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Isolation, antigenicity and immunogenicity of Lleida bat lyssavirus

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Abstract

The lyssaviruses are an important group of viruses that cause a fatal encephalitis termed rabies. The prototypic lyssavirus, rabies virus, is predicted to cause more than 60 000 human fatalities annually. The burden of disease for the other lyssaviruses is undefined. The original reports for the recently described highly divergent Lleida bat lyssavirus were based on the detection of virus sequence alone. The successful isolation of live Lleida bat lyssavirus from the carcass of the original bat and *in vitro* characterization of this novel lyssavirus are described here. In addition, the ability of a human rabies vaccine to confer protective immunity following challenge with this divergent lyssavirus was assessed. Two different doses of Lleida bat lyssavirus were used to challenge vaccinated or naïve mice: a high dose of 100 focus-forming units (f.f.u.) 30 μl^{-1} and a 100-fold dilution of this dose, 1 f.f.u. 30 μl^{-1}. Although all naïve control mice succumbed to the 100 f.f.u. 30 μl^{-1} challenge, 42 % (*n*=5/12) of those infected intracerebrally with 1 f.f.u. 30 μl^{-1} survived the challenge. In the high-challenge-dose group, 42 % of the vaccinated mice survived the challenge (*n*=5/12), whilst at the lower challenge dose, 33 % (*n*=4/12) survived to the end of the experiment. Interestingly, a high proportion of mice demonstrated a measurable virus-neutralizing antibody response, demonstrating that neutralizing antibody titres do not necessarily correlate with the outcome of infection via the intracerebral route. Assessing the ability of existing rabies vaccines to protect against novel divergent lyssaviruses is important for the development of future public health strategies.

INTRODUCTION

The lyssaviruses constitute a group of high-impact viral pathogens that are of importance to both human and animal populations. The genus *Lyssavirus* currently includes 16 officially recognized species, all of which are assumed to be capable of causing rabies in mammals [1]. Whilst rabies virus, the prototype lyssavirus, causes more than 60 000 human deaths annually, the threat to human populations from other lyssaviruses is undefined, with only 13 human lyssavirus-related deaths having been reported [2]. Interestingly, with the exception of Mokola lyssavirus (MOKV) and Ikoma lyssavirus (IKOV), all other lyssavirus species, i.e. rabies lyssavirus (RABV), Lagos bat lyssavirus (LBV), Duvenhage lyssavirus (DUVV), European bat-1 lyssavirus (EBLV-1), European bat-2 lyssavirus (EBLV-2), Australian bat lyssavirus (ABLV), Aravan lyssavirus (ARAV), Khujand lyssavirus (KHUV), Irkut lyssavirus (IRKV), Shimoni bat lyssavirus (SHIBV), Bokeloh bat lyssavirus (BBLV), Gannoruwa bat lyssavirus (GBLV), West Caucasian bat lyssavirus (WCBV) and Lleida bat lyssavirus (LLEBV), have been detected from bat species. RABV is the most commonly reported lyssavirus, both from a terrestrial infection perspective and as a chiropteran pathogen. Indeed, ARAV, KHUV, WCBV, SHBV, IKOV and LLEBV exist as single detections, and as such there is still a complete lack of epidemiological understanding of their range or distribution. With the exception of RABV, the remaining lyssaviruses remain poorly understood in terms of both their epidemiology and their ability to cross the species barrier.

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Abbreviations: APHA, Animal and Plant Health Agency; DFAT, direct fluorescent antibody test; IC, intracerebral; IM, intramuscular; mFAVN, (modified) fluorescent antibody neutralization test; OIE, World Organisation for Animal Health; RTCIT, rabies tissue culture isolation technique; TCSN, tissue culture supernatant.

Four supplementary figures are available with the online version of this article.

The association of bats with the majority of lyssaviruses has led to the hypothesis that bats act as the origin of all lyssaviruses [3, 4]. The human and animal health profile of the lyssaviruses has galvanized the continued detection of novel lyssaviruses in bats. Consequently, the degree of vaccine protection conferred by rabies vaccines to divergent members of the genus *Lyssavirus* is of increasing interest. Lyssavirus species have been grouped into phylogroups, which correlate with cross-neutralization and the ability of vaccine-derived antibodies to neutralize these viruses. In addition, a distinction between viruses for which existing vaccines can confer protective immunity (phylogroup I) and viruses that are not neutralized (phylogroup II) is an essential parameter [5]. However, more divergent species have been proposed to be antigenically distinct from both phylogroup I and II viruses and may form a further phylogroup (III) or several distinct phylogroups [6, 7]. Vaccination and challenge studies in animal models utilizing existing rabies vaccines have demonstrated that the antibody response induced by rabies vaccines provides protection against DUVV [8], the EBLVs [9], BBLV and some of the recently identified Asian lyssaviruses [10], depending on the individual antibody titre achieved [11]. Additionally, reports of LBV- and MOKV-derived clinical disease in rabies-vaccinated companion animals has highlighted the lack of protection against phylogroup II lyssaviruses following vaccination [12–14]. Rabies biologicals were demonstrated to be ineffective against WCBV in both *in vitro* and *in vivo* studies [10, 15, 16]. Assessment of vaccine efficacy has also demonstrated that existing rabies vaccines are also unable to protect against IKOV [17]. In 2011, nucleic acid from a third, highly divergent lyssavirus, termed Lleida bat lyssavirus (LLEBV), was discovered in a common bent-winged bat (*Miniopteris schreibersii*) in Catalonia, Spain [18]. The bat was collected in the city of Lleida and initial evidence for a lyssavirus as the cause of death from lyssavirus antigen detection on brain was confirmed through a short sequence of the viral genome that suggested a highly divergent lyssavirus. However, attempts at the time to isolate the virus were unsuccessful, likely because the bat was stored for months and had been frozen and thawed twice [18]. Since the initial detection of viral nucleic acid a full genome sequence has been determined, and here the isolation of live LLEBV and the *in vitro* characterization of this virus is described. These data demonstrate, as for each of the phylogroup II lyssaviruses, and both IKOV and WCBV, that rabies vaccines are not consistently able to confer protective immunity against LLEBV. Investigations into the development of vaccines that are able to protect against multiple lyssavirus species are of interest [19–22]. The public health importance of lyssaviruses that are present in Eurasia and Africa and for which existing rabies vaccines do not confer protective immunity suggests that consideration should be given for the development of a pan-lyssavirus rabies vaccine [23].

RESULTS

Virus detection and isolation

Following receipt of the bat carcass at the Animal and Plant Health Agency (APHA), a full necropsy was undertaken on the remainder of the original bat carcass (without any brain material). A rabies tissue culture isolation test (RTCIT) was performed using homogenates of each specimen taken at necropsy and molecular tests were conducted on RNA extracted from each sample (Table 1). The bat was supplied after the entire brain had been removed and so only a swab of oral cavity, the cranium and the salivary glands could be tested. All were negative. Concurrent to necropsy, material from the top of the spinal cord was homogenized and inoculated into 3–4-week-old CD1 mice (n=6). The attempted isolation in tissue culture was unsuccessful, with only a small area of antigen being detected in the lung homogenate (Table 1). Nucleic acid was detected in a range of samples taken at post-mortem by both conventional and real time RT-PCR (Table 1). However, attempts to passage the supernatant from the antigen-positive lung homogenate failed. Inoculation of homogenized cervical spinal cord into mice, caused clinical disease in one of the six animals inoculated via the intracerebral (IC) route. Early signs of clinical disease were first suspected on day 12 post-inoculation, with one animal showing signs of nervousness and hyper-excitability. Clinical disease developed over a period of 4 days and included signs of incoordination, intermittent paralysis, hyperactivity and hyper-sexuality. During the clinical period the mouse was monitored and the clinical score progressed gradually from score 1 through to score 3, at which stage the mouse was humanely terminated. The remaining mice (n=5) were monitored until day 28, at which time they were cardiac bled under terminal anaesthesia and humanely terminated. Pathological examination of the brain from the clinical mouse showed good tissue preservation with the cortex, caudate putamen, hippocampus, thalamus, cerebellum and medulla being present. No obvious histopathological changes were observed and the brain was deemed negative by pathological examination.

In vitro assessment of virus growth and neutralization

A full necropsy was undertaken on the clinical mouse and the FAT on the brain material revealed areas of positivity where virus antigen was clearly present (Fig. 1a). RT-PCR and RTCIT were also conducted on a range of tissues taken from the clinical mouse (Table 2). Virus from the original brain homogenate was subjected to eight passes on N2a cells before 100 % infectivity and a titre of 3.3×10^5 focus-forming units (f.f.u.) ml^{-1} was reached. Growth was assessed on BHK cells in a multistep growth curve using a multiplicity of infection of 0.01 (Fig. 1b). The growth curve mirrored that of IKOV, albeit reaching approximately 1 log lower in the final titre [17]. A full genome sequence was generated and the content of both the coding and the non-coding regions of the genome (Fig. S1, available in the online version of this article) and the sequence identity with other

Table 1. Post-mortem molecular testing of samples from the original bat carcass

Y, yes; N, no; NT, not tested; (++), strong positive; (+), weak positive; SB, secondary amplification product; ¼, one well in four.

Sample no.	Sample	RNA ng μl^{-1}	PanLyssavirus hnRT-PCR block assay		SYBR real-time assay Ct values		RTCIT (Y/N)
			1st Round (Y/N)	2nd Round (Y/N)	SYBR	B-actin*	
1	Cervical spinal cord	149.8	Y (++)	Y (++)	34.95	No Ct	N
2	Tongue	243.8	Y (+)	Y (++)	36.63	No Ct	N
3	Pectorial muscle	146.2	Y (++)	Y (++)	34.58	No Ct	N
4	Oral swab	221.9	Y (+)	Y (++)	38.54	37.72	N
5	Lumbar spinal cord	249	Y (++)	Y (++)	31.73	No Ct	N
6	Kidney	276.2	N	Y (+)	No Ct	No Ct	N
7	Skull cavity	155	N	N	No Ct	No Ct	N
8	Intestine S	422.4	Y (++)	Y (++)	33.56	38.69	N
9	Intestine L	240.9	Y (+)	Y (++)	34.06	No Ct	N
10	Salivary gland	271.6	N	N	36.13	No Ct	N
11	Lung	131.7	Y (++) SB	Y (++)	37.14	No Ct	1/4†+
12	Thor spinal cord	223.3	Y (++)	Y (++)	32.29	No Ct	N
13	Liver	430.4	Y (++)	Y (++)	30.9	No Ct	N
14	Skin	320.9	N	N	No Ct	No Ct	N
15	Heart	236.7	N SB	N	35.15	37.59	N
16	Penis	211.7	N	N	No Ct	No Ct	NT
17	Testes	298.2	Y (++)	Y (++)	No Ct	No Ct	NT

*The absence of Cts for B-actin is likely a result of severe sample degradation.

†Isolation in tissue culture was unsuccessful, with only a small area of antigen being detected in the first passage, but not in subsequent passages.

lyssaviruses (Fig. S2) were compared [3, 24]. Phylogenetic alignment demonstrated the divergence of LLEBV when compared to other lyssaviruses, with LLEBV being most closely related to IKOV when using neighbour-joining and maximum likelihood reconstructions with the Kimura-2 parameter and GTR+G+I models, respectively. Regardless of the model or reconstruction used, the resulting tree topologies did not alter (Fig. S3).

Virus stocks were then utilized to develop a modified version of the fluorescent antibody neutralization (FAVN) test (mFAVN), in which LLEBV replaced the standard challenge virus (CVS) in the test [25, 26]. A panel of sera from vaccinated humans (*n*=2;○) and domestic dogs (*n*=10; ●) was then assessed using LLEBV as the challenge virus to determine the ability of each serum sample to neutralize the more divergent virus (Fig. 2). The ability of each serum sample to neutralize CVS was demonstrated, with one sample being negative for RABV neutralizing antibodies. A broad range of titres were included to assess whether even high-titre RABV-neutralizing sera were able to neutralize LLEBV. None of the serum samples tested neutralized LLEBV, with reciprocal titres of 5.66 being achieved for all samples when tested against LLEBV (Fig. 2).

Antigenic typing with a panel of eight N-specific monoclonal antibodies (mAbs) clearly differentiated the isolated virus from all other tested lyssavirus species (Table 3). Notably, only mAb MW187.6.1 reacted positively, albeit with faint staining. A similar picture was observed with the commercial conjugate from SIFIN (Berlin, Germany), a mixture of MW187.6.1; W239.17 and DUV6.15.19, whilst the Fujirebo conjugate yielded clear staining similar to that seen in the original direct fluorescent antibody test (DFAT).

In vivo vaccination challenge study

To assess vaccine protection, mice were vaccinated as described and serological responses were then determined using standard FAVN [26]. All mice seroconverted to a level above 0.5 IU ml^{-1}, the internationally assigned cut-off for neutralization of RABV, and were grouped into two groups based on antibody titre, such that a broad range of titres were present in each group. The mouse serology ranged from ~5 IU ml^{-1} to over 45 IU ml^{-1} (Fig. 3). Mice were challenged on day 28 post-vaccination with either a low or a high dose of LLEBV. Survivorship was monitored and compared to that for groups of unvaccinated mice challenged with the same dose of virus. All unvaccinated mice challenged with 100 f.f.u. 30 μl^{-1} of LLEBV (*n*=12) succumbed to infection between days 8 and 10 (Fig. 4). The mice were assessed according to a clinical score sheet to ensure humane termination of mice exhibiting clinical disease [27]. Clinical disease included piloerection, and a hunched stance leading to intermittent incoordination, hind limb paralysis, hyperactivity and body spasms. At the 100-fold lower dose of inoculation, the time to development of disease was prolonged, with mice succumbing between 10 and 17 days post-inoculation. Forty-two per cent (*n*=5/12) of the mice infected with the lower dose survived infection. From the

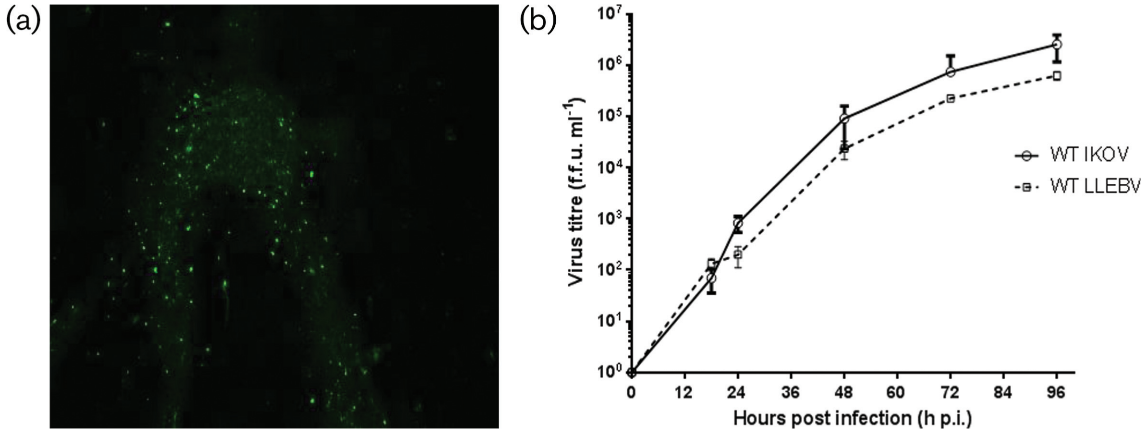

Fig. 1. (a) Detection of LLEBV by the direct fluorescent antibody test and (b) *In vitro* assessment of LLEBV in comparison with Ikoma lyssavirus. BHK cells were infected with virus at a multiplicity of infection of 0.01. An aliquot of cell supernatant was taken at each required time point and titrated in triplicate. Titres were calculated and expressed in focus-forming units (f.f.u.) ml^{-1}. The standard deviations are shown for each virus at each time point with error bars.

vaccinated mice, in the high-challenge-dose group, 42 % of the mice survived challenge (n=5/12) (Fig. 4a), whilst at the lower LLEBV challenge dose, the survival of the vaccinated mice was similar to that of the unvaccinated mice, with 33 % (n=4/12) surviving to the end of the experiment (Fig. 4b).

Serological assessment of mice

All animals that succumbed to infection and were humanely terminated, and those that survived to the end of the experiment, were cardiac bled and sera were assessed using both FAVN and mFAVN with LLEBV. From the groups of unvaccinated 'challenge control', animals the majority of the animals that developed clinical disease did not generate an antibody response that could neutralize either CVS or LLEBV (Fig. 5a). The exception was one mouse from the group infected with the low challenge dose of LLEBV IC that was terminated on day 17 post-infection that had an antibody response that was able to neutralize LLEBV. This individual developed disease a few days later than others from the same group that succumbed. In contrast, 42 % (n=5/12) of the unvaccinated mice challenged with the low dose of LLEBV survived and all seroconverted with sera that were able to neutralize LLEBV but were unable to neutralize CVS (Fig. 5a). From the vaccinated groups the challenge with LLEBV did not have a statistically significant effect on the serological titre developed when assessed for the ability to neutralize CVS. In fact, no significant increase in individual titres was seen while assessing the neutralization of CVS with tail-bleed sera from day 21 and terminal-bleed sera from either clinical mice or those that survived to the end of the experiment (Fig. 5b). Interestingly, when sera from survivors were assessed using the mFAVN with LLEBV, four mice had developed significant LLEBV neutralizing titres, with the remaining unvaccinated but challenged with a high dose of LLEBV mice also demonstrating exposure to LLEBV from their serological status (Fig. 5b). When we assessed sera from the low-challenge-dose groups, the serological status again remained virtually unchanged between the tail-bleed sera data from day 21 and those taken either following the development of clinical disease or following the termination of survivors. An exception to this was one surviving mouse whose reciprocal titre at day 21 was 270, but had increased markedly to over 10 000 upon termination at the end of the experiment as a survivor. Interestingly, this mouse also developed a high neutralizing titre against LLEBV, whilst none of the other mice within this group developed a demonstrable titre. Finally, the observation that the level of a RABV protective neutralizing titre at day 21 titre did not necessarily correlate with survival was of interest (Fig. S4). Here, some mice that had low levels of CVS neutralizing antibodies at day 21 survived, whilst mice with

Table 2. Post-mortem molecular testing on the LLEBV clinical mouse

Sample no.	Sample type	SYBR real-time assay Ct values	
		SYBR	B-actin
1	Spinal cord	21	26
2	Tongue	34/38	26
3	Salivary gland	25	20
4	Lung	30	21
5	Heart	28	24
6	Kidney	33	20
7	Liver	37	25
8	Ovaries	37	20
9	Uterus	38	26
10	Bladder	29	18

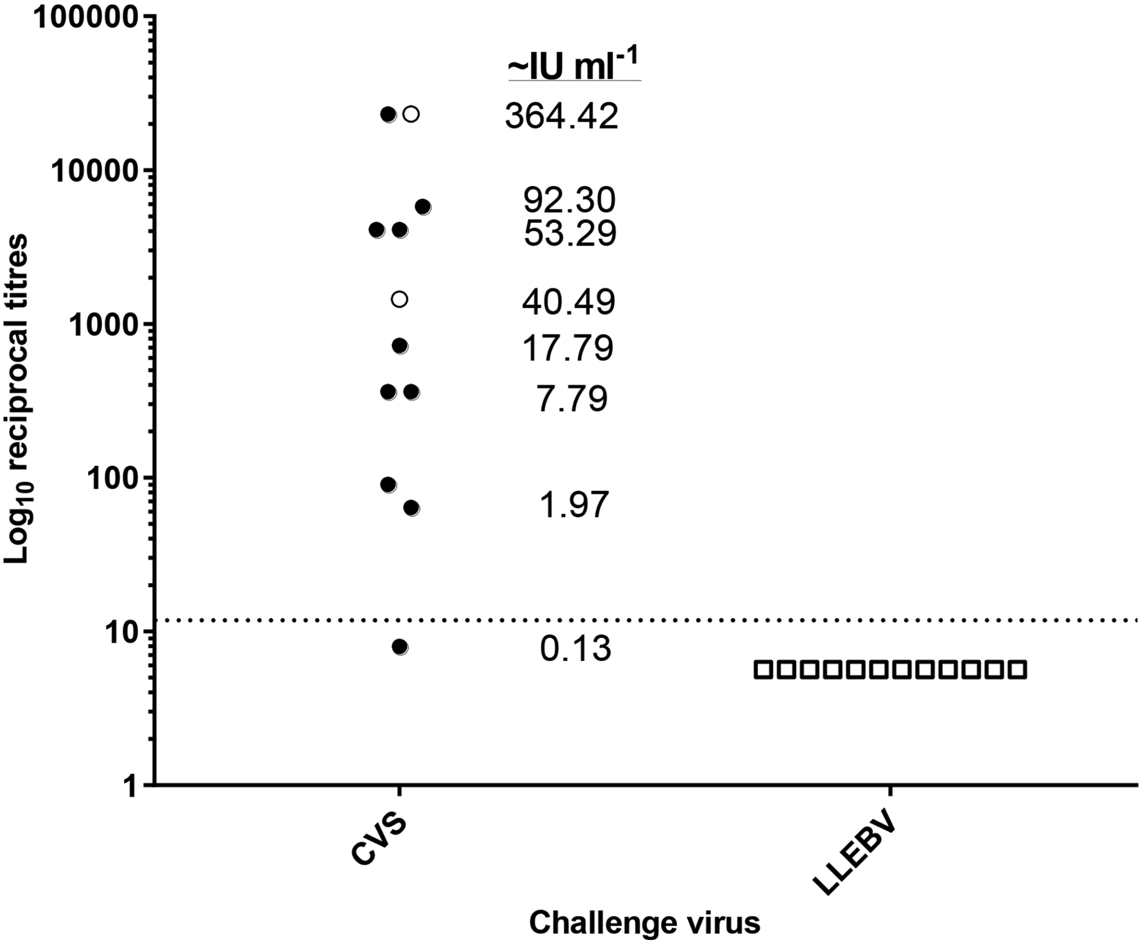

Fig. 2. Assessment of *in vitro* neutralization of LLEBV using a panel of sera from RABV vaccinees. The same panel of sera were tested against the challenge virus strain of RABV and LLEBV. Titres in IU ml^{-1} for neutralizing antibodies against CVS are labelled next to the equivalent reciprocal titre. The cut-off for RABV neutralization, as defined by the WHO, of 0.5 IU ml^{-1} is shown as a dotted line. Human samples are shown as open circles and dog samples are closed circles.

comparatively high titres succumbed to LLEBV challenge (Fig. S4).

DISCUSSION

Novel lyssaviruses continue to be detected and potentially pose a threat to human and animal health globally. The increase in awareness of rabies virus, with the global elimination of dog-mediated human rabies targeted by the World Organisation for Animal Health (OIE), the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) for 2030 [28], means that within the next 20 years dog-mediated rabies may be controlled [1]. With this in mind, the discovery of novel lyssaviruses is of increased interest, as where these viruses are detected, the potential for spillover infection of humans or animals exists [23, 29–32]. LLEBV was initially discovered in 2011, with only nucleic acid detection and sequence data being available. In the present study, live virus has been successfully isolated from the cervical spinal cord of the original bat carcass and stocks of the virus have been generated for virus characterization studies. The assessment of viral RNA in the original bat carcass demonstrated that the oral swab and salivary glands were negative, suggesting that the virus was likely not being excreted at the time of death. As the carcass was submitted without brain material, sections of the spinal cord were sampled. Viral RNA was detected in the available neuronal tissues, as well as in other organs, likely through sampling of innervated regions of each organ. Attempts to isolate virus in tissue culture failed, with only a lung sample being positive for virus antigen following RTCIT, although no live virus could be isolated following attempted passage of this sample. The inoculation of a small region of cervical spinal cord enabled the isolation of live virus following the development of clinical disease in a mouse. Once isolated, *in vitro* characterization demonstrated that LLEBV grows similarly to other lyssaviruses – to low titres when compared to other genera within the *Rhabdoviridae*, such as

Table 3. Reaction pattern of a panel of 10 anti-nucleocapsid monoclonal antibodies and commercial conjugates with LLEBV and selected lyssaviruses

anti-NC mAb	RABV	DUVV	EBLV-1	EBLV-2	ABLV	BBLV	LBV	MOKV	LLEBV
W239.17	+++	+++	+++	+++	+++	+++	+++	+++	–
W187.5	+++	–	–	–	+++	–	–	–	–
W187.11.2	+++	–	–	–	+++	+++	–	–	–
MW187.6.1	+++	+++	–	–	+++	+++	+++	+++	+
MSA6.3	–	–	+++	+++	–	+++	–	+++	NA
LBV7.36	–	–	–	–	–	–	+++	–	–
DUV6.15.19	–	+++	+++	–	–	–	–	–	–
S62.1.2	–	–	+++	+++	–	–	–	–	–
P 41	–	–	–	–	–	–	–	–	–
Z144.88	–	–	–	–	–	–	–	–	–
SIFIN	+++	+++	+++	+++	+++	+++	+++	+++	+
Fujirebio	+++	+++	+	+	+++	+	+++	+++	+++
BIO-RAD	NA	NA	NA	NA	NA	NA	NA	NA	++

vesiculoviruses, where the type viruses, such as vesicular stomatitis virus, can reach considerably higher titres (10^9–10^{12}) *in vitro* and *in vivo* [33]. The reason for this difference is unknown, but may be a factor in the evolutionary direction of lyssaviruses, with their strong neurotropism.

The impact of LLEBV among the genus *Lyssavirus* lies in its genetic and antigenic divergence from other members of the genus, as demonstrated by antigenic typing and phylogeny. The observation that some commercially available anti-N rabies conjugates were only weakly able to detect LLEBV is of importance for diagnostic activities. Of further note, the HAM 5DF123B0 monoclonal antibody (a gift from the Swiss Rabies Centre, Switzerland) used to detect lyssavirus antigen in histopathological specimens was unable to detect LLEBV. Regardless, generic pan-lyssavirus PCR methodologies were able to detect the LLEBV viral RNA and, as such, should molecular tests be adopted by the OIE, they would serve as a useful broadly sensitive diagnostic tool. The ability of diagnostic tools that are used globally to detect this and other highly divergent lyssaviruses should be investigated. From the latter perspective, LLEBV is most closely related to both IKOV and WCBV. As such, it was predicted that the existing rabies vaccines would be unable to afford protection against the virus. Only limited studies have defined any type of pathogenicity or virulence for these viruses. Previous studies with WCBV have demonstrated that inoculation of the big brown bat (*Eptesicus fuscus*) via the oral route was non-viable, with both clinical and serological response being absent following instillation of inocula into the oral cavity. In contrast, peripheral inoculation into the masseter caused seroconversion, demonstrating that the immune system had reacted to the inoculated virus and generated a neutralizing antibody response. The only productive infection observed was following intramuscular inoculation of WCBV to the neck, with 37 % (n=3/8) of infected animals developing clinical disease, and those that did not succumb becoming seropositive from infection [15]. For IKOV, both peripheral and IC inoculation of wild-type IKOV caused clinical disease in a mouse model. Intracerebral inoculation and peripheral inoculation with a high dose of IKOV ($10^{3.8}$TCID$_{50}$ ml^{-1}) caused 100 % mortality, with a detectable dose effect being observed following intramuscular infection, with only 40 % (n=2/5) of mice infected peripherally succumbing to infection with a lower dose [17].

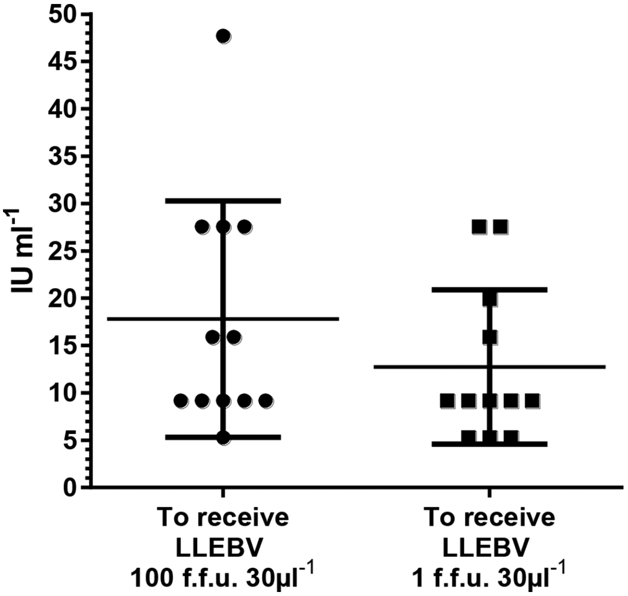

Fig. 3. Post-vaccination serology on day 21 for mice vaccinated with Rabipur on days 0 and 7. All day 21 post-vaccination the titres were assessed by FAVN. Mice were grouped for challenge as demonstrated, with a range of titres being placed in each group to assess protection against a high and a low dose of LLEBV.

Should rabies be eliminated from dogs across the globe, the vaccine protection offered by existing rabies vaccines for the

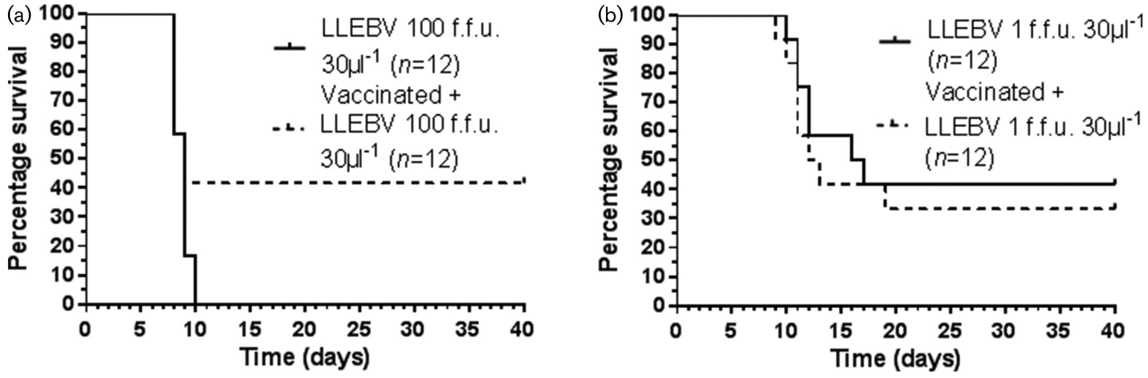

Fig. 4. *In vivo* survivorship following intracranial infection with (a) 100 f.f.u. 30 µl^{-1} and (b) 1 f.f.u. 30 µl^{-1}. Mice were vaccinated 28 days before challenge. Challenge day is day 0 on the graphs presented.

other lyssaviruses requires investigation in a world free of canine-transmitted rabies virus. As such, the genetic divergence between LLEBV, IKOV and WCBV, and their potential ability to host-switch is of global interest. Despite none of the serum samples from vaccinated humans and domestic animals tested neutralizing LLEBV, the vaccination challenge studies demonstrated that when a standardized dose of vaccine was used, some protection was afforded by vaccination when the challenge was with a dose of 100 f.f.u. 30 µl^{-1} LLEBV administered intracerebrally. Previous studies have demonstrated a complete lack of vaccine-derived protection when assessing IKOV pathogenicity, although in those studies the challenge dose was substantially higher. In the present study, a dose that was sufficient to cause clinical disease but lower than previous doses was utilized in an attempt to mimic a more realistic challenge dose. Certainly, other studies have demonstrated that the virus dose utilized in pathogenesis studies, or in vaccination challenge studies as described here, needs to be standardized according to the model being used [34]. As was perhaps expected, the sera from surviving mice in the unvaccinated low-dose-challenge group (42 %; *n*=5/12) showed detectable neutralizing antibody responses against LLEBV. As almost all of the remaining unvaccinated mice that succumbed from either group failed to develop detectable neutralizing antibody responses (Fig. 5a), the principle that neutralizing antibodies are required to clear infection was upheld [35]. However, one mouse that succumbed on day 17 developed a neutralizing

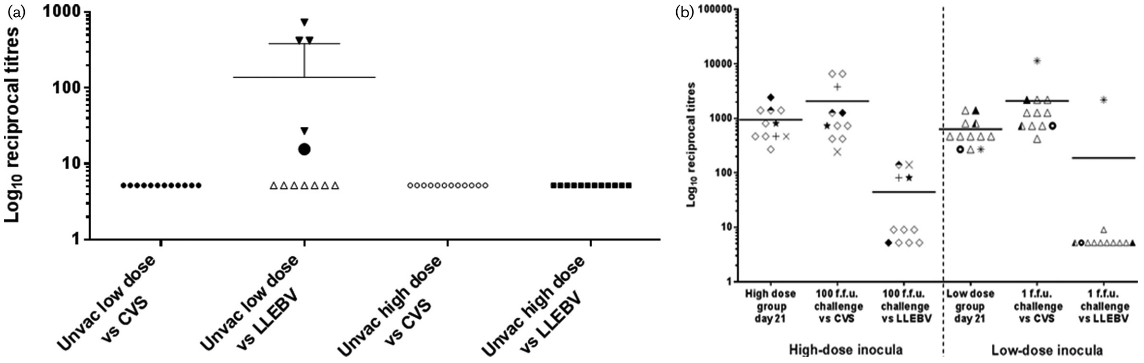

Fig. 5. Assessment of serological status in mice following vaccination and challenge. (a) Unvaccinated mice were terminated at completion of the experiment. All unvaccinated mice that received a high dose of LLEBV succumbed and were serologically negative for neutralizing antibodies against CVS and LLEBV at termination. A few mice that survived infection (▼) with the low dose of LLEBV developed a response that could neutralize LLEBV but not CVS. One mouse that was terminated with clinical disease late post-infection (day 17) also demonstrated a neutralizing antibody response that could neutralize LLEBV by mFAVN (●). (b) Vaccinated and challenged mice. Mice were vaccinated and their day 21 post-vaccination serology is shown. The mice were then challenged and cardiac bled following termination either at a humane endpoint or at the end of the experiment as survivors. Sera from each mouse were tested against CVS and LLEBV to assess their neutralizing antibodies. The open symbols represent mice that developed clinical disease and were terminated. The surviving mice have the same symbol across each dataset within each group to show serological status by neutralization of CVS and LLEBV vs post-vaccination serology. The same shading pattern is used across the groups to link the data from each surviving mouse against each virus tested both before challenge and at the end of the experiment as survivors.

antibody response, but still succumbed to infection. However, it is possible that this mouse may have survived with neurological sequelae if it had not been necessary to terminate it at clinical score 2 according to defined humane endpoints.

In contrast, the fate of the vaccinated animals is somewhat contradictory. Despite the general dogma that increasing neutralizing antibody titre correlates with increased protection, the data here indicate otherwise. The initial *in vitro* assessment of a panel of human and dog sera demonstrated clearly that even sera containing high levels of high neutralizing antibodies were unable to neutralize LLEBV (Fig. 2). *In vivo*, however, of the mice that were vaccinated but developed clinical disease, individual mice had varying post-vaccination anti-CVS neutralizing antibodies (Fig. S4) with higher CVS neutralizing titres not necessarily corresponding with increased likelihood of survival. This reiterates the fact that cell-mediated mechanisms may influence the outcome of survival in vaccinated individuals following challenge with a highly divergent virus that is thought to evade rabies vaccine-induced antibodies. Certainly cell-mediated responses have been associated with clearance in the periphery of fixed rabies strains, although street strains of rabies are thought to use certain mechanisms, such as reduced protein expression, to evade the host responses in the periphery [36]. Overall, there is a general lack of knowledge detailing the host mechanisms for lyssavirus clearance and further studies are warranted. From a dose/response perspective, previous studies that titrated out lyssaviruses to assess the outcomes of initial and multiple exposures of bats and mice to low doses of virus support the observations noted here [37, 38].

The association of LLEBV with a bat species found across Europe is of increased interest, as bat populations and the lyssaviruses they harbour across the European Union (EU) have been the target of surveillance schemes for many years [39–42]. Historically, the discovery of novel lyssaviruses has often been limited to a single isolate. Only having a single isolation for LLEBV limits any epidemiological assessment of risk, although the bat species involved has a wide distribution across North Africa and much of the EU. Interestingly, WCBV is the only other lyssavirus to be detected in *Miniopteris schreibersii*, suggesting that both WCBV and LLEBV have evolved into distinct sub-lineages, and that *Miniopteris schreibersii* should be considered to be a reservoir of these lyssavirus species. However, with repeated detections of other recently described lyssaviruses in the EU [43], it is possible that any future detection of these divergent lyssaviruses could raise the public health importance of these highly fatal viral pathogens, especially if another case of LLEBV or WCBV were to be reported from a bat in Eurasia. Importantly, the lack of consistent vaccine-induced protective immunity conferred by the existing rabies vaccines against more divergent lyssaviruses, including LLEBV, suggests that novel pan-lyssavirus vaccine formulations should be further investigated to ensure that appropriate post-exposure biologicals for human use are available, should they be required in the future.

METHODS

In vitro assessment of the bat carcass

The bat carcass was stored at −80 °C at the Instituto de Salud Carlos III, Madrid, Spain before virus isolation and characterization was undertaken. No brain material remained in the carcass submitted. The original carcass was thawed and an extensive necropsy was conducted, taking sections of the cervical/thoracic and lumbar spinal cord, the tongue, the pectoral muscle, the kidney, the small and the large intestine, the lung and the heart. Once taken, the samples were rapidly homogenized using aliquots of frozen PBS and vigorous shaking. Homogenized samples were assessed for both live virus and viral RNA. The cervical spinal cord section taken was utilized in virus isolation in both cell culture and in mice.

Rabies tissue culture isolation test

Samples taken at post-mortem (Table 1) were assessed for the presence or absence of live virus as described previously [44]. Briefly, murine neuroblastoma cells were split and mixed with sample homogenate, enabling infection where live virus was present. After incubation, cells were fixed and stained with a rabies anti-nucleocapsid FITC-conjugated antibody (Fujirebio Diagnostics, USA). RTCIT-positive material was then further passaged to generate virus stocks and once 100 % infectivity was reached, viral titres were calculated using the Spearman–Kärber method [45]. Viral antigens were visualized in acetone-fixed cells by the DFAT using standard techniques [46] with FITC-conjugated antibody (FITC Anti-Rabies Monoclonal Globulin, Fujirebio Diagnostics, Malvern, USA).

Monoclonal typing

Antigenic typing of LLEBV propagated in cell culture was undertaken using an indirect fluorescent antibody test (iFAT) with a panel of 10 anti-nucleocapsid monoclonal antibodies (N-MAbs) as described previously [47]. Representatives of other lyssavirus species, i.e. RABV, LBV, MOKV, DUVV, EBLV-1, EBLV-2 and ABLV, were used as reference viruses for comparison. Additionally, polyclonal rabbit sera against N (N161-5; [48] and P were tested, as well as commercially available anti-rabies nucleocapsid conjugates (Bio-Rad, Marne, France; Fujirebio Diagnostics, USA; SIFIN, Berlin, Germany).

In vivo studies

All *in vivo* work was undertaken according to UK Home Office regulations under licence 70/7394. All studies were assessed internally by the APHA ethics and statistical committee. For attempted virus isolation, six 3–4-week-old female CD-1 mice were inoculated with homogenized, clarified sample (30 µl) intracerebrally. Mice had access to food and water *ad libitum* and were monitored twice daily for

35 days using an established lyssavirus clinical scoring system [27] to evaluate any behavioural changes consistent with lyssavirus infection. From the first suspicion of clinical disease, they were monitored closely and were terminated humanely either before or upon reaching clinical score 3 [27]. The brains of suspected mice were tested using the direct fluorescent antibody test (DFAT) and positive material was passaged by the RTCIT.

Groups of mice were vaccinated via the intraperitoneal route with 500 µl of a 1 : 20 dilution of the human rabies vaccine (Rabipur) on days 0 and 7 in order to assess the ability of Rabipur to protect against LLEBV. All mice were monitored twice daily for 28 days post-vaccination, and were tail bled on day 21 to assess the serological response to vaccination as described previously [26]. A group of mock-vaccinated mice (PBS) were included to assess the challenge doses administered. On day 28 post-vaccination, 2 groups of 12 female CD1 mice were challenged with either a high-dose tissue culture supernatant (TCSN high dose; 10^2 f.f.u. 30 μl^{-1}) or a 100-fold dilution (low dose; 1 f.f.u. 30 μl^{-1}) by intracranial inoculation, followed by observation twice daily and euthanasia according to an established clinical score system [27]. The mice were humanely terminated and cardiac bled under terminal anaesthesia, either following the development of clinical disease or at the end of the experiment. A full pathological assessment of mice was carried out. Immunohistochemical demonstration of RABV antigens in post-mortem samples from brains of mice was attempted as described previously [49].

Serology

For the assessment of vaccine-derived antibodies, a standard FAVN was undertaken with the CVS strain of rabies as the challenge virus as described previously [26]. A mFAVN was developed and optimized for LLEBV using virus harvested within TCSN [25, 26]. Live virus was titrated to enable an accurate dilution to reach 100 $TCID_{50}$ 50 μl^{-1}. This virus dose was added to serial twofold dilutions of serum in duplicate, with the quantity of virus being checked by back-titration on each test. The sera and virus were incubated with BHK-21 cells for 48 h before fixing in 80 % acetone and staining with FITC-conjugated antibody (Fujirebio Diagnostics). The 50 % endpoint serum dilution was calculated using the Spearman–Kärber method [45]. Serum from uninfected mice was used as a negative control. A panel of serum samples from both human and animal vaccinees were tested for their ability to neutralize LLEBV.

Molecular analyses

Nucleic acids were extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Two different pan-lyssavirus PCR assays were utilized to determine the presence or absence of lyssavirus RNA in both bat and mouse organs at post-mortem. First, a conventional hemi-nested RT-PCR (hnRT-PCR [50], modified by using JW10uni reverse primer, was used [51]. In parallel, a real-time SYBR based PCR assay (Bio-Rad) was also applied to test for the presence of lyssavirus RNA [52]. Amplification using Beta actin specific primers was used as a control for RNA extraction.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References

1. **Fooks AR, Cliquet F, Finke S, Freuling C, Hemachudha T *et al.*** Rabies. *Nat Rev Dis Primers* 2017;3:17091.
2. **Banyard AC, Fooks AR.** The impact of novel lyssavirus discovery. *Microbiol Aust* 2017;38:18–21.
3. **Hayman DT, Fooks AR, Marston DA, Garcia-R JC.** The global phylogeography of lyssaviruses – challenging the 'Out of Africa' hypothesis. *PLoS Negl Trop Dis* 2016;10:e0005266.
4. **Badrane H, Tordo N.** Host switching in *Lyssavirus* history from the Chiroptera to the Carnivora orders. *J Virol* 2001;75:8096–8104.
5. **Badrane H, Bahloul C, Perrin P, Tordo N.** Evidence of two *Lyssavirus* phylogroups with distinct pathogenicity and immunogenicity. *J Virol* 2001;75:3268–3276.
6. **Fooks A.** The challenge of new and emerging lyssaviruses. *Expert Rev Vaccines* 2004;3:333–336.
7. **Kuzmin IV, Hughes GJ, Botvinkin AD, Orciari LA, Rupprecht CE.** Phylogenetic relationships of Irkut and West Caucasian bat viruses within the *Lyssavirus* genus and suggested quantitative criteria based on the N gene sequence for lyssavirus genotype definition. *Virus Res* 2005;111:28–43.
8. **Fekadu M, Shaddock JH, Sanderlin DW, Smith JS.** Efficacy of rabies vaccines against Duvenhage virus isolated from European house bats (*Eptesicus serotinus*), classic rabies and rabies-related viruses. *Vaccine* 1988;6:533–539.
9. **Brookes SM, Parsons G, Johnson N, Mcelhinney LM, Fooks AR.** Rabies human diploid cell vaccine elicits cross-neutralising and cross-protecting immune responses against European and Australian bat lyssaviruses. *Vaccine* 2005;23:4101–4109.
10. **Hanlon CA, Kuzmin IV, Blanton JD, Weldon WC, Manangan JS *et al.*** Efficacy of rabies biologics against new lyssaviruses from Eurasia. *Virus Res* 2005;111:44–54.
11. **Malerczyk C, Freuling C, Gniel D, Giesen A, Selhorst T *et al.*** Cross-neutralization of antibodies induced by vaccination with purified chick embryo cell vaccine (PCECV) against different *Lyssavirus* species. *Hum Vaccin Immunother* 2014;10:2799–2804.
12. **King A, Crick J.** Rabies-related viruses. In: Campbell JB (editor). *Rabies*. Boston: Kluwer Academic Publishers; 1988. pp. 177–200.
13. **Markotter W, van Eeden C, Kuzmin IV, Rupprecht CE, Paweska JT *et al.*** Epidemiology and pathogenicity of African bat lyssaviruses. *Dev Biol* 2008;131:317–325.
14. **Wright E, Temperton NJ, Marston DA, Mcelhinney LM, Fooks AR *et al.*** Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: a cross-species comparison. *J Gen Virol* 2008;89:2204–2213.
15. **Kuzmin IV, Franka R, Rupprecht CE.** Experimental infection of big brown bats (*Eptesicus fuscus*) with West Caucasian bat virus (WCBV). *Dev Biol* 2008;131:327–337.

16. **Kuzmin IV, Botvinkin AD, Poleschuk EM, Orciari LA, Rupprecht CE.** Bat rabies surveillance in the former Soviet Union. *Dev Biol* 2006;125:273–282.
17. **Horton DL, Banyard AC, Marston DA, Wise E, Selden D *et al.*** Antigenic and genetic characterization of a divergent African virus, Ikoma lyssavirus. *J Gen Virol* 2014;95:1025–1032.
18. **Aréchiga Ceballos N, Vázquez Morón S, Berciano JM, Nicolás O, Aznar López C *et al.*** Novel lyssavirus in bat, Spain. *Emerg Infect Dis* 2013;19:793–795.
19. **Bahloul C, Jacob Y, Tordo N, Perrin P.** DNA-based immunization for exploring the enlargement of immunological cross-reactivity against the lyssaviruses. *Vaccine* 1998;16:417–425.
20. **Jallet C, Jacob Y, Bahloul C, Drings A, Desmezieres E *et al.*** Chimeric lyssavirus glycoproteins with increased immunological potential. *J Virol* 1999;73:225–233.
21. **Desmézières E, Jacob Y, Saron MF, Delpeyroux F, Tordo N *et al.*** Lyssavirus glycoproteins expressing immunologically potent foreign B cell and cytotoxic T lymphocyte epitopes as prototypes for multivalent vaccines. *J Gen Virol* 1999;80:2343–2351.
22. **Weyer J, Kuzmin IV, Rupprecht CE, Nel LH.** Cross-protective and cross-reactive immune responses to recombinant vaccinia viruses expressing full-length lyssavirus glycoprotein genes. *Epidemiol Infect* 2008;136:670–678.
23. **Evans JS, Horton DL, Easton AJ, Fooks AR, Banyard AC.** Rabies virus vaccines: is there a need for a pan-lyssavirus vaccine? *Vaccine* 2012;30:7447–7454.
24. **Marston DA, Ellis RJ, Wise EL, Aréchiga-Ceballos N, Freuling CM *et al.*** Complete genome sequence of lleida bat lyssavirus. *Genome Announc* 2017;5:e01427-16.
25. **Brookes SM, Healy DM, Fooks AR.** Ability of rabies vaccine strains to elicit cross-neutralising antibodies. *Dev Biol* 2006;125:185–193.
26. **Cliquet F, Aubert M, Sagné L.** Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. *J Immunol Methods* 1998; 212:79–87.
27. **Healy DM, Brookes SM, Banyard AC, Núñez A, Cosby SL *et al.*** Pathobiology of rabies virus and the European bat lyssaviruses in experimentally infected mice. *Virus Res* 2013;172:46–53.
28. **Fahrion AS, Taylor LH, Torres G, Müller T, Dürr S *et al.*** The road to dog rabies control and elimination-what keeps us from moving faster? *Front Public Health* 2017;5:103.
29. **Fooks AR, Mcelhinney LM, Pounder DJ, Finnegan CJ, Mansfield K *et al.*** Case report: isolation of a European bat lyssavirus type 2a from a fatal human case of rabies encephalitis. *J Med Virol* 2003; 71:281–289.
30. **van Thiel PP, van den Hoek JA, Eftimov F, Tepaske R, Zaaijer HJ *et al.*** Fatal case of human rabies (Duvenhage virus) from a bat in Kenya: The Netherlands, December 2007. *Euro Surveill* 2008;13: 8007.
31. **Lumio J, Hillbom M, Roine R, Ketonen L, Haltia M *et al.*** Human rabies of bat origin in Europe. *Lancet* 1986;1:378.
32. **Gould AR, Hyatt AD, Lunt R, Kattenbelt JA, Hengstberger S *et al.*** Characterisation of a novel lyssavirus isolated from Pteropid bats in Australia. *Virus Res* 1998;54:165–187.
33. **Whelan SP, Ball LA, Barr JN, Wertz GT.** Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. *Proc Natl Acad Sci USA* 1995;92:8388–8392.
34. **Rupprecht CR** (editor). *Lyssavirus Diversity, Immunological Breath and the Fermi-Paradox Revisited. Rabies Serology Meting 2017; 2017 14th-15th June 2017*. Budapest, Hungary.
35. **Hooper DC, Phares TW, Fabis MJ, Roy A.** The production of antibody by invading B cells is required for the clearance of rabies virus from the central nervous system. *PLoS Negl Trop Dis* 2009;3:e535.
36. **Wang ZW, Sarmento L, Wang Y, Li XQ, Dhingra V *et al.*** Attenuated rabies virus activates, while pathogenic rabies virus evades, the host innate immune responses in the central nervous system. *J Virol* 2005;79:12554–12565.
37. **Banyard AC, Healy DM, Brookes SM, Voller K, Hicks DJ *et al.*** Lyssavirus infection: 'low dose, multiple exposure' in the mouse model. *Virus Res* 2014;181:35–42.
38. **Turmelle AS, Jackson FR, Green D, McCracken GF, Rupprecht CE.** Host immunity to repeated rabies virus infection in big brown bats. *J Gen Virol* 2010;91:2360–2366.
39. **Schatz J, Fooks AR, McElhinney L, Horton D, Echevarria J *et al.*** Bat rabies surveillance in Europe. *Zoonoses Public Health* 2013;60: 22–34.
40. **Schatz J, Freuling CM, Auer E, Goharriz H, Harbusch C *et al.*** Enhanced passive bat rabies surveillance in indigenous bat species from Germany–a retrospective study. *PLoS Negl Trop Dis* 2014;8:e2835.
41. **Schatz J, Ohlendorf B, Busse P, Pelz G, Dolch D *et al.*** Twenty years of active bat rabies surveillance in Germany: a detailed analysis and future perspectives. *Epidemiol Infect* 2014;142: 1155–1166.
42. **Wise EL, Marston DA, Banyard AC, Goharriz H, Selden D *et al.*** Passive surveillance of United Kingdom bats for lyssaviruses (2005-2015). *Epidemiol Infect* 2017;145:2445–2457.
43. **Eggerbauer E, Troupin C, Passior K, Pfaff F, Höper D *et al.*** The recently discovered Bokeloh Bat Lyssavirus: insights into its genetic heterogeneity and spatial distribution in Europe and the population genetics of its primary host. *Adv Virus Res* 2017;99: 199–232.
44. **Webster WA.** A tissue culture infection test in routine rabies diagnosis. *Can J Vet Res* 1987;51:367–369.
45. **Aubert M.** Methods for the calculation of titres. In: Meslin FX, kaplan MM and Koprowski H. *Laboratory Techniques in Rabies*. Geneva: World Health Organisation; 1996. pp. 445–459.
46. **Dean DJ, Abelseth MK, Atanasiu P.** The fluorescent antibody test. In: Meslin FX, kaplan MM and Koprowski H (editors). *Laboratory Techniques in Rabies*. Geneva: World Health Organisation; 1996. pp. 88–93.
47. **Schneider LG, Barnard BJH, Schneider HP.** Application of monoclonal antibodies for epidemiological investigations and oral vaccination studies: I- African viruses. In: Kuwert CM, Koprowski H and Bogel K. *Rabies in the Tropics*. Berlin: Springer-Verlag; 1985. pp. 49–53.
48. **Orbanz J, Finke S.** Generation of recombinant European bat lyssavirus type 1 and inter-genotypic compatibility of lyssavirus genotype 1 and 5 antigenome promoters. *Arch Virol* 2010;155: 1631–1641.
49. **Hicks DJ, Núñez A, Banyard AC, Williams A, Ortiz-Pelaez A *et al.*** Differential chemokine responses in the murine brain following lyssavirus infection. *J Comp Pathol* 2013;149:446–462.
50. **Heaton PR, Johnstone P, McElhinney LM, Cowley R, O'Sullivan E *et al.*** Heminested PCR assay for detection of six genotypes of rabies and rabies-related viruses. *J Clin Microbiol* 1997;35: 2762–2766.
51. **Freuling CM, McElhinney LM, Fooks AR, Müller TF.** Gel-based reverse transcription-polymerase chain reaction. In: *Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention*, vol. 2. San Diego and London: Academic Press; 2015. pp. 119–128.
52. **Hayman DT, Banyard AC, Wakeley PR, Harkess G, Marston D *et al.*** A universal real-time assay for the detection of Lyssaviruses. *J Virol Methods* 2011;177:87–93.