Experimental study of European bat lyssavirus type-2 infection in Daubenton's bats (*Myotis daubentonii*)

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European bat lyssavirus type 2 (EBLV-2) can be transmitted from Daubenton's bats to humans and cause rabies. EBLV-2 has been repeatedly isolated from Daubenton's bats in the UK but appears to be present at a low level within the native bat population. This has prompted us to investigate the disease in its natural host under experimental conditions, to assess its virulence, dissemination and likely means of transmission between insectivorous bats. With the exception of direct intracranial inoculation, only one of seven Daubenton's bats inoculated by subdermal inoculation became infected with EBLV-2. Both intramuscular and intranasal inoculation failed to infect the bats. No animal inoculated with EBLV-2 seroconverted during the study period. During infection, virus excretion in saliva (both viral RNA and live virus) was confirmed up to 3 days before the development of rabies. Disease was manifested as a gradual loss of weight prior to the development of paralysis and then death. The highest levels of virus were measured in the brain, with much lower levels of viral genomic RNA detected in the tongue, salivary glands, kidney, lung and heart. These observations are similar to those made in naturally infected Daubenton's bats and this is the first documented report of isolation of EBLV-2 in bat saliva. We conclude that EBLV-2 is most likely transmitted in saliva by a shallow bite.

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INTRODUCTION

A rabies-like virus was first isolated in Europe in 1954 following an incident where a child was bitten on the finger by a bat (Mohr, 1957). Inoculation of brain homogenate from the bat into mice induced progressive fatal encephalitis, and Negri bodies were detected in brain smears. At about the same time, rabies virus was also identified in insectivorous bats in North America (Scatterday, 1954). Sporadic reports of rabies-like virus followed in Europe, usually associated with the Serotine bat (*Eptesicus serotinus*) and mainly from Germany (Seidler *et al.*, 1987). It was only through the development of monoclonal antibody (mAb) typing (Wiktor & Koprowski, 1978) that the lyssaviruses isolated from European bats could be differentiated from classical rabies virus; they were found to be antigenically similar to Duvenhage virus, a lyssavirus isolated exclusively from Africa (Tignor *et al.*, 1977). Both Duvenhage and European lyssaviruses were classified as lyssavirus serotype 4. However, virus-typing using extended panels of mAbs identified a number of European exceptions. The first was a virus isolated from a human case of rabies in a bat ecologist who died in Finland (Lumio *et al.*, 1986), the second were a number of viruses isolated from pond bats (*Myotis dasycneme*) in The Netherlands (Van der Poel *et al.*, 2005). These viruses were tentatively classified as serotype 5 (King *et al.*, 1990). Subsequent genomic sequencing

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confirmed this distinction and led to a revision of lyssavirus classification with two distinct viruses, European bat lyssavirus (EBLV)-1 and EBLV-2, being recognized (Bourhy *et al.*, 1992). The first UK case of EBLV-2 in a Daubenton's bat (*Myotis daubentonii*) was recorded in 1996 (Whitby *et al.*, 2000), with a second case isolated from the same species in 2002 (Johnson *et al.*, 2003). This coincided with a human case in a bat worker in Angus, Scotland (Fooks *et al.*, 2003a). Extensive surveillance in a number of countries in Europe has established that the reservoir species of EBLV-1 is the Serotine bat (Van der Poel *et al.*, 2005; Müller *et al.*, 2007), whilst the reservoir species of EBLV-2 are bats of the Myotis genus, exclusively *M. dascycneme* and *M. daubentonii*. For reasons that are not understood, EBLV-1 is prevalent throughout mainland Europe but not in the UK, whereas EBLV-2 appears to be restricted to the UK, The Netherlands and the Swiss/German border (Freuling *et al.*, 2007).

EBLV-2 has been isolated from six Daubenton's bats in the UK between 1996 and 2007 (Harris *et al.*, 2007) and serosurveys of bat populations throughout England and Scotland suggest that the virus is endemic in the species but prevalence is low (Brookes *et al.*, 2005a). The Daubenton's bat belongs to the family *Vespertilionidae* and is found throughout Europe as far south as Greece, with a northern limit of central Finland. Colonies of Daubenton's bats have been found throughout the UK, where it is the third most abundant bat species after the common pipistrelle (*Pipistrellus pipistrellus*) and the brown long-eared bat (*Plecotus auritis*). All bats within the UK are protected by both national and European legislation, although the Daubenton's bat is not considered to be under threat and numbers may be increasing. After two well-documented cases of spill-over to humans, in both cases causing rabies, it is clear that the virus has the capacity for transmission to humans. In both cases, the individuals were unvaccinated and had recent contact with Daubenton's bats. This implies that bite transmission, in a similar manner to North American bats (Messenger *et al.*, 2002), is the principal means of dissemination of this virus.

Cross-neutralization and cross-protection studies have demonstrated that current anti-rabies vaccines provide protection against EBLV-2 (Brookes *et al.*, 2005b) and all bat handlers in the UK are required to receive vaccination. A limited number of pathogenesis studies have been conducted on the EBLVs using a range of animal models (Vos *et al.*, 2004; Johnson *et al.*, 2006a; Brookes *et al.*, 2007). These have suggested that EBLV-2 is less virulent in animal models including mouse, sheep and fox, when compared directly with EBLV-1, and both are less virulent than rabies virus (RABV). However, it is not clear if this is true for the virus within its reservoir host. The case-reports of EBLV-2-infected bats indicate that unprovoked biting is commonly associated with rabies in bats (Table 1). This contrasts strongly with studies in Spain where apparently healthy bats have been shown to excrete EBLV-1 in saliva (Echevarria *et al.*, 2001; Vazquez *et al.*, 2006), suggesting the possibility that an asymptomatic carrier state could exist in lyssavirus-infected bats. From a public health perspective it is important to confirm or exclude this possibility.

Surveillance of bats for lyssaviruses in the UK demonstrates that EBLV-2 has a unique association with the Daubenton's bat (Table 1) and that this species is likely to be the reservoir host. The principal aim of this study was to investigate the susceptibility of the Daubenton's bat to inoculation with EBLV-2 and to study the spread of virus within its reservoir host.

METHODS

***M. daubentonii*.** Thirty four *M. daubentonii* bats were caught in the north of England under Natural England licence 20062087; these were transferred to Germany for adaptation to captive life and a one month quarantine period to ensure the health of the bats. All handling and invasive procedures were conducted in compliance with the German Animal Welfare Act 2 and 2a and recommendations of the Society for Laboratory Animal Science (GV-SOLAS). Ethical approval was sought from the Federal State of Sachsen-Anhalt, Germany, and the Ethical Review Proceedings committee at the Veterinary Laboratories Agency. Animals were treated with IVOMEC (Merial) to control ecto- and endo-parasites and fed mealworms and water *ad libitum*. A blood sample was taken from each bat from the vena brachialis and tested for virus neutralizing antibodies against EBLV-2 to establish a baseline for comparison to future measurements, using a modified fluorescent antibody virus neutralization test (see below).

Thirty-three bats were assigned into five groups and housed in purpose-built metal-framed cages (75 cm × 75 cm × 75 cm) with fine-mazed wire gauzed side sheets. The remaining bat was not considered to be sufficiently healthy to enter the study and was retained by a bat rehabilitator. Two cages were held within one scantainer (Scanbur), which provided a closed, filtered-air system with constant temperature and humidity. Three scantainers were held within a secure containment laboratory under negative air pressure and controlled access. Animals were inspected daily to monitor their condition and development of disease.

Virus. The EBLV-2 isolate used in this study was obtained from a Daubenton's bat found in Lancashire (Johnson *et al.*, 2003) and designated RV1332. Virus was passaged three times by intracerebral (i.c.) inoculation in female 5-week-old Swiss OF-1 mice (Charles River Laboratories, France) until a titre of 4.9 $\log_{10}(MLD_{50})$ per ml was reached. A 20 % mouse brain homogenate was prepared and clarified by centrifugation at 3200 ***g*** for 10 min, then immediately frozen at −80 °C until required.

Inoculation and monitoring. Four routes of inoculation of 20 µl of EBLV-2 infected mouse brain homogenate were used. Intranasal (i.n.) and i.c. inoculations were performed under light ether anaesthesia. For i.n. inoculation, the inocula was placed on the nasal passage and inhaled. For intramuscular (i.m.) inoculation, either EBLV-2 or negative control mouse brain homogenate was injected into the pectoral muscle. Subdermal (s.d.) inoculation was performed by injecting EBLV-2 homogenate under the skin in the scruff of the neck. This gave a final inoculation dose of 3.2 $\log_{10}(MLD_{50})$ that consistently induced disease in mice through both i.c. and s.d. routes. Lower doses of virus have produced variable outcomes in the murine model. All animals were monitored closely for one hour after inoculation. Subsequently, all animals were observed twice daily for 120 days. Saliva swabs were taken 1 week prior to inoculation and at

Table 1. Cases of EBLV-2 in the UK

Year	Isolation species	Age	Sex	County	Comments	Reference
1996	*M. daubentonii*	Adult	Female	Sussex	On May 30th a Daubenton's bat was found in the cellar of a public house in Newhaven. It was in poor condition and bit a woman who handled the bat. She subsequently received full post-exposure treatment. The bat was humanely killed the same day and was found to be lyssavirus-positive by FAT. This was the first case of EBLV-2 reported in the UK.	Whitby *et al.* (2000)
2002	Human	55 years	Male	Angus	A 55-year-old man was admitted to a Dundee hospital suffering from acute haematemesis and upper limb paraesthesia. On day 5 he became aggressive and required sedation; he died on day 14. He had a history of exposure to bats in the UK and ante-mortem PCR on saliva detected the presence of EBLV-2. This was confirmed on autopsy specimens where virus was recovered from brain tissue.	Fooks *et al.* (2003a)
2002	*M. daubentonii*	Juvenile	Female	Lancashire	On July 7th, a cat brought a bat into a property next to the Lancashire canal. The bat remained in captivity for seven weeks before developing signs of agitation and aggression to its handler. This continued for six days until the bat became uncoordinated and suffered muscular spasms when it was humanely killed.	Johnson *et al.* (2002)
2003	*M. daubentonii*	Adult	Male	Lancashire	On October 27th 2004, a bat was found to be lyssavirus positive on routine diagnosis. The specimen had originally been obtained in September the previous year where a member of the public had seen the bat flying during daylight. After hitting a tree the bat was recovered alive and passed on to bat rehabilitators where it died shortly afterwards, after displaying signs of unprovoked aggression.	Fooks *et al.* (2004a)
2004	*M. daubentonii*	Juvenile	Female	Surrey	On September 17th, a bat was observed in undergrowth during daylight hours in the town of Staines. Members of the Surrey Bat Group were called on the 21st and took the bat into care. The bat was in poor condition and very weak but prone to occasional acts of aggression and biting. The bat died on September 23rd.	Fooks *et al.* (2004b)
2006	*M. daubentonii*	Juvenile	Female	Oxfordshire	On September 12th, a grounded bat was found on the path by the River Thames close to Abingdon. The couple who found the bat cared for it for 24 h before passing it on to a bat rehabilitator. It displayed signs of aggression and was lethargic and dehydrated; it died two days later.	Fooks *et al.* (2006)
2007	*M. daubentonii*	Adult	Female	Shropshire	On August 12th, a bat was found on the floor of a public building. After biting the hand of a member of the public, the bat was transferred to a bat rehabilitation centre. It refused to take food or fluids and was aggressive to the handlers. The bat died later that day. All those who had contact with the bat either had completed full rabies vaccination or were given post-exposure treatment. A subsequent survey of the building identified a colony of Daubenton's bats in the building and entry to the room in which the bat was found was restricted to members of the public.	Harris *et al.* (2007)

weekly intervals, or daily if individual bats showed signs of disease. A cotton swab was held within the mouth until moistened and placed in 0.4 ml minimal essential medium with gentamicin (50 mg ml^{-1}) and amphotericin B (2.5 mg ml^{-1}). All swabs were frozen immediately and stored at −20 °C until processing. Body weight was measured at weekly intervals throughout the study.

Blood samples were taken either from the vena brachialis during the study period, or by cardiac puncture on the development of severe disease (paralysis) or at the completion of the experiment (terminal bleed after euthanasia). Serum samples were prepared and stored at −20 °C until testing. Bat carcasses were frozen immediately and stored at −20 °C until necropsy. One animal from each group was fixed in formalin immediately after death for pathological examination.

Virus detection. The fluorescent antibody test (FAT) was performed following standard protocols (Dean & Abelseth, 1973). Virus isolation from saliva swabs (200 µl) was performed using the rapid tissue culture inoculation test (RTCIT) as described previously (Webster & Casey, 1996). At least three consecutive passages were conducted to prove a negative result by RTCIT.

Detection of EBLV-2 RNA. RNA was extracted from organ samples using Trizol (Invitrogen) following the manufacturer's protocol. RNA was extracted from saliva swabs (200 µl) using an RNeasy column (Qiagen) following the manufacturer's protocol. For detection of virus genome, two RT-PCR methods were utilized. The first was the nested RT-PCR described by Heaton *et al.* (1997). Amplicons were separated on 1 % agarose gels and visualized by ethidium bromide (1 µg μl^{-1}) staining and UV illumination. The second assay was a probe-based real-time (q)RT-PCR assay described by Wakeley *et al.* (2005). This was modified using primers Jw12 (5′-ATGTAACACC-YCTACAATG-3′) and N165-146 (5′-GCAGGGTAYTTRTACT-ACTCATA-3′) where indicated, to incorporate SyBr green using the SyBr green JumpStart Taq ReadyMix assay (Sigma) to enable quantification of genome copies as described by Johnson *et al.* (2006a). β-actin primers (Wakeley *et al.*, 2005) and 18S RNA primers (Ambion) were used to detect host mRNA within samples.

Rapid fluorescent focus inhibition test (RFFIT). Virus neutralizing antibody (VNA) against EBLV-2 was measured with a modified RFFIT (Cox & Schneider, 1976) by replacing challenge virus standard (RABV) with EBLV-2. Samples were tested in duplicate with twofold serum dilutions starting at 1 : 10. A heterologous WHO international standard immunoglobulin (2nd human rabies immunoglobulin preparation, National Institute for Standards and Control, Potters Bar, UK), adjusted to 1.5 IU ml^{-1}, served as a positive control. The VNA titre was expressed as the reciprocal of the serum dilution showing a 50 % reduction in concentration of the EBLV-2 test virus *in vitro*. Sera from bats were assessed prior to inoculation and at the end of the study.

Immunohistochemistry. Following fixation in 10 % buffered formalin, brain, spinal cord, salivary glands, liver, kidney, lung, spleen and intestinal samples were blocked and routinely processed to paraffin wax. Immunohistochemical detection of lyssavirus nucleocapsid was performed as described by Hicks *et al.* (2008). Briefly, serial 4 µm sections were hydrated and successively incubated with hydrogen peroxide block (BHD), normal goat serum block, primary antibody anti-rabies nucleocapsid mAb HAM (obtained from the Swiss Rabies Centre, Bern, Switzerland), biotinylated goat–anti-mouse secondary antibody, avidin–biotin-peroxidase conjugate (Vector Laboratories) and 3,3-diaminobenzidine (Sigma). Sections were then counterstained with Meyer's haematoxylin (Surgipath) and mounted.

RESULTS

Five challenge groups were studied, four receiving $10^{3.2}$ $\log_{10}(MLD_{50})$ EBLV-2 and one receiving a normal mouse brain homogenate. Inoculation of EBLV-2 by the i.c. route resulted in the development of rabies in all bats challenged (Y2495, Y2505, Y2516, Y2556 and Y2566). Clinical signs were registered between 24 and 48 h prior to death and were characterized by an inability to fly and increasing paralysis. No overt signs of aggression between bats were observed during this time, although continuous observation was not possible and such events could have occurred. Weight measurements taken from this group of bats showed that, overall, the bats were losing weight as they progressed through the study, a feature that was observed in all of the groups. However, the mean weight within the i.c.-inoculated group declined sharply, decreasing from 9.76 g shortly after inoculation to 6.65 g at day 12, and coincided with disease (Fig. 1). Of the animals challenged by peripheral routes, only one animal (Y2070) developed rabies, at 33 days following inoculation by the subdermal route. At day 30, it developed disease that progressed rapidly to severe paralysis by day 33 and it was euthanized. Weight of this bat had decreased from 10 g at inoculation to 5.4 g at death. Antigen detection in the brain confirmed that these six animals (Y2495, Y2505, Y2516, Y2556, Y2566 and Y2070) had died of EBLV-2 infection (Table 2). One animal from each group was investigated for lyssavirus antigen using immunohistochemistry. Of five animals investigated this way, only the bat showing clinical signs of disease from the i.c. group (Y2556) showed evidence of lyssavirus nucleoprotein immunolabelling within the brain and spinal cord. Specific immunolabelling was detected in the perikarion and neuronal processes of neurons scattered through most regions of the brain (Fig. 2a, b) and spinal cord segments (Fig. 2c). Immunolabelled neurons were also observed outside the central nervous system in dorsal root ganglia neurons (Fig. 2d) and enteric plexus neurons. No viral nucleoprotein was detected in other tissues, including salivary glands, using this technique.

All animals were screened for neutralizing antibodies prior to inoculation and were shown to be EBLV-2-naive with a

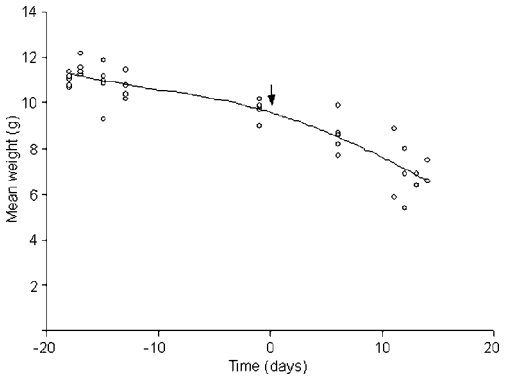

Fig. 1. Weight loss observed in bats infected by i.c. inoculation with EBLV-2. Each value shows the weight of individual bats at different times throughout the experiment. The arrow refers to the day of inoculation (day 0). The smooth line was fitted by 'distance weighted least-squares'.

Table 2. Inoculation of Daubenton's bats with European bat lyssavirus type 2

Inoculation route	Inoculum	Bat identification no.	Sex	Approximate age (years)*	Days to death†	EBLV-2 antigen detected
i.c.	EBLV-2	Y2495	F	2	14	+
		Y2505	F	>3	12	+
		Y2516	F	2	12	+
		Y2556	F	>4	14	+
		Y2563	M	4	−	Died during inoculation
		Y2564	F	>3	−	Died during inoculation
		Y2566	F	1–2	14	+
i.m.	EBLV-2	Y2494	M	2	−	−
		Y2498	F	>3	−	−
		Y2508	F	>2	−	−
		Y2513	M	3	−	−
		Y2517	F	2	−	−
		Y2557	F	>4	−	−
		Y2565	F	2	−	−
i.n.	EBLV-2	Y2287	F	4	−	−
		Y2560	F	3	−	−
		Y2561	M	2–3	−	−
		Y2568	M	?	−	−
		Y2569	M	4	−	−
		Y2572	F	>4	−	−
		Y2573	M	2–3	−	−
s.d.	EBLV-2	Y2410	M	3–4	−	−
		Y2550	F	3–4	−	−
		Y2551	F	>4	117	− (Non-specific death)
		Y2558	F	2–3	−	−
		Y2559	M	>4	−	−
		Y2562	M	2	−	−
		Y2720	M	>4	33	+
i.m.	Mouse brain homogenate	Y2554	F	−	−	−
		Y2553	F	−	−	−
		Y2555	M	−	−	−
		Y2570	M	−	−	−
		Y2571	F	−	114	− (Non-specific death)

*Age not estimated for animals indicated by −.
†Animals that survived to the end of the study period (123 days) indicated by −.

baseline titre of <1 : 10. Subsequent analysis of serum samples demonstrated that no animals, irrespective of whether they developed disease or survived to the end of the study, seroconverted (data not shown).

Two animals died during the 123 day experimental period, although these could not be attributed to EBLV-2 infection and were considered non-specific. The remaining animals survived to the end of the experiment. Both nested RT-PCR and a qRT-PCR assay corroborated the antigen detection assays, with both detecting EBLV-2 genes in all five bats from the i.c. group and the single bat from the s.d. group (Table 3).

To further understand the nature of EBLV-2 disease in its natural host, we investigated the distribution within organs and the salivary excretion of virus by nested RT-PCR. In four animals inoculated i.c., EBLV-2 RNA was not detected in any peripheral tissue (Table 4). Host (18S) RNA presence was confirmed in all but two samples. In contrast, EBLV-2 RNA was detected in the salivary gland, tongue, heart, lung and kidney samples of the single bat that succumbed following s.d. inoculation (Table 4 and Fig. 3a). Furthermore, EBLV-2 RNA was detected in saliva samples taken from this bat between days 28 and 32 (Fig. 3a), preceding the development of clinical signs of rabies by 2 days. The genomic sequence generated from these amplicons demonstrated that all sequences showed 100 % identity with EBLV-2 strain RV1332 (data not shown). This finding was confirmed by virus isolation from saliva swabs taken from this s.d.-inoculated bat on three

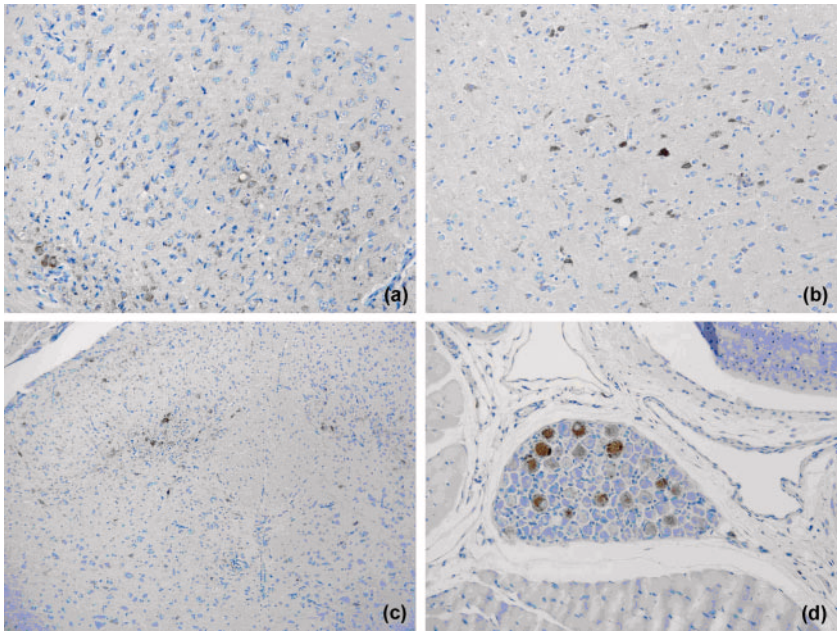

Fig. 2. Immunohistochemical demonstration of lyssavirus nucleocapsid in neurons of the cortex (a), medulla (b), spinal cord (c) and dorsal root ganglion (d) of a Daubenton's bat inoculated intracranially with EBLV-2. Brown staining indicates detection of viral nucleoprotein. Magnification, 100× (c) and 200× (a, b, d).

consecutive days prior to its death. However, this required three cell passages of each sample before virus was detected in the cells, verifying the low levels of viable virus in saliva. Further analysis of organ samples from this bat using qRT-PCR detected EBLV-2 RNA in all samples tested, but at very low levels (Fig. 3b), with the exception of the brain where very high levels of virus were observed. Relatively higher levels of genome copies (100–1000 copies per ml RNA) were found in the salivary gland, tongue, heart, lung and kidney, in agreement with nested RT-PCR detection of viral RNA.

DISCUSSION

The pathogenesis of EBLV-2 has presented a challenging enigma to scientists. The genome organization of EBLV-2 and RABV is very similar (Marston *et al.*, 2007), although distinctly divergent at the sequence level (Bourhy *et al.*, 1992). Whereas RABV causes over 50 000 human deaths a year, is endemic within a range of animal reservoirs and is present throughout the world, EBLV-2 has only been detected in two humans, and is restricted to two bat species in a geographically limited area within Europe. Despite these differences, the virus clearly has the ability to kill animals, including its reservoir species, with disease manifestations that are virtually identical to classical rabies. EBLV-2 also displays a strict neurotropism (Johnson *et al.*, 2006b) with sections of brain and spinal cord showing infected neurons (Fig. 2). The small numbers of natural cases of EBLV-2 also limit our understanding of the pathogenesis of this infection and it is this fact that has prompted us to investigate the virulence of this virus under experimental conditions. Surprisingly, experimental inoculation with a relatively large virus dose did not induce disease in the majority of animals (Table 2). Both the i.m. and i.n. inoculation routes used were ineffective at infecting Daubenton's bats. Only one of seven bats challenged by the s.d. route developed disease. Intensive investigation of this bat's saliva demonstrated that EBLV-2 genome could be detected in this bat up to 2 days before the development of disease. Previous studies that have infected bats with lyssaviruses have demonstrated virus excretion in saliva (Kuzmin *et al.*, 2008) but this study is the first demonstration of EBLV-2, either by detection of viral RNA or virus isolation in bat saliva. However, the levels of viral RNA detected were at the lower level of reliable quantification, suggesting that the amount of virus shed is low, possibly below 100 genome copies per μg extracted RNA. We have only had the opportunity to assess viral load in the saliva of one bat. It is possible that some bats may have a greater virus concentration in the saliva. EBLV-2 genome was also observed in bladder and kidney samples, although at low levels (Fig. 3). Further studies to investigate possible virus secretion in bat urine may identify an alternative means of virus egress and transmission within bats.

The data obtained in this study are limited, with only one of seven bats inoculated by the s.d route developing disease and all bats challenged by the i.m. route surviving to the end of the experiment. This implies that the reservoir species is surprisingly resilient to infection. In most lyssavirus challenge models, the i.m. route is effective at inducing disease (Moreno & Baer, 1980) so the relative differences in efficacy of inoculation may reflect differences in innervation at particular sites in the Daubenton's bat. The inability of EBLV-2 to infect bats by i.n. inoculation repeats a previous observation made for EBLV-1 in *Eptesicus fuscus* bats (Franka *et al.*, 2008). In roosts of

Table 3. Detection of EBLV-2 RNA within the brain of Daubenton's bats inoculated with EBLV-2

Group	Bat identification no.	β-actin*	18S RNA†	qRT-PCR‡	Nested PCR 1st round§	Nested PCR 2nd round‖
i.c	Y2495	+	+	+ (C_t=18.45)	+	+
	Y2505	+	+	+ (C_t=12.93)	+	+
	Y2516	+	+	+ (C_t=13.97)	+	+
	Y2556	+	+	+ (C_t=19.17)	+	+
i.m.	Y2494	+	+	−	−	−
	Y2498	+	+	−	−	−
	Y2508	+	+	−	−	−
	Y2517	+	+	−	−	−
	Y2557	+	+	−	−	−
	Y2565	+	+	−	−	−
i.n.	Y2287	+	+	−	−	−
	Y2560	+	+	−	−	−
	Y2568	+	+	−	−	−
	Y2569	+	+	−	−	−
	Y2572	+	+	−	−	−
	Y2573	+	+	−	−	−
s.d.	Y2410	+	+	−	−	−
	Y2550	+	+	−	−	−
	Y2558	+	+	−	−	−
	Y2559	+	+	−	−	−
	Y2562	+	+	−	−	−
	Y2070	+	+	+ (C_t=14.56)	+	+
Control	Y2554	+	+	−	−	−
	Y2555	+	+	−	−	−
	Y2570	+	+	−	−	−
	Y2571	+	+	−	−	−

*Primers: β act intronic/β act reverse (Wakeley *et al.*, 2005).
†Primers: 18S RNA primers (Ambion, Catalogue no. 1716).
‡Primers: Jw12/N165-146 (Wakeley *et al.*, 2005).
§Primers: Jw12/Jw6 (dpl, e, m) (Heaton *et al.*, 1997).
‖Primers: Jw12/Jw10 (me1, dle2, p) (Heaton *et al.*, 1997).

European bats, in contrast with those observed in bat species in other parts of the world, the number of individuals of species such as the Daubenton's bat is relatively small (<100 individuals). This would not favour aerosol spread and thus EBLV-2 could have evolved away from transmission by this route. When directly compared under experimental conditions, RABV proved more effective than EBLV-2 at infecting mice by the airborne route (Johnson *et al.*, 2006c), although a mouse is not the natural host for lyssaviruses, so these results should be interpreted with caution.

One possible explanation for sustained transmissibility could be that EBLV-2, like the silver-haired bat rabies variant (SHBRV) in North America, has become adapted to replication at the relatively lower body temperatures that are found in the dermis. SHBRV was shown to replicate faster at 34 °C than a RABV variant isolated from a coyote (Morimoto *et al.*, 1996). Superficial bites from Daubenton's bats were believed to be responsible for both human cases of EBLV-2 (Fooks *et al.*, 2003b). Another possible factor could be an extended incubation period. All of the Daubenton's bats in our study developed rabies within 32 days, with the animals inoculated by the i.c. route developing disease between 12 and 14 days. In one case of a naturally infected Daubenton's bat, an incubation period of 8 weeks was reported (Johnson *et al.*, 2003). Although a 90 day observation period was planned, this was extended to 123 days to lengthen the opportunity to detect disease after a longer incubation period; however, none occurred and there was no evidence of salivary excretion of virus without development of disease as has been reported for RABV (Aguilar-Setien *et al.*, 2005) and EBLV-1 (Echevarria *et al.*, 2001). However, much longer incubation periods have been observed for RABV infections (Smith *et al.*, 1991).

Table 4. Distribution of EBLV-2 RNA within organs of bats infected with EBLV-2 as detected by nested RT-PCR

Bat (inoculation group)	Organ	18S	Nested PCR 2nd round*
Y2495 (i.c.)	Submandibular gland	+	−
	Brown fat	+	−
	Salivary gland	+	−
	Tongue	−	−
	Heart	+	−
	Liver	+	−
	Pectoral muscle	+	−
	Liver	−	−
	Kidney	−	−
	Spleen	+	−
	Bladder	−	−
Y2556 (i.c.)	Submandibular gland	+	−
	Brown fat	−	−
	Salivary gland	+	−
	Tongue	+	−
	Heart	+	−
	Lung	+	−
	Pectoral muscle	+	−
	Liver	+	−
	Kidney	+	−
	Spleen	+	−
	Bladder	+	−
Y2505 (i.c.)	Submandibular gland	+	−
	Brown fat	+	−
	Salivary gland	+	−
	Tongue	No sample	No sample
	Heart	+	−
	Lung	+	−
	Pectoral muscle	+	−
	Liver	+	−
	Kidney	+	−
	Spleen	+	−
	Bladder	+	−
Y2516 (i.c.)	Submandibular gland	+	−
	Brown fat	+	−
	Salivary gland	+	−
	Tongue	+	−
	Heart	+	−
	Lung	+	−
	Pectoral muscle	+	−
	Liver	+	−
	Kidney	+	−
	Spleen	+	−
	Bladder	+	−
Y2070 (s.d.)	Submandibular gland	+	−
	Brown fat	+	−
	Salivary gland	+	+
	Tongue	+	+
	Heart	+	+
	Lung	+	+
	Pectoral muscle	+	−
	Liver	+	−
	Kidney	−	+

Table 4. cont.

Bat (inoculation group)	Organ	18S	Nested PCR 2nd round*
	Spleen	+	−
	Bladder	+	−

*All tested samples were negative in the first round PCR.

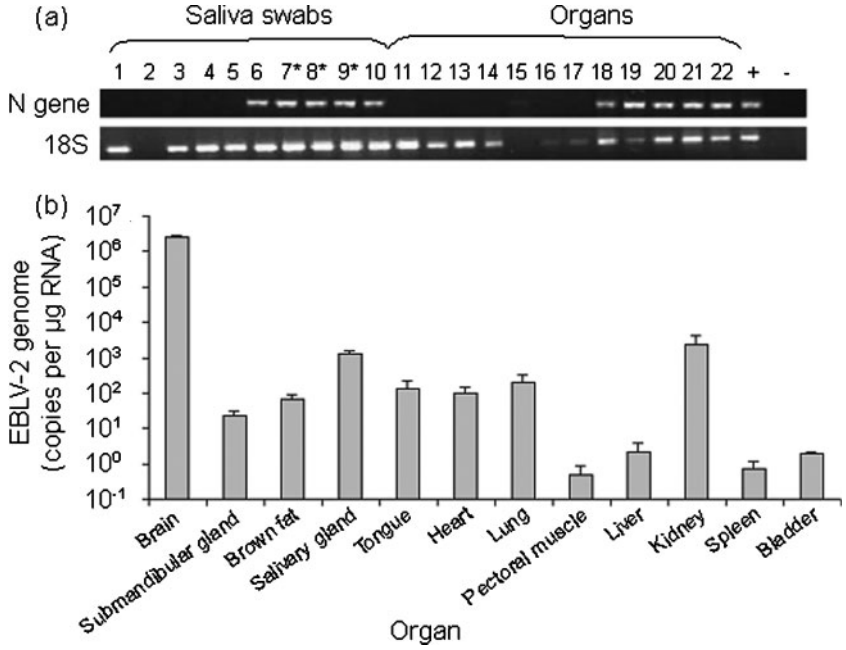

Fig. 3. Organ distribution and salivary excretion of EBLV-2 from a bat inoculated subdermally (Y2070). (a) Agarose gel-based detection of EBLV-2 nucleoprotein amplicons produced by nested RT-PCR of salivary samples (lanes 1–10) and organ samples (11–22). Salivary samples were taken post-inoculation on days 6, 13, 19, 20, 27, 28, 29, 30, 31 and 32 (lanes 1–10, respectively). *, Indicates samples from which virus was isolated. The organ samples were obtained at necropsy after euthanasia and were from the submandibular gland, brown fat, pectoral muscle, liver, kidney, spleen, bladder, brain, salivary gland, tongue, heart and lung (lanes 11–22, respectively). A positive control (+) of challenge virus standard RNA and a control with no template (−) were used. (b) qRT-PCR detection of EBLV-2 genome copies within the organs of bat Y2070.

In the single case of rabies following peripheral inoculation of EBLV-2 registered in our study, viral RNA was detected in saliva 2 days before the development of disease. Similar observations have been made for RABV infection in both insectivorous (Bell *et al.*, 1969) and vampire (Moreno & Baer, 1980) bats and Eurasian bat lyssaviruses in *E. fuscus* (Hughes *et al.*, 2006). This indicates that EBLV-2 shows similar pathogenic characteristics under experimental conditions to other lyssaviruses. Within this experiment, we did not find evidence for an asymptomatic carrier state with virus excretion. However, virus excretion from an apparently healthy bat was observed by detection of viral RNA; the bat then developed clinical signs of rabies over the next 2 days. This reinforces the need for those that have contact with bats to have rabies pre-exposure vaccination and to wear protective gloves to prevent being exposed to a bite.

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