Novel Lyssavirus in Bat, Spain

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A new tentative lyssavirus, Lleida bat lyssavirus, was found in a bent-winged bat (*Miniopterus schreibersii*) in Spain. It does not belong to phylogroups I or II, and it seems to be more closely related to the West Causasian bat virus, and especially to the Ikoma lyssavirus.

Bats have been considered natural hosts of a wide diversity of viruses, including human pathogens such as lyssaviruses, severe acute respiratory syndrome coronavirus, henipavirus, and filoviruses (*1*). Within the genus *Lyssavirus*, 12 species have been described: *Rabies virus* (RABV), *Lagos bat virus* (LBV), *Mokola virus* (MOKV), *Duvenhage virus* (DUVV), *European bat lyssavirus* types 1 and 2 (EBLV-1 and -2), *Australian bat lyssavirus* (ABLV), *Aravan virus* (ARAV), *Khujand virus* (KHUV), *Irkut virus* (IRKV), *West Causasian bat virus* (WCBV), and *Shimoni bat virus* (SHIBV). Two more recently described viruses have not yet been classified: Bokeloh bat lyssavirus (BBLV) (*2*) and Ikoma lyssavirus (IKOV) (*3*).

Bats are the natural reservoirs for most lyssaviruses, and to our knowledge, only MOKV and IKOV have never been detected in bats. RABV is the only virus known to establish epidemiologic cycles in bats and carnivores, and it is responsible for most human infections, mainly transmitted by dogs. The genus *Lyssavirus* comprises at least 2 phylogroups: phylogroup I (RABV, DUVV, EBLV1–2, ABLV, ARAV, IRKV, BBLV, KHUV) and phylogroup II (LBV, MOKV, and SHIBV). Phylogroup III consists of WCBV (*4*). According to a recent phylogenetic reconstruction that included the novel IKOV and was based on a fragment of 405 nt from the nucleoprotein gene, IKOV has proven to be highly divergent (*3*) and probably also forms part of phylogroup III.

During 1977–2011 in Europe, 988 cases of bat rabies were reported to the Rabies Bulletin Europe. Bats of the species *Eptesicus serotinus* and *E. isabellinus*, which account for >95% of cases, are considered the major natural reservoirs of EBLV-1. Several bat species within the genus *Myotis* are reservoirs for EBLV-2, BBLV, and the central Asian lyssaviruses ARAV and KHUV (*5*). WCBV has been isolated in the common bent-winged bat *Miniopterus schreibersii* (*6*). Other bat species might act as eventual hosts, although in Spain, bat rabies has been declared only in *E. isabellinus* bats (*7*). The possibility of a wider host range has been suggested by some surveys on natural bat colonies of other bat species describing neutralizing antibodies and genomic fragments related to EBLV-1 (*8*).

The Study

In July 2011, a bat was found in the City of Lleida and taken to the Wildlife Care Center of Vallcalent (Lleida, Catalonia). The bat arrived lethargic and dehydrated, died soon after admission, and its carcass was frozen at -20°C. On March 12, 2012, as part of the rabies surveillance program in Spain, the bat carcass was received by the National Center of Microbiology, where rabies testing was conducted by 2 generic reverse transcription PCR (RT-PCR) methods for lyssavirus detection (*9*,*10*) and 2 commercial rabies antiserum assays (Bio-Rad Laboratories, Marnes La Coquette, France; and Fujirebio, Inc., Tokyo, Japan) for antigen detection by fluorescent antibody testing.

Brain smears were positive for lyssavirus by RT-PCR and fluorescent antibody testing, and an oropharyngeal swab sample was positive by RT-PCR. Further attempts to isolate the virus by tissue cultures were unsuccessful after 2 blind passages in BHK-21 and murine neuroblastoma cells. The negative results could be explained by the fact that the sample had been stored at –20°C for 8 months and had been frozen and thawed twice before cell culture testing; however, the possibility of the cell lines not being permissive for the virus cannot be excluded.

The bat was morphologically identified as a bent-winged bat (*M. schreibersii*) and genetically identified by cytochrome b sequencing (*11*). The genomic sequence of the corresponding fragment of the diagnostic RT-PCR on the conserved region of the nucleoprotein gene, determined by BLAST (http://blast.ncbi.nlm.nih.gov/), showed no substantial sequence similarity to previously known lyssaviruses.

To determine the identity of the lyssavirus, we sequenced a larger fragment (565 bp), including the variable codifying region of the nucleoprotein gene (GenBank accession number submitted). We reconstructed an overall

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phylogeny of lyssaviruses by using a Bayesian Inference with the first 405 nt of the N-gene and MrBayes version 3.1.2 (http://mrbayes.csit.fsu.edu/). Two simultaneous runs of 10^6 generations were conducted, each with 4 Markov chains, and trees were sampled every 100 generations. The best-fit nucleotide model, GTR + I + G, available in MrBayes,was selected according to the corrected Akaike information criterion. The phylogenetic reconstruction was based on a dataset representative of all known lyssaviruses, including the recently described IKOV. The topology obtained showed that this sequence is more closely related to IKOV and WCBV than to the lyssaviruses in phylogroups I and II (Figure). These results suggest that this sequence tentatively belongs to a new *Lyssavirus* species named after the location of collection, Lleida bat lyssavirus (LLEBV).

The highest nucleotide identity was with IKOV (71.6%), followed by SHIBV (68.6%), IRKV (68.1%), KHUV (67.6%), EBLV-2 (67%–68.2%), ARAV (67.3%), WCBV (67.4%), ABLV (66.6%–67.7%), BBLV (66.1%), LBV (65.7%–68.6%), MOKV (65.7%–67.2%), DUVV (65.5%–65.8%), RABV (64.7–66.4%), and EBLV-1 (63.7%–64%). The lowest nucleotide identity was with the only lyssavirus found in bats of the Iberian Peninsula, EBLV-1. The nucleotide identity among the previously known lyssaviruses was 63.5%–80.0% in this particular fragment, and the lowest identities among strains belonging to the same lyssavirus were 80.4% for ABLV and 79.9% for LBV (the most distant LBV strain has been suggested to be a different lyssavirus) (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/5/12-1071-Techapp1.pdf).

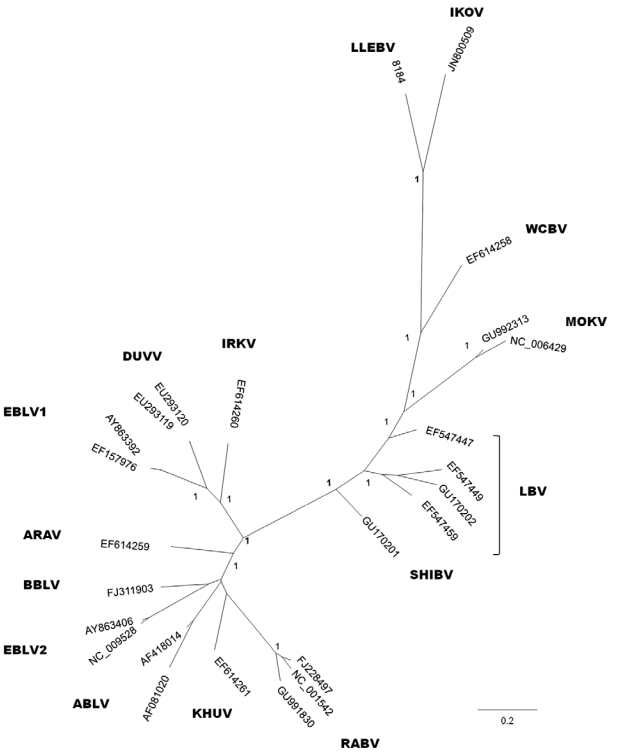

Figure. Phylogenetic reconstruction based on the first 405 nt of the nucleoprotein gene, including all representative lyssaviruses. The tree was obtained by Bayesian inference, and the first 25% of trees were excluded from the analysis as burn-in. Node numbers indicate posterior probabilities. ARAV, Aravan virus; ABLV, Australian bat lyssavirus; BBLV, Bokeloh bat lyssavirus; DUVV, Duvenhage virus; EBLV-1 and EBLV-2, European bat lyssavirus types 1 and 2; IRKV, Irkut virus; KHUV, Khujand virus; LBV, Lagos bat virus (lineages A, B, C, and D); MOKV, Mokola virus; RABV, rabies virus; SHIBV, Shimoni bat virus; WCBV, West Caucasian bat virus; IKOV, Ikoma lyssa virus; LLEBV, Lleida bat lyssavirus (proposed). Scale bar indicates expected number of substitutions per site.

Conclusions

The lyssavirus-specific antigen reactivity and association with a genomic sequence found in a bent-winged bat in northeastern Spain could be derived from the tentative new virus LLEBV. According to our phylogenetic reconstruction, the virus does not seem to belong to phylogroup I, which comprises most bat lyssaviruses, or to the African phylogroup II. The evolutionary relationships between the LLEBV sequence with WCBV and IKOV sequences need to be clarified before it can be determined whether they form >1 different phylogroups.

Of note, the new LLEBV was detected in *M. schreibersii* bats, as was WCBV, the other European lyssavirus outside phylogroup I. The genus *Miniopterus* has traditionally been considered to belong to the family Vespertilionidae as do other bat genera linked to lyssaviruses in Eurasia (*Eptesicus, Myotis,* and *Murina*). However, recent molecular analyses have confirmed that the genus *Miniopterus* belongs to the family Miniopteridae (*12*). *M. schreibersii* bats are migratory, widely distributed across southern Europe and Eurasia. Large numbers (thousands) of these bats overwinter in caves and move in the spring to different and sometimes distant summer roosts for reproduction (*13*). These ecologic features make it relatively easy for an infectious agent to quickly spread out within and among the populations.

Consequently, it is difficult to imagine that WCBV or LLEBV are locally restricted; both could be located far from where they were found. Neutralizing antibodies against WCBV have been found in bats in Africa (*14*). The cumulative description of new bat lyssaviruses in recent years shows the convenience of always using generic amplification primers for rabies diagnosis based on RT-PCR to complement antigen detection.

No human exposure to the new virus has been reported. However, because of the divergence exhibited by LLEBV and IKOV, and the growing evidence of inadequate protection/cross-neutralization against viruses outside phylogroup I, the effectiveness of current rabies vaccines for these viruses remains a concern (*15*).

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Table of contents
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Technical Appendix

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. LLEBV		71,6	68,6	68,1	67,6	68,2	67,0	67,3	67,4	66,6	67,7	65,7	65,9	67,2	68,6	65,7	67,2	66,1	65,8	65,5	65,4	64,7	66,3	63,7	64,0
2. JN80050 IKOV	71,6		63,7	67,4	66,2	65,4	65,4	64,5	69,5	65,3	64,6	66,0	64,0	68,1	64,9	63,5	64,7	66,0	65,0	64,5	64,0	64,2	63,8	65,3	66,0
3. GU170201 SHIBV	68,6	63,7		73,6	73,1	72,3	71,4	75,8	71,1	69,6	71,1	75,8	76,0	76,8	80,0	73,3	74,3	72,8	71,9	71,6	71,1	71,4	72,3	70,9	69,6
4. EF614260 IRKV	68,1	67,4	73,6		73,8	75,8	75,1	72,3	68,6	71,6	74,3	71,1	70,9	72,9	72,6	70,4	72,7	73,6	77,8	77,8	73,6	73,3	70,1	76,8	77,0
5. EF614261 KHUV	68,1	66,7	73,1	73,8		79,8	77,8	77,3	67,5	72,8	79,0	70,1	68,6	72,1	67,9	67,9	67,2	77,3	73,1	72,6	75,6	73,1	72,6	72,3	72,3
6. NC_009528 EBLV2	68,4	65,9	72,3	75,8	79,8		95,6	76,8	70,7	72,8	75,3	71,4	68,9	72,8	70,1	68,4	70,2	78,3	72,8	73,1	72,1	70,6	70,6	72,6	71,6
7. AY863406 EBLV2	67,2	65,7	71,4	75,1	77,8	95,6		76,5	70,4	74,1	75,8	72,3	69,9	73,8	68,9	68,2	68,2	80,0	73,6	73,8	74,1	70,9	70,1	74,8	73,8
8. EF614259 ARAV	67,7	64,7	75,8	72,3	77,3	76,8	76,5		70,6	73,6	76,8	73,6	72,8	73,8	72,3	72,1	73,1	77,3	73,3	73,1	71,9	70,9	73,6	75,3	75,6
9. EF61425 WCBV	67,4	69,6	71,1	68,6	67,7	70,9	70,6	70,6		68,1	69,7	72,8	74,1	71,9	74,1	69,4	71,1	70,7	70,1	69,9	67,2	66,9	66,9	68,1	67,9
10. AF081020 ABLV	66,9	65,9	69,6	71,6	72,8	72,8	74,1	73,6	68,1		84,4	71,9	72,1	72,1	71,1	72,3	70,4	74,8	72,8	73,3	72,6	73,6	72,6	72,8	73,1
11. AF418014 ABLV	67,9	64,9	71,1	74,3	79,0	75,3	75,8	76,8	69,9	84,4		71,1	74,1	72,1	69,6	72,8	72,6	79,8	76,3	75,8	76,0	74,8	74,8	72,6	72,6
12. EF547447 LBV	65,7	66,2	75,8	71,1	70,1	71,4	72,3	73,6	72,8	71,9	71,1		78,0	79,3	79,8	76,8	74,1	71,1	69,6	69,9	74,1	71,4	73,3	71,4	72,3
13. EF547449 LBV	65,9	64,2	76,0	70,9	68,6	68,9	69,9	72,8	74,1	72,1	74,1	78,0		81,2	81,2	75,8	73,6	72,8	73,1	72,6	70,1	70,1	71,9	71,4	70,9
14. GU170202 LBV	67,2	68,6	76,8	73,1	72,1	72,8	73,8	73,8	71,9	72,1	72,1	79,3	81,2		78,8	72,6	71,9	70,4	72,8	72, 1	71,4	72,3	73,1	71,4	72,3
15. EF547459 LBV	68,6	65,2	80,0	72,6	67,9	70,1	68,9	72,3	74,1	71,1	69,6	79,8	81,2	78,8		72,6	73,3	70,1	71,6	70,6	69,6	70,4	71,6	69,6	71,1
16. GU992313 MOKV	65,7	63,5	73,3	70,4	67,9	68,4	68,4	72,1	69,4	72,3	72,8	76,8	75,8	72,6	72,6		88,9	69,9	72,6	72,3	71,4	72,1	71,6	68,1	68,1
17. NC_006429 MOKV	67,2	64,7	74,3	72,8	67,2	70,4	68,4	73,1	71,1	70,4	72,6	74,1	73,6	71,9	73,3	88,9		69,6	72,4	71,9	70,2	70,5	69,9	68,5	68,7
18. JF311903 BBLV	66,4	66,7	72,8	73,6	77,3	78,3	80,0	77,3	70,9	74,8	79,8	71,1	72,8	70,4	70,1	69,9	69,6		72,6	72,3	75,1	72,3	73,1	74,1	73,6
19. EU293119 DUVV	65,9	65,2	71,9	77,8	73,1	72,8	73,6	73,3	70,1	72,8	76,3	69,6	73,1	72,8	71,6	72,6	72,6	72,6		99,0	72,6	70,9	70,6	78,8	78,0
20. EU293120 DUVV	65,7	64,7	71,6	77,8	72,6	73,1	73,8	73,1	69,9	73,3	75,8	69,9	72,6	72,1	70,6	72,3	72,1	72,3	99,0		73,1	70,9	71,1	78,0	77,3
21. FJ228497 RABV	65,4	64,0	71,1	73,6	75,6	72,1	74,1	71,9	67,2	72,6	76,0	74,1	70,1	71,4	69,6	71,4	70,4	75,1	72,6	73,1		92,3	86,2	72,1	71,6
22. NC_001542 RABV	64,7	64,2	71,4	73,3	73,1	70,6	70,9	70,9	66,9	73,6	74,8	71,4	70,1	72,3	70,4	72,1	70,9	72,3	70,9	70,9	92,3		85,4	70,9	69,6
23. GU991830 RABV	66,4	64,0	72,3	70,1	72,6	70,6	70,1	73,6	66,9	72,6	74,8	73,3	72,1	73,1	71,6	71,6	69,9	73,1	70,6	71,1	86,2	85,4		70,6	70,4
24. AY863392 EBLV1	63,7	65,4	70,9	76,8	72,3	72,6	74,8	75,3	68,1	72,8	72,6	71,4	71,4	71,4	69,6	68,1	68,6	74,1	78,8	78,0	72,1	70,9	70,6		95,1
25. EF157976 EBLV1	64,0	66,2	69,6	77,0	72,3	71,6	73,8	75,6	67,9	73,1	72,6	72,3	70,9	72,3	71,1	68,1	68,9	73,6	78,0	77,3	71,6	69,6	70,4	95,1	

Similarity
Identity