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BRIEF REPORT

Repeated detection of European bat lyssavirus type 2 in dead bats found at a single roost site in the UK

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Abstract In August 2007, European bat lyssavirus type 2 (EBLV-2) was isolated from a Daubenton's bat found at Stokesay Castle. In September 2008, another bat from the same vicinity of Stokesay Castle also tested positive for EBLV-2. This is the first occurrence of repeated detection of EBLV-2 from a single site. Here, we report the detection of low levels of viral RNA in various bat organs by qRT-PCR and detection of viral antigen by immunohistochemistry. We also report sequence data from both cases and compare data with those derived from other EBLV-2 isolations in the UK.

Introduction

Cases of European bat lyssaviruses type-1 and -2 (EBLV) continue to occur across Europe. EBLV-1 seems to be restricted to the infection of serotine bats (*Eptesicus serotinus*) [3], although it has been reported in 'spill-over' events into incidental hosts [18, 22, 24]. In comparison to EBLV-1, EBLV-2 has been reported on fewer occasions, having been isolated from both Daubenton's bats (*Myotis daubentonii*) and pond bats (*Myotis dasycneme*) [20]. The discovery of EBLV-2 in a Daubenton's bat in June 1996 in Newhaven, East Sussex, prompted concerns that bat rabies may be present within the UK and, furthermore, that the threat of rabies entering the UK via migratory bats was realistic [28]. Indeed, bat rabies cases in European bats in the mid 1980s indicated a possible spread of the virus, especially in Denmark and the Netherlands. As well as having been isolated in the UK, EBLV-2 isolates have also been reported in Switzerland, Holland and Finland [16, 25]. EBLV-2 infection has also been observed in Daubenton's bats in Germany [6, 19].

Since 1996, EBLV-2 has been identified from several locations across the UK, suggesting that EBLV-2 is endemic at a low level in British bats (Table 1), and in August 2007, a further UK isolate was identified in an adult female Daubenton's bat found by a member of the public at Stokesay Castle, Shropshire [8]. The bat carcass was submitted to the Veterinary Laboratories Agency (VLA) for laboratory testing to confirm the presence of EBLV-2 and was shown to be positive by the fluorescent antibody test (FAT). Confirmatory diagnosis was achieved by PCR using a hemi-nested RT-PCR that detected viral RNA in brain, salivary gland and tongue samples (Fig. 1a). Quantitative RT-PCR (qRT-PCR) was undertaken using previously described methods [27] to determine levels of viral RNA present within different organs of the infected animal (Fig. 1b). High levels of viral RNA were detected in the brain, with lower levels in the salivary glands, stomach and tongue, with RNA also being detected in the intestine and heart (Fig. 1c). Immunohistochemical analyses were restricted to sections of the spinal cord and detected virus antigen in dorsal root ganglia (Fig. 2a).

The original observation of an EBLV-2 positive bat at this site prompted measures to be taken to minimise the risk to members of the public encountering other bats from the roost. Twice-daily checks were implemented to ensure that no injured, sick or dead bats were present in areas of the

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Table 1 Cases of EBLV-2 in the UK

Date tested	Bat reference	Location	Bat species	Sex
30/05/1996	96/19	New Haven, Sussex	*M. daub.*	Adult female (pregnant)
07/07/2002	105/02	Carnforth, Lancashire	*M. daub.*	Juvenile, female
11/11/2002	n/a	Angus, Scotland	Human	Male
28/09/2004	603/04	Staines, Surrey	*M. daub.*	Juvenile, female
26/10/2004	696/04	Blackburn, Lancashire	*M. daub.*	Adult, male
12/09/2006	06/652	Abingdon, Oxfordshire	*M. daub.*	Juvenile, female
12/08/2007	07/762	Stokesay Castle, Shropshire	*M. daub.*	Adult, female
02/05/2008	08/163	Teddington, Surrey	*M. daub.*	Adult, female
25/09/2008	08/1218	Stokesay Castle, Shropshire	*M. daub.*	Juvenile, male

castle open to public access. Importantly, control measures were implemented, including the wearing of protective gloves so that staff could handle a bat with minimal risk during the regular checks before members of the public entered the tower of the castle [2]. Signs were also erected at the entrance of the tower warning members of the public not to handle bats.

In September 2008, a dead bat was discovered on the top floor of the South Tower of Stokesay Castle during one such check. The bat was a juvenile male Daubenton's bat and was submitted to the VLA for routine testing. This bat was also shown to be positive for infection with EBLV-2 by RT-PCR, a 405-base-pair (bp) fragment of the nucleocapsid (N) gene being successfully amplified. Unfortunately, the standard FAT could not be undertaken, as the brain material had decomposed during storage and transit. The remainder of the carcass of this bat was, however, submitted for histopathological examination. Several sections were taken, and despite advanced autolysis, specific labelling was found throughout the spinal cord in neurons, dorsal root ganglia (Fig. 2b) and peripheral nerves. The N-gene PCR product was sequenced in its entirety and found to be 100% identical to the 2007 isolate across the 405-bp region analysed. A phylogenetic analysis of the EBLV-2 isolates across the UK to date was generated using the neighbour-joining method, using Mega 4 software (Fig. 1d). Unfortunately, the carcass of this bat was severely decomposed, and further molecular tests, such as comparative qRT-PCR on a range of organs, could not be performed.

The identification of EBLV-2 positive bats from the same site more than 1 year apart raises several questions regarding the basic transmission and biology of this virus within bat roosts. Recent attempts to undertake scientific studies with bat species regarding the transmission and maintenance of these viruses between bats have resulted in limited conclusions as to how the virus is maintained within colonies. Bite transmission seems the most likely route of transmission, although no direct evidence for this in captive bats infected with EBLVs has been observed [5, 7, 14].

The detection of high levels of virus antigen in the brain of infected bats is typical for these viruses, and generally, where EBLV-2 has been detected in British bats, live virus has been isolated from brain material where samples have not decomposed. Here, we have reported the detection of virus in other tissue types. Presence of virus in these regions is likely to be linked to the degree of innervation, principally by the autonomic nervous system, although quantitation of neuronal involvement within different organs and tissue types to establish a basis for this hypothesis has not been undertaken [4, 12]. However, studies with EBLV-1 infection in the natural host, *Eptesicus serotinus*, showed no substantial pattern of virus distribution in different non-neuronal organs in bats that developed disease [7].

Mechanisms of virus transmission within roosts remain an enigma. For genotype 1 lyssaviruses it has been established that infection of the salivary glands may lead to the secretion of virus in the saliva for several days before the onset of clinical signs. Whilst this is widely documented for larger species, low levels of viable virus or viral RNA detected in saliva swabs tested during experimental studies with different bat lyssaviruses highlight the difficulty in determining the importance of this route of transmission for virus dissemination within a roost [5, 11, 14].

Aerosol spread of virus within a roost would seem feasible, as bats live in very close proximity. However, to date, transmission studies have only been reported with genotype 1 rabies viruses, experimental attempts to transmit EBLV-2 via this route proving unsuccessful [13]. For the 2007 Stokesay Castle case, the detection of EBLV-2 RNA in tongue lends support to the transmission through bite or grooming, although again, this has not been conclusively shown. In 2008, advanced autolysis prevented thorough histological examination or molecular assessment of the tongue. Granular immunolabelling was, however, seen in nerves at the base of the tongue and along the jaw. Unfortunately, no taste buds or epithelial tissue was available for further testing.

The mechanisms of maintenance of EBLVs within bat roosts and transmission between individuals remains

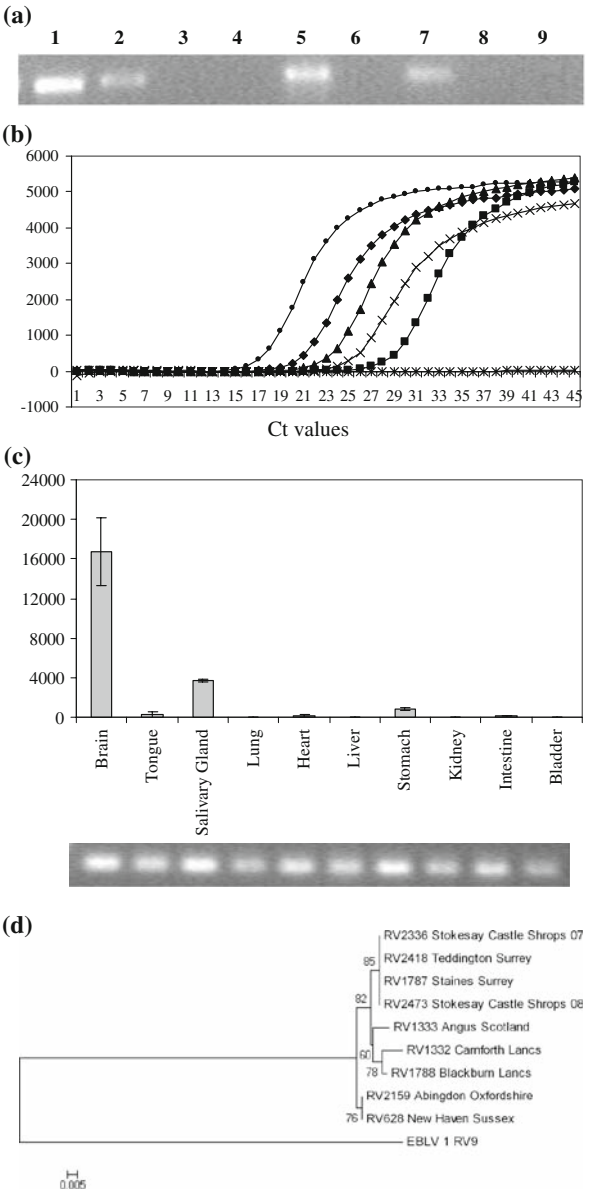

Fig. 1 **a** Hemi-nested second-round PCR results for bat 762/07. First-round PCR (primers JW6 and 12) produced a negative result for all three bat samples tested. However, the second-round PCR (primers JW10 and 12) produced a positive result for the brain and salivary gland [10] (1 brain, 2 salivary gland, 3 tongue, 4 negative control mouse brain, 5 RT positive control, 6 RT negative control, 7 PCR positive control, 8 PCR negative control, 9 PCR negative control for second-round reaction.). **b** Real-time qPCR results for bat 762/07. (*circles* positive control, *diamonds* brain, *triangles* salivary gland, *crosses* stomach, *squares* tongue, *stars* negative control. **c** qRT-PCR highlighting viral RNA present within RNA extracted from different organs taken from bat 762/07. Values are copies per µg. **d** Phylogenetic analysis of a 405-bp fragment of the N-gene from isolates of EBLV-2 across the UK. Evolutionary history was inferred using the neighbour-joining method with the bootstrap consensus tree inferred from 10,000 replicates. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site

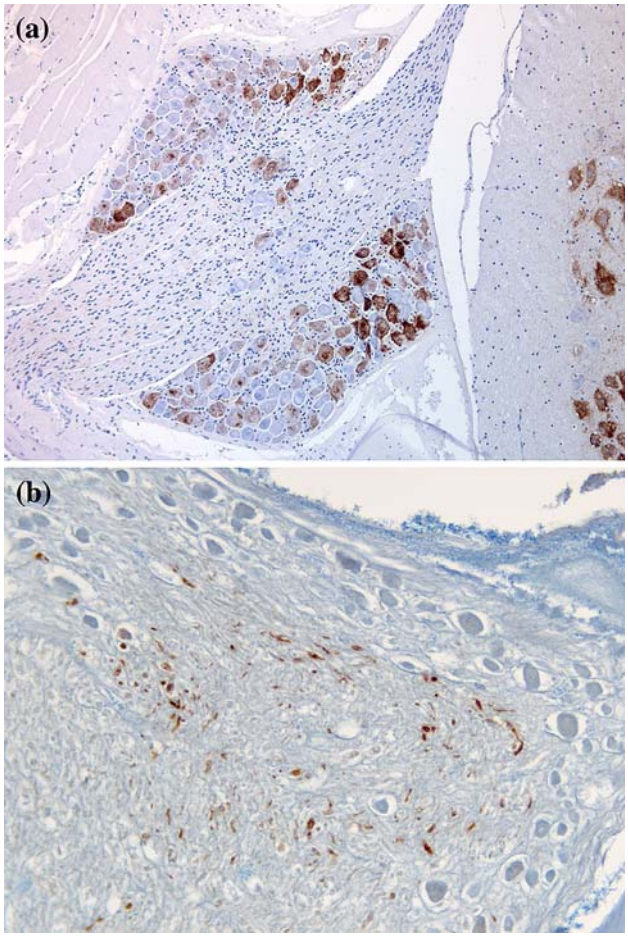

Fig. 2 **a** Immunohistochemical staining of dorsal root ganglia of Bat 07/762. Viral nucleocapsid is stained brown using anti-nucleocapsid protein antibody. (×10 magnification). **b** Immunohistochemical staining of dorsal root ganglia of Bat 08/1218. Viral nucleocapsid is stained brown using anti-nucleocapsid protein antibody (×40 magnification)

unknown, although the 'small vector hypothesis' remains plausible [17]. The restriction of EBLV infection to certain species of bat as well as mechanisms by which individuals are able to survive infection, or at least exposure to the virus, are key questions that remain [26]. Viral load, bite/exposure site and immunological competence of the exposed animal may all affect the outcome of infection. Factors such as seasonal variation, pregnancy, nutritional status and immune status must also play an important part in dictating whether or not bats succumb to infection [21], but currently, our understanding of bat biology and immunology is low. The detection of a large number of viruses within different bat species has highlighted this lack of knowledge [1]. Isolation of a number of zoonotic viruses in bat species, including coronaviruses, astroviruses, henipaviruses and other lyssaviruses, will surely drive further scientific investigation [9, 15, 23, 29]. Clearly,

with EBLV-2 having now been identified on two separate occasions from the same roost, virus is being maintained and transmitted from bat-to-bat by some as yet undefined mechanism. The status of Daubenton's bats, and indeed all bats across the UK, as protected species makes it difficult to undertake investigative studies at such sites. However, serosurveillance of bats are planned at the Stokesay Castle site that will allow determination of the level of seroconversion within this roost and highlight possible transmission mechanisms that will help understand the transmission biology of these elusive viruses and may identify those parameters needed to enhance strategies to combat neuroinvasion and subsequent disease development.

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