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Isolation of Buggy Creek Virus (Togaviridae: Alphavirus) From Field-Collected Eggs of *Oeciacus vicarius* (Hemiptera: Cimicidae)

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Abstract

Alphaviruses (Togaviridae) rarely have been found to be vertically transmitted from female arthropods to their progeny. We report two isolations of Buggy Creek virus (BCRV), an ecologically unusual alphavirus related to western equine encephalomyelitis virus, from field-collected eggs of cimicid swallow bugs (*Oeciacus vicarius* Horvath), the principal vector for BCRV. Ten percent of egg pools were positive for BCRV, and we estimated minimum infection rates to be 1.03 infected eggs per 1,000 tested. The results show potential vertical transmission of BCRV, represent one of the few isolations of any alphavirus from eggs or larvae of insects in the field, and are the first report of any virus in the eggs of cimicid bedbugs. The specialized ecological niche of BCRV in swallow bugs and at cliff swallow (*Petrochelidon pyrrhonota* Vieillot) nesting sites may promote vertical transmission of this virus.

Keywords

Buggy Creek virus; cliff swallow; *Petrochelidon pyrrhonota*; swallow bug; vertical transmission

Some arboviruses are transmitted vertically from infected female arthropods to their offspring, either transovarially or when virus infects a fully developed egg as it is oviposited (Rosen 1988). This mode of transmission has been documented in mosquitoes for various bunyaviruses (Watts et al. 1973, Christensen et al. 1978, Turell et al. 1982) and flaviviruses (Rosen et al. 1978, Aitken et al. 1979, Hardy et al. 1984, Goddard et al. 2003, Phillips and Christensen 2006) in North America, and can potentially contribute to virus persistence within vector populations during winter in temperate latitudes when potential vertebrate hosts are unavailable (e.g., having migrated or hibernated). Vertical transmission is less common, however, in the alphaviruses (Togaviridae: Alphavirus), with several studies finding limited or no evidence (Chamberlain and Sudia 1957, 1961, Thomas 1963, Henderson and Brust 1977, Morris and Srihongse 1978, Rosen 1981, Scherer et al. 1986, Reeves 1990, Reisen 1990, Fulhorst et al. 1994, Reisen et al. 1996).

Here, we report isolation of an alphavirus, Buggy Creek virus (BCRV), from eggs of cimicid swallow bugs (*Oeciacus vicarius* Horvath). BCRV is an ecologically unusual arbovirus

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transmitted primarily by the swallow bug and amplified by the ectoparasitic bug's vertebrate hosts: the cliff swallow (*Petrochelidon pyrrhonota* Vieillot) and house sparrow (*Passer domesticus* L.; Hayes et al. 1977, Rush et al. 1980, Scott et al. 1984, Brown et al. 2001, 2008). BCRV is serologically and phylogenetically related to the alphaviruses in the western equine encephalomyelitis virus (WEEV) complex (Calisher et al. 1988, Hopla et al. 1993, Powers et al. 2001, Pfeffer et al. 2006). This virus can persist in its vectors during the winter months in the temperate latitudes of North America (Hayes et al. 1977, Strickler 2006, Moore et al. 2007), and its prevalence in bug vectors during summer is relatively stable at ≈25% of pools across years (Brown et al. 2001). If BCRV can be maintained in its insect vectors through vertical transmission, this could potentially contribute to its persistence in the absence of potential vertebrate hosts.

Materials and Methods

Study Organisms

BCRV was first isolated in 1980 from swallow bugs collected at a cliff swallow colony along Buggy Creek in Grady County, west central Oklahoma (Loye and Hopla 1983, Hopla et al. 1993). Fort Morgan virus (FMV), which is 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). BCRV has been found primarily in the western Great Plains of North America (Padhi et al. 2008) and always in association with cliff swallow nesting sites. Two lineages of BCRV (designated A and B) co-occur at swallow colonies in our Nebraska study area and differ from each other by >6% at the nucleotide level (Pfeffer et al. 2006, Padhi et al. 2008).

The hematophagous swallow bug is an ectoparasite primarily of cliff swallows and is found throughout the bird's wide geographic range (Brown and Brown 1995); the bugs also parasitize house sparrows that occupy nests in some cliff swallow colonies (Loye 1985, Hopla et al. 1993, Brown et al. 2001). Swallow bugs are nest-based parasites that overwinter in cliff swallows' mud nests or in the cracks and crevices of the nesting substrate near the nests. Infestations can reach 2,600 bugs per nest. The adult bugs begin to reproduce as soon as they feed in the spring. Swallow bugs lay eggs on the inner and outer surfaces of the nests and also on the adjacent substrate (Loye 1985). The eggs are contained within whitish, tubular casings and laid in masses, which are readily visible. Eggs hatch over variable lengths of time, ranging from 3 to 5 (Loye 1985) to 12–20 d (Myers 1928). Bug populations at an active colony site increase throughout the summer, reaching a peak at approximately the time cliff swallows fledge. The bugs seem to be adapted to withstanding long periods of host absence, in some cases persisting at a site not used by cliff swallows for up to at least three consecutive years (Smith and Eads 1978, Loye 1985, Loye and Carroll 1991, Rannala 1995).

Study Site

Our study site was centered at the Cedar Point Biological Station (41°13′ N, 101°39′ W) near Ogallala, in Keith County, along the North and South Platte Rivers, and also included portions of Deuel, Garden, Lincoln, and Morrill counties, southwestern Nebraska. The study area is described in detail by Brown and Brown (1996) and Brown et al. (2008). For this study, we sampled swallow bug eggs at nine cliff swallow colonies extending from near North Platte, Lincoln Co., to near Broadwater, Morrill Co., a span of 190 km. Colony sites studied were concrete culverts underneath roads, where the swallows attach their nests to the vertical walls near the ceiling.

Field Collections

We collected eggs from the outer surface of nests along the bottom of the nest bowl. Using a sterile razor blade, we scraped eggs into vials containing BA-1 diluent, a growth medium

containing M-199 Hank's salts, 1% bovine serum albumin, 0.05 M Tris-HCl (pH 7.5), 0.35 g/liter sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, 1µg/ml Fungizone (Gibco-BRL, Gaithersburg, MD), 20% fetal bovine serum, and sterile distilled water. We attempted to collect ≈100 eggs per vial from several nests and took special care to ensure that no bugs or bug feces inadvertently fell into the vials during collection. Collections were all made in June and July 2007. Each pool likely contained eggs from multiple females, based on the data of Loye (1985), showing that a given female *O. vicarius* lays 7–27 eggs.

For comparison, we examined BCRV prevalence in swallow bugs at the same colony sites in summer 2007. Bugs were collected from cliff swallow nests as described in Moore et al. (2007). In general, we collected at least 1,000 bugs per site and typically sampled 10–30 nests depending on the level of bug infestation at a site. Bugs at most colony sites were sampled once (on one date) during the summer. Bugs were sorted into pools of 100 individuals while alive and frozen at −70°C immediately after sorting.

Virus Screening and Isolation

Egg pools were macerated with a Mixer Mill (MM 31) from Qiagen (Valencia, CA). The homogenate was centrifuged at 11,000 × *g* for 1 min to clarify the supernatant and homogenates subsequently stored at −70°C. A 100-µl aliquot of the supernatant was added to 400 µl of a guanidine thiocyanate–based lysis buffer. After the addition of 400 µl of 100% ethanol, RNA was isolated 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 during reverse transcription-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 the mocycler 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, 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 samples that were initially positive by RT-PCR but failed to be confirmed by plaque assay, we re-extracted RNA from the homogenate and performed a second RT-PCR for confirmation (Moore et al. 2007).

Bug pools were treated similarly, and only those that were positive by both RT-PCR and plaque assay were used in comparisons here. Infection rates for both eggs and bugs were computed using the pooled infection rate method of Biggerstaff (2006).

Lineage Determination

To identify isolates as BCRV and to determine the lineage (A or B) present in positive egg pools, we sequenced 564 bp of the E2 region of the genome (positions 67–630 bp in the *E2* gene) for each positive pool. Protocols and primers used for amplification and sequencing of the E2 region are given in Brown et al. (2008). We constructed a neighbor-joining phylogeny

using the maximum composite likelihood method implemented in MEGA ver. 4 (Tamura et al. 2007). Nodal support was estimated with 1,000 nonparametric bootstrap replicates. We used the previously published phylogeny of the BCRV lineages (Padhi et al. 2008) to assign isolates from eggs to their respective lineages.

Results

We collected 20 egg pools (≈2,000 eggs) from nine swallow colony sites. Two (10.0%) pools were positive for BCRV by both RT-PCR and plaque assay. One pool (from colony CP) produced 50 plaque-forming units (PFU)/ml and the other (from colony WF) produced 15 PFU/ml. These colony sites were 9.0 km apart in Morrill County on the western edge of the study area. None of the multiple negatives we ran in either the RNA extractions or the RT-PCR showed any evidence of BCRV, so we are confident that laboratory cross-contamination cannot explain our results. No samples that were initially positive by RT-PCR but failed to grow plaques were reconfirmed in a second RT-PCR, so those pools were considered negative.

Of 279 swallow bug pools (containing both adults and later instars) collected from the same nine colony sites in summer 2007, 53 (19.0%) were positive for BCRV by both RT-PCR and plaque assay. The two sites with positive egg pools had a significantly higher prevalence of BCRV in bugs (26.2%, $N = 107$ pools) than the seven sites without positive egg pools (14.5%, $N = 172$ pools; $X^2_1 = 5.80$, $P = 0.016$). At colony CP, titers of BCRV within bug pools ranged from 50 to >2,000 PFU/ml, whereas, at colony WF, bug pools ranged from 5 to 845 PFU/ml.

Infection rate for eggs was 1.03/1,000 (95% CI: 0.19–3.40/1,000), based on the 20 egg pools. Infection rate for the 279 bug pools from the same sites was 2.10/1,000 (95% CI: 1.59–2.73/1,000).

Sequencing confirmed the identities of the virus isolates from eggs as BCRV. One positive egg pool contained BCRV of lineage A, and the second one contained BCRV of lineage B.

Discussion

The detection of BCRV in field-collected eggs of *O. vicarius* suggests that this virus can be transmitted vertically. Although vertical (including transovarial) transmission is well documented in various bunyaviruses, rhabdoviruses, and flaviviruses vectored by ticks and mosquitoes (Watts and Eldridge 1975, Mims 1981, Tesh 1984, Turell 1988, Danielová et al. 2002, Nuttall and Labuda 2003), WEEV is the only alphavirus in which vertical transmission has been found, first being detected in mosquito eggs in the laboratory (Kissling et al. 1957, Thomas 1963) and then in field-collected mosquito larvae (Fulhorst et al. 1994, but see Reisen et al. 1996, Kramer et al. 1998). Experimental evidence indicates that most alphaviruses are rarely transmitted directly from a female mosquito to her progeny, if at all (Barnett 1956, Chamberlain et al. 1956, Chamberlain and Sudia 1957, Henderson and Brust 1977, Morris and Srihongse 1978, Sprance 1981, Kay 1982, Clark et al. 1985, Scherer et al. 1986). Experiments are necessary to definitively establish that swallow bug eggs infected with BCRV hatch and produce infected instars, although our finding of BCRV in early-stage instars in other studies (C. Brown et al., unpublished data) suggests that infection of progeny might occur.

In some reported cases of transovarial virus transmission in insects, only viral nucleic acids were transmitted, and no infectious virus could be isolated from eggs or progeny (Bosco et al. 2004). We found infectious BCRV in bug eggs, which is evidence for transmission of more than just viral RNA. The virus titers in eggs were relatively low but within the range of titers found for bug pools at the same colony sites, suggesting that virus can be transmitted vertically in sufficient concentration to be ecologically relevant. In addition, the 95% confidence interval of the pooled infection rate (Biggerstaff 2006) for the egg pools did not include zero, and the

BCRV infection rate was two to five times greater than that found for vertically transmitted WEEV (Fulhorst et al. 1994, Reisen et al. 1996).

One limitation of field-collected samples is that we could not entirely rule out contamination of the egg casing surface by virus after the eggs were laid, perhaps from virus shed from cliff swallows or from bugs. However, we believe the former is unlikely because the eggs were collected from the bottom of the gourd-shaped nests, and cliff swallows (or their feces) rarely if ever contact the outer nest surface there. We took care to avoid getting bug feces in the samples.

Little is known about the potential for hemipterans in general or cimicids in particular to vertically transmit viruses or other pathogens. To our knowledge, this is the first report of a virus in the eggs of any cimicid. However, the human bedbug (*Cimex lectularius*) is known to transmit endosymbiotic bacteria transovarially (Hypša and Aksoy 1997). A major unresolved question in our study is whether vertical transmission of BCRV in swallow bugs represents true transovarial transmission or only infection of fully developed eggs during oviposition.

Swallow bug eggs deposited on substrates (e.g., nest walls, concrete culvert ceilings) do not overwinter successfully, and thus BCRV cannot be maintained in eggs at a site between years. However, female bugs begin laying eggs soon after they take a blood meal in the spring (Usinger 1966, Loye 1985), and thus infected bugs from the previous summer potentially could produce infected eggs at a site even if none of the vertebrate hosts there that year are infectious. This may contribute to the relatively stable persistence of BCRV at given colony sites from year to year when few cliff swallows are viremic or when the number of birds there fluctuates between years. Between consecutive years, virus prevalence at a site is significantly auto-correlated (Brown et al. 2001), and passage of BCRV into bug eggs may be one mechanism contributing to this result. BCRV is perhaps more similar ecologically to the vertically transmitted California group bunyaviruses than to other alphaviruses. Like BCRV, these bunyaviruses (e.g., La Crosse virus) tend to maintain relatively stable occurrence in time and space, and they are also more likely to be vertically transmitted (Reisen 1990). Vertical transmission may be especially important for BCRV, because it seems to amplify poorly in at least some of its typical vertebrate hosts (Huyvaert et al. 2008).

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