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Experimental infection of serotine bats (*Eptesicus serotinus*) with European bat lyssavirus type 1a

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The serotine bat (*Eptesicus serotinus*) accounts for the vast majority of bat rabies cases in Europe and is considered the main reservoir for European bat lyssavirus type 1 (EBLV-1, genotype 5). However, so far the disease has not been investigated in its native host under experimental conditions. To assess viral virulence, dissemination and probable means of transmission, captive bats were infected experimentally with an EBLV-1a virus isolated from a naturally infected conspecific from Germany. Twenty-nine wild caught bats were divided into five groups and inoculated by intracranial (i.c.), intramuscular (i.m.) or subcutaneous (s.c.) injection or by intranasal (i.n.) inoculation to mimic the various potential routes of infection. One group of bats was maintained as uninfected controls. Mortality was highest in the i.c.-infected animals, followed by the s.c. and i.m. groups. Incubation periods varied from 7 to 26 days depending on the route of infection. Rabies did not develop in the i.n. group or in the negative-control group. None of the infected bats seroconverted. Viral antigen was detected in more than 50 % of the taste buds of an i.c.-infected animal. Shedding of viable virus was measured by virus isolation in cell culture for one bat from the s.c. group at 13 and 14 days post-inoculation, i.e. 7 days before death. In conclusion, it is postulated that s.c. inoculation, in nature caused by bites, may be an efficient way of transmitting EBLV-1 among free-living serotine bats.

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INTRODUCTION

Rabies is a viral zoonotic disease that is inevitably fatal in humans and other mammals once clinical signs develop. It is caused by different lyssavirus species of the family *Rhabdoviridae* (Tordo *et al.*, 2004). Of the seven taxonomic species and four putative members of the genus *Lyssavirus*, all have reservoirs in bats except for Mokola virus (WHO, 2005). In Europe, bat rabies is caused by two different lyssaviruses, European bat lyssavirus types 1 (EBLV-1) and 2 (EBLV-2), also referred to as genotypes 5 and 6 (Bourhy *et al.*, 1992). Based on molecular characterization, EBLV-1 can be divided into the sublineages 1a and 1b. Whilst the circulation of EBLV-1a has been demonstrated in many European countries, mainly in central and western Europe, sublineage 1b appears to be somewhat restricted to Spain, France and The Netherlands (Amengual *et al.*, 1997; Davis *et al.*, 2005). However, sporadic findings of EBLV-1b in other countries, such as Germany and Poland, suggest that there is also a wider distribution of subtype 1b (Müller *et al.*, 2007; Smreczak *et al.*, 2007).

Recently, West Caucasian bat lyssavirus (WCBV), a putative member of the genus *Lyssavirus*, with distinct genetic and biological properties, was isolated from a Schreiber's long-fingered bat (*Miniopterus schreibersii*) on

the European side of the Caucasus mountain range (Kuzmin *et al.*, 2005).

The first European bat found to be rabid was located in Hamburg, Germany, in 1954 (Mohr, 1957). From 1977 to 2007, a total of 860 bat rabies cases were reported in Europe (Rabies Bulletin Europe, http://www.who-rabies-bulletin.org/). Most cases have been reported from The Netherlands, followed by Denmark, Germany and Poland, accounting for more than 90 % of all rabid bats recorded over this time period. Bat rabies was also reported in France, Spain, Switzerland, the UK, the Czech Republic, Slovakia, Hungary, Ukraine and Russia (Ondrejkova *et al.*, 2004; Müller *et al.*, 2007). EBLV-2 has been found only sporadically in Europe. It is always associated with *Myotis* bats (*Myotis daubentonii* and *Myotis dasycneme*) and has been detected in the Netherlands, the UK, Switzerland and Germany (Van der Poel *et al.*, 2005; Johnson *et al.*, 2006a; Freuling *et al.*, 2008).

In the Americas, spill-over infection of bat rabies into other terrestrial mammals and humans appears to occur more frequently than EBLV infections (Kuzmin & Rupprecht, 2007). Four laboratory-confirmed human rabies cases associated with both EBLV-1 and -2 have been reported from Europe (Fooks *et al.*, 2003). A further clinical case was reported from the Ukraine, but not confirmed by laboratory investigation (Botvinkin *et al.*, 2005). Among thousands of fox rabies isolates characterized by monoclonal antibody typing, not a single sample showed a pattern distinctive for EBLV-1 or -2. The first spill-over into livestock was reported from Denmark, where sheep were found to be rabid in 1998 and 2002, with EBLV-1 identified as the causative agent (Rønsholt, 2002).

The first wildlife species other than bat infected with EBLV was reported from Germany, where a stone marten with suspicious signs indicative of rabies was subsequently confirmed to be rabid and EBLV-1 was isolated. The isolated virus showed a high sequence identity to viruses that were isolated from serotine bats (*Eptesicus serotinus*) from the same region (Müller *et al.*, 2004). Recently, spill-over infections into domestic cats in France in 2006 and 2007 highlighted the veterinary public health importance of bat rabies (Dacheux *et al.*, 2009), even in countries otherwise considered 'free' of classical rabies caused by rabies virus (RABV).

The vast majority of bat rabies cases in Europe have been identified as EBLV-1. The serotine bat accounts for more than 90 % of all bat rabies cases in Europe and is therefore considered to play a key role in the epidemiology of EBLV-1 (King *et al.*, 2004). The serotine bat has a wide distribution across Europe. It is found through the Palaearctic from the Atlantic to the Pacific seaboards, across the Mediterranean from Portugal eastwards to Turkey, and even extending to regions adjacent to North Africa (Hutson *et al.*, 2008). Current knowledge about the disease in its native host is limited and little is known about its virulence, virus dissemination and the probable means of virus transmission of EBLV-1 among free-living insectivorous bats (Vos *et al.*, 2007). Field studies from Spain found seropositive individuals, and EBLV-1 RNA was detected in oral swabs from *Eptesicus isabellinus*, a bat species recently distinguished from *E. serotinus* (Pérez-Jordá *et al.*, 1995; Echevarría *et al.*, 2001; Vázquez-Morón *et al.*, 2008a, b). Virus transmission among bats causing mild or asymptomatic disease was suggested (Vázquez-Morón *et al.*, 2008a, b).

All European bats are strongly protected by European regulations (EUROBATS agreement, EU Directives 92/43 and 97/62) or by national legislation. Experimental studies with indigenous bat species are therefore restricted. Consequently, the pathogenesis of EBLV-1 has been studied in several non-reservoir species, i.e. mice, dogs, cats, red foxes, ferrets and sheep (Fekadu *et al.*, 1988; Schneider, 1982; Baltazar *et al.*, 1988; Vos *et al.*, 2004a, b; Tjornehoj *et al.*, 2006; Brookes *et al.*, 2007; Picard-Meyer *et al.*, 2008). The susceptibility of bats (*M. daubentonii* and *Myotis brandtii*) to infection with EBLV-1 and another unclassified bat rabies virus isolate has been studied in Russia (Botvinkin *et al.*, 1992). The influence of hibernation on the duration of the incubation period and viral distribution in *Pipistrellus pipistrellus* bats infected with EBLV-1 has also been investigated (Kuzmin *et al.*, 1994). In a recent experiment with EBLV-1, the North American big brown bat (*Eptesicus fuscus*) was chosen as a comparative model because of its close genetic relationship and biological similarities to *E. serotinus* (Franka *et al.*, 2008). This study provided data on the susceptibility of a non-reservoir bat species in response to inoculation with EBLV-1. Injection of virus by the intramuscular (i.m.) route resulted in 50 % mortality in this species. A recent study of EBLV-2 infection in Daubenton's bats (*M. daubentonii*) was the first experimental infection with EBLV in one of its assumed natural hosts (Johnson *et al.*, 2008). In this study, no animals developed disease following i.m. inoculation and a single bat (14 %) developed rabies following subcutaneous (s.c.) inoculation. Provided with these background data, we performed experimental infections in the natural reservoir for EBLV-1, the serotine bat. Different routes of infection were applied to study the pathogenesis of the EBLV-1 in its natural host, the dynamics of the humoral immune response, the distribution of virus in infected bats and in those who had succumbed to the disease, and virus excretion in saliva.

METHODS

E. serotinus. Twenty-nine juvenile or adult common serotine bats of both sexes were captured from various sites in Germany. Some animals that could not be returned to the wild were obtained from bat sanctuaries and zoos. The bats originated from the federal states of Baden-Württemberg, Lower Saxony and Saxony-Anhalt. Ethical approval and exceptional permission was sought prior to the project from the competent authorities in the respective federal states. The bats were quarantined for at least 1 month prior to use and adapted to housing in cages and to artificial feeding. Seventeen days before

inoculation, a blood sample was taken from each bat from the vena uropatagialis and the titre of rabies virus-neutralizing antibodies (VNAs) against EBLV-1 was determined using a modified rapid fluorescent focus inhibition test (RFFIT; Cox & Schneider, 1976). The animals were marked individually with a microchip (UNO Micro ID ISO Transponder 8 mm), inserted subcutaneously under the scruff of the neck. The bats were housed essentially as described elsewhere (Johnson *et al.*, 2008). All handling and invasive procedures were conducted in compliance with the German Animal Welfare Act and the recommendations of the Society for Laboratory Animal Science (GV-SOLAS). In addition, all procedures were reviewed by the Veterinary Laboratories Agency Ethics Committee and approval for the experimental study was given.

Virus. In accordance with a study in *E. fuscus* (Franka *et al.*, 2008), the same EBLV-1a isolate from a serotine bat isolated in Osnabrück, Germany, in 1997 (GenBank accession nos DQ522860 and DQ522892) was used (Müller *et al.*, 2007). The virus was passaged three times by the intracerebral (i.c.) route in ORF1 mice prior to use. Mouse brain suspensions (20 %) were prepared in minimum essential medium (MEM-10; Gibco) supplemented with 10 % fetal calf serum and centrifuged at 3200 ***g*** for 10 min. Aliquots of the supernatant were frozen and used for bat inoculations and titration in mice. The inoculation dose used was 20 µl EBLV-1 ($10^{3.2}$ 50 % mouse lethal dose ml^{-1}) for all groups.

Inoculation and monitoring. Four routes of inoculation were used for infection. For i.m. inoculation, either EBLV-1 or a 10 % negative-control mouse brain homogenate (negative-control group) was injected into the pectoral muscle. S.c. inoculation was performed by injecting EBLV-1 homogenate under the skin in the scruff of the neck. For i.c. inoculation, animals were sedated and inoculum was applied into the left side of the brain. The inoculum for intranasal (i.n.) inoculation was placed in the nasal passage and inhaled. All animals were monitored closely for 1 h after inoculation. Subsequently, the bats were examined visually twice a day for 120 days. Animals were bled on days 35, 68 and 91 post-inoculation (p.i.). Blood (10–100 µl) was collected from the brachial artery or vein, or by cardiac puncture after the animal had been humanely killed. Blood was centrifuged at 5000 ***g*** for 20 min, serum separated and heat inactivated for 30 min at 56 °C and stored at −80 °C until use.

Oral swabs were taken at different time intervals during the experiment. Oral swab samples were collected using sterile cotton-tipped swabs (nerbe plus GmbH) and placed directly into 1 ml MEM-10, 200 µl of which was taken for virus isolation using a rapid tissue cell infection test (RTCIT; Webster & Casey, 1996). The remaining volume was used for RNA extraction and subsequently for the detection of viral nucleic acid by RT-PCR. All bats were weighed weekly after the oral swabs had been taken. Body surface temperatures (neck and chest) of bats were measured in parallel with weighing using an infrared laser thermometer (Fluke 62; Fluke).

All animals that displayed clinical disease during the experimental period or were healthy at the end of the experiment (day 120) were humanely killed. Carcasses were frozen immediately and stored at −20 °C until necropsy. A diseased bat from the i.c. group and a non-diseased animal from the s.c. group were fixed in formalin immediately after death for pathological examination. All other bats were subjected to necropsy and tissues (brain, submandibular and parotid salivary glands, tongue, heart, lung, kidney, liver, spleen and bladder, pectoral muscle and thyroid gland) were collected and stored at −80 °C until further virological examination.

Virus detection. A fluorescent antibody test (FAT) was performed according to standard recommendations (Dean *et al.*, 1996) using a fluorescein isothiocyanate (FITC)-conjugated polyclonal Ig (SIFIN) on all brain samples (i.e. from experimentally infected bats and from mice used for mouse inoculation tests and virus titrations. Infected cell cultures were also tested by FAT. Virus isolation from oral swabs (200 µl) was undertaken using the RTCIT (Webster & Casey, 1996). Briefly, mouse neuroblastoma cells (MNA 42/13; Friedrich-Loeffler-Institute) were inoculated with 200 µl oral swab medium. Three consecutive passages were conducted to confirm a negative result by RTCIT.

In the animals destined for histopathological examination, brain, spinal cord, salivary glands, liver, kidney, lung, spleen and intestinal samples were fixed in 10 % buffered formalin and processed using routine techniques. Immunohistochemical detection of lyssavirus nucleocapsid was undertaken as described elsewhere (Hicks *et al.*, 2009). Briefly, serial 4 µm sections were hydrated and successively incubated with hydrogen peroxide (BHD), normal goat serum, primary anti-rabies nucleocapsid monoclonal antibody HAM (obtained from the Swiss Rabies Centre, Bern, Switzerland), biotinylated goat anti-mouse secondary antibody, avidin–biotin–peroxidase conjugate (Elite; Vector Laboratories) and 3,3′-diaminobenzidine (Sigma Aldrich). Sections were counterstained with Meyer's haematoxylin (Surgipath) and mounted.

RT-PCR and quantitative real-time PCR. RNA extraction of tissue samples and medium from oral swabs was performed using a commercial kit (RNeasy; Qiagen). Amplification and detection of viral RNA were undertaken using a hemi-nested RT-PCR (Heaton *et al.*, 1997). Oral swabs were also tested by real-time PCR (Wakeley *et al.*, 2005). The viral load was estimated from RNA samples extracted from the organs of three bats (IDs 34629, 34841 and 34620) from the s.c. group. A standard curve was generated using purified amplicons generated with primers Jw12 and N165 (Wakeley *et al.*, 2005) and this was used to calculate the absolute number of target copies in a particular sample, as described previously (Johnson *et al.*, 2006a). Target samples and standards were amplified using primers Jw12 and N165, and detected using probe GT5 (Wakeley *et al.*, 2005). Results are expressed as the number of genome copies per 0.04 µg extracted RNA. Positive-control (RNA from mouse brain infected with EBLV-1) and negative-control (water) samples were analysed in parallel in each PCR run. Amplicons from brain tissues were sequenced using standard primers and protocols to confirm virus identity (Heaton *et al.*, 1997).

Modified RFFIT. The presence of VNAs in bat sera was determined with a modified RFFIT (Smith *et al.*, 1973) using a homologous EBLV-1 isolate as test virus (Cox & Schneider, 1976). Sera were tested in twofold dilutions on mouse neuroblastoma cells (MNA 42/13) with a starting dilution of 1 : 10.

RESULTS

Of the 29 individual serotine bats from five different groups, 14 died or were euthanized during the study (120 days). Non-specific deaths occurred in two bats from the i.n. group (IDs 30892 and 31088), two of the s.c. group (IDs 29725 and 25599) and one from the control group (ID 33926). These animals tested negative for rabies by FAT (Table 1). In the i.c. group, all animals (n=5) succumbed to the disease and subsequently tested positive for rabies by FAT (IDs 29614, 31215, 32373 and 34392) or immunohistochemistry (ID 33342). The incubation period ranged from 7 to 13 days p.i. Inoculation of EBLV-1 by the i.m. route resulted in the development of rabies in one (14 %, ID 34550) out of seven inoculated bats. The animal died on day 26 p.i. (Fig. 1). Three animals (42 %) from the s.c. group died between 17 and 20 days after inoculation (IDs

Table 1. Experimental details, course of infection and laboratory results of EBLV-1 infections

Inoculation route/chip ID	Sex	Age (years)	Days to death	FAT	IHC	RT-PCR (brain)	Virus shedding (days p.i.)	Titres of VNAs/days p.i.				
								BS*	33	61	116	123
i.c.												
29614	M	>1	13	+	ND	+	−		Dead			
34392	M	>1	10	+	ND	+	−		Dead			
32373	M	>1	10	+	ND	+	−		Dead			
31215	F	>1	7	+	ND	+	−	<10†	Dead			
33342	M	>1	11	ND	+	ND	−		Dead			
i.n.												
29107	M	>1	−	−	ND	−	−		<10	ND	<10	<10
34526	M	>1	−	−	ND	−	−		<10	<10	<10	<10
29460	M	>	−	−	ND	−	−		<10	<10	<10	<10
30892	M	>1	116‡	−	ND	−	−		<10	<10†	Dead	
31088	F	<1	108‡	−	ND	−	−		<10	<10	Dead	
29033	M	>2	−	−	ND	−	−		<10	ND	<10	<10
i.m.												
32361	M	>1	−	−	ND	−	−		<10	<10	<10	<10
27896	M	>1	−	−	ND	−	−		<10	<10	<10	<10
34550	M	>1	26	+	ND	+	−		Dead			
31263	M	>2	−	−	ND	−	−		<10	ND	<10	<10†
30896	M	>2	−	−	ND	−	−		<10	<10	<10	<10
27030	M	<1	−	−	ND	−	−		<10	<10	<10	<10
32990	M	<1	−	−	ND	−	−		<10	<10	<10	<10
Control												
31923	F	>1	−	−	ND	−	−		<10	ND	ND	ND
32475	M	>1	−	−	ND	−	−		<10	ND	ND	<10
32631	M	>1	−	−	ND	−	−		<10	ND	ND	<10
33926	M	>1	17‡	−	ND	−	−		Dead			
s.c.												
29579	F	>1	−	−	ND	−	−		<10	<10	<10	<10
34620	M	>1	20	+	ND	+	13/14		Dead			
29725	M	>1	17‡	ND	−	ND	−	<10†	Dead			
34629	M	>1	17	+	ND	+	−		Dead			
25599	M	>1	17‡	−	ND	−	−	<10†	Dead			
33841	F	>1	18	+	ND	+	−	<10†	Dead			
31638	F	<1	−	−	ND	−	−		<10	<10	<10	<10

ND, Not determined.
*BS, Blood sample taken on the day of euthanasia.
†Blood clot also tested by RT-PCR.
‡Non-specific deaths.

34629, 33841 and 34620) and tested positive by FAT. Oral swab samples of one positive individual (ID 34620) from this group tested positive by RTCIT on days 13 and 14 p.i. (Table 1). The animal died on day 20 p.i. Rabid bats showed clinical signs with reduced feed intake, tremor, irritability, aggressiveness and paralysis, but acute death also occurred. None of the investigated sera, regardless of the group or the time point of blood sampling, gave a result indicative of the presence of VNAs (Table 1).

The pattern of virus distribution in different organs in diseased bats is shown in Table 2. Besides the brain, viral RNA was most frequently detected in the tongue, thyroid glands and lungs of diseased animals. Viral RNA was also found occasionally in other organs. In the salivary glands, viral RNA was detected only in the group infected by the s.c. route, with all three animals from this group testing positive. Quantification of viral RNA in the different organs of s.c.-inoculated animals showed most genome copies in the salivary gland and to a lesser extent in the thyroid gland (Fig. 2, Table 2). Blood clots from several diseased and non-diseased bats were also tested for the presence of viral RNA but all gave negative results (Table 1).

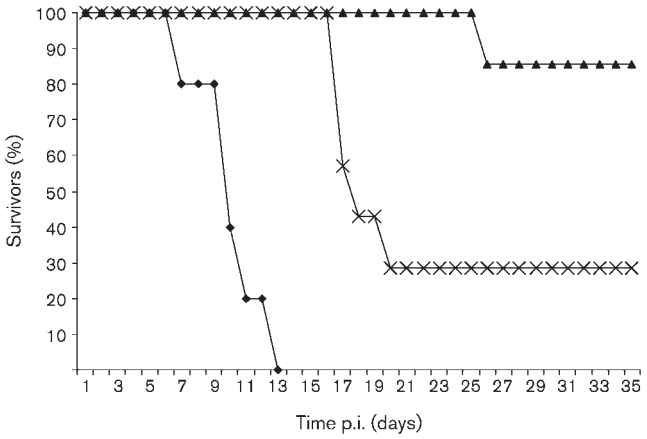

Fig. 1. Survival graph of infected groups (◆, i.c.; ▲, i.m.; ×, s.c.). The remaining groups (i.n., all infected; control, none infected) have been omitted for better visualization.

Immunohistological examination of a bat from the i.c. group (ID 33342) that had succumbed to infection showed viral antigen in both the peripheral and the central nervous system. In the latter, EBLV-1 antigen was detected in all regions examined, including the medulla oblongata, hippocampus and cerebellum (Fig. 3a–c). Virus antigen was also observed throughout the spinal cord in dorsal (sensory) and ventral (motor) horn neurons (Fig. 3d). Occasionally, immunolabelled ventral horn motor neurons showed marked vacuolation of the cytoplasm. In various parts of the peripheral nervous system, viral antigen was detected. Several ganglioneurons as well as sympathetic ganglions displayed immunolabelling. Viral antigen was also detected in nerves in the tongue and salivary gland. In the latter, viral antigen was not detected in the secretory or ductal epithelial cells, but immunolabelling was observed in both extra- and intraglandular nerves. Interestingly, more than 50 % of the papillae in each tongue section showed immunolabelling in the epithelial cells, regardless of the region. Specifically, epithelial cells and cells surrounding the taste buds were found to be positive, as were cells in the lamina propria. In some taste buds, the presence of pyknotic and karyorrhectic debris indicative of degeneration and necrosis of the affected taste bud was observed (Fig. 4a). No significant changes or immunohistological findings were detected in any of the tissues of uninfected animal (Fig. 4b).

In general, in all groups except for the control and i.c. group, bats lost weight as they progressed through the study, albeit without statistical significance (data not shown). However, in all rabid animals (n=9), stable or increasing weights were more common than weight loss, regardless of the route of infection. Body surface temperatures differed widely in individual bats, within groups and between groups. No specific trend could be observed (data not shown).

DISCUSSION

Based on limited observational studies and experimental data of EBLV infection in bats, our understanding of the transmission and pathogenesis of this virus under natural conditions is incomplete. In contrast to previous EBLV-1 studies in *Chiroptera* (Botvinkin *et al.*, 1992; Kuzmin *et al.*, 1994; Franka *et al.*, 2008), the natural host, the serotine bat, was infected experimentally with EBLV-1 in this study to mimic different possible routes of infection. Whilst all animals inoculated by the i.c. route succumbed to the disease, none of the animals in the negative-control group tested positive, thus proving the viability of the inoculum. Only one of the bats inoculated by the i.m. route died of rabies (14 %), whilst three animals (42 %) in the s.c. group

Table 2. Detection by RT-PCR of viral RNA within various organs of serotine bats inoculated with EBLV-1a

In the s.c. group, the number of genome copies per 0.04 μg RNA in peripheral organs is shown.

Group	i.m. (control) (n=0)	i.n. (n=0)	i.m.	s.c.			i.c.			
	–	–	ID 34550	ID 34620	ID 33841	ID 34629	ID 31215	ID 29614	ID 32373	ID 34392
Brain	−	−	+	+	+	+	+	+	+	+
Thyroid gland	−	−	+	770 000	4 320	17 000	+	−	+	−
Salivary gland	−	−	−	159 000	345 000	594 000	−	−	−	−
Heart	−	−	+	26 400	−	−	+	−	+	−
Lung	−	−	+	77 600	256	11 700	+	−	+	+
Pectoral muscle	−	−	+	571	1 000	1 150	+	−	−	−
Liver	−	−	+	12 400	1 890	−	−	−	−	−
Kidney	−	−	+	53 200	409	3 740	+	−	−	−
Spleen	−	−	+	76 400	46 800	−	−	−	−	+
Bladder	−	−	−	9 970	−	−	−	+	−	−
Tongue	−	−	+	88 700	11 900	261	+	+	+	+

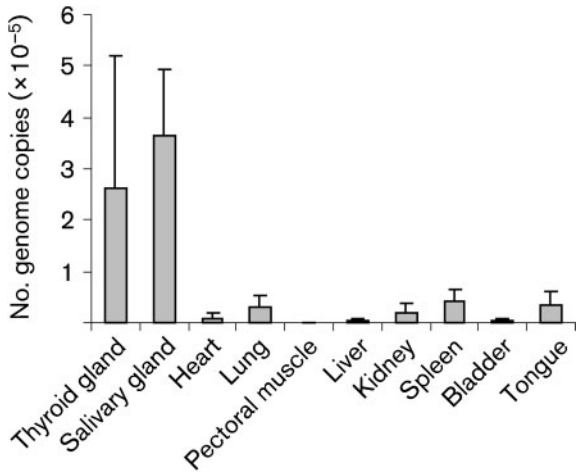

Fig. 2. Quantitative real-time RT-PCR detection of EBLV-1 genome copies within the peripheral organs of rabid bats of the s.c. group. Results are shown as means ± SD. Details of individual values are given in Table 2.

developed the disease (Fig. 1). These findings suggest that i.c. infection is the most efficient way for transmission, followed by s.c. and i.m. inoculation, although animals infected by the s.c. or i.m. route may not necessarily become rabid. As expected, the incubation periods varied depending on the route of inoculation, with the i.c. route leading to the shortest incubation period (7–13 days), followed by s.c. (17–18 days) and i.m. (26 days) (Table 1).

The i.n. route was ineffective in inducing rabies, supporting previous findings in experimental studies with WCBV in *E. fuscus* (Kuzmin *et al.*, 2008), EBLV-1 in *E. fuscus* (Franka *et al.*, 2008) and EBLV-2 in *M. daubentonii* (Johnson *et al.*, 2008). It seems highly unlikely that transmission of EBLVs among European bats occurs via non-bite exposures in nature. However, the i.n. route remains a possibility, especially under particular environmental conditions, as shown experimentally for mice (Johnson *et al.*, 2006b).

The finding that i.m. inoculation is inefficient at inducing the disease in the natural host is noteworthy. Interestingly, i.m. inoculation of EBLV-1 in some mammals other than bats was found to be dose-dependent and a reduced pathogenicity of the virus was shown in experimental studies in ferrets (Vos *et al.*, 2004a). In contrast to this study, EBLV-1 infection via i.m. inoculation in non-reservoir bat species resulted in mortalities of up to 24 % (*M. daubentonii* and *M. brandtii*) and 57 % (*E. fuscus*) (Botvinkin *et al.*, 1992; Franka *et al.*, 2008). Also, in other experimental studies with the new Eurasian lyssavirus genotypes Aravan, Khujand and Irkut viruses, considerably higher mortalities of 75, 60 and 54 %, respectively, were found in *E. fuscus* (Hughes *et al.*, 2006; Kuzmin *et al.*, 2008). It remains to be clarified whether this is due to the infectious dose used, the specific pathogenicity of the viruses or to the differences in the susceptibility of various bat species. In any case, i.m. inoculation was not as efficient at inducing the disease in natural hosts as s.c. inoculation. Similar results have been obtained with EBLV-2 (Johnson *et al.*, 2008). This may indicate an adaptation of bat-related lyssavirus species to their natural hosts. Our hypothesis is supported by the observation that i.m. inoculation of Mexican free-tailed bats (*Tadarida brasiliensis*) with RABV seemed to reduce the proportion of virus-positive salivary glands compared with s.c. infection (Baer & Bales, 1967).

Bites are the presumed route of virus transmissions among bats, causing superficial lesions. To maintain a transmis-

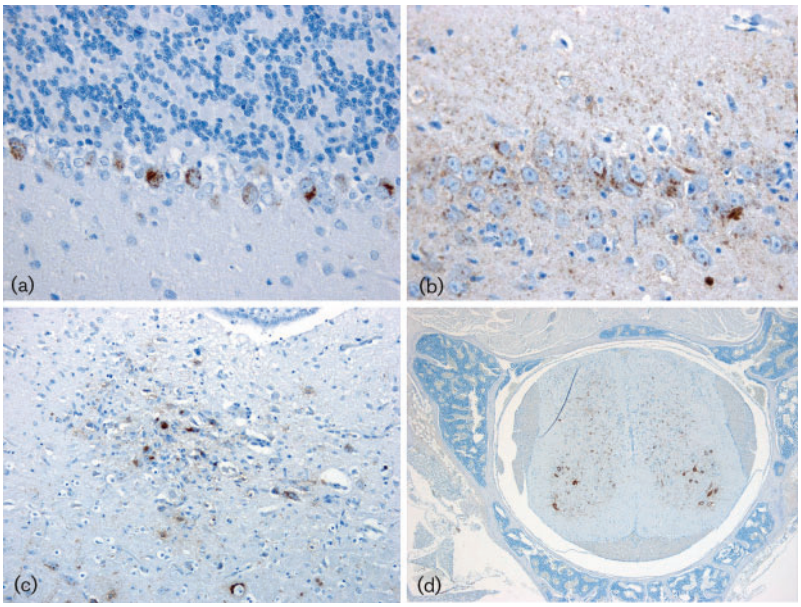

Fig. 3. Detection of viral antigen (brown pigment) in the central nervous system. Immunolabelling of Purkinje cells in the cerebellum (a), numerous neurons in all layers of the hippocampus (b), and cells in the medulla oblongata, associated with neuronal degeneration and gliosis (c) and in the spinal cord (d). Magnification: ×40 (a, b); ×20 (c); ×5 (d)

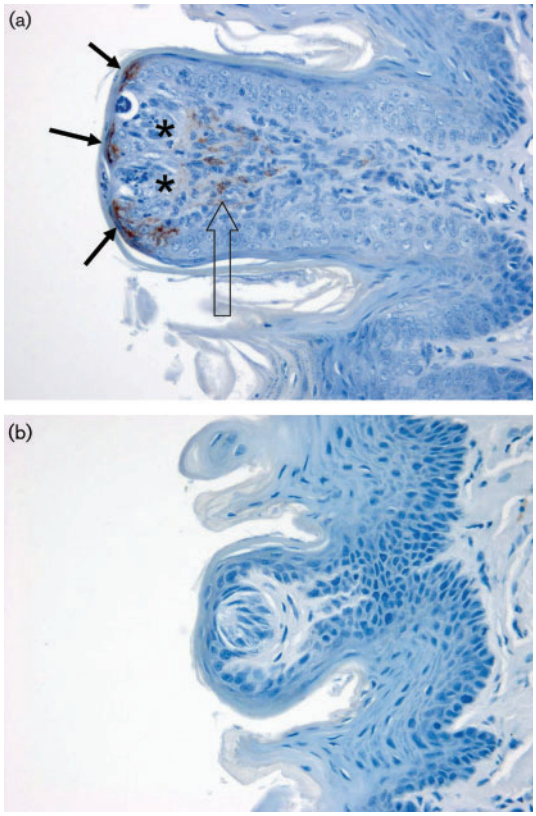

Fig. 4. (a) Immunohistochemical detection of viral antigen in epithelial cells (arrows) and cells in the lamina propria (open arrow) in a taste bud of a rabid individual. Asterisks indicate the presence of pyknotic and karyorrhectic debris within taste buds that have lost their normal architecture, and mononuclear infiltration of underlying tissues. (b) Lingual papilla containing a normal taste bud from a non-rabid animal. Magnification: ×40 (a, b).

sion cycle among free-living serotine bats, EBLV-1 may be adapted to this route of infection by having a higher affinity to certain cell types found intra- or subcutaneously, similar to observations made with silver-haired bats infected with RABV in North America (Morimoto *et al.*, 1996). Further *in vitro* studies with EBLV-1 may clarify the biological properties of this virus with respect to target-cell phenotype and the optimal temperature for virus infection and replication.

The propagation of virus prior to inoculation in mice is unlikely to be responsible for the apparent differences between field observations and this study. Considering that EBLV-1 has one of the lowest mutation rates of RNA viruses (Davis *et al.*, 2005), mutations during propagation are unlikely to occur. This is supported by sequence comparisons of original strains and passaged strains of EBLV-1 where no obvious changes in sequence were detected (C. Freuling, unpublished data). In our study, only one animal was found to shed viable virus via saliva. Also, highly sensitive RT-PCR techniques were not able to detect viral RNA in other saliva samples. The apparent low level of virus shedding and the low mortality among infected bats leave the mechanisms of the transmission cycle in nature enigmatic (Vos *et al.*, 2007). It cannot be ruled out, however, that the amount of shed virus or viral RNA is below the diagnostic threshold of both RTCIT and RT-PCR. Whether such extremely low levels of virus shedding would be able to transmit the infection and cause disease is unclear, as no evidence for secondary transmission has been reported in studies conducted with bats in captivity. Experimental studies with EBLV-1 in *E. fuscus* with various doses suggest that a low dose of virus inoculum is more efficient for virus spread in infected animals (Franka *et al.*, 2008). A 'multiple exposure' theory may explain why EBLV-1 is endemic in serotine bat populations but causes few fatalities, so that an equilibrium exists: frequent biting as part of gregarious behaviour with small dermal scratches could result in exposure to virus. Depending on the number of bites, the virus dose and the status of the immune system, some animals may develop VNAs, as shown in other experimental studies (Franka *et al.*, 2008), and may clear the virus, whilst in others the disease may develop and a transmission cycle may be maintained. Our experimental studies do not provide any evidence for an asymptomatic carrier state or for a prolonged incubation period, which is needed as a prerequisite for this hypothesis. Long incubation periods have not been reported for EBLV-1. However, for EBLV-2, a Daubenton's bat was found to be EBLV-2 positive only after being housed for more than 9 months in captivity (Pajamo *et al.*, 2008).

Generally, virus shedding during experiments of this nature is rarely detected (Hughes *et al.*, 2006; Franka *et al.*, 2008; Johnson *et al.*, 2008). A possible reason for this may be the sampling frequency. If virus shedding occurs only at certain intervals p.i., the chance of finding viable virus or nucleic acid present in saliva may be greatly reduced. This may imply that active lyssavirus surveillance in bat populations focusing on virus detection in live bats alone has limited effectiveness and should be accompanied by serological surveys.

None of the sera of *E. serotinus* in our experiment showed virus-neutralizing activity, whereas in a previous study with EBLV-1 in *E. fuscus*, several animals infected by the i.c., i.m. and s.c. routes developed VNAs before succumbing to the disease and VNAs were also detected in several survivors (Franka *et al.*, 2008). Technical reasons for the observed lack of seroconversion can be excluded, as identical virus from the previous study was used and essentially the same method was applied by the same team. The absence of VNAs is also in contrast to field data from Spain and Germany, where few wild-caught *Eptesicus* bats from selected colonies repeatedly tested serologically positive (Vázquez-Morón *et al.*, 2008a, b; T. Müller, unpublished data). Extremely high virus-neutralizing activity has been found in various other bat species in bat colonies in Spain (Serra-Cobo *et al.*, 2002; Amengual *et al.*, 2007). However, there is no validated standard

serological method available for the measurement of VNAs in bats. Different laboratories use different thresholds for determining positivity and therefore such results should be interpreted with caution. There is an obvious requirement for reference laboratories to standardize protocols for the (sero)surveillance of lyssaviruses in bats.

The absence of detectable levels of VNAs in the sera may also be seen as an indication of a significant role of innate or cellular immunity in the peripheral clearance of EBLV-1 in serotine bats. Another explanation for varying mortality rates and levels of VNAs detected in experimental studies and in the field could be the different conditions, which may have resulted in different levels of distress for the animals involved. Physiological stress can cause dysregulation in antibody production and cell-mediated immune responses (Padgett & Glaser, 2003). However, assays to measure other immune-modulating factors such as cytokines and chemokines as parameters of the immune system have not yet been established for serotine bats but should be considered for further experimental studies.

The results of our experiments and previously published data suggest that virus in bats spreads centripetally from the site of inoculation through nerves and, after reaching the brain (Fig. 3a–c), centrifugally to peripheral tissues and organs (Fig. 3d). In general, no significant pattern of virus distribution in different non-neuronal organs was observed in those bats that developed disease (Table 2). Neither virus nor viral RNA was detected in the brain, salivary gland, other organs or oral swabs of bats that survived the experiments, contradicting any suggested carrier state in bats. In addition, the presence of viral RNA in blood clots as described elsewhere (Serra-Cobo *et al.*, 2002; Amengual *et al.*, 2007) seems to have been refuted by our results.

The virus appears to have a certain affinity to the thyroid gland, as this organ was found to be positive in most rabid animals. Quantitative real-time RT-PCR also revealed that the number of genome copies was very high in the thyroid gland (Fig. 2). This has also been shown in a previous experiment (Franka *et al.*, 2008). Infection of the thyroid gland may cause hormonal imbalances, providing a possible explanation for some of the clinical features associated with the disease (Fooks *et al.*, 2009).

Lung and tongue tissues of six out of seven investigated individuals were positive by RT-PCR. This supports other studies with lyssaviruses in experimentally infected bats, which also found viral RNA or viable virus in tongue and/or lungs (Hughes *et al.*, 2006; Markotter, 2007; Franka *et al.*, 2008; Johnson *et al.*, 2008; Kuzmin *et al.*, 2008). The detection of EBLV-2 in the tongues of infected bats is a consistent finding in naturally infected bats (Johnson *et al.*, 2006a). Findings in the tongue corroborate the immunohistochemical analysis of an i.c.-infected animal, which clearly showed virus antigen in lingual papilla epithelium associated with the taste buds (Fig. 4a). Interestingly, similar findings were observed in *Epomophorus wahlbergi* experimentally infected with Lagos bat virus using staining of tissue with FITC-labelled antibodies (Markotter, 2007). This is a remarkable observation, as the taste buds are intensely innervated and may provide conditions for viral transport and amplification, possibly indicating a different route of virus shedding. It remains to be clarified, however, whether viable virus can be shed through the superficially located taste buds. The isolation of viable virus from the tongues of experimentally infected bats supports this hypothesis, although contamination of the tongue caused by adjacent salivary gland or saliva could not be ruled out completely (Hughes *et al.*, 2006; Markotter, 2007). Furthermore, the hypothetical shedding via epithelial cells in the tongue is supported by the fact that viral antigen was not found in the secretory or ductal epithelial cells of the salivary gland of the same individual. This may also explain the absence of viral RNA in oral swabs from all infected animals tested, indicating very limited virus shedding via saliva. However, the failure to detect virus or viral RNA in the salivary glands or oral swabs of any of the i.c.-infected animals that died could also be attributable to the route of inoculation causing death before extensive dissemination of the virus to peripheral parts of the body. This is partly in accord with experimental data from *E. fuscus* where no viral RNA was detected in the salivary gland of any animal inoculated by the i.c. route at the time of necropsy, although viable virus was detected in the oral swab of one animal, by virus isolation and RT-PCR, 2 days prior to death (Franka *et al.*, 2008). However, the results of quantitative real-time RT-PCR, with most genome copies in peripheral organs found in the salivary gland (Fig. 3, Table 2), indicate the prominent role of this organ in virus transmission. Weight loss as a supposed sign of naturally infected *E. isabellinus* (Vázquez-Morón *et al.*, 2008a, b) was not observed in rabid individuals during this study. Most likely, this discrepancy is due to the fact that, whilst naturally infected animals lose the ability to hunt and reduce their feeding, thus losing weight, experimentally infected animals are fed regularly and maintain their body condition. The resulting difference in body condition could partly influence the clinical course of the infection and may therefore account for some of the observed differences between field and experimental data.

From our observations, we conclude that s.c. inoculation, probably as a result of shallow bites, is an effective way of EBLV-1 transmission among free-living serotine bats. Further research is needed to elucidate this possible new mechanism of virus shedding and transmission for bat lyssaviruses.

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