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Evaluation of the Eastern Cottontail *Sylvilagus floridanus* as an Amplifying Vertebrate Host for Cache Valley Virus (*Bunyaviridae*) in Indiana

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ABSTRACT: To evaluate the importance of eastern cottontails (*Sylvilagus floridanus*) as amplifying hosts for Cache Valley virus (CVV), we tested hunter-provided blood samples from northern Indiana for specific neutralizing (N) antibodies against this mosquito-borne bunyavirus. Samples were collected during the winter of 1994–95. Two seronegative eastern cottontails, captured in July 1995, were also infected with CVV by subcutaneous inoculation, and two others were infected by allowing CVV-infected mosquitoes to feed on them. The results indicate that eastern cottontails probably are not important amplifying hosts for CVV. The prevalence of N antibodies against CVV was low (6.0%, $n=82$) among the hunter-killed animals. Low viremia (<1.8 $\log_{10}$ plaque-forming units/ml) of short duration (1–3 days) were seen in three of four experimentally infected eastern cottontails. The viremias were insufficient for infecting *Coquillettidia perturbans*, a mosquito species commonly found naturally infected with CVV.

Key words: *Bunyaviridae*, Cache Valley virus, *Orthobunyavirus*, serology, *Sylvilagus floridanus*.

Cache Valley virus (CVV), a North American orthobunyavirus (family *Bunyaviridae*, genus *Orthobunyavirus*) with a wide geographic distribution, cycles between mosquitoes and mammals in nature (Calisher et al., 1986). The amplifying vertebrate host(s) is/are not known; however, high prevalence of antibodies against CVV have been reported in domestic and wild ungulates (Kokernot et al., 1969b; Hoff et al., 1973; McLean et al., 1987; Crandell et al., 1989). This virus also has been isolated from horses, cattle, and sheep (McConnell et al., 1987; McLean et al., 1987). Because most large mammals are long-lived and populations of various species can maintain high levels of herd immunity, they generally are not considered adequate amplifying hosts for enzootic arboviruses. Experimental CVV infections of cattle, swine, and goats have shown that these animals develop a very low viremia, probably of insufficient magnitude to infect potential vector mosquitoes (Kokernot et al., 1969a).

Little is known about the prevalence of CVV infections in small mammals. Antibodies to the virus have been detected in woodchucks (*Marmota monax*), raccoons (*Procyon lotor*), gray foxes (*Urocyon cinereoargenteus*), and various rodent species (Kokernot et al., 1969b; Buescher et al., 1970). A number of small animal species have been experimentally infected including raccoons and opossums (*Didelphis virginiana*) (Kokernot et al., 1969b), as well as desert cottontails (*Sylvilagus audubonii*), jackrabbits (*Lepus californicus*), Fresno kangaroo rats (*Dipodomys nitratoides exilis*), California ground squirrels (*Spermophilus beecheyi*), Nelson's antelope ground squirrels (*Ammospermophilus nelsoni*), deer mice (*Peromyscus maniculatus*), and laboratory white mice (*Mus musculus*) (Francy, 1972). The opossums' virus titers were low; however, desert cottontails, Fresno kangaroo rats, and Nelson's antelope ground squirrels were more susceptible to CVV, with two-to-three-day mean viremia titers ranging from 3–5 $\log_{10}$ plaque-forming units (pfu)/ml.

The last three species, those that developed elevated viremia levels, occur in the western United States (Hall, 1981) and could serve as amplifying hosts for CVV

there. However, species of *Sylvilagus* occur in most parts of the North American continent where CVV has been isolated. In the Midwest the eastern cottontail (*Sylvilagus floridanus*) is common (Hall, 1981). Two *Anopheles* spp. (*An. quadrimaculatus* and *An. punctipennis*) and *Coquillettidia perturbans* mosquitoes have been implicated as potentially important Midwestern CVV vectors (Blackmore et al., 1998). *Coquillettidia perturbans* feed on a variety of birds and mammals, including rabbits (Edman, 1971). While these two *Anopheles* spp. feed mostly on large mammals, blood meal analyses have revealed that rabbits also are common blood meal hosts (Kokernot et al., 1969b; Robertson et al., 1993). The objective of this study was to determine, through serosurveillance and experimental infections, whether eastern cottontails could serve as vertebrate amplifying hosts of CVV.

Blood samples for the serosurvey were collected from eastern cottontails in five counties of northern Indiana during December 1994 and January 1995. Local hunters were supplied with Nobuto® (Microfiltration Systems, Dublin, CA, USA) filter paper strips. After these were soaked with blood from harvested animals, strips were air-dried, placed in envelopes containing information about the collector and the site of the kill, and mailed to the University of Notre Dame, Notre Dame, Indiana, USA. In the laboratory, they were stored at 4 C until eluted and tested.

Female *Cq. perturbans* were collected in CO_2-baited CDC miniature light traps (Sudia and Chamberlain, 1962) 2.5 km northwest of Edwardsburg, MI (41.5°N, 86.0°W) in July 1995 and 1996. Old female mosquitoes with worn wings or missing scales were excluded from the experiment. The mosquitoes were held at 21 C and 80% R.H. until use.

Eastern cottontails used in the infection experiment were captured in July 1995 ($n=2$) and June 1996 ($n=2$) by a licensed, professional trapper in New York State and shipped to the University of Notre Dame. The animals were held individually in 91 $cm^3 \times 91$ $cm^3 \times 91$ cm^3 rabbit cages with free access to food and water. A 30 cm^3 nesting box was also supplied to each cage. All work was performed following an experimental protocol approved by the University of Notre Dame IACUC.

The CVV strain used in these experiments (strain MI80-1-450 smb-1, Vero-1), obtained from the Centers for Disease Control and Prevention, Fort Collins, Colorado, USA, was isolated from a horse in St. Joseph County, Michigan, USA, in 1980 (McLean et al., 1987).

Mosquitoes were infected orally with 6.1 $\log_{10}$ pfu/ml virus in a 1:10 CVV/defibrinated rabbit blood mixture as described by Grimstad et al. (1989). To increase the likelihood of feeding, the mosquitoes were sugar-starved 24 hr prior to the experiment. Samples of unfed field-collected females were also frozen (−70 C) and assayed as controls. Fully engorged females were held in an insectary at 27 C and 80% R.H. for 21 days. A 16 hr light:8 hr dark photoperiod was used in all experiments.

The two rabbits trapped in 1995 were infected with CVV by mosquito bite (six infected mosquitoes were removed at random from the holding cage; two fed on one rabbit, four were allowed to feed on the other). Engorged mosquitoes were frozen at −70 C until assayed for virus. In 1996, the rabbits were infected subcutaneously with 5.1 $\log_{10}$ median tissue culture infectious dose ($TCID_{50}$) CVV.

Blood samples were obtained from each eastern cottontail by ear vein puncture for five consecutive days post-infection (PI). The blood was immediately mixed with an equal volume of Medium 199 supplemented with 10% Seru-Max-2, 2.2 g $NaHCO_3$, and 2.5 mg amphotericin B (Sigma Chemical Company, St. Louis, Missouri, USA) and 75 mg gentamycin (J.R.H. Biosciences, Inc., Lenexa, Kansas, USA)/liter (M199) and frozen at −70 C. Blood was collected

TABLE 1. Cache Valley virus viremia and antibody responses in *Sylvilagus floridanus* infected by mosquito bite or by subcutaneous inoculation.

Animal	No. of mosquitoes[a]	Viremia titer[b] (pfu/ml) PID 1	PID 2	PID 3	No. of mosquitoes[c]	Antibody titer[d] PID 9
1	4	0	44	0	50	8
2	2	0	0	0	39	32
3	0	52	0	3	45	16
4	0	0	47	0	42	8

[a] Number of mosquitoes used to infect two of the eastern cottontails (Animals 1, 2). Two animals (Animals 3, 4) were infected by subcutaneous inoculation.

[b] Viremia titers are presented from post-infection day (PID) 1–3.

[c] Average number of mosquitoes refeeding on viremic animals on PID 1–5. None of the mosquitoes tested positive for CVV.

[d] Cache Valley virus neutralizing antibody titers were measured on PID 9.

prior to the mosquito feedings and on day 9 PI to determine whether seroconversion had occurred. These samples were diluted twofold with M199 centrifuged at 3,000 × G for 10 minutes and stored at 4 C until tested.

To establish whether the viremias of the eastern cottontails were sufficiently high to infect vector mosquitoes, field-collected female *Cq. perturbans* were allowed to feed on the rabbits for 30–60 min on days 1 through 5 PI. Fully engorged mosquitoes were held for 48 hr and then stored at −70 C until assayed to allow time for virus penetration into midgut cells and enzyme digestion of unabsorbed virions (Edman and Spielman, 1989). For blood sampling and subsequent mosquito feeding, the animals were sedated intramuscularly with 0.1–0.3 ml of either a combination of Fentanyl (0.4 mg/ml) and Droperidol (20 mg/ml) (Inovar®, Pitman Moore, Mundelin, Illinois, USA) in 1995 or Acepromazine maleate (10 mg/ml) (Fermenta Animal Health Company, Kansas City, Missouri, USA) in 1996.

Rabbit sera from inoculated and hunter-killed rabbits were assayed for CVV antibodies as described by Blackmore and Grimstad (1998). Mosquitoes, the artificial blood meal and rabbit blood samples were assayed for virus by plaque assay on confluent Vero cell cultures (Blackmore et al. 1998; Blackmore and Grimstad, 1998).

Blood samples were obtained from 82 *Sylvilagus floridanus* hunted in five northern Indiana counties. Neutralizing antibodies against CVV were detected in 14% (4/28) of the animals from LaGrange County and 14% (1/7) of the eastern cottontails harvested in Jasper County; no seropositive eastern cottontails were found among those collected in Newton ($n=44$), Porter ($n=1$), or Noble counties ($n=2$).

The six mosquitoes utilized for transmitting the virus to eastern cottontails all had disseminated CVV infections. However, despite being fed upon by multiple mosquitoes capable of transmitting CVV, only low levels of viremia were induced in the two rabbits (Table 1). The viremia was too low to subsequently infect *Cq. perturbans* mosquitoes fed on the viremic animals ($n=50$ mosquitoes/animal/day) (Table 1). The subcutaneously inoculated rabbits also developed a low viremia, and the mosquitoes fed on these animals did not become infected either ($n=45$, 42 mosquitoes/animal/day; Table 1). All eastern cottontails had seroconverted by day 9 PI.

The results of this small study demonstrated that eastern cottontails become infected with CVV in northern Indiana and perhaps elsewhere in the Midwest, but are probably not important as a vertebrate host for this virus. Cache Valley virus is common in the area of Indiana where the serosurvey was conducted. A survey of

white-tailed deer (*Odocoileus virginianus*) from the same region yielded >80% prevalence of N antibodies against CVV (Blackmore and Grimstad, 1998). Neither virus transmission by mosquito bite nor subcutaneous injection produced a sufficient viremia to infect female *Cq. perturbans* feeding on the rabbits despite the high viral dose used in both experiments. In studies with suckling mice, *Cq. perturbans* artificially infected with a dose similar to that which we used to infect mosquitoes in this study had transmission rates exceeding 60% after 21 days of incubation (Blackmore et al., 1998). One or both of the eastern cottontails infected by mosquito bite were therefore most likely infected by more than one mosquito and more than one dose of virus.

White-tailed deer inoculated with 4.7 $\log_{10}$ $TCID_{50}$ CVV produced a 4.0 $\log_{10}$ pfu/ml mean peak viremia (Blackmore and Grimstad, 1998). These findings, the high seroprevalence of CVV antibodies in local deer populations, together with the results from this study suggest that white-tailed deer are an important vertebrate host for CVV in Indiana. However, to reach a definitive conclusion about the role of eastern cottontails in the CVV transmission cycle in the Midwestern US, the virus transmission experiments need to be expanded to include a larger number of preferably locally obtained animals. Difficulties encountered in procuring eastern cottontails locally in numbers during the summer season when *Cq. perturbans* populations emerge must first be resolved as this mosquito species has never been colonized, and thus all studies require use of fresh field-emerged adult females.

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