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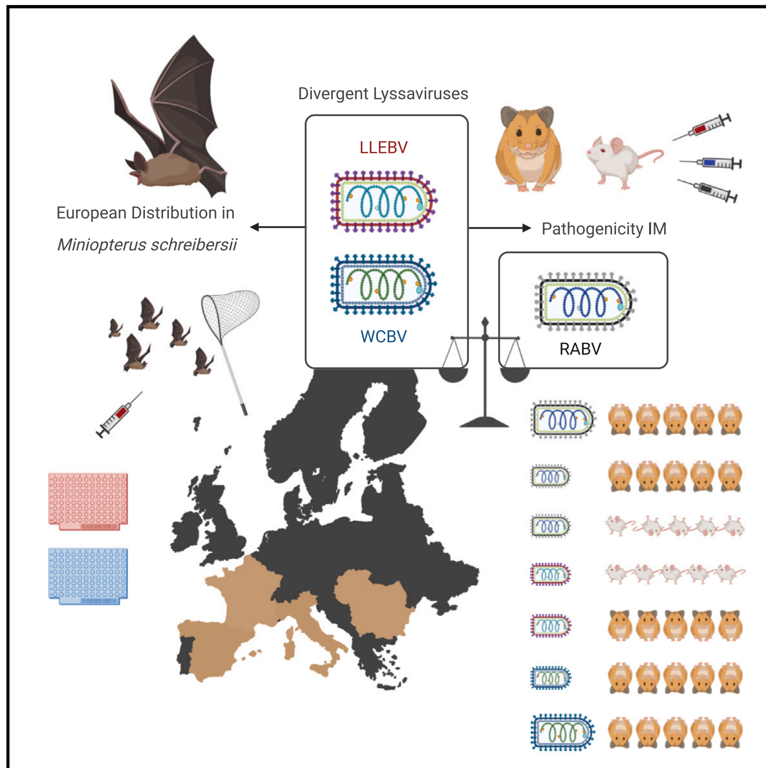
European distribution and intramuscular pathogenicity of divergent lyssaviruses West Caucasian bat virus and Lleida bat lyssavirus

Graphical abstract



Highlights

- Serology showed the wide circulation of WCBV and LLEBV in *M. schreibersii* from Europe
- WCBV caused clinical rabies and 100% lethality in hamsters upon IM infection
- LLEBV showed no IM lethality after 40 and 21 days of experiment in hamsters or mice
- Hamsters activated humoral response against WCBV and LLEBV by day 7 post infection

Authors

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In brief

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European distribution and intramuscular pathogenicity of divergent lyssaviruses West Caucasian bat virus and Lleida bat lyssavirus

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SUMMARY

Among lyssaviruses, West Caucasian bat virus (WCBV) and Lleida bat lyssavirus (LLEBV) raise concern as their divergence from rabies virus leads to the inefficacy of available prophylactic agents. Both viruses were described in the bat *Miniopterus schreibersii*. We investigated the European distribution of WCBV and LLEBV by screening sera from *Miniopterus schreibersii* across eight countries, finding widespread serological evidence and positivity up to 70%. We evaluated the intramuscular lethality of wild type isolates in Syrian hamsters. WCBV induced 100% lethality and a clinical disease compatible with furious rabies. All animals infected with LLEBV remained healthy for 40 days, despite one individual testing positive in the brain. We confirmed LLEBV's intramuscular a-pathogenicity using mice. Infected hamsters developed antibodies by day seven, regardless the virus and the clinical outcome. This study highlights the widespread circulation of WCBV and LLEBV in Europe and suggests differences in neuro-invasiveness and/or pathogenesis that are crucial for risk assessment.

INTRODUCTION

Europe accounts for a wide viral diversity within the genus *Lyssavirus,* with six species different from the prototype rabies virus (RABV) circulating in members of the order Chiroptera,[1,2] including two highly divergent species, West Caucasian bat virus (WCBV) and Lleida bat lyssavirus (LLEBV). However, due to few characterized cases and difficulties in investigating their circulation in the reservoir, we know very little about their eco-pathology, hampering the evaluation of the risk associated with their presence.

For example, the distribution of bat lyssaviruses is currently defined based on the sporadic cases reported by countries, without considering the level and characteristics of the local system of passive surveillance in bats.[3] Of note, the circulation of WCBV in Europe is currently supported by its detection in a cat in Italy,[2] while no cases have been confirmed in the Schreibers' bent-winged bat (*Miniopterus schreibersii*), the species where

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the virus was first described more than twenty years ago in Russia.[4] As a matter of fact, *Miniopterus schreibersii* is mostly cave-dwelling so that individuals are rarely found by humans to be included in the surveillance for lyssaviruses, in Italy or elsewhere in Europe.[3] In 2020, the detection of neutralizing antibodies from Italian colonies supported the circulation of WCBV in these bats.[2] Pitfalls and challenges in serological diagnostic approaches are the possible cross-reactivity between antigenically related viruses and the high level of uncertainties about the antibody mediated immune response to lyssaviruses in bats, including the percentage of individuals seroconverting upon exposure and the duration of detectable antibodies.[3,5] However, the case of WCBV in Italy stresses the importance of active surveillance to unravel the ecology of bat-lyssaviruses, with serological analysis providing higher sensitivity for the detection compared to molecular screening, due to the low prevalence of viruses.[3,6–8]

Bat-lyssaviruses mostly have a precise ecological niche in one or a few related bat species that act as a reservoir and source for spillover events in humans or other animals.[1,9] This provides a unique opportunity to target active surveillance and investigate proactively the geographic distribution of viruses, assuming it may mirror the one of the reservoirs. Generally, each bat species is more susceptible to the infection and prone to the development of symptoms by one specific lyssavirus species. However, the divergent WCBV and LLEBV have been both found in *Miniopterus schreibersii*, in Russia and in Western Europe (Spain and France) respectively.[4,10,11] While the possible segregation of viruses in different subpopulations of *Miniopterus schreibersii* could explain this conundrum,[12] the lack of readable genetic structure and the migratory habits of this bat[13] suggest these viruses most likely co-circulate. Up to date, their actual distribution remains unexplored.

Understanding the likelihood of developing a clinical disease upon exposure is another feature that is highly relevant for risk assessment but is mostly unknown for the lyssaviruses circulating in European bats. While the vast majority of rabies cases are caused by RABV, all eighteen lyssaviruses recognized by the International Committee on the Taxonomy of Viruses (ICTV) are considered able to cause rabies.[12,14] However, only eight species were actually associated with spillover cases in humans or non-flying mammals so far.[1,2,12] In addition, experimental studies support different pathogenicity of lyssaviruses in animal models upon intramuscular (IM) infection, used as a proxy for natural infection through bites.[14–18] Regarding WCBV and LLEBV, previous studies describe high pathogenicity and lethality upon intracerebral (IC) infection.[4,11,19] In addition, the spillover of WCBV in the Italian cat confirms its ability to cause a lethal neurological disease mirroring rabies,[2] corroborating previous experimental evidence in Syrian hamsters.[20] On the other hand, the pathogenicity of LLEBV upon exposure is currently unknown, with no report of natural spill over infection or experimental inoculation different from the IC route.

While the burden associated with the circulation of bat lyssaviruses remains different from RABV, the occurrence of cases in humans and domestic animals highlights the need to enhance our knowledge of the distribution and pathogenicity of these viruses in order to provide a realistic risk assessment. Defining the actual risk related to the circulation of WCBV and LLEBV is particularly relevant because the antigenic distance to RABV leads to the inefficacy of vaccines and monoclonal antibodies available for human and veterinary use as pre and post-exposure prophylaxis.[19,20] In addition, clearer information about their presence and danger is crucial to mitigate the potential conflict between humans and *Miniopterus schreibersii*, whose populations are declining across Europe and require strict protection.[21] This study aims at filling some gaps around WCBV and LLEBV, by investigating their geographical distribution and pathogenicity upon IM infection.

RESULTS

Serological evidence for the circulation of Lleida bat lyssavirus and West Caucasian bat virus in the reservoir host

To define the distribution of WCBV and LLEBV across Europe, we performed a serological screening of *Miniopterus schreibersii*, from which both viruses were first isolated. We investigated 29 roosts from eight countries, stretching over 2.000 km between Bulgaria and Spain, performing 38 sampling campaigns (Figure 1A). By using archive samples, our analyses covered 12 years' time, from 2012 to 2023. Investigations date back to 2012 in Spain, 2015 in France, 2020 in Italy, and 2021 in Hungary, while other countries were investigated once in September 2022 (Table S1).

Figures 1A–1D shows a summary of serological results (extended data in Table S1). We screened 645 sera, including 571 against WCBV, 608 against LLEBV, and 538 against both viruses. Serological analyses showed the presence of neutralizing antibodies for at least one target lyssavirus in 179 (27.8%) individuals, including 142 against WCBV (24.9%) and 65 against LLEBV (10.7%). For both viruses, the oldest positive samples were collected from Spain in 2012.

We detected WCBV in 31/37 (83.8%) occasions, 24/28 (85.7%) sampling sites, and at least once in all countries investigated (Figure 1B). We confirmed maintenance across Europe and within five out of seven sites investigated more than once, with the longest occurrence detected in France and Italy where the virus was detected three years apart. On the other hand, we failed to detect the virus in 2022 in a Spanish colony testing positive 10 years before. The percentage of positivity dropped in the Hungarian colony from 13.3% to 3.7% between May and September 2021, yielding negative results the following year. It is worth highlighting that all sampling campaigns showing no evidence of WCBV included less than 20 samples. Out of 571 samples, 222 (38.9%) were diluted at 1:30. due to the low amount of serum available, potentially missing the detection of sera showing neutralization titers $\geq$1.48 and $\leq$1.95 LogD50/mL, which accounted for 62% of positive individuals (88/142) (Figure 1C).

LLEBV was detected in 15/38 (39.5%) occasions from 11/29 (37.9%) roosts from six countries (Figure 1B). No animals showed antibodies against LLEBV in Slovakia and Romania, where we tested an average of 16 individuals in one and three

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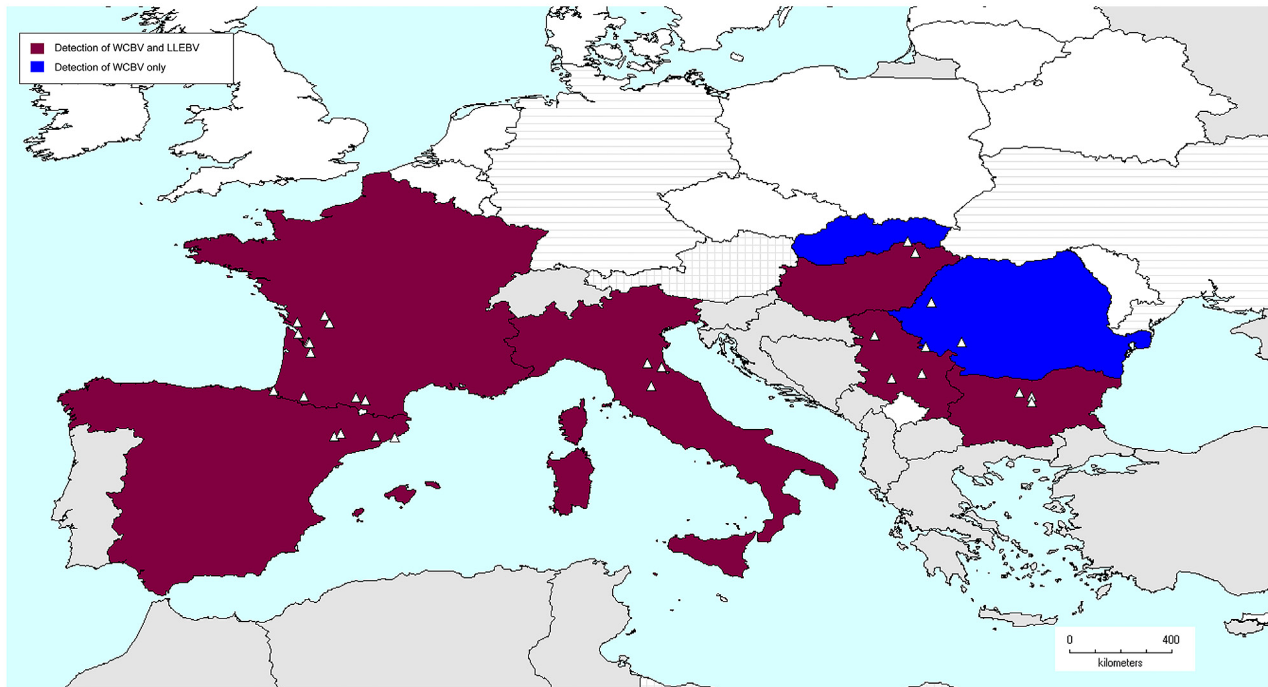

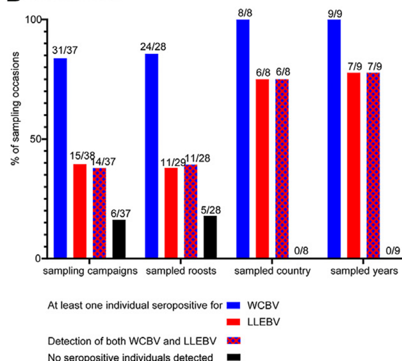

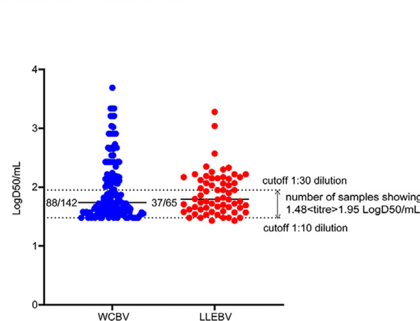

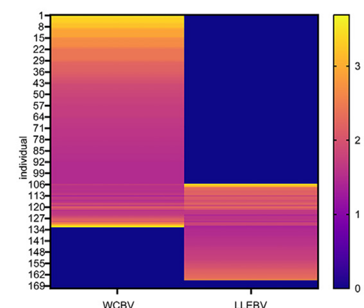

Figure 1. Detection of LLEBV and WCBV in the putative reservoir *Miniopterus schreibersii*

(A) Occurrence of WCBV and LLEBV in European *Miniopterus schreibersii*. Investigated countries are colored in blue if only WCBV was detected at least once or in burgundy if we also confirmed the presence of LLEBV; screened roosts are shown as triangles. Other countries across the distribution of *Miniopterus schreibersii* are colored in gray, with lines or crosses where the species is, respectively, extinct or likely extinct (https://www.iucnredlist.org/species/81633057/195856522, accessed 30-11-23).

(B) Detection rate of WCBV and LLEBV in sampled campaigns, roosts, countries, and years. Bars indicate the % of occasions in which WCBV (blue), LLEBV (red) or both (blue/red pattern) were confirmed by at least one sero-positive individual. Black bars indicate the % of occasions showing no evidence for any virus. Exact numbers are shown above bars.

(C) Distribution of antibody titers against WCBV (blue) and LLEBV (red). The two scattered lines show minimum titers detected using the starting dilution of 1:30 and 1:10. The value within lines indicates the number of sera showing titer 1.48–1.95 LogD50/mL that can only be detected using dilution ≤1:10; d. Heatmap showing the pattern of cross-reactivity of 170 bat sera analyzed for WCBV and LLEBV. Colors shade from blue to yellow upon increasing antibody titers.

sites respectively. The longitudinal sampling of colonies allowed the detection of the maintenance of the virus across three following years in Italy. On the other hand, Spanish, French and Hungarian colonies that were investigated more than once failed to detect the neutralization of LLEBV in at least one occasion. As for WCBV, the sample size of negative campaigns was low (≤25 individuals). Dilution 1:30 was used for 345/608 (56.7%) samples, missing antibodies falling below 1.95 LogD50/mL, estimated around 57% based on overall results (37/65 positive individuals) (Figure 1C).

In 14 out of 37 occasions and five countries, we detected together individuals reacting against WCBV and LLEBV as potential evidence for their co-circulation. Among the 538/645 samples tested in parallel, only 28 (5.2%) showed cross-neutralization,

suggesting the two viral species might have different ecology, for example in terms of seasonality. These individuals showed similar affinity with the two viruses, with main antibody titers ranging from 1.48 to 3.69 (mean 1.97) LogD50/mL against WCBV, and from 1.48 to 3.28 (mean 1.72) LogD50/mL against LLEBV (Figure 1D). On the other hand, six campaigns performed in five roosts from four countries yielded negative results for either virus (Figure 1B).

Statistical analyses showed that the percentage of serological positivity was associated with the year of sampling ($p < 0.001$ for WCBV and LLEBV), but not with the sex or the season (Table S2).

Pathogenicity of Lleida bat lyssavirus and West Caucasian bat virus in comparison with rabies virus in rodent animal models

Syrian hamsters

In order to investigate the risk associated with the circulation of WCBV and LLEBV in the reservoir, we studied their pathogenicity in comparison with RABV after the IM injection of Syrian hamsters. Hamsters exhibited different clinical outcomes and trends of mortality based on the challenge virus and the viral dose (Figure 2; Tables S3–S5 for statistical metrics and raw data). Overall, survival curves for each group showed significant differences from the overall population, supporting the hypothesis that tested viruses have different pathogenicity in the tested animal model ($p < 0.0001$, Log rank (Mantel-Cox) test). However, when comparing WCBV with classical RABV we found no significant difference in survival rates regardless of the infection dose ($p = 0.064$ Log rank (Mantel-Cox) test). The mortality rate of RABV was 100% after the incubation of eight days at a dose of 5.16 LogD50/200μL and ranging 8–15 days lowering the dose to 3.16 LogD50/200μL, with significant differences detected between doses in terms of lethality ($p = 0.014$) and incubation period ($p = 0.048$). All animals infected with WCBV showed similar survival curves regardless of the dose ($p = 0.280$) and died between 7 and 15 and between 8 and 12 days post infection, respectively at 5.16 and 4.16 LogD50/200μL (Figure 2A), with no difference detected between groups ($p = 0.659$). The incubation period did not significantly differ between the two viral species ($p = 0.226$).

Both RABV and WCBV induced a clinical disease compatible with rabies, whose signs started up to two days before reaching the experimental and humanitarian endpoint. Weight loss ranging from 3% to 32% was common as an early sign of infection and was more severe in animals infected with WCBV (mean loss of 7.7% against 5.4% for RABV) albeit this difference between the viruses was not found to be significant ($p = 0.272$) (Figure 2B). The clinical presentation of RABV-infected hamsters included depression, tremors, and progressive paralysis. The infection of hamsters with WCBV was characterized by self-injuries, hyperactivity, incoordination, hypersensitivity to stimuli, vocalizations, and aggressive behaviors (Figure 2C).

All 10 animals infected with 3.75 LogD50/200μL of LLEBV in two independent experiments survived up to 21 and 40 days following the challenge. The 100% survival rate against the 0% of the other tested viruses well explained the overall statistical difference between groups determined in our experiment, with $p < 0.0001$ confirmed in pairwise comparisons between LLEBV and WCBV and between LLEBV and RABV using the Log rank (Mantel-Cox) test, despite the viral dose. LLEBV-infected hamsters appeared clinically healthy throughout the experiment, exception made for sporadic days of depression and self-injuries (Figure 2C). These animals either maintained or increased their weight throughout the experiments, with a mean gain of 6.1% that showed no significant difference compared to the trend observed in non-infected animals ($p = 0.077$) (Figure 2B). This data differed from WCBV and RABV that induced significantly higher weight loss compared to the mock group ($p = 0.049$ and $p = 0.011$ respectively).

We confirmed the infection in the brain with both WCBV and RABV by immunofluorescence (Figure 2D) and real time RT-PCR (Figure 3A and Table S5). Depending on the virus and the dose, RNA reached titers ranging from $1.56*10^5$ to $8.65*10^6$ (mean $2.02*10^6$) copies/μL (Figure 3A, circles). These values fall within the range of $5.01*10^5$–$4.09*10^7$ viral copies/μL identified for WCBV and RABV after the IC infection of mice (Table S6). We were able to quantify mRNA in all positive samples, with titers ranging from $7.42*10^3$ to $6.93*10^5$ (mean $2.36*10^5$) copies/μL, indicating active viral replication (Figure 3A, triangles). The amount of virus found within positive brains greatly differed between experimental groups in terms of total RNA ($p = 0.043$) but not mRNA ($p = 0.101$). In particular, for total RNA we found no difference in the final titer reached by WCBV and RABV injected at the same dose of 5.16 LogD50/200μL ($p = 0.426$) or by RABV injected at scalar doses ($p = 0.095$). On the other hand, the titer significantly decreased when lowering the dose of WCBV ($p = 0.004$). Conversely, the amount of mRNA was similar between groups but significantly decreased when lowering the dose of RABV ($p = 0.032$). The level of mRNA reached in hamsters upon IM infection was significantly lower compared to the one reached after the IC inoculation of mice ($p < 0.0001$) (Table S6).

Surprisingly, one individual infected with LLEBV, euthanized at 40 days, showed evidence for antigen accumulation within the brain (Figure 2D), and $3.64*10^4$ and $9.12*10^2$ viral copies/μL detected for total RNA and mRNA respectively (Table S5). No pairwise comparisons were possible to investigate results from the LLEBV infected group, due to the presence of a single positive data. However, viral RNA and mRNA quantified around two (RNA) and three (mRNA) logarithms lower compared to mean values obtained for other viruses after IM infection (Figures 3A and Table S5), or three (RNA) and four (mRNA) logarithms lower compared to mean values obtained for LLEBV after the IC infection of mice (Table S6). The positive individual seemed to be healthy during the whole experiment, except for a few days when it showed tremors/depression and a decrease in body weight dropping at −19 g between 24 and 28 days post infection (Figure 2C, solid red line). The animal recovered in the following days and was euthanized at the end of the 40-day experiment, weighing only 2 g less than its initial weight (Table S5).

Weekly serological evaluation showed that all lyssaviruses activate the humoral response by day 7 in hamsters regardless of the development of disease, with a significant difference between groups at day 7 ($p = 0.001$) (Figure 3B and Table S4). Individuals infected with LLEBV developed neutralizing antibodies by day seven, exception made from a single individual testing positive by day 14. Titers increased significantly after infection

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Figure 2. Pathogenicity of WCBV and LLEBV in hamsters upon IM infection, in comparison with RABV

(A) Survival curves of hamsters upon the IM inoculation of study viruses over time. Data are represented as mean +/− standard error. Lines are colored in red for LLEBV, blue for WCBV, and black for RABV. Solid versus dashed and gradient shades are used to show decreasing doses of viruses. Bars of standard error are shown for each virus following the same color scheme. The figure has been modified using Adobe Illustrator to correct the superimposition of lines, which hampered the correct evaluation of single curves.

(B) Daily weight's changes presented as mean values for each group +/− standard deviation. Lines are colored as for Figure 2A to identify different viruses and doses. Standard deviation of groups is shown through pointed lines above and below the mean. The solid red line represents the single individual that turned out positive for LLEBV in the brain.

(C) Heatmap showing clinical signs, graded based on the severity toward the blue for the paralytic form and toward the yellow for the furious form.

(D) Representative immunofluorescence staining for the brains of animals infected with RABV and WCBV. While most LLEBV showed negative staining, the figure includes the single brain showing the presence of LLEBV antigen. Images show lyssaviruses nucleoprotein (green) and the DAPI nuclear staining (blue). Scale bar = 25 μm.

from 1.72 to 2.08 (mean 1.86) LogD50/mL at day seven to 2.17–3.57 (mean 2.70) LogD50/mL at day 40 (p = 0.001). A similar trend was observed for WCBV, which induced immunity regardless of the dose in all but one individual by day seven, with titers of neutralizing antibodies ranging from 1.69 to 2.2 (mean 1.8) LogD50/mL. All individuals died before further time points could be investigated (Table S5). While the response of hamsters to the high dose of RABV (2.18–5.55; mean 3.35 LogD50/mL) was significantly higher compared to a similar dose of WCBV (p = 0.036), only one animal developed neutralizing antibodies upon infection with a lower dose of RABV, raising from 1.97 at day seven to 3.17 LogD50/mL at day 14. None of the hamsters showed cross neutralization toward heterologous lyssaviruses (Figure 3C).

Mice

All viruses used in the study were produced and titrated using the IC inoculation of 2 days and 3 weeks mice respectively, confirming their ability to cause a lethal disease using this infection route and animal model. The lethality of undiluted batches was 100% regardless the mice age and clinical signs included tremors, circling, vocalization, and running backward (Figure 4A). The lethality of all viruses decreased upon the dilution of viruses for titration, highlighting a lower lethal dose for the batch of LLEBV used in this study (Figure 4A).

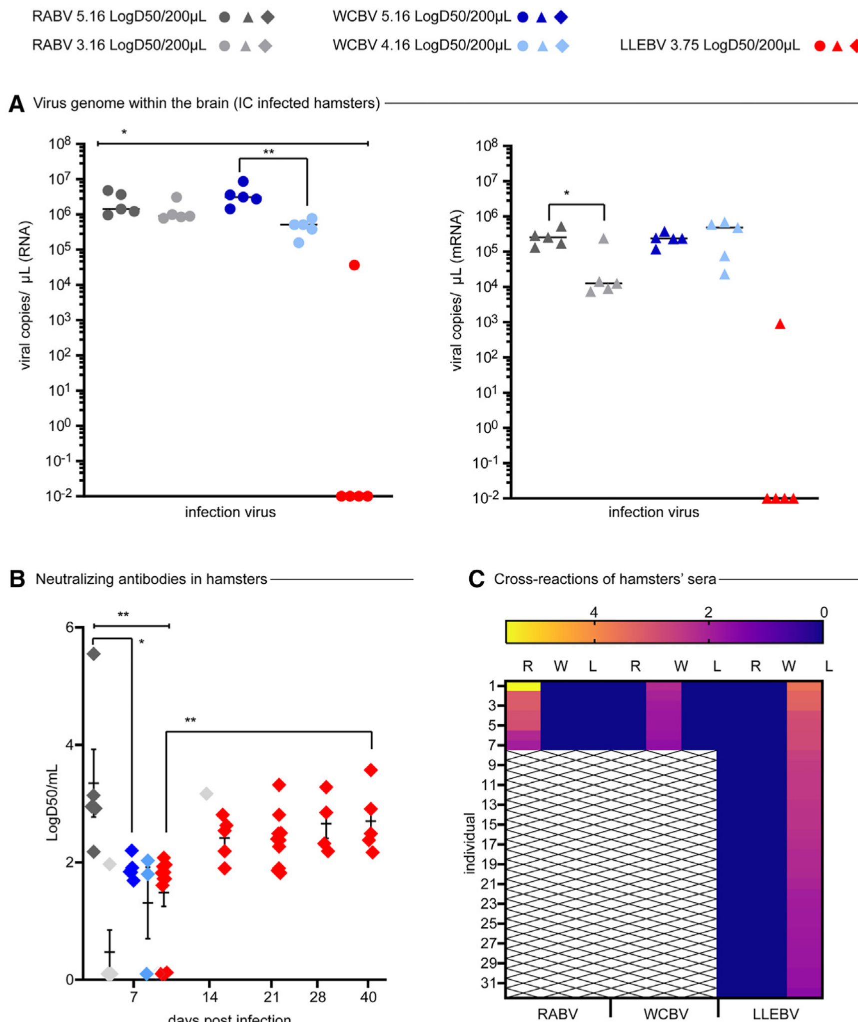

Figure 3. Molecular and serological results from infected hamsters

(A) Viral RNA (circles)/mRNA (triangles) detected in brain samples of experimental hamsters. The panel show individual data and the mean value for each group.

(B) Neutralizing antibodies, determined at day 7 for all viruses and weekly up to 40 days for individuals surviving the infection with LLEBV. The panel shows individual data plus the mean value and standard deviation for each group. Panels a and b use the same coloring scheme of Figure 2 to identify different viruses and doses.

(C) Heatmap showing the cross-reactivity pattern of experimental sera from hamsters with target viruses. Asterisks indicate groups or couples showing significant pairwise differences at 0.05–0.01 (*), 0.001–0.01 (**) or <0.001 (***). See Table S4 for statistics.

Comparing the disease course in mice upon IC infection with undiluted viruses, animals died between 8 and 9 days when inoculated IC at three weeks of age, with no difference determined between viruses (p = 0.728, Log rank (Mantel-Cox) test) (Figure 4B and Table S3). In new-borns, the course of the disease was generally faster but differed between viruses (p = 0.007, Log rank (Mantel-Cox) test) (Table S3), with LLEBV killing all individuals between day 4 and 6, WCBV between day 6 and 8 and RABV showing the highest variance, killing mice between 5 and 16 days (Figure 4B). We quantified the virus in 3-week old mice infected for the determination of mouse intracerebral lethal dose (MICLD50) (Figure 4C). We found significant overall differences in the final RNA (p = 0.0004) but, interestingly, similar values around the mean of $4.8*10^6$ for mRNA, with no differences detected between different viruses and inoculation doses (p = 0.154), mirroring what we then had seen in hamsters (Table S4).

Following the evidence that LLEBV was not able to induce a lethal infection of hamsters following IM injection, we used the murine model to discriminate between the resistance of the hamster model and the inadequacy of the infection route. Our data confirmed that LLEBV and RABV have different lethality upon IM infection in rodents (p = 0.001) (Figure 5A and Table S3). Indeed, LLEBV failed to kill 10 mice older than 8 weeks within 21 days, following IM infection with 3.45 LogD50/100µL, in comparison to RABV that induced lethal infection in 80% (4/5) mice at 3.86 LogD50/100µL. Similarly to what we observed in hamsters, one mouse infected with LLEBV tested positive upon immunofluorescence (Figure 5B) and molecular tests (Figure 5C), despite looking healthy. In particular, the animal showed $1.06*10^6$ viral copies of total RNA/µL, which is around two logarithms higher than the infected hamster and only one logarithm lower compared to IC infection. Regarding mRNA, we found $7.60*10^4$ viral copies, which supports active replication even if the actual value is around two logarithms lower compared to IC infection (Tables S6 and S7). As for total RNA, mRNA was two logarithms higher compared to the infected hamster. These data support the hypothesis that LLEBV has impaired competence in producing infection in both hamsters and mice upon IM inoculation compared to WCBV and RABV.

DISCUSSION

While most of Europe is currently free from the endemic circulation of RABV, results from active and passive surveillance provide evidence for the circulation of other lyssaviruses in bats. While EBLV-1 and EBLV-2 have been largely addressed in terms of ecology and pathology, the reporting of other lyssavirus species remains sporadic.[1] Among these, LLEBV and WCBV have been described only twice each,[2,4,10,11] hampering a solid definition of the reservoir hosts, and no studies investigated their distribution or epidemiology. Our data provide evidence for a wide distribution of WCBV and LLEBV across the geographical range

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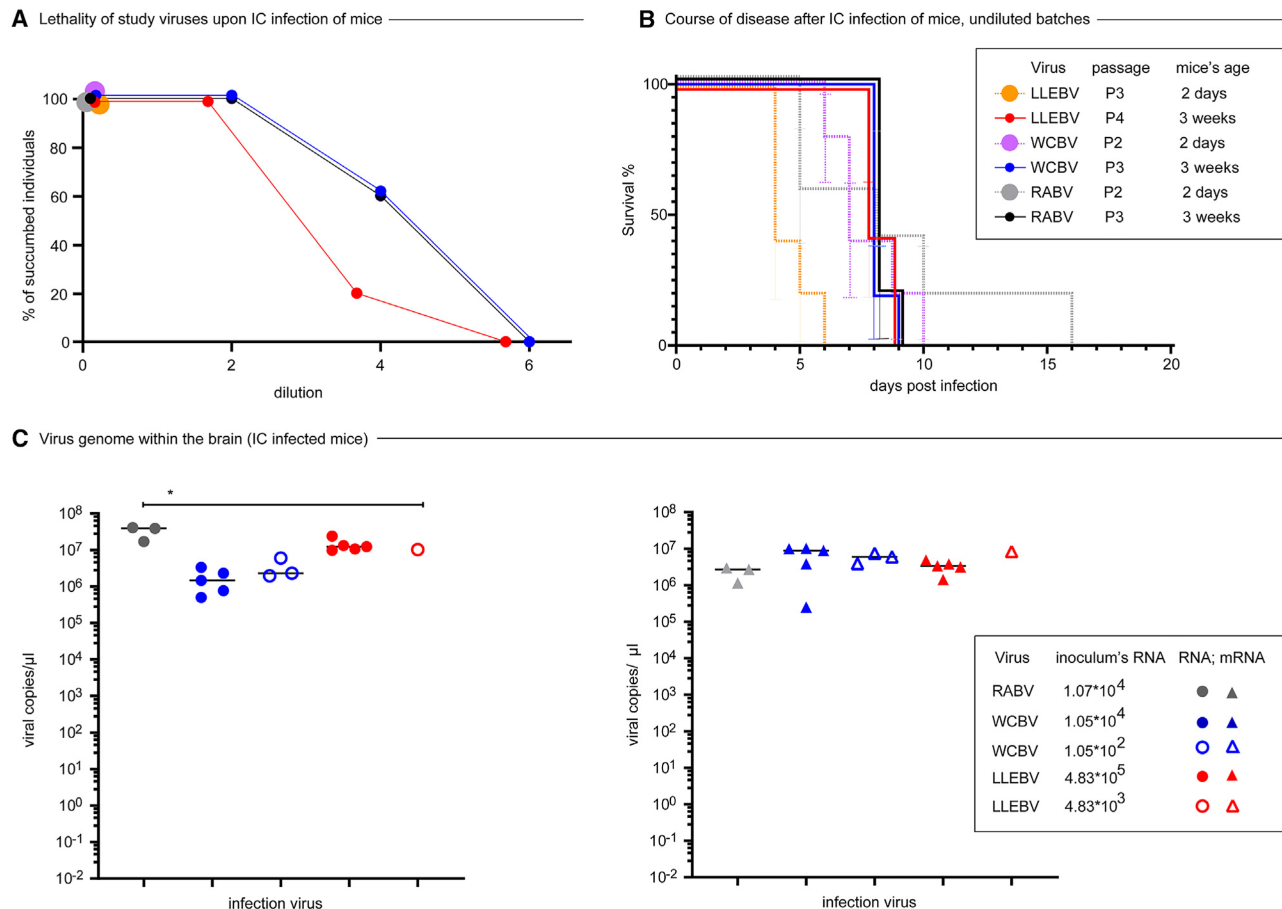

Figure 4. Pathogenicity of WCBV and LLEBV in mice upon IC infection, in comparison with RABV

(A) Lethality of viruses was determined on 2 days mice (undiluted batches only, used for viral production) or 3 weeks mice (serial dilutions, used for viruses' titration). We used red/orange for LLEBV, blue/purple for WCBV, and black/gray for RABV, according to the age of the host, as shown within the figure.

(B) Survival curves of mice upon IC inoculation over time of undiluted batches. Data are represented as mean +/− standard error. Lines are solid for 3-week mice, dashed for 2-day mice, and colored as for Figures 4A and as shown within the figure. Bars of standard error is shown for each virus following the same color scheme. The figure has been modified using Adobe Illustrator to correct the superimposition of lines, which hampered the correct evaluation of single curves.

(C) Viral RNA (circles)/mRNA (triangles) detected in brain samples of IC infected mice, using full colored figures for higher dose and empty ones for lower doses. The panel shows individual data and the mean value for each group. *group showing significant pairwise difference at 0.05–0.01. See Table S4 for statistics.

of *Miniopterus schreibersii,* the bat in which both viruses were first identified.[4,10] In several countries, our finding precedes the identification of viruses themselves, showing the advantage of serological approaches in the surveillance of wildlife diseases.[3,22] Positivity rates for WCBV and LLEBV ranged from 3% to 67% (mean 23%) for both viruses, similar to what had been described in Italy for EBLV-1 sero-surveillance.[3] Previous studies showed inter- and intra-annual variations in lyssavirus sero-prevalence that are crucial to predict peaks that increase the likelihood for viral spillover.[5,23] Unfortunately, the vast heterogeneity of samples used in this study prevented us from investigating the epidemiology of LLEBV and WCBV, including their prevalence based on the geographical area, the year or the season. Indeed, the sampling effort differed across sites in terms of samplings (from a single investigation to yearly samplings across three years), sample size for each campaign (1–60 individuals tested) and amount of sera available for the study, directly affecting the starting dilution and thus the sensitivity of the test (LogD50/mL $\geq$ 1.48 and $\geq$1.95 for dilutions 1:10 or 1:30 respectively). While this heterogeneity of data hampered significant comparisons between the prevalence across sampling campaigns, statistical analyses on single individuals confirmed a significant variation of sero-positivity between years that has been largely described for lyssaviruses in European bats.[5,23,24] On the other hand, the season did not seem to affect our serological results. This finding is consistent with data obtained for EBLV-1 in *Eptesicus serotinus*[8] and for EBLV-2 in *Myotis daubentonii*[23]*,* but differs from previous investigations on *Myotis myotis,* whose lyssavirus followed a strict seasonal pattern, with peaks of amplification after the birth pulse that were consistent across several years.[5] We suggest that longitudinal data from single colonies are still to be collected in order to highlight, if present, a similar signal for WCBV and LLEBV. We also investigated the physiological traits of individuals and

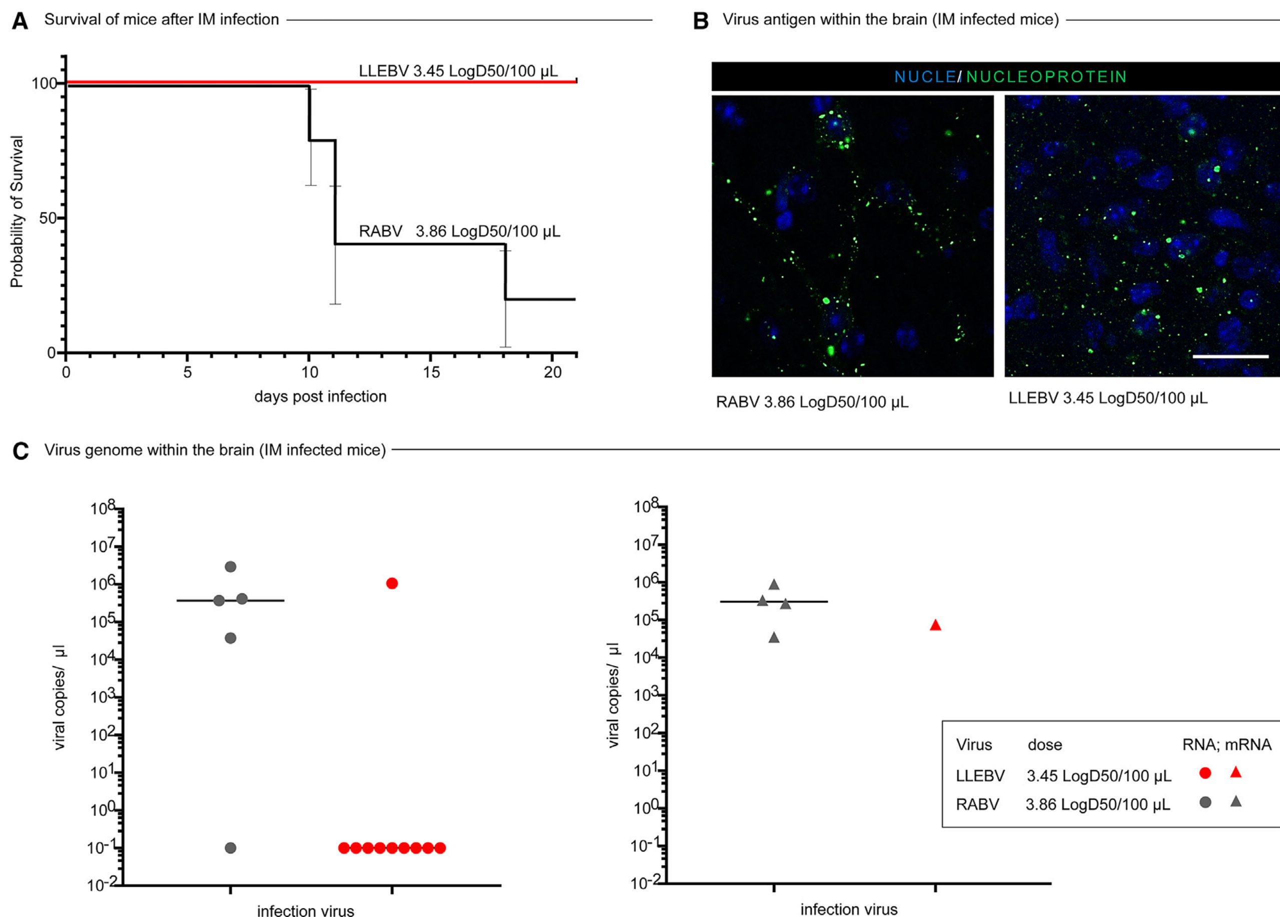

Figure 5. Pathogenicity of WCBV and LLEBV in mice upon IM infection, in comparison with RABV

(A) Survival curves of mice upon IM inoculation over time. Data are represented as mean +/− standard error. Lines are colored in red for LLEBV and black for RABV. Bars of standard error are shown for each virus following the same color scheme. The figure has been modified using Adobe Illustrator to correct the superimposition of lines, which hampered the correct evaluation of single curves.

(B) Representative IF staining for RABV infected animals and the single individual positive for LLEBV. Images show lyssaviruses nucleoprotein (green) and the DAPI nuclear staining (blue). Scale bar = 25 µm.

(C) Viral RNA (circles)/mRNA (triangles) detected in brain samples of mice infected IM. The panel shows individual data and the mean value for each group.

found that sex had no influence over the positivity for lyssavirus, mirroring other studies performed in bats.[5,25] Unfortunately, our dataset lacked several information on animal age and reproductive status, which could not be included as a variable in our model. Based on our serological data we found how a sample size lower than 30 strongly reduced the sensitivity of the screening. Similarly, our data suggest that dilution higher than 1:10 might hamper the detection of weak samples. Therefore, they should both be avoided.

Even if we cannot exclude the role of co-roosting species, which were not tested in this study, the wide distribution and the sustained detection over the years and roosts of *Miniopterus schreibersii* further support its role as reservoir host for both viruses, challenging previous assumptions that single hosts could serve as ecological niches for single lyssaviruses.[12] In addition, the fact that on several occasions we detected positivity against WCBV and LLEBV in different individuals from the same colony or even in the same individual provides a strong indication for their co-circulation. Interestingly, some of the Spanish archive samples used in this study were previously found to neutralize EBLV-1 as well,[26] showing how *Miniopterus schreibersii* is at least exposed and potentially infected. Due to the low amount of serum available, our study did not investigate further the role of *Miniopterus schreibersii* in the ecology of EBLV-1 that remains a topic of interest, especially considering that the majority of cases have been associated with *Eptesicus serotinus/isabellinus* and the virus never identified by molecular tests in the Schreibers' bent winged bat.[1] Indeed our findings pose several open questions, i.e., on the effective host tropism of EBLV-1 and on the role of concurrent infections in the outcome of a lyssavirus infection in bats. Our experimental data and previous investigations found no evidence for serological cross-neutralization between viruses of different phylogroups or between LLEBV and WCBV.[27] Nevertheless, we cannot exclude that a previous immunity, i.e., cell-mediated, has an influence on the disease outcome in single individuals

and more broadly on the dynamics of virus circulation within host populations exposed to different lysaviruses, such as the Schreibers' bent winged bat.

All members of the genus *Lyssavirus* are generally considered able to cause rabies in at least one mammal species, but the pathogenicity of WCBV and LLEBV remained unexplored. The description of a spillover case for WCBV in a cat proved this viral species can be lethal, but the likelihood of developing disease after exposure was not fully assessed[9,12,14]. In this study, we confirmed the ability of WCBV and LLEBV to cause a lethal disease upon IC infection described upon isolation.[4,11,19] While there is no information regarding lethality and clinical signs of WCBV upon IC infection, our data are consistent with previous reports for LLEBV, showing hyperactivity, body spasm and 100% lethality.[19] Results from RT-qPCR support viral replication at this level, with titers mirroring RABV for both viruses, particularly in terms of mRNA.

Conversely, our data support that WCBV and LLEBV have different pathogenicity upon IM infection, which was used as a proxy for bites as the most likely route for natural exposure to bat viruses.[14] In our experiment, all hamsters succumbed after the IM inoculation of WCBV at MICDL50 as low as 4.16 LogD50/200µL. These results corroborate previous evidence extrapolated from experimental studies on the vaccination coverage, where 7/9 and 9/9 unvaccinated controls died in two separate experiments after the IM injection of the Russian isolate at the dose of 5.7 Log50/50µL MICLD50.[20] Based on our data, WCBV mirrors the lethality of RABV, albeit causing a very different clinical presentation. In particular, WCBV disease in hamsters did not cause the paralysis seen for RABV, but resembled most closely the furious presentation generally described for rabies in humans and carnivores, including the cat infection with WCBV.[2] Regardless of its high lethality after IC infection, LLEBV was not able to kill any hamster or mouse inoculated IM. Previous experiments describe a certain variability in rodent's survival depending on the viral species, strain, dose, and infection route.[15–18] Most lyssaviruses show IC lethality up to 100% decreasing for IM infection. The complete IM a-pathogenicity was rarely reported and was most often associated with low doses or several passages in cell lines before use.[15,16,18] Experimental settings also recorded IM reduced lethality at very high doses of Duvenhage virus (DUVV) and Lagos bat virus (LBV). While appearing counterintuitive, this trend can be explained by the production of defective viral particles and/or by increasing chances of triggering effective immune responses.[18,28] In our case, even if we used LLEBV at its highest concentration, the dose fell within the range causing rabies in our setting after infection with WCBV and RABV. These data suggest that LLEBV has an impaired ability to cause rabies compared to WCBV and RABV after IM injection.

One hamster and one mouse were positive for the presence of LLEBV in the brain at 40 and 21 days post infection using two distinct approaches regardless of being clinically healthy. Considering that a progressive weight loss preceded of a few days the onset of neurological signs after the infection with WCBV and RABV, the fact that the LLEBV-positive hamster lost 19 g between 24 and 28 days of infection and recovered afterward does not support a prodromal phase of the disease, but may rather suggest infection and subsequent recovery. Of note, we did not find any positivity among the five hamsters euthanized healthy at 21 days post infection. Unfortunately, we did not investigate whether animals still harbored the virus at a peripheral level, which could indicate this virus has a longer latency compared to other members of the genus. However, previous studies showed that the incubation of lyssaviruses in rodents ranges 7–20 days depending more on the inoculation route and the dose rather than the viral species under study.[17,18,29] While we cannot discriminate between the control of viral replication and the clearance of the virus based on current data, this result is rather surprising for lyssaviruses that are generally considered invariably fatal once they reach the brain. Viral copies were lower compared to WCBV and RABV when infected IM and compared to titers reached by LLEBV upon IC infection. However, the detected amount of virus was consistent with values associated with symptomatic rabies in mice infected with RABV and LBV in other studies.[16] In addition, the identification of viral mRNA supports active replication in the brain. In humans, the few cases described in the literature of survival after the onset of symptoms report detectable neutralizing antibodies in the serum and the cerebrospinal fluid and suggest a critical role of an early humoral immune response.[30] It is then crucial to consider that discrepancies between the IC and IM routes reported in this study might be influenced by the difference in age of the two experimental groups, with the young age of IC infected animals likely resulting in an immature immune system compared to adult animals infected IM.

Experimental data using rodents and bats are controversial in defining any correlation between the survival and development of neutralizing antibodies[15,16,28]. Our data found no correlation between the development of antibodies and the likelihood of survival. However, they suggest that increasing doses result in higher stimulation for the humoral immunity, with 100% of animals sero-converting upon infection with the highest doses used for RABV, WCBV, and LLEBV, decreasing to 20% and 40% lowering the dose of RABV and WCBV respectively. This data highlights likely variations in the immune response of accidental hosts upon different exposure to the viruses. As a matter of fact, neutralizing antibodies against lyssaviruses, rabies included, have been found in humans, domestic animals, and wildlife unvaccinated and apparently healthy, challenging previous dogma about rabies as being inevitably fatal.[31] The finding of neutralizing antibodies in individuals that have been naturally exposed to lyssaviruses would be indeed consistent with the wide sero-positivity found in bats in this study as well as in other programs of active surveillance.[3,6–8] However, the correlation between sero-positivity and infection dynamics in bats is still puzzling, with open questions regarding the infection status, the survival rates, and the susceptibility to the reinfection of sero-positive individuals other than the immunological history of negative ones.[5,6,22,32]

In conclusion, our study shows that two divergent lyssaviruses are widely circulating in southern Europe in the bat *Miniopterus schreibersii*, highlighting a plausible risk for public health. While the highest likelihood of transmission is associated with professionals handling bats, the recent identification of *Miniopterus*

schreibersii in urban settings, likely due to the reduction of suitable natural habitats, translates into increasing risk also for citizen and domestic animals that need to be further addressed.[2] Based on experimental data obtained in mice, both viruses can cause rabies. However, only WCBV was able to induce the disease after intramuscular infection, mirroring the lethality of RABV. On the other hand, this infection route seems not to be effective for LLEBV. More studies in different classes of ages, using different isolates, doses and injection routes are needed to define whether this virus has impaired neuro-invasiveness or a peculiar eco-pathology, which is crucial to manage correctly risks related to its circulation within the reservoir.

Limitations of the study

Our data suggesting that LLEBV has impaired ability in causing rabies compared to WCBV and RABV after IM injection have been obtained only using the French isolate for the challenge. Because previous studies showed marked variations in the lethality of LBV strains, ranging between 0% and 60%,[17] we acknowledge the limitation of using a single isolate. However, differences in the pathogenicity between LBV's strains can be explained by its genetic variability and its association with different bat species in Africa,[33] differently from the high nucleotide identity of 99.7% shared by the two described strains of LLEBV, suggesting a similar behavior upon infection.[11]

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Stefania Leopardi (sleopardi@izsvenezie.it).

Materials availability

Remaining aliquots of viruses, animal samples, and sera used and produced for this study can be available on request but require a completed materials transfer agreement.

Data and code availability

- Data: The summary of bat serological data, characteristics of sampling campaigns, row data for animal experiments, and all details of statistical analyses are reported as supplementary tables (Tables S1–S7).
- Code: Codes for statistical analyses performed in R are available as Supplemental data.
- All reagents, cell lines, and animal lines used in the study are described in the key resources table. The key resources table also provides GenBank accession number of viral strains used for serology and experimental infections and sequences of all primers used in the study.

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AUTHOR CONTRIBUTIONS

Conceptualization: S.L and P.D.B. Data curation: S.L., L.D., J.S.C., Á.Á., F.F., M.C., P.D., and G.K. Formal analysis: S.L., F.F., M.C., P.D., L.K., M.F.P., and B.Z. Funding acquisition: S.L., L.D., J.S.C., D.P., G.K., and P.D.B. Investigation (fieldwork and animal experiments) S.L., J.S.C., Á.Á., B.B, S.B, I.B, M.C., P.D., H.D., F.F., M.L., M.L.R., D.P., D.S., Zs.L., T.G., G.K., and P.D.B. Methodology: S.L., M.C., P.D., and P.D.B. Project administration: P.D.B. Resources: L.D., B.B, S.B, I.B, H.B., H.D., J.S.C., D.P., E.R., D.S., G.K., and P.D.B. Supervision: P.D.B. Validation: P.D., M.C., L.D., and B.Z. Visualization: S.L., M.C., and M.F.P. Writing – original draft: S.L. Writing – review and editing: L.D., J.S.C., H.B., M.C., G.K., and P.D.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Rabies Nucleocapsid Conjugate; lyophilized, adsorbed Used for immunofluorescence and serological tests	Bio-Rad	cat# 3572112
FITC Anti-Rabies Monoclonal Globulin Used for serological tests	Fujirebio	cat# 800-092; RRID: AB_2802166
Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 Used for immunofluorescence tests	Life Technologies	cat# A32731; RRID: AB_2633280
Bacterial and virus strains		
West Caucasian bat virus, strain WCBV_Arezzo_Italy_cat_2020 Host source: cat P2 produced by IC infection of 2 days old mice Used for experiments *in vivo*	Istituto Zooprofilattico Sperimentale delle Venezie	GenBank: MZ501949
West Caucasian bat virus, strain WCBV_Arezzo_Italy_cat_2020 Host source: cat cell adapted Used for serological tests	Istituto Zooprofilattico Sperimentale delle Venezie	GenBank: MZ501949
West Caucasian bat virus, strain RV3384 Host source: *Miniopterus schreibersii* cell adapted Used for serological tests	Insitute Pasteur	GenBank: NC_025377
Rabies virus, strain RABV_Italy_exZanzibar_human_2020 Host source: human P2 produced by IC infection of 2 days old mice Used for experiments *in vivo*	Istituto Zooprofilattico Sperimentale delle Venezie	GenBank: OQ787037
Rabies virus, strain RABV_Italy_exZanzibar_human_2020 Host source: human cell adapted Used for serological tests	Istituto Zooprofilattico Sperimentale delle Venezie	GenBank: OQ787037
Lleida bat virus, strain 131989 Host source: *Miniopterus schreibersii* P3 produced by IC infection of 2 days old mice Used for experiments *in vivo*	Istituto Zooprofilattico Sperimentale delle Venezie. Derived from the amplification of P2, provided by Nancy Laboratory for Rabies and Wildlife, Anses	GenBank: MG983927
Lleida bat virus, strain 131989 Host source: *Miniopterus schreibersii* cell adapted Used for serological tests	Nancy Laboratory for Rabies and Wildlife, Anses	GenBank: MG983927
Lleida bat virus, strain RV3208 Host source: *Miniopterus schreibersii* cell adapted Used for serological tests	Insitute Pasteur	GenBank: NC_031955
Biological samples		
Sera from *Miniopterus schreibersii* collected in Italy in the framework of active surveillance for lyssaviruses	Istituto Zooprofilattico Sperimentale delle Venezie	original samples
Sera from *Miniopterus schreibersii* collected in Hungary, Bulgaria, Serbia, Slovakia, Romania in the framework of active surveillance for Lloviu virus	Univeristy of Pecs	original samples

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sera from *Miniopterus schreibersii* collected in Spain and France in the framework of active surveillance for lyssaviruses	Insitut Pasteur	original samples
rabbit Hyperimmune sera raised against WCBV, strain WCBV_Arezzo_Italy_cat_2020	Istituto Zooprofilattico Sperimentale delle Venezie	EVAg Ref-SKU: 025A-04869
rabbit hyperimmune sera raised against Lleida bat virus, strain 131989	Istituto Zooprofilattico Sperimentale delle Venezie	produced in house code 22R/4376
Chemicals, peptides, and recombinant proteins		
RNA*later*	Thermo Fisher	cat# AM7021
10% neutral-buffered formalin	Bio-optica	cat# 05-01005Q
Critical commercial assays		
Rneasy Mini kit	qiagen	cat# 74104
AgPath-ID™ One-Step RT-PCR Reagents	Life technologies	cat# 4387424
SuperScript II Reverse Transcriptase	Thermo Fisher	cat# 18064014
SsOFast EvaGreen Supermix	Bio-Rad	cat# 172-5201
Experimental models: Cell lines		
BSR (BHK derived cell line)	Institut Pasteur	
Experimental models: Organisms/strains		
LVG Golden Syrian Hamster	Charles River	Strain Code 049
Oligonucleotides		
Probe LN34	https://doi.org/10.1371/journal.pone.0197074	(FAM) AACACCYCTACAATGGA (BHQ1)
LLEBV Sense primer	https://doi.org/10.3390/v15030680	ACGCTTAACAGCTAAAAACYAGAAG
LLEBV Antisense primer	https://doi.org/10.3390/v15030680	CTGGATATTTGTAYTCATAYTGATC
WCBV Sense primer	https://doi.org/10.3390/v13102064	ACGCTTAACAACAAAATCTTATAAG
WCBV Antisense primer	https://doi.org/10.3390/v13102064	CAGGATATTTATATTCATACTGGTC
RABV Sense primer	https://doi.org/10.1371/journal.pone.0197074	ACGCTTAACAACAAAATCADAGAAG
RABV Antisense primer	newly designed	CAGGRTAYTTGTACTCATATTGATC
Probe β-actin	https://doi.org/10.1371/journal.pone.0197074	(FAM) TCCACCTTCCAGCAG ATGTGGATCA (BHQ1)
β-actin Sense primer	https://doi.org/10.1371/journal.pone.0197074	CGATGAAGATCAAGATCATTGC
β-actin Antisense primer	https://doi.org/10.1371/journal.pone.0197074	AAGCATTTGCGGTGGAC
Software and algorithms		
LAS AF software	Leica Application Suite	version 2.7.3.9723
ImageJ	open source Java-based image processing program	
Graphpad Prism	dotmatics	version 10
R	free software	
Biorender	Academic licence online	

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bats

We performed live sampling of bats, targeting individuals belonging to the species *Miniopterus schreibersii,* considered as the most likely reservoir of WCBV and LLEBV. Individuals were randomly captured from their roost and were sampled regardless of their sex,

age and physiological status. However, we deliberately avoided captures during the delicate period ranging one month before and one month after parturition, so that the sampled animals were all at least one month old, weaned and able to fly. Because most of the roosts were maternity colonies, the majority of animals older than one year, classified as adults, were females.

The capture of animals was authorized by national authorities in derogation to Council Directive 92/43/EEC (Supplementary method). Authorizations are the following: 38025 of 13/08/2020 and 6831 of 15/02/2021 granted by the Italian Ministry of Environment and Energy Security; C692660703 from the Departmental Direction of Population Protection, Rhone, France; 2012 from the Department of Climate Action, Food and Rural Agenda of the Generalitat de Catalunya and Directorate General of Natural Environment (SF/151, SF/555, SF/749, SF/123, SF/402, SF/158, SF/0218, SF/0080/2019, SF/0026/2020, SF/0082/21, SF/0096/22, SF/0061/23, SF/0015/24) and Forestry Management of the Government of the Balearic Islands; 2012 to 2021 (CAP 26/2012, CAP 39/2013, ESP 22/2014, CEP 33/2015, CEP 12/2016, CEP 17/2017, CEP 07/2018, CEP 12/2019, CEP 31/2020, CEP 11/2020); Bulgaria: 828/19.03.2020 and 912/02.11.2021 issued by the Bulgarian Ministry of Environment; Serbia: 353-01-3405/2021-04 issued by the Ministry of Environmental Protection, Serbia; Hungary: PE-KTFO/4384-24/2018 and PE-KTFO/1402-11/2022 issued by the National Department of Environmental Protection, Nature Conservation and Waste Management.

In the period between the capture and the release of bats, which lasted for a maximum of 4 h, we maintained them either individually in cotton bags or in groups in soft ventilated bags intended for the transport of pets. We monitored bats for signs of discomfort throughout the whole procedure and provided water after sampling whenever appropriate.

Golden syrian hamster

We used golden Syrian hamsters (*Mesocricetus auratus*) as the main animal model for this project, using individuals bred *in house* at the Istituto Zooprofilattico Sperimentale delle Venezie (Breeding Authorization n°2020/0095 granted 05/08/2020). Parents were purchased from Charles River and belonged to the LVG strain which has been outbred in the company since 1951. Before the study, a veterinarian inspected and tested all the animals for the infection with SARS-CoV-2 and considered them healthy. Animals were then transferred to the experimental facilities under conditions of biosafety level 2 (BSL2) or 3 (BSL3) - depending on the virus - and acclimatized 7 days before starting the experiment. In order to avoid confounding factors related with sex-driven difference in the immune response against viral infection,[34] experimental groups only included females. Because we cannot exclude the influence of sex on the results of this study, this choice should be considered as a limitation to the generalizability of the research. We performed intramuscular (IM) infections at 8–10 weeks of age and a mean weight of 142 g (110–185). Individuals were randomly allocated in their experimental group and were housed in individual cages, using BCU-2 Rat Sealed Negative Pressure and IVC Rat 1800 (Allentown Inc) within BSL3 and BSL2 facilities respectively.

The study design, animal care and procedures followed national and international regulations on the welfare of laboratory animals and were performed in compliance with directive 2010/63/EU (Permit 344/2021-PR, released by the Italian Ministry of Health).

Mice

We performed intracranial (IC) infection of mice to produce and titrate the viral batches used for the pathogenicity study. In particular, we injected two-day old animals for the production and three-week old individuals for the titration. In addition, we employed two experimental groups of mice that were infected IM with RABV and LLEBV between eight and ten weeks of age. All mice were CD1 Swiss Outbred mice (albino) reared *in house* at the Istituto Zooprofilattico Sperimentale delle Venezie (Breeding Authorization n°2011/9980 granted 20/10/2011). Individuals were injected in the experimental facilities under conditions of biosafety level 2 (BSL2) or 3 (BSL3) depending on the virus, housing them in the same systems employed for hamsters. In the case of weaned animals, we employed both females and males that were housed together in groups of five for a maximum of 28 days, after a minimum of two acclimatization days. To perform the infection of two-day old mice, we transferred pregnant females around one week before the expected delivery. Two days after birth, we injected all new-borns of the resulting litters, leaving them altogether with their mothers for 28 days, after which we euthanized the surviving individuals and the adult females.

All procedures were performed in compliance with Directive 2010/63/EU (permit 1006/2016-PR and 491/2020-PR, released by the Italian Ministry of Health).

Microbe strains

In this study, we employed either lyssaviruses produced *in vivo* to investigate their pathogenicity or amplified *in vitro* to perform serological tests. Details of all viruses and strains used are listed in the key resources table.

Viruses produced *in vivo* included one strain of LLEBV (GenBank: MG983927) isolated from *Miniopterus schreibersii* in France in 2018, one strain of WCBV (GenBank: MZ501949) isolated after spilling over a cat in Italy in 2020 and one strain of RABV (Cosmopolitan lineage; GenBank: OQ787037) identified in 2019 in Italy from a human case acquired abroad. The National Reference Center for Rabies isolated the human strain from a salivary sample of the patient after molecular diagnosis to allow whole genome sequencing of the infecting strain.

We produced and titrated the inoculum for the pathogenicity study following the same procedure for all viruses. Viruses were isolated from field samples and passaged once (WCBV and RABV) or twice (LLEBV) through the intracranial (IC) inoculation of two-day-old mice. We performed Whole Genome Sequencing (WGS) to exclude mutation from the original strain and confirmed in all cases 100% identity with the sequences already deposited in GenBank. Brains from the last passage were pooled together, diluted 1:5 in

PBS and homogenated using the TissueLyser (Qiagen). After clarification trough centrifugation, we titrated the final viral batches using the mouse intracranial lethal dose 50 (MICLD50) in three weeks old mice. For each virus, we used three groups of five animals infected IC with 30 μL of viral suspension in scalar dilutions, and determined the MICLD50 over 28 days of experiment using the Reed-Muench formula, expressed as LogD50/mL[1,2].

For serological analyses, we used RABV, WCBV and LLEBV viruses adapted and titrated in BSR cell lines. In Italy, serological tests were performed using the same strains of viruses employed for pathogenicity studies, in France analyses were performed on bat sera using the Russian strain of WCBV (GenBank: NC_025377) and the Spanish strain of LLEBV (GenBank: NC_031955).

Cell lines

We used BSR cell line for serological analyses. BSR are a clone of Baby Hamster Kidney (BHK-21) produced and provided by Pasteur Institute. We tested cells for mycoplasma contamination upon the production of the batch and when put in line for serial amplification.

METHOD DETAILS

Active surveillance in bats

For this study, we captured and sampled individuals of *Miniopterus schreibersii* across its distribution in Europe (Table S1).

In Italy, we sampled three roosts between 2020-23, including the longitudinal screening of two urban sites with increased encroachment with human and domestic animals, as described elsewhere.[2] In all cases, we performed captures within roosts during the day using hand-nets, and placed the animals individually in cotton bags or in groups in soft ventilated bags intended for the transport of pets. We inspected all individuals to confirm the species and to determine their age, sex and physiological status. We collected 20–90 μL of blood from the saphenous (interfemoral) vein under physical restraint, using a 300 μL insulin syringe with a 30 G needle. When sampling was carried out within the roost, we immediately released individuals after the procedure. Otherwise, we placed animals in groups in the ventilated bag and released them back to the roost within a few hours.

We exploited surveillance plans for other diseases and used archive samples to investigate the circulation of divergent lyssaviruses outside Italy. In Hungary, Bulgaria, Serbia, Slovakia and Romania we exploited samples collected in the framework of Lloviu surveillance between 2021-22.[35] We included samples from three campaigns of one site in Hungary and from a single screening of one to three sites in the other countries. Samples from Spain included 24 sera newly collected in 2022 from two sites and archived sera collected in 2012 from three roosts for the surveillance of European bat lyssavirus 1 (EBLV-1), as described elsewhere.[26] A single roost included samples from 2012 to 2022. Finally, we analyzed 59 archived samples collected for conservation purposes between 2015-20 from 11 roosts in France.

Regardless of the country and the team involved in the fieldwork, bats were captured using the best practices for the species, according to the feature of each roost, avoiding hibernation and breeding. Field campaigns were approved by the ethical committees of the University of Barcelona, the University of Pecs, the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), and by the Société Française pour l'Etude et la Protection des Mammifères.

Experimental infection with hamsters and mice

In order to study the pathogenicity of WCBV and LLEBV in comparison with RABV, we performed experimental infections of Syrian hamsters and mice.

We infected adult female Syrian hamsters IM with 200 μL of viral inoculum divided between the gastrocnemius muscles. The study involved six experimental groups of five individuals that were randomly allocated to each experimental group, for a total of 30 hamsters. We infected three groups with high doses of each virus, consisting of MICDL50 5.86 LogD50/mL (neat-dose 5.16 LogD50/200 μL) for WCBV and RABV and of 4.45 LogD50/mL (neat-dose 3.75 LogD50/200 μL) for LLEBV, corresponding to the undiluted original batch. As we surprisingly obtained no mortality with LLEBV, we repeated the experiment with lower doses of other viruses for a more accurate comparison, including WCBV at 4.16 and RABV at 3.16 LogD50/200 μL, and repeated the original experiment with LLEBV, prolonged to 40 days of infection to exclude longer incubation. After infection, we monitored the animals twice a day to spot early signs of distress or rabies-specific signs, including the collection of daily weights. All the staff involved in the experimental study was aware of the group allocation of animals, including the infection virus and dose. In case of clinical rabies, when reaching our humanitarian threshold or after 21 days of experiment, we euthanized the animals using a CO[2] chamber and collected the brain in two identical parts that were fixed in 10% neutral-buffered formalin and collected RNA later (Thermo Fisher) for immunofluorescence and molecular analyses, respectively. We tested all the individuals for the development of neutralizing antibodies upon death or at days 7, 14, 21, 28 and 40, drawing blood from the gingival vein under general anesthesia using a 1mL insulin syringe with a 25 G needle.[36]

We used 15 mice to test the hypothesis that the lack of pathogenicity was related to the host species rather than to the infection route. We performed an IM infection of 10 mice with 100 μL of LLEBV 4.45 LogD50/mL (neat-dose 3.45 LogD50/100 μL). As comparison, we infected five mice with 100 μL of RABV 4.86 LogD50/mL (neat-dose 3.86 LogD50/100 μL). Individual mice were randomly allocated to each experimental group. Upon the development of clinical signs or 21 days post infection, we euthanized animals and confirmed the infection using molecular techniques from brains. No blood was collected from the mice to investigate their immune response to the infection.

Serology

We assessed the presence of virus-neutralizing antibodies in bats and hamsters using the Rapid Fluorescent Foci Inhibition Test (RFFIT) modified as described,[3,37] using as challenge virus either WCBV, LLEBV or field RABV (see key resources table). Sera were diluted 1:10 (bats and hamsters) or 1:30 (bats only) in culture medium according to the available volume, and analyzed on a 3-fold dilution using BSR cells and commercial antibodies (Bio-Rad, Hercules, USA or Fujirebio, Tokyo, Japan). Titers were calculated through the Reed-Muench method and expressed as LogD50/mL. Samples were clearly identified as belonging to one of the six experimental groups using laboratory codes. However, the lab technician was not aware of the infection virus and dose of each group when analyses were performed. Bat samples were analyzed either by the IZSVe (Italy) or the Institut Pasteur, Paris (France), after standardization using rabbit hyper-immune reference sera raised against WCBV (Italian strain) and LLEBV (French strain).

Viral detection

We investigated the presence of viruses within the brain of hamsters and mice infected IM using molecular and immunological techniques, to detect viral RNA and the antigens, respectively. In addition, we investigated the amplification of viruses in the brain by performing a molecular quantification of viruses in the original batches used for the infection and in three-five representative brains of three-week-old mice infected IC for the titration of viruses, including individuals infected with similar copies of viruses.

For the molecular analyses of experimental animals, we stored half brain collected in RNAlater for 24–48 h at +4°C after which the medium was removed and organs were frozen at −80°C until further analysis. We homogenated half brains using the TissueLyser (Qiagen, Hilden, Germany) in RLT lysis buffer and extracted genetic material using the Rneasy Mini kit (Qiagen). We first investigated the presence of total virus RNA using a one-step real-time RT-PCR (rRT-PCR), using AgPath-ID One-Step RT-PCR Reagents (Thermo Fisher, Waltham, USA) and in house developed virus-specific primers that matched the sequences of study viruses to achieve a perfect primer-template complementarity.[38] We determined RNA copies using a standard curve of *in vitro* transcribed RNA. We tested the quality of the sample and the successful extraction by detection of the housekeeping gene β-actin.[38,39] We further investigated the amount of viral mRNA using a two-step real-time RT-PCR as a measure for viral replication. We retrotranscribed into cDNA all the samples, starting from an equal amount of RNA using oligo(dT)-primed SuperScript II Reverse Transcriptase (Thermo Fisher), and performed virus-specific real-time PCRs using primers designed *in house* within the nucleoprotein and SsoFast EvaGreen supermixes (Bio-Rad). We tested all the samples in triplicates against standard curves. Oligonucleotide and probes sequences are listed in the key resources table. Samples were not blinded for these analyses.

We used immunofluorescence to investigate the presence of antigens within 4 μm-thick sections of formalin-fixed paraffin-embedded half-brains of experimental animals. For antigen visualization, we used the FITC-conjugated anti-nucleoprotein (Bio-Rad) for RABV and WCBV and performed indirect IF for LLEBV using a rabbit hyper-immune serum produced and purified *in house* and an anti-rabbit secondary antibody conjugated with an Alexa 488 fluorophore. Images were acquired with Leica TCS SP8 confocal microscope equipped with a CCD camera at 63X enlargement using LAS AF 2.7.3.9723 software and analyzed using ImageJ. Samples were not blinded for these analyses.

QUANTIFICATION AND STATISTICAL ANALYSIS

Active surveillance in bats

The bat sample size included in the study was pre-determined only for the Italian campaigns, where it ranged between 30 and 60 individuals, considered effective in detecting at least one positive sample with 95% confidence based on population size and assuming prevalence as low as 10 and 5% respectively. For other countries, we employed all leftover samples available from other surveillance plans or from the archive of the institutes involved. This resulted in relevant differences in the sampling effort across sites in terms of samplings (from a single investigation to yearly samplings over a period of three years), sample size for each campaign (1–60 individuals tested) and amount of sera available for the study. In turn, we avoided the statistical comparison between the sero-prevalence determined at each location during each sampling time.

On the other hand, we tested the chance for single individuals to be sero-positive for either WCBV or LLEBV upon different variables, by performing a multivariate mixed-effect logistic regression analysis (GLMM) using the glmer function of the lmer4 package in R 4.1.2; odd ratios and respective confidence intervals were computed using the function confint() developed in R (Supplemental data). We used as predictor variables the sex of animals, the year of sampling, and the season; we used sampling country as a random effect. To define the season, we divided the year in four main periods: the "aggregation" phases of individuals in colonies (i.e., aggregation; set between March and April), the "late pregnancy" of females (i.e., pregnancy; May-June), the presence of "pups" (i.e., weaning; July-August) and the "dispersion" of colonies (i.e., swarming; September-October).

Experimental infection with hamsters and mice

We analyzed the lethality of viruses upon IM infection using Kaplan-Meier survival analysis, using Prism version 10 (GraphPad Software, La Jolla, USA) to display the survival proportions of infected hamsters at each time point. We used the Log rank (Mantel-Cox) test to compare curves among different groups. We compared all survival curves at once and performed a subset of comparisons between pairs of infection viruses. We assessed the statistical difference in the incubation period (excluding the group of LLEBV

including only animals euthanized at the end of the experiment), the titer of the virus detected within the brain and the antibody titer upon death of the experimental groups. We compared all groups at once using one way ANOVA with Tukey's multiple comparison test, and pairs of infection viruses and doses, using the Mann-Whitney test. We assessed differences between viruses in the final weight loss induced, using one way ANOVA with Dunnett's test to compare each group to the mock control group. Furthermore, we performed paired comparisons between groups infected with each virus and the non-infected control group, using the Mann-Whitney test. Statistical analyses of experimental data were performed using Prism.