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## Active surveillance of bat rabies in France: a 5-year study (2004-2009)

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### Abstract

Active surveillance of bats in France started in 2004 with an analysis of 18 of the 45 bat species reported in Europe. Rabies antibodies were detected in six indigenous species, mainly in *Eptesicus serotinus* and *Myotis myotis*, suggesting previous contact with the EBLV-1 rabies virus. Nineteen of the 177 tested bats were shown serologically positive in seven sites, particularly in central and south-western France. Neither infectious viral particles nor viral genomes were detected in 173 and 308 tested oral swabs, respectively. The presence of neutralising antibodies in female bats (18.6%) was significantly higher than in males (5.6%).

**Key words:** Rabies, *Lyssavirus*, Bat, epidemiological surveillance, France

**Abbreviations:** BHK-21: Baby hamster kidney cells; EBLV: European bat lyssavirus; RTCIT: rabies tissue culture infection test; FAVNT: fluorescent antibody virus neutralisation test; hnRT-PCR: hemi-nested reverse-transcription polymerase chain reaction; TCID<sub>50</sub>: median tissue culture infective dose; 95% CI: 95% confidence interval.

### 1. Introduction

Rabies is a viral zoonosis that causes progressive and ultimately fatal encephalitis. Rabies is a very old disease and is responsible for approximately 55 000 human deaths per year worldwide (World Health Organisation, 2008). The rabies virus belongs to genus *Lyssavirus*, family Rhabdoviridae and can infect all mammals. The virus is usually transmitted through saliva in a bite from an infected animal.

The *Lyssavirus* genus includes 11 different recognised species (Bourhy et al., 1993; Carstens, 2010), also referred to as genotypes: “classical” rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), Australian bat lyssavirus (ABLV), European bat lyssavirus type 1 (EBLV-1) and European bat lyssavirus type 2 (EBLV-2). Four additional bat viruses (the Irkut, West Caucasian bat, Khujand and Aravan viruses) were recently ratified by the International Committee on Virus Taxonomy (Carstens, 2010). Ten of the 11 above-listed recognised virus species have been isolated from bats; only MOKV has not been isolated from bats (Sabeta et al., 2007).

Since the first case of bat rabies found in 1954, approximately 850 cases of rabid bats infected by EBLV-1 (also known as genotype 5 with two variants EBLV-1a and EBLV-1b) or EBLV-2 (genotype 6) have been reported in Europe (Muller et al., 2007). Most cases of bat rabies have been reported in European countries having a well-established rabies surveillance network (Germany, the Netherlands, Denmark, France and Great Britain). EBLV-1 seems to be adapted to *E. serotinus* (Van der Poel et al., 2000), species in which more than 95% of cases have been reported, while EBLV-2 appears to infect only *Myotis* bat species (*M. daubentonii* and *M. dasycneme*) with 20 cases, of which 10 have been reported in *M. daubentonii* in England (Banyard et al., 2009; Harris et al., 2009). Recently, another species (*E. isabellinus*) sibling species of *E. serotinus* and mainly found in Southern Iberia was shown to be infected with EBLV-1 (Vazquez-Moron et al., 2008b) and sporadic rabies cases have been found in *Pipistrellus pipistrellus*, *Pipistrellus nathusii*, *Plecotus auritus*, *Nyctalus noctula* (Muller et al., 2007) and *Vespertilio murinus* (Selimov et al., 1991).

National (Moutou et al., 2003) and European recommendations (Cliquet et al., 2010; Med Vet Net Working Group, 2005) encourage the continuation and reinforcement of bat rabies research and epidemiological surveillance.

Here, we report results from field studies carried out in France since 2004 in close collaboration with bat specialists, preventively vaccinated against rabies. The purpose of the present study was to investigate the circulation of EBLV-1 among indigenous bat species and identify species involved in the transmission of bat rabies. The seroprevalence of EBLV-1 was investigated in bats from 2004 to 2009 to improve current knowledge on the epidemiology of bats infected by EBLV-1.

## 2. Materials and Methods

### Sampling

From 2004 to 2009, 308 bats belonging to 18 species were captured at 18 sites located around the country (Figure 1). Surveillance was undertaken in several regions chosen for their proximity

to confirmed cases of EBLV-1 infection in *E. serotinus* (Limousin, Centre, Champagne-Ardenne, Lorraine, Midi-Pyrenees and Pays de la Loire). Bats were captured at night in roosts, along commuting routes, at swarming sites or in parturition roosts when the bats left to forage. Bat specialists identified bats to the species level using morphological criteria (Dietz and Von Helversen, 2004; Schober and Grimmberger, 1991). Several parameters were recorded for each bat: sex, reproductive status, age, body weight, forearm length, behaviour and parasite load. Oral swabs were taken to collect saliva from each trapped bat and stored in 1 mL of RNeasy lysis buffer (Qiagen, France), a buffer solution designed for RNA preservation, for subsequent hemi-nested reverse-transcription PCR (hnRT-PCR) (n=308 samples) and/or in a volume of 0.3 mL of DMEM culture (Dulbecco's minimum essential medium, Invitrogen, France) (n=173) for the rabies tissue culture infection test (RTCIT). Blood samples were taken from 185 bats for a modified fluorescent antibody virus neutralisation test (mFAVNt) on EBLV-1. After sampling, all bats were released at the site of capture at night. Capture, handling and sampling were undertaken with authorisation from the French Ministry of the Environment. Bat species, study sites and the number of collected blood and saliva samples are detailed in Table 1.

### **Laboratory methods**

Oral swabs stored in 0.3 mL of culture medium (n = 173) were analysed using RTCIT on murine neuroblastoma cells (ATCC:CCL31) (Barrat et al., 1988) and by RT-PCR (Picard-Meyer et al., 2004). The 135 oral swabs stored in RNeasy lysis buffer were tested only for the presence of EBLV-1 RNA by hnRT-PCR using universal primers (JW12-JW6) in the first round of PCR and specific primers (JW12-JEBL1) for the second round. RNA integrity was verified by amplifying 18S rRNA using a commercial Competimer system (Ambion, France) in each RT-PCR reaction.

To detect EBLV-1-specific neutralising antibodies in blood samples, a modified FAVN test was performed (Cliquet et al., 1998) with an EBLV-1 virus strain (ANSES, N°121411) isolated in France (2000). Samples were tested in threefold dilutions on BHK-21 cells with a starting dilution of 1/27. Controls included uninfected BHK-21 cells, OIE positive dog serum, negative dog serum and back-titration of the specific EBLV-1 virus. Levels of virus-neutralising antibodies are expressed in log D<sub>50</sub>. The threshold of antibody detection was calculated by using the Spearman-Kärber formula and set at 1.67 log D<sub>50</sub>.

The 95% confidence intervals (95% CI) of seroprevalence data were calculated using free open-source R software, version 2.8.1 (R Development Core Team, 2004).

## **3. Results**

### **Presence of EBLV-1 antibodies (mFAVN test)**

From 2004 to 2009, of the 185 bat blood samples, 4 contained insufficient quantities to perform mFAVNt, 177 yielded readable mFAVNt results and 4 were not interpretable.

Neutralising EBLV-1 antibodies were detected in 19 bats (range, 1.67 - 2.99 log D<sub>50</sub>). Of these antibody-positive bats, six species were identified: *E. serotinus* (n=7), *Myotis myotis* (n=5), *Myotis blythii* (n=1), *M. blythii* or *M. myotis* (n=2), *Miniopterus schreibersii* (n=2), *Barbastella barbastellus* (n=1) and *Rhinolophus ferrumequinum* (n=2). Antibodies to EBLV-1 were detected in bats from seven sites, stretching from south-western to central France (Table 2). Infection with EBLV-1a had already been reported in areas. Antibody-negative samples were recorded in central France (sites 6 and 7) and also in north-eastern France (sites 1 to 5), north-western France (site 8) and south-western France (sites 10, 15 and 16).

Of the 19 antibody-positive individuals, 13 were females and 6 were males. On the total bats population, seroprevalence among females (n=13; 18.6% seroprevalence; 95% CI, 10.64-30.02) was significantly higher ( $\chi^2=6.13$ ; P=0.013) than among males (n=6; 5.6% seroprevalence; 95% CI, 2.23-12.30).

#### **Presence of infectious viruses (RTCIT) and viral RNA from oral swabs (hnRT-PCR)**

All RTCIT samples (n=173) were negative for infectious viruses (Table 2). The 308 collected saliva samples were tested for the simultaneous presence of host 18S rRNA and viral EBLV-1 RNA. Host RNA was detected in 88% (n=271) of swabs; none of the tested samples were positive for EBLV-1 RNA (Table 2).

## **Discussion**

The purpose of the present study was to investigate the circulation of EBLV-1 among indigenous bat species in France. This study is the first report of EBLV-1 neutralising antibodies in bats in France (*B. barbastellus*, *E. serotinus*, *M. blythii*, *M. myotis*, *M. schreibersii* and *R. ferrumequinum*). Our results are consistent with previous field studies in Europe.

Several European bat species have been already shown to be serologically positive: *M. myotis*, *M. schreibersii*, *R. ferrumequinum* and *Tadarida teniotis* (Serra-Cobo et al., 2002). Antibodies have previously been detected in *E. serotinus* (Freuling et al., 2009a; Perez-Jorda et al., 1995; Vazquez-Moron et al., 2008a; Vazquez-Moron et al., 2008b) as well as in other species, such as *Plecotus sp.*, *M. natterei* (Klein et al., 2007) or, in southern Spain, *E. isabellinus* (Vazquez-Moron et al., 2008b).

In contrast, some hundreds of serum samples from 17 other bat species have tested serologically negative in Spain, Germany, Belgium (Freuling et al., 2009a) and in France. Seroprevalence is low [0.5 to 3.8%] in Daubenton's bats in the UK (Brookes et al., 2005) but in Belgium no EBLV-2 antibodies have been detected in this species. The main techniques used in Europe to determine the seroprevalence in bats are based on FAVNt, which directly measure the antigen-specific neutralising antibody response. These techniques have been adapted for

bat studies (in terms of screened viruses, sample dilutions and positivity thresholds). The differences in levels of rabies-neutralising antibodies may be due to a lack of harmonization of the serological technique (Cliquet et al., 1998; Freuling et al., 2009a) or to physiological stress in bats that may change the immune response with respect to lyssaviruses (Messenger et al., 2003). The level of detected antibodies by mFAVNt could be the result of serological cross-reactivity (Wright et al., 2010) between Lyssavirus (EBLV-2, WCBV or an yet unknown serologically Lyssavirus), even if the serological test has been adapted to EBLV-1. However, to date and despite the reinforcement of bat rabies passive surveillance, no other rabies virus than EBLV-1 was reported in France.

EBLV-1 antibodies in female bats are more frequent than in males, with respectively 18.6% [95% CI, 10.64-30.02] and 5.6% [95% CI, 2.23-12.30] of seroprevalence. This difference in seroprevalence may arise from the well-known gregarious behaviour of female bats. The presence of antibodies in females suggests that virus transmission occurs within the breeding colonies during social grooming, nursing or olfactory or lingual contact with body fluids. Adult male serotines, known to inhabit maternity colonies, may also play a role in virus transmission (Vos et al., 2007). Reproductive activity, also recently shown to be an important factor affecting rabies seroprevalence, may also play a role in virus transmission (Turmelle et al. 2010). In this study, males from only two bat species (*M. schreibersii* and *M. myotis*) were shown to be serologically positive. However, this observation must be interpreted with caution due to the limited sample size for mFAVNt analysis. Further investigation is needed, particularly in species that may contribute to the dispersal of EBLV-1. *M. myotis* has a large activity space, covering a radius of about 25 km (Schober and Grimmberger, 1991) and the distance between their summer and winter roosts varies between 20 and 253 km. In *M. schreibersii*, seasonal migration distances are greater than 350 km (Schober and Grimmberger, 1991) between Spain and France (Serra-Cobo and Balcells, 1986; Serra-Cobo et al., 1998). Serotine bats are considered to be sedentary and non-migratory, although distances of 10 to 45 km have been reported in central Europe (Havekost, 1960), given their strong philopatry for hibernation and breeding roosts.

The effect of lyssavirus infection in bat populations has still not been resolved and the presence of rabies antibodies in bat sera is difficult to interpret. It is currently unknown whether antibody-positive animals have ever been infectious and have recovered from a rabies infection or whether the presence of antibodies is evidence of a peripheral infection prevented by the immune response of the host (Constantine et al., 1968). Our study suggests that bats previously exposed to EBLVs, have recovered from infection and have developed a neutralising antibody response. This hypothesis is supported by recapture data on EBLV-1 in *M. myotis* species in

Spain, indicating that there may be cycles of infection and persistent immunity (Amengual et al., 2007). Detection of EBLV-1 antibodies in free-ranging bats probably demonstrates that bats have been exposed to rabies virus antigens, reflecting immunity rather than a viral incubation phase or ensuing illness (Shankar et al., 2004). The development of antibodies and subsequent immunity to rabies may be attained via frequent exposure to small viral loads during social contact among bats, through biting, scratching or grooming.

The presence of antibodies in six different bat species suggests that viruses are also transmitted among bats, as previously reported in Europe and in the Americas (Constantine et al., 1968; Serra-Cobo et al., 2002; Shankar et al., 2004; Smith et al., 2006; Turmelle et al., 2010). Bats share their roosts primarily with conspecifics, but also with other bat species (serotine bats are commonly observed with *P. pipistrellus*, *M. myotis*, *Nyctalus noctula* and *Vespertilio murinus* offering many opportunities for interspecific virus transmission (Freuling et al., 2009a; Vos et al., 2007).

In the present study, all tested bats appeared to be “healthy” and none exhibited any obvious clinical signs of rabies. No viral RNA was detected in RT-PCRs performed on oral swabs during the study. These data suggest that the virus was not excreted by the bats at the time of sampling, as demonstrated in several experimental bat infections (Franka et al., 2008; Freuling et al., 2009b; Johnson et al., 2008) and other active surveys (Brookes et al., 2005; Harris et al., 2009; Kuzmin et al., 2008). In contrast to studies in Spain, we did not detect any viral RNA in oral swabs (Echevarria et al., 2001; Vazquez-Moron et al., 2008b). This difference may be due to temporal fluctuations in viral RNA, to insufficient amounts of viral RNA for detection by RT-PCR or to the overall better apparent health status of all tested bats captured in flight.

The results of the present study suggest that serologically positive bats are concentrated in south-western France, where EBLV-1a-infected bats are most frequently encountered, with the first case of EBLV-1a found in 2003. In contrast, EBLV-1b infections and antibodies in bats do not appear to have the same geographical distribution as EBLV-1a. Further studies will be undertaken to determine the detailed distribution of antibody-positive bats in France.

## Conclusion

Continuing active and passive surveillance of bats will help increase knowledge of EBLV-1a and b distribution and epidemiology in France. Furthermore, owing to the numerous serological techniques used in European laboratories working on rabies, there is an urgent need to standardise and harmonise the serological testing of bat rabies in free-ranging bats.



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**Figure 1A:** Location of bat sampling sites in France. Circles indicate the location (number code given in Table 1) and circle size is proportional to the number of bats sampled.

**Figure 1B.** Geographical distribution of the 19 serologically positive bats with the localisation of the 51 EBLV-1-infected serotine bats diagnosed during passive surveillance. In all bats sampled during active surveillance, all saliva samples were negative for EBLV-1 RNA using the hnRT-PCR technique.

1 **Table 1:** Number of saliva (blood) samples collected, by bat species and by study site.  
2

Region	North-eastern France					Central France			North-western France	South-western France								
Species\Site no.	1	2	3	4	5	6	7	9	8	10	11	12	13	14	15	16	17	18
<i>R. euryale</i>										3 (0)			1 (0)				1 (0)	
<i>R. ferrumequinum</i>											3 (3)	1 (0)		2 (1)	2 (2)	2 (2)	9 (2)	2 (2)
<i>R. hipposideros</i>			7 (0)	3 (0)										1 (1)				
<i>B. barbastellus</i>			2 (0)											1 (1)	1 (1)		3 (0)	
<i>E. serotinus</i>						11 (0)	33 (0)	12 (12)			2 (2)	7 (7)		1 (1)	1 (1)		5 (5)	
<i>M. alcathoe</i>		2 (0)																
<i>M. bechsteinii</i>	2 (0)			1(0)	3 (3)												1 (0)	
<i>M. blythii</i>											3 (3)	2 (2)						
<i>M. daubentonii</i>	1 (0)				5 (5)							2 (2)		1 (0)	2 (2)	7 (3)	8 (5)	1 (1)
<i>M. emarginatus</i>	8 (0)				18 (16)												1 (0)	
<i>M. myotis</i>			1 (1)		8 (8)						1 (1)	17 (17)		4 (4)			9 (8)	
<i>M. myotis/blythii</i>											2 (2)	7 (7)				1 (1)		1 (1)
<i>M. mystacinus</i>	1 (0)				0 (0)				2 (2)									
<i>M. nattereri</i>	2 (0)		3(0)		3 (3)												2 (0)	
<i>M. schreibersii</i>													14 (14)	3 (3)			1 (0)	
<i>P. auritus</i>	1 (0)			1(0)	1 (1)				4 (4)								1 (0)	
<i>P. austriacus</i>									15 (12)						1 (1)			
<i>P. kuhlii</i>									1 (1)									
<i>P. pipistrellus</i>					1 (0)				9 (8)						6 (1)		1 (0)	
Total number of saliva samples (blood)	15 (0)	2 (0)	13 (1)	5 (0)	39 (36)	11 (0)	33 (0)	12 (12)	31 (27)	3 (0)	11 (11)	36 (35)	15 (14)	13 (11)	13 (8)	10 (6)	42 (20)	4 (4)

3 †: sites positive for EBLV-1 antibodies. Values give the number of collected saliva samples; the number of collected  
4 blood samples for mFAVN test is given in parentheses.

5 The municipalities of study sites numbered 1 to 18 are as follows: Tincry (1), Montiers sur Saulx (2), Neufchateau-  
6 Rebeville (3), Neufchateau-Landaville (4), Chamarandes-Choignes (5), Saint Loup des Chaumes (6), Saint Amand  
7 Montrond (7), Fontenay le Comte (8), Guéret (9), Lafage (10), Rocamadour (11), Gasques (12), Dunes (13), Loze  
8 (14), Feneyrols (15), Roussayrolles (16), Penne 1 & 2 (17), Saint Antonin Noble Val (18).

**Table 2:** Results of laboratory investigations on the presence of infectious rabies virus (RTCIT), viral RNA (hnRT-PCR) and the detection of EBLV-1 antibodies in bats (mFAVNt).

Species	RTCIT	hnRT-PCR	mFAVN test	Antibody detection in bats (mFAVNt)				Study Sites
				% with antibody	95% CI	F	M	
<i>R. euryale</i>	0 (4)	0 (5)	/	/	/	0 (0)	0 (0)	18
<i>R. hipposideros</i>	0 (11)	0 (11)	0 (1)	0	[0 – 95.5]	0 (1)	0 (0)	
<i>R. ferrumequinum</i>	0 (11)	0 (21)	1 (10)	10	[0.5 – 45.9]	1 (5)	0 (5)	
<i>B. barbastellus</i>	0 (6)	0 (7)	1 (2)	50	[9.5 – 90.5]	1 (2)	0 (0)	14
<i>E. serotinus</i>	0 (18)	0 (72)	7 (28)	25	[11.4 – 45.2]	7 (19)	0 (9)	9, 11, 12
<i>M. alcaethoe</i>	/	0 (1)	/	/	/	0 (0)	0 (0)	11
<i>M. bechsteini</i>	0 (7)	0 (5)	0 (3)	0	[0 – 69.0]	0 (0)	0 (3)	
<i>M. blythii</i>	/	0 (5)	1 (5)	20	[1.0 – 70.1]	1 (2)	0 (3)	
<i>M. daubentonii</i>	0 (15)	0 (22)	0 (17)	0	[0 – 22.9]	0 (5)	0 (12)	12, 14, 17
<i>M. emarginatus</i>	0 (27)	0 (10)	0 (16)	0	[0 – 24.1]	0 (1)	0 (15)	
<i>M. myotis</i>	0 (22)	0 (36)	5 (37)	13.5	[5.1 – 29.6]	2 (19)	3 (18)	
<i>M. myotis/blythii</i>	/	0 (11)	2 (11)	18.2	[3.2 – 52.2]	1 (4)	1 (7)	12, 18
<i>M. mystacinus</i>	0 (3)	0 (3)	0 (2)	0	[0 – 80.2]	0 (0)	0 (2)	13, 14
<i>M. nattereri</i>	0 (10)	0 (7)	0 (3)	0	[0 – 60.9]	0 (2)	0 (1)	
<i>M. schreibersii</i>	0 (4)	0 (18)	2 (17)	11.8	[2.1 – 37.7]	0 (6)	2 (11)	
<i>P. auritus</i>	0 (8)	0 (7)	0 (5)	0	[0 – 53.7]	0 (0)	0 (5)	
<i>P. austriacus</i>	0 (15)	0 (15)	0 (13)	0	[0 – 28.3]	0 (2)	0 (11)	
<i>P. kuhli</i>	0 (1)	/	0 (1)	0	[0 – 95.5]	0 (0)	0 (1)	13, 14
<i>P. pipistrellus</i>	0 (11)	0 (15)	0 (6)	0	[0 – 48.3]	0 (2)	0 (4)	
<b>Total</b>	<b>0/173</b>	<b>0/271</b>	<b>19/177</b>			<b>13 (70)</b>	<b>6 (107)</b>	

Values correspond to the number of positive samples using the RTCIT, hnRT-PCR or mFAVN test; the number given in parentheses gives the total number of tested samples.

Abbreviations: CI: confidence interval; F: female; M: male.



