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Surveillance, Pathogenicity and Shedding of Lyssaviruses

von Antonia Klein

aus Ludwigsfelde

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Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen Fakultät
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TABLE OF CONTENTS

I. INTRODUCTION

The fascinating and continuously expanding genus of lyssaviruses comprises a plethora of different RNA virus species, which are globally distributed and mostly associated with bats, their ancestral principal reservoir hosts (Fooks et al., 2017). Including their best-known representative, the prototypical rabies virus (RABV), all of them are potentially capable of infecting mammals and cause the zoonotic disease rabies. Transmitted via infectious saliva through bites or scratches of infected animals, lyssavirus infections cause an acute progressive encephalomyelitis. Due to the absence of efficacious treatment methods, rabies inevitably leads to death after the onset of symptoms (World Health Organization, 2018).

With one of the highest case fatality rates among all infectious diseases (Rupprecht and Dietzschold, 2017) and predominantly affecting low- and middle-income countries in Asia and Africa, rabies is considered as one of the most prominent neglected tropical diseases (Molyneux et al., 2017). Despite the existence of effective vaccines and adequate post-exposure prophylaxis (PEP), worldwide, more than 59,000 rabies-induced human fatalities are reported annually, with the estimated number of unrecorded cases being considerably higher (Hampson et al., 2015). The vast majority of cases is transmitted through rabid dogs (World Health Organization, 2018), however, human rabies infections associated with bats are also occasionally reported. One essential step towards rabies control and eventual elimination is to increase and refine extensive global rabies surveillance programs combined with data aggregation. Therefore, reliable, rapid and straightforward diagnostic solutions as, for example, the so-called lateral flow devices (LFDs) could contribute to rabies surveillance, in particular in low-resource settings.

In countries where terrestrial rabies has already been successfully eliminated, the continued presence of bat-related rabies still poses a threat to human and animal health and makes a complete elimination rather challenging. To this end, various national surveillance programs for bat associated lyssaviruses have been established to further our understanding of lyssavirus epidemiology, emergence and spread. The rarely though constant emergence of novel lyssavirus species during the last decades highlights the significance of those projects. Furthermore, advanced in-vitro and in-vivo studies of different bat-related lyssavirus species

are crucial for expanding our so far insufficient knowledge on pathogenesis and virus-host interactions to better assess a potential public health risk.

To implement a global rabies control strategy in line with the One Health approach, not only medical education and prevention but also surveillance based on simple and reliable diagnostic methods as well as a risk assessment based on scientific analyses are of utmost importance.

To this end, the present study aims at investigating aspects of surveillance, pathogenicity and virus shedding of different lyssaviruses and thus their impact on public health. The diagnostic performance of several commercially available rapid LFDs used as a rapid point-of-care test for rabies diagnostics and surveillance were comparatively evaluated. Unsatisfying results regarding specificity and sensitivity confirmed previous findings (Eggerbauer et al., 2016), and clearly indicated that strict quality control measures should be followed before these tests can be used for rabies diagnosis in animals. Additionally, enhanced passive bat lyssavirus surveillance in Germany was continued by using a refined sampling scheme and a novel diagnostic approach based on molecular diagnostic methods. In this context, the tenth reported case of the novel Bokeloh bat lyssavirus (BBLV) in Europe was detected.

The primary focus of this work was the assessment of the comparative pathogenicity and virus shedding of bat-related lyssaviruses as opposed to classical RABV. Therefore, in-vivo experimental studies in a standardized mouse model were performed. Based on the resulting data, a novel pathogenicity index to classify lyssaviruses according to their pathogenic phenotype in mice was established. Furthermore, the significance of virus shedding on onward cross-species transmission and potential sustained spillover was investigated. The outcome corroborates field data of very limited transmission of other lyssaviruses (Johnson et al., 2010) to conspecifics or terrestrial species when compared to RABV.

II. LITERATURE REVIEW

1. Lyssaviruses

1.1. Virus Taxonomy

All lyssavirus species form a distinct monophyletic group of negative-sensed and single-stranded RNA viruses that belong to the family *Rhabdoviridae* of the order *Mononegavirales*. At present, the genus *Lyssavirus* comprises 17 different officially recognized virus species (Table 1) according to the International Committee on Taxonomy of Viruses (ICTV) (ICTV, 2017; Walker et al., 2020). Lyssaviruses are assigned to different virus species following specific demarcation criteria, e.g. genetic distances (cut-off of 80 % - 82 % nucleotide identity for the N gene for distinction between species), immunological cross-reactivity, as well as geographical spread and host range (Kuzmin et al., 2005). Two recently identified lyssaviruses, the Kotalahti bat lyssavirus (KBLV) isolated from a bat in Finland in 2017 (Nokireki et al., 2018), and the Matlo bat lyssavirus (MBLV) isolated from bats in South Africa (Coertse et al., 2020; Grobler et al., 2021), await further classification.

Within the genus *Lyssaviruses*, the virus species are distinguished into at least three different phylogroups (Fooks and Jackson, 2020) based on sequence similarities as well as on related antigenic and pathogenic characteristics (Badrane et al., 2001; Kuzmin et al., 2005). Phylogroup I comprises the prototypical Rabies virus (RABV), Aravan virus (ARAV), Australian bat lyssavirus (ABLV), Bokeloh bat lyssavirus (BBLV), Duvenhage virus (DUVV), European bat lyssavirus 1 (EBLV-1) European bat lyssavirus 2 (EBLV-2), Gannoruwa bat lyssavirus (GBLV), Irkut lyssavirus (IRKV), Khujand lyssavirus (KHUV), and Taiwan Bat Lyssavirus (TWBLV), while Lagos bat lyssavirus (LBV), Mokola lyssavirus (MOKV), and Shimoni bat lyssavirus (SHIBV) belong to phylogroup II (Badrane et al., 2001). Due to the distinct phylogenetic relation, it is assumed that the most genetically divergent lyssaviruses including Ikoma virus (IKOV) (Marston et al., 2012), Lleida bat lyssavirus (LLEBV) (Banyard et al., 2018) and West Caucasian bat lyssavirus (WCBV) (Botvinkin et al., 2003; Kuzmin et al., 2005), form a detached phylogroup III (Aréchiga Ceballos et al., 2013; Markotter and Coertse, 2018) and even a potential fourths phylogroup represented by IKOV and LLEBV has already been discussed (Fooks et al., 2014). However, this classification will be subject to constant adaption in response to the increasing diversity of the lyssavirus genus (Fooks, 2004).

Table 1 Overview of lyssavirus taxonomy according to current (as of August 2021) ICTV classification (ICTV, 2020) including distribution, reservoir and phylogroup (modified from (Fooks and Jackson, 2020).

Virus Species	Virus Name	Abbreviation	Distribution	Reservoir (Main Host Species)	Phylogroup
Rabies lyssavirus	**Rabies virus**	RABV	Worldwide except Australia	Several species of wild carnivors and domestic dogs, bats (Americas only)	I
Aravan lyssavirus	**Aravan virus**	ARAV	Asia	*Microchiroptera (Myotis blythi)**	I
Australian bat lyssavirus	**Australian bat lyssavirus**	ABLV	Australia	*Megachiroptera* (Pteropid species) *Microchiroptera (Saccolaimus flavicentris)*	I
Bokeloh bat lyssavirus	**Bokeloh bat lyssavirus**	BBLV	Europe	*Chiroptera (Myotis nattereri)*	I
Duvenhage lyssavirus	**Duvenhage virus**	DUVV	Africa	Several *single cases in Microchiroptera**	I
European bat 1 lyssavirus	**European bat lyssavirus 1**	EBLV-1	Europe	*Microchiroptera (Eptesicus serotinus, E. isabellinus)*	I
European bat 2 lyssavirus	**European bat lyssavirus 2**	EBLV-2	Europe	*Microchiroptera (Myotis dasycneme and Myotis daubentoni)*	I
Gannoruwa bat lyssavirus	**Gannoruwa bat lyssavirus**	GBLV	Asia	*Megachiroptera (Pteropus medius)*	I
Irkut lyssavirus	**Irkut virus**	IRKV	Asia	*Microchiroptera (Murina leucogaster)**	I
Khujand lyssavirus	**Khujand virus**	KHUV	Asia	*Microchiroptera (Myotis daubentoni)**	I
***Kotalahti bat lyssavirus**[#]*	**Kotalahti bat lyssavirus**	KBLV	Europe	*Microchiroptera (Myotis brandti)**	I
Taiwan bat lyssavirus	**Taiwan bat lyssavirus**	TWBV	Asia	*Microchiroptera (Pipistrellus abramus)*	I
Lagos bat lyssavirus	**Lagos bat virus**	LBV	Africa	*several Megachiroptera (e.g. Eidolon helvum)*	II
Mokola lyssavirus	**Mokola virus**	MOKV	Africa	Rodents and carnivors, unknown bat reservoir*	II
Shimoni bat lyssavirus	**Shimoni bat virus**	SHIBV	Africa	*Microchiroptera (Hipposideros commersoni)**	II
West Caucasian bat lyssavirus	**West Caucasian bat virus**	WCBV	Europe	*Microchiroptera (Miniopteris schreibersi)**	III
Lleida bat lyssavirus	**Lleida bat lyssavirus**	LLBV	Europe	*Microchiroptera (Miniopteris schreibersi)**	III/IV?
Ikoma lyssavirus	**Ikoma lyssavirus**	IKOV	Africa	*Civettictis civetta*, unknown bat reservoir*	III/IV?
***Matlo bat lyssavirus**[#]*	**Matlo bat lyssavirus**	MBLV	Africa	*Microchiroptera (Miniopterus natalensis)*	III?

[#]Not yet assigned as lyssavirus species by ICTV; [*]single cases in the respective host where a reservoir has not yet been confirmed

1.2. Morphology of Lyssaviruses

Lyssaviruses but rather all Rhabdoviruses share a characteristic morphology exhibiting one conical and an opposite planar end, shaping a unique bullet -or rod-like virus particle, where also the name *Rhabdoviridae* arose from (Greek: *rhabdos* – rod) (Fooks and Jackson, 2020). The enveloped lyssaviral virions measure in between 183-222 nm in length with an average diameter of 86 nm (Riedel et al., 2019) and contain the coiled helical nucleocapsid core with the RNA backbone.

1.2.1. Viral Genome

The single-stranded and non-segmented viral genomic ribonucleic acid (RNA) of negative-sense polarity is not infectious as direct translation by cellular polymerase is not possible. The length of the lyssavirus genome differs slightly between 11.9 and 12.2 thousand nucleotides, with RABV having the shortest and WCBV having the longest genome (Tordo et al., 1986b; Kuzmin et al., 2008). More than 99 % of the viral genome contain the genetic information for the five structural viral proteins in the order nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large RNA-dependent polymerase (L), which are arranged in a 3' - 5' orientation (Fooks and Jackson, 2020). This strictly conserved sequence is interrupted by non-coding intergenic regions of variable length between open reading frames and flanked by non-coding regulatory leader and trailer sequences at the ends, respectively (Tordo et al., 1986a; Albertini et al., 2011). A particularly long non-coding region between the G- and L-gene, referred to as the pseudogene, was assumed to be a remnant of an additional former gene, as it is transcribed together with the mRNA (messenger RNA) of the G-gene but not translated (Tordo et al., 1986a; Tordo et al., 1986b).

1.2.2. Virion Structure and Viral Proteins

The viral RNA is bound in its full length to the nucleoprotein, forming the ribonucleoprotein (RNP), which is itself associated with the viral RNA-dependent RNA-polymerase complex formed by the large polymerase and its cofactor, the phosphoprotein (Tordo and Poch, 1988; Conzelmann et al., 1990). Those RNP core components are surrounded by the viral matrix protein, which not only interacts with the nucleoprotein but also with a lipid bilayer envelope derived from the host cell as well as with the cytoplasmic component of the glycoprotein.

Thus, the matrix protein and the glycoprotein form the outer envelope around the core structure of the virion (Mebatsion et al., 1999; Guichard et al., 2011) (Figure1).

As a main component of the RNP, apart from the viral RNA, the nucleoprotein has been comprehensively studied due to its antigenic and immunogenic function. Even though the sequence of the nucleoprotein is the most conserved of the five viral proteins within the genus of lyssaviruses (Warrilow et al., 2002; Marston et al., 2007), a certain extent of diversity within short regions in the N gene between different species (Kissi et al., 1995) enabled differentiation on the nucleotide level. The specific configuration of the nucleoprotein embeds the viral RNA; hence, it is protected against enzymatic digestion through host ribonucleases (Albertini et al., 2011). The polymerase complex, which is responsible for the transcription as well as replication process, relies on a catalytic subunit, the large polymerase, which is the by far largest of the lyssavirus proteins (Tordo et al., 1986b). Its non-catalytic but essential cofactor is the dimeric phosphoprotein, a hyperphosphorylated protein that features up to five phosphorylation sites, depending on the respective virus strain (Gupta et al., 2000). It is the most diverse of the lyssavirus proteins (Marston et al., 2007) and apart from its importance for the replication and transcription process, phosphoprotein plays an essential role in host immune evasion as it functions as the main interferon (IFN) antagonist (Rieder and Conzelmann, 2011). It interferes with the innate immune response by either direct IFN antagonism (Brzózka et al., 2006) or by inhibiting IFN signaling pathways and downstream induction of IFN-stimulated genes (Vidy et al., 2005; Brzozka et al., 2006). Even though the matrix protein is of very small size, it is a multifunctional protein which is involved in several functional processes. As part of the viral envelope, it is partly located at the inner surface of the lipid-bilayer envelope and also sticks to the outside of the nucleocapsid, stabilizing the virion structure (Ben Khalifa et al., 2016). Its major function is condensing the RNP core into its typical bullet shape during virus assembly, thus it structurally connects envelope and nucleocapsid (Mebatsion et al., 1999; Ge et al., 2010; Riedel et al., 2020). The matrix protein also has regulatory effects on RNA synthesis, as it balances transcription and replication of the viral RNA (Finke et al., 2003; Finke and Conzelmann, 2003). Notably, the matrix protein by itself is capable of initiating virus budding, however, this exocytotic pathway was observed to be more efficient in the presence of the glycoprotein (Mebatsion et al., 1999). As the sole lyssavirus surface protein, the glycoprotein (Gaudin et al., 1992) is not only involved in virus budding but also responsible for targeting and binding to cellular receptors for cell entry. The

homo-trimeric structure of this transmembrane protein presents spike-like formations on the viral surface that bind to the host cell receptors to mediate cell entry (Gaudin et al., 1992). Therefore, the glycoprotein is responsible for the neurotropism of lyssaviruses and, as it is regarded a major pathogenicity determinant, it has been intensively studied. Due to the fact that it embodies the only target structure for neutralizing antibodies, it does also play an important role in lyssavirus immunity (Cox et al., 1977). The glycoprotein is the only glycosylated lyssavirus protein which is essential for intracellular transport processes and its antigenicity (Yamada et al., 2014). Notably, both the glycoprotein and matrix protein are also capable of inducing cellular apoptosis (Faber et al., 2002; Prehaud et al., 2003).

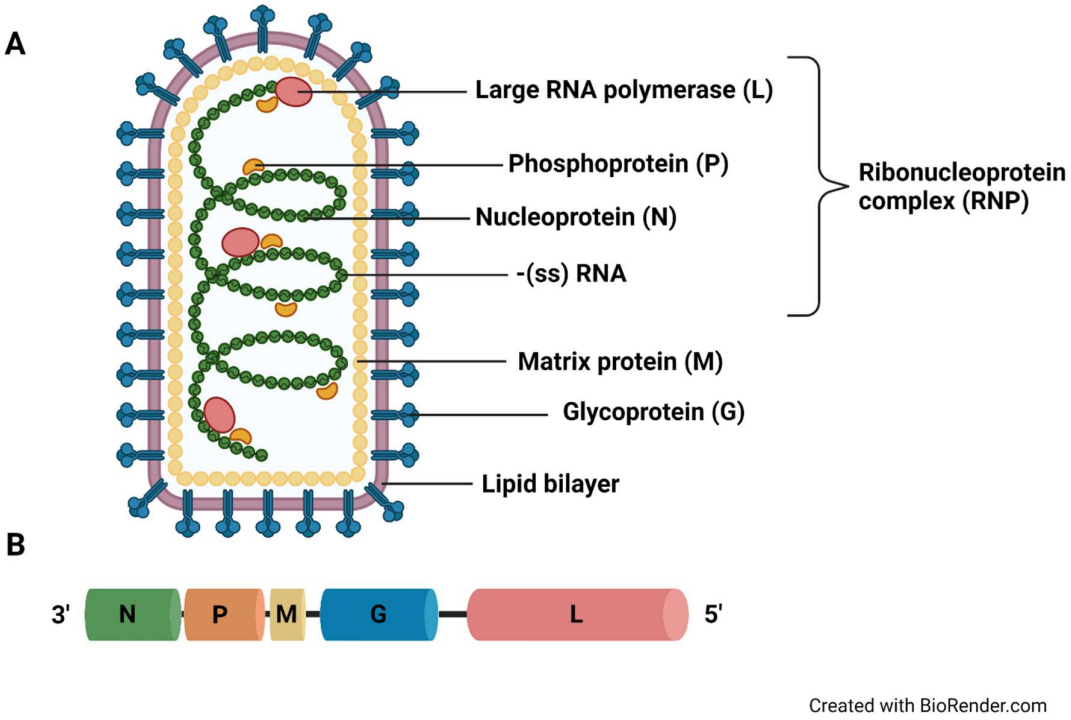

Figure 1 Schematic illustration of the lyssavirus particle **(A)** with its viral proteins; phosphoprotein (P), nucleoprotein (N), large polymerase (L), matrix protein (M) and glycoprotein (G) and the genome organization **(B)**.

1.3. Replication Cycle

For successful lyssavirus replication in the host cell, virus has to attach to cells for initiating receptor mediated endocytosis. Several cellular receptors were shown to interact with the

glycoprotein: the nicotinic acetylcholine receptor (nAChR) (Lentz et al., 1982), the neuronal cell adhesion molecule (NCAM) (Thoulouze et al., 1998), the p75 neurotrophin receptor (p75NTR) (Tuffereau et al., 1998) and the recently described metabotropic glutamate receptor 2 (mGluR2) (Wang et al., 2018). However, since cells without these receptors are also permissive for infection, other receptors must also be involved. After receptor-mediated endocytosis, a pH-dependent conformational change of the glycoprotein induces membrane fusion and thus the uncoating of the virion, releasing the RNP from the endocytic vesicle into the cytoplasm of the host cell (Gaudin et al., 1993). As a next step, the virion-associated large RNA polymerase synthesizes viral mRNA through primary transcription inside the host cell cytoplasm. Except for the glycoprotein, all other viral proteins are translated on free ribosomes within the cytoplasm. The G protein is constructed in the rough endoplasmic reticulum (rER), processed and translocated within the Golgi network, and integrated into the host cell plasma membrane (Tordo and Kouknetzoff, 1993). Subsequent synthesis of viral proteins induces the formation of multiple cytoplasmic inclusion bodies, in neuronal cells also referred to as Negri bodies. However, it has not yet been clearly defined whether those inclusion bodies only comprise an aggregation of viral proteins or whether -what is believed to be most likely- active viral transcription and replication are located within them (Lahaye et al., 2009). The increase of viral matrix protein concentration triggers a shift from transcription to replication activity of the large RNA polymerase, resulting in generation of full-length RNA genomes (Finke and Conzelmann, 2003). New viral core structures are formed with RNPs and the viral polymerase complex, which are subsequently condensed into their helical structures by the matrix protein. In a last step, also mediated by the matrix protein, itself assembles together with the RNP and the glycoprotein at the host cell plasma membrane to form new virus particles, which are in turn released from the host cell through the budding process (Mebatsion et al., 1999).

2. Historical Background

Probably due to its extraordinary disease progression and resulting dreadful clinical picture rabies has always been surrounded by terrifying myths and fascinated human mankind since the ancient times. The ancient Greek were the first to introduce the term “lyssa” for rabies which is assumedly derived either from the word “lysis” (loosing rational faculties), “lykos” (wolf) (Neville, 2004) or “lud” (violent) (Jackson, 2013). Each of those roots suggests a relation to the thousand-year-old association of a madness-derived, furious and animalistic bestial nature for one of the oldest known infectious diseases (Neville, 2004). The term “rabies” itself shall be either of Latin (“rabere” - to rage) or Sanskrit (“rabhas”- to do violence) origin (Baer, 1975). There are not only etymological but also historical indications for that both ancient Greeks and Romans were most probably already aware of a linkage between rabid animals and diseased humans, even though the infectious agent was still unknown. In 100 AD, the roman scholar Celsus actually suspected a poisonous agent within the saliva (Jackson, 2013). However, the described history of rabies traces its origin even further back to around 1900 BC in Mesopotamia. There, the Eshnunna code, an ancient collection of laws, defined that dog-owners whose mad dogs would bite fellow humans had to be fined (Tarantola, 2017). Similar to the situation in Eurasia, there is also a long history of rabies on the African continent (Fooks and Jackson, 2020). In contrast, in the New World it was only after the beginning of the European colonization that dog-mediated rabies spread all over the Americas (Velasco-Villa et al., 2017), and no evidence for rabies in the pre-Columbian era was found (Vos et al., 2011). Meanwhile, in Europe in 1769, the Italian pathologist Giovanni Morgagni who is generally regarded as the father of modern pathological anatomy already hypothesized the neuronal transport of the infectious agent for rabies (Jackson, 2013). Soon thereafter in 1804, Georg Gottfried Zinke was the very first to conduct in-vivo experiments to study disease transmission. He proved that the infectious agent is carried in saliva from one individual to another by infecting healthy animals with saliva from a rabid dog (Wilkinson, 1988). Thereafter, it was regarded a milestone in the history of rabies and its research when Louis Pasteur succeeded in developing the first anti-rabies vaccine. Made out of desiccated nerve tissue of infected rabbits, it served as a precursor for later vaccines and PEPs. It was in 1885 when a little boy bitten by a rabid dog survived because Pasteur subsequently administered him 13 inoculations consisting of nerve tissue from an animal infected with partially

inactivated virus (Pasteur, 1885; Jackson, 2013). Word quickly spread all over the globe, and after reduction of unwanted side-effects about hundreds of thousands of people received PEP over the next decades (Jackson, 2013). Since the days of Pasteur rabies vaccine improvement was eagerly promoted and, furthermore, from the 1950th on mass vaccination programs for dogs lead to considerable progress in the elimination of dog-mediated rabies in many parts of Europe as well as in the Americas (Jackson, 2013; Velasco-Villa et al., 2017). Meanwhile, rabies in wildlife, e.g. foxes, emerged and rapidly spread all over Europe (Wandeler, 2004). Those new hurdles in fighting canine rabies were overcome in the 1970th with the development of oral rabies vaccines packed in baits for practical and efficient distribution. Regarded as a major breakthrough in rabies elimination in wildlife it progressively lead to a strong decline and eventual elimination in vast areas of Europe (Müller et al., 2015). Despite all these milestones and major efforts that have been made, rabies is still endemic in various countries all over the world, mainly as a result of limited resources and a lacking political will (King et al., 2004).

It is often forgotten that, even though bat-related RABV was firstly isolated in the Unites States in the early 1950s, there is historical reference implying the existence of bat associated rabies for centuries, with their exact geographical origin still not being conclusively resolved (Velasco-Villa, Mauldin et al. 2017). During the last decades, a rising awareness towards the linkage between bats and lyssaviruses lead to the detection of several hitherto unknown bat associated viruses on different continents. These, were, in fact, genetically related to RABV and are considered as novel virus species. Starting with the discovery of the Lagos Bat virus (LBV) in Nigeria in 1956 (Boulger and Porterfield, 1958), followed by another two African viruses, Mokola virus (MOKV) in 1986 (Shope et al., 1970) and Duvvenhage virus (DUVV) in 1970 (Meredith et al., 1971) and later accompanied in 2009 by both the Shimoni bat lyssavirus (SHIBV) (Kuzmin et al., 2010) and the Ikoma virus (IKOV) (Marston et al., 2012), five different lyssaviruses have been detected on the African continent alone. Also, there have been uncovered six different species in Asia since the early 1990ths – Aravan virus (ARAV) in 1991 (Kuzmin et al., 1992), Khujand virus (KHUV) in 2001 (Kuzmin et al., 2003), followed by the West Caucasian bat lyssavirus and Irkut virus (IRKV) in 2002 (Botvinkin et al., 2003) and only recently in 2016 the Gannoruwa bat lyssavirus (GBLV) (Gunawardena et al., 2016) as well as the Taiwan bat lyssavirus (TWBL). In Europe, rabies was first documented in a bat from Hamburg in 1954 (Mohr, 1957). The incident aroused scientific attention, thus bringing bat investigation into focus. Consequently, bats were regularly diagnosed with rabies in the following years

(Kappeler, 1989; King et al., 2004). In 1986, European bat lyssavirus 2 (EBLV-2) was first isolated from a Swiss bat biologist (Lumio et al., 1986) but it took until 1990 that a distinction from RABV as well as between European bat lyssavirus 1 and 2 (EBLV-1 and EBLV-2) was made (Dietzschold et al., 1988; King et al., 1990; Montano-Hirose et al., 1990; Bourhy et al., 1999). Due to public health concerns since the identification of EBLV 1 and 2, serious efforts were made to establish surveillance programs in several European countries (Fooks and Jackson, 2020) whereupon, another two lately recognized species were assigned; Bokeloh bat lyssavirus (BBLV) in 2010 (Freuling et al., 2011) and Lleida bat lyssavirus (LLBV) in 2012 (Aréchiga et al., 2012; Aréchiga Ceballos et al., 2013). Even on the Australian continent a representative of the lyssaviruses can be found; the Australian bat lyssavirus (ABLV), discovered in 1996 (Fraser et al., 1996). Just recently, another two novel lyssavirus species were found – the Kotalahti bat lyssavirus (KBLV) in Finland in 2017 (Nokireki et al., 2018) and the Matlo bat lyssavirus (MBLV) in South Africa in 2020 (Coertse et al., 2020). However, both of them are not yet officially approved.

3. Rabies Disease

Bites of infected dogs provoke more than 99 % of human rabies cases of which then again 40 % are children under 15 years of age (World Health Organization, 2018). Once clinical signs develop, the outcome is almost certainly fatal and there are only rarely reported cases of people surviving an infection. Less than 20 adequately documented clinical reports worldwide demonstrate that hitherto no therapeutic approach has proved to be effective and replicable (Jackson, 2016).

3.1. Transmission and Pathogenesis – Lifecycle of Rabies Virus Infection

Lyssaviruses are transmitted wherever there is direct contact between infectious saliva and broken skin surface, which is most commonly seen with bites of infected animals (Fooks et al., 2017). Transmission through scratches or any other superficial skin lesions are a lot less likely but cannot be excluded and are often reported in the context of contact to bats. In any case, even superficial injuries should be treated as urgently as severe wounds (Hemachudha et al., 2002). In fact, it is not uncommon that the potential hazard of those micro lesions is underestimated and resulting exposure remains more or less unnoticed (Messenger et al., 2002b). It has not yet been studied whether such exposures involving only skin or subcutaneous tissue might use different pathways to enter the nervous system (Fooks and Jackson, 2020). It is known, however, that lyssaviruses are able to replicate in the epidermis and dermis (Morimoto et al., 1996) and therefore it is believed that there might exist decisive differences in transmission (Begeman et al., 2017).

Another potential route of transmission through aerosols being exposed to nasal or ocular mucosa has been attributed with only very few human cases (Johnson et al., 2006; Davis et al., 2007). Even though a few studies have investigated this issue, it is still debatable whether airborne transmission plays an actual role as a natural route of infection. If at all, it probably only applies either among bats within one roost living in a narrow space or when other mammals come into close contact with large bat colonies, e.g. in caves (Constantine, 1962, 1966; Constantine et al., 1972; Fooks and Jackson, 2020).

At the site of exposure, lyssaviruses locally invade and initially replicate in skeletal muscle cells, though the latter is not absolutely required (Shankar et al., 1991). Subsequently, peripheral nerves get infected through the motor endplate of neuromuscular junctions and the virus

travels within endosomal vesicles along the axons via fast retrograde transport, which is enabled by microtubules (Tsiang, 1979; Gillet et al., 1986; Lycke and Tsiang, 1987; Gluska et al., 2015; Piccinotti and Whelan, 2016) (Figure 2). After reaching the neuronal cell soma, virus replicates and spreads centripetally via the synaptic cleft to next-order motor neurons of the CNS (central nervous system) on its way along the spinal cord towards the brain (Charlton and Casey, 1979; Ugolini, 2011) (Figure 2). Thereby, lyssaviruses actively evade immune responses (Schnell et al., 2010; Scott and Nel, 2016). Within the brain, virus disseminates and replicates in different brain areas, followed by centrifugal spread into the periphery. Consequently, salivary glands become infected and intermittent shedding of infectious virus via saliva occurs (Charlton et al., 1983; Jackson et al., 1999; Boonsriroj et al., 2016) (Figure 2). Virus shedding has been extensively studied for some domestic animals, with the result that there is no continuous shedding and the onset of viral excretion is also variable (Vaughn et al., 1963; Vaughn et al., 1965; Niezgoda et al., 1998). That is why not every infectious bite triggers a new infection (Constantine, 1962, 1966; Constantine et al., 1972; Fooks and Jackson, 2020). In terms of timing, several studies about virus shedding in bats suggested differing results and the underlying mechanisms are yet unknown (Aguilar-Setien et al., 2005; Franka et al., 2008; Freuling et al., 2009b).

Unlike in-vivo where lyssaviruses almost exclusively infect neuronal cells, in vitro lyssaviruses are capable of infecting various cell types, e.g. primary neurons, microglia, astrocytes and various mammalian cell lines (Seganti et al., 1990; Ray et al., 1997; Weli et al., 2006). Although the underlying courses of this phenomenon are not yet sufficiently investigated (Fooks et al., 2017), the in vitro ability and recent in-vivo observations in microglia and astrocytes might suggest a less pronounced neurotropism (Jackson et al., 2000; Pfefferkorn et al., 2016; Potratz et al., 2020b; Potratz et al., 2020a).

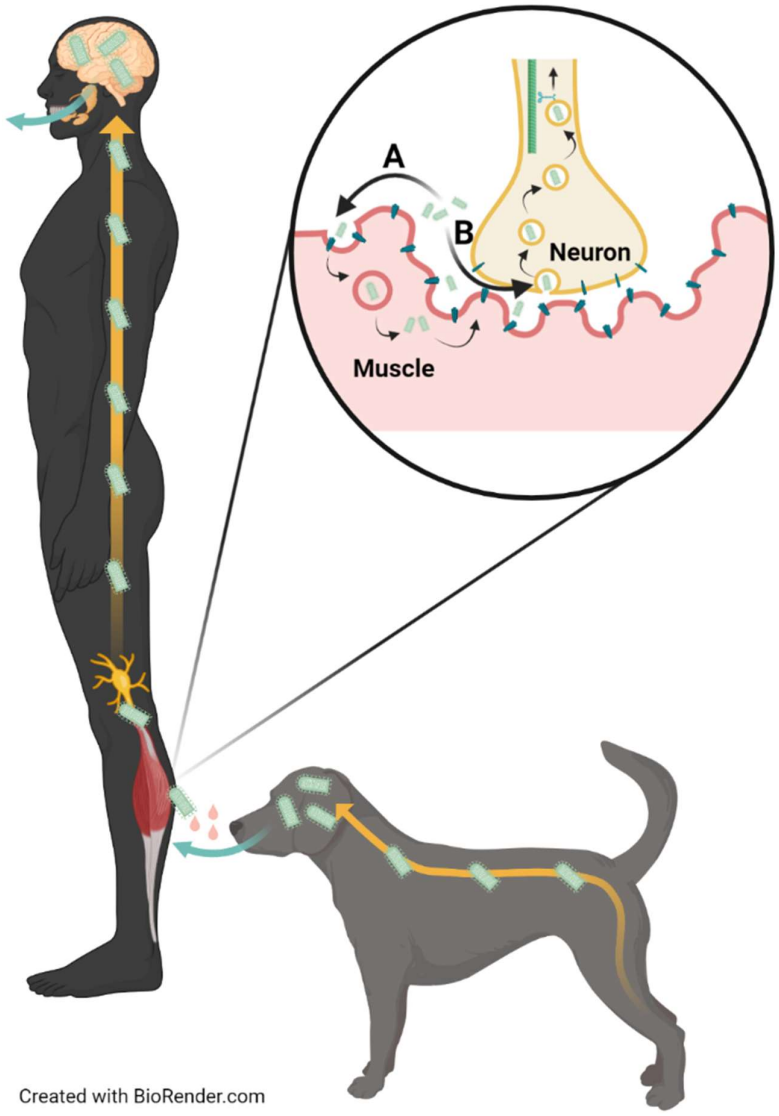

Figure 2 Schematic illustration of transmission and neuroinvasive strategy (insert) of lyssaviruses (modified from (Davis et al., 2015)). Through the bite of an infected animal virus is transmitted via saliva. On the site of exposure virus either initially enters and replicates in muscle cells before invading the neuronal system via neuromuscular junctions **(A)** or directly infects neurons without prior replication **(B)**. Either way entry occurs through receptor mediated endocytosis and subsequent retrograde transport along axons is dynein-mediated. The virus spreads from the peripheral site of exposure to the CNS, traveling along the spinal cord and ascending to the brain causing a fatal encephalitis.

3.2. Clinical Picture

It is challenging to provide a framework that clearly defines the extend of diverse manifestations of rabies disease as described in clinical reports over the last decades (Warrell and Warrell, 2004). After invading the CNS the neurotrophic virus rapidly replicates, leading

to severe pathologic effects on nerve cell physiology resulting in a clinical picture that appears to be relatively similar in animals and humans (Warrell and Warrell, 2004; Hemachudha et al., 2013). Hitherto, no difference in manifestation of clinical signs in relation to particular infected brain areas was found (Tirawatnpong et al., 1989; Laothamatas et al., 2008). Histopathological changes within the brain are absolutely out of proportion to the severity of the clinical picture, what might be explained by viral mechanisms that have adapted to keep damage in the CNS to an absolute minimum (Warrell and Warrell, 2004; Hemachudha et al., 2013). Depending on distinct factors, e.g. viral load or virus strain (Fooks et al., 2017), the incubation period in humans generally varies between three weeks to three months. In rare cases, though, it may also be either exceedingly extended up to several years or reduced down to only a few days (Plotkin, 2000; Johnson et al., 2008a). The lengths of the incubation period does also depend on the site of virus entry and its severity, which is why injuries to the head and neck area as well as on the upper extremities are linked to shorter incubation periods, especially when they are bleeding (Hemachudha et al., 2002). The first stage in disease progression is the short prodromal phase when virus reaches the dorsal-root ganglia causing nonspecific clinical signs of a deterioration of the general health condition with possible signs of pruritus or paresthesia (Hemachudha et al., 2002). Afterwards, patients enter the acute neurological phase and manifest either the furious or paralytic form at a ratio of three to one. While the former is characterized by hypersalivation, alternating confusion and agitation as well as aggression, the latter is described with progressing muscle weakness, paralysis and inspiratory spasms (Hemachudha et al., 2002; Fooks et al., 2017).The case specific clinical picture might differ between individuals but usually leads to death within an average of eight to eleven days from clinical onset, while disease progression of the paralytic form is usually slightly slower compared to the furious form. In terrestrial animals, disease progression and clinical signs are comparable to what is observed for humans. Firstly, an unspecific clinical picture with signs of lethargy or anorexia is present, further developing a rapid deterioration of the general condition and characteristic signs as for example aggressiveness and hypersalivation (Hanlon, 2013). Regardless an early timing of the diagnosis, the prognosis for humans as well as animals stays extremely poor and all manifestations result in coma and death (Fooks et al., 2017). In the rare cases where humans developed rabies disease as a consequence of contact to bats, no uniquely defined clinical course was reported, but rather different manifestations were observed, e.g. paralytic, furious or atypical forms (Hanlon et al., 1989; Samaratunga et al.,

1998; Hemachudha et al., 2002; Messenger et al., 2002a; Nathwani et al., 2003). Furthermore, it is sometimes challenging to differentiate between rabies cases and other conditions where neurological disorders are part of the course of clinical disease, e.g. the human Guillain-Barré syndrome (Hemachudha et al., 2002; Fooks et al., 2017). Therefore, a definite diagnosis should not be made without approved laboratory confirmation (World Health Organization, 2018).

3.3. Pathogenicity

On a molecular level lyssavirus pathogenicity depends on genetic differences with the viral glycoprotein being one major determinant (Ito et al., 2001). It defines the neuro-invasive pathway by enabling virus entry into the host cell (Yan et al., 2002; Sissoëff et al., 2005) and also provides epitopes for virus-neutralizing antibodies (VNAs) (Luo et al., 1998; Mansfield et al., 2004). The capacity of lyssaviruses to cause rabies disease does indeed rely on molecular pathogenicity determinants that are then again depending on the lyssavirus species and more specifically on the individual isolate in question (Badrane et al., 2001; Vos et al., 2007; Kgaladi et al., 2013). Differences between bat-borne RABV strains and classical terrestrial RABV have also been assumed. However, in this regard, knowledge about comparative pathogenicity is fragmentary due to insufficient data (Fuoco et al., 2018; Banyard et al., 2020). Pathogenicity differences within one lyssavirus species have so far been reported for LBV, RABV (Markotter et al., 2009a; Kgaladi et al., 2013) and recently also for EBLV-1 (Eggerbauer et al., 2017a). Further factors determining pathogenicity are the circumstances of infection as inoculation dose and route as well as factors provided by the host itself, i.e. immune status, age and animal species (Soulebot et al., 1982; Banyard et al., 2014b). Especially, regarding a reported substantial variability in pathogenicity in mice between phylogroups I and II, pathogenicity in different mammalian species arouses scientific interest (Banyard et al., 2011). To date, pathogenicity in bats remains enigmatic as knowledge relating thereto is still patchy. There have been reports of clinically silent rabies infections (Ronsholt et al., 1998; Fooks et al., 2003a; Vazquez-Moron et al., 2008) drawing the assumption that bats might coexist with lyssaviruses due to mechanisms controlling viral replication (Baker et al., 2013), and also viral RNA or antiviral antibodies have been detected in healthy appearing bats (Harris et al., 2009; Megali et al., 2010; Schatz et al., 2013b; Leopardi et al., 2018). However, so far, the majority of described cases in bats has been in sick or dead animals and experimental inoculation mostly lead to death (Johnson et al., 2008b; Freuling et al., 2009b).

3.4. Rabies Prevention

In 2018, the World Health Organization (WHO), World Organisation for Animal Health (OIE), Food and Agriculture Organization of the United Nations (FAO) and the Global Alliance for Rabies Control (GARC) joined together to form the United Against Rabies (UAR) consortium to combat dog-mediated human rabies. With the so called "Zero by 30" plan they have established a strategy to globally eradicate dog-mediated rabies within all endemic regions by 2030 (Minghui et al., 2018). To achieve this ambitious goal, it is crucial to combine different approaches, e.g., improved diagnostics and medical infrastructure, reliable surveillance and accurate data acquisition as well as awareness programs. Additionally, to prevent host-switching events from wildlife reservoirs to domestic animals, rabies in wildlife has to be controlled and diminished (Fooks et al., 2017). First and foremost, the primary and most cost-effective prevention strategy for human rabies is interrupting transmission from dogs to humans (Shwiff et al., 2018). Thus firstly, dog vaccination campaigns to decrease rabies incidence within the main reservoir itself and secondly, preventing dog-bites through education and awareness programs on responsible dog ownership for children and adults, are essential. However, due to lack of current data it is questionable whether those programs indeed reduce dog-bites, while meta-analyses about rabies-related education for children revealed only moderate influence (Duperrex et al., 2009; Shen et al., 2017; World Health Organization, 2018). Whenever primary prevention has failed, i.e. in case of suspected exposure, secondary prevention comprising PEP and extensive wound care should be initiated in accordance to the WHO guidelines for risk assessment (World Health Organization, 2018). In case of doubt, PEP should still always be provided as early as possible and a divergence from the protocol is clearly not advised (Wilde et al., 1996). PEP treatment does not only consist of the administration of inactivated rabies vaccines combined with, if indicated, rabies immunoglobulin (RIG), but also of immediate and determined cleansing of the wound using soapy water (Fooks et al., 2017). Another important component is the so called integrated bite case management which implies that, the biting animal in question is first checked for its health and vaccine status and then either accordingly removed if suspected or quarantined until further lab confirmed results (World Health Organization, 2018). Providing that thorough wound care and correct PEP administration were performed, the outcome is almost as effective as pre-exposure prophylaxis (PrEP). Due to the invincible financial burden of PrEP, vaccination is only recommended for certain people at risk, who regularly come in contact

with canines, bats or other suspected species through their occupational or voluntary work. That said, conventional rabies vaccines are believed to confer no protection against virus species of phylogroups II and III. This is because contemporary vaccines usually derived from RABV strains as they were merely designed to combat classical RABV. However, at the same time they also provide protection for genetically and antigenically related virus species. Virus species other than phylogroup I, which in that respect are sufficiently divergent from RABV, manage to escape vaccination induced cross-neutralization (Hanlon et al., 2001; Hanlon et al., 2005; Malerczyk et al., 2014; Evans et al., 2018).

4. Rabies Diagnosis

The diagnosis of rabies is preferably undertaken under well controlled laboratory conditions (Mallewa et al., 2007) with brain material being the specimen of choice, indicating that only post mortem analysis provides a definitive diagnosis. Hereby, most favorable brain areas for collecting samples are Ammon's horn, thalamus, cerebral cortex, cerebellum and, most importantly, the brain stem because viral antigen sometimes exclusively accumulates in this area (Bingham and van der Merwe, 2002; World Health Organization, 2018; OIE, 2021). Multiple sampling from different areas should be done as it increases detection rate. However, for ante mortem diagnosis nuchal skin biopsies including several hair follicles or saliva samples seem most eligible (Fooks et al., 2017; Fooks and Jackson, 2020). Immunostained skin can be tested for viral antigens and both - skin and saliva samples - for the presence of viral RNA via conventional or real-time reverse transcription polymerase chain reaction (RT-PCR/RT-qPCR). However, due to intermittent virus shedding, again, multiple sampling at several timepoints is advisable and negative results in saliva should be interpreted with caution (Fooks et al., 2017). While ante mortem diagnosis would offer the obvious advantage of no unnecessary euthanasia of animals suspected of rabies, it must be noticed that it does not constitute an alternative. As results are not sufficiently reliable it can only confirm an infection rather than it can exclude one (Fooks et al., 2017). Testing for virus specific antibodies in sera or cerebrospinal fluid (CSF) theoretically offers another diagnostic possibility of albeit limited significance as seroconversion usually arises at late stages of infection (Rupprecht and Plotkin, 2013). Of note, whereas an ante mortem (intra vitam) diagnosis is not recommended for animals, it is the only possibility to either exclude or diagnose rabies in humans showing symptoms associated with CNS disorders (World Health Organization, 2018).

4.1. Standard Diagnostic Methods and its Challenges under Low-Resource Conditions

Recommended as a primary step in rabies diagnostics by both OIE and WHO the fluorescent antibody test (FAT) was historically regarded as the gold standard (OIE, 2018; WHO, 2018) and is therefore still most commonly used. It detects viral antigen in infected brain tissue with particularly high sensitivity in fresh and good quality samples in less than two hours (Fooks et al., 2009). Thereby, heat fixed tissue is stained with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody mix and assessed under a fluorescent microscope using ultraviolet light. If viral antigen is present, dust-like and green-fluorescent particles are

visualized (Dean and Abelseth, 1973). Ideally, the time between test implementation and sample extraction should not exceed four hours and no autolysis should have started (OIE, 2018). However, those requirements can hardly be met in regions where there is only scarce laboratory and transport infrastructure. Furthermore, the FAT requires expensive material and equipment as well as experienced staff because results can be somewhat difficult to interpret correctly (Fooks et al., 2017). The rabies tissue-culture infection test (RTCIT) is another conventional diagnostic method commonly used to further confirm questionable results obtained by FAT or its alternatives as prescribed by the OIE (OIE, 2018). Thereby, through isolation from suspected samples and subsequent propagation on cell culture, viable virus is indirectly detected (Rudd et al., 1980). The same also applies to the mouse inoculation test (MIT) with the difference that this particular one is based on virus propagation in live mice that are inoculated intracranially (Webster and Dawson, 1935). Due to animal welfare reasons and a similar sensitivity (Rudd and Trimarchi, 1989; Robardet et al., 2011) the latter has almost completely been replaced by the RTCIT which should accordingly always be prioritized wherever suitable laboratory equipment allows cell cultivation (OIE, 2018). Both MIT and RTCIT offer the advantage of subsequent sequencing and phylogenetic analysis. However, both of them cannot be performed without specially qualified staff as well as appropriately equipped facilities and also require a rather long turnaround time (Fooks et al., 2017). Nowadays it is recommended to supplement conventional methods as FAT and RTCIT with advanced histopathology, serological assays and molecular diagnostics, i.e. RT-PCR (Fooks et al., 2009). Especially viral RNA detection via real-time RT-PCR has enormously increased worldwide since this relatively rapid technique also demonstrates a very high diagnostic sensitivity as well as specificity and is now also regarded as primary test (OIE, 2018). Initial doubts concerning a reduced reliability of molecular tests proofed unfounded since it was already shown that their results are consistent with those obtained through RTCIT (Robardet et al., 2011). Both conventional and real-time RT-PCR offer the possibility to either directly or via sequencing further identify the particular virus species. Contrary to the conventional gel-based RT-PCR, there is a lower risk for potential cross-contamination due to the applied one-closed-tube system within the real-time RT-PCR application process. Nonetheless, as all of the aforementioned methods, it likewise requires cost-intensive materials that are often unaffordable for routine diagnostic laboratories in less developed areas (WHO 2019). Further challenges arise wherever there are no sufficient financial and administrative resources for

adequate validation and routine quality control of laboratory equipment and procedures as this might lead to inaccurate application of diagnostic methods. Likewise, there are additional problems regarding sample acquisition, transportation and storage, including the maintenance of appropriate cold chains (Voupawoe et al., 2021).

4.2. Alternative Methods for Rabies Diagnosis

To some extent several alternative methods for sample collection and storage as well as diagnostic tests facilitate or even enable rabies diagnosis and surveillance in regions with restricted medical and veterinary infrastructure. Under specific biosafety measures, brain samples are typically collected through the open cranium. Where those safety precautions cannot be taken, either the route via the occipital foramen (Barrat and Blancou, 1988) or the retro-orbital route (Montaño Hirose et al., 1991) also offer the possibility for brain sample acquisition under a reduced risk for injury or contamination. To circumvent potential problems concerning the integrity of the cold chain, other preservative techniques may be applicable. During shipment and storage, samples can be either formalin-fixed or preserved in a mixture of 50 % glycerol and phosphate buffered saline (PBS). The former method, however, brings up the disadvantage of a reduced sensitivity in subsequent diagnostics (Warner et al., 1997). In surroundings with relatively high temperatures it is still advisable to refrigerate glycerol-preserved samples (OIE, 2018). The direct immunohistochemical test (dRIT) has been developed as a low-cost alternative diagnostic method to the FAT and visualizes rabies virus particles by using biotinylated antibodies while only relying on a light microscope (Lembo et al., 2006). Despite the simplification, a certain standard laboratory environment is still required. To facilitate diagnosis even more, the concept of rapid immunodiagnostic tests (RIDT), also called lateral flow devices, was established for rabies disease and firstly evaluated in 2007. The test principle is based on immunochromatography with gold conjugated capture antibodies on a nitrocellulose membrane (Kang et al., 2007) (Figure 3). Of all currently available diagnostic alternatives, LFDs stand out as the most user-friendly and provide the shortest processing time. Additionally, they require minimal staff training and are cost effective point of care diagnostics with the only but serious disadvantage of presently still lacking validation due to unreliable performance, albeit at different scale, depending on sample quality and the respective manufacturer (O'Farrell, 2015; Eggerbauer et al., 2016). To overcome financial limitations regarding technical equipment for detection of viral RNA,

another two novel and inexpensive diagnostic approaches were developed. Both techniques, the nucleic acid sequence-based amplification (NASBA) (Sugiyama et al., 2003) and the reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Notomi et al., 2015), amplify and subsequently detect RNA of lyssaviruses under isothermal conditions without DNA template denaturation, thus eliminating the use of thermal cyclers (Nagamine et al., 2001). Both offer the opportunity for extremely efficient and easy-to-use ante-mortem testing in saliva and CSF in more humble surroundings. While they are supposed to gain higher sensitivities than other diagnostic ante-mortem options, keeping the high level in specificity seems challenging when using RT-LAMP and further improvement on primers is necessary (Wacharapluesadee and Hemachudha, 2001; Boldbaatar et al., 2009; Saitou et al., 2010).

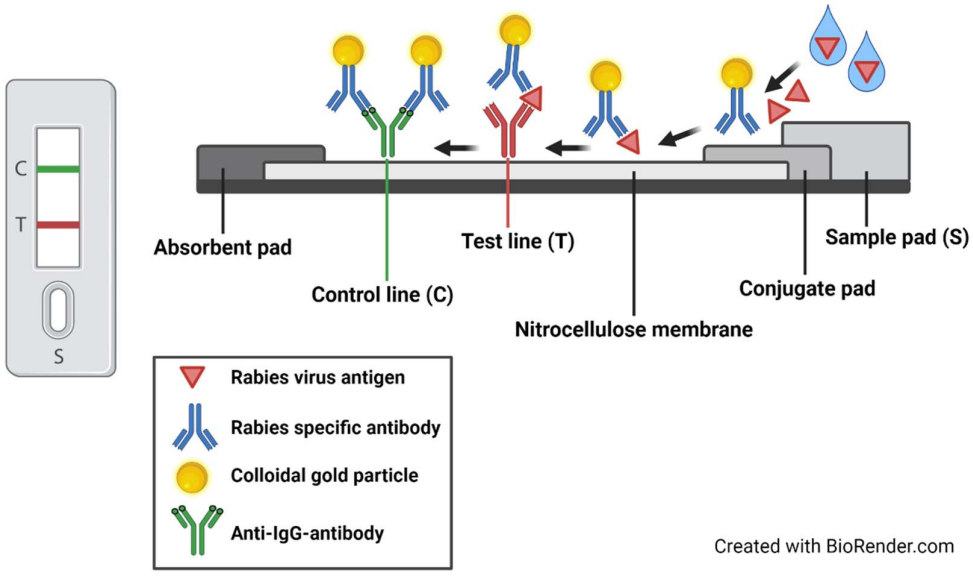

Figure 3 Schematic illustration of the LFD test principle (modified from (Kang et al., 2007)). Colloidal gold conjugated antibodies capture the antigen within a sample. The antigen–antibody complex binds to a second detection antibody fixed at the test zone “T“ on a nitrocellulose membrane, showing a colored line for a positive sample. A colored line at the control line “C” confirms the functionality of the respective device.

5. Lyssavirus Epidemiology

Lyssaviruses are globally distributed and endemic on all continents except Antarctica. Annually, more than 59.000 human fatalities are estimated, whereas approximately 98 % of those cases can be traced back to dog-transmitted terrestrial RABV (WHO, 2018). The relatively complex lyssavirus epidemiology comprises their potential ability to infect all mammals while being maintained by a limited number of divers and almost always spatially bound reservoir species. Their epidemiological characteristics like intrareservoir transmission, spillover and host-shift events as well as their extraordinary transmission cycle and their specific adaptive capacities in the course of evolution allow a presumably ongoing switch between distinct host populations (Fooks and Jackson, 2020).

5.1. Terrestrial Lyssaviruses

In accordance to the guidelines of the World Organisation for Animal Health (OIE), Germany has officially declared freedom from terrestrial rabies in 2008. To gain this rabies-free status, specific requirements must be met. Above all, within an ongoing system of a surveillance program, no RABV case shall be reported for the last 24 months (OIE - World Organisation for Animal Health, 2019). While most countries of the Western World have successfully eliminated terrestrial rabies in domestic dogs and wildlife, the burden of dog-mediated rabies still lies on many countries in Africa and Asia where more than 99 % of all reported human rabies deaths occur (OIE, 2018; WHO, 2018). However, due to insufficient surveillance, many cases might never end up on statistical records and, as a consequence, the rabies-induced death rate in these regions is believed to be far higher than official numbers estimate (Taylor et al., 2017).

In contrast to bats, terrestrial lyssavirus reservoir species do not meet the classical definition of a reservoir host in terms of an organism that harbors the pathogen as the source of infection but does not show any signs of illness itself. Instead, it is rather some sort of maintenance where the pathogenic agents, despite the deadly outbreak of the disease, are passed on to the next individuum in time (Zhang et al., 2008; Fooks and Jackson, 2020). Wildlife reservoir species for RABV almost all belong to the *Carnivora* order and include red foxes (*Vulpes Vulpes*), arctic foxes (*Vulpes lagopus*), grey foxes (*Urocyon cinereoargenteus*), bat-eared foxes (*Otocyon megalotis*), raccoon dogs (*Nyctereutes procyonoides*), jackals (*Canis adustus*, *Canis*

mesomelas, *Canis aureus*), coyotes (*Canis latrans*), raccoons (*Procyon lotor*), mongooses (*Herpestes auropunctatus*), ferret badgers (*Melogale moschata*), skunks (*Mephitis mephitis* and *Spilogale spp.*) and coatis (*Nasua nasua*) (World Health Organization, 2018). Also *Primates* were identified to comprise another recently spreading rabies reservoir – the in South America occurring marmosets (*Callithrix jacchus*) (Favoretto et al., 2001; Kotait et al., 2018).

5.2. Bat-Related Lyssaviruses in a Global Perspective

Even though the scientific perspective concentrates on mesocarnivores as the main rabies reservoirs, the role of *Chiroptera* as ancestral reservoir hosts has come increasingly to the fore during the last decades (Banyard et al., 2011). Bats feature several fascinating and unique biological traits, e.g. relatively long lifespan, hibernation or high immune tolerance, which enable them to act as ideal reservoir hosts for a plethora of viral zoonotic pathogens including lyssaviruses (Badrane and Tordo, 2001; Calisher et al., 2006; Banyard et al., 2020; Irving et al., 2021). Even though bats make up more than approximately 20 % of all existing mammalian species, they are considerably different to other mammals, not only due to their unique ability to fly. Moreover, they present a vast intraspecies diversity, which is also reflected by the variety of lyssaviruses respectively adapted to their particular host (Banyard et al., 2020). Although bat derived lyssaviruses can be found all across the globe, the spatially restricted geographical distribution of particular lyssavirus species in comparison to classical terrestrial RABV, which is globally omnipresent, remains one of two remarkable conundrums (Marston et al., 2018) (Figure 4). While bat associated RABV solely circulates in New World bats but is lacking evidence of existence in chiropteran species in every other part of the world, none of the other bat-related lyssaviruses has yet been found in the Americas, which outlines the second conundrum. Instead, non-RABV lyssaviruses, even though all mammals are theoretically susceptible (Banyard et al., 2013), are usually restricted to a very few and mostly even only one bat host species they have been steadily co-evolving with over time. In contrast, RABV seems to have a very broad host spectrum and occurs in multiple terrestrial Carnivores as well as in Chiropterans in the Americas (Marston et al., 2018) (Figure 4). The evolutionary development of lyssaviruses has not been sufficiently reconstructed yet, though it is assumed that bat-borne lyssaviruses existed long before terrestrial RABV has evolved (Rupprecht et al., 2017). Interestingly, only RABV infection leads to sustained spillovers, whereas bat-related lyssavirus spillovers to conspecifics or non-flying mammals hardly ever occur. If so, they

usually seem to result in dead ends as perpetuation has not been observed yet (Johnson et al., 2010). Those sporadic spillover infections of non-RABVs from bats to humans and animals have so far been reported for seven different virus species from all over the globe. One officially recognized case of human spillover with IRKV was reported from Russia and yet another three possible cases were described in Russia as well as China. Moreover, in Australia, three distinct human rabies cases and another two spillover events to horses were described for ABLV (Banyard et al., 2020). In Europe, EBLV-1 caused three human cases, with one being just recently diagnosed (Regnault et al., 2021), and more spillover to sheep, cat and stone marten, whereas there were two solely human cases confirmed for EBLV-2 (World Health Organization, 2018). Out of the five lyssaviruses detected in Africa, MOKV and IKOV were not yet assigned to a specific bat species. The reservoir for MOKV remains mysterious as it has so far been isolated from bats as well as shrews, cats and dogs and also from two human cases (World Health Organization, 2018). To date, IKOV has exclusively been isolated from an African civet but its close phylogenetic relation to LLBV and WCBV indicates a possible bat derived origin (Horton et al., 2014). LBV is widely spread but in contrast to DUVV with its three associated human fatalities, LBV only caused several spillovers to dogs, cats and a water mongoose (Markotter et al., 2006; World Health Organization, 2018). As aforementioned, bats in the Americas are only associated with distinct enzootic RABV variants and in comparison, to the Old World, inter-species transmission within the bat community as well as cross-species transmissions to carnivores are observed more frequently (Velasco-Villa et al., 2006; Piñero et al., 2012). RABV transmission among bats appears to affect different bat species at different scales and is presumably dependent on geographic range overlap as well as on genetic relation of the hosts (Streicker et al., 2010). Quite a few sustained spillovers of bat associated RABV have already been recorded for several wild carnivores, as for instance for raccoons, and they also seem to increase over time (Wallace et al., 2014). Correspondingly, the number of human cases involved in bat rabies is also higher than compared to the Old World (Wallace et al., 2014). Notably, in North America, the vast majority of human rabies cases acquired from wildlife was related to contact with bats, primarily to either silver-haired bats (*Lasionycteris noctivagans*), tricolored bats (*Perimyotis subflavus*) or Brazilian free-tailed bats (*Tadarida brasiliensis*) (Serres et al., 2008; Ma et al., 2020). Moreover, in South America the situation is different again, as one indigenous hematophagous species, the common vampire bat

(*Desmodus rotundus*), facilitates spillover to cattle and humans due to its dietary trait, particularly in remote regions in Peru and Brazil (Schneider et al., 2009; Streicker et al., 2012).

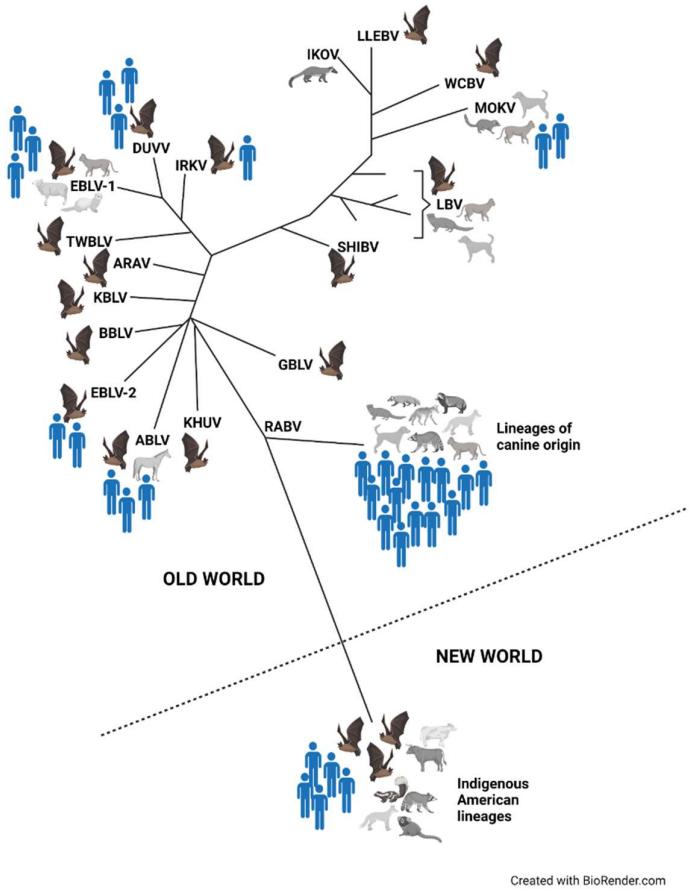

Figure 4 Schematic illustration of phylogenetic relatedness of the different lyssavirus species, their host reservoir restrictions and reported spillover events (modified from (Marston et al., 2018)).

6. Bat Rabies Surveillance

The fact that both EBLV-1 and EBLV-2 have already caused at least four human fatalities, emphasizes their threat to European public health (Fooks et al., 2003a). In order to better assess and subsequently reduce this public health risk, surveillance programs were established throughout Europe. Those substantially contribute to a more comprehensive understanding about geographical distribution, prevalence and epidemiology of bat related lyssaviruses (Schatz et al., 2013a).

6.1. Active and Passive Surveillance

While passive surveillance investigates the existence of lyssaviruses, i.e. viral RNA or viable virus, in diseased or dead bats only, active surveillance, also sometimes referred to as targeted surveillance, is focusing on sampling free-living indigenous bat populations. Either the detection of VNAs in serum samples is investigated or saliva and oropharyngeal swabs are tested for viral RNA or infectious virus (Harris et al., 2009; Schatz et al., 2013b). This surveillance method has been implemented for the first time in the Americas (Constantine et al., 1968; Trimarchi and Debbie, 1977; Steece and Altenbach, 1989) and was then gradually adopted in European research (Echevarria et al., 2001; Med Vet Net Working Group, 2005; Harris et al., 2009; Presetnik et al., 2010; Picard-Meyer et al., 2011). Even though active surveillance indicates the occurrence of bat lyssaviruses in regions where they have not yet been detected, the imbalance between scientific benefit and the massive logistic effort lead to a decrease in active surveillance (Schatz et al., 2013a). While passive surveillance in routine diagnostics does solely focus on submitted cases with suspected human or animal contact, a wider range of possible cases from a more diverse background can be depicted by so called enhanced passive surveillance as established in Germany in 1998 (Schatz et al., 2014). It has proved to be the method of choice and has ever since been used for various European studies in order to monitor bat rabies occurrence and distribution (Schatz et al., 2013b).

6.2. Surveillance in Europe with Focus on Germany

European recommendations encourage continuous surveillance of bat lyssaviruses at a national level in order to support bat rabies research in a public health context (Med Vet Net Working Group, 2005). Between 1977 and 2021, a total of 1335 bat rabies cases have been recorded in the WHO Rabies Bulletin for Europe, thus representing 0.5 % of all reported rabies

cases (Rabies Bulletin Europe, 2021) (Figure 5). At present, all currently recognized 51 European bat species are listed as endangered and hence protected species according to the EUROBATS agreement (Hutson, A.M., Marnell, F. & Petermann, 2019). Thus, conservational endeavors affect and partly limit bat-related lyssavirus surveillance and provoke a very heterogenous European surveillance landscape with different methodologies being used (Schatz et al., 2013a). It is therefore not surprising that the by far highest case rates are reported from countries with well-developed surveillance networks. With 348 reported cases, Germany comes second after The Netherlands with 388 bat rabies cases and is followed by Denmark, Poland and France (Rabies Bulletin Europe, 2021). The majority of German cases accumulates in the northern lowlands (Figure 5) where there is a particularly high density of Serotine bats (*Eptesicus serotinus*) (Boye et al., 1998). They are regarded the reservoir bat species for EBLV-1 (Schatz et al., 2014) since more than 95 % of all known EBLV-1 cases were detected in Serotine bats (Jakava-Viljanen et al., 2010). The Isabelline serotine bat (*Eptesicus isabellinus*) has been identified as another reservoir for EBLV-1 (Vazquez-Moron et al., 2011) and sporadic cases have also been found in other bat species with the Common pipistrelle (*Pipistrellus pipistrellus*) being only one example (Müller et al., 2007). Whereas EBLV-1 is the causative agent for the vast majority of the European bat rabies cases (Schatz et al., 2013a), a comparatively small number of 34 confirmed EBLV-2 cases was so far mainly reported in Daubenton's bats (*Myotis daubentonii*) from the United Kingdom, Norway, Finland, and Switzerland (McElhinney et al., 2018). EBLV-2 also occasionally occurs in Daubenton's bats in Germany and has additionally been identified in Pond bats (*Myotis dasycneme*) in The Netherlands (Figure 5), making those two bat species presumable EBLV-2 reservoirs (Van der Poel, W. H. M. et al., 2005; Schatz et al., 2013a). Another four distinct lyssaviruses have been isolated from European bats, whereby only BBLV is present in Germany, where six out of nine European cases were reported since its discovery in 2010. Except one, all of those cases were isolated from the Natterer's bat (*Myotis nattereri*) which is therefore believed to be the only reservoir species (Freuling et al., 2011; Eggerbauer et al., 2017b). Both WCBV from the Caucasus region and LLBV detected in Spain and France were sporadically described in Common bent-winged bats (*Miniopterus schreibersii*) (Botvinkin et al., 2003; Kuzmin et al., 2005; Picard-Meyer et al., 2019). A first case of the tentative novel KBLV in Finland was associated with the Brandt's bat (*Myotis brandtii*) (Nokireki et al., 2018; Calvelage et al., 2021).

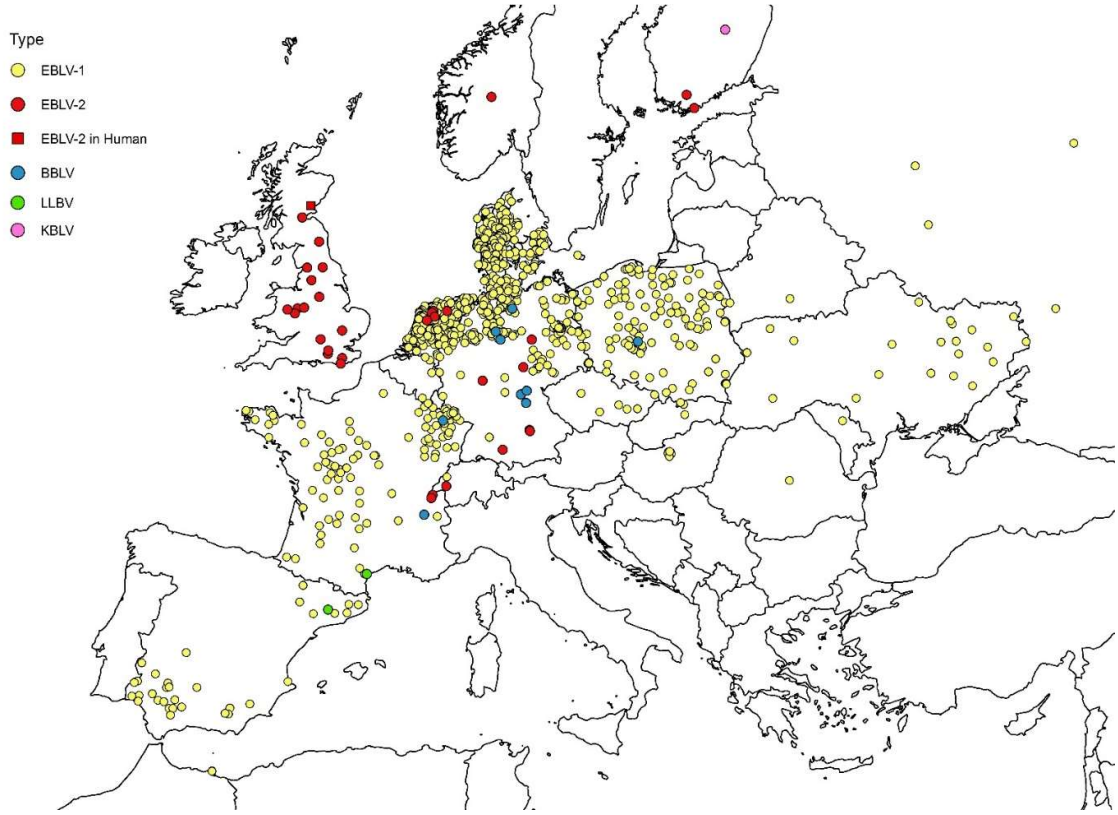

Figure 5 Distribution of bat rabies cases caused by the respective lyssavirus species in Europe between 1977 and 2018 (WCBV was detected in Caucasia, which is not depicted on this map). Source: WHO Collaborating Centre for Rabies Surveillance and Research, FLI, 2019

III. OBJECTIVES

Although, lyssaviruses are the causative agents of one of the oldest infectious diseases known to mankind and there are efficacious vaccines already existing for more than 100 years, remarkable scientific issues concerning various lyssavirus research areas have not yet been sufficiently addressed. Taking particularly the vast diversity of the lyssavirus genus into consideration, knowledge gaps regarding e.g. virus emergence and spread, pathophysiology or diagnostics and treatment, need to be closed. This study defined three objectives to contribute to an improved understanding in the aforementioned fields.

Comparatively evaluating the performance of LFDs as an alternative diagnostic tool in low resource settings:

Against the background of very limited diagnostic capacities especially in developing countries in Africa and Asia where rabies is still endemic, there is a huge demand for low-cost, rapid and user-friendly diagnostic alternatives. Commercially available lateral flow devices offer the potential to bridge this gap, however, previous studies published alarming results regarding their sensitivity as well as specificity and many more still lack reliable validation. Nevertheless, there is an increasing trend to bring more of these tests onto the market. Therefore, in an attempt to assess whether the diagnostic performance of commercial LFDs has substantially improved, a further five different LFDs that were currently available on the market was comparatively evaluated using a broad panel of samples within a multi-centered study including several FAO/OIE laboratories.

Continuing surveillance of bat lyssavirus occurrence and distribution in Germany:

A second part of this work focused on proceeding with the enhanced passive bat lyssavirus surveillance program in Germany from 2018 until 2020, which had initially been established in 1998. The aim of the present study was to improve the surveillance scheme by refining the diagnostic approach through routine use of molecular diagnostic methods as screening tools on the one hand and a novel minimally invasive procedure for sampling specimen on the other hand. Analyzing the current situation of lyssavirus distribution in Germany is essential for a better understanding of lyssavirus emergence and spread, especially against

the background of novel bat-related lyssaviruses continuously emerging in Europe (e.g. KBLV) as well as worldwide (e.g. MBLV).

Comparatively assessing pathogenicity and virus shedding of different lyssavirus species:

In respect to the lyssavirus diversity and the constant identification of novel lyssavirus species as mentioned above, a universal assessment of the presumed highly variable lyssavirus pathogenicity is challenging and the present knowledge those evaluations are based on remains patchy. So far, varying experimental conditions of previous studies prevented a broader direct and reliable comparison. It was therefore attempted in this thesis to develop a matrix to classify lyssaviruses in relation to their pathogenicity. Therefore, comparable parameters of 13 different phylogroup I isolates needed to be assessed through in-vivo studies using a standardized mouse model. In order to investigate whether virus shedding through saliva can indeed be considered a key factor for sustained spillover, it is furthermore intended to include the analysis of active virus shedding through animals infected during the in-vivo experiments.

With the one-health approach in mind, this thesis partly aims at contributing to “Zero-by-30” where improving surveillance of terrestrial rabies is one important pillar. Additionally, identifying and understanding differences between different lyssavirus species is necessary to face even further challenges since the prevention of host shifts back into susceptible terrestrial species.

IV. PUBLICATIONS

Each manuscript, including its figures and tables, is presented in the style of the respected journal where it was published/submitted. References of each publication are presented in the style of the respective journal at the end of each manuscript and are therefore as well as the respective abbreviations used in each manuscript not included in the relevant sections at the end of this document. Where corresponding supplementary material was published, it directly follows the respective reference section of each manuscript.

1. Publication I: Further Evidence of Inadequate Quality in Lateral Flow Devices Commercially Offered for the Diagnosis of Rabies

Antonia Klein [1], Anna Fahrion [1], Stefan Finke [1], Marina Eyngor [2], Shiri Novak [2], Boris Yakobson [2], Ernest Ngoepe [3], Baby Phahladira [3], Claude Sabeta [3], Paola De Benedictis [4], Morgane Gourlaouen [4], Lillian A. Orciari [5], Pamela A. Yager [5], Crystal M. Gigante [5], M. Kimberly Knowles [6], Christine Fehlner-Gardiner [6], Alexandre Servat [7], Florence Cliquet [7], Denise Marston [8], Lorraine M. McElhinney [8], Trudy Johnson [8], Anthony R. Fooks [8], Thomas Müller [1] and Conrad M. Freuling [1]

[1]Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Institute of Molecular Virology and Cell Biology, 17493 Greifswald-Insel Riems, Germany

[2]Kimron Veterinary Institute (KVI), Veterinary Services and Animal Health, P.O. Box 12, Beit Dagan 50250, Israel

[3]Onderstepoort Veterinary Institute (OVI), Rabies Unit, Private Bag X05, Onderstepoort 0110, South Africa

[4]Istituto Zooprofilattico Sperimentale delle Venezie, FAO Reference Centre for Rabies, Viale dell'Università, 10, 35020-Legnaro (PD), Italy

[5]Centers for Disease Control and Prevention (CDC), Poxvirus and Rabies Branch, Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Diseases, Atlanta, GA 30329, USA

[6]Canadian Food Inspection Agency, Centre of Expertise for Rabies, Ottawa Laboratory Fallowfield, 3851 Fallowfield Road, Nepean, ON K2H 8P9, Canada

[7]French Agency for Food, Environmental and Occupational Health and Safety (Anses), Laboratory for Rabies and Wildlife, Domaine de Pixérécourt, 54220 Malzéville CEDEX, France

[8]Animal and Plant Health Agency (APHA), Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK

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Communication

Further Evidence of Inadequate Quality in Lateral Flow Devices Commercially Offered for the Diagnosis of Rabies

Antonia Klein [1], Anna Fahrion [1], Stefan Finke [1], Marina Eyngor [2], Shiri Novak [2], Boris Yakobson [2], Ernest Ngoepe [3], Baby Phahladira [3], Claude Sabeta [3], Paola De Benedictis [4], Morgane Gourlaouen [4], Lillian A. Orciari [5], Pamela A. Yager [5], Crystal M. Gigante [5], M. Kimberly Knowles [6], Christine Fehlner-Gardiner [6], Alexandre Servat [7], Florence Cliquet [7], Denise Marston [8], Lorraine M. McElhinney [8], Trudy Johnson [8], Anthony R. Fooks [8], Thomas Müller [1] and Conrad M. Freuling [1,*]

1 Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Institute of Molecular Virology and Cell Biology, 17493 Greifswald-Insel Riems, Germany; Antonia.Klein@fli.de (A.K.); Anna.Fahrion@fli.de (A.F.); Stefan.Finke@fli.de (S.F.); Thomas.Mueller@fli.de (T.M.)
2 Kimron Veterinary Institute (KVI), Veterinary Services and Animal Health, P.O. Box 12, Beit Dagan 50250, Israel; MarinaL@moag.gov.il (M.E.); shirin@moag.gov.il (S.N.); boris.yakobson@gmail.com (B.Y.)
3 Onderstepoort Veterinary Institute (OVI), Rabies Unit, Private Bag X05, Onderstepoort 0110, South Africa; NgoepeE@arc.agric.za (E.N.); PhahladiraB@arc.agric.za (B.P.); sabetac@arc.agric.za (C.S.)
4 Istituto Zooprofilattico Sperimentale delle Venezie, FAO Reference Centre for Rabies, Viale dell'Università, 10, 35020-Legnaro (PD), Italy; pdebenedictis@izsvenezie.it (P.D.B.); MGourlaouen@izsvenezie.it (M.G.)
5 Centers for Disease Control and Prevention (CDC), Poxvirus and Rabies Branch, Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Diseases, Atlanta, GA 30329, USA; lao0@cdc.gov (L.A.O.); pay1@cdc.gov (P.A.Y.); lzu1@cdc.gov (C.M.G.)
6 Canadian Food Inspection Agency, Centre of Expertise for Rabies, Ottawa Laboratory Fallowfield, 3851 Fallowfield Road, Nepean, ON K2H 8P9, Canada; kim.knowles@canada.ca (M.K.K.); christine.fehlner-gardiner@canada.ca (C.F.-G.)
7 French Agency for Food, Environmental and Occupational Health and Safety (Anses), Laboratory for Rabies and Wildlife, Domaine de Pixérécourt, 54220 Malzéville CEDEX, France; Alexandre.servat@anses.fr (A.S.); florence.cliquet@anses.fr (F.C.)
8 Animal and Plant Health Agency (APHA), Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK; Denise.Marston@apha.gov.uk (D.M.); Lorraine.McElhinney@apha.gov.uk (L.M.M.); Trudy.Johnson@apha.gov.uk (T.J.); Tony.Fooks@apha.gov.uk (A.R.F.)
* Correspondence: Conrad.Freuling@fli.de; Tel.: +49-3835171660

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Abstract: As a neglected zoonotic disease, rabies causes approximately 5.9×10^4 human deaths annually, primarily affecting low- and middle-income countries in Asia and Africa. In those regions, insufficient surveillance is hampering adequate medical intervention and is driving the vicious cycle of neglect. Where resources to provide laboratory disease confirmation are limited, there is a need for user-friendly and low-cost reliable diagnostic tools that do not rely on specialized laboratory facilities. Lateral flow devices (LFD) offer an alternative to conventional diagnostic methods and may strengthen control efforts in low-resource settings. Five different commercially available LFDs were compared in a multi-centered study with respect to their diagnostic sensitivity and their agreement with standard rabies diagnostic techniques. Our evaluation was conducted by several international reference laboratories using a broad panel of samples. The overall sensitivities ranged from 0% up to 62%, depending on the LFD manufacturer, with substantial variation between the different laboratories. Samples with high antigen content and high relative viral load tended to test positive more often in the Anigen/Bionote test, the latter being the one with the best performance. Still, the overall unsatisfactory findings corroborate a previous study and indicate a persistent lack of appropriate test

validation and quality control. At present, the tested kits are not suitable for in-field use for rabies diagnosis, especially not for suspect animals where human contact has been identified, as an incorrect negative diagnosis may result in human casualties. This study points out the discrepancy between the enormous need for such a diagnostic tool on the one hand, and on the other hand, a number of already existing tests that are not yet ready for use.

Keywords: rabies; diagnostics; lateral flow devices; validation

1. Introduction

Rabies is one of the most important yet neglected zoonotic diseases, causing approximately 5.9×10^4 human deaths annually and primarily affecting low- and middle-income countries in Asia and Africa [1,2]. The deadly encephalitis is caused by different lyssaviruses, with the prototypical rabies virus (RABV) being their best-known representative and responsible for the vast majority of human cases [3]. All lyssaviruses belong to the Rhabdoviridae family in the order Mononegavirales [4]. The genome encodes for the five different viral proteins: nucleoprotein N, phosphoprotein P, matrix protein M, glycoprotein G, and the large polymerase L. All of these proteins are essential for virus replication and virus spread [5].

The virus is transmitted via infectious saliva, usually through bites or scratches of infected animals [3]. Dogs are the main source of human rabies, with approximately 99% of all human cases attributed to infections from rabid dogs [2]. Although resulting in a lethal disease, human rabies is completely preventable through vaccination of the dog reservoir to eliminate its source as well as through adequate and timely post-exposure prophylaxis (PEP), consisting of wound care and rabies vaccination in combination, when indicated, with rabies immunoglobulin [2]. At present, the United Against Rabies (UAR) consortium, comprising of the international organizations World Health Organization (WHO), World Organisation for Animal Health (OIE), Food and Agriculture Organization of the United Nations (FAO) and the Global Alliance for Rabies Control (GARC), have established a global strategic plan to reduce the burden of rabies, with the goal of reaching zero human deaths due to dog-mediated rabies by 2030 [6]. One of the pillars of this plan is to increase and harmonize rabies surveillance, ideally based on laboratory confirmation of clinically suspect animals to provide evidence-based guidance [7], i.e., both for bite case management and guidance for individual treatment as well as for general data aggregation to inform policymakers.

To this end, post mortem techniques recommended for the detection of the disease in animals [8] include the direct fluorescent antibody test (DFA), which was previously considered the gold standard and is still used as the reference method in most laboratories. The DFA is based on detecting viral antigen in brain impressions stained with fluorophore-conjugated antibodies by the use of fluorescence microscopy [9]. When biotin-conjugated antibodies are used in a direct rapid immunochemical test (DRIT), this technique offers the advantage that viral antigen can also be detected using light microscopy [10,11]. Various RT-PCRs have been established and validated to identify the presence of viral RNA [8]. Other tests, used mainly as confirmatory techniques, are based on virus isolation in cell culture, i.e., the rapid tissue culture infection test (RTCIT, [12]) or in mice [13]. Replacement of the latter by RTCIT is desirable for several reasons, including ethical considerations [8].

Rabies particularly occurs in regions of Africa and Asia that have limited access to healthcare or veterinary services [14], including adequate laboratory facilities with staff with high-level scientific expertise. Furthermore, often there are logistical constraints for sample shipment and storage, e.g., maintenance of a cold chain to ensure reliable results under tropical and subtropical conditions. Besides the fact that medical intervention, i.e., PEP, is not targeted without laboratory confirmation, lack of surveillance data is also one essential component that drives the vicious circle of neglect. Inaccurately low human and animal rabies case numbers belie the strong need for action [15,16].

Where laboratory-based disease confirmation is limited, there is a high demand for user-friendly and reliable low-cost diagnostic tools that rely neither on specific laboratory facilities nor on compound logistics. Lateral flow devices (LFDs), also known as rapid immunodiagnostic tests (RIDTs), immunodiagnostic assays or immunochromatographic strip tests, offer a promising alternative to conventional diagnostic methods and have the potential to strengthen prevention and control efforts in low-resource settings [17]. These LFDs are principally based on colloidal gold conjugated monoclonal antibodies that capture the antigen within a sample. The antigen–antibody complex thereupon binds to a second detection antibody that is fixed at the test zone "T" on a nitrocellulose membrane, showing a colored line for a positive sample [18]. With their relative ease of test performance, which requires minimal staff training or scientific expertise, ambient storage temperature and minimal processing time, LFDs show great potential for in-field use for rabies diagnosis. Accordingly, the increased use of LFDs could help to overcome the limitations of disease detection and improve surveillance. However, prior to the adoption of such technology, the quality of these devices must be rigorously tested since life-saving PEP decisions may be contingent on results. False negative test results are unacceptable for a disease with case fatality of nearly 100%.

The Anigen/Bionote LFD is the only rabies test for which scientific evaluation has been published, showing promising results [19–29]. However, in a study from 2016, six different LFDs, including the Anigen/Bionote test, were evaluated with very unsatisfying results concerning their diagnostic reliability [30].

Since the completion of these previous studies, numerous LFDs marketed for rabies diagnosis have become available on the market. Based on current knowledge, none of those have passed any kind of national or international quality control or licensure procedures, such as in the United States [31] or Germany [32], indicating a lack of data regarding their sensitivity and specificity beyond the data from the test insert. We therefore assessed the diagnostic performance of these currently available LFDs in addition to Anigen/Bionote in relation to DFA and RT-qPCR using a comprehensively broad panel of samples within a multi-centered study. Furthermore, we determined the target of the diagnostic antibody used in the LFDs by assessing the reaction towards standardized samples consisting of transfected cells expressing only glycoprotein G, nucleoprotein N, matrix protein M, and phosphoprotein P, respectively.

2. Materials and Methods

2.1. Commercial LFD Test Kits for Rabies Diagnosis in Brain Material

To complement the analysis of a previous study [30], different commercial LFD test kits were selected based on availability and country of origin. The selected kits originated from five different countries and were purchased online. The acquired test kits were the Anigen Rapid Rabies Ag test kit (Bionote, Hwaseong-si, Korea; LOT NO: 1801DDO19), Intermedical Rapid Test Device Rabies Ag (Intermedical Diagnostics, Villaricca, Italy; LOT NO: Q007011701), LilliTest Rapid Rabies Ag test kit (Lillidale, Wimborne, United Kingdom; LOT NO: LRR041801), Elabscience rabies virus antibodies rapid test (Elabscience Biotechnology Inc., China/USA; LOT NO: AK0018JAN24057) and Span Biotech rapid rabies test (Span Biotec Ltd., Shenzhen, China; LOT NO: R04415970151). Each test kit consists of one test device, a cotton swab, a buffer solution tube or dropper bottle, and a small disposable pipette. All test kits require only little to no experience in laboratory work and can easily be performed after following the manufacturer's user manuals; the Anigen kit provides a pictorial illustration of the most important steps and all kits provided illustrations of possible test results.

2.2. Participating Laboratories

Eight different OIE and FAO international reference laboratories for rabies were invited to participate voluntarily in this inter-laboratory comparison, from the following countries: Canada (Canadian Food Inspection Agency, CFIA), France (French Agency for Food, Environmental and

Occupational Health & Safety, ANSES), Germany (Friedrich-Loeffler-Institut, FLI), Israel (Kimron Veterinary Institute, KVI), Italy (Istituto Zooprofilattico Sperimentale delle Venezie, FAO reference centre), South Africa (Onderstepoort Veterinary Institute, OVI), United Kingdom (Animal and Plant Health Agency, APHA), and USA (Centers for Disease Control and Prevention, CDC). Due to the limited numbers of kits, they were split amongst the participating laboratories, but could not be distributed equally to all. In general, the laboratories received at least ten tests per manufacturer and independently decided on the samples they included in the study.

2.3. Sensitivity Analyses

Diagnostic sensitivity of the commercial LFDs in comparison with DFA and RTqPCR results were investigated using a panel consisting of 132 different samples from already existing collections of frozen brain specimens. Each laboratory tested selected samples from their own collections, and no animals were used for this study.

All samples contained fresh or archived naturally infected brains or mouse brain homogenates generated from field strains after mouse inoculation. The panel comprised of 26 different genetic lineages of all major RABV genetic clusters (Arctic/Arctic-like, Asian, Cosmopolitan, New World), originating from 30 countries. Only RABV positive samples (N = 105) were taken into account for sensitivity analyses. All invalid test results were excluded from analysis, while faint lines were still considered as positive. The sensitivity was calculated using GraphPad Prism (Version 7), with confidence limits calculated according to Clopper and Pearson [33].

In addition to RABV variants, European bat lyssavirus type 1 (EBLV-1), European bat lyssavirus type 2 (EBLV-2), Bokeloh bat lyssavirus (BBLV), and Lleida bat lyssavirus (LLBV) infected mouse brains were included in this study at APHA.

For each sample, the DFA was performed according to the standard operating procedures of the respective laboratory, with results being quantified using a four-plus scoring system. Additionally, samples were subjected to real-time RT-PCR for confirmation and to determine the relative viral load with different RT-qPCR assays (Supplementary Table S1). For reasons of data evaluation, the ct-values were stratified into three different groups: a ct-value that exceeded 25 was considered as "low", whereas a ct-value between 15 and 25 was regarded as "high", and a ct-value below 15 was considered as a "very high" viral load.

All participating laboratories tested their own selected samples with the different LFDs by strictly following the manufacturers' instructions. Briefly, a cotton swab was inserted into a 10% brain tissue suspension until saturated and then placed into the buffer solution where it was thoroughly mixed for about ten seconds. Between two and four drops of the buffer solution were then added to the sample pad using the disposable pipette. The reading was done 10 to 15 min afterwards, as recommended by the manufacturers. The test lines on the strips were classified by using a binomial plus/minus scoring system representing either a positive result when a red stripe appeared in the test line "T" or a negative outcome when the test line was not visible. The test was considered valid by the appearance of a red line on the control area "C" (Supplementary Figure S1). Also, non-infected brain homogenates (N = 20) were included (Supplementary Table S1).

2.4. Identification of the Binding Target of Antibodies Used in LFDs

For determining the binding target of the antibodies used on the test strips, HEK293T cells were transfected at FLI in six-well plates with 6 µg expression plasmid pCAGGS coding for the four RABV genes, N, P, M, and G, as described before [34,35]. After 24 h, the cells were suspended in 500 µl PBS. Samples from two wells were mixed and the cells were subsequently pelleted by 5 min centrifugation (1000× *g*; room temperature). The pellets were resuspended in 200 µL PBS buffer and vortexed. Eventually, 15 µL of the cell suspension were added to the buffer of the test kit and the test was evaluated as described before. This analysis was performed in duplicate per plasmid per test kit.

3. Results

3.1. Diagnostic Sensitivity and Specificity of Five LFD Test Kits

None of the negative samples tested positive in any test; thus all tests demonstrated a specificity of 100%. Test sensitivity was highly variable, ranging between 0% and 62% (Figure 1a,b, Table 1), depending on the type of LFDs. Specifically, the Span Biotech kit detected none of 105 RABV positive samples, resulting in a sensitivity of 0%. With values ranging between 1% and 3%, the Lillidale and Intermedical test kits exhibited similarly low sensitivities, whereas the Elabscience kit showed a moderately higher sensitivity of 20% (95% CI: 12.8% to 30.1%, Table 1).

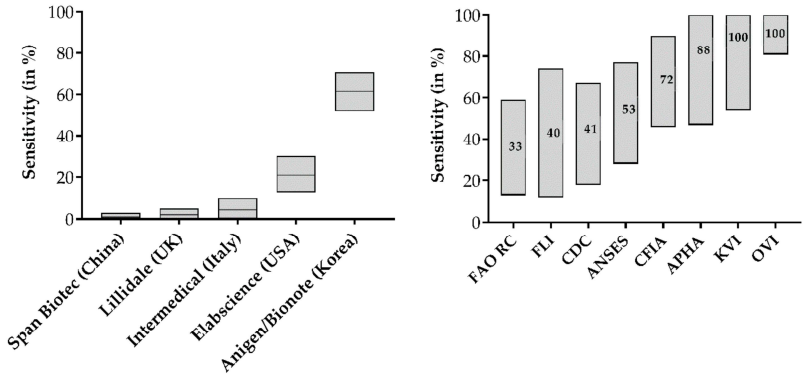

Figure 1. Overall sensitivities of tested different LFDs for confirmed positive samples (**a**), and the sensitivities for the Anigen/Bionote test at the different laboratories (**b**). The confidence limits are indicated as shaded boxes.

Table 1. Summary of results for the different LFD tests for rabies positive and negative samples.

Manufacturer	RABV Pos		RABV Neg		Sensitivity	95% CI
	LFD Pos	LFD Neg	LFD Pos	LFD Neg		
Span Biotec (China)	0	105	0	12	0%	0% to 3%
Lillidale (UK)	1	103	0	12	1%	0.2% to 5%
Intermedical (Italy)	2	66	0	11	3%	0.3% to 10%
Elabscience (China/USA)	19	74	0	12	20%	12.8% to 30.1%
Anigen/Bionote (Korea)	69	43	0	16	62%	51.9% to 70.6%

The overall sensitivity of the Anigen/Bionote test for RABV was 62% (95% CI: 51.9% to 70.6%). For further analyses concerning the test agreement with DFA and RT-qPCR, only the results of Anigen/Bionote were taken into account as the sensitivities of the other four LFDs were too low to draw any further conclusions.

3.2. Anigen/Bionote in Interlaboratory Comparison and Agreement with DFA and RT-qPCR

When applying a post hoc stratification for factors (covariates), the Anigen/Bionote LFD test kit demonstrated a variable sensitivity between 33% and 100% in different participating laboratories (Figure 1a,b). While the sensitivity was 100% in the panels tested at KVI in Israel and OVI in South Africa, in other laboratories, much lower sensitivities were observed (Figure 1b). Specimens with a

high antigen load (3+ and 4+) as measured by DFA were more likely to also test positive with the Anigen/Bionote tests than the ones with a low antigen load (+ and 2+, Figure 2a,b). The difference in the resulting sensitivities for the antigen content was not statistically significant (Fischer's exact test, $p = 0.31$). Similarly, the sensitivity of the Anigen/Bionote test was highest (87%) in samples containing very high viral RNA loads (ct-value <15), while it decreased in samples with less RNA content (49% of ct-value 15–25, 17% of ct-value >25). Additionally, non-RABV lyssavirus positive samples, i.e., EBLV-1, EBLV-2, LLBV, and BBLV, were included among samples at APHA. All except for LLBV were detected as positive only by the Anigen/Bionote test.

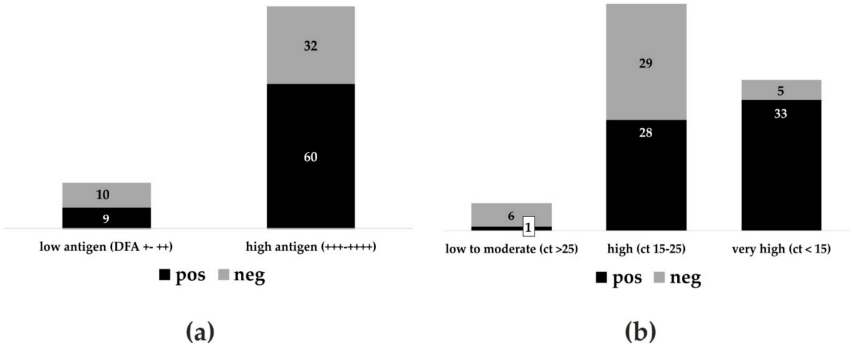

Figure 2. Diagnostic performance of the Anigen/Bionote LFD in relation to the antigen content as measured by DFA (**a**) and the relative viral RNA content as measured by RT-qPCR (**b**). Results are shown in absolute numbers.

3.3. Identification of the Binding Target of Antibodies Used in LFDs

When testing the various LFDs with different viral proteins, Span Biotech and Lillidale showed no reaction at all, whereas Intermedical and Elabscience tested positive when G-gene transfected cells were used, while Anigen/Bionote reacted specifically with N-gene transfected cells.

Of note, in the test zone "T" of Anigen/Bionote, a strong red line was clearly visible, while on the Intermedical test strips, the test line was barely visible. Also for Elabscience, where two different batches were used, a marked difference in the visibility and intensity of the test line was observed.

4. Discussion

In this study, different LFDs for rabies diagnosis were evaluated in regard to their diagnostic sensitivity and their agreement with DFA and RT-qPCR in order to ascertain their suitability as point-of-care diagnostics in routine surveillance. Historically, DFA was regarded as the gold standard; however, with recent updates to recommend tests by both OIE and WHO, both DFA and RT-qPCR approaches can be used as a primary diagnostic test for rabies since both demonstrate a very high (>95%) diagnostic sensitivity and specificity [8].

With sensitivities of the LFDs ranging between 0% and 62%, the outcome of our investigation confirms previous comparative analysis where different LFDs, including the Anigen/Bionote kit, showed unsatisfactory results. Although in that study the Anigen/Bionote performed the best, sub-optimal performance was still observed [30].

Apart from the Anigen/Bionote test kit, the results of the other four test kits did not differ much between the different laboratories since there were only three detections among all samples. Lillidale, Span Biotech, and Intermedical consistently failed in every laboratory. In contrast, Anigen/Biotech showed a wide range of sensitivities between 33% and 100% (Figure 1a,b, Table 1). This is perplexing,

and may partly reflect the differences of our study with other published data where sensitivities of the Anigen/Bionote LFD test kit ranged between 91% and 100% [19–29].

With the explicit aim to include a broad diversity of RABV isolates, with respect to geographical origin, host species, and genetic background of RABV, we included different genetic lineages of all major genetic clusters [36] from most parts of rabies endemic areas. Unfortunately, none of these parameters provided any correlation to the outcome of the test result. For instance, members of one specific genetic lineage tested both positive and negative with the Anigen/Bionote test. In principle, LFD tests are based on antibody recognition of the target analyte, in our case, the lyssavirus antigen, and the performance of the test is linked to the specific characteristics of the binding antibody. No information is available on the target antigen by the manufacturers except for Anigen/Bionote [28], and our analyses using transfected cells demonstrated that only one kit recognized N protein as a target, whereas two kits detected G-protein. Because of the conserved structure of the nucleoprotein and the abundance in clinical specimens, antibodies targeting this protein are generally used for diagnostic purposes, i.e., for the DFA and the DRIT [9,11]. Conversely, it is much more difficult to verify the presence of G-protein in the brain of infected animals. Therefore, G antigen is not considered a sufficiently sensitive target for detection. In fact, this may be one reason why only the N-targeting Anigen/Bionote showed the highest sensitivities as opposed to all other tests (Figure 1a, Table 1). The absence of any reaction with transfected cells in the Span Biotech and Lillidale kits is striking and correlates to their absolute failure in detecting rabies.

Only the Anigen/Bionote tests were able to also detect three other non-RABV lyssavirus positive samples, i.e., EBLV-1, EBLV-2, and BBLV. Previous studies had also shown that the reactivity of this test is not limited to RABV, with the detection of both Phylogroup I [23,27] and Phylogroup II [21] viruses noted. With this broad reactivity, it is unlikely that the genetic background of the analyte plays an important role in modifying the test performance of the Anigen/Bionote. Parameters that influenced the likelihood of test agreement with the established DFA and RT-qPCR methods were a high antigen as well as high viral RNA content, indicating an effect of disease progression on the test performance, which would be a severe limitation of these tests. Such effects of different populations on test characteristics are often seen in validation studies [37].

Still, the lack of test agreement with the DFA and RT-qPCR remains puzzling against the background that most of the samples used for the interlaboratory comparison had already been confirmed highly positive in DFA.

For reasons that also remain unknown, fresh samples from the field, used at KVI in Israel and OVI in South Africa, tended to yield better agreements with standard methods than archived samples, even though these were tested positive in RT-qPCR and DFA after years of storage. This phenomenon was observed previously with laboratory [30] and field investigations [19]. The latter study also demonstrated an increased sensitivity when modifying the manufacturer's instruction and eliminating the first dilution step, an effect also observed under laboratory conditions [30]. In fact, the sensitivity was also increased when the test protocol by Lechenne et al. 2016, whereby the initial dilution step is omitted, was additionally applied at the FAO reference centre in the present study (Supplementary Table S1). Together these data suggest that a modification of the manufacturer's instructions may increase sensitivity of the Anigen/Bionote test kit.

Another inter-laboratory comparison with two LFDs, including the Anigen/Bionote, was recently performed using a panel of ten anonymized samples of experimentally infected mouse brains [20]. For a single lot of Anigen/Bionote test kits, an overall concordance of results of 100% was achieved amongst the participating laboratories, and in comparison to the DFA, for RABV-infected tissue. However, these results must be interpreted carefully regarding the diagnostic sensitivity of the test as the sample size was limited, and did not necessarily reflect the variation in antigen load that would be observed in a diagnostic laboratory. Furthermore, the sample dilution used was lower than that found in the manufacturer's instructions. In the same laboratory (ANSES), the sensitivity of the Anigen/Bionote kit in the present study was below average (Figure 1b), supporting previous concerns about batch-to-batch

variation in the quality of those tests [30], and a trend towards reduced sensitivity on samples with lower antigen content, as demonstrated here.

In the context of shortages in rabies diagnostics in low resource settings, the development of rapid, reasonably priced, and user-friendly solutions to detect rabies virus in brain material could be a major step towards disease control and elimination. However, high quality is crucial as false-negative test results would not only promote negligence in a global epidemiological context but may discourage exposed individuals from seeking prompt medical care including PEP, putting them at an important risk of fatal outcome. Additionally, even though the specificity was high in this and other studies, false positive outcomes are also unacceptable as they lead to an incorrect distribution of expensive and limited medical resources and biologics.

With the increasing demand for point of care diagnostics, a growing but non-transparent landscape of commercially available LFDs for rabies diagnosis has emerged. It is not clear how many of the approximately 15 LFD test kits which are currently available online are actually sold under different brand names while they may originate from only a few manufacturers. Furthermore, none of the manufacturers provided detailed insight into the principles on which their tests are based, i.e., the specific antibodies they use on the test strip.

This lack of transparency, proper validation, and thus reliability limits the use of LFDs and the results of our study advocate for stricter quality controls and approval procedures, especially when taking the international goal to reach zero human deaths due to dog-mediated rabies by 2030 into account. In some countries, for example in Germany, where diagnostics for notifiable animal diseases need to obtain marketing authorization, such tests would not fulfill the respective criteria.

There are clear guidelines for the development and validation of tests for veterinary diagnostics. Following these guidelines, a change of procedures after licensure, as it has been done in some field studies (e.g., [19]), would not be acceptable [38]. Another major component that had been addressed before [19,30] is the manufacturers' instructions, which should be more specific for sampling and sample preparation, and secondly, concern the use of saliva instead of brain material. Except for Anigen/Bionote, all other tests still mention saliva as an analyte, which is likely to give false negative results because of intermittent shedding of virus in saliva or limited amount of virus in saliva below the limit of detection [39]. The declaration of some manufacturers that the purpose of the kit is "for research only" does not absolve them from their responsibilities in offering a test with an appropriate quality as those tests are most likely bought by customers other than research scientists. In response to the results obtained, all manufacturers were contacted but the response received was limited to no responses at all. Intermedical, Elabscience, and Lillidale sent new batches for re-testing and after confirming the limited quality, at least Lillidale guaranteed to stop sales.

5. Conclusions

The increasing use of LFDs as a basis for surveillance, disease control, and medical intervention [19] indicates the need to overcome resource- and operational limitations in current rabies diagnosis. Nevertheless, massive improvements need to happen before tests, even the Anigen/Bionote test, can be unconditionally recommended by the OIE. Simply encouraging the producers to substantially improve and assure the quality of their test kits, as done by the previous study [30], did not show any significant development or changes. Therefore, the quality and evaluation of those tests should be controlled through standardized approval procedures following OIE recommendations [38]. In order to increase the pressure on manufacturers, for instance, only those kits that have passed such licensure should be used in the frame of UAR-supported rabies control programs.

Supplementary Materials: The following are available online at http://www.mdpi.com/2414-6366/5/1/13/s1, Figure S1: positive direct fluorescent antibody test (DFA, a), positive direct rapid immunochemical test (DRIT, b) and negative LFD result (Anigen/Bionote, c) of a rabid fox (A17-3454 AZ) infected with the South Central Skunk RABV variant, a and b: magnification = 200×; Table S1: Test results and details of samples tested.

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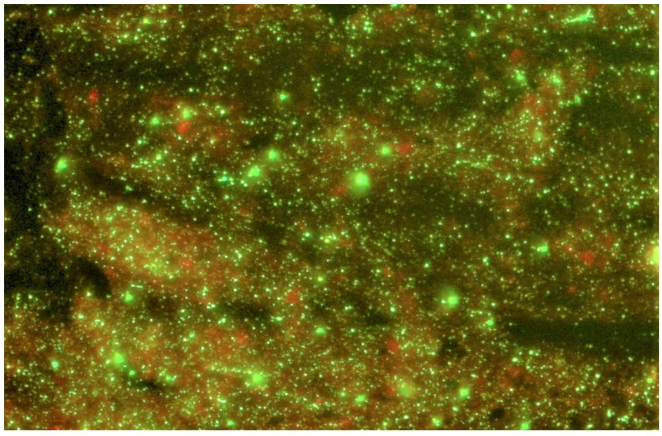
Supplementary Material:

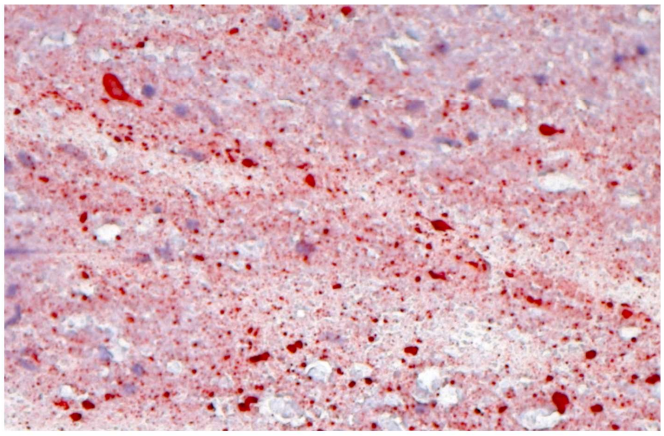
Figure S1: **positive direct fluorescent antibody test (DFA, a), positive direct rapid immunochemical test (DRIT, b) and negative LFD result (Anigen/Bionote, c) of a rabid fox (A17-3454 AZ) infected with the South Central Skunk RABV variant, a and b: magnification = 200×;**

Table S1: **Test results and details of samples tested.**

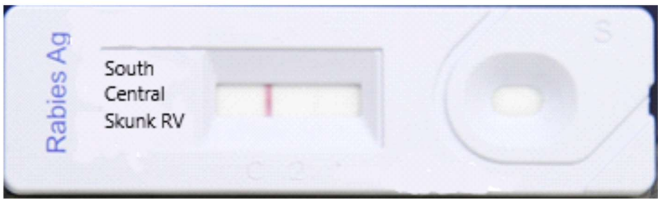
a)



b)



c)



Lab	LabImgID	host	virus	lineage	origin	DFA	qRTmegPCRmegResult	Viral load	InterMedical	Antigen/Bionote	Antigen/Bionote modified	Elabscience	Rabies Ag (Span Biotech)	Lillidale
ANSES	DR-1793	cat	-	-	Luxembourg	neg	no Ct		neg	neg		not tested	neg	neg
ANSES	DR-1788	fox	-	-	Luxembourg	neg	no Ct		neg	neg		not tested	neg	neg
ANSES	DR-1784	fox	-	-	Luxembourg	neg	no Ct		neg	neg		neg	neg	neg
ANSES	GS-5 bat 05-18 (1/50)	fox @mouse	RABV	Cosmopolitan lineage-WE	France	2	19.70	high (15-25)	neg	neg		neg	neg	neg
ANSES	GS-5 bat 03-18 (1/30)	fox @mouse	RABV	Cosmopolitan lineage-WE	France	3	18.90	high (15-25)	neg	neg		neg	neg	neg
ANSES	GS-7 bat 14-18	fox @mouse	RABV	Cosmopolitan lineage-WE	France	3	10	very high (5-15)	neg	pos		(pos) Doubtful	neg	neg
ANSES	RABV Maroc bat 12-17	dog @mouse	RABV	Cosmopolitan-Africa-1	Morocco	3	17.50	high (15-25)	neg	pos		neg	neg	neg
ANSES	Shevaplus bat 10-16	fox @mouse	RABV	Cosmopolitan lineage-NEE	Slovakia	3	18.80	high (15-25)	neg	pos		neg	neg	neg
ANSES	DR-1778	reindeer	RABV	Arctic Fox RV	Norway	3	28.20	low	neg	neg		neg	neg	neg
ANSES	DR-0914	fox	RABV	Cosmopolitan lineage-EE	Albania	1	16.40	high (15-25)	neg	pos		neg	neg	neg
ANSES	DR-1068	fox	RABV	Vaccine strain	Hungaria	1	25.60	low	neg	neg		neg	neg	neg
ANSES	DR-1037	cattle	RABV	Vaccine strain	Romania	2	14.50	very high (5-15)	neg	pos		neg	neg	neg
ANSES	DR-1333	fox	RABV	Cosmopolitan lineage-NEE	Romania	1	16.80	high (15-25)	neg	neg		neg	neg	neg
ANSES	DR-1335	fox	RABV	Cosmopolitan lineage-NEE	Romania	2	16.40	high (15-25)	neg	neg		neg	neg	neg
ANSES	DR-1343	cat	RABV	Cosmopolitan lineage-NEE	Moldova	2	17.30	high (15-25)	neg	neg		neg	neg	neg
ANSES	DR-1790	fox	RABV	Cosmopolitan lineage-C	Lituania	3	11.40	very high (5-15)	neg	pos		neg	neg	neg
ANSES	DR-0641	dog	RABV	Cosmopolitan lineage-EE	Greece	1	19.60	high (15-25)	neg	pos		neg	neg	neg
ANSES	DR-A627	fox	RABV	Cosmopolitan lineage-EE	Greece	1	18.20	high (15-25)	neg	pos		neg	neg	neg
ANSES	DR-0653	fox	RABV	Cosmopolitan lineage-EE	Greece	2	19.10	high (15-25)	neg	pos		neg	neg	neg
ANSES	DR-A654	fox	RABV	Cosmopolitan lineage-EE	Greece	1	18.60	high (15-25)	neg	neg		neg	neg	neg
Italy	10MR/5493-15	WILDCAT	RABV	AFRICA 3	BOTSWANA	4	17.20	high (15-25)	not tested	pos	pos	not tested	neg	neg
Italy	10MR/5493-8	DOG	RABV	COSMOPOLITAN (EX-AFRICA 1)	BOTSWANA	4	19.16	high (15-25)	not tested	pos	pos	not tested	neg	neg
Italy	10MR/5493-10	BOVINE	RABV	COSMOPOLITAN (EX-AFRICA 1)	BOTSWANA	4	14.30	very high (5-15)	not tested	pos	pos	not tested	neg	neg
Italy	RS13/266-4	BOVINE	RABV	COSMOPOLITAN (EX-AFRICA 1)	UGANDA	4	20.66	high (15-25)	not tested	neg	pos	not tested	neg	neg
Italy	RS13/266-5	DOG	RABV	COSMOPOLITAN (EX-AFRICA 1)	UGANDA	4	16.65	high (15-25)	not tested	neg	pos	not tested	neg	neg
Italy	RS13/266-12	DOG	RABV	COSMOPOLITAN (EX-AFRICA 1)	UGANDA	4	20.32	high (15-25)	not tested	neg	pos	not tested	neg	neg
Italy	RS13/116-8	BOVINE	RABV	American-Indigenous (vampire bat)	Brazil	4	22.28	high (15-25)	not tested	neg	pos	not tested	neg	neg
Italy	RS13/116-17	BOVINE	RABV	American-Indigenous (vampire bat)	Brazil	4	22.14	high (15-25)	not tested	neg	pos	not tested	neg	neg
Italy	RS13/116-38	BOVINE	RABV	American-Indigenous (vampire bat)	Brazil	4	23.56	high (15-25)	not tested	neg	pos	not tested	neg	neg
Italy	RS13/1249	mice replicated (ex-BAT)	RABV	American-Indigenous (insectivorous bat)	Brazil	4	23.19	high (15-25)	not tested	neg	pos	not tested	neg	neg
Italy	RS13/3609-5	PIOTOS FLAMUS	RABV	American-Indigenous (insectivorous bat)	Brazil	4	not tested		not tested	pos	pos	not tested	neg	neg
Italy	10RD/2322	FOX	RABV	Italy 1	Italy	4	not tested		not tested	neg	neg	not tested	neg	neg
Italy	10RD/1450	FOX	RABV	Italy 2	Italy	4	22.82	high (15-25)	not tested	neg	pos	not tested	neg	neg
Italy	10RD/1000	FOX	RABV	Italy 2a	Italy	4	16.50	high (15-25)	not tested	neg	pos	not tested	neg	neg
Italy	07VAR/6235-3	DOG?	RABV	AFRICA 2	Mauritania	4	not tested		not tested	pos	pos	not tested	neg	neg
Italy	07VAR/6097 (253)	DOG?	RABV	AFRICA 2	Niger	4	not tested		not tested	neg	neg	not tested	neg	neg
Italy	18RCV666-2	DOG	RABV	AFRICA 2	Liberia	4	not tested		not tested	neg	pos	not tested	neg	neg
Italy	18RCV666-4	DOG	RABV	AFRICA 2	Liberia	4	not tested		not tested	pos	pos	not tested	neg	neg
Italy	18RCV666-1	DOG			Liberia	neg	no CT		not tested	neg	neg	not tested	neg	neg
Italy	RS13/266-11	DOG			UGANDA	neg	not tested		not tested	neg	neg	not tested	neg	neg

KVI Israel	336706	Jackal	RABV	Cosmopolitan (Middle East)	Israel	4	11,90	very high (5-15)	neg	pos	pos	not tested	not tested	
KVI Israel	320155	Dog	RABV	Cosmopolitan (Middle East)	Israel	4	12,30	very high (5-15)	neg	pos	neg	not tested	not tested	
KVI Israel	315175	Cattle	RABV	Cosmopolitan (Middle East)	Israel	4	9,10	very high (5-15)	neg	pos	pos	not tested	not tested	
KVI Israel	318829	Sheep	RABV	Cosmopolitan (Middle East)	Israel	4	10,50	very high (5-15)	neg	pos	pos	not tested	not tested	
KVI Israel	305400	Cat	RABV	Cosmopolitan (Middle East)	Israel	4	12,90	very high (5-15)	neg	pos	neg	not tested	not tested	
KVI Israel	317604	Badger	RABV	Cosmopolitan (Middle East)	Israel	4	11,10	very high (5-15)	neg	pos	pos	not tested	not tested	
KVI Israel	337355	Jackal	-	-		neg	no CT		neg	neg	neg	not tested	not tested	
KVI Israel	337447	Cat	-	-		neg	no CT		neg	neg	neg	not tested	not tested	
KVI Israel	337443	Dog	-	-		neg	no CT		neg	neg	neg	not tested	not tested	
KVI Israel	337217	Cattle	-	-		neg	no CT		neg	neg	neg	not tested	not tested	
CDC	A19-0514	Sheep	NA	NA	Georgia, USA	neg	no CT		invalid test	neg	neg	neg	neg	
CDC	A19-0070	Raccoon	RABV	Eastern Raccoon RV	Tennessee, USA	4	20,88	high (15-25)	neg	pos (weak)	neg	neg	neg	
CDC	A19-0069	Skunk	RABV	North Central Skunk RV	Tennessee, USA	4	21,77	high (15-25)	neg	neg	neg	neg	neg	
CDC	A19-0484	Dog	RABV	Cosmolitan Dog (AF-4) RV	Egypt (translocated to KS USA)	4	26,04	low	neg	neg	neg	neg	neg	
CDC	A18-1142	Bovine	RABV	Arizona Gray Fox RV	Arizona, USA	4	22,81	high (15-25)	neg	neg	neg	neg	neg	
CDC	A18-1690	Dog	RABV	Tadarida brasiliensis (Tb) RV	Georgia, USA	4	21,97	high (15-25)	neg	pos (weak)	neg	neg	neg	
CDC	A18-1274	Goat	RABV	North Central Skunk RV	North Dakota, USA	4	23,10	high (15-25)	neg	neg	neg	neg	neg	
CDC	A18-0238	Cat	RABV	Lasiurus cinereus RV	North Carolina, USA	4	25,37	low	invalid test	neg	neg	neg	neg	
CDC	A18-1123	Dog	RABV	Eastern Raccoon RV	Delaware, USA	4	22,99	high (15-25)	neg	neg	neg	neg	neg	
CDC	A17-3454	Fox	RABV	South Central Skunk RV	Arizona, USA	4	20,28	high (15-25)	invalid test	neg	neg	neg	neg	
CDC	A19-0072	Raccoon	NA	NA	Kentucky, USA	neg	no CT		neg	neg	neg	neg	neg	
CDC	A18-1145	Dog	NA	NA	Virgin Islands (US Territory)	neg	no CT		neg	neg	neg	neg	neg	
CDC	A18-2091	Raccoon	RABV	Eastern Raccoon RV	Georgia, USA	4	20,32	high (15-25)	neg	pos (weak)	neg	neg	neg	
CDC	A18-3256	Fox	RABV	Arctic Fox RV	Alaska, USA	4	23,25	high (15-25)	neg	pos (weak)	neg	neg	neg	
CDC	A17-4048	Skunk	RABV	Western Spotted Skunk RV	Colorado, USA	4	21,98	high (15-25)	neg	neg	neg	neg	neg	
CDC	A17-4100	Fox	RABV	Arizona Gray Fox RV	Arizona, USA	4	24,60	high (15-25)	neg	neg	neg	neg	neg	
CDC	A17-4241	Dog	RABV	Cosmolitan Dog (AF-4) RV	Egypt (translocated to CT USA)	4	22,93	high (15-25)	neg	pos (weak)	neg	neg	neg	
CDC	A17-3417	Fox	RABV	South Central Skunk RV	New Mexico, USA	4	19,31	high (15-25)	neg	pos (weak)	neg	neg	neg	
CDC	A15-2820 (15-3409)	Gray Fox	RABV	Myotis sp RV	Oregon, USA	4	25,62	low	invalid test	(pos) Doubtful	neg	neg	neg	
CDC	A15-2815 (13-01325)	Fox	RABV	Myotis sp RV	Oregon, USA	4	26,33	low	neg	neg	neg	neg	neg	
OVI	001/19	Bovine	RABV	Mongoose variant	Cloculan, Free State, SA	3	23,72	high (15-25)	neg	pos	neg	neg	neg	
OVI	002/19	Mongoose	RABV	Mongoose variant	Colliary, North West, SA	4	13,41	very high (5-15)	neg	pos	pos	neg	neg	
OVI	044/19	Jackal	RABV	canid variant	Knopfontein, North West, SA	4	24,17	high (15-25)	pos	pos	pos	neg	neg	
OVI	045/19	Dog	RABV	canid variant	Jappi, Limpopo, SA	4	15,05	high (15-25)	neg	pos	neg	neg	neg	
OVI	049/19	Jackal	RABV	canid variant	Naledi, North West, SA	4	12,85	very high (5-15)	pos	pos	neg	neg	neg	
OVI	065/19	Dog	RABV	canid variant	Polokwane, Limpopo, SA	4	10,82	very high (5-15)	neg	pos	neg	neg	neg	
OVI	076/19	Bovine	RABV	canid variant	Ganyesa, North West, SA	4	20,71	high (15-25)	neg	pos	neg?	neg	neg	
OVI	082/19	Meerkat	RABV	Mongoose variant	Ladybrand, Free State, SA	4	13,16	very high (5-15)	neg	pos	neg	neg	neg	
OVI	384/18	Dog	RABV	canid variant	Nkomazi, Mpumalanga, SA	4	24,48	high (15-25)	neg	pos	neg	neg	neg	
OVI	391/18	Dog	RABV	canid variant	Klerksdorp, North West, SA	4	19,51	high (15-25)	neg	pos	neg	neg	neg	
OVI	396/18	Rhino	RABV	canid variant	Windhoek, Namibia	4	13,20	very high (5-15)	neg	pos	neg	neg	neg	
OVI	505/18	Dog	RABV	canid variant	Schweizersiedler, NW, SA	4	12,11	very high (5-15)	neg	pos	neg	neg	neg	
OVI	541/18	Bat Eared fox	RABV	canid variant	Kuruman, Northern Cape, SA	4	20,89	high (15-25)	neg	pos	neg	neg	neg	
OVI	551/18	Dog	RABV	canid variant	Madidi, North West, SA	4	20,88	high (15-25)	neg	pos	neg	neg	neg	
OVI	555/18	Dog	RABV	canid variant	Tzaneen, Limpopo, SA	4	19,74	high (15-25)	neg	pos	neg	neg	1	

OVI	557/18	Dog	RABV	canid variant	Bushbuckridge, MP, SA	4	20,13	high (15-25)	neg	pos	neg	neg	neg
OVI	560/18	Dog	RABV	canid variant	Makhado, Limpopo, SA	4	25,94	low	neg	pos	neg	neg	neg
OVI						**neg**	no cT		not tested	**neg**	**neg**	**neg**	**neg**
OVI						**neg**	no cT		not tested	**neg**	**neg**	**neg**	**neg**
OVI	V113 (H/119/86/D University of Berne)	dog	RABV	Sri Lankan dog	Sri Lanka	4	7,10	very high (5-15)	not tested	+	+	**neg**	**neg**
OVI	OLF-18-00127	dog	RABV	Arctic Fox RV	Canada	1	20,10	high (15-25)	not tested	**neg**	**neg**	**neg**	**neg**
OVI	OLF-17-00722	dog	RABV	Arctic Fox RV	Canada	1	17,90	high (15-25)	not tested	**neg**	**neg**	**neg**	**neg**
OVI	OLF-17-00710	dog	RABV	Arctic Fox RV	Canada	1	18,20	high (15-25)	not tested	**neg**	**neg**	**neg**	**neg**
OVI	V120 (82 University of Berne)	dog	RABV	Nepal dog	Nepal	3	6,10	very high (5-15)	not tested	+	+	**neg**	**neg**
OVI	V050 (RV424 Central Veterinary Lab, Weybridge GB)	yellow mongoose (MBr pass)	RABV	Africa mongoose	RSA	4	7,70	very high (5-15)	not tested	+	+	**neg**	**neg**
OVI	V218 (RR4-73 Texas Department of State Health Services)	coyote (MBr pass)	RABV	Mexican dog/coyote	Mexico	4	9,30	very high (5-15)	not tested	+	+	**neg**	**neg**
OVI	OLF-18-01902	dog	RABV	Arctic Fox RV	Canada	3	9,70	very high (5-15)	not tested	+	+	**neg**	**neg**
OVI	OLF-18-01669	skunk	RABV	BBCAN1	Canada	4	11,90	very high (5-15)	not tested	+	**neg**	**neg**	**neg**
OVI	OLF-18-1582	little brown bat (Myotis lucifugus)	RABV	MYCAN	Canada	4	13,80	very high (5-15)	not tested	**neg**	**neg**	**neg**	**neg**
OVI	V737 (00R025 Gyeongsang National Univeristy, Korea)	raccoon dog	RABV	Korea raccoon dog	Korea	4	12,90	very high (5-15)	not tested	+	**neg**	**neg**	**neg**
OVI	V650 (T97-0372 California Department of Public Health)	striped skunk	RABV	California skunk	USA	3	16,50	high (15-25)	not tested	+	+	**neg**	**neg**
OVI	V903 (Zbonosis Control Center Sao Paulo Brazil 1989)	dog	RABV	BRL-1a	Brazil	2	10,60	very high (5-15)	not tested	+	+	**neg**	**neg**
OVI	V908 (Zbonosis Control Center Sao Paulo Brazil 1997)	Histiotus velatus (MBr pass)	RABV	BRL-2	Brazil	3	14,80	very high (5-15)	not tested	+	**neg**	**neg**	**neg**
OVI	V905 (Zbonosis Control Center Sao Paulo Brazil 1999)	horse	RABV	BRL-3	Brazil	1	18,80	high (15-25)	not tested	**neg**	**neg**	**neg**	**neg**
OVI	OLF-16-00270	raccoon	RABV	MidAtlantic Raccoon	Canada	3	9,80	very high (5-15)	not tested	+	**neg**	**neg**	**neg**
OVI	OLF-17-00231	raccoon	RABV	MidAtlantic Raccoon	Canada	3	12,50	very high (5-15)	not tested	+	**neg**	**neg**	**neg**
OVI	OLF-18-01803	striped skunk	RABV	Western skunk	USA	4	9,90	very high (5-15)	not tested	+	+	**neg**	**neg**
APHA	RV50	Bat	RABV		USA	4	20,97	high (15-25)	neg	neg	neg	neg	neg
APHA	RV61	Arc-like	RABV	Arc-like	India	3	16,17	high (15-25)	neg	+	neg	neg	neg
APHA	RV313	Fox	RABV	cosmopolitan	Germany	3	16,10	high (15-25)	Not tested	(1) Doubtful	neg	neg	neg
APHA	RV334		RABV	Vaccine strain	China	2	16,57	high (15-25)	Not Tested	pos	(1) Doubtful	neg	neg
APHA	RV410		RABV	Africa 3	RSA	4	18,73	high (15-25)	neg	pos	(1) Doubtful	neg	neg
APHA	RV437	Raccoon Dog	RABV	cosmopolitan	Estonia	3	17,65	high (15-25)	neg	(1) Doubtful	(1) Doubtful	neg	neg
APHA	RV1237	Deer	RABV	cosmopolitan	Serbia	2	18,71	high (15-25)	Not Tested	(1) Doubtful	neg	neg	neg
APHA	RV2417	Dog	RABV		Sri Lanka	2	13,34	very high (5-15)	neg	pos	neg	neg	neg
APHA	RV20	bat	EBLV-1	subtype 1a	Germany	3	17,92		neg	(1) Doubtful	neg	invalid test	neg
APHA	RV628	bat	EBLV-2		UK	2	not tested		neg	+	neg	neg	neg
APHA	RV2507	bat	BBLV		Germany	1	not tested		neg	+	neg	neg	neg
APHA	RV3208	bat	LLEBV		Spain	2	27,28		neg	neg	neg	neg	neg
APHA	NA	Mouse	-ve			neg	no cT		neg	neg	neg	neg	neg
APHA	NA	Mouse	-ve			neg	no cT		neg	neg	neg	neg	neg
FLI	13315	horse	RABV	cosmopolitan	Germany	4	13,45	very high (5-15)	n/a	neg	neg	neg	neg
FLI	5013	fox	RABV	cosmopolitan	Germany	4	15,00	very high (5-15)	neg	neg	neg	neg	neg
FLI	35005	cat	RABV	cosmopolitan	Germany	4	12,88	very high (5-15)	neg	+	+	neg	neg
FLI	5481	sheep	RABV	cosmopolitan	Germany	3	16,10	high (15-25)	neg	neg	neg	neg	neg
FLI	5465	fox	RABV	cosmopolitan	Germany	4	13,03	very high (5-15)	neg	+	neg	neg	neg
FLI	11571	fox	RABV	cosmopolitan	Germany	4	14,13	very high (5-15)	neg	neg	neg	neg	not tested
FLI	11565	fox	RABV	cosmopolitan	Germany	4	13,93	very high (5-15)	neg	+	neg	neg	neg
FLI	13638	fox	RABV	cosmopolitan	Germany	4	14,54	very high (5-15)	neg	neg	neg	neg	neg
FLI	31181	deer	RABV	cosmopolitan	Germany	3	13,41	very high (5-15)	neg	+	neg	neg	neg
FLI	31827	dog	RABV	cosmopolitan	Germany	4	18,22	high (15-25)	neg	neg	neg	not tested	not tested
FLI	5483	fox	RABV	cosmopolitan	Germany	2	23,65	high (15-25)	Not tested	not tested	not tested	neg	neg

2. Publication II: Retrospective Enhanced Bat Lyssavirus Surveillance in Germany between 2018–2020

Antonia Klein [1], Sten Calvelage [2], Kore Schlottau [2], Bernd Hoffmann [2], Elisa Eggerbauer [3], Thomas Müller [3] and Conrad M. Freuling [1]

[1]Friedrich-Loeffler-Institut (FLI), 17493 Greifswald-Insel Riems, Germany

[2]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut (FLI), 17493 Greifswald-Insel Riems, Germany

[3]Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut (FLI), WHO Collaborating Centre for Rabies Surveillance and Research, OIE Reference Laboratory for Rabies, 17493 Greifswald-Insel Riems, Germany

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Communication

Retrospective Enhanced Bat Lyssavirus Surveillance in Germany between 2018–2020

Antonia Klein [1], Sten Calvelage [2], Kore Schlottau [2], Bernd Hoffmann [2], Elisa Eggerbauer [3], Thomas Müller [3] and Conrad M. Freuling [1,*]

[1] Friedrich-Loeffler-Institut (FLI), 17493 Greifswald-Insel Riems, Germany; antonia.klein@fli.de
[2] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut (FLI), 17493 Greifswald-Insel Riems, Germany; Sten.Calvelage@fli.de (S.C.); kore.schlottau@fli.de (K.S.); bernd.hoffmann@fli.de (B.H.)
[3] Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut (FLI), WHO Collaborating Centre for Rabies Surveillance and Research, OIE Reference Laboratory for Rabies, 17493 Greifswald-Insel Riems, Germany; Elisaeggerbauer@gmx.de (E.E.); Thomas.Mueller@fli.de (T.M.)
* Correspondence: conrad.freuling@fli.de

Abstract: Lyssaviruses are the causative agents for rabies, a zoonotic and fatal disease. Bats are the ancestral reservoir host for lyssaviruses, and at least three different lyssaviruses have been found in bats from Germany. Across Europe, novel lyssaviruses were identified in bats recently and occasional spillover infections in other mammals and human cases highlight their public health relevance. Here, we report the results from an enhanced passive bat rabies surveillance that encompasses samples without human contact that would not be tested under routine conditions. To this end, 1236 bat brain samples obtained between 2018 and 2020 were screened for lyssaviruses via several RT-qPCR assays. European bat lyssavirus type 1 (EBLV-1) was dominant, with 15 positives exclusively found in serotine bats (*Eptesicus serotinus*) from northern Germany. Additionally, when an archived set of bat samples that had tested negative for rabies by the FAT were screened in the process of assay validation, four samples tested EBLV-1 positive, including two detected in *Pipistrellus pipistrellus*. Subsequent phylogenetic analysis of 17 full genomes assigned all except one of these viruses to the A1 cluster of the EBLV-1a sub-lineage. Furthermore, we report here another Bokeloh bat lyssavirus (BBLV) infection in a Natterer's bat (*Myotis nattereri*) found in Lower Saxony, the tenth reported case of this novel bat lyssavirus.

Keywords: bat lyssavirus; bat rabies surveillance; European bat lyssavirus 1 (EBLV-1); Bokeloh bat lyssavirus (BBLV); zoonosis

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1. Introduction

Bats (Chiroptera) have been identified or were suspected of being reservoir hosts for a plethora of viruses including those with a zoonotic potential [1]. Among the latter, there are pathogens of high concern like Ebolaviruses, Henipaviruses, Coronaviruses and Lyssaviruses [2–6]. Interestingly, rabies is the oldest known bat associated infection in humans. Rabies in bats was first identified in the Americas, but has ever since been found on all continents except Antarctica [7]. The causative agents are different lyssaviruses of the family *Rhabdoviridae* within the order *Mononegavirales* [8]. Of note, with two exceptions, all of the 18 known lyssaviruses are associated with bats, their assumed ancestral primary reservoir hosts [9].

Six distinct lyssaviruses have been isolated from European bats, whereby the majority of reported cases is caused by European bat lyssavirus type 1 (EBLV-1) [10]. European bat lyssavirus type 2 (EBLV-2) was identified in only two dozen cases [11], while West Caucasian bat lyssavirus (WCBV) and Lleida bat lyssavirus (LLBV) were only isolated sporadically. Bokeloh bat lyssavirus (BBLV) [12] and Kotalahti bat lyssavirus (KBLV) [13] further extended the diversity of lyssaviruses found in European bats.

EBLV-1, EBLV-2 and BBLV are presently known to circulate in bats in Germany [10]. EBLV-1 caused the majority of the 346 reported German bat rabies cases [14], and has mainly been associated with the serotine bat (*Eptesicus serotinus*) [15]. In contrast, EBLV-2 was only isolated five times from Daubenton's bat (*Myotis daubentonii*) [11]. BBLV was first discovered in a Natterer's bat (*Myotis nattereri*) in Lower Saxony in 2010 [12]. Ever since, it has been isolated several times from this species in Germany, France and Poland [16,17], suggesting that the Natterer's bat is the reservoir host species.

All bat lyssaviruses are potentially capable of infecting other mammals including humans and cause the fatal disease rabies. In fact, in Europe bat lyssaviruses were identified in spillover infections in cats [18], sheep [19], and a stone marten [20]. Also, human rabies cases caused by EBLV-1 [21,22] and EBLV-2 [23,24] infections were confirmed, thus highlighting the zoonotic potential and public health importance. Therefore, to advance our understanding on epidemiology of bat-related lyssaviruses, surveillance activities are ongoing across Europe [15]. Against this background, we here report recent results from an enhanced passive surveillance scheme in Germany. By using molecular methods as opposed to previously applied fluorescent antibody test (FAT), we could detect several EBLV-1 cases, including two in the common pipistrelle (*Pipistrellus pipistrellus*). Additionally, we identified the tenth case of BBLV in a Natterer's bat.

2. Materials and Methods

2.1. Bat Samples

Dead found bats are regularly collected by local bat biologists, private bat handlers, different wildlife care centers as well as nature conservation institutions, and are stored under frozen conditions. Upon request and by providing cool boxes for shipment, bats were submitted to the Friedrich-Loeffler-Institute (FLI) for lyssavirus diagnosis. Information regarding bat species, geographical origin, and sex, was provided by the bat handlers. Where information on the bat species was missing, bats were determined to genus or species level by external morphological characteristics [25,26]. A total of 117 individuals could not be clearly specified due to a decomposed condition.

Additionally, an archived set of bat samples that had been tested negative for rabies by the FAT [15] was screened in the process of assay validation.

2.2. Brain Sample Generation

Brain tissue from bats was sampled by puncturing the *foramen occipitale magnum* using a syringe and a 0.90 × 40 mm cannula. Initially, brain tissue was aspirated and flushed out into Eppendorf vials using cell culture media. Then the cranial cavity was repeatedly flushed with cell culture media and aspirated. Here, a mixture of equal volumes of Eagle MEM (Hanks' balanced salts solution) and Eagle MEM (Earle's balanced salts solution) medium, supplemented with 10% fetal bovine serum was used. Extracted brain tissue was homogenized in a volume of 1000 µL cell culture media and stored at −80 °C until further testing.

2.3. RNA Extraction

Total RNA was extracted from 100 µL of brain suspension in a BioSprint 96 magnetic particle processor (Qiagen, Hilden, Germany) using the NucleoMagVet kit (Macherey & Nagel, Düren, Germany) according to the manufacturer's instructions. A final volume of 100 µL nucleic acid was generated.

2.4. Virus Detection by RT-qPCR

For the detection of lyssaviral RNA, a double-check approach was used [27]. On the one hand, a pan-lyssa real-time RT-PCR targeting both the N- and L-gene with Resolight as intercalating dye was conducted. The RT-PCR reaction was prepared using the OneStep RT-PCR kit (Qiagen), adjusted to a volume of 12.5 µL, with 2.5 µL of extracted nucleic acid added. The reaction included 10 min at 45 °C for reverse transcription and 10 min at 95 °C

for activation, followed by 45 cycles of 15 s at 95 °C for denaturation, 20 s at 56 °C for annealing and 30 s at 72 °C for elongation, respectively.

To specifically detect RNA of EBLV-1, EBLV-2 and BBLV a modification of the R14-assay [27], i.e., the RABV probe was omitted, the EBLV-1 probe was FAM-labelled, and ß-Actin-mix2-HEX was included as internal control assay. To this end, the AgPath-ID One-Step RT-PCR kit (Thermo Fisher Scientific, Waltham, MA, USA) was applied in a total volume of 12.5 µL including 2.5 µL of beforehand extracted nucleic acid were added to 10 µL of the master mix [27].

All RT-qPCRs were run on a BioRad CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Negative (RNA isolation control, no template control) and positive (EBLV-1, EBLV-2, BBLV) controls were analyzed in parallel with each PCR run.

2.5. Virus Isolation in Cell Culture

Virus isolation was attempted for all brain suspension samples that had initially been tested positive for lyssaviral RNA using the rabies tissue-culture infection test (RTCIT) as described before [28]. Briefly, bat brain suspensions were centrifuged and 500 µL supernatant was equally mixed with 10^6 mouse neuroblastoma cells (NA42/43; CCLV-RIE 0229, Collection of Cell Lines in Veterinary Medicine (CCLV) at the FLI, Riems) and incubated at 37 °C with 5% CO_2 for 30 min. Cells were maintained in a mixture of equal volumes of Eagle MEM (Hanks' balanced salts solution) and Eagle MEM (Earle's balanced salts solution) medium, and supplemented with 10% fetal bovine serum and 1% Pen-Strep (10,000 U/mL).

After further centrifugation, cell pellets were resuspended in T25 cell culture flasks and incubated for three to four days under the same conditions as stated above. Additionally, a control dish was set up in parallel for each passage. After three to four days the control was fixed, stained with a fluorescein isothiocyanate (FITC) conjugated polyclonal antibody (SIFIN, Berlin, Germany), washed and checked for the presence of virus. If viral antigen was detected, the test result was declared positive. A sample was considered negative after three consecutive serial passages without viral growth.

2.6. NGS Sample Processing

Preparation of Ion Torrent compatible sequencing libraries was conducted according to an adapted version of the NGS-based metagenomics pathogen detection workflow published by Wylezich et al. [29]. In short, homogenized brain material was combined with 1 mL Trizol and subsequently treated with chloroform. For sample 45369, a mixture of 250 µL cell culture supernatant and 750 µL Trizol LS was used instead. After centrifugation, 400 µL of the aqueous phase was used for RNA extraction on a KingFisher Flex platform (Thermo Fisher Scientific, Waltham, MA, USA) in combination with the RNAdvance Tissue Kit (Beckman Coulter, Brea, CA, USA) and included DNase I digestion step. Double stranded cDNA was generated from 350 ng total RNA under usage of the SuperScript™ IV First-Strand cDNA Synthesis System (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) and the NEBNext® Ultra™ II Non-Directional RNA Second Strand Synthesis Module (New England Biolabs, Ipswich, MA, USA). After ultrasonic fragmentation on a Covaris M220 (Covaris, Brighton, UK), ds cDNA was converted to Ion Torrent compatible libraries utilizing the GeneRead L Core Kit (Qiagen) in combination with IonXpress barcode adaptors (Thermo Fischer Scientific) followed by a size selection step targeting for library fragments of approx. 500 bp size. Sample processing steps related to cDNA generation, library preparation and size selection were conducted on a Biomek 4000 automated liquid handler (Beckman Coulter). Subsequently, sequencing libraries were quality controlled (2100 Bioanalyzer, High sensitivity DNA Kit, Agilent Technologies, Santa Clara, CA, USA) and quantified (QIAseq Library Quant Assay Kit, Qiagen) to ensure optimal sequencing results. Libraries were sequenced on an Ion Torrent S5XL instrument (Thermo Fisher Scientific) utilizing Ion 530 chips and reagents according to the manufacturer's instructions.

Processing of samples 5668, 31955, 23549 and 23157 was adjusted considering the highly decomposed state of the original sample material. For these samples, RNA extraction was conducted utilizing the RNeasy Mini Kit (Qiagen) and an on-column DNase I digestion step. Subsequently, cDNA was generated using the cDNA synthesis system kit (Roche Diagnostic, Rotkreuz, Switzerland) in combination with random hexamer primers (Roche Diagnostic). After library preparation, small library fragments (~200 bp size) were separated from standard size fragments (~500 bp) in the size selection step and kept for further processing instead of being discarded. Standard libraries (500 bp) derived from samples 5668 and 23157 as well as small fragments (200 bp) of sample 23157 were amplified using the GeneRead DNA Amp L Kit (Qiagen). Amplified libraries were purified twice with a 1.2× volume of Agencourt AMPure XP beads (Beckman Coulter) to remove any interfering substances and remaining adapter dimers. Sequencing of small fragment libraries was realized on Ion 540 chips and reagents according to the manufacturer's instructions.

2.7. Generation of Full Genome Sequences and Phylogenetic Analysis

Raw sequencing data were automatically adapter trimmed by the Ion Torrent Software Suite (v.5.12.1) and subsequently mapped against the EBLV-1 reference sequence (NC_009527) using the 454 Sequencing System Software v3.0 (Roche). Full genome sequences were obtained by de novo assembly of full or partial mapped reads and annotated with Geneious Prime (2021.0.1, build 2020-12-01). Phylogenetic analyses were conducted with IQ-TREE (v. 1.6.5) under usage of the ultrafast bootstrap approximation approach (100.000 ultrafast bootstrap) and enabled ModelFinder feature [30] for maximum-likelihood phylogenetic tree construction (best-fit model: GTR+F+R2). Therefore, a dataset of 127 EBLV-1 full genome sequences was investigated encompassing the newly generated German EBLV-1 full genome sequences and 111 sequences from previously published datasets. German cases with only partial genomes were excluded from phylogenetic analysis.

3. Results

General Surveillance

During a period of 30 months a total of 1236 bats were sampled and investigated under this scheme, comprising of animals that have been collected within and before this study period, with the oldest sample originating from 2004. Samples were received from ten different participating German federal states, with the majority of dead bats originating from Lower Saxony (*N* = 464), followed by Berlin (*N* = 252) and Baden-Wuerttemberg (*N* = 167). The sample set encompassed 18 different bat species from the family *Vespertilionidae*, with the *Pipistrellus pipistrellus* (*N* = 625) being the most frequently sampled bat species, followed by *Eptesicus serotinus* (*N* = 96) and *Nyctalus noctula* (*N* = 89) (Table 1). For 9.5% of all bats (*N* = 117) the species could not be determined. Of all analyzed bats with known gender, 54% were male and 46% were female.

In total, 16 samples tested positive for lyssaviral RNA by RT-qPCR (Tables 1 and 2). All those specimens tested positive in the N-gene pan-lyssa PCR, and were confirmed to the virus species level by the specific R14 RT-qPCR assays. Virus isolation was not successful in two cases and sequencing data received from one of those samples were insufficient to obtain a virus genome sequence.

The vast majority of positive specimens was found in bats from Lower Saxony (*N* = 7) and Berlin (*N* = 6), in contrast to only one lyssavirus infection detected in Brandenburg, Saxony-Anhalt and Saxony, respectively (Figure 1). Despite a relatively high number of submitted animals, no lyssaviral RNA was detected in bats from Baden-Wuertemberg in the ongoing study (Figure 1, Table 1), but in samples that were screened retrospectively (Table 2). Viruses characterized as EBLV-1 were predominately detected in serotine bats, and in two common pipistrelles (Tables 1 and 2).

Table 1. Details of passive bat rabies surveillance. Numbers of animals investigated per species and federal state. Lyssavirus-positive cases are indicated (in brackets). All viruses were characterized as EBLV-1, except for one case in the Natterer's bat. Abbreviations for German federal states: Baden-Wuerttemberg (BW), Bavaria (BY), Berlin (BE), Brandenburg (BB), Hesse (HE), Lower Saxony (NI), Mecklenburg-Western Pomerania (MV), Northrhine-Westphalia (NW), Saxony-Anhalt (ST), Saxony (SN).

Species	BB	BE	BW	BY	HE	MV	NI	NW	SN	ST	Total
Barbastella barbastellus	1						1		1		3
Eptesicus nilssonii							3				3
Eptesicus serotinus	22 (1)	35 (6)	1		2		22 (6)		5 (1)	9(1)	96 (15)
Myotis ssp.							7				7
Myotis bechsteinii			1								1
Myotis brandtii					3		28				31
Myotis daubentonii	4	2	2				18		4	3	33
Myotis myotis	3	1	3		1		2		2		12
Myotis mystacinus	2		8		1		32		2		45
Myotis nattereri	8	4			1		12 (1) #		2	3	31 (1)
Nyctalus ssp.										2	2
Nyctalus leisleri		2			11		2		1		16
Nyctalus noctula	22	35	1			5	11	2	13		89
Pipistrellus kuhlii	1					1					2
Pipistrellus nathusii	7	2	9		1		13		5	1	38
Pipistrellus pipistrellus	52	152	73	1	44	2	251		28	22	625
Pipistrellus pygmaeus	10						3		1	1	15
Plecotus ssp.							1			1	2
Plecotus auritus	4	1	3		1		15		3	4	31
Plecotus austriacus	2	1			1		2				6
Vespertilio murinus	3	9	2		1		8		7	1	31
unspecified	4	8	64		5		32		1	3	117
total	**145 (1)**	**252 (6)**	**167**	**1**	**72**	**8**	**463 (7)**	**2**	**75 (1)**	**50 (1)**	**1236 (16)**

Characterized as BBLV.

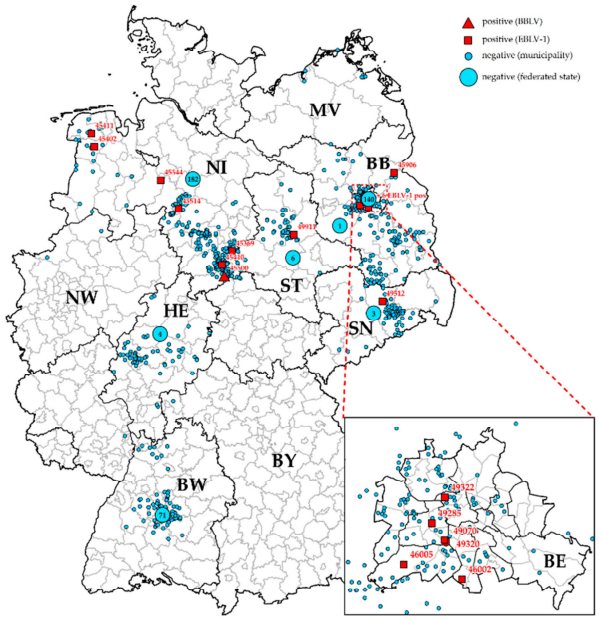

Figure 1. Spatial distribution of analyzed bat specimen, with positive cases indicated (red). Numbers in

larger circles correspond to specimen for which detailed information on the origin was not available. Red dashed box: area of Berlin enlarged to visualize the distribution of samples. Abbreviations for German federal states: Baden-Wuerttemberg (BW), Bavaria (BY), Berlin (BE), Brandenburg (BB), Hesse (HE), Lower Saxony (NI), Mecklenburg-Western Pomerania (MV), Northrhine-Westphalia (NW), Saxony-Anhalt (ST), Saxony (SN).

PCR-positive samples were subjected to next generation sequencing, resulting in the generation of complete/nearly complete genome sequences for most samples (Table 2). Subsequent phylogenetic analyses of the newly generated German EBLV-1 sequences revealed the grouping of the majority of the investigated cases within the A1 cluster of the EBLV-1a sub-lineage (Figure 2A), as proposed [31], and exhibited a sequence identity of 98.7% between the 15 considered German sequences. Furthermore, these cases were represented in three distinct phylogenetic groups within the A1 cluster. Nearly exclusively formed by German isolates, the first group included five of the newly generated EBLV-1 sequences (sample 46002, 49320, 46005, 49322 and 49512) distributed over the eastern part of Germany (Berlin, Saxony) as well as a single Polish EBLV-1 case. A second group encompassed sequences of new and already published German cases that were mainly found in central regions of the country (samples 5668, 23549, 49285, 49911 and 45514). Lastly, a third group of German and Dutch cases was extended by four new German EBLV-1 viruses (sample 45410, 45402, 45411 and 45544) from areas near the German-Dutch border. Interestingly, despite its geographic location in central Germany, sample 45369 was separated from other German cases and clustered closely with a Slovakian EBLV-1a sequence. Besides EBLV-1a, one of the investigated cases (sample 49070) was identified as member of the EBLV-1b sub-lineage, clustering closely with a previously found German EBLV-1b case from the year 2008 from Halle/Saale (20174GER, Figure 2A).

The single bat that tested BBLV positive was found dead in the area of Herzberg, District of Göttingen, in southern Lower Saxony (GPS-coordinates: 51°39′5.218″ N/10°20′16.687″ E) and was identified as a female Natterer's bat. Full genome sequencing revealed 99.7% sequence identity with a BBLV case detected earlier in Kronach, Bavaria in 2015 [17]. The close genetic relationship is illustrated in Figure 2B.

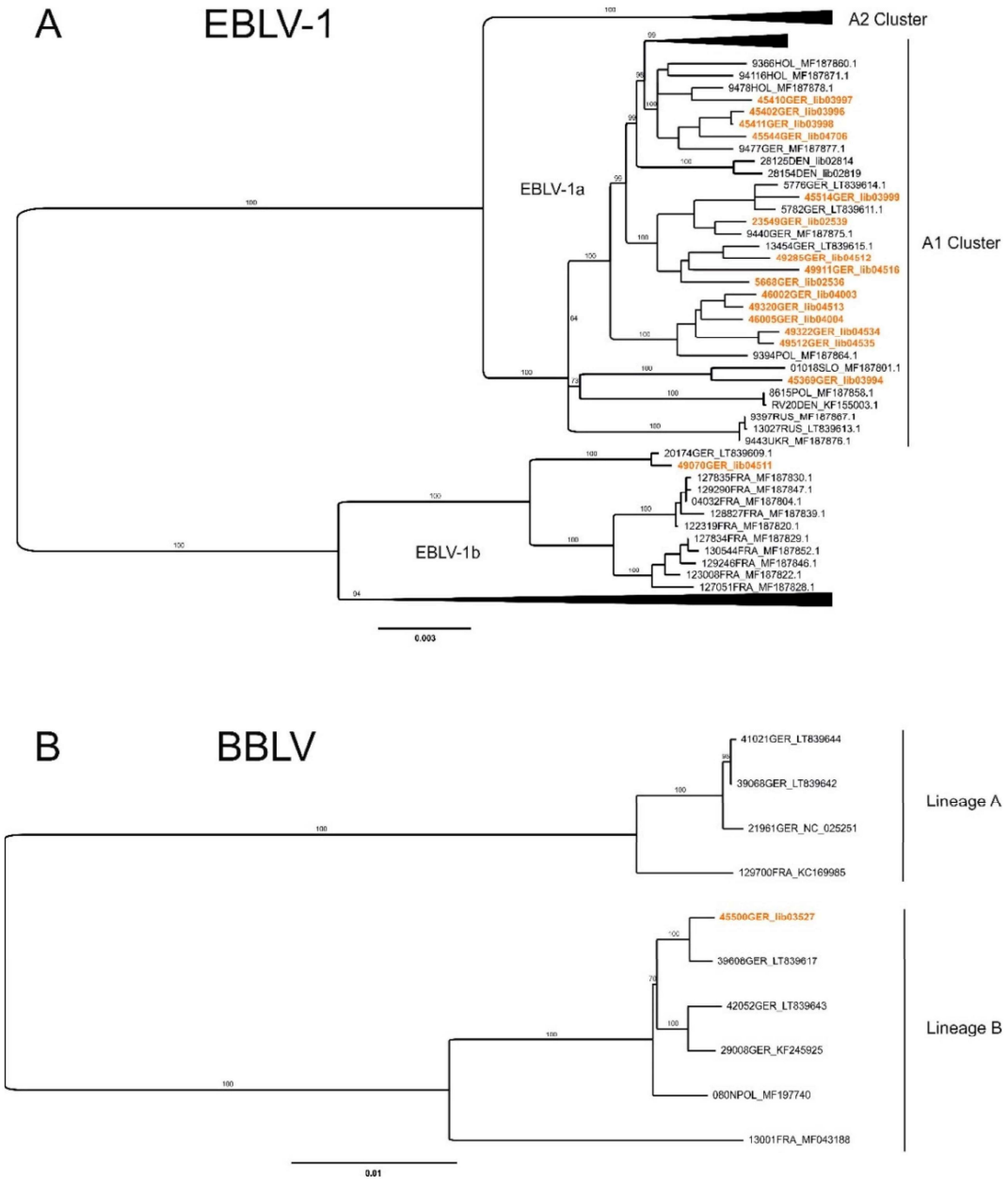

Figure 2. Mid-point rooted maximum-likelihood phylogenetic trees showing (**A**) the genetic diversity of 127 EBLV-1 full genome sequences originating from nine different countries, and (**B**) all available full genome sequences for BBLV. All newly generated full genome sequences (see Table 2) are indicated (orange).

Table 2. Details for bat samples that tested positive for EBLV-1 and BBLV. Cq-Values for the different RT-PCR assays are provided. For sample 45906, NGS only generated two viral reads and the lyssavirus species was determined with PanLyssavirus hn-RT-PCR [32] and subsequent Sanger sequencing. Abbreviations for German federal states: Baden-Wuerttemberg (BW), Berlin (BE), Brandenburg (BB), Lower Saxony (NI), Mecklenburg-Western Pomerania (MV), Saxony-Anhalt (ST), Saxony (SN).

Lab-ID	Collection Date	Host	Sex	Location	Virus	R14 EBLV-1	R14 EBLV-2	R14 BBLV	Pan-N	Pan-L	Virus Isolation	Library Number	Genome Length	Sequence
45402	July 18	*E. serotinus*	f	Holtland (NI)	EBLV-1a	13.84	-	-	30.95	36.19	+	Lib03996	11,962	Nearly Complete
45410	n.a.	*E. serotinus*	m	n.a. (NI)	EBLV-1a	14.45	-	-	26.85	32.19	+	Lib03997	11,953	Nearly Complete
45411	May 16	*E. serotinus*	m	Westerende Holzloog (NI)	EBLV-1a	15.29	-	-	31.72	39.62	+	Lib03998	11,963	Nearly Complete
45514	August 16	*E. serotinus*	m	Wunstorf (NI)	EBLV-1a	9.17	-	-	43.60	40.03	+	Lib03999	11,965	Nearly Complete
45544	August 12	*E. serotinus*	m	Bruchausen-Vilsen (NI)	EBLV-1a	9.77	-	-	39.18	32.39	+	Lib04706	11,966	Complete
46002	August 18	*E. serotinus*	f	Berlin-Marienfelde (BE)	EBLV-1a	14.76	-	-	35.95	37.02	+	Lib04003	11,966	Complete
46005	May 18	*E. serotinus*	m	Berlin-Nikolassee (BE)	EBLV-1a	17.92	-	-	34.68	37.51	+	Lib04004	11,962	Nearly Complete
49070	August 19	*E. serotinus*	f	Berlin-Friedenau (BE)	EBLV-1b	12.02	-	-	27.10	37.05	+	Lib04511	11,967	Complete
49285	September 19	*E. serotinus*	m	Berlin-Witzleben (BE)	EBLV-1a	12.59	-	-	27.40	28.47	+	Lib04512	11,966	Complete
49320	2017	*E. serotinus*	m	Berlin-Friedenau (BE)	EBLV-1a	15.05	-	-	27.70	30.01	+	Lib04513	11,967	Complete
49911	June 19	*E. serotinus*	m	Gommern (ST)	EBLV-1a	12.03	-	-	28.74	29.79	+	Lib04516	11,958	Nearly Complete
49322	2017	*E. serotinus*	f	Berlin-Wedding (BE)	EBLV-1a	9.65	-	-	27.75	28.75	+	Lib04534	11,966	Complete
49512	August 17	*E. serotinus*	f	Niederau (SN)	EBLV-1a	12.07	-	-	28.18	31.51	-	Lib04535	11,964	Nearly Complete
45369	May 16	*E. serotinus*	f	Göttingerode (NI)	EBLV-1a	20.68	-	-	35.51	37.45	+	Lib03994	11,965	Nearly Complete
45906	September 04	*E. serotinus*	f	Falkenberg (BB)	EBLV-1a	24.79	-	-	36.28	-	-	n.a.	n.a.	n.a.
45500	June 14	*M. nattereri*	f	Herzberg (NI)	BBLV	-	-	12.75	44.21	30.06	+	Lib03527	11,896	Nearly Complete
5668	August 2000	*E. serotinus*	n.a.	Kubbelkow (MV)	EBLV-1a	28.65	-	-	33.52	-	+	Lib02536	11,966	Complete
31955	June 12	*P. pipistrellus*	m	Tübingen (BW)	EBLV-1a	-	-	-	32.83	-	+	Lib02538	n.a.	Partial
23549	June 07	*E. serotinus*	m	Halle/Saale (ST)	EBLV-1a	26.92	-	-	29.89	34.1	+	Lib02539	11,966	Complete
23157	April 10	*P. pipistrellus*	m	Nürtingen (BW)	EBLV-1a	-	-	-	34.66	-	+	Lib02580	n.a.	Partial

4. Discussion

This study provides novel insight into the epidemiology of bat-related lyssaviruses in Germany. To this end, more than 1000 bats were sampled and analyzed over a period of two and a half years, yielding results comparable to previous studies with similar focus [10,15]. In Germany, routine bat rabies surveillance performed by regional veterinary laboratories is focused on bats associated to human contact or which show signs of clinical disease suggestive of rabies [33]. While this surveillance scheme is important for the immediate public health intervention, it is inherently biased. Therefore, this sample set should be complemented by enhanced passive surveillance, i.e., the integration of dead found bats without human contact (e.g., found in caves, forests, etc.), as recommended before [15,34]. This allows for a higher sampling intensity and provides a better picture of the occurrence and distribution of bat lyssaviruses. In our study, we supported submissions by providing shipment material and covering the costs for transportation. Also, by non-destructive sampling outside the BSL-3 facility, we could offer to return bats that tested negative. These facts may have led to higher willingness of bat handlers for sample submissions. Unfortunately, this scheme could not be applied uniformly across Germany, as can be seen from the origin of the submissions (Figure 1). The practical implementation was hampered by different constraints on various levels, including, e.g., the heterogeneous landscape of bat conservation in Germany, and different regulations in federal states on the conservation and archival of endangered and protected species.

The number of submitted individuals per bat species varied, ranging from one animal (*Myotis bechsteinii*) to 625 (*Pipistrellus pipistrellus*). This variation may be reflective of population numbers of particular bat species, which, however, are difficult to estimate. Similar to previous surveillance studies from Europe [15,35–37], the common pipistrelle was the most frequently submitted bat species, which is consistent with the fact that it is one of the most abundant synanthropic European bat species [26]. Taken together, the results of our study need to be carefully assessed and should not be considered representative for the respective bat species.

By investigating dead found bats the animals can also be screened for other pathogens and viruses besides lyssaviruses including, for example, Coronaviruses. Recent findings of novel lyssaviruses, e.g., KBLV in Finland [13] and Matlo bat lyssavirus (MBLV) in South Africa [38], confirm the necessity for such surveillance studies. While negative results do not exclude the presence of lyssaviruses in the bat population, positive samples and isolated viruses thereof are essential for further characterizations, including phylogenetics, pathogenesis in animal models and cross-neutralization by available vaccines. This contributes to a risk assessment for novel bat lyssaviruses, as exemplified for BBLV [39], LLBV [40] and KBLV [41].

Historically, the FAT was regarded as the gold standard in rabies diagnostics but recently recommendations by both the WHO and OIE were updated, allowing the use of RT-qPCR as a primary diagnostic test since it also demonstrates a very high diagnostic sensitivity and specificity [42]. Consequently, we changed the previous screening strategy for bat-associated lyssaviruses to using different RT-qPCR assays for a more convenient and therefore faster analysis. Our approach for non-destructive sampling was based on previous recommendations for surveillance in larger mammals [43]. Due to the fact that the bat carcasses were often in a state of decomposition and subject to freeze-thawing, bat brains were mostly liquefied. Therefore, aspiration was easily performed, and in fact, sufficient material could be obtained as visually checked and confirmed by beta-actin results of the PCR. If the diagnostic sensitivity was lowered by the sampling technique, which cannot be completely ruled out, this is outweighed by the increased submissions and sensitivity of the molecular techniques used.

Screening each sample in a double-check approach allows a diagnostic maximum in finding known and potentially novel lyssavirus species. Especially in bat surveillance, working with poor quality samples and additionally very small amounts is a common case, where molecular methods offer a higher sensitivity [44]. This is exemplified by the

additional EBLV-1 cases identified in samples (Table 2) that initially tested FAT-negative in a previous retrospective study [15].

The predominance of EBLV-1 (94% of all positive bats) corroborate results of previous bat rabies surveillance studies [15,45]. Also, the positivity rate of 16% in serotine bats is comparable to results observed in a previous German enhanced passive surveillance study, where 13% of all tested serotine bats were found to be EBLV-1-positive [15]. Similarly, in Spain (*Eptesicus isabellinus*) and the Netherlands a positivity rate for EBLV-1 of about 20% was reported [34,46]. Spillover infections of EBLV-1 to bat species other than *E. serotinus* and *E. isabellinus* are rarely found [10,15,47,48]. Spillover infections of EBLV-1 into bats other than *E. serotinus* could not be detected in the submitted samples between 2018−2020. However, screening of a large number of bat samples that had initially tested negative by FAT [15] by using molecular methods revealed two cases in common pipistrelles (Table 2). Interestingly, those bats were found in the southeastern federal state of Baden-Wurttemberg, a region without known cases of EBLV-1. The results demonstrate that spillover events can also be observed in regions with hitherto undetected occurrence of EBLV-1.

The spatial distribution of EBLV-1 positive bats generally confirmed previous patterns of distribution, with the majority of cases found in the North of Germany [15,32]. This was explained by higher population density of serotine bats in this region which seems to support the intraspecies transmission and virus maintenance [32]. Interestingly, six positive cases were detected in the urban area of Berlin. This apparent aggregation of cases is likely biased by the fact that the number of submitted samples from this area was very high. Whether this is due to higher abundance of the serotine bat, or the increased encounters of bats by members of the public, which is likely for this synanthropic bat species, is arguable [49].

Genetically, all except one EBLV-1 isolate can be assigned to sub-lineage EBLV-1a, which is considered to exhibit a relatively higher genetic homogeneity compared to EBLV-1b [50]. However, a higher phylogeographic segregation of EBLV-1a sequences with the A1 cluster can be observed, similar to a recent analysis on Danish EBVL-1 samples [48]. EBLV-1b occurrence in Germany is centered in the west, close to the border with France [15]. Here, we report an additional EBLV-1b case in the eastern part of Germany, which supports the assumption that this sub-lineage is distributed beyond its known expansion in western European countries like Spain, France and the Netherlands [31].

Within our study, we identified the tenth BBLV case, which is the seventh case in Germany and the fourth case in Lower Saxony isolated from a Natterer's bat. Since its first detection in 2010 in Germany [12], BBLV was found several times in Germany, France and Poland [16,17]. The fact that it was again isolated from the same bat species supports the hypothesis of the Natterer's bat representing the reservoir host species.

Interestingly, the BBLV from Herzberg in Lower Saxony is genetically closer related to BBLV detected in Kronach, Bavaria in 2015 [51] than to other cases found in Lower Saxony. The isolate from Kronach is again closely related to an isolate from Poland [16]. The apparent discrepancies between phylogenetic grouping and geographic origin are difficult to explain [17]. Also, the fact that BBLV has only recently been discovered but ever since appears to be more prevalent than, for example, EBLV-2, is puzzling and cannot be explained by increased surveillance activities. Further investigations would be needed to elucidate these phenomena.

5. Conclusions

Bat rabies surveillance is only operative where dedicated people involved in bat conservation, biology, research, etc., are working together with veterinary scientists in a true One Health approach. Without their additional effort and motivation, such studies would not be feasible, and we would like to reiterate our acknowledgement to all parties and numerous individuals that contributed bat specimen.

While taking the limitations of passive surveillance data into account, nonetheless it is essential for the identification of known and novel pathogens, as exemplified by the discovery of BBLV [12] and KBLV [13].

The results of our study support that enhanced passive bat rabies surveillance can gain sensitivity by applying RT-qPCR screening. The methodology is also more convenient and could offer a higher throughput. We therefore recommend a nationwide and eventually European enhanced passive surveillance via RT-qPCR-screening complementary to testing suspected bats with human contact.

Biased sampling, as in this enhanced passive surveillance scheme, cannot fully reflect the true prevalence and the correct epidemiological bat rabies situation. The 1.2% positivity across all species is similar to values found in retrospective studies in France [52]. While this value may appear to be of a relatively low level, rabies in bats poses a potential veterinary and public health risk. This risk is especially eminent for people handling bats for research or conservation reasons. Mitigating measures should include preventing bites by, e.g., using gloves and adequate pre- and post-exposure prophylactic treatments according to international and national guidelines.

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3. Publication III: Comparative Pathogenesis of Different Phylogroup I Bat Lyssaviruses in a Standardized Mouse Model

Antonia Klein [1], Elisa Eggerbauer [2], Madlin Potratz [2], Luca M. Zaeck [2], Sten Calvelage [3], Stefan Finke [2], Thomas Müller [2], Conrad M. Freuling [1]

[1]Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany

[2]Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany

[3]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut (FLI), Greifswald-Insel Riems, Germany

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RESEARCH ARTICLE

Comparative pathogenesis of different phylogroup I bat lyssaviruses in a standardized mouse model

Antonia Klein[1], Elisa Eggerbauer[2], Madlin Potratz[2], Luca M. Zaeck[2], Sten Calvelage[3], Stefan Finke[2], Thomas Müller[2], Conrad M. Freuling[1]*

1 Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany, **2** Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany, **3** Institute of Diagnostic Virology, Federal Research Institute for Animal Health, Friedrich-Loeffler-Institut (FLI), Greifswald-Insel Riems, Germany

* conrad.freuling@fli.de

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Abstract

A plethora of bat-associated lyssaviruses potentially capable of causing the fatal disease rabies are known today. Transmitted via infectious saliva, occasionally-reported spillover infections from bats to other mammals demonstrate the permeability of the species-barrier and highlight the zoonotic potential of bat-related lyssaviruses. However, it is still unknown whether and, if so, to what extent, viruses from different lyssavirus species vary in their pathogenic potential. In order to characterize and systematically compare a broader group of lyssavirus isolates for their viral replication kinetics, pathogenicity, and virus release through saliva-associated virus shedding, we used a mouse infection model comprising a low (10^2 $TCID_{50}$) and a high (10^5 $TCID_{50}$) inoculation dose as well as three different inoculation routes (intramuscular, intranasal, intracranial). Clinical signs, incubation periods, and survival were investigated. Based on the latter two parameters, a novel pathogenicity matrix was introduced to classify lyssavirus isolates. Using a total of 13 isolates from ten different virus species, this pathogenicity index varied within and between virus species. Interestingly, Irkut virus (IRKV) and Bokeloh bat lyssavirus (BBLV) obtained higher pathogenicity scores (1.14 for IRKV and 1.06 for BBLV) compared to rabies virus (RABV) isolates ranging between 0.19 and 0.85. Also, clinical signs differed significantly between RABV and other bat lyssaviruses. Altogether, our findings suggest a high diversity among lyssavirus isolates concerning survival, incubation period, and clinical signs. Virus shedding significantly differed between RABVs and other lyssaviruses. Our results demonstrated that active shedding of infectious virus was exclusively associated with two RABV isolates (92% for RABV-DogA and 67% for RABV-Insectbat), thus providing a potential explanation as to why sustained spillovers are solely attributed to RABVs. Interestingly, 3D imaging of a selected panel of brain samples from bat-associated lyssaviruses demonstrated a significantly increased percentage of infected astrocytes in mice inoculated with IRKV (10.03%; SD ±7.39) compared to RABV-Vampbat (2.23%; SD±2.4), and BBLV (0.78%; SD±1.51), while only individual infected cells were identified in mice infected with Duvenhage virus (DUVV).

Competing interests: The authors have declared that no competing interests exist.

These results corroborate previous studies on RABV that suggest a role of astrocyte infection in the pathogenicity of lyssaviruses.

Author summary

Globally, there are at present 17 different officially recognized lyssavirus species posing a potential threat for human and animal health. Bats have been identified as carriers for the vast majority of those zoonotic viruses, which cause the fatal disease rabies and are transmitted through infectious saliva. The occurrence of sporadic spillover events where lyssaviruses are spread from bats to other mammalian species highlights the importance of studying pathogenicity and virus shedding in regard to a potentially sustained onward cross-species transmission. Therefore, as part of this study, we compared 13 different isolates from ten lyssavirus species in a standardized mouse infection model, focusing on clinical signs, incubation periods, and survival. Based on the latter two, a novel pathogenicity index to classify different lyssavirus species was established. This pathogenicity index varied within and between different lyssavirus species and revealed a higher ranking of other bat-related lyssaviruses in comparison to the tested Rabies virus (RABV) isolates. Altogether, our results demonstrate a high diversity among the investigated isolates concerning pathogenicity and clinical picture. Furthermore, we comparatively analyzed virus shedding via saliva and while there was no indication towards a reduced pathogenicity of bat-associated lyssaviruses as opposed to RABV, shedding was increased in RABV isolates. Additionally, we investigated neuronal cell tropism and revealed that bat lyssaviruses are not only capable of infecting neurons but also astrocytes.

1. Introduction

The *Lyssavirus* genus of the family *Rhabdoviridae* within the order *Mononegavirales* comprises highly neurotropic, single negative-strand RNA viruses [1], which are capable of causing rabies, an acute and invariably fatal viral encephalitis [2]. At present, 17 lyssavirus species are recognized as separate taxonomic entities [1]. Based on antigenic divergence and phylogenetic relationships, lyssavirus species can be grouped into phylogroups [3]. Phylogroup I include the prototypical rabies virus (RABV), Aravan virus (ARAV), Australian bat lyssavirus (ABLV), Bokeloh bat lyssavirus (BBLV), Duvenhage virus (DUVV), European bat lyssavirus 1 (EBLV-1), European bat lyssavirus 2 (EBLV-2), Gannoruwa bat lyssavirus (GBLV), Irkut lyssavirus (IRKV), Khujand lyssavirus (KHUV), and Taiwan Bat Lyssavirus (TWBLV), while Lagos bat lyssavirus (LBV), Mokola lyssavirus (MOKV), and Shimoni bat lyssavirus (SHIBV) are members of phylogroup II. Based on phylogenetic distance, the most genetically divergent lyssaviruses, including Ikoma virus (IKOV), Lleida bat lyssavirus (LLEBV), and West Caucasian bat lyssavirus (WCBV), have been tentatively classified within a dispersed phylogroup III [4]. Two new lyssaviruses found in Europe, the Kotalahti bat lyssavirus (KBLV) [5], and Africa, the Matlo bat lyssavirus (MBLV) [6], are not yet approved as new virus species. While almost all lyssavirus species are strongly associated with chiropteran hosts [4], RABV is the only lyssavirus maintained in many different species of mesocarnivores around the world. Exceptions include the circulation of RABV in multiple species of bats in the New World and the reported role of a small primate, the marmoset, as an RABV reservoir in Brazil [2,7]. Most bat

lyssaviruses seem to be restricted to a limited number of reservoir host species they have been steadily co-evolving with over time [8].

For RABV, transmission, particularly from vampire bats, to other non-bat mammals is common in the Americas [9]. Nonetheless, sporadic spillover infections of other bat lyssaviruses to non-bat mammal species, including humans, emphasize the threat these viruses pose for both human and animal health [10,11]. In contrast to phylogroup I lyssaviruses, failure of protection against the more divergent phylogroup II and III lyssaviruses has been demonstrated for all commercially available vaccines [3,12–14]. While antigenicity and phylogeny of lyssaviruses have been well studied, the comparative pathogenicity of bat lyssavirus isolates in different species is of scientific interest, particularly against the background of a highly variable pathogenicity between phylogroups observed in mice [15]. Partly, pathogenicity of bat lyssaviruses was tested in ferrets [16], foxes [17–19], raccoon dogs [18,20–22], cats [18,21–28], dogs [18,21,22,24,25,29–32], and skunks [18,21,22,33]. In most of these studies and also when mice were used, either only a limited number of bat lyssavirus species were compared or different viral variants of one particular lyssavirus species have been studied [34,34–40]. Varying experimental designs and conditions often prevent a reliable and broader comparative assessment of the pathogenicity of bat lyssaviruses from these studies. However, in many of the aforementioned studies, the results regarding limited pathogenicity of bat lyssaviruses in non-bat mammals seem to contradict reported bat lyssavirus-borne human fatalities [10].

Moreover, it has not yet been clarified why cross-species transmissions to either humans or animals are more often seen with bat-associated RABVs [8] compared to other bat lyssaviruses for which such events seem to be relatively rare [10]. While the underlying mechanisms of cross-species transmissions are not yet completely understood, virus shedding is assumed to be a key factor, particularly for sustained spillovers [41]. Therefore, it is of importance to understand whether potential spillover hosts are shedding virus and can subsequently transmit it to conspecifics or other mammals, including humans. Such assessment of the potential impact of bat-associated lyssaviruses on public health is particularly challenging but of great importance. To this end, we compared the pathogenicity of 13 phylogroup I lyssaviruses in a standardized mouse model using different inoculation doses and routes. Furthermore, shedding of virus in saliva of infected mice was measured in order to assess the likelihood of onward transmission. Based on obtained pathogenicity data, we further developed a pathogenicity index for comparison and classification of lyssavirus-induced pathogenicity. Since it was shown that the degree of central nervous system resident astrocyte infection differed in RABV field strains compared to laboratory-adapted fixed virus strains, potentially affecting their pathogenicity [42], the cell tropism of a selected lyssavirus panel from diseased mice was analyzed. Even though the restricted number of lyssaviruses analyzed for astrocyte infection hinders a full comparison, the results support previous studies of RABV on the association of astrocyte tropism and pathogenicity.

2. Material and methods

2.1. Ethics statement

The experimental work in mice strictly followed the European guidelines on animal welfare and care according to the authority of the Federation of European Laboratory Animal Science Associations (FELASA) [43]. Animal experiments were evaluated, reviewed, and approved by the animal welfare committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, LALLF M-V/TSD 7221.3–2.1-002/11; M-V/TSD/7221.3-2-001/18) and supervised by the commissioner for animal welfare at the Friedrich-

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

Lab-ID	Name	Virus species	Year	Host	Origin	Accession Number
5989	RABV-DogA	RABV	2002	dog (*Canis lupus familiaris*)	Azerbaijan	LN879480
13205	RABV-Raccoon	RABV	1981	raccoon (*Procyon lotor*)	United States	MN862283
34886	RABV-Vampbat	RABV	1973	vampire bat (*Desmodus rotundus*)	Latin America	PRJEB46947
13240	RABV-Insectbat	RABV	1986	big brown bat (*Eptesicus fuscus*)	Canada	PRJEB46947
13027	EBLV-1	EBLV-1	1982	human (Yuli virus)	Russia	LT839613
16618	EBLV-2	EBLV-2	2007	daubenton's bat (*Myotis daubentonii*)	Germany	KY688138
29008[#]	BBLV	BBLV	2010	natterer's bat (Myotis Nattererii)	Germany	KF245925
46579	ARAV	ARAV	1991	lesser mouse-eared bat (*Myotis blythii*)	Kyrgyzstan	EF614259
46580	KHUV	KHUV	2001	whiskered bat (*Myotis mystacinus*)	Tajikistan	NC025385
46582	IRKV	IRKV	2002	greater tube-nosed bat (*Murina leucogaster*)	Russia	NC020809
39663	GBLV	GBLV	2016	indian flying fox (*Pteropus medius*)	Sri Lanka	KU244267
12862	DUVV	DUVV	1971	human	South Africa	EU293119
13849	ABLV	ABLV	1986	human	Australia	AF418014

[#]A recombinant virus was used.

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Loeffler-Institut (FLI) representing the Institutional Animal Care and Use Committee (IACUC).

2.2. Viruses and cells

A total of 13 virus isolates representing ten different phylogroup I lyssaviruses originating from Europe (EBLV-1, EBLV-2, BBLV), Asia (ARAV, KHUV, IRKV, GBLV), Africa (DUVV), Australia (ABLV), and the Americas (RABV) were included in this study. Regarding the latter, two bat-related RABV isolates, one from an insectivorous (RABV-Insectbat) and one from a hematophagous bat (RABV-Vampbat) (Table 1), were selected. For EBLV-1, DUVV, and ABLV, isolates from human cases were used. The Asian bat lyssaviruses ARAV, IRKV, GBLV and KHUV were kindly provided by the Animal Plant and Health Agency (APHA), Weybridge, United Kingdom through the European Virus Archive global (EVAg). All other isolates originated from the virus archive of the FLI, Riems, Germany. For comparison, two representatives of classical non-bat RABVs, one being an isolate from a dog from Azerbaijan (RABV--DogA) and the other being a wildlife variant isolated from a raccoon from North America (RABV-Raccoon), both of which had been used in previous infection studies in raccoons [20], were included. Cell lines used in this study were obtained from the Collection of Cell Lines in Veterinary Medicine (CCLV; FLI, Riems, Germany). Mouse neuroblastoma cells (Na 42/13, CCLV-RIE 0229) maintained in a mixture of equal volumes of Eagle MEM (Hanks' balanced salts solution) and MEM (Earle's balanced salts solution) medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively) were used for propagation of virus stocks, titration, viral replication kinetics, and virus isolation.

2.3. Full genome sequencing

All viruses taken from archived samples were subjected to full genome sequencing essentially as described before [44]. Briefly, RNA was automatically extracted on a KingFisher Flex platform (Thermo Fisher Scientific, Waltham, MA, USA) using the RNAdvance Tissue Kit (Beckmann Coulter, Brea, CA, USA). Double stranded cDNA was generated from 350 ng total RNA using the SuperScript IV First-Strand cDNA Synthesis System (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) and the NEBNext Ultra II Non-Directional RNA Second Strand

Synthesis Module (New England Biolabs, Ipswich, MA, USA). After conversion into cDNA, fragmentation was achieved by ultrasonication on a Covaris M220 (Covaris, Brighton, UK). Subsequently, Ion Torrent-specific sequencing libraries were generated using the GeneRead L Core Kit (Qiagen, Hilden, Germany) together with IonXpress barcode adaptors (Thermo Fisher Scientific). After quantification (QIAseq Library Quant Assay Kit, Qiagen) and quality control (2100 Bioanalyzer, High sensitivity DNA Kit, Agilent Technologies, Santa Clara, CA, USA) of the libraries, sequencing was performed on an Ion Torrent S5XL instrument utilizing Ion 530 chips and reagents according to the manufacturer's instructions.

2.4. Viral propagation and replication kinetics

In order to generate virus stocks for inoculation, Na 42/13 cells were infected at a multiplicity of infection (MOI) of 0.001, and incubated at 37˚ C and 5% CO_2. Depending on the viral strain, supernatants were harvested 72 to 168 hours post-infection (hpi) when 100% of the cell monolayer was infected. Infection was assessed using a control dish stained with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody mix (SIFIN, Germany/Fujirebio, Belgium). For growth curves, Na 42/13 cells were infected at an MOI of 0.001. Cell culture supernatants were harvested at 0, 16, 24, 48, 72, 96, and 168 hpi. The virus titers (tissue culture infective dose 50—$TCID_{50}$) were determined by endpoint titration of three technical replicates and subsequent calculation by the Spearman-Karber method [45].

2.5. Animal experiments

For the experimental studies, three- to four-week-old BALB/c mice from a commercial breeder (Charles River, Germany) were used. Animals were randomly assigned to groups and housed in individual, labelled cages with water and food provided ad libitum. Per lyssavirus isolate, six mice each were inoculated intramuscularly (i.m.) in the femoral muscle using a high (10^5 $TCID_{50}$/30 μl) and a low (10^2 $TCID_{50}$/30 μl) viral dose. Additionally, one group of six and one of three mice was inoculated intranasally (i.n.) with 10^2 $TCID_{50}$/10 μl and intracranially (i.c.) with 10^2 $TCID_{50}$/30 μl, respectively. Mock-infected control groups for each administration route were inoculated with 10 μl or 30 μl of cell culture medium respectively. Animals were monitored for 21 days post-infection (dpi). Body weight and clinical signs were recorded daily for each animal using clinical scores (S2 Table). If animals showed more than one clinical sign at a given time point, the most prominent one dominating the physical condition was recorded. Once mice developed clinical signs, they were checked twice a day. Animals were humanely euthanized at the humane endpoint or after 21 days by cervical dislocation under anesthesia with isoflurane (Isofluran CP, CP Pharma, Germany). Immediately before euthanasia, oropharyngeal swabs were taken of all mice that succumbed to infection using dry sterile cotton swabs (Nerbe plus GmbH & Co. KG, Germany), which were placed into 1500 μl of cell culture medium supplemented with penicillin/streptomycin as described above. Upon euthanasia, salivary glands and brain samples of all animals were taken and stored at -80˚ C until further processing.

2.6. Diagnostic assays

Presence of lyssavirus antigen in heat-fixed brain tissue samples was detected by direct fluorescence antibody test (DFA) using FITC-conjugated monoclonal antibodies (SIFIN, Germany and Fujirebio, Belgium) as well as defined positive and negative controls [46].

Brain, salivary gland and oropharyngeal swab samples were used to detect lyssaviral RNA. Briefly, organ samples were homogenized in 1000 μl cell culture medium using a TissueLyser (Qiagen, Germany) with a 3 mm steal bead. Homogenates as well as oral swabs were

centrifuged at 3750 x g for 10 minutes. Viral RNA was then extracted from the supernatant (100 μl) using the NucleoMagVet kit (Macherey&Nagel, Germany) according to the manufacturer's instructions in a KingFisher/BioSprint 96 magnetic particle processor (Qiagen, Germany). Viral RNA was detected by an RT-qPCR targeting the N-gene using the R14-assay (RABV-, EBLV-1-, EBLV-2-, and BBLV) and the R13-assay (ABLV, DUVV) [47,48]. For GBLV, primers and probe were specifically designed (S1 Table) and the protocol was run separately but with the same conditions as below. The PCR mastermix was prepared using the AgPath-ID One-step RT-PCR kit (Thermo Fisher Scientific, USA) in a volume of 10 μl including 0.5 μl of β-Actin-mix2-HEX as internal control and 2.5 μl of extracted RNA. The reaction was performed for 10 minutes at 45˚ C for reverse transcription and 10 minutes at 95˚ C for activation, followed by 42 cycles of 15 seconds at 95˚ C for denaturation, 20 seconds at 56˚ C for annealing and 30 seconds at 72˚ C for elongation. Fluorescence was measured during the annealing phase. RNA specific for ARAV, IRKV and KHUV was detected using the pan-lyssa realtime PCR targeting both the N- and L-gene [47,48]. Here, the RT-qPCR reaction was prepared using the OneStep RT-PCR kit (Qiagen, Germany), adjusted to a volume of 12.5 μl with an internal control mastermix, based on β-Actin, running in parallel. The reaction consisted of 10 minutes at 45˚ C for reverse transcription and 10 minutes at 95˚ C for activation, followed by 45 cycles of 15 seconds at 95˚ C for denaturation, 20 seconds at 56˚ C for annealing and 30 seconds at 72˚ C for elongation. All RT-qPCRs were performed on a BioRad CFX96 Real-Time System (Bio-Rad, USA).

RT-qPCR positive salivary glands and oral swab samples were subjected to virus isolation in cell culture using the rabies tissue-culture infection test (RTCIT) [49]. Briefly, either the respective swap sample or supernatant from the homogenized organ suspension prepared using cell culture media as described above and supplemented with 10% fetal bovine serum and 200 U/ml und 200 μg/ml penicillin/streptomycin, was mixed with dextran-pretreated Na 42/13 cell suspension at an equal ratio. The mixture was then incubated at 37˚ C and 5% CO_2 for 30 minutes and centrifuged at 1250 x g for 10 minutes. The obtained cell pellets were resuspended in T25 cell culture flasks and incubated for three to four days at 37˚ C and 5% CO_2. A control-dish was fixed, stained with a commercial FITC-conjugated monoclonal antibody conjugate (SIFIN, Germany/Fujirebio, Belgium), washed and microscopically analyzed for the presence of virus. Three consecutive serial passages were used to confirm a negative result.

2.7. Antibodies for immunofluorescence imaging of solvent-cleared brain sections

To detect bat lyssavirus antigen in infected brains, a polyclonal rabbit serum against recombinant RABV P protein (P160-5, 1:3,000 in PTwH [0.2% Tween 20 in PBS with 10 μg/ml heparin]) was used [50]. The following commercial primary antibodies were used: chicken anti-GFAP (Thermo Fisher, USA; #PA1-1004, RRID:AB_1074620, 1:1,500 in PTwH) and guinea pig anti-NeuN (Synaptic Systems, Germany; #266004, RRID:AB_2619988, 1:800 in PTwH). Donkey anti-rabbit Alexa Fluor 568 (Thermo Fisher, USA; #A10042, RRID:AB_2534017) and donkey anti-guinea pig Alexa Fluor 647 (Dianova, Germany; #706-605-148, RRID: AB_2340476) were used as secondary antibodies, each at a dilution of 1:500 in PTwH.

2.8. Immunostaining and clearing of brain tissue samples

Immunostaining and clearing protocols from previous reports [51–53] were modified and performed as described previously [54]. All incubation steps were performed on an orbital shaker. Briefly, the brains fixed with paraformaldehyde (PFA) were cut into 1 mm thick slices using a vibratome (Leica Biosystems, Germany, VT1200S). After bleaching with 5% H_2O_2/PBS

overnight at 4˚ C the samples were pre-permeabilized (0.2% Triton X-100/PBS) twice for 3 h each at 37˚ C and then permeabilized (0.2% Triton X-100/20% DMSO/0.3 M glycine/PBS) for 2 days at 37˚ C. After subsequent blocking (0.2% Triton X-100/10% DMSO /6% donkey serum/PBS) at 37˚ C for further 48 h, primary antibodies diluted in 3% donkey serum/5% DMSO/PTwH were added for a total of 7 days at 37˚ C. After 3.5 days, the antibody solution was renewed once. Subsequently, the samples were washed with PTwH four times with increasing intervals, leaving the final wash on overnight. Secondary antibodies were diluted in 3% donkey serum/PTwH and incubation was performed as described for the primary antibodies. After further washing with PTwH four times, leaving the final wash on overnight, the samples were dehydrated in a graded ethanol series (30%, 50%, 70% in aqua ad iniectabilia [pH 9–9,5] and twice 100%; each for ≥6 h) at 4˚ C. Subsequently, they were delipidated for 2 h in *n*-hexane at room temperature. Gradually replacing the *n*-hexane with ethyl cinnamate (ECi), they were then incubated in ECi until optically transparent. For confocal laser-scanning microscopy analysis, the cleared samples were embedded in 3D-printed imaging chambers as described before [55].

2.9. Confocal laser-scanning microscopy and image processing

Confocal z-stacks were acquired with a Leica DMI 6000 TCS SP5 confocal laser-scanning microscope equipped with a 40×/1.10 water immersion HC PL APO objective using the Leica Application Suite Advanced Fluorescence software (v2.7.3.9723). Fluorescence was acquired sequentially between lines with a pinhole diameter of 1 Airy unit and a z-step size of 0.5 μm.

The quantification of infected neurons and astrocytes in 1 mm thick brain sections was done as according to previous description [42]. To this end, confocal image stacks were split into individual channels using Fiji, an ImageJ (v1.52h) distribution package [56]. After bleach correction (simple ratio, background intensity 5.0) brightness and contrast were adjusted for each channel. The 3D objects counter plugin was used to identify objects in each channel [57]. The resulting objects map was then overlaid with the RABV P channel to detect and count infected objects. Visualization was done using arivis vision4D (v3.4.0).

2.10. Statistical analysis

Survival of mice was displayed in Kaplan-Meier curves and statistically analyzed by log-rank (Mantel-Cox) test. Significant differences in the means of incubation periods and astrocyte tropism were assessed using ordinary one-way ANOVA with Tukey's multiple comparison test. All statistical analyses were performed using Prism version 8 (GraphPad Software, La Jolla, USA).

2.11. Calculation of intramuscular pathogenicity index (IMPI)

For the calculation of the IMPI, we followed the example of the intracerebral pathogenicity index (ICPI) test for Newcastle disease virus [58] with the following modifications: The clinical observations of individual animals recorded every 12 to 24 hours over a period of up to 21 days were transferred to a daily rating scheme. Mice were scored as follows: 0 if healthy; 1 if sick, and 2 if dead. Dead individuals were scored as 2 at each of the remaining daily observations after death. For calculation of the IMPI, only animals from the i.m.-inoculated groups were included. The intramuscular pathogenicity index was then calculated based on the following formula: cumulative score for sick animals + cumulative score of dead animals / 126 (number of animals x days of observation, i.e. 6 x 21). The index is determined as the mean score per mouse over a 21-day-period, i.e. very pathogenic viruses showing high and less pathogenic ones showing lower indices. A minimum index value of 0 corresponds to absolutely

apathogenic animals throughout the complete observation period while a maximum index value of 2 would be reached if all mice died at day 1 post-infection.

3. Results

3.1. In vitro replication kinetics

In mouse neuroblastoma cells (Na 42/13), the tested lyssaviruses replicated to maximum titers ranging from 10^5 $TCID_{50}$/ml (ABLV) to $10^{7.75}$ $TCID_{50}$/ml (RABV-Vampbat) after 168 hours (Fig 1). RABV-Vampbat, IRKV, GBLV, RABV-DogA, and EBLV-1 exhibited titers around 10^7 $TCID_{50}$/ml and higher, while the titers for the rest of the isolates ranged between 10^5 and 10^6 $TCID_{50}$/ml. IRKV and EBLV-1 showed the fastest replication with measurable titers obtained already after 16 and 24 hpi, while all other isolates yielded measurable titers either after 48 or 72 hpi. The RABV-Raccoon variant in particular exhibited a slow replication kinetic; replication started after 72 hpi but with comparably low titers even after 96 hpi (Fig 1).

3.2. Survival rates

The survival rates of mice investigated according to the aforementioned experimental setup (Fig 2) considerably differed depending on the lyssavirus, the route of infection, and the

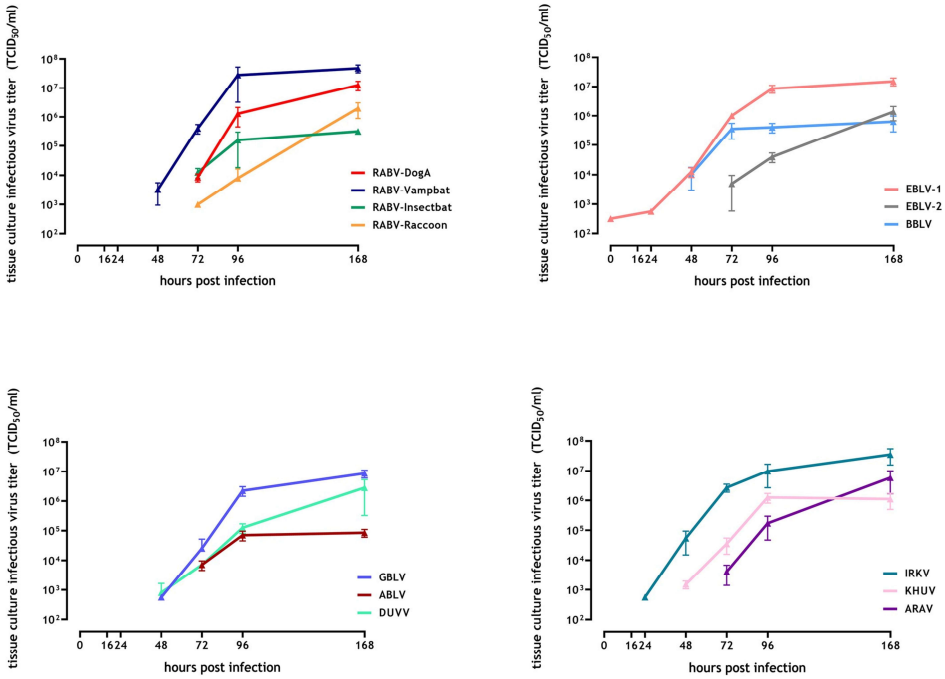

Fig 1. In vitro replication kinetics of lyssavirus isolates in Na 42/13 cells infected with a MOI of 0.001. The mean and standard errors of three replicates are indicated.

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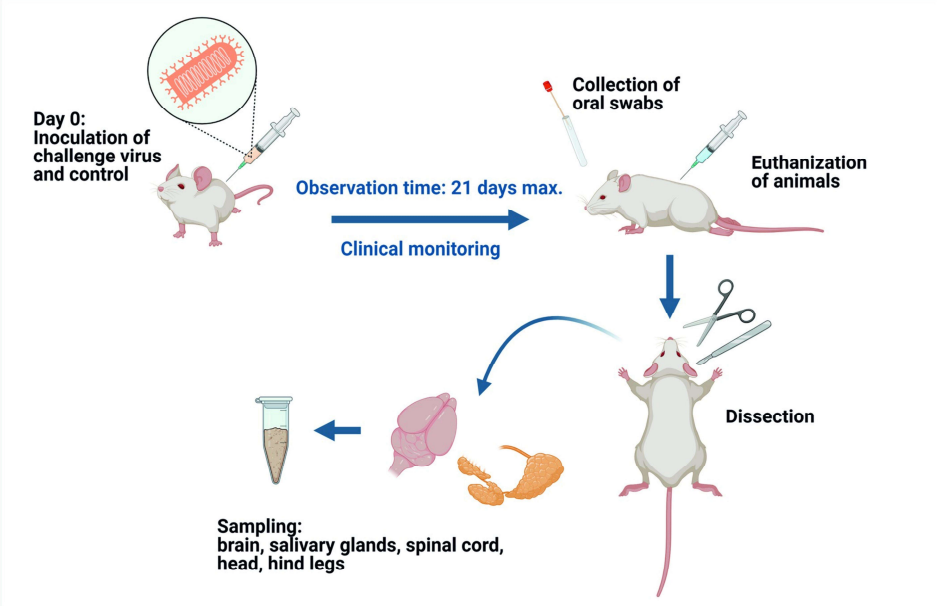

Fig 2. Experimental setup. Outline of the animal experiment and sample collection.

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infectious dose (Fig 3). In the low dose i.m. groups, survival rates varied between 100% (RABV-Raccoon, EBLV-1) and 0% (BBLV) (Fig 3A). All mice i.m. infected with a high dose of ARAV, GBLV, IRKV, RABV-DogA and BBLV developed clinical signs and had to be euthanized. In contrast, 67% and 50% of mice survived following high dose inoculation with ABLV and RABV-Raccoon, respectively, and all animals survived after DUVV infection (Fig 3B). In contrast, when comparing bat lyssaviruses with classical RABV strains, there was no significant difference in survival rates among i.m. low dose ($p = 0.0862$, Log-rank (Mantel-Cox) test) and high dose ($p = 0.8761$, Log-rank (Mantel-Cox) test) infected groups. All mock-infected control mice did not develop any clinical signs and survived until the end of the observation period. All mice inoculated i.c. with the different isolates developed clinical signs and were euthanized, except one mouse inoculated with BBLV, which survived until the end of the observation period (S1A Fig). The survival rate in groups inoculated i.n. with the lyssavirus isolates varied between 33%, (EBLV-1, EBLV-2 and BBLV) and 100%, (RABV-Racoon, RABV-Insectbat, RABV-Vampbat and IRKV, S1B Fig).

3.3. Incubation periods

In the i.m. low dose groups, the longest incubation periods were observed for mice inoculated with the reference strain RABV-DogA (mean: 17 days, SD±3) and with RABV-Vampbat (mean: 17 days, SD±0). However, in the group inoculated with RABV-Vampbat only a single animal developed clinical signs at all, the same applied to mice inoculated with DUVV.

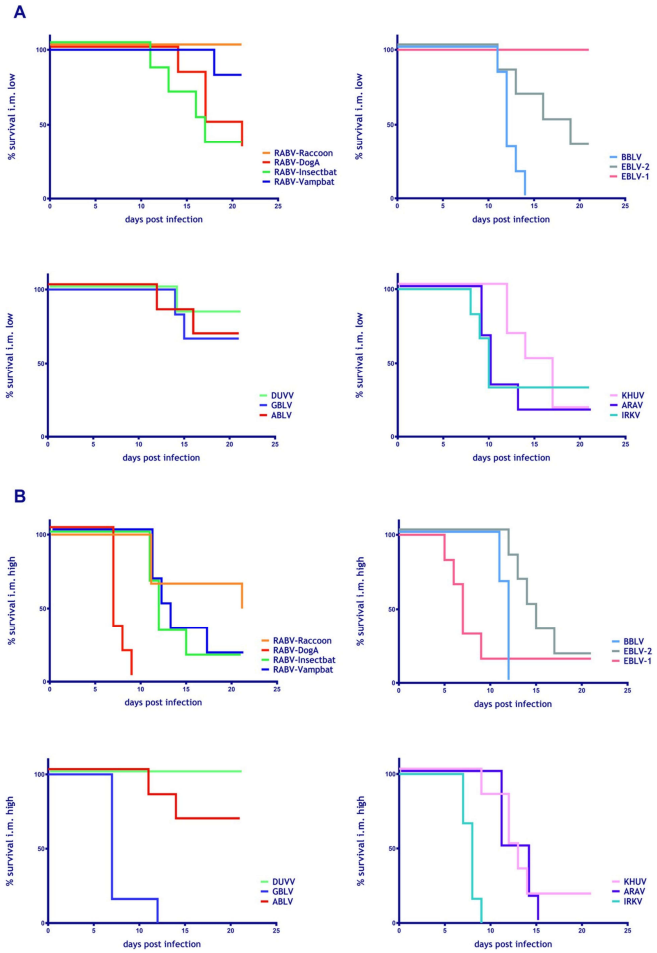

Fig 3. Kaplan-Meyer survival plots of the individual isolates following i.m. infection with low (A) and high doses (B). Six BALB/c mice were inoculated per group. Mock-infected control mice did not develop any clinical signs and, hence, were omitted for better visualization.

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Incubation periods of mice inoculated with BBLV (mean: 10 days, SD±1), ARAV (mean: 9 days, SD±1.8), IRKV (mean: 8 days, SD±0.5) were significantly shorter compared to RABV--DogA (Fig 4A). In contrast, all mice infected with RABV-Raccoon and EBLV-1 survived until 21 dpi showing no clinical signs at all. Differences in incubation periods in the i.m. high dose groups were more pronounced. Mice inoculated with RABV-DogA (mean: 7 days, SD±0.8),

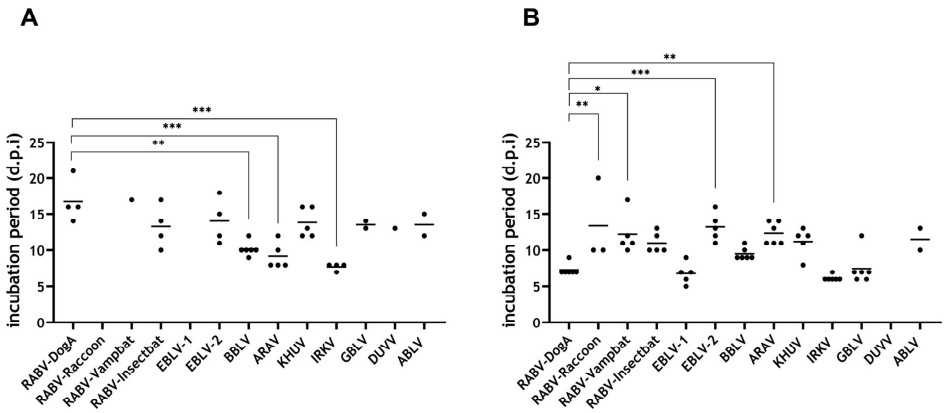

Fig 4. Incubation periods after low dose (A) and high dose (B) i.m. infection. Mean values are provided as horizontal lines. Statistical differences between the mean of RABV-DogA as a reference challenge strain and the means of other lyssaviruses are indicated (* $p \leq .05$; ** $p \leq .01$; *** $p \leq .001$; ordinary one-way ANOVA with Tukey's multiple comparison test).

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EBLV-1 (mean: 7 days, SD±1.5), BBLV (mean: 9 days, SD±0.8), IRKV (mean: 6 days, SD±0.4) and GBLV (mean: 8 days, SD±2.3) displayed clinical signs earlier (mean <10 days) than the remaining groups (Fig 4B). Regarding classical and bat-associated RABVs, the mean incubation for RABV-DogA was significantly different to those for RABV-Raccoon (mean: 13 days, SD±5.8) and RABV-Vampbat (mean: 12 days, SD±2.8) (Fig 4B). None of the mice infected with DUVV (high dose; i.m.) showed clinical signs until the end of the observation period.

3.4. Clinical signs

Independent of either the bat lyssavirus isolates or the classical RABV variants used, mice that succumbed to infection displayed clinical pictures suggestive of rabies. In general, clinical signs summarized as a progressive deterioration of the general health condition included decreased activity, hunched back, ruffled fur, weight loss, and lethargy or loss of alertness. Other clinically evident signs comprised paralysis, paresis, spasms, convulsive seizures, tremor, pruritus, aggressiveness, tameness, moving in circles, or extremely increased uncoordinated movements (Fig 5). For i.m. infected mice, disease progression after onset of clinical signs was either peracute with rapid development of clinical signs within <12 hours from healthy to apathetic, or disease duration was comparatively slower starting with mild, unspecific signs evolving into the full clinical picture within two to three days. The former was more frequently observed in mice inoculated with IRKV or KHUV and the latter was particularly common in mice inoculated with BBLV.

After i.m. infection, 41% of diseased mice had a deterioration of their general condition as the most prominent clinical sign, while 52% showed paralysis and paresis. The latter was more pronounced in GBLV (100%), ARAV (91%), EBLV-2 (89%) and IRKV (80%), whereas EBLV-1 and DUVV are outliers in the non-RABV lyssaviruses with no paresis/paralysis observed in diseased mice. Generally, with 63% over 25%, paresis/paralysis was significantly increased in non-RABV after i.m. infection of mice ($\chi^2 = 21.16$, $p<0.0001$). Also, paresis/paralysis occurred generally less often in i.c. (11%)- and i.n. (14%)-infected mice (Fig 5).

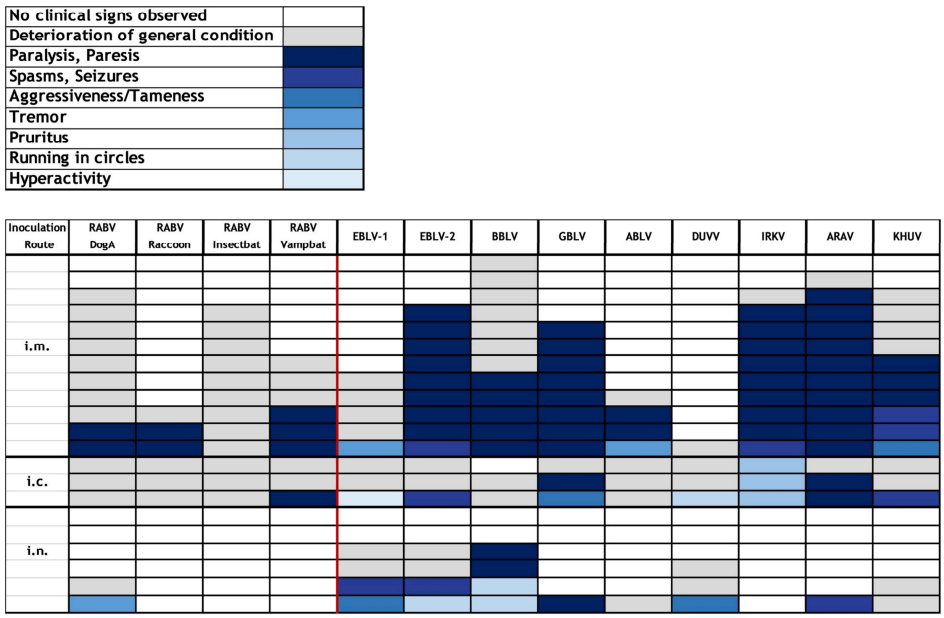

Fig 5. Clinical signs in individual mice inoculated with the indicated lyssaviruses by the i.m. (n = 156; 78 high dose and 78 low dose), i.c. (n = 39) or i.n. (n – 78) route of infection. The color code per cell represents the predominant clinical sign for each individual mouse before euthanasia or death. For clarity, high and low dose i.m. infections were combined.

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3.5. Index-based comparative pathogenicity

To allow for a ranking in pathogenicity, we implemented a novel intramuscular pathogenicity index (IMPI), which takes clinical signs and deaths/euthanasia of all i.m.-inoculated (dose-independently) mice into account. If all infected mice died at day 1, the IMPI would be 2, whereas it would be 0 if all mice survived with no clinical score. Depending on the lyssavirus used, indices ranging between 1.14 and 0.07 were obtained (Fig 6). The IMPI scored highest for IRKV and BBLV (>1) compared to the classical RABV-DogA (0.85). In contrast, RABV--Raccoon and DUVV had the lowest score (<0.19) (Fig 6).

3.6. Infection of neuron and astrocytes by selected bat lyssaviruses

Since pathogenic RABV have recently been shown to exhibit a specific astroglia tropism [42], we analyzed whether other lyssaviruses could infect central nervous system resident astrocytes to a comparable extent and whether differences in the pathogenicity correlate with astroglia infection levels. Therefore, the brain cell tropism of selected bat lyssaviruses with a high (IRKV, BBLV) and a low (RABV-Vampbat, DUVV) pathogenicity index (Fig 6) was investigated by 3D immunofluorescence imaging and quantitative analysis of infected neurons and astrocytes (Fig 7).

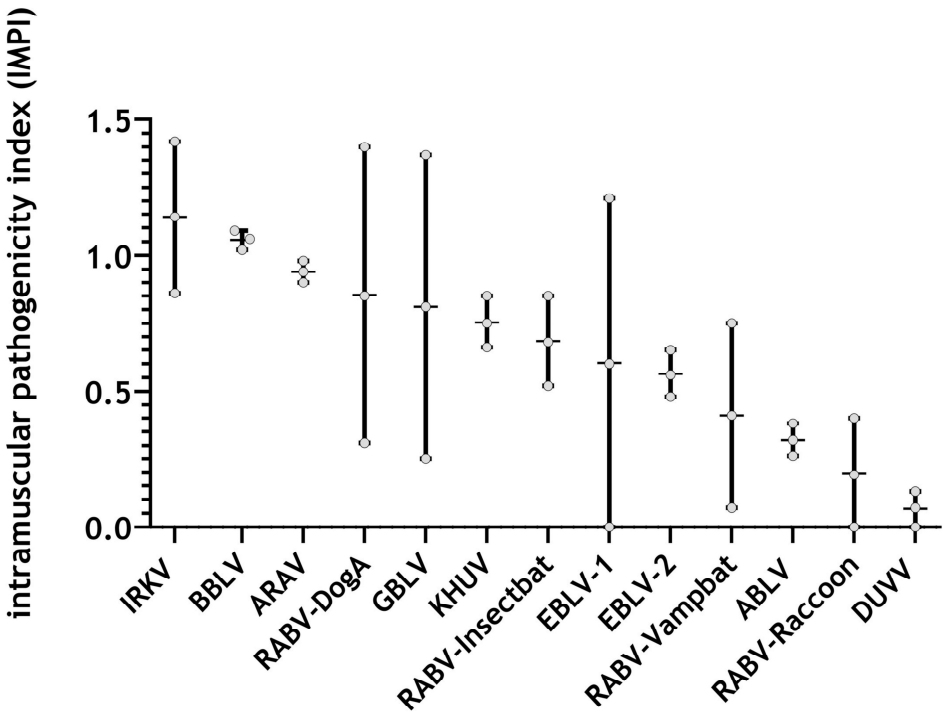

Fig 6. Intramuscular pathogenicity index (IMPI) of the different lyssavirus isolates obtained in the mouse model. Depicted are mean pathogenicity indices (median bar) of combined datasets of i.m. low (lower values) and high dose (upper values) infected animals. A maximum index value of 2 would be reached if all mice died at day 1 post-infection.

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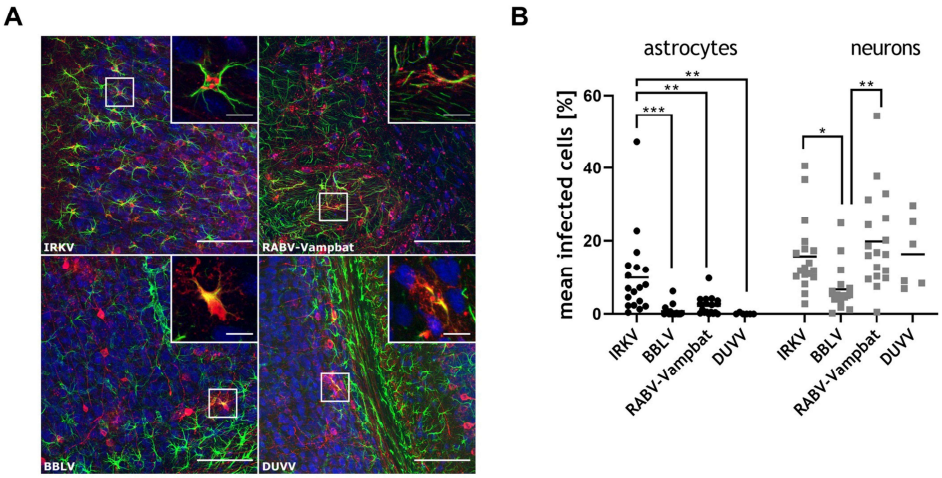
Intramuscular infections with IRKV, RABV-Vampbat, BBLV and DUVV resulted in a mean of 15.77% (Standard Deviation, SD±8.54), 19.83% (SD±12.59), 7.45% (SD±6.05), and 16.4% (SD±8.81) virus-positive neurons, respectively, demonstrating that IRKV and RABV-Vampbat had significantly higher levels of neuron infection compared to BBLV in clinically diseased mice (Fig 7B and 7C). Concerning astroglia infection, IRKV-infected mice featured the highest percentage of infected astrocytes (10.03%; SD±7.39). While astrocyte infection was lower in RABV-Vampbat (2.23%; SD±2.4)- and BBLV (0.78%; SD±1.51)-infected mice, only individual infected cells were identified in the DUVV-infected mice (Fig 7B and 7C).

3.7. Virus shedding–detection of viral RNA and viable virus in salivary glands and oral swabs

The detection of viral RNA in salivary glands and oral swabs in mice differed depending on the lyssavirus species used for inoculation. Positivity rates for all salivary gland and oral swab samples were highest for RABV-DogA (100%), followed by RABV-Insectbat (92%),



C

	Neurons				Astrocytes			
Virus	Counted	Infected	Mean (%)	SD	Counted	Infected	Mean (%)	SD
IRKV	14139.00	2121.00	15.77	8.54	8624.00	748.00	10.03	7.39
RABV Vampbat	12068.00	2338.00	19.83	12.59	9429.00	178.00	2.23	2.40
BBLV	17487.00	1295.00	7.45	6.05	11210.00	61.00	0.78	1.51
DUVV	5115.00	759.00	16.40	8.81	3560.00	3.00	0.08	0.18

Fig 7. Comparison of the astrocyte tropism of different bat lyssaviruses with a high and low IMPI. A) Indirect immunofluorescence of solvent-cleared brain sections demonstrates the presence of lyssavirus phosphoprotein P (red), neurons (blue, marker: NeuN) and astrocytes (green, marker: glial fibrillary acidic protein, GFAP). Insets show RABV P accumulation (red) at GFAP-positive cells (green). x, y = 387.5 μm, 387.5 μm; z = 65.5 μm (IRKV), 36.5 μm (RABV-Vampbat), 64.5 μm (BBLV), 65.5 μm (DUVV). Scale bar = 100 μm (overview), 15 μm (inset). **B)** Percentage of infected astrocytes (black dots) and neurons (gray squares). Per virus, 3 to 11 × 10^3 astrocytes and 5 to 17 × 10^3 neurons were counted in independent confocal z-stacks in two animals per isolate (one animal for DUVV). Each dot represents the ratio of infected astrocytes or neurons in an analyzed z-stack. Mean values are provided as horizontal lines. Statistical comparisons of the means between the different groups are indicated for those with a statistically significant difference (* $p \leq .05$; ** $p \leq .01$; *** $p \leq .001$; ordinary one-way ANOVA with Tukey's multiple comparison test). **C)** Corresponding data table for the measurements.

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RABV-Raccoon (33%) and RABV-Vampbat (33%) (Fig 8A). Independent of the route of infection, 97% of diseased mice inoculated with RABV strains were positive for viral RNA in salivary glands, and in 72% of mice viral RNA was also detected in the corresponding oral swabs (Fig 8B). In contrast, mice infected with non-RABV bat lyssaviruses exhibited significantly lower positivity rates ($p<0.0001$, Fischer's exact test), i.e. in only 50% of the diseased animals, salivary glands were positive for viral RNA, and 12% exhibited both virus RNA-positive salivary glands and oral swabs (Fig 8B). Regarding the presence of infectious virus, a similar pattern was observed when all routes of infection were considered (Fig 8C and 8D). When grouped together, infectious virus could be isolated in 86% of salivary gland samples from mice that succumbed to RABV, while infectious virus shedding, as determined by virus isolation from both salivary glands and oral swabs, was 47% overall, with the highest proportion in

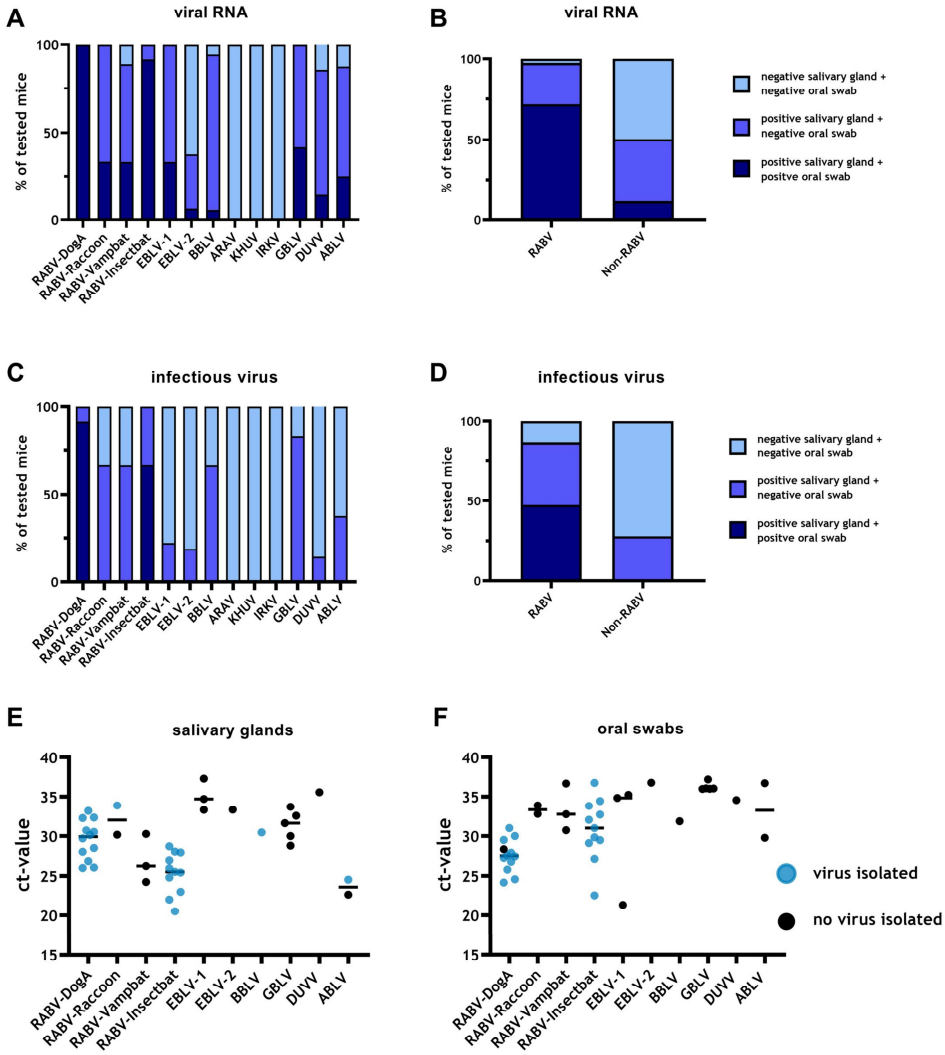

Fig 8. Comparison of virus shedding in lyssavirus-infected mice. Percentage of animals positive/negative for viral RNA (A, B) and viable virus (C, D) in either salivary glands or oral swabs or both according to individual lyssaviruses (A, C) or grouped according to RABVs and non-RABV bat lyssaviruses (B, D). **Correlation between ct-values as obtained in RT-qPCR and results of virus isolation in salivary glands (E) and oral swabs (F).** Here, only animals were considered were active shedding (positive salivary gland and positive corresponding oral swab) was observed. Individual ct-values are shown and the mean is indicated. Successful virus isolations in cell culture are highlighted.

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RABV-DogA (92%) and RABV-Insectbat (67%). While BBLV- and GBLV-diseased mice had positive salivary glands at frequencies of 83% and 67%, respectively (Fig 8C), no virus was isolated from oral swabs. None of the salivary glands or oral swabs from ARAV-, IRKV-, and KHUV-infected mice were positive for viral RNA or infectious virus (Fig 8A and 8C). Combined, similar to the presence of viral RNA, mice infected with non-RABV bat lyssaviruses exhibited significantly lower ($p<0.0001$, Fischer's exact test) positivity rates for infectious virus isolation as compared to RABV-infected mice (Fig 8D).

When data were analyzed according to inoculation dose and route, shedding of infectious virus was observed more often in mice diseased after i.c. inoculation (51%), followed by i.m. high dose (41%), i.n. (35%), and i.m. low dose (29%). The mean ct-values were significantly lower in samples with successful virus isolation from salivary glands ($p = 0.003$, unpaired t-test, Fig 8E) and corresponding oral swabs ($p = 0.003$, unpaired t-test, Fig 8F) as opposed to unsuccessful virus isolation. This observation is mainly driven by the low ct-values observed in RABV-Insectbat and RABV-DogA.

4. Discussion

Although lyssaviruses comprise a genetically close group of viruses, which all cause the disease rabies, differences in their phenotype may indicate different risks for veterinary and public health. In our assessment, 13 different lyssaviruses exhibited differences in their replication kinetics in terms of the growth dynamic and the final virus titer. As such, *in vivo* replication kinetics may reflect replication in the animal host and thus may explain differences seen in incubation periods and pathogenicity among individual lyssaviruses. Interestingly, relatively short incubation periods (IRKV, EBLV-1) (Fig 4A and 4B) or relatively high (IRKV, BBLV) or low pathogenicity indices (ABLV) (Fig 6) in the animal model also correlate with the replication kinetics (Fig 1).

Similarly, the number of animals that survived infection with the individual lyssaviruses varied (Fig 3A and 3B). Survival was not associated with belonging to classical RABV as opposed to non-RABV bat lyssaviruses. There was considerable variation seen in incubation periods across lyssavirus species as well as between animals of individual groups (Fig 4A and 4B). However, we have no evidence that bat-associated lyssaviruses cause longer incubation periods in mice compared to observations made in epidemiological bat models [59,60], and supporting case studies [61,62].

The perception and subsequent recording of clinical signs might be biased due to the clinical score being applied for animal welfare reasons and due to the temporally restricted observation scheme. However, all investigated lyssaviruses caused a clinical picture that led to euthanisia or death in mice, albeit at different scale. We observed the clinical outcome to be predominantly dependent on the virus species but also on the route of application. The fact that clinical signs such as paralysis and spasms were more pronounced after i.m. inoculation corroborates previous findings [34]. On the other hand, the observation that mice infected i.m. with non-RABV bat lyssaviruses were more likely to develop spasms and paralysis is interesting but requires further investigation. While lyssavirus species-dependent clinical signs were reported before [36], in summary, no clear pattern in regard to particular lyssavirus species was evident in our study (Fig 5).

Overall, the variation in pathogenicity factors shown in our study highlights the complexity and difficulty to establish a holistic concept for the classification of lyssaviruses. To integrate these factors from our standardized in vivo model, we applied an intramuscular pathogenicity index for lyssaviruses, and thereby demonstrated a high diversity across phylogroup I lyssaviruses that had not been shown to such an extent before. Remarkably, non-RABV bat

lyssavirus isolates are among those with the highest IMPI (Fig 6), questioning previous suggestions that bat-related lyssaviruses are less pathogenic [63]. Interestingly, IRKV demonstrated the highest pathogenicity in a ferret model as well in comparison to the other bat-associated lyssaviruses KHUV and ARAV [13]. Moreover, the fact that current RABV-based biologicals provide only partial protection against IRKV challenge [13,64] further emphasizes the higher risk for a lethal outcome associated to IRKV infections. Even though EBLV-1, DUVV, and IRKV have all caused human rabies cases [10,65], no elevated pathogenicity was found in our model using isolates from human cases (EBLV1, DUVV) (Fig 6). Of note, the Yuli isolate we used here is the only available EBLV-1 isolated from a fatal human case [66].

Using 3D high-resolution confocal laser-scanning microscopy, the CNS cell tropism for bat-related lyssaviruses was analyzed here for the first time and provides new insights into their capability to infect astrocytes (Fig 7). A comparison between RABV field isolates and lab-adapted strains revealed that astrocyte infection after i.m. inoculation is associated with field strains and, thus, might be a potential pathogenicity determinant [42]. In our analyses using representatives of bat-associated lyssaviruses, mice infected with IRKV, as a representative for high pathogenicity (IMPI = 1.14), had significantly higher proportions of infected astrocytes than RABV-Vampbat (IMPI = 0.41)- and BBLV (IMPI = 1.06)-infected mice. Interestingly, only sporadic astrocyte infection was found in DUVV (IMPI = 0.07)-infected mice. Even though the restricted number of lyssaviruses analyzed for astrocyte infection hinders a full comparison, the results support previous studies of RABV on the association of astrocyte tropism and pathogenicity. Additional virus isolates and strains have to be analyzed in further studies to confirm the role of astrocyte tropism in lyssavirus pathogenicity. However, different virus kinetics and astrocyte-related innate immune reactions may affect the progression kinetics, immune pathogenicity, and further spread of the virus to peripheral salivary glands. The latter may represent a key issue in terms of virus transmission and maintenance in host populations. In our analyses, virus shedding was not demonstrated in IRKV-infected mice. In general, virus shedding represents a striking discrepancy between RABV and other bat lyssaviruses, as virus shedding was significantly reduced in non-RABV bat lyssaviruses in the mouse model. Interestingly, while shedding was highest for the dog rabies strain RABV-DogA, bat-related RABV isolates and a raccoon RABV variant demonstrated a lower percentage of active shedding (Fig 8A–8D). Of note, the raccoon RABV lineage in the Americas is a result from an ancient sustained spillover event from bats [67].

By our definition, active shedding is the successful virus isolation from salivary glands and the respective oral swab sample. Of note, a positive result from an oral swab may not necessarily correspond to viral excretion in saliva itself because the swab could also contain desquamated cells, including infected neurons, which may not necessarily have been excreted naturally. However, technically it was not possible to extract only saliva and the same methodology was used for all isolates. Discrepant results in virus isolation in salivary gland samples and corresponding oral swabs can be explained by the fact that neurons innervating the glands are infected, without excreting virus in the lumen of the gland. The reason for the limited shedding of bat-associated lyssaviruses may be a yet unknown block or barrier in virus distribution in the salivary gland of non bat mammals. Another explanation could be intermittent shedding, i.e. samples might have been taken at time points when virus was temporarily not shed, but the salivary gland was still virus positive. Nevertheless, intermittent shedding does also apply for RABV strains and consequently it does not completely explain the differences. For some isolates the disease duration in mice was very short so that the centripetal spread of virus may not have reached the salivary gland before death or euthanasia. Interestingly, neither viral RNA nor infectious virus could not be found in salivary glands or oral swabs for the three virus species IRKV, KHUV, and ARAV. Furthermore, there was a gradient for virus shedding

with lowest percentage of shedding in low dose i.m. infected animals, and highest in i.c. inoculated ones. While the mean ct-value in successfully isolated samples was significantly lower, there was a high range in ct-values between 20 and 37 (Fig 8E and 8F), and no threshold for successful isolation could be defined.

In any case, our shedding results could mimic the capability of transmission from a spillover host to another conspecific as the prerequisite of a sustained spillover [41], and thereby contribute to an overall risk assessment for lyssaviruses. Our shedding results in the mouse model confirm previous assumptions based on field observations that the potential for sustained spillovers is highest in RABV as opposed to other bat lyssaviruses [8]. However, a further component of successful virus transmission is the initial shedding potential in the respective reservoir host. Animal models have greatly improved our understanding of virus-host interactions. When studying virus-host interactions of bat-related lyssaviruses, experimental studies in bats as their primary reservoir hosts would be ideal. While the results of those studies can infer the pathobiology in the host, they are particularly challenging due to their demanding housing conditions, difficult handling, conservation issues and partly strict protection status [68]. Furthermore, they are less suitable to assess the pathogenicity in non-reservoir hosts. Therefore, for reasons of comparison, the selection of reliable and standardized alternative animal models is of great importance in the study of pathogenicity factors. While mice are considered a standard model for studying lyssavirus pathogenicity [69], in fact little attention has been paid to comparability. Here, our model facilitates a direct comparison of pathogenicity data of 13 different phylogroup I bat lyssaviruses by using a consistent approach regarding experimental conditions, e.g. cells for viral propagation, mouse breed, viral doses, inoculation routes as well as observation times and scoring schemes. We thereby optimized an in vivo mouse model established for EBLV-1 [34] by including data on virus shedding. Furthermore, we established a novel matrix for comparing pathogenicity based on clinical parameters, the intramuscular pathogenicity index IMPI. Such indices are commonly used for avian influenza viruses and Newcastle disease viruses to directly infer the potential for causing disease in animals and thus relate to the respective veterinary control measures. In our case for lyssaviruses, the index can also be used to summarize the pathogenic potential of the individual lyssavirus isolate.

We included i.n. and i.c. inoculation routes in our assessment (S1 Fig), with i.c. primarily used as a positive infection control with the low dose virus inoculum, whereas i.n. application was tested as it was speculated before that virus transmission via aerosols could contribute to disease spread among bats and in spillover infection [68,70]. While i.n. application led to infection of some animals (S1B Fig) likely via the olfactory pathway [71], no clear indication for a specific role in bat lyssaviruses was found (Fig 5), supporting experimental studies in bats [72,73]. Therefore, we then focused our analyses on pathogenicity to i.m. inoculation as the most likely route of virus infection. For virus shedding, we wanted to assess the virus' potential to be transmitted when an animal develops disease, and therefore all inoculation routes were considered. Nevertheless, there are some limitations in our study. The number of mice used was kept to a minimum, respecting animal welfare guidelines on the 3R principle [74]. Also, propagation of viruses in cell culture was a necessary requirement to obtain sufficient virus stocks for the experiments. To minimize the possibility of adaption to cell culture that may influence the results obtained, we used Na 42/13 cells, a mouse neuroblastoma cell line considered to be primary target cells for lyssaviruses, and kept the number of passages as low as possible. Also, the likelihood of adaptive mutation is regarded low since lyssaviruses, i.e. RABV [75] and EBLV-1 [76] have among the lowest mutation rates of RNA viruses [77]. The full genome sequences derived from passaged material did not give evidence of nucleotide exchanges compared to other sequences of the primary isolates.

5. Conclusion

Here, we have comparatively assessed the pathogenicity and virulence of a wide diversity of lyssaviruses belonging to phylogroup I using a standardized and systematic approach. Interestingly, we found in our model bat-associated lyssaviruses, which are more pathogenic and virulent than a classical RABV challenge strain. In fact, no tendency towards a generally reduced pathogenicity of bat-associated lyssaviruses as opposed to classical RABV can be confirmed and thus each isolate should be considered individually concerning its pathogenicity. In contrast to RABV, we could not determine virus shedding in all other lyssavirus-infected mice. This indicates a limited potential of those lyssaviruses to spread beyond the initial spillover-host, and may explain the absence of onward transmission in non-RABV bat lyssaviruses.

Supporting information

S1 Table. Details of the primer and probe mix for GBLV. The reaction conditions were identical to those used for EBLV-1, BBLV and EBLV-2.
(DOCX)

S2 Table. Clinical score sheet for mice, ranging from zero up to five.
(DOCX)

S1 Fig. Kaplan-Maier survival plots of the individual isolates following i.c. infection (six Balb/c mice were inoculated per group) (A) and i.n. infection (three Balb/c mice were inoculated group) (B). Mock-infected control mice did not develop any clinical signs and, hence, were omitted for better visualization.
(TIF)

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Author Contributions

Conceptualization: Thomas Müller.

Formal analysis: Antonia Klein, Madlin Potratz, Luca M. Zaeck, Stefan Finke, Conrad M. Freuling.

Funding acquisition: Thomas Müller, Conrad M. Freuling.

Investigation: Antonia Klein, Elisa Eggerbauer, Madlin Potratz, Luca M. Zaeck, Sten Calvelage, Stefan Finke, Thomas Müller, Conrad M. Freuling.

Methodology: Stefan Finke, Thomas Müller, Conrad M. Freuling.

Resources: Stefan Finke.

Supervision: Thomas Müller.

Visualization: Antonia Klein, Luca M. Zaeck, Conrad M. Freuling.

Writing – original draft: Antonia Klein, Conrad M. Freuling.

Writing – review & editing: Antonia Klein, Elisa Eggerbauer, Madlin Potratz, Luca M. Zaeck, Sten Calvelage, Stefan Finke, Thomas Müller, Conrad M. Freuling.

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Supplementary Material:

S1 Table: Details of the primer and probe mix for GBLV. The reaction conditions were identical to those used for EBLV-1, BBLV and EBLV-2.

S1 Fig: Clinical score sheet for mice, ranging from zero up to five.

S2 Fig: Kaplan-Maier survival plots of the individual isolates following i.c. infection (six Balb/c mice were inoculated per group) (A) and i.n. infection (three Balb/c mice were inoculated group) (B). Mock-infected control mice did not develop any clinical signs and, hence, were omitted for better visualization.

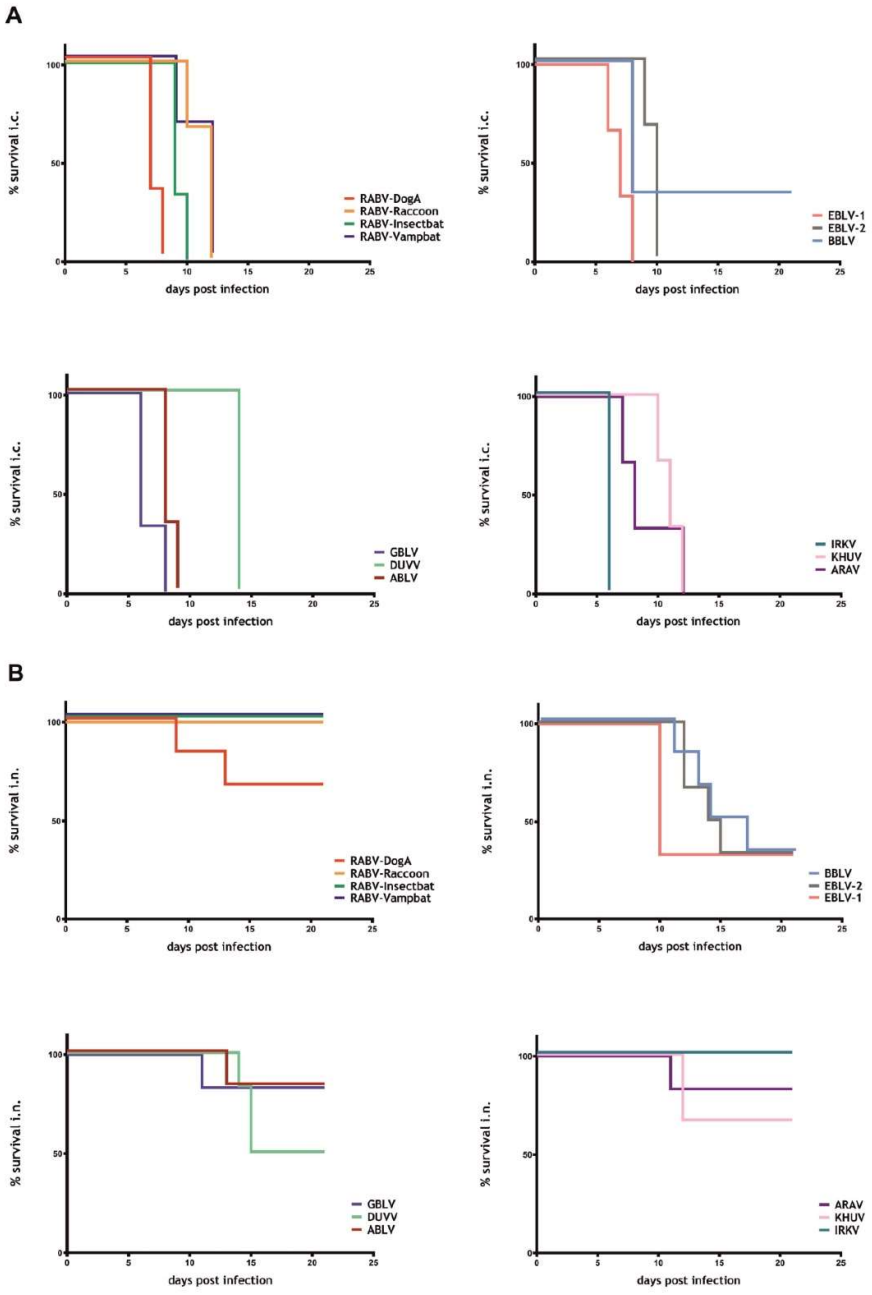
S1 Table:

Volume	**Oligo** (Concentration)	**Sequence Primer/Probe** (5`- 3`)
20.0 µl	**GBLV_for** (100 pmol/µl)	CCGGCGATTAGAGATCAAAAG
20.0 µl	**GBLV_rev** (100 pmol/µl)	CATTCCAGACAGAACGGAC
5.0 µl	**GBLV_HEX probe** (100 pmol/µl)	HEX-CCTAGTATAACTCTTGGTAAGGCCCCAGAT-BHQ1
155.0 µl	0.1 x TE (pH 8.0)	
200.0 µl	**Primer-probe-mix**	

S1 Fig:

Score	Clinical Signs	Instructions / Humane Endpoints
0	• Healthy/Normal	Daily observation
1	• Ruffled fur • Hunched back	Shorten observation intervals to 12h
2	• Slowed movements • Circular movements • Weight loss ≥ 15%	Euthanasia within the next 8 hours maximum
3	• Tremor • Wobbly gait • Seizures • Weight loss ≥ 20%	Immediate Euthanasia
4	• Signs of paralysis or spasms • Weight loss ≥ 25%	Immediate Euthanasia
5	• Coma/Death	Immediate Euthanasia

S2 Fig:


A
% survival i.c.
days post infection
RABV-DogA
RABV-Raccoon
RABV-Insectbat
RABV-Vampbat
EBLV-1
EBLV-2
BBLV
GBLV
DUVV
ABLV
IRKV
KHUV
ARAV
B
% survival i.n.
RABV-DogA
RABV-Raccoon
RABV-Insectbat
RABV-Vampbat
BBLV
EBLV-2
EBLV-1
GBLV
DUVV
ABLV
ARAV
KHUV
IRKV

V. RESULTS AND DISCUSSION

Comparative evaluation of different LFDs as an alternative diagnostic tool in low resource settings

The WHO considers rabies a neglected disease due to its enormous human and financial burden that largely affects the poorest regions of the world where there is only limited access to either vaccines or PEP with additionally very low or even no vaccination coverage for stray or domestic dogs (Hampson et al., 2015). Even though it is actually an entirely preventable disease, a complex combination of first and foremost economic but also logistical as well as political aspects leads to a global persistence that is still lasting in the 21st century. Furthermore, massive discrepancies between Asian and African retrospective active surveillance studies and official reports indicate that published numbers of human case prevalence are most likely severely underestimated. Many people that are affected do not have the opportunity to seek medical care after an incident and thus die at home (Cleaveland et al., 2002; Hampson et al., 2008; Ly et al., 2009; Deressa et al., 2010; Tenzin et al., 2011; Hossain et al., 2012; Suraweera et al., 2012). The resulting inaccurately low case statistics of canine rabies-endemic countries are responsible that too little attention is given to rabies prevention in public health programs (Hampson et al., 2015). Additional deficits in suitable technical capacities for rabies surveillance in animals in less developed areas might also result in a decline of motivation to control the spread of the disease, leading to a vicious circle of neglect and ignorance (Taylor et al., 2017). Generally, the lack of adequate modern diagnostic and laboratory equipment hampers reliable rabies diagnosis in animals and humans (Fooks et al., 2009). In this context, the development of rapid, cost-effective and uncomplicated diagnostic solutions for the detection of rabies, presents a possible answer to the question how one can put disease control and elimination into action in regions with highest demands but lowest resources. However, any test for rabies needs to meet quality criteria as regards specificity and sensitivity. As for the latter, false-negative test results reinforce negligence in a global epidemiological context and also discourage exposed individuals from seeking quick medical care which might be essential for their survival. The rising demand for such point-of-care tests as well as their relatively simple operating and, hence, manufacturing principle implies a flood of countless but often non-transparent LFD offers engulfing the market.

Moreover, it is not apparent how many of these tests are actually produced by the exact same manufacturers but are sold under different brand names. There is also a lack of transparency concerning the test principle they are based on, i.e., what kind of antibody they used for what target epitope, and often there is no reference neither on any sort of validation nor any approval procedures they might have to pass. However, they are all commercially available and can be ordered online without any further restrictions.

Since a previous comparative analysis of different LFDs revealed unsatisfactory results regarding their diagnostic reliability (Eggerbauer et al., 2016), this study aimed at expanding the evaluation work. For that matter, another five different rapid immunodiagnostic tests, including the Anigen/Bionote kit, were evaluated by assessing their diagnostic sensitivity according to the manufacturers' instructions and their agreement with FAT and real-time RT-PCR in order to verify their suitability as point-of-care diagnostics in routine surveillance and veterinary services. In the present study, previous results were confirmed by the partly highly disappointing outcome that the performance of the tested commercial LFDs was very poor with sensitivities broadly ranging between 0 % and 62 %. Although, the repeated evaluation of the Anigen/Bionote test only yielded sub-optimal results it still performed by far best. To ensure a high evaluation standard, this analysis was based on a multi-centered approach that involved eight different OIE and FAO rabies reference laboratories using a large sample set. The latter included a broad diversity of RABV and other lyssavirus isolates. For the broad panel of isolates, different host species, a diverse geographical origin and different genetic lineages of all major genetic clusters (Fischer et al., 2018) were considered. However, any concluding patterns that may explain the outcome were not found.

Apart from the Anigen/Bionote kit, the poor results of the other four test kits did not differ much between the different laboratories since almost all of them failed completely in each category neither detecting rabies in fresh field nor in archived samples. Only the Anigen/Bionote test kit showed a wide range of sensitivities ranging between 33 % and 100 %. For reason unknown yet, fresh field samples that were tested in two of the participating laboratories (KVI in Israel and OVI in South Africa) achieved better agreement with the standard diagnostic methods than archived samples. Prior to this study this phenomenon has already been reported (Eggerbauer et al., 2016; Léchenne et al., 2016) but its definite cause still remains puzzling as the majority of samples used for this study had been confirmed

positive beforehand by standard rabies diagnostic tests, i.e. FAT, RT-PCR and RTCIT. As was shown for the Anigen/Bionote test kit high antigen as well as high viral RNA content seem to be important factors that influence the likelihood of test agreement with the conventional methods FAT and RT-qPCR, indicating a direct dependency ratio between disease progression and test performance. Even though the Anigen/Bionote test gained better results compared to other test kits, those findings still strictly limit its applicability and suggest caution. Its wide range in sensitivities obtained in different independent laboratories only partly corroborates findings of numerous other studies that have also evaluated this specific test kit in different environments and under diverse conditions, claiming its sensitivities somewhere between 91 % and 100 % (Kang et al., 2007; Nishizono et al., 2008; Markotter et al., 2009b; Servat et al., 2012; Yang et al., 2012; Reta et al., 2013; Voehl and Saturday, 2014; Pranoti et al., 2015; Léchenne et al., 2016; Certoma et al., 2018; Servat et al., 2019; Tenzin et al., 2020). When only considering those results, a restricted use of the Anigen/Bionote test kit might be acceptable in combination with further laboratory confirmation of negative results, which is generally advised. However, referring to this study's results, an in-field use is not advisable yet.

The fact that with the exception of the Anigen/Bionote test kit all other tested LFD kits completely failed is even more alarming. Substantial improvements are required before unconditional use of these tests in the frame of national rabies surveillance systems can be recommended, especially when used for animals with suspect human contact. Manufacturers' quality controls seem lacking and when directly approached, only limited to no response was received. Moreover, the manufacturers have already been advised to improve their instruction manuals with regard to more detailed instructions for sampling and sample preparation as well as to exclude saliva as accepted alternative sample material (Eggerbauer et al., 2016; Léchenne et al., 2016). Since saliva is inadequate as an analyte due to possible intermittent virus shedding and a lower viral load that might be below the limit of detection, only brain material should be used as an appropriate analyte (Hanlon, 2013). Especially when taking the international goal to reach zero human deaths due to dog-mediated rabies by 2030 into account, primarily relying on the producers' individual responsibilities is not sufficient enough. Quality control through standardized approval procedures following OIE recommendations (OIE, 2013) is strongly advised. Nevertheless, those LFDs should be appreciated as an essential tool for rabies diagnosis in resource-poor surroundings where point-of-care use strengthens surveillance and supports potentially lifesaving decision making.

Therefore, they should urgently be optimized by adjusting sensitivities to those of standard diagnostics and avoiding possible batch-to-batch variation as every single improvement eventually leads to a better disease control.

Retrospective enhanced bat lyssavirus surveillance in Germany between 2018–2020

To date, only a few transmissions from bats to other mammals and two human cases each for EBLV-1 (Johnson et al., 2010; Regnault et al., 2021) and EBLV-2 (Lumio et al., 1986; Fooks et al., 2003b), respectively, have been recorded in Europe. Even though the occurrence of bat rabies seems to remain on a consistently low level, the true number of transmissions might be higher, thus posing a potential veterinary and public health risk (Banyard et al., 2014a; Smreczak et al., 2018). To constantly assess the current state of prevalence and distribution of bat-related lyssaviruses in Germany, enhanced passive surveillance is conducted, encompassing samples that would not be tested under routine conditions. To this end, in a continued effort 1238 bats were sampled and analyzed between 2018 and 2020. Thereby, multiple EBLV-1 cases as well as the tenth BBLV case in Europe were detected by the use of molecular techniques.

In this study, opposed to other previously conducted enhanced passive surveillance studies (Schatz et al., 2013a; Schatz et al., 2014), the sampling scheme was changed to a non-destructive method based on recommendations for sampling in terrestrial mammals (Barrat, 1996). It was often requested by bat handlers to keep carcasses intact and either return negative tested ones to natural collections or forward them to other research projects. In order to meet those requirements and thereof expecting a higher contribution to sample submission, we followed a minimal invasive approach using cannula and syringe to aspirate brain material through the *foramen magnum occipitale*, thus causing only minimal damage. Moreover, in accordance with updated OIE diagnostic standards (World Organisation for Animal Health, 2021) and with the purpose of gaining a higher throughput, instead of using the FAT what had originally been regarded the gold standard in rabies diagnostic (OIE, 2018), lyssavirus screening was optimized by applying two different RT-qPCR assays in a double-check approach (Fischer et al., 2014). Thus, it was possible to initially scan a significantly higher number of specimens in a reduced turnaround time with multiple target detection while simultaneously enabling virus typing. In bat rabies surveillance where quality and quantity of samples often is quite low, applying RT-qPCRs offers a higher sensitivity and thus a higher

reliability in detecting low viral loads of known or potentially novel virus species that might otherwise not be identified (McElhinney et al., 2014). The detection of EBLV-1 cases by RT-qPCR in bats that during repeated investigation initially tested negative by conventional techniques, e.g. FAT (Schatz et al., 2014), supports this concept. To overcome limitations in the diagnostic range of the used modified R14-assay, which only detects EBLV-1, EBLV-2 and BBLV, the analysis was combined with a pan-lyssa RT-qPCR, targeting both the N- and L-gene. Similarly, to the use of the FAT but on a molecular level, this combination offers the possibility to detect novel or divergent lyssavirus species.

In Germany, rabies is classified a notifiable disease, irrespective of the animal species infected (Verordnung zum Schutz gegen die Tollwut (Tollwut-Verordnung), 2010 (BGBl. I S. 1313)). However, commonly, only suspected bats with human contact are sent to responsible regional authorities for laboratory testing. Retrospective studies underline that complementing this automatically biased routine diagnostic surveillance scheme by enhanced passive surveillance adds a significant value (Schatz et al., 2014). Thereby, bats that were found diseased, dead or moribund and were subsequently collected by bad handlers or nature conservationists are included. Hence, an overall more realistic picture of natural prevalence can be drawn. Nevertheless, even when both systems are combined, the correct bat rabies prevalence cannot be determined as the sampled bat species assortment does not necessarily reflect the natural bat population. Moreover, albeit major efforts were made, an equally distribution of collection sites with submissions from all across Germany was not yet achieved. However, this is only influenceable to a limited extend, as a study participation of the different federal states is largely dependent on the willingness and contribution of the respective regional bat experts. The heterogeneous landscape of bat conservation in Germany, and different regulations in federal states on the conservation and archival of endangered and protected species lead to sampling difficulties and makes an equal geographical distribution quite challenging. Hereby, it is of particular interest and importance to establish and maintain contacts to bat associated stakeholder groups whose active contributory work forms the basis of every enhanced passive surveillance program. This collaboration could be further expanded in the sense of a more detailed data acquisition regarding the collected bats. Generally, a closer cooperation between bat rabies surveillance programs and, bat ecology and conservation research could be a possible future approach to complement surveillance strategies. Particularly, in the scope of both climate change and other anthropogenic factors it is hypothesized by different studies

that shifting environmental conditions for bats might lead to geographical range expansion outside of typical territories (Lundy et al., 2010; Sherwin et al., 2013; Ancillotto et al., 2016) such as already expected for vampire bats from South America (Hayes and Piaggio, 2018). Thus, a variety of different factors such as a potential decrease in distances towards densely populated areas or reduction in bat health might facilitate possible emergence and spread of a various number of distinct zoonotic viruses including lyssaviruses (Daszak et al., 2013; Hayes and Piaggio, 2018; Beyer et al., 2021; Yuen et al., 2021). Therefore, a more extensive cooperation between different research fields, e.g. lyssavirus epidemiology and bat ecology, offers the chance to put the One Health concept into practice to better face future challenges. As bats are naturally not constraint by national boundaries, throughout Europe, considerable efforts should be made to strengthen and expand the hitherto relatively heterogeneous bat rabies surveillance structure (Schatz et al., 2013a) in favor of a more comprehensively and Europe-wide understanding of lyssavirus dynamics. Recent findings of novel lyssaviruses within surveillance programs not only in Europe, e.g. KBLV (Nokireki et al., 2018) but also in Africa, e.g. MBLV (Coertse et al., 2020; Grobler et al., 2021) support the need for such surveillance studies. Further detections of already known lyssaviruses with so far only single reported cases could also help to either confirm or question assumptions regarding the respective host species or even resolve questions to yet unknown reservoirs such as for IKOV or MOKV in Africa.

While the absence of positive findings during surveillance in a certain region or country does not necessarily guarantee a general absence of lyssaviruses, detecting positive cases and isolating viruses thereof is essential for a continuously adapted risk assessment. Following a novel virus isolation, investing in further characterizations concerning phylogenetics, pathogenesis in animal models and cross-neutralization by available vaccines, is necessary for the development of adequate response. This has already been exemplified for BBLV (Nolden et al., 2014), LLBV (Banyard et al., 2018) and KBLV (Shipley et al., 2021). To also assess threats posed by other zoonotic bat-related viruses, samples from the enhanced passive surveillance could be tested for additional pathogens such as coronaviruses, making the surveillance system even more efficient. In other parts of the world this could also involve other concerning bat-borne viruses with zoonotic character including henipaviruses or ebolaviruses (Li et al., 2005; Wibbelt et al., 2010; Clayton et al., 2013; Rougeron et al., 2015; Letko et al., 2020).

In this study, the fact that 94 % of all lyssavirus positive bats were found infected with EBLV-1 corroborate results of other surveillance studies (McElhinney et al., 2013; Schatz et al., 2014). Similarly, the determined positivity rate of 16 % in serotine bats is comparable to results gained for EBLV-1 in previous studies from Germany, Spain and The Netherlands (Echevarria et al., 2001; Van der Poel, W. H. M. et al., 2005; Schatz et al., 2014). Minor deviations between the study findings might derive from different sampling schemes and diagnostic methods applied. No spillover infections of EBLV-1 into bats other than *E. serotinus* were found in the samples submitted between 2018-2020. However, a repeated RT-qPCR screening of additional bat samples that had initially been tested negative by FAT, revealed two cases in common pipistrelles. Remarkably, the latter were collected in the southeast of Germany, a region where no EBLV-1 cases had ever been reported before, thus demonstrating the occurrence of spillover events in regions where lyssaviruses have not yet been detected at all. Hitherto, the majority of EBLV-1-positive cases was found in the northwest of Germany, supporting previously established distribution patterns that located serotine bats infected with EBLV-1 mainly in the German northern lowlands (Müller et al., 2007; Schatz et al., 2014). This was explained by high serotine bat population density appearing in this region which seems to increase the intraspecies transmission and virus maintenance (Müller et al., 2007). Although the 1.2 % positivity rate for lyssaviruses across all investigated bat species as determined in this study might appear to be relatively low, it still poses a potential veterinary and public health hazard. This concern particularly addresses people who regularly handle bats, emphasizing the need for adequate prophylactic treatments according to international and national guidelines.

This study also revealed the tenth European BBLV case, which is the seventh case in Germany and the fourth case in Lower Saxony which were all isolated from *M. nattereri*. The repeated detection of BBLV within a short time proves its endemic occurrence in indigenous bat populations in Germany and Europe. Since the first detection of BBLV as a novel lyssavirus in 2010 in Germany (Freuling et al., 2011), it was thereafter found several times in Germany, France and Poland (Eggerbauer et al., 2017b; Smreczak et al., 2018). The fact that it was again isolated from the same bat species as in all other cases supports the hypothesis of the Natterer's bat representing the only reservoir host species. Most strikingly, this BBLV case from Lower Saxony is genetically closer related to a BBLV case detected in Kronach, Bavaria in 2015 (Freuling et al., 2013) than to others found in Lower Saxony. The isolate from Bavaria is

again closely related to the one found in Poland (Smreczak et al., 2018). It seems puzzling how isolates found within large geographical distances can cluster so closely together and show such a high sequence identity. A possible explanation might be that the natterer's bat, initially believed to be a sedentary species, rather tends to be a facultative migrant according to increasing evidence. Usually, they fly no more than 50 km for seasonal movement between summer and winter roosts but long distance migration over several hundred kilometers has also been observed (Hutterer et al., 2005). Against this background, the respective BBLV outliers may be, however, due to those yet rarely observed long distance movements. It is also striking that since its sudden emergence BBLV appears to be more prevalent than, for example, EBLV-2. Even though, all those cases were detected in countries with a, by comparison, relatively high level of lyssavirus surveillance where by definition more positive findings are expected, the heterogeneous European surveillance landscape alone cannot explain the high number of novel cases. In fact, lately there have been only very few changes regarding surveillance activities in Europe and, nevertheless, BBLV has been detected quite regularly during the last ten years but never before and especially not in relation to Natterer's bats (Eggerbauer et al., 2017b). Consequently, establishing and expanding a broad surveillance network throughout Europe may contribute to answering those and many more questions about lyssavirus epidemiology.

Comparative assessment of pathogenicity and virus shedding of different lyssavirus species

The growing variety of lyssavirus species that indeed form a genetically rather close group raises questions as to what extent they are of either similar or distinct nature in terms of pathogenicity and clinical manifestation. Moreover, the very rarely seen sustained spillovers after cross-species transmission of bat-associated lyssaviruses, evoke questions in terms of potential differences in virus shedding. To find answers to those questions the phenotypic characteristics of 13 lyssavirus isolates of phylogroup I were assessed by using virus replication in cell culture, a standardized mouse infection model and 3D high-resolution imaging. Thereby, the focus was put on comparing bat-related virus species with classical RABV strains. The analysis found no significant differences between RABV and other bat-associated lyssaviruses in the survival of mice, however, there was a striking difference in virus shedding, which was limited in mice inoculated with bat-related strains.

At present, pathogenicity studies in reservoir hosts and in animal models still play an essential role in characterizing individual viruses and understanding their virus-host interactions. Under ideal circumstances animal experimental studies were conducted in bats as the primary lyssavirus reservoir hosts. However, this has proved to be particularly challenging due to their demanding housing conditions, difficult handling and partly strict protection status (Banyard et al., 2020). The mouse infection model offers an alternative and is considered a standard in studying lyssavirus pathogenicity. Furthermore, it is an appropriate representative model for spillover hosts (Fooks and Jackson, 2020). Therefore, in this study, a mouse infection model initially established for comparative analyses of pathogenicity factors in different EBLV-1 strains (Eggerbauer et al., 2017a) was chosen and optimized in such a manner that saliva-associated virus shedding was additionally investigated. Previous studies on the pathogenicity of non-RABV bat-related lyssaviruses so far mainly focused on comparing either single isolates within one species (Markotter et al., 2009a; Eggerbauer et al., 2017a) or included only a restricted number of different virus species, commonly concentrating on European Lyssaviruses (Hicks et al., 2009; Healy et al., 2013; Kgaladi et al., 2013; Banyard et al., 2014b). A resulting variety in experimental conditions across all studies prevents from a reliable and systematic comparison. Here, these limitations were overcome by analyzing all available phylogroup I lyssaviruses under the exact same experimental conditions, including terrestrial and bat-associated RABVs as reference strains.

Incubation periods and survival varied within and between distinct lyssavirus species but there was no evidence found that neither longer incubation periods nor a smaller number of surviving individuals was exclusively related to non-RABV bat-associated viruses as opposed to RABVs. Concerning this matter, expectations towards a reduced pathogenicity of bat-related lyssaviruses had been raised beforehand due to observations made in epidemiological bat models (George et al., 2011; Blackwood et al., 2013), and supporting case studies (Moore and Raymond, 1970; Pajamo et al., 2008). The complex variation in pathogenicity factors was a challenge for the desirable systematic comparability the experiment aimed at. Therefore, the gained clinical parameters from the standardized in-vivo model were used to develop a novel matrix in order to classify different lyssavirus isolates in regard to their pathogenicity. Focusing on intramuscularly inoculated animals, those calculations were inspired by other indices already existing for Newcastle disease virus (NDV) or Avian influenza virus (AIV), but were slightly modified to fit the rabies virus induced disease progression. Differently to NDV

or AIV, the here established intramuscular pathogenicity index (IMPI) does not assign different isolates with respect to their potential to cause disease in animals and thus eventually induces veterinary control measures. The IMPI should be much more perceived as a tool for ranking an individual lyssavirus isolate according to its pathogenic potential allowing a holistic perspective, with the overall aim to classify each newly acquired isolate in the exact same procedure. Thus, part of this thesis demonstrated a hitherto unprecedented structural and systematic assessment of the high diversity of phylogroup I lyssaviruses. However, it should be considered that careful interpretation is necessary when transferring results that have been obtained through testing pathogenicity of a single isolate to the complete virus species, as it might be deceptive (Eggerbauer et al., 2017a). It is somehow surprising that, among those isolates with the highest intramuscular pathogenicity index non-RABV bat lyssaviruses such as IRKV, BBLV or ARAV lead the ranking and hence question previous suggestions that bat-related lyssaviruses are less pathogenic (Freuling et al., 2009a). Although, not directly relatable or justifiable with those observations, the fact that conventional RABV-based vaccines provide only partial protection against these viruses (Hanlon et al., 2005; Liu et al., 2013) further emphasizes the higher risk for a lethal outcome associated to particular phylogroup I virus infections. This should be considered, when assessing risks for working with those isolates. Interestingly, survival of mice post infection, incubation periods as well as high or low pathogenicity indices partly correlate with replication kinetics of the particular viruses in-vitro.

Since differentiation in CNS-resident astrocyte infection between RABV field strains and laboratory-adapted fixed RABV strains has already been shown and was suggested to potentially influence pathogenicity (Potratz et al., 2020a), here, high-resolution imaging of a selected panel of brain samples from bat-related lyssaviruses was conducted to investigate whether there might be similar findings that offer a possible explanation for the detected differences in pathogenicity between other lyssaviruses. Interestingly, the few selected lyssaviruses studied (IRKV, BBLV, DUVV and RABV-Vampbat) seemed to have different abilities to infect astroglia. A significantly increased percentage of infected astrocytes in IRKV-inoculated mice could be demonstrated, while only very few infected cells were identified in the DUVV-inoculated mice, implying a probable correlation with respect to the established IMPI. Immune pathogenicity and further spread of virus towards the periphery might be affected and also explain why, in this analysis, no shedding was reported for IRKV. However, in order to verify these observations, a higher number of isolates and strains would need to

be investigated as for this study only four different isolates were compared for a first overview.

For all mice that succumbed to the infection during the experiment, the development of clinical signs suggestive of rabies was observed, albeit at different scale. As already reported from a previous study (Eggerbauer et al., 2017a) apart from a deterioration of the animals' general health condition, paralysis or paresis as well as spasms and convulsive seizures were predominantly recorded during the observation period. Notably, mice infected intramuscularly with non-RABV bat lyssaviruses seemed to be more likely to develop spasms and paralysis compared to mice infected with classical and bat-related RABVs. For reasons of animal welfare, more detailed investigation concerning disease progression and clinical picture was challenging as, on the one hand,the gradual evolvement of further clinical signs might have been missed due to timely euthanization when reaching the humane endpoint score. On the other hand, determining the clinical score was solely based on subjective and temporarily limited observation as well as measuring body weight without conducting a complete general or neurological examination. Thus, potential patterns concerning the appearance of clinical signs in regard to particular virus species should be interpreted with caution and require further investigation, although lyssavirus species-dependent clinical signs have already been reported before (Healy et al., 2013).

When shedding was evaluated, a striking discrepancy between RABV and other bat lyssaviruses became apparent. For mice infected with RABV-related isolates, shedding was significantly increased compared to other lyssaviruses. As shedding is assumed a key factor for the likelihood of onward transmission to other mammalian species, these findings may offer a possible explanation why cross-species transmission followed by a sustained spillover is observed more often in the field for classical RABV compared to other lyssavirus species (Marston et al., 2018). In contrast, bat-related lyssavirus spillovers to conspecifics or non-flying mammals occur very rarely and if so, they are typically associated with bat-borne RABVs. However, mostly they seem to result in a dead end host and perpetuation for non-RABV lyssavirus species has not yet been observed at all (Johnson et al., 2010). Interestingly, active shedding, which is here defined as the successful virus isolation from salivary glands and the respective oral swab sample, was only observed for the classical terrestrial RABV variant derived from a dog and for one strain isolated from an insectivorous big brown bat (*Eptesicus*

fuscus). Those experimentally gained results might provide evidence and explanation for observations made in an RABV outbreak in skunks in North America that was believed to be related to *E. fuscus (Leslie et al., 2006). A*gain, this highlights the common knowledge about numerous transmissions followed by adaptions of classical rabies to carnivores. Thus, it somehow seems puzzling that there is nevertheless variation within RABV variants as no evidence for active shedding was observed for other RABVs, at least within this animal experiment. Though, mice infected with all other bat-associated lyssaviruses included in this study, showed significantly decreased to no virus shedding. However, it has to be mentioned that discrepancies in virus shedding might appear due to different factors e.g., intermittent shedding, extremely short disease duration, desquamated infected neurons on oral swab samples or a yet unknown blockage of virus distribution in salivary glands. On the one hand, this may lead to a bias in either direction. On the other, it may simultaneously reflect the natural circumstances of the shedding event. In any case, those results reported here contribute to an overall risk assessment for particularly non-RABV bat-related lyssaviruses by mimicking a probable transmission from a spillover host to another conspecific as the first step towards a sustained spillover (Mollentze et al., 2014).

In summary, the potential of non-RABV lyssaviruses to spread beyond the initial spillover-host is estimated to be very low, thus explaining the absence of onward transmission and adaption to new host reservoirs. For the purpose of a pathogenicity-based risk assessment, each isolate should be considered individually since no tendency towards a generally reduced pathogenicity of bat-associated lyssaviruses as opposed to classical RABV was confirmed in this thesis.

VI. SUMMARY

Rabies is a fatal neglected zoonosis caused by representatives of the genus lyssaviruses which are usually transmitted via infectious saliva from diseased animals and can be associated with an estimated number of 59.000 human deaths annually. In the great majority of cases, rabies infection can be attributed to dog bites with its main burden lying on developing countries in Africa and Asia. There the absence of adequate transportation systems, suitable laboratory facilities and trained staff hamper rabies surveillance as well as disease detection and hence, disease control. Under these conditions, rapid and low-cost immunochromatographic assays, i.e. lateral flow devices (LFDs) offer the possibility for user-friendly point-of-care diagnosis to increase surveillance and improve control and prevention efforts. In fact, there is a growing but intransparent landscape of commercially available LFDs, which lack appropriate validation, and previous study results indicate deficient test reliability. Therefore, a part of this thesis addresses the comparative evaluation of the diagnostic performance of five different, currently available, LFDs in comparison to conventional diagnostics as the fluorescent antibody test (FAT) and real time RT-PCR. A comprehensively broad panel of samples was analyzed within a multi-centered approach with different participating international reference laboratories. Overall, the determined test sensitivities ranged from 0 % (Span-Biotech) up to 62 % (Anigen/Bionote). However, the Anigen/Bionote test kit demonstrated a significantly better performance, although as well not yet satisfactory and with substantial variation between different laboratories. Thereby, samples with high antigen content and high relative viral load tended to test positive more often. Despite the enormous need for those tests and their already existing commercial availability, at present, none of the tested kits are suitable for in-field use and securing an authorized central quality control is strongly advised.

Apart from dogs as the main terrestrial reservoir species for the prototypical lyssavirus RABV, various bat species from all over the world host a growing variety of other lyssaviruses that are also being capable of causing rabies. Specialized bat rabies surveillance programs are conducted to constantly assess the potential public health threat that is triggered by sporadic spillover infections to other mammals and humans. Thus, collecting information on distribution and ecology of these viruses in their chiropteran hosts is of continuous importance, albeit challenging due to their strictly protective status in Europe. Therefore, a second part of this thesis reports on surveillance activities in more than 1.200 dead found bats

from Germany between 2018 and 2020. Using a novel minimal invasive technique for sampling followed by molecular detection, 16 positive bat brains were identified as well as another four additional positive cases from an archived sample set. For the majority of cases viable virus was successfully isolated and next generation sequencing generated full or partial genomes of almost all isolates. Both PCR and sequence analyses revealed that all of them were identified as European bat lyssavirus 1 (EBLV-1) except one case, which was identified as Bokeloh bat lyssavirus (BBLV) isolated from a *Myotis nattereri* found in Lower Saxony. This finding presents the tenth case of this novel lyssavirus in European bats. Apart from two EBLV-1-positive cases that were isolated from *Pipistrellus pipistrellus* from Baden-Württemberg, all others were assigned to the known reservoir *Eptesicus serotinus*.

Not only in Europe but also in many other parts of the world the extensive development of bat-related rabies surveillance strategies, lead to an increased detection of novel lyssavirus species particularly over the last decade. However, in comparison to terrestrial rabies that had been known since ancient times, bat-associated lyssaviruses have been far less in the focus of rabies research. Thus, many aspects in respect to virus-host-interaction, transmission and maintenance have not yet been sufficiently investigated. It is still not clearly evident, if at all, to what extent, distinct lyssavirus species vary in their pathogenic potential. To expand that knowledge, a third part of this thesis comparatively investigated several aspects concerning pathogenicity and saliva-associated virus shedding of other bat-borne phylogroup I lyssaviruses compared to terrestrial and bat associated rabies virus variants in a standardized mouse infection model. Using 13 different isolates from ten different virus species, the achieved results suggest a high diversity in lyssavirus pathogenicity with no significant difference between classical rabies virus (RABV) and other bat lyssaviruses observed in the survival of mice. However, there was a striking tendency towards decreased virus shedding of other bat lyssaviruses. As virus shedding is assumed to be a key factor for the likelihood of onward transmission to other mammalian species, these findings may offer a possible explanation as to why cross-species transmission is more often seen with classical and bat-related rabies compared to other lyssavirus species. In order to classify lyssaviruses regarding their pathogenicity, this thesis proposes a novel matrix based on parameters such as incubation time, disease duration and survival. Within that newly established pathogenicity index bat-related lyssaviruses such as Irkut virus (IRKV) or BBLV remarkably obtained higher scores than different RABV isolates, questioning the suggestion of a generally reduced

pathogenicity of bat-associated viruses as opposed to RABV. Interestingly, high resolution imaging could show that astrocyte infection was increased in a virus with the highest pathogenicity index, thus supporting the role of astrocyte infection in the pathogenicity of lyssaviruses, as already suggested in previous studies.

VII. ZUSAMMENFASSUNG

Die fast immer tödlich verlaufende Tollwut wird als sogenannte vernachlässigte (neglected) Zoonose eingestuft. Hervorgerufen wird Tollwut durch Vertreter der Gattung Lyssaviren, welche für gewöhnlich durch infektiösen Speichel erkrankter Tiere auf den Menschen übertragen werden. Jährlich versterben schätzungsweise 59.000 Menschen an Tollwut und die überwiegende Mehrzahl der Tollwutinfektionen steht in Zusammenhang mit einem Hundebiss, wovon insbesondere Kinder in Entwicklungsländern Afrikas und Asiens betroffen sind. Meist herrscht in diesen Ländern ein Mangel an geschultem Personal, Transportmöglichkeiten und adäquater Laboreinrichtung, was die Erkennung, Kontrolle und Bekämpfung der Tollwut stark beeinträchtigt. Unter den vor Ort herrschenden Bedingungen kann die Tollwutüberwachung durch sogenannte Lateral flow devices (LFDs), die eine schnelle und günstige Tollwutdiagnostik ermöglichen, unterstützt werden. Zwar ist eine wachsende Anzahl von Tollwut-LFDs kommerziell erhältlich, jedoch mangelt es an transparenten Validierungsdaten und Zulassungen. Schon vorherige Studien haben Defizite in der Zuverlässigkeit der LFDs angedeutet. Deshalb befasst sich die vorliegende Arbeit mit der vergleichenden Evaluierung fünf weiterer momentan erhältlicher LFDs. Dabei wird deren diagnostische Leistung konventionellen Methoden, wie dem Fluorescent Antibody Test (FAT) und der PCR gegenübergestellt. Hierbei wurde ein multizentrischer Validierungssansatz verfolgt, wobei auf ein umfangreiches Probenmaterial mehrerer internationaler Referenzlabore zurückgegriffen werden konnte. Insgesamt lagen die dabei festgestellten diagnostischen Sensivitäten zwischen 0 % (Span Biotech) und 62 % (Anigen/Bionote). Bei letzterem haben sich auch große Unterschiede zwischen den Ergebnissen der einzelnen Labore ergeben. Es wurde zudem festgestellt, dass die Senistivität positiv mit einem hohen Antigengehalt und einer großen Viruslast korreliert. Zusammenfassend lässt sich sagen, dass keines der hier untersuchten Testkits für den Einsatz unter Feldbedingungen geeignet erscheint. Vor dem Hintergrund des großen Bedarfs nach solchen Tests und den strengen Anforderungen hinsichtlich Spezifität und Sensitivität ist die Einführung einer übergeordneten Qualitätskontrolle beziehungsweise eines Zulassungsverfahrens wie in Deutschland empfehlenswert.

Fledertiere (*Chiroptera*) stellen das Reservoir für eine wachsende Vielfalt von Lyssaviren dar, die alle Tollwut hervorrufen können. Zur Überwachung der Fledermaustollwut wurde ein

spezielles Monitoring eingerichtet, um somit potentielle Gefahren für die öffentliche Gesundheit kontinuierlich erkennen und abschätzen zu können. Dafür ist es notwendig, Information zur Verbreitung sowie zur Ökologie der Viren in Bezug auf ihre Wirte zu sammeln und auszuwerten. Dabei stellt es eine besondere Herausforderung dar, dass alle in Deutschland vorkommenden Fledermäuse unter Naturschutz stehen. Der zweite Teil der vorliegenden Arbeit widmet sich daher der Untersuchung und Auswertung der zwischen 2018 und 2020 im Rahmen der erweiterten passiven Überwachung der Tollwut bei Fledermäusen gesammelten Fledermaus-Totfunde. Mittles eines neu angewandten minimal-invasiven Probenentnahmeverfahrens und molekulardiagnostischer Methoden wurden mehr als 1.200 Gehirnproben gescreent und dabei 16 positive Tiere und zusätzlich vier weitere in Archivproben einer vorangegangenen Studie entdeckt. Zumeist konnte infektiöses Virus isoliert und mittels der Next Generation Sequencing-Technologie vollständige oder partielle Genomsequenzen generiert werden. Sowohl real-time PCR als auch phylogenetische Analysen zeigten, dass bis auf einen einzigen Fall alle anderen isolierten Viren dem Europäischen-Lyssavirus Typ 1 (EBLV-1) zugeordnet werden können. Dabei wurde das Virus in zwei *Pipistrellus pipistrellus* aus Baden-Württemberg nachgewiesen und in allen anderen Fällen aus dem bekannten Reservoir *Eptesicus serotinus*. Darüber hinaus wurde ein weiteres Bokeloh-Fledermaus-Lyssavirus (BBLV) bei einer in Niedersachsen gefundenen *Myotis nattereri* nachgewiesen. Dabei handelt es sich um den zehnten Nachweis dieser Virusspezies in Europa

Sowohl in Europa als auch in vielen anderen Teilen der Welt hat die umfangreiche Weiterentwicklung in der Überwachung der Fledermaustollwut über das letzte Jahrzehnt hinweg zur Entdeckung vieler weiterer neuartiger Lyssaviren geführt. Dennoch standen die fledermausadaptierten Lyssaviren bisher weniger im Fokus der Wissenschaft als die bereits seit der Antike bekannte klassische Tollwut. Somit sind viele Themenbereiche, wie die Virus-Wirt-Interaktion, die Übertragung und Verbreitung der fledermausadaptierten Lyssaviren bis heute nicht hinreichend erforscht. Des Weiteren ist auch noch nicht abschließend geklärt, in welchem Umfang sich verschiedene Lyssavirusspezies in ihrer Pathogenität unterscheiden. Der dritte Teil der vorliegenden Arbeit vergleicht diesbezüglich verschiedene Aspekte und untersucht außerdem, ob fledermausübertragene Lyssaviren im standardisierten Mausmodel seltener über den Speichel ausgeschieden werden als klassische Tollwutviren. Es wurden 13 verschiedenen Isolate, welche zehn unterschiedliche Virusspezies umfassen, untersucht. Dabei konnte kein signifikanter Unterschied in Bezug auf die Pathogenität von klassischen

Rabies Virus Isolaten (RABVs) im Vergleich zu fledermausadaptierten Lyssaviren festgestellt werden, wohl aber eine hohe Diversität bezüglich der einzelnen Isolate untereinander. Interessanterweise zeigen die Daten des Versuches, dass nur RABV-assoziierte Isolate im Speichel infizierter Mäuse ausgeschieden wurden. Für fledermausadaptierte Lyssaviren hingegen konnte keine vollständige Virusausscheidung nachgewiesen werden. Diese Ergebnisse könnten eine mögliche Erklärung dafür liefern, weshalb bei diesen Lyssaviren nur vereinzelte Spillover-Infektionen nachgewiesen wurden und keine Weiterverbreitung in anderen Spezies beobachtet werden konnte. Somit wird die These unterstützt, dass RABV mit der Fähigkeit, die Speziesbarriere zu durchbrechen, eine Ausnahme darstellt. Im Rahmen dieser Arbeit wurde weiterhin eine neue Pathogenitätsmatrix entwickelt, welche unterschiedliche Parameter, wie Inkubationszeit, Krankheitsdauer sowie die Überlebensrate berücksichtigt. Das Ergebnis zweifelt die bisherige Annahme, dass fledermausassoziierte Lyssaviren weniger pathogen sind als klassische Tollwutviren, stark an. Die im Vergleich zu verschiedenen RABV Isolaten höheren Indices von zum Beispiel Irkut Virus (IRKV) oder BBLV verdeutlichen dies. Durch die Anwendung eines modernen 3D-Imaging Verfahrens konnte außerdem gezeigt werden, dass fledermausadaptierte Lyssaviren in der Lage sind, neben Neuronen auch Astrozyten zu infizieren. Dabei wurde deutlich, dass Isolate mit einem höheren Pathogenitätsindex mehr Astrozyten infizieren als solche mit einem niedrigen Pathogenitätsindex, was die Theorie unterstreicht, dass der Zelltropismus eine wichtige Pathogenitätsdeterminante darstellt.

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IX. APPENDIX

1. Abbreviations

ABLV	Australian bat lyssavirus
ARAV	Aravan virus
AIV	Avian influenza virus
BBLV	Bokeloh bat lyssavirus
CNS	Central nervous system
CFS	Cerebrospinal fluid
dRIT	Direct rapid immunohistochemical test
DUVV	Duvenhage virus
EBLV-1	European bat lyssavirus 1
EBLV-2	European bat lyssavirus 2
FAO	Food and Agriculture Organization of the United Nations
FAT	Fluorescent antibody test
FITC	Fluorophore conjugated
FLI	Friedrich-Loeffler-Institut
G	Glycoprotein
GARC	Global Alliance for Rabies Control
GBLV	Gannoruwa bat lyssavirus
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
IKOV	Ikoma virus
IMPI	Intramuscular pathogenicity index
IRKV	Irkut virus
KBLV	Kotalahti bat lyssavirus
KHUV	Khujand virus
LBV	Lagos bat virus
L	Large RNA polymerase
LFD	Lateral flow device
LLBV	Lleida bat lyssavirus

M	Matrix protein
MBLV	Matlo bat lyssavirus
mGluR2	Metabotropic glutamate receptor 2
MIT	Mouse inoculation test
MOKV	Mokola virus
mRNA	Messenger RNA
N	Nucleoprotein
nAchR	Nicotinic acetylcholine receptor
NASBA	Nucleic acid sequence based amplification
NCAM	Neuronal cell adhesion molecule
NDV	Newcastle disease virus
nm	Nanometer
OIE	World Organisation for Animal Health
P	Phosphoprotein
p75NTR	p75 neurotrophin receptor
PBS	Phosphate-buffered saline
PEP	Post-exposure prophylaxis
PrEP	Pre-exposure prophylaxis
RABV	Rabis virus
rER	Rough endoplasmic reticulum
RIDT	Rapid immunodiagnostic test
RIG	Rabies immunoglobulin
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RTCIT	Rabies tissue culture infection test
RT-LAMP	Reverse transcription loop mediated isothermal amplification
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Quantitative real time RT-PCR
SHIBV	Shimoni bat virus
ss	Single-stranded
TWBL	Taiwan bat lyssavirus
UAR	United Against Rabies

VNA	Virus-neutralizing antibody
WCBV	West Caucasian bat virus
WHO	World Health Organization

2. Figures

3. Tables

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