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Exemplary investigations of squirrels and shrews as relevant virus reservoir hosts

von

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Contents

Chapter 1: Introduction

1 Introduction

Rodentia is the most numerous and most diversified order of mammals, representing about 43 % of the total number of mammalian species. They are distributed almost worldwide and inhabit a broad range of habitats including forest, desert, agricultural land, and urbanized regions (Wilson, Lacher Jr, and Mittermeier 2016).

The order Eulipotyphla, includes more than 400 species, comprising hedgehogs and gymnures (family Erinaceidae), solenodons (family Solenodontidae), the desmans, moles, and shrew-like moles (family Talpidae) and true shrews (family Soricidae), of which the most feed almost exclusively on insects. Equally to rodents they are globally distributed and inhabit all continents, except Antarctica (Wilson and Mittermeier 2018).

Due to their close proximity to humans, rodents and insectivores, i.e. shrews, often function as reservoir hosts of numerous zoonotic viruses. They serve as a connection between humans, domestic animals, wildlife and e.g. arthropod vectors (Meerburg, Singleton, and Kijlstra 2009). They can spread viruses either between the respective reservoir hosts or to accidental dead-end hosts.

Various molecular methods and workflows have been developed for the identification of novel viruses or virus variants, directly without prior agent cultivation or after virus isolation. The choice of the optimal method depends on the aim of the study, the agents to be detected and their genome organization. Common methods are conventional polymerase chain reactions (PCRs) in different formats (e.g. generic or nested format), quantitative reverse transcription polymerase chain reaction (RT-qPCR) and High-Throughput sequencing (HTS), which can be applied separately or in combination, e.g. in a sophisticated workflow.

Even if the implementation of new technologies, like HTS and optimized workflows in combination with classical PCR methods resulted in numerous discoveries of novel viruses in the recent past (Supplement, Table S1), still only a small portion of the whole number of different viruses (so-called ´virome´) in rodents is known (Drewes et al. 2017; Wu et al. 2018). In congruence to this, knowledge about pathogens in squirrels and shrews is also scarce.

Therefore, the studies described in this thesis present the characterization of reservoir hosts, exemplary in squirrels and shrews. In addition, results from selected approaches for the search and detection of novel viruses in these reservoir hosts are reported. In conclusion, this work contributes to the expansion of the important

knowledge on viruses in rodents and shrews and their function as relevant reservoir hosts.

In the literature review, general knowledge about reservoir hosts, the current knowledge on the virus diversity in rodents, workflows used for virus identification, and orthoborna-, polyoma-, and herpesviruses will be reviewed in more detail.

Chapter 2: Literature review

2 Literature review

2.1. Rodents and other small mammals and their role as reservoirs

Rodentia is the most numerous mammalian taxon. Most rodents are small animals with compact bodies, short extremities and long tails, and display a great variability in their body weight dimensions. While the capybara (*Hydrochoerus hydrochaeris*) is the largest living rodent in the world and can weigh up to 79 kg, the great majority of rodents weigh less than 100 g (Wilson, Lacher Jr, and Mittermeier 2016).

Rodents developed different adaptations and survival strategies through historical evolutionary processes, which led to their numerous and pervasive presence. These strategies include e.g. physical adaptations concerning their tooth morphology, and extremely high reproduction rates. As an example, in case of the house mouse (*Mus musculus*) a single female produces between 5-10 litters annually, of which each consist of 5-6 offspring. The latter are in turn able to reproduce at approximately 30 days of age. This exceptional enormous breeding capabilities enable a high adaptivity to changing environmental conditions (Wilson, Lacher Jr, and Mittermeier 2016).

The morphology of most species belonging to the order Eulipotyphla is characterized by a rather small body size, pointy noses, sharp teeth and relatively small eyes. Shrews are small-bodied mammals that have particularly high metabolic rates that cause them to be constantly active throughout day and night and requires continues food intake. Moles are insectivores which are specialized for digging and spend the majority of their time underground in extensive burrow systems. Hedgehogs and gymnures are closely related and belong to the family Erinaceidae. These small to moderately sized mammals are native to Africa, Eurasia and South East Asia. Only two species of solenodons remain today and these live on the island of Hispaniola (*Solenodon paradoxus*) and in Cuba (*Solenodon cubanus*). They have ancient lineages and are poisonous, nocturnal, burrowing, insectivorous mammals, which have larger bodies than moles and shrews. Their body size reaches around 30 cm and they weigh between 0.7 and 1.0 kg (Wilson and Mittermeier 2018).

There exist different and controversially discussed definitions of the term ‘reservoir’ but most definitions share the following characteristics, which will also be applied for the actual studies: i) reservoir hosts are chronically infested with the agent, ii) they harbour and maintain it, iii) they are able to transmit it to other individuals, but iv) they do not show any clinical signs (Haydon et al. 2002). Viruses are often highly specific

concerning their hosts and target cells within the host that they infect (Reece et al. 2011). However, in addition to the reservoir hosts some viruses infect also accidental (spill-over) hosts. The latter are normally not able to further transmit the virus in the context of its natural replication cycle and in the majority of cases the accidental hosts develop clinical signs and might even die due to the infection (Blood and Studdert 1998).

Besides bats, which are well studied reservoir hosts for many pathogens, rodents and insectivores, i.e. shrews, also play an important role as reservoir hosts for a variety of zoonotic and non-zoonotic viruses. Possible routes for virus transmission are either direct contacts like biting or scratching or indirect contact through infected faeces, urine or saliva (e.g. hantaviruses), or through the alimentary route by contaminated water or food products. Common examples for rodents as reservoir hosts include e.g. different orthohantaviruses, such as Dobrava-Belgrade virus (Klempa et al. 2003), Tula virus (Plyusnin et al. 1994) and Seoul virus (Lee et al. 1980), and Cowpox virus (Hoffmann, Franke, et al. 2015) (Table 1).

Of course there are also rodent-associated viruses that are considered to be non-zoonotic, like e.g. the squirrel adenovirus 1 (SqAdV-1) (Abendroth et al. 2017) and different rodent polyomaviruses, such as the bank vole polyomavirus (Nainys et al. 2015). Rodents and shrews can also be reservoirs for vector-borne pathogens, like e.g. the tick-borne encephalitis virus (Gritsun, Lashkevich, and Gould 2003) (Table 1). In this scenario the animals carry the pathogen and serve as potential virus source for arthropods with vector function that feed on them, but they do not play a direct role in transmission to humans.

New technologies contribute to increase the number of known reservoir hosts, e.g. Han et al. used machine learning for processing data about biology, ecology, and life history traits of selected rodents. These data can be used for the prediction of potential novel reservoir hosts of zoonotic diseases (Han et al. 2015).

Living in close surrounding of humans in general strengthens the idea of small mammals as present infection source and therefore potential risk for humans, domestic animals and wildlife (Meerburg, Singleton, and Kijlstra 2009).

Table 1: Overview about confirmed reservoir species for selected viruses.

Genome organization	Virus family	Virus species	Reservoir species	Host family	Reference
dsDNA[a] (linear)	Poxviridae	Cowpox virus	*Microtus arvalis, Myodes glareolus*	Cricetidae	(Hoffmann, Franke, et al. 2015; Weber et al. 2020)
+ssRNA[b] (non-segmented)	Hepe-viridae	Rat hepatitis E virus	*Rattus norvegicus, R. rattus*	Muridae	(Ryll et al. 2017)
	Flavi-viridae	bank vole hepacivirus	*Myodes glareolus*	Cricetidae	(Drexler et al. 2013)
		Tick-borne encephalitis virus (TBEV)	*Myodes glareolus*	Cricetidae	(Lopez et al. 1996)
-ssRNA[c] (non-segmented)	Borna-viridae	BoDV-1	*Crocidura leucodon*	Soricidae	(Bourg et al. 2013; Hilbe et al. 2006; Nobach et al. 2015)
-ssRNA (segmented)	Hanta-viridae	Puumala virus	*Myodes glareolus*	Cricetidae	(Brummer-Korvenkontio et al. 1980)
		Tula virus	*Microtus arvalis*	Cricetidae	(Plyusnin et al. 1994)
		Seoul virus	*Rattus norvegicus*	Muridae	(Lee et al. 1980)
		Dobrava-Belgrade virus, genotyp Dobrava	*Apodemus flavicollis*	Muridae	(Avsic-Zupanc et al. 1992)
		Dobrava-Belgrade virus, genotyp Kurkino	*Apodemus agrarius*	Muridae	(Klempa et al. 2004)
		Dobrava-Belgrade virus, genotyp Saaremaa	*Apodemus agrarius*	Muridae	(Nemirov et al. 1999)
		Dobrava-Belgrade virus, genotyp Sochi	*Apodemus ponticus*	Muridae	(Klempa et al. 2008)
		Hantaan virus	*Apodemus agrarius*	Muridae	(Lee, Lee, and Johnson 1978)

[a] double-stranded deoxyribonucleic acid

[b] single-stranded ribonucleic acid of positive polarity

[c] single-stranded ribonucleic acid of negative polarity

2.2. Current knowledge on the virus diversity in rodents and other small mammals

Virus diversity, concerning the number of viruses and their genetic variability, is extremely large (Anthony et al. 2013). Research efforts are therefore ongoing to analyse virus diversity in more detail, especially in their natural reservoir hosts, and novel virus-reservoir-relationships are discovered by using novel detection technologies (Han et al. 2015).

In this regard, Anthony et al. used the Indian Flying Fox (*Pteropus giganteus*) to estimate the diversity of viruses in this species and predict the possible viral diversity in mammals. According to the author's speculative results, it is extrapolated that in mammalian species a minimum of around 320,000 new viruses is waiting for discovery. In addition according to Anthony and colleagues the following limitations need to be considered concerning this assumption: it is assumed that all 5,486 described mammalian species harbour an average of 58 viruses belonging to the nine families of interest (as estimated in *P. giganteus*) (Anthony et al. 2013). Furthermore, it is hypothesized that all these viruses exhibit 100 % host specificity (Anthony et al. 2013). As these postulations cannot be generalized for all virus species, the number of unknown viruses is probably even much higher.

Modern molecular methods, especially the availability of HTS-instruments and sequencing services in the last decade, allowed a rapid increase in the detection rate of novel viruses (Supplement, Table S1). This makes not only an important contribution to an increased scientific knowledge, but is also important for the identification of disease aetiologies and implementation of control measures.

2.3. Workflows for the identification and genetic characterization of viruses

2.3.1. Initiation

Initiation for such a workflow can be the occurrence of a disease of unknown aetiology, where the aim is to determine the potential aetiology of the disease. Furthermore, metagenomic 'open-view' or pan/generic PCR/reverse transcription PCR (RT-PCR) screenings of a large set of samples are used for the search for novel pathogens.

A HTS-based attempt was followed e.g. in case of the detection of a SqAdV-1 in Eurasian red squirrels, *Sciurus vulgaris*, after death of an adult squirrel with enteritis in 2013 (Abendroth et al. 2017; Wernike et al. 2018) and a novel respirovirus in a Sri

Lankan Giant squirrel (*Ratufa macroura*). The latter caused most likely the death of a squirrel, that showed typical lesions associated with pneumonia (Forth et al. 2018).
The second approach, testing with 'open view' methods for the possible presence of novel viruses without initial relation to a certain disease, was also applied on Norway rats (*Rattus norvegicus*) and other rodents and shrews before, like reported e.g. by Firth et al., Johne et al., Sachsenröder et al., and Wu et al. (Firth et al. 2014; Johne et al. 2019; Sachsenröder et al. 2014; Wu et al. 2018) (Supplement, Table S1). Furthermore, the generic RT-PCR approach was successfully used for the identification of novel hepato-, hepaci-, hepe- and hepadnaviruses in rodents and shrews (Drexler et al. 2015; Drexler et al. 2013; Johne et al. 2010; Rasche et al. 2019; Ryll et al. 2019).

2.3.2. General workflows

Different workflows can be applied for the detection of novel viruses. Essential steps in such workflows are the choice of appropriate samples and the nucleic acid preparation, followed by a variety of molecular methods for virus detection and finally -if possible- the complete genome determination (Figure 1).

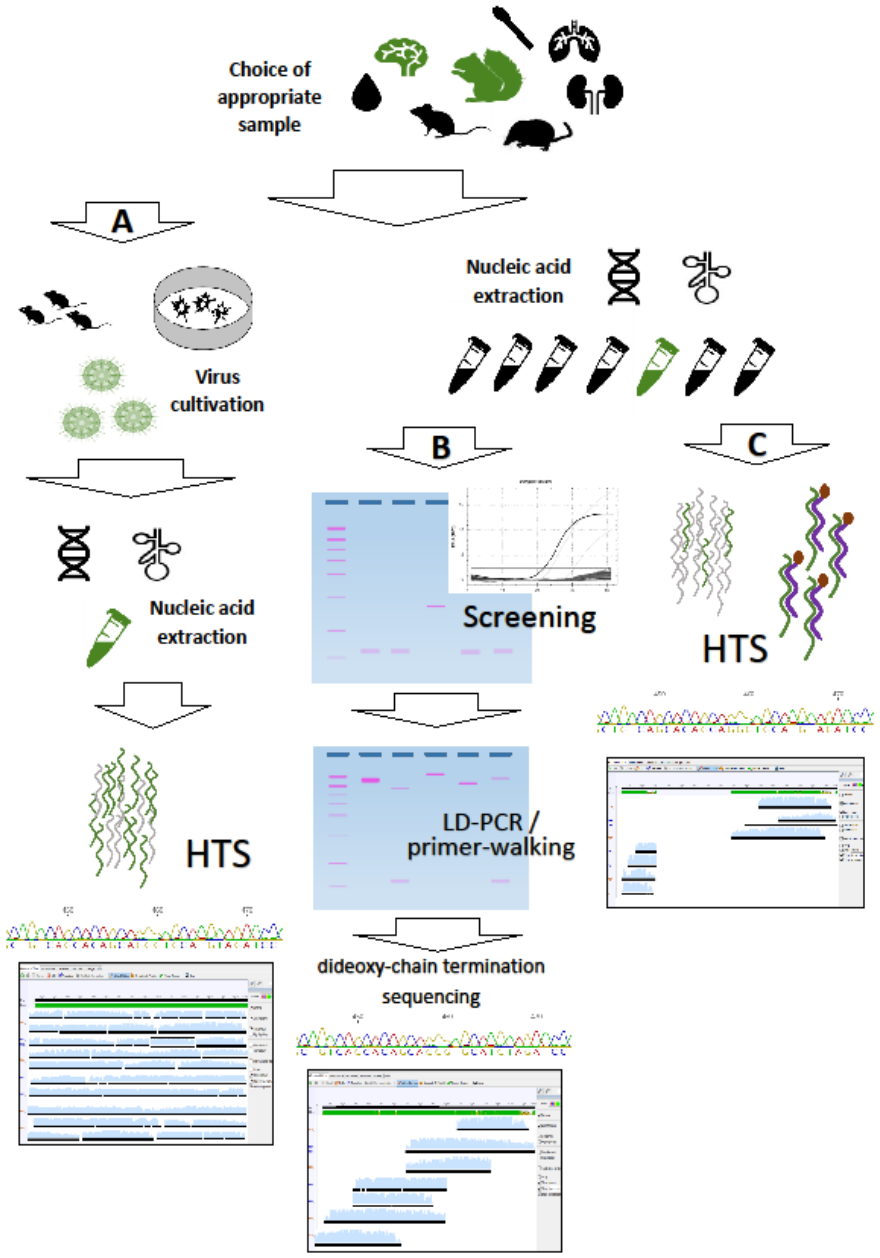


Figure 1: Schematic of exemplary workflows for virus detection. (A): virus cultivation prior to virus detection with High-Throughput Sequencing (HTS); **(B)**: classical approach including the screening with polymerase chain reactions (PCRs/RT-PCRs) in different formats, long distance-PCR (LD-PCR), primer-walking and dideoxy-chain termination sequencing; **(C)**: metagenomic HTS approach with hybrid-capture enrichment; samples potentially including a novel virus are indicated in green; source of pictures: Microsoft Powerpoint Pictogramms, http://phylopic.org, Geneious version 2019.2 (Biomatters, available from https://www.geneious.com).

2.3.3. Sampling and sample preparation

The initial and very important step of the workflows for virus detection consists of the right choice of samples. Sampling depends on the initiation of the investigation. While in case of diseased animals with unknown aetiology the focus lies on organs with pathological peculiarities, for metagenomics or broad generic PCR/RT-PCR screenings more or less all tissues can be used. Blood, due to viremia, or faeces are frequently used for the detection of different viruses. In cases where a virus detection approach is done with stool samples it needs to be verified whether the found pathogens belong really to the tested host or whether it originates from a ´cargo´-host in the intestine. E.g. evidence of a murine virus in faeces of a raptor might be due to the fact that the bird has eaten a mouse before. Non-invasive sampling, such as collection of swab samples, is a common tool for testing live animals. The outcome of the detection of a given virus is markedly determined by the selection of appropriate samples, e.g. in case of bornaviruses there is very good evidence that brain material is the most suitable sample (Hoffmann, Tappe, et al. 2015; Schlottau, Hoffmann, et al. 2017) and in case of hepatitis viruses liver is the sample of choice (Ryll et al. 2017).

The workflow continues either after virus isolation or directly without initial agent cultivation. Virus cultivation (Figure 1A) is done for virus enrichment and can be performed either *in vivo* by giving the homogenized and filtrated sample into an animal, such as a suckling mouse or *in vitro* by placing the sample onto cell cultures. The choosen cell culture of course needs to be susceptible for the virus to be detected. Common cell lines used for virus cultivation are BHK-21 cells (baby hamster kidney cells), Vero cells (simian kidney cells) or PK-15 cells (pig kidney cells) and recently also special reservoir-derived cell lines were established as optimized cell culture models for the investigation of these viruses *in vitro* (Binder et al. 2019; Eckerle, Lenk, and Ulrich 2014; Essbauer et al. 2011).

2.3.4. Nucleic acid extraction

The next important step is nucleic acid extraction. Various methods are available for this purpose, including different commercial kits and automatic extraction machines. Nucleic acid can also be extracted using a combination of commercial kits and an automatic purification system. Following extraction, the obtained ribonucleic acid (RNA)/ deoxyribonucleic acid (DNA), RNA alone or DNA alone can be used for a

variety of different molecular detection methods. These methods differ in sensitivity, specificity and price and therefore for each project the best suitable method has to be defined. Common molecular detection assays include conventional PCR, RT-PCR, RT-qPCR (Figure 1B) and HTS (Figure 1C) and will be reviewed in the following sections.

2.3.5. Molecular methods for initial detection of viruses

The molecular detection workflow for a virus infection is usually based on a pathogen-specific attempt, using pathogen-specific PCR or RT-PCR. Variants of the general PCR approach include e.g. nested PCR or quantitative PCR (qPCR) or corresponding RT-PCR-formats. For a broad detection of related viruses generic or panPCRs can be used (Figure 1B).

In order to ensure successful nucleic acid extraction and a sufficient sample quality, additional internal controls (ICs), such as the enhanced green fluorescent protein (eGFP) gene (Hoffmann et al. 2006) and/or primers and probes targeting a house-keeping gene, e.g. the beta actin gene (Toussaint et al. 2007), are added to the PCR mastermix. In addition, in each round of all applied PCRs no template controls (NTCs), in form e.g. of H_2O, are carried along to identify possible contaminations.

HTS technologies have revolutionized the possibilities for pathogen identification, especially in cases of unknown disease aetiology (Figure 1C). It is an often used method for ‘open view’-approaches in virus detection, enables rapid and deep sequencing and has the potential to detect not only viruses, but also bacteria, fungi and parasites in parallel (Barzon et al. 2013; Lipkin and Anthony 2015).

2.3.6. Determination of a complete viral genome

In addition to the metagenomic approach of HTS, this methodology is frequently used for complete genome determination (´whole-genome sequencing´= WGS). In this context HTS can be combined with a microarray (Abendroth et al. 2017) or hybrid-capture enrichment (Gaudin and Desnues 2018) (Figure 1C).

Alternatively, LD-PCR allows amplification of up to 30 kilobase pairs (kbp) and beyond (Figure 1B). For performing complete genome amplification of the novel polyomaviruses (PyVs), LD-PCR in nested format using specific primers that were derived from the sequences amplified with the initial generic PCR has been

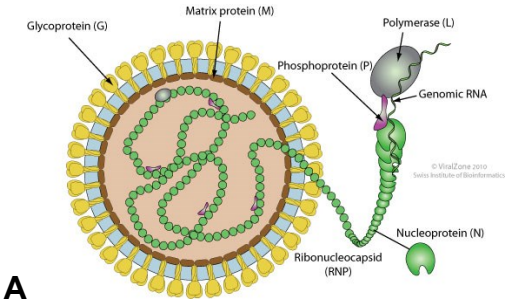
established (Leendertz et al. 2011). The produced amplification products had a size of approximately 5 kbp.

Following the LD-PCR a primer-walking strategy can be applied for sequencing complete genomes of the novel PyVs (Figure 1B).

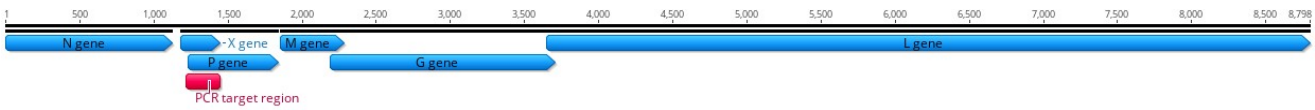
2.4. Orthobornaviruses and their reservoirs

The genus *Orthobornavirus* belongs to the order *Mononegavirales* in the family *Bornaviridae*. One differentiates between the species *Mammalian orthobornavirus 1*, including the classical borna disease virus 1 and 2 (BoDV-1 and 2), the species *Mammalian orthobornavirus 2*, represented by the variegated squirrel bornavirus 1 (VSBV-1), a snake bornavirus (*Elapid 1 orthobornavirus*) and different avian bornaviruses (e.g. *Passeriform 1 and 2 orthobornavirus, Psittaciform 1 and 2 bornavirus* and *Waterbird 1 bornavirus*) (Afonso et al. 2016; Briese et al. 1994).

The single-stranded ribonucleic acid genome has a length of about 8.9 kilobases (kb) and is of negative polarity (-ssRNA). The non-segmented genome compromises six partially overlapping open reading frames (ORFs) which encode the following proteins: N = nucleoprotein, X = X protein, P = phosphoprotein, M = matrix protein, G = glycoprotein and L = polymerase protein (Briese et al. 1994). The virions are spherically structured and have a diameter between 85-125 nm (Danner, Heubeck, and Mayr 1978) (Figure 2).



A



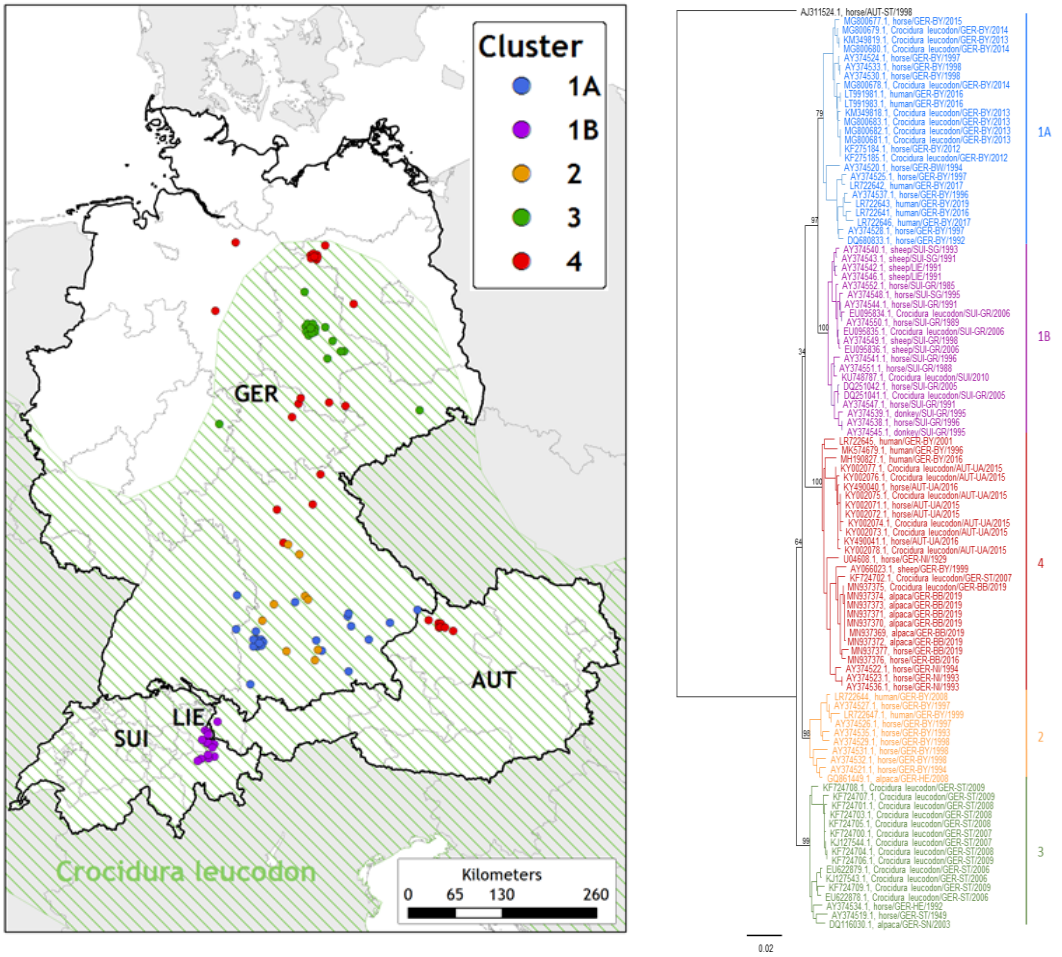
B

Figure 2: Virion structure and genome organization of orthobornaviruses. (A): Schematic view of a bornavirus virion (available from Viral Zone, Swiss Institute of

Bioinformatics), **(B)**: Schematic genome organization of an orthobornavirus; the target region of the respective BoDV-1 specific, VSBV-1 specific or panBornavirus-RT-qPCRs is located within the X (X protein) and P (phosphoprotein) gene and marked in red; the figure was prepared using Geneious version 2019.2 (Biomatters, available from https://www.geneious.com); the exemplary used bornavirus genome (VSBV-1, complete genome) is available from GenBank under accession number LN713680.

Infection with BoDV-1 causes an immune-mediated non-suppurative encephalitis and chronic progressive meningoencephalitis with neurological clinical signs such as behavioural changes, apathy and movement disorders, e.g. ataxia (Caplazi et al. 1999; Dürrwald et al. 2016; Richt and Rott 2001; Schmidt 1952). Such neurologic disease, caused by BoDV-1, is mainly known in domestic mammals, such as horses, sheep, goats and cattle but since recently also in humans (Caplazi and Ehrensperger 1998; Caplazi et al. 1999; Dürrwald et al. 2016; Korn et al. 2018; Niller et al. 2020; Richt and Rott 2001; Schlottau et al. 2018; Schmidt 1952). In addition, rare infections of New World camelids are reported (Jacobsen et al. 2010). All these species are (accidental) dead-end hosts and do therefore not contribute to spreading of the virus. The bicolored white-toothed shrew, *Crocidura leucodon*, is the only known reservoir host of BoDV-1 (Bourg et al. 2013; Hilbe et al. 2006; Nobach et al. 2015) (Figure 3) and the majority of reported BoDV-1 cases is located in regions that overlap with the natural distribution range of this species (Figure 4A). Furthermore, there is evidence for a remarkable relation between the geographical origin of samples and similarities of BoDV-1 sequences leading to characteristic cluster designations as illustrated by the repeatedly used significant colour code in the map (Figure 4A) and the corresponding phylogenetic tree (Figure 4B). This clustering happens regardless of the species, but in congruence with the geographical origin of the samples. The virus is endemic in shrew populations in parts of Southern and Central Germany, Austria, Liechtenstein and Switzerland (Bourg et al. 2013; Dürrwald et al. 2014; Hilbe et al. 2006; Weissenböck et al. 2017). Assays for detection of orthobornaviruses comprise a broadrange orthobornavirus RT-qPCR detecting different known orthobornaviruses (Schlottau et al. 2018), a VSBV-1-specific and a BoDV-1-specific RT-qPCR, targeting the X and P genes (Hoffmann, Tappe, et al. 2015; Schlottau et al. 2018) (Figure 2B).

Figure 3: Photo of the bicolored white-toothed shrew, *Crocidura leucodon*. Species confirmed with Cytochrom *b* gene analysis, sample ID: KS19/446; photo taken by Vanessa Schulze.



A **B**

Figure 4: Geographic distribution of confirmed BoDV-1 infections compared to the distribution range of *Crocidura leucodon* and corresponding phylogenetic tree.

(A): Confirmed BoDV-1 infections (represented with circles), are shown in combination with the distribution range of the bicolored white-toothed shrew, *Crocidura leucodon* (represented in green shading, data available from IUCN Red List); the map was designed using ArcGIS Desktop 10.5.1 (ESRI, Redlands, CA, USA); **(B)**: phylogenetic analysis of partial BoDV-1 sequences from endemic regions; the tree building was performed using Neighbor-Joining algorithm and Jukes-Cantor distance model in Geneious version 2019.2 (Biomatters, available from https://www.geneious.com); the tree was rooted using sequence BoDV-2 No/98 (AJ311524); the sequences are denoted by GenBank accession number, Latin taxonomic names or common names of their hosts, country and federal state of origin of the sample and year of infection; GER = Germany, SUI = Switzerland, LIE = Liechtenstein, AUT = Austria; BoDV-1 data were available from Briese et al. 1994,

Vahlenkamp et al. 2002, Kolodziejek et al. 2005, Kolodziejek et al. 2006, Dürrwald et al. 2006, Hilbe et al. 2006, Dürrwald et al. 2007, Puorger et al. 2010, Dürrwald et al. 2014, Rubbenstroth et al. 2016, Weissenböck et al. 2017, Korn et al. 2018, Schlottau et al. 2018, Niller et al. 2020, and archived GenBank sequences; colours indicate regional BoDV-1 sequence clusters; colour code: blue = Cluster 1A, violet = Cluster 1B, orange = Cluster 2, green = Cluster 3, red = Cluster 4.

In this context, it is important to mention that five years ago, a novel zoonotic bornavirus, the variegated squirrel bornavirus 1 (VSBV-1), was discovered. It is associated with cases of fatal encephalitis in three German squirrel breeders (Hoffmann, Tappe, et al. 2015) and an animal care taker in a German zoo (Tappe et al. 2018). Virus sequences with high similarities were found in brain samples from the four patients and in organ tissue of their exotic squirrels. Five squirrel species, all belonging to the family Sciuridae, were so far identified to harbour VSBV-1 RNA: Prevost's squirrel (*Callosciurus prevostii)*, Finlayson's squirrel (*Callosciurus finlaysonii*), Variegated squirrel (*Sciurus variegatoides*), red-tailed squirrel (*Sciurus granatensis*), and Swinhoei's striped squirrel (*Tamiops swinhoei*). Highest viral genome loads were found in the central nervous system. There are no clinical signs of infection or relevant pathological alterations in the infected squirrels and transmission occurs most probably direct through scratches and bites or indirect by excretions like faeces or urine (Schlottau, Hoffmann, et al. 2017; Schlottau, Jenckel, et al. 2017).

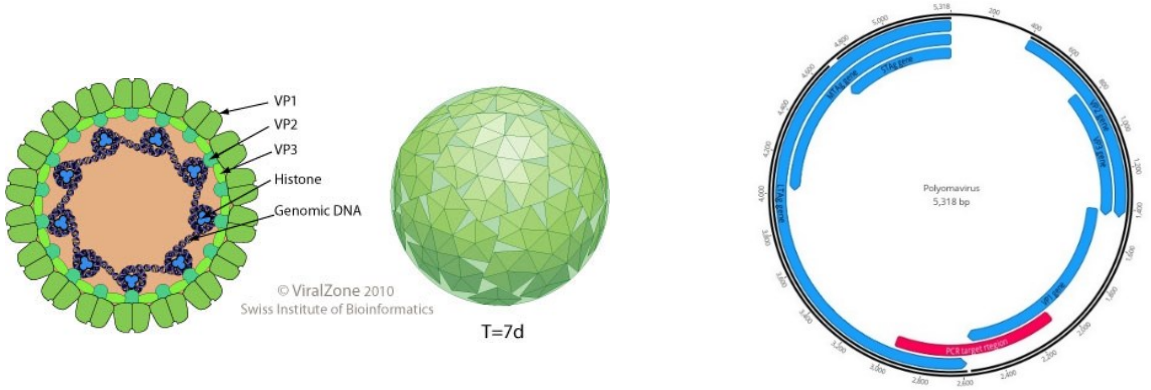
The identification of recent fatalities in humans due to BoDV-1 and the re-evaluation of old human cases of lethal encephalitides with unexplained aetiology, clearly indicate that BoDV-1 is zoonotic (Korn et al. 2018; Niller et al. 2020; Schlottau et al. 2018). Therefore, both viruses, VSBV-1 as well as BoDV-1, are considered as zoonotic. There is report about antiviral efficacy of ribavirin through reducing the virus proliferation, e.g. in infected gerbils (Lee et al. 2008), but in the end there is still no effective prophylaxis or therapy for orthobornaviruses available (Richt and Rott 2001).

2.5. (Non-zoonotic) polyoma- and herpesviruses

2.5.1. Polyomaviruses

Taxonomically, polyomaviruses (PyVs) belong to the family *Polyomaviridae*. This family comprises around 100 members, including 14 human PyVs. PyVs have been identified in many hosts, including humans, nonhuman primates, rodents, cattle, bats, birds, and fish (Gjoerup and Chang 2010; Moens, Krumbholz, et al. 2017).

PyVs are non-enveloped viruses with a circular double-stranded deoxyribonucleic acid (dsDNA) genome. The viral genome has a size of around 5 to 5.5 kbp and is surrounded by an icosahedral capsid (Figure 5A). The genome organization of mammalian PyVs is very similar and the genome is divided into three functional regions: the early region, the late region and the non-coding control region (NCCR). The early transcriptional region encodes regulatory proteins, known as tumor antigens, including large T-antigen (LTAg) and small T-antigen (STAg), and is predominantly expressed early during the infection cycle. The counter-clockwise oriented late transcriptional region comprises the genetic information for the capsid proteins viral protein (VP) 1, VP2, and VP3 and is mainly transcribed after the viral DNA replication begins. Early and late regions are separated by the NCCR, which consists of the origin of replication and the transcription control region (TCR) and regulates DNA replication and transcription from the early and late promoters (Moens, Krumbholz, et al. 2017) (Figure 5B).



A **B**

Figure 5: Virion structure and genome organization of polyomaviruses. (A): Schematic view of a polyomavirus virion (available from Viral Zone, Swiss Institute of Bioinformatics), **(B)**: Schematic genome organization of polyomaviruses; the target region of the conventional generic PCR is located within the VP1 gene and marked in red; the figure was prepared using Geneious version 2019.2 (Biomatters, available

from https://www.geneious.com); the exemplary used polyomavirus genome (Polyomavirus spp. strain, *Rattus norvegicus*, complete genome) is available from GenBank under accession number MK372231.

LTAg is a multifunctional protein that participates in viral DNA replication and transcription. Distinct regions of the protein display different activities required for DNA replication, including adenosine-triphosphatase (ATPase), DNA helicase, specific DNA binding and recruitment of cellular DNA replication proteins (Gjoerup and Chang 2010; Imperiale 2001; Moens, Van Ghelue, and Johannessen 2007). It can cause an abnormal stimulation of the cell cycle and is therefore involved in oncogenic transformation (Arrington 2001; Gjoerup and Chang 2010; Moens, Van Ghelue, and Johannessen 2007).
STAg is a cysteine-rich protein, that is generated by alternative splicing of the early transcript. This protein shares the first approximately 80 amino-terminal residues with LTAg and seems to provide a helper function for LTAg by supporting viral replication and activation of the viral promoter (Moens 2001; Rundell and Parakati 2001).
In addition to LTAg and STAg, which are universal and expressed by all known PyVs, in genomes of a few PyVs, e.g. the murine PyV (species: *Mus musculus* polyomavirus 1), there is also a middle T-antigen (MTAg) encoded. MTAg is essential for viral proliferation and also involved in tumor formation (Freund et al. 1992).

Mammalian PyVs are assigned to four distinct genera within the family *Polyomaviridae*:
Alpha-, *Beta*-, *Gamma*- and *Deltapolyomavirus* (Calvignac-Spencer et al. 2016; Moens, Calvignac-Spencer, et al. 2017):

Alphapolyomavirus. The genus *Alphapolyomavirus* includes more than 40 species with five members that infect humans. Members of other PyV species infect apes, monkeys, bats, rodents and other mammals. Alphapolyomaviruses have transforming activity *in vitro* and reveal tumorigenic capacity in laboratory animals. Merkel cell polyomavirus (MCPyV) is the only currently known human PyV that is associated with a tumor in humans (Feng et al. 2008; Spurgeon and Lambert 2013). The type species of this genus is *Mus musculus* polyomavirus 1 (Calvignac-Spencer et al. 2016).

Betapolyomavirus. More than 30 already discovered species, infecting mammals, belong to the genus *Betapolyomavirus*. They include in total four human PyVs, among these are the intensively studied human BK polyomavirus (BKPyV; species *Human polyomavirus 1*) and JC polyomavirus (JCPyV; species *Human polyomavirus 2*) which are associated with nephropathy and progressive multifocal leukoencephalopathy, respectively (Calvignac-Spencer et al. 2016; Gardner et al. 1971; Padgett et al. 1971). The type species of this genus is *Macaca mulatta* polyomavirus 1 (SV40, *simian virus 40*) (Arrington 2001; Calvignac-Spencer et al. 2016; Sweet and Hilleman 1960).

Gammapolyomavirus. This genus comprises nine species, which all infect birds. Some of them cause severe illness and even death, but oncogenicity has not been observed. The type species of this genus is Aves polyomavirus 1 (previous: *Budgerigar fledgling* polyomavirus) (Calvignac-Spencer et al. 2016).

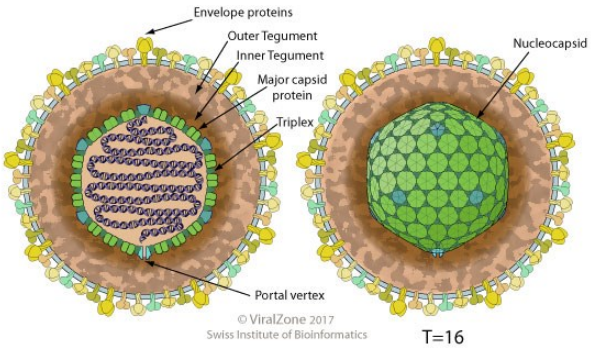
Deltapolyomavirus. This genus consists of four human polyomaviruses: the species *Human polyomavirus 6* and *7*, that exhibit skin tropism, and *Human polyomavirus 10* and *11* (MW polyomavirus and STL polyomavirus), that are commonly detected in the gastrointestinal tract (Calvignac-Spencer et al. 2016; Lim et al. 2013; Schowalter et al. 2010; Siebrasse et al. 2012).

2.5.2. Herpesviruses

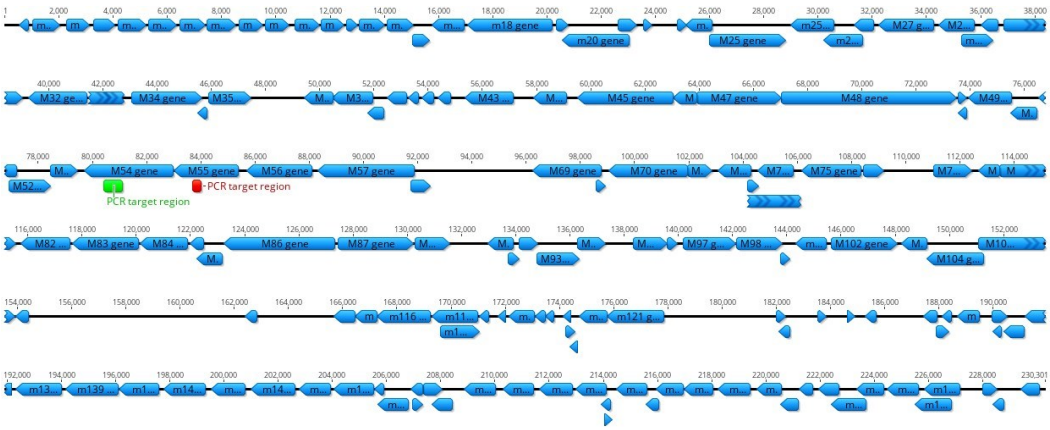
Herpesviruses (HVs; family *Herpesviridae*) are a family of large enveloped dsDNA viruses that infect many vertebrates including birds, reptiles, humans, and nonhuman primates (Davison et al. 2009). Besides the family *Herpesviridae* the order *Herpesvirales* also comprises the families *Alloherpesviridae*, which include four genera infecting fish, e.g. salmonids, and frogs, and *Malacoherpesviridae* with two genera infecting molluscs (King et al. 2018).

Herpesvirus particles consist of a core that contains the linear dsDNA genome ranging from 110 - 290 kbp in length. The core constitutes together with its capsid the icosahedral nucleocapsid. Between the nucleocapsid and the outer lipid envelope there is a tegument layer embedding proteins, mostly glycoproteins, that are required for viral entry, replication and egress (Davison et al. 2009; Guo et al. 2010;

Mettenleiter, Klupp, and Granzow 2009) (Figure 6A). The glycoprotein spikes on the viral surface play an important role for attachment and entry via cell surface receptors (Grünewald et al. 2003; Heldwein and Krummenacher 2008). Genomes of members of the *Herpesviridae* comprise between 70 and 200 protein-coding genes (Pellett and Roizman 2013) (Figure 6B).



A



B

Figure 6: Virion structure and genome organization of herpesviruses. (A): Schematic view of a herpesvirus virion (available from Viral Zone, Swiss Institute of Bioinformatics), **(B)**: Schematic genome organization of the Herpesviridae; the target region of the generic herpesvirus PCR is located within the DNA polymerase (DPOL) gene (marked in green) and the target region of the generic panBeta- and panGamma-PCRs are located within the glycoprotein B (gB) gene (marked in red); the figure was prepared using Geneious version 2019.2 (Biomatters, available from https://www.geneious.com); the exemplary used betaherpesvirus genome (Murine

cytomegalovirus, strain K181, complete genome) is available from GenBank under accession number AM886412.

In addition to virion morphology, all HVs share the strategic capacity to establish a state of latency within the infected host. During latency viral gene expression is restricted and during maintenance of the latent state no production of infectious virus takes place. After reactivation, infectious virus is again produced and spreads to infect other susceptible individuals. Herpesviruses can cause a variety of medical conditions in humans and animals, including cancerous processes. The family *Herpesviridae* contains three distinct subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. These subfamilies differ in genetic structure, sequence and cell type tropism of their members (Davison et al. 2009; Pellett and Roizman 2013).

Alphaherpesvirinae. Members of this subfamily have a relatively short replication cycle, a variable host range, and usually cause rapid damage of cultured cells. They establish a latency state primarily in neurons of sensory ganglia (Davison et al. 2009; Pellett and Roizman 2013). This subfamily comprises four genera: i) *Simplexvirus*, with e.g. human herpes simplex virus 1 (HSV-1; species: *Human alphaherpesvirus 1*) and bovine herpesvirus 2 (BoHV-2; bovine mammillitis virus; species: *bovine alphaherpesvirus 2*); ii) *Varicellovirus* with the human varicella-zoster virus (HHV-3; species: *Human alphaherpesvirus 3*), bovine herpesviruses 1 and 5 (BoHV-1, infectious bovine rhinotracheitis virus; BoHV-5, bovine encephalitis herpesvirus; species: *Bovine alphaherpesvirus 1* and *5*), and equine herpesviruses 1 and 4 (EHV-1, EHV-4; species: *Equid herpesvirus 1* and *4*); iii) genus *Iltovirus* with the infectious laryngotracheitis virus (species: *Gallid Herpesvirus 1*) as typical representative; and iv) *Mardivirus* with Marek's disease virus (species: *Gallid Herpesvirus 2*) as type member (King et al. 2018).

Betaherpesvirinae. Betaherpesviruses (BHVs) generally include larger genomes (>200 kbp) than either the alpha- or the gammaherpesviruses, and are characterised by extended replication cycles and strict host specificity (Davison et al. 2009; Pellett and Roizman 2013). This subfamily contains the genera *Cytomegalovirus* (type member human cytomegalovirus; species: *Human betaherpesvirus 5*),

Muromegalovirus (type member murine cytomegalovirus; species: *Murid betaherpesvirus 1*), *Roseolovirus* (type member human herpesvirus 6; species: *Human betaherpesvirus 6*) and *Proboscivirus* (type member Elephantid Herpesvirus 1; species: *Elephantid betaherpesvirus 1*) (King et al. 2018).

Gammaherpesvirinae. Gammaherpesviruses (GHVs) comprise viruses with transforming potential and preferential association to lymphocytes (Davison et al. 2009; Pellett and Roizman 2013). They are grouped into the genera *Lymphocryptovirus* (type member Epstein-Barr Virus; species *Human gammaherpesvirus 4*), *Rhadinovirus* (type member Kaposi's sarcoma herpesvirus; species *Human gammaherpesvirus 8*), *Macavirus* (type member malignant catarrhal fever virus; species *Alcelaphine Herpesvirus 1*) and *Percavirus* (type member Equine herpesvirus 2; species: *Equid gammaherpesvirus 2*) (King et al. 2018).

2.6. Virus-host evolution

In general RNA viruses display a remarkably higher mutation rate than DNA viruses (Holmes 2009). One explanation for that is the 'proofreading capability' of DNA polymerases, which enables them to correct errors made during replication, resulting in overall reduced mutation rates compared to RNA polymerases (Garcia-Diaz and Bebenek 2007). While cross-species transmission is the principle mechanism of viral emergence mainly of RNA viruses, DNA viruses carry out longterm virus-host co-divergence in many cases (Holmes 2009; Woolhouse et al. 2002). These co-evolutionary processes allow optimal adaptations between host and virus and create optimal replication conditions for the virus. Indications for co-evolution of viruses and their hosts are e.g. shown for PyVs (Ehlers et al. 2019) and the co-evolution model, typical for DNA viruses, also holds true for the majority of HVs (Davison 2002).

In line with these principles, orthobornaviruses, belonging to the RNA viruses, are also present in several hosts, which might be traced back to events of transspecies-transmission for evolutionary reasons in the past. They show extremely high sequence conservation and therefore cluster in line with their geographic origin but independent of the host species, where the sequences were isolated from (Figure 4B).

Chapter 3: Study objectives

3 Study objectives

Although a lot of virus screening and reservoir host investigations were performed in a variety of small mammal species, including different rodents and shrews, the current knowledge on viruses in squirrels and the role of squirrels as virus reservoirs is scarce. In addition, the virus occurrence of BoDV-1 in the bicolored white-toothed shrew, *Crocidura leucodon*, as reservoir host and in alpacas as (accidental) dead-end hosts needs to be characterised in more detail. Therefore, the objectives of this study are the following:

3.1. Searching for squirrels as reservoir of the VSBV-1 and for novel polyoma- and herpesviruses in squirrels

Screening squirrel samples for the presence of orthobornaviruses was supposed to shed light on the role of squirrels as potential virus reservoir hosts. In addition, the search for novel polyoma- and herpesviruses with conventional PCRs should expand the knowledge on virus diversity in these neglected species.

3.2. Characterization of the interactions between reservoir host and (accidental) dead-end hosts during an exemplary BoDV-1 outbreak

The discovery of a bornavirus disease outbreak in an alpaca herd in Northwest Brandenburg, Germany led to further analyses of pest rodents from this alpaca farm. Based on this example possible interactions between the virus reservoir host bicolored white-toothed shrew, *Crocidura leucodon*, and the accidental, but obviously highly susceptible, dead-end host alpaca should be characterised in more detail.

Chapter 4: Results

4 Results

The reference section of each manuscript/the results are presented in the style of the respective journal and are not included at the end of this document. The numeration of figures and tables corresponds to the published form of each manuscript.

4.1. Publication 1

Search for polyoma-, herpes-, and bornaviruses in squirrels of the family Sciuridae

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Virology Journal

RESEARCH Open Access

Search for polyoma-, herpes-, and bornaviruses in squirrels of the family Sciuridae

Vanessa Schulze[1], Peter W. W. Lurz[2], Nicola Ferrari[3], Claudia Romeo[3], Michael A. Steele[4], Shealyn Marino[4], Maria Vittoria Mazzamuto[5], Sébastien Calvignac-Spencer[6], Kore Schlottau[7], Martin Beer[7], Rainer G. Ulrich[1,8*] and Bernhard Ehlers[9*]

Abstract

Background: Squirrels (family Sciuridae) are globally distributed members of the order Rodentia with wildlife occurrence in indigenous and non-indigenous regions (as invasive species) and frequent presence in zoological gardens and other holdings. Multiple species introductions, strong inter-species competition as well as the recent discovery of a novel zoonotic bornavirus resulted in increased research interest on squirrel pathogens. Therefore we aimed to test a variety of squirrel species for representatives of three virus families.

Methods: Several species of the squirrel subfamilies Sciurinae, Callosciurinae and Xerinae were tested for the presence of polyomaviruses (PyVs; family *Polyomaviridae*) and herpesviruses (HVs; family *Herpesviridae*), using generic nested polymerase chain reaction (PCR) with specificity for the PyV VP1 gene and the HV DNA polymerase (DPOL) gene, respectively. Selected animals were tested for the presence of bornaviruses (family *Bornaviridae*), using both a broad-range orthobornavirus- and a variegated squirrel bornavirus 1 (VSBV-1)-specific reverse transcription-quantitative PCR (RT-qPCR).

Results: In addition to previously detected bornavirus RNA-positive squirrels no more animals tested positive in this study, but four novel PyVs, four novel betaherpesviruses (BHVs) and six novel gammaherpesviruses (GHVs) were identified. For three PyVs, complete genomes could be amplified with long-distance PCR (LD-PCR). Splice sites of the PyV genomes were predicted in silico for large T antigen, small T antigen, and VP2 coding sequences, and experimentally confirmed in Vero and NIH/3T3 cells. Attempts to extend the HV DPOL sequences in upstream direction resulted in contiguous sequences of around 3.3 kilobase pairs for one BHV and two GHVs. Phylogenetic analysis allocated the novel squirrel PyVs to the genera *Alpha-* and *Betapolyomavirus*, the BHVs to the genus *Muromegalovirus*, and the GHVs to the genera *Rhadinovirus* and *Macavirus*.

(Continued on next page)

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(Continued from previous page)
Conclusions: This is the first report on molecular identification and sequence characterization of PyVs and HVs and the detection of bornavirus coinfections with PyVs or HVs in two squirrel species. Multiple detection of PyVs and HVs in certain squirrel species exclusively indicate their potential host association to a single squirrel species. The novel PyVs and HVs might serve for a better understanding of virus evolution in invading host species in the future.

Keywords: Bornavirus, Polyomavirus, Betaherpesvirus, Gammaherpesvirus, Large T, Small T, VP2, Splicing, Squirrel

Background

Squirrels (family Sciuridae) are members of the order Rodentia, and with the exception of Antarctica, distributed globally on all continents. They occur both in the Old and in the New world, and squirrel species diversity is particularly high in Africa and Asia [1]. Squirrels are generally distinguished as ground, flying (gliding) or tree squirrels. With respect to the latter, the Eurasian red squirrel (*Sciurus vulgaris*) is the dominant squirrel species across the Palaearctic [2, 3]. In contrast to the North American red or pine squirrel (*Tamiasciurus hudsonicus*) which is a boreal coniferous species [1], the Eurasian red squirrel originally occupied all available forest habitats in the absence of other tree squirrels across most of its range.

The Eastern grey squirrel (*Sciurus carolinensis*) is a broadleaf specialist and was originally distributed in the eastern deciduous forests of Northern America [4]. However, the species has been translocated repeatedly within North America and globally to Europe, South Africa and Australia (e.g. see [4, 5]). The multiple introductions and subsequent translocations had devastating consequences for local Eurasian red squirrel populations in deciduous and mixed forest landscapes in Great Britain, Ireland and Italy leading to large-scale declines as a result of competition for resources and introduced pathogens [6–8]. With regard to pathogens, the replacement of native Eurasian red squirrels by the Eastern grey squirrels in the British Isles is highly accelerated by a squirrelpox virus (e.g. [9]). However, the resulting research interest on pathogens also resulted in the identification of other potential disease threats to red squirrels such as leprosy bacilli [10] and adenovirus [11–13]. Although squirrel adenovirus has been known for many years, it was only recently that the complete genome of squirrel adenovirus 1 (SqAdV-1) was determined and found to indicate a close relationship between British and Continental European red squirrel populations [11, 13].

Prevost's squirrels (*Callosciurus prevostii*) and Pallas's squirrels (*Callosciurus erythraeus*) belong to the subfamily Callosciurinae. Both species are native to South-East Asia and Pallas's squirrels were introduced to Italy, France, Belgium and the Netherlands. They typically represent non-native, invasive tree squirrel species that arrived in Europe via pet trade [14–16]. The variegated squirrel (*Sciurus variegatoides*), a species of dry tropical forests in Central America, is another tree squirrel that appears popular among squirrel breeders [17], perhaps due to its great variability in coat colour [18]. Whilst there has been some progress in recognition of potential threats to wildlife and people from introduced species (e.g. [19, 20]), little information is available about viral and bacterial pathogens and parasites in squirrels and the role of squirrels in population dynamics, competitive interactions or as reservoirs for zoonotic diseases.

Recently a novel zoonotic bornavirus, variegated squirrel bornavirus 1 (VSBV-1), was detected in five different squirrel species from private holdings and zoos, including *Sciurus variegatoides* and *Callosciurus prevostii* and is associated with cases of fatal encephalitis of their breeders and care takers [17, 21–23]. Bornaviruses (family *Bornaviridae*) are enveloped spherically structured viruses with a single-stranded RNA genome of negative polarity (size around 8.9 kilobases (kb)). Bornaviruses have been identified in a wide range of hosts, e.g. mammals, birds and reptiles [24]. Members of the genus *Orthobornavirus* infecting mammals are assigned to two species: *Mammalian 1 orthobornavirus* (Borna Disease Virus 1 and 2; BoDV-1 and BoDV-2) and *Mammalian 2 orthobornavirus* (VSBV-1). These viruses have pathogenic potential for humans and other mammals. BoDV-1 is the causative agent of Borna disease, an often fatal neurologic condition of horses, sheep and other domestic mammals. Recently, its zoonotic potential has been demonstrated by molecular and immunohistochemical detection of several BoDV-1-induced fatal encephalitis cases in humans [25–28].

Polyomaviruses (PyVs; family *Polyomaviridae*) and herpesviruses (HVs; family *Herpesviridae*) both comprise a plethora of viruses many of which are pathogenic for humans and animals. In members of some mammalian orders (e.g. primates, artiodactyls or rodents) many PyVs and HVs have been identified, some of which are well studied [29–33]. Despite this knowledge, information on PyVs and HVs is still scarce for many mammalian families. This holds particularly true for the family Sciuridae. To our knowledge, there is no report describing the occurrence of PyVs in members of the Sciuridae. From the 1980s there exist descriptions of HVs in a thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) [34],

and *Citellus* spp. (now *Spermophilus*) [35, 36] but these reports are based only on electron microscopy and cytopathogenic effects in infected cell cultures, which were assumed to be typical for HVs, despite the lack of sequence data.

PyVs are small non-enveloped viruses with an icosahedral capsid and a circular double-stranded DNA genome that consists of approximately 5 kilobase pairs (kbp). The family *Polyomaviridae* comprises around 100 members, including 14 that infect humans. PyVs have been found in many hosts, including humans, nonhuman primates, rodents, cattle, bats, birds, and fish [31]. PyVs cause subclinical infections, as well as acute systemic diseases, the latter mainly in immune-compromised individuals. Some PyVs have transforming activity in vitro and reveal tumorigenic capacity in laboratory animals. Merkel cell polyomavirus (MCPyV) is the first human PyV that is associated with a tumor in humans [37, 38].

The genomic organization of mammalian PyVs comprises three regions: The early transcriptional region, the late transcriptional region and the non-coding control region (NCCR). The early region encodes regulatory proteins, including large T-antigen (LTAg) and small T-antigen (STAg). The counter clock-wise oriented late region encodes the structural proteins VP1, VP2, and VP3. Early and late regions are separated by the NCCR, which controls DNA replication and transcription from the early and late promoters [39]. LTAg and STAg are involved in viral transcription and replication. LTAg induces the synthesis phase of cells and can cause an abnormal stimulation of the cell cycle and tumor formation [31, 39, 40]. Mammalian PyVs are assigned to three distinct genera within the family *Polyomaviridae*: *Alpha-*, *Beta-*, and *Deltapolyomavirus* [41].

HVs are a family of large, enveloped viruses with a double-stranded DNA genome (length: 110–295 kbp) that infect many vertebrates, including humans and nonhuman primates [29]. Mammalian HVs are divided into three distinct subfamilies within family *Herpesviridae*: *Alphaherpesvirinae*, *Betaherpesvirinae* (BHVs) and *Gammaherpesvirinae* (GHVs). All HVs share the capacity to establish a state of latency resulting in lifelong association with the infected host. After reactivation, an infectious virus is produced and spreads to other susceptible individuals [29, 42]. Herpesviruses cause a variety of diseases in humans and animals, including some cancers.

As bornaviruses have so far only been identified in five of the > 280 sciurid species, and sciurid PyVs and HVs are not yet known, we sought to improve our knowledge on such viruses in the Sciuridae family and performed a molecular survey in squirrels of different species and subfamilies. Spleen and lung samples of 238 animals from five countries (Canada, USA, Italy, UK and Germany) were tested with generic nested PCRs [43–49] for the identification of PyVs and HVs. Brain samples from 126 of these animals originating from four countries (Canada, USA, Italy and UK) were analyzed with broad-range orthobornavirus RT-qPCR for the generic detection of orthobornaviruses and a VSBV-1-specific reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In case of successful PyV sequence detection, we aimed at generating complete genome sequences, and in case of HV sequence detection, we wanted to amplify and sequence a genome segment, which comprises around 3.4 kbp and extends from the glycoprotein B (gB) gene to the DNA polymerase (DPOL) gene. This approach led to the discovery of four PyVs and 10 HVs (four BHVs and six GHVs).

Methods

Sample collection and nucleic acid preparation

Squirrels ($n = 126$) of five species belonging to the family Sciuridae were collected in four different countries (Canada, USA, Italy and UK), dissected according to standard protocols and brain, lung and spleen samples of these animals were available for screening for all three viruses. In addition, lung and spleen samples from another 112 squirrels belonging to another three species were available from previous bornavirus studies [17, 21, 22]. Thus, a total of 361 organ samples (243 spleen samples and 118 lung samples) were available for PyV and HV analyses (Tables 1, 2 and 3).

RNA extraction was performed using the KingFisher™ Flex Purification System (Thermo Fisher) in combination with the Nucleo Mag Vet Kit (Macherey Nagel) following the instructions of the manufacturer. DNA extraction was performed using EURx GeneMatrix Tissue DNA Purification Kit (Roboklon), Qiagen DNeasy Blood & Tissue-Kit (Qiagen) and the KingFisher™ Flex Purification System (Thermo Fisher) in combination with the Nucleo Mag Vet Kit (Macherey Nagel). DNA preparations were stored at − 20 °C and RNA preparations at − 80 °C. During the nucleic extraction processes negative controls were carried along to monitor for potential contaminations. Morphological species identification was confirmed for all samples by cytochrome *b* PCR and sequencing according to a previously described protocol ([50], data not shown). Prior to nucleic acid extraction no attempts concerning virus isolation in cell culture were done.

Bornavirus screening of squirrel brain samples

Brain samples of the newly dissected 126 squirrels were screened with broad-range and VSBV-1 specific RT-qPCRs (Additional file 1) as described previously [17, 28]. The other animals ($n = 112$) have already been investigated before [17, 21, 22].

Table 1 Bornavirus reverse transcription - quantitative polymerase chain reaction (RT-qPCR) analysis of squirrels from wildlife and holdings

Host taxonomic name (subfamily, species)	Host common name	n positive/ total n tested in this study	RT-qPCR positive/ total n tested in previous studies [17, 21, 22]
Sciurinae			
Sciurus carolinensis	Eastern grey squirrel	0/77[a]	0/11[a]
Sciurus variegatoides	Variegated squirrel		7/7[b]
Sciurus vulgaris	Eurasian red squirrel		0/77[a]
Tamiasciurus hudsonicus	American red squirrel	0/1[a]	
Xerinae			
Urocitellus richardsonii	Richardson's ground squirrel	0/11[a]	
Tamias striatus	Eastern chipmunk	0/2[a]	
Callosciurinae			
Callosciurus erythraeus	Pallas's squirrel	0/35[a]	
Callosciurus prevostii	Prevost's squirrel		10/17[b]
total		**0/126**	**17/112**

[a]all individuals originated from wildlife
[b]all individuals originated from holdings

Identification of polyomaviruses and herpesviruses with generic PCR assays

For identification of PyVs and HVs generic nested PCRs were performed, that broadly detect a partial VP1 coding sequence (CDS) of PyVs (Additional files 1 and 2) or a fragment of the DPOL gene of HVs (Open reading frame 09 (ORF09) of GHV; ORF UL54 of BHV) (Additional file 1 and Additional file 3) in the second PCR round. Both nested PCRs were performed as carried out previously [46, 48].

Amplification of gB sequences of betaherpesviruses and gammaherpesviruses with generic PCR

For amplification of gB sequences of BHVs (ORF UL55) and GHVs (ORF08), we used subfamily-specific nested primer sets (Additional file 1) essentially as described previously [51, 52]. The scheme of multi-level PCR analysis used for squirrel BHVs and GHVs is shown in Additional file 3.

Nested long-distance PCR with virus-specific primers

For all PyVs, specific nested primers (Additional file 1) were selected tail-to-tail from the sequences amplified with generic PCR. They were used for the amplification and sequencing of the remaining parts of the circular genomes (approximately 5 kbp). For all HVs, for which both gB and DPOL sequences could be amplified, nested primers (Additional file 1) were selected that were specific for each virus and used in long-distance PCR (LD-PCR) for the amplification and sequencing of the sequence that spans the gap between the partial gB and DPOL sequences. LD-PCR was performed with the TaKaRa-Ex PCR system (Takara Bio Inc.), according to the manufacturer's instructions. After the first PCR round, a 2 µl aliquot of the reaction mix was used as template in the second-round reaction.

Hemi-nested herpesvirus DPOL PCR

In cases where only the generic HV PCR was successful (and not gB PCR and/or LD-PCR), the short DPOL sequence was extended in upstream direction with hemi-nested PCR, using the outer sense primer (285 DFA) of the generic HV PCR and two virus-specific antisense primers for amplification of approximately 480 base pairs (bp) (Additional file 1). The hemi-nested DPOL PCR was carried out as described above for generic HV PCR.

Based on these extended sequences we were able to design again virus-specific primers that were used for re-screening of all samples of the respective species.

Nested PCR with polyomavirus-specific primers

For all novel PyVs, for which full genomes could be assembled, nested primer sets (Additional file 1) were selected. They were used for amplification of sequences of approximately 800 bp that encompass the short overlap between the sequences generated from the generic PCR fragments and the LD-PCR fragments of the respective PyV genome. PCR was performed in a total volume of 25 µl with 0.4 µl (2 units) Applied Biosystems AmpliTaq Gold DNA Polymerase (Thermo Fisher), 25 pmol of each primer, 200 µM dideoxynucleoside triphosphates (dNTPs), 2 mM $MgCl_2$, 5% dimethyl sulfoxide (DMSO) and 250 ng of sample DNA as template. A thermocycler from Biometra was used under the following cycling conditions: activation of the polymerase at 95 °C for 12 min and 45 cycles of denaturation at 95 °C for 30 s, annealing at 61 °C for 30 s, and elongation at 72 °C for 5 min, followed by a final extension step at 72 °C for 30

Table 2 Geographic origin of identified polyomaviruses in members of the Sciuridae and detailed PCR results

Host taxonomic name (subfamily, species)	Host common name	Origin[a]	Polyomavirus	Organs tested with PCRs	n tested squirrels	n tested samples	n samples positive in generic nested PCR	n samples positive in virus specific nested PCR	n animals positive in generic or specific nested PCR
Sciurinae									
Sciurus carolinensis	Eastern grey squirrel	Penicuik, Scotland	ScarPyV1	lung, spleen	6	11	1	8	6
		Anwoth, Scotland		lung, spleen	3	6	0	0	0
		Kirkbright, Scotland		lung, spleen	4	8	0	0	0
		Dumfries, Scotland	ScarPyV1	spleen	13	13	n. d.[b]	9	9
		Borders Region, Scotland	ScarPyV1	spleen	13	13	n. d.	4	4
		Brampton, England	ScarPyV1	spleen	10	10	n. d.	9	9
		Pennsylvania, USA	ScarPyV1	lung	5	5	0	2	2
		Piedmont Region, Italy	ScarPyV1	lung, spleen	30	45	0	1	1
		Lomardy Region, Italy		lung, spleen	4	7	0	0	0
subtotal					**88**	**118**	**1**	**33**	**31 (35.2%)**
Sciurus vulgaris	Eurasian red squirrel	Scotland (road mortality)		lung, spleen	7	12	0	-[c]	0
		Isle of Arran, Scotland		lung, spleen	12	13	0	–	0
		Neustadt, Germany		lung, spleen	1	2	0	–	0
		Munich, Germany		lung, spleen	16	22	0	–	0
		Waiblingen, Germany		lung, spleen	2	4	0	–	0
		Heidelberg, Germany		spleen	1	1	0	–	0
		Bad König, Germany		lung, spleen	2	4	0	–	0
		Dresden, Germany		lung, spleen	1	2	0	–	0
		Wentorf, Germany		lung, spleen	1	2	0	–	0
		Billigheim, Germany		lung, spleen	1	2	0	–	0
		Rödermark, Germany		lung, spleen	6	12	0	–	0
		Leutenbach, Germany		lung, spleen	1	2	0	–	0
		Bonn, Germany		lung	1	1	0	–	0
		Bad Nauheim, Germany		lung	1	1	0	–	0
		Oberhausen, Germany		lung	1	1	0	–	0
		Berlin, Germany		lung, spleen	4	8	0	–	0
		Stuttgart, Germany		lung, spleen	6	12	0	–	0
		Görlitz, Germany		lung, spleen	6	12	0	–	0
		Sternenfels, Germany		lung, spleen	7	13	0	–	0
subtotal					**77**	**126**	**0**		**0**

Table 2 Geographic origin of identified polyomaviruses in members of the Sciuridae and detailed PCR results *(Continued)*

Host taxonomic name (subfamily, species)	Host common name	Origin[a]	Polyomavirus	Organs tested with PCRs	n tested squirrels	n tested samples	n samples positive in generic nested PCR	n samples positive in virus specific nested PCR	n animals positive in generic or specific nested PCR
Sciurus variegatoides	Variegated squirrel	Germany	SvarPyV1	spleen	7	7	1	–	1 **(14.3%)**
Tamiasciurus hudsonicus	American red squirrel	Pennsylvania, USA		lung	1	1	0	–	0
Xerinae									
Urocitellus richardsonii	Richardson's ground squirrel	Winnipeg, Canada		spleen	11	11	0	–	0
Tamias striatus	Eastern Chipmunk	Pennsylvania, USA		lung, spleen	2	2	0	–	0
Callosciurinae									
Callosciurus erythraeus	Pallas's squirrel	Lombardy Region, Italy	CeryPyV1	lung, spleen	35	44	8	18	15 **(42.9%)**
Callosciurus prevostii	Prevost's squirrel	Germany	CprePyV1	spleen	17	17	3	4	4 **(23.5%)**
total					**238**	**326**	**13**	**55**	**51**

[a]all animals originated from wildlife, except variegated squirrels and Prevost's squirrels originating from holdings in Germany
[b]*n. d.* not done
[c]- = specific nested PCR was not possible because no polyomavirus was detected in generic PCR or specific nested primers could not be designed because LD-PCR was unsuccessful

Table 3 Geographic origin of identified herpesviruses in members of the Sciuridae and detailed PCR results

Host taxonomic name (subfamily, species)	Host common name	Origin[a]	Herpesvirus	Organs tested with PCRs	n tested squirrels	n tested samples	n samples positive in generic PCR	n samples positive in virus specific nested PCR	n animals positive in generic or specific nested PCR
Sciurinae									
Sciurus carolinensis	Eastern grey squirrel	Penicuik, Scotland	ScarGHV1	lung, spleen	6	11	8	n. d.[b]	5
		Anwoth, Scotland		lung, spleen	3	6	0	-[c]	0
		Kirkbright, Scotland	ScarGHV1	lung, spleen	4	8	2	n. d.	1
		Dumfries, Scotland	ScarGHV1	spleen	13	13	2	n. d.	2
			ScarGHV2	spleen			1	n. d.	1
		Borders Region, Scotland	ScarGHV1	spleen	13	13	3	n. d.	3
		Brampton, England	ScarGHV1	spleen	10	10	9	n. d.	9
		Pennsylvania, USA	ScarGHV1	lung	5	5	3	n. d.	3
			ScarGHV2	lung			1	0	1
		Piedmont Region, Italy	ScarBHV1	lung, spleen	30	45	2	4	4
		Lombardy Region, Italy	ScarBHV1	lung, spleen	4	7	0	1	1
subtotal		**ScarGHV1**			**88**	**118**	**29**	**n. d.**	**28 (31.8%)**
		ScarGHV2			**88**	**118**	**2**	**0**	**2 (2.3%)**
		ScarBHV1			**88**	**118**	**2**	**5**	**5 (5.7%)**
Sciurus vulgaris	Eurasian red squirrel	Scotland (Scotland (road mortality))	SvulBHV1	lung, spleen	7	12	9	n. d.	6
		Isle of Arran, Scotland	SvulBHV1	lung, spleen	12	13	7	n. d.	7
		Neustadt, Germany		lung, spleen	1	2	0	–	0
		Munich, Germany	SvulBHV1	lung, spleen	16	22	9	n. d.	7
		Waiblingen, Germany		lung, spleen	2	4	0	–	0
		Heidelberg, Germany		spleen	1	1	0	–	0
		Bad König, Germany		lung, spleen	2	4	0	–	0
		Dresden, Germany		lung, spleen	1	2	0	–	0
		Wentorf, Germany	SvulBHV1	lung, spleen	1	2	1	n. d.	1
		Billigheim, Germany		lung, spleen	1	2	0	–	0
		Rödermark, Germany	SvulBHV1	lung, spleen	6	12	3	n. d.	2
		Leutenbach, Germany	SvulBHV1	lung, spleen	1	2	1	n. d.	1
		Bonn, Germany	SvulBHV1	lung	1	1	1	n. d.	1
		Bad Nauheim, Germany		lung	1	1	0	–	0
		Oberhausen, Germany		lung	1	1	0	–	0
		Berlin, Germany	SvulBHV1	lung, spleen	4	8	3	n. d.	2

Table 3 Geographic origin of identified herpesviruses in members of the Sciuridae and detailed PCR results *(Continued)*

Host taxonomic name (subfamily, species)	Host common name	Origin[a]	Herpesvirus	Organs tested with PCRs	n tested squirrels	n tested samples	n samples positive in generic PCR	n samples positive in virus specific nested PCR	n animals positive in generic or specific nested PCR
		Stuttgart, Germany	SvulBHV1	lung, spleen	6	12	1	n. d.	1
		Görlitz, Germany	SvulBHV1	lung, spleen	6	12	2	n. d.	2
		Sternenfels, Germany	SvulBHV1	lung, spleen	7	13	2	n. d.	1
subtotal					**77**	**126**	**39**		**31 (40.3%)**
Sciurus variegatoides	Variegated squirrel	Germany		spleen	7	7	0	–	0
Tamiasciurus hudsonicus	American red squirrel	Pennsylvania, USA		lung	1	1	0	–	0
Xerinae									
Urocitellus richardsonii	Richardson's ground squirrel	Winnipeg, Canada	UricGHV1	spleen	11	11	10	n. d.	10 **(90.9%)**
Tamias striatus	Eastern Chipmunk	Pennsylvania, USA	TstrGHV1	lung, spleen	2	2	2	2	2 **(100.0%)**
Callosciurinae									
Callosciurus erythraeus	Pallas's squirrel	Lombardy Region, Italy	CeryBHV1	lung, spleen	35	44	18	11	23 **(65.7%)**
			CeryGHV1	lung, spleen	35	44	2	5	6 **(14.3%)**
Callosciurus prevostii	Prevost's squirrel	Germany	CpreBHV1	spleen	17	17	1	1	1 **(5.9%)**
			CpreGHV1	spleen	17	17	1	1	1 **(5.9%)**
total					**238**	**326**	**104**	**27**	**108**

[a]all animals originated from wildlife, except variegated squirrels and Prevost's squirrels originating from holdings in Germany
[b]*n. d.* not done
[c]– = specific nested PCR was not possible because no herpesvirus was detected in generic PCR

min. For the second PCR round, a 1 µl aliquot of the first-round reaction mix was used as a template. The primers were also used for more sensitive re-screening all samples of the respective host species.

Nested PCR with herpesvirus-specific primers

For all novel HVs, for which extended sequences could be determined, nested primer sets were selected for re-screening all samples of the respective host species (Additional file 1). They were used as described above for specific PyV amplification (annealing temperatures listed in Additional file 1).

RT-PCR and PCR controls

For each round of all nested PCRs and the RT-qPCR of our screening, no-template controls (PCR-grade H_2O) and extraction controls were carried along to detect any possible contaminations. These analyses were negative for all PCRs and RT-qPCRs. As positive PCR controls, DNA extracts of samples were used that tested positive for BHVs, GHVs or PyVs in previous studies.

PCR product purification and sequencing

All PCR products were purified with MSB® Spin PCRapace (Stratec), according to the manufacturer's instruction and directly sequenced using the BigDye Terminator v3.1 system (Life Technologies) on an Applied Biosystems 3500xL DX Genetic Analyzer (Thermo Fisher). The LD-PCR products were sequenced by a classical primer walking strategy (primers not listed).

Synthesis of polyomavirus early and late region

Early or late region plus flanking sequences (approximately 3.1 kbp) of *Sciurus carolinensis* polyomavirus 1 (ScarPyV1; GenBank accession number MK671101) were commercially synthesized and delivered as recombinant plasmids (Biomatik). They were named pScarPyV1early and pScarPyV1late and transformed into competent *Escherichia coli* DH5 alpha cells (Thermo Fisher). Plasmid DNA was extracted with Invisorb® Spin Plasmid Mini Two (Stratec) according to the manufacturer's instruction.

Cell lines

Vero cells C1008 (monkey kidney cells; European Collection of Authenticated Cell Cultures (ECACC) # 85020206) were cultured in standard high-glucose Dulbecco's minimal Eagle medium (DMEM, Thermo Fisher) containing 10% fetal calf serum (FCS) (PAN Biotech) and 1% penicillin/streptomycin (Thermo Fisher). For NIH/3T3 cells (mouse embryo fibroblast cells; American Tissue Culture Collection, ATCC®, CRL-1658™) the same medium was used, except that 5% FCS (PAN Biotech) was added. Both cell lines were cultivated at 37 °C and 5% CO_2. DNA extracts of cell aliquots were tested with PCR for absence of mycoplasma contamination [53]. Primers are listed in Additional file 1.

Transfection of cells

As described previously [54], cells were transfected with 1 µg DNA of plasmid pScarPyV1early or plasmid pScarPyV1late, using X-tremeGENE HP DNA Transfection Reagent (Roche Applied Biosciences). Before transfection, Vero cells were seeded in a volume of 500 µl cell culture medium in tissue culture plates with 24 wells (Sarstedt). For NIH/3T3 cells, Cell+ plates with 24 wells and a special Cell+ growth surface for sensitive adherent cells (Sarstedt) were used. Transfection procedures were performed 24 h after seeding according to the manufacturer's instructions.

RNA extraction and cDNA synthesis

Total RNA was isolated on 0, 1, 2.5, and 6 days post transfection of recombinant plasmid DNA using the NucleoSpin® RNA-Kit (Macherey Nagel) according to the manufacturer's instructions. DNA was removed by an additional Turbo DNA-free DNase treatment (Thermo Fisher). RNA concentrations were determined with the NanoDrop 8000 device (Thermo Fisher) at 260 nm. Synthesis of cDNA was carried out with 500 ng RNA using SuperScript II Reverse Transcriptase (Thermo Fisher) and oligo(dT)16 primers (Roche Applied Bioscience).

PCR for identification of introns

To identify introns in the early and late region of ScarPyV1, PCR was performed with cDNA as a template, using primers specific for ScarPyV1 that bind in flanking regions of predicted introns (Additional file 1). PCR was performed with 2.5 µl cDNA in a total volume of 25 µl with 0.2 µl (1 unit) Applied Biosystems AmpliTaq Gold DNA Polymerase (Thermo Fisher), 1 µM of each primer, 200 µM dNTP PCR Mix (Metabion), 2 mM $MgCl_2$ and 5% DMSO (Merck). All PCR products were purified with MSB® Spin PCRapace (Stratec), according to the manufacturer's instructions. Sequencing reactions were performed as described above.

Bioinformatics and phylogenetic analysis

For the datasets we selected reference viral genomes representing all currently recognized species in the family/subfamily considered as well as additional viruses whose genomes represented distinct viral lineages discussed in the literature but still not integrated into the official taxonomy (sensu International Committee on Taxonomy of Viruses (ICTV)). For PyVs, this represented 109 viruses; for BHVs, 21 viruses; and for GHVs, 39 viruses. We extracted the LTAg and VP1 (PyV) or DPOL and gB (BHV and GHV) coding sequences from these genomes as well as from the novel viruses we

identified in squirrels using Geneious v11.1.5 [55]. For each coding sequence, sequences were translated into amino acid sequences and aligned using Muscle [56] as implemented in Seaview v4 [57]. Conserved amino acid blocks were then selected using Gblocks as implemented in Seaview, using options for a less stringent selection: allow smaller final blocks, allow gap positions within the final blocks and allow less strict flanking positions [58]. The final amino acid sequence alignments comprised 260 (VP1), 517 (LTAg), 628 (DPOL BHV), 298 (gB BHV), 622 (DPOL GHV) and 282 (gB GHV) positions, respectively.

For phylogenetic analyses of all datasets we first ran Maximum-Likelihood (ML) analyses using PhyML v3 with smart model selection (PhyML-SMS) using the Bayesian information criterion and a tree search using subtree pruning and regrafting [57, 59, 60]. Branch robustness was estimated using Shimodaira-Hasegawa-like approximate likelihood ratio tests (SH-like aLRT) [61]. The PyV and GHV ML trees were rooted with TempEst v1.5 by minimizing the variance of root-to-tip distances [62]; the BHV ML trees were rooted using roseolovirus outgroups. We then ran Bayesian Markov chain Monte Carlo (BMCMC) runs using BEAST v1.10.4 [63]. For each alignment, we used the amino acid substitution model identified by PhyML-SMS, an uncorrelated relaxed clock (lognormal) model and a speciation model (birth-death) as a tree prior. The output of multiple BMCMC runs was examined for convergence and appropriate sampling of the posterior using Tracer v1.7.1 [64], before being merged using LogCombiner v1.10.4 (distributed with BEAST). The maximum clade credibility (MCC) tree was identified from the posterior set of trees (PST) and annotated with TreeAnnotator v1.10.4 (also distributed with BEAST). Branch robustness was estimated based on their posterior probability in the PST.

Results

Bornavirus analysis

In previous studies, brain and oral swab samples of 112 squirrels belonging to various species were investigated with a broad-range orthobornavirus RT-qPCR and VSBV-1-specific RT-qPCR. VSBV-1-RNA was detected in brain and/or oral swab samples of 7/7 individuals from holdings of variegated squirrels, in samples of 10/17 Prevost's squirrels and a few other species [17, 21, 22], but not in any brain samples of the 77 tested Eurasian red squirrels and in any of the 11 Eastern grey squirrels [17, 21, 22]. Here we investigated a total of 126 brain samples from wildlife-derived squirrels from four countries, but did not find any positive specimens (Table 1).

Identification of novel polyomaviruses and herpesviruses

To identify PyVs and HVs in squirrels, we analyzed 238 squirrels (spleen, $n = 208$; lung, $n = 118$) of eight different species (Tables 2 and 3). First we screened with generic nested PCR that targets a short fragment of the major capsid VP1 CDS of mammalian PyVs (Additional file 1 and Additional file 2) [43, 47–49] and generic nested PCR with specificity for the highly conserved DPOL gene of mammalian HVs (Additional file 1 and Additional file 3) [44–46]. Products of expected length were sequenced and sequences analyzed using Nucleotide Basic Local Alignment Search Tool (BLASTn). Thirteen samples of twelve animals were positive for yet unknown PyVs (Table 2) and 104 samples of 89 animals were positive for yet unknown HVs (Table 3) as revealed by BLASTn analysis (data not shown). In total, four novel PyVs, and 10 novel HVs (four BHVs and six GHVs) were identified and tentatively named: *Sciurus carolinensis* polyomavirus 1 (ScarPyV1), *Sciurus carolinensis* betaherpesvirus 1 (ScarBHV1), *Sciurus carolinensis* gammaherpesvirus 1 and 2 (ScarGHV1 and ScarGHV2), *Sciurus variegatoides* polyomavirus 1 (SvarPyV1), *Sciurus vulgaris* betaherpesvirus 1 (SvulBHV1), *Callosciurus erythraeus* polyomavirus 1 (CeryPyV1), *Callosciurus erythraeus* betaherpesvirus 1 (CeryBHV1), *Callosciurus erythraeus* gammaherpesvirus 1 (CeryGHV1), *Callosciurus prevostii* polyomavirus 1 (CprePyV1), *Callosciurus prevostii* betaherpesvirus 1 (CpreBHV1), *Callosciurus prevostii* gammaherpesvirus 1 (CpreGHV1), *Urocitellus richardsonii* gammaherpesvirus 1 (UricGHV1) and *Tamias striatus* gammaherpesvirus 1 (TstrGHV1).

In PyV detection with generic PCR, one of 118 spleen samples and one of 88 lung samples from Eastern grey squirrels (*Sciurus carolinensis*) were positive for ScarPyV1, 1/7 spleen samples of variegated squirrels (*Sciurus variegatoides*) was positive for SvarPyV1, 8/44 tested samples (6x spleen and 1x spleen and lung) from 35 Pallas's squirrels (*Callosciurus erythraeus*) originating from Italy were positive for CeryPyV1, and 3/17 spleen samples of Prevost's squirrels (*Callosciurus prevostii*) were positive for CprePyV1 (Table 2).

In HV tests of 118 spleen and lung samples of *Sciurus carolinensis* from the UK, Italy and USA with generic PCR, we identified 20 animals from the UK as ScarGHV1-positive (16x in spleen and 4x in spleen and lung), three Eastern grey squirrels from the USA as ScarGHV1-positive in lung and two individuals as ScarGHV2-positive, one from the UK in spleen and one from the USA in lung. ScarBHV1 was detected in lung samples of two animals from Italy. Testing 77 *Sciurus vulgaris* revealed 18 SvulBHV1-positive individuals from Germany (1x in spleen, 12x in lung and 5x in spleen and lung) and 13 from the UK (1x in spleen, 9x in lung and 3x in spleen and lung). We identified 10 out of 11 Richardson's ground squirrels (*Urocitellus richardsonii*) to be positive for UricGHV-1 in spleen and 2/2 Eastern chipmunks (*Tamias striatus*) positive for TstrGHV1, one in spleen and one in lung. In wild *Callosciurus*

erythraeus from Italy as well as *Callosciurus prevostii* from a holding in Germany, we discovered both a BHV and a GHV. Upon testing a total of 35 *Callosciurus erythraeus*, we identified 16 individuals as CeryBHV1-positive (12x in spleen, 2x in lung and 2x in spleen and lung) and two animals as CeryGHV1-positive (1x in spleen and 1x in lung). Within the 17 Prevost's squirrels, one spleen each was detected to be positive for CpreBHV1 or CpreGHV1 (Table 3).

We did not detect any PyVs in 77 Eurasian red squirrels, 11 Richardson's ground squirrels and two Eastern chipmunks and no HVs in seven spleen samples of variegated squirrels from German holdings. In one lung sample of an American red squirrel (*Tamiasciurus hudsonicus*) from the USA, neither PyVs nor HVs were detected (Tables 2 and 3).

Based on extended sequences that we generated for three PyVs and eight HVs (see below) we selected specific nested primers (Additional file 1) for more sensitive screening and re-tested all samples of the respective species. By this approach we observed the following prevalences: ScarPyV1 was detected in 35.2% (31/88 squirrels, 27x positive in spleen, 2x lung and 2x in spleen and lung) of *Sciurus carolinensis*, SvarPyV1 in 14.3% (1/7 spleen samples positive) of *Sciurus variegatoides*, CeryPyV1 in 42.9% (15/35 squirrels, 12x positive in spleen and 3x in spleen and lung) of *Callosciurus erythraeus* and CprePyV in 23.5% (4/17 squirrels positive in spleen) of *Callosciurus prevostii* (Table 2). For the herpesviruses, the ScarGHV1 prevalence of the *Sciurus carolinensis* from the UK was 40.8% (20/49 squirrels, 16x positive in spleen and 4x in spleen and lung) and for *Sciurus carolinensis* from the USA prevalence was 60.0% (3/5 spleen samples positive). The second gammaherpesvirus of *Sciurus carolinensis* (ScarGHV2) was detected in 2.0% (1/49 squirrels positive in spleen) and 20.0% (1/5 lung samples) of the samples from Britain and the USA, respectively. The average prevalence of ScarBHV1 amounts 5.7% (5/88 squirrels positive in spleen) and was only found in Eastern grey squirrels from Italy. The *Sciurus vulgaris* from the UK revealed a SvulBHV1-prevalence of 68.4% (13/19 squirrels, 1x positive in spleen, 9x in lung and 3x in spleen and lung) and those from Germany, 31.0% (18/58 squirrels, 1x positive in spleen, 12x in lung and 5x in spleen and lung). In *Callosciurus erythraeus* from Italy, the prevalence of CeryBHV1 was 65.7% (23/35 squirrels, 19x positive in spleen, 1x in lung and 3x in spleen and lung) and that of CeryGHV1 was 14.3% (6/35 squirrels, 5x positive in spleen and 1x in spleen and lung). Richardson's ground squirrels from Canada showed a very high prevalence (90.9%, 10/11 spleen samples positive) of UricGHV1. The herpesvirus prevalence in Prevost's squirrels was 5.9%: Two of seventeen samples were positive, one for CpreBHV1 and the other for CpreGHV1 (Table 3).

In this study we identified one *Sciurus variegatoides* (sample #10291) from a holding in Germany with a co-infection of VSBV-1 and SvarPyV1 and four *Callosciurus prevostii* (samples #10295, #10296, #10303 and #10304) from German holdings that were infected with VSBV-1 and CprePyV1.

Amplification of complete polyomavirus genomes

Complete genomes of ScarPyV1, CeryPyV1, and CprePyV1 were generated by nested LD-PCR using specific tail-to-tail primers (Fig. 1, Additional file 1 and Additional file 2). The LD-PCR products of around 5.2 kbp were sequenced by classical primer-walking (primers not listed) and sequences of the (i) initial generic PCR products, (ii) LD-PCR products, and (iii) specific PCR fragments mentioned above were assembled to final circular genome sequences (Fig. 1). Full PyV genomes or partial PyV sequences are listed with countries of origin and GenBank accession numbers in Table 4.

ORF analysis with Geneious 11.1.5. software revealed the typical PyV genome organization with CDS for the viral capsid proteins VP1, VP2 and VP3, the regulatory proteins STAg and LTAg (but not middle T antigen (MTag)) on the opposite strand, and the NCCR (Fig. 1). As the LTAg CDS of all other mammalian PyVs and some of the STAg CDS are interrupted by an intron, we searched for such introns in the squirrel PyV genomes in silico and experimentally, as described in detail below. In addition, the VP2 ORF of CprePyV1 and ScarPyV1 was found to be interrupted by two (CprePyV1) or three (ScarPyV1) stop codons (not shown). Therefore, splicing of the VP2-encoding late mRNA was predicted and analyzed experimentally in cell culture. In upstream direction of the VP2 coding sequence ScarPyV1 and CprePyV1 display an additional ORF in their genome. In a few PyVs of the genus *Betapolyomavirus*, e.g. SV40 and BK virus, the so-called agnoprotein is encoded at this position. Agnoprotein was identified as a regulatory protein required for efficient virus proliferation. However, the proteins putatively encoded by ScarPyV1 and CprePyV1 do not show similarities with the known agnoproteins, and their function is currently unknown.

Identification of splice sites in early and late regions of the novel polyomaviruses

Splice donor and acceptor sites with high Human Splicing Finder (HSF) rating (75–95) and conserved in sequence and position compared to related PyVs were identified in LTAg CDS of all three squirrel PyVs. In addition, splice sites were predicted for STAg mRNA of CprePyV1, with the splice donor site interrupting the stop codon. The introns of STAg mRNA of ScarPyV1 and CeryPyV1 were found to be located after the stop codon. Finally, an intron was predicted for the VP2 CDS

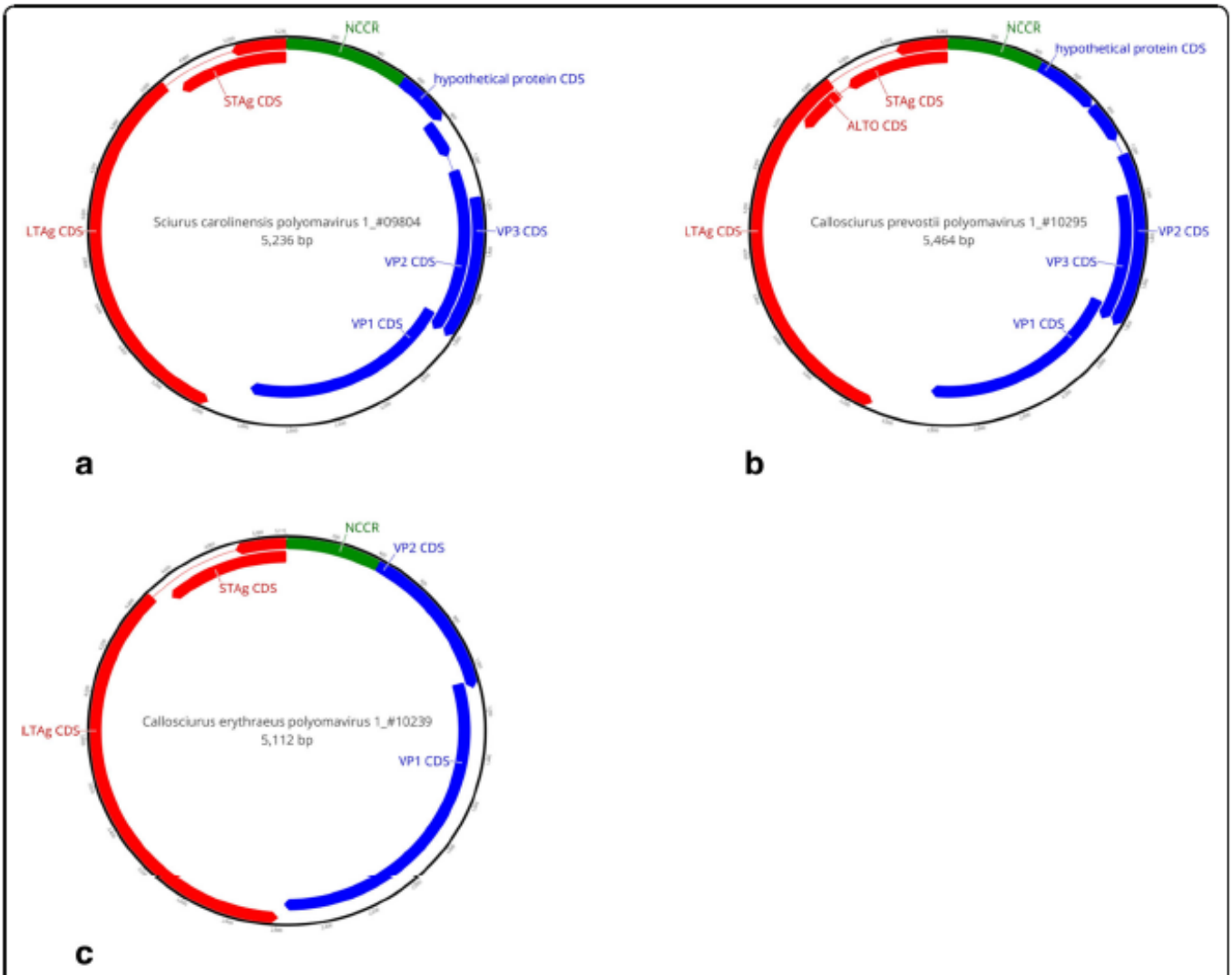

Fig. 1 Genome organization of *Sciurus carolinensis* PyV1 (**a**), *Callosciurus prevostii* PyV1 (**b**) and *Callosciurus erythraeus* PyV1 (**c**). Putative coding regions for VP1, VP2 and VP3 are marked by blue bars; putative STAg antigen and LTAg antigen coding regions are marked by red bars, each with arrow head indicating direction of transcription. Thin lines demarcate introns. Non-coding control region (NCCR) is marked by green bars. An additional hypothetical protein CDS was predicted in the genomes of ScarPyV1 and CprePyV1

of CprePyV1 and ScarPyV1 explaining the occurrence of an interrupted CDS (Fig. 2 and Table 5).

Experimental confirmation of the splice sites predicted for ScarPyV1 was performed with an approach that was used previously for splice site analysis of two human PyVs [54]. First we transfected either the early or late region with flanking sequences of ScarPyV1 (from sample #9804) into monkey Vero cells and murine NIH/3T3 cells, isolated mRNA at the day of transfection and at days 1, 2.5 and 6 after transfection, and synthesized cDNA. Thereafter, a nested PCR was performed with primers that flank the putative introns (Additional file 1). By sequencing of the PCR products, the splice sites predicted for both the early (STAg and LTAg CDS) and the late region (VP2 CDS) were confirmed in Vero and NIH/3T3 cells as described in detail below.

With two nested PCRs that respectively span 300 bp and 500 bp in the early region around the predicted STAg intron, a spliced mRNA was detected in Vero and NIH/3T3 cells that displays an unspliced CDS of 519 bp and encodes the predicted STAg of 172 amino acids (aa). Behind the stop codon (nucleotide (nt) 517–519), a short intron of 72 nt was observed (Fig. 2a and Table 5). With PCR that spans 800 bp in the early region around the predicted LTAg intron, a spliced mRNA was detected in Vero and NIH/3T3 cells from which a spliced CDS of 1.935 kbp (exon 1 and 2 in frame +1) was inferred. It encodes an LTAg of 644 aa (Table 5 and Fig. 2a). The ScarPyV1-encoded TAgs share the 81 N-terminal aa.

Finally, we performed a PCR that spans 400 bp in the late region around the predicted VP2 intron. A spliced

Table 4 Novel polyoma- and herpesvirus sequences deposited in GenBank

Virus name	Country of origin	Sample ID	Sequence length (bp)	Complete genome	GenBank accession number
CeryPyV1	Italy	#10239, #10271, #10275	5112	+	MK671087, MK671088, MK671089
CprePyV1	Germany[a]	#10295, #10296, #10304	5464	+	MK883808, MK883809, MK883810
ScarPyV1	UK	#9804, #9982, #10018	5236, 5237, 5237	+	MK671101, MK671096, MK671097
SvarPyV1	Germany[a]	#10291	213		MK671090
CeryBHV1	Italy	#10257, #10259, #10262	478		MK957142, MK957143, MK957144
CpreBHV1	Germany[a]	#10298	478		MN037512
ScarBHV1	Italy	#10197	412		MN047451
SvulBHV1	UK	#9807, #9808, #9813, #9824	3336, 3336, 3327, 3336		MK671091, MK671092, MK671093, MK671094
	Germany	#9886	3442		MK671095
CeryGHV1	Italy	#10276	472		MK957139
CpreGHV1	Germany[a]	#10305	166		not archivable in GenBank (< 200 bp)
ScarGHV1	UK	#9783, #9800, #9802	3334		MK671098, MK671099, MK671100
ScarGHV2	USA	#10179	166		not archivable in GenBank (< 200 bp)
UricGHV1	Canada	#10168, #10170, #10171, #10173, #10174, #10175	3212		MK671102, MK671103, MK671104, MK671105, MK671106, MK671107
TstrGHV1	USA	#10182, #10183	443		MK957140, MK957141

[a]animals originate from holdings

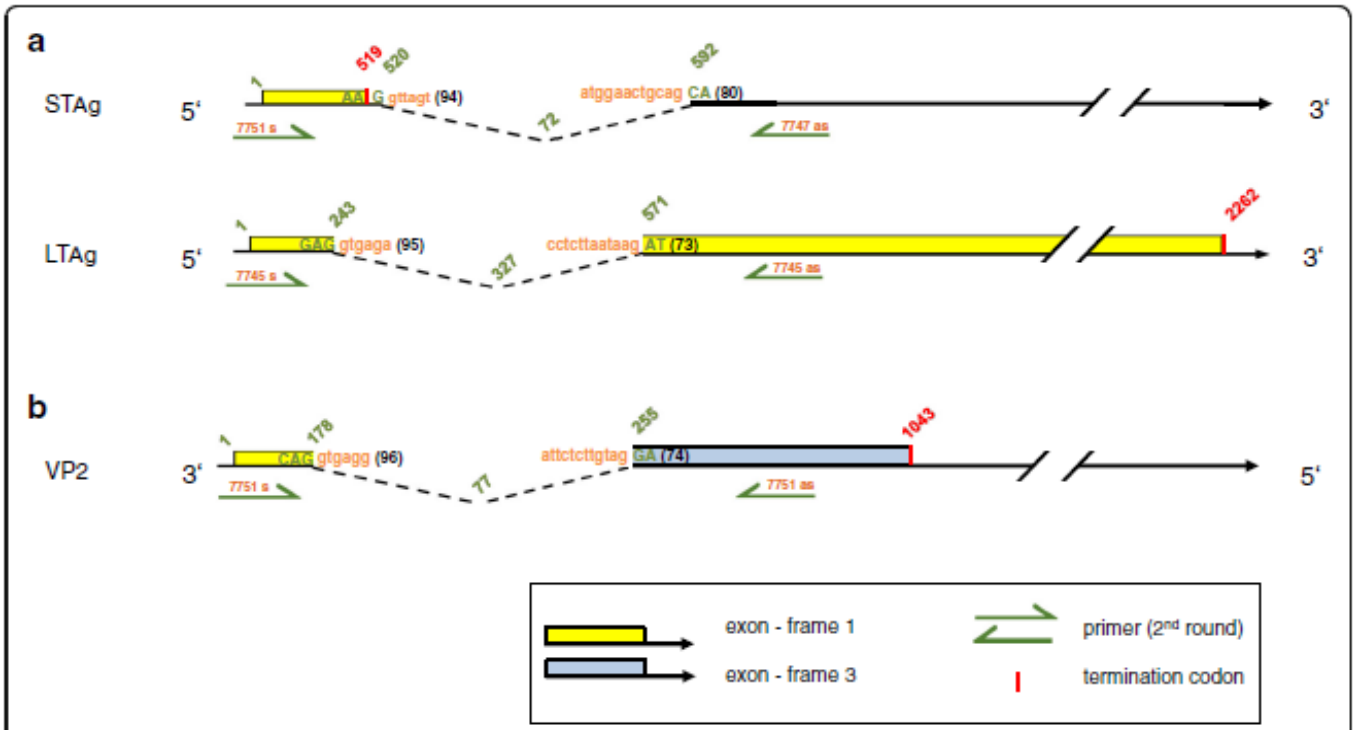

Fig. 2 Scheme of experimentally identified spliced mRNAs of the early (**a**) and late (**b**) region of *Sciurus carolinensis* polyomavirus 1. Experimentally identified STAg, LTAg and VP2 exon sequences are depicted as colored bars, introns as dotted lines, and second-round primers (s = sense, as = antisense) for intron identification with green arrows. Exon sequences are written in green capitals and intron sequences in orange lower case. Their probability predicted by the Human Splicing Finder 3.1. is shown in brackets (score 0–100). The nucleotide positions in green font shown at intron/exon borders represent the first or last nucleotide of the exons. The nucleotide positions in red font represent the last nucleotide of the termination codon

Table 5 Splice donor and acceptor sites in the early and late regions of the novel polyomaviruses

Polyomavirus	CDS[a]	Splice donor site EXON|intron[b]	Splice acceptor site intron|EXON[b]
Sciurus carolinensis polyomavirus 1	STAg	AAG|gttagt (94)	atggaactgcag|CA (80)
	LTAg	GAG|gtgaga (95)	cctcttaataag|AT (73)
	VP2	CAG|gtgagg (96)	attctcttgtag|GA (85)
Callosciurus prevostii polyomavirus 1	STAg	ATG|gtgagt (93)	taccttaacag|AT (85)
	LTAg	GAG|gtaaaa (83)	taccttaacag|AT (85)
	VP2	GAG|gtaaga (96)	ttcctttgtag|GA (87)
Callosciurus erythraeus polyomavirus 1	LTAg	GAG|gtacgt (89)	tgcttctttcag|GA (93)

[a]CDS coding sequence
[b]score (0–100; in brackets) of similarity to the splice consensus site generated by the Human Splicing Finder 3.1

CDS was amplified from cDNA that displays two exons (Table 5 and Fig. 2b).

The experimental results generated for ScarPyV1 were completely in line with the theoretical predictions, i.e., all three predicted introns were detected in both tested cell lines and at different time points of harvesting. The splice sites predicted for LTAg, STAg, and VP2 CDS of CprePyV1 (Table 5) were not studied experimentally because the CprePyV1 genome is closely related to that of ScarPyV1 (see phylogenetic tree analysis below) and the splice sites of both PyVs are conserved in position and sequence. Likewise, splice sites predicted for CeryPyV1 were not studied experimentally as they are conserved in position and sequence with those of the closely related *Philantomba monticola* PyV1, whose sites were experimentally confirmed earlier [65].

Determination of glycoprotein B and extended DNA polymerase nucleotide sequences of herpesviruses

To extend the DPOL gene sequence information for each discovered HV in upstream direction into the adjacent gB CDS, all samples HV-positive in the DPOL PCR were re-evaluated with generic PCRs that were reported earlier [44, 51, 52, 66] to target the gB sequences of either BHVs or GHVs (Additional file 1 and Additional file 3). These generic gB PCRs were less sensitive compared to the generic DPOL PCR, as we amplified a partial gB sequence of ScarGHV1 from only one lung sample of 20 spleen and seven lung samples that had been positive in DPOL PCR. SvulBHV1 gB sequence was amplified from two spleen and three lung samples of 39 DPOL PCR-positive samples and UricGHV1 gB sequence was amplified from all 10 DPOL PCR-positive spleen samples. Next, we closed the sequence gap between the gB and the DPOL sequence (approximately 3.2 kbp) for each of the three HVs with LD-PCR and sequenced the gB-to-DPOL product by classical primer walking. This led to five continuous SvulBHV1 sequences with a length of 3.327–3.442 kbp, three ScarGHV1 sequences of 3.334 kbp, and five UricGHV1 sequences of 3.212 kbp (Table 4).

For ScarBHV1, ScarGHV2, TstrGHV1, CpreBHV1, CpreGHV1, CeryBHV1 and CeryGHV1 the generic gB PCR did not produce the desired gB fragment. Therefore we extended the short DPOL sequences from around 170 bp to > 400 bp by using the outer sense primer of the generic DPOL PCR (285-S DFA; Additional file 1) in combination with two virus-specific antisense primers (Additional file 1) in hemi-nested PCR format. This approach was successful for ScarBHV1 (412 bp; from one sample), CeryBHV1 (478 bp; from three samples), CeryGHV1 (472 bp from one sample), CpreBHV1 (478 bp; from one sample) and TstrGHV-1 (443 bp from two samples), and failed for CpreGHV1 and ScarGHV2. The partial HV sequences are listed with countries of origin and GenBank accession numbers in Table 4.

Phylogenetic analysis of conserved amino acid blocks of the polyomavirus LTAg and VP1 sequences

For evolutionary conclusions and taxonomical classification phylogenetic analyses were done. The ML and MCC trees based on PyV LTAg aa sequences (Fig. 3 and Additional file 4) are quite similar and allow the following conclusions: the novel PyVs can be tentatively assigned to different genera within the family *Polyomaviridae*, as ScarPyV1 and CprePyV1 nest within the diversity of genus *Betapolyomavirus* and CeryPyV1 in the genus *Alphapolyomavirus*. Within the betapolyomaviruses the three ScarPyV1 (GenBank Accession numbers MK671096, MK671097 and MK671101) and the three CprePyV1 (GenBank Accession numbers MK883808 - MK883810) cluster together in a well-supported monophyletic group, which also comprises another rodent polyomavirus, Glis glis polyomavirus 1 (GgliPyV1, GenBank Accession number MG701352), and *Delphinus delphis* polyomavirus 1 (DdelPyV1, GenBank Accession number KC594077). The close evolutionary relationship of these viruses is strengthened by the observation that their VP2 CDS is interrupted by an intron. This splicing event within the VP2 CDS has been either experimentally verified

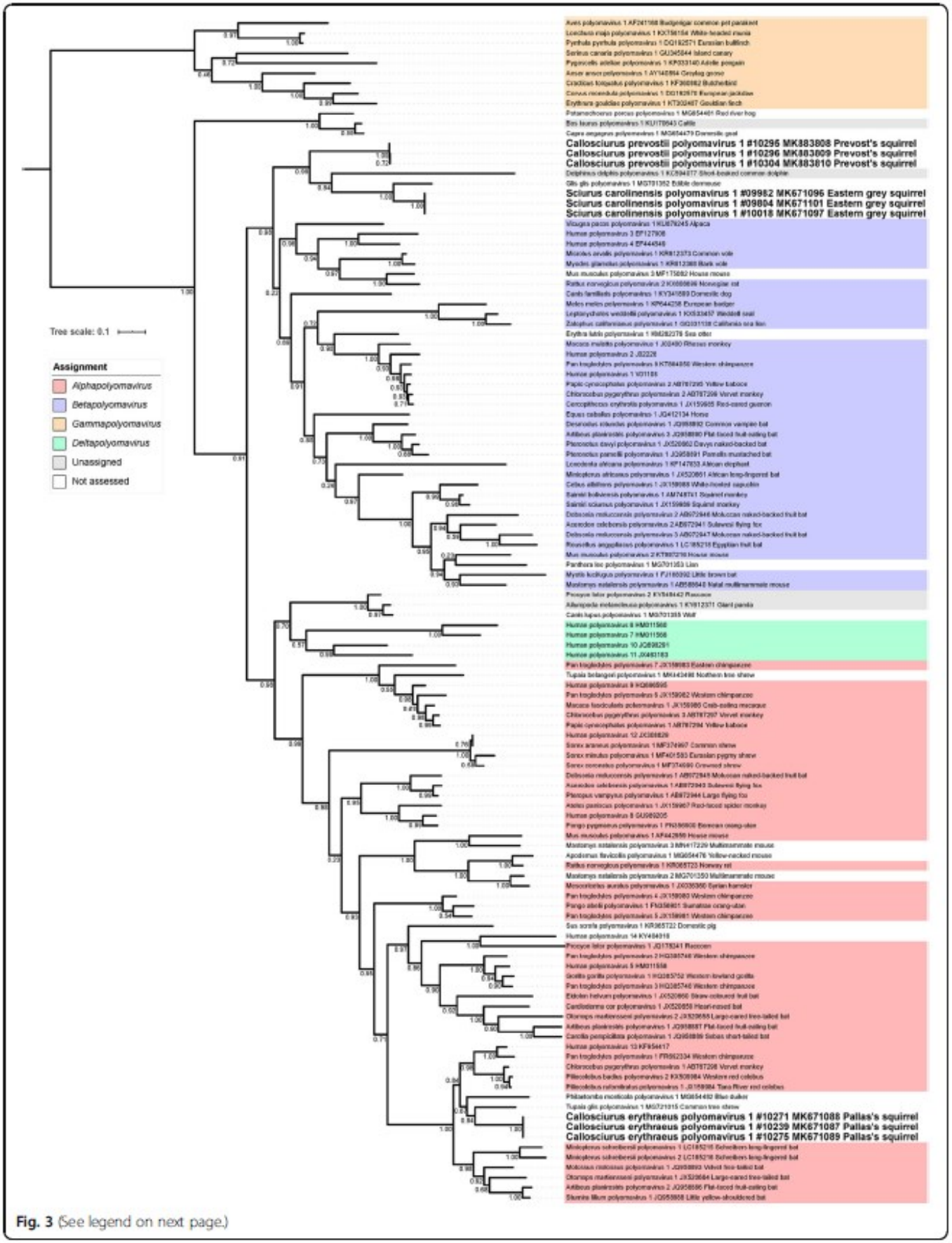

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 Maximum-likelihood (ML) tree analysis of polyomaviruses based on conserved amino acid blocks of the polyomavirus LTAg sequences. Phylogenetic relationships of polyomaviruses, including classification of the novel viruses, based on conserved amino acid blocks of LTAg sequence. Polyomaviruses are denoted by Latin taxonomic names of their hosts, GenBank accession number, common name of the species and – in case of the new viruses – sample ID. For International Committee on Taxonomy of Viruses (ICTV)-recognized species, virus genera are indicated by colors. Viruses newly identified in this study are given in bold font. Branch support was assessed using Shimodaira-Hasagawa-like approximate likelihood ratio tests (SH-like aLRT)

(*Sciurus carolinensis* polyomavirus 1 (this study) and Glis glis polyomavirus 1 [65]) or predicted in silico for *Delphinus delphis* polyomavirus 1, and *Callosciurus prevostii* polyomavirus 1 as the VP2 CDS of all clade members comprise highly conserved splice donor and acceptor motifs with high score (>75) in the Human Splicing Finder 3.1.

All three representatives of the novel CeryPyV1 cluster together within the genus *Alphapolyomavirus*. Although their exact phylogenetic placement is uncertain, they belong to a well-supported clade of polyomaviruses infecting hosts of the orders Artiodactyla, Chiroptera, Primates and Scandentia (Fig. 3 and Additional file 4).

As expected and reported many times, the VP1-based ML and MCC trees (Additional file 5 and Additional file 6) support clades that differ from those delineating genera in the LTAg-based analyses [41]. In these trees, ScarPyV1 and CprePyV1 also formed a weakly supported monophyletic group with GgliPyV1 and DdelPyV1 but this group also included *Canis familiaris* polyomavirus 1 (GenBank Accession number KY341899). This virus may also have a spliced VP2 as it shares conserved VP2 splice sites with the other four polyomaviruses. The evolutionary position of SvarPyV1 (from the variegated squirrel) had to be allocated in the phylogenetic trees based on VP1 (Additional file 5 and Additional file 6), because only a partial VP1 sequence was identified. The single SvarPyV1 sequence clusters within the genus *Alphapolyomavirus* and is a sister virus to a PyV group consisting of an organ-utan and two chimpanzee PyVs. This phylogenetic allocation and the tentative host association of SvarPyV1 will need confirmation, once additional SvarPyV1 sequences from several animals and a complete SvarPyV1 LTAg sequence are available.

Phylogenetic analysis of conserved amino acid blocks of herpesvirus partial DPOL and gB sequences

The ML and MCC trees based on partial DPOL sequences of BHVs (Fig. 4 and Additional file 7) are very similar and show that the four identified squirrel BHVs form a separate cluster that is associated with moderate support to a clade comprising rodent HVs of the genus *Muromegalovirus* and other currently unclassified rodent HVs. The ML and MCC trees based on partial gB (Additional file 8 and Additional file 9) are very similar

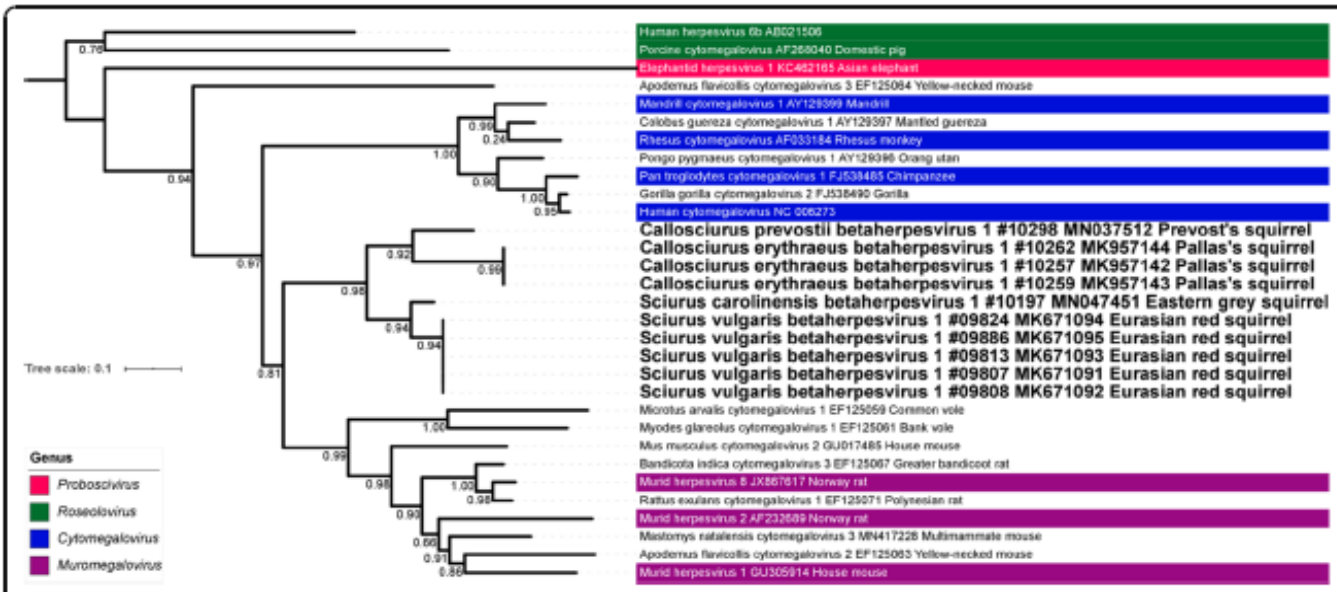

Fig. 4 Maximum-likelihood (ML) tree analysis of betaherpesviruses based on conserved amino acid blocks of the DPOL sequences. Phylogenetic relationships of betaherpesviruses, including classification of the novel viruses, based on conserved amino acid blocks of DPOL sequence. Betaherpesviruses are denoted by Latin taxonomic names or common names of their hosts, GenBank accession number, common name of the species and – in case of the new viruses – sample ID. For ICTV-recognized species, virus genera are indicated by colors. Viruses newly identified in this study are given in bold font. Branch support was assessed using Shimodaira-Hasagawa-like approximate likelihood ratio tests (SH-like aLRT)

to the DPOL-based trees but only the *Sciurus vulgaris* betaherpesvirus 1 sequences are included as for the other three BHVs gB sequences were not available.

The ML and MCC phylogenetic trees based on partial DPOL (Fig. 5 and Additional file 10) and partial gB of GHVs (Additional file 11 and Additional file 12) are also quite similar and show that the squirrel GHVs form two separate groups. One group nests within GHVs of the genus *Rhadinovirus* and forms a weakly supported clade with a group of rodent HVs (in all trees) and a rhadinovirus of the South American tapir (only in DPOL-based trees). The other squirrel HV group nests within a clade that comprises ungulate GHVs of the genus *Macavirus*, a GHV of African elephant and the human Epstein-Barr virus (species *Human herpes virus 4*).

Discussion

The screening of more than 200 squirrels from holdings and sampled wildlife, either indigenous or introduced species, from five countries on two continents resulted in the discovery of four novel PyVs, four novel BHVs and six novel GHVs. Phylogenetic analysis allocated the novel squirrel PyVs to the genera *Alphapolyomavirus* and *Betapolyomavirus*, the novel BHVs to the herpesvirus genus *Muromegalovirus*, and the GHVs to the genera *Rhadinovirus* and *Macavirus*. This first description of PyVs, BHVs and GHVs in squirrels of different species increased our knowledge (e.g. [51, 65] on the diversity of PyVs and HVs in rodents. In particular, some of these viruses represent novel highly divergent lineages or sublineages in the corresponding phylogenetic trees.

The results of our PyV and HV investigations furthermore show that (i) the virus-specific nested PCRs have a higher sensitivity than the generic PCRs, thereby increasing the number of PyV- and HV-positive samples and positive individuals and (ii) virus detection was more frequent in spleen, compared to lung samples. The adapted methods were used here for a first prevalence estimation for some of these viruses, allowing an initial comparison of the prevalences in indigenous and introduced populations.

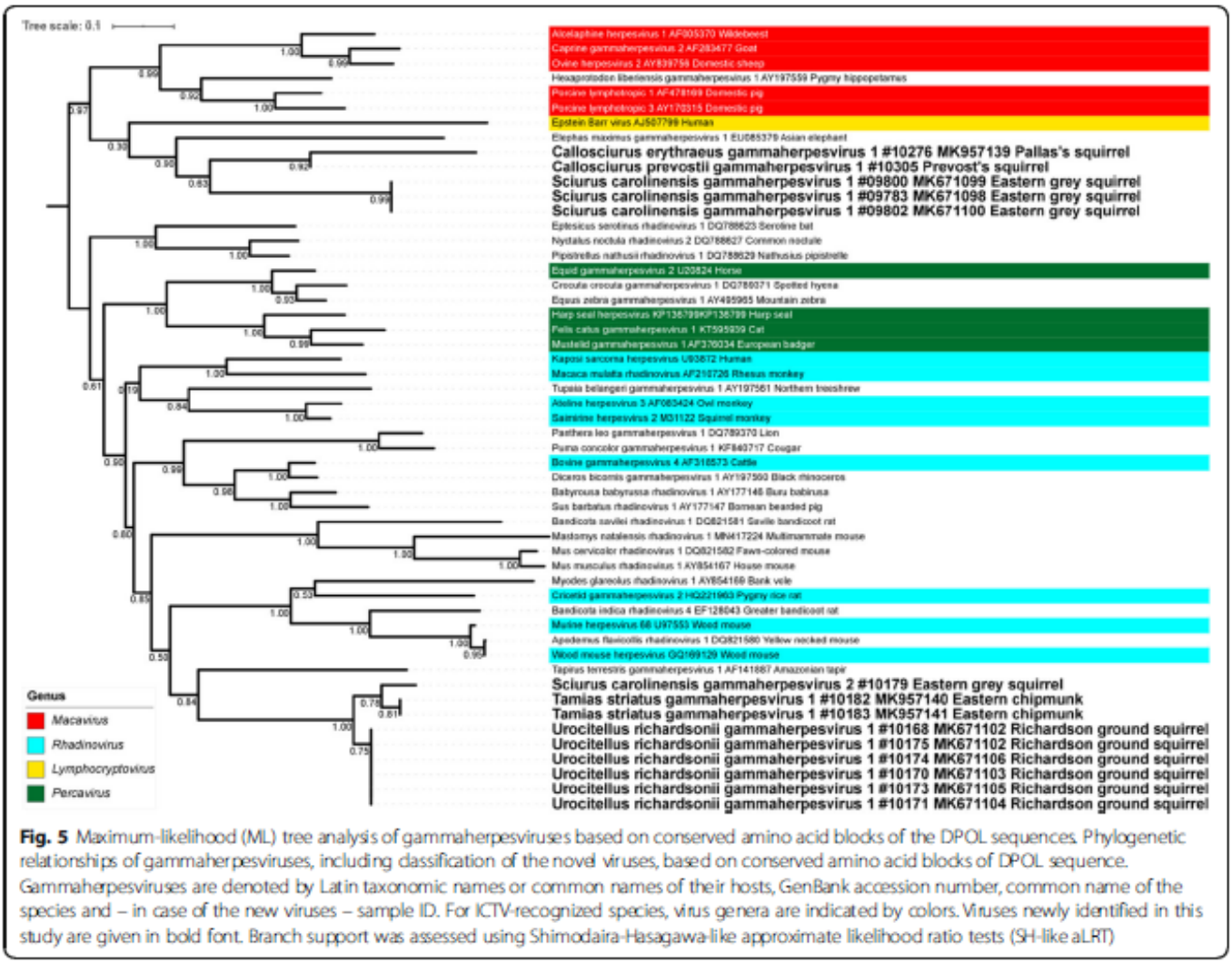

Fig. 5 Maximum-likelihood (ML) tree analysis of gammaherpesviruses based on conserved amino acid blocks of the DPOL sequences. Phylogenetic relationships of gammaherpesviruses, including classification of the novel viruses, based on conserved amino acid blocks of DPOL sequence. Gammaherpesviruses are denoted by Latin taxonomic names or common names of their hosts, GenBank accession number, common name of the species and – in case of the new viruses – sample ID. For ICTV-recognized species, virus genera are indicated by colors. Viruses newly identified in this study are given in bold font. Branch support was assessed using Shimodaira-Hasagawa-like approximate likelihood ratio tests (SH-like aLRT)

Based on the prevalence of the different viruses and their detection in exclusively only one respective squirrel species, it is likely that the viruses are all species-specific and were detected in their respective natural hosts. The fact that the novel squirrel PyVs and HVs or closely related ones were not detected previously in other host species may further strengthen this assumption of host specificity. These investigations thereby improve our knowledge on the host specificity of PyVs and HVs [29, 41].

Our study indicated a broad geographical distribution of some of the novel viruses: ScarPyV1 was detected in the Eastern Grey squirrel from the original range in North America, but also in introduced populations in Great Britain and Italy. Similarly, both ScarGHV1 and ScarGHV2 were detected in the original North American and the introduced British populations. The detection of SvulBHV1 in Eurasian red squirrels from Germany and Scotland might be explained by the long, interlinked history of Eurasian red squirrels on the British Isles with other European populations as discussed previously for the detection of Eurasian red squirrel-associated Squirrel adenovirus 1 (SqAdV-1) strains of high sequence similarity in Germany and Scotland [13].

For three PyVs complete genomes were generated and splice sites were experimentally determined for one of these novel viruses. As reported earlier [65], such experimental determination is critically important for annotation of coding PyV sequences. The search for the reservoir of VSBV-1 within this study resulted in solely negative findings, although additional squirrel species were investigated. This confirms results of our previous study that the Eastern grey squirrel is most likely not the reservoir of bornaviruses [21] and suggests that the Pallas's squirrel (*Callosciurus erythraeus*) is also not a reservoir host for known bornaviruses, at least in the investigated introduced Italian population. It is still unclear and requires further investigation why squirrels of two different subfamilies in German private and zoo holdings which were imported from different geographic origins (*Sciurus variegatoides* and *Callosciurus prevostii*) harbor VSBV-1 sequences [21] of such high similarity. Further screening approaches, including squirrels and other small mammals, will focus on the identification of other possible reservoir hosts of orthobornaviruses in the future. Thereby, it should be evaluated if (i) VSBV-1 was imported with squirrels from Central America or South East Asia and afterwards spread in the German squirrel holdings and holdings in The Netherlands and Croatia, or if (ii) another, yet unknown reservoir host of VSBV-1 exists in Central Europe. Furthermore, experimental infection studies will be done to evaluate the VSBV-1 susceptibility of different squirrel species.

In this study we identified SvarPyV1 in a *Sciurus variegatoides* from a German holding that was tested positive for VSBV-1 in a previous study and CprePyV1 in four *Callosciurus prevostii* from German holdings that were also tested VSBV-1-positive before [17, 21, 22]. These observations indicate viral coinfections in squirrels, confirming results from previous studies in other rodents [67–69]. Evidence for bi-directional interplay of viral and/or bacterial agents, e.g. altering the host's susceptibility, the disease progression, severity of the disease and the host's immune response in rodents have been reported [70, 71]. However, it is currently unknown if the agents investigated in the current study affect each other, and we have no direct evidence if the detected agents are causing clinical signs or pathologic alterations in the investigated squirrels.

Conclusions

This is the first report on molecular identification and sequence characterization of PyVs and HVs in rodents of the family Sciuridae. These findings will allow further targeted screenings of squirrels of the investigated species to analyze the role that these novel viruses play on the population dynamics and competitive interactions in wildlife squirrel populations. Furthermore, the origin of these novel viruses and their spatially and temporally driven evolution in indigenous and introduced populations of grey squirrels, Pallas's squirrels and Prevost's squirrels and questions regarding the interactions of different agents in squirrels are interesting topics for future investigations.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12985-020-01310-4.

Additional file 1. PCR assays for detection of borna-, polyoma- and herpesviruses and mycoplasma.

Additional file 2. Flow chart of multi-level PCR analysis for detection of squirrel polyomaviruses. Generic nested VP1 PCR (second-round product displayed as magenta-coloured bar) with degenerate primers was conducted. For full genome amplification, this was followed by specific nested long-distance PCR (LD-PCR; second-round product of approximately 5 kbp shown as red bar) and overlapping standard nested PCR (second-round product of approximately 800 bp shown as green bar) with specific primers. Grey bars represent coding sequences, black bar the non-coding control region.

Additional file 3. Flow chart of multi-level PCR analysis for detection of squirrel herpesviruses. Generic nested DPOL PCR (product: bar in magenta) with degenerate primers was carried out. For extended sequence determination, this was followed by generic gB PCR (blue) with degenerate primers and subsequent long-distance PCR (LD-PCR) (red) with specific primers. Products of the second PCR rounds are shown. The sequences of the generic DPOL PCR product and the extended DPOL PCR product build a contiguous sequence of 0.4–0.5 kbp (black). The sequences of the generic gB and the generic DPOL PCR product build together with the LD-PCR-derived sequence a contiguous sequence of approximately 3.3 kbp (black). On top of the figure, coding sequences are displayed by grey bars. The arrow heads indicate the direction of transcription.

Additional file 4. Maximum clade credibility tree analysis of polyomaviruses based on conserved amino acid blocks of the LTAg sequences. Phylogenetic relationships of polyomaviruses, including classification of the novel viruses, based on conserved amino acid blocks of LTAg sequence. Branch support values displayed at the nodes correspond to their posterior probability. For further details see legend of Fig. 3.

Additional file 5. Maximum likelihood tree analysis of polyomaviruses based on conserved amino acid blocks of the VP1 sequences. Phylogenetic relationships of polyomaviruses, including classification of the novel viruses, based on conserved amino acid blocks of VP1 sequence. Branch support values displayed at the nodes were assessed using Shimodaira-Hasagawa-like approximate likelihood ratio tests (SH-like aLRT). For further details see legend of Fig. 3.

Additional file 6. Maximum clade credibility tree analysis of polyomaviruses based on conserved amino acid blocks of the VP1 sequences. Phylogenetic relationships of polyomaviruses, including classification of the novel viruses, based on conserved amino acid blocks of VP1 sequence. Branch support values displayed at the nodes correspond to their posterior probability. For further details see legend of Fig. 3.

Additional file 7. Maximum clade credibility tree analysis of betaherpesviruses based on conserved amino acid blocks of the DPOL sequences. Phylogenetic relationships of betaherpesviruses, including classification of the novel viruses, based on conserved amino acid blocks of DPOL sequence. Branch support values displayed at the nodes correspond to their posterior probability. For further explanation see legend of Fig. 4.

Additional file 8. Maximum likelihood tree analysis of betaherpesviruses based on conserved amino acid blocks of the gB sequences. Phylogenetic relationships of betaherpesviruses, including classification of the novel viruses, based on conserved amino acid blocks of gB sequence. Branch support values displayed at the nodes were assessed using Shimodaira-Hasagawa-like approximate likelihood ratio tests (SH-like aLRT). For further explanation see legend of Fig. 4.

Additional file 9. Maximum clade credibility tree analysis of betaherpesviruses based on conserved amino acid blocks of the gB sequences. Phylogenetic relationships of betaherpesviruses, including classification of the novel viruses, based on conserved amino acid blocks of gB sequence. Branch support values displayed at the nodes correspond to their posterior probability. For further explanation see legend of Fig. 4.

Additional file 10. Maximum clade credibility tree analysis of gammaherpesviruses based on conserved amino acid blocks of the DPOL sequences. Phylogenetic relationships of gammaherpesviruses, including classification of the novel viruses, based on conserved amino acid blocks of DPOL sequence. Branch support values displayed at the nodes correspond to their posterior probability. For further explanation see legend of Fig. 5.

Additional file 11. Maximum likelihood tree analysis of gammaherpesviruses based on conserved amino acid blocks of the gB sequences. Phylogenetic relationships of gammaherpesviruses, including classification of the novel viruses, based on conserved amino acid blocks of gB sequence. Branch support values displayed at the nodes were assessed using Shimodaira-Hasagawa-like approximate likelihood ratio tests (SH-like aLRT). For further explanation see legend of Fig. 5.

Additional file 12. Maximum clade credibility tree analysis of gammaherpesviruses based on conserved amino acid blocks of the gB sequences. Phylogenetic relationships of gammaherpesviruses, including classification of the novel viruses, based on conserved amino acid blocks of gB sequence. Branch support values displayed at the nodes correspond to their posterior probability. For further explanation see legend of Fig. 5.

Abbreviations

aa: Amino acid; as: Antisense; BHVs: Betaherpesviruses; BLASTn: Nucleotide Basic Local Alignment Search Tool; BMCMC: Bayesian Markov chain Monte Carlo; BoDV-1: Borna Disease Virus 1; BoDV-2: Borna Disease Virus 2; bp: Base pairs; CDS: Coding sequence; CeryBHV1: *Callosciurus erythraeus* betaherpesvirus 1; CeryGHV1: *Callosciurus erythraeus* gammaherpesvirus 1; CeryPyV1: *Callosciurus erythraeus* polyomavirus 1; CpreBHV1: *Callosciurus prevostii* betaherpesvirus 1; CpreGHV1: *Callosciurus prevostii* gammaherpesvirus 1; CprePyV1: *Callosciurus prevostii* polyomavirus 1; DdelPyV1: *Delphinus delphis* polyomavirus 1; DMEM: Dulbecco's minimal Eagle medium; DMSO: Dimethyl sulfoxide; dNTP: Dideoxynucleoside triphosphate; DPOL: DNA polymerase; ECACC: European Collection of Authenticated Cell Cultures; FCS: Fetal calf serum; gB: Glycoprotein B; GgliPyV1: Glis glis polyomavirus 1; GHVs: Gammaherpesviruses; HSF: Human Splicing Finder; HVs: Herpesviruses; ICTV: International Committee on Taxonomy of Viruses; kb: Kilobases; kbp: Kilobase pairs; LD-PCR: Long-distance PCR; LTAg: Large T-antigen; MCC: Maximum clade credibility; MCPyV: Merkel cell polyomavirus; ML: Maximum likelihood; MTAg: Middle T-antigen; NCCR: Non-coding control region; nt: Nucleotide; ORF: Open reading frame; PCR: Polymerase chain reaction; PhyML-SMS: PhyML v3 with smart model selection; PST: Posterior set of trees; PyVs: Polyomaviruses; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; s: Sense; ScarBHV1: *Sciurus carolinensis* betaherpesvirus 1; ScarGHV1: *Sciurus carolinensis* gammaherpesvirus 1; ScarGHV2: *Sciurus carolinensis* gammaherpesvirus 2; ScarPyV1: *Sciurus carolinensis* polyomavirus 1; SH-like aLRT: Shimodaira-Hasegawa-like approximate likelihood ratio test; SqAdV-1: Squirrel adenovirus 1; STAg: Small T-antigen; SvarPyV1: *Sciurus variegatoides* polyomavirus 1; SvulBHV1: *Sciurus vulgaris* betaherpesvirus 1; TstrGHV1: *Tamias striatus* gammaherpesvirus 1; UricGHV1: *Urocitellus richardsonii* gammaherpesvirus 1; VSBV-1: Variegated squirrel bornavirus 1

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Authors' contributions

VS did data curation, formal analysis, sample acquisition, investigation of the samples for bornavirus, PyVs and HVs, visualization of the data and was the major contributor in writing the manuscript and editing. PWWL helped organizing samples in the UK, and supported writing the squirrel background part in the original draft and through review and editing. NF and CR provided samples from Italy and were part of the review and editing process. MAS and SM sent samples from the USA and helped review and editing. MVM was involved in the squirrel collection in Italy and supported through review and editing. SCS did the phylogenetic analyses and tree reconstruction. KS investigated squirrel samples for bornavirus belonging to previous studies, did formal analysis, validation of the data and review and editing. MB was responsible for funding acquisition, project administration, supervision and review and editing of the manuscript. RGU took part in the conceptualization, funding acquisition, project administration, supervision, data validation, writing the original draft and review and editing. BE contributed to the conceptualization, data curation, formal analysis, methodology, supervision, data validation, writing the original draft and supporting the review and editing process. The author(s) read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.
Sequences of the novel viruses have been deposited in GenBank with accession numbers MK671087, MK671088, MK671089, MK671090, MK671091, MK671092, MK671093, MK671094, MK671095, MK671096, MK671097, MK671098, MK671099, MK671100, MK671101, MK671102, MK671103, MK671104, MK671105, MK671106, MK671107, MK883808, MK883809, MK883810, MK957139, MK957140, MK957141, MK957142, MK957143, MK957144, MN037512, MN047451.

Ethics approval and consent to participate
Animal carcasses investigated in this study were collected as part of population monitoring in the context of field research and were from animals that had been found dead, died at wildlife rescue centers, or had been humanely culled as part of invasive species or disease control efforts. No animals were directly sacrificed for the purpose of this study. Therefore, the need for ethical approval was not applicable. All relevant guidelines for the use and handling of animals in scientific studies were strictly followed.

Consent for publication
N/A

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

4.2. Publication 2

Borna disease outbreak with high mortality in an alpaca herd in a previously unreported endemic area in Germany

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Borna disease outbreak with high mortality in an alpaca herd in a previously unreported endemic area in Germany

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Abstract

Borna disease virus 1 (BoDV-1) is the causative agent of Borna disease, an often fatal neurologic condition of domestic mammals, including New World camelids, in endemic areas in Central Europe. Recently, BoDV-1 gained further attention by the confirmation of fatal zoonotic infections in humans. Although Borna disease and BoDV-1 have been described already over the past decades, comprehensive reports of Borna disease outbreaks in domestic animals employing state-of-the-art diagnostic methods are missing. Here, we report a series of BoDV-1 infections in a herd of 27 alpacas (*Vicugna pacos*) in the federal state of Brandenburg, Germany, which resulted in eleven fatalities (41%) within ten months. Clinical courses ranged from sudden death without previous clinical signs to acute or chronic neurologic disease with death occurring after up to six months. All animals that underwent necropsy exhibited a non-suppurative encephalitis. In addition, six apparently healthy seropositive individuals were identified within the herd, suggesting subclinical BoDV-1 infections. In infected animals, BoDV-1 RNA and antigen were mainly restricted to the central nervous system and the eye, and sporadically detectable in large peripheral nerves and neuronal structures in other tissues. Pest control measures on the farm resulted in the collection of a BoDV-1-positive bicoloured white-toothed shrew (*Crocidura leucodon*), while all other trapped small mammals were negative. A phylogeographic analysis of BoDV-1 sequences from the alpacas, the shrew and BoDV-1-positive equine cases from the same region in Brandenburg revealed a previously unreported endemic area of BoDV-1 cluster 4 in North-Western Brandenburg. In conclusion, alpacas appear to be highly susceptible to BoDV-1 infection and display a highly variable clinical picture ranging from peracute death to subclinical forms. In addition to horses and sheep, they can serve as sensitive sentinels used for the identification of endemic areas.

KEYWORDS
alpaca, Borna disease virus 1 (BoDV-1), *Bornaviridae*, *Crocidura leucodon*, encephalitis, reservoir

Schulze and Große contributed equally to this work.

1 | INTRODUCTION

Borna disease virus 1 (BoDV-1, species *Mammalian 1 orthobornavirus*, family *Bornaviridae*, order *Mononegavirales*) is the causative agent of Borna disease, an often fatal neurologic condition of horses, sheep and other domestic mammals (Dürrwald, Nowotny, Beer, & Kuhn, 2016; Richt & Rott, 2001). Recently, the zoonotic potential of the virus has been demonstrated by molecular and immunohistochemical detection of several BoDV-1-induced fatal encephalitis cases in humans (Coras, Korn, Kuerten, Huttner, & Ensser, 2019; Korn et al., 2018; Liesche et al., 2019; Niller et al., 2020; Rubbenstroth, Schlottau, Schwemmle, Rissland, & Beer, 2019; Schlottau et al., 2018).

The known BoDV-1 reservoir is the bicoloured white-toothed shrew (*Crocidura leucodon*). The virus is endemic in shrew populations in parts of Southern and Eastern Germany, Austria, Liechtenstein and Switzerland (Bourg et al., 2013; Dürrwald, Kolodziejek, Weissenböck, & Nowotny, 2014; Hilbe et al., 2006; Weissenböck et al., 2017). Incidentally, the virus can be transmitted to a broad range of other mammals, including humans, horses, sheep and New World camelids (Caplazi et al., 1999; Dürrwald et al., 2016; Jacobsen et al., 2010; Korn et al., 2018; Schlottau et al., 2018). These species serve as dead-end hosts to the virus, in which it is strictly neurotropic and induces an immune-mediated non-suppurative encephalitis leading to neurologic deficits, including behavioural abnormalities, apathy, somnolence-like conditions, ataxia and central blindness (Caplazi et al., 1999; Dürrwald et al., 2016; Richt & Rott, 2001; Schmidt, 1951). BoDV-1 infection and Borna disease in New World camelids, such as alpacas (*Vicugna pacos*) and llamas (*Lama glama*), have been described sporadically in the past, but comprehensive reports on their susceptibility and role as BoDV-1 hosts are lacking (Altmann, Kronberger, Schüppel, Lippmann, & Altmann, 1976; Jacobsen et al., 2010; Kobera, 2016; Kobera & Pöhle, 2004; Schüppel, Kinne, & Reinacher, 1994).

New World camelids, which are originally native to the Andean plateau (Altiplano) in South America, have gained considerable popularity in private husbandries worldwide since the 1980s. Alpacas are mainly kept for their fine quality fibre, but also as companion and therapy animals and tourist attractions (Gauly, Vaughan, & Cebra, 2018). In Germany, the number of alpacas registered in the 'Llama & Alpaca Registries Europe' (LAREU; https://www.lareu.org/), Europe´s largest New World camelid database, has increased continuously from less than 500 animals in 2008 to nearly 7,000 individuals in 2019 (Christian Kiesling, personal communication).

Here, we describe a Borna disease outbreak with high mortality on an alpaca farm in the federal state of Brandenburg, Germany. The report provides comprehensive data on the course of disease, neuropathology, diagnostic measures, viral tissue distribution and epidemiology.

2 | MATERIALS AND METHODS

2.1 | Origin of samples

The samples analysed in this study originated from an alpaca farm in the north-western part of the federal state of Brandenburg, Germany. Since 2010, the farm owned an alpaca herd that was housed on two different premises sized 1 hectare (ha) and 0.6 ha (premises 1 and 2, respectively), which are located approximately 1 km apart. In addition, the farm harboured five goats (*Capra aegagrus hircus*), five wallabies (*Macropus agilis*) and approximately 50 chickens (*Gallus gallus*) on premise 1 and a cat (*Felis silvestris catus*) on premise 2. Before 2010, sheep had been kept on the same grasslands.

At the onset of the outbreak in December 2018, the alpaca herd consisted of 22 animals (eight adult males, M1 to M8; twelve adult females, F1 to F12; and two juvenile males, J1 and J2). Two additional adult stallions (M9 and M10) and one adult mare (F13) were newly introduced during April to May 2019 and two foals (J3 and J4) were born in May and August 2019, respectively.

Serum samples were collected from all alpacas on 2 April, 12 June, 11 and September 2019. At these time points, nasal, conjunctival and oral swabs as well as faecal samples were collected from selected diseased and/or seropositive alpacas. Additional sera and/or cerebrospinal fluid (CSF) samples were available from alpacas M1, M4, M5 and M6 and from three healthy goats. Furthermore, serum samples were collected from two persons, who lived on the farm and had regular contact to the diseased animals.

Necropsy was performed on six euthanized or perished animals (M1 to M6) and tissue samples were collected for histopathological analysis, detection of viral antigen and RNA. Furthermore, archived formalin-fixed paraffin-embedded (FFPE) kidney and liver tissue or brain tissue were available from two additional alpacas from the farm, which had died in January 2015 (A1) and August 2016 (A4), respectively.

Frozen brain tissue from two BoDV-1-positive horses (*Equus caballus*) originating from the same region in North-Western Brandenburg in 2016 and 2019 were used for sequencing of BoDV-1 genomes and phylogenetic analysis.

Non-fixed tissue and serum samples were stored at −80°C or −20°C, respectively, for further analysis.

2.2 | Trapping of small mammals

For pest control measures, small mammals were trapped on the private ground of the farm using snap traps during April to August 2019. Approximately 150 traps were baited with a mixture of peanut butter with oat flakes and bacon and distributed over both premises of the alpaca farm, focussing predominantly on premise 1. Collected carcasses were stored at −20°C and transported to the laboratory on dry ice. Dissection followed a standardized protocol and samples were immediately stored at −20°C until further processing and analysis. Species were identified by analysis of partial cytochrome *b* gene sequences according to a previously described method (Schlegel et al., 2012).

2.3 | Detection of BoDV-1 RNA by RT-qPCR

Nucleic acid from fresh-frozen tissue samples, swabs and/or CSF from alpacas, horses and trapped small mammals was extracted using the Nucleo Mag Vet Kit (Macherey & Nagel, Düren, Germany) and KingFisher™

Flex Purification System (Thermo Fisher Scientific) according to the manufacturer´s instructions, with or without a prior extraction step with Trizol or Trizol LS reagent (Life Technologies). RNA from FFPE tissue was extracted as described elsewhere (Boos, Nobach, Failing, Eickmann, & Herden, 2019). A defined copy number of in vitro-transcribed RNA of the eGFP gene was added to each sample during RNA extraction as an internal control (Hoffmann, Depner, Schirrmeier, & Beer, 2006).

BoDV-1 RNA was detected by two RT-qPCR assays (BoDV-1 mix 1 and mix 6) detecting the BoDV-1 phosphoprotein (P) and matrix protein (M) gene, respectively. Samples from small mammals were additionally analysed by RT-qPCR panBorna mix 7.2, which is designed to detect a broad spectrum of orthobornaviruses. All tests were performed as described previously (Schlottau et al., 2018) and primer sequences are provided in Table S1. Primers and probes targeting the beta actin gene (Toussaint, Sailleau, Breard, Zientara, & De Clercq, 2007) and the eGFP RNA (Hoffmann et al., 2006) were employed to assess the RNA quality and the efficacy of RNA extraction and RT-qPCR, respectively. Results were determined as cycle of quantification (Cq) values. RNA dilutions from a persistently BoDV-2-infected cell culture were used as positive controls and for calibration of Cq values from independent RT-qPCR runs.

2.4 | Metagenome analysis by high-throughput sequencing (HTS) analysis

To identify the potential pathogen, nucleic acids extracted from FFPE tissue from different areas of the central nervous system of animal M1 and from frozen brain samples of animal M2 were analysed by high-throughput sequencing (HTS) using a metagenomics approach. Briefly, extracted RNA was reverse-transcribed using a combination of SuperScript™ IV First-Strand Synthesis System (Invitrogen) and NEBNext® Ultra II Non-Directional RNA Second Strand Synthesis Module (New England Biolabs). The cDNA was fragmented to 200 base pairs (bp) length (FFPE material of animal M1) or 550 bp length (frozen material of animal M2) using an M220 Focused-ultrasonicator (Covaris). Afterwards, cDNA libraries were prepared as described previously (Forth et al., 2019; Wylezich, Papa, Beer, & Höper, 2018). During size exclusion, fragments smaller than 400 bp were additionally retained and purified two times with 1.2× Agencourt Ampure XP Beads (Beckman Coulter). Quality control and library quantification were performed following previously described protocols (Wylezich et al., 2018) and the libraries (lib03059-3066) sequenced together on an Ion 540 Chip using an Ion S5 XL instrument (Thermo Fisher Scientific). The data sets containing 2.7–8.5 million reads were analysed using the metagenomics software pipeline RIEMS (Scheuch, Höper, & Beer, 2015).

2.5 | Sequencing of complete and partial BoDV-1 genomes and phylogenetic analysis

Complete or partial BoDV-1 genome sequences from FFPE material of two alpacas (A4, M1) and frozen brain from a BoDV-1-infected shrew were generated by HTS following library preparation as described above. BoDV-1 reads were mapped to a BoDV-1 reference genome (GenBank accession number U04608) using GS Reference Mapper (Newbler version 3.0; Roche).

Partial BoDV-1 genomes (positions 20 to 2,291 according to the complete BoDV-1 genome; U04608) from fresh-frozen tissues were determined by Sanger sequencing of overlapping RT-PCR products. Primers used for amplification and sequencing are shown in Table S1. Sequencing was performed by Eurofins (Cologne).

BoDV-1 sequences generated during this study are available from INSDC databases under accession numbers MN937369 to MN937377.

A phylogenetic tree of partial BoDV-1 genomes covering the complete nucleoprotein (N), X protein and P genes (1,824 nucleotides, representing genome positions 54 to 1,877 of BoDV-1 reference genome U04608) was built to include all sequences generated during this study together with GenBank-derived sequences originating from domestic mammals, humans and shrews from the endemic areas in Central Europe. The analysis was performed using neighbor-joining algorithm and Jukes–Cantor distance model in Geneious 11.1.5 software (Biomatters).

2.6 | Detection of BoDV-1-reactive antibodies using an indirect immunofluorescence assay

All sera and CSF samples from alpacas and goats were tested for the presence of bornavirus-reactive antibodies by indirect immunofluorescence assay (iIFA) using a modification of previously described protocols (Schlottau et al., 2018; Zimmermann, Rinder, Kaspers, Staeheli, & Rubbenstroth, 2014). Briefly, confluent overnight culture of either non-infected Vero cells or non-infected Vero cells mixed with 30% Vero cells persistently infected with BoDV-1 isolate Z65-1 (Schlottau et al., 2018) were air-dried for 2.5 hr and subsequently heat-fixed for two hours at 80°C. Twofold dilution series of samples were prepared in Tris-HCl buffer with Tween 20 (T9039; Sigma-Aldrich) and 50 µl of each dilution was added in parallel to the BoDV-1-positive and BoDV-1-negative wells. After incubation for one hour, the plates were washed three times with phosphate-buffered saline (PBS), followed by incubation with goat anti-llama-IgG DyLight488 (Agrisera) or donkey anti-goat-IgG Alexa Fluor 488 conjugate (Jackson Immunoresearch) for another hour. After a final washing cycle, the assays were analysed by fluorescence microscopy. For each serum dilution, BoDV-1-positive and BoDV-1-negative wells were compared. Wells were considered positive, when the expected 30% BoDV-1-positive cells stained markedly brighter than the background staining of uninfected cells in the same well and in the corresponding bornavirus-negative control well. Human serum samples were analysed using a slightly modified procedure as published by Tappe et al. (2019). All tests were performed with a minimal dilution factor of 20. Samples showing no specific signal were assigned a titre of <20.

2.7 | Histopathology and BoDV-1 antigen detection by immunohistochemistry (IHC)

Tissue samples were collected from seven alpacas (A1, A4 and M1 to M6). All tissue samples were immersion-fixed in 4% formaldehyde for up to 48 hr. Formaldehyde-fixed tissues were embedded in paraffin, cut at 1-µm thickness and subsequently stained with haematoxylin and eosin for histological examination.

For immunohistochemical detection of BoDV-1 antigen, sections of FFPE tissue from each animal were dewaxed in xylene, followed by rehydration in descending concentrations of ethanol. After blocking of endogenous peroxidase, slides were incubated with either polyclonal rabbit anti-BoDV-1 P (4,000-fold diluted) or N serum (8,000-fold diluted) overnight at 4°C (Zimmermann et al., 2014). Irrelevant, immunopurified normal rabbit immunoglobulin (Ig) (BioGenex) in PBS at the same dilution served as negative control. Sections were incubated with biotinylated goat anti-rabbit-IgG antibody (200-fold diluted; Vector) for 30 min and subsequently treated with an avidin–biotin–peroxidase complex (ABC; Vector) for 30 min at room temperature. Following exposure to 3,3′-diaminobenzidine tetrahydrochloride (DAB) for eight minutes, slides were counterstained with haematoxylin, dehydrated in ascending concentrations of ethanol, cleared in xylene, and coverslipped.

An alpaca brain without signs of encephalitis originating from a herd without history of BoDV-1 infection served as negative control and a brain of a horse with confirmed Borna disease served as positive control.

3 | RESULTS

3.1 | Identification of BoDV-1 as the cause of fatalities on an alpaca farm in Brandenburg, Germany

A series of fatalities was reported in a herd of totally 27 alpacas from North-Western Brandenburg. Eleven alpacas died during December 2018 to October 2019 following clinical courses ranging from peracute death to chronic neurologic signs (Figure 1; Table 1). Necropsy was performed for six of these animals that had died during March to October 2019.

Due to the lack of a typical clinical presentation reported for the initial cases, the first two investigated cases (M1 and M2) were subjected to metagenomic analysis as part of a study aiming at the identification of unknown viral causes of encephalitis. The analysis led to the detection of BoDV-1 RNA in central nervous system (CNS) samples of both animals. In FFPE tissue from animal M1, the number of reads classified as BoDV-1 ranged from 2,835 to 37,933 (representing 0.1%–0.5% of the total data sets), whereas only up to 324 BoDV-1 reads (0.005% of the data set) were detected in frozen tissues of animal M2. No other pathogens potentially causing the observed clinical signs and microscopic lesions were identified by metagenomic analysis. The diagnosis was confirmed by BoDV-1-specific RT-qPCR and by detection of BoDV-1 N and P antigen by IHC in the brains of both animals as well as for the subsequently necropsied alpacas M3 to M6 (Table 1). Necropsy was not performed for any of the five remaining alpacas that had died during the outbreak (F1 to F4 and J1) and, thus, CNS tissue from these animals was not available for BoDV-1 detection (Table 1).

Five additional alpacas of the farm had died prior to the described outbreak, between January 2015 and January 2018. Archived FFPE brain tissue was available from a subadult stallion (A4) that had died in August 2016 with non-suppurative encephalitis following neurologic disease. Strikingly, detection of BoDV-1 RNA and antigen in this sample confirmed a BoDV-1 infection (Table 1). Furthermore, FFPE liver and kidney tissues were available for stallion A1 that had died in January 2015. IHC staining indicated the presence of bornavirus N and P antigen in renal nerve fibres of this animal (data not shown) but RT-qPCR failed to detect viral RNA. Archived brain tissue was not available for further confirmation of the infection.

3.2 | Course of Borna disease outbreak

The farm possesses two separate grasslands (premises 1 and 2) located approximately 1 km apart. Usually, the adult stallions were kept on premise 1 and the mares and foals on premise 2. However, from 30 September 2018 on, the two groups had switched premises for 3 months. The first fatality of the current outbreak occurred on premise 1 on 27 December. An adult mare (F1) was euthanized after showing non-specific clinical signs, such as anorexia and apathy,

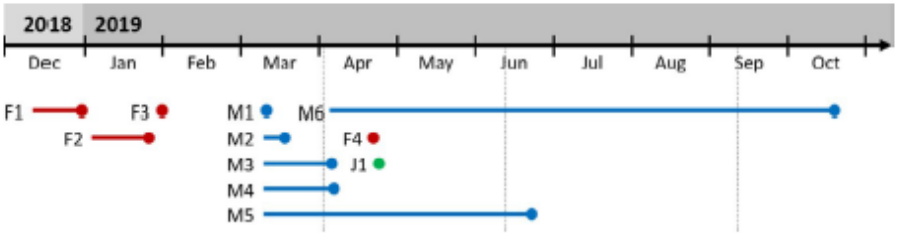


FIGURE 1 Course of disease outbreak on the BoDV-1-affected alpaca farm. Coloured horizontal lines and dots represent duration of disease and time point of death, respectively, of individual animals. Grey dotted vertical lines represent the time points of blood sampling of the complete herd. F = adult female (red), M = adult male (blue) and J = juvenile (green)

TABLE 1 Overview of all fatalities of alpacas reported on the farm from 2010 to 2019

Animal	Sex	Age	Beginning of disease	Death	Duration of disease (days)	Clinical course and signs	BoDV-1 detection [a]
A1	m	3 y.	Unknown	6 January 2015	/	Emaciation, massive endoparasitosis	(pos)[b]
A2	w	/	Unknown	17 February 2016	/	Unknown	/
A3	m	/	Unknown	March 2016	/	Unknown	/
A4	m	1 y.	Unknown	18 August 2016	/	Cachexia, neurologic signs	pos
A5	w	/	Unknown	3 January 2018	/	Unknown	/
F1	f	12 y.	11 December 2018	28 December 2018	17	Reduced general condition, inactivity	/
F2	f	6 y.	2 January 2019	23 January 2019	21	Ataxia, hoisted lips and nostrils, dysphagia, anorexia, weakness	/
F3	f	4 y.	29 January 2019	29 January 2019	0	Sudden death, dyspnoea	/
M1	m	2 y.	9 March 2019	9 March 2019	0	Sudden death	pos
M2	m	9 y.	9 March 2019	16 March 2019	7	Depression, recumbency, weakness	pos
M3	m	7 y.	9 March 2019	3 April 2019	25	Ataxia, head shaking, hypersensitivity, weakness	pos
M4	m	6 y.	9 March 2019	4 April 2019	26	Apathy, ataxia, tremor of the head, later seizures	pos
F4	f	4 y.	20 April 2019	20 April 2019	0	sudden death	/
J1	m	1 y.	22 April 2019	22 April 2019	0	Sudden death	/
M5	m	8 y.	9 March 2019	19 June 2019	102	Ataxia, lack of coordination, opisthotonus, torticollis, head shaking, food material impacted in the cheek, polydipsia, progressing apathy	pos
M6	m	6 y.	4 April 2019	14 October 2019	193	Circle movements, depression, head shaking and severe apathy after six months	pos

Abbreviations: (/), information or sample not available; A, archived; F/f, female; J, juvenile; M/m, male; pos, positive, y., year(s).

[a]Confirmation of BoDV-1 infection by detection of BoDV-1 RNA by RT-qPCR and bornavirus antigen via immunohistochemistry (IHC) from brain tissue.

[b]Only FFPE kidney and liver tissues were available for animal A1. BoDV-1 antigen was detectable by IHC in renal nerve fibres, but detection of viral RNA by RT-qPCR failed.

for 2 weeks (Figure 1; Table 1). Thereafter, the housing of the two groups was reversed again on 1 January 2019 and the adult stallions were transferred back to premise 1. Two further mares (F2 and F3), now located on premise 2, died on 23 and 29 January, respectively. Animal F2 had suffered from neurologic disease for three weeks, whereas F3 died peracutely without showing prior clinical signs (Figure 1; Table 1).

On 9 March, two days after immunization of all alpacas with an inactivated clostridia toxoid vaccine, an adult stallion (M1) on premise 1 died suddenly without previous signs of disease. On the same day, four further stallions were reported to exhibit behavioural changes and neurologic signs (Figure 1; Table 1). These four animals died or were euthanized on 16 March (M2), 3 April (M3), 4 April (M4) and 19 June (M5). An additional adult stallion (M6) on premise 1 showed behavioural changes and slight apathy starting on 4 April. After five months of mild disease, its condition markedly exacerbated during October, requiring euthanasia on 14 October. Furthermore, two sudden fatalities occurred in an adult mare (F4) and a male foal (J1) on premise 2 on 20 and 22 April, respectively (Figure 1; Table 1). The remaining 16 alpacas (M7 to M10, F5 to F13, J2 to J4) as well as all goats, wallabies, chickens and the cat remained apparently healthy throughout the observation period.

3.3 | Clinical signs exhibited by diseased alpacas

Diseased animals during this outbreak exhibited a broad spectrum of clinical signs and varying courses of disease, ranging from sudden fatalities (animals F3, F4, M1, J1) to death after several weeks to months of disease (animals F1, F2, M2 to M6; Table 1).

Animals suffering from sudden fatalities were usually reported as clinically healthy until few hours before death. Animals with acute-to-chronic courses of disease exhibited varying degrees of neurologic signs and behavioural abnormalities, such as polydipsia, hoisted lips and nostrils, dysphagia, incoordination, ataxia, circle movements, opisthotonus, torticollis, tremor, seizures and paralysis,

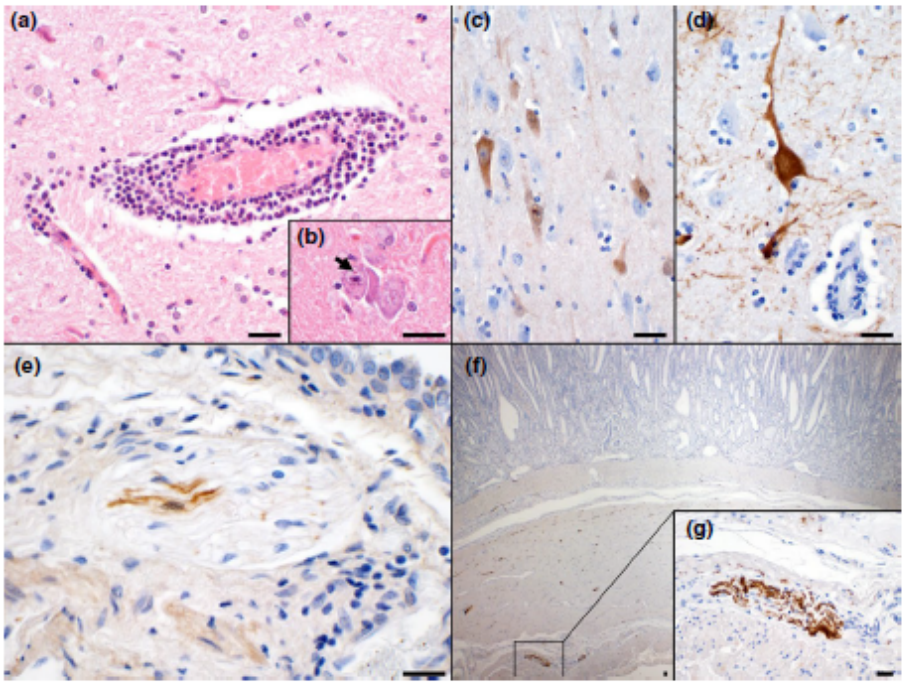


FIGURE 2 BoDV-1 antigen detection in the nervous system of alpacas. (a) A moderate to severe, chronic, multifocal, non-suppurative encephalitis with lymphocytic perivascular cuffing and (b) occasionally distinct, eosinophilic, intranuclear viral inclusions (Joest–Degen inclusion bodies, arrow) was diagnosed (animals M2 and M5, respectively; HE staining). (c) BoDV-1 phosphoprotein and (d) BoDV-1 nucleoprotein were immunohistochemically detected in neurons and axons of the cerebrum (M4). In peripheral organs such as (e) the nasal mucosa of animal M3 and (f,g) the intestine of animal M5. BoDV-1 nucleoprotein was localized in peripheral nerve fibres. Scale bar: 20 µm

but also non-specific signs, such as apathy, anorexia and progressive weight loss. Some animals showed phases of temporary recovery with subsequent exacerbation of the disease.

Two stallions (M4 and M5) had been hospitalized at the Clinic for Ruminants and Swine of the Freie Universität Berlin and were therefore clinically examined in more detail. Animal M4 had been submitted to the clinic on 9 March, when after exhibiting prominent clinical signs. The animal was weak, showed ataxia and progressively diminishing feed intake. Within the following 3 weeks, it developed head tremor and seizures, particularly during handling. Clinical chemistry did not reveal alterations, but differential blood count revealed a left-shift. May–Grünwald–Giemsa-stained blood smears revealed characteristic haemoplasma-like organisms in the cytoplasm or attached to the cell wall of erythrocytes. The organisms were identified as *Mycoplasma haemolamae* by PCR. Antibiotic treatment was initiated with procaine penicillin (40,000 IU/kg daily) because of initially suspected clostridial enterotoxemia and later changed to oxytetracycline (three applications of 20 mg/kg at three-day intervals) after confirmation of *M. haemolamae* infection, but neither treatment resulted in clinical improvement. Following a single dexamethasone dose (2 mg/kg), the animal became considerably more active for three days but its condition progressively exacerbated thereafter, requiring euthanasia on 4 April.

Starting on 9 March, stallion M5 showed stereotypic behaviour, dysphagia and polydipsia for about one week. Thereafter, the behavioural changes subsided but the animal progressively lost weight and became less active. Neurologic signs, including opisthotonus, torticollis, head tremor and ataxia, considerably exacerbated from the beginning of June on. Clinical chemistry and haematology did not reveal marked alterations. The alpaca was admitted to the clinic on 17 June and euthanized two days later.

3.4 | Microscopic lesions in BoDV-1-infected alpacas

Necropsy of the seven confirmed BoDV-1-positive alpacas (A4 and M1 to M6) revealed no relevant macroscopic lesions, but a chronic, multifocal, non-suppurative meningoencephalitis with lymphocytic perivascular cuffing (Figure 2a) and occasional intranuclear Joest–Degen inclusion bodies (Figure 2b) was diagnosed histologically in all animals. The severity of the inflammation ranged from mild (A4, M5, M6) to moderate or severe (M1 to M4). No other significant lesions were identified in any of the examined tissues.

3.5 | BoDV-1 tissue distribution

The tissue distribution of BoDV-1 RNA was determined by semi-quantitative RT-qPCR of tissue samples available for animals M1 to M6 (Figure 3). Highest levels were usually detectable in hippocampus, olfactory bulb and brain stem, followed by cerebellum, spinal cord, optic nerve and retina. Only in animal M6, viral RNA was not found widely distributed in the CNS but restricted mainly to olfactory bulb, hippocampus and eye (Figure 3). Low-to-moderate RNA levels were sporadically detected in peripheral nerves, nasal mucosa and/or salivary glands of alpacas M3, M5 and M6 (Figure 3). Viral RNA was found widely distributed in the organs of animal M5, including lacrimal glands, adrenal gland, gastrointestinal tract and urinary bladder, but it was not detectable in any other peripheral site tested for M1 to M4 and M6 (Figure 3).

Immunohistochemical analysis confirmed BoDV-1 antigen in the brains of all investigated animals. Viral antigen was predominantly

Tissue		Animal:	M1	M2	M3	M4	M5	M6
		Day of death:	March 9	March 16	April 3	April 4	June 19	Oct 14
Brain	Olfactory bulb		13.3	19.2	11.2	13.0	13.1	25.0
	Hippocampus		18.2	17.1	12.0	11.5	12.6	24.0
	Brain stem		13.4	21.1	14.0	14.2	13.7	neg
	Hypophysis						24.9	neg
	Cerebellum		15.1		14.7	18.2	18.4	neg
Spinal cord	Spinal cord (cervical)		14.0		15.4	15.8	13.9	neg
	Spinal cord (thoracal)				23.8	21.9	22.1	neg
	Spinal cord (lumbal)				23.2	23.2	24.9	neg
Eye	Optic nerve (right)				15.1	16.1	18.6	28.2
	Optic nerve (left)			25.1	15.3	19.2	21.2	26.4
	Retina (right)				21.1	28.0	22.8	21.0
	Retina (left)				23.8	25.7	30.1	22.8
Peripheral nerves	Trigeminal nerve & ganglion (right)						17.1	neg
	Trigeminal nerve & ganglion (left)						20.5	27.4
	Facial nerve (right)				26.2	33.3	26.4	neg
	Facial nerve (left)				28.9	neg	26.9	neg
	Brachial plexus (right)				31.2	neg	25.6	neg
	Brachial plexus (left)				neg	31.9	28.2	neg
	Sciatic nerve (right)				neg	neg	31.3	neg
	Sciatic nerve (left)				neg	neg	29.6	neg
Nose & glands	Nasal muscosa (right)				25.5	neg	27.0	neg
	Nasal muscosa (left)				27.0	neg	28.7	neg
	Lacrimal gland (right)						25.8	26.0
	Lacrimal gland (left)						28.9	neg
	Parotid gland (right)						29.3	neg
	Parotid gland (left)						24.6	neg
Other organs	Kidney				neg	neg	neg	neg
	Liver		neg		neg	neg	neg	neg
	Spleen				neg	neg	neg	neg
	Lung				neg	neg	neg	neg
	Heart, septum				neg	neg	neg	neg
	Heart, sinoatrial node				neg	neg	24.0	neg
	Adrenal gland (right)				neg	neg	32.0	neg
	Adrenal gland (left)				neg		25.2	neg
	Stomach				neg	neg	28.2	neg
	Jejunum		neg	neg	neg	neg	27.0	neg
	Colon		neg	neg	neg	neg	28.5	neg
	Urinary bladder						27.3	neg

FIGURE 3 Tissue distribution of BoDV-1 RNA in deceased alpacas. BoDV-1 RNA in six alpacas with available tissue samples (M1 to M6) was detected using semi-quantitative RT-qPCR. Results are presented as minimal cycle of quantification (Cq) value of two RT-qPCR assays (BoDV-1 mix 1 and mix 6) used in parallel. Low Cq values (dark green shadings) represent high viral RNA loads. neg = no BoDV-1 RNA detectable (Cq value > 37)

identified in neurons (Figure 2c and d). In consistence with the RT-qPCR results, BoDV-1 antigen was occasionally detected in peripheral nerves of the nasal mucosa of animals such as M3 (Figure 2e) and in neuronal structures within several organs of animal M5, including the gastrointestinal tract (Figure 2f and g).

Despite the presence of BoDV-1 infection in brain, eye and occasionally nasal mucosa, viral RNA was not detectable in CSF or nasal swabs collected from BoDV-1-positive animals and only very low levels were detected in two conjunctival swabs (Table 2).

3.6 | Screening for bornavirus-reactive antibodies in animals and humans

Serum samples were collected from all alpacas on 2 April, 12 June and 11 September 2019 for detection of BoDV-1-reactive antibodies by IIFA (Table 3). Additional sera and/or CSF samples were available from the diseased alpacas M1 and M4 to M6 (Figure 4).

The four stallions that exhibited a subacute or chronic course of disease (M3 to M6) developed moderate-to-high levels of

bornavirus-reactive antibodies (titres 320–1,600; Table 3). Animal M4 remained seronegative for at least nine days after the onset of disease and reached moderate titres (160–320) from day 16–26 of disease (Figure 4). A CSF sample collected during euthanasia at day 26 showed a similar titre of 400. Animal M5 was first sampled three weeks after the onset of disease, when it already showed high levels of bornavirus-reactive antibodies (titre 1,600). High antibody titres were also detected in three subsequent serum samples from M5 collected during the last week before death (titres 800–1,600) and in a CSF sample (titre 1,280) collected during euthanasia after more than three months of disease (Figure 4). Animal M6 had been tested negative two days before onset of disease and was first tested positive 10 weeks later, reaching a titre of 640 after five months of disease. Notably, a serum sample collected at the day of euthanasia tested negative (Figure 4), while a CSF sample from the same time point was weakly positive (titre 80).

Stallion M1, which had died peracutely, tested likewise positive (titre 320) in a single available serum sample collected at necropsy (data not shown), whereas bornavirus-reactive antibodies were not detectable in sera from animals F4 and J1 collected three weeks before their sudden deaths (Table 3).

Strikingly, seroconversion was observed also for three stallions and three mares that stayed apparently healthy throughout the study period (M7, M8, M10, F6, F8 and F11), reaching maximal titres of 80 to 1,280. Stallion M10 was newly introduced into the farm in May 2019 and first tested positive in September 2019 (Table 3). All remaining alpacas, including the newborn foal J3 originating from the weakly seropositive mare F6, remained seronegative (Table 3). Likewise, bornavirus-reactive antibodies were not detectable in sera collected from three healthy goats on 2 April and from two healthy humans living on the farm (data not shown).

3.7 | BoDV-1 screening of small mammals trapped on the farm premises

During pest control measures on the farm from April to August 2019, 36 small mammals were trapped by the owner and submitted for analysis. The tested animals included two shrews (a bicoloured white-toothed shrew, *Crocidura leucodon* and a common shrew, *Sorex araneus*; order Soricomorpha) and 34 rodents (11 house mice, *Mus musculus*, 16 yellow-necked mice, *Apodemus flavicollis*, five striped field mice, *Apodemus agrarius*, and two common voles, *Microtus arvalis*; order Rodentia). Brain tissue of these animals was used for viral screening, since high viral loads had been reported in the CNS of BoDV-1-infected shrews (Bourg et al., 2013; Dürrwald et al., 2014; Hilbe et al., 2006; Nobach et al., 2015; Puorger et al., 2010; Weissenböck et al., 2017). BoDV-1 RNA was detected in the brain of the bicoloured white-toothed shrew that had been trapped on 29 May on the grassland of premise 1. In contrast, no bornavirus RNA was detectable in the brain of any of the other small mammals.

3.8 | Phylogeny and geographic distribution of BoDV-1 infections in Brandenburg

Complete or partial BoDV-1 sequences were generated from six alpacas for which brain tissue had been available for analysis

TABLE 2 Analysis of BoDV-1 RNA by RT-qPCR in samples collected *intra vitam* from diseased and/or seropositive alpacas

Animal	Day of sampling	CSF	Nasal swabs	Conjunctival swabs	Oral swab	Faeces
M3	2 April	/	neg/neg	neg/neg	/	neg/neg
M4	4 April	neg/neg	neg/neg	**36.7**/neg	/	/
M5	2 April	/	neg/neg	neg/neg	/	neg/neg
	12 June	neg/neg	neg/neg	neg/neg	/	/
M6	11 September	/	neg/neg	neg/neg	neg/neg	/
	14 October	neg/neg	neg/neg	neg/neg	neg/neg	/
M7	12 June	/	neg/neg	neg/**34.2**	/	/
	11 September	/	neg/neg	neg/neg	neg/neg	/
M8	12 June	/	neg/neg	neg/neg	/	/
	11 September	/	neg/neg	neg/neg	neg/neg	/
F6	12 June	/	neg/neg	neg/neg	/	/
	11 September	/	neg/neg	neg/neg	neg/neg	/
F8	11 September	/	neg/neg	neg/neg	neg/neg	/
F11	11 September	/	neg/neg	neg/neg	neg/neg	/

Note: Results are presented as cycle of quantification (Cq) value determined by two semi-quantitative RT-qPCR assays used in parallel (BoDV-1 mix 1 and mix 6).

Abbreviations: (/), sample not available; CSF, cerebrospinal fluid; neg, negative (Cq value > 37).

Positive results are depicted in bold.

TABLE 3 Bornavirus-reactive antibodies in sera of healthy and diseased alpacas

Animal	Sex	Age	Beginning of disease	Death	BoDV-1 detection[a]	Anti-BoDV-1 iIFA titres[b]		
						2 April	12 June	11 September
M3	m	7 y.	9 March	3 April	pos.	**1,280**	/	/
M4	m	6 y.	9 March	4 April	pos.	**320**	/	/
M5	m	8 y.	9 March	19 June	pos.	**1,600**	**1,600**	/
M6	m	6 y.	4 April	14 October	pos.	<20	**160**	**640**
F4	f	4 y.	20 April	20 April	/	<20	/	/
J1	m	1 y.	22 April	22 April	/	<20	/	/
M7	m	8 y.	-	-	/	**160**	**160**	**160**
M8	m	3 y.	-	-	/	**1,280**	**320**	**320**
M9[c]	m	7 y.	-	-	/	/	<20	<20
M10[c]	m	3 y.	-	-	/	/	<20	**160**
F5	f	10 y.	-	-	/	<20	<20	<20
F6	f	9 y.	-	-	/	**80**	**40**	**160**
F7	f	1 y.	-	-	/	<20	<20	<20
F8	f	9 y.	-	-	/	<20	**80**	**80**
F9	f	6 y.	-	-	/	<20	<20	<20
F10	f	6 y.	-	-	/	<20	<20	<20
F11	f	11 y.	-	-	/	<20	**80**	**320**
F12	f	7 y.	-	-	/	<20	<20	<20
F13[c]	f	3 y.	-	-	/	/	<20	<20
J2	m	1 y.	-	-	/	<20	<20	<20
J3[c]	m	4 mo.	-	-	/	/	<20	<20
J4[c]	f	1 mo.	-	-	/	/	/	<20

Abbreviations: (/), sample not available; f, female; m, male; mo., months; y., years.

Positive results are depicted in bold.

[a]Detection of BoDV-1 RNA by RT-qPCR and bornavirus antigen by immunohistochemistry from brain tissue (if available).

[b]Detection of bornavirus-reactive antibodies by indirect immunofluorescence assay (iIFA). Samples were tested at the lowest dilution of 20-fold. Titres of samples without specific signal were regarded as < 20.

[c]Animals were introduced or born during May to August 2019.

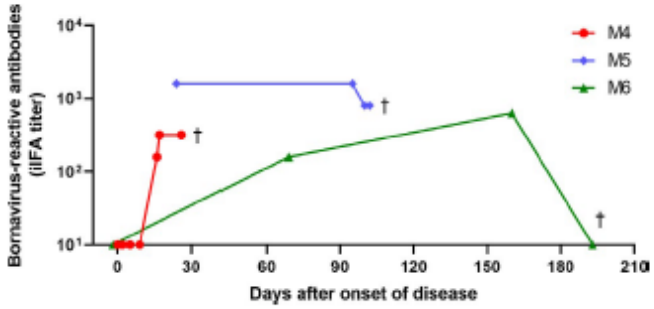
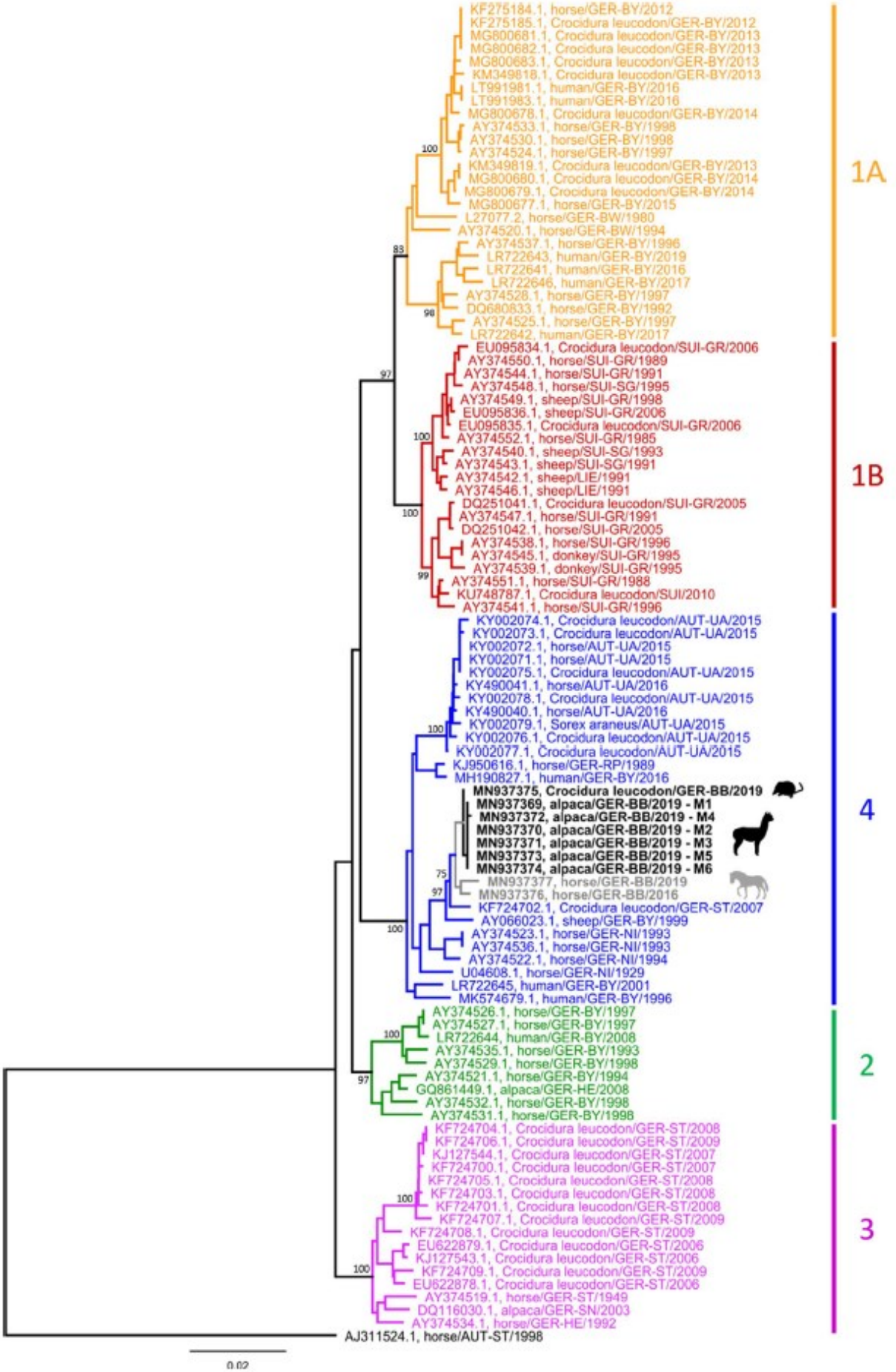


FIGURE 4 Course of bornavirus-reactive antibody titres in three BoDV-1-infected alpacas (M4 to M6). BoDV-1-reactive antibodies were determined by indirect immunofluorescence assays (iIFA). The lower limit of the y-axis represents the detection limit of the assay. †The last sample collected from each animal represents the day of death

(M1 to M6) and from the BoDV-1-positive shrew trapped on the farm. The seven sequences were almost identical to each other, with only up to two nucleotide alterations within the 2,237 nucleotide positions shared by all sequences (99.91%–100% pairwise nucleotide sequence identity). The complete viral genomes available from alpaca M1 (MN937369) and from the BoDV-1-infected shrew (MN937375) differed at six nucleotide positions (99.93% nucleotide sequence identity; data not shown). Phylogenetic analysis revealed the sequences to belong to BoDV-1 cluster 4 (Figure 5). Strikingly, the most closely related sequences originated from recently analysed samples from two BoDV-1-infected horses from North-Western Brandenburg in 2016 and 2019 (MN937376 and MN937377, respectively; Figures 5 and 6), which shared 99.0%–99.5% nucleotide sequence identity with the sequences from the alpaca farm.

Due to the high degree of RNA degradation, HTS from the FFPE brain tissue of animal A4 revealed only a few short reads that were nearly identical to the sequences from the other alpacas and the shrew (data not shown).


KF275184.1, horse/GER-BY/2012
KF275185.1, Crocidura leucodon/GER-BY/2012
MG800681.1, Crocidura leucodon/GER-BY/2013
MG800682.1, Crocidura leucodon/GER-BY/2013
MG800683.1, Crocidura leucodon/GER-BY/2013
KM349818.1, Crocidura leucodon/GER-BY/2013
LT991981.1, human/GER-BY/2016
LT991983.1, human/GER-BY/2016
MG800678.1, Crocidura leucodon/GER-BY/2014
AY374533.1, horse/GER-BY/1998
AY374530.1, horse/GER-BY/1998
AY374524.1, horse/GER-BY/1997
KM349819.1, Crocidura leucodon/GER-BY/2013
MG800680.1, Crocidura leucodon/GER-BY/2014
MG800679.1, Crocidura leucodon/GER-BY/2014
MG800677.1, horse/GER-BY/2015
L27077.2, horse/GER-BW/1980
AY374520.1, horse/GER-BW/1994
AY374537.1, horse/GER-BY/1996
LR722643, human/GER-BY/2019
LR722641, human/GER-BY/2016
LR722646, human/GER-BY/2017
AY374528.1, horse/GER-BY/1997
DQ680833.1, horse/GER-BY/1992
AY374525.1, horse/GER-BY/1997
LR722642, human/GER-BY/2017
1A
EU095834.1, Crocidura leucodon/SUI-GR/2006
AY374550.1, horse/SUI-GR/1989
AY374544.1, horse/SUI-GR/1991
AY374548.1, horse/SUI-SG/1995
AY374549.1, sheep/SUI-GR/1998
EU095836.1, sheep/SUI-GR/2006
EU095835.1, Crocidura leucodon/SUI-GR/2006
AY374552.1, horse/SUI-GR/1985
AY374540.1, sheep/SUI-SG/1993
AY374543.1, sheep/SUI-SG/1991
AY374542.1, sheep/LIE/1991
AY374546.1, sheep/LIE/1991
DQ251041.1, Crocidura leucodon/SUI-GR/2005
AY374547.1, horse/SUI-GR/1991
DQ251042.1, horse/SUI-GR/2005
AY374538.1, horse/SUI-GR/1996
AY374545.1, donkey/SUI-GR/1995
AY374539.1, donkey/SUI-GR/1995
AY374551.1, horse/SUI-GR/1988
KU748787.1, Crocidura leucodon/SUI/2010
AY374541.1, horse/SUI-GR/1996
1B
KY002074.1, Crocidura leucodon/AUT-UA/2015
KY002073.1, Crocidura leucodon/AUT-UA/2015
KY002072.1, horse/AUT-UA/2015
KY002071.1, horse/AUT-UA/2015
KY002075.1, Crocidura leucodon/AUT-UA/2015
KY490041.1, horse/AUT-UA/2016
KY002078.1, Crocidura leucodon/AUT-UA/2015
KY490040.1, horse/AUT-UA/2016
KY002079.1, Sorex araneus/AUT-UA/2015
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KJ950616.1, horse/GER-RP/1989
MH190827.1, human/GER-BY/2016
MN937375, Crocidura leucodon/GER-BB/2019
MN937369, alpaca/GER-BB/2019 - M1
MN937372, alpaca/GER-BB/2019 - M4
MN937370, alpaca/GER-BB/2019 - M2
MN937371, alpaca/GER-BB/2019 - M3
MN937373, alpaca/GER-BB/2019 - M5
MN937374, alpaca/GER-BB/2019 - M6
MN937377, horse/GER-BB/2019
MN937376, horse/GER-BB/2016
KF724702.1, Crocidura leucodon/GER-ST/2007
AY066023.1, sheep/GER-BY/1999
AY374523.1, horse/GER-NI/1993
AY374536.1, horse/GER-NI/1993
AY374522.1, horse/GER-NI/1994
U04608.1, horse/GER-NI/1929
LR722645, human/GER-BY/2001
MK574679.1, human/GER-BY/1996
4
AY374526.1, horse/GER-BY/1997
AY374527.1, horse/GER-BY/1997
LR722644, human/GER-BY/2008
AY374535.1, horse/GER-BY/1993
AY374529.1, horse/GER-BY/1998
AY374521.1, horse/GER-BY/1994
GQ861449.1, alpaca/GER-HE/2008
AY374532.1, horse/GER-BY/1998
AY374531.1, horse/GER-BY/1998
2
KF724704.1, Crocidura leucodon/GER-ST/2008
KF724706.1, Crocidura leucodon/GER-ST/2009
KJ127544.1, Crocidura leucodon/GER-ST/2007
KF724700.1, Crocidura leucodon/GER-ST/2007
KF724705.1, Crocidura leucodon/GER-ST/2008
KF724703.1, Crocidura leucodon/GER-ST/2008
KF724701.1, Crocidura leucodon/GER-ST/2008
KF724707.1, Crocidura leucodon/GER-ST/2009
KF724708.1, Crocidura leucodon/GER-ST/2009
EU622879.1, Crocidura leucodon/GER-ST/2006
KJ127543.1, Crocidura leucodon/GER-ST/2006
KF724709.1, Crocidura leucodon/GER-ST/2009
EU622878.1, Crocidura leucodon/GER-ST/2006
AY374519.1, horse/GER-ST/1949
DQ116030.1, alpaca/GER-SN/2003
AY374534.1, horse/GER-HE/1992
3
AJ311524.1, horse/AUT-ST/1998
0.02

FIGURE 5 Phylogenetic analysis of BoDV-1 sequences from the endemic areas. Phylogenetic analysis of partial BoDV-1 sequences (1,824 nucleotides, representing genome positions 54–1877 of BoDV-1 reference genome U04608) from the endemic regions in Germany, Austria, Switzerland and Liechtenstein was performed using neighbor-joining algorithm and Jukes–Cantor distance model in Geneious 11.1.5 and the tree was rooted with sequence BoDV-2 No/98 (AJ311524). Novel sequences originating from the alpaca farm (black) and from horses in Brandenburg (grey) are depicted in bold. Values at branches represent support in 1,000 bootstrap replicates. Only bootstrap values ≥ 70 at major branches are shown. Germany (GER): BB = Brandenburg, BY = Bavaria, BW = Baden-Wuerttemberg, HE = Hesse, NI = Lower Saxony, RP = Rhineland-Palatinate, SN = Saxony, ST = Saxony-Anhalt; Switzerland (SUI): GR = Grisons, SG = St. Gall; Liechtenstein (LIE); Austria (AUT): UA = Upper Austria, ST = Styria. Cluster designations, host and geographic origin are indicated according to previously published work (Dürrwald, Kolodziejek, Herzog, & Nowotny, 2007; Dürrwald et al., 2014; Kolodziejek et al., 2005; Niller et al., 2020; Nobach et al., 2015; Schlottau et al., 2018; Weissenböck et al., 2017)

4 | DISCUSSION

Here, we describe detailed investigations on a severe Borna disease outbreak on an alpaca farm in Brandenburg, Germany. During the outbreak, eleven out of totally 27 alpacas (40.7%) of the herd had died within a period of ten months. Although diagnostic material allowing BoDV-1 detection was available only from six of these animals, it is conceivable that also the remaining deaths in this outbreak were associated with BoDV-1 infection, since only few fatalities due to other causes had been reported on the farm prior to December 2018. A similarly high mortality of approximately 30% within 14 months had been reported for the only previously described Borna disease outbreak in a New World camelid herd (Schüppel et al., 1994), possibly suggesting a high susceptibility of these species. In contrast, the mortality in horses and sheep is usually low and often restricted to one or few individuals of an otherwise unaffected herd (Caplazi et al., 1999; Metzler, Frei, & Danner, 1976; Richt & Rott, 2001; Vahlenkamp, Konrath, Weber, & Muller, 2002).

The alpacas that developed disease and eventually died during this outbreak showed a highly variable disease progression, ranging from sudden death to chronic disease. The animals exhibited typical neurologic signs and behavioural changes, as well as unspecific clinical courses characterized mainly by loss of appetite and progressive weight loss. Similarly diverse forms of Borna disease have been reported not only for New World camelids, but also for other dead-end hosts, such as equids and sheep, and the time until death has been described to vary from few days to several months after disease onset (Altmann et al., 1976; Caplazi et al., 1999; Heinig, 1964; Jacobsen et al., 2010; Katz et al., 1998; Kobera, 2016; Matthias, 1958; Priestnall et al., 2011; Rott & Becht, 1995; Vahlenkamp et al., 2002; Weissenböck et al., 2017). These findings further emphasize that BoDV-1 has to be considered as a possible cause not only for neurologic disorders but also for untypical clinical presentations in potential dead-end hosts in known endemic areas.

In addition to the clinically affected animals, we identified six alpacas that developed bornavirus-reactive antibodies but remained free of apparent clinical disease throughout the study period. Seropositive animals without clinical signs of Borna disease have been previously reported from BoDV-1-affected sheep herds (Metzler, Ehrensperger, & Danner, 1979; Vahlenkamp et al., 2002). Whether such seroconversion results from subclinical virus persistence or from an abortive infection remains elusive.

Four alpacas had seroconverted in June 2019 or later, including a stallion that had been newly introduced into the herd in May 2019 and had been kept only on premise 1. This observation and the occurrence of a BoDV-1-infected bicoloured white-toothed shrew trapped on premise 1 in May 2019 indicate that the infection source had been present on premise 1 and that the exposure lasted until at least early summer. It remains unknown if exposure to BoDV-1 had also occurred on premise 2 or if the mares and foals that died or seroconverted on this premise had encountered the infection already during their stay on premise 1 at the beginning of the outbreak. Information on the incubation period of Borna disease in naturally infected non-reservoir hosts is scarce. In experimentally BoDV-1-infected horses and sheep, the time point of first clinical signs is highly variable, ranging from less than two weeks to several months after intracranial or intranasal inoculation (Heinig, 1964; Katz et al., 1998; Matthias, 1958; Mayr & Danner, 1974; Nitzschke, 1963; Schmidt, 1951). Similar incubation periods may be assumed for natural infections, since a BoDV-1-infected alpaca and horse were reported to develop Borna disease two and five months, respectively, after translocation from endemic regions in Germany to locations outside the known endemic areas (Jacobsen et al., 2010; Priestnall et al., 2011).

The factors determining the incubation period of Borna disease and the fate of persistently infected animals are poorly understood. In this study, the disease occurred simultaneously in five adult alpaca stallions 2 days after immunization with an adjuvanted clostridia vaccine. However, vaccination of all mares and foals, including three seropositive individuals, with the same vaccine in September 2019 did not induce apparent disease. Borna disease is known to result from immunopathogenesis driven by virus-specific T lymphocytes (Bilzer & Stitz, 1994; Richt et al., 1997; Richt, Stitz, Wekerle, & Rott, 1989; Stitz, Bilzer, & Planz, 2002). Triggering clinical signs by vaccine-induced non-specific activation of immune responses appears therefore possible. In the past, provocation of Borna disease by vaccinating BoDV-1-infected horses and sheep with inactivated bacterial vaccines has been discussed, but experimental evidence is lacking (Matthias, 1958).

BoDV-1 is known to possess a broad cell tropism in bicoloured white-toothed shrews, its known reservoir host, leading to shedding of infectious virus (Dürrwald et al., 2014; Hilbe et al., 2006; Nobach et al., 2015; Puorger et al., 2010; Weissenböck et al., 2017). In contrast,

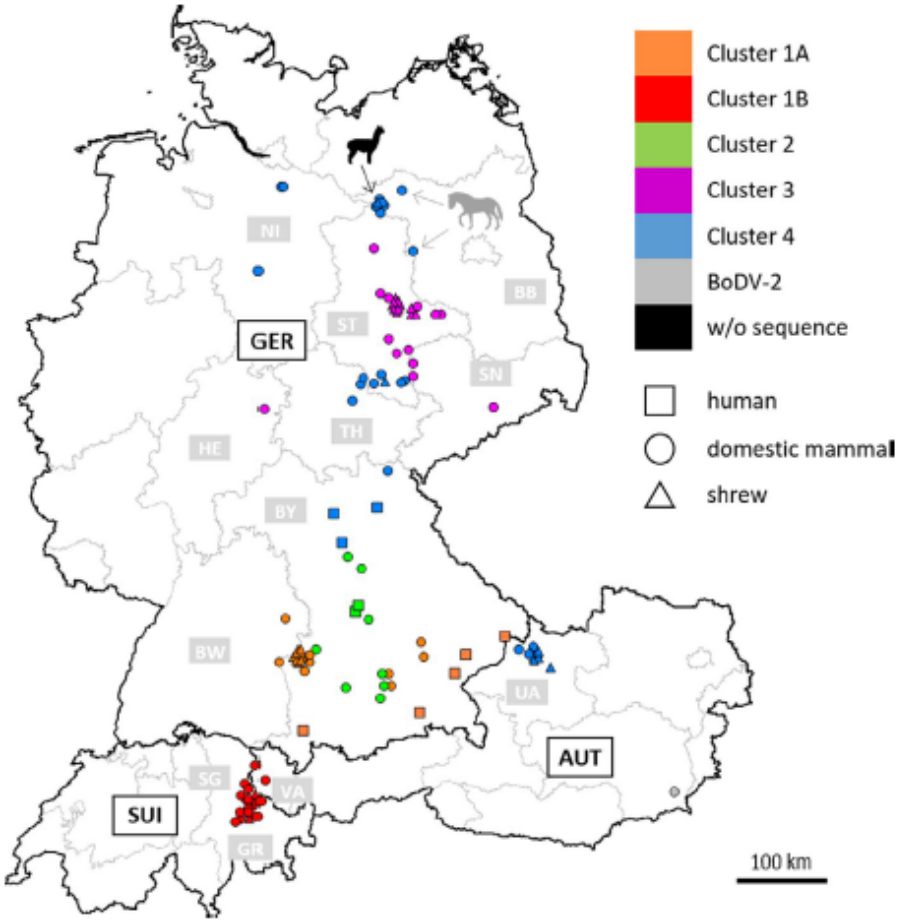

FIGURE 6 Geographic distribution of confirmed BoDV-1 infections of animals and humans. BoDV-1 infections detected in alpacas and horses in Brandenburg reported in this study are presented together with published sequence-confirmed BoDV-1 infections of shrews (triangles), domestic mammals (circles) and humans (squares) with available geographic localization (Dürrwald et al., 2007, 2014; Kolodziejek et al., 2005; Niller et al., 2020; Nobach et al., 2015; Weissenböck et al., 2017). Colours represent regional BoDV-1 sequence clusters (see Figure 5). Germany (GER): BB = Brandenburg, BY = Bavaria, BW = Baden-Wuerttemberg, HE = Hesse, NI = Lower Saxony, SN = Saxony, ST = Saxony-Anhalt, TH = Thuringia; Switzerland (SUI): GR = Grisons, SG = St. Gall; Austria (AUT): UA = Upper Austria, VA = Vorarlberg. w/o = without

it is described as strictly neurotropic and almost exclusively restricted to the CNS in incidentally or experimentally infected immunocompetent non-reservoir hosts (Bilzer, Planz, Lipkin, & Stitz, 1995; Enbergs, Vahlenkamp, Kipar, & Müller, 2001; Herzog, Kompter, Frese, & Rott, 1984; Korn et al., 2018; Liesche et al., 2019; Lipkin, Briese, & Hornig, 2011; Richt & Rott, 2001; Schlottau et al., 2018; Zwick, Seifried, & Witte, 1927). While RT-qPCR confirmed the predominantly CNS-restricted BoDV-1 distribution for the majority of the analysed alpacas, one animal had clearly detectable levels of viral RNA widely distributed in its peripheral nerves and additional tissues, such as nasal mucosa, salivary glands, adrenal gland and gastrointestinal tract, possibly suggesting a retrograde spread of the virus during the particularly long course of disease of this individual. However, IHC analysis confirmed viral antigen in peripheral organs to be clearly restricted to neuronal structures, indicating viral shedding by infected alpacas to be an unlikely event. In congruence, viral RNA was hardly detectable in mucosal swabs collected from several animals with confirmed or suspected BoDV-1 infection during this study. No bornavirus-reactive antibodies were demonstrated in humans who lived on the farm and had close contact to the diseased animals, underscoring that infected dead-end accidental hosts do not transmit the virus to people.

To date, the bicoloured white-toothed shrew is the only known reservoir of BoDV-1 (Bourg et al., 2013; Dürrwald et al., 2014; Hilbe et al., 2006; Nobach et al., 2015; Puorger et al., 2010; Weissenböck et al., 2017). Despite extensive testing of other small mammals within known endemic areas, only a single common shrew tested positive for BoDV-1 (Weissenböck et al., 2017). In congruence with these findings, BoDV-1 infection was detected only in the single bicoloured white-toothed shrew during this study, while a common shrew and all rodents collected during pest control measures on the farm tested negative.

The bicoloured white-toothed shrew is an insectivorous small mammal that feeds on beetles, their larvae and molluscs. They prefer dry open habitats in a dispersal area ranging from Western Europe to Ukraine and Southern Russia (Burgin & He, 2018; Krapp, 1990). Territories of bicoloured white-toothed shrews are usually small with diameters of only up to 120 m and their activity range rarely exceeds 1 km (Burgin & He, 2018). This territory-bound behaviour may well explain the restriction of BoDV-1 infection to populations only in particular parts of Central Europe with apparently little tendency of spreading to neighbouring populations to the East and West (Dürrwald et al., 2014; Weissenböck et al., 2017).

While BoDV-1 is well known to be endemic in parts of Eastern and Southern Germany, the federal state of Brandenburg had not been confirmed as a BoDV-1-endemic region in the published literature prior to this work (Dürrwald et al., 2014; Kolodziejek et al., 2005; Weissenböck et al., 2017). Initial epidemiological investigations on the alpaca farm identified several potential links to known endemic regions in Bavaria. These links included a temporary stay of

the complete herd in Bavaria during a flood in their home region in summer 2013 and the import of hay from Bavaria in the winters of 2015/16 and 2017/18. However, the detection of a BoDV-1-positive shrew on the alpaca farm, which possessed a BoDV-1 sequence that was virtually identical to those identified in the alpacas, as well as the detection of closely related BoDV-1 sequences from horses in the same region, unequivocally confirmed a local infection source. Thus, a previously not described endemic area exists in North-Western Brandenburg. The retrospective identification of a BoDV-1-infected alpaca from the herd, which had died in 2016, and a further possibly infected animal in 2015 indicated a previous exposure to the virus already several years before the current outbreak.

The precise route of BoDV-1 transmission from the infected reservoir to spillover hosts, including alpacas, remains elusive. Experimentally, intranasal inoculation of rats, horses and sheep resulted in persistent infection and disease (Carbone, Duchala, Griffin, Kincaid, & Narayan, 1987; Heinig, 1964; Kupke et al., 2019; Matthias, 1958; Morales, Herzog, Kompter, Frese, & Rott, 1988). In rats, viral entrance was shown to occur via olfactory receptor cells and epithelial cells in the nasal and pharyngeal mucosa followed by spread to the CNS via the olfactory nerve (Kupke et al., 2019; Morales et al., 1988). Likewise, the virus may reach the brain by intra-axonal transport in peripheral nerves after inoculation of rats into the footpad (Carbone et al., 1987).

In summary, alpacas appear to be highly susceptible to BoDV-1 infection and they exhibit highly variable progression of infection, including atypical courses of Borna disease, such as sudden fatalities, as well as apparently subclinical infections. This study led to the identification of a previously undescribed endemic area in Northern Germany. The BoDV-1 susceptibility and growing numbers in Europe may render New World camelids ideal sentinels for the identification of BoDV-1 risk areas for domestic animals and humans.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

ETHICAL APPROVAL

Sera, cerebrospinal fluid (CSF) and swab samples were collected from alpacas and goats during this outbreak for veterinary diagnostic purposes. Handling and sampling of live animals were performed by trained personnel, with animal safety and welfare as first priority. Organ samples from alpacas and horses originated from animals that had died or had to be euthanized due to animal welfare reasons. Small mammal carcasses originated from pest control measures. No animals were killed for the purpose of this study. Informed consent was obtained from the human subjects, who were serologically screened for bornavirus-reactive antibodies in this study. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. All relevant guidelines for the use of animals in scientific studies were followed.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Chapter 5: Discussion

5 Discussion

5.1. Squirrels as hosts of the Mammalian orthobornavirus 2

Previous studies identified the following five squirrel species harbouring VSBV-1: the variegated squirrel (*Sciurus variegatoides*), the red-tailed squirrel (*Sciurus granatensis*), the Prevost's squirrel (*Callosciurus prevostii*), the Finlayson's squirrel (*Callosciurus finlaysonii*) and the Swinhoei's striped squirrel (*Tamiops swinhoei*) (Schlottau, Hoffmann, et al. 2017; Schlottau, Jenckel, et al. 2017).

Through adding the previous not-tested squirrel species Northern flying squirrel (*Glaucomys sabrinus*), Richardson's ground squirrel (*Urocitellus richardsonii*), Franklin's ground squirrel (*Poliocitellus franklinii*) and Thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) (**Publication 1**, Table 1; unpublished data) the spectrum of investigated squirrels was extended to in total 19 squirrel species from seven different countries (Germany, the Netherlands, Croatia, United Kingdom, Italy, Canada, and the USA). But despite extension of the range of squirrel species tested in the recent study compared to the previous studies (Schlottau, Hoffmann, et al. 2017; Schlottau, Jenckel, et al. 2017) it was not possible to identify any more positive individuals. These results raise again the question whether there is at all a local host, respectively reservoir host that spreads the virus within Germany, respectively Europe, or whether the virus was imported with one of those exotic animals in the course of pet trade. Recent findings suggest that there might have been one certain squirrel breeder, to which all infected squirrels can be traced back (Tappe, Frank, Homeier-Bachmann, et al. 2019).

To determine this issue more precisely investigations of rodents and other small mammals from the originating countries of these species (especially *Sciurus variegatoides* from Central America and *Callosciurus prevostii* from South East Asia) need to be part of the future work. In addition, animal trials with different squirrel species could help to analyse the susceptibility of different squirrel species. It could also be possible that the source of the initial introduction of the infection into the exotic squirrel breedings in Germany was not directly the exotic pet squirrels but that there was an alternative reservoir involved that was not included in all previous investigations and that infected the squirrels. But despite this hypothesis, there is evidence that squirrels are the viral reservoirs for VSBV-1 as they act as typical reservoir hosts without clinical signs (Schlottau, Hoffmann, et al. 2017; Schlottau, Jenckel, et al. 2017).

5.2. Novel polyomaviruses in squirrels

During this study the spectrum of known host-specific polyomaviruses could be extended by four novel PyVs: *Sciurus carolinensis* polyomavirus 1 (ScarPyV1), *Sciurus variegatoides* polyomavirus 1 (SvarPyV1), *Callosciurus prevostii* polyomavirus 1 (CprePyV1) and *Callosciurus erythraeus* polyomavirus 1 (CeryPyV1). For three of them complete genomes were generated (**Publication 1,** Figure 1 and Table 4). Looking more into detail, on the complete genome level there are numerous nucleotide exchanges within the three ScarPyV1 genomes (#9804 = 5,236 bp, #9982 and #10018 = 5,237 bp), which lead to an identity of only 98.8 %. The majority of these sequence differences were observed in LTAg and VP1 coding sequences (CDS) and the NCCR as previously shown for variants of *Sorex araneus* PyV 1 (Gedvilaite et al. 2017).

As a very interesting finding, due to a more detailed analysis of the VP 1 gene, it was possible to identify two variants of the ScarPyV1. For differentiation between the two variants a defined part of the genome (comprising position 577 to 972 of the in total 1068 base pair (bp)-long VP1 gene) was choosen. In this part of the genome there are six nucleotide exchanges at the positions 591, 738, 781, 852, 867 and 930 (Figure 7). All but one of these nucleotide exchanges are silent mutations and therefore do not cause changes on amino acid level. However, the adenine/guanine-nucleotide exchange at position 781 results in substitution of the amino acid isoleucine (variant a) by valine (variant b) and strengthens the idea of two separate variants of this virus.

It is also remarkable that the occurrence of the two different variants can be clearly traced back to the geographical origin of the samples in the UK: variant a exclusively occured in Brampton and Dumfries and variant b was only found in individuals from Penicuik and the Borders Region of Scotland. But there is no obvious geographical barrier, e.g. a river, that might explain the geographically separate detection of these variants. The VP1 variants were not only detected in the three available complete genomes but were also observed in larger sample sets with screening PCR using virus-specific nested primers that target the VP1 CDS (Figure 7). In contrast to all other samples belonging to variant b, in sample #10104 there is exclusively an additional thymine/cytosine-nucleotide exchange at position 930. Similar findings of two single nucleotide substitutions related to the geographical origin of the samples were found in sequences of *Mus musculus* Rhadinovirus 1 (MmusRHV1) differing in

analysed house mice from Afghanistan, Germany and Great Britain (Gertler et al. 2017).

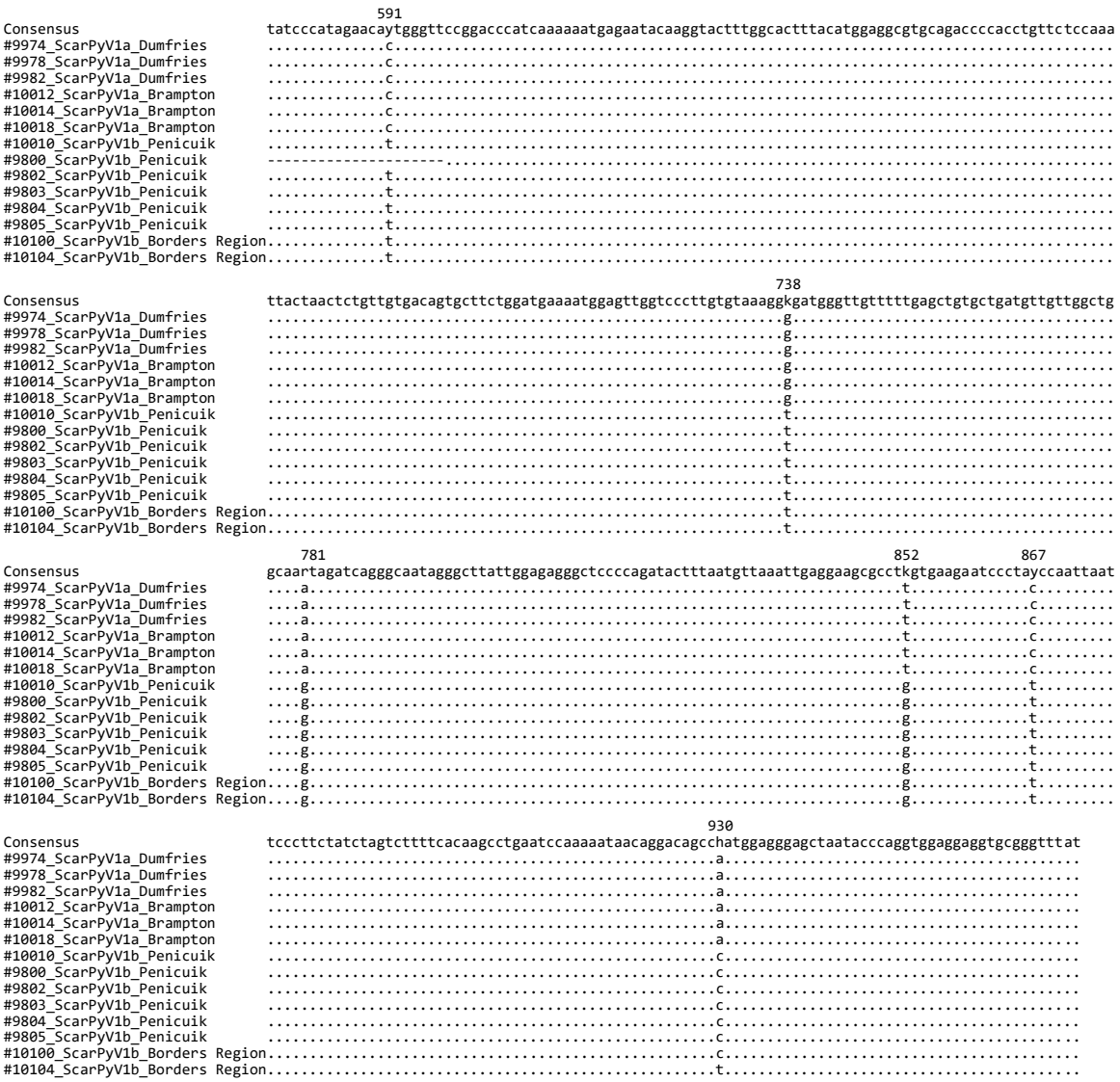

Figure 7: Comparison of the *Sciurus carolinensis* polyomavirus 1 (ScarPyV1) variants a and b in the VP1 gene. Identical nucleotides are marked with dots and differences in the two variants are indicated with the corresponding nucleotide at these positions; the position numbers on top of the figure refer to the complete viral protein (VP) 1 gene (length 1068 bp); sequence names are denoted by sample number, virus variant and geographical origin of the sample; figure was prepared in Geneious version 2019.2 (Biomatters Auckland, New Zealand, available from https://www.geneious.com).

As another noteworth finding the experimentally confirmed and *in silico* predicted splicing patterns of the novel PyVs correspond with the localization of the virus sequences within the phylogenetic trees. The only known viruses with experimentally confirmed splicing events within the VP2 gene, *Glis glis* polyomavirus 1 (GgliPyV1) (Ehlers et al. 2019) and ScarPyV1 (**Publication 1**, Figure 2), are clustering next to each other in the phylogenetic tree (**Publication 1**, Figure 3). In line with this finding also *Delphinus delphis* PyV1, a virus for which the conserved splicing motifs in the VP2 region can also be theoretically identified through analyses with the Human Splicing Finder 3.1., is located next to these sequences. The same scenario can be found in another cluster of the tree, where CeryPyV1 clusters besides *Philantomba monticola* PyV1 and *Tupaia glis* PyV 1 (**Publication 1,** Figure 3). For the latter splicing events were again already experimentally confirmed (Ehlers et al. 2019) and for CeryPyV1 analyses with the Human Splicing Finder 3.1. within the actual studies allow the theoretical prediction of identical splicing motifs.

Within the last decade the number of known polyomaviruses increased rapidly. The majority of novel PyVs was exclusively found in a certain single species and can therefore be classified as strictly host-specific. In congruence with this the novel PyVs were also exclusively found in single squirrel species (**Publication 1**). A great variety of mammalian PyVs is known, also in rodents and shrews, such as the common vole (*Microtus arvalis*) and the bank vole (*Myodes glareolus*) (Nainys et al. 2015), the nutria (*Myocastor coypus*) (da Silva et al. 2018) and the common shrew (*Sorex araneus*), pygmy shrew (*Sorex minutus*) and crowned shrew (*Sorex coronatus*) (Gedvilaite et al. 2017). In addition, a recently published study about the discovery and further characterization of 16 novel polyomaviruses included the following rodent viruses: Norway rat (*Rattus norvegicus* polyomavirus 1, RnorPyV1), yellow-necked mouse (*Apodemus flavicollis* polyomavirus 1, AflaPyV1), edible dormouse (*Glis glis* polyomavirus 1, GgliPyV1) and multimammate mouse (*Mastomys natalensis* polyomavirus 2, MnatPyV2) (Ehlers et al. 2019).

Future work should focus on the transmission routes and pathogenicity of PyVs in animals. In contrast to some of the human PyVs it can be assumed that PyVs in animals are apathogenic and cause no obvious clinical signs. One example showing the opposite is Aves PyV 1 (previous: *Budgerigar fledgling* polyomavirus) that is clearly connected to inflammatory disease in psittacine birds, especially in young

budgerigar (Ma et al. 2019). In consideration of the latter more detailed studies, e.g. animal trials, on the pathogenicity of PyVs in animals are required in the future.

5.3. Novel herpesviruses in squirrels

Herpesviruses are known to infect a variety of different hosts, including Mammalia, Reptilia, Aves, and different fish species (Ehlers 2008). In the recent study the following four novel BHVs and six novel GHVs were identified: *Sciurus carolinensis* betaherpesvirus 1 (ScarBHV1), *Sciurus carolinensis* gammaherpesvirus 1/2 (ScarGHV1/2), *Sciurus vulgaris* betaherpesvirus 1 (SvulBHV1), *Callosciurus prevostii* betaherpesvirus 1 (CpreBHV1), *Callosciurus prevostii* gammaherpesvirus 1 (CpreGHV1), *Callosciurus erythraeus* betaherpesvirus 1 (CeryBHV1), *Callosciurus erythraeus* gammaherpesvirus 1 (CeryGHV1), *Urocitellus richardsonii* gammaherpesvirus 1 (UricGHV1) and *Tamias striatus* gammaherpesvirus 1 (TstrGHV1). These findings contribute to the extension of the important knowledge of DNA viruses in rodents.

The family *Herpesviridae* is one of the largest virus families and includes viruses that infect a broad spectrum of mammalian hosts. But there are nevertheless differences concerning which host species are represented in the different herpesvirus subfamilies. In humans, representatives of all three subfamilies, alpha-, beta-, and gammaherpesviruses are found, while other host species seem to lack herpesviruses of a certain subfamily. For example, in rodents only beta- and gammaherpesviruses have been found, but no alphaherpesviruses (Ehlers et al. 2007; Prepens et al. 2007). This is also in line with findings of this actual study in the squirrels. Finding viruses of a certain subfamily only in samples of certain species but not in others may be due to evolutionary developments, i.e. some herpesvirus species may have existed in the past but died out later, or alternatively have never developed in certain host species. As one of the key factors the choice of appropriate samples can also influence the outcome of the virus detection. E.g. while in this study only spleen and lung samples were used for the screening for novel PyVs and HVs in squirrels, in a previous PyV screening of small mammals a broader sample panel, which additionally included lymph node, liver, kidney and chest cavity fluid, was used (Gedvilaite et al. 2017). Furthermore, the localization of virus latency should be taken into consideration for the choice of the best suited sample. Especially for human viruses, that are intensively studied, latency localizations are known, e.g. BKPyV

(*human polyomavirus 1*) is found in kidney tissue (Gardner et al. 1971) and JCPyV (*human polyomavirus 2*) in the brain (Padgett et al. 1971). The absence of alphaherpesviruses in rodents could therefore also be related to the choice of sample material. Taking into consideration that the human representatives of these subfamily, *herpes simplex virus 1* and *2*, potentially lead to encephalitis (Roizman, Knipe, and Whitley 2013), brain material could also be a suitable sample for virus detection in rodents.

Similar to PyVs it would be interesting to find out more about possible clinical or pathologic alterations in squirrels caused by HV infections. This information could also contribute to a possible optimization of the choice of samples for future studies. No detection of the same virus in different species reinforces the notion of a strong host-specificity and rare host switches in HVs.

5.4. Co-infections in squirrels

In this study squirrels infected with multiple ‘pathogens’ were identified (**Publication 1**): on the one hand detection of a SvarPyV1 coinfection in a *Sciurus variegatoides* from a German holding that was tested positive for VSBV-1 in a previous study (Hoffmann, Tappe, et al. 2015; Schlottau, Hoffmann, et al. 2017; Schlottau, Jenckel, et al. 2017) and on the other hand CprePyV1 coinfections in four *Callosciurus prevostii* that were also tested VSBV-1-positive before (Hoffmann, Tappe, et al. 2015; Schlottau, Hoffmann, et al. 2017; Schlottau, Jenckel, et al. 2017). These data provide an indication that coinfections exist in squirrels and findings in other rodents confirm this assumption (Cvetko et al. 2006; Schmidt et al. 2014; Tadin et al. 2012). There are publications about similar findings in rats. Different studies performing broad range metagenomic HTS apporaches with stool samples from rats, showed the co-existence of a large variety of viral pathogens, even if the number of reads belonging to a particular virus family, varied remarkably (Firth et al. 2014; Sachsenröder et al. 2014). There are also references about modifications, such as influencing the susceptibility of the host, its immune response or the disease progression and severity of the disease, caused by different viral and/or bacterial agents (McAfee et al. 2015; Seki et al. 2004). But further investigations are needed to evaluate if this kind of potential is also available in the ‘pathogens’ investigated in the current study. This should be one topic of the future work.

5.5. The reservoir of BoDV-1

Investigation of small mammals from an alpaca farm in the North-Western part of the federal state of Brandenburg, Germany led to the detection of a BoDV-1-positive bicolored white-toothed shrew, *Crocidura leucodon* (**Publication 2**, Table S2). Despite also testing another 35 trapped small mammals from the alpaca farm and a still unpublished screening of another approximately 5,000 small mammals, including more than 30 species (unpublished data), no other small mammal species could be identified to harbour BoDV-1 or other orthobornaviruses that could have been detected with the broadrange panBorna-RTqPCR. Similar efforts including testing of 257 bat brain samples for orthobornaviruses, did not reveal any other hosts (Nobach and Herden 2020), although bats are in general known to play an important role as reservoirs for many other pathogens. All these negative test results, plus a still unpublished study about another eight positive *Crocidura leucodon* from different BoDV-1 endemic regions, confirmed this species to be up to now the only known reservoir host of BoDV-1 (Bourg et al. 2013; Hilbe et al. 2006; Nobach et al. 2015). Interestingly, at the farm where the pest rodents originated from a severe BoDV-1 outbreak affected also the alpacas. Therefore, this case was a great possibility to exemplary look more into the interactions between virus reservoir and (accidental) dead-end hosts. The study reveals alpacas to be obviously highly susceptible for BoDV-1 (**Publication 2**). It is remarkable that, while in the majority of cases where other dead-end hosts, e.g. horses, are affected, only single individuals from a holding are infected and the rest remains healthy, on the alpaca farm the scenario was remarkably different. A large number of animals from this herd died due to the virus infection and also new infections were observed during the study period on the farm. With identification of these borna disease cases the previously known endemic region of BoDV-1 should be extended in North-Eastern direction. New infections during the investigation period indicate that the reservoir host was still present on the farm. Due to the collected data alpaca-to-alpaca-transmission can be most likely excluded, but there is evidence that transmission/spill-over infections occurred, which make the bicolored white-toothed shrew, *Crocidura leucodon*, the infection source for the alpacas. Despite these postulations, detailed and reliable data on transmission routes are missing. Possible ways of transmission include direct contact in form of biting or scratching as well as indirect transmission via contaminated aerosols, excretions, such as urine or saliva, or contaminated water or food products (e.g.

sometimes it happens that mouse carcasses are by mistake included into hay bales during the manufacturing process).

Furthermore, additional detailed phylogeographic analyses may improve our knowledge about the geographic distribution of the virus and allow a more clearly separation of the different phylogenetic BoDV-1 clusters within Germany and its neighbouring countries. Based on the experiences that were made in the recent study, the future work should more concentrate on a targeted collection of small mammals in BoDV-1 endemic regions and/or in the surrounding of human fatalities due to BoDV-1, the discovery of novel bornaviruses in other species and on gaining more detailed knowledge about the known reservoir host. These data should shed light on potential transmission routes between the shrews and their accidental hosts, which may also be beneficial for establishing a better risk assessment and implementing of preventive measures to avoid new infections. As BoDV-1 recently also raised awareness as a fatal zoonosis, appropriate prevention measures should comprise e.g. providing more information to humans, especially those living with potential dead-end hosts of this virus, living in BoDV-1 endemic regions and those living within the distribution area of *Crocidura leucodon*. In this context also more enlighting of the society is needed and it is important to raise awareness of human physicians, farmers and veterinarians to bornavirus being a possible etiological differential diagnosis in cases of encephalitis. Functional interdisciplinary cooperation between farm owners and research institutions is very important and can also contribute to efforts concerning the development of treatments and vaccines for this up to now not-curable disease. Some of these issues will be part of the future work of the zoonotic bornavirus consortium (ZooBoCo).

Chapter 6: Summary

6 Summary

Rodents and other small mammals, such as shrews, are important reservoirs for numerous zoonotic pathogens. In addition, also various non-zoonotic agents such as polyomaviruses and herpesviruses have been described in these species. The development and broad availability of new methods, in particular High-Throughput Sequencing, has led to a growing number of newly discovered viruses in recent years. This knowledge about novel viruses is of great importance for the development of appropriate diagnostics and control or prevention measures. Nevertheless, it is assumed that only a very small part of the existing viruses is known so far and that the number of other currently unknown pathogens is enormous.

The aim of the present work was to exemplary investigate the reservoir function of squirrels and shrews for selected viruses.

These investigations led to the discovery and further characterization of novel polyomaviruses and beta- and gammaherpesviruses in different squirrel species of the family Sciuridae with conventional polymerase chain reactions (PCRs) and subsequent sequencing using a primer-walking strategy. Full genomes of three squirrel polyomaviruses could be generated this way. In the course of further characterization, splice products of the early and late gene region were identified for *Sciurus carolinensis* polyomavirus 1 (ScarPyV1). Furthermore, the application of polyomavirus-, betaherpesvirus- and gammaherpesvirus-specific PCRs led to a first prevalence estimation of the occurrence of these viruses in their potential reservoir hosts. The multiple detection of the pathogens and high prevalences within the selected species provided indications for the conspicuous host association of these viruses.

In addition, the role of the bicolored white-toothed shrew, *Crocidura leucodon*, in transmission processes of zoonotic bornaviruses was further analysed based on an outbreak of the Borna disease on an alpaca farm in the federal state of Brandenburg, Germany. The present results identify Brandenburg as a previously undescribed endemic area for the borna disease virus 1 (BoDV-1) and confirm the bicolored white-toothed shrew, *Crocidura leucodon,* as the so far only known reservoir host for BoDV-1.

In summary, the data collected in this thesis contribute to a deeper understanding of the virus diversity in squirrels and the remarkable host specificity of the analysed

example viruses. In addition, the studies provide important insights into the role of squirrels as potential virus reservoirs, as well as the bicolored white-toothed shrew as known reservoir host for BoDV-1

Chapter 7: Zusammenfassung

7 Zusammenfassung

Nagetiere und andere Kleinsäuger, wie Spitzmäuse, stellen wichtige Reservoire für zahlreiche Zoonoseerreger dar. Darüber hinaus sind bei diesen Arten auch verschiedene nicht-zoonotische Erreger, wie Polyoma- und Herpesviren beschrieben worden. Die Entwicklung und breite Verfügbarkeit von neuen Methoden, insbesondere die Hochdurchsatz-Sequenzierung, führte in den vergangenen Jahren zu einer wachsenden Zahl neuentdeckter Viren. Diese Kenntnisse über neue Viren sind von großer Bedeutung für die Entwicklung von geeigneter Diagnostik und Kontroll- oder Präventionsmaßnahmen. Nichtsdestotrotz wird davon ausgegangen, dass bisher nur ein sehr kleiner Teil der existierenden Viren bekannt und die Zahl an weiteren derzeit noch unbekannten Erregern enorm ist.

Das Ziel der vorliegenden Arbeit bestand in der exemplarischen Untersuchung der Reservoirfunktion von Hörnchen und Spitzmäusen für ausgewählte Viren.

Diese Untersuchungen führten zur Entdeckung und weiteren Charakterisierung von neuen Polyoma-, und Betaherpesviren und Gammaherpesviren in verschiedenen Hörnchenspezies der Familie Sciuridae mithilfe von konventionellen Polymerase-Kettenreaktionen (PCRs) und anschließender Sequenzierung mittels Primer-Walking-Strategie. Für drei Hörnchen-Polyomaviren konnten auf diese Weise Vollgenome generiert werden. Im Rahmen der weiteren Charakterisierung wurden für *Sciurus carolinensis* Polyomavirus 1 (ScarPyV 1) Spleiß-Produkte der frühen und späten Genregion identifiziert. Des Weiteren führte die Verwendung von Polyomavirus-, Betaherpesvirus- und Gammaherpesvirus-spezifischen PCRs zu einer ersten Prävalenzabschätzung des Vorkommens dieser Viren in ihren potentiellen Reservoirwirten. Der multiple Nachweis der Erreger und die hohe Prävalenz innerhalb der ausgewählten Arten lieferten Hinweise zur spezifischen Wirtsassoziation dieser Viren.

Darüber hinaus wurde anhand eines Ausbruchs der Borna'schen Krankheit in einer Alpakahaltung in Brandenburg, die Rolle der Feldspitzmaus, *Crocidura leucodon*, im Rahmen des Transmissionsgeschehens von zoonotischen Bornaviren weiter analysiert. Die vorliegenden Ergebnisse identifizieren Brandenburg als ein vorher nicht beschriebenes Endemiegebiet für das Borna Disease Virus 1 (BoDV-1) und bestätigen die Feldspitzmaus, *Crocidura leucodon*, als einzigen bisher bekannten Reservoirwirt für BoDV-1.

Zusammenfassend tragen die in dieser Dissertation erhobenen Daten zu einem tieferen Verständnis der Virusvielfalt in Hörnchen und der stark ausgeprägten Wirtsspezifizität der Beispielviren bei. Darüberhinaus liefern die Studien wichtige Erkenntnisse zur Rolle von Hörnchen als potenzielle Virusreservoire, sowie zur Feldspitzmaus, *Crocidura leucodon*, als bekanntem Reservoir für BoDV-1.

Chapter 8: References

8 References

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Chapter 9: Supplement

9 Supplement

9.1. Table S1: Overview about recently discovered viruses associated with rodents and shrews.

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
ssDNA[a] (linear)	*Parvoviridae*		RtCb-ParV-HeB2014-2	*Cricetulus barabensis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtCb-ParV-SX2015	*Cricetulus barabensis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtMr-ParV-JL2014-3	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtMr-ParV-JL2014-1	*Myodes rutilus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtMr-ParV-JL2014-2	*Myodes rutilus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtPl-ParV-Tibet2015	*Phaiomys leucurus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtAa-ParV-HuN2015	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtMc-ParV-YN2014	*Mus caroli*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtNn-ParV-SAX2015	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtNn-ParV-HuB2015	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtNe-ParV-YN2014	*Niviventer eha*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtNf-ParV-HaiN2015	*Niviventer fulvescens*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
			RtRrs-ParV-YN2014	*Rattus andamanensis*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtRn-ParV-GZ2016	*Rattus nitidus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtRn-ParV-ZJ2016	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rat bocavirus	-	*Rattus norvegicus*	Muridae	HTS[b] (Illumina MiSeq instrument), conventional PCR	(Lau et al. 2017; Sachsenröder et al. 2014)
			RtCd-ParV-HeB2014	*Spermophilus dauricus*	Sciuridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
ssDNA (circular)	*Circoviridae*	Rodent circovirus	RtCb-CV-1/HeB2014	*Cricetulus barabensis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtCb-CV-2/HeB2014	*Cricetulus barabensis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtCb-CV-3/HeB2014	*Cricetulus barabensis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtMc-CV-1/Tibet2014	*Microtus clarkei*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtMc-CV-2/Tibet2014	*Microtus clarkei*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtAs-CV/IM2014	*Allactaga sibirica*	Dipodidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtDs-CV/IM2014	*Dipus sagitta*	Dipodidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		Rodent circovirus	RtAc-CV-1/GZ2015	*Apodemus chevrieri*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtAc-CV-2/GZ2015	*Apodemus chevrieri*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtAd-CV/SAX2015	*Apodemus draco*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtBi-CV-1/FJ2015	*Bandicota indica*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtBi-CV-2/FJ2015	*Bandicota indica*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtNe-CV/YN2013	*Niviventer eha*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtNf-CV/HaiN2015	*Niviventer fulvescens*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtRf-CV-1/YN2013	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtRf-CV-2/YN2013	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	RtRs-CV/YN2013	*Rattus andamanensis*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent circovirus	Shrew-CV/Tibet2014	*Sorex araneus*	Soricidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
dsDNA[c] (circular)	*Papilloma-viridae*	Rodent papillomavirus	RtAc-PV/GZ2015	*Apodemus chevrieri*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Apodemus sylvaticus papillomavirus 1	AsPyV 1	*Apodemus sylvaticus*	Muridae	Rolling circle amplification (RCA) and subsequent dideoxy-chain termination sequencing	(Schulz et al. 2012)
		laboratory mouse papillomavirus	MusPV	*Mus musculus*	Muridae	Rolling circle amplification (RCA) and subsequent dideoxy-chain termination sequencing	(Joh et al. 2011)
		Rattus norvegicus papilloma-virus 1/2	RnPV 1/2	*Rattus norvegicus*	Muridae	Rolling circle amplification (RCA) and subsequent dideoxy-chain termination sequencing	(Schulz et al. 2012; Schulz et al. 2009)
		Rodent papillomavirus	RtRn-PV/GD2014	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
dsDNA (circular)	*Polyoma-viridae*	Common vole PyV	CVPyV	*Microtus arvalis*	Cricetidae	conventional PCR (nested format)	(Nainys et al. 2015)
		Bank vole PyV	BVPyV	*Myodes glareolus*	Cricetidae	conventional PCR (nested format)	(Nainys et al. 2015)
		Myocaster coypus polyomavirus 1	McPyV 1	*Myocaster coypus*	Echimyidae	HTS (Illumina MiSeq instrument)	(da Silva et al. 2018)
		Glis glis polyomavirus 1	GgliPyV 1	*Glis glis*	Gliridae	conventional PCR (nested format)	(Ehlers et al. 2019)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		Apodemus flavicollis polyomavirus 1	AflaPyV 1	*Apodemus flavicollis*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2019)
		Mastomys natalensis polyomavirus 1	MnatPyV 1	*Mastomys natalensis*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2019)
		Mus musculus polyomavirus 3	MmusPyV 3	*Mus musuculus*	Muridae	Illumina (HiSeq 2500 system)	(Williams et al. 2018)
		Rattus norvegicus polyomavirus 1	RnorPyV 1	*Rattus norvegicus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2019)
		Rattus norvegicus polyomavirus 2	RatPyV 2	*Rattus norvegicus*	Muridae	Illumina (HiSeq 3000 system)	(Besch-Williford et al. 2017)
		Sorex araneus polyomavirus 1	SaraPyV 1	*Sorex araneus*	Soricidae	conventional PCR (nested format)	(Gedvilaite et al. 2017)
		Sorex coronatus polyomavirus 1	ScorPyV1	*Sorex coronatus*	Soricidae	conventional PCR (nested format)	(Gedvilaite et al. 2017)
		Sorex minutus polyomavirus 1	SminPyV 1	*Sorex minutus*	Soricidae	conventional PCR (nested format)	(Gedvilaite et al. 2017)
dsDNA (linear)	*Adenoviridae*	Rodent adenovirus	RtCb-AdV/HeB2014	*Cricetulus barabensis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent adenovirus	RtCb-AdV/SX2014	*Cricetulus barabensis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent adenovirus	RtEc-AdV/YN2013	*Eothenomys custos*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent adenovirus	RtRn-AdV/GX2016	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		Squirrel Adenovirus 1	SqAdV-1	*Sciurus vulgaris*	Sciuridae	pan-viral microarray (PVM) (version Biochip 6.2) and subsequent HTS (Illumina MiSeq instrument)	(Abendroth et al. 2017; Wernike et al. 2018)
		Rodent adenovirus	RtCd-AdV/HeB2014	*Spermophilus dauricus*	Sciuridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent adenovirus	Shrew-AdV/YN2013	*Crocidura dracula*	Soricidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent adenovirus	Shrew-AdV/ZJ2016	*Suncus murinus*	Soricidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
dsDNA (linear)	*Herpesviridae (Betaherpes-virinae)*	Arvicola terrestris cytomegalovirus 1	AterCMV 1	*Arvicola terrestris*	Cricetidae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Microtus agrestis cytomegalovirus 1	MagrCMV 1	*Microtus agrestis*	Cricetidae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Microtus arvalis cytomegalovirus 1	MarvCMV 1	*Microtus arvalis*	Cricetidae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Myodes glareolus cytomegalovirus 1	MglaCMV 1	*Myodes glareolus*	Cricetidae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Ondatra zibethicus cytomegalovirus 1	OzibCMV 1	*Ondatra zibethicus*	Cricetidae	conventional PCR (nested format)	(Ehlers et al. 2007)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		Apodemus flavicollis cytotomegalovirus 1/2/3	AflaCMV 1/2/3	*Apodemus flavicollis*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Apodemus sylvaticus cytomegalovirus 1	AsylCMV 1	*Apodemus sylvaticus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Bandicota indica cytomegalovirus 1/2/3/4	BindCMV 1/2/3/4	*Bandicota indica*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Mus cervicolor cytomegalovirus 1	McerCMV 1	*Mus cervicolor*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Murine cytomegalovirus	MCMV	*Mus musculus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Rattus exulans cytomegalovirus 1	RexuCMV 1	*Rattus exulans*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Rat cytomegalovirus (strain England)	RVMV-E	*Rattus norvegicus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Rat cytomegalovirus (strain Maastricht)	RCMV-M	*Rattus norvegicus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Rattus rattus cytomegalovirus 1	RratCMV 1	*Rattus rattus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Rattus tiomanicus cytomegalovirus 1	RtioCMV 1	*Rattus tiomanicus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
dsDNA (linear)	*Herpesviridae (Gammaherpesvirinae)*	Microtus agrestis rhadinovirus 1	MagrRHV 1	*Microtus agrestis*	Cricetidae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Myodes glareolus rhadinovirus 1	MglaRHV 1	*Myodes glareolus*	Cricetidae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Apodemus agrarius rhadinovirus 1	AagrRHV 1	*Apodemus agrarius*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Murine gammaherpesvirus	MHV-68	*Apodemus agrarius, A. flavicollis, A. sylvaticus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Apodemus flavicollis rhadinovirus 1	AflaRHV 1	*Apodemus flavicollis*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Apodemus sylvaticus rhadinovirus 1	AsylRHV 1	*Apodemus sylvaticus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Bandicota indica rhadinovirus 1/2/3/4	BindRHV 1/2/3/4	*Bandicota indica*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Bandicota savilei rhadinovirus 1	BsavRHV 1	*Bandicota savilei*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Mus cervicolor rhadinovirus 1	McerRHV 1	*Mus cervicolor*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Mus musculus rhadinovirus 1	MmusRHV 1	*Mus musculus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Rattus exulans rhadinovirus 1/2	RexuRHV 1/2	*Rattus exulans*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		Rattus norvegicus rhadinovirus 1/2	RnorRHV 1/2	*Rattus norvegicus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Rattus rattus rhadinovirus 1/2/3	RratRHV 1/2/3	*Rattus rattus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
		Rattus tiomanicus rhadinovirus 1/2	RtioRHV 1/2	*Rattus tiomanicus*	Muridae	conventional PCR (nested format)	(Ehlers et al. 2007)
dsDNA (linear)	*Poxviridae*	Squirrelpox virus	SQPV	*Sciurus carolinensis, Sc. vulgaris*	Sciuridae	electron microscopy, RT-qPCR	(Atkin et al. 2010; Sainsbury et al. 2008)
dsDNA with reverse transcriptase (linear)	*Hepadna-viridae*	Shrew hepatitis B viruses	HBVs	*Crocidura grandiceps*	Soricidae	conventional PCR (nested format)	(Rasche et al. 2019)
				Crocidura olivieri	Soricidae	conventional PCR (nested format)	(Rasche et al. 2019)
				Sorex araneus	Soricidae	conventional PCR (nested format)	(Rasche et al. 2019)
				Sorex coronatus	Soricidae	conventional PCR (nested format)	(Rasche et al. 2019)
		shrew hepadnavirus	SHBV	*Anourosorex squamipes, Crocidura attenuata, Croc. lasiura*	Soricidae	conventional PCR	(Nie et al. 2019)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
+ssRNA[d] (non-segmented)	*Arteriviridae*	Rodent arterivirus	RtClon-Aterivirus/ NX2015	*Cricetulus longicaudatus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent arterivirus	RtEi-Arterivirus/ SX2014	*Eothenomys inez*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent arterivirus	RtClan-Arterivirus/ GZ2015	*Eothenomys melanogaster*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent arterivirus	RtMc-Arterivirus/ Tibet2014	*Microtus clarkei*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent arterivirus	RtMruf-Arterivirus/ JL2014	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent arterivirus	RtDs-Arterivirus-4/ IM2014	*Dipus sagitta*	Dipodidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent arterivirus	RtDs-Arterivirus-1/ IM2014	*Dipus sagitta*	Dipodidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
+ssRNA (non-segmented)	*Astroviridae*	Rodent astrovirus	RtCb-AstV /HeB2014	*Cricetulus barabensis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtEc-AstV-1/ YN2013	*Eothenomys custos*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtEc-AstV-2/ YN2013	*Eothenomys custos*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		Rodent astrovirus	RtMg-AstV/ XJ2015	*Microtus gregalis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtMc-AstV/ Tibet2014	*Microtus clarkei*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtMf-AstV/ FJ2015	*Microtus fortis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtMruf-AstV-1/JL2014	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtMruf-AstV-2/JL2014	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtMruf-AstV-3/JL2014	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtTt-AstV/ SD2016	*Tscherskia triton*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtAa-AstV-2/ SX2014	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtAa-AstV-1/ SX2014	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtAa-AstV/ XJ2015	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtAa-AstV/ GZ2015	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtAa-AstV/ HuN2015	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtAa-AstV-1/ SD2016	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtAa-AstV-2/ SD2016	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		Rodent astrovirus	RtAc-AstV/GZ2015	*Apodemus chevrieri*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al.2018)
		Rodent astrovirus	RtAp-AstV/ Tibet2014	*Apodemus peninsulae*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtAs-AstV/YN2013	*Apodemus syhaticus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtBi-AstV/FJ2015	*Bandicota indica*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtMm-AstV/IM2014	*Meriones meridianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtMc-AstV/YN2013	*Mus caroli*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtMm-AstV/GD2015	*Mus musculus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtMm-AstV/ZJ2016	*Mus musculus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtMp-AstV/YN2013	*Mus pahari*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtNn-AstV/ Tibet2014	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtNn-AstV/ SAX2015	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtNn-AstV /HuN2015	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtNn-AstV-1/SAX2015	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		Rodent astrovirus	RtNn-AstV-2/SAX2015	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtNn-AstV/ZJ2016	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtNe-AstV/YN2013	*Niviventer eha*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRs-AstV/YN2013	*Rattus andamanensis*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRr-AstV-1/HaiN2015	*Rattus andamanensis*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRr-AstV-2/HaiN2015	*Rattus andamanensis*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRl-AstV-1/HaiN2015	*Rattus losea*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRl-AstV-2/HaiN2015	*Rattus losea*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRl-AstV-1/GD2015	*Rattus losea*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRl-AstV-2/GD2015	*Rattus losea*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRnit-AstV/GZ2015	*Rattus nitidus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRn-AstV/YN2013	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRn-AstV-1/GD2015	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		Rodent astrovirus	RtRn-AstV-2/GD2015	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRn-AstV/ZJ2016	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRf-AstV-1/YN2013	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRf-AstV-2/YN2013	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRf-AstV-3/YN2013	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRf-AstV-4/YN2013	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRf-AstV-5/YN2013	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRf-AstV/ZJ2016	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	RtRf-AstV/GX2016	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	Shrew-AstV/SAX2015	*Sorex araneus*	Soricidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	Shrew-AstV/GX2016	*Sorex araneus*	Soricidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent astrovirus	Shrew-AstV/ZJ2016	*Suncus murinus*	Soricidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
+ssRNA (non-segmented)	*Caliciviridae*	rodent/Manhattan/2013 sapovirus 1/2	Ro-SaV 1/2	*Rattus norvegicus*	Muridae	HTS (Ion Torrent)	(Firth et al. 2014)
		Shrew calicivirus	Shrew-CalV/Tibet2014	*Sorex araneus*	Soricidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
+ssRNA (non-segmented)	*Coronaviridae*	Rodent coronavirus	RtClan-CoV/GZ2015	*Eothenomys melanogaster*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Microtus agrestis alphacoronavirus	-	*Microtus agrestis*	Cricetidae	conventional PCR	(Tsoleridis et al. 2016)
		Rodent coronavirus	RtMg-CoV/XJ2015	*Microtus gregalis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Myodes glareolus alphacoronavirus		*Myodes glareolus*	Cricetidae	conventional PCR	(Tsoleridis et al. 2016)
		Rodent coronavirus	RtMruf-CoV-1/JL2014	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtMruf-CoV-2/JL2014	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtMruf-CoV/HLJ2015	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtMrut-CoV/JL2014	*Myodes rutilus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtAs-CoV/IM2014	*Allactaga sibirica*	Dipodidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtAa-CoV/SX2014	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtAa-CoV/XJ2015	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		Rodent coronavirus	RtAa-CoV/GZ2015	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtAa-CoV/SD2016	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtAd-CoV/SX2014	*Apodemus draco*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtAp-CoV/Tibet2014	*Apodemus peninsulae*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtAp-CoV/SAX2015	*Apodemus peninsulae*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtAs-CoV/XJ2015	*Apodemus syhaticus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtBi-CoV/FJ2015	*Bandicota indica*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtMm-CoV-1/IM2014	*Meriones meridianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtMm-CoV-2/IM2014	*Meriones meridianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtMm-CoV/XJ2015	*Meriones meridianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtMc-CoV-1/YN2013	*Mus caroli*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtMc-CoV-2/YN2013	*Mus caroli*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtMm-CoV/GD2015	*Mus musculus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtNn-CoV/SX2014	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		Rodent coronavirus	RtNn-CoV/ Tibet2014	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtNn-CoV/ HuB2014	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtNn-CoV/ SAX2015	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtNe-CoV/ Tibet2014	*Niviventer eha*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtRr-CoV/ HaiN2015	*Rattus andamanensis*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtRl-CoV/ FJ2015	*Rattus losea*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtRn-CoV/ YN2013	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rattus norvegicus alphacoronavirus	-	*Rattus norvegicus*	Muridae	conventional PCR	(Tsoleridis et al. 2016)
		Rodent coronavirus	RtRnit-CoV/ GZ2015	*Rattus nitidus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtRf-CoV-1/ YN2013	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtRf-CoV-2/ YN2013	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent coronavirus	RtRf-CoV/ GX2016	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Sorex araneus alphacoronavirus	-	*Sorex araneus*	Soricidae	conventional PCR	(Tsoleridis et al. 2016)
		Rodent coronavirus	Shrew-CoV/ Tibet2014	*Sorex araneus*	Soricidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
+ssRNA (non-segmented)	*Flaviviridae*	Rodent pestivirus	RtCb-HCV/ HeB2014	*Cricetulus barabensis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent pestivirus	RtMc-HCV/ Tibet2014	*Microtus clarkei*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent pestivirus	RtMg-TBEV /XJ2015	*Microtus gregalis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent pestivirus	RtAs-HCV/ IM2014	*Allactaga sibirica*	Dipodidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent pestivirus	RtDs-HCV/ IM2014	*Dipus sagitta*	Dipodidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent pestivirus	RtAd-PestV/ SAX2015	*Apodemus draco*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent pestivirus	RtAp-PestV/ JL2014	*Apodemus peninsulae*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent pestivirus	RtMm-HCV/ IM2014	*Meriones meridianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent pestivirus	RtNn-PestV/ HuB2014	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent pestivirus	RtNn-PestV/ SAX2015	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent pestivirus	RtNe-PestV/ SC2014	*Niviventer ex-celsior*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Norway rat hepacivirus 1/2	NrHV 1/2	*Rattus norvegicus*	Muridae	HTS (Ion Torrent)	(Firth et al. 2014)
		Norway rat pegivirus	NrPgV	*Rattus norvegicus*	Muridae	HTS (Ion Torrent)	(Firth et al. 2014)
		Norway rat pestivirus	NrPV	*Rattus norvegicus*	Muridae	HTS (Ion Torrent)	(Firth et al. 2014)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
+ssRNA (non-segmented)	*Hepeviridae*	-	RtCb-HEV/ HeB2014	*Cricetulus barabensis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		-	RtCm-HEV/ XJ2016	*Cricetulus migratorius*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		-	RtEi-HEV/ SX2016	*Eothenomys inez*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		-	RtCl-HEV /GZ2016	*Eothenomys melanogaster*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Common vole-associated hepatitis E virus	cvHEV	*Microtus arvalis*	Cricetidae	RT-PCR (nested format)	(Ryll et al. 2019)
		-	RtMg-HEV/ XJ2016	*Microtus gregalis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		-	RtMr-HEV/ HLJ2016	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		-	RtAa-HEV/ JL2014	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		-	RtRn-HEV/ ZJ2016	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rat hepatitis E virus	ratHEV	*Rattus norvegicus, R. rattus*	Muridae	real-time and conventional RT-PCR (nested format)	(Ryll et al. 2017)
		-	RtRf-HEV/ YN2014	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
+ssRNA (non-segmented)	*Picornaviridae*		Rodent/Ee/PicoV/NX2015	*Caryomys eva*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtCb-PicoV/HeB2014	*Cricetulus barabensis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			Rodent/Mc/PicoV/Tibet2015	*Microtus clarkei*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtMrut-PicoV/JL2014-1	*Myodes rutilus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtMrut-PicoV/JL2014-2	*Myodes rutilus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtMruf-PicoV/JL2014-1	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtMruf-PicoV/JL2014-2	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtMruf-PicoV/JL2014-3	*Myodes rufocanus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			Rodent/Ds/PicoV/IM2014	*Dipus sagitta*	Dipodidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtAc-PicoV/GZ2016	*Apodemus chevrieri*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtAs-PicoV/XJ2016	*Apodemus sylvaticus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtRn-PicoV/YN2014	*Mus caroli*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtMp-PicoV/YN2014	*Mus pahari*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
			Rodent/CK/PicoV/Tibet2014	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			Rodent/Rn/PicoV/SX2015_1	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			Rodent/Rn/PicoV/SX2015_2	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtNn-PicoV/HuB2015-1	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtNn-PicoV/HuB2015-2	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtNn-PicoV/HuB2015-3	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtNn-PicoV/SAX2016	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			RtRrs-PicoV/YN2014	*Rattus andamanen-sis*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
			Rodent/RL/PicoV/FJ2015	*Rattus losea*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rat picornavirus	-	*Rattus norvegicus*	Muridae	HTS (Illumina MiSeq instrument)	(Sachsenröder et al. 2014)
		Norway rat kobuvirus 1/2	NrKoV 1/2	*Rattus norvegicus*	Muridae	HTS (Ion Torrent)	(Firth et al. 2014)
		Norway rat hunnivirus	NrHuV	*Rattus norvegicus*	Muridae	HTS (Ion Torrent)	(Firth et al. 2014)
		Manhattan rat parechovirus	MPeV	*Rattus norvegicus*	Muridae	HTS (Ion Torrent)	(Firth et al. 2014)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
		rodent picornavirus	RPV	*Rattus norvegicus*	Muridae	HTS (Ion Torrent)	(Firth et al. 2014)
			RtRn-PicoV/ GD2015	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
-ssRNA[e] (non-segmented)	*Bornaviridae*	Variegated squirrel bornavirus 1	VSBV-1	*Callosciurus prevostii, Cs. finlaysonii, Sciurus variegatoides, Sc. granatensis, Tamiops swinhoei*	Sciuridae	HTS (Illumina MiSeq instrument)	(Hoffmann, Tappe, et al. 2015)
-ssRNA (non-segmented)	*Paramyxo-viridae*	Rodent paramyxovirus	RtAp-ParaV/ NX2015	*Apodemus peninsulae*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Giant Squirrel respirovirus	GSqRV	*Ratufa macruora*	Sciuridae	HTS (Illumina MiSeq instrument)	(Forth et al. 2018)
-ssRNA (segmented)	*Arenaviridae*	Rodent arenavirus	RtDs-AreV/ IM2014	*Dipus sagitta*	Dipodidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent arenavirus	RtMc-AreV/ YN2014	*Mus caroli*	Muridae	Illumina (HiSeq 2500 system)	Wu et al. 2018
		Rodent arenavirus	RtRl-AreV/ HuN2015	*Rattus losea*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent arenavirus	RtRn-AreV/ YN2014	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent arenavirus	RtRn-AreV/ ZJ2016	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent arenavirus	RtRf-AreV/ YN2014	*Rattus tanezumi*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
-ssRNA (segmented)	*Hantaviridae*	Rodent hantavirus	RtCe-HV/NX2015	*Caryomys eva*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent hantavirus	RtCl-HV/GZ2015	*Eothenomys melanogaster*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Traemmersee orthohantavirus	-	*Microtus agrestis*	Cricetidae	RT-PCR	(Jeske et al. 2019)
		Rodent hantavirus	RtMg-HV/XJ2015	*Microtus gregalis*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent hantavirus	RtMrut-HV/JL2014	*Myodes rutilus*	Cricetidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent hantavirus	RtDs-HV/IM2014	*Dipus sagitta*	Dipodidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent hantavirus	RtAa-HV/SD2016	*Apodemus agrarius*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent hantavirus	RtAp-HV/JL2014	*Apodemus peninsulae*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent hantavirus	RtNn-HV/NX2015	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent hantavirus	RtNn-HV/SAX2015	*Niviventer confucianus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent hantavirus	RtNn-HV/HuN2015	*Niviventer niviventer*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent hantavirus	RtRn-HV/GD2015	*Rattus norvegicus*	Muridae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent hantavirus	Shrew-HV/Tibet2014	*Sorex araneus*	Soricidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)
		Rodent hantavirus	Shrew-HV/SX2014	*Sorex araneus*	Soricidae	Illumina (HiSeq 2500 system)	(Wu et al. 2018)

Table S1 (continued)

Genome organization	Family (Subfamily)	Virus	Abbreviation	Host	Host family	Detection method	Reference
dsRNA[f] (segmented)	*Reoviridae*	Rat rotavirus	-	*Rattus norvegicus*	Muridae	HTS (Illumina MiSeq instrument)	(Sachsenröder et al. 2014)
		shrew rotavirus A	RVA	*Sorex araneus*	Soricidae	RT-PCR	(Johne et al. 2019)
		shrew rotavirus C	RVC	*Sorex araneus*	Soricidae	RT-PCR	(Johne et al. 2019)
		shrew rotavirus H	RVH	*Sorex araneus*	Soricidae	RT-PCR	(Johne et al. 2019)

[a] single-stranded deoxyribonucleic acid

[b] High-Throughput Sequencing

[c] double-stranded deoxyribonucleic acid

[d] single-stranded ribonucleic acid of positive polarity

[e] single-stranded ribonucleic acid of negative polarity

[f] double-stranded ribonucleic acid

9.2. List of abbreviations

AflaPyV1	Apodemus flavicollis polyomavirus 1
ATPase	adenosine-triphosphatase
AUT	Austria
BHV	betaherpesvirus
BKPyV	BK polyomavirus
BoDV-1/2	Borna disease virus 1/2
BoHV-1/2/5	bovines herpesvirus 1/2/5
bp	base pair
CDS	coding sequence
CeryBHV1	Callosciururs erythraeus betaherpesvirus 1
CeryGHV1	Callosciurus erythraeus gammaherpesvirus 1
CeryPyV1	Callosciurus erythraeus polyomavirus 1
CpreBHV1	Callosciurus prevostii betaherpesvirus 1
CpreGHV1	Callosciurus prevostii gammaherpesvirus 1
CprePyV1	Callosciurus prevostii polyomavirus 1
DNA	deoxyribonucleic acid
DPOL	DNA polymerase
dsDNA	double-stranded deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
eGFP	enhanced green fluorescent protein
EHV-1/4	equine herpesvirus 1/4
FCS	fetal calf serum
G	glycoprotein
gB	glycoprotein B

GgliPyV1	Glis glis polyomavirus 1
GHV	gammaherpesvirus
HHV-3	human alphaherpesvirus 3
HSV-1	herpes simplex virus 1
HTS	High-Throughput Sequencing
HV	herpesviruses
IC	internal control
JCPyV	JC polyomavirus
kb	kilobases
kbp	kilobase pairs
L	polymerase protein
LD-PCR	long distance-PCR
LIE	Liechtenstein
LTAg	large T-antigen
M	matrix protein
MCPyV	Merkel cell polyomavirus
MmusRHV1	Mus musculus rhadinovirus 1
MnatPyV2	Mastomys natalensis polyomavirus 2
MTAg	middle T-antigen
N	nucleoprotein
NCCR	non-coding control region
NTC	no template control
ORF	open reading frame
P	phosphoprotein

PCR	polymerase chain reaction
PyV	polyomavirus
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RnorPyV1	Rattus norvegicus polyomavirus 1
RT-PCR	reverse transcription PCR
RT-qPCR	quantitative reverse transcription polymerase chain reaction
ScarBHV1	Sciurus carolinensis betaherpesvirus 1
ScarGHV1/2	Sciurus carolinensis gammaherpesvirus 1/2
ScarPyV1	Sciurus carolinensis polyomavirus 1
SqAdV-1	Squirrel adenovirus 1
SQPV	Squirrel poxvirus
ssDNA	single-stranded deoxyribonucleic acid
+ssRNA	single-stranded ribonucleic acid of positive polarity
-ssRNA	single-stranded ribonucleic acid of negative polarity
STAg	small T-antigen
SUI	Switzerland
SV40	simian virus 40
SvarPyV1	Sciurus variegatoides polyomavirus 1
SvulBHV1	Sciurus vulgaris betaherpesvirus 1
TCR	transcription control region
TstrGHV1	Tamias striatus gammaherpesvirus 1
UricGHV1	Urocitellus richardsonii gammaherpesvirus 1
VP	viral protein

VSBV-1	variegated squirrel bornavirus 1		
WGS	whole-genome sequencing		
X	X protein		
ZooBoCo	zoonotic	bornavirus	consortium

9.3. List of figures

9.4. List of tables

Chapter 10: Acknowledgement

10 Acknowledgement

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