

Epidemiology of bat rabies in Germany

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Summary. Rabies in European bats was first reported in Germany in 1954. In concordance with Denmark and the Netherlands, Germany has reported one of the highest numbers ($n = 187$) of European bat lyssavirus (EBLV)-positive cases in bats in Europe so far (1954–2005). A combined descriptive epidemiological and phylogenetic analysis on bat rabies and prevailing EBLVs is presented, comprising the past 50 years. So far, only the two lineages of EBLV-1 (genotype 5), a and b, have been detected. Although only 50% of the rabies-positive bats have been identified by species, the Serotine bat (*Eptesicus serotinus*) is the bat species most frequently infected. Single rabies cases have also been detected in a further five indigenous bat species. There is proven evidence for a substantial bias in the frequency of bat rabies cases in the north of Germany, with an endemic cluster in the northwesternmost low-lying plain areas adjacent to the Netherlands and Denmark. Improvements to bat rabies surveillance and research are discussed.

Introduction

Rabies-like disease in European bats is caused by negative-strand RNA viruses of the order *Mononegavirales*, family *Rhabdoviridae*, genus *Lyssavirus*, species *European bat lyssavirus 1* and *European bat lyssavirus 2*, representing genotypes 5 and 6, respectively, and was first reported in 1954. The description of the first case in Hamburg, Germany, describes a biting incident from a bat that was captured but not formally identified [24]. Mouse inoculation tests on the brain material from the bat demonstrated rabies-like disease, and the brains from those mice were

subsequently NEGRI body positive. During the following 30 years, an additional five cases occurred infrequently in the north of Germany [2, 18, 33, 35, 48]. After 1985, the number of reports of rabid bats increased dramatically, coincident with the first diagnosis of the disease in Denmark and the report of a human case of bat-acquired rabies in Europe [23, 25]. These cases raised awareness of a potential public health threat and led to an increase in lyssavirus surveillance of bats in other European countries [6, 11, 16, 17, 31, 32, 46]. The number of rabid bats peaked in Europe, with 142 cases between 1986 and 1987, mainly from the Netherlands, Denmark and the coastal regions of northern Germany. Antigenic analysis using monoclonal antibodies directed against the nucleoprotein and glycoprotein of rabies virus indicated that the viruses isolated from European bats were distinct from the viruses found in rabid terrestrial animals and bats from the Americas, but more closely related to another lyssavirus, Duvenhage virus [35]. Subsequent genomic characterization has further differentiated European rabies viruses of bats into a separate group, European bat lyssavirus (EBLV). Within this group there is a further division into two subtypes, 1 and 2, also referred to as genotypes [3, 4]. Phylogenetic analysis has identified two lineages within the EBLV-1 genotype [1], which is firmly supported by a degree of geographical separation. EBLV-1a is distributed over northern Europe between the Netherlands and Russia [1, 9], whilst EBLV-1b is found only in Western Europe along an axis from Spain to the Netherlands. Numerous isolations of EBLV-1 have been made in Spain from a variety of species [11, 30, 41], whilst France has reported 13 cases of EBLV-1b and a single case of EBLV-1a [31]. The second EBLV genotype, EBLV-2, has also been subdivided into two lineages, a and b, although there is less support for this due to the considerably fewer isolations reported for this virus [17, 20, 46].

European bat lyssavirus type 1 (EBLV-1) has been isolated from bats from throughout Europe and has a specific association with the Serotine bat (*Eptesicus serotinus*). In contrast, EBLV2 is more commonly associated with the species of Myotis bats (*M. daubentonii* and *M. dasycneme*) and has been isolated from bats in the Netherlands, United Kingdom and Switzerland in recent years [17, 20, 22, 50]. Both EBLV types cause rabies-like disease in bats and in spill-over hosts [26, 45]. There has been a single confirmed case of EBLV-1 in humans [39] and two human deaths due to EBLV-2 infection [14, 15, 23], emphasising European bat lyssaviruses as an emerging zoonosis [13]. EBLV-1 spillover has also been reported in sheep [26, 44, 45].

From 1977 to 2004, a total of 729 cases of bat rabies were detected in Europe and reported to the WHO Collaborating Centre for Rabies Surveillance and Research at the Friedrich-Loeffler-Institute, Germany. The majority of positive bats originated from Denmark, followed by the Netherlands, Germany and Poland, accounting for more than 90% of all positive bats recorded for this time period. In contrast, only 50 bat rabies cases were detected in other European countries, mainly in France and Spain. So far, isolated cases have also been reported in Switzerland, Great Britain, the Czech Republic, Slovakia, Hungary, the Ukraine and Russia.

Despite the large numbers of EBLV cases reported from Germany, there has been no comprehensive epidemiological analysis and also no reported study on these viruses since 1987 [37]. Where German EBLV isolates have been included, only a limited number of cases have been used to illustrate EBLV in the wider context of the distribution of EBLV throughout Europe [1, 3, 9]. In this paper, the epidemiology of bat rabies in Germany has been investigated and compared to the situation in other European countries since 1985. Furthermore, this is the first study to survey EBLV cases in Germany during the past 50 years to assess the extent of virus distribution and host range. In addition, we have included forty-one isolates of German origin from the last 10 years (1994–2004) in a phylogenetic analysis using a partial sequence of the EBLV nucleoprotein gene. In this paper we report the discovery of EBLV-1b in Germany.

Materials and methods

Data source and test assays

In Germany, rabies diagnosis has been the responsibility of the individual federal states, of which there are sixteen. Suspect or rabid bats are submitted for testing to the regional veterinary laboratories. EBLV viral antigen was detected in the brain using the 'gold standard' test, the fluorescent antibody test (FAT), using commercially available polyclonal FITC-labelled anti-rabies conjugates (Behring, Marburg; SIFIN, Berlin, Germany) [10]. For FAT-suspect and FAT-negative bats with human exposure, virus isolation using the rabies tissue culture infection test (RTCIT) was undertaken as described elsewhere [47]. Information, including location, year found, the result of the FAT and RTCIT, and the bat species when reported, were recorded for each sample. Because rabies is a notifiable disease in Germany, data were subsequently forwarded to the national rabies data bank established at the national reference laboratory (NRL) for rabies at the WHO Collaborating Centre for Rabies Surveillance and Research in Tübingen (1977–1995) and Wusterhausen at the Friedrich-Loeffler-Institute (1995–date), Germany [27]. Brain tissues or virus isolates of EBLV-positive bats were requested to be submitted to the NRL for further characterization in an indirect FAT using a panel of 10 anti-nucleocapsid monoclonal antibodies able to distinguish EBLV-1 and -2 as described elsewhere [35]. Identification of bats into species was done using morphological features using the guidelines as described [36].

Data exploration

For epidemiological analysis, these data are limited to the number of bat rabies cases reported (T+) during regular rabies surveillance programmes from 1954 until 2005. Additional cases based on retrospective studies including bats obtained from (private) collections were not included. Additionally, all data from federal state regional veterinary laboratories about bats submitted for testing (N) were included. With these figures the effect of influential variables on the ratio T+/N can be investigated using a logistic regression model. Analyses were conducted with respect to geographical locations. For this purpose, the whole territory of Germany was classified according to elevation into two categories using an arbitrary threshold [below (north) and above (south) 300 meters above sea level] and the number of submissions and positive cases allocated accordingly. Additionally, the areas below 300 meters above sea level were further classified into 2 regions (northwest and northeast). In two separate statistical tests, the hypothesis of significant differences in geographical bat rabies prevalence was examined. The SAS V9.1 was used to perform all the statistical calculations. Both variables were treated as

Table 1. EBLV-1 isolates from Germany cases phylogenetically analysed in this study

Sample no.	Region	Date	Species	Origin	GenBank accession no.	
					Partial N	N-P Region
9437GER	Bremen	1987	<i>E. serotinus</i>	Bremen	U89459	–
RV9	Hamburg	1968	<i>E. serotinus</i>	Hamburg	AY062082	DQ522895
9908	Hamburg	2004	NR	Hamburg	DQ522858	–
9398GER	Lower Saxony	1970	<i>E. serotinus</i>	Stade	U89457	–
RV145	Lower Saxony	1978	NR (bat)	Cuxhaven	DQ522879	DQ522893
9436GER	Lower Saxony	1986	<i>E. serotinus</i>	Stade	U89458	–
9477GER	Lower Saxony	1986	<i>E. serotinus</i>	Nienburg	U89469	–
9439GER	Lower Saxony	1989	<i>E. serotinus</i>	Bad Iberg	U89461	–
9440GER	Lower Saxony	1989	<i>E. serotinus</i>	Braunschweig	U89462	–
9441GER	Lower Saxony	1990	<i>E. serotinus</i>	Walsrode	U89463	–
5250	Lower Saxony	1994	<i>P. pipistrellus</i>	Hannover	DQ522867	–
5226	Lower Saxony	1996	<i>P. auritus</i>	Lingen	DQ522862	–
932	Lower Saxony	1998	<i>E. serotinus</i>	Breddenburg	DQ522866	–
934 (RV1423)	Lower Saxony	1998	<i>E. serotinus</i>	Osnabruck	DQ522860	DQ522892
998	Lower Saxony	1998	<i>E. serotinus</i>	Emden	DQ522865	–
992	Lower Saxony	1998	<i>E. serotinus</i>	Moordorf	DQ522868	–
989	Lower Saxony	1998	<i>E. serotinus</i>	Aurich	DQ522881	–
959	Lower Saxony	1998	<i>E. serotinus</i>	Aurich	DQ522882	–
933	Lower Saxony	1998	<i>E. serotinus</i>	Oldendorf	DQ522864	–
2136	Lower Saxony	1998	<i>E. serotinus</i>	Hannover	DQ522883	–
3135	Lower Saxony	1998	<i>E. serotinus</i>	Hannover	DQ522878	–
5300	Lower Saxony	1999	<i>E. serotinus</i>	Emden	DQ522876	–
5304	Lower Saxony	1999	<i>E. serotinus</i>	Emden	DQ522874	–
7467	Lower Saxony	1999	<i>E. serotinus</i>	Emden	DQ522873	–
7471	Lower Saxony	1999	<i>E. serotinus</i>	Emden	DQ522869	–
5009	Lower Saxony	1999	NR	Hannover	DQ522859	DQ522887
5665	Lower Saxony	2001	NR	Osnabrück	DQ522863	DQ522888
8482	Lower Saxony	2003	NR	Winsen/Luhe	DQ522877	–
8624	Lower Saxony	2003	<i>E. serotinus</i>	Osterholz-Scharmbeck	DQ522884	DQ522890
9588	Lower Saxony	2004	<i>E. serotinus</i>	Hannover	DQ522880	–
10850	Lower Saxony	2004	<i>E. serotinus</i>	Emden	DQ522861	DQ522885
5254	Lower Saxony	NR	<i>E. serotinus</i>	Osterode	DQ522875	–
RV11	Lower Saxony	NR	NR (bat)	Bremerhaven	AY062083	–
9396GER	Mecklenburg Western-Pomerania	1985	<i>E. serotinus</i>	Rostock	U89467	–
5006	Saarland	2000	NR	Wadgassen	DQ522857	DQ522886
4895	Saxony-Anhalt	2000	NR	Freyburg	DQ522872	–
8215	Saxony-Anhalt	2002	<i>E. serotinus</i>	Halle-Kröllwitz	DQ522871	DQ522889
9442GER	Schleswig-Holstein	1990	<i>E. serotinus</i>	Ratzeburg	U89464	–
9481GER	Schleswig-Holstein	1990	<i>E. serotinus</i>	Lubeck	U89475	–
9438GER	Schleswig-Holstein	1988	<i>E. serotinus</i>	Neumünster	U89460	–
3132	Thuringia	1999	<i>E. serotinus</i>	Jena	DQ522870	–

NR Data not recorded

discrete influential variables. Multiple post hoc comparisons are conducted with Bonferroni – correction of the p -values [42]. Bats submitted for testing and EBLV-positive bats were plotted geographically on a map with the software “RegioGraph” (Macon Markt und Konzept, Waghäusel, Germany).

Phylogenetic analysis

Virus samples from rabid bats ($n = 41$) were obtained from a virus archive established at the NRL. Those viruses were either submitted to the NRL between 1994 and 2004 or obtained during a retrospective study (Table 1). Virus was propagated from the brain of each sample in murine neuroblastoma cells as previously described [26]. Tissue culture supernatants were stored at -80°C . Total RNA was purified from $200\ \mu\text{l}$ infected neuroblastoma culture medium using the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. RNA was eluted in a total volume of $50\ \mu\text{l}$ and stored at -70°C .

Reverse transcription was performed on $2\ \mu\text{l}$ aliquots of RNA as previously described [19] using the primer Jw12 (5'-ATGTAACACC[C/T]CTACAATG-3'). Briefly, cDNA was generated with moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Southampton, UK) for 1 h at 42°C in a volume of $10\ \mu\text{l}$. The reaction was then chilled on ice and diluted ten-fold with HPLC-grade water.

Two amplifications were performed on two separate sequences of the EBLV-1 genome. The first amplified a 606-base-pair (bp) sequence at the start of the nucleoprotein gene [19], using primers Jw12 and Jw6e (5'-CAGTTGGCACACATCTTGTG-3'). The second amplified a 374-bp sequence spanning the end of the nucleoprotein (N) to the start of the phosphoprotein

Table 2. EBLV-1 N gene sequences from European countries other than Germany

Sample no.	Country of origin	Date	Species	Region	GenBank accession no.
9366HOL	The Netherlands	1992	<i>E. serotinus</i>	Goor	AY863359
9372HOL	The Netherlands	1992	<i>E. serotinus</i>	Valthermond	AY863360
9478HOL	The Netherlands	1989	<i>E. serotinus</i>	Rolde	AY863361
9480HOL	The Netherlands	1987	<i>E. serotinus</i>	Bellingwolde	AY863362
94116HOL	The Netherlands	1989	<i>E. serotinus</i>	Jubbega	AY863363
02017HOL	The Netherlands	2000	<i>E. serotinus</i>	Soest	AY863364
02018HOL	The Netherlands	2000	<i>E. serotinus</i>	Leiden	AY863365
02020HOL	The Netherlands	1999	<i>E. serotinus</i>	Woerden	AY863366
02021HOL	The Netherlands	1998	<i>E. serotinus</i>	Leens	AY863367
02022HOL	The Netherlands	1998	<i>E. serotinus</i>	Oldeberkoop	AY863368
8615POL	Poland	1985	<i>E. serotinus</i>	Gdansk	AY863369
9394POL	Poland	1990	<i>E. serotinus</i>	Ketrzyn	AY863370
03002FRA	France	2003	<i>E. serotinus</i>	Angers	AY863381
9479DEN	Denmark	1987	<i>E. serotinus</i>	Horsens	AY863373
94110DEN	Denmark	1987	<i>E. serotinus</i>	Christiansfeld	AY863374
02010DEN	Denmark	1995	<i>E. serotinus</i>	Northeast Jutland	AY863375
02011DEN	Denmark	1997	<i>E. serotinus</i>	Zealand	AY863376
02012DEN	Denmark	1999	<i>E. serotinus</i>	Southwest Jutland	AY863377
02013DEN	Denmark	1999	<i>E. serotinus</i>	Southwest Jutland	AY863378
02015DEN	Denmark	2000	<i>E. serotinus</i>	South Jutland	AY863379
02016DEN	Denmark	2002	Sheep	Southwest Jutland	AY863380

(P) also known as the N-P intergenic region [26] using primers EBL 1161 (5'-AAGAGCTA GGATTACGAGG-3') and EBL 1534 (5'-GACAAAGATCTTGCTCATGA-3'). Amplifications were carried out with Amplitaq (Promega) using the conditions of 94 °C for 10 min, then 40 cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 90 sec. A 10 µl-aliquot of each reaction was separated on a 1.8% agarose gel and visualised by ethidium bromide staining under UV illumination. Sequencing was undertaken using a 5 µl aliquot taken directly from the amplification reaction. Reactions were primed using both flanking primers in separate reactions with the Big Dye sequencing kit (Applied Biosystems, Warrington, UK). Sequencing reactions were analysed using a commercial supplier (Lark Technologies Inc, Takeley, UK).

Sequence analysis was performed using the Lasergene 6 package (DNASTar Inc., Madison, USA), and phylogenetic analysis was undertaken using the maximum likelihood method as previously described [19]. Further sequences were obtained from GenBank or held at the Rabies Archive, Veterinary Laboratories Agency, UK (Table 2).

Results

Detection and distribution of EBLV-1 cases in Germany

Since 1985, a total of 843 indigenous bats have been examined for the presence of lyssaviruses in Germany, of which 181 (22.5%) tested positive. This equals about 9 positive rabid bats per year on average. A total number of 187 rabies-positive bats has been reported nationwide since 1954 (Fig. 1).

A routine identification of bats submitted for testing into species was undertaken only for rabies-positive bats. Of the 187 bat rabies cases reported, the species of 132 bats (70.5%), was undetermined. Only 57 cases included the species of bat involved, of which 92.5% occurred in the Serotine bat (*Eptesicus serotinus*). Single rabies cases were reported in the Mouse-eared bat (*Myotis myotis*), the

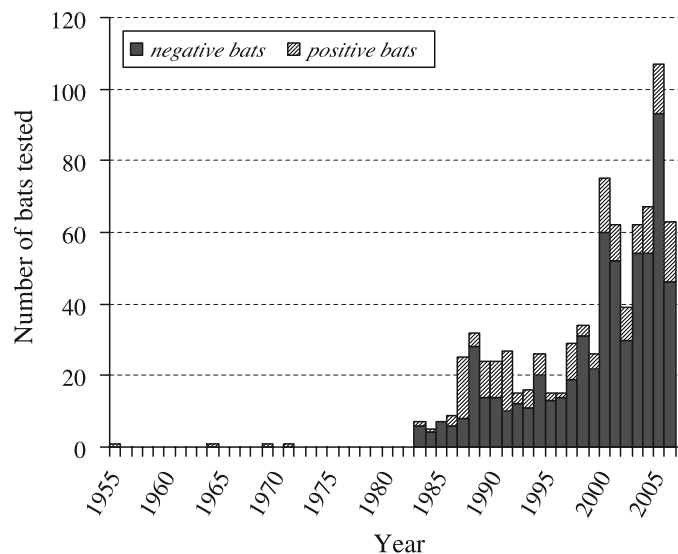


Fig. 1. Submissions of bats for routine rabies testing and number of positive bats in Germany during the past 50 years

Table 3. Reaction pattern of a panel of 10 anti-nucleocapsid monoclonal antibodies with selected lyssaviruses (genotypes) and bat rabies virus isolates from Germany

Rabies virus	Monoclonal antibody										
	genotype	W239.17	W187.5	W187.11.2	MW187.6.1	MSA6.3	LBV7.36	DUV6.15.19	862.1.2	P41	Z144.88
Laboratory strains	1	+	+	+	+	-	-	-	-	-	+
Oral rabies virus vaccine strains	1	+	-	+	-	-	-	-	-	-	+
Atypical fox variant	1	+	-	+	+	-	-	-	-	-	-
European fox strain	1	+	+	+	+	-	-	-	-	-	-
Dog mediated strains	1	+	+	+	+	-	-	-	-	-	+
Polar strains	1	+	+	+	+	-	-	-	-	-	+
Mokola virus	2	+	-	-	+	-	+	-	-	-	-
Lagos bat virus	3	+	-	-	+	+	-	-	-	-	-
Duvenhage virus	4	+	-	-	+	-	-	+	-	-	-
EBLV-1	5	+	-	-	-	+	-	+	+	-	-
EBLV-2	6	+	-	-	+	+	-	+	-	-	-
100 German EBLV-isolates		+	-	-	-	+	-	+	+	-	-

Daubenton's bat (*Myotis daubentoni*), the Pipistrelle bat (*Pipistrellus pipistrellus*), the Noctule bat (*Nyctalus noctula*), and the Nathusius' pipistrelle bat (*Pipistrellus nathusii*). One hundred of the viruses isolated have been further characterised with a panel of 10 anti-nucleocapsid monoclonal antibodies, and all have produced a reaction pattern indicative of EBLV-1 (genotype 5) (Table 3).

During the past 20 years, bats were submitted for testing from all German federal states with the highest number of bats investigated from the federal states of Lower Saxony ($n = 140$), Saxony ($n = 130$), Schleswig-Holstein ($n = 110$) and Brandenburg ($n = 90$) followed by Mecklenburg Western-Pommerania ($n = 60$) and Berlin ($n = 50$). In contrast, in 6 and 4 of the remaining states, the samples sizes were less than 50 and 20, respectively (Fig. 2A, B). A geographical analysis of the total rabies-positive cases in bats between 1954 and 2005, however, suggests a bias in the frequency of cases in the federal states of Lower Saxony ($n = 75$), Schleswig-Holstein ($n = 49$) and the capital, Berlin ($n = 20$). Three further states have sporadic cases of bat rabies, namely Saxony-Anhalt ($n = 9$), Brandenburg ($n = 7$) and Mecklenburg Western-Pomerania ($n = 6$). All of these states are situated in the northern parts of Germany (Fig. 2C). In seven federal states, fewer than 5 cases were reported per annum, suggesting a sampling/reporting bias. In Bavaria, Hesse and Rhineland Palatinate, no bat rabies cases have been reported so far.

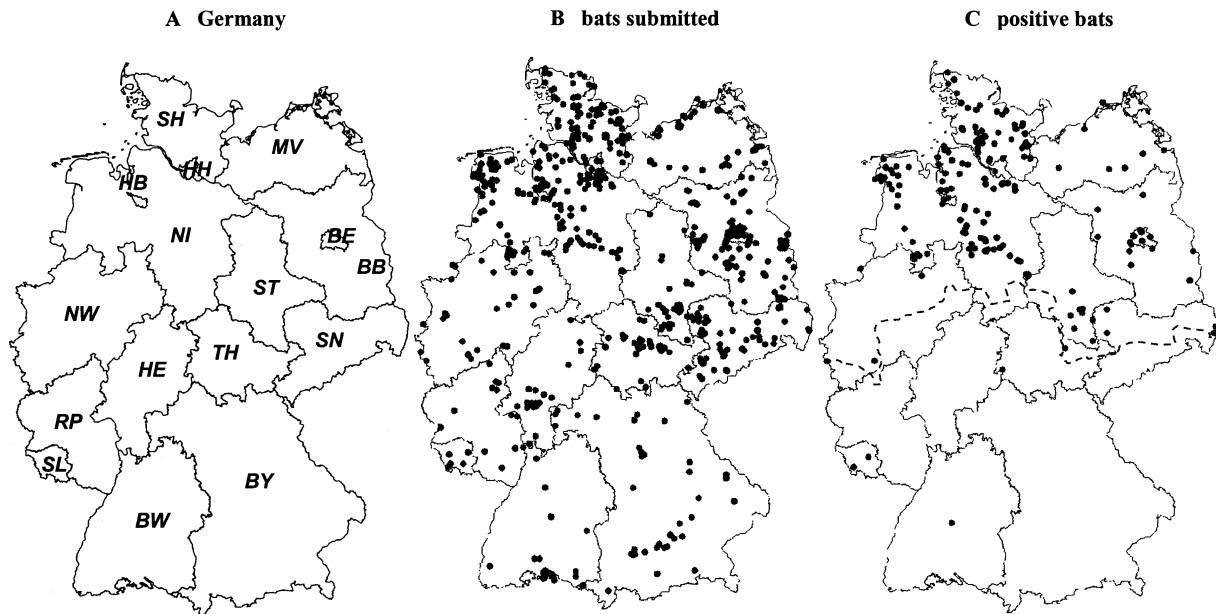


Fig. 2. Bat rabies in Germany: **A** political map of Germany: *SH* Schleswig-Holstein; *NI* Lower Saxony; *HB* Bremen; *HH* Hamburg; *NW* North Rhine Westphalia; *HE* Hessen; *RP* Rhineland Palatinate; *BW* Baden-Württemberg; *BY* Bavaria; *SL* Saarland; *BE* Berlin; *BB* Brandenburg; *MV* Mecklenburg Western Pomerania; *SN* Saxony; *ST* Saxony-Anhalt; *TH* Thuringia; **B** Total of bat submissions between 1985–2005 ($n = 843$) from each federal state, **C** Distribution of EBLV-1-positive bats between 1954–2005 ($n = 187$). The dashed line represents a division between land below 300 m (above the line) and land above 300 m (below the line).

Another observation made from the geographical distribution of rabies-positive bats is that more than 96% of the reported bat rabies cases in Germany are located in areas above the 51st latitude. In general, a significant difference of bat rabies prevalence was detected ($p < 0.0001$), with higher prevalence in areas below 300 m above sea level. In the latter case, significant differences in bat rabies prevalence ($p < 0.001$) were detected between regions in the northwest and northeast as well as between the northwest and south, suggesting the existence of an endemic cluster in the northwest of the country. Interestingly, a few positive bats were also reported along the common borders with neighbouring countries in the west (The Netherlands, Belgium, France) and east (Poland) (Fig. 2C).

Phylogenetic analysis of German EBLV-1 isolates

Using a limited panel of isolates ($n = 9$) from various geographical locations within Germany and two EBLV-1b isolates from France (RV266) and Spain (RV119), with the genotype 1 Pasteur virus sequence acting as an outgroup, comparison of a site at the 5' end of the nucleoprotein gene coding sequence (331 bp, Fig. 3A) with a sequence from the N-P intergenic region (331 bp, Fig. 3B) demonstrated limited variation in the general structure of the tree. All but one German isolate clustered exclusively with the subgroup, EBLV-1a. However, isolate 5006 from Saarland clustered with the EBLV-1b sequences in both phylogenetic trees. As a consequence of using rabies virus as an outgroup, the definition of the

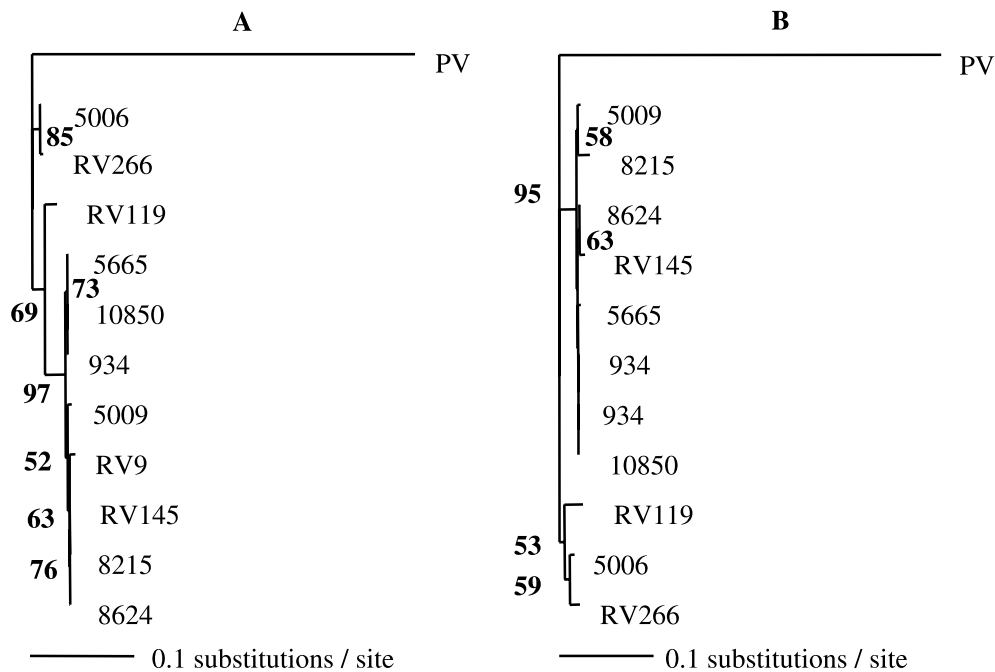
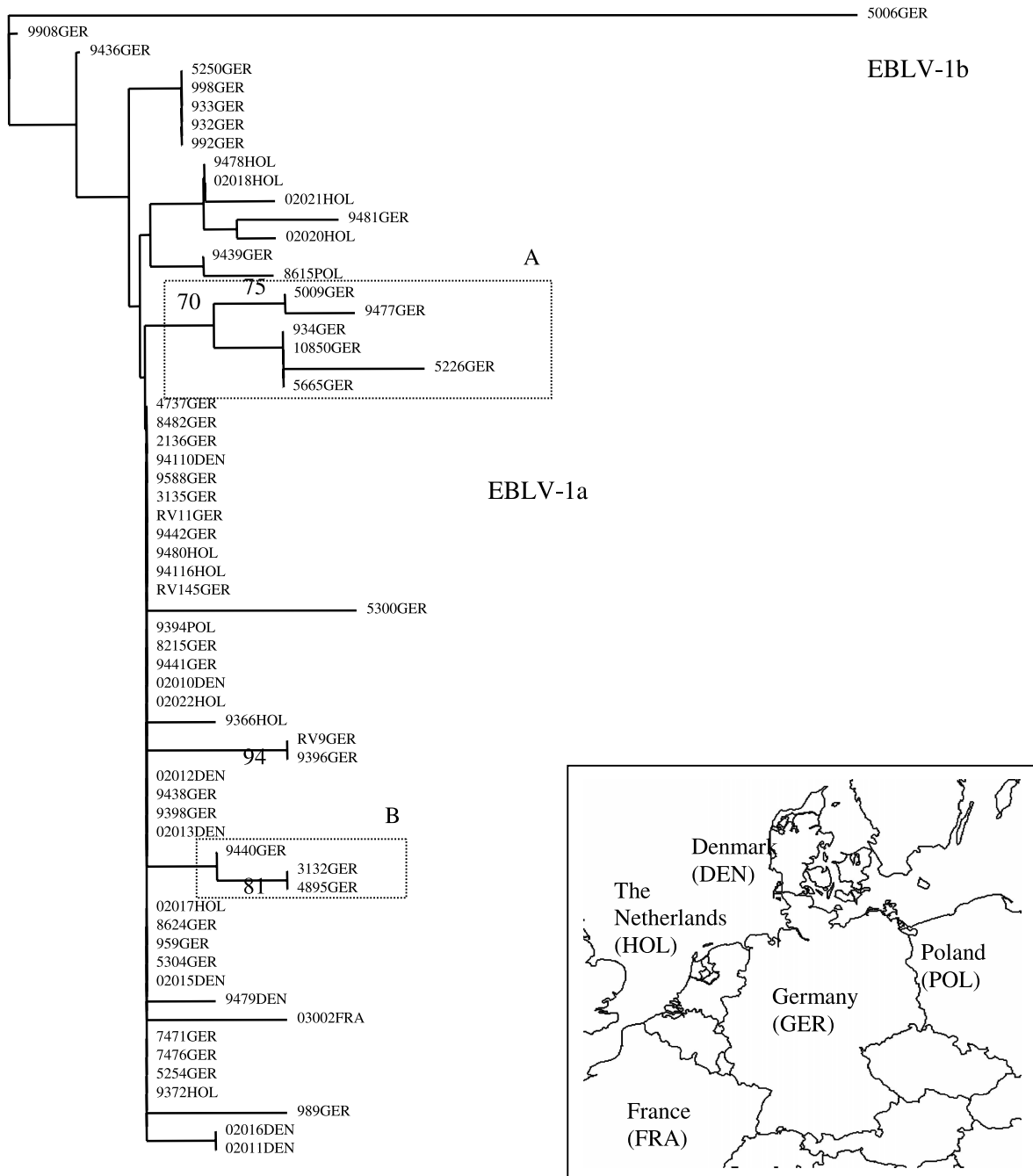


Fig. 3. Phylogenetic comparison of a limited panel of EBLV-1 isolates using 331 bp of the nucleoprotein gene **A** or the nucleoprotein-phosphoprotein intergenic region **B**. Bootstrap values greater than 50 are included



0.01 substitutions / site

Fig. 4. Phylogenetic comparison of EBLV-1 isolates from Germany and northern Europe using 331 bp of the viral nucleoprotein gene using isolate 5006 (EBLV-1b) as the outgroup. Bootstrap values greater than 65 are included

branching structure was very limited. The phylogeny based on the nucleoprotein sequence appeared to give the strongest bootstrap support, and this region was used to analyse the full panel of German EBLV-1 isolates, but using the EBLV-1b isolate 5006GER as an outgroup.

Phylogenetic analysis of the complete German panel with isolates from neighbouring countries (Fig. 4) revealed a large number of isolates with identical sequences. Again, all isolates, with the exception of isolate 5006, were shown to belong to the EBLV-1a group. Often, sequences from isolates separated by a number of years were identical. Examples of these are the isolates 9398GER and 9588GER (Table 1). Two small groups emerge from this panel. One group (Fig. 4, box A) contains isolates exclusively from Lower Saxony, again with a group of viruses isolated between 1986 and 2004. The second (Fig. 4, box B) contains isolates from three federal states, Thuringia, Saxony-Anhalt, and Lower Saxony. However, the bootstrap support is poor for this group (<70%). As reported by previous authors, the phylogeny does not appear to be influenced by geography with identical or similar sequences being recovered from distant locations [9].

Discussion

Germany has reported one of the highest numbers (187) of EBLV-positive cases in bats in Europe during the past 50 years. Recently, an additional 20 cases were identified during retrospective studies (Müller, unpublished). Usable bat rabies data have been obtained only since the second half of 1985 when Germany and other western European countries started more intensive surveillance [37]. Findings in subsequent years prompted the initiation of regular bat rabies surveillance programmes in many German federal states and resulted in an increasing number of bats submitted for testing and bats found positive during the past 20 years (Fig. 1). However, passive surveillance was and still is severely hindered as a result of Council Directive 92/43/EEC of the European Union on the conservation of natural habitats and of wild fauna and flora, the German Species Protection Act (2005), and federal state legislation that both changed the status of indigenous bats to highly protected and restricted the handling and submission of rabies-susceptible bats [7, 12]. Therefore, surveillance on bat rabies in Germany has relied on limited and opportunistic sampling. In fact, the number of positive cases did not only depend on the actual rabies incidence but of course also on the active support of local bat conservationists and on the number of animals handed in for diagnosis. The latter is supported by a significant correlation between the number of positive bats and the total number of bats examined in Germany. Hence, the estimated prevalence (22%) is potentially biased with infected bats likely to be more susceptible to the sampling process than non-infected animals because of behavioural alterations.

More than 24 of the 39 known European bat species occur in Germany [5]. The Serotine bat accounts for 92.5% of all cases in Germany in which the bat species was determined [36]. Although many other species have been examined, federal states that have reported bat rabies cases often failed to provide detailed information on which species were submitted and involved. This makes an epidemiological

analysis on distribution and incidence very problematic. Nevertheless, all of the 8 bat species but *Myotis dasycneme* found to be infected with EBLVs in Europe since 1985 using standard FAT, e.g. *Eptesicus serotinus*, *Myotis myotis*, *Myotis daubentoni*, *Pipistrellus pipistrellus*, *Nyctalus noctula*, *Pipistrellus nathusii*, and the Brown long-eared bat (*Plecotus auritus*), have also been reported, often exclusively, from Germany. Rabies was diagnosed in the latter during a retrospective study in Germany, and not during the regular rabies surveillance programme (Müller, unpublished results). During recent studies throughout Europe, EBLV antigen was detected in the brains of five additional species using the more sensitive nested RT-PCR method; Natterer's bat (*Myotis nattereri*), bent-winged bat (*Miniopterus schreibersii*), greater horseshoe bat (*Rhinolophus ferrumequinum*) and Barbastelle bat (*Barbastella barbastellus*) [41, Müller, unpublished]. Additionally, EBLV antibodies were detected in blood samples of many bat species, including the Daubenton's bat [6] and the European free-tailed bat (*Tadarida teniotis*) [41]. Also, a lyssavirus-positive Parti-coloured bat (*Vespertilio murinus*) was reported from the Ukraine [38]. Virus typing using monoclonal antibodies identified 100 of the 187 reported bat rabies cases in Germany as infection with EBLV-1. The detection of an EBLV-2 of German origin as described elsewhere [8] cannot be confirmed by our study.

The phylogenetic analysis of a short genomic fragment (331 bp) of the nucleoprotein gene coding sequence clearly demonstrates that the majority of German isolates are EBLV-1a (Fig. 4). Even if a sequence from the N-P intergenic region had been used it would have resulted only in a limited variation in the general structure of the tree (Fig. 3). As observed by previous authors who have based phylogenetic analysis on the nucleoprotein gene sequence [3, 19], bootstrap values for much of the tree were low due to the limited variability within the two subtypes. However, longer sequences (> 1000 bp) of the N and G genes have also been unable to demonstrate significant variation within this subgroup, suggesting that the virus is highly adapted to the principal host, *E. serotinus* [9]. This is supported by the fact that in our analysis sequences from isolations separated by a number of years were often identical (Fig. 4, box A, B). Interestingly, the phylogenetic analysis confirms for the first time the presence of EBLV-1b in Germany. The fact that the virus was isolated from a bat from Saarland in the southernmost part of Germany is evidence for an epidemiological relationship to EBLV-1b infections occurring in Serotine bats from France (Fig. 3) [31].

Transmission of both EBLV-1 and EBLV-2 from bats to humans has been reported [2, 14, 23, 39, 40]. Also, natural infections of sheep (*Ovis aries*) with EBLV-1 genotypes have been documented from Denmark in 1998 and 2002 [34, 44, 45]. A rabies case in a stone marten (*Martes foina*) from Germany, however, is the first confirmed spillover of an EBLV-1 into terrestrial wildlife in Europe [26]. However, the present available data show that the transmission of the disease caused by EBLV infection from bats to other mammalian hosts are limited to rare incidents: over 10,000 terrestrial animals diagnosed rabies positive have also been examined for the presence of EBLV-1 and EBLV-2, but all yielded negative results [28].

The existing differences in bat rabies surveillance among the German federal states (Fig. 2B) might not only reflect different levels of veterinary and public awareness over time but might also be a result of a subliminal conflict between bat conservation and public health. Nevertheless, there is a remarkable bias in the frequency of bat rabies cases in the northernmost parts of the country (Fig. 2C). This bias is reflected in the limited number of German EBLV-1 isolates included in previous phylogenetic studies [1, 9] and the overwhelming majority of isolates included in the present study (Table 1). Another common feature is the link to the Serotine bat, first identified as the host species in an early case from the city of Jena in 1963 [33]. The longevity of the virus in northwest Germany suggests that the ecological conditions for virus persistence are optimal in this area of the country [43]. One factor which may support this is the density of susceptible hosts. Although little detailed information is available for the density of *Eptesicus serotinus* in Germany, the federal states of Lower Saxony and Schleswig-Holstein are believed to have the largest populations of this species in Germany [5]. Furthermore, the geographical features of northern Germany, flat rural/urban landscape with some forest, is shared with its neighbours, the Netherlands, southern Denmark and Poland, three countries that have identified large numbers of EBLV-1 cases [28, 29]. This type of landscape provides an ideal habitat for Serotine bats, which favour open country with many roost sites, often associated with human dwellings. The topography of Germany is divided into three parts from north to south. The north of the country is low-lying plain, a second area is termed the central German uplands with increasing elevation, whilst the south of the country is dominated by the Alps mountain range. All but four EBLV-1 samples was isolated from north of a contour line dividing the northern plain and the central uplands (Fig. 2C). One of those single exceptions was the EBLV-1b isolate from the southwestern federal state of Saarland.

This topographical observation could in part explain the distribution of EBLV-1 in Europe. Certainly, the contiguous lowlands between the Netherlands, Germany and Denmark could allow free movement of bats and thus EBLV-1 throughout this region and give rise to the phylogenetic picture observed in this study (Fig. 4) and in others [9, 43]. Throughout Europe, EBLV-1a has been recovered primarily from Serotine bats along a north-east axis between the Netherlands and Russia, whereas EBLV-1b is found along an axis but running north-south between the Netherlands and Spain [3]. With the exception of a single isolation of EBLV-1a in central France [31], the segregation of these two lineages appears complete, with a possible interface in the Netherlands. The Serotine bat is distributed throughout Europe, but EBLV-1 has not been isolated from a number of European countries despite extensive surveillance for bat rabies in both the UK and Switzerland [21, 49]. If the topography of Europe is overlaid on the distribution of EBLV-1 isolations, the vast majority are, as in Germany, from sites below 300 m above sea level, which is considered a favourable habitat for the Serotine bat, probably indicating a density dependence of EBLV-1 incidence (Fig. 2). Amengual et al. [1] and Davis et al. [9] have suggested that EBLV-1a and b entered Europe from North Africa on two occasions but from two different

directions. The observations of EBLV-1 distribution in Germany might support this hypothesis, however, further investigations are needed, as the data from Europe are too limited and patchy.

We propose that further surveillance for EBLV in Europe should be linked to an assessment of bat densities to provide a complete model for virus-host interactions between EBLV-1 and the Serotine bat and EBLV-2 and *Myotis* species. Furthermore, there is a need to standardise surveillance across federal and national borders to ensure continuity and data consistency, especially the speciation of infected bats. This will require increased collaboration between veterinary authorities and bat conservationists in the future. Attempts should also be made to include bats from private collections in the surveillance and to establish active surveillance in free-living bat colonies where possible.

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