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1 Susceptibility of sheep to European Bat Lyssavirus types -1 and -2 infection: A
2 clinical pathogenesis study

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23 These data have been presented at the 143rd American Veterinary Medicine
24 Association (AVMA) in Hawaii in July 2006 and at the XVII Rabies In The
25 America's (RITA) meeting in Brazil in October 2006.

1 **Abstract**

2 European bat lyssaviruses (EBLVs) have been known to cross the species barrier
3 from their native bat host to other terrestrial mammals. In this study, we have
4 confirmed EBLV-1 and EBLV-2 susceptibility in sheep (*Ovis ammon*) following
5 intracranial and peripheral (intramuscular) inoculation. Notably, mild clinical
6 disease was observed in those exposed to virus via the intramuscular route.
7 Following the intramuscular challenge, 75% of the animals infected with EBLV-1
8 and 100% of those that were challenged with EBLV-2 developed clinical signs of
9 rabies and then recovered during the 94-day observation period. Disease
10 pathogenesis also varied substantially between the two viruses. Infection with
11 EBLV-1 resulted in peracute clinical signs, which are suggestive of motor neurone
12 involvement. Antibody induction was observed and substantial inflammatory
13 infiltrate in the brain. In contrast, more antigen was detected in the EBLV-2
14 infected sheep brains but less inflammatory infiltrate and no virus neutralising
15 antibody was evident. The latter involved a more protracted disease that was
16 behaviour orientated. A high infectious dose was required to establish EBLV
17 infection under experimental conditions (≥ 5.0 logs/ml) but the infectious dose in
18 field cases remains unknown. These data confirm that sheep are susceptible to
19 infection with EBLV but that there is variability in pathogenesis including
20 neuroinvasiveness that varies with the route of infection. This study suggests that
21 inter-species animal-to-animal transmission of a bat variant of rabies virus to a
22 terrestrial mammal host may be limited, and may not always result in fatal
23 encephalitis.

24 Keywords – EBLV, bat, lyssavirus, rabies, sheep, virus

1 Introduction

2
3 Viruses capable of causing clinical rabies belong to the genus *Lyssavirus*,
4 family *Rhabdoviridae*. Rabies is a notifiable disease in bats as well as other
5 terrestrial mammals (WHO, 2005). Many species of bat are known to be
6 reservoir hosts for these lyssavirus variants or genotypes, with the single
7 exception of Mokola virus (MOKV, genotype 3), which has not been isolated from
8 a bat host (Harris et al., 2006). In the Americas the bat-borne rabies ecological
9 niche is occupied by classical rabies virus (RABV, genotype 1), in Africa it is
10 Lagos bat virus (LBV, genotype 2) and Duvenhage virus (DUVV, genotype 4) and
11 Australian bat lyssavirus (ABLV, genotype 7) in Australasia. In Europe, this niche
12 is occupied by the European Bat Lyssaviruses (EBLVs types -1 and -2,
13 genotypes 5 and 6), which predominantly reside in three bat species: *Eptesicus*
14 *serotinus*, *Myotis daubentonii* and *M. dasycneme* (Amengual et al., 1997; Fooks
15 et al. 2003a). In addition, there have been four, as yet unclassified lyssaviruses,
16 detected in other bat species from Eurasia (Botvinkin et al., 2003; Fooks et al.,
17 2004).

18 Apart from their natural reservoir hosts, all genotypes have been known to
19 cause clinical disease in humans with the exception of LBV (Warrell and Warrell,
20 2004). There have been three confirmed cases of human rabies as a result of
21 EBLV-1 infection and possibly an additional three where infection was suspected
22 but the virus was never isolated for genotype confirmation (Anonymous, 1986;
23 Selimov et al., 1989; Botvinkin et al., 2005). EBLV-2 has caused lethal infections
24 in two human cases (Lumio et al., 1986; Fooks et al., 2003b).

25 Lyssaviruses of bat origin have also caused lethal infection in domestic
26 pets, livestock and wildlife (McColl et al., 2000; Mayen, 2003). Of the European
27 bat variants of rabies virus, EBLV-1 has been recorded in terrestrial mammals.
28 Surprisingly however, EBLV-2 has not been detected in other mammals.
29 Between 1998-2002, EBLV-1 was diagnosed in five Danish sheep suffering from
30 clinical CNS signs (Ronsholt et al. 1998; Ronsholt., 2002; Muller et al., 2004).
31 The animals originated from four different herds within a 40 km range and 1 of 69
32 animals analysed from two herds had EBLV-1 neutralising antibodies (Ronsholt,
33 2002; Tjørnehoj et al., 2006). The first case involved 4 sheep from individual
34 herds, the virus isolate was EBLV-1a which was identical to the bat rabies virus
35 isolated sporadically from Danish bats. The case, however, was complicated by
36 the detection of listeria, which can also result in neurological disease. A second
37 sheep case (n=1), in 2002 from the same region, showed classical signs of rabies
38 infection. The virus was shown to be homologous to previous sheep and bat
39 isolates from Denmark (Tjørnehoj et al., 2006). Various mammals including,
40 sheep, foxes, ferrets, cats and dogs have been shown to be susceptible to
41 experimental EBLV-1 infection (Soria Baltazar et al., 1988; Fekadu, 1988; Vos et
42 al., 2004a; Vos et al., 2004b; Tjørnehoj et al., 2006).

43 The clinical picture of natural rabies in sheep includes changes in
44 behaviour, which includes: aggression, wool chewing, head butting, drooling,
45 restlessness, depression, muzzle and head tremors, sexual excitement, in-
46 coordination and paralysis. The incubation period is in the region of 10 days
47 following exposure and morbidity is approximately 3 days (Whitney and Stratton,
48 2003). A limited number of experimental studies of classical rabies virus infection
49 of sheep have been reported. The clinical outcome of these studies was similar
50 to that seen in naturally-occurring cases of rabies, with incubation times between

1 10-40 days and morbidity around 3 days. In some cases, death occurred
2 peracutely with no clinical signs reported (Soria Baltazar et al., 1992; Baltazar
3 and Blancou, 1995). In one experimental study, greater than 70% of the sheep
4 developed rabies following an intra-muscular inoculation of RABV in a dose
5 dependant manner (Hudson et al., 1996). The disease manifests as the furious
6 form in 80% of the animals, however in some animals the clinical signs were
7 reportedly subtle. Virus was recovered in the CNS and the salivary glands,
8 however only 30% of animals produced an antibody response and 50-60% of
9 animals were protected by vaccination (Baltazar and Blancou, 1995).

10 The earliest experimental inoculation of larger mammals with EBLV-1, then
11 referred to as Danish bat virus, was undertaken by Soria Baltazar and others
12 (1988). Five sheep were inoculated with EBLV-1 by the IM route (masseter
13 muscle). No clinical signs, deaths or antibody responses were recorded over a
14 45 day observation period. In a second group of sheep receiving a higher dose of
15 virus, 1 of 5 succumbed to infection with clinical signs between days 22-30.
16 Clinical signs included reduced food intake, weight loss, prostration and death. In
17 this second group, 3 of the 5 sheep developed low titre antibody levels (0.03-0.42
18 IU/ml) when tested against challenge virus standard (CVS), which included the
19 animal that developed disease. Live virus was detected in the hippocampus of
20 the animal that died but not in other neuronal tissue or in the other sheep that did
21 not develop clinical disease. No virus was detected in the saliva. A recent
22 experimental challenge of sheep with EBLV-1 showed that the animals developed
23 neutralising antibodies following a low dose challenge but did not develop clinical
24 disease over a 33-week observation period (Tjornehoj et al. 2006).

25 The ability of EBLV-1 to infect livestock naturally and yet the failure of a
26 number of experimental attempts to induce disease following peripheral
27 inoculation suggests that this subject requires further investigation. The study
28 reported here describes the susceptibility of sheep to both EBLV-1 and EBLV-2
29 and the course of clinical pathogenesis. The principal aim of this study was to
30 assess the neuroinvasiveness of EBLV-1 and EBLV-2, thereby representing inter-
31 species animal-to-animal transmission of a bat variant of rabies virus in a sheep
32 model.

33

2.0 Materials and Methods

2.1 Viruses and preparation of viral stocks

Both viruses, EBLV-1 and EBLV-2, were obtained from original bat brain tissue. The EBLV-1 isolate (Ref. 934/RV1423) was isolated from an *E. serotinus*, from Osnabrück, Lower Saxony, Germany during September 1997 and the EBLV-2 isolate (Ref. RV1332) from a *M. daubentonii* from Carnforth, Lancashire, UK during September 2002. The original bat brain material was passaged three times through mice (OF1, Charles River Ltd, France) by IC inoculation to amplify the virus and obtain the required quantity of virus stock material. A 20% (w/v) mouse brain homogenate (PBS-antibiotic buffer: Penicillin, Streptomycin, Nystatin at 50/2/2 ug/mL) was generated and clarified by low speed centrifugation (~800g) for 10 min. The supernatant was harvested and aliquots of 1.5 ml stored at -80°C until use. An aliquot was thawed and mouse infectious dose (ID₅₀)-IC, FP and tissue culture titration performed to determine the relative virus titre of the inoculum. The titres for EBLV-1 were 5.0, 3.2 and 7.8, and 5.0, 2.0 and 5.8 (log₁₀/ml) for EBLV-2.

2.2 Experimental sheep

The experimental sheep (*Ovis ammon*) were of the Deutsches Schwarzköpfiges Fleischschaf (German Black Head Mutton Sheep) breed. All the sheep were female, aged between 1-2 years from a single flock. None of the animals had been previously vaccinated and all were sero-negative for rabies prior to the experimental procedures (day -51). The animals were randomly assigned to the virus and route groups; four sheep per virus (EBLV-1 or EBLV-2) per inoculation route (IC or IM) plus two control-mock infected animals per inoculation route.

2.3 Experimental infection protocols

All animals were blood sampled (5 ml) prior to commencing the infection experiments for base line serology (day -51). The sheep were individually anaesthetised with 0.25 ml of a 1:1 mixture of 10% ketamine (Serumwerk Bernburg AG, Germany) and 2% xylazine (WdT AG, Garbsen, Germany), the inoculations undertaken as described below and allowed to recover.

2.3.1 Intracranial direct inoculation of the brain

The right hand side of the skull was trepanated for the IC inoculation and the inoculum (1 x 1 ml) delivered to the rostral cortex approximately 20 mm behind the frontal sinus to a depth of approximately 5 mm.

2.3.2 Intramuscular inoculation

For the IM inoculation, the dose (2 x 0.5ml) was delivered to both the left and right side of the head in the rostral portion of the masticatory muscle.

2.3.3 Mock infected control animals

For both IC and IM routes, the control animals received either PBS or normal mouse brain homogenate (20%) as described above.

2.3.4 Sample collection during the experimental period.

Each animal was observed twice daily following the inoculation for the development of altered behaviour and the clinical signs that were documented via clinical score cards. Blood samples (5 ml) were collected initially every 4 days

1 and then periodically as indicated (Figures 1aandb). Serum samples were
2 harvested and stored at -20°C until required for serology. Oral swab samples
3 (saliva) were collected every 2 days and then periodically (5-7 days) and stored in
4 transport medium - 2.0ml MEM plus antibiotics (50mg/l gentamicin and 2.5 mg/l
5 amphotericine) and stored at -70°C until required for virus isolation and genome
6 detection (PCR). Animals were humanely euthanased on the development of
7 overt disease or at the termination of the experiment (IC at d20 and IM at d94)
8 with intravenous T61 (Intervet, Germany) following ketamine / xylazin sedation
9 and immediately underwent necropsy. Tissues (brain – cortex, cerebellum,
10 medulla oblongata and hippocampus, spinal cord, trigeminal ganglion, facial
11 nerve, parotid and submandibular salivary glands, tongue, tonsil, lung, heart,
12 liver, kidney, mandibular, parotid and superficial cranial lymph nodes, rumen,
13 bladder and spleen) were harvested and stored frozen until required with the
14 exception of one complete brain per group of animals that was fixed in 4%
15 formalin for histopathology processing and fresh brain and / or CNS tissue for
16 direct antigen detection (FAT).

17 18 *2.4 Sample analysis*

19 *2.4.1 Serology*

20 Sera were tested to determine the level of specific (EBLV-1 or EBLV-2, reference
21 RV20 and RV628) neutralising antibody using the modified fluorescent antibody
22 tests (mFAVNs) as described previously (Brookes et al., 2005a). Each titre was
23 expressed as OIE IU/ml.

24 25 *2.4.2 Virology*

26 *2.4.2.1 Antigen and live virus detection*

27 The detection of antigen by direct fluorescent antibody test (FAT) was undertaken
28 on the same or the next day on acetone fixed brain smears to establish the cause
29 of disease using a commercially available conjugate (FITC-labelled polyclonal
30 anti-serum from Behring, Marburg; DIFIN, Berlin, Germany) as per the
31 manufacturer's instructions and Dean et al. (1996). The detection of live virus
32 was undertaken on previously frozen samples using the rabies tissue culture
33 inoculation test (RTCIT) using mouse neuroblastoma cells (N2A) as described
34 previously (Webster and Casey, 1996).

35 36 *2.4.2.2 Genome detection (PCR)*

37 RNA (viral and host) was extracted from previously stored tissue using the
38 RNeasy Kit (Qiagen, Hamburg, Germany) as described previously (Muller et al.
39 2004), and held at -70°C until specific amplification by standard PCR to detect
40 lyssavirus and host cell ribosomal RNA (Heaton et al. 1997; Smith et al. 2000).
41 In addition, quantitative real-time PCR analysis was undertaken on selected
42 tissues. The RNA concentration of each sample was quantified using a NanoDrop
43 WD-1000 spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE,
44 USA) and reverse transcribed using primer EBLV1Nf (5'-
45 AAGGTTGATGTAGCATTGGC-3') for EBLV-1 and primer EBLVNa (5'-
46 CCTGGCAGATGATGGGAC-3') for EBLV-2 using previously published
47 techniques (Heaton et al., 1997). EBLV-1 challenge samples were then amplified
48 with primers EBLV1Nf and EBLV1Nr (5'-ACTAGAATACGTCTTGTGAG-3'),
49 whilst EBLV-2 challenge samples were amplified with primers EBLVNf and
50 EBLVNr (5'- GCCTTTTATCTTGGATCACT -3'). Both primer pairs amplify a 219

1 base pair (bp) and a 221bp amplicon, respectively, that is directly upstream of the
2 nucleoprotein termination codon. All quantitative PCRs were undertaken using
3 the SYBR Green JumpStart Taq Ready Mix (Sigma, Saint Louis, Mo, USA)
4 following the manufacturers recommendations. Quantitation of RNA was
5 obtained through comparison to a standard curve generated by amplification of a
6 target of known concentration as described previously (Johnson et al., 2006).
7 Results were expressed as genome copies per nanogram of input RNA.

8 9 *2.4.3 Pathology and Immunohistochemistry (IHC).*

10 *2.4.3.1 Tissue processing*

11 Tissue samples from all animals were fixed in 4% phosphate-buffered
12 formaldehyde and processed for paraffin-embedding. Paraffin-wax sections (3
13 μm) were dewaxed and stained with haematoxylin and eosin. To analyze
14 distribution of EBLV antigens within the brain, sections were mounted on
15 SuperFrost[®] plus microscope slides (Menzel, Braunschweig, Germany), dewaxed
16 and rehydrated. The sections were incubated with a mouse monoclonal antibody
17 against the lyssaviral nucleoprotein antigen (Swiss MAB-HAM, kindly provided by
18 the Swiss Rabies Centre) at a dilution of 1:800 in Tris-buffered-saline (TBS, 0.1 M
19 Tris-base, 0.9% NaCl, pH 7.6). A biotinylated goat anti-mouse IgG1 (Vector,
20 Burlingame, CA) was used as linker-antibody for the avidin-biotin-complex (ABC-)
21 method. A nonspecific primary antibody (anti ILTV gp60, kindly provided by J.
22 Veits, FLI) and mock infected control sheep tissue acted as negative controls and
23 were analysed in parallel. By means of the ABC-method and an
24 immunoperoxidase kit (Vectastain Elite ABC Kit, Vector), a brown-red signal was
25 obtained from the substrate 3-amino-9-ethylcarbazole (DAKO AEC substrate-
26 chromogen system; Dako, Carpinteria, CA, USA). The sections were
27 counterstained with Mayer's haematoxylin, and sealed with aqueous medium
28 (Aquatex; Merck, Darmstadt, Germany).

29 30 *2.4 Statistical analysis*

31 Data comparisons of the clinical scores were undertaken using either the
32 Student's T-test (two sided, unequal variance for the number of clinical days and
33 qPCR) or AOVA (analysis of variance).

34

3.0 Results

3.1 EBLV susceptibility in sheep following CNS (IC) exposure

3.1.1 Clinical disease

All animals exposed to either EBLV-1 (n=4) or EBLV-2 (n=4) via direct CNS exposure succumbed to clinical rabies as confirmed by the detection of antigen in the brain (cortex cerebellum, hippocampus and medulla oblongata) using the FAT and other assays (see sections 3.1.3 below). The incubation periods and number of days that the animals were clinically ill varied depending on the virus used in the inoculum. EBLV-1 inoculated sheep had a short morbidity period of 1-2 days (mean 1.25) and all four animals were euthanased by days 9 or 10 post-challenge (Table 2). EBLV-2 inoculated sheep had a distinct protracted clinical illness, 3-9 days (mean 5.5, $p=0.004$) and all four animals were euthanased between days 13-20 post-challenge (Table 1). Following a prodromal period of 11-14 days, two of the four EBLV-2 infected animals showed clinical signs (Table 2) that waxed and waned, between 12-20 dpi. There was an average of five days where mild clinical signs were recorded during a 6 or 9 day observation period. The other two sheep that received EBLV-2 had a similar prodromal phase of 10-14 days followed by a shorter continuous clinical period of either 3 or 4 days.

Disease in both EBLV-1 and EBLV-2 infected sheep manifested as 'furious' rather than 'paralytic' rabies (Table 2a). The EBLV-1 IC infected sheep had a higher mean clinical score (overall mean=2.25, mean/day=0.28 – data not shown) and clinical signs suggesting motor neurone involvement (Table 2a). In contrast, EBLV-2 infected animals had a lower mean clinical score (overall mean=1.97, mean/day=0.11 – data not shown) and clinical signs orientated towards behavioural changes (Table 2a). Analysis of variance indicated that the difference between the overall clinical score for EBLV-1 and EBLV-2 infected sheep was not significant ($p=0.523$), however, the mean clinical scores for each clinical period (onset of clinical signs to termination) were significantly higher ($p<0.001$) in EBLV-1 (0.82) infected sheep compared to EBLV-2 (0.36) infected animals.

3.1.2 Serology

Serum was collected from each of the animals every 4 days until euthanasia, as indicated (Figure 1a) and assayed to determine the level of virus neutralising antibody (VNA). Only one animal (EBLV-1D) had antibody levels above both the control-mock infected animals and the day zero samples from all animals prior to inoculation (0.87 IU/ml). This animal attained a level of 2.6 IU/ml on days 4 and 8, but became clinically ill on day 9 and was euthanased. None of the other animals appeared to sero-convert in that they did not attain a titre above that of the control-mock and / or day zero sera (n=56, mean 0.25, range 0.1-0.87 IU/ml).

3.1.3 Virology – Antigen, live virus and genome detection.

Viral antigen was detected in the infected sheep brains by FAT. This assay was not undertaken on non-neuronal tissue. Live virus was detected using the RTCIT virus isolation assay and was undertaken on those samples that were positive for the presence of virus genome by reverse transcriptase PCR. Positive RTCIT results were obtained for brain, spinal cord and trigeminal ganglion samples in all EBLV IC inoculated sheep where material was available (6 of 8, as two whole brains were fixed in buffered formalin for IHC). In the majority of the animals, both EBLV-1 and EBLV-2 infected, the brain regions; cortex, cerebellum, medulla

1 oblongata and hippocampus, were positive by all three techniques (FAT, RTCIT
2 and PCR) as were the spinal cord and trigeminal ganglion. Other peripheral
3 nerve material was negative, as were extra-neural organs and tissues. The one
4 exception was a positive parotid salivary gland in EBLV-2 sheep F.
5 Real time quantitative PCR (qPCR) was undertaken on the four brain regions,
6 spinal cord (SC), trigeminal ganglia (TG) and the parotid salivary gland based on
7 routine PCR data. For both EBLV-1 and EBLV-2 IC inoculated sheep, the
8 number of virus genome copies appeared substantially higher in the cortex and
9 hippocampus (10-30 fold) than in other regions of the CNS (Fig. 2). This elevation
10 was not statistically significant due to the limited numbers of experimental sheep
11 analysed from each group (n=3) and the degree of variation between individual
12 samples. However, these data demonstrated the wide dissemination of virus
13 genome within the infected sheep brain.

14 15 *3.1.4 Pathology and IHC*

16 *Gross pathology.* At necropsy animals of both IC groups (EBLV-1 and EBLV-2)
17 displayed mild erosive cutaneous lesions that were attributed to the ataxia and
18 mechanical trauma of the animals prior to death.

19 20 *Histopathology*

21 Histopathologic examination of ovine brain sections of both groups revealed mild
22 to severe, subacute, diffuse, non-suppurative meningoencephalitis that varied in
23 its degree depending on the location analyzed (Table 3). Histopathologically,
24 meningoencephalitis consisted of perivascular cuffing with numerous
25 lymphocytes and fewer macrophages, multifocal infiltration of the neutrophils with
26 microglial and astrocytic cells (gliosis), neuronal degeneration and necrosis and
27 rarefaction with pallor and disruption of neuropil by clear spaces. Sections of the
28 rostral cerebrum (G1) that covered the olfactory tract, primary motor and
29 somatosensory cortex displayed minimal (animals EBLV-1 B and D, EBLV-2 E
30 and F) to severe encephalitis (animal EBLV-1 A). Sections of the cerebrum with
31 thalamus, piriform lobe, amygdala, optical tract, cerebral cortex (G2) or the
32 hippocampus, dentate gyrus, choroid plexus and cerebral cortex (G3) showed
33 moderate to severe meningoencephalitis in all sheep. Sections of the brain stem
34 and the cerebellum (G4) displayed minimal lesions in the cerebellum of all
35 animals (Fig. 3 A and B) and mild (animals numbers EBLV-1 A, B and D, EBLV-2
36 E and G) or moderate lesions in the brainstem. Animals inoculated with EBLV-2
37 displayed one or more 5-15 μm large round, brightly eosinophilic, intracytoplasmic
38 inclusion bodies (IB) with a clear halo in neurons at all levels, whereas in the
39 EBLV-1 inoculated sheep, inclusion bodies were rarely detected.

40 41 *Immunohistochemistry (IHC).*

42 Immunohistochemistry detection of the nucleoprotein revealed in all animals
43 strong granular intracytoplasmic signals in neurons and to a lesser extend in glial
44 cells (Figure 3 C-J). Distribution of antigen corresponded with the histologic
45 lesions in G2, G3 and G1 of EBLV-2 infected animals (Figure 3). In G1 of EBLV-1
46 infected animals mild (animals numbers EBLV-1 B and D) to severe (animal
47 EBLV-1 A) meningoencephalitis was not associated with detectable viral antigen
48 (Fig. 3C). Furthermore, mild meningoencephalitis in the cerebellum (G4) of EBLV-
49 2 infected animals was co-localized with abundant granular to globoid
50 intracytoplasmic antigen (IB) in numerous Purkinje cells. In the EBLV-1-group,

1 viral antigen was present in a small number of Purkinje cells of animal EBLV-1 A
2 only.

4 *EBLV susceptibility in sheep following peripheral (IM) exposure*

5 *3.2.1 Clinical disease*

6 Only one animal of four inoculated with EBLV-1 died (day 12) during the 94 days
7 of observation whereas none of the sheep that received EBLV-2 succumbed to
8 clinical disease (Table 1). The single sheep that did succumb had a morbidity
9 period of two days prior to death and clinical signs (Table 2b) that were less
10 severe than that of the IC inoculated sheep (Table 2a). This one animal was
11 found to be positive by PCR only and this was later confirmed by qPCR in the
12 trigeminal ganglion tissue and antigen detection by IHC in brain sections (see
13 below). In following the original study protocol, one brain from each experimental
14 group was used for histopathology analysis on formalin fixed brain regions
15 including IHC. For this reason, conventional assays including FAT and PCR were
16 not undertaken on this sheep brain.

17 The remaining three sheep that had received EBLV-1 and all four EBLV-2
18 inoculated animals, had a protracted illness that waxed and waned for a period of
19 either 39 or 66 days respectively, followed by complete recovery until day 94
20 when the experiment was terminated. The clinical signs in the IM sheep were
21 different to those of the IC inoculated sheep and between EBLV-1 and EBLV-2
22 inoculated animals (Tables 2a and b). The nature of the protracted illness was
23 also dissimilar with the number of days that surviving sheep were clinically ill
24 being significantly different from those sheep infected with EBLV-1 when
25 compared to EBLV-2 ($p=0.006$). EBLV-1 infected animals had mild clinical signs
26 between days 4-43 with an average of 12.3 days (range 10-14 days) of ill health.
27 In the EBLV-2 infected animals this period was between days 4-70 with an
28 average of only 4.25 days (range 1-7 days) with clinical signs. The overall clinical
29 score range was also lower for EBLV-2 infected sheep indicating that the clinical
30 syndrome was shorter and less severe.

31 The EBLV-1 IM infected sheep had a higher mean clinical score (overall mean =
32 1.4, mean / day = 0.04 – data not shown) and clinical signs suggesting motor
33 neurone involvement (Table 2b). EBLV-2 infected animals had a lower mean
34 clinical score (overall mean = 1.18, mean / day = 0.07 – data not shown) and
35 clinical signs orientated more towards behavioural changes (Table 2b). Analysis
36 of variance indicated that the difference between the overall clinical score for
37 EBLV-1 was substantially higher than that in EBLV-2 IM infected sheep ($p=0.09$).
38 The mean clinical scores per day were also significantly different ($p<0.001$) with
39 EBLV-2 being higher than EBLV-1 as a result of the difference in the total number
40 of clinical days per virus group (EBLV-1 $n = 36$, EBLV-2 $n = 17$). However, this
41 result is reversed if the score for each clinical period (waxing and waning days)
42 was used as the denominator. For EBLV-1, the denominator was 0.04 / day over
43 41 days whilst EBLV-2 was 0.03 / day over 37 days ($p=0.019$).

44 Overall, EBLV-2 clinical disease was less severe than that of EBLV-1.

46 *3.2.2 Serology*

47 Serum was collected to determine the level of VNA from the IM inoculated
48 animals every 4 days until euthanasia (Figure 1b). One EBLV-1 challenged sheep
49 (C) that died did not have a significant antibody level. The titre was never above
50 that of the mock infected animals or the day zero samples (0.87 IU/ml). In

1 contrast, the other three EBLV-1IM inoculated animals did develop antibody
2 levels above the threshold of 1.0 IU/ml. Two animals reached a level of 1.5 IU/ml
3 and the third 4.5 IU/ml on several sampling days, between days 12-56 concurrent
4 with the clinical period (days 4-43). The EBLV-2 inoculated animals did not sero-
5 convert above the threshold level during the 94 day observation period, including
6 the times of mild clinical signs.

7 8 **3.2.3 Virology – Antigen, virus and viral RNA detection**

9 All animals that survived (7 of 8) until day 94 were euthanased at the termination
10 of the experiment and underwent necropsy. All of the animals were negative in all
11 assays to detect viral antigen by FAT, live virus by RTCIT and viral RNA by PCR.
12 Two tissue samples, the spinal cord and trigeminal ganglion were PCR positive in
13 the one animal (EBLV-1 challenged sheep C) that died during the peripheral
14 inoculation study. The amplified product was detected at both first and second
15 round PCR. Real time qPCR confirmed the standard PCR results. A comparison
16 of the genome copies per nanogram of RNA (gRNA/ng) for these tissues from
17 EBLV-1 IC infected animals indicated that there was a two fold higher signal in
18 the trigeminal ganglion (TG) compared to the spinal cord (SC). The variation
19 amongst the four animals indicated that this result was not significant ($p>0.05$,
20 data not shown). These two tissues (TG and SC) from the single EBLV-1 IM
21 animal had a low copy number (<500) of gRNA/ng (data not shown). The fold
22 differences between IC and IM inoculated animals for TG and SC were 20 and 6
23 fold lower respectively, however due to the small sample sizes a statistically
24 meaningful comparison was not undertaken.

25 26 **3.2.4 Pathology and IHC**

27 The gross pathology of the EBLV-1 IM brain was similar to that of the IC with both
28 perivascular cuffing and gliosis demonstrable (Fig. 4a). The IHC illustrated the
29 presence of antigen distributed throughout neurons of the cortex (Fig. 4b).

30 31 **4.0 Discussion**

32 This study indicates that sheep are highly susceptible to disease with
33 EBLVs following neuroinvasion (Table 1). However, as shown by previous
34 studies with EBLV-1 and here for EBLV-2, this group of viruses show a limited
35 capacity to induce lethal disease when inoculated at a peripheral site. These
36 observations have been made in challenge studies using a range of animal
37 models. In one experimental study, ferrets were exposed to a low dose (4.0 logs
38 FFU-TC/ml) peripheral (intra-muscular, IM) challenge with EBLV-1 or -2. None of
39 the ferrets were susceptible to the challenge confirming a similar study in which
40 only 20% of mice challenged with EBLV-1 or -2 developed clinical disease (Vos
41 et al. 2004a). One critical factor between our study and previous studies was the
42 virus dose used in each experiment. In order to induce disease via the peripheral
43 route in sheep from which the animals do not recover the dose required was ≥ 5.0
44 \log_{10} IC-MID₅₀/ml. This is equivalent to 6-8 \log_{10} TCID₅₀/ml (see section 2.1),
45 which correlates with a 100-1000 fold increase in the challenge dose used in our
46 study compared with that used in previous large animal model studies (Tjørnehoj
47 et al., 2006; Vos et al., 2004a; Vos et al., 2004b). However, we have
48 demonstrated that mice were experimentally susceptible (20-80%) to EBLV-2 via
49 the peripheral route if the virus was introduced into the plantar surface of the hind
50 foot (FP) at a range of doses equivalent to <3.0 \log_{10} IC-MID₅₀/ml. The

1 invasiveness indices for these viruses were low (1.6 and 1.2 for EBLV-1 and -2
2 respectively) when the murine susceptibility for intracranial (IC) and FP
3 administered EBLV were compared (Vos et al., 2004a; Brookes et al., 2005a). In
4 contrast to previous studies, disease signs suggestive of rabies were noted in
5 sheep peripherally inoculated during the study period, but which did not lead to
6 fatal disease. The cause of these neurological sequelae, including tremors,
7 chomping and frequent licking of lips (Table 2b), are unknown and require further
8 investigation to provide a conclusive link to the inoculated virus. The single
9 exception to this was an animal infected with EBLV-1 that developed rabies and
10 died after 12 days. This suggests that those animals found to be infected with
11 EBLV-1 naturally could have resulted from a bite from a rabid bat.

12 Differences have been noted in the levels of VNA between this and may be
13 previous studies may be a result of variation in the assay method used to
14 determine neutralisation. We employed modified FAVN assays that use
15 genotype matched virus in the VNA test, for example sera from EBLV-1
16 inoculated sheep were tested on an EBLV-1 specific FAVN. Other workers have
17 used CVS and there is significant difference in the results depending on the test
18 virus used (VLA, unpublished data; Soria Baltazar et al., 1988; Tjornehoj et al.,
19 2006). The traditional 0.5 IU/ml indication of a positive serological assay is also
20 only based on CVS using genotype 1 induced antibodies. In our laboratory, this is
21 not equivalent when using the EBLVs (genotypes 5 and 6) as the basis of the
22 VNA test. The cut-off level chosen in this study (1.0 IU/ml), for both EBLV-1 and
23 EBLV-2, was chosen based on the pre-bleeds of the experimental sheep (n=20,
24 mean =0.36, range 0.1-0.87 IU/ml) and results for 'naïve' British sheep sera
25 (n=38, EBLV-1 mean=0.35, range 0.1-1.9; EBLV-2 mean=0.11, range 0.02-0.87).
26 In a previous study, animals with clinical neurological signs 'suspect scrapie'
27 (n=25) were substantially higher for EBLV-1 (mean 0.73, p=0.068) but not EBLV-
28 2 (mean 0.12, p=0.786) compared to 'naïve' animals (unpublished data). A total
29 of 4 experimental animals (2 IC and 2 IM) had pre-bleed results of 0.87 IU/ml
30 which appears to have been non-specific as their day 4 and 8 sera were ≤ 0.5
31 IU/ml. One animal (EBLV-1 D – IC) appeared to have a rapid response,
32 increasing from 0.38 to 2.6 IU/ml between days zero and four, which appears to
33 be earlier than expected for a primary immune response. Positive immune
34 responses in the IM inoculated animals (EBLV-1) were not observed until days 12
35 or 20. EBLV-1 positive sheep have not been recorded in Germany, however pre-
36 exposure to EBLV-1 or a related virus in the case of the one IC EBLV-1
37 inoculated animal although unlikely cannot be excluded.

38 The histopathology and IHC results were similar for both EBLV-1 and
39 EBLV-2, and for the route of infection, albeit the IM study was limited to one
40 animal only. The diseased brain tissue was characterised by diffuse
41 meningoencephalitis with gliosis and perivascular cuffing as observed in EBLV-1
42 infected cats and dogs (Fekadu et al., 1988). Viral antigen was also observed in
43 neurons throughout the various regions of the brain. There was a suggestion of
44 antigen distribution differences in the cerebellum and hippocampus; the signal
45 appeared less intense and more focal in EBLV-2 compared to that in EBLV-1
46 infected tissue. In general, the number of infected cells and intensity of staining
47 was greater for EBLV-1 than EBLV-2. Inclusion bodies that were similar to rabies-
48 specific Negri bodies were observed in EBLV-2 infected cells but rarely in EBLV-1
49 infected tissue, and inflammation was more prevalent in EBLV-2 than EBLV-1.
50 Inflammation of the brain in the form of gliosis and perivascular cuffing were also

1 observed in those sheep that succumb to clinical disease; EBLV-1 IC and IM and
2 EBLV-2 IC inoculations. In those sheep that recovered, however, tissue was not
3 harvested during the clinical periods as the principal objective of this study was to
4 observe the final outcome of the challenge and therefore brain pathology and the
5 presence of virus could not be assessed in the waxing clinical phases. At the
6 termination of the experiments, brain morphology in all survivors was normal.

7 We observed very limited centrifugal dissemination (brain to peripheral
8 tissues) of EBLVs in the infected sheep. This observation was in contrast to
9 similar data for the detection of EBLV-2 in the natural bat host. In these studies,
10 EBLV-2 was widely disseminated in organs and tissues of Daubenton's bats at
11 the time of death (Johnson et al. 2006). In addition, the excretion of lyssavirus in
12 naturally infected bat colonies has not been extensively studied. On rare
13 occasions, EBLV-1 RNA has been detected from oral swabs of Serotine bats in
14 Spain, however live virus was not isolated (Echevarria et al., 2001). In an
15 unpublished study from Germany, viable EBLV-1 has been isolated from oral
16 swabs of naturally infected Serotine bats. EBLV-2 has not been detected in
17 swabs from *Myotis* bats, neither infected bats or those that were antibody positive
18 (Brookes et al., 2005b). However, EBLV-2 RNA has been quantified in oral
19 associated tissues such as the tongue and salivary glands (Johnson et al.,
20 2006). EBLV-1 and -2 are only rarely detected on the oral swabs or salivary
21 glands of infected mice. It is un-clear whether virus transmission following a bat
22 bite would deliver the minimal experimental infectious EBLV dose and for the
23 virus not to be detectable during attempts at live virus isolation. The pathogenesis
24 and disease transmission of EBLVs in their natural hosts are not fully understood
25 and further challenge studies with these viruses in bats should provide more
26 information on the mode of virus transmission. Moreover, there is a lack of
27 knowledge in our understanding of the natural circumstances under which these
28 viruses cross the species barrier. It is probable, yet unproven, that EBLVs have
29 host-adapted to their chiropteran host and therefore do not easily cross the
30 species barrier. The risk of an epizootic of rabies in domestic livestock from an
31 EBLV spillover and the possibility of animal-animal transmission is therefore low.

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Table 1

Survival following IC or IM inoculation of sheep with EBLV-1 or EBLV-2

Virus	FAT	No. of Survivors	Time to Death (d.p.i)	Morbidity period (days)
EBLV-1 IC	+	0/4	9-10	1-2
EBLV-2 IC	+	0/4	13-20	3-9
Mock IC	-	2/2	NA	NA
EBLV-1 IM	-/+*	3 [*] /4	12	3
EBLV-2 IM	-	4/4	NA	NA
Mock IM	-	2/2	NA	NA

*PCR positive for the trigeminal ganglia and spinal cord sample and IHC on brain sections.

Table 2a
Clinical signs in EBLV-1 and EBLV-2 (IC) infected sheep

Clinical progress	EBLV-1 Per-acute	EBLV-2 Protracted
Clinical signs	Frequent licking of lips Tremors – lips / eyelids Unnatural head posture Rigidity / body tremors Nervousness Limb dysfunction / signs of paresis Rotations Exhaustion / fatigue	Reduced food intake Breathlessness Anxiety – running Hypersalivation Frequent licking of lips Tremors – lips / eyelids Un-coordinated head posture Aggressiveness Nervousness Limb dysfunction / signs of paresis
Overall clinical score range	1-5	1-4
Overall dysfunction	Motor	Behavioural

Table 2b
Clinical signs in EBLV-1 and EBLV-2 (IM) infected sheep

	EBLV-1	EBLV-2
Clinical progress	Per-acute n=1, death Protracted n=3,	Protracted n=4,
Clinical signs	Frequent licking lips & chomping Tremors Nervousness / Anxious Salivation Breathlessness Reduced food intake	Breathlessness Anxiety – jumpy Frequent licking of lips Tremors – tail Anxious / standing alone Nervousness Laying down Wool pulling Fawning
Overall clinical score range	1-3	1-2
Overall dysfunction	Motor	Behavioural

Table 3

Histopathology and immunohistochemistry detection of lyssavirus antigen in the brains of sheep after IC inoculation with EBLV1 or EBLV2.

Animal No.	EBLV-1				EBLV-2			
	A	B	C	D	E	F	G	H
G1	+++/-	+/-	++/●	+/-	+/●	+/●●	++/●●	++/●
G2	++/●●	++/●●	++/●●	++/●●	++/●●	+/●●	+++/●●●	++/●●
G3	+++/●	++/●	++/●●	++/●	++/●●●	++/●●●	+++/●●●	+++/●●●
G4	+/●	+/-	+/●	+/-	+/●●	++/●●	+/●	++/●
Inclusion bodies:								
Purkinje cells	0	0	+*	0	+	+	+	0
Other neurons	0	0	0	0	+	+	+	0

Brain locations:

G1= cerebrum with olfactory tract, primary motor and somatosensory cortex

G2= cerebrum with thalamus, piriform lobe, amygdala, optical tract, cerebral cortex

G3= cerebrum with hippocampus, dentate gyrus, choroid plexus

G4= brain stem and cerebellum

(<http://www.msu.edu/user/brains/sheepatlas>)

Histopathology score:

- no alterations, + mild, ++ moderate and +++ severe meningoencephalitis.

Immunohistochemistry score:

- no antigen positive neurons, ● few, ●● moderate and ●●● numerous antigen positive neurons.

Inclusion body score: 0 - no inclusion bodies, + > numerous inclusion bodies per cell, +* rare & not well defined

Figure 3

Histopathology and immunohistochemical detection of EBLV-1 and 2 at different brain locations of sheep after IC inoculation. For definition of G1-G4 see the key for Table 3. **(A-B)** H&E, **(C-J)** IHC / ABC-method with haematoxylin counterstain. Bar = 25 µm.

A - G4, EBLV-1A. Perivascular cuffing of small vessels in the cerebellum.

B - G4, EBLV-2F. Purkinje cells with one or more eosinophilic intracytoplasmic inclusion bodies.

C - G1, EBLV-1A. Olfactory tract with mild gliosis, perivascular cuffing.

D - G1, EBLV-2F. Moderate number of neurons in the olfactory tract with granular intracytoplasmic staining for lyssaviral antigen.

E - G2, EBLV-1A. Thalamic neuron with strong intracytoplasmic staining of the perikaryon and neurites.

F - G2, EBLV-2F. Thalamic neurones with intracytoplasmic staining associated with mild gliosis and perivascular cuffing.

G - G3, EBLV-1A. Mild gliosis and perivascular cuffing in the dentate gyros without associated lyssaviral antigen.

H - G3, EBLV-2F. Infection of numerous neurons of the dentate gyros with neuronal degeneration and gliosis.

I - G4, EBLV-1A. Few degenerated Purkinje cells with intracytoplasmic antigen.

J - G4, EBLV-2F. Purkinje cells with moderate amounts of intracytoplasmic granular lyssaviral antigen.

Figure 4

Histopathology and immunohistochemical detection of EBLV-1 in the G2 location of one sheep after IM inoculation. **(A)** HE, **(B)** ABC-method with haematoxylin counterstain. Bar = 25 μ m.

A: EBLV-1C (IM) . Severe encephalitis with perivascular cuffing of small vessels, moderate neuronal degeneration and gliosis in the cerebellum.

B: EBLV-1C (IM). Thalamic neurones and their axons with strong intracytoplasmic staining.

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Figure 1a

Virus specific neutralising antibody response in IC inoculated sheep

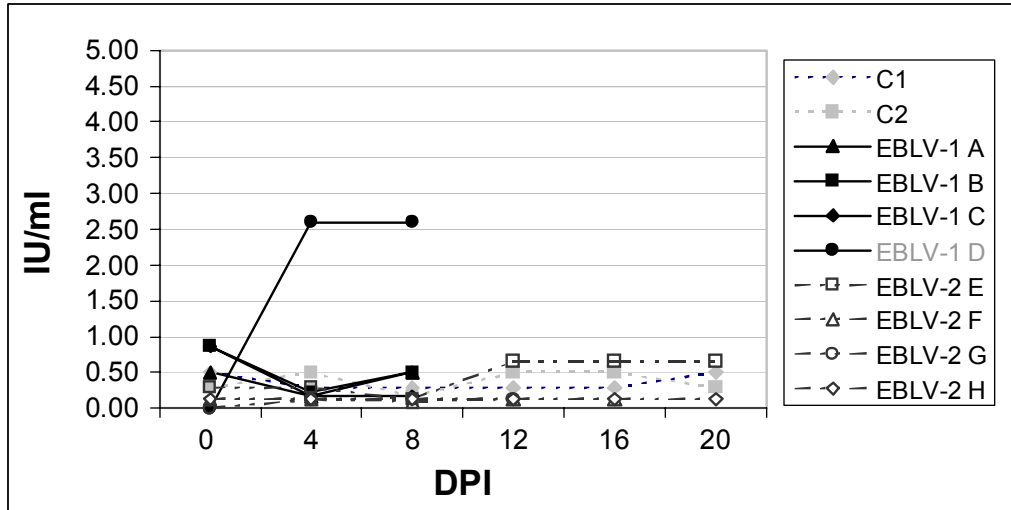
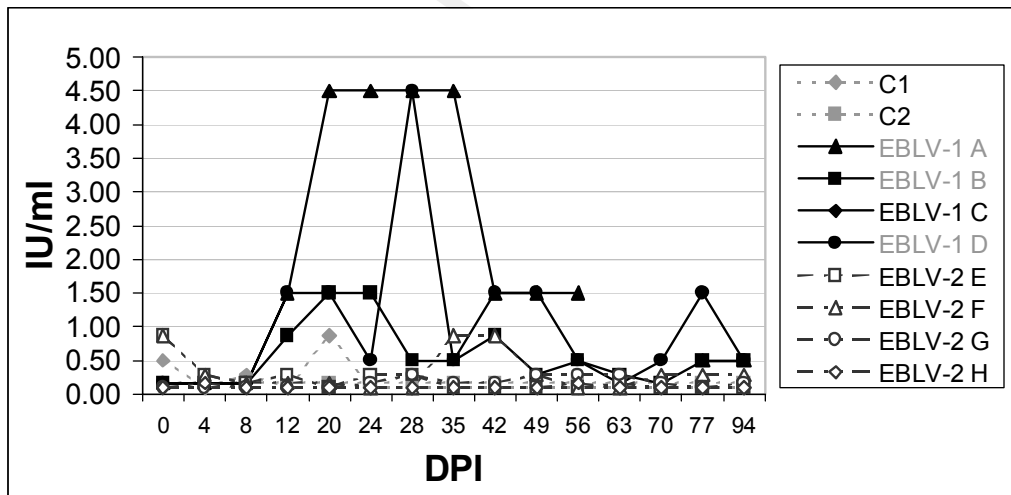


Figure 1b

Virus specific neutralising antibody response in IM inoculated sheep



Legend:

C1, C2 – control 1 and 2;

DPI – days post infection

Figure 2

Quantitative PCR of four brain regions, the spinal cord and trigeminal ganglia, from EBLV-1 (A) and EBLV-2 (B) IC inoculated animals.

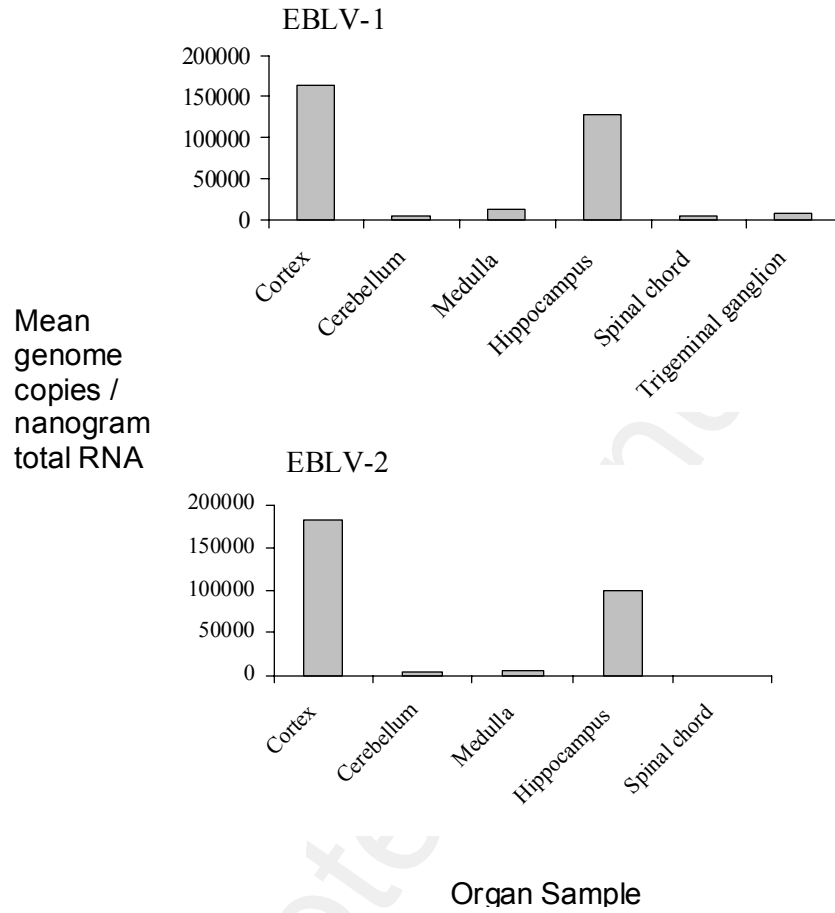


Figure 3 - Lyssavirus immunohistochemistry on EBLV-1 and EBLV-2 infected sheep brain following IC inoculation.

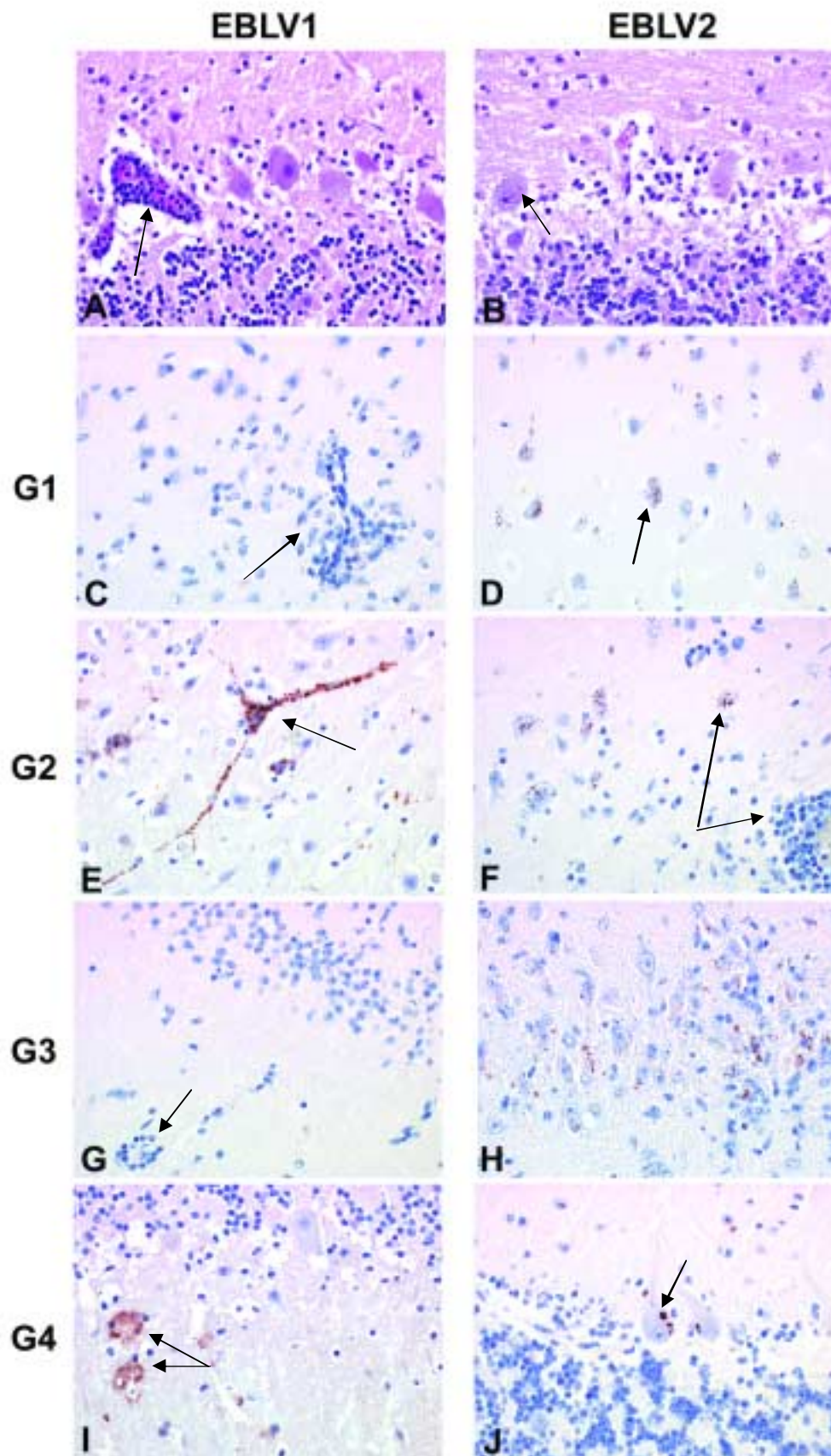


Figure 4 - Lyssavirus immunohistochemistry on an EBLV-1 infected sheep brain following IM inoculation.

