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Rabies Outbreak in Captive Big Brown Bats (*Eptesicus fuscus*) Used in a White-nose Syndrome Vaccine Trial

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ABSTRACT: An outbreak of rabies occurred in a captive colony of wild-caught big brown bats (*Eptesicus fuscus*). Five of 27 bats exhibited signs of rabies virus infection 22–51 d after capture or 18–22 d after contact with the index case. Rabid bats showed weight loss, aggression, increased vocalization, hypersalivation, and refusal of food. Antigenic typing and virus sequencing confirmed that all five bats were infected with an identical rabies virus variant that circulates in *E. fuscus* in the US. Two bats with no signs of rabies virus infection were seropositive for rabies virus-neutralizing antibodies; the brains of these bats had no detectable viral proteins by the direct fluorescence antibody test. We suspect bat-to-bat transmission of rabies virus occurred among our bats because all rabies-infected bats were confined to the cage housing the index case and were infected with viruses having identical sequences of the entire rabies nucleoprotein gene. This outbreak illustrates the risk of rabies virus infection in captive bats and highlights the need for researchers using bats to assume that all wild bats could be infected with rabies virus.

Key words: Bats, *Eptesicus fuscus*, rabies, virus-neutralizing antibodies.

White-nose syndrome (WNS), an emerging fungal disease caused by *Pseudogymnoascus destructans* (*Pd*), has killed millions of North American insect-eating bats (Blehert et al. 2009; Turner et al. 2011). Research is underway to evaluate methods, including vaccination, to control WNS in bats. This research often requires direct contact with bats, putting personnel at risk of rabies virus infection.

As part of a WNS vaccine study, 27 big brown bats (*Eptesicus fuscus*) were collected from a maternity colony at a private residence in Dane County, Wisconsin, US, in August 2017. Six adult females (A/F), 12 volant juvenile females (J/F), and nine volant juvenile males (J/M) were captured in harp traps and identified with numbered arm bands. Age was estimated based on the degree of fusion of the phalangeal epiphyses (Brunet-Rossinni and Wilkinson 2009). All bats appeared to be healthy based on physical examination on arrival at US Geological Survey National Wildlife Health Center (NWHC). Bats were separated by sex and housed in groups (Table 1) in 40×76×122 cm nylon mesh cages; towels were hung in cages to provide roosting spaces. Bats were maintained on a diet of live mealworms (*Tenebrio molitor*) raised on a diet of grains, vitamins, minerals, and fresh vegetables (Barnard et al. 2011). Mealworms and fresh water were available ad libitum in dishes in cages. Bats were weighed periodically and hand-fed as needed to maintain weights. All animal handling and sampling procedures were approved by the US Geological Survey NWHC Animal Care and Use Committee (ACUC; Protocol EP170719); bats were quarantined and acclimated to captivity for 2 wk prior to the study start as required by the ACUC. No changes in the use of personal protective equipment or handling procedures occurred after the quarantine period, and animal care staff considered all bats potentially infected with rabies virus. On day 14 of captivity, baseline serum samples were collected from adult bats but not from juvenile bats. Because they had not hibernated previously, we assumed that juvenile bats had not been exposed to *Pd*. Bats were randomly assigned to treatment groups while maintaining equal sex and age ratios. On day 16, bats were inoculated with experimental recombinant raccoon poxviruses expressing *Pd* fungal proteins, developed with collaborators at the

TABLE 1. Cage assignments and rabies diagnoses of female captive wild big brown bats (*Eptesicus fuscus*) that experienced an outbreak of rabies during a white-nose syndrome vaccine study. Letters under days indicate cage assignment. Main cages A and C were 42×76×122 cm; isolation cages E, F, G, J, K were 32×38×61 cm. Day 0 is the day of capture. Bats were vaccinated on day 16 and separated into 2 cages by treatment. Sick bats were euthanized. Surviving bats remained in cages indicated on day 86 until they were placed in a hibernation chamber on day 111. Age: A = adult, J = juvenile; ++ = rabies virus detected by direct fluorescent antibody test in bat euthanized on that day; — = no rabies virus detected.

				Cage assignment and rabies diagnosis by day											
No.	Age	Treatment	0	16	17	22	33	40	43	44	48	51	56	62	86
583	J	Vaccine	A												
585	A	Vaccine	A			E[b]									
587	A	Vaccine	A		E[b]						—				
709	A	Vaccine	A		E[b]										
593	J	Vaccine	A							J[c]			++		
706	J	Vaccine	A					++							
707	J	Vaccine	A												
708	J	Vaccine	A					G[c]	++						
710[a]	A	Vaccine	A			F[c]	++								
713	J	Vaccine	A												
714	J	Vaccine	A									—			
715	J	Vaccine	A									++			
589	A	Control	A	C											
500	J	Control	A	C											
588	J	Control	A	C										K[c]	C[d]
594	A	Control	A	C											
704	J	Control	A	C											
705	J	Control	A	C											

[a] Index case.

[b] Moved to smaller cage to receive additional hand-feeding because of weight loss.

[c] Isolated in smaller cage because of wounds or behavior suspicious of rabies virus infection.

[d] Moved back to main cage because abnormal behavior resolved and rabies virus infection was no longer considered probable.

University of Wisconsin, Madison, or sterile water. Vaccinated and control bats, separated by sex, were placed into separate cages (Table 1). Serum samples were collected from all bats at least once after treatment prior to being placed into hibernation on day 111 (Table 2).

On days 18–19, dishes in cage A, housing female vaccinates, were knocked down, and bats were increasingly vocal. Fighting among several bats was noted during weighing on day 22. Bat 710 (A/F) had fresh wounds near her eyes, was very vocal and aggressive, and had lost 4.3 g over the previous week. She was placed in a separate cage to enable individual observation and hand-feeding (Table 1). Over the next 10 days, 710 became anorexic and refused to be hand-fed mealworms. She continued to be vocal and aggressive, biting anything within reach including herself. She was often seen outside of the towel roost during the day, either on the ground or climbing around on the cage netting or appearing to drink from water dishes. On day 33, she had lost more than 7 g over 2 wk and was euthanized due to suspected rabies. The brain was positive for rabies virus (RABV) by the direct fluorescent antibody test (DFA; Centers for Disease Control and Prevention 2016; Table 2), and antigenic typing with 20 nucleoprotein-specific monoclonal antibodies confirmed a variant that circulates in *E. fuscus* in the US (Smith 1989).

In the next week, two more bats (706 and 708, both J/F) from cage A showed signs suspicious of rabies, including weight loss,

TABLE 2. Results of tests for rabies virus neutralizing antibodies in serum using a micro-neutralization, rapid fluorescent focus inhibition test (microRFFIT) and rabies virus in brain tissue using a direct fluorescent antibody test (DFA) of female captive wild big brown bats (*Eptesicus fuscus*) used in white-nose syndrome vaccine study that experienced an outbreak of rabies. A = adult; J = juvenile; NA = not applicable; NT = not tested.

		microRFFIT					
No.	Age	Day	Titer	IU/mL	Result	DFA	Day euthanized
710[a]	A	14	<1:10	<0.1	Negative	Positive	33
706	J	40	1:1635	6.4	Positive	Positive	40
708	J	42	1:396	1.5	Positive	Positive	42
587[ab]	A	48	<1:10	<0.1	Negative	Negative	48
714	J	51	<1:10	<0.1	Negative	Negative	51
715	J	51	1:511	2	Positive	Positive	51
593	J	56	1:684	3	Positive	Positive	56
585[a]	A	48	<1:10	<0.1	Negative	NT	243[c]
709[a]	A	48	<1:10	<0.1	Negative	NT	243[c]
583	J	71	<1:10	<0.1	Negative	NT	243[c]
707	J	71	1:79	0.3	Positive	Negative	243[c]
713	J	NA	NA	NA	NA	NT	243[c]
589[a]	A	43	<1:10	<0.1	Negative	NT	243[c]
594[a]	A	48	<1:10	<0.1	Negative	NT	243[c]
500	J	71	<1:10	<0.1	Negative	NT	243[c]
588	J	43	<1:10	<0.1	Negative	NT	243[c]
704	J	48	<1:10	<0.1	Negative	NT	243[c]
705	J	48	1:137	0.5	Positive	Negative	243[c]
		71	1:327	1.3	Positive		

[a] Baseline samples were taken from adults only on day 14, and all were negative for RVNA.

[b] Bat 587 died under anesthesia.

[c] End of study, includes 132 days of hibernation.

aggression, increased vocalization, and hypersalivation and were euthanatized on days 40 and 43, respectively (Table 1). Both bats were RABV positive by DFA. Rabies virus was isolated from the brains of these two bats, and sequence analysis of the entire nucleoprotein gene showed they were identical to the rabies virus isolated from the index case.

On day 44, bat 593 (J/F) from cage A showed weight loss, developed an abscess on the right forearm, and was placed in a separate cage (Table 1). The abscess was lanced and flushed with chlorhexidine. Although the abscess was resolving, on day 55 she was observed hanging abnormally with open mouth breathing. Crackles were auscultated in both lung fields. The bat had lost more than 7 g body weight and was euthanatized with a suspicion of septic pneumonia or rabies. The bat was RABV positive by DFA. Rabies virus isolated from the brain of this bat was identical to the rabies virus isolated from the index case by sequence analysis of the entire nucleoprotein gene.

On day 51, bats in cage A appeared to be more aggressive. Bat 715 (J/F) exhibited aggression, hypersalivation, refusal of food, and weight loss of 4.7 g over one week. She was euthanatized and tested positive for RABV by DFA (Table 2). Rabies virus isolated from the brain of this bat was identical to the rabies virus isolated from the index case by sequence analysis of the entire nucleoprotein gene. Bat 714 (J/F) had a bite wound on the right patagium and showed signs suggestive of RABV infection, including mild weight loss, increased vocalization, and aggression. The bat was euthanatized, but no RABV was detected in the brain by DFA.

On day 58, bat 702 (J/M, vaccinate) from Cage B, was euthanatized because of increased vocalization, aggression, abnormal locomotion, and hypersensitivity to stimulation; its weight was stable. No RABV was detected in the brain by DFA.

Other bats exhibited behavior suggesting RABV infection. Bat 585 (A/F), a vaccinate that had been placed in a separate cage on day 22 with two other bats to receive additional hand-feeding because of weight loss developed an abscess on the right forearm on day 48, which resolved with treatment. The bat showed increased vocalization and aggression but continued to gain weight. Bat 583 (J/F), a vaccinate that remained in cage A for the entire study, briefly showed aggression. Bat 588 (J/F), a control, exhibited a brief period of aggression, vocalization, and weight loss. Because these bats gained weight over the course of the study, their aggression and vocalization were ultimately attributed to aversion to being handled rather than RABV infection.

Serum samples were tested for rabies virus-neutralizing antibodies (RVNA) by the microneutralization, rapid fluorescent focus inhibition test (microRFFIT; Smith and Gilbert 2017; Table 2) for small sample volumes. None of the 6 A/F bats were seropositive at the initial sampling (day 14). We were unable to obtain a terminal blood sample from the index case, but four other bats that were positive for RABV infection by DFA had positive RVNA titers on the day of euthanasia (Table 2). Two additional bats (both J/F; one vaccinated and one control) had positive RVNA titers at days 48 and 71 but showed no clinical signs of RABV infection (Table 2). The control bat (705) had been separated from the group containing the index case on day 16; the positive vaccinate (707) remained in cage A for the entire study period (Table 1). No RABV was detected by DFA in the brains of these two bats. No male bats, which were housed separately from the females and, thus, the index rabies case, had positive RVNA titers (data not shown).

Overall, five rabid bats showed signs of weight loss, increased vocalization, aggression, hypersalivation, and refusal of food. All rabid bats were female vaccinates housed together with 13 other bats for at least 16 d (Table 1), and all but the index case were juveniles. Even though all five bats were infected with RABV having identical sequences of the entire nucleoprotein gene, it is unclear if bat-to-bat transmission occurred during captivity or if bats were already infected at the time of capture. The incubation period of RABV infection in bats is highly variable, from 13 to 267 d (Jackson et al. 2008; Davis et al. 2012b). In addition, bats can be infectious for several days prior to showing clinical signs (Davis et al. 2012b). Bats in our study showed signs of infection 22–51 d after capture or 18–22 d after initial contact with the index case. Once bat-to-bat aggression was seen in cage A, bats in question were isolated to facilitate close observation and limit injuries to cage mates. Several bats developed abscesses along wing bones presumably because of bite wounds, the primary route of transmission of RABV among mammals; one of these bats (593) became rabid. Other studies have reported clinical rabies in captive wild big brown bats (Moore 1970; Shankar et al. 2004; Davis et al. 2012a, b), including possible bat-to-bat transmission (Davis et al. 2012a). Because all rabid bats came from the cage housing the index case and were infected with RABV having identical sequences of the entire nucleoprotein gene, we suspect that bat-to-bat transmission of RABV occurred among our bats, resulting in a higher number of cases than has been previously reported in captive bats. If infection had been acquired prior to capture, we would expect rabies cases to have been more uniformly distributed among bats in all cages.

None of the male bats (all juveniles) in our colony had positive RVNA titers or developed rabies; two juvenile females that did not develop signs of RABV infection had positive titers indicating exposure to the virus, prior to or during captivity (Table 2). Titers to RVNA are transient in bats (Jackson et al. 2008; Turmelle et al. 2010; O'Shea et al. 2014) and are not reliable for predicting susceptibility to development of clinical infection (Davis et al.

2013). Many rabid bats remain seronegative during the incubation and clinical phases of the infection but develop RVNA titers during the terminal phase (Jackson et al. 2008). Four of our rabid bats had RVNA titers (0.3–6.4 IU/mL) at the time of euthanasia. Bat 710, the index case in our report, was seronegative on day 14 and showed initial signs of rabies infection on day 22, but we were unable to obtain a serum sample at euthanasia on day 33, so we do not know if this bat seroconverted. In another study, a big brown bat that developed clinical rabies during captivity remained seronegative, although it shed virus one week before showing signs (Davis et al. 2012b). Unfortunately, oral swabs for virus isolation were not taken from our bats, so we do not know if any bats were shedding virus.

The first indication of disturbance among our bats occurred 2 d after inoculation with live raccoon poxvirus-vectored vaccine to *Pd*. It is unclear if clinical rabies was related to vaccination. Although all the rabid bats had been inoculated with the experimental vaccine, they were also housed with the index case, putting them at greater risk of becoming infected. In another experimental study, one bat developed rabies 10 d after inoculation with West Nile virus suggesting that the stress of West Nile virus exposure decreased the bat's ability to fight off RABV infection (Davis et al. 2005, 2012a). The vaccine virus used in our study is highly attenuated, and though it is possible that inoculation with live raccoon poxvirus altered the immune response in our bats allowing RABV infection to become clinical, the period (2 d) between vaccination and obvious signs of aggression among bats in the affected cage was very short. Stress associated with captivity and atypical food may also have impacted susceptibility to rabies development.

While several questions remain unanswered concerning RABV infection in our big brown bats, this outbreak illustrates the risk of viral infection in captive bats. Although our quarantine period was only 2 wk, other studies have used quarantine periods of 1–8 mo for wild caught bats (Davis et al. 2007, 2012a; Turmelle et al. 2010). Because the incubation period of RABV infection in bats is highly variable, cases can still occur even after long quarantine periods of 6–8 mo (Moore and Raymond 1970; Davis et al. 2012a, b). Shorter quarantine periods may be more practical when it is important to consider the cost and difficulties of maintaining colonies of captive bats for research studies. Researchers using bats need to assume all wild bats could be infected with RABV, be vigilant for signs of rabies, and adapt when cases disrupt planned studies.

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