the pathogenicity following i.n. infection depends on a variety of factors, for example the amount and exact administration of the inoculum [59]. In our study the pathogenicity of different isolates of the same lyssavirus species were investigated under the same conditions and differences in pathogenicity were still observed. Intranasal inoculation is an artificial infection route, even though it has been proposed for transmission of lyssaviruses in bats [60], but was never proven to happen under natural conditions [61].

Aside from differences in pathogenicity, significantly longer incubation periods were observed following i.m. inoculation with two particular isolates when high doses were used (Fig 1b). Interestingly, both isolates have particular genetic characteristics, i.e. insertion in the NP-region. The longer incubation period of isolate 5006_EBLV-1b_ins also correlated with slow replication and low titres in one step replication kinetics. Whether this is due to the AA exchange S176P in the phospoprotein (S3 and S4 Tables) which might have an influence on interferon antagonism [62] or the observed six nucleotide insertion (AAAAGA) in the N-gene UTR is debatable (25). It needs to be clarified whether the insertion in front of the transcription termination signal (TTS) has an influence on termination and downstream transcription. In any case, this insertion seems to have no disadvantage for virus transmission and host maintenance under natural conditions as this insertion has been found in several subsequent EBLV-1b isolates from France, Germany [25, 26] and the Netherlands (B.Kooi, pers. Communication). Generally, 7As in the TTS are considered optimal for termination of transcription [63]. The single A insertion in Isolate 5776_EBLV-1a-ins leading to an 8A TTS affects transcription termination at the N and P gene border.

The incubation periods between the different inoculation routes and doses varied for the same isolates. In several cases the incubation periods following i.m. inoculation with high doses were even shorter than following i.c. inoculation. This is interesting since after i.m. inoculation the virus needs to travel from hind limb to the central nervous system in order to reach its main replication site. An explanation may be the dose of infection, whereby a thousandfold higher dose was used for i.m. compared to i.c. inoculation. This is corroborated by the fact that incubation periods for 5989_RABV_dog_azerb and 5782_EBLV-1a_del following i.m. inoculation with low doses were significantly longer compared to i.c. inoculation (p-values: 0.0021 and 0.0023, resp., Fig 2a, 2b and 2c).

Clinical signs depended on the inoculation route as well as on the virus species. As for the latter, mice inoculated i.m. with the RABV isolates showed signs like ruffled fur, hunched back, slowed movements, tremor and paralysis whereas EBLV-1 infected mice displayed agitation and spasms. A difference between RABV and EBLV-1 had been described before [28], although here some RABV infected mice also exhibited sings of furious rabies.

Mice inoculated i.c. usually developed general clinical signs, whereas mice inoculated i.m. showed spasms and mice inoculated i.n. displayed aggressive behavior and circular movement.

This is likely a result of the specific virus distribution in peripheral neurons and brain following the different routes of infection. Indeed, IHC analyses revealed that after i.n. inoculation antigen was more prevalent in the olfactory bulb and the overall brain, compared to i.m. inoculated mice. As for the olfactory bulb, it is comprehensible that there is more antigen present following i.n. compared to i.m. inoculation as virus has to travel through this brain section in order to infect the other parts of the brain. However, this does not explain the similar amount of antigen present in the medulla for both inoculation routes. Perhaps, clinical signs appear at a later stage of infection after i.n. infection when the virus is already present in most parts of the brain compared to i.m. infection. This would also explain the overall higher antigen content following i.n. infection compared to i.m. infection.

Following i.n. infection the fact that none of the mice seroconverted is not surprising since it was shown for RABV that sensory neurons in the olfactory mucosa are infected and the virus travels directly to the olfactory bulb [64], obviously without measurable interaction with the immune system. In contrast, a larger percentage of mice seroconverted following i.m. inoculation with high doses compared to low dose inoculation. Generally, there was no correlation between the serological response and the outcome of infection. In survivors that did not seroconvert, it is likely that the innate immune response was able to clear the virus without triggering an adaptive immune response (S3 Fig).

Full genome sequences were generated for all viruses used in this study and thus the number of available sequences more than doubled. Sequence analyses confirmed previously discovered unique indels in the selected EBLV-1 isolates. Additionally, NGS provided evidence for the presence of another single nt insertion in the G-UTR of isolate 5006_EBLV-1b_ins. The reported high sequence identity within EBLV-1 of >96.7% based on partial N-gene sequences [23, 24] was confirmed on full genome sequence level with the isolates from this study having an identity of 95.2% or higher.

On protein level, only two aa exchanges were found in known pathogenicity determining sites (S3 and S4 Tables). Their potential effect and the effect of the other 69 observed aa exchanges particularly those that resulted in a change of the respective aa property on the results of this study is difficult to assess. Against the background that most pathogenicity markers were determined using attenuated RABV strains, this needs further investigation.

The glycosylation site at position 319 in the glycoprotein was found in all isolates used in this study and is known to be conserved in at least seven lyssavirus species [30]. One additional glycosylation site was found at position 37 in the RABV isolates and a third glycosylation site is present in the isolate 35009_RABV_CVS at position 204. Wildtype RABV strains have usually two glycosylation sites but can acquire more during cell culture passage resulting in up to four glycosylation sites in certain fixed RABV strains [65]. Fixed RABV strains have certain advantages compared to wildtype viruses, e.g. fixed clinical picture, incubation periods and mortality rates [66]. Unfortunately, fixed virus strains are often attenuated following peripheral inoculation compared to wildtype viruses likely due to cell culture or host adaptation [67, 68]. This may explain why the fixed RABV strain 35009_RABV_CVS was highly attenuated compared to a wildtype isolate 5989_RABV_dog_azerb in this study (Fig 2b and 2c).

In order to generate virus for inoculation, all viruses used in our study had to be passaged in cell culture offering the possibility for adaption. However, none of the sequences derived from passaged material indicated nucleotide exchanges compared to previously generated partial sequences of the primary isolate. Furthermore, indels have not been described as result of cell-culture adaptation of lyssaviruses. Functionally, isolate 13027_EBLV-1_Yuli, which had the longest passage history of 11 passages on MNA cells, was still highly pathogenic following peripheral inoculation (Fig 2a).

Conclusion

Although EBLV-1 isolates display very high sequence conservation, significant differences in pathogenicity as well as in incubation periods were found. Thus, transfer of results obtained with single isolates to the whole lyssavirus species can be misleading. The cause of these differences can only be speculated, as data concerning pathogenicity determinants, especially for EBLV-1, are insufficient. Here further studies using reverse genetics are warranted to confirm the role of indels as well as SNPs. To this end, isolate 13454_EBLV-1a_ref already available as recombinant virus [37], was included in the study. Retrospectively, results indicating reduced pathogenicity obtained with EBLV-1 isolates in previous studies have to be carefully interpreted. Thus, the results emphasize the need for proper post-exposure prophylaxis in case of any severe exposure to the reservoir hosts of lyssaviruses.

Supporting information

S1 Fig. a) two step and b) one step replication kinetics of the isolates used in the study.
(PDF)

S2 Fig. Survival curves of the mice following i.c. inoculation.
(PDF)

S3 Fig. Percentage seroconversion for the different inoculation routes following inoculation a) with EBLV-1 isolates and b) with RABV isolates. Percentage of seroconverted mice for the individual isolates can be seen following i.m. inoculation with c) high doses and d) low doses.
(PDF)

S1 Table. Clinical score sheet of the mice, ranging from zero up to four.
(PDF)

S2 Table. The 28 potentially significant amino acid exchanges in the proteins of the EBLV-1 isolates and their frequency in other EBLV-1 as well as in RABV isolates.
(PDF)

S3 Table. Summary of known pathogenicity determining sites and the protein sequences found in the RABV as well as in the EBLV-1 isolates used in this study.
(PDF)

S4 Table. Summary of previous pathogenicity studies with EBLV-1 as well as details to their experimental design. 13454* is identical to 13454_EBLV-1a_ref used in this study.
(PDF)

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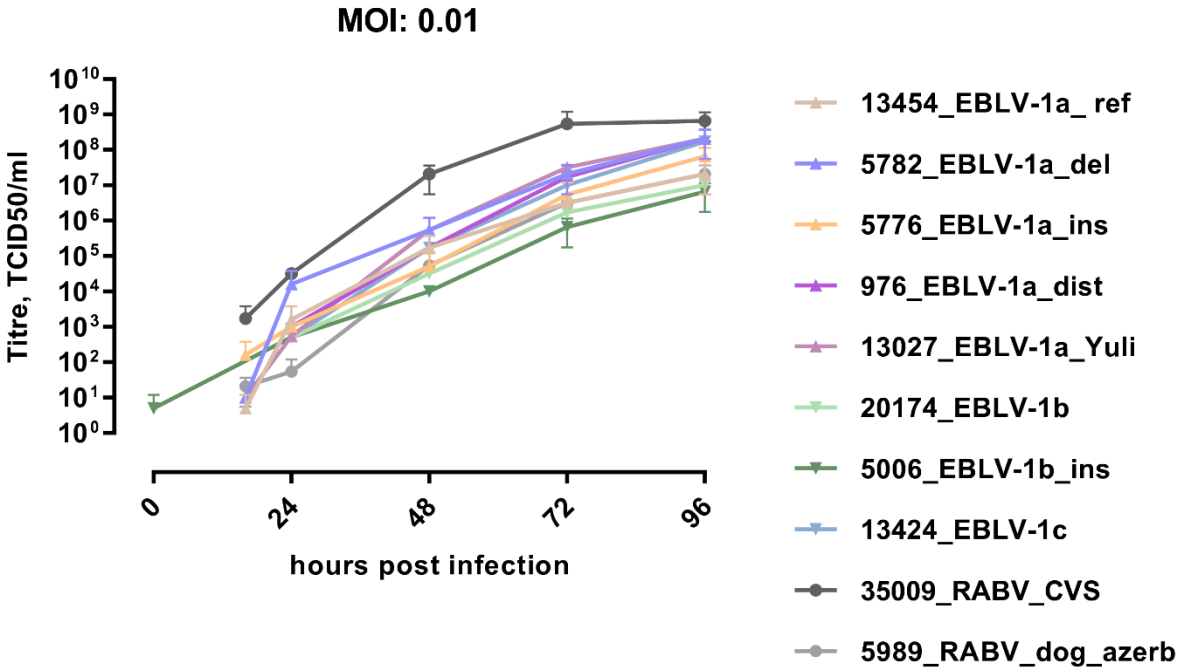
Supplementary Table 1: Clinical score sheet of the mice, ranging from zero up to four.

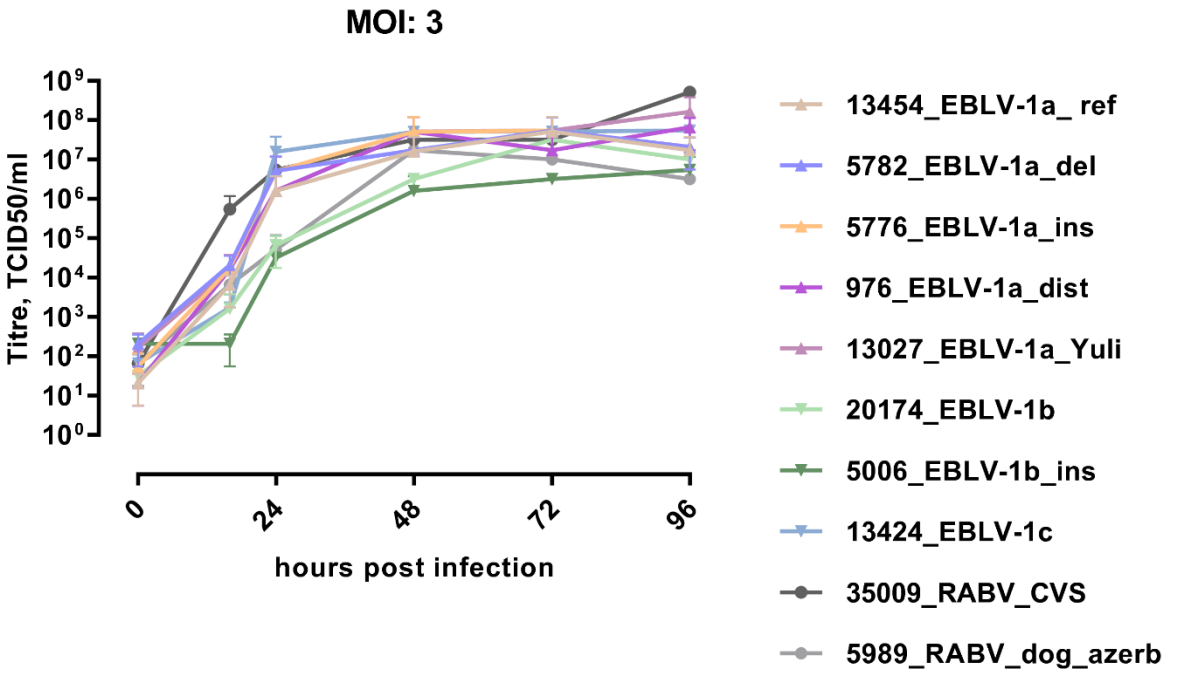
Score	Symptoms
0	• healthy
1	• ruffled fur • hunched back • hypermetria in inoculated limb • wobbly gait in inoculated limb • calm
2	• ruffled fur • hunched back • slow movements • wobbly gait both hind limbs • jumpy • tame
3	• paralysis or spasms in hind limbs • agressiveness • biting of objects and other mice • automutilation • circular movements
4	• death

Supplementary Figure 1: a) two step and b) one step replication kinetics of the isolates used in the study.

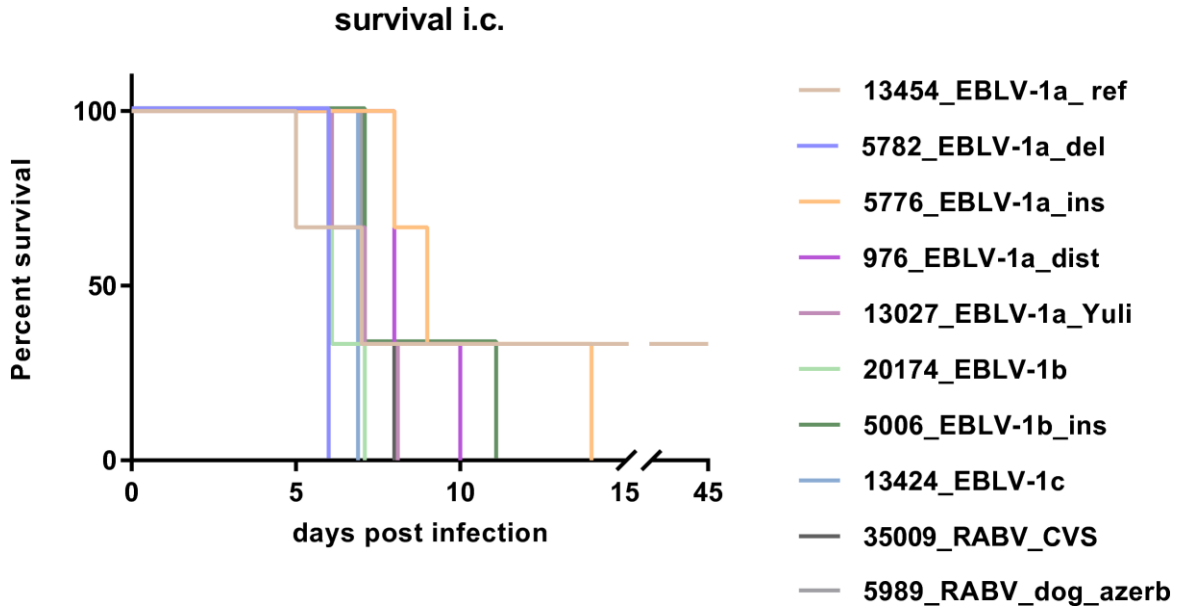
a)



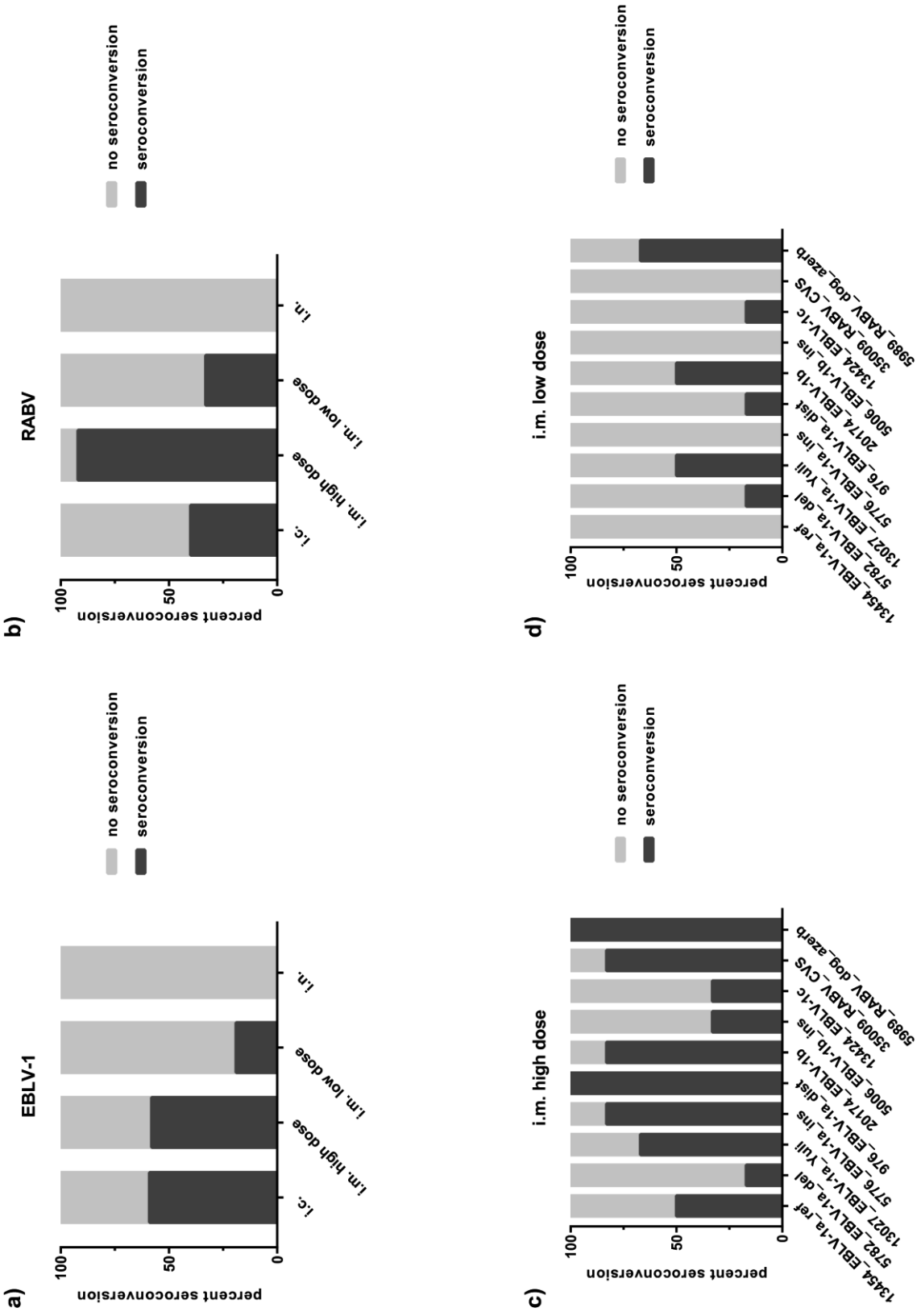
b)



Supplementary Figure 2: Survival curves of the mice following i.c. inoculation.



Supplementary Figure 3: Percentage seroconversion for the different inoculation routes following inoculation a) with EBLV-1 isolates and b) with RABV isolates. Percentage of seroconverted mice for the individual isolates can be seen following i.m. inoculation with c) high doses and d) low doses.



Supplementary Table 2: The 28 potentially significant amino acid exchanges in the proteins of the EBLV-1 isolates and their frequency in other EBLV-1 as well as in RABV isolates.

Gene	N-gene			P-gene							M-gene					
Position	90	112		61	140	141	156	157	176	29	56	85	121	140	163	
13454_EBLV-1a_ref	T	H		Y	L	Q	T	A	S	D	H	E	I	Q	H	
5782_EBLV-1a_del	.	.		.	.	.	A	.	.	.	N	G	.	.	.	
5776_EBLV-1a_ins	.	.		.	.	.	A	.	.	.	N	.	.	.	.	
976_EBLV-1a_dist	.	.		.	.	.	A	.	.	.	N	G	.	K	.	
13027_EBLV-1a_Yuli	.	.		.	.	.	A	.	.	Y	N	G	R	.	.	
20174_EBLV-1b	.	N		.	P	H	A	T	.	.	N	G	.	.	.	
5006_EBLV-1b_ins	.	N		C	P	H	A	T	P	.	N	G	.	.	.	
13424_EBLV-1c	I	N		.	P	H	A	T	.	.	N	G	.	.	P	
Other EBLV-1 isolates	I: 0%	N:45.1%; S:1.2%		C: 0%	P: 28.6%	H: 28.6%	T: 42.9%	T: 28.6%	P: 0%	Y: 0%	H: 0%	E: 0%	R: 0%	K: 0%	P: 0%	
Number of AA sequences (EBLV-1)	82 sequences			7 sequences						9 sequences						
RABV-isolates	T: 91.8%; N:3.9%; I:2.8%; V:1.3%; A:0.2%	K:82.9; R:16.4; M:0.4; N&Q: 0.1%		G: 50.6%; E: 21.3%; K: 17.2%; R: 4.7%; D: 4.5%; T: 1.2%; A: 0.2%	S: 78%; P: 20.1%; V: 1.2%; F: 0.5%; A: 0.2%	S: 80.1%; L: 18.5%; T: 1.2%; V: 0.2%	E: 97.7%; D: 0.5%; K: 0.2%;	T: 90.6%; A: 5.0%; P: 1.6%; V,S: 0.5%; M: 0.2%	A: 99.1%; V: 0.5%; T: 0.3%	D: 100%	N: 99.6% K, S: 0.2%	G: 96.9%; W: 3.1%	I: 99.0%; V:1.0%	Q: 100%	H: 100%	
Number of AA sequences (RABV)	2557 sequences			576 sequences						520 sequences						

Gene	G-gene									L-gene						
Position	-1	23	80	167	256	336	496	501	132	860	1075	1786	2097	2101
13454_EBLV-1a_ref	A	S	T	I	Q	K	S	A	K	Y	H	A	Q	K
5782_EBLV-1a_del	.	R	.	.	.	.	L	.	.	.	.	.	.	.
5776_EBLV-1a_ins	.	.	.	.	.	.	.	.	.	.	.	.	.	.
976_EBLV-1a_dist	.	.	I	.	.	.	.	.	.	.	.	.	.	.
13027_EBLV-1a_Yuli	.	.	.	.	.	.	.	.	N	.	.	.	.	.
20174_EBLV-1b	G	.	.	.	.	T	.	T	.	F	.	T	K	.
5006_EBLV-1b_ins	G	.	.	T	.	T	.	T	.	F	.	T	K	Q
13424_EBLV-1c	G	T	.	.	R	T	.	T	.	F	N	T	K	.
Other EBLV-1 isolates	G: 39.3%	R: 5.4%, T:0%	I: 0%	T: 3.6%	R: 0%	T: 39.3%	L: 0%	T: 39.3%, V: 1.8%	N: 0%	F: 28.6%	N: 0%	T: 28.6%	K: 28.6%	Q: 14.3%
Number of AA sequences (EBLV-1)	56 sequences								7 sequences					
RABV-isolates	G: 99.9%; W: 0.1%	S: 99.4%; R: 0.2%; N,G: 0.1%	T: 100%	T: 99.2%; A: 0.6%; I: 0.1%	Q: 96.8%; R; 2.8%; K: 0.4%	N: 93.2%; D: 3.8%; S: 2.9%; G: 0.1%	S: 82.9%; L: 14.4%; Y: 2.4%; V: 0.1%	G: 85.7%; S: 13.1%; A,D: 0.4%; N: 0.2%; C: 0.1%	K: 85.1%; R: 14.3%; N: 0.6%	F: 99.4%; Y: 0.3%; X: 0.3%	H: 99.4%; X: 0.6%	Q: 85.7%; K: 11.0%; R: 3.0%; H: 0.3%	G: 73.5%; R: 20.4%; K: 6.1%	R: 60.4%; K: 39.6%
Number of AA sequences (RABV)	2264 sequences								327 sequences (RABV-Alignment +1AA)					

Supplementary Table 3: Summary of known pathogenicity determining sites and the protein sequences found in the RABV as well as in the EBLV-1 isolates used in this study.

	Nucleoprotein		Phosphoprotein			Matrixprotein			
Reference	(Masatani, Ito et al. 2011)	(Kgaladi, Wright et al. 2013)	(Kgaladi, Wright et al. 2013)	(Rieder, Brzozka et al. 2011)	(Brzozka, Finke et al. 2006)	(Wirblich, Tan et al. 2008)	(Kgaladi, Wright et al. 2013)	(Gholami, Kassis et al. 2008)	(Mita, Shimizu et al. 2008)
position	AA273	AA394	AA 144-148	AA176-186	AA289-298	AA35-38	AA77	AA81	AA95
13454_EBLV-1a_ref	F	F	KSTQT	SQESSGPPGLD	QDDLNRYLAY	PPEY	S	G	V
5782_EBLV-1a_del	F	F	KSTQT	SQESSGPPGLD	QDDLNRYLAY	PPEY	S	G	V
5776_EBLV-1a_ins	F	F	KSTQT	SQESSGPPGLD	QDDLNRYLAY	PPEY	S	G	V
976_EBLV-1a_dist	F	F	KSTQT	SQESSGPPGLD	QDDLNRYLAY	PPEY	S	G	V
13027_EBLV-1a_Yuli	F	F	KSTQT	SQESSGPPGLD	QDDLNRYLAY	PPEY	S	G	V
20174_EBLV-1b	F	F	KSTQT	SQESSGPPGLD	QDDLNRYLAY	PPEY	S	G	V
5006_EBLV-1b_ins	F	F	KSTQT	PQESSGPPGLD	QDDLNRYLAY	PPEY	S	G	V
13424_EBLV-1c	F	F	KSTQT	SQESSGPPGLD	QDDLNRYLAY	PPEY	S	G	V
35009_RABV_CVS	F	Y	KSTQT	AQVAPGPPALE	QDDLNRYTSC	PPEY	R	E	V
5989_RABV_dog_azerb	F	Y	KSTQT	AQAASGPPALE	QDDLNRYASC	PPEY	R	E	V

	Glycoprotein								
Reference	(Faber, Faber et al. 2005)	(Lentz, Wilson et al. 1984)	(Takayama-Ito, Ito et al. 2006)	(Kgaladi, Wright et al. 2013)	(Kgaladi, Wright et al. 2013)	(Langevin and Tuffereau 2002)	(Kgaladi, Wright et al. 2013)	(Tuffereau, Leblois et al. 1989)	(Prehaud, Wolff et al. 2010)
position	AA 194	AA198	AA242	AA255	AA268	AA318	AA352	AA330-333	AA502-506
13454_EBLV-1a_ref	T	K	S	D	I	I	H	KSVR	TGES
5782_EBLV-1a_del	T	K	S	D	I	I	H	KSVR	TGES
5776_EBLV-1a_ins	T	K	S	D	I	I	H	KSVR	TGES
976_EBLV-1a_dist	T	K	S	D	I	I	H	KSVR	TGES
13027_EBLV-1a_Yuli	T	K	S	D	I	I	H	KSVR	TGES
20174_EBLV-1b	T	K	S	D	I	I	H	KSVR	TSES
5006_EBLV-1b_ins	T	K	S	D	I	I	H	KSVR	TSES
13424_EBLV-1c	T	K	S	D	I	I	H	KSVR	TSES
35009_RABV_CVS	N	K	A	D	I	F	H	KSVR	EIRL
5989_RABV_dog_azerb	N	K	A	D	I	F	H	KSVR	ETRL

	Large protein				
	(Tian, Luo et al. 2015)				
	Reference position	AA1685	AA1797	AA1829	AA1867
13454_EBLV-1a_ref		K	D	K	E
5782_EBLV-1a_del		K	D	K	E
5776_EBLV-1a_ins		K	D	K	E
976_EBLV-1a_dist		K	D	K	E
13027_EBLV-1a_Yuli		K	D	K	E
20174_EBLV-1b		K	D	K	E
5006_EBLV-1b_ins		K	D	K	E
13424_EBLV-1c		K	D	K	E
35009_RABV_CVS		K	D	K	E
5989_RABV_dog_azerb		K	D	K	E

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Supplementary Table 4: Summary of previous pathogenicity studies with EBLV-1 as well as details to their experimental design. 13454* is identical to 13454_EBLV-1a_ref used in this study.

Isolate	cells for virus propagation	Mice	Age of mice	Number per group	Inoculation dose	Inoculation route	Incubation time (days)	Mortality	Other	Reference
13454*	MNA	CD1-mice	-	5	10^4,5 FFU	i.m., M. glutaeus	8-9	100%	-	(Vos, Müller et al. 2004)
13454*	MNA	CD1-mice	-	5	10^2,5 FFU	i.m., M. glutaeus	11-15	60%	-	(Vos, Müller et al. 2004)
Stade isolate	BSR	OF1 Swiss mice	4 weeks	8-9	10^7 PFU	i.m., hind leg	7-14	>75%	-	(Montano-Hirose, Lafage et al. 1993)
RV20 (bat denmark)	BHK	RIII inbred albino	3-4 weeks	5	25 MLD50	i.c./ f.p.	-	100%/ 40%	10^6 TCID50 (i.c. 10^4.5 MLD50, f.p. 10^2.9 MLD50)	(Brookes, Parsons et al. 2005)
RV20 (bat denmark)	BHK	RIII inbred albino	3-4 weeks	5	2.5 MLD50	i.c./ f.p.	-	100%/ 20%	10^6 TCID50 (i.c. 10^4.5 MLD50, f.p. 10^2.9 MLD50)	(Brookes, Parsons et al. 2005)
RV20 (bat denmark)	BHK	RIII inbred albino	3-4 weeks	5	0.25 MLD50	i.c./ f.p.	-	60%/ 0%	10^6 TCID50 (i.c. 10^4.5 MLD50, f.p. 10^2.9 MLD50)	(Brookes, Parsons et al. 2005)
RV1423	-	CD1 mice	5 weeks	25	8*10^2 infectious virus particles	f.p.	-	32%	-	(Hicks, Nunez et al. 2013)
RV1423	-	OF1 Swiss mice	5 weeks	7	5 MLD50	f.p.	8-11	100%	-	(Healy, Brookes et al. 2013)
RV1423	-	OF1 Swiss mice	6 weeks	13	5 MLD50	f.p.	7-13	100%	-	(Hicks, Nuncz et al. 2009)
EBL1Fra	-	BALB/c and C3H	6-8 weeks	8	10^5 MICLD50	i.m. tigh	13-17	100%	-	(Badrane, Bahloul et al. 2001)
-	Neuro-2a, BHK21-C13	BALB/c	7-8 weeks	-	3x10^7 MICLD50	i.m. hind leg	9-13 (surv. time)	100%	-	(Perrin, DeFranco et al. 1996)

FFU: Foci forming units, PFU: Plaque forming units; MLD50: Mouse lethal dose 50; MICLD50: Mouse intracerebral lethal dose 50; i.m.: intramuscular; f.p.: footpad;

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5.2. The recently discovered Bokeloh bat lyssavirus – Insights into its genetic heterogeneity and spatial distribution in Europe and the population genetics of its primary host

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The manuscript is presented in the form accepted for publication and has its own content and reference sections. References and abbreviations from the manuscript are not included in the relevant sections at the beginning and the end of this document.

Figures and tables are numbered individually within the manuscript and correspond to the published form.

Contents

Abstract

In 2010, a novel lyssavirus named Bokeloh bat lyssavirus (BBLV) was isolated from a Natterer's bat (*Myotis nattereri*) in Germany. Two further viruses were isolated in the same country and in France in recent years, all from the same bat species and all found in moribund or dead bats. Here we report the description and the full-length genome sequence of five additional BBLV isolates from Germany (n=4) and France (n=1). Interestingly, all of them were isolated from the Natterer's bat, except one from Germany, which was found in a common Pipistrelle bat (*Pipistrellus pipistrellus*), a widespread and abundant bat species in Europe. The latter represents the first case of transmission of BBLV to another bat species. Phylogenetic analysis clearly demonstrated the presence of two different lineages among this lyssavirus species: lineages A and B. The spatial distribution of these two lineages remains puzzling, as both of them comprised isolates from France and Germany; although clustering of isolates was observed on a regional scale, especially in Germany. Phylogenetic analysis based on the mitochondrial cytochrome *b* (CYTB) gene from positive Natterer's bat did not suggest a circulation of the respective BBLV sublineages in specific Natterer's bat subspecies, as all of them were shown to belong to the *Myotis nattereri* sensu stricto clade/subspecies and were closely related (German and French positive bats). At the bat host level, we demonstrated that the distribution of BBLV at the late stage of the disease seems large and massive, as viral RNA was detected in many different organs.

1. Introduction

1.1. Rabies and bats

Rabies is an acute, progressive incurable viral encephalitis that is transmitted following bites or scratches by infected mammals. Whilst the name rabies is derived from the Latin name for ‘madness’, the old Greeks derived the word lyssa, from lud or "violent"; this root is used in the name of the genus *Lyssavirus* (Jackson, 2013). Lyssaviruses, the causative agents of rabies, are negative single strand RNA viruses of the *Rhabdoviridae* family, and alongside with the *Bornaviridae*, *Filoviridae*, *Paramyxoviridae*, *Pneumoviridae*, *Mymonaviridae*, *Nyamiviridae* and *Sunviridae* forms the order Mononegavirales (Afonso et al., 2016). They are currently classified into 14 officially recognized (ICTV, 2015) and two yet unassigned species (Arechiga Ceballos et al., 2013; Gunawardena et al., 2016). All lyssaviruses appear or are suspected to follow a rabies like pathogenesis (Davis et al., 2015). With the advent of novel molecular techniques, the *Rhabdoviridae* family was extended (Dietzgen et al., 2017). In this chapter we summarize the current situation in the genus *Lyssavirus* and with Bokeloh bat lyssavirus (BBLV) provide a recent example how the genus diversifies.

While rabies caused by the archetypical rabies virus (RABV, belonging to Rabies lyssavirus species) has been known for millennia (Jackson, 2013), causing tens of thousands of human deaths per year (Hampson et al., 2015), the association of rabies with bats (*Chiroptera*) was first demonstrated in the New World in the early 20th Century (Pawan, 1936). Since the appearance of first descriptions of RABV in bats in the Americas, other divergent lyssavirus species have been detected in a wide range of chiropteran hosts, and bats are the recognized reservoirs for the majority of lyssaviruses (Banyard et al., 2013). Furthermore, there is strong ecological as well as phylogenetic support that bat derived viruses have evolved long before those RABV of terrestrial carnivore origin (Badrane and Tordo, 2001; Troupin et al., 2016). However, the evolutionary history of lyssaviruses within bats is unresolved. For instance, the absence of non-RABV lyssaviruses in bats in the Americas, and the diversity of bat lyssaviruses elsewhere in the Old World remain enigmatic and may indicate an ancient co-evolution and dispersal of lyssaviruses and their reservoir hosts, i.e. primarily bats (Rupprecht et al., 2017).

1.2. Bat rabies in the Americas

Rabies in bats in the Americas is in some aspects different to the Old World. As introduced before, the sole causative agents are exclusively variants of classical RABV. However, rabies virus is found in many species of bat in the Americas (Banyard et al., 2013; Constantine, 2009; Sodre et al., 2010), and it was demonstrated that particular bat species are associated with specific RABV variants (Hughes et al., 2005; Streicker et al., 2010; Velasco-Villa et al., 2006). Among bats, cross-species transmissions (CSTs) seem to be related to the relative genetic distance of the host (Streicker et al., 2010). Also, bat RABV variants caused a historical sustained CST in raccoons (Velasco-Villa et al., 2008), and more recent CST in striped skunks (*Mephitis mephitis*), red foxes (*Vulpes vulpes*) and gray foxes (*Urocyon cinereoargenteus*) (Daoust et al., 1996; Kuzmin et al., 2012; Leslie et al., 2006). A bat rabies variant was also responsible for rabies cases in white-nosed coati (*Nasua narica*) (Arechiga-Ceballos et al., 2010).

In Latin America, besides insectivorous and frugivorous bat species, the common vampire bat (*Desmodus rotundus*), one of three blood-feeding or hematophagous bats found exclusively in this region, acts as a reservoir for rabies (Johnson et al., 2014). This behaviour facilitates virus transmission not only to livestock, particularly cattle (Arellano-Sota, 1988; Johnson et al., 2014), but also causes human cases, particularly in remote areas with limited access to medical intervention (Condori-Condori et al., 2013; Meynard et al., 2012; Stoner-Duncan et al., 2014; Streicker et al., 2012).

1.3. Bat rabies in Asia and Australia

Rabies in bats in the Asian part of Russia were described already in the 1970s. However, tracing back of these early isolates could not confirm the presence of bat associated lyssaviruses (Kuzmin et al., 2006a). The first well documented and verified isolation of a lyssavirus from a bat occurred in 1991, when a novel lyssavirus, named Aravan virus (ARAV) was isolated from a lesser mouse-eared bat (*Myotis blythii*) in Kyrghyzstan. The discovery of Khujand virus (KHUV) in a whiskered bat (*Myotis mystacinus*) followed in 2001 (Kuzmin et al., 2003). One year later another novel lyssavirus virus, Irkut virus (IRKV) was discovered in the Irkutsk province of Russia, when a bat caught indoors died approximately 10 days later after

developing clinical signs (Botvinkin et al., 2003). IRKV is insofar distinctive from the other Asian lyssaviruses as further cases were reported in China in a greater tubenosed bat (*Murina leucogaster*) (Liu et al., 2013), as well as a human rabies case in 2007 in the Russian Far East (Leonova et al., 2009). Most recently, Gannoruwa bat lyssavirus (GBLV) was isolated from Indian flying foxes (*Pteropus medius*) in Sri Lanka, after 62 grounded and dead found bats were tested for the presence of lyssaviruses (Gunawardena et al., 2016). Further surveillance and search for bat pathogens including lyssaviruses will likely expand the diversity and range of bat-associated lyssaviruses in Asia. In fact, serological surveys of bats indicate the presence of lyssaviruses in several countries in Southeast Asia, i.e. the Philippines (Arguin et al., 2002), Cambodia (Reynes et al., 2004), Thailand (Lumlertdacha et al., 2005), Bangladesh (Kuzmin et al., 2006b), China (Jiang et al., 2010) and Vietnam (Nguyen et al., 2014).

Australian bat lyssavirus (ABLV) was initially discovered in the brain of a black flying fox (*Pteropus alecto*) and is predominantly associated with fruit eating bat species (Fraser et al., 1996; Weir et al., 2014). Interestingly, ABLV infection has only been confirmed by virus isolation in one insectivorous species, the yellow-bellied sheath-tailed bat (*Saccolaimus flaviventris*). Genetically, ABLV is also delineated into two lineages associated with either fruit bats or insectivorous bats, respectively (Barrat, 2004). ABLV infection caused three known human cases in Queensland, Australia (Johnson et al., 2010), and spill-over infections were identified in two horses with neurological disease (Annand and Reid, 2014).

1.4. Bat rabies in Africa

Of note, the first indication of the circulation of lyssaviruses other than RABV was demonstrated by early virological investigations in Africa (Boulger and Porterfield, 1958; Shope et al., 1970), and the virus isolated from a straw-coloured fruit bat (*Eidolon helvum*) in Nigeria in 1956 was named Lagos bat virus (LBV). LBV infections were reported from several African countries, including detections in other bat species, dogs, cats and one water mongoose (Markotter et al., 2006; Sabeta et al., 2007). Analysis of the virus phylogeny suggests the existence of four major LBV genetic lineages. The genetic distances between some lineages is greater than those established as a demarcation criterion for individual lyssavirus species (Markotter et al., 2008). Serosurveys revealed a high seroprevalence of antibodies against LBV, and thus indicated the circulation of LBV in various fruit bat species

across parts of Africa (Dzikwi et al., 2010; Freuling et al., 2015; Hayman et al., 2008; Hayman et al., 2012; Kalemba et al., 2017; Kuzmin et al., 2008a).

Closely related to LBV is Shimoni bat virus (SHIBV), which was isolated from a dead Commerson's leaf-nosed bat (*Hipposideros commersoni*) during a search for bat associated pathogens in Kenya (Kuzmin et al., 2010). Comparative serological surveys in *Rousettus aegyptiacus* bats and *Hipposideros commersoni* bats in sympatric roosts in Kenya suggested that *H. commersoni* is a primary host species of SHIBV (Kuzmin et al., 2011).

Duvenhage virus (DUVV) was isolated in 1970 in South Africa following the death of a human that was bitten by an insectivorous bat (Meredith et al., 1971). Sporadically, DUVV was reported from insectivorous bats in Southern Africa (Paweska et al., 2006; Weyer et al., 2011) and caused two additional fatal human infections in South Africa (Paweska et al., 2006) and in the Netherlands (following a contamination in Kenya)(Van Thiel et al., 2008).

1.5. Bat rabies in Europe

In Europe, the first rabid bat was diagnosed in 1954 in Germany (Mohr, 1957). Other and only sporadic reports indicated the presence of bat associated rabies in Europe until the 1980s (Müller et al., 2007), when two human rabies cases due to bat contact sparked surveillance efforts in many European countries (Johnson et al., 2010). From 1977 until 2016, bat rabies was confirmed in 1180 cases during bat rabies surveillance activities across Europe (Source: Rabies Bulletin Europe database, found at: http://www.who-rabies-bulletin.org/ accessed May 2017). A recent study evaluating all available data on bat rabies surveillance across Europe concluded that bat rabies surveillance is not evenly conducted (Schatz et al., 2013). Therefore, while cases detected in bats indicate the circulation among bats in that region, the absence of evidence is not the evidence of absence.

Molecular characterization initially demonstrated that viruses isolated from European bats and human cases belong to two genetically diverse lyssaviruses, European bat lyssavirus type 1 (EBLV-1) and European bat lyssavirus type 2 (EBLV-2), belonging to *European bat 1 lyssavirus* and *European bat 2 lyssavirus* species, respectively (Amengual et al., 1997; Bourhy et al., 1993; Davis et al., 2005). While serotine bats (*Eptesicus serotinus* and *E. isabellinus*) are considered the primary reservoir hosts for EBLV-1, rabies cases in Daubenton's bats (*Myotis daubentonii*) and pond bats (*Myotis dasycneme*) were genetically characterized as

EBLV-2 (Banyard et al., 2013). Besides the European bat lyssaviruses, two other unrelated bat lyssaviruses were also isolated from common bent-winged bats (*Miniopterus schreibersii*) in the Caucasus region (West Caucasian bat lyssavirus –WCBV- belonging to *West Caucasian bat lyssavirus* species, (Kuzmin et al., 2008b)) and in Spain (Lleida bat lyssavirus, LLEBV, (Arechiga Ceballos et al., 2013) belonging to the *Lleida bat lyssavirus* species), respectively.

In contrast to the Americas, CSTs are relatively rare in Europe. However, EBLV-1 CSTs have been documented in a number of occasions, including in a Dutch colony of captive Egyptian fruit bats (*Rousettus aegyptiacus*) (Wellenberg et al., 2002). Also, EBLV-1 has been detected in sheep from Denmark (Ronsholt, 2002), in a stone marten (*Martes foina*) in Germany (Müller et al., 2004) and two domestic cats in France (Dacheux et al., 2009).

Sporadic human rabies cases following a bat bite have been described in Europe. In 1977, the first human rabies case associated with a bat bite in Europe was reported in the Ukraine (Kuzmin et al., 2006a). Another confirmed case in Russia that has been characterized was transmitted from a bat and occurred in 1985 (Selimov et al., 1989). The virus responsible for this infection was isolated and shown to be EBLV-1 (Amengual et al., 1997).

A Swiss biologist who had multiple bat bites died in Finland in 1985. Rabies as a diagnosis was confirmed by laboratory tests and EBLV-2 was isolated for the first time (Lumio et al., 1986). The second confirmed human rabies case with this lyssavirus was a 56-year-old bat conservationist from Angus, Scotland who died in November 2002 (Fooks et al., 2003). In all cases, the individual infected had a history of close contact with bats and none had received vaccination against rabies.

2. BBLV - a novel bat lyssavirus isolated first in Germany

In 2010, another novel lyssavirus named Bokeloh bat lyssavirus (BBLV) was isolated from a Natterer's bat (*Myotis nattereri*) in Germany. The presence of lyssavirus antigens in the diseased bat was confirmed using fluorescence antibody test and immunohistochemistry with standard rabies biologics (Freuling et al., 2011). Two further viruses were isolated in

Germany and in France, respectively, 2 years later, all from the same bat species and all found in moribund or dead bats (Freuling et al., 2013; Picard-Meyer et al., 2013).

Animal experimental studies demonstrated that BBLV was pathogenic for mice via intracranial and intramuscular inoculation routes, causing fatal encephalitis irrespective of the isolate used (Nolden et al., 2014). As typical to lyssaviruses during infection BBLV formed intracytoplasmic inclusions, detected by staining with FITC-conjugated anti-nucleocapsid monoclonal antibodies. Kaplan-Meyer survival plots were significantly different to EBLV-2 and similar to EBLV-1, although BBLV is on a genetic level more closely related to the former (Nolden et al., 2014). The distinction between BBLV and other lyssavirus species was also evident in the antigenic patterns in reactions with anti-nucleocapsid monoclonal antibodies. This difference was supported by antigenic cartography where BBLV was separated from all characterized lyssaviruses, but related to phylogroup I viruses (Nolden et al., 2014). While the genome organization and sequence relationships are consistent with the classification as a lyssavirus, BBLV nucleotide sequences demonstrate 80% and 79% identity to the sequence of the most similar viruses KHUV and EBLV-2, respectively. Altogether, genetic distance, phylogenetic reconstructions, antigenic patterns and ecologic features were sufficiently different to existing lyssavirus species, and the creation of the *Bokeloh bat lyssavirus* species was approved (ICTV, 2015).

2.1. The Natterer's bat – the presumed primary host of BBLV

For better understanding of virus-host interactions, i.e. between BBLV and the Natterer's bat, knowledge on their ecological and phylogenetic characteristics are essential. *M. nattereri* (Kuhl, 1818), synonym *Myotis escalerae* (Cabrera, 1904), is a middle-sized, crevice-dwelling, nocturnal, insectivorous European bat species of the Western Palearctic belonging to the genus *Myotis*, subfamily *Myotinae*, chiropteran family *Vespertilionidae* of the suborder Vespertilioniformes. This bat was first described by the German naturalist and zoologist Heinrich Kuhl in 1818, who named it in honour of his Austrian colleague Johann Natterer (Beolens et al., 2009). Ancestors of *M. nattereri* can be traced back millions of years ago till the Pliocene (Sigé and Legendre, 1983). Paleontological evidence suggests an evolutionary segregation and subsequent development and adaptation of several subspecies at the beginning of the Holocene-Pleistocene (Horacek and Hanak, 1983; Hutterer et al., 2012). Its geographic range extends throughout most of Europe, from the Atlantic Ocean

and the Mediterranean basin up to latitude of 60 degree north (Sweden, Finland) into western Russia, and Ukraine (Figure 1). Populations of *M. nattereri* also occur in western and south-western Asia Minor, the Caucasus region, the coastal regions of the Near East, the Kopetdag Mountains (Turkmenia, Iran) and northern Kazakhstan. Its historic range included Norway, in which for reasons unknown it is no longer found anymore. Presumably, Natterer's bats do not occur on the islands of Sardinia, Malta and Kreta (Hutson et al., 2008; Topal, 2011). It can also be found in the western Atlas and coastal zones north-west Africa, although few records from north Africa indicate that the population is likely to be relatively small (Hutson et al., 2008).

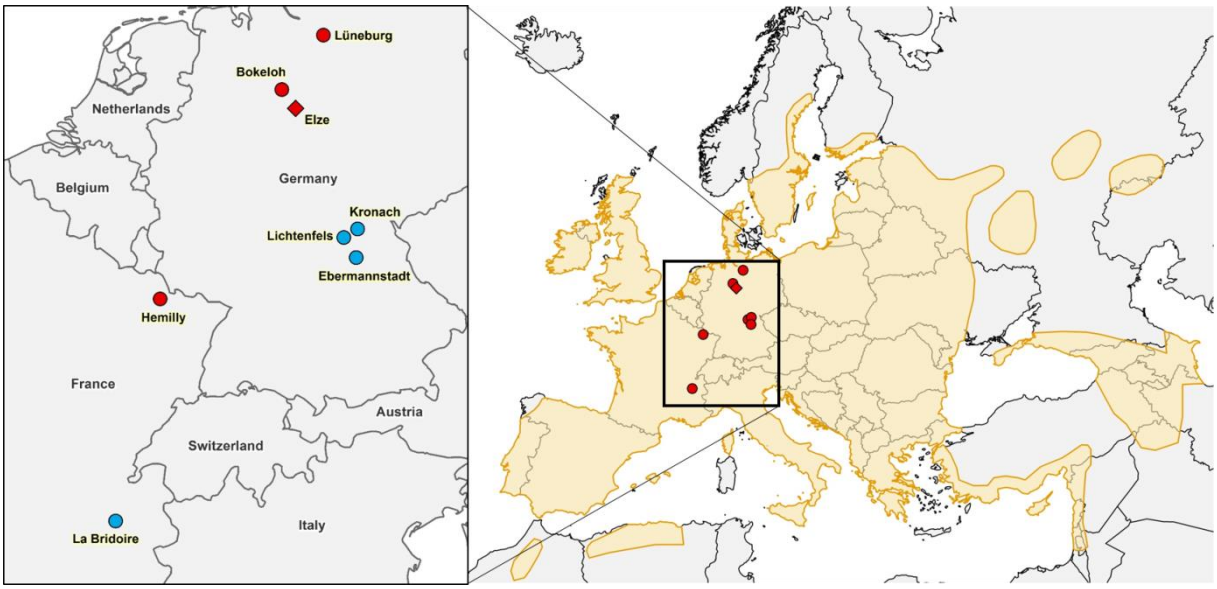

Fig. 1. Geographic origin of the BBLV isolates (left) in combination with the range (right in yellow) of the presumed reservoir species, the Natterer's bat (*Myotis nattereri*). The case associated with a pipistrelle bat (*Pipistrellus pipistrellus*) is indicated (diamond). Also, the delineation into the two genetic lineages A (red) and B (blue) is shown in the left box. Source for the range data: *Myotis nattereri* In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1. http://www.iucnredlist.org. Downloaded on 30 April 2017.

Although *M. nattereri* occurs in various habitats from sea level up to an altitude of about 2,000 meters, in the middle European inland it is predominantly found in open countryside with scattered broad-leaved woodland and close to water bodies such as tree-lined river

corridors (Smith, 2001) (http://www.eurobats.org). Natterer's bats are generally considered a sedentary species (Schober and Grimmberger, 1998). In general, roost switching seems to be very common among *M. nattereri*, especially in summer times (Smith, 2001) with transboundary migration flows in border areas of European countries. Bat banding data from 12 European countries reveal that migration distances between maternity, swarming and hibernation roosts usually vary according to region and climatic conditions (Hutterer et al., 2005). While in southern Europe due to the moderate climate during winter times populations of Natterer's bats are believed not to be forced to move from one roost to another (DePaz et al., 1986), migration distances between those roost sites of their northern conspecifics are reported to range between 14 and 90 km, on average (Topal, 2011). However, there is increasing evidence that this species is at least facultative migrant suggesting that this bat species is more a regional migrant than a true sedentary species (Hutterer et al., 2005). Based on registered one-way flights and return flights from winter to summer roost and between winter, summer and swarming sites some individuals are known to have covered longer distances. In Germany, long distance records (return flight from wintering roost) of 157 km (Haensel, 2004) and up to 266 km and even 327 km in a male and female for one-way flights, respectively, have been described. These unusual long movements are believed to be dispersal flights (Steffens et al., 2005).

In the course of history, for the *Natterer's bat* species, several subspecies and other potentially related species have been identified and proposed (for review see (Topal, 2011)). However, differentiating closely related species from another can represent a challenge (Padial et al., 2010; Shaffer and Thomson, 2007), because traditional taxonomy based on morphological characteristics can be misleading by the presence of phenotypic plesiomorphism or adaptative convergence (Salicini et al., 2011). With the advent of sophisticated genetic tools, molecular phylogenetics have been widely used to survey biological diversity of mammals, in particular *Chiroptera*, with results shedding new light on molecular systematics and taxonomy of species and their biogeography. In fact, the genetic biodiversity of the Natterer's bat seems to be much more complex and is still not fully understood. Recent phylogenetic analysis based on nuclear and mitochondrial microsatellite loci revealed new phylogenetic relationships within the *M. nattereri* species complex (also referred to as *M. nattereri sensu lato*) and among closely related species (Puechmaille et al., 2012). Evidence is mounting that there is much higher genetic variation in the *M. nattereri*

species complex than previously thought. *M. nattereri* in the Western Mediterranean, France and Germany, for example, was shown to be a paraphyletic group that is composed of well-differentiated lineages across regions, which have a narrower geographic distribution with potentially overlapping contact zones (Halczok et al., 2017; Ibanez et al., 2006; Puechmaille et al., 2012; Salicini et al., 2011). These lineages match clades partially described previously, e.g. *M. nattereri sensu stricto*, *M. escalerae*, clade A corresponding to *Myotis sp. A* and clade B corresponding to *Myotis sp. B* (Garcia-Mudarra et al., 2009; Ibanez et al., 2006; Mayer et al., 2007). According to the unified species concept which is proposing separately evolving metapopulation lineages as the only necessary property of species (De Queiroz, 2007) some of these novel phylogenetic *M. nattereri* lineages could be considered as new cryptic species (Puechmaille et al., 2012; Salicini et al., 2011; Salicini et al., 2013). More research is needed however, to further substantiate these observations.

3. Further investigation the presence of BBLV in bats in Germany and in France

3.1. Bat lyssavirus surveillance

In both countries, France and Germany, rabies surveillance in bats is based on a passive surveillance system. In Germany, due to the federal structure, animal disease detection including routine bat rabies surveillance is performed at regional veterinary laboratories focussing on clinically suspect animals and especially those with human contact. Furthermore, in order to increase submission numbers an enhanced passive surveillance was initiated in 1998 (Schatz et al., 2014), whereby dead bats from different sources and collections, e.g. conservation agencies, universities, private collections or museums were acquired. The submitting bat handlers morphologically delineated all submitted bats into species using phenotypical features (Dietz and von Helversen, 2004). In France, all bats associated with human contact are submitted for analysis to the National Reference Center for Rabies (NRC-R), at Institut Pasteur, Paris, whereas dead found bats without human contact are investigated by the National Reference Laboratory for Rabies, Anses-Malzeville.

3.2. Postmortem diagnosis of rabies in bats

Similar to other rabies suspected animals, the *postmortem* diagnosis of rabies in bats is based on standard virological techniques. With the advent of molecular diagnostics, the rapid detection, confirmation, and differentiation of bat rabies isolates became possible and is additionally applied. (Box 1).

Box 1. Rabies diagnostics

Postmortem rabies diagnosis of bats is usually performed on brain samples using the fluorescent antibody test (FAT) as described (Dean et al., 1996). In brief, brain smears are fixed on slides and stained with fluorescein isothiocyanate (FITC) conjugates (Bio-Rad, Marnes-la-Coquette, France and SIFIN, Berlin, Germany). All FAT results in France and positive or questionable results in Germany are confirmed by the rabies tissue culture infection test (RTCIT) using mouse neuroblastoma cell lines (Neuro-2a, ATCC CCL-131 or MNA 42/13, FLI Cat. No.0229, respectively), essentially as described (Bourhy et al., 1989; Webster and Casey, 1996). RTCIT was performed using clarified brain suspension and homogenates from various other organs where available (Table 2).

Viral RNA detection: RNA isolation in France was performed following manufacturer's recommendations, from the brain suspensions or directly from organs following a digestion step (Dacheux et al., 2008), after immersion into 1mL of TriReagent or 0.8 mL of TriReagent LS (Molecular Research Center) respectively. Diluted RNA (1:10, in nuclease-free water) was subjected to real-time RT-PCR (RT-qPCR) as previously described (Dacheux et al., 2016). In short, two RT-qPCRs were performed. The first was a probe-based (TaqMan) RT-qPCR for detecting the RABV species (pan-RABV RT-qPCR) and in the second RT-qPCR an intercalating dye (SYBR Green) was used for the detection of other lyssavirus species (pan-lyssa RT-qPCR). Furthermore, two conventional hemi-nested RT-PCRs targeting the nucleoprotein and the polymerase genes were performed as described (Dacheux et al., 2008; Davis et al., 2005), followed by Sanger sequencing of the respective amplicons for genotyping. In Germany 250 µl of the brain and organ homogenates were immersed into 750 µl of peqGOLD TriFast (peqlab) and RNA extraction was performed according to manufacturer's instructions. The resulting RNA was eluted in 20 µl of nuclease-free water and subjected to multiplex R14 RT-qPCR as described elsewhere (Fischer et al., 2014) using the AgPath-IDTM One-Step RT-PCR Kit (Applied-Biosystems).

3.3. Characterization of positive specimens

Historically, lyssaviruses were delineated based on reaction patterns of anti-nucleocapsid monoclonal antibodies (Schneider et al., 1985). The initial BBLV isolate was also subject to typing, confirming the antigenic distinction between BBLV and other lyssavirus species (Freuling et al., 2011). Furthermore, sequence comparisons allow for the inference of phylogenetic relationships and have been applied for lyssaviruses, including BBLV (Freuling et al., 2011; Picard-Meyer et al., 2013). Besides classical Sanger sequencing for bat species verification, next-generation sequencing (NGS) was applied to obtain full-length genome sequences of all available BBLVs (Box 2).

Box 2. Sequencing

For high quality full-length genome sequencing of the BBLV isolates the protocol described by (Nolden 2016) can be applied. Briefly, extracted RNA is transcribed into cDNA using the cDNA synthesis system kit (Roche) together with random hexamer primers (Roche). Subsequently, either Illumina or Ion Torrent compatible libraries are generated using the respective adapters. After size-selection, libraries are quantified and sequenced either on an Illumina MiSeq (Illumina) or Ion Torrent PGM (Life Technologies) instrument using MiSeq reagent kit v3 (Illumina) and HiQ-View chemistry (Life Technologies), respectively. Application of an iterative mapping and de-novo assembly approach using 454 Sequencing Systems Software [version 3.0; Roche] generates reliable BBLV consensus sequences from each isolate. Subsequently, the consensus sequences are annotated for instance using the Geneious software package (Kearse et al., 2012).

An alternative sequencing protocol was described (Dacheux et al., 2010; Joffret et al., 2013). Here, total RNA is reverse transcribed using Superscript III reverse transcriptase with random hexamers (Invitrogen) and amplified using QuantiTect Whole Transcriptome kit (Qiagen). Amplified cDNA is fragmented and libraries are prepared using KAPA Library Preparation Kit (Kapa Biosystems). The resulting libraries are subsequently sequenced on Illumina MiSeq running in 325 nt single-end modus. The obtained sequencing reads are pre-processed to remove low quality or artificial bases using AlienTrimmer (Criscuolo and Brisse, 2013) as implemented in Galaxy (Goecks et al., 2010). To obtain viral genome sequences, trimmed reads are mapped along appropriate reference sequences using CLC Genomics Assembly Cell (CLC bio) as implemented in Galaxy. Annotation of the derived

consensus sequence is performed using Sequencher 5.2.4 software (Gene Codes Corporation).

For bat species determination partial or full cytochrome *b* (CYTB) sequences are used. These are usually obtained by Sanger sequencing essentially as described previously (Bickham et al., 2004; Schatz et al., 2014). To this end, wing membrane samples or brain material are used which are lysed overnight. Alternatively, CYTB-DNA is extracted from a patagium biopsy using the using DNeasy Blood and Tissue kit (Qiagen). CYTB-DNA is then amplified using specific primer pairs (CytB Uni fw and CytB Uni rev or LGL-765- F and LGL-766-R). The obtained PCR fragments are seperated in a 1% agarose gel stained with ethidium bromide, purified using the QIAquick Gel Extraction Kit (Qiagen) and sequenced.

Regardless of the protocol used, sequences are made publicly available by submission to one of the INSDC (International Nucleotide Sequence Database Collaboration) databases. From these databases, the sequences can be retrieved using their individual accessions (see for instance table 1). Alternatively, at the European Nucleotide Archive (part of the INSDC) sequences can combined into studies and be retrieved using a single study accession (see for instance PRJEB20392 for a number of BBLV and related CYTB sequences).

3.4. Confirmation of further cases of BBLV

In Germany, from 1998 until 2016 a total of 5591 bats were tested in the frame of enhanced passive surveillance, with the most frequently submitted bat species being the common pipistrelle bat (*Pipistrellus pipistrellus*) and the noctule bat (*Nyctalus noctula*). Of all samples, 67 tested positive for lyssavirus antigen representing primarily EBLV-1 isolated from its reservoir host, the serotine bat. Also, a few infections in other bat species i.e., the common pipistrelle bat, the Nathusius' pipistrelle bat (*Pipistrellus nathusii*) and the brown long-eared bat (*Plecotus auritus*) were found as reported before (Schatz et al., 2014). During the same period, five cases of BBLV infection were observed in Natterer's bats (*M. nattereri*, Table 1, Fig. 1) and an additional case of BBLV infection was observed in the common pipistrelle bat, representing the first case of BBLV in a species other than a Natterer's bat. The bat was initially found injured during daytime in December 2015 and was taken to a regional bat conservationist for rehabilitation and eventual return to the wild, but died 3 days later

without clear signs of a disease. The bat was subject of sampling in the frame of retrospective bat lyssavirus surveillance, and initial fluorescent antibody test results were corroborated by real-time RT-PCR specific for BBLV.

In France during a 28-year period (from 1989 to 2016), a total of 546 dead bat specimens suspected of human exposure were received at the NRC-R for rabies diagnosis. Of those, 17 were confirmed to be rabid after *postmortem* diagnosis and found to be infected by EBLV-1, with 4 EBLV subtype a and 12 EBLV subtype b. All of them were serotine bats (*E. serotinus*), except for one in 2005 which was identified as a common pipistrelle bat (*P. pipistrellus*) and for which viral RNA (EBLV-1b) and viral antigens by ELISA were detected (data from the NRC-R). In 2013, a case of BBLV infection was diagnosed in a Natterer's bat (*M. nattereri*) in La Bridoire (Table 1, Fig. 1). It represented the second case of BBLV infection in France in a Natterer's bat, the first one being detected in 2012 (Picard-Meyer et al., 2013). Similar to the German BBLV isolates, all these isolates were detected using classical techniques for the post-mortem diagnosis of rabies.

3.5. Virus distribution in BBLV infected bats

Different tissue samples were tested for virus isolation by RTCIT and for viral RNA detection by RT-qPCR from BBLV infected bat specimens. Viable virus was isolated from all tested organs from the bat from Kronach (39608) and in 5/8 organs of the common pipistrelle bat from Elze (39068) (Table 2). In contrast, virus isolation was successful in four out of ten organs from the bat from Ebermannstadt (42052). Similarly to virus isolation, lyssavirus RNA was detected by RT-qPCR in all tested organs in the bat from Kronach (39607) and Elze (39068), and in 8/10 tested organs for bats from Ebermannstadt (42052) (Table 2). For the BBLV positive bat from La Bridoire (13001FRA), virus could only be isolated from brain and lyssavirus RNA were detected in brain and kidney (Table 2).

Isolate-No.	Species	Date	Location	Clinical signs	Duration of clinical stage	Virus Accession Numbers	Reference
21961	*Myotis nattereri*	Feb 10	Bokeloh, Germany	aggressively, directly approaching any moving object, vigorously trying to bite, and screaming ferociously, general weakness, lethargy, and paralysis. After the first 3 days of the clinical course, the bat stopped drinking and eating	10 days	JF311903	Freuling et al., 2010
KC169985		Jun 12	Hemilly, France	paralysis, weight loss	>2 days	KC169985	Picard-Meyer et al., 2013
29008/29028		Oct 12	Lichtenfels, Germany	prominent biting behavior,aggressiveness, tremor of the wings and was unwilling to drink	6 days	KF245925	Freuling et al., 2013
13001FRA		Aug 13	La Bridoire, France	Found dead by two young children, which further received a post-exposure prophylaxis	N/A	MF043188	this chapter
39607/39608		May 15	Kronach, Germany	Animal was found grounded and was transferred to a rehabilitation centre where it showed overt agressive behavior. Eventually, the animal died and was submitted for rabies testing	N/A	LT839617	
39068	*Pipistrellus pipistrellus*	Dec 15	Elze, Germany	animal was found grounded during daytime and was cared for in a rehabilitation centre. Eventually, the animal refused to eat and drink and died without other clinical signs of rabies	>3days	LT839642	
41021	*Myotis nattereri*	Aug 16	Lüneburg, Germany	difficulty in swallowing, refused to eat and drink, motion stimulated vocalization, defensive wing beating	9 days	LT839644	
42052/42053		Aug 16	Ebermannsstadt, Germany	animal was found grounded and died the same day, following refusal to drink and eat	>1day	LT839643	

Table 1: Details on BBLV isolates including e.g. host, clinical signs and accession numbers.

Organ	Isolate			
	39068 (Elze)	39608 (Kronach)	13001FRA (La Bridoire)	42052 (Ebermannstadt)
Brain	+/+	+/+	+/+	+/+
Tongue	+/+	+/+	-/-	+/+
Kidney	+/-	+/+	+/-	+/-
Pectoral muscle	+/+	+/+	-/-	+/+
Lung	+/+	+/+	-/-	+/-
Heart	+/+	+/+	-/-	+/-
Liver	+/-	+/+	n.a.	+/-
Spleen	+/-	+/+	n.a.	-/-
Salivary gland	n.a.	+/+	n.a.	-/+
Bladder	n.a.	+/+	n.a.	n.a.
Intestine	n.a.	n.a.	-/-	+/-

Table 2: Virological investigations on tissue samples for different bats infected with BBLV (RT-PCR/RTCIT); n.a. = not analyzed

3.6.Sequence and phylogenetic analyses

Full-length genome sequences were obtained using NGS for the five new BBLV isolates (Box 2) and were analyzed together with the three sequences already available (Table 1). Phylogenetic analysis of these eight genomes showed a differentiation of BBLV sequences into two lineages. Lineage A included BBLV isolates 21961, 39068, and 41021 from lower Saxony, Germany and the isolate KC169985 from Hemilly, France, whereas lineage B included BBLV isolates 29008, 39607, and 42052 from Bavaria, Germany and the isolate 13001FRA from La Bridoire, France (Figs. 1-3). Nucleotide identity within lineage A was above 98.8% and within lineage B above 96.8%. The heterogeneity of 3.2% in lineage B is primarily caused by the genetically divergent isolate 13001FRA from La Bridoire, France, whereas the BBLV isolates from Bavaria (29008, 39608, and 42052) have a nucleotide identity of above 99.2%. The same applies to lineage A with the BBLV isolates from lower Saxony (21961, 39068, and 41021) having a nucleotide identity above 99.7%. Nucleotide

identities between the two lineages varied between 92.7% and 93.2%, depending on the isolates.

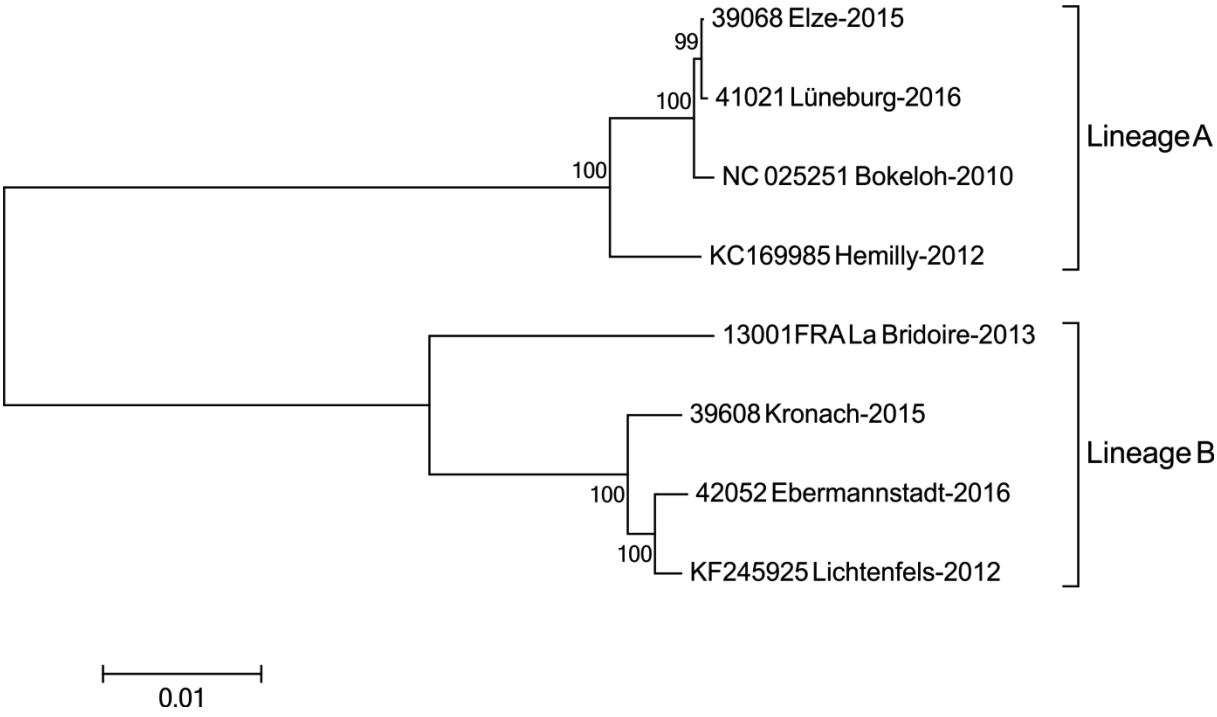

Fig. 2. Phylogenetic relationship of BBLV isolates from available full-length genome sequences using Maximum Likelihood method. Following an alignment with MAFFT (Katoh and Standley, 2013), the genetic distances were calculated using GTR+G as the optimal substitution model with 1000 bootstrap replications as implemented in MEGA6 (Tamura et al., 2013). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. BBLV can be differentiated into two lineages: lineage A included BBLV isolates 21961, 39068, and 41021 from lower Saxony, Germany and the isolate from Hemilly, France, whereas lineage B included BBLV isolates 29008, 39608, and 42052 from Bavaria, Germany and the isolate 13001FRA from La Bridoire, France.

4.Discussion

Since its first and recent discovery in 2010 in Bokeloh, Germany, five additional cases of BBLV were found in Germany and two in France. In contrast, most other recently discovered lyssaviruses from insectivorous bats, i.e. ARAV, KHUV, West Caucasian bat virus (WCBV), SHIBV and LLEBV, have all been detected only once (Banyard et al., 2013). Diagnostically, as with other lyssaviruses, all BBLV isolates have been detected by techniques used for the *postmortem* diagnosis of rabies, based on antinucleocapsid antibodies conjugated with fluorescent dyes. Evidently, modern molecular tools offer advantages, e.g. increased speed, sensitivity, and possibilities for genetic characterization (Fischer et al., 2012; Fooks et al., 2012). Specifically, RT-qPCRs dedicated to large spectrum detection of lyssaviruses are now available and suitable for the diagnosis of emerging lyssaviruses such as BBLV (Dacheux et al., 2016). In addition, multiplex RT-qPCRs have improved the differentiation of bat rabies into the individual bat lyssavirus species (Fischer et al., 2014). Also, it was demonstrated before that undirected molecular detection methods were useful in identifying novel BBLV cases. The bat brain from the BBLV case from Lichtenfels, Germany (29008) was subjected to microarray analysis and in parallel to next generation sequencing (Freuling et al., 2013). Similarly, the brain sample of the bat infected with the French isolate 13001FRA was tested using a novel high-density resequencing microarray (called VirID), based on the two previous versions of the PathogenID microarray (Dacheux et al., 2010), and was successfully detected (data not shown). As for the previous versions, this detection was possible with the presence of conserved nucleotide region in the polymerase genes of prototype viruses comprising seven species of lyssavirus (namely RABV, LBV, MOKV, DUVV, EBLV-1, EBLV-2 and ABLV) and an artificial consensus sequence based on all of these species.

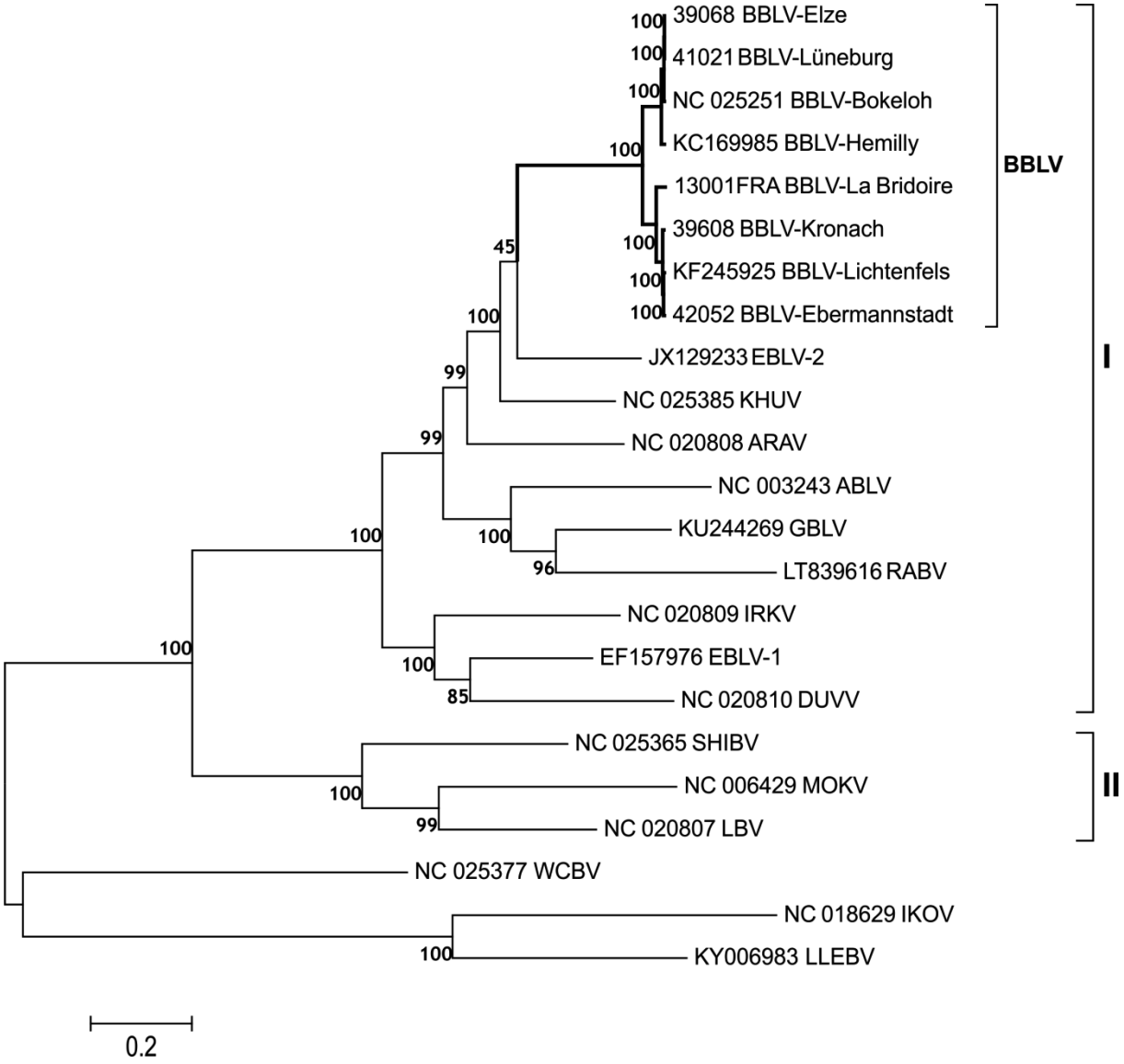

Fig. 3. Phylogenetic relationship of all lyssavirus species representatives and all BBLV isolates inferred using the Maximum Likelihood method based on the GTR model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, and bootstrap values (1000 replication) shown next to the branches. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Virus species' associations to phylogroups I and II are also indicated.

Phylogenetic analysis performed on the full-length genome sequences of all BBLV isolates known to date clearly demonstrated the presence of two different lineages among this lyssavirus species: lineages A and B, with nucleotide identities between them above 92%.

The spatial distribution of these two lineages is puzzling, as both lineages comprise isolates from France and Germany. However, on a regional scale all isolates from Bavaria cluster closely together, and the same can be observed for the isolates from Lower Saxony, while the French viruses appear more distinct (Figs. 1 and 2).

At the bat host level, the distribution of BBLV lyssavirus at the late stage of the disease seems large and massive, because viral RNA and infectious virus were detected in several tissues and organs, including tongue, kidney, pectoral muscle, lung, heart, liver, spleen, salivary glands, bladder and intestine, confirming earlier studies in one bat from Lichtenfels, Germany (Freuling et al., 2013). This large distribution was found for Natterer's bats as well as for the common pipistrelle bat. An exception was observed with the French bat, for which the presence of viral RNA was observed only in kidney and brain. This is probably the consequence of the poor general condition of this bat carcass.

The sudden emergence of BBLV is somewhat puzzling as surveillance activities across Europe and particularly in Germany and in France have not much changed in the last few years. In fact, in Europe, more than 1,000 bat rabies cases have been reported, and no significant changes in reported numbers have been observed in recent years (Fig. 4).

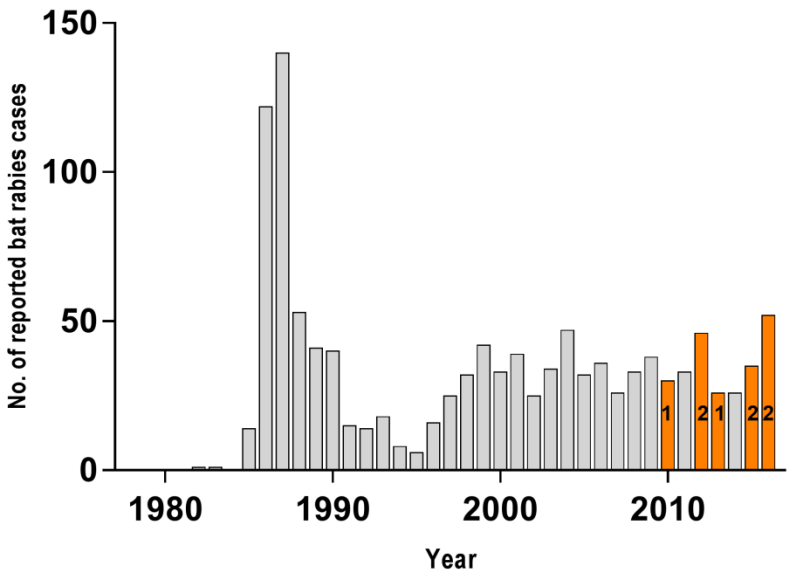

Fig. 4. Graph showing the annual number of bat rabies cases in Europe as reported to the database of the WHO Rabies Bulletin Europe. The years in which BBLV was detected are highlighted and the number of BBLV cases per year are indicated.

Although surveillance efforts were further encouraged by the Eurobats agreement in 2006 (Anon, 2006), bat rabies surveillance in Europe remained heterogeneous (Schatz et al., 2013). This may have contributed to the fact that all BBLV isolates were discovered in France and Germany, two countries in which continuous bat rabies surveillance has been performed for decades (Picard-Meyer et al., 2014; Schatz et al., 2014). However, heterogeneous surveillance on a spatial level does not explain the number of novel cases of BBLV observed. Also, there is no indication that bat rabies cases in Natterer's bats were found in the past that had not been characterized (Brass, 1994; Kappeler, 1989; Schatz et al., 2013).

In Germany, besides routine surveillance targeting suspect animals and public health related incidents, in 1998 enhanced passive surveillance was started to include bats, which would otherwise be missed for lyssavirus surveillance. In total, the combined surveillance efforts resulted in sampling of 8,310 bats, with the most submitted bat species being the common pipistrelle. Although the pipistrelle bat is not considered as a primary host for any known bat lyssavirus species, three lyssavirus infections were discovered in this bat species. One was a case of EBLV-1 infection as described before (Schatz et al., 2014). A similar infection of EBLV-1 in common pipistrelle bat was observed in France (data from the NRC-R). The other was the case of BBLV in a common pipistrelle from Elze described here. The latter represents the first evidence of BBLV in another bat species apart from the Natterer's bat. All other cases of BBLV were discovered in Natterer's bats, leading to the assumption that this bat species is the primary host for BBLV. When material was available, bats species were determined by partial CYTB gene sequencing, since bat species determination due to morphological features can be challenging, especially when dealing with cryptic species. Also, when carcasses arrive in the laboratory, they may be incomplete or in a state of degradation, thus making it difficult to verify the bat species. Indeed, the Natterer's bat from Ebermannstadt, Germany was initially thought to be a Daubenton's bat and only phylogenetic analysis of partial CYTB sequence revealed that it was in fact a Natterer's bat (Fig. 5). Besides bat speciation, the combined molecular analysis of both host and virus is also essential for drawing further epidemiological conclusions, as exemplified for RABV in arctic foxes from Greenland (Hanke et al., 2016).

As indicated previously, full-length analyses demonstrated that two distinct lineages of BBLV circulate among European Natterer's bats. On the host side, recent phylogenetic analyses

revealed a much higher genetic variation in the *M. nattereri* species complex than previously thought. In Central Europe, *M. nattereri* was shown to be a paraphyletic group that is composed of four well-differentiated lineages, i.e. *M. nattereri sensu stricto*, *M. nattereri escalerae*, *M. nattereri sp. A*, and *M. nattereri sp. B*. (Halczok et al., 2017; Ibanez et al., 2006; Puechmaille et al., 2012; Salicini et al., 2011; Salicini et al., 2013). All Natterer's bats found positive for BBLV belong to the subspecies *M. nattereri sensu stricto* (Fig. 5), which is not surprising, as this subspecies is known to occur in Germany and France (Salicini et al., 2011; Salicini et al., 2013). However, the distinct two lineages of BBLV do not seem to be related to a circulation in specific Natterer's bat subspecies.

In a recent study correlating genetic distances of Natterer's bats and their associated astroviruses, identical astrovirus haplotypes were not shared between different sampling regions suggesting that astroviruses are mostly transmitted among host colonies at regional scale. The fact that some virus haplotypes found in several different regions had high genetic similarity implied that occasional transmission across regions occurred (Halczok et al., 2017). However, against the background that this bat species is a regional migrant species (Hutterer et al., 2005), the same can be assumed for BBLV, where the respective outliers could be a result of yet not documented long-distance migration. Evidently, for BBLV this analysis is restricted to the available dataset and further isolations may support this delineation. Interestingly, the same observation was made with EBLV-1 in serotine bats where almost identical nucleotide sequences from within certain geographical regions of the Netherlands and Germany indicate genomic stability during the transmission cycle of these virus variants but with occasional geographic spread or intermixing (Freuling et al., 2012; Poel et al., 2005). However, on a European scale there seems to be no clear correlation between migration behaviour of lyssavirus reservoir species and the phylogeographical clustering of virus isolates. For EBLV-1, the primary host serotine bat is a rather sedentary species, which is not reflected in the clustering of isolates (Davis et al., 2005). On the other hand, the Daubenton's bat as the reservoir for EBLV-2 can migrate over larger distances. However, sequence analysis for EBLV-2 revealed a strong geographical clustering (Jakava-Viljanen et al., 2010). Further research, including and combining host biology, migration data, as well as molecular characterization of host and virus is essential to understand transmission dynamics and gain further insights into the epidemiology of bat-virus interaction. This may contribute to predict virus spread and establish risk assessments for virus transmission to humans.

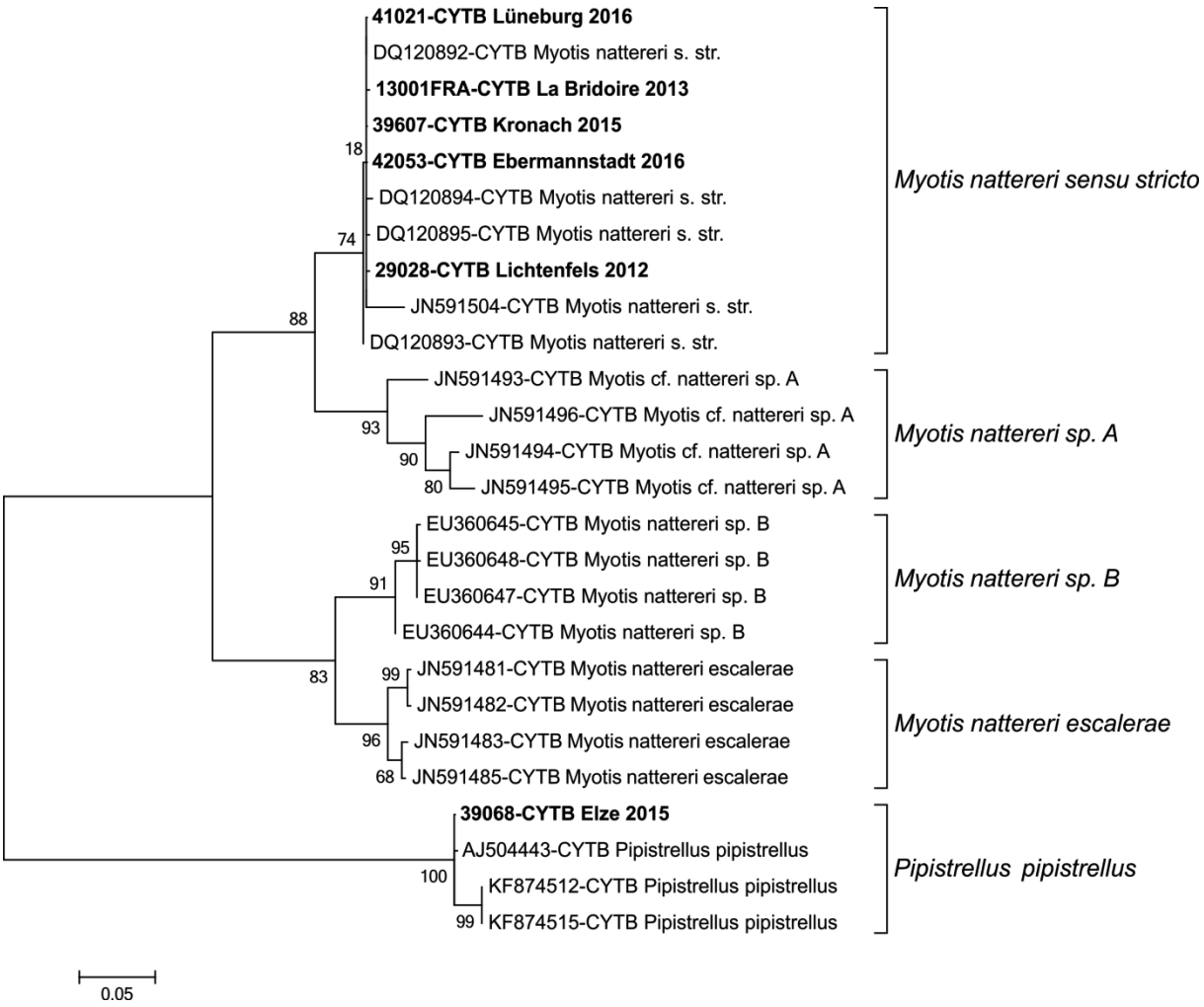

Fig. 5. Phylogenetic relationship of partial CYTB sequences (484 nt) of bats found positive for BBLV and representatives of Myotis nattereri clades (Salicini et al., 2011; Salicini et al., 2013). While the bat from Elze (Lower Saxony) was identified as a common pipistrelle bat (Pipistrellus pipistrellus), all other bats found positive for BBLV were closely related and classified as Natterer's bats (Myotis nattereri), and more precisely belong to the Myotis nattereri sensu stricto clade/subspecies. Phylogenetic analysis was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (HKY) with invariant sites (G) conducted in MEGA6 (Tamura et al., 2013). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, and bootstrap values (1000 replication) shown next to the branches.

BBLV was shown to be as pathogenic in mice as EBLV-1 (Nolden et al., 2014) and it can be assumed that BBLV infections in humans potentially lead to an encephalitis eventually causing death. Therefore, BBLV alongside with the other known bat lyssaviruses in Europe

represents a low but undeniable public health threat. Unlike other remote regions in the world where people do not get into close contacts with bats, in Europe research and species conservation programs require the handling of bats by bat workers. While in the eastern parts of Germany the cumulative number of bat handlings from 2000-2010 for serotine bats were 1,260, those for *M. daubentonii* and *M. dasycneme* were 22,245 and 1086, respectively, with 37,140 by far the most handlings were recorded for the Natterer's bat (Bat Marking Centre, Dresden). This underlines the importance of adequate prophylaxis for bat handlers. In addition, accidental exposure to bats could occur for individuals other than bat handlers. For example in 2015, a total of 128 patients consulted an antirabic clinic (ARC) in France after being exposed to a bat in France metropolitan, representing 2.6% of the total number of patients consulting such clinic (n=8,336) (data from NRC-R, available at (Pasteur, 2017)). Some of these patients could be exposed to laboratory confirmed rabid bats, as it was the case for the two children who were in contact with the BBLV infected bat from La Bridoire, France. Considering the relatedness of BBLV to other members of the phylogroup I lyssaviruses (Figure 3) it appears likely that persons who received rabies prophylaxis will be protected. In fact, a recent study showed that available human inactivated vaccines elicit an antibody response that cross-neutralizes BBLV and therefore, are considered to confer protection against BBLV and other European bat lyssaviruses (Malerczyk et al., 2014; Nolden et al., 2014). Similarly, serum samples from French patients preventively vaccinated against rabies were analysed for the potency of neutralization against the CVS rabies strain (used as the reference virus) and 13001FRA isolate adapted to cell-culture. Seroneutralization of BBLV was observed, but with a 5.8-fold decrease compared to CVS (Fig. 6). In any case, as there are currently no other vaccines available (Evans et al., 2012), consistent preventive vaccination of all persons working with bats and PEP of all persons exposed to bats should be given priority as recommended in France (Haut Conseil de la santé publique, 2013; Lafeuille et al., 2005) and Germany (Delere et al., 2011).

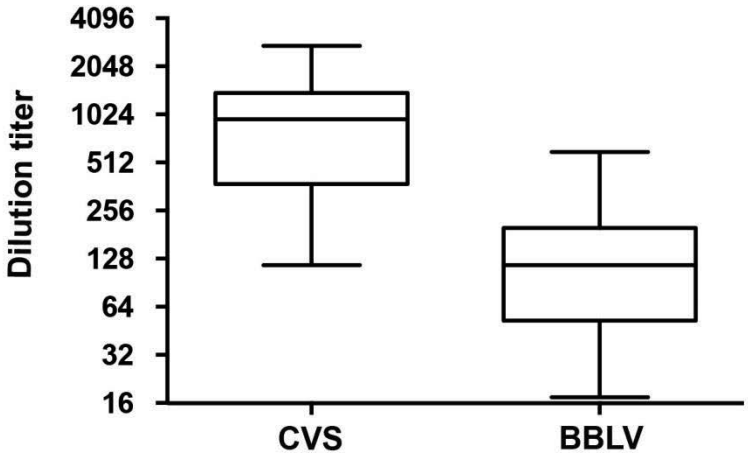

Fig. 6. Comparison of seroneutralization against RABV (CVS) and BBLV (13001FRA) with 37 sera of patients vaccinated against rabies using a modified RFFIT (Feyssaguet et al., 2007). The dilution value to obtain a 50% viral inhibition is visualized as box-plots, with the first, third quartiles are indicated at the bottom, and top of the box, respectively, and the median is indicated with the band. The neutralizing activity was found to be significantly lower (paired Student t test, p value < 0.05) against BBLV (mean value = 157, standard deviation = 137) compared to CVS (mean value = 907, standard deviation = 623), with a 5.8 fold decrease in the titre dilution.

Despite the recent discovery of a novel bat lyssavirus, lyssaviruses have been characterized in only a small proportion of recognized species (Banyard et al., 2013). Future surveillance of bat-associated pathogens using highly sensitive and novel high-throughput technologies, e.g. NGS (Dacheux et al., 2014) or pan viral microarrays, will likely increase the number of detected pathogens including novel lyssavirus species. This may also lead to further discoveries of BBLV in countries along the distribution of the Natterer's bat.

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5.3. Evaluation of six commercially available rapid immunochromatographic tests for the diagnosis of rabies in brain material

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RESEARCH ARTICLE

Evaluation of Six Commercially Available Rapid Immunochromatographic Tests for the Diagnosis of Rabies in Brain Material

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Abstract

Rabies is a neglected zoonotic disease that causes an estimated 60,000 human deaths annually. The main burden lies on developing countries in Asia and Africa, where surveillance and disease detection is hampered by absence of adequate laboratory facilities and/or the difficulties of submitting samples from remote areas to laboratories. Under these conditions, easy-to-use tests such as immunochromatographic assays, i.e. lateral flow devices (LFD), may increase surveillance and improve control efforts. Several LFDs for rabies diagnosis are available but, except for one, there are no data regarding their performance. Therefore, we compared six commercially available LFDs for diagnostic and analytical sensitivity, as well as their specificity and their diagnostic agreement with standard rabies diagnostic techniques using different sample sets, including experimentally infected animals and several sets of field samples. Using field samples the sensitivities ranged between 0% up to 100% depending on the LFD and the samples, while for experimentally infected animals the maximum sensitivity was 32%. Positive results in LFD could be further validated using RT-qPCR and sequencing. In summary, in our study none of the tests investigated proved to be satisfactory, although the results somewhat contradict previous studies, indicating batch to batch variation. The high number of false negative results reiterates the necessity to perform a proper test validation before being marketed and used in the field. In this respect, marketing authorization and batch release control could secure a sufficient quality for these alternative tests, which could then fulfil their potential.

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Author Summary

Despite being preventable with adequate biologicals, rabies still causes an estimated 60,000 human deaths annually. The main burden lies on developing countries in Asia and Africa, where dog rabies surveillance is hampered by laboratory confirmation of disease due to a number of reasons, including laboratory infrastructure and logistics. Lateral flow devices (LFD) may increase surveillance and improve control efforts. Several LFDs for rabies diagnosis are available but, except for one, there are no data available regarding their performance. Therefore, we compared six commercially available LFDs for diagnostic and analytical sensitivity. With sensitivities ranging from 0% up to 100% depending on the LFD and the samples, none of the tests investigated proved to be satisfactory, and the results somewhat contradict previous studies, indicating batch to batch variation. The high number of false negative results reiterates the necessity to perform a proper test validation before being marketed and used in the field. Only when sufficient quality is assured for these alternative tests, then they can fulfil their potential. In this respect, we demonstrated that positive results in LFD can be further validated and characterized using RT-qPCR and sequencing.

Introduction

Rabies is an important zoonotic disease and exhibits the highest case fatality rate of any infectious disease in humans. Infection is usually transmitted by bites via saliva and it is invariably fatal once clinical signs develop. The etiological agents of the disease are the different lyssavirus species of the order *Mononegavirales*, family *Rhabdoviridae* [1]. The prototypical rabies virus (RABV) transmitted by dogs is responsible for an estimated 60,000 human deaths per year, especially in Asia and Africa [2,3]. The gold standard for rabies diagnosis is the fluorescence antibody test (FAT) [4], which is internationally approved by OIE and WHO. Briefly, brain tissue is fixed on slides, stained with fluorophore conjugated antibodies and examined under a fluorescence microscope. Confirmatory tests are virus isolation in cell culture (Webster and Casey, 1996) and the mouse inoculation test (Koprowski, 1996), the latter no longer being recommended by international organizations (OIE/WHO). Alternative diagnostics include various assays to detect viral RNA or antigen [5,6].

However, particularly in those countries that are most affected the lack of resources results in inadequate availability of equipment, chemicals and trained staff. Also, the maintenance of a cold chain during shipment of samples is difficult especially in tropical and subtropical countries, and hampers the use of these standard laboratory tests [7]. Unfortunately, the resulting inadequate rabies surveillance contributes to a cycle of neglect with a very limited number of laboratory confirmed human and animal rabies cases and thus an underestimation of the real impact of this neglected zoonotic disease, particularly in Africa and Asia [8]. Therefore, WHO has called for better tests for the rapid and economical diagnosis of RABV, without loss of sensitivity or specificity [2]. One approach to address this issue is the development of tests for the diagnosis of rabies that are relatively easy to perform, e.g. the direct rapid immunohistochemical test (dRIT), which was developed as an alternative to FAT using light microscopy [9]. Another approach is lateral flow devices (LFDs), also called rapid immunodiagnostic tests (RIDTs), immunodiagnostic assays or immunochromatographic strip tests that are interesting insofar as they have potential for field use. They are rapid and easy to use without the need for special training for implementation and evaluation. Another advantage is that these tests have

no special storage requirements in terms of temperature, i.e. they can be shipped and stored at ambient room temperatures.

Their basic principle behind such tests is the fluid migration of a sample along a nitrocellulose membrane [10]. Gold conjugated antibodies bind to antigen in the sample and the antigen-antibody complex is then immobilized by a second antibody which is fixed on the test strip [11]. LFDs are applied in many different fields [10,12] including the diagnosis of viral human and animal diseases, e.g foot-and-mouth disease [13], avian influenza [14], Ebola virus disease [15], porcine epidemic diarrhea [16], Hepatitis C [17], and respiratory syncytial virus infection [18]. Recently, LFDs for rabies detection were developed and proof of principle studies yielded good results regarding sensitivity and specificity [19,20], raising hope of extending rabies diagnostic capacity in resource-limited settings [6,9]. Since then only one prototype LFD [19] was extensively evaluated, including its diagnostic range, indicating that the test is able to detect rabies and non RABV-lyssaviruses in field samples [21–24]. The routine use of LFDs for rabies diagnosis, however, is hampered by the lack of data regarding its sensitivity and specificity compared to standard diagnostic assays. In addition to the initially published prototype LFDs numerous other rabies LFDs are also commercially available for diagnostic use. Unfortunately, they have never been comprehensively analysed. Therefore, following WHO recommendations, six commercially available LFDs were compared in this study for their diagnostic and analytical sensitivity, as well as specificity, in comparison with FAT and PCR using a range of samples from experimentally infected animals and field samples.

Materials and Methods

Commercial LFD test kits

Six different commercial LFD test kits for rabies, i.e. Vet-o-test Rabies Ag (BioGen Technologies, Germany; LOT NO: AI191301), Anigen Rapid Rabies Ag Test kit (Bionote, Korea; LOT NO: 1801088), Quicking Pet Rapid Test (Quicking Biotech, China; LOT NO: G140210303), Rapid Rabies Ag Test Kit (Creative Diagnostics, USA; LOT NO: CD8921), Rabies Virus Ag Rapid test (Green Spring, China; LOT NO: 20140210), and quickVET Rabies Antigen Rapid test (Ubio, India; LOT NO: UB0131303).), were identified based on literature and internet searches and purchased. The price per test including tax and shipment varied between 3.14€ and 10.12€.

Sensitivity and specificity

Sensitivity and specificity of the commercial LFDs were tested using three different sets of samples from already-existing collections of brain specimens, i.e no animals were used in this study.

Samples from sample set I and sample set II were obtained from the virus archive of the Friedrich-Loeffler-Institut (FLI). Sample set I comprised 51 samples from different parts of the brain of 17 raccoons experimentally infected with three virulent primary host adapted RABV-isolates from a European red fox, Eurasian dog and North American raccoon (Table 1) [25]. Sample set II contained 31 samples from different naturally infected brains, or mouse brain homogenates generated from field strains after mouse inoculation test (MIT), representing five different lyssavirus species. In addition to RABV variants of differing geographical origin, these species were European bat lyssavirus type 1 (EBLV-1), European bat lyssavirus type 2 (EBLV-2), Duvenhage virus (DUVV) and Bokeloh bat lyssavirus (BBLV), each of which was represented by at least one sample. The RABV field strains originated from North and South America, Asia and Europe (Table 2). Specificity was determined using five non infected brain

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Table 1. Diagnostic results of experimentally infected raccoons for different parts of the brain[#].

Strain	Animal	Lab-ID	Material	FAT-Result	Genome copies/µl	BioGen	Bionote	Quicking	Creative Diagnostics	Green Spring	Ubio
RABV (dog Azerbaijan)	1	26052	AH	-	1.31E+05	-	-	-	+	-	-
	1	26053	C	+++	2.08E+05	-	-	-	+	-	-
	1	26054	MO	+++	1.52E+06	-	+	-	++	+	-
	2	26056	AH	++++	4.74E+04	-	-	-	+	-	-
	2	26057	C	++++	5.97E+04	-	-	-	+	-	-
	2	26058	MO	++++	3.56E+06	-	++	-	++	++	-
	3	26060	AH	-	6.60E+01	-	-	-	-	-	-
	3	26061	C	-	1.10E+05	-	-	-	-	-	-
	3	26062	MO	++	2.48E+05	-	-	-	-	-	-
	4	26064	AH	++++	1.68E+05	-	-	-	-	-	-
	4	26065	C	+++	4.67E+05	-	-	-	-	-	-
	4	26066	MO	++++	1.52E+07	-	+	-	++	+	-
	5	26068	AH	-	1.78E+04	-	-	-	-	-	-
	5	26069	C	++	9.19E+04	-	-	-	-	+	-
	5	26070	MO	+++	1.67E+06	-	-	-	-	-	-
	6	26072	AH	+++	1.40E+05	-	-	-	-	-	-
	6	26073	C	+++	4.17E+05	-	-	-	+	+	-
	6	26074	MO	++++	4.31E+06	-	+	-	++	++	-
RABV (fox Europe)	7	26076	AH	-	2.94E+01	-	-	-	-	-	-
	7	26077	C	-	7.03E+00	-	-	-	-	-	-
	7	26078	MO	-	2.26E+00	-	-	-	-	-	-
	8	26080	AH	+	7.49E+03	-	-	-	-	-	-
	8	26081	C	+++	3.58E+05	-	-	-	+	-	-
	8	26082	MO	++	2.19E+05	-	-	-	-	-	-
	9	26084	AH	+	4.80E+04	-	-	-	-	-	-
	9	26085	C	+++	1.21E+06	-	-	-	-	-	-
	9	26086	MO	++	4.52E+05	-	-	-	-	-	-
	10	26088	AH	+	7.67E+03	-	-	-	-	-	-
	10	26089	C	++	1.41E+05	-	-	-	-	-	-
	10	26090	MO	+++	2.39E+05	-	-	-	-	-	-
	11	26092	AH	+	8.28E+03	-	-	-	-	-	-
	11	26093	C	++	1.33E+05	-	-	-	-	-	-
	11	26094	MO	+++	2.25E+04	-	-	-	-	-	-
	12	26096	AH	+	4.93E+04	-	-	-	-	-	-
	12	26097	C	++++	1.13E+06	-	-	-	+	-	-
	12	26098	MO	+++	5.87E+05	-	-	-	-	-	-
RABV (raccoon USA)	13	26100	AH	+++	1.32E+03	-	-	-	+	+	-
	13	26101	C	++++	1.22E+04	-	+	-	++	++	-
	13	26102	MO	++++	2.27E+04	+	++	-	+++	+++	-
	14	26104	AH	++++	1.69E+04	-	-	-	-	-	-
	14	26105	C	+++	5.72E+03	-	-	-	-	-	-
	14	26106	MO	++++	3.35E+03	-	+	-	+	+	-
	15	26108	AH	+++	2.53E+03	-	-	-	-	-	-
	15	26109	C	+++	4.57E+04	-	-	-	-	-	-

(*Continued*)

Table 1. (*Continued*)

Strain	Animal	Lab-ID	Material	FAT-Result	Genome copies/μl	BioGen	Bionote	Quicking	Creative Diagnostics	Green Spring	Ubio
	15	26110	MO	+++	5.21E+02	-	-	-	-	-	-
	16	26112	AH	++	3.59E+01	-	-	-	-	-	-
	16	26113	C	++	5.41E+02	-	-	-	-	-	-
	16	26114	MO	++++	7.35E+00	-	-	-	-	-	-
	17	26116	AH	+	6.35E+01	-	-	-	-	-	-
	17	26117	C	+++	4.54E+02	-	-	-	-	-	-
	17	26118	MO	++	3.35E+02	-	-	-	-	-	-

[#]: AH = Ammon's horn, C = cerebellum, MO = medulla oblongata

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homogenates. For both sample sets FAT was repeated for each sample essentially as previously described [4] using a four-plus scoring system. Additionally, brain material was subjected to real-time RT-PCR for confirmation and to determine the viral genome load. The quantification was performed essentially as described before [26]. Briefly, a synthetic artificial control encoding corresponding fragments of RABV, EBLV-1, EBLV-2, and BBLV was used to generate a standard curve with the R14 multiplex RT-PCR so that cq-values could be transformed into genome copies per μl template, i.e. 50mg of brain. For other lyssavirus species, the N-gene based pan-lyssa system was used [26]. Testing of those samples was conducted at the national reference laboratory for rabies at FLI, Germany. Sample set III comprised 20 brain samples of naturally infected animals including seven different animal species obtained from six different provinces of the Republic of South Africa (RSA) during rabies routine surveillance in 2015. These samples were tested with both FAT and the respective LFDs (Table 3). Test specificity was determined using 10 negative field samples. Testing of the African samples was conducted at the OIE reference laboratory at Onderstepoort Veterinary Institute, RSA using the same LOT number for each of the LFD kits tested.

For all tests a preparation of a 10% brain homogenate (in PBS) was required after which the manufacturers' instructions were followed. Briefly, a cotton swab was inserted into the brain suspension until saturated and then placed into the buffer solution where it was thoroughly mixed. Between two and four drops of the buffer solution were then added to the sample inlet using the disposable dropper. For the Creative Diagnostics test kit, no sample buffer was provided and PBS was used instead. The readout was made 10 min afterwards, as recommended by the manufacturers. The test and control lines on the strips were separately classified by two individuals using a three-plus scoring system representing the intensity of the reaction in the test line area.

Analytical sensitivity

To mimic low antigen content in a potential rabid brain sample (analytical sensitivity) a two-fold positive-in-negative brain homogenate dilution series was prepared. From each of those prediluted preparations different brain suspensions in buffer were again derived, i.e. neat/undiluted, 40%, 20%, and 10%. Subsequently, the produced brain suspensions of each prediluted positive brain sample were tested by mixing 100μl with 100μl of buffer and adding 100μl to the test. Additionally, brain suspensions were subjected to real-time RT-PCR to determine the viral genome load as described above.

Table 2. Diagnostic results of archived field samples.

Lab-ID	Species	Year	Origin	Viral species	Lineage	Material	FAT-Result	Genome copies/µl	BioGen	Bionote	Quicking	Creative Diagnostics	Green Spring	Ubio
34202*	Dog	1985	Yugoslavia	RABV	Cosmopolitan (WE)	brain	++++	2.49E+07	-	+	+	++	++	-
13491*	Dog	1981	Ethiopia	RABV	Africa 1	brain	+++	1.33E+07	-	-	-	+	-	-
34203*	Wolf	1999	Yugoslavia	RABV	Cosmopolitan (WE)	brain	+++	1.28E+07	-	-	-	+	-	-
13099*	Dog	1974	Taiwan	RABV	South-East Asia	brain	++++	1.21E+07	-	++	-	+++	++	-
13255*	Human	1979	Chile	RABV	Cosmopolitan	brain	+++	3.44E+08	-	+++	-	+++	+++	-
8192	Fox	2003	Bosnia-Herzegovina	RABV	Cosmopolitan (WE)	brain	++++	2.70E+06	-	-	-	+	-	-
3139	Fox	1999	Germany	RABV	Cosmopolitan (WE)	brain	++++	5.83E+06	-	-	-	-	-	-
13133	Cat	1982	Nigeria	RABV	Africa 1	MP#	+	1.16E+06	-	+	-	++	+	-
13242	Bat	1966	South America	RABV	American bat variant	brain	++++	2.28E+07	-	-	-	+	-	-
13209	Mongoose	1980	South America	RABV	Cosmopolitan	MP#	++++	1.44E+07	-	-	-	+	-	-
13206	Raccoon	1981	North America	RABV	raccoon variant	MP#	+++-++++	9.81E+02	-	-	-	+	-	-
13200	Skunk	1981	USA	RABV	skunk variant	brain	++	1.14E+07	-	-	-	-	-	-
4131	Fox	1999	Czech Republic	RABV	Cosmopolitan (WE)	brain	+++	2.64E+06	-	-	-	++	+	-
13117	Dog	1983	Algeria	RABV	Africa 1	brain	++++	9.14E+07	-	-	-	++	+	-
4134	Fox	1999	Czech Republic	RABV	Cosmopolitan (WE)	brain	++++	1.97E+06	-	-	-	+	+	-
13056	Dog	1984	Turkey	RABV	Middle East	MP#	++++	1.31E+06	-	+	-	+	+	-
13112	Human	1974	Malaysia	RABV	South-East Asia	MP#	+++	3.64E+07	-	++	-	++	+++	-
13208	Vampire bat	1980	South America	RABV	American bat variant	MP#	++	1.59E+06	-	++	-	++	+	-
13015	Arctic fox	1981	Norway	RABV	Arctic	brain	++++	1.88E+06	-	+	-	-	-	-
13017	Arctic fox	1981	Norway	RABV	Arctic	brain	++	4.50E+05	-	-	-	+	++	-
16854	Fox	2007	Kosovo	RABV	Cosmopolitan (WE)	brain	+	5.78E+06	-	+	-	+	-	-
13512	-	1982	South Africa	RABV	Cosmopolitan (SAD vaccine strain)	brain	++-+++	7.18E+07	-	-	-	-	+	-
13114	Human	1974	Malaysia	RABV	South-East Asia	brain	++++	7.99E+06	-	-	-	-	+	-
13093	Camel	1994	Emirates	RABV	Cosmopolitan (ME)	brain	++	4.50E+06	-	++	-	+++	+	-
20299	Cattle	2008	Iraq	RABV	Cosmopolitan (ME)	brain	++++	1.24E+07	-	++	+	++	++	-
2498	Cat	1999	Germany	RABV	Cosmopolitan (WE)	brain	(+)	3.76E+00	-	-	-	-	-	-
10280	Sheep	2004	experimental	EBLV-1	-	brain	+++	8.50E+06	-	-	-	-	-	-
10270	Sheep	2004	experimental	EBLV-2	-	brain	+	2.13E+04	-	-	-	-	-	-
34494	Bat	2010	Germany	BBLV	-	MP#	++++	3.91E+07	-	+	-	+	-	-
34495	Bat	2012	Germany	BBLV	-	MP#	++++	3.28E+07	-	+	-	-	-	-
12861	Human	1974	South Africa	DUVV	-	brain	++++	1.04E+08	-	-	-	-	-	-
33341	Wolf	2014	Germany	NC#		brain	-	0.00E+00	-	-	-	-	-	-
33342	Wolf	2015	Germany	NC#		brain	-	0.00E+00	-	-	-	-	-	-
33343	Wolf	2016	Germany	NC#		brain	-	0.00E+00	-	-	-	-	-	-

(*Continued*)

Table 2. (*Continued*)

Lab-ID	Species	Year	Origin	Viral species	Lineage	Material	FAT-Result	Genome copies/µl	BioGen	Bionote	Quicking	Creative Diagnostics	Green Spring	Ubio
33344	Wolf	2017	Germany	NC[#]		brain	-	0.00E+00	-	-	-	-	-	-
33345	Wolf	2018	Germany	NC[#]		brain	-	0.00E+00	-	-	-	-	-	-

[#]NC = negative controls, MP = mouse brain,

* = real-time RT-PCR was performed using RNA extracted from LFD-strips

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Table 3. Diagnostic results of South African field samples.

Lab-ID	Species	Year	Origin	Virus species*	FAT-Result#	Genome copies/µl	BioGen	Bionote	Quicking	Creative Diagnostics	Green Spring	Ubio
06/15	Yellow mongoose	2015	Free State	RABV	+	8.43E+04	+	+	-	-	-	-
75/15	Yellow mongoose	2015	Mpumalanga	RABV	+	4.51E+07	+	+	-	+	+	-
102/15	Jackal	2015	North West	RABV	+	1.40E+08	+	+	-	+	+	-
110/15	Jackal	2015	North West	RABV	+	6.35E+07	+	+	-	+	+	-
149/15	Civet	2015	Mpumalanga	RABV	+	1.74E+08	+	+	-	+	-	-
14/15	Feline	2015	Limpopo	RABV	+	2.10E+07	+	+	-	+	+	-
15/15	Caracal	2015	Limpopo	RABV	+	1.15E+08	+	+	-	-	+	-
38/15	Feline	2015	Free State	RABV	+	1.73E+08	+	+	+	+	+	+
113/15	Hyena	2015	North West	RABV	+	1.92E+07	+	+	-	+	+	-
130/15	Jackal	2015	Limpopo	RABV	+	1.27E+08	+	+	-	+	-	-
36/15	Bovine	2015	Limpopo	RABV	+	4.90E+08	+	+	+	+	+	+
56/15	Bovine	2015	Free State	RABV	+	3.00E+05	+	+	-	+	+	-
139/15	Bovine	2015	Free State	RABV	+	6.68E+04	+	+	-	+	+	-
146/15	Bovine	2015	North West	RABV	+	1.79E+08	+	+	-	+	+	-
153/15	Bovine	2015	North West	RABV	+	1.00E+08	+	+	+	+	+	-
41/15	Canine	2015	Limpopo	RABV	+	5.56E+07	-	+	-	+	+	-
42/15	Canine	2015	Mpumalanga	RABV	+	7.50E+06	-	+	+	-	-	-
55/15	Canine	2015	Free State	RABV	+	3.06E+06	-	+	-	-	+	-
66/15	Wild dog	2015	North West	RABV	+	2.39E+07	+	+	-	+	+	-
125/15	Canine	2015	North West	RABV	+	2.69E+07	+	+	+	+	+	-
03/15	Canine	2015	North West	NC	-	NA	-	-	-	-	-	-
17/15	Dassie	2015	Free State	NC	-	NA	-	-	-	-	-	-
20/15	Yellow mongoose	2015	Limpopo	NC	-	NA	-	-	-	-	-	-
22/15	Sable Antelope	2015	Limpopo	NC	-	NA	-	-	-	-	-	-
26/15	Honey badger	2015	Mpumalanga	NC	-	NA	-	-	-	-	-	-
54/15	Bovine	2015	Free State	NC	-	NA	-	-	-	-	-	-
63/15	Canine	2015	Western Cape	NC	-	NA	-	-	-	-	-	-
78/15	Giraffe	2015	Mpumalanga	NC	-	NA	-	-	-	-	-	-
132/15	Feline	2015	Gauteng	NC	-	NA	-	-	-	-	-	-
136/15	Bovine	2015	Limpopo	NC	-	NA	-	-	-	-	-	-

*NC = negative controls

#FAT and the LFDs were only regarded positive (+) or negative (-) without any scoring the intensity

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Virus characterization from RABV positive LFD test strips

To investigate whether further characterization of virus in LFD test strips is possible, RNA was extracted from 30 randomly selected LFD test strips which had been stored at room temperature for six weeks. A square piece of approximately 5mm length, in the area where the test line appears, was excised, and immersed in 1ml of TriZol (Invitrogen). RNA extraction was done following manufacturer's instructions, followed by real-time RT-PCR essentially as described

[27]. Exemplarily, five of the RNA samples originating from Bionote test strips were amplified using a conventional PCR assay for subsequent partial nucleoprotein sequencing [28].

Biosafety issues

To assess the potential presence of viable virus on the LFD, each buffer solution supplied with the test kits was tested for virus inactivation. Briefly, buffer/brain suspensions were prepared from two rabies positive samples as for use on the LFDs. A volume of 0.5 ml of those suspensions was then subjected to virus isolation in cell culture using the rabies tissue culture infection test (RTCIT) [29]. Additionally, strips of all LFDs used, except the Bionote, with a positive sample were excised 10 minutes and one hour after use and added to the prepared cell suspensions for virus isolation in cell culture. Three consecutive serial passages were considered confirmative for a negative result.

Results

Diagnostic sensitivity and specificity

In experimentally infected raccoons from sample set I, 44 out of 51 brain samples were positive in FAT with fluorescence scores ranging between + and ++++, whereas all samples tested positive using RT-qPCR. Most of the FAT negatives comprised samples from the Ammon's horn. The amount of RNA per sample as determined by real-time RT-PCR ranged from 2.26 up to $1.52^{*}10^{7}$ mean genome copies/μl template. The lowest amount of RNA in a FAT positive sample was 7.35 mean copies/μl template. Four of the seven FAT-negative samples had RNA content of $6.60^{*}10^{1}$ mean genome copies/μl template or lower. The remaining three FAT-negative samples contained more than $1.78^{*}10^{4}$ mean genome copies/μl template of RNA. Generally, the strength of agreement between results obtained by individual commercial LFDs and FAT with brain samples from experimentally infected raccoons was considered to be 'poor'. Of the 44 FAT positive samples, none tested positive using the test kits of Ubio and Quicking and one sample only tested positive using BioGen (Kappa = 0.006, 95% CI: -0.007–0.020). The other test kits detected more samples, with Bionote displaying a positive result for seven samples (Kappa = 0.049; 95% CI: -0.000–0.099), Green spring for 10 (Kappa = 0.075; 95% CI: 0.007–0.143) and Creative diagnostics for 14 samples (Kappa = 0.064; 95% CI: -0.052–0.180) (Table 1). Another sample was positive with the Creative diagnostics test kit but negative using FAT, at an RNA-content of $1.31^{*}10^{5}$ mean copies/μl template. The lowest amount of viral RNA in a sample that tested positive in an LFD was $1.32^{*}10^{3}$ mean copies/μl template.

Of 31 field samples from sample set II 30 tested positive and one inconclusive using FAT, while all were positive by pan-lyssa real-time RT-PCR. The amount of lyssaviral RNA in the samples ranged from $9.81^{*}10^{2}$ mean copies/μl template up to $3.44^{*}10^{8}$ mean copies/μl template per sample excluding one sample. Here the amount of RNA was 3.76 mean copies/μl template, presenting with only unspecific fluorescence in FAT. In contrast, all FAT positive samples were negative using Ubio and BioGen (Kappa = -0.0283; 95% CI: -0.067–0.021). Quicking displayed positive results for two samples (Kappa = -0.028; 95% CI: -0.059–0.053), while Bionote and Green spring detected 13 (Kappa = 0.085; 95% CI: -0.012–0.320) and 15 (Kappa = 0.196; 95% CI: 0.004–0.387) FAT positive samples, respectively. With 21 FAT positive samples recognized (Kappa = 0.364; 95% CI: 0.094–0.633) by the Creative diagnostics test, the correlation was considered 'fair' (Table 2). No LFD displayed a positive result with the sample that showed inconclusive fluorescence in FAT. Lyssavirus species other than RABV were negative in all LFDs except for BBLV. Creative diagnostics was able to detect one and Bionote both BBLV positive samples. All LFDs displayed a negative result for the five rabies negative samples resulting in a specificity of 100%.

With field samples from South Africa (Sample set III) all LFDs displayed a negative result for the ten rabies negative samples resulting in a specificity of 100%. The correlation between results obtained by FAT and individual commercial LFDs ranged between perfect and poor. Bionote and BioGen showed the best test results. While the correlation between FAT and Bionote was perfect, it was considered 'good' for BioGen, Green spring and Creative diagnostics. Compared to FAT, BioGen displayed a positive result for 17 (Kappa = 0.791, 95% CI: 0.571–1.000) South African samples. Green spring and Creative diagnostics each recognized 16 (Kappa = 0.727; 95% CI: 0.488–0.967) RABV positive field samples. In contrast, Quicking and Ubio detected only five (Kappa = 0.182; 95% CI: 0.013 to 0.350) and two (Kappa = 0.069; 95% CI: -0.030–0.168) FAT positive samples, respectively (Table 3).

Analytical sensitivity

All 'spiked' brain-suspensions were positive using FAT (+—++++) and real-time RT-PCR. The amount of RNA in brain suspensions decreased as the dilution factor increased, starting in the undiluted positive brain at $1.24^{*}10^{7}$ mean genome copies/μl template and finishing with $1.60^{*}10^{5}$ mean genome copies/μl template at a dilution step of 1:128, which was the highest dilution factor used. The cut-off point up to which the LFDs were able to detect the positive brain varied, as can be seen in Table 4. Many of the test results for Bionote and Ubio could not be analyzed, since the samples did not reach either the test line or the control line. Ubio did not display a single positive result.

Virus characterization from RABV positive LFD test strips

The real-time RT-PCR was positive for all 30 LFD test strips with Cq values ranging between 19.12 and 37.11. Partial sequencing of the N-gene was successful for two out of 5 samples tested. When comparing the Cq values derived directly from the samples with the mean Cq values from the test strips, an increase between 11.82 and 13.51 was observed.

Biosafety

Viable virus could be detected after mixing of RABV positive samples with the buffer solutions of Quicking, Green spring and Ubio. Also, one virus isolation was positive when the buffer solution of BioGen was used, while no positive results were obtained with Bionote buffer. After 10 minutes all test strips except Quicking still contained viable virus, but after one hour only the test strip of Creative diagnostics still contained infectious virus particles.

Discussion

Because the gold standard of rabies diagnosis, i.e. FAT requires expensive equipment e.g. a fluorescence microscope, consumables and well trained technicians to obtain high sensitivity and specificity, it is often not applied in many endemic areas. LFDs could fundamentally facilitate and enhance rabies surveillance under these settings. The first study to evaluate a commercial rabies LFD (Bionote) for sensitivity and specificity yielded good results, but the authors concluded that the LFD should only be used for research purposes until validated or authorized for use by OIE or WHO [19]. In recent years, further studies concentrated on the Bionote LFD showing its potential to detect lyssaviruses from Africa, Asia, and Europe. With this test, sensitivities compared to FAT ranged between 91% and 100% [21–24,30,31].

Here, we compared the performance of six commercially available rabies LFDs using identical sample sets. Interestingly, sensitivity varied considerably depending on the sample set used. Clearly, sensitivity of all rabies LFDs for sample set I and sample set II were generally below

Table 4. Sensitivity assessment based using various dilutions of rabid brain (Lab-ID: 20299, n.a. = non analysable).

Dilution	FAT-Result	Genome copies/µl	Brain suspension	BioGen	Bionote	Quicking	Creative Diagnostics	Green Spring	Ubio
neat	++++	1.24E+07	neat	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			40%	+	+++	++	+++	++	n.a.
			20%	+	+++	++	+++	+++	-
			10%	-	++	+	++	++	-
1:2	++++	4.12E+06	neat	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			40%	+	n.a.	n.a.	+	n.a.	n.a.
			20%	-	n.a.	+	+	+	-
			10%	-	+	+	+	+	n.a.
1:4	++++	3.36E+06	neat	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			40%	-	n.a.	+	+	+	n.a.
			20%	-	n.a.	+	+	+	-
			10%	-	+	+	+	+	-
1:8	+++	2.67E+06	neat	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			40%	-	n.a.	+	+	+	n.a.
			20%	-	n.a.	+	+	+	-
			10%	-	+	-	-	+	-
1:16	++-+++	1.52E+06	neat	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			40%	-	n.a.	-	n.a.	+	n.a.
			20%	-	n.a.	-	-	+	n.a.
			10%	-	n.a.	-	-	-	n.a.
1:32	++	7.53E+05	neat	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			40%	-	n.a.	-	-	+	n.a.
			20%	-	n.a.	-	-	+	n.a.
			10%	-	-	-	-	-	-
1:64	+	4.33E+05	neat	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			40%	-	n.a.	n.a.	n.a.	+	n.a.
			20%	-	n.a.	-	-	-	-
			10%	-	-	-	-	-	-
1:128	+	1.60E+05	neat	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			40%	-	n.a.	-	-	-	n.a.
			20%	-	n.a.	-	-	+	n.a.
			10%	-	-	-	-	-	n.a.

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expectations with a poor correlation with FAT and three tests completely failing. Originating from experimentally infected raccoons (Sample set I), the reduced sensitivity can in part be explained by the fact that for animal welfare reasons animals had to be euthanized at the onset of the first clinical signs when in some parts of the brain only little or no antigen was detectable, while viral RNA was already found [25] (Table 1). Generally, the overall RNA viral load of sample set I was lower (mean: 6.49 x 10^5, range: 2.26 to 1.52 x 10^7) than for sample set II (mean: 2.87 x 10^7; range: 9.81 x 10^2–3.44 x 10^8) and III (mean: 8.82 x 10^7; range: 6.68 x 10^4–4.9 x 10^8) where field samples mostly comprised animals that had died from the disease. Nonetheless, even samples with a high antigen load tested negative using LFDs, clearly indicating that they are unsuitable.

Comparison of performance with field samples from sample set II and sample set III, the test agreement between the individual LFDs and FAT seems contradictory. For reasons that remain unknown, particularly the LFDs from Bionote, BioGen, Green spring and Creative

diagnostics showed a much better test agreement with the field sample set III from South Africa. Those results were largely confirmed when the sample set III was re-tested at FLI (S4 Table).

In additions to different RABV variants, EBLV-1, EBLV-2, DUVV, and BBLV were also included in the test panel of sample set II. In previous studies, different batches of the Bionote test had demonstrated its potential to detect lyssaviruses other than RABV from Africa and Europe [21,23], so in principle it seemed possible. However, except for BBLV, which was detected by two tests, none of the non-RABV samples tested positive. Given the high diversity in lyssaviruses [32], a broad reactivity of antibodies for capture and recognition would be ideal. This should also encompass bat lyssaviruses, as a failure to recognize lyssavirus variants could result in an incomplete picture of the epidemiological situation.

Reasons for the unsatisfactory performance of the commercial rabies LFDs could be manifold. Batch-to-batch variation could be a possible explanation for the relatively low sensitivity obtained. For example, we observed a considerably lower sensitivity of the Bionote LFD batch (41.4%) compared to previous studies [21–24,30,31] including our own results from the year 2008 (S1 Table). Similar observations were made with another batch of the Bionote tested recently in Italy (S2 Table). Also, for another test (BioGen) two different batches were analyzed and while both showed sensitivities below twenty percent, a difference in sensitivity was observed (S3 and S4 Tables).

In all tests analyzed faint reactions at the test line area were observed that made a clear differentiation of positive reactions by eyesight impossible. As this could have been indicative of low concentration of rabies or lyssavirus antigen, initially those reactions were evaluated as questionable. When testing negative samples, however, those lines also occurred occasionally and were therefore considered negative. Even with the aid of photographic technology the assessment could not be improved, thus pointing to the fact that only properly visible lines should be regarded as positive. For pen-side tests to be used directly in the field the latter option is the only solution. Another disadvantage that can be noticed in sandwich assay format LFDs is that signal generation on the test line may be compromised when the concentration of target exceeds a certain critical value [33]. Here, an excess of rabies or lyssavirus antigen could be responsible for the poor or absent signal. This possibility, however, can be excluded as in our study we clearly demonstrated that the analytical sensitivity for the tested LFDs using a pre-diluted brain suspension was generally poor with higher diluent factors having a negative influence.

Another factor influencing the sensitivity of the LFDs in this study could be the manufacturer's instruction. Although they were all very similar, for the Bionote LFD the preparation of the samples to be tested differed between the original publication [19] and others. This is partly attributable to changes in the respective leaflet over time as shown before [22] or to other modifications being applied. For instance, eight samples from the Italian sample set that initially tested negative were positive when a modified protocol, i.e. without the first dilution step, only using the vial with buffer provided by the kit, was used (S2 Table), as had been recommended for field use [34].

Generally, a weak point of all manufacturers' instructions was that they were not very precise regarding sample preparation, in particular the amount of brain tissue to be diluted in buffer solutions. Some instructions for instance indicated correctly to collect small pieces from different brain regions, as would be recommended for FAT. Under field conditions this may cause problems. To allow comparability, in this study we prepared one 10% brain suspension of each sample which was then used for all LFDs. Alternatively, obtaining a mixed brain sample via the occipital foramen of animals using a straw [35] could be used if animals without human contact are to be tested.

Some tests also claimed that the LFD could be used to detect virus in saliva. Because of intermittent shedding of virus, saliva-based rabies diagnosis is per se inappropriate and should be discouraged [2]. We therefore omitted to test this, also because of a lack of samples from naturally or experimentally infected animals. But even when mimicking the shedding of virus in saliva using cell culture supernatant of virus propagations, e.g. CVS ($10^{6.3}$, $10^{6.5}$ $TCID_{50}$/ml), EBLV-1 ($10^{6.2}$ $TCID_{50}$/ml), and EBLV-2 ($10^{4.3}$ $TCID_{50}$/ml) using the Bionote test in addition to field samples (S1 Table) only the undiluted supernatant and for EBLV-1a 1:10 dilution could be detected (S5 Table).

One potential disadvantage of using an LFD is its simple yes-or-no answer without further characterization of the virus. Here, we have shown that viral RNA can be stored and eventually extracted from the strip using standard procedures, similar to what has been shown for other RNA-viruses, e.g. [13–16]. If samples are additionally tested this allows not only for a confirmation of the results, but also further characterization of virus isolates. We detected viral RNA using real-time RT-PCR after six weeks of storage at room temperature. Thus strips could be easily shipped by regular mail to a specialized laboratory, e.g. to a national reference laboratory or to an internationally approved laboratory. This approach was successfully applied following our recommendation in a field trial in Ndjamena, Chad [34]. Even sequencing of the partial N-gene is possible; however this was only the case for two out of five samples. This could be explained by the six week storage of the tests at room temperature, which probably led to RNA degradation. The resulting RNA fragments may have been long enough for real-time RT-PCR but not always for sequencing, where a longer RNA fragment is needed.

Even though one LFD strip was positive in RTCIT after one hour at room temperature, generally strips can be regarded as non-infectious, as a contamination of mucous membranes is highly unlikely. However, the buffer used in those test kits should contain a virus inactivating substance, as it does for Bionote, to exclude any potential infectivity.

Conclusions

Based on the need to improve rabies surveillance in many remote endemic areas, LFDs would be one promising alternative to laboratory testing. However, with their current limitations commercially available rabies LFDs cannot be recommended for routine diagnosis and surveillance. In particular, if animals were involved in a biting incident to a human being, false negative results may induce the patient and the doctor to refrain from appropriate post-exposure prophylaxis (PEP). Although the leaflet may explain that the results of these tests are to be confirmed by a reference method, this may not be followed and given that the cost of PEP equals a high proportion of the income in developing countries, PEP may be omitted, thus causing unnecessary deaths.

Generally, the observed limited sensitivity indicates a lack of quality control. Quality control is essentially establishing adequate performance characteristics (sensitivity, specificity, negative predictive value, positive predictive value, cross reactivity, etc.) of a given test [10,12]. Thorough validation including various circulating variants of RABV and other lyssaviruses has been recommended before those tests could be relied upon and be used as an alternative for the gold standard FAT [6]. However, it should be the responsibility of the producers and not of the customers to install a rigorous quality control system before the tests are released on the market. In some countries, e.g. Germany, any test used for the detection of a notifiable animal disease needs to obtain marketing authorization. None of the tests studied would have met the requirements for this marketing authorization and thus would not be allowed to be marketed in Germany.

This study is not meant to discredit the use of LFDs for rabies diagnosis but rather to encourage producers to substantially improve and assure the quality of their products. In principle, if those tests show a high sensitivity and specificity they could be very valuable and with their advantages in e.g. speed, easiness and storage without maintaining a cold chain could help to improve rabies detection in some parts of the world.

Supporting Information

S1 Table. Diagnostic results of archived field samples tested in 2008 using the Bionote LFD (Cat.No.:RG 18–01; Lot NO.:1801029).
(PDF)

S2 Table. Diagnostic results of archived field samples tested in Italy using the Bionote LFD (Lot NO.: 1801077, 1801081).
(PDF)

S3 Table. Comparison of two batches of BioGen LFD (Batch 1: Lot NO: AI191301, Batch 2: Lot NO: AI191402) using archived field samples (MP = mouse brain).
(PDF)

S4 Table. Comparison of between results obtained with sample set III in the laboratories at Onderstepoort (SA) and Friedrich-Loeffler-Institut (FLI).
(PDF)

S5 Table. Results of tissue culture supernatant tested in 2008 using the Bionote LFD (Cat. No.:RG 18–01; Lot NO.:1801029).
(PDF)

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Author Contributions

Conceived and designed the experiments: TCM TM BH CMF. Performed the experiments: EE KS CMF ECN CTS PdB. Analyzed the data: EE TM CMF. Wrote the paper: TCM TM EE BH CMF.

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Supplementary Table 1: Diagnostic results of archived field samples tested in 2008 using the Bionote LFD (Cat.No.:RG 18-01; Lot NO.:1801029)

Lab-ID	species	year	Origin	Viral species	Lineage	Material	FAT-Result	Bionote
148	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
149	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
150	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
151	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
152	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
153	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	-
154	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
155	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
156	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	-
280	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
281	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
282	Sheep	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	-
284	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
285	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
286	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
287	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
288	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
289	Marten	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
290	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
291	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
292	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
293	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
904	Dog	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	+
1390	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	-
1391	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	-
1392	Fox	1998	Germany	RABV	Cosmopolitan (WE)	brain	+	-
5989	Dog	2002	Aserbaijan	RABV	Cosmopolitan (WE)	brain	+	+
16854	Fox	2007	Kosovo	RABV	Cosmopolitan (WE)	brain	+	+
16862	Fox	2007	Kosovo	RABV	Cosmopolitan (WE)	brain	+	+
17039	Fox	2007	experimental	RABV	Cosmopolitan	brain	+	+
17040	Fox	2007	experimental	RABV	Cosmopolitan	brain	+	+
6214	Ferret	2002	experimental	EBLV-1	-	brain	+	+
6215	Ferret	2002	experimental	EBLV-1	-	brain	+	+
6216	Ferret	2002	experimental	EBLV-1	-	brain	+	+
6217	Ferret	2002	experimental	EBLV-1	-	brain	+	+
10280	Sheep	2004	experimental	EBLV-1	-	brain	+	+
10282	Sheep	2004	experimental	EBLV-1	-	brain	+	+
10285	Sheep	2004	experimental	EBLV-1	-	brain	+	+
10271	Sheep	2004	experimental	EBLV-2	-	brain	+	+
10274	Sheep	2004	experimental	EBLV-2	-	brain	+	+
10277	Sheep	2004	experimental	EBLV-2	-	brain	+	+

FAT and the LFDs were only regarded positive (+) or negative (-) without scoring the intensity

Supplementary Table 2: Diagnostic results of archived field samples tested in Italy using the Bionote LFD (Lot NO.: 1801077, 1801081)

ID number	Animal species	Origin	Viral species	Lineage	FAT result	RIDT result	RTCIT	RT-PCR
303/2010	badger	Italy	RABV	Cosmopolitan (WE)	pos	neg	pos	pos
450/2010	cat	Italy	RABV	Cosmopolitan (WE)	pos	neg	pos	pos
527/2010	roe deer	Italy	RABV	Cosmopolitan (WE)	pos	pos	pos	pos
836/2010	equine	Italy	RABV	Cosmopolitan (WE)	pos	neg	pos	pos
1546/2010	cat	Italy	RABV	Cosmopolitan (WE)	pos	pos	pos	pos
2174/2010	red fox	Italy	RABV	Cosmopolitan (WE)	pos	neg	pos	pos
2176/2010	red fox	Italy	RABV	Cosmopolitan (WE)	pos	neg	pos	pos
2177/2010	red fox	Italy	RABV	Cosmopolitan (WE)	pos	pos	pos	pos
2418/2010	roe deer	Italy	RABV	Cosmopolitan (WE)	pos	pos	pos	pos
2944/2010	red fox	Italy	RABV	Cosmopolitan (WE)	pos	pos	pos	pos
3144/2010	stone marten	Italy	RABV	Cosmopolitan (WE)	pos	neg	pos	pos
6944/2009	red fox	Italy	RABV	Cosmopolitan (WE)	pos	neg*	pos	pos
7024/2009	red fox	Italy	RABV	Cosmopolitan (WE)	pos	neg	pos	pos
117/1996	human (ex-dog)	Italy (ex-Nepal)	RABV	Arctic-like 1	pos	neg*	pos	pos
3570/2011	human (ex-dog)	Italy (ex-India)	RABV	Arctic-like 1	pos	neg*	neg	pos
1920/2005	dog	Mauritania	RABV	Africa 2	pos	neg	pos	pos
2019/2006	dog	Mauritania	RABV	Africa 2	pos	neg	pos	pos
2029/2007	dog	Mauritania	RABV	Africa 2	pos	neg	pos	pos
2049/2007	goat	Mauritania	RABV	Africa 2	pos	neg	pos	pos
1916/2005	goat	Mauritania	RABV	Africa 2	pos	neg	pos	pos
2871/2009	bovine	Botswana	RABV	Cosmopolitan (Africa 1)	pos	neg*	pos	pos
4125/2009	bovine	Botswana	RABV	Cosmopolitan (Africa 1)	pos	neg	pos	pos
3580/2009	dog	Botswana	RABV	Cosmopolitan (Africa 1)	pos	pos	pos	pos
3416/2009	goat	Botswana	RABV	Cosmopolitan (Africa 1)	pos	pos	pos	pos
5980/2009	dog	Botswana	RABV	Cosmopolitan (Africa 1)	pos	pos	pos	pos
6665/2009	honey badger	Botswana	RABV	Cosmopolitan (Africa 3)	pos	neg*	pos	pos
251/2007	dog	Niger	RABV	Africa 2	pos	neg	pos	pos
252/2007	dog	Niger	RABV	Africa 2	pos	neg	pos	pos
246/2007	dog	Niger	RABV	Africa 2	pos	neg	pos	pos
247/2007	dog	Niger	RABV	Africa 2	pos	neg	pos	pos
137/2007	dog	Burkina Faso	RABV	Africa 2	pos	neg	pos	pos
70/2007	dog	Burkina Faso	RABV	Africa 2	pos	neg	pos	pos
20/2007	dog	Burkina Faso	RABV	Africa 2	pos	pos	pos	pos
19/2007	dog	Burkina Faso	RABV	Africa 2	pos	neg	pos	pos
21/2007	dog	Burkina Faso	RABV	Africa 2	pos	neg	pos	pos
37/2007	dog	Burkina Faso	RABV	Africa 2	pos	pos	pos	pos
144/2007	dog	Burkina Faso	RABV	Africa 2	pos	neg	pos	pos
124/2007	dog	Burkina Faso	RABV	Africa 2	pos	pos	pos	pos
36/2007	dog	Burkina Faso	RABV	Africa 2	pos	neg	pos	pos
139/2007	dog	Burkina Faso	RABV	Africa 2	pos	pos	pos	pos
49/2007	dog	Burkina Faso	RABV	Africa 2	pos	neg	pos	pos
28/2007	dog	Burkina Faso	RABV	Africa 2	pos	neg	pos	pos
4314/1993	badger	Italy	RABV	Cosmopolitan (WE)	pos	neg	n.e.	pos
786/1993	chamois	Italy	RABV	Cosmopolitan (WE)	pos	neg	n.e.	pos
629/1993	cat	Italy	RABV	Cosmopolitan (WE)	pos	neg	n.e.	pos
4313/1993	marten	Italy	RABV	Cosmopolitan (WE)	pos	neg	n.e.	pos
4241/1993	red fox	Italy	RABV	Cosmopolitan (WE)	pos	neg	n.e.	pos
EURL PT2012/1 (Ariana 1991)	dog	Tunisia	RABV	Cosmopolitan (Africa 1)	pos	neg	pos	pos
EURL PT2012/2 (EBL2-VLA P3)	mouse (ex M. daubentonii)	UK	EBLV-2		pos	neg	pos	pos
EURL PT2012/3 (201020958)	mouse (ex dog)	Spain	RABV	Cosmopolitan (Africa 1)	pos	pos	pos	pos
EURL PT2012/7 (GS7)	red fox	France	RABV	Cosmopolitan (WE)	pos	neg	pos	pos
EURL PT2012/8 (GS7)	red fox	France	RABV	Cosmopolitan (WE)	pos	neg	pos	pos
EURL PT2012/9 (R75)	mouse (ex E. serotinus)	Spain	EBLV-1		pos	neg	pos	pos
351/2010	bovine	Brazil	RABV	American indigenous	pos	neg*	n.e.	pos
299/2010	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
134/2010	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
451/2010	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
87/2010	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
227/2010	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
251/2010	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
211/2010	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
9/2010	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
77/2010	bovine	Brazil	RABV	American indigenous	pos	pos	n.e.	pos

ID number	Animal species	Origin	Viral species	Lineage	FAT result	RIDT result	RTCIT	RT-PCR
158/2011	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
125/2011	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
285/2011	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
218/2011	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
101/2011	equine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
283/2011	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
303/2011	bovine	Brazil	RABV	American indigenous	pos	pos	n.e.	pos
62/2011	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
320/2011	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
196/2011	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
144/2011	bovine	Brazil	RABV	American indigenous	pos	neg	n.e.	pos
5B1/2011	kinkajou	Brazil	RABV	American indigenous	pos	neg*	pos	pos
343/2011	equine	Brazil	RABV	American indigenous	pos	neg*	n.e.	pos

*Samples resulted positive when tested with a different protocol which skipped the first dilution step

Supplementary Table 3: Comparison of two batches of BioGen LFD (Batch 1: Lot NO: AI191301, Batch 2: Lot NO: AI191402) using archived field samples (MP=mouse brain)

Lab-ID	species	year	Origin	Viral species	Lineage	Material	FAT-Result	Batch 1	Batch 2
34202	dog	1985	Yugoslavia	RABV	Cosmopolitan (WE)	brain	++++	++	+
13491	dog	1981	Ethiopia	RABV	Africa 1	brain	+++	-	-
34203	wolf	1999	Yugoslavia	RABV	Cosmopolitan (WE)	brain	+++	-	-
13099	dog	1974	Taiwan	RABV	South-East Asia	brain	++++	+	-
13255	human	1979	Chile	RABV	Cosmopolitan	brain	+++	++	+
8192	fox	2003	Bosnia-Herzegovina	RABV	Cosmopolitan (WE)	brain	++++	-	-
3139	fox	1999	Germany	RABV	Cosmopolitan (WE)	brain	++++	-	-
13133	cat	1982	Nigeria	RABV	Africa 1	MP	+	-	-
13242	bat	1966	South America	RABV	Cosmopolitan	brain	++++	-	-
13209	mongoose	1980	South America	RABV	Cosmopolitan	MP	++++	-	-
12861	human	1974	South-Africa	DUVV	-	brain	++++	-	-
13206	raccoon	1981	North America	RABV	raccoon variant	MP	+++-++++	-	-
10280	sheep	2004	experimental	EBLV-1	-	brain	+++	-	-
13200	skunk	1981	USA	RABV	skunk variant	brain	++	-	-
13117	dog	1983	Algeria	RABV	Africa 1	brain	++++	-	-
4134	fox	1999	Czech Republic	RABV	Cosmopolitan (WE)	brain	++++	-	-
13056	dog	1984	Turkey	RABV	Middle East	brain	++++	-	-
13208	vampire bat	1980	America	RABV	American bat variant	MP	++	-	-
13015	arctic fox	1981	Norway	RABV	Arctic	brain	++++	-	-
10270	sheep	2004	experimental	EBLV-2	-	brain	+	-	-
13017	arctic fox	1981	Norway	RABV	Arctic	brain	++	-	-
16854	fox	2007	Kosovo	RABV	Cosmopolitan (WE)	brain	+	-	-
13512	-	1982	South America	RABV	Cosmopolitan (SAD vaccine strain)	brain	++-+++	-	-
13114	human	1974	Malaysia	RABV	South-East Asia	brain	++++	+	-
2498	cat	1999	Germany	RABV	Cosmopolitan (WE)	brain	(+)	-	-
34494	mouse	2010	Germany	BBLV_1	-	brain	+	+	-
34495	mouse	2012	Germany	BBLV_2	-	brain	+	-	-

Supplementary Table 4: Comparison of between results obtained with sample set III in the laboratories at Onderstepoort (SA) and Friedrich-Loeffler-Institut (FLI)

							BioGen		Bionote	Quicking		Green Spring		Ubio	
Lab-ID	Species	Year	Origin	Virus species	Lineage	FAT-Result	SA	FLI (new batch)	SA	SA	FLI	SA	FLI	SA	FLI
06/15	Yellow mongoose	2015	Free State	RABV	nd	+	+	-	+	-	-	-	-	-	NA
75/15	Yellow mongoose	2015	Mpumalanga	RABV	Mongoose	+	+	-	+	-	-	+	+++	-	-
102/15	Jackal	2015	North West	RABV	Canid	+	+	-	+	-	-	+	++	-	-
110/15	Jackal	2015	North West	RABV	nd	+	+	-	+	-	-	+	+++	-	-
149/15	Civet	2015	Mpumalanga	RABV	Canid	+	+	-	+	-	+	-	++	-	-
14/15	Feline	2015	Limpopo	RABV	Canid	+	+	-	+	-	-	+	+	-	-
15/15	Caracal	2015	Limpopo	RABV	Canid	+	+	-	+	-	-	+	+	-	+
38/15	Feline	2015	Free State	RABV	Canid	+	+	-	+	+	-	+	++	+	-
113/15	Canine	2015	North West	RABV	Canid	+	+	-	+	-	-	+	++	-	-
130/15	Jackal	2015	Limpopo	RABV	nd	+	+	-	+	-	-	-	++	-	-
36/15	Bovine	2015	Limpopo	RABV	Canid	+	+	+	+	+	+	+	++	+	-
56/15	Bovine	2015	Free State	RABV	Mongoose	+	+	-	+	-	-	+	++	-	-
139/15	Bovine	2015	Free State	RABV	nd	+	+	-	+	-	-	+	++	-	-
146/15	Bovine	2015	North West	RABV	nd	+	+	+	+	-	-	+	++	-	-
153/15	Bovine	2015	North West	RABV	nd	+	+	-	+	+	-	+	++	-	-
41/15	Canine	2015	Limpopo	RABV	Canid	+	-	-	+	-	-	+	+	-	-
42/15	Canine	2015	Mpumalanga	RABV	Canid	+	-	-	+	+	-	-	-	-	-
55/15	Canine	2015	Free State	RABV	nd	+	-	-	+	-	-	+	-	-	-
66/15	Canine	2015	North West	RABV	Canid	+	+	-	+	-	-	+	+	-	-
125/15	Canine	2015	North West	RABV	nd	+	+	-	+	+	-	+	+	-	-
03/15	Canine	2015	North West	NC		-	-	-	-	-	-	-	-	-	-
17/15	Dassie	2015	Free State	NC		-	-	-	-	-	-	-	-	-	-
20/15	Yellow mongoose	2015	Limpopo	NC		-	-	-	-	-	-	-	+	-	-
22/15	Sable Antelope	2015	Limpopo	NC		-	-	-	-	-	-	-	-	-	-
26/15	Honey badger	2015	Mpumalanga	NC		-	-	-	-	-	-	-	-	-	-
54/15	Bovine	2015	Free State	NC		-	-	-	-	-	-	-	-	-	-
63/15	Canine	2015	Western Cape	NC		-	-	-	-	-	-	-	-	-	-
78/15	Giraffe	2015	Mpumalanga	NC		-	-	-	-	-	-	-	-	-	-
132/15	Feline	2015	Gauteng	NC		-	-	-	-	-	-	-	-	-	-
136/15	Bovine	2015	Limpopo	NC		-	-	-	-	-	-	-	+	-	-

Supplementary Table 5: Results of tissue culture supernatant tested in 2008 using the Bionote LFD (Cat.No.:RG 18-01; Lot NO.:1801029)

Virus	Titre (TCID50/ml)	Undiluted	1:10	1:100	1:1000	1:10000	1:100000	1:1000000
CVS	$10^{6.5}$	+	-	-	-	-	-	-
EBLV-1	$10^{6.2}$	+	+	-	-	-	-	-
EBLV-2	$10^{4.2}$	+	-	-	-	-	-	-

6.Discussion

Rabies still poses a human health threat almost worldwide. The greatest burden is carried by Asia and Africa, where dog-mediated rabies is responsible for the majority of human deaths caused by rabies (WHO, 2013a). Rabies surveillance in these regions is hampered by challenges in rabies diagnosis (Banyard et al., 2013). Lyssaviruses also circulate in bat populations and have been detected in many areas of the World including Europe (Schatz et al., 2013a). Although bat rabies surveillance in parts of Europe give an insight into the epidemiology of bat lyssaviruses, little is known about the transmission and maintenance of lyssaviruses in bat colonies (Freuling et al., 2009a). Previous surveillance revealed that EBLV-1 is the most common lyssavirus present in Europe and that there is genomic variation within this lyssavirus species in the form of sublineages and indels (Freuling et al., 2012, Johnson et al., 2007, Amengual et al., 1997). Human deaths due to bat mediated rabies in Europe have been reported and therefore it is important to monitor the epidemiological situation (Fooks et al., 2003a). Furthermore, investigation of the respective lyssaviruses, e.g. their pathogenicity, is important to assess their zoonotic potential.

Assessment of EBLV-1 pathogenicity:

The low spill-over rate of EBLV-1 from bats to animals and humans compared to RABV, where spill-over infections occur more frequently, presents a conundrum (Johnson et al., 2010, Kuzmin et al., 2012, Schatz et al., 2014a). Possible explanations include differences in host densities and contact rates of the reservoirs with terrestrial animals and humans, as well as the properties of the viruses (Freuling et al., 2009a). Pathogenicity studies in various animal species indicate that their susceptibility to EBLV-1 may be limited, however the results were obtained using single isolates (Cliquet et al., 2009, Brookes et al., 2007, Tjornehoj et al., 2006, Vos et al., 2004b, Vos et al., 2004a). Previously the pathogenicity of LBV was underestimated, based on limited data for only one isolate that indicated an apathogenic phenotype, following peripheral inoculation (Badrane et al., 2001). However, later investigations with other LBV isolates showed that this was not true (Markotter et al., 2008).

Therefore, in order to assess the impact of natural genetic variation on EBLV-1 pathogenicity, selected isolates were compared under standardised conditions in a mouse model. The results demonstrate that differences between the EBLV-1 isolates exist, regarding their pathogenicity as well as their incubation periods. These results need to be considered for the interpretation of previous studies, including studies where the pathogenicity of different lyssaviruses was compared. In previous experimental studies, all lyssaviruses present in Germany were shown to be pathogenic. EBLV-1 displayed higher mortalities in mice and ferrets following peripheral inoculation compared to EBLV-2, while the pathogenicity of EBLV-1 in mice was comparable to BBLV (Vos et al., 2004a, Nolden et al., 2014). The EBLV-1 isolate used by Vos et al. is identical to the EBLV-1 isolate 13454_EBLV-1a, while in the study by Nolden et al. another EBLV-1 isolate was investigated, but under the same experimental conditions as in this thesis. This second EBLV-1 isolate displayed comparable pathogenicity to isolate 5782_EBLV-1a_del (Nolden et al., 2014). The other seven EBLV-1 isolates investigated in this thesis were less pathogenic, with 100% survival rates after intramuscular (i.m.) inoculation with low doses. This suggests, that at least for this animal model, i.e. 3 week old Balb/c mice, EBLV-2 and BBLV are in fact more pathogenic than EBLV-1. Interestingly isolate 13454_EBLV-1_ref, less pathogenic than EBLV-2 in Balb/c mice, displayed higher mortalities in mice and ferrets following peripheral inoculation (Vos et al., 2004a). However, different EBLV-2 isolates might have been used in the two investigations and there might be differences in the pathogenicity of the isolates. Therefore, investigation of these and other EBLV-2 and BBLV isolates under the same experimental conditions are warranted. Furthermore, different animal models were used in those studies, i.e. Balb/c mice, CD1-mice and ferrets (Nolden et al., 2014, Vos et al., 2004a), and it is known that the pathogenicity of lyssaviruses depends on the animal species (Jackson and Fu, 2013). Since in this thesis, a very sensitive model, i.e. not fully immunocompetent mice, was used to detect even slight differences in the properties of the isolates, it is unclear whether these differences also apply to other animal species, including the natural reservoir for EBLV-1. Therefore, these results can provide only an indication how these viruses might behave in their reservoirs or in humans. Two studies assessed the pathogenicity of EBLV-1 in bats, i.e. in the serotine bat, as the reservoir species, and the big brown bat (*Eptesicus fuscus*), a bat species related to the serotine bat. In both studies, following peripheral inoculation with EBLV-1 mortalities were below 57% depending on

inoculation route and dose (Freuling et al., 2009b, Franka et al., 2008). To determine if the EBLV-1 isolates would also display differences in pathogenicity, when inoculated into their reservoir host, further studies are warranted, but difficult to implement due to the protected status of European bats (UNEP/EUROBATS, 1994).

A correlation between the inoculation route, the clinical signs as well as the virus distribution in the brain was observed, with the virus distribution in different brain regions, as well as the clinical signs being dependent upon the inoculation route. Although previous studies examined distribution of EBLV-1, EBLV-2 and RABV in different brain regions, the viruses were always inoculated into the left hind footpad. Thus the impact of the inoculation route on virus distribution was not assessed (Hicks et al., 2009, Healy et al., 2013). In these studies, virus antigen was most frequently observed in the pons and rostral medulla, followed by the cortex 3, hypothalamus and thalamus (Hicks et al., 2009). The highest quantity of virus antigen was present in the pons and rostral Medulla (Healy et al., 2013). No difference between the lyssavirus species could be observed (Hicks et al., 2009, Healy et al., 2013). These results are concordant to the results presented here, as following i.m. inoculation the medulla contained most virus antigen and no difference in virus distribution between the RABV isolate and the EBLV-1 isolates could be observed. The high antigen content in the medulla following intranasal (i.n.) inoculation could be due to the development of clinical signs at a later stage of infection. Another possibility would be that the virus following i.n. inoculation does not just spread via the olfactory nerve, but also via sensory neurons of the ophthalmic nerve and subsequent trigeminal nerve as described previously (Lafay et al., 1991, Hronovsky and Benda, 1969a). In one study, following i.n. inoculation with EBLV-2 and RABV, virus was detected in the lung, stomach and tongue, indicating that the inoculum gets swallowed and aspirated, which could result in multiple routes of entry (Johnson et al., 2006). Although the inoculum used in this thesis was considerably smaller (10µl compared to 30µl), the possibility of another entry route cannot be entirely ruled out (Johnson et al., 2006).

While a number of i.m. infected mice developed antibodies, there was a complete absence of seroconversion following i.n. inoculation. Although this had been described previously in tested survivors of a mouse study after inoculation with EBLV-2 and RABV, survivors of a study where guinea pigs were infected via aerosol with a RABV strain developed antibodies

(Hronovsky and Benda, 1969b, Johnson et al., 2006). The absence of antibodies supports the theory, that after i.n. inoculation the virus directly enters the nervous system via olfactory or sensory neurons, without inducing an immune response. Generally no correlation between survival and seroconversion of the mice was observed following i.m. inoculation with EBLV-1, which is concordant with previous results, where even after four subsequent multiple peripheral exposures of mice to EBLV-1, EBLV-2 and RABV over a period of ca. 3 months seroconversion in survivors did not always occur (Banyard et al., 2014b). Similar observations were also made in bats as the reservoir hosts of lyssaviruses. Following s.c. infection of big brown bats and serotine bats with EBLV-1, seroconversion of all or none of the infected animals was observed, respectively (Franka et al., 2008, Freuling et al., 2009b). Furthermore, antibody titres in bats seem to drop within a year, as demonstrated in studies using ABLV and RABV (McColl et al., 2002, Turmelle et al., 2010).

The results suggest, that seroconversion after exposure to lyssaviruses does not always occur in mice and bats, and that in the latter antibody titres tend to drop relatively soon following infection, although the animals are still protected (Turmelle et al., 2010). This needs to be taken into consideration for the interpretation of serological results during active surveillance studies in bats (Banyard et al., 2011). The seroprevalence of lyssavirus antibodies in bats in Europe varies (Schatz et al., 2013a). Estimating the prevalence of bat lyssaviruses in Europe from these serologic results is difficult, considering that there is evidence, that seroconversion following exposure to lyssaviruses does not always occur and is not of long duration. Coupled with afore mentioned difficulties, i.e. non-standardized serological test procedures and cross-neutralization of phylogroup 1 lyssaviruses, active surveillance can provide only limited information (Freuling et al., 2009a, Schatz et al., 2014a).

Enhanced passive bat rabies surveillance in Germany:

Passive bat rabies surveillance is the method of choice to obtain information on bat rabies epidemiology (Freuling et al., 2009a, Schatz et al., 2014a). The continued isolation of EBLV-1, EBLV-2 and BBLV shows that routine surveillance as well as enhanced passive bat rabies surveillance contribute to the understanding of the epidemiology of bat lyssaviruses in

Germany. Although the majority of bat rabies cases were detected during routine surveillance, enhanced passive bat rabies surveillance provides additional information, through the detection of bat rabies cases which would otherwise be missed, and also gives a better estimate of the true prevalence of bat lyssaviruses in Germany. For example the spill-over case of BBLV in the common pipistrelle in 2015, described in this thesis, was only detected during enhanced passive bat rabies surveillance. The discovery of two further BBLV cases within a year doubled the number of BBLV cases detected in Germany. This sudden emergence of BBLV represents somewhat of a mystery. BBLV seems to be as or even higher pathogenic than EBLV-1, at least in the mouse model used here. As human spill-over cases have been described for EBLV-1 (Fooks et al., 2003a, Selimov et al., 1989), the zoonotic potential of BBLV should also not be underestimated. BBLV RNA was present in various organs of the BBLV infected bats, suggesting wide-spread virus distribution at the host level. Similar observations were made in naturally EBLV-1 infected serotine bats, EBLV-2 infected Daubenton's bats and RABV infected non-hematophagous bats (Schatz et al., 2014b, Allendorf et al., 2012). Interestingly, in an EBLV-1 spill-over case of a Nathusius' pipistrelle bat, virus RNA was only detected in the heart and replication competent virus could be isolated only from the brain (Schatz et al., 2014b). In comparison, BBLV RNA was detected in all tested organs and viable virus in 5 out of 8 organs of the common pipistrelle bat. These results indicate that lyssavirus distribution from the central nervous system to various peripheral organs is apparently common for natural infected bats. Virus was isolated from the salivary glands of two investigated bats and since the presence of viable virus in the salivary glands is of outmost importance for onward transmission, this supports the potential of BBLV to cause spill-over infections.

The pathogenicity of BBLV has been investigated using two isolates (21961-Bokeloh, 29008-Lichtenfels) with a nucleotide identity of 92.9%, which cluster into the two separate sublineages A and B proposed here. No difference in the pathogenicity of the BBLV isolates, was observed (Nolden et al., 2014) although one AA exchange (His523Gln) in the glycoprotein is present in a potential pathogenicity determining site (Babault et al., 2011, Prehaud et al., 2010). This AA exchange is so far only present in BBLV isolates from Lower Saxony. No other AA exchanges were observed in known pathogenicity determining sites of the BBLV isolates (For references see S4 table, EBLV-1 paper). Overall the data obtained so far provide no indication that BBLV isolates differ in their pathogenicity, although the same

was true for EBLV-1 where differences in the pathogenicity of isolates were discovered. Here, continuing surveillance for BBLV and investigation of novel isolates is of importance.

Passive bat rabies surveillance is also important for determining the diversity and evolution of lyssavirus isolates by genetic analyses and in vitro and in vivo studies. By isolation and archiving of isolates, these are available for further investigations, e.g. determination of the impact of genetic variation on the pathogenicity as described in the previous section. The results of such investigations provide important information for the assessment of the human health threat posed by endemic lyssaviruses. Bat rabies control is not feasible in Europe and has so far only been performed for RABV in Vampire bats in America (Kuzmin and Rupprecht, 2015). These programs focus of on the reduction or elimination of vampire bat populations through culling, but there are strong indications that these measures are counterproductive and in the long term result in a dispersal of the bats and reduction in seroprevalence (Johnson et al., 2014). Population reduction in European bats would be ineffective and is due to their protected status not an option (Kuzmin and Rupprecht, 2015).

LFDs as a tool for rabies surveillance in developing countries:

Control of dog-mediated rabies and subsequent elimination has already been performed successfully. This could greatly reduce the number of human rabies cases, as rabid dogs present the main source for human infection (Müller et al., 2012). However, in developing countries in Asia and Africa, which are most affected, diagnosing rabies is challenging (Banyard et al., 2013). Therefore, LFDs as an alternative diagnostic tool for rabies were developed and six commercially available LFDs were evaluated in this thesis.

Unfortunately, the performance of the LFDs was not satisfactory, as test agreement of the LFDs, with the gold standard FAT, for experimentally infected animals and archived field samples were low (Kappa < 0.37). Only for field samples from South Africa test agreement between four of the LFDs and FAT was good to perfect (Kappa 0.72-1.00). Overall, investigated LFDs displayed insufficient sensitivities and reproducibility. These are known weaknesses of traditionally designed LFDs and can be caused by the materials incorporated in the LFD, their treatment and the final assembly of the test strip (O'Farrell, 2013). For example the nitrocellulose membrane and its treatment, as well as the conjugate pad

material and the many processing steps required for conjugate application can have a notable impact on the reproducibility of LFDs (O'Farrell, 2013, Posthuma-Trumpie et al., 2009). There was no information regarding composition, the treatment of the components, and the antibodies used of the investigated LFDs, except for the Bionote LFD (Kang et al., 2007).

In other studies, evaluating the Bionote test, sensitivities and specificities of 85%-100% and 93%-100% were observed respectively (Table 3). In the results presented here, a discrepancy in the sensitivities of the Bionote test was also seen between and within batches, considering the different sample sets, previous testing at the Friedrich-Loeffler-Institute (FLI) and the results from Italy. Although there are differences between the published studies, i.e. different samples and batches of the test used and test implementation and evaluation by different individuals, the simplicity of the test system should enable it to function independently from these factors. After all, the simple nature of the LFDs should be one of their strengths (O'Farrell, 2015).

A very interesting feature of the LFDs first demonstrated here and later confirmed in another study (Lechenne et al., 2016), is that RNA can be stored in the used test strips for a longer period of time, i.e. six weeks. As test strips can be considered no longer infectious one hour after use, they could be sent to a laboratory for lyssavirus characterisation using RT-PCR or sequencing. However, cooperation with a laboratory containing the necessary facilities is mandatory and results are not immediately available. An alternative would be a multiplex LFD which is able to differentiate between the lyssavirus species. As multiplexing on one test strip provides several challenges, separate strips with specific antibodies for the different lyssaviruses might be a solution (Li and Macdonald, 2016). This would on the other hand increase the cost for testing, as more LFDs are needed for rabies diagnosis. As there are often difficulties with the funding of rabies surveillance in developing countries, a single LFD with a broad reacting antibody for the detection of preferably all lyssaviruses might be the better option. In these regions the focus is on dog rabies surveillance and elimination, since most human rabies cases are dog-mediated. As more than 99% of rabies cases in terrestrial mammals are caused by RABV, the identification of the lyssavirus species is not a necessity for rabies elimination in these animals. One region where a multiplex LFD might be appropriate would be Africa, where phylogroup 2 lyssaviruses, i.e. MOKV and LBV, are

endemic. Since for both lyssaviruses infections of companion animals, i.e. cats and dogs, have been reported (Sabeta et al., 2007, Markotter et al., 2006), a LFD which is able to differentiate between phylogroups 1 and 2 would be useful, especially if a human exposure has occurred.

Publication of the data presented here, resulted in an echo of the media, including a press release by the journal Public Library of Science Neglected Tropical Diseases (PLOS NTDs), which was picked up by other news agencies, i.e. ScienceDaily, Labmedica, Scimex, EurekAlert and the New York Times (McNeil Jr., 2016, Labmedica International staff writers, 2016, PLOS Neglected Tropical Diseases, 2016, PLOS, 2016b, PLOS, 2016a). Furthermore, in a search for continued availability of LFDs used in this study in May 2017 only four out of the six LFDs were found, with two (Creative diagnostics, Biogen) apparently no longer available. An extended search for other commercially available LFDs using the keywords “rapid rabies test” resulted in the discovery of thirteen additional LFDs now commercially available (Appendix Table 1). However, there is hardly any information regarding their composition with sometimes confusing descriptions, e.g. what sample to use, if the tests aim at the detection of rabies antigen or antibodies, or mention of other infectious agents indicating copy and paste of the test description (see Table 4, No. 2, 4, 13). Still, the number of tests available highlight the interest in and need for a rapid test system for rabies diagnosis and prove that LFDs in general are promising candidates for field diagnosis of rabies.

As mentioned previously, the development of a functional, i.e. sensitive, specific and reliable, LFD would greatly improve rabies surveillance and subsequent elimination in developing countries, but test development is only the first step. As far as information is provided within the manufacturer’s instruction, apart from Bionote, three further LFDs were developed for rabies diagnosis, which were never commercialized and can therefore not be acquired by countries in need (Ahmed et al., 2012, Kasempimolporn et al., 2011, Nishizono et al., 2008). Therefore, besides test development and extensive validation, collaboration with a company is important to make the test commercially available.

Conclusion:

Rabies presents a human health threat which, although the elimination of dog-mediated rabies would reduce it immensely, can never be erased due to the presence of bat lyssaviruses. Since bat rabies elimination is not feasible for various reasons, monitoring bat lyssaviruses in the scope of preferably passive surveillance is the only option. This enables a regular assessment of bat lyssaviruses epidemiology and the resulting possible human health threat, and is therefore extensively performed in Germany. Furthermore, assessing the properties of the viruses themselves is of importance to identify and quantify possible sources and risks of transmission and infection. Elimination of dog-mediated human rabies is aimed to be achieved by 2030, but beforehand several major challenges, e.g. concerning rabies diagnosis, need to be overcome.

7.Summary

Rabies, a zoonotic disease known since ancient times, is caused by lyssaviruses and almost always fatal once clinical signs develop. Although most of Europe, including Germany, is free of terrestrial rabies, bat rabies is still present. Surveillance in Europe showed that most bat rabies cases are caused by European bat lyssavirus 1 (EBLV-1). For this virus spill-over infections in various terrestrial mammals including humans have been described. Two genetic sublineages of EBLV-1 exist and a third one has been proposed. Furthermore, insertions and deletions were found in the genomes of selected isolates. Since it was proven for other lyssaviruses that genetic variation can have an impact on the pathogenicity of the respective isolates, it was investigated if the same is true for EBLV-1. Differences in the pathogenicity of investigated isolates were discovered after intranasal and intramuscular inoculation, although at the moment it is impossible to determine the exact cause. Upon comparison of the different inoculation routes, i.e. intracranial, intramuscular and intranasal, differences in the clinical signs of the mice as well as in the virus distribution within the brain were discovered.

The isolates used in the pathogenicity study were obtained in the scope of passive surveillance, providing another example of the benefit of such programs. Furthermore, surveillance is important to assess the potential human health threat of endemic bat rabies by providing epidemiological data. Passive surveillance led to the detection of 362 bat rabies cases in Germany until 2016. The majority were caused by EBLV-1, but five cases of European bat lyssavirus 2 (EBLV-2) and six cases of Bokeloh bat lyssavirus (BBLV) were also discovered, including in 2015 the first case of BBLV in another bat species apart from its presumed reservoir host the Natterer's bat. Interestingly within the same year two additional cases of BBLV were found in Germany, doubling the number of BBLV cases there from three to six. Eight BBLV isolates were so far detected altogether and a division of the isolates into two genetic lineages A and B was possible. At the host level there is a massive distribution of virus to the peripheral organs. This sudden emergence of BBLV, despite similar surveillance intensity over the past years, is puzzling.

The majority of human rabies cases are caused by rabid dogs and occur prominently in Asia and Africa, resulting in an estimated 59000 human deaths per year. In these regions rabies

surveillance is hindered by several factors including challenges in rabies diagnosis. Therefore, alternative test methods to the standard diagnostic tests for rabies were developed, including Lateral flow devices (LFDs), which have potential for field use. However, the comparison of six commercially available LFDs performed here revealed that the tests have major deficits regarding their sensitivity and reproducibility.

8.Zusammenfassung

Die Tollwut ist eine zoonotische Erkrankung, die durch Lyssaviren verursacht wird, welche mit dem Auftreten klinischer Symptome fast immer tödlich endet. Obwohl große Teile Europas inklusive Deutschland frei von terrestrischer Tollwut sind, spielt die Fledermaustollwut in diesen Gebieten eine wichtige Rolle. Die Fledermaustollwutüberwachung in Europa hat gezeigt, dass das Europäische Fledermaustollwutvirus 1 (EBLV-1) für die meisten Fälle verantwortlich ist. Vereinzelte Infektionen anderer Tierarten und des Menschen wurden beschrieben. Während für EBLV-1 zwei genetisch unterschiedliche Entwicklungslinien anerkannt sind, ist eine dritte derzeit noch in Diskussion. Bei einigen wenigen EBLV-1 Isolaten wurden bislang einzigartige Insertionen und Deletionen in bestimmten Bereichen des Genoms beschrieben. Wie für andere Lyssaviren bereits gezeigt wurde, können genetische Variationen die Pathogenität beeinflussen. Daher bestand ein wesentlicher Teil dieser Arbeit darin, herauszufinden, ob dies auch bei derartigen EBLV-1 Isolaten zutrifft. Unterschiede in der Pathogenität der untersuchten Isolate wurden nach intranasaler und intramuskulärer Inokulation von Mäusen gefunden. Allerdings ist unklar, welcher dieser genetischen Unterschiede dafür verantwortlich ist. Beim Vergleich der unterschiedlichen Inokulationsrouten, d.h. intrakranial, intramuskulär und intranasal, wurden Unterschiede im klinischen Bild der inokulierten Mäuse sowie der Virusverteilung im Gehirn gefunden.

Die für die Pathogenitätsstudie verwendeten EBLV-1 Isolate wurden im Rahmen der passiven Fledermaustollwutüberwachung gefunden und stellen damit ein Beispiel für den Nutzen solcher Programme dar. Darüber hinaus ist die Überwachung und anschließende Auswertung epidemiologischer Daten eine wichtige Voraussetzung für die Risikobewertung hinsichtlich einer potentiellen Gefährdung des Menschen durch endemische Fledermaustollwut. Im Rahmen der passiven Überwachung wurden bis zum Jahr 2016 insgesamt 362 Fledermaustollwutfälle in Deutschland diagnostiziert. Während EBLV-1 für den Großteil dieser Fälle verantwortlich war, entfielen fünf Fälle auf das Europäische Fledermaustollwutvirus 2 (EBLV-2) und sechs Fälle auf das Bokeloh Fledermaustollwutvirus (BBLV). Einer dieser durch BBLV verursachten Tollwutfälle wurde 2015 in einer anderen Fledermausart als dem vermuteten Reservoir, der Fransenfledermaus, gefunden. Interessanterweise trat innerhalb eines Jahres eine unerwartete Häufung von BBLV Fällen

auf. Insgesamt sind bislang acht BBLV-Isolate nachgewiesen worden, welche in zwei unterschiedliche Entwicklungslinien, A und B, eingeteilt werden können.

Tollwütige Hunde sind für die überwältigende Mehrheit der schätzungsweise 59000 humanen Tollwutfälle in Asien und Afrika verantwortlich. In diesen Regionen wird die Tollwutüberwachung stark durch gesundheitspolitische und infrastrukturelle Probleme, aber auch fehlende technische Ausrüstung und Engpässe in der Tollwutdiagnostik beeinflusst. Aus diesem Grund wurden Point-of-care Tests, wie z.B. Lateral flow devices (LFD), als alternative Verfahren zu standardisierten diagnostischen Tollwuttestmethoden entwickelt, die jedoch bislang nicht ausreichend validiert sind. Die vergleichende Evaluierung sechs kommerziell erhältlicher Tollwut-LFDs ergab, dass diese Tests große Defizite hinsichtlich ihrer Sensitivität und Reproduzierbarkeit aufweisen.

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10.Appendix

No.	Name	Country	Source
1	Dianotech Rapid Rabies Ag Test Card	China	http://www.lyncmed.com/product/997.htm
2	Raysonbio Rabies Virus Antigen Rapid Test Kit	China	http://www.globalsources.com/si/AS/Jiangsu-Rayson/6008850358162/pdtl/Rabies-Virus-RV-Antigen-Rapid-Test-Kit/1132150614.htm
3	Rohi Biotechnology Rabies Virus Ag Rapid Test	China	http://rohibio.en.alibaba.com/product/60387345482-802442170/Manufacturer_of_Rabies_Virus_Ag_Rapid_Test_with_ISO_certification.html
4	Finder Canine Rabies Virus (RBV) Antigen Test Strip	China	http://www-finderbio-com-en.sell.ecer.com/pz6e0794a-canine-rabies-virus-rbv-antigen-test-strip-colloidal-gold.html
5	Intas Rabies Antigen Rapid Test Kit	India	http://www.weiku.com/products/14615294/Rabies_Antigen_Rapid_Test_Kit.html
6	Diavetra Rabies antigen test	India	http://www.toboc.com/images/pdf/764982.pdf
7	LilliTest Rapid Rabies Ag Test Kit	England	http://diagnostics.lillidale.co.uk/wp-content/uploads/2016/08/LilliTest-Rabies-Ag-Test-Kit.pdf
8	Swissavans rabies antigen test Kit	Switzerland	http://www.swissavans.com/domains/swissavans_com/data/free_docs/RapidTests_PI_de.pdf
9	InterMedical Rapid Test Device Rabies Ag	Italy	http://www.intermedical.it/en/products/veterinary/
10	MINITEST Rabies Ag kit	Austria/ France	http://biomedica.eval3.css4you.at/fileadmin/data/VetRapidTests_2011-06_AT_CZ_HU_PL.pdf
11	Reagen rabies virus antigen rapid Test Kit	USA	http://www.bio-equip.cn/enshow1equip.asp?equipid=58335&division=2535
12	Medigen Rabies Ag Test Kit	USA	http://www.stallionpublishers.com/publications/1122/p/medigen_one_step_rapid_test_kitv2.pdf
13	Abgenome Ag Rapid Canine Rabies Virus Test Kit	unknown	https://www.dhgate.com/store/product/crv-ag-rapid-canine-rabies-virus-test-one/255686682.html

Appendix Table 1: Summary of other rapid rabies test for the detection of rabies virus antigen.

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