



Assessing the occurrence of the novel zoonotic variegated squirrel bornavirus 1 in captive squirrels in Germany – A prevalence study

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Abstract

The newly described zoonotic variegated squirrel bornavirus 1 (VSBV-1) in German squirrel holdings has been associated with the death of three private owners and one zoo animal caretaker (confirmed cases). Epidemiological investigations were severely impeded by the general lack of data on holdings of the putative reservoir hosts, the family Sciuridae. To fill this lack of data for detailed epidemiological investigations of the captive squirrel population, a register of private and zoological squirrel holdings was established. The findings show a broad variety of kept species and their frequency distribution. By contacting the different stakeholders via Web-based social groups and societies, information passed in both directions so that disease awareness could be raised and participants could be recruited for further studies. Cross-sectional studies revealed a prevalence of VSBV-1-positive subpopulations of 0% (95% CI 0%–6.2%) among private squirrel collections and 1.9% (95% CI: 0%–9.9%) among zoos in Germany. The approach presented here can be transferred to other populations of non-traditional pets, which may be equally difficult to monitor, in the case of an emerging zoonotic infectious disease.

KEYWORDS

bornavirus, exotic pets, prevalence, squirrel, VSBV-1, zoonoses

1 | INTRODUCTION

More than three quarters of human infectious diseases that emerged in recent decades are estimated to be zoonotic (Taylor et al., 2001). At the same time, the popularity of non-traditional, exotic pets of various species is increasing worldwide according to trade statistics (Bush et al., 2014; Sung et al., 2018) and growing sales figures in the supply markets (Zentralverband Zoologischer Fachbetriebe Deutschlands e.V., 2020). For several diseases, for example

salmonellosis (Weiss et al., 2011), cow pox (Becker et al., 2009), monkey pox (Bernard et al., 2006) and rabies (Chomel et al., 2007), trade, contact and ownership of non-traditional pets were found to be the sources of local outbreaks of human infections. However, in many cases, the potential hazards associated with the ownership of or contact with these exotic animals are unexplored and therefore largely unknown to caretakers, veterinarians, physicians and public health workers (Kuiken et al., 2005). Epidemiologic investigations and risk assessment are often challenging, as data on the presence,

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ownership or trading routes of exotic animals are scarce and usually not available to the competent veterinary authorities (Karesh et al., 2005). Our epidemiological approach deals with a novel zoonotic pathogen of largely unknown features in a neglected niche of human-animal interface. Facing globalization and human population growth, this becomes of increasing importance within our modern one health comprehension (Marano et al., 2007; Webster et al., 2016).

The variegated squirrel bornavirus 1 (VSBV-1) is a novel zoonotic pathogen and forms its own species, the *Mammalian 2 orthobornavirus*, in the family *Bornaviridae* (Amarasinghe et al. (2019). In at least four cases, it caused progressive fatal encephalitis in infected human patients in Germany (Hoffmann et al., 2015; Tappe et al., 2018). All known cases of VSBV-1-associated human disease were linked to the husbandry of various exotic squirrel species. When captive squirrels were examined, virus was found not only in neuronal tissue, but also in various organs including those with excretional and secretional functions without causing any clinical signs (Schlottau, Jenckel, et al., 2017). Accordingly, the squirrels are thought to be the reservoir host for this zoonotic virus (Schlottau, Hoffmann, et al., 2017). In European holdings, the Central American variegated squirrels (*Sciurus variegatoides* Ogilby) and the South-East Asian Prevost's squirrels (*Callosciurus prevostii* Desmarest) were predominantly affected. In addition, a Finlayson squirrel (*Callosciurus finlaysoni* Horsfield), a Swinhoe's striped squirrel (*Tamiops swinhoei* Milne-Edwards) and a red-tailed squirrel (*Sciurus granatensis* Humboldt) were found positive (Schlottau, Hoffmann, et al., 2017; Schlottau, Jenckel, et al., 2017). These five species are members of two different subfamilies of the family *Sciuridae*, the *Sciurinae* and the *Callosciurinae*, and they originate in the tropics and subtropics of two different continents, America and Asia, respectively (Steppan et al., 2004). Therefore, the origin of the virus is uncertain. Besides an extra-European origin, a 'non-squirrel' regional virus reservoir of European origin is discussed, as described for the Borna disease virus 1, which is hosted by the bicoloured white-toothed shrew (*Crocidura leucodon* Hermann) (Durrwald et al., 2014). Indigenous Eurasian squirrels (*Sciurus vulgaris* Linnaeus) or free-ranging neozootic populations of Eastern grey squirrels (*Sciurus carolinensis* Gmelin) and Pallas's squirrels (*Callosciurus erythraeus* Pallas) in Europe were so far not found infected with VSBV-1 (Schlottau, Jenckel, et al., 2017) (Schulze et al., 2020).

In the aftermath of the detection of VSBV-1, epidemiological investigations among private breeders and owners of squirrels were undertaken in Germany. A core network of affected owners and of captive tropical squirrels was assembled (Tappe et al., 2019), and the testing of a growing number of squirrels revealed more infected animals. However, a systematic approach to sampling was not possible. As the screened holdings were selected by a certain degree of contact between the owners and included predominantly similar species, the VSBV-1 prevalence among captive squirrels in Germany could not be reliably estimated.

Accordingly, an approach was chosen, which is similar to those recommended for hard-to-survey populations (Tourangeau, 2014). Objectives were the determination of the size of the population at

Impacts

- This survey resulted in the first and most comprehensive data set on captive squirrels in zoos and private holdings in Germany.
- With the owners' support, insight into characteristics of squirrel holdings was gained, whereas at the same time, a communication channel for public health information was established.
- Subsequent cross-sectional studies revealed a prevalence of 0% (95% CI 0%–6.2%) positive subpopulations among private squirrel collections and 1.9% (95% CI: 0%–9.9%) among zoos in Germany.

risk and the estimation of the prevalence of VSBV-1 within this target population as the most important parameters. To this end, a register of squirrel owners in Germany was implemented, which elucidates several features of squirrel husbandries in the country. On this basis, we conducted a cross-sectional study to assess the prevalence of VSBV-1 in captive squirrels in Germany in private holdings and zoos.

2 | MATERIALS AND METHODS

2.1 | Registration process

Captive animals of all species of the family *Sciuridae* were regarded as the population at risk due to the unknown interspecific susceptibility for VSBV-1. Two major populations were identified: squirrels in private hands and those kept in zoological gardens. Considerations for separate investigations were the limited numbers of zoos versus the potentially not completely recordable private holdings, different levels of accessibility of owners, their compliance and the documentation of animal movements. Furthermore, public and occupational health concerns were considered.

To assess the size and characteristics of the population at risk in the private husbandry sector, the squirrel owners were identified as the best source of information. To address this community, an online approach was chosen for a simple and fast access to a broad spectrum of potential participants. Possible sources of bias in a Web survey are the coverage of Internet use and the sensitivity for self-selection (Bethlehem, 2010). Although the coverage of Internet use in Germany is over 90% (German Federal Statistical Office, 2018), the possible under-coverage in the target population was addressed by a parallel offline strategy. Different online platforms of breeders and owners of squirrels and other small mammals were identified and explored. If they had been active within the last six months prior to the launch of the online survey in January 2018, general information on VSBV-1, the project and the planned study, and the rationale for participation in the online survey were shared. The same information was promoted in specialized German

magazines for squirrel owners or enthusiasts of rodents and other small mammals (Allendorf et al., 2017, 2018) and by visiting meetings of small mammal breeder societies and exotic animal markets. The online post included a link to a questionnaire for self-registration of the squirrel owners. Offline information on the pathogen was presented, and leaflets were distributed including a QR-coded link to the online questionnaire and contact addresses.

The questionnaire (Supplements) was designed as a basic version for registration, from which an extended version with more detailed questions on the husbandry condition and management could be reached after completion. With the basic version, information was gathered on (a) species and number of squirrels kept, (b) the purpose of holding (hobby or breeding), (c) documentation of movements of bred animals and (d) personal data (name and address). Informed consent was obtained from all participants. Finally, the participants could register for a mailing list with information on VSBV-1 and for the involvement in the subsequent diagnostic study.

Concomitantly, online platforms for animal trade, squirrel-specific groups, adverts in special magazines and Web-based exotic animal markets were screened for current offers of squirrels of any species. Sellers were registered passively with the data available on the website and the offered species. They were informed about the purpose of the investigation and motivated to participate in the survey, as were all people who had sent in samples for VSBV-1 diagnostics to the Friedrich-Loeffler-Institut. These diagnostic services had been offered since 2015.

For the zoo sector, a publicly available online database on German zoological inventories (<https://www.zootierliste.de>, 2018-04-01) and the inventories listed in the Zoo Information Management System (ZIMS) for German zoos with one or more records on sciurid species were pre-screened and the entries confirmed or corrected by phone calls, emails or personal contact. Meetings of national and international zoological societies and small mammal specialist groups were visited to spread information and acquire participating institutions.

In general, individual squirrel species were kept separately, whereas the exchange between enclosures within one holding for breeding and other reasons was frequent for animals of the same species. Therefore, all individuals of one species were presumed to form one epidemiologic unit, further on referred to as a subpopulation, if one owner/breeder/institution kept more than one species.

Registered private and zoological populations were compared with the only known and presumably selection-biased data set (Schlottau, Hoffmann, et al., 2017; Schlottau, Jenckel, et al., 2017), further referred to as the pilot cohort. This pilot cohort included the squirrels of 33 private breeders or owners and 19 zoological gardens investigated since the first detection of VSBV-1 in three private holdings in 2015 until the beginning of 2017. On subpopulation level, it consisted of 59 subpopulations from 40 private holdings and 27 subpopulations from 19 zoological institutions with a focus on variegated squirrels and *Callosciurus* species. Other species were examined on a smaller scale. The screening results of the pilot cohort showed that at least one positive individual was detected in nine out of 59 private (15.3%) and four out of 27 zoological (14.8%) subpopulations.

2.2 | Sampling

For the subsequent cross-sectional studies on subpopulation level within both populations, sample sizes were calculated by the formula.

$$n = \left(\frac{Z \cdot \sqrt{p \cdot (1 - p)}}{e} \right)^2 = \frac{Z^2 \cdot p \cdot (1 - p)}{e^2}$$

with Z representing the value from standard normal distribution that corresponds to the desired confidence level (Z = 1.96 for 95% CI), p as the estimated true prevalence and e as the desired precision (Cochran, 1977).

The sample size for estimating the prevalence in the private and zoo population was calculated for a confidence interval of 95% and different precision values. For the private population, a precision of 10% was chosen to take the expected lower compliance into account; for the zoo population, the common precision of 5% was maintained.

The prevalence estimate p was taken from the pilot cohort. To generate less biased estimators from these pilot data, the proportions p_i of positive subpopulations within each species (i.e. stratum) were adjusted to the assumed true frequency distribution of all species as measured throughout the registration process by applying the formula.

$$p(\text{adj}) = \sum p_i \cdot \frac{r_i}{R}$$

with r_i representing the number of subpopulations registered within each stratum and R as the sum of all registered subpopulations (Thrusfield et al., 2018). This then led to an estimate of $p(\text{adj}) = 6.5\%$ (95% CI 1.7%–16.1%) respectively $p(\text{adj}) = 3.2\%$ (95% CI 0.05%–18.1%) as the adjusted prevalence of VSBV-1 in the years 2015–2017.

For the zoo population, the following correction for a finite population size was applied to the sample size n resulting from the equation above (Thrusfield & Christley, 2018):

$$n(\text{cor}) = \frac{N \cdot n}{(N + n)}$$

with N as the sum of all registered subpopulations (pilot plus new register).

2.3 | Detection of VSBV-1 RNA

All registered owners and zoos were contacted and motivated to sample all their squirrels by dry oral swabs and/or faecal samples if possible. Previous studies had shown equal suitability of both sample types (Schlottau, Hoffmann, et al., 2017). If no individual swabs or faecal samples could be taken, pooled faecal samples were collected from the enclosure of the respective subpopulation. Informed consent was obtained from all owners and institutions participating in this study.

Registration process

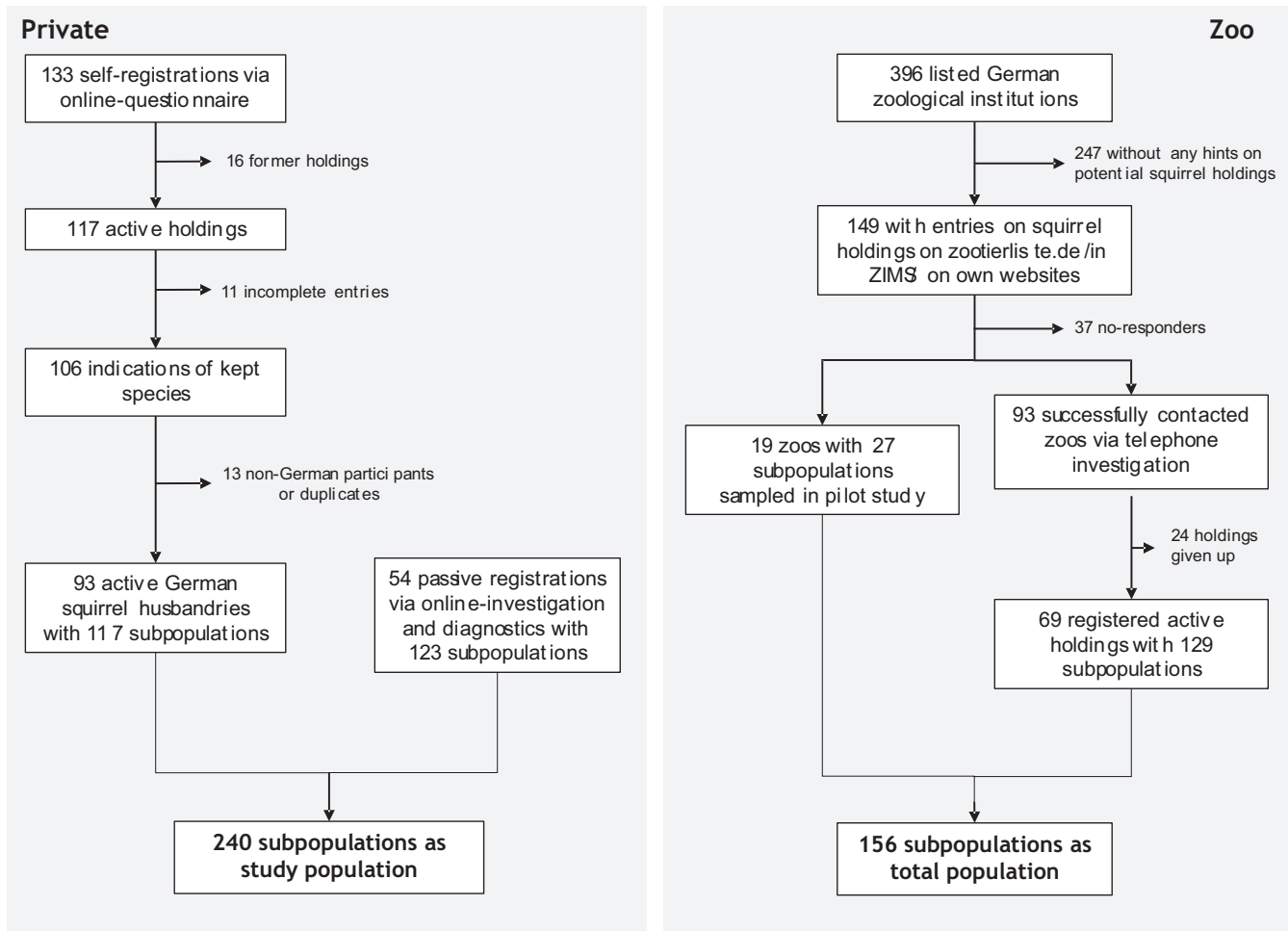


FIGURE 1 Comparative CONSORT diagram on the different registration procedures for private (left side) and zoo populations (right side) of squirrel subpopulations in Germany

Samples were analysed by real-time quantitative reverse transcription-PCR (RT-qPCR) using the system established in former studies (Hoffmann et al., 2015; Schlottau, Hoffmann, et al., 2017; Schlottau, Jenckel, et al., 2017). Briefly, in an extraction step with TRIzol reagent (Life Technologies), RNA was extracted from swabs or faecal samples using the NucleoMag Vet Kit (Macherey & Nagel) and the KingFisher™ Flex Purification System (Thermo Fisher Scientific) according to the manufacturer's instructions. Subsequently, two RT-qPCR assays were performed on a Bio-Rad CFX96 Cycler (Bio-Rad), detecting VSBV-1-specific RNA (VSBV-1-Mix 10) and a broad spectrum of other orthobornaviruses (panBorna Mix 7.2). The samples were analysed along with positive, negative and RNA isolation controls. The analytical sensitivity and diagnostic specificity of the assays have been shown to come close to 100% (Schlottau, Hoffmann, et al., 2017).

2.4 | Statistical analyses

To estimate the apparent prevalence, the samples were stratified by subpopulation. Data processing and statistical analyses were

performed using R Version 3.5.1.; the packages `utils`, `propCI`, `dplyr`, `stats`, `treemap` and `ggplot2` were used for descriptive statistics, calculations and graphical visualization. To determine whether the observed frequencies of the study and the pilot data followed the same distribution, Pearson's chi-squared goodness-of-fit test was performed. Confidence intervals were calculated using the Clopper-Pearson exact method (Clopper et al., 1934).

3 | RESULTS

3.1 | Private sector

3.1.1 | Study population

In the private sector, the online questionnaire was completed by 133 participants, of which 117 stated that they kept one or more squirrels at the time, when the study was conducted. Ninety-three German participants answered the question on the species of squirrels kept, of which eleven kept more than one species. With this, data on 117

subpopulations were collected. To this self-registration data, passively compiled data on owners and breeders, for example by incoming diagnostic requests ($n = 39$) and by offline and online presentation of squirrel holdings ($n = 84$), were merged. Taken together, information on 240 subpopulations from 147 different private holdings was collected (for further details, refer Figure 1).

Twenty-three different species were kept by private owners (Figure 2 and Table 1). The most common species were Swinhoe's striped squirrels (23.9% of all registered subpopulations) and Siberian chipmunks (21.0%), followed by the indigenous Eurasian squirrel (16.8%). Together, these three species accounted for more than 60% of all registered subpopulations. The species of highest interest concerning previous VSBV-1 occurrence, the variegated squirrel and the Prevost's squirrel, were far less common, each constituting about 5% of the squirrel subpopulations. In 35 of the holdings (23.8%), two or more different species were kept. Ten holdings housed both Central American squirrels (*S. variegatoides*, *S. granatensis*) and South-East Asian squirrels (*Callosciurus* spp.).

All subpopulations registered using this open approach were considered as a proxy for the total population of squirrels in private hands. Hence, the frequency distribution of species served as the basis for further calculations. The frequencies differed significantly between the subpopulations of the pilot cohort and those of the main survey for the private squirrel holdings (Table 1; *Pearson's chi-squared goodness-of-fit test*, $X^2(df = 3, N = 280) = 15.206, p = .002$).

3.1.2 | Sampling

In brief, the sample size calculation revealed that sampling of at least 24 subpopulations was required. The data were stratified by the frequency of different species in the registered subpopulations. Between March 2018 and January 2020, samples of overall 353 individual animals from 58 different subpopulations were received

and analysed. This sample exceeded the minimum sample size and reflected approximately the expected stratification (Table 1).

3.1.3 | Analysis

During the cross-sectional study, VSBV-1 RNA was not detected in any of the tested samples (Table 1) and thus not in any of the source subpopulations. This leads to an apparent prevalence of 0% (95% CI 0%–6.2%) of VSBV-1 in private subpopulations. Both the apparent prevalence of the time period 2018–20 and the adjusted prevalence from 2015 to 17 (according to the registry data) are depicted in Figure 4.

3.2 | Zoo sector

3.2.1 | Study population

In the zoo sector, 150 out of 396 officially listed institutions were identified as potentially keeping squirrels, 19 of which were already included in the pilot cohort. In the telephone and mail follow-up, 93 of the remaining 131 institutions responded. Of these, 24 stated to have given up squirrel husbandry. The 69 remaining institutions were asked about their current stock of squirrels. In total, 130 subpopulations were registered. As there is—in contrast to the private sector—a limited and countable number of subpopulations of squirrels in German zoos, both pilot (if still existing) and newly registered subpopulations were aggregated into a total population of 156 subpopulations (Figure 1). The frequency distribution of subpopulations in the pilot cohort differed significantly from that of the entire zoological population (Table 2; *Pearson's chi-squared goodness-of-fit test*, $X^2(df = 3, N = 156) = 21.164, p = 9.735 \times 10^{-5}$).

Species in the German private population

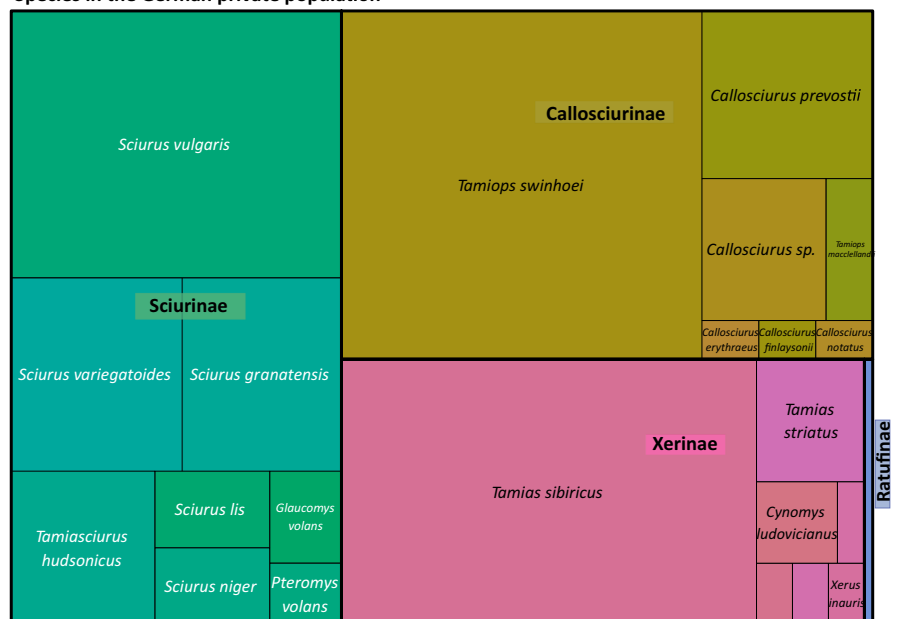


FIGURE 2 Treemap of the species frequency of the registered sciurid subpopulations in private holdings in Germany ($n = 240$)

Subfamily	Registered	Pilot		Study	
		Sampled	Positive	Sampled	Positive
Callosciurinae					
<i>Callosciurus erythraeus</i>	1	1	0	—	
<i>Callosciurus finlaysonii</i>	1	1	1	1	0
<i>Callosciurus notatus</i>	1	—		1	0
<i>Callosciurus prevostii</i>	13	10	2	6	0
<i>Callosciurus</i> sp.	8	1	0	2	0
<i>Tamiops maclellandii</i>	3	—		—	
<i>Tamiops swinhoi</i>	57	13	1	15	0
Ratufinae					
<i>Ratufa macroura</i>	1	—		—	
Sciurinae					
<i>Glaucomys volans</i>	3	—		2	0
<i>Pteromys volans</i>	2	1	0	1	0
<i>Sciurus granatensis</i>	14	6	1	1	0
<i>Sciurus lis</i>	4	—		—	
<i>Sciurus niger</i>	4	1	0	1	0
<i>Sciurus variegatoides</i>	15	13	4	5	0
<i>Sciurus vulgaris</i>	40	9	0	8	0
<i>Tamiasciurus hudsonicus</i>	10	—		—	
Xerinae					
<i>Cynomys ludovicianus</i>	3	1	0	—	
<i>Paraxerus cepapi</i>	1	—		—	
<i>Sciurotamias davidianus</i>	1	—		1	0
<i>Spermophilus tridecemlineatus</i>	1	—		—	
<i>Tamias sibiricus</i>	50	1	0	9	0
<i>Tamias striatus</i>	6	1	0	5	0
<i>Xerus inauris</i>	1	—		—	
Total	240	59	9	58	0

Note: Frequency distribution of subpopulations in the squirrel population in private holdings registered and sampled during this study (2018–2020) including results on the presence of VSBV-1 in comparison with the pilot data on private squirrel holdings retrieved from the VSBV-1 screening studies (2015–2017) (Schlottau, Hoffmann, et al., 2017; Schlottau, Jenckel, et al., 2017).

In the zoological institutions, 26 different sciurid species were kept (Figure 3 and Table 2). In 39 institutions (57% of all registered), two or more different species were kept. Thirty of these institutions (77%) housed subpopulations of two or more subfamilies. The most common species (25% of all subpopulations) were the prairie dog (*Cynomys ludovicianus* Ord) of the subfamily of ground squirrels (Xerinae), followed by the Eurasian red squirrel (15%) and the Swinhoe's striped squirrel (13%). Prevost's squirrels are currently kept by 9% of the registered zoos; variegated squirrels are among the rarely kept species with only five records (3%).

3.2.2 | Sampling

As described above, sample size calculation was adjusted to a limited population size of $N = 156$. The calculation resulted in at least 37

TABLE 1 Squirrel subpopulations in private holdings—register and cross-sectional study on the presence of VSBV-1

subpopulations required to be sampled. Sampling was stratified by the frequency of different species within the registered subpopulations. Between March 2018 and January 2020, samples of overall 330 individual animals from 54 different subpopulations were analysed (Table 2).

3.2.3 | Analysis

VSBV-1 RNA was detected in samples from one individual Prevost's squirrel, which leads to an apparent prevalence of 1.9% (95% CI 0%–9.9%) VSBV-1-positive squirrel subpopulations in zoos (Table 2). Both the apparent prevalence of the time period 2018–20 and the adjusted prevalence from 2015 to 17 (according to the registry data) are depicted in Figure 4.

The only VSBV-1-positive individual originated from a subpopulation that had been affected already in the pilot study

TABLE 2 Squirrel subpopulations in zoos—register and cross-sectional study on the presence of VSBV-1

Subfamily	Registered	Pilot		Study	
		Sampled	Positive	Sampled	Positive
Callosciurinae					
<i>Callosciurus erythraeus</i>	1	—	—	—	—
<i>Callosciurus prevostii</i>	12	10	4	6	1
<i>Tamiops mccllellandii</i>	1	—	—	1	0
<i>Tamiops swinhoi</i>	23	5	0	13	0
Ratufinae					
<i>Ratufa macroura</i>	1	1	0	1	0
Sciurinae					
<i>Microsciurus</i> sp.	—	1	0	—	—
<i>Pteromys volans</i>	1	—	—	—	—
<i>Sciurus anomalus</i>	1	—	—	—	—
<i>Sciurus granatensis</i>	2	—	—	—	—
<i>Sciurus lis</i>	1	—	—	—	—
<i>Sciurus niger</i>	1	—	—	—	—
<i>Sciurus variegatoides</i>	4	3	0	1	0
<i>Sciurus vulgaris</i>	19	2	0	3	0
<i>Sciurus yucatanensis</i>	1	—	—	—	—
<i>Tamiasciurus hudsonicus</i>	8	1	0	2	0
Xerinae					
<i>Ammospermophilus harrisi</i>	1	—	—	—	—
<i>Cynomys ludovicianus</i>	43	1	0	15	0
<i>Heliosciurus rufobrachium</i>	1	—	—	1	0
<i>Marmota bobak</i>	2	—	—	—	—
<i>Marmota marmota</i>	5	—	—	2	0
<i>Paraxerus cepapi</i>	2	—	—	1	0
<i>Sciurotamias davidianus</i>	7	2	0	3	0
<i>Spermophilus citellus</i>	4	—	—	—	—
<i>Tamias sibiricus</i>	5	—	—	2	0
<i>Tamias striatus</i>	2	—	—	—	—
<i>Xerus inauris</i>	8	1	—	2	0
Total	156	27	4	53	1

Note: Frequency distribution of subpopulations in the squirrel population kept in zoos registered and sampled during this study (2018–2020) including results on the presence of VSBV-1 in comparison with the pilot data on private squirrel holdings retrieved from the VSBV-1 screening studies (2015–2017)(Schlottau, Hoffmann, et al., 2017; Schlottau, Jenckel, et al., 2017).

conducted in 2016 (Schlottau, Jenckel, et al., 2017). After removal of its positively tested partner animal from the holding in 2016, the remaining squirrel had not been retested until its trauma-related death in December 2019. *Post-mortem* examination revealed high loads of VSBV-1 RNA in the brain. Furthermore, bornavirus-reactive antibodies were detected by serological testing. Subsequently, the zoological garden decided to give up the Prevost's squirrel holding, and the new partner animal, which had been introduced in 2018, was euthanized. The analysis of tissue and serum samples of the partner animal revealed neither VSBV-1

RNA nor bornavirus-reactive antibodies. The VSBV-1-positive animal had no contact with further squirrels.

4 | DISCUSSION

The registers of squirrel holdings established in this study allow new and comprehensive insights into the populations at risk and form the first data set of this kind. While earlier back-tracing investigations helped to minimize exposure of humans and squirrels, the

Species in the German zoo population

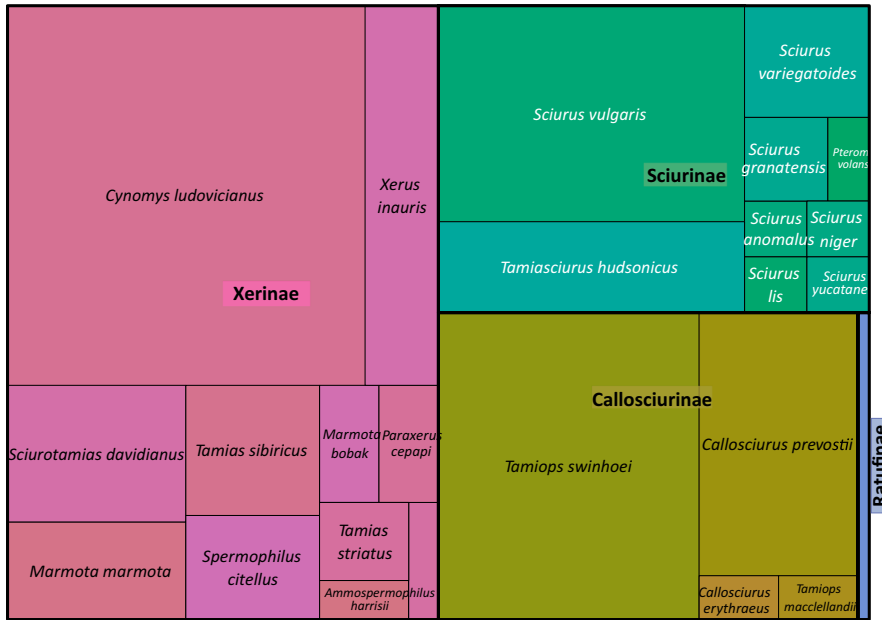


FIGURE 3 Treemap of the frequency of all sciurid subpopulations in the zoo population in Germany (n = 156)

registers allowed us to plan and conduct cross-sectional studies in the squirrel populations in captivity in Germany. Important for the compliance in general was the zoonotic potential of the pathogen and the interest to maintain a healthy and non-hazardous population by dealing only with negative tested squirrels. This was supported by the availability of in vivo diagnostics of non-invasive samples of squirrels. As an additional benefit, the outreach to the squirrel owners and zoos with relevant information on the pathogen and its disease combined with recommendations on good practice regarding the husbandry management may have led to an increase in awareness among the human population at risk when handling their animals.

The approach chosen for the registration of the private population was based on voluntarily participation. However, it was tried to approach potential participants in different ways to guarantee access for all existing holdings and to prevent the initial exclusion of certain groups. Self-selection of participating owners and breeders might have led to a selection bias resulting in an over-representation of owners/breeders with certain characteristics or interests. On the other hand, an under-representation of owners involved in illegal activities such as black market deals and illegal trafficking has to be expected. Due to this limitation, the total number of squirrel husbandries in private households remains unknown. Additionally, an information bias cannot be excluded, as the questionnaire was

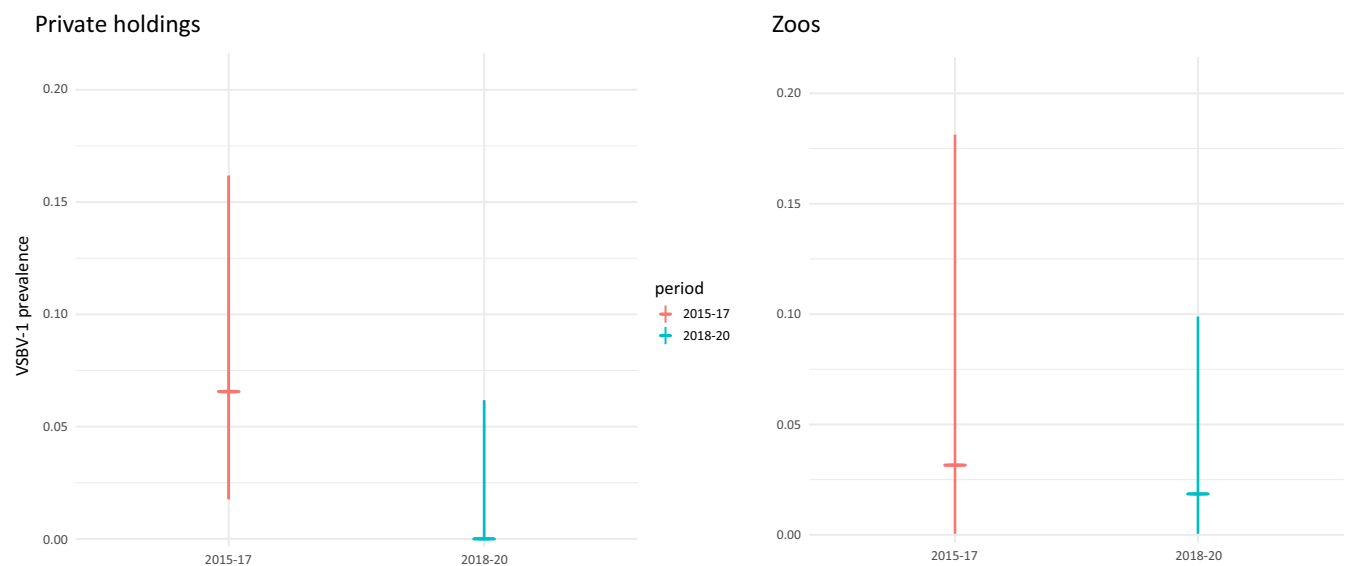


FIGURE 4 VSBV-1 prevalence estimates for squirrels in private holdings (left) and zoos (right) in Germany for the present cross-sectional study (2018–2020) compared with the adjusted VSBV-1 prevalence assessed using the pilot screening data (2015–2017). Bars indicate 95% Clopper-Pearson exact confidence intervals

answered unsupervised and anonymously, without verification of the input. False inputs were discouraged by informing on the strictly anonymized data policy. In contrast to the private sector, the registered zoos can be assumed almost complete, with very few missing data of institutions that could not be contacted or failed to reply. Here, limitations in terms of selection and compliance were staffing levels of the institution, which likely correlates with its size and its financial equipment.

The also voluntary cross-sectional study revealed no additional positive squirrel subpopulations. Initially, the measures of local veterinary authorities applied to the holdings of the primary occurrences of VSBV-1 included culling of the entire stock in at least one instance. Owners who were aware of that fact may have been more reluctant to participate. Moreover, holdings of illegally imported squirrels may not have found entrance in the sampling pool as mentioned above. The result may therefore be selection-biased. The relatively low expectations for owner's compliance in general and the planned high accepted error of 10% revealed to be conservative, as the included subpopulations exceeded the required number by far. This demonstrated the success of the recruiting plan by providing detailed information and subsequent voluntary diagnostic testing of kept squirrels. Moreover, it led to a more precise prevalence estimate. Nonetheless, the assumption of the upper limit of the confidence interval, 6.2%, as the worst-case apparent prevalence is recommendable.

For the zoo population, the required sample size was also exceeded, which underscores the assumption of a high compliance among the institutions. According to the results of telephone interviews, only small zoos ran by one person or a family had no time or staff for sampling. These institutions often housed European squirrels, explaining the gap in sampling within this subpopulation.

Given the suspected lifelong persistent infection of sciurids and the lack of therapeutic agents, euthanasia of VSBV-1-infected individuals might be the only possibility to eliminate the hazard from squirrel colonies. The success of this strategy can be delineated from the decrease in VSBV-1 prevalence in both populations, when comparing the register-adjusted prevalence from the pilot study (2015–2017; 3.2%, 95% CI 0.05%–18.1%) to the apparent prevalence in the cross-sectional study (2018–2020; 1.9%, 95% CI 0%–9.9%). Considering the relatively large time intervals of the surveys, this decrease also suggests a low reproduction ratio of VSBV-1 infections within the squirrel population in captivity, although the exact mode of transmission is not yet understood. This observation may be beneficial for the further reduction in the risk of VSBV-1 infections in humans and animals in Germany.

The only positive subpopulation of Prevost's squirrels was also found to be affected in the pilot study (Schlottau, Jenckel, et al., 2017). After removing the positive animal, the subpopulation was not monitored until December 2019, when sampling revealed the remaining contact animal of the previously positive animal to be VSBV-1-infected. This illustrates the importance of regular sampling, especially of those species known to be susceptible, to minimize the risk of transmission to humans. It should be noted that, this

institution (like every other affected holding) had also been advised by the Friedrich-Loeffler-Institut to (re)test their squirrel population every 3 months.

In summary, the observed low prevalence and the presumptively low infection rate within the subpopulations may indicate a controllable and bounded outbreak of still unknown origin. But also, it proves the still existing risk of positive squirrels in German holdings. Taking into account the high lethality of human VSBV-1 infections, the importance of a thorough surveillance—for example regular diagnostics—of at least the known susceptible species is not debatable. If not enforceable by the authorities, especially the zoos and private breeders selling their animals should feel responsible to insure the reduction or elimination of all occupational and public health risks.

5 | CONCLUSION

With this study, the insight into squirrel husbandry in Germany, certainly one of many niches in animal-human interface, is broadened. Simultaneously the occurrence of the new emerging zoonotic VSBV-1, causing a fatal disease in humans, is elucidated. Initially, the populations at risk had to be defined, before the prevalence studies could be conceptualized and conducted. Due to lacking regulation, estimating and reaching the population at risk was challenging and time-consuming. The deficiency of data regarding not just squirrel but most exotic animal ownership is inherent in the German system and in other countries of the world. The described approach may serve as a blueprint for epidemiologic assessment of emerging diseases in exotic animals. However, a profound solution would be the implementation of a general unified registration system for exotic pets. From a one health perspective, it is urgent to increase the surveillance of trade and ownership of exotic pets to prevent the potential introduction of and exposure to EID in general (Reaser et al., 2008). Traded and kept under the radar, animals carrying medium to highly virulent pathogens could easily become a hazard for human public health.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

VA, MB, FJC and TH designed the study. VA carried out the survey. DR, KS and DH carried out the laboratory analysis of the samples. VA, CF and SA conceived and performed statistical analysis. VA drafted the manuscript. MB, FJC and TH supervised the study. DR,

KS, SA, CF, MB, FJC and TH provided critical feedback and helped shape the research, analysis and manuscript.

ETHICAL APPROVAL

Informed consent was obtained from all owners and institutions participating in this study.

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