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1 First case of lethal encephalitis in Western Europe due to European bat lyssavirus type 1

2 Béatrice Regnault<sup>1,2</sup>, Bruno Evrard<sup>3,4</sup>, Isabelle Plu<sup>5,6</sup>, Laurent Dacheux<sup>7</sup>, Eric Troadec<sup>1,2</sup> Pascal Cozette<sup>7</sup>,

3 Delphine Chrétien<sup>1,2</sup>, Mathilde Duchesne<sup>8</sup>, Jean-Michel Vallat<sup>9</sup>, Anne Jamet<sup>10</sup>, Marianne Leruez<sup>10</sup>,

4 Philippe Pérot<sup>1,2</sup>, Hervé Bourhy<sup>7</sup>, Marc Eloit<sup>1,2,11\*</sup>, Danielle Seilhean<sup>5,7\*</sup>

5

6 1 Pathogen Discovery Laboratory, Institut Pasteur, Paris, France

7 2 The OIE Collaborating Center for the detection and identification in humans of emerging animal  
8 pathogens, Institut Pasteur, Paris, France)

9 3 Medical-Surgical Intensive Care Unit, Dupuytren University Hospital, Limoges, France

10 4 Inserm CIC 1435 and UMR 1092, Dupuytren Teaching Hospital, Limoges, France

11 5 Sorbonne Université, Brain Institute (ICM; INSERM, UMRS 1127; CNRS, UMR 7225), Paris, France

12 6 Département de Neuropathologie Raymond Escourolle, AP-HP-Sorbonne, Groupe Hospitalier Pitié-  
13 Salpêtrière, Paris, France

14 7 Lyssavirus Epidemiology and Neuropathology Unit, National Reference Centre for Rabies, WHO  
15 Collaborative Centre for Reference and Research on Rabies, Institut Pasteur, Paris, France.

16 8 Pathology Department, Dupuytren University Hospital, Limoges, France

17 9 Neurology Department, Dupuytren University Hospital, Limoges, France

18 10 Department of Clinical Microbiology, Necker Enfants-Malades Hospital, AP-HP, Centre Université  
19 de Paris, Paris, France

20 11 Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort, France

21

22

23 \*Danielle Seilhan and Marc Eloit are co-senior authors

24 Corresponding author : Marc Eloit, Pathogen Discovery Laboratory, Institut Pasteur, 28 rue du Dr Roux,  
25 75015 Paris, France marc.eloit@pasteur.fr

26 Alternate corresponding author: Danielle Seilhean, Département de Neuropathologie Raymond  
27 Escourolle, AP-HP-Sorbonne, Groupe Hospitalier Pitié-Salpêtrière, Paris, France,  
28 [danielle.seilhean@aphp.fr](mailto:danielle.seilhean@aphp.fr)

29

## 30 Keywords

31 Encephalitis; European bat lyssavirus type 1; Lyssavirus; Metatranscriptomics; Next Generation  
32 Sequencing

## 33 Running title

34 EBLV-1a Lyssavirus lethal Encephalitis

## 35 Keypoints

36 European Bat Lyssavirus type 1 is prevalent in bats in Europe. We present the first human case  
37 identified in Western Europe. Unless vaccinated against rabies, the public should avoid direct  
38 interactions with bats.

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43 conceptualisation and methodology: BE, AJ, ML, PP, ME, DS

44 investigation: BR, BE, IP, LD, ET, PC, DC, MD, JMV, PP, ME, DS

45 supervision: DS, ME

46 writing original draft ME, BR, BE, DS

47 review and editing : BR, BE, IP, LD, AJ, PP, HB

48

49

## 50 Abstract

### 51 Background

52 Inaccurate diagnosis of encephalitis is a major issue as immunosuppressive treatments can be  
53 deleterious in case of viral infection. The European bat lyssavirus type 1, a virus related to rabies virus,  
54 is endemic in European bats. No human case has yet been reported in Western Europe. A 59 year-old  
55 patient without specific past medical history died from encephalitis. A colony of bats lived in an  
56 outbuilding of his house. No diagnosis was made using standard procedures.

### 57 Methods.

58 We used a Next Generation Sequencing (NGS) based transcriptomic protocol to search for pathogens  
59 in autopsy samples (meninges and brain frontal lobe). Results were confirmed by PCR and by antibody  
60 testing in serum. Immunocytochemistry was used to characterize inflammatory cells and viral antigens in  
61 brain lesions. Cells and mice were inoculated with brain extracts for virus isolation.

### 62 Results

63 The patient's brain lesions were severe and diffuse in white and gray matter. Perivascular inflammatory  
64 infiltrates were abundant and rich in plasma cells. NGS identified European bat lyssavirus type 1a in  
65 brain, which was confirmed by PCR. A high titer of neutralizing antibodies was found in serum. No viral  
66 antigen was detected and the virus could not be isolated by cell culture or by mouse inoculation.

### 67 Conclusions

68 The patient died from European bat lyssavirus type 1a infection. NGS was key to identifying this  
69 unexpected viral etiology in an epidemiological context that did not suggest rabies. People exposed to  
70 bats should be strongly advised to be vaccinated with rabies vaccines, which are effective against EBLV-  
71 1.

72

73

## 74 Introduction

75 Encephalitides are defined by inflammation of the brain parenchyma and are mostly of infectious  
76 origin, but the underlying pathogen frequently remains unknown[1]. In addition, inflammation may be  
77 associated with autoimmunity (e.g. autoantibodies against neurotransmitter receptors[2]), which is, in  
78 some cases, due to an infectious trigger[3]. *In vivo*, blood and cerebrospinal fluid (CSF) based assays,  
79 although used routinely for diagnostic purpose, are also associated with poor performance. Although  
80 difficult to access, cerebral tissue is the most informative sample in encephalitides[4], while CSF and  
81 other clinical samples are surrogates, more readily available in clinical practice. Here, using agnostic  
82 metatranscriptomics procedures, we analyzed the brain tissue from a fatal case of encephalitis with  
83 unusual pathological features for which a viral etiology was suspected. We found evidence of infection  
84 by the European bat lyssavirus type 1a (EBLV-1a), a rabies-related lyssavirus, which widely circulates  
85 in some European bats (mainly the common serotine or *Eptesicus serotinus*). This case demonstrates  
86 that the risk of bat lyssavirus transmission to human exists in Europe.

## 87 Methods

### 88 Case history

89 A 59-year-old patient without specific past medical history was hospitalized in July 2019 for temporal  
90 headache, phono- and photophobia, vomiting, dysarthria and swallowing disorders, which had started  
91 3 days earlier. His family reported that he had had asthenia without weight loss or anorexia for a few  
92 months. No travel outside France was noted in the previous two years. The patient lived in central  
93 France in a rural wooded area. After the cause of the death was established, the family reported that  
94 in an outbuilding of his house lived a colony of bats. At admission, physical examination showed mild  
95 fever (38.5°C), paralysis of the oculomotor (III), left abducens (VI) and facial (VII) nerves, salivary stasis  
96 and dysphonia. No other sensory or motor deficit was noticed. The main clinical and non-clinical

97 findings are described in Table 1. A few hours after admission, the patient developed an acute coma  
98 for which mechanical ventilation was introduced. Suspecting meningoencephalitis, a treatment with  
99 acyclovir (800 mg/8h) and amoxicillin (12g/24h) was started. In the two following days, a complete  
100 paralysis of all cranial nerves was evidenced associated with the progression to a non-reactive coma  
101 and clinical seizures. The electroencephalogram (EEG) showed no status epilepticus under  
102 leviteracetam but a poor cerebral activity. Because immune encephalitis was suspected, treatment  
103 with intravenous immunoglobulins (32 g/day for 5 days) and steroids (methylprednisolone 1 g/day for  
104 3 days, followed by 2 mg/kg/day) were started. Serial EEG monitoring showed a severe worsening of  
105 electrical activity over a few weeks with diffuse theta frequency and delta frequency organization,  
106 potentially compatible with necrotizing encephalitis. MRI findings at day 5 and 23 post-admission in  
107 the ICU are described in Fig. 1. ICU evolution was characterized by a non-reactive coma without any  
108 improvement despite absence of any status epilepticus. Fifty-one days after admission, in the face of  
109 persistent coma, the patient died following withdrawal of life-sustaining therapy and an autopsy was  
110 performed in accordance with current French regulation, after obtaining informed consent from the  
111 patient's family and query of the National Refusal Register (Agence de la Biomedecine).

112 A full description of pathological findings is presented as Supplementary data. In brief, the brain was  
113 edematous. At microscopic examination, the lesions were diffuse, observed in both white and grey  
114 matter, and in all of the samples. They were characterized by abundant inflammatory perivascular  
115 infiltrates, astrocytic gliosis and microglial activation, in contrast with limited necrosis [Fig. 2]. The  
116 perivascular infiltrates were rich in plasma cells [Fig. 2]. The cerebellum was severely damaged with  
117 loss of Purkinje cells. The roots of the cranial pairs were atrophic.

118 Despite the absence of a cytopathogenic effect, including Negri's bodies, the lesions were not  
119 evocative of autoimmune encephalitis, but rather suggested a viral origin due to the involvement of  
120 both white and grey matter and the spread of plasma cell infiltrates. For this reason, frozen samples  
121 were sent to the collaborative diagnostic platform of the Institut Pasteur and Necker Hospital for  
122 further analysis aimed at identifying a pathogenic agent.

123 **Post Autopsy Diagnostic Methods** We used Next Generation Sequencing (NGS)-based transcriptomic  
124 analysis to search for viral transcripts in meninges and brain frontal lobe. This technique is agnostic  
125 and provides sequences of both the human transcriptome and pathogen, the latter of which that may  
126 be identified by similarity with known microbes. RNA from meninges and brain frontal lobe samples  
127 were extracted and used to construct a cDNA library and sequenced using the Illumina technology.  
128 Sequences were aligned after translation against a comprehensive viral protein reference database  
129 (details in supplementary data). We completed the viral genome gaps by applying standard molecular  
130 biology methods (supplementary data). Once the pathogen identified, we used previously described  
131 PCRs (Supplementary data and [5–7]) to confirm the presence of virus from necropsy samples and CSF.  
132 Classical reference methods for the *post-mortem* diagnosis of rabies were performed on non-fixed  
133 meninges and brain samples. They included antigen detection by the direct fluorescent antibody test  
134 (FAT)[8]) and virus isolation with the rapid tissue culture infection test (RTCIT)[9] and the mouse  
135 inoculation test (MIT)[10]. Electronic microscopy was conducted as described in Supplementary data.  
136 Detection of neutralizing antibody against rabies virus (RABV, CVS strain) and against rabies-related  
137 EBLV-1 lyssavirus (EBLV-1b, strain 8918FRA) was performed by a modified rapid fluorescent focus  
138 inhibition test (RFFIT)[11] on serum and CSF samples.

## 139 Results

140 We found sequences in meninges and brain frontal lobe samples covering the quasi-full length of a  
141 genome related to EBLV-1a lyssavirus (Genbank accession number MW551946). Based on  
142 phylogenetic analysis (Fig. 3), the closest relatives were other EBLV-1a strains from bats identified in  
143 France[12]. Based on PCR results, the viral load was around 500-fold higher in the frontal lobe than in  
144 meninges. In contrast, no viral RNA was detected in CSF sampled on the day of hospitalization and 34  
145 days later. Immunochemical analyses of multiple paraffin-embedded brain samples using a polyclonal  
146 antiserum detecting EBLV-1a was negative, but electronic microscopy suggested the presence of  
147 rhabdovirus particles (Supplementary data). Infectious virus was not detected from these samples. A



148 high titer (1/1200) of antibodies neutralizing EBLV-1 virus was detected in the serum sampled the day  
149 of hospitalization, but not detected in the CSF samples collected the same day and 34 days later. These  
150 serum antibodies were unable to neutralize RABV.

## 151 Discussion

152 Members of the *Lyssavirus* genus, *Rhabdoviridae* family, are the causative agents of rabies, a  
153 progressive viral encephalitis transmitted from infected animals by bites and scratches, or licking of  
154 mucous or wounds. Rabies virus (RABV) transmitted by non-flying terrestrial mammals has now  
155 disappeared from Western and central Europe following efficient control measures. Various other  
156 lyssaviruses, however, still circulate in bats. To date, five different lyssaviruses have been identified in  
157 Western Europe: (EBLV-1) hosted by the common serotine (*Eptesicus serotinus*), European bat  
158 lyssavirus type 2 (EBLV-2) in Daubenton's (*Myotis daubentonii*) and pond (*Myotis dasycneme*) bats, the  
159 Bokeloh bat lyssavirus (BBLV) in Natterer's bat (*Myotis nattereri*)[13], the Lleida bat lyssavirus (LLEBV)  
160 in bent-winged bats (*Miniopterus schreibersii*) (review in[14]) and the Kotalahti bat lyssavirus (KBLV)  
161 in Brandt's bat (*Myotis brandtii*) in Finland [15]. EBLV-1 is widely spread in European and North African  
162 bat colonies[12,16,17]. Its importance in public health has been raised, as spillovers of EBLV-1 to  
163 terrestrial mammals have been described in sheep in Denmark[18], in two cats in France[19], in a stone  
164 marten (*Martes foina*) in Germany[20] and in Egyptian fruit bats (*Roussetus aegyptiacus*)[21]. To date,  
165 however, only a single human EBLV-1 case (Yuli strain, EBLV-1a lineage[22], occurring in Belgorod,  
166 Russia in 1985, has been described, though an earlier one was suspected but not characterized in  
167 Voroshilovgrad, Ukraine in 1977[23]. Therefore, the patient described in the present study represents  
168 the first identified in Western Europe, more than 35 years after the previous one(s) in Eastern Europe.  
169 We provide the first neuropathological description of the panencephalitis caused by the virus,  
170 characterized by dense plasmocytic infiltrates.

171 The lack of detectable viral antigen in brain sections and the failure to isolate live virus are puzzling.

172 Indeed, upon inoculation of mice with the Yuli strain (the only known EBLV-1a human strain), viral

173 antigens were observed to accumulate in the brain, as is the case for other EBLV-1 strains [24]. Viral  
174 detection in natural cases, however, seems to be more challenging. Indeed, fatal encephalitis in cats  
175 after natural exposure to EBLV-1 has proven difficult to diagnose, as little if any antigen has been  
176 detected by immunofluorescence. Moreover, virus isolation was inefficient or even impossible in cell  
177 culture or in mice [19]. Similarly, identification of EBLV-1 antigens and virus isolation from naturally  
178 infected sheep and stone martens have also been difficult[18,20]. The absence of virus isolation in our  
179 study could have various causes, possibly in relation to the type (meninge and cortex), size or  
180 conservation of brain samples, or to sample processing to dilute inhibitors. Likewise, isolation of KBLV  
181 has been unsuccessful in cell culture using standard procedures[25]. According to his family, the  
182 patient had been suffering from asthenia for several months prior to hospitalization. It is therefore  
183 possible that brain neuroinvasion preceded the acute symptoms by several months. The viral load may  
184 have become undetectable despite the persistence of viral genomes, considering the additional delay  
185 of about two months, due to management in an intensive care unit, between the onset of major  
186 symptoms and death. EBLV-1 replication was nevertheless evidenced in the present study by the  
187 presence of antibodies neutralizing the virus in serum collected at the onset of symptoms. Antibodies  
188 elicited by EBLV-1 infection have little or no capacity to cross-neutralize RABV, as shown both in our  
189 study and previously in sheep[18]. Antibodies neutralizing lyssaviruses (RABV) have already been  
190 identified in asymptomatic humans exposed to vampire bat bites, which shows that antibody  
191 responses can be elicited in case of prolonged incubation times or clearance of the virus[26].

192 Serotine bats were the most likely hosts responsible in France for human exposure to EBLV-1[27].  
193 Transversal surveys and longitudinal capture-recapture studies have shown that infected bats may  
194 remain healthy[28,29] in contrast to earlier findings[30]. Transmission between bats seems to occur  
195 mainly by biting and is inefficient per intranasal route[30]. It is reasonable to speculate that the same  
196 applies in spillover conditions including transmission to humans. According to his family, the patient  
197 had handled a dead or sick bat from the colony living near his house at least on one occasion. The  
198 family was unaware of any cases of biting, but this type of event would probably not have been

199 reported to the family given their unease regarding the accommodation of the bat colony. The  
200 entrance to the colony was condemned after the patient's death and any investigation regarding bat  
201 species and virological status unfortunately proved impossible.

202 Inaccurate diagnosis of encephalitis is a major issue as immunosuppressive treatments can be  
203 deleterious in case of viral infection. Metranscriptomics NGS was key in identifying this unexpected  
204 viral etiology in an epidemiological context that did not suggest rabies, which is an additional  
205 demonstration of the efficacy of such techniques when applied to brain tissues in encephalitis  
206 cases[31]. The patient had no known comorbidities and in particular was not immunocompromised.  
207 Our results suggest that the risk of EBLV-1 transmission to human, even if low, persists and could be  
208 higher than previously perceived. Most EBLV-1 cases in bats are probably not detected (69 cases only  
209 detected in Western Europe in 2019-2020, eurl-rabies.anses.fr), but surveys indicate that the virus  
210 circulates in bat colonies[32–34] and sometimes lead to high mortality[35]. The public should be  
211 reminded to avoid direct interactions with bats. In a context where the disappearance of rabies from  
212 terrestrial mammals in Western Europe is reducing the frequency of vaccination among professionals  
213 exposed to wildlife, people exposed to bats should be strongly incited to be vaccinated with rabies  
214 vaccines, which are effective against EBLV-1[36], and their antibody titer regulatory monitored [27]  
215 according to national recommendations[37].

## 216 Notes

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- 309

## Figure legends

### **Figure 1: Representative images of MRI scans at 5 and 23 days of ICU admission**

At day 5, Fluid Attenuated Inversion Recovery (FLAIR) transverse images showed an apparently normal cerebellum (A) whereas a well demarcated zone of high intensity was visible at the bottom of the pons (B). At that stage, the thalamus and caudate nucleus were slightly hyperintense bilaterally (C) and the other part of the brain seemed normal (D). At day 23, there was an obvious edema of the cerebellum (E) and the high intensity signal of the pons had diffused to the dentate nucleus of cerebellum bilaterally (F). The brain was overtly swollen with an increased high intensity signal of the lenticular nuclei and insula (G). This high intensity signal seemed to have diffused to the whole brain (H).

### **Figure 2: Histopathology of necropsy samples**

Abundant inflammatory perivascular infiltrate in the caudate nucleus (a: hematoxylin eosin staining, x10), composed of T CD3+ and B CD20 + lymphocytes, among which a high number of large plasma cells (b: hematoxylin eosin staining, x400). Astrocytic gliosis, microglial activation, and neuronal loss in the caudate nucleus (c: hematoxylin eosin staining, x400). Atrophy of a nerve root in the medulla oblongata (d: hematoxylin eosin staining x100). Immunohistochemistry against rabies was negative (e: caudate nucleus, x200) whereas the cerebral cortex of a rabid patient was positive (f: cerebral cortex x200).

### **Figure 3: Phylogenetic analysis**

Phylogenetic tree based on full-genome sequences of representatives of the lyssavirus species belonging to the Phylogroup I. Clades are collapsed into triangles representative of species diversity. The sequence of Hum1\_FRA clusters into the clade EBLV1-a.



Table 1: Main clinical and non-clinical findings

<b>Physical examination</b>	<b>Imaging</b>	<b>Biology</b>
Paralysis of the oculomotor (III), left abducens (VI) and facial (VII) nerves	CT scan: normal	CSF: turbid liquid with 26 WBC/mm <sup>3</sup> (84% lymphocytes), high protein (1.43 g/l) and normal glucose level
Salivary stasis	MRI: hyperintensity at the posterior part of the pons on the axial Fluid Attenuated Inversion Recovery (FLAIR) sequence	Gram stain and multiplex PCR (Meningitidis/encephalitis panel, FilmArray Biomérieux, Marcy l'Etoile) negative.
Dysphonia		Immune encephalitis screening negative (anti-aquaporin-4 antibody, anti-MOG and antineuronal antibodies)

Figure 1

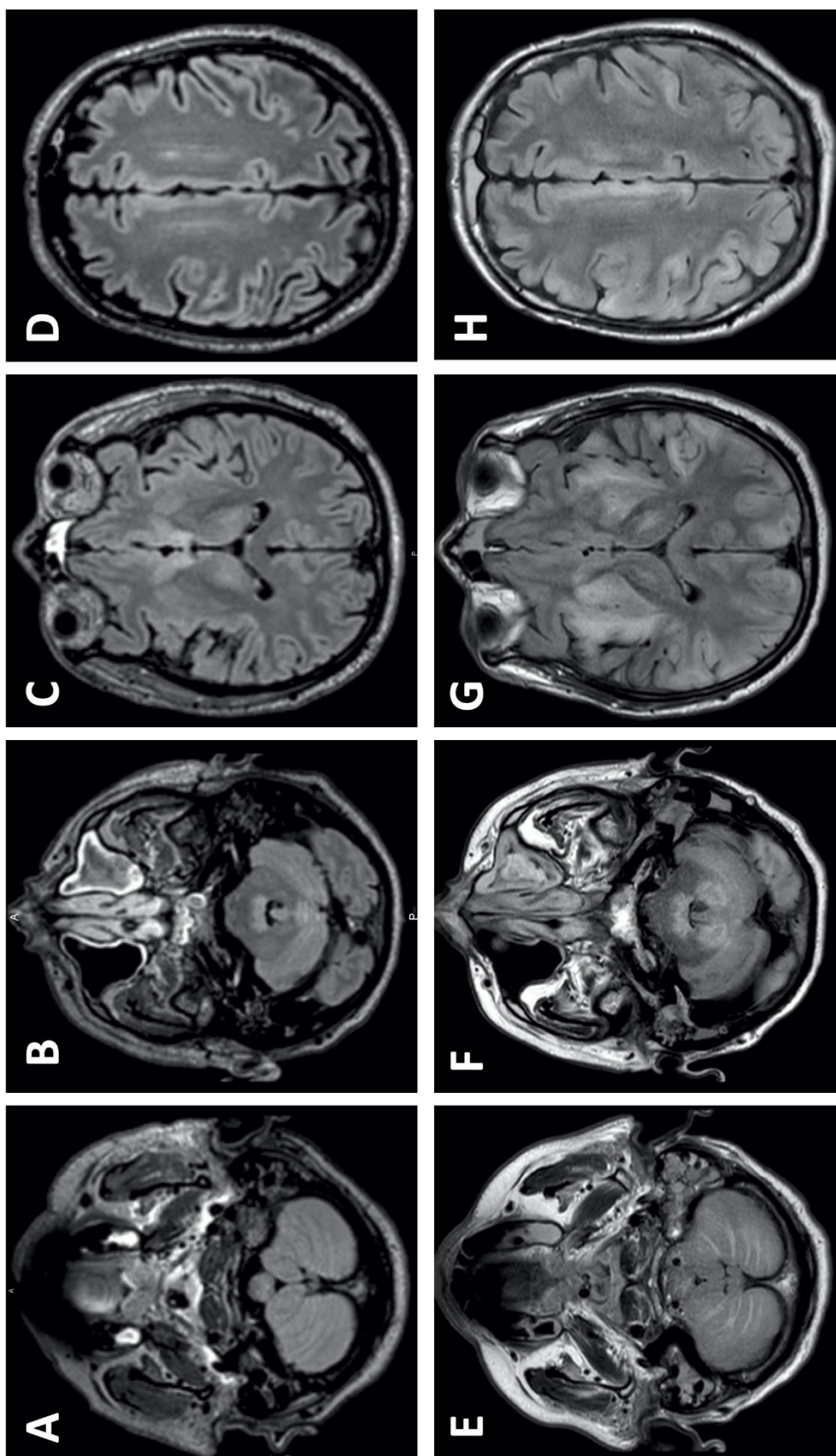


Figure 2

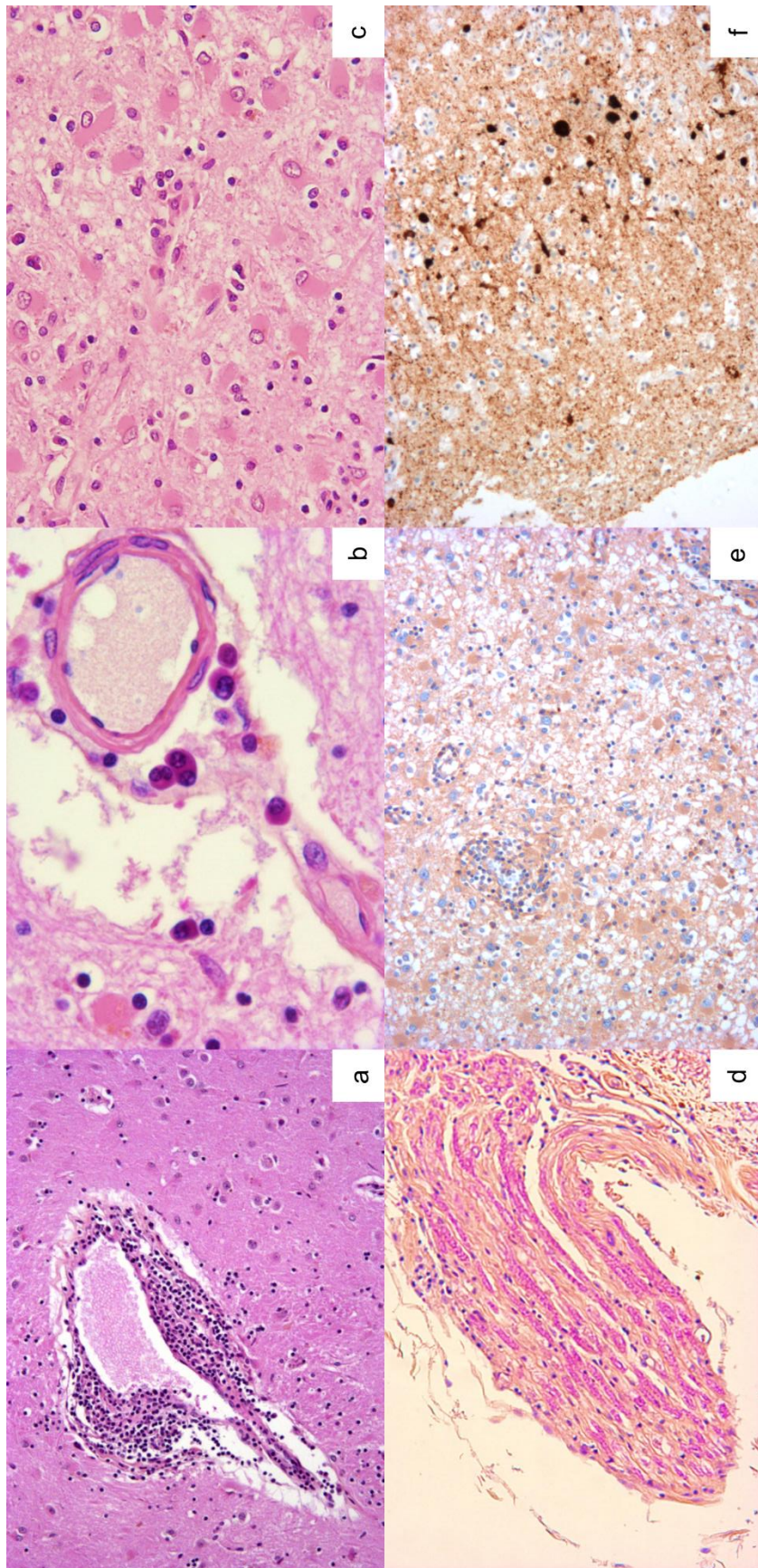
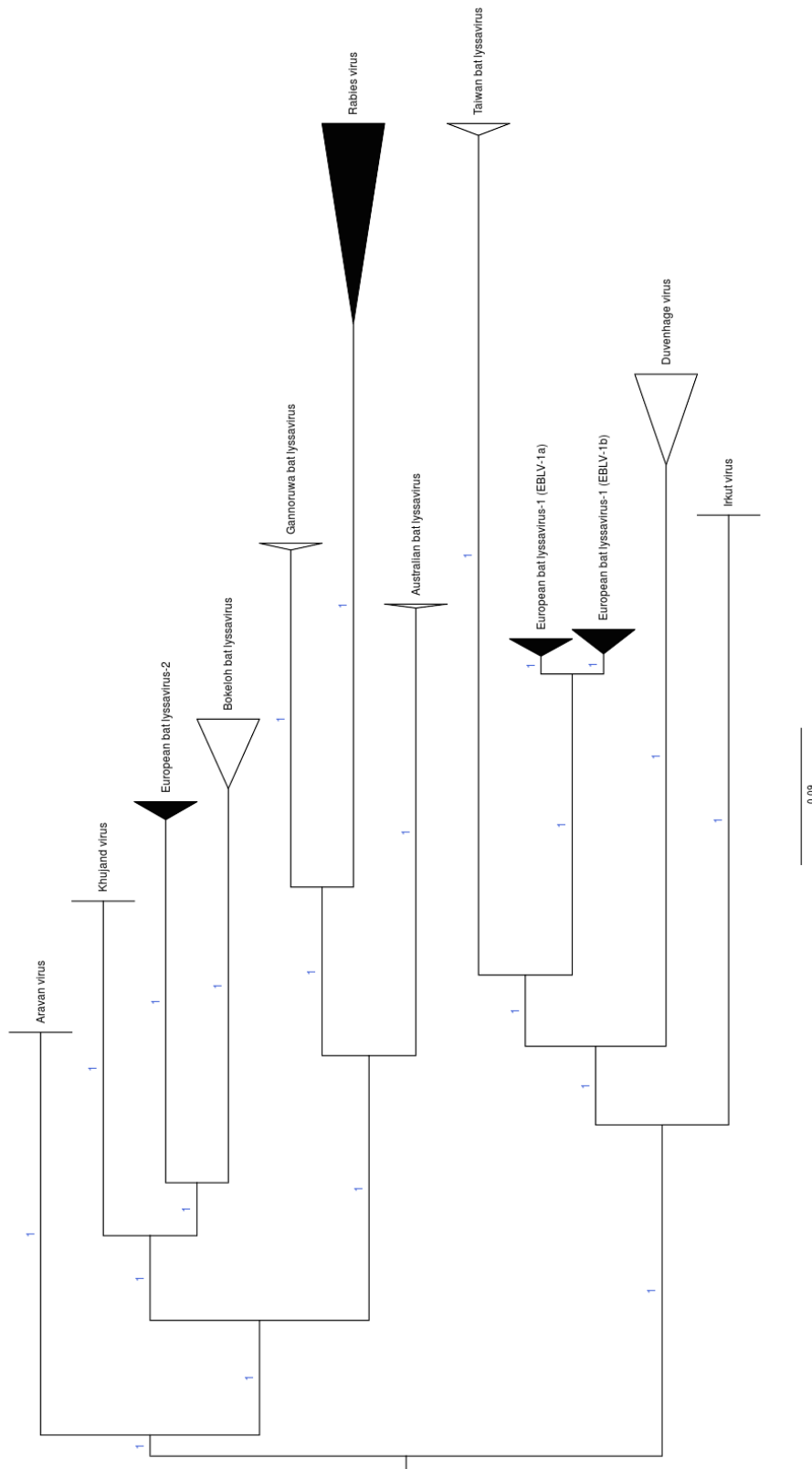


Figure 3



## **Supplementary material**

### **METHODS**

Metatranscriptomics  
Reverse-transcription  
Extraction of nucleic acids from CSFs  
Quantitative PCR  
Genome Finishing  
Nested-PCR  
Immunochemistry  
Transmission electron microscopy  
Phylogenetic analyses

### **SUPPLEMENTARY PATHOLOGICAL DATA**

#### **SUPPLEMENTARY TABLES**

Table S1. List of primers used in quantitative PCR

Table S2. List of primers used in PCR

Table S3: Immunohistochemical methods.

Table S4: List of Accession numbers of EBLV1, subgenotypes, Date of isolation, Country of isolation

#### **SUPPLEMENTARY IMAGES**

Figure S1: Transmission electron micrograph

Figure S2: Phylogenetic analysis

#### **REFERENCES**

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### **METHODS**

#### **Metatranscriptomics**

Nucleic acids from meninges and brain frontal lobe samples were extracted after a bead-beating step with the MagNA Lyser using the MagNA Pure Compact RNA Isolation kit (Roche Molecular Systems, Inc.) including a DNase treatment. Total RNA was used to construct a cDNA library with the SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian (Takara Bio, kit v.2).

A total of 49 and 94 raw million reads of 150 bp for meninges and frontal lobe sample respectively were processed with an agnostic in-house bioinformatics pipeline as described<sup>1</sup>. Sequences were aligned after translation against a viral protein reference comprehensive database (RVDB-prot.V20.0)<sup>2</sup>. Ninety percent coverage of the genome was obtained by NGS from the frontal lobe sample.

#### **Reverse-transcription**

Reverse-transcription was carried out using the SuperScript IV First-Strand Synthesis System kit (Invitrogen) according to the manufacturer's instructions with random primers, 440 ng RNA input for the brain frontal lobe sample and 316 ng RNA input for the meninges sample.

#### **Extraction of nucleic acids from CSFs**

Each CSF (July, August) was mixed with ATL buffer and DX reagent into a Pathogen Lysis Tube L (QIAGEN). Bead beating was carried out twice 60 sec at full speed with 1 min resting time on ice on Minilys (Bertin). Linear acrylamide (Ambion), Proteinase K and VXL buffer (INDICAL Bioscience) were added to CSF after the bead beating step, and incubated at room temperature for 15 min. Lysate was transferred into a QIAamp mini column (INDICAL Bioscience) and purified according to the manufacturer's instructions, with a DNase treatment on the column after the binding step. Purified RNA was eluted into 50 µL of elution buffer.

### Quantitative PCR

qPCR was performed in SybrGreen technology using FastStart Essential DNA Green Master kit (Roche Diagnostic) with 45 cycles of amplification, specific primers at 0.5 µM final concentration and 1 µL of cDNA. Four sets of primers were used to detect viruses [Table S1]. Each reaction was run in duplicate on a LightCycler® 96 instrument (Roche Diagnostic). Results were normalized using the human beta-actin housekeeping gene.

Table S1. List of primers used in quantitative PCR

Primer	Sequence (5' => 3')	Region	Size	Reference
<b>EBLV1-G-F</b>	TGA TAA CAG AGG CAG AGA CC	G	317 pb	this study
<b>EBLV1-G-R</b>	ACA ACA TAT GGC ACT CGG			
<b>EBLV1-N-F</b>	ACC AAT GTG GCA GAT AGA ATG G	N	315 pb	
<b>EBLV1-N-R</b>	TCT TCC TCG AAG TTC TTG TGG			
<b>1671 P_2F</b>	CTG AGG ATA TTA AGA GGC TCA	P	686 pb	3
<b>2357 P_2R</b>	GCC YAR TTT CGC CGA ATT GAC			
<b>4712 GL_2F</b>	ATA TCT GTG CTT GCC CTT CT	G-L	763 pb	
<b>5475 GL_2R</b>	CCA CCG GAT CAT CGT AAA CC			

### Genome Finishing

Complete genome was obtained by conventional PCR using Phusion high fidelity DNA polymerase (Thermo Fischer Scientific FINNZYMES). Primers were designed according to our NGS sequences and a 45 cycles of amplification round was performed. The end of 5'UTR anti-genome region was obtained by RACE-PCR using 5'/3' RACE Kit 2nd Generation (Roche). After running into a 2 % agarose gel, amplicons were purified by NucleoSpin Gel & PCR Clean-up kit (Macherey-Nagel) and sequenced by Sanger sequencing.

### Nested PCR

For testing CSFs, a nested PCR was performed using Phusion high fidelity enzyme kit (Thermo Fischer Scientific FINNZYMES) and sets primers [Table 2]. Thermal Cycling conditions were as follows : first round PCR at 98°C for 30 s, 45 cycles at 98°C for 15 s, annealing temperature according to the T<sub>m</sub> of primers for 30 s and 72°C for 1min, then a final extension at 72°C for 7 min. Second round PCR was performed in the same conditions with *annealing temperature* according to primers' T<sub>m</sub>.

Table S2. List of primers used in PCR

Primer	Sequence (5' => 3')	Tm	Tm for PCR	Region	Size	Reference
1617 P_1F	TGG AGG ATA GTC AAG CCC AC	59 °C	52 °C	P	769 bp	3
2386 P_1R	TAT CTG TTK ARA TCA TCTY GC	53 °C				
1671 P_2F	CTG AGG ATA TTA AGA GGC TCA	56 °C	54 °C	P	686 bp	
2357 P_2R	GCC YAR TTT CGC CGA ATT GAC	60 °C				
4633 GL_1F	TCA CCT TCC AGA CAC CCA	56 °C	55 °C	G-L	896 bp	
5502 GL_1R	TCA GGT CTG CTT CTG GCT CA	59 °C				
4712 GL_2F	ATA TCT GTG CTT GCC CTT CT	55 °C	54 °C	G-L	763 bp	
5475 GL_2R	CCA CCG GAT CAT CGT AAA CC	59 °C				

## Immunocytochemistry

The immunostaining was performed by an automate (Ventana BenchMark Stainer, Roche). The biotinylated secondary antibody was included in the detection kit (Ventana Medical Systems Basic DAB Detection Kit 250-001). The streptavidin–biotin-peroxidase complex was revealed by diaminobenzidine. Antibodies used for immunohistochemistry and pretreatments are listed in the table S3.

Table S3: Immunohistochemical methods.

Antigen	Poly/monoclonal	Producer	Immunogen	Clone	Pretreatment	Dilution	Incubation Time (minutes)
CD163	Monoclonal (mouse)	Cell Marque®	Acute phase-regulated transmembrane protein on monocytes	MRQ-26	CC1	1:50	32
CD20	Monoclonal (mouse)	Dako®	Transmembrane protein expressed on B cells	L26	CC1	1:100	32
CD3	Monoclonal (rabbit)	Ventana®	Epsilon chain of the human CD3	2GV6	CC1	prediluted	32
CD4	Monoclonal (mouse)	Dako®	T helper cells	4B12	CC1	1:40	60
CD8	Monoclonal (mouse)	Dako®	Suppressor/cytotoxic T cells	C8/144B	CC1	prediluted	32
GFAP	Monoclonal (mouse)	Dako®	Glial Fibrillary Acidic Protein	6F2	CC1	1:500	28
CMV	Monoclonal (mouse)	Agilent®	Early nuclear protein	CCH2 + DDG9	protease	1:50	32
HSV1	Monoclonal (mouse)	Cell Marque®	Nuclear proteins	10A3	CC1	prediluted	32
VZV	Monoclonal (mouse)	Monosan®		7 clone cocktail (SG1-1, SG1-SG4, NCP-1, IE-62)	CC1	prediluted	32
Measles	Polyclonal (rabbit)	Diagomics®	Fusion protein		CC1	1:100	60
Rabies	Polyclonal (rabbit)	Biorad	Rabies virus nucleocapsid	3572112	CC2	1:100	92

CC1, a proprietary high pH (=8) buffer; CC2, a proprietary low pH (=6) buffer.

## Transmission electron microscopy

Samples from brain stored during several months after autopsy were taken from macroscopically abnormal areas of the white matter. These samples were fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate



buffer, post-fixed in 1% osmium tetroxide and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined using a JEOL (Flash 1400) electron microscope at 120 KeV.

### Phylogenetic analyses

Phylogenetic analyses were performed using NGPhylogeny.fr<sup>4</sup>. The glycoprotein complete CDS of the French EBLV1a sequence (Hum1\_FRA) was aligned along with 91 other representative sequences of sublineages *EBLV1a* and *EBLV1b* using MAFFT (Multiple Alignment using Fast Fourier Transform) aligner with default parameters. Tree inferences were constructed with PhyML using approximate Bayes branch supports. The phylogenetic tree was visualised by using FigTree (version 1.4.4).

### SUPPLEMENTARY PATHOLOGICAL DATA

The brain was edematous (1575 grams). There was no herniation, atrophy or focal lesion. After formalin fixation, sampling and paraffin embedding, 5 µm thick sections were stained with hematoxylin and eosin. Additional immunohistochemistry analyses were performed (as described in Supplementary methods). At microscopic examination, the lesions were diffuse, observed in both white matter and grey matter, and in all the samples. They were characterized by abundant inflammatory perivascular infiltrates, astrocytic gliosis, microglial activation, contrasting with few necrosis [Fig. 2]. Lymphoid cells in the leptomeningeal space were relatively few in number, contrasting with the abundance of perivascular intra-parenchymal infiltrates. The latter were composed of T and B lymphocytes, among which a high number of large plasma cells [Fig. 2]. There was no noticeable imbalance between the number of CD8 and CD4 T lymphocytes.

The cerebellum was one of the most severely damaged region with white matter pallor, axonal loss and astrocytic gliosis. There was a loss of Purkinje cells associated with hyperplasia of the Bergmann glia. In the brainstem, the perivascular infiltrates were abundant. The roots of the cranial pairs were atrophic. The *substantia nigra* and *locus caeruleus* were relatively spared.

In the supra-tentorial area, the lesions were also diffuse, affecting white and gray matters with a predominance in the cortical and striatal gray matter. The lesions were necrotic in the occipital region. A spongy state was observed in the visual cortex, in temporal region and in the head of the caudate nucleus. Elsewhere, diffuse microglial activation was associated with neuronal loss and astrocytic gliosis. Perivascular inflammatory infiltrates were abundant and rich in plasma cells [Fig. 2]. Examination of the Ammon's horns showed neuronal rarefaction without necrosis. The thalamus and the mammillary body appeared to be spared. Cytopathogenic effect, including Negri's bodies, was not seen. Immunohistochemistry against herpes simplex virus 1, varicella-zoster, cytomegalovirus, measles virus, and rabies virus were negative.

### SUPPLEMENTARY TABLES

Table S4. List of Accession numbers, lineage, Date of isolation, Country of isolation

Accession number	Sublineage	Isolate	Country of isolation	City of isolation	Date of isolation	Source	Host
KF155003.1	EBLV-1a	RV20	Denmark	Køge	1986	-	Eptesicus serotinus
MF187802.1	EBLV-1a	02016DEN	Denmark	Janderup	2002	Mouse	Eptesicus serotinus
MF187803.1	EBLV-1a	03002FRA	France	Chemellier	30/01/2003	Original brain	Eptesicus serotinus
MF187809.1	EBLV-1a	08120FRA	France	Fontenay-le-Comte	15/01/2008	Original brain	Eptesicus serotinus
MF187811.1	EBLV-1a	09034FRA	France	Dignac	16/09/2009	Original brain	Eptesicus serotinus
MF187821.1	EBLV-1a	122938	France	Guéret	26/08/2002	Mouse	Eptesicus serotinus
MF187823.1	EBLV-1a	123801	France	Carmaux	22/10/2003	Original brain	Eptesicus serotinus

MF187824.1	EBLV-1a	124193	France	Guéret	29/06/2004	Original brain	Eptesicus serotinus
MF187826.1	EBLV-1a	124489	France	Vaux sur Mer	13/09/2004	Mouse	Eptesicus serotinus
MF187831.1	EBLV-1a	128210	France	Le Haillan	04/09/2008	Original brain	Eptesicus serotinus
MF187840.1	EBLV-1a	129051	France	La Crèche	24/08/2010	Original brain	Eptesicus serotinus
MF187841.1	EBLV-1a	129055	France	Rochefort sur Mer	27/08/2010	Mouse	Eptesicus serotinus
MF187843.1	EBLV-1a	129090	France	Champagne	04/10/2010	Mouse	Eptesicus serotinus
MF187855.1	EBLV-1a	130904	France	Clisson	18/05/2015	Mouse	Eptesicus serotinus
MF187856.1	EBLV-1a	131054	France	Chenommet	06/02/2015	Mouse	Eptesicus serotinus
EU626552.1	EBLV-1a	07240FRA	France	Fontenay-le-Comte	2007	cat	cat
LT839610.1	EBLV-1a	976	Germany	-	1992	-	Pipistrellus nathusii
LT839611.1	EBLV-1a	5782	Germany	-	2001	-	unknown
LT839614.1	EBLV-1a	5776	Germany	-	2001	-	unknown
LT839615.1	EBLV-1a	13454	Germany	-	2000	-	Eptesicus serotinus
MF187865.1	EBLV-1a	9395GER	Germany	Hamburg	1968	Mouse	Eptesicus serotinus
MF187866.1	EBLV-1a	9396GER	Germany	Rostock	1985	Mouse	Eptesicus serotinus
MF187868.1	EBLV-1a	9399GER	Germany	Bremerhaven	1982	Mouse	Eptesicus serotinus
MF187873.1	EBLV-1a	9436GER	Germany	Stade	18/09/1986	Mouse	Eptesicus serotinus
MF187874.1	EBLV-1a	9438GER	Germany	Neumunster	août-88	Mouse	Eptesicus serotinus
MF187875.1	EBLV-1a	9440GER	Germany	Brauschweig	24/07/1989	Mouse	Eptesicus serotinus
MF187877.1	EBLV-1a	9477GER	Germany	Nienburg	28/08/1986	Mouse	Eptesicus serotinus
MF187860.1	EBLV-1a	9366HOL	Netherlands	Goor	17/06/1992	Mouse	Eptesicus serotinus
MF187871.1	EBLV-1a	94116HOL	Netherlands	Jubbega	14/08/1987	Mouse	Eptesicus serotinus
MF187878.1	EBLV-1a	9478HOL	Netherlands	Rolde	27/08/1987	Mouse	Eptesicus serotinus
MF187879.1	EBLV-1a	9480HOL	Netherlands	Bellingwolde	16/07/1987	Mouse	Eptesicus serotinus
MF187858.1	EBLV-1a	8615POL	Poland	Gdansk	1985	Mouse	Eptesicus serotinus
MF187864.1	EBLV-1a	9394POL	Poland	Ketrzyn	1990	Mouse	Eptesicus serotinus
LT839613.1	EBLV-1a	13027	Russia	-	1982	human	-
MF187867.1	EBLV-1a	9397RUS	Russia	Belgorod	1985	Mouse	Eptesicus serotinus
MF187801.1	EBLV-1a	01018SLO	Slovakia	Presov	08/02/2001	Mouse	Eptesicus serotinus
MF187876.1	EBLV-1a	9443UKR	Ukraine	Volyn Region	août-87	Mouse	Eptesicus serotinus
MF187804.1	EBLV-1b	04032FRA	France	Bourges	10/09/2004	Original brain	Eptesicus serotinus
MF187805.1	EBLV-1b	05001FRA	France	Souesmes	17/05/2005	Original brain	Eptesicus serotinus
MF187806.1	EBLV-1b	06001FRA	France	Ourches-sur-Meuse	16/05/2006	Original brain	Eptesicus serotinus
MF187807.1	EBLV-1b	06002FRA	France	Bourges	08/06/2006	Original brain	Eptesicus serotinus
MF187808.1	EBLV-1b	07058FRA	France	Sainte Méline	25/05/2007	Original brain	Eptesicus serotinus
MF187810.1	EBLV-1b	08341FRA	France	Aillant-sur-Tholon	25/09/2008	Original brain	Eptesicus serotinus
MF187812.1	EBLV-1b	78983	France	Bainville sur Madon	04/10/1989	Mouse	Eptesicus serotinus
MF187813.1	EBLV-1b	107251	France	Bourges	16/10/1995	Original brain	Eptesicus serotinus
MF187814.1	EBLV-1b	113852	France	Champigneulles	14/03/1997	Original brain	Eptesicus serotinus
MF187815.1	EBLV-1b	116883	France	Morlaix	18/03/1998	Original brain	Eptesicus serotinus
MF187816.1	EBLV-1b	120914	France	Plouneour-Menez	27/03/2000	Original brain	Eptesicus serotinus
MF187817.1	EBLV-1b	121411	France	Fouesnant	25/09/2000	Mouse	Eptesicus serotinus
MF187818.1	EBLV-1b	121633	France	Joinville	13/12/2000	Original brain	Eptesicus serotinus
MF187819.1	EBLV-1b	122154	France	Waville	22/08/2001	Mouse	Eptesicus serotinus
MF187820.1	EBLV-1b	122319	France	Vallon en Sully	28/09/2001	Original brain	Eptesicus serotinus
MF187822.1	EBLV-1b	123008	France	Lurcy Levis	06/09/2002	Mouse	Eptesicus serotinus
MF187825.1	EBLV-1b	124345	France	Guénin	19/08/2004	Original brain	Eptesicus serotinus
MF187827.1	EBLV-1b	126669	France	Arradon	27/10/2005	Mouse	Eptesicus serotinus
MF187828.1	EBLV-1b	127051	France	Crosses	12/07/2006	Mouse	Eptesicus serotinus
MF187829.1	EBLV-1b	127834	France	Bourges	13/11/2007	Original brain	Eptesicus serotinus

MF187830.1	EBLV-1b	127835	France	Bourges	13/11/2007	Original brain	Eptesicus serotinus
MF187832.1	EBLV-1b	128633	France	Ancy sur Moselle	30/06/2009	Original brain	Eptesicus serotinus
MF187833.1	EBLV-1b	128635	France	Ancy sur Moselle	30/06/2009	Original brain	Eptesicus serotinus
MF187834.1	EBLV-1b	128637	France	Ancy sur Moselle	30/06/2009	Original brain	Eptesicus serotinus
MF187835.1	EBLV-1b	128665	France	Ancy sur Moselle	07/07/2009	Original brain	Eptesicus serotinus
MF187836.1	EBLV-1b	128681	France	Ancy sur Moselle	13/07/2009	Original brain	Eptesicus serotinus
MF187837.1	EBLV-1b	128683	France	Mars la Tour	17/07/2009	Original brain	Eptesicus serotinus
MF187838.1	EBLV-1b	128708	France	Lure	31/07/2009	Original brain	Eptesicus serotinus
MF187839.1	EBLV-1b	128827	France	Bourges	23/10/2009	Original brain	Eptesicus serotinus
MF187842.1	EBLV-1b	129087	France	Recologne	24/09/2010	Original brain	Eptesicus serotinus
MF187844.1	EBLV-1b	129116	France	Sécheval	08/10/2010	Original brain	Eptesicus serotinus
MF187845.1	EBLV-1b	129123	France	Guingamp	12/10/2010	Original brain	Eptesicus serotinus
MF187846.1	EBLV-1b	129246	France	Monceaux le Comte	29/04/2011	Original brain	Eptesicus serotinus
MF187847.1	EBLV-1b	129290	France	Jouet sur l'Aubois	24/05/2011	Original brain	Eptesicus serotinus
MF187848.1	EBLV-1b	129394	France	Pagny-sur-Moselle	27/07/2011	Mouse	Eptesicus serotinus
MF187849.1	EBLV-1b	129396	France	Billy sous les Côtes	28/07/2011	Mouse	Eptesicus serotinus
MF187850.1	EBLV-1b	129409	France	Pagny-sur-Moselle	09/08/2011	Original brain	Eptesicus serotinus
MF187851.1	EBLV-1b	129428	France	Belleville sur Meuse	12/08/2011	Original brain	Eptesicus serotinus
MF187852.1	EBLV-1b	130544	France	Saint-Martin-d'Auxigny	03/06/2014	Mouse	Eptesicus serotinus
MF187853.1	EBLV-1b	130576	France	Nexon	10/09/2014	Mouse	Eptesicus serotinus
MF187854.1	EBLV-1b	130662	France	Cour-Cheverny	24/06/2014	Mouse	Eptesicus serotinus
MF187857.1	EBLV-1b	15007FRA	France	Gouvieux	04/06/2015	Original brain	Eptesicus serotinus
MF187859.1	EBLV-1b	8918FRA	France	Briey	11/09/1989	Original brain	Eptesicus serotinus
MF187861.1	EBLV-1b	9367HOL	Netherlands	Wassenaar	10/07/1992	Mouse	Eptesicus serotinus
MF187862.1	EBLV-1b	9376HOL	Netherlands	Moerkapelle	08/06/1993	Mouse	Eptesicus serotinus
MF187863.1	EBLV-1b	9377HOL	Netherlands	Apeldoorn	08/06/1993	Mouse	Eptesicus serotinus
MF187869.1	EBLV-1b	94113HOL	Netherlands	Schagen	20/07/1989	Mouse	Eptesicus serotinus
MF187870.1	EBLV-1b	94115HOL	Netherlands	Bovenkarspel	17/08/1989	Mouse	Eptesicus serotinus
KP241939.1	EBLV-1b	RV2416	Spain	Granada	2007	Mouse	Eptesicus isabellinus
MF187872.1	EBLV-1b	94285SPA	Spain	Granada	sept-94	Mouse	Eptesicus serotinus
MF187880.1	EBLV-1b	9483SPA	Spain	Granada	1987	Mouse	Eptesicus serotinus
LT839609.1	EBLV-1b	20174	Germany	-	2008	-	Eptesicus serotinus
LT839612.1	EBLV-1b	5006	Germany	Wadgassen	2000	-	Eptesicus serotinus
LT839608.1	EBLV-1c	13424	Spain	-	1989	-	unknown

## SUPPLEMENTARY FIGURES

Figure S1: Transmission electron micrograph

Micrograph of a lesion located behind the right putamen showing cytoplasmic viral particles that look like rods (white "r") (diameter (50–100 nm) and length (100–300 nm); some are bullet-shaped (b) with limiting trilaminar membranes (white arrows). Most have variable shapes, more or less long or oval, as they are arranged in all planes of space (scale bar: 200 nm). A high resolution image is available as a separate file.

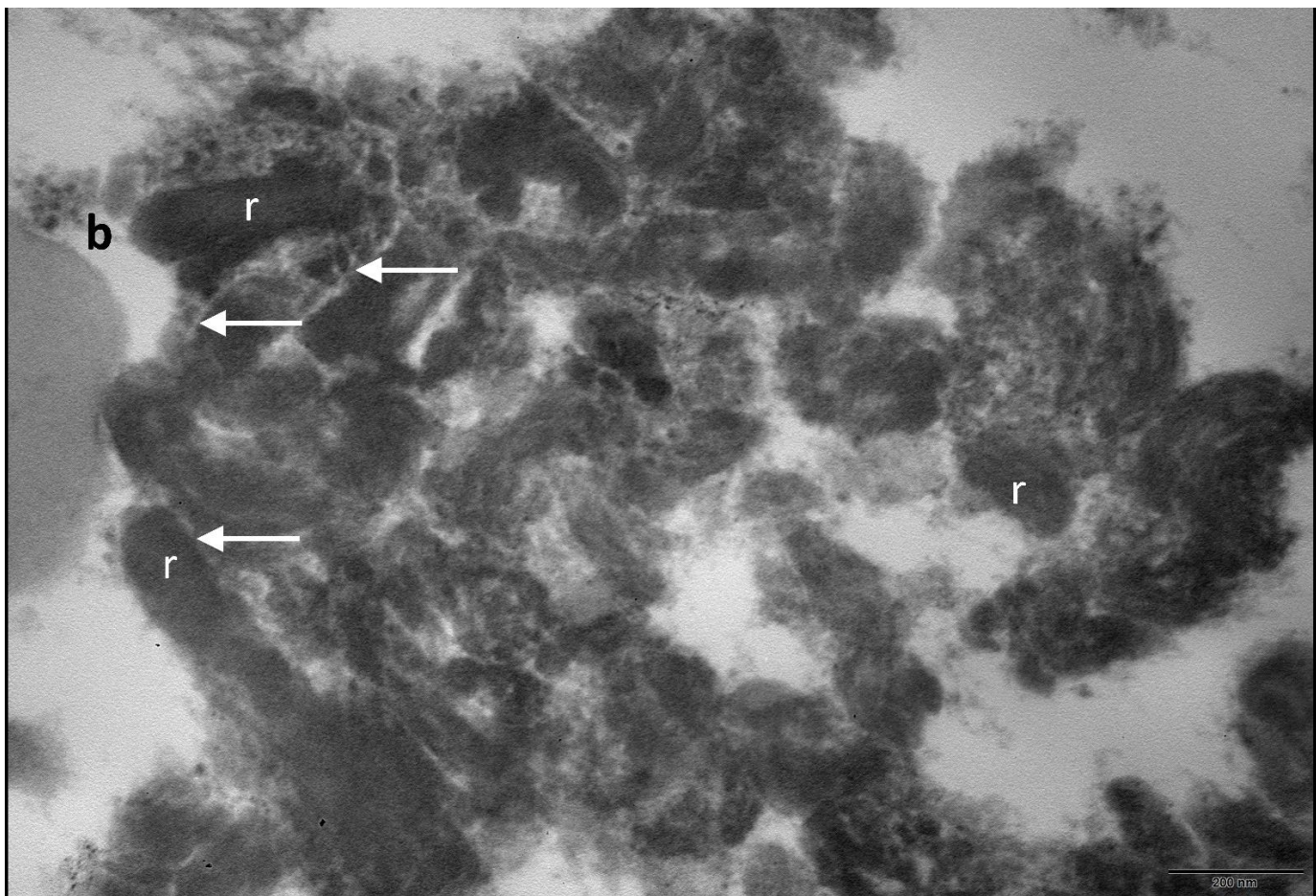
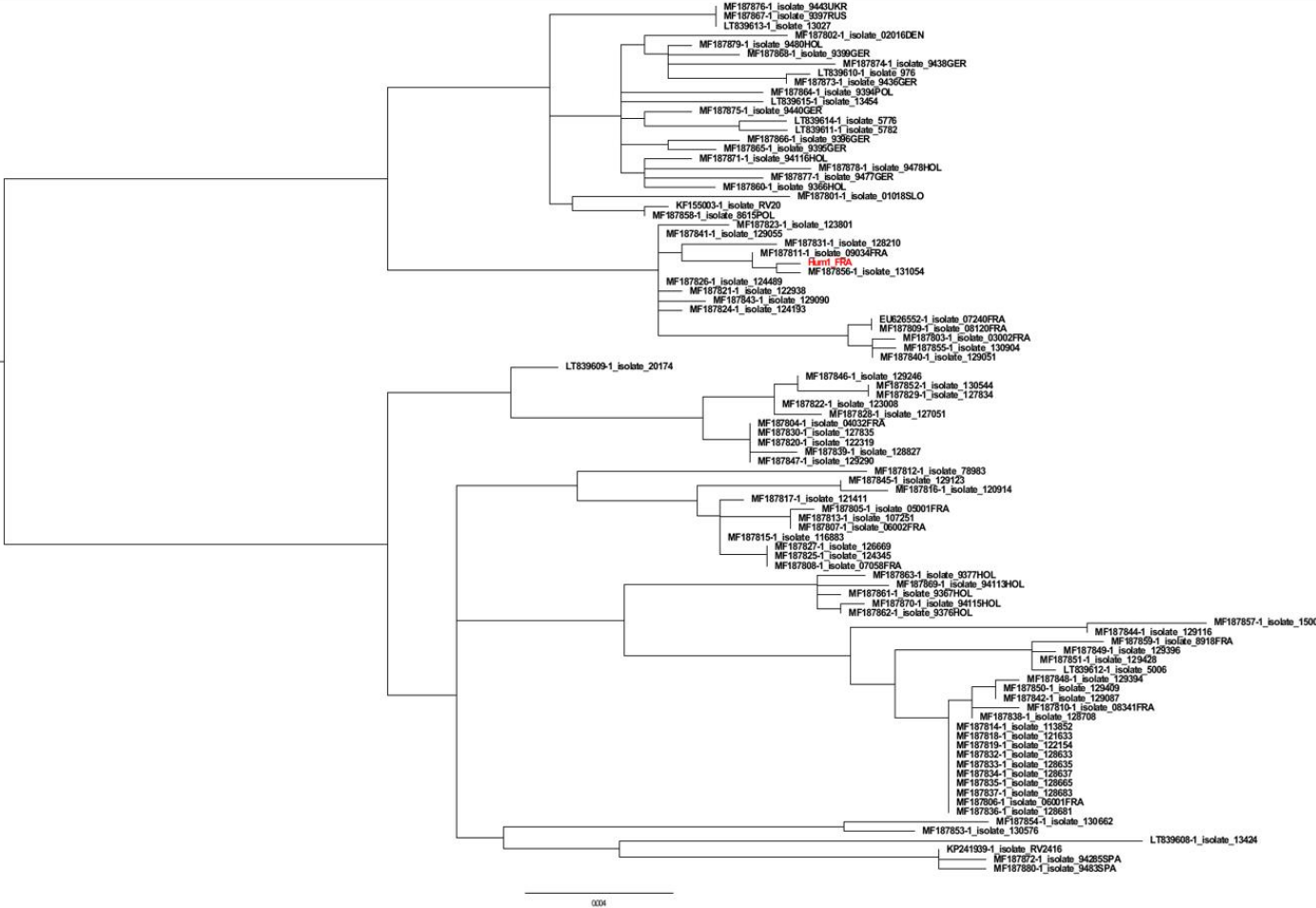


Figure S2: Phylogenetic analysis



The whole genome of the French EBLV-1a sequence (Hum1\_FRA) was aligned along with 91 other representative sequences of EBLV1a and EBLV1b listed in table S4.

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