Naturally Acquired Rabies Virus Infections in Wild-Caught Bats

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Abstract

The study of a zoonotic disease requires an understanding of the disease incidence in animal reservoirs. Rabies incidence in bats submitted to diagnostic laboratories does not accurately reflect the true incidence in wild bat populations as a bias exists for testing bats that have been in contact with humans or pets. This article details the rabies incidence in two species of bats collected from natural settings without such bias. In this study, brain smears from 0.6% and 2.5% of wild-caught and apparently healthy *Tadarida brasiliensis* and *Eptesicus fuscus*, respectively, were positive for rabies virus (RV) antigen. Conversely, 92% of the grounded *T. brasiliensis* were positive for RV. Serology performed on captive colony and sick bats reveal an immune response to rabies. This work illustrates the complex interplay between immunity, disease state, and the conundrum of RV maintenance in bats.

Key Words: Antibodies—Bats—Rabies—Viral isolation.

Introduction

IT HAS BEEN ESTIMATED that <1% of wild bats in endemic regions are infected with rabies virus (RV) at any given time (Childs 2002). Prevalence data from diagnostic laboratories are not a true indicator of rabies incidence among wild bats as most of these animals had been in contact with humans or domestic animals or were displaying abnormal behaviors such as being grounded or flying during the day. Depending on the geographic location, the species most commonly submitted to diagnostic laboratories are the big brown bat, *Eptesicus fuscus*, and the Mexican free-tailed bat, *Tadarida brasiliensis*. Approximately 17% of *E. fuscus* and 16% of *T. brasiliensis* submitted to diagnostic laboratories in Colorado and Texas were rabies positive (Pape et al. 1999, Rohde et al. 2004). In New York, 1,241 of the 36,506 (3.3%) *E. fuscus* submitted to the New York State Diagnostic Laboratory diagnostic between 1988 and 2007 were found to be rabid (NYSDOH 2009). Conversely, the number of bats reported rabid from field studies is very low, and large outbreaks have not been reported (Constantine 1978, Steece and Altenbach 1989). Unlike healthy bats, which typically avoid interaction with humans, it is the bat that comes into contact with humans which is submitted to public health laboratories, consequently explaining the discrepancy between rabies in wild bat populations and rabies in bats submitted to public health laboratories.

The reasons for the low intraspecies transmission rate of RV within bat populations remain unknown. Constantine (1988) hypothesized that animals that develop clinical rabies are more likely to be immunocompromised than animals that do not. Alternately, highly virulent RV variants may be fatal to bats before the centrifugal migration of virus to the salivary glands, thereby preventing the bat from transmitting the virus (Brass 1994a). It is also possible that juvenile bats will be exposed to RV while they still possess maternal antibodies to RV, resulting in the development of active immunity rather than infection (Steece and Altenbach 1989).

The studies described here were designed to estimate the number of rabid bats in a wild population, the percentage of rabid bats with infectious virus in their salivary glands, and the presence of anti-rabies viral neutralizing antibodies (VNA) in wild bat populations. These data will provide insight into the maintenance of RV in bat populations and how it may affect public health.

Materials and Methods

All experimental procedures and animal care at Colorado State University (CSU) were performed in compliance with CSU's Institutional Animal Care and Use Committee.

Bats were obtained from three sources:

Colorado State Department of Public Health and Environment: Rabid bats submitted to the Colorado State

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Department of Public Health and Environment (CDPHE) during the years 2000–2004 were provided to CSU to determine the presence or absence of RV antigen in salivary gland tissue. Paired parotid and mandibular salivary glands and a small amount of the remaining brain tissue were collected from all bats; both tissues were processed to detect RV antigen using a standard direct fluorescent antibody (DFA) test (Dean et al. 1996).

Captive colonies of *E. fuscus* and *T. brasiliensis*: From 2001 to 2005, four colonies of *E. fuscus* were established at CSU. The *E. fuscus* were removed from buildings or caught in mist nets placed near feeding sites. Between 2002 and 2003 two colonies of *T. brasiliensis* were established at CSU. All bats were tested for the presence or absence of VNA when brought into the captive colony. These bats were captured at a bridge roost site located in Travis County, TX. Bats were captured during September, ~5–6 months after their northern migration from Mexico. During quarantine, bats were observed two to four times a day. All bats that died or were euthanized while in captivity were necropsied; paired parotid and mandibular salivary glands and brain tissue were collected from all bats; both tissues were processed to detect RV antigen using the DFA test to verify the presence or absence of RV infection.

Grounded *T. brasiliensis*, Travis County, TX: During 2004, downed (grounded) bats were collected daily between 8 and 11 am over 7 days from under a bridge in Travis County, TX. During 2005, grounded bats were collected twice a day for 7 days at the same site as in 2004; morning collections began at ~9 am and continued through 12 pm, and evening collection began before emergence, ~7 pm, and continued until 2 am. Within 30 min of collection, bats were anesthetized with isoflurane, and blood was collected through cardiocentesis. After clotting, the blood samples were centrifuged and serum frozen on dry ice. The bats were euthanized, and carcasses placed on dry ice. All samples were returned to CSU for processing.

Antigen detection in brains and salivary glands

At necropsy, brains and paired parotid and mandibular salivary glands were removed from each bat. Brain tissue was examined by the DFA test to verify rabid individuals. Rabies diagnostic conjugates employed were as follows: Light Diagnostics™ Rabies DFA Reagent (Catalog No. 5100) or Fujirubio Diagnostics, Inc. Centocor FITC-Anti-Rabies Monoclonal Globulin (Catalog No. 800-090) anti-rabies conjugates. Salivary glands were minced using iridectomy scissors and milled in a Ten-broeck tissue grinder, and a sample of the processed salivary gland tissue was smeared on a glass slide and stained as described for brain. The remaining tissue was processed for virus isolation.

Virus isolation and titration

Virus was isolated from the parotid and mandibular salivary glands as described in Rudd and Trimarchi (1987, 1989). Homogenates of salivary glands (10%w/v) were made with a Ten-broeck tissue grinder using 1.0 mL of growth medium (GM; Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 2.0 mM glutamate, and 100 IU penicillin G, 50 µg streptomycin, and 2.5 mg amphotericin B/mL) as a diluent. Salivary gland homogenates were frozen at –80°C. Viral isolation and titrations were performed in 96-well plates

using murine neuroblastoma (NA) cells (C-1300) (Rudd and Trimarchi 1989). Briefly, for virus isolation, 50 µL of test inoculum was added to 150 µL of GM containing 5×10^5 NA cells in individual wells of a 96-well plate. For viral titrations, 5×10^5 NA cells were added to 10-fold dilutions of the original test inoculum. The plates were incubated in 5% CO₂ at 37°C for 72 h. The medium was aspirated, and the wells were washed twice with PBS, air-dried, and fixed with 75% aqueous acetone at –20°C for 1 h to overnight. The fixative was removed, plates were allowed to air-dry, and rabies diagnostic conjugate (50 µL/well) was added and incubated for 30 min at 37°C. Optimal working dilutions of rabies diagnostic conjugate were determined for each lot of conjugate. Wells were given two 5 min PBS washes and 50 µL of PBS was added to each well. Fluorescent foci were examined on a Nikon fluorescent microscope at 150× and 450×. Tissue culture infectious doses (TCID₅₀) were calculated by the method of Reed and Muench (1938).

Serologic analysis

Sera were analyzed for anti-rabies VNA employing the tissue culture serum neutralization test using NA cells (Trimarchi et al. 1996).

Results

Captive colonies of *E. fuscus*

Four colonies of captive *E. fuscus* bats were established between 2001 and 2004; each colony was quarantined for ~6 months after capture. The 2001 colony consisted of 35 bats, 2 of which developed clinical rabies while in captivity. The first bat developed signs consistent with RV infection ~4 weeks after introduction into the captive colony, was euthanized, and found to be rabid. Sixteen days later, a second bat developed clinical signs compatible with RV infection. This bat was also euthanized and found to be rabies positive by the DFA. RV was isolated from the salivary glands of both bats. The rabies serology results are not available for all bats entering the 2001 captive colony.

The second colony of *E. fuscus* consisted of 89 bats. One of these bats developed rabies 135 days after introduction into the captive colony, which was 10 days after inoculation with West Nile virus. The salivary glands from this bat were not available for virus isolation. Five of the 98 bats were seropositive upon entering the colony and remained healthy throughout the quarantine period.

The third colony was composed of 12 *E. fuscus* bats, none of which developed clinical rabies. The fourth colony was composed of 63 *E. fuscus* bats; two developed clinical signs compatible with RV infection. Bat 2 became aggressive and ataxic after 132 days in captivity; Bat 5, which was housed with the first bat that developed rabies, developed clinical signs of rabies 190 days after admission into the captive colony, 58 days after the first bat. RV antigen was detected in the brains of both animals by the DFA, but virus was not isolated from salivary gland suspensions. All bats were negative for rabies VNA upon entering the captive colony.

From the 199 *E. fuscus* brought into the captive colonies during 2001–2004, 5 (2.5%) developed rabies during the 6-month quarantine period. Of the five bats that developed rabies while in the captive colony, at least three bats were

naturally infected in the wild and two may have been exposed while in captivity (Table 1). The two bats that subsequently developed rabies while in captivity were healthy when their cage mates developed clinical illness. In some instances, fighting was noted between the ill and healthy bats. It could not be determined if these two bats were infected before capture or were infected by their rabid cage mates.

T. brasiliensis from Travis County, TX

Between 2002 and 2003, *T. brasiliensis* were collected in Travis County, TX, and maintained in a captive colony at CSU. In 2002, at the time of capture, one bat was easily removed by reaching up into a bridge crevasse. This bat was unresponsive, showed mild paralysis, and was euthanized 12 h later. The bat was positive for rabies antigen by DFA. The remaining 15 bats were healthy throughout the 14-month study. All bats were negative for VNA upon entering the captive colony.

During 2004, a total of 81 downed bats were collected from under a bridge in Travis County, TX. Of these animals, 70 (86%) were diagnosed as rabid by DFA on brain tissue. RV was isolated from the salivary glands of 19 (27%) of the 70 rabies-positive bats. Of the 62 rabid bats from which adequate serum was available for testing, 33 (53%) were positive for the presence of serum anti-rabies VNA. Serum was collected from 11 of the rabies-negative bats and 3 (27%) of these bats were positive for VNA. The number of bats collected each day and the percentage of rabid bats are presented in Table 2.

During 2005, a total of 240 downed bats were collected from under the bridge in Travis County, TX. Of these animals, 226 (94%) were diagnosed as rabid by the DFA on brain tissue. RV was isolated in cell culture from 43% of the salivary glands removed from all bats. RV was not isolated from the salivary glands of any of the bats that were negative for RV in the brain (Table 2). Serum samples adequate for testing were collected from 186 rabies-positive bats and 100 (54%) were positive for VNA. Serum was available from ten rabies-negative bats and four were positive for VNA.

Bats from state public health laboratory

Between 2000 and 2004, 36 rabid bats, representing five bat species, were received from the CDPHE. RV antigen was observed in the brain of all bats. Twenty-two bats (61%) had

RV antigen in the salivary glands. In 16 of the 17 bats from which RV was isolated from the salivary glands, RV antigen was also present in the salivary glands. Table 3. The ability to isolate virus from salivary glands did not always correlate with the amount of viral antigen in the brain of individual bats. However, virus was not isolated from the salivary glands of those bats when only a small amount of antigen was found in the brain. Virus was isolated from the salivary glands of one *Nyctinomops macrotis* yet antigen was not observed. This discrepancy may be the result of tissue lost during processing.

Discussion

The absence of large rabies outbreaks in bat populations has not been widely studied, but several hypotheses have been proposed, including coadaptation, variations in the infectivity and virulence of bat RV variants, and immunocompetence of the host (Brass 1994b, Badrane and Tordo 2001, Hughes et al. 2005). By employing the use of molecular clocks, Badrane and Tordo (2001) proposed coadaptation over the last 11,000 years between bats and a now-extinct lyssavirus. More recent studies suggest that the currently circulating RVs in bats and carnivores emerged from a common ancestor less than 1000 years ago (Davis et al. 2006, Bourhy et al. 2008, Nadin-Davis et al. 2010). Differences in virulence between RV variants may be the result of the alterations in the expression of viral glycoprotein (Sarmiento et al. 2005). The relationship between immune function and host susceptibility remains unclear.

Approximately 2.5% of healthy, wild-caught *E. fuscus* developed rabies during the 4 years of our study, demonstrating the low frequency of RV circulating within bat colonies. However, Pape et al. (1999) reported that 15% of bats submitted to the CDPHE were rabies positive. It is noteworthy that all of bats submitted for the Pape et al. (1999) study had a history of abnormal behavior, or confirmed or suspected contact with a human and/or domestic pet. Similarly, 92% of the grounded *T. brasiliensis* were rabies positive. The discrepancies may be explained by the conditions under which the animals were submitted. Bats submitted to public health laboratories are often reported to have abnormal behavior such as flying during the day, aggression, or being found on the ground. Likewise, grounded *T. brasiliensis* are not

TABLE 1. RABIES VIRUS IN CAPTIVE COLONIES OF *EPTESICUS FUSCUS*

Year	Relationship between the year, total number of bats, and bats developing rabies while in captivity				
	2001	2002	2003	2004	Total
Number of bats in colony	35	89	12	63	199
Number of bats developing rabies (% of total)	2 (6)	1 (1)	0	2 (4)	5 (2.5)
Average duration bats were maintained in captive colony before developing clinical signs	37 days	134 days	N/A	161 days	
Number of bats from which RV was isolated from salivary glands (% of rabid)	2 (100)	ND	0 (0)	0 (0)	2 (40)
Duration bats were maintained in captive colony	6 months	13 months	18 months	18 months	
Percent of rabid bats that were seropositive when entering the colony	ND	6 ^a	0	0	2.5

Eptesicus fuscus were collected in Fort Collins, CO, during September–October. Bats were brought into a captive colony and maintained for at least 6 months.

^aThese bats remained healthy for the duration of the quarantine period.

NA, not available; ND, not done; RV, rabies virus.

TABLE 2. *TADARIDA BRASILIENSIS* WERE COLLECTED IN TRAVIS COUNTY, TX, DURING THE FALL OF 2004 AND 2005

Date collected	No. of bats collected	No. (%) of dFAT-positive bats ^a	No. (%) of SG from which virus could be isolated ^b	No. (%) of rabies-positive >0.2IU ^c	No. (%) of rabies-negative >0.2IU ^c
9-11-04pm	12	11 (92)	1 (10)	3 (27)	0
9-12-04pm	9	8 (88)	2 (25)	2 (33)	0
9-13-04pm	5	5 (100)	1 (20)	4 (80)	ND
9-14-04pm	14	13 (93)	5 (38)	6 (50)	1 (100)
9-15-04pm	24	19 (79)	6 (32)	9 (64)	2 (40)
9-16-04pm	14	9 (64)	4 (44)	6 (55)	0
9-17-04pm	3	3 (100)	0 (0)	3 (100)	ND
2004 total	81	70 (86)	19 (27)	33 (53)	3 (27)
9-25-05 am	11	9 (82)	2 (22)	3 (50)	ND
9-25-05 pm	25	24 (96)	7 (29)	12 (50)	0
9-26-05 am	6	6 (100)	0 (0)	4 (67)	0
9-26-05 pm	21	20 (95)	10 (50)	11 (55)	1 (100)
9-27-05 am	19	17 (89)	2 (12)	10 (59)	0
9-27-05 pm	27	27 (100)	17 (65)	11 (41)	0
9-28-05 am	12	11 (92)	6 (60)	8 (73)	0
9-28-05 pm	27	26 (96)	18 (69)	2 (50)	ND
9-29-05 am	8	7 (88)	1 (14)	6 (75)	1 (100)
9-29-05 pm	19	18 (95)	9 (50)	10 (59)	0
9-30-05 am	14	12 (86)	2 (17)	1 (100)	ND
9-30-05 pm	31	30 (97)	19 (63)	15 (56)	0
10-1-05 am	20	18 (90)	5 (28)	7 (58)	2 (100)
2005 Total	240	226 (94)	98 (43)	100 (54)	4 (40)
2 year total	321	296 (92)	117 (40)	133 (54)	7 (33)

^aThe dFAT employed brain tissue only.^bThis includes SG from positive bats only.^cSerum was not available for all bats.

dFAT, direct fluorescent antibody test; SG, salivary glands.

representative of the general healthy population, and thus data would be skewed toward rabid bats. Conversely, our wild-caught *E. fuscus* were healthy at the time of capture and hence the smaller percentage of bats that developed rabies. Beteke et al. (2008) reported that the daily average number of bats at this bridge site during the nonwinter months is ~750,000. By extrapolating this data using the total population, ~0.7% of all healthy *T. brasiliensis* at this site will develop rabies during their 153-day residence. When we include the total *T. brasiliensis* population at this site, the estimated incidence of rabies in healthy, wild-caught *T. brasiliensis* at this

bridge site is similar to our findings of rabies in healthy, wild-caught *E. fuscus*.

The lack of VNA in the bats incubating rabies demonstrates the difficulties predicting which bat(s), if any, have had previous exposure to RV. The duration of rabies incubation in bats is highly variable, ranging from less than 14 days to over 290 days (Jackson et al. 2008, A. Davis, unpublished data). Variable incubation times may be the result of location of exposure, variant to which the animal is exposed, and immunocompetence of the animal. In our study, one bat developed rabies after experimental inoculation with West Nile Virus (Davis et al. 2005). It is possible that the stress of West Nile Virus exposure decreased the hosts' ability to adequately suppress and eliminate the RV infection to which the bat had been exposed before entering the captive colony.

With the exception of five *E. fuscus* in our 2002 colony, none of the other *E. fuscus* were positive for anti-rabies VNA when entering the colony. In our 2004 study with *T. brasiliensis*, rabies-positive bats were more likely to be seropositive as compared to the rabies-negative bats, 53% and 27%, respectively. The results of our 2005 study were similar: Fifty-four percent of the rabies-positive and 40% of the rabies-negative *T. brasiliensis* were positive for VNA. The percentage of rabies-negative bats positive for VNA suggests that bats that develop VNA may have been exposed to a less pathogenic variant, a smaller amount of virus, or exposed at a distal or less innervated site. The unpredictability of circulating VNA during RV infections is poorly understood. Due to the neurotropic nature of the rabies infection, the virus may evade the immune system preventing activation of the humoral immune response. Sarmiento et al. (2005) reported the pathogenicity of RV is

TABLE 3. RABIES VIRUS IN THE SALIVARY GLANDS OF FIVE COLORADO BAT SPECIES

Bat species	No. of bats ^a	RV ag in SG ^b	VI from SG ^c
<i>Myotis</i> spp.	1	1 (100)	1 (100)
<i>E. fuscus</i>	22	13 (59)	9 (41)
<i>Lasiurus cinereus</i>	8	6 (75)	4 (50)
<i>Lasionycteris noctivagans</i>	1	1 (100)	1 (100)
<i>Nyctinomops macrotis</i>	4	1 (25)	2 (50) ^d

^aBats received from the CDPHE that were rabies positive based on brain tissue tested with the dFAT at CDPHE and CSU.^bNumber and (percentage) of bats that had RV antigen present in their salivary glands.^cNumber and (percentage) of rabid bats that had infectious virus present in the salivary glands.^dIn one *N. macrotis*, RV antigen was not found in the salivary glands, yet RV was isolated.

CDPHE, Colorado State Department of Public Health and Environment; CSU, Colorado State University; VI, virus isolation.

inversely correlated with the amount of viral glycoprotein produced during infection, and thus lack of VNA would not be surprising after inoculation with a highly pathogenic virus.

Considerably, fewer grounded bats were collected in 2004 than in 2005. Although we were unable to ascertain a single reason for the discrepancy, we anticipate the variation was multifactorial. In 2005, bats were collected in the morning, after returning to the bridge site, and in the evening, after emergence. Terrestrial wildlife lives underneath the bridge site and in surrounding areas; thus, it is possible that grounded bats were removed from the collection site before our evening collection. A higher percentage of the grounded bats collected in 2005 were rabid as compared to bats collected in 2004, 94% and 86%, respectively. The higher incidence of grounded bats and the increased percentage of rabid bats at the bridge site during the 2005 collection may have been the result of a more susceptible bat population or circulation of a more virulent RV variant.

Although rabies is typically transmitted through saliva, RV antigen and/or infectious virus is not always present in the salivary glands of infected animals. RV antigen was present in the salivary glands of 69% of rabid *T. brasiliensis* collected and 61% of the bats submitted by the CDPHE. From salivary glands in which RV antigen was present, RV isolated from the salivary glands of 40% of the rabid *T. brasiliensis* tested and 73% of the salivary glands of rabid bats provided by the CDPHE. The lack of RV antigen suggests that virus may not have disseminated to the salivary glands before death. Further, infection with a more pathogenic virus may result in death before centrifugal dissemination to the salivary glands. Of the rabid *T. brasiliensis* collected during 2005, RV antigen was more likely to be present in the salivary glands of bats without detectable VNA than bats with VNA, 85% and 62%, respectively. The role of VNA in viral dissemination is unclear and more research is required to increase our understanding of the relationship between the immune response and viral movement.

The absence of rabies outbreaks in bat populations is likely the consequence of multiple factors. Anti-rabies VNA may decrease the susceptibility of individual bat, thereby reducing the number of rabid bats in a given population. The degree of virulence of an RV variant may be associated with the percentage of bats in a given population with VNA. Further, the number of seropositive bats may be dependent upon the population's overall health. This interplay of pathogen versus host requires further study if we are to improve our understanding of the evolution of RV infections in bats.

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Disclosure Statement

The authors have declared that no competing interests exist.

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