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LYSSAVIRUS IN *EPTESICUS SEROTINUS* (CHIROPTERA: VESPERTILIONIDAE)

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ABSTRACT: To determine the prevalence of European Bat Lyssavirus 1 (EBL1), antibodies plasma samples were obtained from 175 serotine bats (*Eptesicus serotinus*) from four colonies in southern Spain between September of 1991 and September 1992. Five bats were detected with EBL1 virus in one colony in 1989. The prevalence of antibodies rose to 74% in one of the colonies studied (Villarrasa) in the spring of 1992. After a few months the prevalence declined to under 10%. Individuals with a high antibody level in the spring (up to ED_{50} = 280) had very low titers or no antibodies in the following summer and autumn.

Key words: *Lyssavirus* spp., serotine bat, *Eptesicus serotinus*, antibodies, prevalence.

INTRODUCTION

The first report of *Lyssavirus* spp. in bats in Europe was from Germany in 1954 (World Health Organization, 1986). In 1985 the virus was identified as belonging to serotype 4 of the genus *Lyssavirus*, closely related to the Duvenhage strain; until then this strain had been isolated only in South Africa (World Health Organization, 1986). Since 1985, cases have increased; 379 cases were reported between 1985 and 1989. Although nine different species of infected bats have been found, the serotine bat (*Eptesicus serotinus*) has composed 88% of the total tested and 95% of the specimens which could be identified (Kappeler, 1989).

Montaño-Hirose et al. (1990) and Bourhy et al. (1992) found that the virus infecting European bats differed from the Duvenhage and rabies virus, and the European strains have been assigned to a group of unclassified *Lyssavirus* called European Bat Lyssavirus (EBL). This group contains two distinct viruses: EBL1 primarily infects *E. serotinus* and EBL2 primarily infects *Myotis dasycneme* (Montaño-Hirose et al., 1990). The two subtypes have infected bats and at least three humans (Montaño-Hirose et al., 1990; Bourhy et al., 1992).

Knowledge of the epizootiology of *Lyssavirus* spp. infection in insectivorous bats is scant and is based exclusively on studies carried out in North America on rabies serotype 1. The epizootiology of bat rabies depends on the ecological and behavioral characteristics of the bats (Kaplan, 1985), which influences the maintenance of the virus in cycles independent of those of other species of bats and terrestrial mammals (Smith et al., 1990).

Our objective was to determine the prevalence of lyssavirus antibodies in *E. serotinus* bats in southern Spain over three seasons.

MATERIALS AND METHODS

The study was conducted in four colonies of *E. serotinus* in southern Spain. Villarrasa (Huelva) (37°25′N, 6°36′W) was a breeding colony of 100 to 200 females; Paterna del Campo (Huelva) (37°29′N, 6°32′W) was a breeding colony of about 20 females; Aznalcóllar (Sevilla) (37°32′N, 6°18′W) was a breeding colony of about 100 females; and Gerena (Sevilla) (37°31′N, 6°11′W) was composed of five to 10 individual males and females. The four locations lie approximately in a line ENE between Villarrasa and Gerena. The distance between the extreme colonies was 48 km and between adjacent colonies varied between 10 and 20 km.

To study the temporal pattern of the disease, blood samples were collected from bats at the breeding roosts. When possible, blood samples were obtained three different times from each colony: the first in spring (20 May to 5 June), before the pups were born; the second one in summer (20 July to 5 August), when the young

TABLE 1. Prevalence of lyssavirus antibody at the different colonies and seasons in southern Spain, 1991 to 1992, along with titers (ED_{50}) among the positive cases.

Site		Autumn[a] 1991	Spring[b] 1992	Summer[c] 1992	Autumn[d] 1992
Villarrasa	Number samples	16	35	39	21
	Prevalence	19%	74%	7.7%	4.8%
	ED_{50} (mean ± SD)	16 ± 10	139 ± 280	38 ± 24	9
	Range	9–28	9–1,412	11–56	9
Aznalcóllar	Number samples	ND[e]	16	21	10
	Prevalence	ND	0%	9.5%	0%
	ED_{50} (mean ± SD)	ND	NA[f]	15 ± 8	NA
	Range	ND	NA	9–21	NA
Paterna del Campo	Number samples	ND	ND	10	ND
	Prevalence	ND	ND	0%	ND
	ED_{50} (mean ± SD)	ND	ND	NA	ND
	Range	ND	ND	NA	ND
Gerena	Number samples	ND	4	ND	3
	Prevalence	ND	25%	ND	0%
	ED_{50} (mean ± SD)	ND	11	ND	NA
	Range	ND	11	ND	NA

[a] 25 September to 10 October.
[b] 20 May to 5 June.
[c] 20 July to 5 August.
[d] 24 and 25 September.
[e] Not done.
[f] Not applicable.

started to fly; and the third one in autumn (25 September to 10 October) before the last bats left the breeding roosts. All samples were collected between September 1991 and September 1992. With the exception of the Gerena colony, where the occasional adult male was captured, the individuals sampled were exclusively adult females and young of both sexes born that year.

The captures were made with locally made 3 × 1 × 0.3 m polyethylene bag traps with a metallic shell or mist nets (Kunz and Kurta, 1988) set at dusk when bats were about to leave the breeding refuges; the age (Anthony, 1988) and sex (Racey, 1988) were recorded, and the bats were marked with a numbered band (Lambournes Ltd., Birmingham, United Kingdom).

Blood (0.125 ml) was extracted by cardiac puncture (Kunz and Nagy, 1988), with 1 ml syringe and 0.4 by 12 mm needle (Terumo Europe, Leuven, Belgium), to yield 0.04 ml of blood plasma per specimen. The animals were restrained by hand. Each individual was released 15 minutes after completion of the procedure. At Villarrasa, blood was extracted from 21% of the bats captured in September 1991, 56% in May 1992, 45% in July 1992, and 64% in September 1994; blood was sampled from all individuals captured in the other colonies (Table 1). All blood samples (n = 175) were stored in tubes with heparin, and were stored at 4 C until the plasma was separated at 2,500 rpm for 10 min in a centrifuge. The plasma was stored in labeled vials at −20 C until tested.

The blood plasma samples were evaluated using the rapid fluorescent focus inhibition test (RFFIT) (Bourhy and Sureau, 1990) to determine the presence of rabies neutralizing antibodies. The tests were carried out in 96-well microtiter plates (Becton Dickinson Labware, Lincoln Park, New Jersey, USA). A viral strain isolated by inoculation in neuroblastoma cells (Muñoz-Cervera and Muñoz-Navarro, 1991) in a *E. serotinus* from the Villarrasa colony in July 1989 was used as a challenge virus; after passing the virus in mouse neuroblastomas several times, a fixed viral strain was obtained, of $10^{4.15}$ fluorescent focus doses infecting 50% of the cells (FFD_{50}).

Following heat inactivation at 56 C for 30 min, four serial dilutions of each serum sample (1:5, 1:25, 1:125, 1:625) each were mixed with a constant quantity of 50 FFD_{50}/0.05 ml of the control virus. The virus-plasma mixture was incubated for 90 min at 35 C, 5% CO_2 and 90% humidity. Cell suspension of Baby Hamster Kidney-21 cells, cell clone S13 (BHK_{21} S_{13}) (obtained from the Virolog Department, Carlos III Institute, Majadahonda, Madrid, Spain), contain-

ing 10^6 cells/ml with 10 μg/ml of diethylaminoethyl (DEAE) Dextran (Pharmacia AB Laboratory Separation Division, Uppsala, Sweden) were added to each virus-plasma mixture. The BHK_{21} S_{13} cells were propagated in Glasgow minimum essential medium (Gibco Ltd., Paisley, Scotland) and supplemented with 10% fetal calf serum (Gibco Ltd.), 10% tryptose phosphate broth (Difco Laboratories, Detroit, Michigan, USA), 200 IU/ml of penicillin (Level Laboratories, Barcelona, Spain), and 100 μg/ml of streptomycin (Cepa, Madrid, Spain). The microtiter plates then were incubated for 18 hr at 35 C, 5% CO_2 and 90% humidity. Afterwards, the cellular monolayers that had formed were fixed with acetone (80%) in distilled water for 30 min at 4 C, washed, and covered with antinucleocapside rabies antibodies marked with fluorescein isothiocyanate (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France).

Three dilutions of challenge virus were used for testing the titrations: 50 FFD_{50}, 10 positive fields with five to 10 fluorescent cells in each one; 5 FFD_{50}, five to 10 positive fields with one to five fluorescent cells in each one; and 0.5 FFD_{50}, five or fewer positive fields with one or more fluorescent cells in each one.

Each dilution of serum received 50 to 100 FFD_{50} of challenge virus. Presence of anti-EBL1 antibodies was determined by counting the fluorescent foci under an Optiphot ultraviolet microscope (Nikon, Tokyo, Japan) at ×100. As we did not have a control serum with anti-EBL1 antibodies, the titers (serum neutralization endpoint titer) were expressed as effective dose 50% (ED_{50}), defined as the highest serum dilution in which there is a 50% reduction in the number of fluorescent foci (Wallis et al., 1981); the ED_{50} was calculated using the Kärber method (Grist et al., 1979).

Starting in 1989 the 10 individuals found dead or ill, or which suffered serious damage (forearm fracture) during handling, in the Villarrasa colony, were collected for analysis of lyssavirus presence in the brains. Specimens were euthanised with sodium pentothal (Abbot, Madrid, Spain) given intraperitoneally; the dose was 35 mg per kg body mass. We also evaluated the blood of three of these 10 bats for the presence of rabies neutralizing antibodies in blood during the summer 1991. The brains were tested for rabies antigen using the direct immunofluorescence method (Dean and Abelseth, 1973). The results obtained were confirmed by the mouse inoculation test (Koprowski, 1973) and also with the inoculation in neuroblastoma cell lines (Neuro 2a) (Muñoz-Cervera and Muñoz-Navarro, 1991).

Annual minimum survivorship for adult and subadult females in the Villarrasa colony between 1989 and 1991 was estimated as the percentage of marked individuals still alive in the subsequent year.

This study was conducted under a permit issued by Agencia de Medio Ambiente de Andalucia, Sevilla, Spain.

The statistical analyses were done with Fischer's exact test (FREQ procedure) for prevalence and survival comparisons, and non-parametric analysis for rank with Tukey multiple range test (GLM procedure) for the titrations (SAS Institute Inc., 1989). At an individual level, the titration between consecutive samplings was independent (probability of Spearman correlation coefficient ≤ 0 was 0.975). For this reason we considered the different samplings carried out on the same individual as independent cases.

The Gerena and Paterna del Campo colonies were not considered in the statistical analyses due to the low number of individuals captured.

RESULTS

In May 1989, a 1-yr-old *E. serotinus* from Villarrasa colony was unable to fly; it was tested for rabies and was positive in all three tests. In July 1989 the brains of four other specimens, all less than 1-mo-old, were positive by direct immunofluorescence and cultivated cell inoculation; however, two of them were negative in the mouse inoculation test. Of the latter four specimens, only one was found dead; the others appeared to be in good health. Brains of four adult bats in 1991 and one in 1992 from Villarrasa colony were tested and found negative.

Antibodies were detected in three of the four colonies (Table 1). For all sampling periods, there were low antibody prevalences and titers except for those samples obtained in the spring of 1992 in Villarrasa, in which the prevalence ($P < 0.001$) and antibody titers ($F = 24.35$, df = 6, $P = 0.0001$; Tukey multiple range test) were significantly different. The spring 1992 sampling at Villarrasa was characterized by very high prevalence (74%) and mean titer (139 ED_{50}). Furthermore, half of the individuals had titers greater than 30 ED_{50}. These values dropped rapidly in a period of 2 mo to a prevalence of 10% and a mean titer of 38 ED_{50} (Table 1).

Twenty-two bats from the Villarrasa colony were captured repeatedly during the study period (19 twice and three on three occasions each). Six individuals captured without detectable antibodies in the autumn of 1991 were recaptured on the next spring with mean (±SD) titers of 308 ± 549 ED_{50} (range 11 to 1412); one of these bats, with a titer of 56 ED_{50} in spring, was recaptured the next summer and had no detectable antibodies. In spring 1992, we recorded seven individuals, with mean (±SD) titers of 65 ± 97 ED_{50} (range 9 to 280), that had a decline in their titers at the next summer or autumn; two other individuals had no antibodies. Eight bats captured before (n = 3) or after (n = 5) spring 1992 and recaptured later, did not have appreciable changes. At the first capture time, the mean (±SD) was 3 ± 5 ED_{50} (range 0 to 11); none had antibodies at the time of recapture.

Two individuals negative in the rabies antigen detection test (one captured in the summer of 1991 and the other in the spring of 1992) had antibody titers of 56 ED_{50}.

When we compared the two age groups considered (young of the year; all others) in each sampling period and by colony, we observed no significant differences in either the prevalence or the mean antibody titers, despite the fact that serum antibodies have never been detected in young specimens. The sample sizes for Villarrasa were eight young and eight adults in autumn 1991; 20 young and 19 adults in summer 1992; and five young and 16 adults in autumn 1992. Aznalcóllar, nine young and 12 adults in summer 1992. For Paterna del Campo, we collected seven young and three adults in the summer of 1992.

Bat survival in the Villarrasa colony was lower in 1989 for both young and adults alike. In 1989, 14 (33%) of 42 young and 71 (49%) of 145 adults survived. In 1990, 16 (55%) of 29 young and 50 (83%) of 60 adults survived. In 1991, 30 (61%) of 49 young and 70 (75%) of 93 adults survived. Annual differences were significant (P = 0.039) only for adults.

DISCUSSION

The prevalence of antibodies obtained in spring 1992 in the Villarrasa colony together with the individual development of antibody titers could be interpreted to be evidence for an epizootic, with a strong increase from low titers (in autumn 1991) followed by a rapid fall, in scarcely 2 mo, to titers similar to the previous autumn. However, the only individual examined in spring 1992 was negative for antigen in the brain; moreover, a correlation between antibody titers and the development of the rabies infection has not been established (Trimarchi, 1978).

Annual variations in prevalence based on a direct immunofluorescence test on brain tissue have been described in European *E. serotinus* with the EBL1 virus (Gaede, 1992).

During the study period we registered a high prevalence of lyssavirus antibodies and titers in only one *E. serotinus* sample. In *Myotis lucifugus*, which has colonies of similar size to *E. serotinus*, rabies serum antibodies prevalence also is low (Schowalter and Trimarchi, unpublished, cited in Rosatte, 1985). In contrast, Steece and Altenbach (1989) reported a constant prevalence of >60% rabies serum antibodies in *Tadarida brasiliensis*. These differences could be due to the conditions in which the rabies virus is maintained in the enormous colonies of *T. brasiliensis*. (Steece and Altenbach, 1989).

The fact that only the Villarrasa colony had lyssavirus infections in 1992 is evidence of existence of cycles independent of rabies infection in each colony; this circumstance would be favored by the strong philopatry of the young females and the scarce contact between the different colonies (J. L. Pérez-Jordá and C. Ibáñez, unpubl.). Antigenic variations of rabies virus (serotype 1) have been found in different populations of *Eptesicus fuscus* in North America, a sedentary species like *E. serotinus* (Smith, 1989). Smith (1989) proposed that, in this case, the virus would be

maintained within certain territorial boundaries or colonies via intraspecific transmissions, which would contribute to a compartmentalisation of the host-virus system.

Constantine (1986) found that rabies viral infection can occur just days after birth in *T. brasiliensis*. We believe that the same happens with EBL1, as demonstrated in the summer of 1989 by the four individuals from Villarrasa which, at less than 1-mo-old, already were infected.

The presence of the EBL1 virus in the brains of all five bats examined is evidence that in the spring and summer of 1989 there was a significant infectious epizootic in the Villarrasa colony; however, the probability of obtaining antigen presence in the brain in all three individuals chosen at random among 187 captured animals during a non-epizootic period was very low.

The effect of lyssavirus infection in populations of bats still has not been resolved. Many authors have shown that the effect of the disease on these populations is limited (Pybus, 1986; Grauballe et al., 1987; Juedes, 1987; Burnett, 1989). We found that animals with high lyssavirus antibody titers were recaptured months later with very low levels, and that in the case of two specimens with relatively high titers, the EBL1 virus was not found in their brains. This could be because *E. serotinus* also is capable of overcoming the infection. Steece and Altenbach (1989) concluded that *T. brasiliensis* is capable of recuperating from the infection of the rabies virus (serotype 1). However, in the bat populations of our study, we propose that the disease probably caused a major increase of mortality in the 1989 epizootic. Exceptional mortality increases in bats living in temperate zones generally are associated with adverse climatic factors, particularly long winters and cold springs (Gillette and Kimbrough, 1970; Ransome, 1989). Since the winter of 1989 to 1990 and the spring of 1990 were the mildest of the entire study period in southern Spain, it seems that other factors such as virus infection could have caused the decline in the survival rate to 35 and 45%, among adults and young, respectively.

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