

Susceptibility of *Myotis daubentonii* (Daubenton's bat) to European Bat Lyssavirus Type 2 (EBLV2)

Introduction

The following proposal discusses the scientific rationale and procedures in which we propose to investigate European bat lyssavirus type 2 (EBLV-2) infection in the Daubenton's bat (*Myotis daubentonii*). It provides supporting information for an application for a project licence to capture Daubenton's bats in England.

This study forms part of a larger Defra funded project to study transmission and host response to infection with EBLV's (grant number SE0524) and involves a European collaboration between two UK governmental agencies, the Veterinary Laboratories Agency (VLA) and the [REDACTED] and a number of groups in Germany (see study protocol for further details). An initial study of EBLV-1 infection in wild-caught North American big-brown bats (*Eptesicus fuscus*) has already been completed.

A discussion on this work has been instigated by Defra with the Bat Conservation Trust (BCT) and aspects of the project have been reviewed by experts acting on behalf of the BCT.

If further information is required concerning the contribution this study will make to UK policy development on EBLV-2 or the virus challenge study, please contact:

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Project purpose

Rationale

This work aims to describe, for the first time, the quantified development of a European bat lyssavirus (EBLV-2) infection in its native British host. This is vital for an informed scientific contribution to government policy formation and is the key underpinning study to three related streams of research. Firstly, this work will demonstrate the susceptibility of Daubenton's bats to a variety of virus challenges and describe how many succumb to the disease (key in describing the effects of endemic lyssavirus on bat conservation and necessary for predictive epidemiological modelling). This will provide a valuable opportunity to record the early signs and manifestations of disease in bats. Secondly, it will describe the proportion of bats that secrete live virus and therefore act as vectors of the disease to con-specifics or other native wildlife (key in an assessment of the human risks and of predictive epidemiological modelling). Thirdly, it will provide the context against which current and future government funded surveillance programmes for lyssaviruses in British bats can be interpreted. Current surveillance work is collecting data but we are currently unsure of the relationships between a sub-clinical infection (sufficient to raise antibodies), virus production and the incidence of full-blown clinical disease ([REDACTED]). The absence of this information limits the interpretive power of the research.

Without this proposed study researchers and policy makers will be reduced to using ad-hoc anecdotal evidence from the sporadic incidence of EBLV2 in the wild (often unsatisfactory as the bat is usually recovered already dead) and evidence gathered in other countries using bat / lyssavirus models inappropriate to the UK. Of particular concern in this respect is that the pathology of 'classical rabies' (RABV) in US bats, appears to show unexpected combinations of symptoms. Little is known about the pathology of the European bat lyssaviruses and whilst similar unexpected pathologies might be anticipated there is also limited evidence that suggests that there may also be distinct differences in the pathology of EBLVs in their co-evolved European host bat species. This work, partly as a result of its diverse national partnership, and partly because of necessity will probably help form policy in a number of states or other Pan-European organisations (e.g. EUROBATS), and a justification of its utility should be at the international scale.

Methodology

Methodology – overview

No more than 60 Daubenton's bats, caught from the wild in England, will be held in an appropriate and humane laboratory context, and infected with an English isolate of EBLV-2 or used as non-infected controls. A number of virus challenge routes will be used to further explore potential infection mechanisms and the development of pathology; all are potential routes of infection within wild populations of bats. The bats will be monitored continually to examine for detectable signs of infection (behaviour -scored on a predetermined scale, blood test for antibodies and saliva swabs for live virus). Bats will be held for a minimum of 120 days (30 days quarantine to establish baseline serology and at least 90 days during the challenge study) and would be humanely killed at the end of the study. If a bat shows signs of distress (especially those behaviours considered to be symptoms of a pathological lyssavirus infection), then it will be humanely killed to limit suffering.

In trying to ensure that we use bat / lyssavirus experimental models that most closely approximate the wild state in the UK, we feel that it is essential that wild English bats be used in conjunction with the EBLV-2 strain held at the VLA, one cultured and amplified from a wild English bat (██████████). Unfortunately, the experimental protocol requires that the bats be infected with EBLV-2, and that these bats must therefore be considered potentially infective. As a result, the bats must be held appropriately (i.e. in a biological isolation unit and at a level of security appropriate for rabies) for a considerable period (at least 90 days) and killed at the end of the experiment. The only research facility identified as capable and competent to perform this work is in Germany, necessitating the transport of the bats initially to the ██████████, for adaptation to captive life and then to the infectious challenge facility at ██████████ in ██████████. A small P3 challenge facility is available at the Veterinary Laboratories Agency but does not have the capacity to perform this study and continue statutory testing and research on rabies and rabies-related viruses.

Methodology – Capture of bats

Daubenton's bats will be caught in England using one or a combination of hand netting, cone trapping, harp trapping and mist netting. Ideally the bats retained for study should be adult females and so catching will be limited to a period during which post-lactating bats are taken from a large maternity roost; typically the last week of July or the first few weeks of August (though this will depend upon a site assessment prior to catching).

An experienced handler will check bats, and only those appearing healthy will be retained for study. Individual bats will be selected on the following basis: Adults will always be selected over juveniles; Females will always be selected over males; Females considered to be pregnant or still lactating will not be selected. All unselected bats will be released immediately and returned to the roost. Ideally bats would be taken upon their return to the roost after foraging (i.e. approaching dawn), thereby ensuring that they will have fed and hydrated normally and would not be subjected to a period without food (whilst in transit) greatly in excess of that experienced on a daily basis. If bats were to be caught on emergence,

then they would be offered mealworms and water in the first hours of their capture until the complete experimental cohort has been captured.

Alternative seasonal timings for the collection of bats do not appear to exist. We feel that collection of bats from hibernacula, even if this were feasible, produces an unacceptably high level of disturbance on other hibernating Daubenton's and bats of other species, and considering the numbers required for the study, this would also be almost impossible. The collection of bats just after their emergence from hibernacula, when they are at their physiologically most vulnerable, we also consider unacceptable. In addition the collection of bats from after mid-March creates the complexity of some being pregnant which raised multiple ethical, practical and scientific issues, again which preclude this as a possible option. Thus the only window of opportunity once the young have been fledged is at the end of the breeding season whilst the adults are still available in the maternity roost.

Methodology – criteria for selecting roosts from which to remove bats.

One point worthy of debate is the location from which bats should be taken. Three possible alternative scenarios present themselves. All of the bats could be taken from one roost. Bats could be accumulated from captures made at a number of roosts in the same locale, or bats could be accumulated from captures made at a number of roosts made in a number of well-separated locations. Of concern is the balance of negative effects on the individual bat, the community in the individual roost(s) and the greater local bat population.

Considering first the individual bat: the necessary randomised allocation of bats to treatment groups, and the concern that this might prompt biting between individuals from different social groups strongly indicates that bats should only be taken from a single roost. By mixing bats from different roosts we not only produce some 'unnecessary' distress whilst bats 'get to know each other', but we also risk confounding the experimental protocol by encouraging biting and thus unwanted viral transmission. Thus under this consideration the ideal would be to take bats from a single roost, followed by the collection of bats from nearby roosts (who may either be distantly related or share some common community), with the collection of bats from disparate locations an unattractive option.

Considering second the dynamics of the individual roost(s) from which bats would be drawn. Assuming the removal of individuals from a roost will represent a 'trauma' to that roost – regardless of the numbers removed or the proportion of individuals taken, any option limiting this to one roost is preferable. However, in terms of establishing recovery over successive seasons, there is also a strong argument for only taking a small to moderate proportion of bats, ensuring that plenty are left to reproduce and return roost number back to 'normal'. Of additional value here is the consideration that large roosts are presumably more favourable than small ones (considering the qualities of the target roost, local forage and the availability of alternative roosts), and that population recovery is more certain for a 'large' roost – regardless of the number or proportion removed – than it would be for an equivalent 'small roost'. Thus the ideal roost under this consideration would be a single large roost from which the removal of 60 bats would represent a small proportion, but that the removal of bats from a single roost even where this was a more significant proportion is preferable to the use of multiple 'small' roosts.

Considering thirdly the dynamics of the local bat community. Regardless of the size of the roosts from which bats are taken, the conservation of a viable local

community of Daubenton's bats is paramount. Even if all of the bats be removed from a roost of 60, and we do not consider this to be an option, the presence of a thriving local population that will use the vacated roost space and draw additional benefit from it, is preferable to removing sufficient bats from a locale (regardless of the number of roosts from which they are drawn) that then jeopardises local population recovery. Thus a key piece of information necessary for a fully informed criterion (i.e. an authoritative and complete description of the bat population in an area) is likely to be missing for most known roosts across the county. In this context, a large roost once again appears to be the most sensible option, since within that one community alone we are confident that we are leaving sufficient bats to permit population recovery. If a suite of smaller roosts were to be used, it would need to be conditional upon the knowledge that the larger local population was thriving. It is thus clear that the selection of the roost(s) from which bats may be taken will require the careful survey of both the actual roost sites and surrounding locale to ensure that both the roost is large enough to permit all of the bats to be taken from one location, and that the locale hosts a thriving population in addition to the selected roost.

Ideally, the roost should have no known history of sero-conversion for any lyssaviruses, though as in practice so little is known of the true extent of lyssavirus infection, this is a limited constraint applicable to only a few locations in England. Finally, we consider this to be a piece of work that will be viewed by some in the bat working community as controversial. We therefore suggest that the selected site should either be unknown to local groups (ideally) or at least not used as part of regular bat group emergence counts. To this end we also request that the usual licence requirement to co-ordinate English Nature licensable research work with the local group be omitted on this licence.

Methodology - transit

We intend to organise the capture and transport of the bats within a 24hr period, so that they suffer the minimum of distress caused by the transport. To facilitate this we intend to closely co-ordinate that catching, transport and housing activities and ensure that all licenses and permissions are in place to permit this. The bats will be held together and transported immediately to [REDACTED] ideally by plane. Regardless of the time of capture bats will be provided with water throughout their period of transit.

References

[REDACTED]

[REDACTED]



Methodology – Laboratory protocol

Susceptibility of *Myotis daubentonii* (Daubenton's bat) to European Bat Lyssavirus Type 2 (EBLV-2) following different routes of infection*.

Responsible Personnel:

██████████ VLA – Weybridge, United Kingdom

██████████ FLI – ██████████, Germany

██████████ ██████████ Germany

* This study is to be conducted in Germany and is not covered by the Animals (Scientific Procedures) Act [ASPA] of 1986, and thus UK Home Office licensing, however this protocol has been reviewed by the Veterinary Laboratories Agency Ethical Review Committee using the same guidelines and has been deemed acceptable. The protocol and study has also been reviewed by the local ethical committee at the ██████████ and approved.

2. Test substance information

2.1 Test virus

Denomination:	European Bat Lyssavirus Type 2 (EBLV-2)
Supplier:	VLA-Weybridge
Origin:	The virus was isolated from the brain of a naturally infected Daubenton bat from Lancashire in the United Kingdom in September 2002 ([REDACTED]). This isolate has been named RV1332. The virus isolate has been passaged from original material by intracranial inoculation in newborn mice three times, and an inoculum containing 20% brain suspension in phosphate buffered saline / antibiotics prepared. This was transferred to the Federal Research Centre for Virus Diseases of Animals, [REDACTED] in July 2004.
Test virus preparation:	The virus isolate of the 20 % brain suspension was passaged 3-times i.c. in OF1 inbred mice
Identification:	RV1332 MBP3
Intended use:	Research
Concentration:	$10^{4.9}$ MLD ₅₀ / ml
Storage:	at -20°C for long term storage and +2°C to +8°C after thawing. Test item should be used within 6 hours of thawing.

2.2 Placebo control

Denomination:	Mouse brain suspension (rabies-negative)
Supplier:	VLA - Weybridge
Identification:	NMB
Storage:	At -20°C for long term storage and +2°C to +8°C after thawing. Test item should be used within 6 hours of thawing at room temperature

3. Methods and Study design

3.1 Test system

Species: Daubenton's bat (*Myotis daubentonii*)

[REDACTED]

[REDACTED]

Origin: Wild caught

Transport: To be arranged

Quarantine:

[REDACTED] GERMANY

Quarantine period: 1 month

Number of animals: A maximum of 60 individuals.

Identification: Animals will ringed on capture in the UK for subsequent identification purposes.

Specifications: None of the animals showed any symptom of disease when captured.
During the quarantine period a blood sample will be collected from all animals and investigated for the presence of EBLV-2 neutralising antibodies (RFFIT).
The animals will also receive Ivomec to control / treat possible parasite infections

3.2 Experimental design

This study will be conducted under GMP conditions. The housing conditions of the animals meet the conditions as stated in the German Animal Welfare Act §2&2a and the recommendations of the GV-SOLAS (Society for Laboratory Animal Science).

Study design

Group	Sample size	Inoculation dose (MLD50/ml)	Dose (ml)	Route of administration
1	10	NMB	20 µl	Control (i.m.)
2	10	...10 ^{4.9} ..	20 µl	i.c.
3	10	...10 ^{4.9} ..	20 µl	i.m.
4	10	...10 ^{4.9} ..	20 µl	i.n.
5	10	...10 ^{4.9} ..	20 µl	intradermal

Justification of inoculation route: As a positive control 10 animals will receive an intracranial inoculation of EBLV which is known to induce rabies-like disease in newborn mice. A negative control group of 5 animals will receive an intramuscular inoculation of rabies-negative mouse brain suspension. Two test groups of 10 animals each will receive the same dose intramuscularly (i.m.) or intranasally (i.n.) and a positive control group (n = 10) will receive the same dose intracranially. These i.m and i.n. routes have been selected to mimic possible routes of infection; i.m. – transmission through biting and and i.n. – aerosol transmission: The latter is much debated and it is important to clarify this possible route of infection for EBLV. The i.c. control group is necessary in the event that extraneural challenge does not induce disease.

Justification of dose level: This infectious dose is known to infect mice although the susceptibility of any particular dose of EBLV-2 in bats is not known. Hence, it was decided to use the highest dose possible as it is important that some bats do indeed develop rabies so that the course of the pathology and the sequential development of serological and viral markers can be followed.

3.3 Animal husbandry

Housing: The study will be performed in an animal isolation unit (IE.), of the Experimental Animal Facility of [REDACTED]. The air ventilation system of the Experimental Animal Facility consists of three filter systems; filter class EU5, EU8 and EU11. This is to prevent bacterial and viral organisms being transported through the ventilation system from one room to the other. Furthermore, a EU3 and E13 filter have been incorporated in the exit air tract of every isolation unit. The filtered air in the room is exchanged 15 times per hour. An under pressure of 20Pa exists in the isolation units. The room will be illuminated (12h light – 12h dark regiment)

Caging: Every group will be kept separately in metal-framed cages (size: 75 x 75 x 50cm) with fine-mazed wire-gauzed side-sheets, except for the front side which consists of two sliding synthetic doors. Two cages are kept in one scantainer, each scantainer is provided with a closed system of filtered air at constant temperature and

humidity throughout the experiment to maintain constant physiological and immunological responses to infection.

The closed system of filtered air is necessary to prevent any build-up of infectious particles in the isolation room as a result of potential virus shedding by the infected animals (prevention of aerosol transmission) and thus protect animal handlers from protection from exposure to infectious agents.

Paper sheets are placed on the bottom of the cage and regularly replaced. In every cage two towels are suspended from the top; this to offer the animals hiding places.

Diet & Water: A food bowl with smooth surface will be placed in every cage; for every bat approximately 15-20 mealworms per day will be provided. Furthermore, Korvimin vitamin will be scattered on top of the worms once per month. Finally, every animal will receive once a month a drop of Nutrica vitamin paste directly administered in the mouth. Water will be available ad libitum.

Contaminants: no contaminants are known to be present in the diet or water at levels, which might interfere with achieving the objective of the study.

3.4 Pre-treatment procedures

Animals will be subjected to a quarantine period at the [REDACTED] of least one month during which they will be monitored daily for any signs of ill health. Furthermore, the animals will be adapted to their new food source upon arrival. Also, the animals will be bled during quarantine to detect possible VNA against EBLV-2.

Identification of the cages: Respective cages will be clearly labelled using appropriate cage identification labels.

Allocation to treatment groups: On arrival at [REDACTED] animals will be randomly allocated into treatment groups consisting of between 5 and 10 animals per test group, although the gender of the animals must be taken into account.

3.5 Administration of test virus

All bats will receive 20µl EBLV-2 (...10^{4.9} ... MLD50/ml), except for the control group that will receive 20µl of a rabies-negative mouse brain suspension

Group 1: control

The animals in the control group will receive the rabies-negative brain suspension by the i.m.-route. The animals will not be sedated. The virus material is injected into the Pectoral muscle (see below)

Group 2: intra-cerebrally

The animals will be injected i.c. under anaesthetic sedation.

Group 3: intra-muscularly

The animals will not be sedated. The virus material is injected in the Pectoral muscle (see below)

Group 4: intra-nasally

The animals will be slightly sedated using anaesthetic sedation and the virus will be slowly added into a single nostril of the animal.

Group 5: intradermal (dependent on bat numbers)

The animals will not be sedated. The virus material is infected under the skin covering the chest (dependent on agreement with [REDACTED]).

The following articles on bat inoculation with rabies were found following a literature search:

Publication	Species	Virus	Muscle (or description)
Moreno & Baer, Am. J. Trop. Hyg (1980) 29, 254-259	<i>Desmodus rotundus</i>	Rabies	Pectoral region
Reid & Jackson, J. Neurovirology (2001) 7, 511-517	<i>Artibeus jamaicensis</i>	Rabies (CVS variants)	Right masseter muscle
Aguilar-Setien et al., J. Wild. Dis. (2002) 38, 539-544	<i>Desmodus rotundus</i>	Rabies	Muscle at the site of the scapular cartilage
MaColl et.al., Aust Vet J. (2002) 80, 636-641	<i>Pteropterus poliocephalus</i>	ABLV and Rabies (virus derived from <i>E. fuscus</i>)	Left extensor carpi radialis
Almeida et. al., Epidemiol Infect (2005) 133, 523-527	<i>Desmodus rotundus</i>	Rabies	Pectoral muscle

3.6 Observation

Clinical signs: Animals will be monitored twice daily for a period 90 days post infection.

To control the health status of the animals, body weight will be checked regularly; for *Myotis daubentonii* the lower threshold will be 5 grams.

To prevent unnecessary suffering bats will be humanely killed when the first signs of severe disease develop. An earlier study identified a number of disease stages in an insectivorous bat model of EBLV-1 infection:

- phase 1: an animal is found hanging separately from the other animals, it is not attempting to conceal itself behind sheeting and is in full view
- phase 2: an animal was not able to climb up the walls therefore its' movements were restricted to the bottom of the cage
- phase 3: an animal becomes paralysed and was not able to move around anymore; remains at one position (biting reflex still possible)

- phase 4: an animal is lying on its back paralysed, however, it was still able to slap its wings when agitated
- phase 5: death

Animals discovered with phases 2/3 signs and above will be humanely killed.

In the event of death, dead animals will be removed from the cages as soon as they are detected and stored at -20°C

The study will be terminated 90 days from the death of the last animal in each group.

3.7 Sampling

During the observation period the animals will be bled once per month and also saliva samples will be taken.

Collection of blood samples:

The animals will be bled prior to inoculation to screen for neutralising antibodies against EBLV-2 and to establish a baseline for future measurements.

Thereafter they will be bled once a month to monitor the development of an antibody response to inoculation.

If an animal is found dead no attempt should be made to remove blood. If an animal develops severe clinical signs and must be sacrificed then an attempt should be made (subject to veterinary approval) to take a cardiac bleed prior to death.

Description of methodology:

Blood samples can be drawn from two different parts of the body, e.g. (i) from the artery or vena brachialis (propatagium) and (ii) capillary from the uropatagium.

Artery or vena brachialis

For blood sampling the bat has to be held tight in one hand with the bat's face upward. Subsequently, the wing is stretched carefully and the propatagium is wiped clean and locally disinfected with a sanitary towel soaked in 96% alcohol to prevent infections. Immediately afterwards, the artery or vena brachialis is punctured next to the humerus distal epiphysis close to the elbow joint (sulcus) using a small needle. In any case an injury of the elbow joint should be avoided. The leaking blood is collected in an Eppendorf tube using a pipette.

Afterwards, a sterilized piece of cellulose (or sanitary towel) is pressed for 30 seconds on the wound to stop bleeding. Haemostasis is to be controlled prior to the release of the animals. Subsequently, the bats are offered 10% glucose-solution to compensate the blood loss and to rapidly assimilate compounds for energy. Tubes containing blood are stored at 4°C for a few hours. Samples are centrifuged for 10 minutes at 3,000 rpm, and the serum is extracted with a pipette. The blood samples are centrifuged and the serum stored at -20°C prior to further examination.

Collection of saliva samples:

The animals will be saliva swabbed prior to inoculation.

There after animals will be swabbed at 1 week intervals to detect possible virus excretion in the absence of symptoms. If animals develop clinical signs of rabies, swabbing should be attempted (subject to veterinary approval) on a daily basis to detect possible virus excretion during disease.

To assess the potential risk to public health it is very important to know if the animals shed the virus in their saliva, and if so, during which period. Hence, saliva swabs will be taken at regularly intervals. A saliva swab will be taken at weekly intervals. When the animals show clinical signs of infection daily swabs will be taken, if possible.

Description of methodology...

The oral cavity is swabbed with a cotton swab (Salivette) for 1 – 1.5 minutes. The The swab is subsequently placed in a holding tube that contained 0.4 ml MEM/SNT and a mixture of gentamicin (50mg/l) and amphotericin B (2.5 mg/l). The tubes will be stored at -20°C until further examination. The tubes are stored at -20°C until further examination (RT-PCR, RTCIT).

3.8 Pathology

Sacrificed animals will be treated in two ways, either immediately frozen for processing at FLI- [REDACTED] or placed intact in 10% buffered formalin. The following procedure should be followed to allocate animals to either course:

The first animal to be sacrificed from each group will be frozen at -20°C.

The second animal to be sacrificed from each group will be place in formalin.

Animals 3 to 5 should be frozen.

Animal 6 should be placed in formalin.

The remaining carcasses will be placed at -20°C.

Frozen carcasses will be kept at -20°C and formalin fixed carcasses will be stored at 4°C. On completion of the study all carcasses will be transported to FLI- [REDACTED].

Fixed carcasses will be stored for a minimum of 2 weeks and sent on the VLA-Weybridge (Histopathology).



3.9 Test assays

Table 1: Tissue samples to be taken at necropsy and subsequent diagnostic investigations.

A suggested protocol (frozen samples only) is to produce an organ homogenate in 0.4ml tissue culture medium and extract RNA from 0.2ml for RT-PCR analysis.

Positive samples will proceed to RTCIT analysis

Tissue	FAT	RT PCR	sequencing	histopathology	RTCIT	SNT
Serum						X
Saliva		X	N=2	X	X	
Cortex	X	X	VLA	X	X	
cerebellum	X	X		X	X	
Spinal cord (optional)		X		X		
Eye (optional)		X		X		
Salivary glands		X		X	X	
tongue		X		X		
Tonsil (optional)		X		X		
Trachea		X		X		
Lung		X		X		
Heart		X		X		
Liver		X		X		
Kidney		X		X	X	
Stomach		X		X		
Bladder		X		X	X	
Spleen		X		X		

	= not done
	= depending on RT-PCR result

3.10 Data handling

All test data will be collated by the institute performing the test. Subsequently this will be collected into a single spreadsheet file and circulated between the collaborating groups.