Overwintering of Infectious Buggy Creek Virus (Togaviridae: Alphavirus) in *Oeciacus vicarius* (Hemiptera: Cimicidae) in North Dakota

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ABSTRACT Arboviruses have seldom been found overwintering in adult vectors at northern latitudes in North America. Buggy Creek virus (BCRV; Togaviridae, *Alphavirus*) is an ecologically unusual arbovirus vectored principally by the cimicid swallow bug (*Oeciacus vicarius* Horvath). The ectoparasitic bugs reside year-round in the mud nests of their host, the cliff swallow (*Petrochelidon pyrrhonota* Vieillot). We report successful overwintering of infectious BCRV in bugs at a field site in western North Dakota, where mid-winter temperatures routinely reach −11 to −15°C. Approximately 21% of bug pools were positive for virus in early spring just before the cliff swallows' return to their nesting colonies; this proportion did not differ significantly from that in summer at active cliff swallow nesting colonies in the same study area. Fewer of the isolates in early spring were cytopathic on Vero cells, and those that were infectious showed less plaque formation than did summer samples. The results show that infectious BCRV commonly overwinters in the adult stages of its vector at northern latitudes in North America.

KEY WORDS Buggy Creek virus, cliff swallow, *Oeciacus vicarius*, *Petrochelidon pyrrhonota*, swallow bug

Most arboviruses at temperate latitudes undergo interrupted transmission during the winter months when their arthropod vectors die off or have reduced activity levels. Continued summer transmission therefore requires that these viruses either overwinter locally or are reintroduced each season by immigrant vertebrate hosts or vectors (Reeves 1974, 1990; Rosen 1987; Reisen 1990; White et al. 2005). Massive efforts to isolate various arboviruses from adult mosquitoes in California during the mid-winter months produced negative results, suggesting that many arboviruses do not commonly overwinter in mosquito vectors (Reeves et al. 1958; Reeves 1974, 1990; Reisen et al. 2006). Among the alphaviruses (Togaviridae), empirical evidence for virus overwintering in adult arthropod vectors at temperate latitudes in central and northern North America is limited to a single report of western equine encephalomyelitis virus (WEEV) in Colorado (Blackmore and Winn 1956).

Buggy Creek virus (BCRV; Togaviridae, *Alphavirus*) is an unusual arbovirus within the WEEV complex of alphaviruses (Calisher et al. 1988, Hopla et al. 1993, Pfeffer et al. 2006, Padhi et al. 2008) that is transmitted primarily by the cimicid swallow bug (Hemiptera: Cimicidae: *Oeciacus vicarius* Horvath) and amplified by the ectoparasitic bug's main avian hosts: the colonially nesting cliff swallow (*Petrochelidon pyrrhonota* Vieillot) and introduced house sparrow (*Passer domesticus* L.) (Hayes et al. 1977; Rush et al. 1980; Scott et al. 1984; Brown et al. 2001, 2007). Fort Morgan virus, also associated with cliff swallows and swallow bugs (Hayes et al. 1977, Calisher et al. 1980, Scott et al. 1984), is a strain of BCRV (Pfeffer et al. 2006, Padhi et al. 2008). Swallow bugs are wingless and remain in or near nests at the swallow nesting colonies throughout the year. The relatively constant summer prevalence of BCRV in bug vectors across years at a study site in Nebraska (Brown et al. 2001) suggests that this virus might be maintained in its sedentary vector during the winter months. To investigate the ability of BCRV to overwinter in cold climates, we tested swallow bugs for BCRV infection in early spring at a northern latitude site in western North Dakota before the arrival of the swallow hosts.

Materials and Methods

Study Site. The study site was in western North Dakota near the towns of Bowman (46°10′ N, 103°24′ W) and Dickinson (46°33′ N, 102°47′ W) and included swallow colony sites in Adams, Bowman, Hettinger, and Stark counties. These colony sites were situated exclusively in concrete box-shaped culverts underneath county roads and state highways.

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Table 1. Prevalence of BCRV by RT-PCR in pools of swallow bugs collected at five cliff swallow colony sites in western North Dakota in early spring (April) 2007 compared with prevalence at those same sites the previous and subsequent summers (in June)

Site no. (latitude-longitude)	June 2006		April 2007		June 2007	
	% pools positive	*N*	% pools positive	*N*	% pools positive	*N*
1 (46°00.969′ N, 102°41.231′ W)	50.0	6	100.0	1	—	—
2 (46°01.516′ N, 102°42.715′ W)	13.3	15	11.1	18	—	—
3 (46°00.128′ N, 102°32.047′ W)	75.9	29	37.5	8	57.1	14
4 (46°10.186′ N, 103°13.057′ W)	25.8	31	0.0	1	28.6	7
5 (46°00.498′ N, 102°39.881′ W)	16.7	12	20.0	25	—	—

Field Collections of Bugs. Overwintering bugs were collected on 23 April 2007 from five colony sites that had contained active cliff swallow nests the previous summer. We scraped old nests off the culvert wall to expose aggregations of wintering bugs that were wedged between the nest and concrete substrate. We brushed these bugs off the wall into a collecting jar and picked out others individually from the nest fragments. No cliff swallows had yet arrived at the study colonies from their South American winter range on the April sampling date, and none of the bugs were engorged with blood. Based on the relatively large difference between the width of the head versus abdomen (Usinger 1966), all of the bugs seemed to be adults.

We also collected bugs on 21–23 June 2006 and 27–30 June 2007 from the same colony and neighboring sites within the same area. Summer collections were done only at active sites (with cliff swallows present), where we brushed bugs off the outsides of nests (see Moore et al. 2007).

Bugs were transported at outside ambient temperature to the laboratory, where they were sorted into pools of 100 individuals while alive and frozen immediately at −70°C.

Virus Screening and Isolation. Egg pools were macerated with a Mixer Mill (MM 31) from Qiagen (Valencia, CA.). Homogenates were clarified by centrifugation at 11,000*g* for 1 min and subsequently stored at −70°C. For RNA extraction, a 100-μl aliquot of the supernatant of each homogenate was added to 400 μl of a guanidine thiocyanate–based lysis buffer. After the addition of 400 μl of 100% ethanol, RNA was extracted using the QIAmp Viral RNA Mini Kit (Qiagen) following the manufacturer's protocol, modified by increasing the amount of buffer AVE (water) to yield 100 μl total RNA per sample. A negative control (water in place of supernatant but otherwise treated the same) was placed between every five samples during extraction and maintained in the same position for reverse transcriptase-polymerase chain reaction (RT-PCR). A positive BCRV control also was included in each extraction.

RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen) following the manufacturer's protocol. Primers and themocycler conditions are given in Moore et al. (2007). A portion (6.5 μl) of each amplification product was electrophoresed on a 4% Nusieve/agarose gel, together with a BCRV amplicon and a 100-bp cDNA ladder, to identify BCRV-positive pools.

Bug pools that were positive by RT-PCR were subjected to plaque assay on Vero cells. We added 100 μl of each supernatant in duplicate to a confluent monolayer of Vero cells in a six-well cell culture plate and incubated it for 1 h at 37°C in 5% CO_2, overlaid each monolayer with 3 ml 0.5% agarose in yeast extract–lactalbumin overlay medium supplemented with 2,240 mg/liter sodium bicarbonate and 292 mg/liter L-glutamine, and returned the plate to the incubator. A second 3-ml overlay, prepared as before but supplemented with 0.004% neutral red dye, was added after 2-d incubation for plaque visualization. Plaques were scored on the third and fourth days of incubation. For RT-PCR–positive samples that showed no plaques on Vero cells, we re-extracted RNA from the remaining swallow bug homogenate and performed another RT-PCR assay to verify the presence of noncytopathic (noninfectious) viral RNA (Moore et al. 2007).

Results

Collectively, 20.8% of the bug pools ($N = 53$) from the five colony sites sampled in April 2007 were positive for BCRV by RT-PCR. At least one positive pool was found at four of the five colony sites sampled (Table 1). For all sites sampled during June 2006 and 2007, 22.5 ($N = 182$) and 15.6% ($N = 96$) of bug pools, respectively, were positive for BCRV by RT-PCR. The percentage of pools positive did not differ significantly between the three sampling periods ($\chi^2_2 = 1.87$, $P = 0.39$) or when April was compared with the two June periods together ($\chi^2_1 = 0.01$, $P = 0.92$). For three individual colony sites for which we had early-spring sample sizes greater than one pool, we found no significant difference in the percentage of pools positive between the early-spring and summer samples at two of these sites (sites 2 and 5; $\chi^2_1 = 0.0$, $P \geq 0.81$), whereas at the third site (site 3), there was significantly less virus in early spring than in summer ($\chi^2_1 = 6.7$, $P = 0.01$; Table 1).

Of the 11 pools positive for BCRV by RT-PCR from April 2007, 45.5% were cytopathic on Vero cells compared with 97.5% of positive pools from June 2006 ($N = 40$) and 86.7% of positive pools from June 2007 ($N = 15$). The percentage of cytopathic positives was significantly lower in April than in the two June periods combined ($\chi^2_1 = 18.8$, $P < 0.001$). The highest PFU found for an April sample was 45 PFU/ml. In contrast, 23 of the June samples showed >2,000 PFU/ml.

Discussion

In an earlier study, we verified that the North Dakota virus isolates were BCRV by sequencing them and finding that they were all BCRV lineage B (Padhi et al. 2008). Our finding of cytopathic (i.e., infectious) BCRV in swallow bugs in early spring just before the cliff swallows' return to the North Dakota nesting colonies showed the apparent capacity of this virus to survive the winter at relatively northern latitudes in North America. We are confident that the isolates found were overwintering virus and not ones recently reintroduced to the study area by cliff swallows, because (1) no cliff swallows were observed by us anywhere in the study area at the time of sampling, (2) cliff swallow nesting does not typically start in North Dakota until May, with the earliest active nest recorded in the state on 8 May (Stewart 1975), (3) there is no empirical evidence of reintroduction of BCRV by returning cliff swallows in spring at sites farther south in the Great Plains (Hayes et al. 1977, O'Brien et al. 2008), and (4) none of the harvested bugs showed evidence of recent blood feeding (i.e., none had a dark blood spot visible in the abdomen), indicating that none had fed on any house sparrows that might have been present in the colonies (and we observed no sparrows on our April collecting visit).

Western North Dakota represents a relatively inhospitable environment for overwintering of arboviruses in adult insects. During the 3 coldest mo of the winter, December–February, average minimum temperatures (as measured at Dickinson; NWS 2008) are −13, −15, and −11°C, respectively, with a record low of −44°C recorded for February. Although the concrete culvert walls on which the bugs clustered (and the birds' mud nests) may have moderated these temperatures, it seems likely that this is among the colder natural environments in which arboviruses persist in adult arthropods. Previous reports of arbovirus overwintering in adult insects (Blackmore and Winn 1956, Bailey et al. 1978, Nasci et al. 2001, Farajollahi et al. 2005) involved mosquitoes in buildings and mines in which indoor temperatures during winter were likely above freezing.

We found no difference in prevalence of viral RNA-positive bugs in April versus June, suggesting that infected bugs do not suffer survival costs in winter and/or that the prevalence of virus does not decline in bugs during their winter dormancy. However, the overwintering samples produced fewer plaques, with less than one half of these isolates showing cytopathicity compared with 85% or more of pools collected in summer. The reduced cytopathicity of the April samples may reflect both lower virus titers at that time of year and the fact that those bugs had not recently blood-fed at the time of collection. Reisen et al. (2002) found that metabolic changes associated with the termination of diapause and digestion of a blood meal increased viral titers of WEEV and St. Louis encephalitis virus in overwintering *Culex tarsalis* Coquillett, and other studies showed that blood feeding was necessary to produce infectious virus in overwintering mosquitoes (Reeves et al. 1958, Bailey et al. 1978). The same result may apply to BCRV in swallow bugs.

Although a number of arboviruses may overwinter in eggs of arthropods through vertical transmission (Tesh 1984, Rosen 1987), relatively few apparently overwinter in adult arthropods at northern latitudes. Eggs of swallow bugs are not known to overwinter in cold climates (Usinger 1966). BCRV's ability to commonly overwinter in its adult vector allows it to persist at colony sites from year to year even when its principal amplifying host (the cliff swallow) completely vacates the colony sites on an annual basis and does not seem to regularly reintroduce the virus to these sites in spring (O'Brien et al. 2008).

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