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Research

Experimental Infection of *Amblyomma americanum* (Acari: Ixodidae) With Bourbon Virus (Orthomyxoviridae: *Thogotovirus*)

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Abstract

Following the recent discovery of Bourbon virus (BRBV) as a human pathogen, and the isolation of the virus from *Amblyomma americanum* (L.) collected near the location of a fatal human case, we undertook a series of experiments to assess the laboratory vector competence of this tick species for BRBV. Larval ticks were infected using an immersion technique, and transstadial transmission of virus to the nymphal and then to the adult stages was demonstrated. Transstadially infected nymphs transmitted virus to adult ticks at very high rates during cofeeding, indicating the presence of infectious virus in the saliva of engorging ticks. Vertical transmission by transstadially infected females to their progeny occurred, but at a low rate. Rabbits fed on by infected ticks of all active life stages developed high titers of antibody to the virus, demonstrating host exposure to BRBV antigens/live virus during tick blood feeding. These results demonstrate that *A. americanum* is a competent vector of BRBV and indicate that cofeeding could be critical for enzootic maintenance.

Key words: *Amblyomma americanum*, Bourbon virus, transstadial transmission, cofeeding transmission, vertical transmission

Bourbon virus (BRBV; family *Orthomyxoviridae*, genus *Thogotovirus*) is a recently discovered human pathogen that was first isolated in 2014 from a hospitalized adult male resident of Bourbon County in eastern Kansas (Kosoy et al. 2015). The previously healthy case-patient, who subsequently died of his illness, had reported numerous tick bites several days before the onset of symptoms. Assays for several tick-borne bacterial pathogens, babesiosis, and Heartland virus (HRTV; family *Phenuiviridae*, genus *Banyangvirus*) were negative. However, during a plaque-reduction neutralization test (PRNT) of the patient's serum for HRTV, plaques not consistent with HRTV were observed (Kosoy et al. 2015). Further analysis revealed a previously unrecognized orthomyxovirus most closely related to Dhori virus (DHOV) in the genus *Thogotovirus* (Kosoy et al. 2015, Lambert et al. 2015). The virus, named BRBV after the county of residence of the patient, is the first human pathogenic *Thogotovirus* to be recognized in the New World.

Following discovery of a novel, potentially tick-borne virus in Kansas, our group at the Centers for Disease Control and Prevention (CDC) retested ticks originally collected during 2013 as part of a HRTV surveillance project in northwestern Missouri, ~240 km from Bourbon County (Savage et al. 2016, 2017). In total, 3,073 tick pools containing 39,096 ticks representing 5 species were tested. Two species, *Amblyomma american* (L.) and *Dermacentor variabilis* (Say) (Acari: Ixodidae), represented 97.6 and 2.3%, respectively, of ticks collected. Bourbon virus was detected in three pools of *Amblyomma americanum* (L.), including one pool of four male adults and two pools each containing 25 nymphs.

During 2015 and 2016, tick surveillance was undertaken in Bourbon County and adjacent southern Linn County by our group at CDC and collaborators in the State. In 2015, 20,639 ticks were collected; of which, 99.5% were *A. americanum*, and one pool containing five *A. americanum* female adults was positive for BRBV (Savage et al. 2018a). In 2016, a total of 14,193 ticks, 99.5% *A. americanum*, were collected, but BRBV was not detected in any tick pools (Savage et al. 2018b).

Although BRBV was detected at a low prevalence in these collections, the detection of virus in some tick pools, the report of recent tick bites in the fatal human case-patient, and the association of ticks with other thogotoviruses (Haig et al. 1965, Anderson and Casals 1973, see Hubalek and Rudolf 2012 for review) suggested that ticks

may play a role in endemic maintenance of BRBV in the environment and in transmission of the virus to humans and other vertebrates. Thus, we undertook experiments to assess the vector competence of *A. americanum* for BRBV using as a model our study of the vector competence of this tick species for HRTV (Godsey et al. 2016). The specific goals of our study were 1) to assess our ability to experimentally infect laboratory-reared larval ticks with the virus, 2) to determine whether transstadial transmission of virus to the nymphal stage, and then to the adult stage, occurred following blood feeding and molting, 3) to assess nonviremic or cofeeding transmission from infected ticks to uninfected ticks feeding in close proximity to each other (Jones et al. 1987), and 4) to determine whether infected females transmit virus vertically to their progeny.

Materials and Methods

Experimental Animals

Larval *A. americanum* were obtained from a colony maintained at Oklahoma State University (Stillwater, OK), or from the Rickettsial Zoonoses Branch, CDC, Atlanta, GA (Troughton and Levin 2007), and arrived as eggs or hatched larvae, in either case with the spent female. The female was tested by RT–PCR for BRBV RNA before offspring were used for transmission experiments. Ticks were maintained in glass desiccators at 23°C, 95–98% relative humidity, and a 16:8 (L:D) h photoperiod.

Ticks were fed on outbred female New Zealand white rabbits (*Oryctolagus cuniculus*; Charles River, Wilmington, MA). Rabbits were housed in individual cages, and a single rabbit was used for each tick feeding trial. Rabbits were only used once for blood feeding to prevent rabbit antitick immunity from affecting tick survival. Rabbits were sedated and euthanized by cardiac exsanguination followed by barbiturate overdose at the end of each experimental trial.

Virus

Two strains of BRBV were used in transmission experiments in order to detect possible differences in infectivity by different geographical strains. Strain MO13-2499 was isolated from a pool of 25 *A. americanum* nymphs collected in Gentry County, MO, on 24 July 2013, and had been passed once in human hepatoma cell line 7 (HUH-7) cells (Savage et al. 2017). Strain KS15-1735 was isolated from a pool of five female *A. americanum* collected in Bourbon County, KS, on 5 June 2015, and had been passed once in HUH-7 cells (Savage et al. 2018a). Both strains were passaged an additional time in Vero cells to produce high titered stock for immersion experiments.

Tick Engorgement on Rabbits

Ticks were fed on rabbits using a previously published technique (Levin and Schumacher 2016). Rabbits were sedated using Ketamine (35 mg/kg) and Xylazine (5 mg/kg) and the rabbit's dorsum was clean-shaved. A double-layer feeding bag consisting of an inner layer of nylon hosiery material and an outer layer of 8.0-cm-diameter cotton stockinette (Alba Health, Rockwood, TN) was glued to the rabbit's back using a veterinary adhesive (Kamar Inc., Zionsville, IN). The bag was glued on ~24 h before ticks were placed in the bag and was carefully examined for gaps through which ticks could escape. The open tops of the inner and outer bags were twisted shut and secured with rubber bands. Rabbits were fitted with an Elizabethan collar (Gralen Co., Chino, CA), and their hind legs were hobbled using 12.7-mm cloth adhesive tape to prevent damage to feeding bags by grooming. On varying days between days 1–7, and on days 10, 14, 21, and 28 after ticks were placed on a rabbit, blood samples (0.5 ml) were drawn from the rabbit's ear vein, centrifuged in 0.6-ml serum separator tubes, and sera were frozen at –80°C until tested for BRBV RNA and virus. Oral swabs were also collected for virus testing using a polyester-tipped applicator stick, and the tip immersed in 1.0 ml of chilled 1% bovine albumin (BA-1) diluent (Lanciotti et al. 2000) supplemented with an additional 100 units/ml of penicillin, 100 µg/ml streptomycin, 1 µg/ml fungizone, and 1 µl/ml of polymixin B sulfate and frozen at –80°C.

Prior to tick feeding experiments on rabbits, needle inoculations of rabbits with BRBV were performed to ascertain whether rabbits developed a viremia or shed virus orally or rectally. One rabbit each was subcutaneously inoculated with 10^4 plaque-forming units (PFU) of one of the two BRBV strains suspended in 200 µl of phosphate-buffered saline (PBS). Blood and oral swabs were taken as described above. Additionally, rectal swabs were also taken using the same type of applicator sticks as described above, but the tip used for rectal swabbing was moistened in PBS before the swab sample was taken. On day 28, both rabbits received another 10^4 PFU of BRBV, and blood, oral, and rectal swabs were collected on days 35 and 42.

In total, 10 rabbits were used in this study (Fig. 1). Rabbits 1 and 2 were each needle-inoculated with one of the two strains of BRBV as described above. Rabbits 3, 4, and 7 were fed on by virus-immersed larvae. Rabbits 5 and 6 were fed on by nymphs molted from larvae fed on rabbit 3, and samples of engorged nymphs and resulting adults were tested for BRBV to detect transstadial transmission. Rabbit 8 was fed upon by nymphs molted from larvae fed on rabbit 7 to produce adults for a vertical transmission trial. Rabbit 9 was fed upon by noninfected colony male and female ticks and by putatively infected nymphs from rabbit 7 to determine whether virus transmission by cofeeding occurred. Rabbit 10 was fed on by adults molted from nymphs fed on rabbit 8, and resulting larvae were tested for vertical transmission.

Tick Infection

Larval ticks were exposed to BRBV using an immersion technique originally developed for infecting *Ixodes scapularis* (Say) with the Lyme disease spirochete *Borrelia burgdorferi* (Policastro and Schwan 2003) and adapted for infecting *I. scapularis* with Langat virus (*Flaviviridae*: *Flavivirus*; Mitzel et al. 2007) and by our group for infecting larval *A. americanum* with HRTV (Godsey et al. 2016). Stocks of BRBV strains MO13-2499 and KS15-1735 (titers of $10^{8.5}$ PFU/ml and $10^{8.4}$ PFU/ml, respectively) were diluted to $10^{6.0}$ or $10^{7.0}$ PFU/ml in cold Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY), and in three different trials, 5-ml aliquots were added to vials containing the larval offspring of one female (~2,000 larvae per vial). Immersed larvae were incubated at 34°C for 1.5 h, then the virus suspension was aspirated off and a 0.5-ml aliquot was frozen at –80°C for later titration. Larvae were washed 2× with cold PBS and then dried at low humidity at 23°C for approximately 24 h before being placed in the tick feeding bag. Three immersion trials were conducted at different viral titers to determine the optimal titer for infecting large numbers of larvae. Engorged larvae were allowed to molt, and a sample of the resulting nymphs was tested for BRBV. Nymphs from the third immersion trial were used in the experiments described below. The progression of these experiments is shown in Fig. 1.

Cofeeding Experiment

Twelve female and 12 male colony adults (virus recipients) were placed on rabbit 9 on day 0. Twenty-five putatively infected nymphs

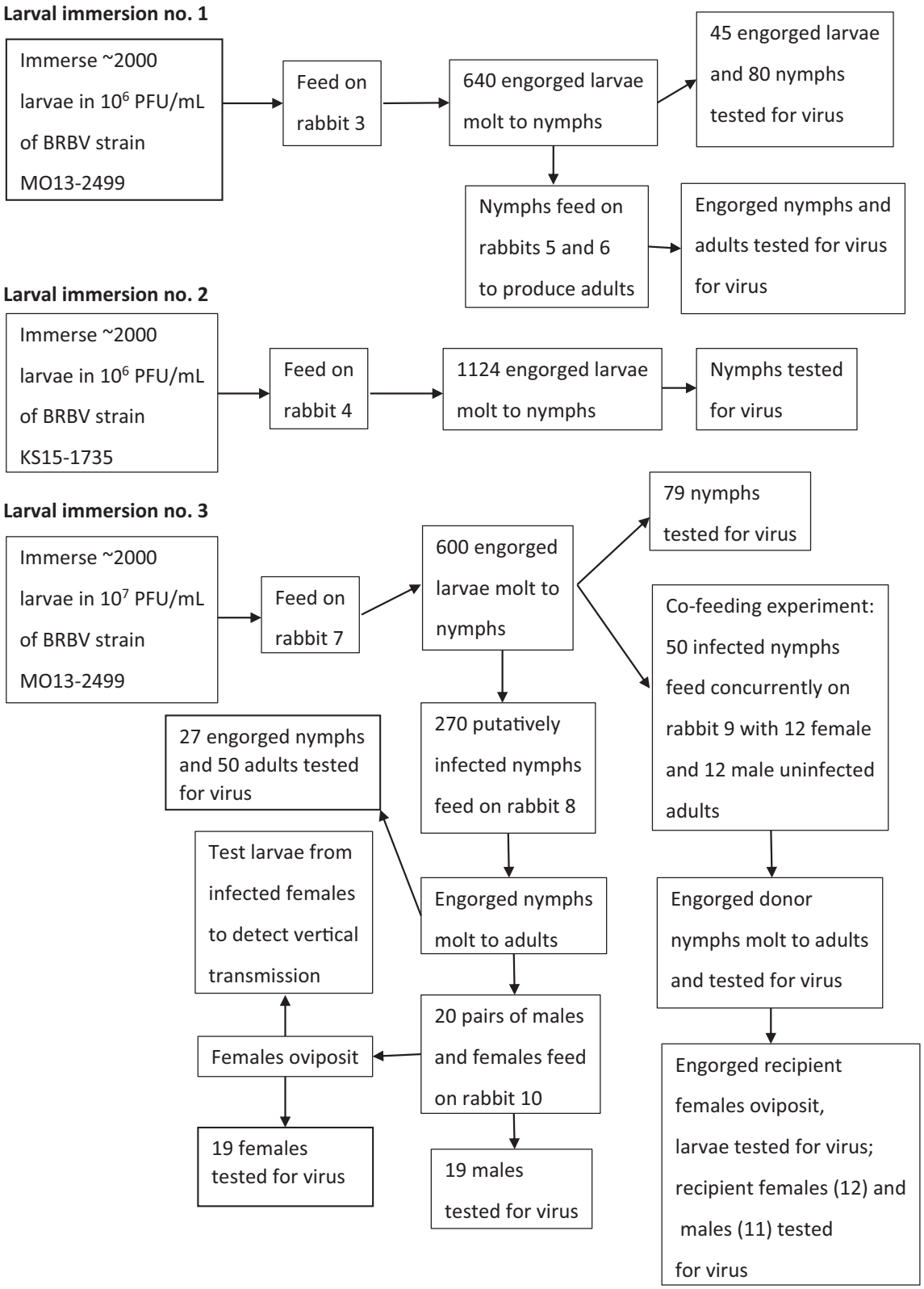

Fig. 1. Progression of experiments for three larval immersion trials.

(virus donors), molted from immersed larvae fed on rabbit 7, were placed in the feeding bag on day 5 and again on day 7 (50 nymphs total). The feeding bag was examined daily and engorged detached nymphs or females were removed. Engorged donor nymphs were placed in vials (≤10 per vial) and stored in a desiccator jar until they molted to adults, then were frozen 27–33 d post-detachment at –80°C for virus testing. Engorged recipient female ticks were placed in individual vials and stored in a desiccator jar until oviposition

occurred. Following oviposition, the female was removed and frozen at –80°C for virus testing. As females still retained a substantial volume of blood following oviposition, the triturated females were diluted 1/5 with DMEM for virus testing. Eggs were allowed to hatch and resulting larvae were tested for vertical transmission of virus in pools of ≤100 larvae per pool. Male ticks were removed when observed to be detached, or when the last female ticks were removed, and were frozen 18 d later for virus testing.

Vertical Transmission Experiment.

Of the nymphs that molted from immersed larvae fed on rabbit 7, 270 were fed on rabbit 8 to produce adults. Twenty males and 20 females were placed on rabbit 10 to mate and feed. Larvae produced by engorged females from rabbit 10, and from recipient females fed on rabbit 9, were frozen in pools of ≤100 larvae when egg hatching appeared to be completed until tested for virus.

Virus Testing

Ticks, and blood serum, oral swabs, and rectal swabs collected from rabbits were tested for BRBV by both real-time RT–PCR and by Vero cell plaque assay. Individual ticks or tick pools were processed, and RNA extracted from a 100-µl sample of serum, swab, or tick suspension, as previously described (Savage et al. 2018a). Sequence and reporter information for the BRBV primer/probe sets used for virus detection and confirmation were described by Lambert et al. (2015). Samples were screened for BRBV using primer/probe set nucleoprotein (NP) 1. Positive pools were confirmed by re-extracting RNA from the original tick homogenate and testing with the NP1 primer/probe set, and with the polymerase basic (PB) 1 primer/probe set. Pools were considered positive for BRBV if crossing threshold (CT) values for both primer/probe sets were ≤37. Test conditions for RT–PCR were as previously described (Savage et al. 2017).

Samples positive by RT–PCR were further tested by plaque assay to attempt virus isolation using a protocol similar to one previously published (Miller et al. 1989). Briefly, 100 µl of each sample were added to duplicate wells of confluent monolayers of Vero cells in six-well plates, incubated for 1 h at 37°C and then covered with overlay media containing 0.5% agarose. After 48 h, a second overlay containing neutral red was added to aid visualization of plaques, and wells were checked for plaques on days 3–5.

Serological Analysis

Rabbits inoculated with BRBV or used for feeding virus-immersed larvae or later stages post-molt were tested for neutralizing antibodies to BRBV by PRNT (90% plaque-reduction threshold; Lambert et al. 2015). Briefly, serial twofold dilutions (from a 1:5 starting dilution) of heat inactivated (56°C for 30 m) sera collected on day 0 (before tick feeding) and at euthanasia (day 29–42 post-exposure to virus) were incubated with approximately 100 PFU of a human isolate of BRBV (Kosoy et al. 2015). Samples were then added to confluent monolayers of Vero cells in six-well plates, incubated 1 h at 37°C, and then covered with overlay media and observed for plaques as described above.

Results

Rabbits Exposed to BRBV by Needle Inoculation

All blood sera, and oral and rectal swabs, collected from rabbits 1 and 2, needle-inoculated with BRBV strains MO13-2499 and KS15-1735, were negative for virus by both RT–PCR and by plaque assay. Both rabbits did, however, show serologic conversion in blood samples collected at euthanasia 42 d post-inoculation, with $PRNT_{90}$ titers of 1:1,280 and 1:2,560, respectively (Table 1).

Larval Immersion Trials

Three larval immersion trials were conducted followed by rabbit feeding (Table 2). The titer of the immersion fluid in the first trial was $10^{6.0}$ PFU/ml of MO13-2499, and immersed larvae were fed on rabbit 3. In total, 1,717 engorged larvae were recovered. Forty-five of these were tested for virus, and all were negative by RT–PCR and plaque assay. In total, 640 larvae molted into nymphs. Eighty nymphs were tested individually for virus to determine transstadial transmission from the larval to the nymphal stage, and two (2.5%) were positive by both assays. Remaining nymphs were fed on rabbits 5 and 6 and molted into 228 females and 167 males, which were tested for BRBV in 124 pools of ≤6 adults per pool. Two of 45 (4%) pools of females and 0/45 pools of males from the rabbit 5 feed were positive by RT–PCR and plaque assay, demonstrating transstadial transmission to the adult stage. From the feed on rabbit 6, 1/19 (5%) female pools and 1/15 (7%) male pools were positive by RT–PCR, but only the female pool was positive by plaque assay.

In the second immersion trial, larvae were immersed in $10^{6.0}$ PFU/ml of strain KS15-1735 and fed on rabbit 4. In total, 2,626 engorged larvae were recovered, of which 1,124 molted to nymphs. No engorged larvae were tested for virus but 280 nymphs were tested individually and all were negative. The remaining surviving nymphs (808) were subsequently tested in 81 pools of ≤10 nymphs/pool, and one pool (1%) was positive by both RT–PCR and plaque assays.

For the third immersion trial, larvae were immersed in $10^{7.0}$ PFU/ml of strain MO13-2499, then fed on rabbit 7. In total, 761 engorged larvae were recovered and 600 molted into nymphs. Seventy-nine nymphs were tested individually for BRBV and all were positive by

Table 1. Serologic conversion in rabbits exposed to Bourbon virus by needle inoculation or blood feeding by infected ticks

Rabbit no.	BRBV exposure route	Bled postexposure day	PRNT titer
1	Needle inoculation	42	1:1,280
2	Needle inoculation	42	1:2,560
3	Tick feeding: first larval immersion	30	1:320
4	Tick feeding: second larval immersion	29	1:1,280
5	Tick feeding: nymphs from rabbit 3	30	1:2,560
6	Tick feeding: nymphs from rabbit 3	29	1:1,280
7	Tick feeding: third larval immersion	34	1:1,280
8	Tick feeding: nymphs from rabbit 7	30	1:1,280
9	Tick feeding: nymphs from rabbit 7 for cofeeding trial	30	1:1,280
10	Tick feeding: Adults from rabbit 8	30	1:640

PRNT, Plaque Reduction Neutralization Test (see Materials and Methods).

Table 2. Summary of results of three Bourbon virus larval immersion trials

Experiment	Virus titer	Rabbit no.	No. infected[a]/no. tested (%)
Larval immersion #1	$10^{6.0}$ PFU/ml	3	0/45 (0%) engorged larvae; 2/80 nymphs (2.5%)
Immersion #1 nymph feed		5, 6	3/64 female pools[b] (5%); 1/60 male pools[b] (2%)
Larval immersion #2	$10^{6.0}$ PFU/ml	4	0/280 nymphs (0%); 1/80 nymph pools[c] (1%)
Larval immersion #3	$10^{7.0}$ PFU/ml	7	79/79 nymphs (100%)
Immersion #3 nymph feed		8	26/27 engorged nymphs (96%); 50/50 adults[d] (100%)

[a]Infection status determined by RT–PCR, Vero cell plaque assay, or both.
[b]Adults tested in pools of ≤6 individuals.
[c]Nymphs tested in pools of ≤10 individuals.
[d]25 males + 25 females.

RT–PCR while 66 (84%) were positive by plaque assay. CT values were generally high, with samples having CT values ranging from 28.1 to 35.4 using the PB1 confirmatory primer set, thus suggesting a low titer of virus present. This is confirmed by the plaque assay results, where the maximum titer of BRBV per tick was $10^{2.6}$ PFU.

Of the 270 nymphs from the third immersion trial that were fed on rabbit 8 to produce adults 27 engorged nymphs were tested for BRBV and 26 were positive by RT–PCR, but all were negative by plaque assay. Following the adult molt 25 males and 25 females were tested for virus and all were positive by RT–PCR, and 9/25 males (36%) and 14/25 females (56%) were positive by plaque assay, though plaque counts were low.

Cofeeding Experiment

Of 50 virus donor nymphs from the third immersion placed on rabbit 9, a total of 47 engorged nymphs were recovered and were allowed to molt to the adult stage, yielding 25 females and 19 males. Ten of 25 females (40%) were virus positive by RT–PCR, whereas 5/25 (20%) were also positive by plaque assay. None of 19 males molted from virus donor nymphs was positive by RT–PCR or plaque assay. Among virus recipient adults 12/12 females were RT–PCR positive, whereas 11/12 (92%) were also plaque positive. Six of 11 (55%) surviving recipient males were RT–PCR positive, with CT values ranging from 28.2 to 33.5, and four of these males (36%) were also plaque positive.

Vertical Transmission Experiment

Six of 12 engorged recipient females fed on rabbit 9 for the cofeeding experiment oviposited but only 2 of these produced large egg batches. Eggs from each female were allowed to hatch and resulting larvae were frozen approximately 2 wk after the estimated end of hatching. The 2 large egg batches resulted in 26 larval pools from female #13 and 17 larval pools from female #17a (Table 3). Three of the 25 (12%) larval pools from female #13 were RT–PCR positive (one pool of extracted RNA was accidentally discarded before RT–PCR testing), but at high Ct values (35.5–36.7), whereas all 26 pools were negative by plaque assay. Of 17 larval pools from female #17a, 1 was RT–PCR positive, and this pool was also plaque positive. Smaller egg batches from two other females (#17b and #19) hatched and yielded a total of 541 larvae which were tested in six pools. All were negative for BRBV in both RT–PCR and plaque assays. Eggs from the remaining two females failed to hatch. Unhatched egg batches from five females were tested for virus without attempting to count the eggs. Two of five egg batches, from females #16 and #17b, were positive for BRBV by RT–PCR, and one of these batches was positive by plaque assay (data not shown).

Of 20 male and 20 female ticks resulting from larval immersion #3, and allowed to mate and feed upon rabbit 10, 19 of each gender were recovered. Eighteen of 19 males were positive for BRBV by RT–PCR and those 18 were also positive by plaque assay. Fourteen of 19 females were recovered alive and were placed in individual vials for oviposition. Following oviposition, all females were tested for BRBV. All 14 surviving females were positive by RT–PCR and 5 were plaque positive. Two of 5 females recovered dead were virus positive by RT–PCR and one of these also produced plaques. Four of the 14 surviving females (a, d, g, h) oviposited producing a total of 55 pools containing 5,266 larvae (1–28 pools per female). All 55 larval pools were negative for BRBV by both RT–PCR and plaque assay (Table 3). Unhatched egg batches from three females were tested for BRBV but were negative by both assays.

Table 3. Detection of Bourbon virus in offspring of infected female *Amblyomma americanum*

Rabbit no.	Female no.	No. of pools (no. larvae)	No. RT–PCR positive (%)	No. plaque assay positive (%)
9	13	26 (2,536)	3/25[a] (12%)	0/26 (0%)
	17a	17 (1,614)	1/17 (6%)	1/17 (6%)
	17b	5 (471)	0/5 (0%)	0/5 (0%)
	19	1 (70)	0/1 (0%)	0/1 (0%)
	Total	49 (4,651)	4/48 (8%)	1/49 (2%)
10	a, d, g, h combined	55 (5,266)	0/55 (0%)	0/55 (0%)

[a]One sample lost.

Rabbit Serology

All 10 rabbits, whether needle-inoculated with BRBV or fed on by infected ticks of all active life stages, developed antibodies to BRBV, with titers ranging from 1:320 to 1:2,560 (Table 1).

Discussion

We were able to infect *A. americanum* larvae using the immersion technique, but infection rates were low in the resulting nymphs (2.5% in a sample of nymphs tested) and adults (0–7%) in the first two immersion trials using $10^{6.0}$ PFU/ml of virus. However, when the titer of the immersion fluid was increased to $10^{7.0}$ PFU/ml in the third immersion trial, all of the tested nymphs molted from immersed larvae were virus positive. When the remaining nymphs were fed on rabbits for the cofeeding trial, or to produce adults for the vertical transmission trial, a substantial percentage of the resulting adults

were infected, indicating that transstadial transmission among life stages was efficient and an important virus maintenance mechanism.

In the cofeeding trial 40% of donor nymphs that molted to females were BRBV positive, but, interestingly, none of the nymphs that molted into males was positive. Among virus recipient ticks all of the engorged females became infected while approximately half of male ticks did. It may seem surprising that male ticks became infected as males do not fully engorge on host blood as do females. Rather, they ingest small amounts of blood to stimulate spermatogenesis. However, infection of male *Dermacentor marginatus* (Sulzer) (Acari: Ixodidae), *Dermacentor reticulatus* (Fabricius) (Acari: Ixodidae), and *Rhipicephalus* spp. with the phleboviruses, Palma virus and Bhanja virus (Family *Phenuiviridae*), during cofeeding experiments has been reported (Labuda et al. 1997). The relatively low CT values, and the detection of live virus in some males in our study 18 d after removal from the rabbit, indicates active replication of BRBV in the males. This, combined with the duration of adult feeding and mating, averaging 12 d, suggests the possibility that virus could disseminate within the male and be transferred venerably to the female during mating.

The efficient transmission of BRBV among cofeeding ticks is in line with the finding of nonviremic or cofeeding transmission of the related Thogoto virus (THOV) by *Rhipicephalus appendiculatus* (Fabricius) (Acari: Ixodidae) (Jones et al. 1987). In continuing studies, both *R. appendiculatus* and *A. variegatum* Fabricius (Acari: Ixodidae) were equally susceptible to infection with THOV and equally effective as infected cofeeding donor nymphs (i.e., there was no significant difference in the number of infected recipient nymphs of either species), but as virus donors, adult *A. variegatum* transmitted virus to substantially fewer virus recipients (Jones et al. 1990).

In contrast to cofeeding infection rates, vertical transmission by infected *A. americanum* was not efficient. In one trial using females infected by cofeeding, only 8% of 48 larval pools tested were virus positive. However, only 6 of 12 engorged females oviposited, and only 2 produced large numbers of eggs. In the main vertical transmission trial using adult ticks resulting from the third larval immersion, none of 55 larval pools was positive. Again, in this trial, only 4 of 14 engorged females oviposited and only 2 of them produced a large number of eggs. This experiment should be repeated using infected and uninfected adults from the same source. If infected females show a reduced level of oviposition compared to uninfected females this would suggest that BRBV may negatively affect fecundity of *A. americanum*.

The distribution and natural history of BRBV are poorly understood. Only a small number of human cases have been identified, occurring in the midwestern and southern states (https://www.cdc.gov/ncezid/dvbd/bourbon/#where). Surveillance and testing of potential tick vectors have only occurred in small areas of northwestern Missouri (Savage et al. 2017) and southeastern Kansas (Savage et al. 2018a, b). In both of these areas, *A. americanum* accounted for >97% of the ticks collected, and accounted for all of the virus isolates obtained. Other hematophagous arthropods have not been studied. DHOV and Batken virus, both closely related to BRBV, have been isolated from mosquitoes in Europe and central Asia (Labuda and Nuttall 2004, Hubalek and Rudolf 2012), but their role, if any, in transmission of those viruses is unknown. Thogoto virus, the type species of the Thogoto virus genus, has been isolated from several genera of ticks in Africa and southern Europe, is recognized to infect domestic cattle and goats, and is associated with abortion in infected ewes (Labuda and Nuttall 2004). A recent survey of blood sera collected from a variety of wild and domestic animals residing in northwestern Missouri found high levels of neutralizing antibody to BRBV in white-tailed deer and raccoons, common vertebrate hosts for *A. americanum*, and lower prevalences in domestic dogs, eastern cottontail rabbits, and horses (Jackson et al. 2019). Whether any of these mammal species are competent reservoir or amplifier hosts has not been determined. Antibodies to BRBV were not detected in domestic cats, fox squirrels, or Virginia opossum, nor in 26 avian species tested. Future studies should include cattle, sheep, and goats in order to determine exposure to infected ticks and to detect any disease that may be associated with BRBV infection.

In summary, the results described herein indicate that *A. americanum* is a competent vector of BRBV. The reported data have demonstrated transstadial transmission of BRBV from the larval to the nymphal stage and from the nymphal stage to the adult stage of the tick, and a high rate of transmission of virus from infected to uninfected ticks by cofeeding, suggesting that cofeeding may be an important mechanism for viral maintenance in the environment. Although vertical transmission occurred at a very low level, any infected larvae produced by this mechanism may provide additional cofeeding transmission. The development of high antibody titers in rabbits fed upon by larvae, nymphs, and adults is further evidence of viral infection of the tick's salivary glands and of horizontal transmission during blood feeding by all of these life stages. Although cofeeding transmission alone may be sufficient for endemic maintenance of BRBV given adequate tick population densities, if vertebrate species that develop viremias of sufficient magnitude to infect engorging ticks can be identified, then horizontal transmission may also be a factor in viral amplification and maintenance. Another route of transmission that should be explored is venereal transmission of virus from infected male ticks to uninfected females.

The results of this study have implications for the public health response to BRBV infection of humans. Public education programs should stress individual behaviors that reduce the risk of tick attachment and virus transmission. Surveys of ticks to obtain local population density estimates, and to obtain specimens for virus testing, could be combined with serologic studies of wild and domestic mammals (Jackson et al. 2019) to assess disease risk to local residents. These surveys could also identify neighborhoods at the suburban/rural interface, and public recreation areas such as parks, where tick control might be feasible.

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References Cited

Anderson, C. R., and J. Casals. 1973. Dhori virus, a new agent isolated from *Hyalomma dromedarii* in India. Indian J. Med. Res. 61: 1416–1420.

Godsey, M. S., H. M. Savage, K. L. Burkhalter, A. M. Bosco-Lauth, and M. J. Delorey. 2016. Transmission of heartland virus (Bunyaviridae:

Phlebovirus) by experimentally infected *Amblyomma americanum* (Acari: Ixodidae). J. Med. Entomol. 53: 1226–1233.

Haig, D. A., J. P. Woodall, and D. Danskin. 1965. Thogoto virus: a hitherto underscribed agent isolated from ticks in kenya. J. Gen. Microbiol. 38: 389–394.

Hubalek, Z., and I. Rudolf. 2012. Tick-borne viruses in Europe. Parasitol. Res. 111: 9–36.

Jackson, K. C., T. Gidlewski, J. J. Root, A. M. Bosco-Lauth, R. R. Lash, J. R. Harmon, A. C. Brault, N. A. Panella, W. L. Nicholson, and N. Komar. 2019. Bourbon virus in wild and domestic animals, Missouri, USA, 2012–2013. Emerg. Infect. Dis. 25:1752–1753.

Jones, L. D., C. R. Davies, G. M. Steele, and P. A. Nuttall. 1987. A novel mode of arbovirus transmission involving a nonviraemic host. Science 237: 775–777.

Jones, L. D., C. R. Davies, T. Williams, J. Cory, and P. A. Nuttall. 1990. Non-viraemic transmission of Thogoto virus: vector efficiency of *Rhipicephalus appendiculatus* and *Amblyomma variegatum*. Trans Royal Soc. Trop. Med. Hyg. 84: 846–848.

Kosoy, O. I., A. J. Lambert, D. J. Hawkinson, D. M. Pastula, C. S. Goldsmith, D. C. Hunt, and J. E. Staples. 2015. Novel Thogotovirus species associated with febrile illness and death, United States, 2014. Emerg. Infect. Dis. 21:760–764.

Lanciotti, R. S., A. J. Kerst, R. S. Nasci, M. S. Godsey, C. J. Mitchell, H. M. Savage, N. Komar, N. A. Panella, B. C. Allen, K. E. Volpe, et al. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J. Clin. Microbiol. 38: 4066–4071.

Labuda, M., M. J. Alves, E. Eleckova, O. Kosuch, and A. R. Filipe. 1997. Acta Virol. 41: 325–328.

Labuda, M., and P. A. Nuttall. 2004. Tick-borne viruses. Parasitology 129(Suppl): S221–S245.

Lambert, A. J., J. O. Velez, A. C. Brault, A. E. Calvert, L. Bell-Sakyi, A. M. Bosco-Lauth, J. E. Staples, and O. I. Kosoy. 2015. Molecular, serological and in vitro culture-based characterization of Bourbon virus, a newly described human pathogen of the genus *Thogotovirus*. J. Clin. Virol. 73: 127–132.

Levin, M. L., and L. B. Schumacher. 2016. Manual for maintenance of multi-host ixodid ticks in the laboratory. Exp. Appl. Acarol. 70: 343–367.

Miller, B. R., C. J. Mitchell, and M. E. Ballinger. 1989. Replication, tissue tropisms and transmission of yellow fever virus in *Aedes albopictus*. Trans. R. Soc. Trop. Med. Hyg. 83: 252–255.

Mitzel, D. N., J. B. Wolfinbarger, R. D. Long, M. Masnick, S. M. Best, and M. E. Bloom. 2007. Tick-borne flavivirus infection in *Ixodes scapularis* larvae: development of a novel method for synchronous viral infection of ticks. Virology 365: 410–418.

Policastro, P. F., and T. G. Schwan. 2003. Experimental infection of *Ixodes scapularis* larvae (Acari: Ixodidae) by immersion in low passage cultures of *Borrelia burgdorferi*. J. Med. Entomol. 40: 364–370.

Savage, H. M., M. S. Godsey, Jr, N. A. Panella, K. L. Burkhalter, D. C. Ashley, R. R. Lash, B. Ramsay, T. Patterson, and W. L. Nicholson. 2016. Surveillance for Heartland virus (Bunyaviridae: *Phlebovirus*) in Missouri during 2013: first detection of virus in adults of *Amblyomma americanum* (Acari: Ixodidae). J. Med. Entomol. 53: 607–612.

Savage, H. M., K. L. Burkhalter, M. S. Godsey, Jr., N. A. Panella, D. C. Ashley, W. L. Nicholson, and A. J. Lambert. 2017. Bourbon virus in field-collected ticks, Missouri, USA. Emerg. Infect. Dis. 23: 2017–2022.

Savage, H. M., M. S. Godsey, Jr., N. A. Panella, K. L. Burkhalter, J. Manford, I. C. Trevino-Garrison, A. Straily, S. Wilson, J. Bowen, and R. K. Raghavan. 2018a. Surveillance for tick-borne viruses near the location of a fatal human case of Bourbon virus (Family Orthomyxoviridae: Genus *Thogotovirus*) in Eastern Kansas, 2015. J. Med. Entomol. 55: 701–705.

Savage, H. M., M. S. Godsey, Jr., J. Tatman, K. L. Burkhalter, A. Hamm, N. A. Panella, A. Ghosh, and R. K. Raghavan. 2018b. Surveillance for Heartland and Bourbon viruses in Eastern Kansas, June 2016. J. Med. Entomol. 55: 1613–1616.

Troughton, D. R., and M. L. Levin. 2007. Life cycles of seven ixodid tick species (Acari: Ixodidae) under standardized laboratory conditions. J. Med. Entomol. 44: 732–740.