

Experimental Infection of White-Tailed Deer (*Odocoileus virginianus*) with Heartland Virus

Lorelei L. Clarke,¹ Mark G. Ruder,² Daniel Mead,² and Elizabeth W. Howerth^{1*}

¹Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, Georgia;

²Southeastern Cooperative Wildlife Disease Study, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, Georgia

Abstract. Heartland virus (HRTV) is a phlebovirus suspected to be transmitted by *Amblyomma americanum*, commonly known as the lone star tick, and reported to cause illness in humans, which is characterized by thrombocytopenia and leukopenia. Heartland virus-reactive antibodies have been detected in a variety of wildlife species including white-tailed deer (WTD). To better understand the potential role of deer in the epidemiology of HRTV, we experimentally inoculated five WTD fawns with HRTV and monitored for clinical disease, viremia, virus shedding, and seroconversion. None of the animals showed signs of clinical disease, and there was no detectable viremia or virus shedding post-inoculation. Two wild-caught fawns entered the study with preexisting antibody titers against HRTV. All animals showed minimal immune responses against HRTV after needle inoculation. In conclusion, this study does not indicate that WTD are a likely reservoir for HRTV in natural settings.

Heartland virus (HRTV; family Bunyviridae, genus *Phlebovirus*) is an emerging zoonotic pathogen first recognized in 2009 after causing febrile disease and thrombocytopenia/leukopenia in two people in the United States.¹ There have been 30 documented human cases of HRTV-associated disease occurring in nine states,^{2–4} and the virus has been detected in large mononuclear cells in the leukocytic fraction of the blood and bone marrow of infected human patients¹ and postmortem in the spleen and lymph nodes.³ The virus is most likely transmitted by ticks, namely *Amblyomma americanum*, and has been isolated from wild-caught ticks in the vicinity of the first reported human cases.^{5,6}

Antibodies reactive with HRTV have been detected in free-ranging white-tailed deer (WTD) (*Odocoileus virginianus*), moose (*Alces alces*), raccoons (*Procyon lotor*), and coyotes (*Canis latrans*) in the central and eastern United States.^{7–9} Heartland virus, however, has not been detected in any naturally infected, seropositive animals to date.^{1,3,9} Experimental HRTV infections have been attempted in raccoons, goats, chickens, rabbits, hamsters, C57BL/6 mice, and Ag129 mice.¹⁰ None of these species developed detectable viremia or pathologic changes with the exception of Ag129 mice, which are interferon $\alpha/\beta/\gamma$ receptor deficient.¹⁰

Several factors suggest WTD could be a host for HRTV, including the detection of seropositive WTD,⁸ the likelihood of *A. americanum* to feed on deer,¹¹ and the high density and widespread geographic range of WTD populations.¹² White-tailed deer are known to be exposed naturally and experimentally susceptible to systemic infection by viruses of the related *Orthobunyavirus* genus, such as Jamestown Canyon virus and La Crosse virus, without the development of clinical disease.^{13–16} To better understand the potential of WTD to serve as hosts for HRTV, we experimentally inoculated WTD fawns with HRTV and monitored for clinical disease, viremia, viral shedding, and seroconversion.

The HRTV isolate, “original Missouri 2009 strain,” used for inoculations and methods for all laboratory assays was obtained from the Centers for Disease Control (Fort Collins,

CO). The isolate was passaged once on Vero E6 cells and was diluted to an inoculation dose of 10^6 tissue culture infectious dose (TCID₅₀) in 1 mL Minimum Essential Medium™ (Sigma-Aldrich, Darmstadt, Germany) with added 3% bovine serum albumin and 5% antibiotics and antimycotics (virus media). White-tailed deer fawns were acquired from the Georgia Department of Natural Resources ($N = 3$) and the Whitehall Deer Research Facility at the University of Georgia ($N = 2$). Fawns were acquired at 1–4 weeks of age and housed indoors in a secure ABSL-2 facility until five clinically healthy fawns were acquired. Fawns were bottle-fed a commercial deer milk replacer and were approximately 4–5 weeks of age at the time of inoculation. All animal handling and care was in compliance with approved Institutional Animal Care and Use Committee protocols.

Serum samples were collected before inoculation to screen for neutralizing antibodies against HRTV, and all fawns were treated for ticks using a weight-appropriate topical application of Parastar® Plus (Elanco, Greenfield, IN; 9.8% fipronil, 5.2% cyphenothrin). On day 0, the fawns were weighed and then sedated (intramuscular: 0.75–2 mg/kg xylazine and 2–3 mg/kg ketamine). Fawns were then injected intradermally with 10^6 TCID₅₀ of HRTV in 1 mL of virus media at multiple sites (~100 μ L/site) in a shaved area of the neck to simulate a tick bite. Fawns were implanted with subcutaneous temperature-sensing microchips to monitor for febrile response.

Fawns were monitored twice daily for clinical signs, febrile response, and assessment of body weight and standardized body condition scores. Oronasal and rectal swabs along with 6 mL of blood were collected on 0, 1, 2, 3, 4, 5, 6, 7, and 14 days postinoculation (dpi). Blood samples were divided between ethylenediaminetetraacetic acid and additive-free tubes. Whole blood was centrifuged at 500 rcf for 6 minutes for separation and collection of plasma, whereas an aliquot of the remaining cellular portion was washed with phosphate buffered saline three times and sonicated. Additive-free tubes were centrifuged at 1,000 rcf for 10 minutes for separation and collection of serum. Serum, plasma, and sonicated cellular fraction (100 μ L aliquots) were inoculated onto Vero E6 cell cultures in 12-well plates and incubated at 37°C. The plates were observed daily for cytopathic effect (CPE) for 7 days, at which point, the isolates were passaged again on Vero E6 cells. The sonicated cellular fraction was frozen and inoculated a second time onto Vero E6 cell cultures in a similar manner except the

*Address correspondence to Elizabeth W. Howerth, Department of Pathology, University of Georgia, 501 D.W. Brooks Dr., Athens, GA 30602. E-mail: howerth@uga.edu

plates were observed daily for 10 days. Oronasal and rectal swabs were collected in 1 mL virus media, centrifuged at 1,000 rcf for 10 minutes, and the supernatant was similarly inoculated onto Vero E6 cell cultures in 12-well plates and incubated at 37°C with daily observation for CPE for 7 days and then passaged a second time.

All animals were euthanized via intravascular injection of pentobarbital and necropsied after final swab and blood collection on 14 dpi. Tissues collected included brain, lungs, heart, liver, kidneys, prescapular lymph nodes, thymus, and skin (at injection site). Tissue samples were collected in 1 mL virus media and were minced using a Tissue-Tearor™ (Biospec Products Inc, Barlesville, OK), vortexed, centrifuged at 1,000 rcf for 10 minutes, and 100 µL of the supernatant plated on Vero E6 cells in 12-well plates for 7 days with daily observation for CPE as described previously.

Heartland virus–neutralizing antibodies were quantified using serum microneutralization. Serum samples were heat-inactivated by incubating at 56°C for 30 minutes, then 2-fold serially diluted and incubated with 100 TCID₅₀ of virus suspension in 96-well microtiter plates for 1 hour at 37°C. The wells were then overlaid with 150 µL of Vero E6 cells. The plates were incubated at 37°C and observed for CPE daily for 5 days. Samples were run in duplicate and neutralization endpoint titers were calculated using the Reed and Muench method.¹⁷

RNA was extracted from 200 µL aliquots of serum and whole blood samples from all fawns collected at previously described time points using the Thermo Fisher Scientific KingFisher™ Duo Prime Purification System robot (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. Viral stock with a titer of 10⁵ TCID₅₀ was used as a positive control. Quantitative real-time RT-PCR was performed using the protocol described by Savage et al.⁵ using the Ambion AgPath ID™ One-step RT-PCR Kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions. Reactions were conducted on Step One Plus Real-Time PCR System (Applied Biosystems, Inc.).

None of the five fawns developed clinical signs during the experimental period and there was no detectable febrile response. At necropsy, all animals were in good physical condition and had no grossly evident lesions. Heartland virus was not detected by RT-PCR from serum or whole blood, and was not detected by virus isolation from serum, plasma, whole blood, oronasal swabs, or rectal swabs at any time point. Virus was not isolated from tissues collected at necropsy.

Results of the serologic testing are summarized in Table 1. Two fawns had HRTV–neutralizing antibodies before the start of the study, one of which was a fairly robust titer. At 14 dpi, all fawns had detectable HRTV–neutralizing antibodies, but titers in the three previously seronegative animals were relatively

TABLE 1

Serum neutralization titers of fawns experimentally inoculated with Heartland virus. Titers are expressed as a reciprocal of the dilution used

	Approximate age (weeks)	Gender	Origin	Day 0 titer	Day 14 titer
Fawn 1	4	F	WHDRL*	< 4	16
Fawn 2	5	M	GA DNR†	8	16
Fawn 3	5	F	GA DNR	< 4	8
Fawn 4	4	M	WHDRL	< 4	16
Fawn 5	4	F	GA DNR	64	128

* Whitehall Deer Research Laboratory.

† Georgia Department of Natural Resources.

low. The animals that were originally seropositive remained seropositive with slight increases in titers.

As in previous vertebrate experimental infections with HRTV,¹⁰ our experimental animals had no evidence of disease or virus replication. One fawn in our study had a robust neutralizing antibody titer before experimental inoculation. This animal was wild-caught and approximately 1 week of age when acquired, making colostrum the most likely source of the antibodies. High antibody titers specific for HRTV have been previously documented in WTD^{8,9}; however, cross-reacting antibodies to closely related viruses cannot be ruled out in these fawns. This cross-reactivity may confound potential serologic tests and may even offer cross-protective immunity to animals subsequently infected with a related virus.

In contrast to evidence in vertebrates, experimental work has shown that ticks can be vertically and horizontally infected.^{6,18} Virus-immersed *A. americanum* larvae as well as nymphs and adults molted from immersed larvae were fed on rabbits, producing low neutralizing antibody titers in affected animals, indicating that HRTV infection of ticks persists transstadially.¹⁸ Horizontal transmission of virus by infected ticks to uninfected ticks co-feeding on a nonviremic host was also described in that study.

Ticks may, therefore, represent a reservoir and vector for HRTV. Components of tick saliva have been shown to have profound effects in modulating host immune responses, influencing transmission and disease course of other arboviruses.^{19,20} These effects are often local to the site of the tick bite, which may explain why seroconversion occurs without a detectable systemic infection in experimentally infected vertebrates.¹⁰ Other factors, such as the frequency of infected tick feeding or a critical tick population density, may also play a role in seroconversion.

In conclusion, although WTD do not appear to be a likely reservoir of the virus in natural settings, they may still play a role in the maintenance of the tick population and as a nonviremic host for horizontal transmission among co-feeding ticks.

Received December 8, 2017. Accepted for publication January 16, 2018.

Published online February 26, 2018.

Acknowledgments: We thank the University of Georgia Animal Resources staff at the College of Veterinary Medicine for their logistical support and use of the BSL2Ag facilities. We thank Deborah Carter and Dr. Charlie Bahnson for their help in fawn husbandry and sample collection. We thank Drs. Nicholas Komar and Aaron Brault at the Arboviral Diseases Branch of the Centers for Disease Control in Fort Collins, CO, for their assistance in project design and the HRTV isolate used in the study.

Financial support: The research in this article was supported by a grant jointly funded by the University of Georgia, College of Veterinary Medicine, Office of the Vice President for Research, the Department of Pathology, and the Southeastern Cooperative Wildlife Disease Study.

Authors' addresses: Lorelei L. Clarke and Elizabeth W. Howerth, Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, GA, E-mails: lclarke@uga.edu and howerth@uga.edu. Mark G. Ruder and Daniel Mead, Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, University of Georgia, Athens, GA, E-mails: mgruder@uga.edu and dmead@uga.edu.

REFERENCES

- McMullan LK et al., 2012. A new phlebovirus associated with severe febrile illness in Missouri. *N Engl J Med* 367: 834–841.

2. Pastula DM, Turabelidze G, Yates KF, Jones TF, Lambert AJ, Panella AJ, Kosoy OI, Velez JO, Fischer M, Staples JE, 2014. Notes from the field: Heartland virus disease—United States 2012–2013. *MMWR Morb Mortal Wkly Rep* 63: 270–271.
3. Muehlenbachs A et al., 2014. Heartland virus-associated death in Tennessee. *Clin Infect Dis* 59: 845–850.
4. Centers for Disease Control, 2017. *Heartland Virus*. CDC. Available at: <https://www.cdc.gov/heartland-virus/index.html>. Accessed September 6, 2017.
5. Savage HM, Godsey MS Jr, Lambert A, Panella NA, Burkhalter KL, Harmon JR, Lash RR, Ashley DC, Nicholson WL, 2013. First detection of Heartland virus (Bunyaviridae: Phlebovirus) from field collected arthropods. *Am J Trop Med Hyg* 89: 445–452.
6. Savage HM, Godsey MS Jr, Panella NA, Burkhalter KL, Ashley DC, Lash RR, Ramsay B, Patterson T, Nicholson WL, 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.
7. Xing Z et al., 2013. Novel bunyavirus in domestic and captive farmed animals, Minnesota, USA. *Emerg Infect Dis* 19: 1487–1489.
8. Riemersma KK, Komar N, 2015. Heartland virus neutralizing antibodies in vertebrate wildlife, United States, 2009–2014. *Emerg Infect Dis* 21: 1830–1833.
9. Bosco-Lauth AM et al., 2015. Serological investigation of Heartland virus (Bunyaviridae: Phlebovirus) exposure in wild and domestic animal adjacent to human case sites in Missouri 2012–2013. *Am J Trop Med Hyg* 92: 1163–1167.
10. Bosco-Lauth AM, Calvert AE, Root JJ, Gidlewski T, Bird BH, Bowen RA, Muehlenbachs A, Zaki SR, Brault AC, 2016. Vertebrate host susceptibility to Heartland virus. *Emerg Infect Dis* 22: 2070–2077.
11. Kollars TM, Durden LA, Masters EJ, Oliver JH, 1997. Some factors affecting infestation of white-tailed deer by blacklegged ticks and winter ticks (Acari: Ixodidae) in southeastern Missouri. *J Med Entomol* 34: 372–375.
12. Quality Deer Management Association, 2016. *White-Tailed Deer Density Map*. QDMA. Available at: <https://www.qdma.com/>. Accessed January 15, 2018.
13. Blackmore CGM, Grimstad PR, 1998. Cache Valley and Potosi viruses (Bunyaviridae) in white-tailed deer (*Odocoileus virginianus*): experimental infections and antibody prevalence in natural populations. *Am J Trop Med Hyg* 59: 704–709.
14. Nagayama JN, Komar N, Levine JF, Biggerstaff B, Apperson CS, 2001. Bunyavirus infections in North Carolina white-tailed deer (*Odocoileus virginianus*). *Vector Borne Zoonotic Dis* 1: 169–171.
15. Neitzel DF, Grimstad PR, 1991. Serological evidence of California group and Cache Valley virus infection in Minnesota white-tailed deer. *J Wildl Dis* 27: 230–237.
16. Osorio JE, Godsey MS, Defoliart GR, Yuill TM, 1996. La Crosse viremia in white-tailed deer and chipmunks exposed by injection or mosquito bite. *Am J Trop Med Hyg* 54: 338–342.
17. Reed LJ, Muench H, 1938. A simple method of estimating fifty per cent endpoints. *Am J Epidemiol* 27: 493–497.
18. Godsey MS Jr, Savage HM, Burkhalter KL, Bosco-Lauth AM, Delorey MJ, 2016. Transmission of Heartland virus (Bunyaviridae: Phlebovirus) by experimentally infected *Amblyomma americanum* (Acari: Ixodidae). *J Med Entomol* 52: 1226–1233.
19. Hermance ME, Thangamani S, 2015. Tick saliva enhances Powassan virus transmission to the host, influencing its dissemination and the course of disease. *J Virol* 89: 7852–7860.
20. Jones LD, Davies CR, Steele GM, Nuttall PA, 1987. A novel mode of arbovirus transmission involving a nonviremic host. *Science* 237: 775–777.