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BRIEF REPORT

# Natural infection of vertebrate hosts by different lineages of Buggy Creek virus (family *Togaviridae*, genus *Alphavirus*)

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**Abstract** Buggy Creek virus (BCRV; family *Togaviridae*, genus *Alphavirus*) is an arbovirus transmitted by the ectoparasitic swallow bug (Hemiptera: Cimicidae: *Oeciacus vicarius*) to cliff swallows (*Petrochelidon pyrrhonota*) and house sparrows (*Passer domesticus*). BCRV occurs in two lineages (A and B) that are sympatric in bird nesting colonies in the central Great Plains, USA. Previous work on lineages isolated exclusively from swallow bugs suggested that lineage A relies on amplification by avian hosts, in contrast to lineage B, which is maintained mostly among bugs. We report the first data on the BCRV lineages isolated from vertebrate hosts under natural conditions. Lineage A was overrepresented among isolates from nestling house sparrows, relative to the proportions of the two lineages found in unfed bug vectors at the same site at the start of the summer transmission season. Haplotype diversity of each lineage was higher in bugs than in sparrows, indicating reduced genetic diversity of virus amplified in the vertebrate host. BCRV appears to have diverged into two lineages based on different modes of transmission.

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Buggy Creek virus (BCRV; family *Togaviridae*, genus *Alphavirus*) is an ecologically unusual arbovirus transmitted by the ectoparasitic swallow bug (Hemiptera: Cimicidae: *Oeciacus vicarius*) to the bug's principal avian host, the cliff swallow (*Petrochelidon pyrrhonota*). The virus is also transmitted to the house sparrow (*Passer domesticus*), an invasive exotic species of European origin that sometimes usurps the swallows' mud nests [1–6]. BCRV and its variant Fort Morgan virus [7, 8] occur locally at swallow colonies across the western Great Plains of North America [9], and a similar virus was recently isolated from California [10]. BCRV exists as two distinct lineages (A and B) that are about 6% divergent at the nucleotide level [11]. Lineage A appears to be more southerly and lineage B more northerly, with both occurring sympatrically in Nebraska and Colorado [9, 11].

BCRV is frequently isolated from swallow bugs [4–6, 12]. Differences between bugs infected with the two lineages at our Nebraska study site suggest that the lineages have diverged ecologically in their transmission strategies [13]. Lineage A tends to occur at more recently established colonies, at sites with more house sparrows, and in bugs more likely to disperse on birds, than lineage B. Unlike in lineage B, the haplotype diversity of lineage A increases in larger bird colonies, and lineage A peaks in seasonal occurrence when nestling birds are most numerous [13]. Lineage A strains isolated from bugs are more cytopathic

on mammalian cells than viruses of lineage B, and lineage A is more likely to persist through the winter in bugs at sites containing resident house sparrows [13, 14]. These differences between the lineages suggest that BCRV lineage A has evolved into a bird-associated virus that is relatively dependent on avian hosts for amplification in a conventional arbovirus transmission cycle, whereas lineage B is a bug-adapted strain that is transmitted mostly among bugs, perhaps through vertical transmission [15]. This ecological divergence may derive from the recent arrival of house sparrows at cliff swallow colonies [13].

Despite considerable information on the ecology of the BCRV lineages in bugs, little is known about how the different lineages infect avian hosts. Work that did not characterize isolates to lineage showed that house sparrows in general amplify BCRV more effectively than do cliff swallows, with nestling sparrows exhibiting higher infection prevalence and higher maximum virus titers than swallows [16]. Based on the previous bug studies [13, 14], we predicted that BCRV lineage A should be more likely than lineage B to (1) infect vertebrate hosts such as house sparrows and (2) replicate in those infected. To test this hypothesis, we sampled nestling house sparrows for BCRV isolates and determined whether the distribution of BCRV lineages A and B in the birds differed from that in bugs at the same location at the beginning of the summer transmission season. We also measured and compared viral genetic diversity of the lineages by host type (bird or bug).

Our study site was a concrete culvert (41°34.106′N, 102°46.576′W) underneath U. S. Highway 26 east of Broadwater, Morrill County, western Nebraska. Cliff swallows attached their mud nests inside the culvert on the walls just below the ceiling. In the summer of 2006, 195 cliff swallow nests and an undetermined number of house sparrow nests were active at this site. On 11 February and 11 April, 2007, we sampled the overwintering swallow bugs there for BCRV [14]. We collected 4–5 old nests or nest fragments on each occasion. Cliff swallows had not yet arrived in the study area from their South American wintering areas on those dates. House sparrows roost in old swallow nests during the winter, and some sparrows were seen at the colony site on 11 April; however, none of the bugs collected had recently fed (known by the absence of a dark blood spot visible in the abdomen). We sifted through the nest fragments by hand, sorted the bugs into pools of 100 while alive, and froze them at −70°C until virus screening. This particular colony site was unusual and selected for this study because it had roughly equal proportions of the two BCRV lineages in mid to late winter. At most bird colony sites, lineage A predominates in winter and early-spring bug samples [14], making field study of potential differences in transmission difficult.

In the summer of 2007, 35 cliff swallow nests and 20 house sparrow nests were active in the culvert. From 28 May to 29 July 2007, we sampled nestling house sparrows (≥4 days old) for virus by removing birds of known age (determined by marking nests and monitoring hatching dates) and taking 0.1 mL of blood by jugular venipuncture. Birds were banded with USGS bands and returned to their nests. Blood was placed in 0.4 mL of BA-1 diluent [12], stored on wet ice in the field, and clarified by centrifugation, and the supernatant was stored at −70°C until analysis. Because of age-related changes in viremia titers for nestlings [16], in analyzing titers, we used only samples taken from nestlings 10 days old or older, which is the age at which titer levels of recently infected birds tend to stabilize. Titers were measured for birds that had become infected within the previous 4 days, these individuals having tested negative for BCRV at earlier ages.

Processing of swallow bug pools was done as described by Moore et al. [12]. For bird samples, viral RNA was extracted from sera by first adding 25 μL of thawed sera in BA-1 diluent to 100 μL of a guanidine-thiocyanate-based lysis buffer [17]. RNA was extracted using a QIAmp Viral RNA Mini Kit (Qiagen, Valencia, California, USA), following the manufacturer's protocol. For screening samples to detect virus, RT-PCR was performed on bird and bug samples using a OneStep RT-PCR Kit (Qiagen) with BCRV-specific primers that yielded a 208-bp fragment from the E2 region of the viral genome [12].

Samples that were initially BCRV-positive by RT-PCR were subjected to plaque assay on Vero cells, as described by Huyvaert et al. [18]. Plaques were scored on day 3 after Vero cell infection. Viremia titers were determined for bird sera by serial dilution. Samples that did not show a cytopathic effect on Vero cells were subjected to re-extraction and repeat RT-PCR to confirm the presence of viral RNA in the sample.

Lineages of all BCRV isolates from bugs and house sparrows were determined by constructing a neighbor-joining (NJ) tree based on sequences of the first 333 bp from the E2 glycoprotein-coding gene, using the maximum composite likelihood method in MEGA [19]. Nodal supports were estimated with 1,000 bootstrap replicates. Previously defined sequences characterized to lineage were used as reference [6, 13, 14]. Lineages could be unambiguously assigned based on the 333-bp region; 23 nucleotide sites within this alignment were unique to lineages A and B (Table 1). Further details on determining lineages are provided in Brown et al. [6, 13]. Sequences used in this study are available in GenBank (accession numbers GU597973–GU598046).

For genetic diversity estimates, we used only E2 sequences with 696 bp and excluded six sequences with shorter fragments. Haplotype (h) and nucleotide ($\pi$)

**Table 1** Nucleotide sites unique to Buggy Creek virus lineages A and B at sites 1–333 of the E2 glycoprotein-coding gene

| Lineage | Substitution site | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9 | 21 | 48 | 57 | 69 | 72 | 78 | 81 | 84 | 99 | 114 | 144 | 165 | 180 | 183 | 207 | 258 | 291 | 297 | 304 | 306 | 318 | 321 |
| A | **A** | **A** | **C** | **T** | **A** | **C** | **C** | **C** | **T** | **T** | **C** | **T** | **G** | **T** | **C** | **T** | **T** | **A** | **C** | **T** | **A** | **T** | **G** |
| B | **G** | **G** | **T** | **C** | **G** | **A** | **T** | **T** | **C** | **C** | **T** | **C** | **A** | **C** | **A** | **C** | **C** | **T** | **T** | **C** | **G** | **C** | **T** |

**Table 2** Comparison of parameters associated with Buggy Creek virus detected in swallow bug vectors and nestling house sparrow hosts at a colony site in Morrill County, western Nebraska, in 2007

| | BCRV in bugs ($n = 37$ isolates) | BCRV in sparrows ($n = 27$ isolates) |
|---|---|---|
| Prevalence of lineage A (%) | 40.5 | 74.1 |
| Prevalence of lineage B (%) | 59.5 | 25.9 |
| Lineage A that was non-cytopathic (%)[a] | – | 5.0 |
| Lineage B that was non-cytopathic (%)[a] | – | 28.6 |
| Mean (±SE) titer of lineage A isolates (PFU/mL)[a,b] | – | 3.53 $\log_{10}$ (±0.93) |
| Mean (±SE) titer of lineage B isolates (PFU/mL)[a,b] | – | 2.20 $\log_{10}$ (±1.28) |
| Lineage A haplotype diversity (h) (±SE) | 0.6605 (±0.0728) | 0.1333 (±0.1123) |
| Lineage B haplotype diversity (h) (±SE) | 0.9091 (±0.0562) | 0.0000 (±0.0000) |
| Lineage A nucleotide diversity ($\pi$) (±SE) | 0.001231 (±0.000953) | 0.001275 ± 0.001225 |
| Lineage B nucleotide diversity ($\pi$) (±SE) | 0.002285 (±0.001593) | 0.000000 (±0.000000) |
| Mean $d_n/d_s$ ($\omega$) (95% confidence interval) | 0.088406 (0.040307–0.165183) | 0.021347 (0.003543–0.066146) |
| Number of transitions ($T_s$) | 45 | 39 |
| Number of transversions ($T_v$) | 6 | 6 |

[a] BCRV of either lineage in mid winter and early spring bug samples is rarely cytopathic in Vero cell assays [14], which also precluded determining titers in bug samples

[b] Measured only for 7 lineage A isolates and 4 lineage B isolates from birds ≥10 days old

diversities for each lineage from bugs and sparrows were estimated using ARLEQUIN [20]. The same program was used to estimate the number of transitions ($T_s$) and transversions ($T_v$) for each data set. The ratio of nonsynonymous ($d_n$) to silent substitutions ($d_s$) ($d_n/d_s = \omega$) was estimated using DATAMONKEY [21]. The maximum-likelihood-based codon substitution models in the codeml of PAML [22] and the random effects likelihood (REL) approach in DATAMONKEY [21] were used to determine whether any specific codons in either bugs or birds were under positive selection. The best-fitting nucleotide substitution model for each data set was determined with the Akaike information criterion as implemented in MODELTEST [23], and this model was used to reconstruct the input tree for PAML with PhyML [24].

BCRV was isolated from 37 pools of bugs collected in February and April 2007 (Table 2). The ratio of lineage A to B did not differ significantly from a 50:50 ratio among the unfed bugs collected in winter (binomial test, $P = 0.97$). We isolated BCRV from sera of 27 nestling house sparrows from 13 different nests at this site in summer 2007 (Table 2). The percentage distributions of the two lineages in birds versus bugs were significantly different ($\chi^2_1 = 7.08$, $P = 0.008$), with disproportionately more lineage A isolates in nestling house sparrows than in unfed bugs at the same site (Table 2). There was a larger percentage of lineage B than lineage A isolates from sparrows that did not exhibit plaque formation on Vero cells (having been detected only by RT-PCR; Table 2), but this difference was not quite statistically significant ($\chi^2_1 = 2.92$, $P = 0.088$). The mean titer for the lineage A isolates was higher than that for the lineage B isolates (Table 2), but this difference was not significant (Wilcoxon test, $Z = -0.57$, $P = 0.58$).

These results are consistent with BCRV lineage A being more associated with birds than is lineage B. Despite both lineages being present in equal amounts (and if anything, with slightly less lineage A) in swallow bugs at the start of the virus transmission season, lineage A was apparently transmitted to house sparrows at a ~3:1 ratio and to a significantly greater extent than would be expected based on its relative abundance in bugs (Table 2). This supported our first prediction that lineage A should preferentially infect vertebrate hosts. A greater percentage of the lineage B isolates taken from sparrows were non-cytopathic on Vero cells than were lineage A isolates, and lineage B

isolates did not produce as high an average titer in nestlings, although these differences were not statistically significant. Nevertheless, the trend in each case supported the prediction that lineage A should show greater replication in vertebrate hosts than lineage B. The possibility that lineage B was equally transmitted to nestling sparrows but caused high mortality prior to day 4, when we first screened these birds for virus, seems unlikely, given the fact that we rarely found nestlings less than 4 days old dead in nests.

If one BCRV lineage is preferentially transmitted to and amplified in house sparrows, genetic diversity of BCRV in the vertebrate host should be less than in the bug vectors (in which both lineages occurred equally). This was supported by haplotype diversity ($h$) measures for both lineages: in each case, $h$ was higher in bugs than in birds (Table 2). These differences were significant ($P < 0.05$) based on the non-overlapping confidence intervals (Table 2). This was particularly apparent for lineage B, in which only one haplotype was found among the seven infected house sparrows. Diversity at the nucleotide level ($\pi$), however, did not differ significantly between bugs and birds for the two lineages (Table 2). The higher apparent genetic diversity of BCRV in bugs versus house sparrows probably cannot be attributed to multiple infected bugs within single pools [25], because the infection rate for bugs at this colony site, calculated according to Biggerstaff [26], was only 2.93/1,000 bugs (CI 2.04–4.10).

The numbers of transitions and transversions were broadly similar in bugs and birds (Table 2). Although there were more transitions in bugs, overall, there was no strong evidence for a host-dependent bias in mutation type for BCRV. Non-synonymous variation in mutations ($\omega$) was low in both bugs and sparrows (Table 2) and did not differ significantly ($P > 0.05$) based on confidence interval overlap. As with other arboviruses [25], the low values suggest strong purifying selection on BCRV, but the higher $\omega$ for bugs might indicate slightly relaxed constraints on sequence variation in the vectors relative to avian hosts. While likelihood ratio tests [22] did not suggest evidence for positive selection of any codons, an REL approach [21, 27] indicated that site 218 was positively selected (with posterior probability 0.97) in lineage A only. This site coded for methionine in some bug sequences and threonine in others but was always methionine in the bird sequences. The functional significance of this change, if any, is unknown.

The few studies of genetic diversity of arboviruses in vectors versus their vertebrate hosts in vivo have in some cases reported reduced diversity in vertebrates, as we found for BCRV, and in other cases no differences between vectors and vertebrate hosts [25, 28, 29]. The differential infection and/or replication rates by lineage that we found for BCRV is, to our knowledge, the first such report for an arbovirus under natural conditions. Greater bug-to-bird transmission of lineage A and greater replication of that lineage by vertebrate hosts would presumably select against lineage B in the absence of its ability to persist and replicate in the vectors [13, 14]. BCRV is the only North American arbovirus known to have sympatric lineages that differ so markedly in ecology [13] and apparent transmission strategies. This system provides a unique opportunity for further study of the evolutionary divergence of these lineages under natural conditions.

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