Persistence of Buggy Creek Virus (*Togaviridae*, *Alphavirus*) for Two Years in Unfed Swallow Bugs (Hemiptera: Cimicidae: *Oeciacus vicarius*)

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ABSTRACT Alphaviruses (*Togaviridae*) have rarely been found to persist for long in the adult insects that serve as their vectors. The ectoparasitic swallow bug (Hemiptera: Cimicidae: *Oeciacus vicarius* Horvath), the vector for Buggy Creek virus (BCRV; *Togaviridae*, *Alphavirus*), lives year-round in the mud nests of its host, the cliff swallow (*Petrochelidon pyrrhonota* Vieillot). We measured the prevalence of BCRV in swallow bugs at sites with cliff swallows present and at the same sites after cliff swallows had been absent for 2 yr. We collected bugs directly from cliff swallow nests in the field and screened bug pools with BCRV-specific real-time-polymerase chain reaction (RT-PCR) and plaque assay. At two colony sites last occupied by birds 2 yr earlier, we found 12.5 and 55.6% of bug pools positive for BCRV RNA by RT-PCR. Infection rates (per 1,000 bugs) for these sites were 1.32 and 7.39. RNA prevalence in the unfed bugs was not significantly different from that in fed bugs 2 yr earlier at the same sites. The RNA-positive samples from unfed bugs failed to yield cytopathic BCRV by Vero-cell plaque assay. However, viral RNA concentrations did not differ between unfed bugs and bugs at active sites, and over 84% of positive bug pools were cytopathic to Vero cells 4–5 wk later, after cliff swallows moved into one of the colony sites. These data demonstrate the persistence of potentially infectious BCRV in unfed swallow bugs for at least 2 yr in nature.

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

One critical variable in understanding the transmission dynamics of most arboviruses is the extent to which virus persists in either the vector or host for extended periods (Reeves 1974, 1990, White et al. 2005, Wilson et al. 2008). Long-term persistence potentially allows arboviruses to survive when local conditions (e.g., winter climate in the Northern hemisphere) interrupt transmission. The ability of viruses to persist as chronic infections in vertebrate hosts (Levine et al. 1994, Kuno 2001) or in eggs or larvae of vectors (Rosen 1981, Tesh 1984, Turell 1988) has been studied extensively, but less is known about how long most arboviruses are routinely maintained in adult arthropods. Virus persistence has been best documented in some of the relatively long-lived ticks; for example, tick-borne encephalitis (TBE) viruses (*Flaviviridae*, *Flavivirus*) in Eurasia persist in unfed *Ixodes* ticks through the winter, and adults have been found capable of maintaining infectious virus for up to a year in the field (Grešíková and Nosek 1967, Nosek and Grulich 1967). *Ornithodoros* ticks infected with TBE viruses in the laboratory were able to transmit virus up to 3 yr later (Turell et al. 2004). Colorado tick fever virus (*Reoviridae*, *Coltivirus*) has been detected in *Dermacentor andersoni* (Stiles) (Acari: Ixodidae) ticks in the field up to 2 yr after initial infection (Eads and Smith 1983).

Long-term arbovirus persistence has rarely been documented in the more short-lived dipteran vectors, such as mosquitoes, which typically live at most only a few months, even when undergoing winter diapause (Briegel and Kaiser 1973, Mitchell 1979, Bailey et al. 1982). Because the alphaviruses (*Togaviridae*, *Alphavirus*) are mostly transmitted by mosquitoes (Strauss and Strauss 1994), in most situations these viruses do not have the ecological potential to be maintained in the adult vectors for extended periods. Furthermore, alphavirus infection can reduce survival of some mosquitoes (Scott and Lorenz 1998, Moncayo et al. 2000, Mahmood et al. 2004), potentially preventing long-term virus persistence. Only a few reports of alphaviruses apparently surviving for several (winter) months in adult mosquitoes exist (Blackmore and Winn 1956, Reeves et al. 1958, Bellamy et al. 1967).

The swallow bug (Hemiptera: Cimicidae: *Oeciacus vicarius* Horvath) is the only known vector for Buggy Creek virus (BCRV), an alphavirus in the western equine encephalomyelitis virus (WEEV) complex (Hayes et al. 1977, Rush et al. 1980, Hopla et al. 1993,

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Table 1. Summary of sampling and BCRV prevalence (by RT-PCR) in swallow bugs at two cliff swallow colony sites in Keith County, NE, where site usage by birds allowed study of whether BCRV persisted for longer than 1 yr

Sampling date	Swallows present	Bug pools positive (%)	IR (skewness-corrected 95% CI) per 1,000 bugs	ΔC_t range	RT-PCR positives forming plaques (%)
Site CS					
28 June 2007	Yes	5.3 ($n = 38$)	0.53 (0.10–1.75)	3.8, 10.9	100
Summer 2008	No	—	—	—	—
24 May 2009	No	12.5 ($n = 40$)	1.32 (0.49–2.93)	13.7–17.5	0
Site NN					
Summer 2003	Yes	—	—	—	—
9, 31 May 2004	No	38.9 ($n = 54$)	4.85 (3.10–7.36)	0.5–16.9	38.1
14 May 2005	No	55.6 ($n = 9$)	7.39 (2.83–17.79)	9.1–21.0	0
6, 29 June; 7 July 2005	Yes	27.7 ($n = 47$)	3.19 (1.79–5.36)	3.6–17.8	84.6

Moore et al. 2007, Brown et al. 2008). Swallow bugs transmit BCRV to their avian hosts, the cliff swallow (*Petrochelidon pyrrhonota* Vieillot) and the house sparrow (*Passer domesticus* L.; Brown et al. 2009b, O'Brien 2009), during blood meal acquisition. The ectoparasitic swallow bugs routinely overwinter in the mud nests of the colonially nesting cliff swallow, and bugs frequently maintain infectious BCRV throughout the winter in the western Great Plains (Brown et al. 2009a, 2010). If a nesting site is unused by swallows and sparrows in a particular breeding season, some bugs will survive into that summer and maintain detectable BCRV infections (Moore et al. 2007) for a year without having fed on blood. However, the capacity of this virus to persist for even longer periods in this insect vector is unknown. Because bugs have been recorded routinely living up to 3 (and occasionally even 4) yr in the absence of a blood meal in the field (Smith and Eads 1978, Loye and Carroll 1991, Rannala 1995), we investigated whether bugs could maintain BCRV for longer than a year under natural conditions. If so, this virus would have the potential to survive a summer in which no birds occupy a given cliff swallow colony site and to resume transmission among birds when they return a year later, without having to be reintroduced to the site.

Materials and Methods

Study Site. Our BCRV studies were conducted in western Nebraska where we have studied cliff swallows and their ectoparasites for almost 30 yr (Brown and Brown 1996; Brown et al. 2001, 2008, 2009b). Since our work on BCRV began in 1998, only two colony sites have had bird usage patterns appropriate for studying the long-term persistence of BCRV in swallow bugs. Both colony sites were in concrete culverts ≈1 km apart underneath the Union Pacific Railroad tracks northwest of Keystone, Keith County, NE. One site, designated NN (41°15.783′ N, 101°40.955′ W), was constructed in 1994 and was first used by cliff swallows in 2000 (one nest only) and by larger numbers in 2001 (90 nests). The second site, designated CS (41°15.794′ N, 101°40.258′ W), was constructed in 1997 and was first used by cliff swallows in 2002 (335 nests). The birds attached their mud nests inside the culverts on the walls just below the ceiling at each site.

Field Methods. Swallow bugs clustering at the entrances of abandoned, intact cliff swallow nests were collected from site NN (from all nests) when it was inactive on 9 May 2004, 31 May 2004, and 14 May 2005 and from site CS (from about half the nests) on 24 May 2009 (Table 1) by brushing the bugs into a wide-mouthed collecting jar. We supplemented bug collections at CS by scraping remnants of five nests into a bag and sorting through the nest chunks.

Swallow bugs were collected from site NN (385 active nests) when birds were nesting on 6 June, 29 June, and 7 July 2005 and from site CS (565 active nests) on 28 June 2007 (Table 1). Bugs from these colonies were obtained by brushing them off the outside bowl of active nests (below the entrance); some bugs that had wedged into the lacunas between nests were also taken. In all cases, bugs were transported to the laboratory, sorted into pools of 100 individuals while alive, and then frozen at −70°C.

We looked for recent blood-feeding activity in the bugs by examining the opaque abdomen for presence of clotted blood. Age of bugs (i.e., adults versus instars) was determined from the head:abdomen width ratio (Usinger 1966). We monitored use of the two sites by any nesting cliff swallows or house sparrows by making several visits to each vacant colony site during the 2004 and 2005 (NN) and 2008 and 2009 (CS) bird nesting seasons. Use of abandoned nests by house sparrows was further evaluated on each visit by inspecting each nest for the bulky grass lining added by sparrows.

Virus Screening. Swallow bug pools were processed as described by Brown et al. (2008). RNA was extracted from bug homogenates and assayed for BCRV RNA by RT-PCR amplification of a segment of the E2 region of the viral genome using BCRV-specific primers (Moore et al. 2007). Pools that were positive in the initial RT-PCR were confirmed with a multiplex real-time RT-PCR assay (Brown et al. 2009b). This resulted in a normalized C_t value (cycle number at which the fluorescence exceeded a predefined threshold), expressed as ΔC_t, and calculated by subtracting the C_t value obtained for an external control (human beta-2-microglobulin gene) from that of the putative BCRV amplicon in each reaction. This method corrected for variations in the efficiency of RT-PCR (Bustin and Nolan 2004, Hugget et al. 2005, Gilsbach et al. 2006),

and meant that as the ΔC_t value increased for a sample, the concentration of BCRV RNA of that sample relative to others decreased.

Samples that confirmed BCRV-positive by RT-PCR were tested by plaque assay on Vero cells (Moore et al. 2007). Cells were examined for plaque formation on day 3 after inoculation. Samples that did not produce plaques were reextracted and RT-PCR repeated to confirm presence of viral RNA in the sample.

Infection rates (IR) were computed with PooledInfRate, version 3.0 (Biggerstaff 2007), and are given as the number of bugs infected (with the skewness-corrected 95% CI) per 1,000 bugs. Calculations of IR considered positive pools to be those positive by RT-PCR.

Results

All of the bugs collected from inactive colony sites (no birds present; Table 1) were unfed adults with no evidence of recent blood meals. Many of the bugs collected at the sites when active (birds present; Table 1) contained blood, and these samples consisted of both adults and nymphal instars.

At site CS (Table 1), the difference in BCRV RNA prevalence in bug pools between 2007 (birds present and bugs fed) and in 2009 (no birds and unfed bugs) was not significant ($\chi^2_1 = 1.25$, $P = 0.26$), and the IRs were not significantly different, based on the overlapping CIs. We could not statistically compare ΔC_t values from the two sampling periods because there were only two positives in 2007 (Table 1). Both of the 2007 positives exhibited plaque formation in Vero cells, at >2,000 plaque forming units (PFU)/ml and 27.5 PFU/ml, respectively. None of those from 2009 formed plaques (Table 1).

At site NN (Table 1), there was no significant difference in BCRV RNA prevalence in bug pools between the mid May 2005 samples and those of 2004 (no birds present and bugs unfed in both cases) ($\chi^2_1 = 0.88$, $P = 0.35$). The IRs for these two periods also did not differ significantly. The positive pools in 2004 that formed plaques in Vero-cell assays ranged from 1.0 to 76.0 PFU/ml. None of those from mid May 2005 formed plaques (Table 1). ΔC_t values (Table 1) for the 2004 positives were not significantly different from those in mid May 2005 (Wilcoxon test, $Z = 1.43$, $P = 0.15$). Bug samples were not available for 2003 when the site was used by cliff swallows (360 nests).

Cliff swallows returned to site NN on 18 May 2005, 4 d after we took the mid May samples (Table 1). BCRV RNA prevalence in bug pools taken at the site after it became active in 2005 (fed bugs) was not significantly different from prevalence when the site was not active in 2004–2005 (unfed bugs) ($\chi^2_1 = 2.18$, $P = 0.14$), although the highest prevalence and highest IR was in mid May 2005 from unfed bugs just before the birds returned (Table 1). Of the pools from NN after it became active that formed plaques in Vero cells, seven had >2,000 PFU/ml. ΔC_t values for the positives in 2005 when NN was active and bugs were fed (Table 1) were not significantly different from those for the unfed bugs in 2004–2005 combined ($Z = 0.28$, $P = 0.78$) or from the unfed bugs in 2005 alone ($Z = 1.18$, $P = 0.24$).

We detected no bird nesting activity at site CS at any time during the summer in either 2008 or 2009. Site NN had no bird nesting activity at any time in 2004 and remained inactive until 18 May 2005. House sparrows have been absent from both colony sites since they were first constructed, and they were not detected during this study. The colonies are isolated and surrounded by Sandhills prairie and unsuitable for house sparrows that prefer nesting sites near human structures.

Discussion

Our data demonstrated that swallow bugs in nature can maintain BCRV without blood-feeding for at least 2 yr. At site CS, the bug collection we made on 28 June 2007 was during the period that nestling cliff swallows were fledging, and the bugs at this site could not have taken a blood meal thereafter much later than early July 2007 (the end of the swallow nesting period there). Therefore, the bugs we collected on 24 May 2009 (Table 1) had not fed for at least 22.5 mo. Similarly, at site NN, bugs collected in mid May 2005 (Table 1) would not have blood-fed since July 2003, ≈22 mo earlier. At both sites, the bugs had survived two full Nebraska winters in which the average daily low temperatures for the coldest months (December–February) were −11.5, −13, and −9.8°C (Brown et al. 2010). We are confident that no cliff swallows or house sparrows had been present in the interim periods to provide blood meals to these bugs. Bugs maintained BCRV infection for ≈10 mo in the laboratory (Rush et al. 1980) and 12 mo in the field (Moore et al. 2007); new data from our current study revealed that BCRV can persist for at least double that time in situ. Neither prevalence nor viral RNA concentrations in the unfed bug pools differed from those for bugs at active sites, suggesting no reduction in the number of infected bugs per pool with time.

Even though we did not isolate infectious (i.e., cytopathic) BCRV from the pools of bugs unfed for 2 yr, for three reasons we suspect that our RT-PCR detections indicated intact virus presence and not merely fragments of viral RNA. (1) First, the concentration of infectious viral particles may simply have been below the level of detection by plaque assay (Bailey et al. 1978, Reisen et al. 2002), although that we did not find a difference in RNA concentrations between positive pools that formed plaques and those that did not means that detection alone probably cannot explain these results. (2) The reduced cytopathicity of the unfed-bug samples is consistent with other data (Moore et al. 2007; Brown et al. 2009a,b, 2010) routinely showing low to no plaque growth for samples positive for BCRV by RT-PCR taken in the winter season or from inactive colony sites, yet at these sites infectious BCRV is easily recovered from bugs as soon as cliff swallows arrive. The same pattern was seen in this study at site NN: though no cytopathic positives

occurred on 14 May 2005 (Table 1) when it was still inactive, after birds arrived a few days later, infectious BCRV was detected in bugs when we next sampled on 6 June, with most of the RT-PCR positives obtained there in the succeeding month being cytopathic (Table 1). (3) Sequencing of noncytopathic RT-PCR positives from winter bugs has produced complete E2 gene sequences, suggesting that noncytopathic positives are not merely RNA fragments (Brown et al. 2010).

Why so many RT-PCR BCRV positives do not form plaques in Vero-cell assay when the bugs are not feeding (even in the warm summer months) remains a puzzling feature of this particular virus. Blood-feeding may stimulate physiological changes in the arthropod vectors that affect virus in some way (perhaps by activating virus replication) and lead to plaque formation, both in BCRV and other arboviruses (Bailey et al. 1978, Reisen et al. 2002, Moore et al. 2007, Brown et al. 2010). The dramatic increase in plaque formation in pools containing blood-fed bugs at NN, compared with blood-deplete bugs there 4–5 wk earlier, and other data on BCRV (Moore et al. 2007, Brown et al. 2010) are consistent with an effect of blood-feeding per se on detection of infectious virus. A similar pattern has been documented for the TBE viruses, in which virus becomes progressively more difficult to detect the longer the vectors are unfed but begins to replicate rapidly after ticks feed (Korenberg 2000).

The capacity of BCRV to persist for 2 yr at sites unused by birds probably contributes to the annual spatiotemporal stability of this virus at Nebraska cliff swallow colony sites. With virus apparently remaining in these bugs as long as many of them live, once virus is introduced to a colony site (Brown et al. 2007, 2008), it probably remains there in the bugs and does not require frequent introduction to a site by birds or vectors to sustain transmission cycles. There is little evidence that the first cliff swallows arriving in the spring at colony sites introduce BCRV to sites (O'Brien et al. 2008), so, for example, the resurgence in infectious BCRV at site NN in June and July 2005 cannot be attributed to birds' introducing it. Even when no cliff swallows occupy a colony site in a given summer and the bugs have no access to blood meals, the data reported here illustrate that BCRV is unlikely to disappear from the site provided some bugs survive, and it retains its ability to replicate at least in bugs once they resume blood-feeding.

Swallow bugs are unusual among insect vectors of arboviruses in being so long-lived. For example, we collected two swallow bugs from a colony site in our study area that had last been used by cliff swallows 7 yr earlier, and there was no evidence that house sparrows had been present in the interim (C. B. and S. Strickler, unpublished data). Swallow bugs as vectors more resemble ticks in many ways than other insects, including in their longevity (Sonenshine and Mather 1994, Randolph 1998).

These data indicate that BCRV persists for longer in its adult vector than do other alphaviruses, and the field data suggest no cost to swallow bugs of being infected with BCRV or maintaining it for long periods. BCRV is closely related phylogenetically to WEEV and Highlands J virus (Powers et al. 2001, Padhi et al. 2008), and thus these alphaviruses may have the potential to also persist in insects for extended periods of time should they switch to a more long-lived vector.

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