Transovarial Transmission of Heartland Virus by Invasive Asian Longhorned Ticks under Laboratory Conditions

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We demonstrated experimental acquisition and transmission of Heartland bandavirus by *Haemaphysalis longicornis* ticks. Virus was detected in tick salivary gland and midgut tissues. A total of 80% of mice exposed to 1 infected tick seroconverted, suggesting horizontal transmission. *H. longicornis* ticks can transmit the virus in the transovarial mode.

The Asian longhorned tick, *Haemaphysalis longicornis*, is an ixodid tick native to Southeast Asia that was reported in the United States during 2017 and has since been found in 17 states (*1*,*2*). In its native range, this tick is the main vector of Dabie bandavirus (*3*) (formerly severe fever with thrombocytopenia syndrome virus), the agent that causes severe human illnesses characterized by high fever, thrombocytopenia, leukopenia, and multiorgan dysfunction (*4*).

Dabie bandavirus is closely related genetically to Heartland bandavirus (HRTV) (*5*), an emerging North American virus reported during 2012 after 2 men in Missouri, USA, showed febrile illness with fatigue, thrombocytopenia, and leukopenia after exposure to ticks (*6*). Because the current geographic range and the predicted range expansion of invasive *H. longicornis* ticks overlap considerably with human cases of HRTV, including Missouri (*7*,*8*), this study was designed to assess the ability of this invasive tick species to maintain and transmit HRTV.

The Study

We selected 74 female *H. longicornis* ticks from an HRTV-free colony into experimental and control groups. We microinjected 50 ticks with 300 focus-forming units of HRTV into the anal pore and 24 ticks with an equivalent volume of Dulbecco modified Eagle medium into the anal pore (Appendix, https://wwwnc.cdc.gov/EID/article/28/3/21-0973-App1.pdf). We dissected *t*icks at 14, 21, 28, and 40 days postinjection (dpi) and collected the salivary glands, midgut, and carcass of each tick. We screened tick samples for HRTV RNA by using quantitative reverse transcription PCR (qRT-PCR) (Table 1; Figure 1). No samples taken from media-injected ticks screened positive for HRTV (Table 1). For virus-injected ticks, HRTV RNA titers followed a general trend across each organ, and titers peaked at 21 dpi (Figure 1).

To screen HRTV-microinjected ticks for infectious virions, we collected ticks at 14, 21, 28, and 40 dpi and individually homogenized them. We cultured tick homogenates in triplicate on Vero E6 cells, and titered infectious virus by using a focus-forming assay (FFA). All ticks from each time point produced foci, indicating the presence of infectious virions in the tick body at each interval (Table 1).

We selected an additional 26 female *H. longicornis* ticks to evaluate horizontal transmission of HRTV to BALB/c mice. We microinjected 16 ticks with HRTV and the remaining 10 with Dulbecco modified Eagle medium. At 40 dpi, mice were infested with the microinjected ticks at a ratio of 1 tick/mouse. Five of the HRTV-injected ticks and 5 medium-injected ticks attached and fed on the mice to repletion. After feeding was complete, we removed engorged ticks and housed them individually to aid oviposition. We monitored mice daily for clinical signs of disease. We collected blood from the mice at −1, 7, and 14 days after tick attachment. Mice were subjected to necropsy at 28 days after attachment, and we collected liver, spleen, kidney, brain, blood, and testis samples. We screened blood and organ samples for HRTV RNA by qRT-PCR. No HRTV RNA was detected in any blood or organs collected from the mice.

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Table 1. Rate of detection of HRTV RNA by qRT-PCR and infectious HRTV by FFA in adult *Haemaphysalis longicornis* ticks at 14, 21, 28, and 40 dpi*

Procedure	Real-time qRT-PCR detection of HRTV RNA, no. positive/no. tested (%)			FFA titration of HRTV, whole tick
	Salivary glands	Midgut	Carcass	
Medium injected	0/15 (0)	0/15 (0)	0/15 (0)	0/9 (0)
HRTV-injected 14 dpi	4/8 (50)	8/8 (100)	8/8 (100)	5/5 (100)
HRTV-injected 21 dpi	7/8 (88)	8/8 (100)	8/8 (100)	5/5 (100)
HRTV-injected 28 dpi	6/8 (75)	8/8 (100)	8/8 (100)	5/5 (100)
HRTV-injected 40 dpi	3/6 (50)	6/6 (100)	6/6 (100)	4/5 (80)

*dpi, days postinjection; FFA, focus-forming assay; HRTV, Heartland virus; qRT-PCR, quantitative reverse transcription PCR.

We screened serum from the terminal blood samples to determine whether mice seroconverted relative to HRTV. We assayed each serum sample on 2 independent occasions. In brief, we assayed diluted serum samples by using HRTV-infected Vero E6 cells as antigens. Four of the 5 mice fed upon by a single HRTV-injected tick were positive for HRTV-specific antibodies. We detected antibodies up to a serum dilution of 1:1,600 for 3 mice and 1:800 for 1 mouse. None of the 5 mice fed upon by media-injected ticks were positive for HRTV-specific antibodies. Likewise, none of the age-matched, sex-matched, preimmune mouse serum demonstrated an antibody response to HRTV.

After each fed female tick completed oviposition, we removed the fed female carcass from the egg mass and homogenized the carcass. We screened the carcasses for HRTV RNA by qRT-PCR, and 5/5 HRTV-injected female carcasses were positive for HRTV RNA (Table 2; Figure 2). The media-injected fed female carcasses were negative for HRTV. We also removed 3 pools of 50 eggs/egg mass to screen for HRTV RNA. All 15 egg pools from HRTV-injected ticks were positive for HRTV RNA (Table 2; Figure 2). Egg pools from the media-injected ticks had no HRTV RNA.

We repeated this analysis for pools of larvae (4 pools of 50 larvae derived from each fed female) after hatching. All larvae clutches derived from HRTV-injected females were positive for HRTV RNA. To screen for infectious virions in larvae, we homogenized pools of 150 larvae from each clutch and cultured them on Vero E6 cells. We titered the infectious virus by using FFA, and all 5 clutches derived from HRTV-injected females were positive for infectious HRTV (Table 2).

Conclusions

We demonstrated experimental acquisition and transmission of HRTV by *H. longicornis* ticks after microinjection of the anal pore with HRTV. Although not a natural route of virus acquisition for ticks, microinjection of the anal pore is an established and reproducible procedure that delivers specific quantities of virus into the alimentary canal of the tick, the first organ system that virus contacts in naturally infected ticks (*9*). Microinjected ticks showed viral RNA titers peaking at 21 dpi in salivary glands, midguts, and carcasses, suggesting that HRTV replication took place within these organs between 14 and 21 dpi.

Maintenance of infectious HRTV virions for several weeks after microinjection suggests that an artificially infected tick is capable of transmitting HRTV to vertebrate hosts on which it feeds long after viral acquisition. Although the mice exposed to HRTV-infected ticks did not show clinical signs of disease and viral RNA was not detected in any mouse tissues, the absence of disease in these immunocompetent mice was expected; previously, only immunocompromised Ag129 mice have shown detectable viremia, clinical signs of HRTV infection, and death (*10*). Seroconversion of 4/5 mice exposed to an individual HRTV-infected *H. longicornis* tick suggests horizontal transmission of HRTV. Future studies should con-

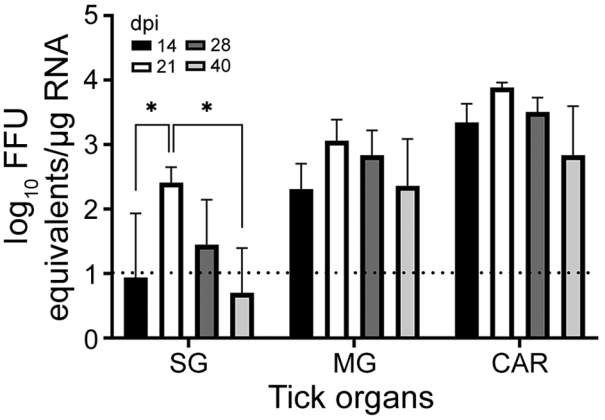

Figure 1. Detection of Heartland virus (HRTV) RNA by real-time, quantitative reverse transcription PCR reaction of HRTV-injected *Haemaphysalis longicornis* ticks. Ticks were dissected at 14, 21, 28, and 40 dpi. Tick organs were screened individually. Viral load data are expressed as FFU equivalents per microgram of RNA after normalization to a standard curve. Data were not normally distributed and are presented as medians with interquartile ranges. Statistical significance was determined by using Kruskal-Wallis tests followed by the Dunn test. Limit of detection was ≈10 FFU equivalents/µg RNA. *p<0.05. CAR, carcass; dpi, days postinjection; FFU, focus-forming units; MG, midgut; SG, salivary glands.

Table 2. Rate of detection of HRTV RNA by qRT-PCR and infectious HRTV by FFA in fed *Haemaphysalis longicornis* adult tick carcasses, tick eggs, and tick larvae*

Procedure	Real-time qRT-PCR detection of HRTV RNA, no. positive/no. tested (%)			FFA titration of HRTV, larvae pools‡
	Fed adult carcasses	Egg pools†	Larvae pools†	
Medium injected	0/5 (0)	0/15 (0)	0/20 (0)	0/5 (0)
HRTV-injected	5/5 (100)	15/15 (100)	20/20 (100)	5/5 (100)

*FFA, focus-forming assay; HRTV, Heartland virus; qRT-PCR, quantitative reverse transcription PCR.
† Egg and larvae pools contained 50 eggs or larvae/pool.
‡Larvae pools contained 150 larvae/pool.

firm the presence of infectious virions in tick saliva to eliminate the possibility of seroconversion caused by transmission of noninfectious HRTV antigens during tick feeding.

We also showed transovarial transmission of HRTV in *H. longicornis* ticks by detection of HRTV RNA in eggs and larvae derived from HRTV-infected mother ticks. Furthermore, we demonstrated the presence of infectious virions in larvae after hatching. The North American strain of the tick is parthenogenetic, a foremost public health concern because 1 female can reproduce asexually to establish and sustain local populations (*11*). Because *H. longicornis* ticks are a 3-host tick and a host generalist (*12*), the possibility of invasive *H. longicornis* ticks acquiring HRTV by cofeeding with infected ticks or by feeding on a viremic host further highlights the potential of the tick to efficiently disseminate the virus. This distinction becomes more crucial because the tick can withstand a wide range of climates (*7*,*13*). Further studies should be conducted to demonstrate whether the tick can transmit HRTV during co-feeding with other ticks because this would be a major factor in promoting the environmental spread of the virus.

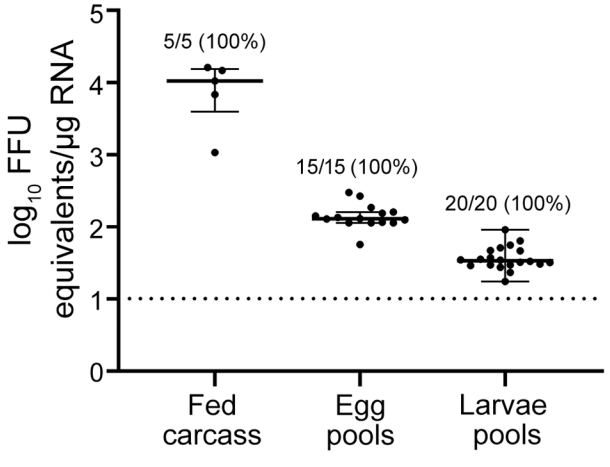

Figure 2. Scatter plot demonstrating detection of Heartland virus (HRTV) by real-time, quantitative reverse transcription PCR. Data were not normally distributed and are presented as medians with interquartile ranges. Fed female carcasses, egg pools, and larvae pools were screened for viral RNA. Egg pools and larvae pools were composed of 50 eggs or larvae per pool. Limit of detection was ≈10 FFU equivalents/μg RNA. FFU, focus-forming units.

The predicted spread of *H. longicornis* ticks in the United States shares a geographic range with states in which HRTV has already been reported in *Amblyomma americanum* ticks and wildlife (*7*,*8*,*14*). The introduction of a new vector species could amplify transmission in natural foci, resulting increased HRTV disease cases in humans.

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Appendix

Materials and Methods

Ethics

All studies including mice were conducted in animal biosafety level 3 (ABSL-3) facilities. Bloodmeals were provided to uninfected ticks for colony maintenance by feeding on rabbits in animal biosafety level 2 (ABSL-2) facilities. Work involving mice and rabbits was conducted with animal use protocols approved by the University of South Alabama Institutional Animal Care and Use Committee (nos. 1619216–2 and 1619233–2)

Cells and Virus

A culture of African green monkey kidney (Vero E6) cells was maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cell culture was incubated at 37°C in an atmosphere of 5% CO_2. Heartland virus (HRTV) strain MO-4 was acquired from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch (Galveston, TX, USA). The stock had previously been passaged once on DH82 cells, once on Vero cells, and once in suckling mouse brain. The stock was then passaged on Vero E6 cells 3 times. Focus-forming assays (FFAs) were used to determine stock virus titers as described (*1*).

Ticks and Animals

Adult female *Haemaphysalis longicornis* ticks (BEI Resources, https://www.beiresources.org) were maintained by feeding on New Zealand White rabbits (Charles River Laboratories, https://www.criver.com). Uninfected ticks were housed under arthropod containment level 2 (ACL-2) conditions. Infected ticks were housed under ACL-3 conditions in a negative pressure containment enclosure (bioBUBBLE Inc.,

https://biobubble.com). ACL-2 and ACL-3 facilities were maintained at 23°C and on a 16:8 light:dark cycle. Tick vials were housed in a high-humidity desiccator at ≥90% relative humidity.

Male and female BALB/c mice were obtained from The Jackson Laboratory (https://www.jax.org). Mice acclimated to the environment for a minimum of 5 days before experimentation. Mice were 10 weeks old upon the start of tick infestation and ranged from 10 to 11 weeks old at the time of tick attachment and initiation of tick feeding. Mice were weighed before and after capsule attachment and were randomly assigned to each infection group. After tick capsules were attached to mice, each mouse was individually housed in ventilated cage systems. A 12:12 light:dark cycle was maintained for all cages. Temperature and humidity were closely regulated for the cage system. Food and water were provided to each mouse ad libitum.

Tick Viral Replication Kinetics

Infection of Adult Ticks by Anal Pore Microinjection

Adult female *H. longicornis* ticks were microinjected in the anal pore with HRTV or medium as described (*2*). In brief, infection by microinjection was accomplished by injecting 475 nL of virus stock containing ≈300 FFUs of HRTV into the anal aperture of the immobilized tick by using a digitally-controlled microinjector with a footswitch, glass microneedles, and a dissecting microscope. An equivalent volume of DMEM was used to microinject ticks in the mock-infected control group. All microinjected ticks were the same generation and same time post-molt (i.e., 6 weeks post-molting from nymph to adult). After microinjection, all ticks were maintained in the ACL-3. Tick viability was monitored twice daily for 4 days after microinjection.

Tick Dissection

At 14, 21, 28, and 40 days postinjection, ticks were dissected from the virus-injected and the media-injected cohorts. Salivary glands and midgut were collected from each tick, and the remaining tick organs, including the exoskeleton, were retained and designated as carcass.

RNA Extraction from Tick Tissues

Tick tissues (salivary glands and midguts) were homogenized in 100 μL TRIzol reagent by using a pellet pestle mixer (Thermo Fisher Scientific, https://www.thermofisher.com). Tick carcasses were homogenized in TRIzol reagent with sterile metal beads in a TissueLyser II (QIAGEN, https://www.qiagen.com) at 30 hz for 3 minutes. A hybrid of TRIzol and QIAGEN

RNeasy Mini Kit protocols was used for tissue RNA extractions because it has been demonstrated that these combined protocols inactivate virus and yield high-quality RNA (*3*). In brief, chloroform was added to tissue homogenate at 0.2 mL chloroform/1 mL homogenate. The samples were shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes. Samples were centrifuged at 12,000 × *g* at 4°C for 15 minutes. The aqueous phase was removed and was mixed with 1 volume of 70% ethanol by pipetting. The samples were added to an Rneasy Mini Column (QIAGEN), and the protocol for the kit was followed. Total RNA was eluted from the column with 30 μL of nuclease-free water. RNA quantity and purity were determined by using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

Detection of HRTV RNA in Tick Tissues by Real-Time Quantitative Reverse Transcription PCR

Absolute quantification of viral loads in tick tissue samples were determined by using a real-time quantitative reverse transcription PCR (qRT-PCR) as described (*4*). In brief, viral RNA loads are expressed on a log10 scale as FFU equivalents per microgram of RNA after normalization to a standard curve produced by using serial 10-fold dilutions of viral RNA from known quantities of infectious virus to estimate viral burden. The qRT-PCR was performed by using forward (5′-CCTTTGGTCCACATTGATTG-3′) and reverse (5′-CACTGATTCCACAGGCAGAT-3′) primers specific to the HRTV spike (S) gene (*5*). An HRTV S gene probe (5′-56-FAM/TGGATGCCTATTCCCTTTGGCAA/36-TAMSp-3′) was also used. When performing a qRT-PCR, a standard amount of sample RNA was added to the appropriate wells of a 96-well PCR plate. Reagents from the iTaq Universal SYBR Green One-Step Probes Kit (BioRad Laboratories, https://www.bio-rad.com), 10 μmol/L of forward and reverse primers, and probe specific to the HRTV S gene were added to the wells. The total reaction volume per well was 20 μL. Plates were sealed and run on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) at the following cycle settings: 10 minutes at 50°C; 10 seconds at 95°C; and 30 seconds for 45 cycles at 60°C.

Infectious HRTV Whole Tick FFA

Whole adult ticks were harvested at 14, 21, 28, and 40 dpi and frozen at −80°C. One pool of 150 larvae hatched from each fed female was harvested ≈2–3 weeks post-hatching and processed by using the FFA. For FFA of tick viral loads, whole ticks were individually homogenized in DMEM supplemented with 2% fetal bovine serum, 1% penicillin/streptomycin,

and 1% fungizone by using sterile metal beads in a Bead Ruptor 96 Tissue Lyser (OMNI International, https://us.omni-inc.com). Larval pools were also individually homogenized as described above. Tick homogenate was centrifuged at 5,000 × *g* at room temperature for 5 minutes. The clarified tick homogenate was then aliquoted into new sterile tubes, and 65 μL of undiluted tick homogenate was used to infect Vero E6 cells seeded in 48-well plates. For adult whole ticks, plates were infected in triplicate. For larval pools, plates were infected in duplicate. Viral titers in adult and larval *H. longicornis* tick samples were determined by using FFA as described (*2*).

HRTV Transmission from Tick to Mouse

Tick infestations on mice were performed by using capsules made of 2-ml cryotubes. The base of each tube was cut to leave ≈3 mm of the remaining tube below the screw-cap lid. The top of the lid was cut to enable an opening when secured. The base of the capsule was attached to the upper dorsum of each mouse by using athletic tape adhered with livestock Kamar glue (Kamar Inc., https://www.enasco.com). After placing ticks inside the capsules, a piece of fine mesh fabric was placed under the capsule lid before closing to enable tick containment and air circulation. Masking tape was used to secure each capsule lid. Capsule integrity was checked daily throughout the tick infestations, and capsules were reinforced with additional adhesive and bandages as needed.

Bodyweights and clinical observations were documented daily for each mouse after addition of 1 tick to each capsule. Ticks that did not attach to mice were removed from the capsule after 1 week. Engorged ticks were removed from capsules upon completion of feeding and detachment from the skin. Blood samples were collected from each mouse by submandibular bleed at −1, 7, and 14 days postattachment under isoflurane anesthesia. Blood was also collected during necropsy by terminal cardiac bleed. Changes in appearance, respiration, neurologic, and behavior were documented daily. When mice reached the study endpoint (28 days postattachment), euthanasia was performed by isoflurane overdose, followed by cervical dislocation and terminal cardiac bleed. Tissues harvested during necropsy were fixed at room temperature in 10% neutral-buffered formalin for 72 hours. Formalin was replaced after 24 hours. Necropsies of mock-infected and HRTV-infected mice were conducted under ABSL-3 conditions.

The following tissues were harvested from mice after undergoing necropsy and were stored in TRIzol Reagent (Invitrogen Life Technologies, https://www.thermofisher.com): spleen, liver, kidney, testes, and brain. Blood collected during submandibular bleeds was also stored in TRIzol Reagent. Terminal mouse blood was divided between TRIzol Reagent and serum separation tubes for storage. Mouse tissues were homogenized in a bead beater system as described above. Homogenized mouse tissues and blood were screened for viral load by qRT-PCR as described above.

An in-house immunoassay was performed to detect HRTV antibodies in serum samples from mice fed upon by HRTV-infected ticks. Vero E6 cells were infected with HRTV at a multiplicity of infection of 0.1 in 48-well plates. After a 1-hour incubation, the virus inoculum was removed, and the cells were incubated for 2 days in 2% fetal bovine serum in DMEM. The plates were fixed with 1:1 methanol:acetone for 30 minutes and air dried. The plates were washed 3 times with phosphate-buffered saline, 0.1% Tween 20 (PBST) and blocked with a 5% goat serum solution. Mouse serum was serially diluted 2-fold from 1:25 to 1:25,600. A total of 65 μL mouse serum dilution was added to the designated plate well and incubated for 1 hour. The plates were washed in PBST and then stained with a secondary goat anti-mouse horseradish peroxidase–conjugated antibody for 1 hour.

Plates were washed with PBST after secondary antibody staining. To develop the plates, 65 μL of aminoethyl carbazole developing solution (ImmPACT AEC Kit, Vector Laboratories, https://vectorlabs.com) was applied to each well according to the product instructions. Plates were wrapped in foil and developed for 30 minutes. The plates were submerged in water to stop development. Presence of HRTV antibodies were confirmed by identifying the lowest serum dilution for which a signal was observed. Comparative immunoassays were performed by using naïve preimmune serum from age-matched sex-matched mice following the same protocol. Every immunoassay included a HRTV-specific antibody positive control (in the form of mouse hyperimmune ascitic fluid) to confirm that the Vero E6 cells were infected and displayed HRTV antigen. To confirm that the secondary antibody did not bind nonspecifically to cellular components, secondary antibody only (i.e., no primary antibody) controls were included for each immunoassay. In addition, every immunoassay included mock-infected Vero E6 cells that were exposed to HRTV mouse hyperimmune ascitic fluid positive control antibody.

HRTV Transovarial Transmission

Engorged ticks were individually housed after feeding on mice. At 14 days (±1 day) post-oviposition, 3 pools of 50 eggs were collected from each egg mass. After the remaining eggs of each clutch had hatched, 4 pools of 50 larvae were collected from each clutch. Each egg and larvae pool was homogenized, and RNA was extracted from the homogenates as described above. Egg and larvae pools were screened for viral loads by using qRT-PCR as described above. For titration of infectious HRTV in larvae by FFA, pools of 150 larvae were homogenized, clarified, and cultured on Vero E6 cells as described above.

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