

First Detection of Heartland Virus (Bunyaviridae: *Phlebovirus*) from Field Collected Arthropods

Harry M. Savage,* Marvin S. Godsey Jr., Amy Lambert, Nicholas A. Panella, Kristen L. Burkhalter,
Jessica R. Harmon, R. Ryan Lash, David C. Ashley, and William L. Nicholson

Centers for Disease Control and Prevention, Fort Collins, Colorado; Centers for Disease Control and Prevention, Atlanta, Georgia;
Department of Biology, Missouri Western State University, St. Joseph, Missouri

Abstract. Heartland virus (HRTV), the first pathogenic *Phlebovirus* (Family: Bunyaviridae) discovered in the United States, was recently described from two Missouri farmers. In 2012, we collected 56,428 ticks representing three species at 12 sites including both patients' farms. *Amblyomma americanum* and *Dermacentor variabilis* accounted for nearly all ticks collected. Ten pools composed of deplete nymphs of *A. americanum* collected at a patient farm and a nearby conservation area were reverse transcription-polymerase chain reaction positive, and eight pools yielded viable viruses. Sequence data from the nonstructural protein of the Small segment indicates that tick strains and human strains are very similar, $\geq 97.6\%$ sequence identity. This is the first study to isolate HRTV from field-collected arthropods and to implicate ticks as potential vectors. *Amblyomma americanum* likely becomes infected by feeding on viremic hosts during the larval stage, and transmission to humans occurs during the spring and early summer when nymphs are abundant and actively host seeking.

INTRODUCTION

The family Bunyaviridae includes a diverse array of viruses infecting animals and plants.¹ The family is composed of five genera, three of which, *Orthobunyavirus*, *Nairovirus*, and *Phlebovirus*, include viruses that are arthropod transmitted and cause human and/or animal disease.² A fourth genus, *Hantavirus*, includes viruses of rodents that lack an arthropod vector, but may be transmitted to humans as aerosols. The genus *Topovirus* includes viruses that affect plants and are transmitted by thrips (Insecta: Thysanoptera).

The genus *Phlebovirus* includes human and animal disease agents vectored by a wide array of arthropods including phlebotomine sandflies (Diptera: Psychodidae), mosquitoes (Diptera: Culicidae), and ticks (Acari: Ixodidae and Argasidae). In June 2009, two men residing on geographically distant farms in northwestern Missouri were admitted to a regional hospital with Ehrlichiosis-like symptoms³; both men reported tick exposure and removal of embedded ticks. Upon culture for the detection of *Ehrlichia chaffeensis*, a new *Phlebovirus* was isolated from both case-patients and tentatively named Heartland virus (HRTV).³ Whole genome sequencing suggested that HRTV was a member of a tick-borne group of the genus *Phlebovirus* and most closely related to a recently described virus from China called severe fever with thrombocytopenia syndrome virus (SFTSV), which is thought to be transmitted by ticks.^{3–5}

The goals of this project were to collect and test arthropods (ticks and mosquitoes) to determine if HRTV persisted at the two case-patients' residences, and to incriminate potential arthropod vectors for this newly described human pathogen. Additionally, we sampled similar farms not associated with HRTV and public lands managed by the Missouri Department of Conservation in northwestern Missouri to determine the geographic distribution of the virus and its prevalence in potential vector species.

MATERIALS AND METHODS

Study area. Ticks were collected at three sites (sites 1, 2a, 2b) on two farms owned by case-patients who became ill after

infection with HRTV in 2009,³ at three sites on two farms not associated with human disease, and at six sites located on five Missouri Department of Conservation areas (MCA) (Figure 1, Table 1). These locations fell within an area composed of four contiguous counties: Andrew, Holt, Nodaway, and Worth. Topographically, the study area is characterized by limited local relief with elevations ranging from their lowest along the Missouri River and the Missouri River flood plain (~250 m) to their highest in northern Nodaway County (~370 m).⁶ Steep bluffs are found along the fringes of the Missouri River floodplain, but the broader multicounty area consists of plains and open low hills topped with loess soils formed by historically wide ranging tallgrass prairies. The multicounty area is part of the broader Western Corn Belt Plains ecoregion. Little of the natural tallgrass prairie ecosystem remains today; the area is largely composed of cropland with corn, soybeans, and other feed grains, along with small-scale cattle farming. Forested areas are primarily composed of white oak-red oak woodland and bur-oak mixed woodland occurring along drainages where the slope of the land is too steep for cultivation.⁷

Tick collections. In 2012, tick collections occurred over 3 week-long time periods, April 16–20, June 18–22, and August 6–10. Ticks were collected primarily by flagging and by use of carbon dioxide (CO₂)-baited tick traps, and less frequently by manual removal from domestic animals. Flags were made by securing a reversible summer infant flannel (68.6 cm by 91.4 cm) waterproof pad (Kmart, Hoffman Estates, IL) to bamboo poles or wooden utility handles.⁸ Tick traps were made from 1.89 L plastic food containers with holes added to dissipate CO₂. Tick traps were baited with ~0.5 k of dry ice and placed in the center of an ~0.7 m² piece of white flannel (Jo-Ann Fabric and Craft, Hudson, Ohio) anchored by rocks or sticks for a period of 2–3 hr. With the permission of the owners, ticks were removed from horses and dogs at farms owned by the two case-patients and on one farm not associated with human disease. Ticks were transferred from flags, CO₂-baited traps, and domestic animals into 16 mL glass Wheaton snap cap vials (Fisher Scientific, Pittsburg, PA) with a solid plaster of Paris/charcoal base. The base was moistened with a small amount of water and vials were covered with a piece of latex that was stretched over the mouth of the vial and held in place by the snap lid with the center removed.

*Address correspondence to Harry M. Savage, Centers for Disease Control and Prevention, 3156 Rampart Road, Fort Collins, CO 80521. E-mail: HMS1@cdc.gov

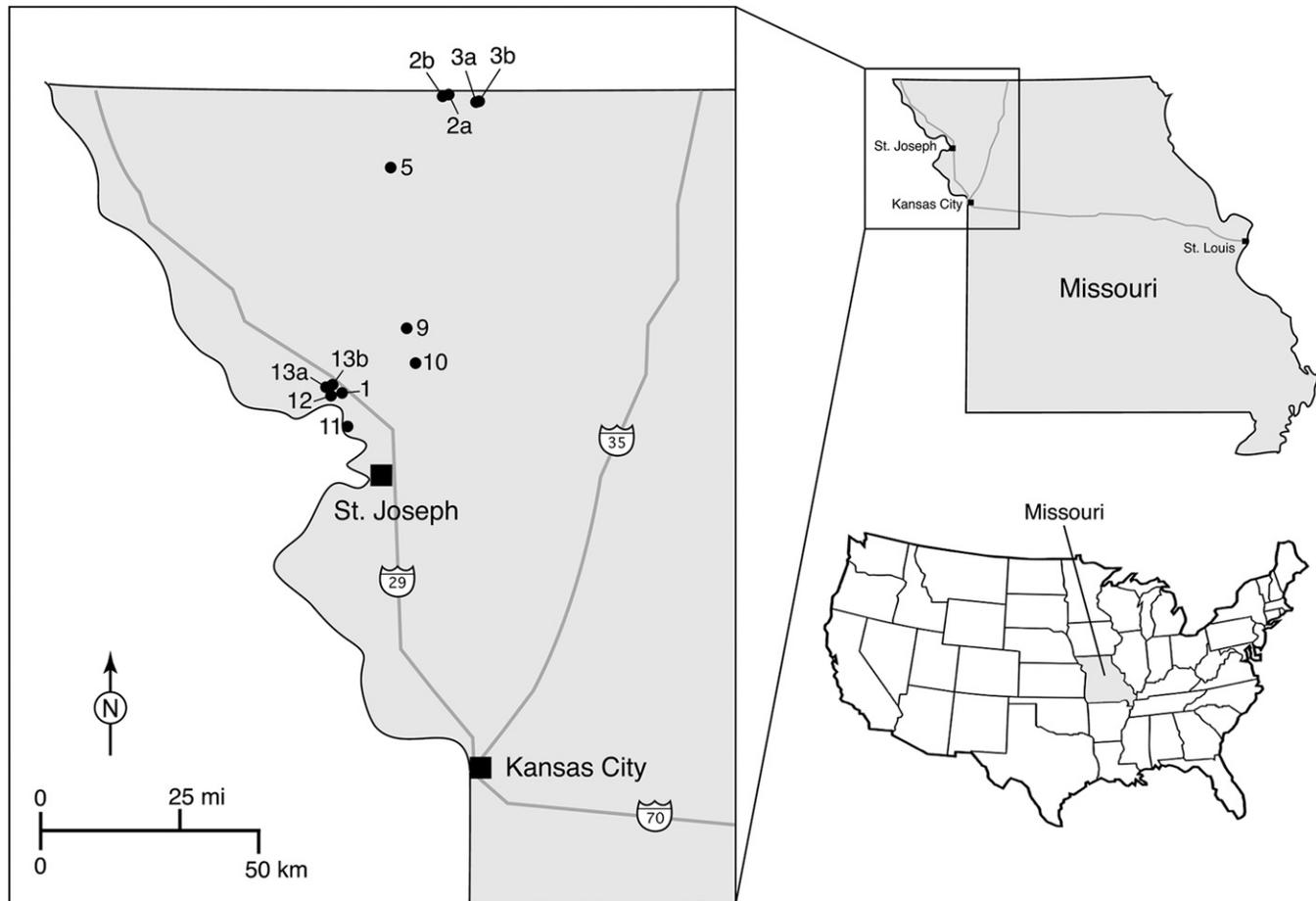


FIGURE 1. Map of northwestern Missouri indicating location of 12 arthropod sampling sites on 9 properties sampled during 2012. See Table 1 for details.

Ticks were inserted through a small slit cut in the latex. Once the collection was completed, or the vial filled, the latex was rapidly replaced with fine mesh to block tick exit and allow ventilation. Vials were placed in Whirl-Pak bags (Daigger, Vernon Hills, IL) with moist cotton balls to maintain humidity, and held in a cooler for transport to the field laboratory. For productive CO₂-baited traps with large numbers of ticks, the cloth was rapidly folded and placed into a large, labeled zip-lock bag for transport to the field laboratory.⁹ Glass field vials and zip lock bags were chilled and ticks transferred to labeled cryotubes.

In August, large numbers of larval ticks were present and collected on flags and occasionally on cloths from CO₂-traps. Larval ticks, when abundant, were removed from flags and cloths using a new method developed by W. L. Nicholson that used a washable sticky lint roller, Sticky Buddy (www.stickybuddy.com). Ticks attached to the roller were washed off with warm water into paper coffee filters supported by plastic coffee filter holders placed over a plastic container to collect water. Ticks moving upward within the coffee filters were washed downward with a water stream from hand-held squirt bottles. The bottom portion of the coffee filter with ticks

TABLE 1
Location of tick sampling sites in northwestern Missouri

Site number	Site name	County	Latitude, °N	Longitude, °W
1	Farm 1	Andrew	NA*	NA
2a	Farm 2a	Nodaway	NA	NA
2b	Farm 2b	Nodaway	NA	NA
3a	Farm 3a	Worth	NA	NA
3b	Farm 3b	Worth	NA	NA
5	Farm 5	Nodaway	NA	NA
9	James D. Christie Conservation Area	Andrew	40.06347	-94.7989
10	Happy Holler Lake Conservation Area	Andrew	39.98789	-94.7678
11	Worthwine Island Conservation Area	Andrew	39.85484	-94.93295
12	Monkey Mountain Conservation Area	Andrew	39.91867	-95.0066
13a	Honey Creek Conservation Area	Andrew	39.94505	-94.98667
13b	Honey Creek Conservation Area	Andrew	39.95394	-94.97101

*NA = detailed locations for private properties are not available, see Figure 1 for general location.

was twisted closed and rapidly cut or torn and placed into labeled cryotubes. Cryotubes from all sources were held on dry ice until shipped to Centers for Disease Control and Prevention (CDC), Fort Collins, CO for processing.

Tick identification and grinding. Ticks were identified to species, sex, and stage using dissecting microscopes on refrigerated chill tables using taxonomic keys.^{10–16} Ticks were grouped into pools by site, collection date, collection type, species, sex, and stage. Maximum pool size for engorged ticks was one, five for deplete adult ticks, 25 for nymphs, and 100 for larvae. Tick pools were homogenized in chilled 2 or 7 mL glass TenBroeck grinders (Fisher Scientific) with alundum bedding material (Fisher Scientific) as an abrasive for 5–10 sec. One mL of chilled bovine albumin-1 (BA-1) solution¹⁷ was added and the ticks ground to completion. The tick homogenates were poured into 2 mL microtubes (Axygen, Union City, CA) and centrifuged at 4°C and 5,013 RCF for 4 min. A 125 µL aliquot of the clarified supernatant was transferred to an identically labeled 1.6 mL microfuge tube for RNA/DNA extraction.

Primer design and selection. For quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR), primers and probes were designed to target the Small (S) segment of the HRTV genome using the Primer Express software package (PE Applied Biosystems, Foster City, CA). Candidate primers and probes were selected by referring to nucleic acid sequence data that were previously derived from two human isolates of HRTV, MO-4 and MO-7.³ Selected primer-probe sets were then evaluated for relative sensitivity by application to RNAs that were extracted from 10-fold dilutions of the MO-4 strain of the virus. Primer-probe sets that demonstrated the highest relative sensitivity for the detection of HRTV were chosen for application to RNAs that were extracted from collected ticks (Table 2).

To screen tick pools for divergent HRTV strains and related viruses, and to allow for result confirmation from more than one amplification platform, broadly reactive screening and confirmatory RT-PCR primers were also designed according to an alignment of the nonstructural protein (NSs) open reading frames (ORFs) of the S segments of HRTV³ and the related Chinese SFTSV HB-29 strain (NC 018137) using the Megalign function of the Lasergene software package (DNASTAR, Madison, WI). Primer sets that target regions of maximum homology were then evaluated by application to RNAs that were extracted from HRTV and SFTSV. Upon gel-based

discrimination, two primer sets that demonstrated the ability to amplify target-sized, virus-specific amplicons from both HRTV and SFTSV RNAs were selected for application in this study (Table 2).

RNA extraction and virus detection. Viral RNA was extracted from a 100-µL aliquot of supernatant taken from each tick pool using the Qiagen QIAmp Virus BioRobot 9604 kit (Qiagen, Inc., Valencia CA) on a Qiagen BioRobot Universal platform according to the manufacturer's protocol. Quantitative real-time RT-PCR was performed by adding 5 µL of each extracted RNA to 50 pmol of each primer and 10 pmol of probe included in HRTV primer-probe set 1 (Table 2) in a 50 µL total reaction using the Qiagen QuantiTect Probe RT-PCR kit (Qiagen) according to the manufacturer's instructions. Reactions were conducted on Bio-Rad's CFX96 Touch real-time PCR Detection System (Bio-Rad, Hercules, CA) using the following cycling conditions: 1 cycle of 50°C for 30 min, 1 cycle of 95°C for 10 min, and 45 cycles of 95°C for 15 sec and 60°C for 1 min. Positive pools were confirmed by re-extracting RNA from the original tick homogenate and performing the quantitative real-time RT-PCR assay with two primer-probe sets, primer-probe sets 1 and 4 (Table 2), using reaction conditions that were described previously in independent reactions.

All tick pools were also assayed with broadly reactive screening primers designed as described previously (Table 2), using the standard RT-PCR protocol described below to detect related viruses or divergent HRTV strains.

To generate nucleotide sequence data for phylogenetic analyses, traditional RT-PCR amplification was conducted on positive pools through the application of confirmatory RT-PCR primers (Table 2). Fifty pmol of the forward and reverse primer (Table 2) and 5 µL of RNA were added to a 50 µL total reaction volume using the Qiagen One Step RT-PCR Kit (Qiagen) according to manufacturer's recommendations. Reactions were conducted using the following cycling conditions: 1 cycle of 50°C for 30 min and 95°C for 15 min, followed by 55 cycles of 94°C for 30 sec, 50°C for 1 min and 72°C for 2 min. Reactions were terminated with a final extension step of 72°C for 10 min. Five µL of product from each reaction was then evaluated by electrophoresis in a 2% agarose gel in 40 mM Tris-acetate-1 mM EDTA buffer. The DNA bands were visualized by ethidium bromide staining and UV trans-illumination. A preliminary positive result was

TABLE 2

Oligonucleotide primers and probes used in Heartland virus (HRTV) detection, screening, and confirmation and sequencing of virus from arthropod samples*

Genomic target	Primer name	Genome position	Sequence (5'-3')	Amplicon size (bp)
HRTV Small (S) segment	HRTV1-forward	361	TGCAGGCTGCTCATTTATTC	86
	HRTV1-reverse	427	CCTGTGGAAGAAACCTCTCC	
	HRTV1-probe†	399	CCTGACCTGTCTCGACTGCCCA	
HRTV S segment	HRTV4-forward	1186	CCTTTGGTCCACATTGATTG	107
	HRTV4-reverse	1273	CACTGATCCACAGGCAGAT	
	HRTV4-probe†	1222	TGGATGCCTATTCCCTTTGGCAA	
HRTV and SFTSV‡ S segments	PhleboScrF	547	GTGGCTCTCCTCCAAATTGAGTCACTT	310
	PhleboScrR§	857	CCATCTGGGGAAAGGACTGGCCAGC	
HRTV and SFTSV S segments	PhleboConF	187	TTGGCAATCCCAACAATCCTCTACA	670
	PhleboConR	857	CCATCTGGGGAAAGGACTGGCCAGC	

*Real-time reverse transcription-polymerase chain reaction (RT-PCR) assay primer/probe sets HRTV1 and HRTV4 (upper two), standard RT-PCR screening (Scr) primers (third), and standard RT-PCR confirmatory (Con) and sequencing primers (bottom).

†Real-time probes were labeled with the FAM reporter dye at the 5' end and Black Hole Quencher 1 dye at the 3' end

‡Severe fever with thrombocytopenia syndrome virus.

§Reverse primers PhleboScrR and PhleboConR are identical.

determined by the presence of a target-sized DNA band on the gel. DNAs were extracted from 670 bp target-sized bands using the Qiaquick Gel Extraction Kit (Qiagen). To verify the identity of amplified cDNAs, 3.2 pmol of the forward and reverse confirmatory primers (Table 2) were added in independent reactions along with 50 ng of purified DNA and ABI BigDye Terminator V3.1 ready reaction cycle sequencing mix (Applied Biosystems, Carlsbad, CA) in a total volume of 20 μ L/reaction. Sequencing reactions were conducted using recommended cycling conditions (Applied Biosystems). Nucleotide sequences were determined by running purified sequencing reactions on the ABI 3130 genetic analyzer (Applied Biosystems). Using these methods, multiple nucleotide sequences were generated for each positive tick pool, as well as for the isolates derived from these pools. Generated nucleotide sequences were confirmed by the re-extraction of RNAs from tick homogenates, followed by re-amplification of cDNAs and the generation of additional nucleotide sequences using methods described previously.

Analyses of nucleotide sequence data from ticks and comparison to human isolates. Multiple nucleotide sequences were aligned in both the 5' and 3' directions for each positive tick pool or isolate using SeqMan software (DNASTAR). Following editing, the resultant consensus sequences were then reviewed for intact ORFs and subjected to National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) analyses to verify the identity of the cDNAs at the genus level using the EditSeq function of Lasergene software (DNASTAR, <http://blast.ncbi.nlm.nih.gov>).¹⁸ Alignments were conducted using ClustalW (MEGA 5). Phylogenetic analyses were then conducted on the tick-derived partial NSs ORFs along with those of other diverse phleboviruses using MEGA 5 software (Figure 2).¹⁹ Evolutionary histories were inferred using both minimum evolution (ME) and maximum likelihood (ML) methods with 2,000 replicates for bootstrap testing of each grouping.^{19–23} Trees generated by both methods displayed nearly identical topologies with

comparable bootstrap values for groupings therefore, the ML tree is displayed here (Figure 2).

Plaque assays to detect viable virus. Tick homogenates from RT-PCR positive samples were tested for the presence of viable virus using VeroE6 cell-culture plaque assay in six-well plates similar to a previously published protocol²⁴; for each RT-PCR-positive pool, two wells were inoculated, each with 100 μ L of clarified supernatant. The second overlay with neutral red was applied on Day 5 post-infection. Wells were inspected and plaques counted on Days 6–8 post-infection. On Day 8 post-infection, positive wells were harvested in 1 mL BA-1 supplemented with 20% Fetal Bovine Serum.

Mosquito collections, identification, and testing. Mosquitoes were collected with CDC-light traps baited with dry ice from August 6 to 10, 2012. Multiple traps were placed at four sites including both case-patient farms (sites 1, 2a, 2b) and one site located on Honey Creek Conservation Area, 13b (Table 1, Figure 1). Mosquitoes were anesthetized with dry ice each morning, placed in labeled cryotubes and held on dry ice until shipped to CDC for processing.

Mosquitoes were identified on refrigerated chill tables using a dissecting microscope and a standard reference²⁵ and pooled by species, sex, and site. One copper-coated BB and 1.75 mL BA-1 were added to each pool in a 2.0 mL snap-cap tube and homogenized using a Qiagen Mixer Mill MM 300 for 4 min at 25-cycles/sec. After centrifugation for 3 min at 5,013 RCF, an aliquot of supernatant from each pool was transferred to a 1.7 mL microcentrifuge tube. The RNA was extracted from each aliquot in a 96-well format using the Qiagen BioRobot Universal, eluted in 100 μ L of elution buffer and tested for HRTV by real-time RT-PCR and with screening primers by standard RT-PCR as described previously for the tick pools (Table 2). In addition, mosquito pools were screened by standard RT-PCR for flaviviruses using NS5 gene primers FU2 and cFD3,²⁶ alphaviruses (Lanciotti R, CDC, unpublished primers), and viruses of the genus *Orthobunyavirus* using the multiplex primers and thermocycler

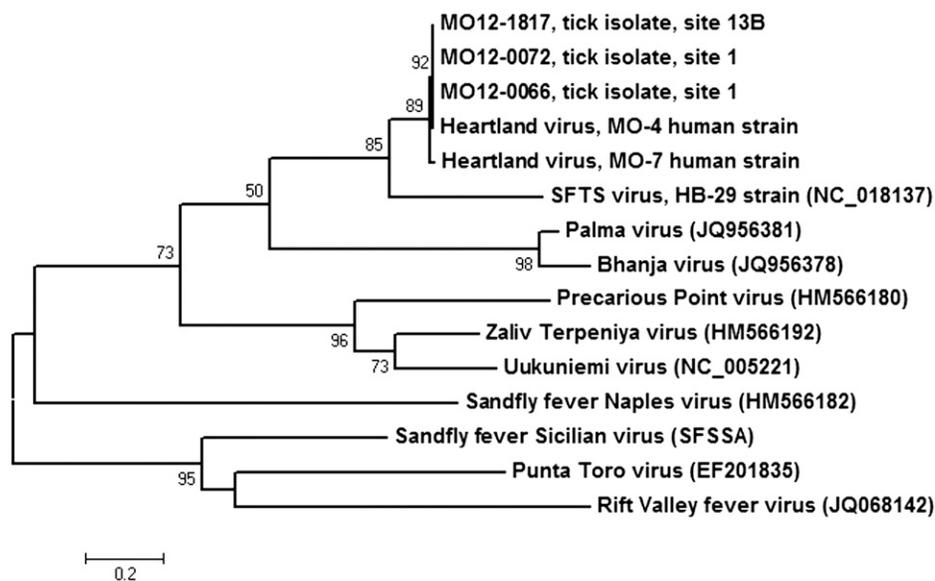


FIGURE 2. Unrooted phylogenetic tree based upon partial nonstructural protein (NSs) open reading frame of the Small (S) segment nucleotide sequences (~670 bp) for representative viruses of the genus *Phlebovirus* inferred by the maximum likelihood (ML) method. Tree is drawn to scale with branch lengths representing the number of nucleotide substitutions per site. Bootstrap values expressed as percentage of 2,000 replicates. GenBank accession numbers for non-Heartland virus strains appear next to virus names.

TABLE 3
Number of collections per site and number of ticks collected by site, species, and stage

Site	Number of collections†	<i>Amblyomma americanum</i>			<i>Dermacentor variabilis</i>			<i>Ixodes dentatus</i> *	Total
		Adults	Nymphs	Larvae	Adults	Nymphs	Larvae		
1	15 [3]	1,385	4,617	19,952	626	6	0	2	26,588
2a	9 [2]	67	262	971	371	5	0		1,676
2b	2 [2]	1	178	6,132	1	0	0		6,312
3a	1	13	6	0	38	0	0		57
3b	2	171	487	0	114	0	6	5	783
5	6	237	471	0	177	1	0		886
9	2	57	147	0	23	0	0		227
10	1 [1]	7	87	8,644	19	0	0		8,757
11	1	1	5	0	0	0	0		6
12	2	36	286	0	2	0	0		324
13a	2 [1]	10	16	3,139	0	0	0		3,165
13b	1 [1]	2	709	6,922	14	0	0		7,647
Total	44	1,987	7,271	45,760	1,385	12	6	7	56,428

* All stages combined, see text.

† Total number of collections [numbers of collections in August].

protocol previously described.²⁷ All standard RT-PCR products were subjected to electrophoresis in 1.2% ethidium bromide-stained pre-cast agarose gels (Invitrogen E-Gel Pre-cast Agarose Electrophoresis System) and evaluated for the presence of appropriately sized bands.

RESULTS

Tick collections and abundance. The 56,428 ticks representing three species were collected at 12 sites on nine properties (Figure 1, Table 1). *Amblyomma americanum* (L.) and *Dermacentor variabilis* (Say) accounted for nearly all ticks collected (99.99%), and the only other species identified was *Ixodes dentatus* Marx (Table 3). Tick collection effort was not standardized across sites; instead it focused on two farms associated with human illness, sites 1–2 (Table 3). However, the overall number of ticks collected at a site corresponds largely to the collection effort made during the August sampling period when larval *A. americanum* ticks were very abundant (Table 3).

Amblyomma americanum was by far the most frequently encountered tick and represented 97.5% (55,018 of 56,428) of collected ticks. Adults, nymphs, and larvae of *A. americanum* were collected during each of the three collection intervals; however, the relative abundance of each stage varied temporally. Adults of *A. americanum* were most abundant in April (1300, 65.43%) and June (677, 34.07%) collections, but were rare in August collections (10, 0.50%). The number of nymphs collected also decreased from April to August, but

more gradually than observed for adults, with 3,400 (46.76%) collected in April, 2,326 (31.99%) in June, and 1,545 (21.25%) in August. In contrast, larvae of *A. americanum* were very rare to sporadic in April (10, 0.02%) and June (275, 0.60%) collections, but extremely abundant in August collections (45,475, 99.38%).

Dermacentor variabilis (Say) was the second most frequently encountered tick representing 2.5% (1,403 of 56,428) of collected ticks. Nearly all *D. variabilis* specimens collected were adults (1,385 of 1,403) and our collection methods failed to detect significant numbers of nymphs and larvae of this species. Adult *D. variabilis* collections were greatest in June (905, 65.34%), moderate in April (430, 31.05%), and lowest in August (50, 3.61%). Nymphs were encountered at low numbers in all three collections, but were also most abundant in June (7, 58.33%). Larvae of *D. variabilis* were collected only in April at site 3B (Table 3).

Virus testing of tick pools and infection rate (IR). All 56,428 ticks were processed, and tested for virus in 2,113 pools. Ten pools were real-time RT-PCR positive for HRTV using two primer sets, and with standard RT-PCR screening primers (Tables 2 and 4). Eight of the 10 positive pools yielded viable viruses in VeroE6 plaque assays. All pools yielding viable virus were collected from Farm 1 in April (Table 4).

All RT-PCR positive pools were composed of nymphs of *Amblyomma americanum* collected at two nearby sites, site 1 and Honey Creek Conservation Area, site 13b (Table 1, Figure 1). These two sites are separated by a distance of ~3.09 km. Nine positive pools were collected at site 1, eight in

TABLE 4

Information on Heartland virus positive tick pools detected and confirmed by reverse transcription-polymerase chain reaction (RT-PCR) in 2012

Pool number	Site	Species	Stage	Number specimens	Collection date	Collection method	GenBank accession*
MO-2012-65	1	<i>A. americanum</i>	nymph	20	April 18	flag	KC466555
MO-2012-66	1	<i>A. americanum</i>	nymph	20	April 18	flag	KC466556
MO-2012-67	1	<i>A. americanum</i>	nymph	20	April 18	flag	KC466557
MO-2012-71	1	<i>A. americanum</i>	nymph	25	April 18	flag	KC466558
MO-2012-72	1	<i>A. americanum</i>	nymph	25	April 18	flag	KC466559
MO-2012-73	1	<i>A. americanum</i>	nymph	25	April 18	flag	KC466560
MO-2012-74	1	<i>A. americanum</i>	nymph	25	April 18	flag	KC466561
MO-2012-75	1	<i>A. americanum</i>	nymph	25	April 18	flag	KC466562
MO-2012-1096	1	<i>A. americanum</i>	nymph	16	June 20	CO ₂ -trap	No data†
MO-2012-1817	13b	<i>A. americanum</i>	nymph	25	August 7	flag	KC466563

* ~670 bp region of the NSs ORF of the S segment.

† No sequence data obtained from sample.

April and one in June. The single positive pool collected at site 13b was composed of nymphs collected in August. The maximum likelihood estimate (MLE)²⁸ of the infection rate (IR) in nymphs of *A. americanum* from site 1 was 1.99 (95% confidence interval [CI] = 0.97–3.64) per 1,000 during 2012, 3.91 (1.83–7.43) for April collections and 0.47 (0.03–2.30) for June collections. The MLE of the IR in nymphs of *A. americanum* from site 13b in August was 1.41 (0.08–6.87).

Testing of 1,385 adults (IR = 0, 95% CI = 0.00–2.75) and 19,952 larvae (IR = 0, 95% CI = 0.00–0.19) of *A. americanum* from site 1, and testing of 2 adults and 6,922 larvae (IR = 0, 95% CI = 0.00–0.54) of *A. americanum* from site 13b produced negative results. Ninety-five percent CI for difference of proportions²⁸ between the IR for nymphs and larvae conducted separately for site 1 (95% CI = 0.58–6.61) and site 13b (95% CI = 0.13–20.16) show that neither CI includes zero, indicating that IR in nymphs and larvae at both sites are significantly different. The CI data on larvae are presented with the caveat that larvae are often collected in clumps that represent the remnants of egg batches, and therefore larvae are not independent random samples.

Sequencing and phylogenetic analysis. Nine of the 10 RT-PCR positive tick pools yielded sequence for a ~670 bp region of the NSs of the S segment and sequences were deposited in GenBank (Table 4). Positive identification of all nine tick isolates as HRTV was supported by an overall 97.6% or greater nucleotide sequence identity shared among the tick isolates and both human strains, MO-4 (Site 1) and MO-7 (Site 2). All tick-associated sequences, which were obtained from site 1 and nearby site 13b, are slightly more similar to MO-4 than MO-7 and share a 99.2% or greater nucleotide sequence identity with MO-4. This high degree of similarity is also reflected at the amino acid level with 99.5% or greater amino acid sequence identity shared among tick strains and MO-4 in the amplified region. All tick isolates and MO-4 share a Proline at position 207 within the amino acid sequence of the NSs ORF, whereas MO-7 displays a Serine at this position. Site 1 and Site 2 are separated by a distance of ~74.27 km. Six genotypes were represented among the nine tick nucleotide sequences. Tick strain MO-12-1817, obtained from ticks collected at site 13b, possessed a unique but silent nucleotide change (T for C) relative to all other tick and human sequences at position 261 within the nucleotide sequence of the NSs ORF.

The ML tree (Figure 2) complements the previous analyses with strong bootstrap support for the lineage containing the tick isolates and MO-4 (92%), and the lineage containing all known HRTV isolates (89%). Sequences from tick isolates obtained from April collections at site 1 are very similar; therefore, only two representative tick-derived sequences from April were included in the phylogenetic analysis. The SFTSV appears as the sister group to HRTV with 85% bootstrap support. The HRTV-SFTSV lineage is joined subsequently by other tick-borne viruses, Palma, Bhanja, Precarious Point, Zaliv Terpeniya, and Uukuniemi. Viruses vectored by mosquitoes (Rift Valley fever virus) and sandflies are located more distantly from HRTV and SFTSV in the tree.

Mosquito collections. Mosquito collections from 54 trap-nights, conducted from August 6 to 10, 2012, yielded only 758 specimens representing 12 species. The species and number collected/tested were: *Culex erraticus* (Dyar and Knab), 581; *Anopheles quadrimaculatus* Say, 52; *Aedes vexans* (Meigen),

38; *Cx. tarsalis* (Newstead), 24; *Ae. triseriatus* (Say), 20; *An. punctipennis* (Say), 20; *Cx. salinarius* Coquillett, 11; *Ae. albopictus* (Skuse), 4; *Cx. restuans* Theobald, 3; *Ae. atropalpus* (Coquillett), 2; *Ae. japonicus japonicus* (Theobald), 2; and *Coquillettia pertubans* (Walker), 1.

Testing for HRTV, flaviviruses, alphaviruses, and the genus *Orthobunyavirus* of Bunyaviridae failed to detect viral RNA in mosquitoes.

DISCUSSION

We collected ticks and mosquitoes on farms owned by the two case-patients who had previously been hospitalized because of infection with HRTV, a newly described *Phlebovirus*. Our goal was to incriminate potential arthropod vectors for this newly described human pathogen. Additionally, we sampled similar farms not associated with HRTV disease and public property in northwestern Missouri to determine the geographic distribution of the virus and its prevalence in potential vector species.

Amblyomma americanum and *D. variabilis* were the two most frequently encountered ticks on case-patient properties and represented nearly all ticks collected from 12 sites in northwestern Missouri. Our tick species and abundance data are similar to those reported from previous tick surveys from southern areas of Missouri.^{29–31}

Ten pools composed of nymphs of *A. americanum* were RT-PCR positive for HRTV, nine produced sequence information on a ~670 bp region of the NSs protein of the S segment, and eight yielded viable virus.

Testing of other life-history stages and species, including 1,987 *A. americanum* adults, 45,760 *A. americanum* larvae and 1,385 adults of *D. variabilis* resulted in negative results. All virus-positive pools were composed of deplete host-seeking nymphs of *A. americanum* and nine of the 10 positive pools were collected on the property of a case-patient. The detection of virus in questing nymphs suggests that *A. americanum* becomes infected with virus by feeding on viremic hosts during the larval stage in the late summer and fall, and that infected larvae apparently transmit the virus transstadially to the nymphal stage. In areas such as Missouri where warm fall temperatures allow for transformation³² HRTV overwinters in deplete nymphs. Transmission of virus from infected nymphs to humans is thought to occur during the spring and early summer when nymphs are abundant, and actively host-seeking. Both case-patients became ill in early-to-mid June after bites by unidentified ticks of a size compatible with nymphs. In a separate study across Missouri, nymphs of *A. americanum* were the most abundant tick collected.²⁹ Although additional field work and laboratory studies are needed to better understand the transmission cycle, our preliminary data suggest that transstadial transmission of virus from nymphs to adults, and vertical transmission from infected adults to eggs and then larvae are rare.

Based on sequencing of a ~670 bp region of the NSs ORF of the S segment, all tick and human HRTV strains are very closely related to each other with an overall 97.6% or greater nucleotide sequence identity. The tick strains collected at site 1 and nearby site 13b were more similar to the human isolate (MO-4) obtained from a case-patient that resided at site 1 (Figures 1 and 2), than the human strain from site 2 (MO-7). All tick isolates and MO-4 share a Proline at position 207

within the amino acid sequence of the NSs ORF, whereas MO-7 displays a Serine at this position. This amino acid difference among site 1 and 13b strains and the human strain from site 2 could be associated with the geographic distance between sites, ~74.27 km. Future isolations of HRTV from ticks collected at site 2 and other sites in Missouri would allow for an assessment of the relationship between geographic distance and virus differentiation. We did not detect HRTV in ticks collected from site 2. This failure is likely associated with ecological differences that resulted in low numbers of *A. americanum* nymphs being collected at this site and isolated rainfall and high wind events that interfered with collections during the April trip.

Testing of 5,900 mosquitoes in China⁵ and our limited samples from Missouri failed to detect SFTSV or HRTV, respectively. Although previous work on mosquitoes provided negative results, future work on potential insect vectors including phlebotomine sandflies and mosquitoes is merited.

In the phylogenetic analysis, HRTV appears as a sister group to the recently described SFTSV from China (Figure 2). Disease associated with SFTSV infection has been reported from China and Japan. In China, SFTSV is believed to be vectored primarily by the tick *Haemaphysalis longicornis* Neumann based on frequent detection of virus in field collected specimens.⁵ Recently, SFTSV was also detected, but at lower rates, in *Rhipicephalus microplus* (Canestrini) in China.³³ The placement of HRTV as a sister group to SFTSV (Figure 2), and the location of the combined HRTV–SFTSV lineage among a number of tick-borne viruses suggest that HRTV is tick transmitted. Additional data that supports tick transmission to humans includes the observation that both case-patients reported multiple tick bites before development of symptoms and our detection of HRTV in 10 pools of deplete nymphs of *A. americanum*, nine of which were collected on the property of a HRTV case-patient. Future experimental vector competence and field studies on *A. americanum* and other tick species are essential to document if transstadial transmission and/or vertical transmission from infected adult female ticks to eggs and larvae are epidemiologically important. Studies to document the seroprevalence of HRTV among domestic and wild animals, including common hosts of *A. americanum* such as deer (*Odocoileus virginianus* Zimmermann), and turkeys (*Meleagris gallopavo* L.), would lead to the identification of potential vertebrate hosts.

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Authors' addresses: Harry M. Savage, Marvin S. Godsey Jr., Amy Lambert, Nicholas A. Panella, and Kristen L. Burkhalter, Arbovirus Diseases Branch, Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, CO, E-mails: hms1@cdc.gov, mjg9@cdc.gov, ahk7@cdc.gov, nap4@cdc.gov, and ktb3@cdc.gov. Jessica R. Harmon, R. Ryan Lash, and William L. Nicholson, Rickettsial Zoonoses Branch, Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Atlanta, GA, E-mails: jharmon@cdc.gov, rlash@cdc.gov, and wnicolson@cdc.gov. David C. Ashley, Missouri Western State University, St. Joseph, MO, E-mail: ashley@missouriwestern.edu.

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