

## Research Article

# Rabies in Spain. A Peculiarity in Eurasia

Patricia Mingo-Casas<sup>1</sup>, Virginia Sandonis<sup>1</sup>, Sonia Vázquez-Morón<sup>1</sup>, José Miguel Berciano<sup>1</sup>, Javier Juste<sup>2,3</sup>, Juan E. Echevarría<sup>1,3\*</sup>

<sup>1</sup>National Center for Microbiology, Instituto de Salud Carlos III, Spain

<sup>2</sup>Estación Biológica de Doñana, CSIC, Spain

<sup>3</sup>CIBER de Epidemiología y Salud Pública. CIBERESP, Spain

## \*Corresponding author

Juan E. Echevarría Mayo, National Center for Microbiology, Instituto de Salud Carlos III; Carretera de, Majadahonda-Pozuelo km 2: Majadahonda 28220, Madrid, Spain, Tel: 34-91-822-3676; Email: jeecheva@isciii.es

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## Abstract

Spain accounts for some natural and political features which have influenced the situation of rabies, making it peculiar in the context of Europe. Dog rabies was eradicated from Spain in 1965 and the European vulpine epizootic was stopped by the French Oral Rabies Vaccination programs (ORVs) and it never crossed the Pyrenees this fact implying the first peculiarity. The possibility of the movement of infected animals from endemic territories in North Africa into the Spanish territories of Ceuta and Melilla by land, causing imported cases regularly is another peculiarity of rabies in Spain. Indeed, the existence of an intense maritime traffic from these endemic territories into the Spanish mainland is another peculiar feature which constitutes a permanent high risk for rabies introduction in Spain. European bat lyssavirus 1 is the most frequently lyssavirus found infecting bats in Spain, as occurs in most European countries. *Eptesicus serotinus* accounts for more than the 95% of infected bats declared in Europe and are considered the natural reservoir of EBLV-1. However, in the southern half of the Iberian Peninsula, *E. serotinus* is replaced by the sibling specie *Eptesicus isabellinus*, which has been found frequently infected by EBLV-1. *E. isabellinus* is present also in the other side of the Gibraltar Strait along a Mediterranean strip in Northern Africa. This constitutes another peculiarity of rabies in Spain. Finally, a new putative lyssavirus called Lleida Bat Lyssavirus (LLEBV) has been reported from a *Miniopterus scheibersii* bat from Catalonia. It still remains as another peculiarity of rabies in Spain.

## Keywords

- Rabies
- Lyssavirus
- EBLV-1
- Bats
- *Eptesicus serotinus*

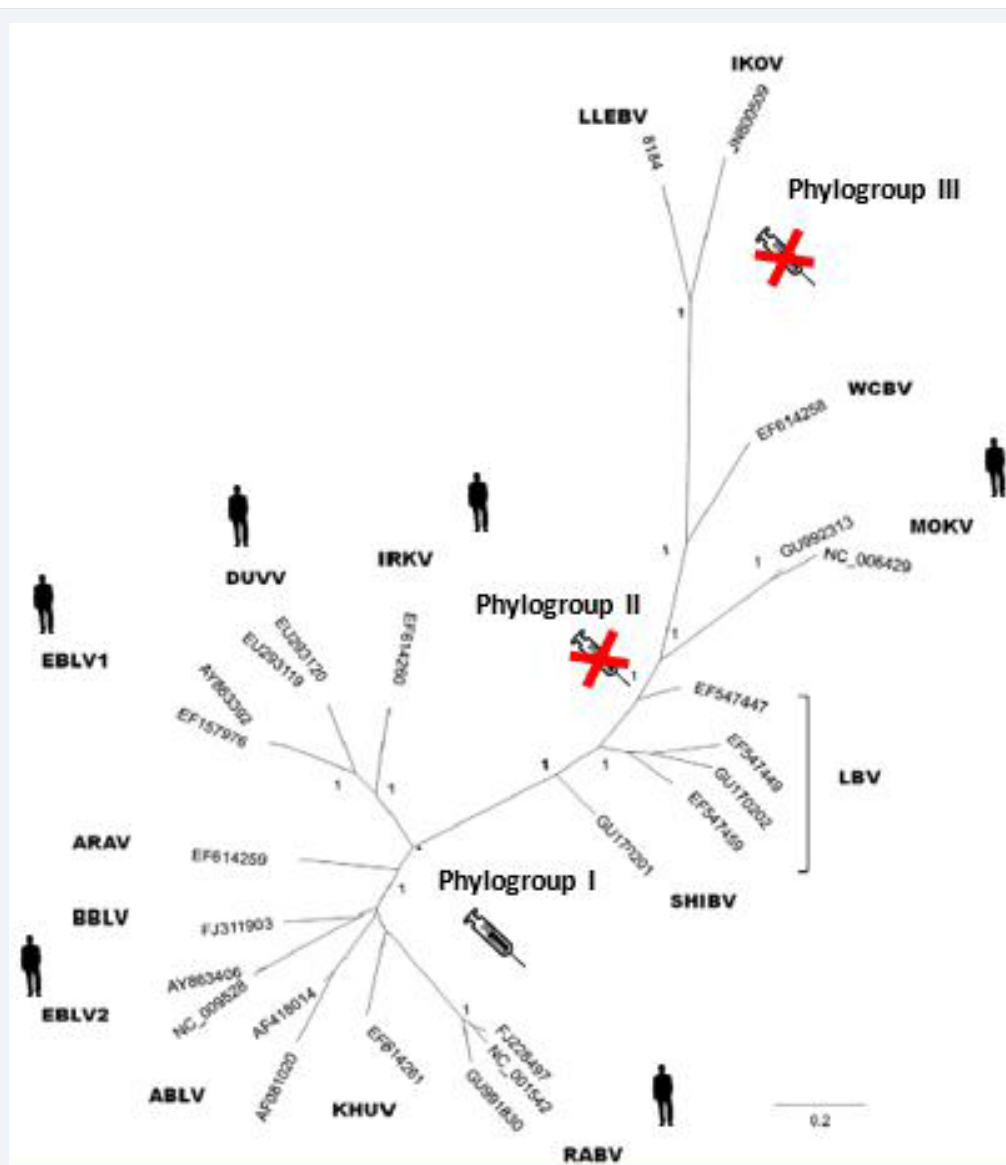
## INTRODUCTION

Rabies is one of the most feared zoonoses due to its extreme and dramatic lethality. It is known since, at least, the Greek ancient world [1] although some previous allusions to dog "madness" found on Babylonian and pre-Babylonian scripts suggest an even more ancient knowledge of the disease. It is caused by up to sixteen different viruses within the genus Lyssavirus [2,3]. They belong to the family *Rhabdoviridae*. Their single-stranded RNA genome of negative sense encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the RNA-dependent RNA polymerase (L). Most mammals can be infected by lyssaviruses and develop rabies, but only those from the orders *Carnivora* (dogs, skunks, racoons, etc) and *Chiroptera* (bats) are reservoirs able to maintain long-term transmission chains enabling the virus to persist. Most lyssaviruses are hosted by bats, which are considered the ancestral reservoirs of the genus. Lyssaviruses would have further switched to carnivores [4]. Classical rabies virus (RABV) is transmitted by dogs and accounts for virtually all human cases. Occasional cases have been caused by other lyssaviruses [3]. The annual toll of human rabies is estimated in about 59.000 deaths, mostly in Subsaharian Africa and the Indian subcontinent, despite lyssaviruses being present worldwide [5].

The reason for this dramatic toll is the insufficient attention and resources dedicated to rabies control in these developing areas. In fact, vaccines have been available since the end of the nineteenth century for the control of animal rabies and pre and post-exposure immune-prophylaxis in humans. For that reason, rabies is one of the neglected diseases recognised by WHO (<http://www.who.int/mediacentre/factsheets/fs099/en/>).

Fourteen of the sixteen lyssaviruses currently recognised, or proposed to the ICTV (<https://talk.ictvonline.org/taxonomy/>), are hosted by bats. Rabies Virus (RABV) is the only one of them linked also to terrestrial mammals. In fact, RABV is present only in bats from America where other lyssaviruses are absent. Lyssaviruses are divided into three different Phylogroups (Figure 1). Most of those that have been found infecting humans belongs to phylogroup I, and are neutralised by vaccine induced antibodies. However, lyssaviruses belonging to phylogroups other than I do not show cross neutralisation with RABV and consequently are not covered by the current vaccines, which contains RABV as immunogen [3].

Eurasia accounts for nine of the sixteen currently recognised or proposed species of lyssaviruses. It has been hypothesized to be their original land instead of Africa, as it was previously



**Figure 1** Lyssavirus phylogenetic relationships. Vaccine preventable and non- preventable Lyssavirus are shown as well as those that have caused human infections. Taken and modify from (19).

proposed [6], although it has been challenged out recently [7]. Seven Eurasian lyssaviruses, all belonging to phylogroup I, are linked to vespertilionid bats from temperate areas: the European bat lyssavirus 1 (EBLV-1), European bat lyssavirus 2 (EBLV-2), Bokeloh bat lyssavirus (BBLV), Aravan lyssavirus (ARAV), Khujand lyssavirus (KHUV), Irkut lyssavirus (IRKV). The Gannoruwa bat lyssavirus (GBLV) has been recently described from fruit bats in the Tropics [8]. The West Caucasian bat lyssavirus (WCBV) and Lleida bat lyssavirus (LLEBV), which are linked to bent-wing bats of the family *Miniopteriidae*, form the phylogenetically distant phylogroup III together with the African Ikoma bat lyssavirus (IKOV) [9].

Eurasian lyssaviruses have caused five rabies cases in humans. Two cases were infected by EBLV-2, other two by EBLV-1 [10], and the last one was caused by IRKV [11]. Lyssavirus cross-species transmission has also been observed in sheep, a cat, as well as in a

stone marten, all them infected by EBLV-1 [12,13,14]. None of the phylogroup III lyssaviruses has been ever found infecting humans. The Iberian Peninsula constitutes the South-Western edge of the Eurasian continent. It is separated from Morocco in the African Continent by just the fourteen kilometres (Km) sea channel namely the Strait of Gibraltar. On the other extreme, the Iberian Peninsula relates to the rest of Europe through a four hundred km land strip at the north-east, which is occupied by the Pyrenees mountains, except for lowland coastal passes at the two edges. This mountain range makes up a significant natural barrier and a limitation for wildlife's connections between the Iberian Peninsula and the rest of Europe. Three different countries are found within this peninsular territory, Spain, Portugal and Andorra. Spain also comprises the insular territories of the Balearic Islands at the Mediterranean Sea and the Canary Islands at the Atlantic Ocean, as well as two autonomous cities (Ceuta and Melilla) placed in North Africa and bordering with Morocco.

Spain is a member state of the European Union and part of the free movement area of Schengen, which was implemented in 1995. At the same time, Spain supports a heavy constant traffic of people, vehicles and goods across its southern maritime and terrestrial borders with Africa. All these peculiar natural and political features have influenced the situation of rabies in Spain.

### Rabies in dogs

Rabies was endemic in dogs across all Europe until it was progressively eliminated during the XX century [1], showing the feasibility of dog rabies elimination as a target when enough resources are dedicated to it. However, during the decade of the forties, a fox epizooty started at the border between Russia and Poland, and spread across Europe and at its maximum extension in the early nineties reached Southern France. At this point, some European governments started campaigns of fox vaccination with oral vaccines disposed in baits, which have turned back vulpine rabies almost to the original area. Dog rabies was eradicated from Spain in 1965 and the European vulpine epizootic was stopped by the French Oral Rabies Vaccination programs (ORVs) and it never crossed the Pyrenees; this fact implying the first peculiarity about rabies in Spain (<http://www.who-rabies-bulletin.org/>); [1].

Nevertheless, during 1975 a dog rabies outbreak was declared on the province of Málaga, at the southern coast of Spain. The origin of the outbreak could not be traced, but an importation from North Africa remains as the most likely hypothesis due to the geographical proximity and the intense maritime traffic with this area. The outbreak took four years to be controlled and caused 129 animal cases and one human fatality [1]. This rabies outbreak mainly affected domestic animals, particularly dogs and cats. However, in 1977, when the outbreak was almost controlled, the diagnosis of two cases in foxes raised up the alarm due to a potential spread into the fox population that would have led to the establishment of a sylvatic rabies cycle. Fortunately, it never happened [1]. An insufficient fox population density or a reduced sensitivity of the fox to that particular RABV strain causing the outbreak to remain as the most plausible hypothetical explanations. The last endemic human case of rabies in the Iberian Peninsula occurred during that outbreak. No more human cases were declared, until an imported case from Morocco was diagnosed in 2014 in Madrid.

After this outbreak in Málaga, dog rabies cases have been declared regularly only in the North African Spanish cities of Ceuta and Melilla (Table 1). A total of 122 cases have been declared from 1987 to date, out of them 78.7% of them from Melilla. Most of these cases (93,4%) were diagnosed in dogs. The temporal distribution along this period (1987-2016) shows time periods free of cases longer than the regular incubation period for rabies in the presence of a strict surveillance system, evidencing a pattern of consecutive events of importation without sustained circulation into the cities. This is in agreement with the fact that vaccination of dogs, cats and ferrets is encouraged by the authorities and free of charge for the owners in both cities. The imported strains in each city belong to different Moroccan lineages circulating in the adjacent areas. Interestingly, some of the lineages imported during the eighties and early nineties in both cities, seem to have become extinct [15]. Although, both cities have been free of human rabies since the last two

cases were declared in Melilla in 1979, an imported case was diagnosed in Ceuta in 2004 in an Austrian tourist who acquired the infection in Morocco [16]. The possibility of the movement of infected animals from endemic territories in North Africa into Spanish territory by land is another peculiarity of rabies in Spain. There is evidence of several cases of illegal importation of infected dogs from Morocco into Spain, that were transported by car across the Iberian Peninsula during the incubation period that develop disease and cause epidemiological alerts in their countries of destination, mostly France but also Germany [17]. Another dog case was imported from Morocco to Spain and then to The Netherlands by aircraft [18]. The existence of intense car traffic from territories endemic to canine rabies into the Spanish mainland is another peculiar feature which constitutes a permanent substantial risk for rabies introduction in Spain [19]. In fact, on year 2013 a rabid dog was imported into the city of Toledo, 68 Km far from Madrid, causing injuries to several people and an epidemiological alert which was extended for six months. The dog was vaccinated by the owners in Spain but introduced into Morocco few days after without following the regulations for pet movements to endemic countries. The dog became infected in Morocco and returned illegally back into Spain four months after to cause the alert. A total of 245 people required post-exposure immune prophylaxis and 335 animal aggressions were notified and studied. No other infected animal was detected and the rabies-free status was restored for Spain on December 2013 (<http://www.who-rabies-bulletin.org/>); [20,21].

### Bat Rabies

The Iberian Peninsula accounts for a large proportion of the total bat diversity found in Europe. According to the most recent systematic classification within the Order Chiroptera, four different families are found in Europe: *Molossidae* (one species), *Miniopteridae* (one species), *Rhinolophidae* (five species) and *Vespertilionidae* (36 species) (modified from [22]), all them present in Spain. The actual official number of species recognized in Spain is 34 ([http://secemu.org/wp-content/uploads/2016/02/LISTADO-IB%C3%89RICO-DE-QUIR%C3%93PTEROS\\_todas-las-lenguas\\_.pdf](http://secemu.org/wp-content/uploads/2016/02/LISTADO-IB%C3%89RICO-DE-QUIR%C3%93PTEROS_todas-las-lenguas_.pdf))

More than 95% of the rabid bats detected in Europe were serotine bats, *Eptesicus serotinus*, infected by EBLV-1, which is covered by rabies vaccines. Thirty five of the thirty-six infected bats detected in Spain to now were also infected by EBLV-1 [23,24]. However, the Iberian Peninsula is the only area of Europe with two different reservoir bat species of genus *Eptesicus*. In the southern half of the Iberian Peninsula, *E. serotinus* is replaced by the sibling specie, *E. isabellinus*, which is present also in the other side of the Gibraltar Strait along a Mediterranean strip in Northern Africa [25]. This constitutes another peculiarity of rabies in Spain. The existence of genetic flow between the populations of *E. isabellinus* from Southern Spain and Northern Morocco has been documented [26] opening the possibility of the presence of EBLV-1 in Africa, which had been previously hypothesized. In fact, it was even suggested that Africa could be the original area of dispersion for EBLV-1 [27]. Interestingly, the Strait of Gibraltar does not seem to be a barrier itself to gene flow for some other bat species, which show very similar genetic signature on both sides of the Strait between the African and European populations [28].

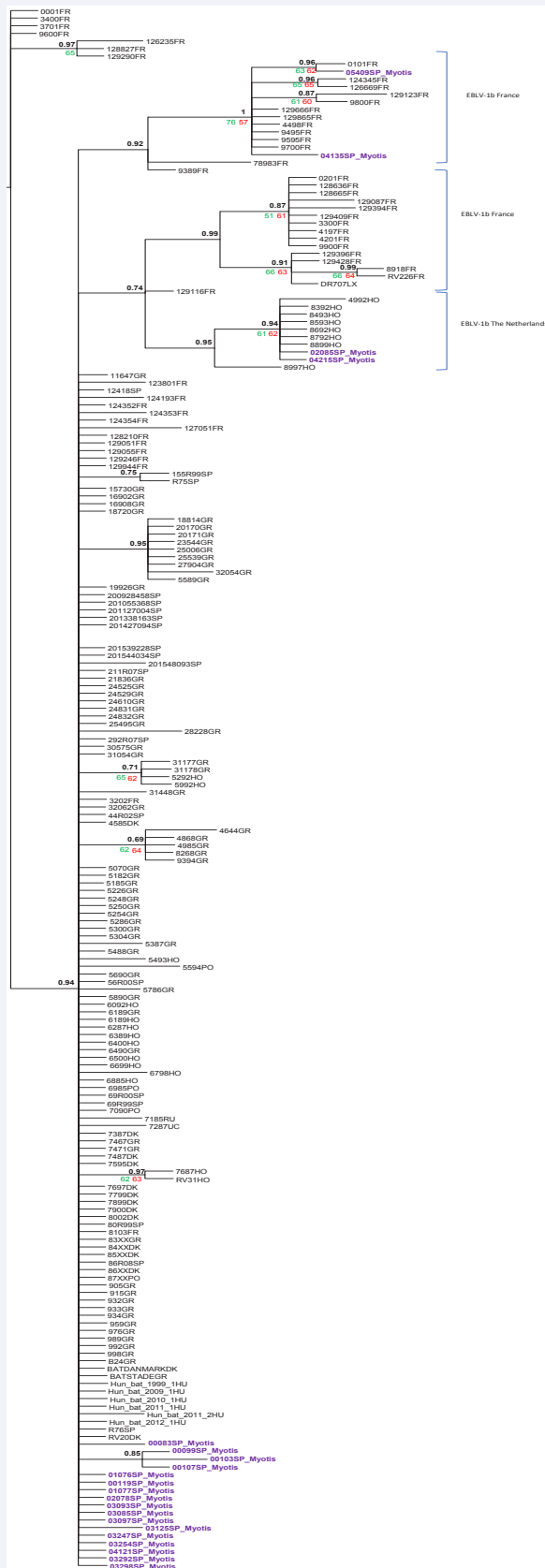
**Table 1:** Total cases of animal rabies declared into the Spanish North African cities of Ceuta and Melilla.

Year	Ceuta	Melilla	Total
1987	6 (3 dogs, 3 cats)	2 dogs	8
1988	1 dog	3 dogs	4
1989	0	1 dog	1
1990	0	6 dogs	6
1991	5 dogs	3 dogs	8
1992	5 dogs	7 dogs	12
1993	1 dog	4 dogs	5
1994	0	2 dogs	2
1995	0	6 (5 dogs, 1 cat)	6
1996	1 dog	0	1
1997	0	5 (4 dogs, 1 horse)	5
1998	3 dogs	4 dogs	7
1999	0	3 dogs	3
2000	0	2 dogs	2
2001	0	9 dogs	9
2002	0	7 (6 dogs, 1 horse)	7
2003	0	1 dog	1
2004	0	1 dog	1
2005	0	1 dog	1
2006	1 dog	0	1
2007	0	0	0
2008	1 dog	1 dog	2
2009	1 dog	1 dog	3
2010	0	2 dogs	2
2011	0	0	0
2012	1 dog	2 dogs	3
2013	0	6 (5 dogs, 1 fox)	6
2014	0	4 dogs	4
2015	0	5 dogs, 1 cat	6
2016	0	6 dogs	6

EBLV-1 is divided in two main lineages or subtypes: EBLV-1a and EBLV-1b [29]. The first subtype is mainly found along the northern edge of the distribution area of EBLV-1 in Europe, in an east-west axis from The Netherlands to Ukraine [29], although EBLV-1a has also been recently described from Southern France [30]. In contrast, the subtype EBLV-1b has been reported from France, southern Germany and The Netherlands [29].

EBLV-1 has been declared so far, only from *E. isabellinus* in Spain, forming an exclusive monophyletic clade related to the EBLV-1b subtype but with a not well defined phylogenetic position within [24]. The dynamics of the EBLV-1 infection in *E. isabellinus* in bat colonies from Andalusia (southern Spain) has been studied in the context of different studies based on the capture and further release of bats after being sampled for oro-pharyngeal swabs and blood. The saliva and blood samples were tested in the lab for viral RNA and EBLV-1-specific antibodies [31-34]. The results of these studies strongly support the idea that this virus follows endemic patterns of circulation within *E. isabellinus* maternal colonies, which behave as close communities for viral circulation. The most general expression of viral infection seems to be generally mild with bats positive for EBLV-1 RNA captured in flight showing a normal behaviour, body condition index and seroprevalences up to 25% in some of the colonies tested, evidencing long-term survival to

infection. Consequently, rabies does not seem to be the most usual expression of EBLV-1 infection in *E. isabellinus*. Similarly, both EBLV-1 specific antibodies, as well as EBLV-1 RNA in oro-pharyngeal swabs, have been reported from several colonies of *E. serotinus* in Germany [35]. The authors did not observed survival of any bat after transmission of EBLV-1 to *E. serotinus* under experimental conditions [36]. Finally, an *E. isabellinus* bat captured in flight which died during handling, tested positive in the brain by florescence antibody test (FAT), suggesting a kind of mild neurological infection. In fact, the pathological study did not show severe injuries, according to what it was expected from the FAT images [31, 32]. Similar results were reported after an outbreak of EBLV-1 infection in a captive colony of *Rousettus aegyptiacus* in a zoo in The Netherlands. The authors found several survivors showing reactivity for EBLV-1 in their brain weeks after the outbreak of rabies [37]. *R. aegyptiacus* is an African fruit bat species that tolerates and reproduce in captivity and is kept in zoos and exhibition parks along Europe. Although, the captive bats should descend from other locally captive bats, they could also have been illegally imported from Africa. In fact, a case of infection by Lagos Bat virus on one of these bats was declared in France [38]. Moreover, this bat species is also the natural reservoir of Marburg virus [39]. In the nineties of the past century several groups of *R. aegyptiacus* were established naturalised in the island of Tenerife (Canary Islands), probably



**Figure 2** Bayesian midpoint-rooted phylogenetic reconstruction of a 123 bp fragment of the N gene of all EBLV-1 strains associated to *Eptesicus* sp along Europe(black) and to *Myotis myotis* from a single cave in the island on Mallorca (42) (blue). Tree nodes show posterior probability (black) after two runs with  $10^6$  generations each. All used sequences are available in GenBank.



after escaping from a captive colony kept in an exhibition park ([http://www.mapama.gob.es/es/biodiversidad/temas/conservacion-de-especies/Rousettus\\_aegyptiacus\\_2013\\_tcm7-307158.pdf](http://www.mapama.gob.es/es/biodiversidad/temas/conservacion-de-especies/Rousettus_aegyptiacus_2013_tcm7-307158.pdf)) Fortunately, the control programs seem to have been successful, and the environmental authorities of the Canary Islands consider the specie eradicated from the wildlife at the present.

Generally, EBLV-1 has been rarely found in other species different than *Eptesicus serotinus* and *E. isabellinus* in the context of the passive surveillance system [29]. There are just a few cases of EBLV-1 on *Pipistrellus nathusii*, *Pipistrellus pipistrellus* and *Plecotus auritus* after enhanced passive surveillance in Germany, which are interpreted as spill-over events from *E. serotinus* [40]. In contrast, several studies reported, not only EBLV-1 antibodies, but also EBLV-1 RNA in blood pellets and brain of many other bat species collected in Spain [41-44]. There are similar reports of EBLV-1 antibodies, in bat species other than *Eptesicus sp* in Germany [35] and France [45]. The passive surveillance system focus on bats that, first show abnormal behaviour and second, interact with humans. Consequently, it is biased to synanthropic species as *P. pipistrellus*, *P. pygmaeus*, *E. serotinus* or *E. isabellinus*, as well as to individuals suffering neurologic symptoms. This could explain why EBLV-1 has never been diagnosed on species that show EBLV-1 antibodies on directed studies, but are underrepresented in the passive surveillance system. However, this is unlikely the explanation for species as *P. pipistrellus* or *P. pygmaeus* with thousands of individuals tested in diagnosis laboratories under passive surveillance, unless EBLV-1 does not cause severe symptoms in these species. Alternatively, these antibodies could be directed against unknown lyssavirus species which would be cross-reactive with EBLV-1 on antibody detection assays, but not reactive with FAT reagents, which does not seem very feasible. Despite the Serra-Cobo et al., report, amplification of short fragments of EBLV-1 RNA on 43 blood clots and 12 organs of bats belonging to seven different non-*Eptesicus* species captured in Spain [43], they do not provide with details on the amplified sequences, other than the closest BLAST match was EBLV-1. In a previous work on a specific *Myotis myotis* colony in a cave in the island of Mallorca, the frequent amplification of short fragments of EBLV-1 RNA was also reported in several individuals [42]. If we construct a phylogenetic tree using these sequences together with all sequences available in GenBank from *E. serotinus* and *E. isabellinus* along Europe (Appendix 1), the observed variability is quite surprising (Figure 2). In fact, the phylogenetic reconstruction recovers some groups within the EBLV-1b subtype with significant support (despite the small length of the amplified fragment), as well as other more local clusters. In this reconstruction, the EBLV sequences detected in a single colony of *M. myotis* from an island 170 km offshore from the continent, exhibit apparently similar variability than all detected sequences in *Eptesicus sp* for whole European continent. This is a fact that needs detailed clarification, as well as that this research group would make available the sequences from viruses published in more recent works and that are not provided.

The most striking and surprising finding on lyssavirus passive surveillance in Spain arose on year 2012, when a bent-wing bat (*M. schreibersii*) from the city of Lleida in Catalonia tested positive for FAT and lyssavirus-generic RT-PCR. The

nucleoprotein sequence suggested it was infected by a new lyssavirus which was named Lleida bat lyssavirus (LLEV). The most related species was IKOV, an African virus isolated from a civet. The second closest member of the genus was WCBV which was described originated from the same bat species in the northern slope of the Caucasus, in Russia. LLEV is very distant lineage from phylogroup I and consequently it could not be cross-reactive with vaccine induced antibodies, as it is the case for all the remaining non-phylogroup I lyssaviruses tested to now. This is an aspect that needs urgent clarification. Recently, the phylogenetic position of LLEV originally estimated from the N gene, has been confirmed with the entire genome [9,46]. LLEV still remains as a peculiarity of rabies in Spain. Despite EBLV-2, BBLV, ARAV, KHUJ and WCBV have been never detected in Spain, the reservoir species are present in Spain ([http://www.mapama.gob.es/es/biodiversidad/temas/inventarios-nacionales/pbl\\_ieet\\_mami\\_04\\_mamiferos\\_espana\\_tcm7-329832.pdf](http://www.mapama.gob.es/es/biodiversidad/temas/inventarios-nacionales/pbl_ieet_mami_04_mamiferos_espana_tcm7-329832.pdf)). However, none of them is well represented in the passive surveillance system [29]. Enhancement of the system to include these species, as well as active surveillance focused of them would be necessary to completely clarify if these lyssaviruses are present in Spain or not.

## CONCLUSIONS

To sum up, it seems that the Pyrenees could have hampered the establishment in the Iberian Peninsula of the fox rabies epizootic that affected most Europe, before the implementation of fox vaccination programmes with oral vaccine on baits in France, pushed it back definitively. Dog rabies was eliminated from Spain on 1965, but an outbreak was declared in the south-east from 1975 to 1978. Spain remains as a rabies-free Country after that, but infected dogs are frequently imported into Ceuta and Melilla from the adjacent endemic territories. The intense traffic movements from North Africa across the southern Spanish borders makes it difficult to control the illegal importation of infected dogs into the Spanish mainland, which remains as the highest risk factor for a potential reintroduction of this rabies. Spain is also the only place where LLEV has been detected and the Iberian Peninsula is the only part of Europe with two different reservoir species of the genus *Eptesicus* for EBLV-1. The southern Iberian populations of *E. isabellinus* are known to carry the virus and on the other hand, they may possibly keep genetic flow with the Moroccan populations. In fact, the Strait of Gibraltar constitutes an unsurmountable barrier for just a few bat species and therefore, it could have been the door for the expansion either of African lyssaviruses into Europe or of Eurasian lyssaviruses into Africa.

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