

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

SE0524 Laboratory Protocol*

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.

1. Introduction

1.1 Objectives

The rabies situation in Europe has changed drastically in the last 25 years. As a result of oral vaccination campaigns of foxes, terrestrial rabies due to genotype 1 Lyssavirus (RABV) has been eradicated from large areas of Europe. Meanwhile, two other lyssaviruses have been identified: European Bat Lyssavirus type 1 and 2 (EBLV-1 and EBLV-2). Experimental susceptibility studies with EBLV have been conducted in several terrestrial mammal species. Unfortunately, only limited knowledge on the susceptibility of the natural EBLV-hosts, insectivorous bats, to EBLV is available. Many questions remain unanswered; how long is the incubation period, how and under which circumstances is the virus transmitted, etc. In this study we propose to evaluate the susceptibility and pathology associated with an EBLV-2 infection in *Myotis Daubentonii*, the reservoir host, following different routes of infection.

1.2 Testing facilities

Animal test facility

██████████, Germany

Head of Study: ██████████

Head of Animal Facility: ██████████

Diagnostic facilities

Veterinary Laboratory Agency – Weybridge (VLA)
New Haw, Addlestone, Surrey KT15 3NB, United Kingdom

Head of Study: ██████████

██████████, Germany

Head of Study: ██████████

Study Coordinator:

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Veterinary Laboratory Agency – Weybridge (VLA)
New Haw, Addlestone, Surrey KT15 3NB, United Kingdom

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's bat from Lancashire in the United Kingdom in September 2002 (██████████). 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, ██████████ 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: Flight (Heathrow to Frankfurt)

Quarantine:

[REDACTED]

GERMANY

Quarantine period: 1 month

Number of animals: 34 individuals.

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

Specifications: None of the animals show 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 (MLD50/20µl)	Dose (µl)	Route of administration
1	6	NMB	20 µl	Control (i.m.)
2	7	...10 ^{3.2} ..	20 µl	i.c.
3	7	...10 ^{3.2} ..	20 µl	i.m.
4	7	...10 ^{3.2} ..	20 µl	i.n.
5	7	...10 ^{3.2} ..	20 µl	subdermal

Justification of inoculation route:

A negative control group of 6 animals will receive an intramuscular inoculation of rabies-negative mouse brain suspension. This group will determine what effect, if any, the non-viral component of the inoculum has following injection on the Daubenton's bat.

A positive control of 7 animals will receive an intracranial (i.c.) inoculation of EBLV-2, which is known to induce rabies-like disease in newborn mice. The i.c. control group is necessary in the event that extraneural challenge does not induce disease.

A test group of 7 animals will receive an intramuscular (i.m.) inoculation of EBLV-2 to mimic possible transmission through a deep bite.

A test group of 7 animals will receive an intranasal (i.n.) inoculation of EBLV-2 to mimic possible transmission through inhalation of virus. The latter is much debated and it is important to clarify this possible route of infection for EBLV.

A test group of 7 animals will receive a subdermal (s.d.) to mimic possible transmission through a shallow bite. This route is also particularly effective in experimental inoculations of mice

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 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: Prior to arrival at [REDACTED] animals will be allocated into treatment groups consisting of 6/7 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: Subdermal (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 ██████████).

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. Neurovirol (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>Pteropus poliocephalus</i>	ABLV and Rabies (<i>virus derived from 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 post infection.

To monitor 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 a pre-bleed will be taken and a terminal bleed following euthanasia. Saliva samples will also be taken at regular intervals.

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. A final terminal bleed will be taken to assess seroconversion during the experiment. Due to the small size of the Daubenton's bat, bleeding on a more frequent basis was not considered for welfare reasons.

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 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 placed in formalin (i.e. one animal from each group should be fixed).

All subsequent animals should be stored at -20°C.


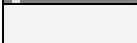
Frozen carcasses will be kept at -20°C and formalin fixed carcasses will be stored at 4°C. On completion of the study all carcasses will be transported to FLI- [REDACTED]. Fixed carcasses will be stored for a minimum of 2 weeks and sent on the VLA-Weybridge (Histopathology).

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.