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RABIES IN A CAPTIVE COLONY OF BIG BROWN BATS (*EPTESICUS FUSCUS*)

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ABSTRACT: Our research has focused on the ecology of commensal populations of big brown bats (*Eptesicus fuscus*) in Fort Collins, Colorado (USA), in relation to rabies virus (RV) transmission. We captured 35 big brown bats (*Eptesicus fuscus*) in late summer 2001 and held them captive for 4.8 mo. The bats were initially placed in an indoor cage for 1 mo then segregated into groups of two to six per cage. Two of the bats succumbed to rabies virus (RV) within the first month of capture. Despite group housing, all of the remaining bats were healthy over the course of the investigation; none developed rabies, although one of the rabid bats was observed to bite her cage mates. Reverse transcription–polymerase chain reaction (RT-PCR) and Taqman® real-time PCR analysis of the RNA derived from the brain tissue, salivary glands, and oral swab samples confirmed RV infection in the dead bats. Rabies virus was also isolated from the brain tissue upon passage in mouse neuroblastoma cells. Nucleotide sequence analysis of the RV nucleoprotein (N) gene showed 100% identity with the N gene sequence of a 1985 *E. fuscus* isolate from El Paso County, Colorado. Bat sera obtained six times throughout the study were assayed for RV neutralizing antibodies using the rapid fluorescent focus inhibition test. The RV neutralizing activity in the serum was associated with the IgG component, which was purified by binding to protein G Sepharose. Five bats were RV seropositive prior to their capture and maintained titers throughout captivity. Two adult bats seroconverted during captivity. Two volant juvenile bats had detectable RV antibody titers at the first serum collection but were negative thereafter. Four seronegative bats responded to a RV vaccine administration with high titers of RV antibodies. A serologic survey of big brown bats in the roost from which one of the captive rabid bats had originated showed a significant rise in seroprevalence during 2002.

Key words: Big brown bats, *Eptesicus fuscus,* rabies, rabies virus, virus neutralizing antibodies.

INTRODUCTION

Molecular epidemiologic studies indicate that a majority of human rabies cases in the United States during the past half century is attributed to rabies virus (RV) variants associated with insectivorous bats (Messenger et al., 2002). As oral vaccination programs progressively reduce the number of rabies cases in geographically restricted terrestrial hosts (Smith, 1996; Nunan et al., 2002), bats may eventually emerge to become a more important RV reservoir with spillover infection to humans and other mammals (Messenger et al., 2003). Alterations of the landscape due to urban and suburban sprawl, expanding human populations, or resource use may reduce both roosting and foraging habitat of bats (Pierson, 1998). However, some species of bats in the United States have adapted to roost in structures built by humans (Kunz and Reynolds, 2004), and the potential for contacts with people and domestic animals may have increased in such species.

Big brown bats (*Eptesicus fuscus*) are one of the most common species of bats to inhabit buildings across the United States (Kunz and Reynolds, 2004). Reports of rabies infection in these bats occur regularly (Krebs et al., 2002). The study we report herein is part of a multiyear project undertaken in Fort Collins, Colorado (USA), to characterize the ecology of an urban population of big brown bats in relation to RV transmission. Big brown bats are commonly submitted for diagnosis in Colorado, where rabies is predominantly a disease of bats (Pape et al., 1999). Two of the goals of the study were to assess the prevalence of rabies in bats by sampling

saliva for RV isolation and detection of viral nucleic acid, and to establish longitudinal histories of exposure to RV by repeat serologic sampling of free-ranging bats. Several investigations have detected antibodies to RV, or to closely related lyssaviruses, among wild bat populations (Baer and Smith, 1991). For example, the presence of RV and neutralizing antibodies was demonstrated in big brown and little brown bats (*Myotis lucifugus*) in New York State (Trimarchi and Debbie, 1977). A high prevalence of RV-specific antibodies and a low prevalence of active infection in the Mexican free-tailed bat (*Tadarida brasiliensis*) suggested that these bats were frequently exposed to RV infection (Steece and Altenbach, 1989). The presence of antibodies but an absence of active infection among bat populations in the Philippines suggested previous exposure to an unknown lyssavirus (Arguin et al., 2002). Also, specific neutralizing antibodies to European bat lyssavirus (EBL1) were detected in several European species of bats. Recapture of seropositive bats over several years suggested that these bats did not succumb to EBL1 exposure (Serra-Cobo et al., 2002). Thus, renewed studies of infectious diseases in bats are warranted.

In the current study, wild big brown bats were captured for experimental RV research. Subsequent detection of naturally infected rabid bats in the colony during the acclimation period altered this primary intention. This article describes the characteristics of the captive bat colony relevant to RV exposure. The objectives of this study were to (1) observe bats for signs of RV infection and transmission, (2) monitor the potential induction of RV neutralizing antibodies, (3) establish an association between RV neutralization activity and the IgG fraction in the serum, (4) genetically characterize RV variants, (5) evaluate selected tissues for evidence of infection, and (6) compare the results of different methods of RV detection for application in future studies of RV pathogenesis. We also present serologic sampling results for antibodies to RV in wild big brown bats at a roost that was identified as the origin of one of the captive rabid bats.

MATERIALS AND METHODS

Animals

All experimental procedures and animal care at Colorado State University (CSU) were performed in compliance with CSU's Institutional Animal Care and Use Committee. We captured 35 big brown bats using mist nets set at foraging and drinking sites, or collected from roosts in buildings, in the Fort Collins area between 29 August and 7 September 2001. Bats were held captive in an animal biosafety level 3 facility. Passive integrated transponder (PIT) tags (Avid Inc., Norco, California, USA) were inserted subdermally into each bat to allow for individual identification (O'Shea et al., in press). The captive colony (bat numbers 993–1030) included 18 volant juvenile females (F/J), 15 adult females (F/A [eight were postlactating]), one volant juvenile male (M/J), and one adult male (M/A). All the juvenile bats were weaned and volant and were distinguishable as juveniles on the basis of the degree of closure of the phalangeal epiphyses (Anthony, 1988). We placed the bats in a common indoor cage, where they roosted in clusters and were exposed to each other. Bats were fed mealworms once daily and had free access to water. A month later, the bats were separated into groups of two to six and housed in eight smaller cubicles (0.6×0.6×0.6 m). Blood samples were obtained six times between 5 October 2001 and 21 February 2002, and the sera were assayed for RV neutralizing antibody. In addition, a swab sample in minimal essential medium supplemented with 10% fetal bovine serum (MEM-10) was collected from the oral cavity of each bat five times between 5 October 2001 and 7 January 2002. Swabs were stored at −80 C for subsequent detection of RV genome using a reverse transcriptase–polymerase chain reaction (RT-PCR) assay. Four seronegative bats (Table 1, cage C) were vaccinated on 11 January 2002 with an intraperitoneal inoculation of 0.5 ml of Defensor 3 vaccine (Pfizer, Inc., Lincoln, Nebraska, USA), and sera were collected on 21 February 2002 for RV antibody analysis. All remaining bats were euthanized by intraperitoneal injection of pentobarbital (80 mg/kg body weight) on 21 February 2002. The brain tissue was tested for the presence of RV antigen by the direct fluorescent antibody (DFA) test, as described by Dean et al. (1996), using fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (mAb) conjugate (Fujira-

TABLE 1. Rabies virus antibody titers in seropositive captive big brown bats, 2001–02.

			Dates of blood collection					
			2001				2002	
Cage	Bat No.	Sex/age[a]	October 5	October 26	November 16	December 7	January 11	February 21
B[b]	1,018	F/A[c]	227	223	148	163	161	139
C	995[d]	F/J	<[e]	<	<	<	<	453
	998[d]	F/A	<	<	<	<	<	269
	1,004[d]	F/A	<	<	<	<	<	1,321
	1,028[d]	F/J	<	<	<	<	<	114
D	1,000	F/A[c]	NC[e]	217	228	380	243	453
	1,002	F/J	69	<	<	<	<	<
F	1,007	F/J	46	<	<	<	<	<
F	1,019	F/J	NC	127	116	133	157	139
	1,029	F/J	520	444	234	469	480	426
G	1,012	F/A[c]	<	26	28	36	62	32
H	1,020	F/A[c]	<	8	11	19	12	7
	1,023	M/J	30	<	18	8	23	7

[a] F/A = female adult; F/J = female/volant juvenile; M/J = male/volant juvenile.
[b] Cage B also housed the second rabid bat, 993, an adult female with a terminal rabies virus antibody titer of 280 on 13 October 2001.
[c] Postlactating female.
[d] Bats vaccinated with rabies virus vaccine on 11 January 2002.
[e] < = titer less than 5, NC = not collected.

bio Diagnostics, Inc., Malvern, Pennsylvania, USA).

As part of a larger study, we captured, tagged, and sampled big brown bats at multiple locations in Fort Collins in the summer of 2001 and 2002 for a serologic survey for RV antibodies. Here we report results of a serologic survey for RV antibodies in bats from one of these locations, a maternity roost, wherein the first rabid bat that died in captivity was captured. Bats were captured in mist nets set near the emergence points on the evening of 23 July and 1 and 10 August 2001, and again on 11 and 26 June, 10 and 24 July, and 6 August 2002. Seventy-seven bats (45 adult females and 32 volant juveniles) and 115 bats (73 adult females and 42 volant juveniles) were sampled in 2001 and 2002, respectively. Captured bats were removed from mist nets, kept isolated from contact with one another during handling, transported to the laboratory, marked with PIT tags, sampled for blood, and released at the capture site on the same night.

Detection of anti-RV antibodies

Blood samples were obtained from interfemoral veins in the tail membrane of bats under isoflurane anesthesia; blood was collected into heparinized microcapillary tubes following vein puncture with a sterile 25-gauge needle (Kunz and Nagy, 1988). Blood was centrifuged immediately after collection and sera stored at −70 C until assayed. The rapid fluorescent focus inhibition test (RFFIT) was performed to measure virus neutralizing antibodies (VNA) induced by the RV glycoprotein (Smith et al., 1996). Bat serum was heated at 56 C for 30 min to inactivate complement and was serially fivefold diluted prior to addition of a constant amount of RV (challenge dose), 50 fluorescing foci doses (FFD) of challenge virus standard (CVS) −11 (50 FFD_{50}/0.1 ml). The virus infection in mouse neuroblastoma cells was monitored using FITC-conjugated RV mAb (Fujirabio Diagnostics, Inc.). The RFFIT results were expressed as endpoint titers. A positive VNA titer is considered complete neutralization of challenge dose by a serum dilution of 1:5 (Smith et al., 1996). Titers less than 5 were considered negative.

Specificity of RV neutralizing antibodies

To demonstrate the specificity of the RFFIT and the association of RV neutralizing activity within the IgG fraction, IgG was purified from seropositive and seronegative bat sera, using protein G Sepharose 4 fast flow gel (Amersham Biosciences, Piscataway, New Jersey, USA), and tested in the RFFIT. In brief, a gel suspension in 20 mM sodium phosphate buffer, pH 7.0, was mixed with serum and incubated for 4 hr at room temperature. The mixture was loaded onto a spin column and washed three times with 200 μl of buffer, each time by centrifuging

at 325 × G for 5 min in a microcentrifuge. The bound IgG was eluted with 100 μl of 0.1 M glycine buffer, pH 2.5, and the eluate was neutralized with 10 μl of 1 M Tris-HCl, pH 9.0, to preserve the activity of acid labile IgGs. The bat serum and IgG derived from an equivalent amount of serum were tested in the RFFIT for their ability to neutralize RV, compared with neutralization activity in positive and negative control samples. The bat and human IgGs were examined by polyacrylamide gel electrophoresis (PAGE) under reducing and nonreducing conditions using ready gels (Bio-Rad Laboratories, Hercules, California, USA).

RNA extraction and RT-PCR

Total RNA was extracted from tissue homogenates and oral swab samples and analyzed for the presence of RV genomic RNA using RT-PCR. An aliquot (~100 μl) of the oral swab, brain, and salivary gland homogenates was mixed with 100 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, and 0.65% NP-40) followed by the addition of 1 ml of Trizol® reagent (Invitrogen, Carlsbad, California); total RNA extraction was carried out according to the manufacturer's instructions. Reagent controls were included in the RNA extractions. Five picomoles of primer 21F (5′-ATGTAACACCCCTACAATG-3′) specific for RV genomic RNA at the start of the N gene were mixed with a 5-μl aliquot of RNA sample, and the mixture was denatured for 1 min at 94 C and chilled on ice. The RT reactions (20 μl) were performed for 90 min at 42 C in a 1× incubation buffer containing RNA-primer complex, 20 nmoles of premixed deoxyribonucleotides, 16 U of RNase Inhibitor, and 8 U of avian myeloblastosis virus reverse transcriptase (Roche Diagnostics, Indianapolis, Indiana, USA).

A primary PCR designed to amplify a 210-bp amplicon of the RV N gene was performed in a 100-μl volume containing 20 μl of RT reaction product and 80 μl of buffer (8 μmol of Tris-HCl [pH 8.3], 25 pmoles of primer 20R [5′-AGCTTGGCTGCATTCATGCC-3′], 20 pmoles of primer 21F, and 2.5 U of Taq DNA polymerase). The thermal cycler was set to 1 cycle of 94 C for 1 min, 40 cycles of 94 C for 30 sec, 37 C for 30 sec, and 72 C for 90 sec, followed by 1 cycle of 72 C for 7 min. Following primary PCR, a heminested PCR was designed to amplify a 122-bp amplicon of the RV N gene. Ten microliters of primary PCR product were added to 90 μl of 1× PCR buffer containing 135 nmoles of $MgCl_2$, 18 nmoles of premixed deoxyribonucleotides, 25 pmoles each of primers 23F (5′-CAATATGAGTACAAGTACCCGGC-3′) and 20R, and 2.5 U of Taq polymerase. Heminested PCR was performed under conditions described above for primary PCR, and 10 μl of PCR products were analyzed on a composite gel containing 3% NuSieve agarose and 1% SeaKem agarose (both from BMA Products, Rockland, Maine, USA).

Taqman® real-time PCR

For detection of the RV genome in oral swabs, a Taqman® real-time PCR was designed to amplify a 122-bp fragment (nucleotides 73 to 194) of the RV N gene, using primers 23F and 20R. The reporter FAM™ and nonfluorescent quencher TAMRA™ dyes formed 5′ and 3′ modifications, respectively, of the 23-mer probe sequence, nucleotides 112 to 134 of the N gene, 6FAM-AAGCCCAGTATAACCTTAGGAAA-TAMRA. In brief, Taqman® PCR reactions were performed in 25-μl of PCR buffer containing 12.5 μl of 2× Taqman® Universal PCR Master Mix (Applied Biosystems, Foster City, California), 22.5 pmoles each of forward and reverse primers, 6.25 pmoles of probe, and 1–5 μl of either cDNA or a primary PCR product as target template. An ABI Prism™ 7700 Sequence Detector (Applied Biosystems) was set for 1 cycle at 50 C for 2 min, 1 cycle at 95 C for 10 min, and 40 cycles of denaturation at 95 C for 15 sec and annealing/extension at 50 C for 1 min. The RT reaction with the RV N gene sequence-specific primer 21F generated cDNAs for Taqman® PCR. To increase the sensitivity of Taqman® PCR, cDNAs were subjected to a primary PCR with 21F and 20R primers, and the resulting 210-bp PCR product was used as the template. The threshold cycle or C_t values in the Taqman® PCR assay correspond to the PCR cycle number, when reaction fluorescence rises above a threshold value. A C_t value of 40 indicated a negative reaction, and values below 40 indicated positive reactions. The C_t values are expressed as mean of triplicates±standard deviation.

Sequence analyses

Total RNA was reverse transcribed with primer 21F, to yield RV-specific cDNA. Using primers 21F and 304R (5′-TTGACGAAGATCTTGCTCAT-3′), a 1,480-bp PCR product was amplified. The DNA was purified and the nucleotide sequence of the RV N gene (1,350 bp) was determined by sequencing on an ABI Prism™ 377 DNA sequencer (Applied Biosystems), using 3.2 pmoles of reverse primers 304R and 20R; forward primers 21F, 23F, and 700F (5′-CACAGTTGTCACTGCTTATG-3′); and 100 ng of DNA as template.

Rabies virus isolation

Oral swabs, salivary gland, and brain samples from rabid bats were homogenized in 0.2 ml of MEM-10, mixed with a 0.5-ml suspension containing 7.5×10^5 mouse neuroblastoma cells, and incubated for 15 min at 37 C in a T25 cell culture flask. Six milliliters of MEM-10 were added to the flask and gently mixed, and 2.4 ml of the mixture was taken out and divided into an 8-well Labtek® chamber slide (Nalge Nunc, Inc., Rochester, New York, USA). Incubation was continued for 48 to 72 hr in a humidified incubator set to 0.5% CO_2 and 37 C. Following incubation, slides were rinsed with 0.01 M phosphate-buffered saline (pH 7.5), fixed in acetone at −20 C, and stained with FITC-conjugated RV antibody. If no RV antigen was detected on the slides, the T25 cultures were passaged in 3–4 days, and the cells were reexamined again by DFA. The flasks were examined for two to three passages before being discarded.

RESULTS

Thirty-five big brown bats were captured between 29 August and 7 September 2001. Each bat had visible scars on the wing (range 1–24, $\bar{X}=9.7\pm6.2$ SD) compatible with bite wounds, the primary mechanism for RV transmission. On the afternoon of 28 September one bat (999, F/J) was found alive in a food bowl at the bottom of the common cage. The bat was replaced in a box within the common cage and remained in the box for the next 3 days. On October 1, the bat was weighed and had lost body mass. The bat became sensitive to noise, bit aggressively when handled, and refused water. On 2 October the bat was moribund and was euthanized. The bat's brain tissue was positive for RV antigen by the DFA test. As determined from the date of capture, this bat had a minimum incubation period of 4 wk.

On 4 October the remaining bats in the enclosure were segregated into eight cages and closely monitored for serologic, behavioral, and clinical signs during the next 4.5 mo. On 11 and 12 October, a second bat (993, cage B) became progressively vocal and aggressive and attacked and bit each of its cage mates. This bat was bled and euthanized after it was observed having seizures in its cage. The brain and salivary gland tissues were positive for RV antigen by DFA test. These rabid bats were captured from different locations in Fort Collins, Colorado, one in flight over the Cache la Poudre River and one at a roost in an owner-occupied home.

The RV antibody titers in sera obtained from bats at six time points are shown in Table 1. The amount of sera available was variable (usually less than 30 μl) and resulted in diverse initial dilutions and somewhat unconventional titers (Table 1). Of the 35 bats, 20 were seronegative and 13 had positive antibody titers. Blood was not collected for serology from the first rabid bat. The second rabid bat had a terminal RV antibody titer of 280. Seven bats displayed RV neutralizing antibodies that were maintained during captivity. Although some bats had high titers of RV antibody (>100), bat 1023 had a low titer. Two bats (1012 and 1020) appeared to seroconvert while in captivity. Two volant juvenile bats (1002 and 1007) had moderately high levels of RV antibodies at the first blood collection, but antibodies were undetectable in subsequent samplings. In contrast, bat 1023, also a volant juvenile, was initially seropositive but became seronegative and then showed detectable levels of antibody in subsequent samplings. The vaccinated bats showed seroconversion with high titers of RV antibodies. Antibody titers in four of nine bats that seroconverted were within a similar titer range as in bats that were vaccinated (Table 1). Except for the rabid bats, the brain tissues from all remaining bats in the colony were negative for RV antigen by the DFA test at the time of euthanasia.

To demonstrate the specific component in the serum associated with neutralizing activity, human and bat sera and the purified IgGs were tested by the RFFIT. The RV seropositive human and seropositive bat (1000) sera and seronegative bat (1003) sera showed titers of 1,634, 97, and <5, respectively, and the IgGs derived from equivalent amounts of these sera

TABLE 2. Comparison of results of polymerase chain reaction (PCR), heminested PCR, and Taqman PCR assays and rabies virus isolation.

Technique	Bat No. 999		Bat No. 993				
				Salivary gland		Oral swab	
	Brain tissue	Salivary gland (submaxillary)	Brain tissue	Submaxillary	Parotid	5 Oct 2001	13 Oct 2001
Primary PCR[a]	+[b]	+	+	+	−[b]	−	−
Heminested PCR[c]	+	+	+	+	+	+	+
Taqman PCR[d]	+	−	+	−	−	−	−
Taqman PCR[e]	ND[b]	+	ND	+	+	+	+
Virus isolation[f]	+	+	+	+	ND	−	−

[a] Primary PCR (cDNA template) to amplify a 210-bp PCR product.
[b] + = positive; − = negative; ND = not determined.
[c] Heminested PCR (210-bp primary PCR product template) to amplify a 122-bp PCR product.
[d] Taqman® PCR (cDNA template).
[e] Taqman® PCR (210-bp primary PCR product template).
[f] Virus isolation in mouse neuroblastoma cells.

showed titers of 431, 59, and <5, respectively. The IgGs derived from the seropositive bat and human sera neutralized RV, whereas the IgG from the seronegative bat did not. The bat and human IgGs, when analyzed on a 4–20% PAGE under nonreducing conditions, showed bands with molecular weights of ~150 Kd, typical of a whole IgG molecule. Under reducing conditions, the IgGs showed bands with molecular weights of 50 and 25 Kd, typical of heavy- and light-chain polypeptides of IgG.

Assays were compared for the ability to detect RV and RV nucleic acid in tissues and swab samples from the rabid bats (Table 2). The primary PCR amplification using primers 21F and 304R produced a 1,480-bp DNA fragment that included the entire RV N gene. This fragment amplified only in the RNA derived from the brain tissue. The salivary gland and oral swab specimens were negative. However, a primary PCR amplification using primers 21F and 20R that produced a 210-bp amplicon in the N-terminal region of the RV N gene was positive with the salivary gland samples. Oral swab specimens were positive for the RV genome only when assayed by a heminested PCR, which produced a 122-bp amplicon when using primers 23F and 20R and a 210-bp primary PCR product as template (Table 2). The oral swab specimens collected from the remaining healthy bats, five times between 5 October 2001 and 7 January 2002, were all negative for the RV genome in the RT-PCR assay.

The Taqman® real-time PCR assay was as sensitive as the primary PCR when using cDNAs from the brain tissue as template, but not from salivary glands or oral swab specimens. The C_t values (mean of triplicates±standard deviation) were 20.11±0.13 and 21.36±0.34 for the brain tissue from the two rabid bats. The submaxillary glands from both bats were RV negative (C_t: 40). Likewise, the parotid glands and oral swab specimens collected on 5 and 13 October 2001, from the second rabid bat were also negative (C_t: 40). However, with the use of the 210-bp primary PCR product as template, the RV genome was detected in the salivary glands and the oral swab specimens (Table 2). A C_t value of 19.22±0.09 was observed for the submaxillary gland from the first rabid bat. The C_t values for the submaxillary and parotid glands from the second rabid bat were 20.09±0.8 and 19.64±0.56, respectively. The observed C_t values for the oral swab specimens dated 5 and 13 October 2001 from the second rabid bat were 26.25±1.06 and 20.64±0.16, respectively.

The N gene of these bat RV isolates had identical nucleotide sequences and showed complete identity with another bat

RV variant isolated from an *E. fuscus* in El Paso County, Colorado, in 1985 (GenBank accession no. AY039228). Attempts to isolate RV from the brain and submaxillary glands of the two rabid bats in mouse neuroblastoma cell cultures (passage 1) were successful (Table 2). Although the oral swab specimens from bat 993 had tested positive by nested PCR, virus isolation was unsuccessful, even at the third passage of cells.

Bats captured at the roost where the first rabid bat had originated had a low seroprevalence in 2001: 2% of adult females (one of 45, endpoint titer of 94) and 3% of volant juveniles (one of 32, endpoint titer of 98) were seropositive. By 2002, seroprevalence at this roost increased to 23% in adult female bats (17 of 73; endpoint titer range of 14–381, $\chi^2_{1\ df}=8.0$ with Yates correction for continuity, $P<0.005$). The 3% to 14% increase in seroprevalence in volant juveniles (six of 42; endpoint titer range of 15–267) was not significant ($\chi^2_{1\ df}=1.5$ with Yates correction, $P>0.05$). Twenty-two individually marked big brown bats were sampled at this colony during both summers and all were seronegative in 2001. However, in 2002, three of these bats (14%) had seroconverted (endpoint titers of 14, 178, and 297). The other 19 bats remained seronegative in 2002. A large number of individuals were sampled in 2002 that were not sampled in 2001. Among the 51 bats sampled only in 2002, 14 were seropositive (27%).

DISCUSSION

Rabies is enzootic in several bat species in all contiguous areas of the United States. Despite its historical description since the 1950s in the United States, relatively little is understood about transmission and maintenance of rabies among bats. Most information on this topic stems from molecular epidemiology or from passive surveillance studies. Molecular studies have revealed multiple independent reservoirs for rabies in several species of insectivorous bats based on the identification of specific viruses in different bat species each transmitting a distinct RV variant (Smith, 1996). Variants transmitted by a migratory bat species, for example, the Mexican freetail bat and the silver-haired bat (*Lasionycteris noctivagans*), can be found throughout the migratory range of these species that may extend over thousands of miles. In contrast, within the same bat species, relative geographic identity may exist for certain RV variants. For example, the eastern and western populations of the big brown bat contain distinct variants (Smith, 1996). The 100% nucleotide sequence identity of the RV N gene found in our rabid bats with a 1985 isolate from elsewhere in Colorado suggests relative stability of this variant over a 16-yr period.

The big brown bat is a ubiquitous nonmigratory species found throughout much of North America and commonly roosts in homes and buildings (Kunz and Reynolds, 2004). Individual female bats are exceptionally loyal to their natal roosts, habitually returning year after year. About 10–30% of the immature volant females and up to 72% of adult females return to the natal roost the following spring (Kurta and Baker, 1990). Big brown bats are the most common species submitted for rabies testing in Colorado and one of the most commonly submitted bats in the rest of the United States (Pape et al., 1999; Krebs et al., 2002). Although bats represented only 10% of the national total, they accounted for 98% of all the animal rabies cases in Colorado between 1977 and 1996. Passive surveillance records indicate that 15% (685) of the 4,470 bats tested in Colorado were diagnosed with rabies; 48% (2,135) of the total tested were big brown bats and 363 (17%) of these were rabid (Pape et al., 1999). In the United States, *E. fuscus* is the species of bat most commonly reported with rabies (Mondul et al., 2003).

Given this background, our original intention was to bring free-ranging big brown bats into captivity for RV pathogenesis research. These plans were not carried

out because two bats during the acclimation phase and the other bats were potentially exposed to RV. Both affected bats may have been incubating RV when captured. However, with only a 2-wk period between occurrences it is more likely that the infections were independent events. Concordant with clinical observations and the DFA results, the heminested PCR, Taqman® real-time PCR, and virus isolation all confirmed RV infection in these bats. The heminested PCR demonstrated the presence of the RV RNA in the brain, salivary glands, and oral swab samples. Results of the primary Taqman® assay had positive C_t values only for the cDNA templates derived from brain samples. Heminested PCR was more sensitive than the primary Taqman® assay. However, after performing a primary PCR with the cDNAs from salivary glands and oral swab specimens, the primary PCR amplicons, when used as templates in the Taqman® assay, produced positive C_t values. A major advantage of the Taqman® assay is that it compares favorably with the nested PCR, reduces the overall time requirement, reduces false positives, and obviates the use of agarose gels in the analysis. Smith et al. (2002) found the Taqman® assay to be a sensitive diagnostic tool in the detection of Australian bat lyssavirus in clinical samples, although primer design and sequence variations are key facts for consideration (Hughes et al., 2004). Additional sampling will be required to ascertain whether this procedure may be routinely used during field sampling to determine RV shedding in bats.

The rabid bats displayed classic signs of rabies. Although the second bat was observed to bite all three cage mates, none of them developed apparent disease, seroconverted, or showed an anamnestic response (Table 1). Of relevance, only one of 45 adult female bats (2%) screened from the capture site of the first rabid bat was seropositive in the summer of 2001, but 17 of 73 adult female bats (23%) from the same roost were seropositive in the summer of 2002. This rise in the fraction of seropositve bats may be indicative of RV transmission late in the summer of 2001 or in the spring of 2002. A large number of bats were sampled in 2002 that were not sampled in 2001; the percentage seropositivity did not increase in bats captured in 2001 and 2002. However, bats captured in 2002 had a higher proportion of seropositivity than those captured in both years. Since bats did not remain at this roost during the winter of 2001, it is possible that a group of seropositive bats may have moved into the colony in 2002.

Fatal RV infections may not be common among bats despite established stable infection cycles via bites, and immunity may be acquired through recurrent exposures to RV during close contact between individuals. Intraspecies spread of rabies may cause acute disease in some bats, while in others an abortive infection may result in seroconversion. For instance, in a study of insectivorous bats in New York State, 278 big brown bats and 333 little brown bats showed a low occurrence of active RV infection (3% and 0.3%, respectively). In contrast, seroprevalence (10% and 2%, respectively) of RV neutralizing antibodies was higher (Trimarchi and Debbie, 1977). Unlike past serologic studies in bats, our adaptation of the RFFIT to assay small quantities of blood coupled with marking, release, and recapture of individual bats at the roost may allow greater insight into the dynamics of RV transmission in bats.

Among the many unanswered questions of the dynamics of RV transmission and pathogenesis in various mammals is whether animals that demonstrate a high titer of RV neutralizing antibodies in the absence of disease reflect acquired natural immunity and protection. For example, rabies in raccoons (*Procyon lotor*) exhibits a complex transmission pattern in that animals may lack discernable clinical signs, may or may not show RV antibody titers, may become rabid with no detectable antibody titers, or may succumb to rabies with detectable antibody titers during ter-

minal phases of the disease (Niezgoda et al., 2002). Detection of RV antibody in free-ranging raccoons is probably indicative of an exposure to RV antigen reflective of immunity rather than to the viral incubation phase or ensuing illness. Similarly, among a sample of 4,754 mongooses (*Herpestes auropunctatus*) from Grenada, 2% were rabid, whereas of the 1,675 mongooses tested for RV neutralizing antibody, 30% were positive, suggesting that animals had acquired natural RV immunity without developing disease (Everard et al., 1981). Moreover, immunity to rabies has reportedly been achieved through frequent exposures to small viral loads during social contact among Serengeti hyena (*Crocuta crocuta*) (East et al., 2001). Thus, as in other mammals, an outbreak of rabies in a bat roost may drive induction of immunity due to nonlethal exposures.

Many host-virus interactions have an environmental component that affects transmission patterns. Although mechanisms are poorly understood, strong colonial behavior and clustering within cramped roosts may influence RV transmission and exacerbation of rabies in bats through intraspecific aggression and biting. All bats caught for the captive colony had small, tooth-sized puncture scars on the wings when brought in from the field. Virus transmission may be possible via proximate contact between bats through biting, scratching, or grooming, which may lead to development of RV antibodies. Seropositivity in a colony may simply demonstrate that exposure has occurred, whereas an elevated seroprevalence with correspondingly high titers may be indicative of a recent outbreak. Determination of recent versus past exposure to RV entails comparison of RV-specific serum IgM versus IgG levels. Antibody titers in four of nine naturally exposed bats and experimentally vaccinated bats were comparable; this suggests that exposure to a nonlethal dose of virus could elicit substantial RV antibody responses.

Detection of RV antibody does not necessarily mean direct exposure to RV. Juvenile bats may acquire passive immunity via maternally transferred antibodies. Two volant juveniles had moderately high titers of RV neutralizing antibodies when first sampled, but antibodies were undetectable at subsequent samplings. These bats may have had waning levels of maternally transferred antibodies. One volant juvenile bat (1023) was initially seropositive and then was negative but in subsequent samplings had detectable levels of antibody, suggesting that this particular bat may have been exposed to RV while in captivity. Although some bats (1012 and 1020) had low titers of antibodies, they were true seropositives and may have seroconverted in captivity. The extent of exposure to RV could also be a factor for these differences.

Immune responses to viral antigens include production of complement-dependent, complement-independent, neutralizing, and nonneutralizing antibodies, depending on the immunodominant epitopes. Historically, it was uncertain what specific component (virus-specific neutralizing antibodies or some nonspecific factors, such as virus inhibitors in the serum) brings about virus neutralization in the RFFIT. Purification of IgG from a RV-seropositive bat and its subsequent activity in the RFFIT demonstrated that the IgG fraction in the serum, rather than any nonspecific components, neutralizes RV. The present study, together with field serology data, highlights some of the complexities of RV maintenance and transmission. The observations of rabies in a captive colony of bats indicate a low proportion of bats with evidence of active RV infection, lack of transmission of infection to cage mates, and a high seroprevalence that presumably may provide protection from subsequent lethal infection. The scenario in the captive bat colony may bear close resemblance to the events occurring in free-ranging big brown bat populations. Thus, population dynamics, colony interchanges, and movements of bats may be key factors influencing disease emergence and per-

petuation in this species. Additional research is required to document the ontogeny of herd immunity, verify its role in disease abrogation, and characterize in greater detail the ecological dynamics of RV transmission in wild populations of big brown bats.

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