Focal persistence and phylodynamics of Heartland virus in Georgia

Authors: Stephanie Bellman[1,2,3], Nima Shariatzadeh[3], Tyshawn Ferrell[3,4,5], Audrey Long[2], Leah Aeschleman[2], Ellie Fausett[1,2], Tim Walsh[1,2], Seana Cleary[1,2], Isabella Roeske[2], Erick Ojeda[1], Madison Schwab[3,6], Hannah Dakanay[3], Sam R Telford III[7], Heidi K Goethert[7], Gonzalo Vazquez-Prokopec[2], Anne Piantadosi[3,8*]

Affiliations:

1. Gangarosa Department of Environmental Health, Rollins School of Public Health, Emory University, Atlanta, GA, US
2. Department of Environmental Sciences, Emory University, Atlanta, GA, US
3. Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA, US
4. Department of Microbiology and Immunology, Emory University, Atlanta, GA, US
5. Graduate Program in Biochemistry, Cell, and Developmental Biology, Emory University, Atlanta, GA, US
6. Graduate Program in Population Biology, Ecology, and Evolution, Emory University, Atlanta, GA, US
7. Department of Infectious Disease and Global Health, Cumming School of Veterinary Medicine, Tufts University, Grafton, MA, US
8. Department of Medicine, Division of Infectious Diseases, Emory University, Atlanta, GA, US

* Corresponding author contact:

Anne Piantadosi MD, PhD
Department of Pathology and Laboratory Medicine
Emory University School of Medicine
101 Woodruff Circle
Atlanta, GA 30322
404-712-9005
anne.piantadosi@emory.edu

Abstract

Heartland virus (HRTV) is an emerging tick-bone virus associated with severe illness in the U.S. There are large gaps in knowledge of HRTV diversity, evolution, and transmission due to a paucity of HRTV-positive samples and genome sequences. We identified a focal site of HRTV-positive *Amblyomma americanum* ticks in central Georgia and developed a novel multiplex-amplicon sequencing assay to generate full HRTV genome sequences. By screening over 21,000 field-collected ticks from 2021-2023, we identified six positive pools. Five were collected from the site in central Georgia where our group first detected HRTV-positive ticks in 2019, and one from a site in western Georgia approximately 175 km away. The HRTV genome sequences from Georgia were highly related, even across this distance and over five years. Reference HRTV genome sequences from across the U.S. were also geographically clustered. Time-scaled phylogenetic analysis suggested recent spread of HRTV in the U.S., with all available sequences sharing a common ancestor within the last 300 years, and sequences from Georgia sharing a common ancestor within the last 40 years. Our observed spatial clustering of HRTV and the high degree of genetic conservation in our persistent focus suggest the importance of small spatial dynamics in HRTV transmission ecology.

Author Summary

Heartland virus (HRTV) was first discovered in humans in 2009 and has since caused over 60 cases of severe and fatal disease in the United States. HRTV is transmitted by the lone star tick, *Amblyomma americanum*, across the Southeast, East coast, and Midwest. Little information is known about how this virus circulates and changes across time and space due to a lack of genetic data. Here, we created a new procedure to generate more genetic sequence data for HRTV and

64 collected over 21,000 ticks to screen for HRTV across three years in Georgia. We generated 6
65 new HRTV sequences and compared them to existing sequences from our group in Georgia, and
66 across the country, finding evidence of regional clustering of HRTV and highly related HRTV
67 across time in Georgia. Our analyses additionally found that this virus was likely introduced to
68 the U.S. in the last 300 years. Our study provides new context and information in understanding
69 the landscape and transmission of HRTV in the U.S.
70

71 **Introduction**

72 In 2009, two farmers in northwestern Missouri presented with symptoms similar to ehrlichiosis
73 and were found to have a novel pathogen, Heartland virus (HRTV).(1) Originally categorized as
74 a *Phlebovirus*, HRTV was recategorized to the genus *Bandavirus* in the family *Phenuiviridae,*
75 order *Bunyavirales* in 2020.(2) HRTV is vector-borne and transmitted by the lone star tick
76 (*Amblyomma americanum*), whose range spans the majority of the eastern half of the United
77 States.(3, 4) To date, HRTV has been associated with over sixty cases of human disease in
78 fourteen U.S. states and has an observed case fatality rate of 5-10%.(5)
79

80 HRTV, like other bunyaviruses, has a segmented negative-sense single-stranded RNA genome
81 with three segments.(1) The L segment encodes the RNA-dependent RNA polymerase (RdRp)
82 and is 6.4 kb long. The M segment encodes two viral glycoproteins (Gn, Gc) and is 3.4 kb long.
83 Lastly, the S segment employs an ambisense strategy to encode the nucleoprotein (Np) and
84 nonstructural protein (NSs) in 1.7 kb. HRTV is most closely related to Dabie bandavirus (DBV)
85 (previously known as Severe Fever and Thrombocytopenia Syndrome virus, SFTSV) which
86 circulates in China, Japan, South Korea, Vietnam and Taiwan.(6) While different genotypes of

87 DBV have been documented across Asia(7) and have been associated with different
88 pathogenicity in humans,(8) very little is known about the genotypic and phenotypic diversity of
89 HRTV. This knowledge gap stems from limited surveillance, low HRTV prevalence in ticks, (9-
90 11) and few detected human cases.(12)
91
92 HRTV has been identified in *A. americanum* populations in eight states across the U.S., spanning
93 the Northeast, Southeast, and Midwest.(13) Prior surveillance studies for HRTV in ticks have
94 been episodic, typically responding to newly discovered human cases, and continuing for one to
95 two years.(9-11, 14) Few studies have continued surveillance beyond two years, and there is very
96 little HRTV genome sequence data available, creating a gap in understanding the enzootic
97 transmission and evolutionary dynamics of HRTV.
98
99 The objective of this study was to begin to fill this knowledge gap through long term surveillance
100 at established HRTV-positive tick sites in central Georgia (GA), where our group initially
101 detected HRTV in 2019.(15) In our prior study, we found a minimum infection rate of 0.46/1000
102 HRTV-positive *A. americanum* ticks at two locations where HRTV seropositivity was previously
103 reported in deer populations,(16) and adjacent to the county where a human case was
104 reported.(12) In the present study, we build upon this by performing extended HRTV
105 surveillance in GA across multiple years. We use a novel multiplex amplicon sequencing
106 approach to generate full HRTV genome sequences and analyze phylogenetic and
107 phylogeographic relationships of HRTV in GA and across the U.S., gaining insight into potential
108 mechanisms of virus transmission and evolution.
109

Results

Six HRTV positive tick pools were detected across central and western Georgia, U.S., from 2021 to 2023

In total, 21,386 ticks were collected across the three year period: 7,687 in 2021, 3,444 in 2022, and 10,255 in 2023 (Table 1). Most (20,959, 98%) were *A. americanum* adults or nymphs. Six pools of *A. americanum* ticks tested positive for Heartland virus: 1 pool of 19 nymphs from 2021, 2 pools of 27 nymphs and 28 nymphs from 2022, and 3 pools of 25 nymphs, 5 adult females, and 25 nymphs in 2023 (Table 2). Five of the six HRTV-positive pools were collected at ST road site (ST) in central GA, and the sixth was collected at Chattahoochee Bend State Park (CB) in 2023 (Figure 1). The minimum infection rates (MIRs) calculated for each site and year were 0.33 per 1000 nymphs at ST in 2021, 1.97 per 1000 nymphs at ST in 2022, 0.59 per 1000 nymphs at ST in 2023, 2.56 per 1000 adults at ST in 2023 and 0.46 per 1000 nymphs at CB in 2023.

Table 1. Species and life stage of ticks collected during field sampling from 2021-2023

Species	Stage	2021	2022	2023
Amblyomma americanum	Adult	1984	600	1109
Amblyomma americanum	Nymph	5584	2708	8974
Dermacentor variabilis	Adult	100	54	19
Amblyomma maculatum	Adult	19	13	14
Ixodes scapularis	Adult	0	66	64
Ixodes species	Nymph	0	0	8
Haemaphysalis longicornis	Nymph	0	3	67
Total		7687	3444	10255

Table 2. Heartland positive pool characteristics

Year	Lab Code	Location	Date Collected	Number of Ticks	Life Stage
2019*	23	KH	4/28/19	5	Adult (Female)
2019*	26	KH	4/28/19	25	Nymph
2019*	504	ST	6/14/19	5	Adult (Male)

2021	404	ST	6/15/21	19	Nymph
2022	100	ST	5/12/22	27	Nymph
2022	187	ST	6/10/22	28	Nymph
2023	56F	ST	5/12/23	25	Nymph
2023	432R	ST	6/16/23	5	Adult (Female)
2023	642T	CB	7/19/23	25	Nymph

All pools consist of *Amblyomma americanum*. Location abbreviations: KH = KH road site, ST = ST road site, CB = Chattahoochee Bend State Park. *2019 HRTV-positive pools were originally published in Romer et al.(15)

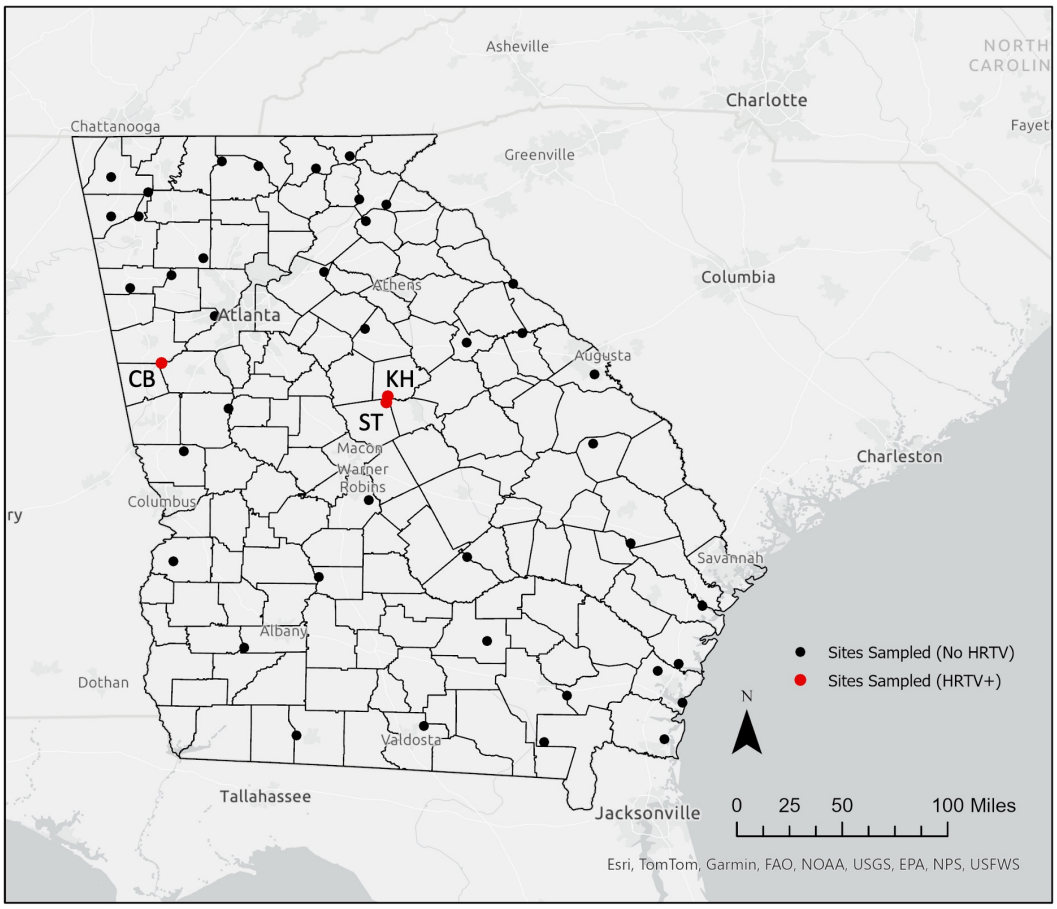


Figure 1. Map of collection sites visited from 2021-2023. State and county boundaries for Georgia are highlighted in black. Black dots represent locations sampled where HRTV positive pools of ticks were not found. Red dots represent locations where HRTV positive pools were found with each location labeled with its two letter site code (KH = KH road site, ST = ST road site, CB = Chattahoochee Bend State Park). Though no positive pools of HRTV were found from 2021-2023, KH is still highlighted due to presence of positive ticks from 2019 detected by our group as originally documented in Romer et al(15) and described in Table 2. Map created in ArcGIS Pro v3.1.0.

141 HRTV sequences from GA are highly related across space and time

142 Full HRTV genome sequences were generated using our novel HRTV-specific multiplex
143 amplicon protocol, with 97-100% coverage achieved across all three segments from the six
144 positive tick pools. Including the three HRTV positive tick pools collected and sequenced by our
145 group in 2019 from sites ST and KH,(15) the nine GA sequences were highly similar with 99.52-
146 99.99% nucleotide identity across the entire genome and similar identity relationships across the
147 individual segments (Table 3). The largest genetic differences were seen between the sample
148 collected at site CB in 2023 (Sample 642T) and other samples from GA, collected approximately
149 175 km away. Sample 642T differed from the other GA sequences by 44-51 single nucleotide
150 polymorphisms (SNPs), while the sequences from sites KH and ST differed from one another by
151 only 1-27 SNPs (Table 3). Few non-synonymous SNPs were found in sample 642T: 4 (of 24
152 total SNPs) in the L segment, 1 (of 13) in the M segment, and none in the S segment.
153 Interestingly, while three of these non-synonymous SNPs were unique to sample 642T, two
154 SNPs in the L segment (T168A and R183K) were shared with the other twelve reference
155 sequences obtained from across the country.

156
157
158
159

Table 3. Pairwise genetic distances between HRTV sequences in Georgia

Location		Site KH		Site ST					
		HRTV-23_T_GA_KH_2019	HRTV-26_T_GA_KH_2019	HRTV-100_T_GA_ST_2022	HRTV-404_T_GA_ST_2021	HRTV-187_T_GA_ST_2022	HRTV-504_T_GA_ST_2019	HRTV-056F_T_GA_ST_2023	HRTV-432R_T_GA_ST_2023
Site KH	HRTV-26_T_GA_KH_2019	3 (99.97%)							
Site ST	HRTV-100_T_GA_ST_2022	10 (99.91%)	7 (99.93%)						
	HRTV-404_T_GA_ST_2021	8 (99.92%)	7 (99.93%)	2 (99.98%)					
	HRTV-187_T_GA_ST_2022	11 (99.9%)	8 (99.92%)	1 (99.99%)	3 (99.97%)				
	HRTV-504_T_GA_ST_2019	23 (99.78%)	22 (99.79%)	23 (99.78%)	21 (99.8%)	24 (99.77%)			
	HRTV-056F_T_GA_ST_2023	11 (99.9%)	10 (99.91%)	7 (99.93%)	7 (99.93%)	8 (99.92%)	26 (99.75%)		
	HRTV-432R_T_GA_ST_2023	12 (99.89%)	11 (99.9%)	8 (99.92%)	10 (99.91%)	9 (99.91%)	27 (99.74%)	7 (99.93%)	
Site CB	HRTV-642T_T_GA_CB_2023	47 (99.56%)	44 (99.58%)	46 (99.56%)	46 (99.56%)	47 (99.56%)	51 (99.52%)	47 (99.56%)	46 (99.56%)

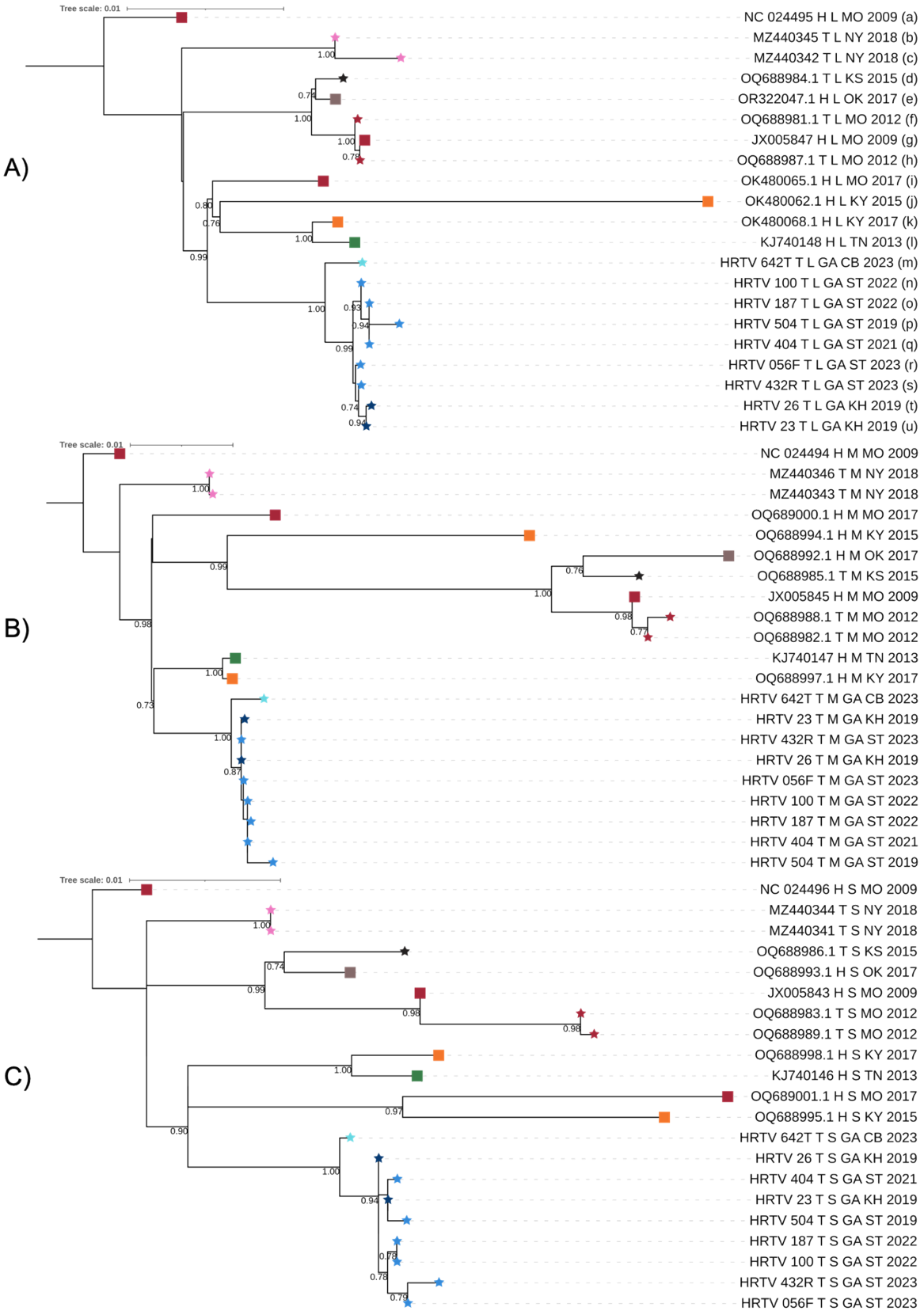
Number of SNPs and percent nucleotide identity is displayed across the concatenated genome of the three segments excluding gaps. Background color gradient represents nucleotide identity with darker colors representing higher nucleotide identity. Sites are delineated by bolded lines.

When comparing all twenty-one HRTV sequences from the U.S., the nucleotide identity ranged from 92.76-99.99% across the three segments, equating to between 1 and 782 SNP differences (Supplemental Table S5). Across the HRTV genome, the GA sequences shared 70 SNPs (35 in L, 20 in M, 12 in S), 6 of which (1 in L, 2 in M, 3 in S) were non-synonymous. While the GA sequences were all derived from tick pools, the twelve available reference sequences from GenBank were from both tick pools (5) and human infections (7). There were no SNPs common to HRTV sequences from only ticks or only humans.

U.S. HRTV sequences demonstrate geographic clustering and potential reassortment

The 21 U.S. HRTV sequences generally clustered geographically on maximum-likelihood phylogenetic trees for each segment (Figure 2). Across all trees, the GA sequences clustered together and the sample collected at western site CB (HRTV 642T) was the most divergent. The two sequences from NY ticks similarly clustered together across all segments. Furthermore, samples from neighboring states generally clustered together (OK/KS/MO and TN/KY) in each segment, regardless of date of sample collection or host. The Mantel test also supported geographic clustering, with a correlation of 0.53 (p-value=0.001) between genetic distance and spatial distance.

While the overall phylogenetic structure was similar across segments, we observed two examples of potential reassortment between segments. First, sequences OQ688994-5, collected from a human case in KY in 2015, clustered with the other KY and TN sequences in the L segment but with the MO, OK, and KS clade in the M segment, and with a single MO sequence in the S segment, all with moderate to high ultrafast bootstrap support (0.76, 0.99, 0.97 respectively). The second example of potential reassortment is from our sample 432R, collected at site ST in 2023. It clusters with the site KH sequences from 2019 (23 and 26) in the L segment, but with the other site ST sequences (187, 100, 056F) in the S segment, with moderate ultrafast bootstrap support (0.74, 0.79). These differences in tree topology may indicate reassortment, as has been reported for the related Dabie bandavirus. RDP4 was also used to assess for reassortment events between the HRTV segments and did not identify any events with high certainty.


A)
Tree scale: 0.01
NC 024495 H L MO 2009 (a)
MZ440345 T L NY 2018 (b)
MZ440342 T L NY 2018 (c)
OQ688984.1 T L KS 2015 (d)
OR322047.1 H L OK 2017 (e)
OQ688981.1 T L MO 2012 (f)
JX005847 H L MO 2009 (g)
OQ688987.1 T L MO 2012 (h)
OK480065.1 H L MO 2017 (i)
OK480062.1 H L KY 2015 (j)
OK480068.1 H L KY 2017 (k)
KJ740148 H L TN 2013 (l)
HRTV 642T T L GA CB 2023 (m)
HRTV 100 T L GA ST 2022 (n)
HRTV 187 T L GA ST 2022 (o)
HRTV 504 T L GA ST 2019 (p)
HRTV 404 T L GA ST 2021 (q)
HRTV 056F T L GA ST 2023 (r)
HRTV 432R T L GA ST 2023 (s)
HRTV 26 T L GA KH 2019 (t)
HRTV 23 T L GA KH 2019 (u)
B)
Tree scale: 0.01
NC 024494 H M MO 2009
MZ440346 T M NY 2018
MZ440343 T M NY 2018
OQ689000.1 H M MO 2017
OQ688994.1 H M KY 2015
OQ688992.1 H M OK 2017
OQ688985.1 T M KS 2015
JX005845 H M MO 2009
OQ688988.1 T M MO 2012
OQ688982.1 T M MO 2012
KJ740147 H M TN 2013
OQ688997.1 H M KY 2017
HRTV 642T T M GA CB 2023
HRTV 23 T M GA KH 2019
HRTV 432R T M GA ST 2023
HRTV 26 T M GA KH 2019
HRTV 056F T M GA ST 2023
HRTV 100 T M GA ST 2022
HRTV 187 T M GA ST 2022
HRTV 404 T M GA ST 2021
HRTV 504 T M GA ST 2019
C)
Tree scale: 0.01
NC 024496 H S MO 2009
MZ440344 T S NY 2018
MZ440341 T S NY 2018
OQ688986.1 T S KS 2015
OQ688993.1 H S OK 2017
JX005843 H S MO 2009
OQ688983.1 T S MO 2012
OQ688989.1 T S MO 2012
OQ688998.1 H S KY 2017
KJ740146 H S TN 2013
OQ689001.1 H S MO 2017
OQ688995.1 H S KY 2015
HRTV 642T T S GA CB 2023
HRTV 26 T S GA KH 2019
HRTV 404 T S GA ST 2021
HRTV 23 T S GA KH 2019
HRTV 504 T S GA ST 2019
HRTV 187 T S GA ST 2022
HRTV 100 T S GA ST 2022
HRTV 432R T S GA ST 2023
HRTV 056F T S GA ST 2023

198

D)

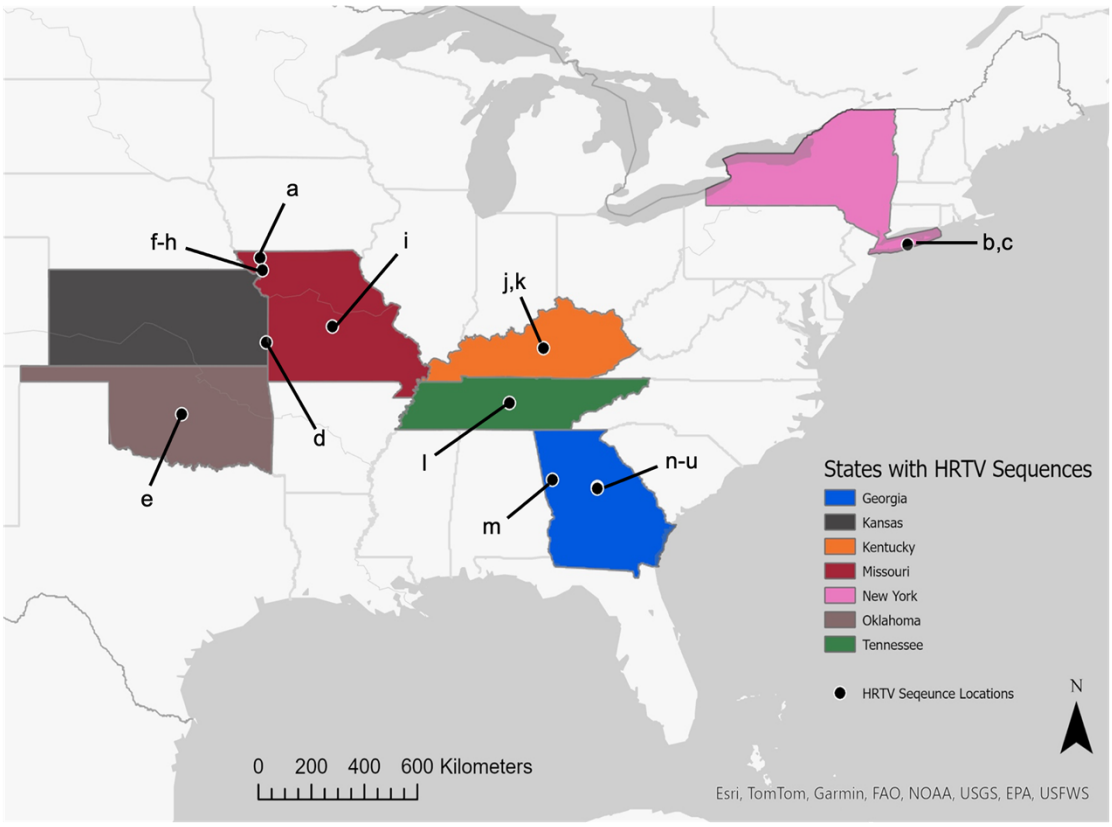


Figure 2: Phylogenetic and geographic relationships of HRTV sequences in the U.S. Segments were aligned separately, and maximum likelihood trees are displayed for the L segment (A), M segment (B), and S segment (C). Reference sequences from GenBank were named by the accession code followed by T (tick sample) or H (human sample), the segment, the state abbreviation for the collection location, and the year of collection. Sequences generated in this study were named by HRTV, sample number, T, GA, collection site, and year collected. All trees were rooted on the reference sequence from MO Patient 1. Ultrafast bootstrap values above 0.7 are displayed on the tree. Tips are labeled with a star for tick samples and square for human samples. The tip color indicates the location of collection. D) A map of the Eastern United States with states colored as in the phylogenetic trees (panels A-C). Locations for each sequence included are marked with a black dot. Dots are placed where either 1) sequences locations were documented in the literature(9, 11, 17) or 2) in the center of the state/county reported when finer resolution location data was unavailable. Dots are additionally marked with a-u and correspond to the sequences in the tree marked in panel A. Map created in ArcGIS Pro v3.1.0.

<u>Contemporary HRTV strains in the U.S. emerged within the last 300 years</u>

Time-scaled Bayesian phylogenetic analysis showed an evolutionary rate of 1.1 $x10^{-4}$ substitutions/site/year (s/s/y) in the L segment and 1.8 $x10^{-4}$ s/s/y in the M segment; this was not

219 performed for the S segment given the lower temporal signal observed. Based on the L segment,
220 the time to most recent common ancestor (tMRCA) for the GA clade was 23 years ago (95%
221 HPD 12-42); the tMRCA for the GA clade and its nearest neighbor, the MO/KY/TN clade, was
222 85 years ago (95% HPD 46-153); and the overall root of the tree was 105 years ago (95% HPD
223 57-187) (Figure 3). Based on the M segment, the tMRCA for the GA clade was 16 years ago
224 (95% HPD 8-29); the tMRCA for the GA clade and its nearest neighbor, the KY/TN clade, was
225 56 years ago (95% HPD 31-99); and the overall root of the tree was 150 years ago (95% HPD
226 78-286) (Figure 3).
227

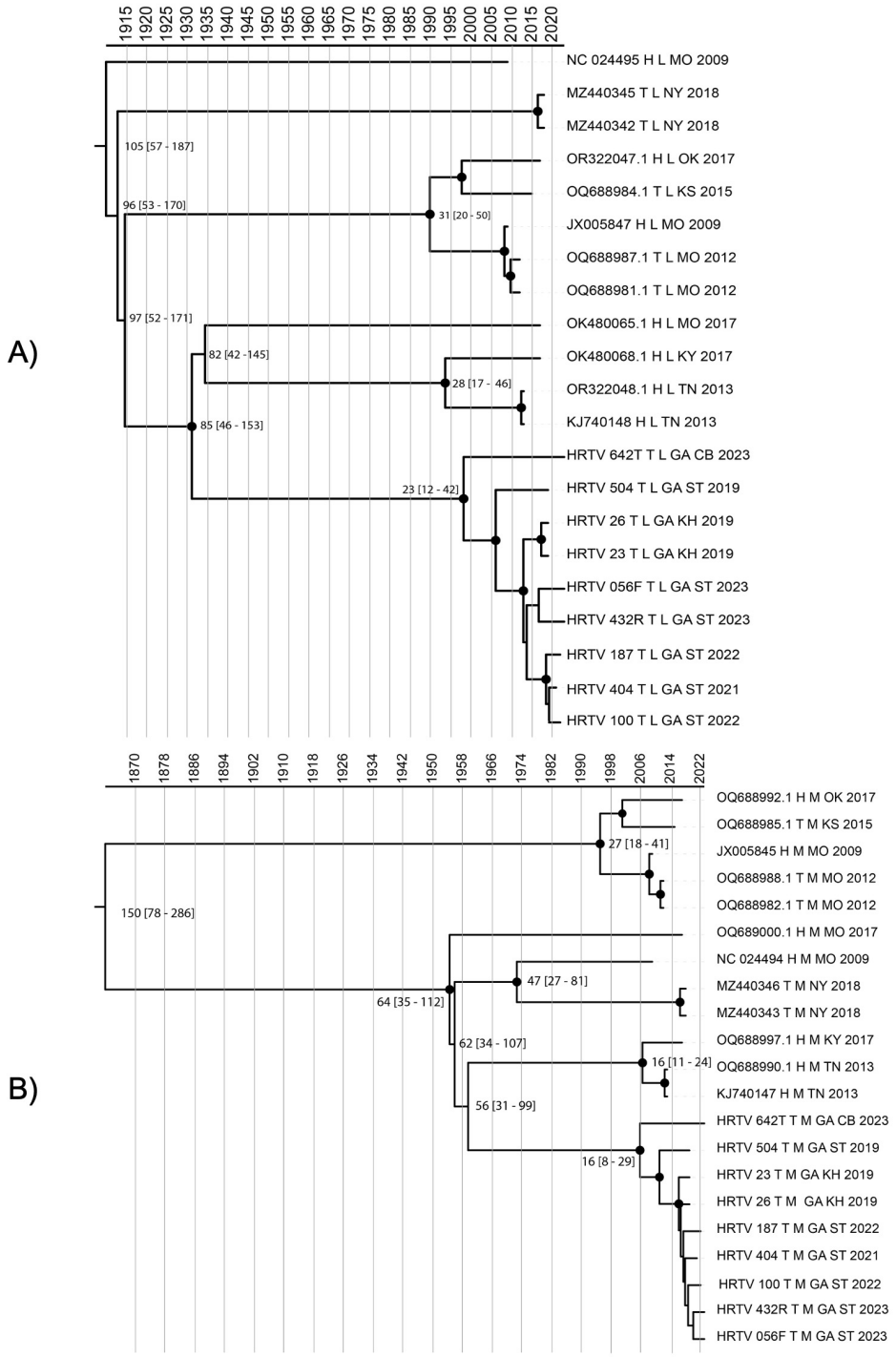


Figure 3: Time-scaled Bayesian phylogenetic tree of HRTV segments L (A) and M (B). Nodes are labeled with median time to recent common ancestor (tMRCA) with [95% HPD].

232 *Amblyomma americanum* ticks feed on a variety of wildlife in Georgia

233 To begin to evaluate potential reservoir species for HRTV, a total of 12 *A. americanum* pools
234 from GA underwent host bloodmeal analysis using retrotransposon qPCR. Each HRTV-positive
235 pool was matched with an HRTV-negative pool with ticks of the same life stage, collection site,
236 and collection date (Table 4) . The HRTV-positive pools of nymphs from ST (samples 404, 187,
237 100, and 56F) tested positive for birds, deer, rabbits, and squirrels/chipmunks. Their matched
238 controls from HRTV-negative pools had similar results, with two exceptions: HRTV-negative
239 pool 403 was positive for rabbit while its matched HRTV-positive pool 404 was not, and HRTV-
240 positive pool 56F was positive for squirrel/chipmunk, while its matched HRTV-negative pool
241 55E was not. Both pools of adult ticks from site ST tested negative for most hosts, other than
242 rabbit in the HRTV-negative pool. Finally, the nymph pools from CB both tested positive for
243 deer, and the HRTV-negative pool additionally tested positive for squirrels/chipmunks.

244

245 **Table 4. Host Bloodmeal PCR results**

Sample Name	HRTV Status	Tick Stage (n)	Location, Year	Rabbit PCR	Bird PCR	Squirrel/ Chipmunk PCR	Deer PCR
403	-	Nymph (25)	ST, 2021	+	+	-	+
404	+	Nymph (19)	ST, 2021	-	+	-	+
186	-	Nymph (27)	ST, 2022	-	-	-	+
187	+	Nymph (28)	ST, 2022	-	-	-	+
99	-	Nymph (26)	ST, 2022	+	+	+	+
100	+	Nymph (27)	ST, 2022	+	+	+	+
55E	-	Nymph (25)	ST, 2023	+	+	-	+
56F	+	Nymph (25)	ST, 2023	+	+	+	+
432R	+	Adult (5)	ST, 2023	-	-	-	-
433S	-	Adult (5)	ST, 2023	+	-	-	-
641S	-	Nymph (25)	CB, 2023	-	-	+	+
642T	+	Nymph (25)	CB, 2023	-	-	-	+

246 The following species with no positive results were not included in the table: mice, voles,
247 shrews, skunks, and opossums.

Discussion

We identified a persistent focus of genetically conserved HRTV in central Georgia. We collected over 21,000 ticks across three years and found minimum infection rates (MIRs) ranging from 0.33 per 1000 nymphs in 2021 to 2.56 per 1000 adults in 2023, compatible with our prior work(15) and other studies.(17, 18) Using a novel multiplex amplicon sequencing assay, we generated six new HRTV genome sequences and examined phylogenetic relationships across multiple years and locations.

One key finding was the very high genetic conservation of HRTV from ticks collected at a focal site in central GA across five years. Studies of Powassan virus, an unrelated tick-borne virus also endemic to the U.S., have similarly reported foci of genetically conserved viruses that were stable across multiple years, but have also reported geographic dispersal of closely-related POWV strains.(19-21) We did not observe geographic dispersal of closely-related HRTV strains, instead we observed phylogenetic clustering by location throughout the U.S., though it is notable that sequence data are limited, with only 12 reference sequences available from elsewhere in the U.S. and our 9 from Georgia.

A second key finding was that time-scaled phylogenetic analysis indicated recent spread of HRTV in the U.S., with all 21 sequences sharing a common ancestor within the last 300 years, and sequences from Georgia sharing a common ancestor within the last 40 years. This timeline is compatible with historical patterns of *A. americanum* prevalence.(22) In the 18th century, dense forest supported high populations of deer and associated ticks, however deforestation and decimation of deer populations in the 19th century led to large-scale *A. americanum* range

272 retraction. The resulting fracturing of tick populations could have generated geographically
273 isolated HRTV lineages that diverged over the last several hundred years. Supporting this, there
274 is evidence for geographic population structure within *A. americanum* ticks between different
275 regions of the U.S.(23)
276
277 Overall, our phylodynamic results for HRTV mirror what has been reported for the closely
278 related Dabie bandavirus (DBV) in Asia. Our estimated evolutionary rate for HRTV, 1.1-1.8
279 $x10^{-4}$ s/s/y, is very similar to the DBV literature, where studies report rates ranging from 1.07
280 $x10^{-4}$ to 1.09 $x10^{-3}$ s/s/y across the three segments, with most estimates around 2 $x10^{-4}$.(24-28)
281 Similar to our results, many DBV studies have shown distinct genotypes clustering by
282 geographic region.(24, 29-31) Interestingly, however, several recent studies also report mixing of
283 DBV genotypes across different countries in Asia,(7, 28) which is thought to be due to migratory
284 bird movement carrying the DBV vector, *Haemaphysalis longicornis*, across longer
285 distances.(32)
286
287 Despite the small number of HRTV sequences, our phylogenetic analysis suggests the possibility
288 of reassortment between genome segments, a common mechanism of increasing genetic diversity
289 in bunyaviruses and other segmented viruses.(33) Reassortment has been frequently described
290 for DBV, allowing new genotypes to emerge after co-infection events.(7, 8, 34, 35) This is
291 important because reassorted viruses have the ability to overcome host barriers and have the
292 potential to generate strains of higher pathogenicity as seen in influenza virus.(33, 36) Because
293 reassortment requires multiple viruses to infect the same cell, it would be expected to occur less
294 frequently in viruses with low prevalence. Thus, our findings are surprising in light of the

295 reported low HRTV prevalences in our study and others, underscoring the need for expanded
296 surveillance and genetic studies of HRTV throughout the U.S.
297
298 Our observation of high HRTV genetic conservation at a focal site across multiple years suggests
299 several possible hypotheses about the enzootic cycle of HRTV, which remains largely
300 unknown.(3) One possibility is that closely related viruses may be sustained within the
301 population through transovarial transmission (also referred to as vertical transmission), as has
302 been documented in experimental studies of HRTV.(37) In this scenario, infected female *A.*
303 *americanum* would lay eggs at the site, passing on virus to their larvae, which would feed on
304 small-range hosts and remain within the site to molt and be captured as nymphs the following
305 year. Vertical transmission typically augments other transmission mechanisms and has not been
306 proposed as a sole mechanism for persistence of other tick-borne viruses.(38) Testing larval
307 populations for HRTV is an important next step in investigating this mechanism.
308
309 A second potential mechanism for HRTV focality involves propagation of virus through
310 cofeeding transmission, which has also been documented experimentally for HRTV(37) and is
311 important in other tick-borne viruses such as tick-borne encephalitis virus (TBEV).(39) In this
312 case, infected *A. americanum* feed next to uninfected ticks, leading to HRTV transmission
313 through infection of local skin and leukocytes. Co-feeding transmission provides a role for hosts
314 that are not competent for viremic infection themselves, as demonstrated for *Borrelia* species
315 that feed on hosts whose innate complement systems prevent systemic infection.(40) Cofeeding
316 on hosts with limited range could explain the persistence of highly related viruses across multiple
317 years.

318 Finally, HRTV could propagate through horizontal transmission by ticks feeding on infected
319 reservoir hosts. Serosurveys and experimental infections investigating potential HRTV reservoir
320 hosts have not yet identified any candidates.(41, 42) Evidence of seropositivity has been found in
321 multiple wildlife species with white-tailed deer, raccoons, horses, and dogs showing the highest
322 rates of neutralizing antibodies across studies.(3, 16, 43) However, experimental infection of
323 multiple wildlife hosts including white-tailed deer, raccoons, goats, chickens, rabbits, hamsters,
324 and C57BL/6 mice have failed to produce detectable viremia, except in an interferon receptor-
325 deficient mouse model.(41) Together, this information suggests that these animals may be
326 incidental hosts, and there is not yet evidence for a viremic reservoir host to support horizontal
327 transmission of HRTV. If there is a role for horizontal or cofeeding transmission in the HRTV
328 enzootic cycle, our HRTV phylogenetic analysis suggests the importance of geographically
329 restricted hosts. Our host bloodmeal analysis provides some possibilities, as *A. americanum* ticks
330 at our focal site in central Georgia fed on deer and rabbits (which do not support HRTV viremia
331 in experimental infection studies), as well as squirrels/chipmunks and birds (which have not been
332 evaluated in experimental infection studies). While we did not observe a clear difference
333 between HRTV-positive and negative samples, this analysis was limited by our analysis of tick
334 pools rather than individual ticks, and it is possible that yet-untested hosts are important.
335
336 In conclusion, through extensive field work and the use of a novel multiplex amplicon
337 sequencing assay, we have gained important insight into HRTV diversity and evolution. Key
338 findings are that HRTV sequences from a focal site in GA were highly related across time; that
339 there is strong geographic clustering of HRTV across the U.S. with this attribute being more
340 important than host or year; and that all available HRTV sequences share a common ancestor

341 within the recent past, suggesting that contemporary strains of HRTV emerged within the last
342 300 years. Our work offers potential insights into the enzootic cycle of HRTV and provides a
343 foundation for further investigation.
344
345
346 **Materials and Methods**

347 Field collections

348 Ticks were collected from March-August 2021-2023, corresponding to the seasonality of
349 *Amblyomma americanum* in Georgia.(44) Collection locations varied by year: in 2021, repeated
350 sampling was conducted at the two sites where our group detected Heartland virus in 2019(15);
351 in 2022, sampling was performed in forty-three locations across the state(45); and in 2023, five
352 locations with the highest density of *A. americanum* from the previous years were repeatedly
353 sampled to maximize potential HRTV detection. Free flagging was conducted at each site
354 according to previously described protocols,(46) with the addition of transect sampling during
355 2022.(45) Ticks were transported live in plastic vials to the laboratory and microscopically
356 identified with taxonomic keys.(47, 48)
357

358 Tick processing, RNA extraction, and qRT-PCR

359 Surface disinfection was conducted on ticks in field vials using a wash of 10% bleach for five
360 minutes, 3% hydrogen peroxide for five minutes, and three rinses of distilled water for five
361 minutes each. The ticks were pooled in groups of approximately five adults or twenty-five
362 nymphs per species per site. Differing amounts of BA-1 diluent (1× medium 199 with Hanks
363 balanced salt solution, 0.05 mole/L Tris buffer [pH 7.6], 1% bovine serum albumin, 0.35 g
364 sodium bicarbonate/L, 100 µg/L streptomycin, 1 µg/mL amphotericin B) were added to each

365 pool based on the number of ticks in the pool (1 ml for >1 adult or >10 nymphs, 0.5 mL for 1
366 adult or <10 nymphs) to preserve concentrations of the ground ticks in media. Pools were ground
367 thoroughly using a 7-mL glass TenBroeck grinder (Fisher Scientific) with alundum bedding
368 material (Fisher Scientific) as an abrasive.
369
370 Tick homogenates underwent RNA extraction (QIAmp TNA Extraction Kit) following the
371 manufacturer's protocol (Supplemental Table S1). Extracted RNA was tested using quantitative
372 reverse-transcriptase PCR (qRT-PCR) with primer-probe set 1 for the S segment of HRTV
373 described by Savage et al.(10) The AgPath-ID One-Step RT-PCR kit (Thermo Fisher Scientific)
374 was used as per the manufacturer's instructions, with cycling conditions of 45°C for 10 minutes,
375 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 60°C for 45 seconds. The reaction
376 mix and primer and probe sequences are available in Supplemental Table S2. Samples were
377 tested in duplicate with positive controls (extracted HRTV virus stock from Romer et al.(15))
378 and negative controls (water) on each plate. As a quality control check for RNA extraction, tick
379 actin RT-qPCR was run in parallel using the Applied Biosystems Power SYBR Green RNA-to-
380 Ct 1-step PCR Kit (Fisher Scientific) with primers, probes, and reaction conditions listed in
381 Supplemental Table S3.
382
383 <u>Infection rate estimation</u>
384 The minimum infection rate (MIR) of HRTV was estimated by site and year by dividing the
385 number of positive pools at a location by the number of *A. americanum* of the same life stage
386 collected at the site and year and multiplying by 1,000.
387

388 HRTV multiplex amplicon sequencing primer design

389 Multiplex amplicon sequencing primers for HRTV were designed with primalscheme
390 (v1.4.1)(49) using an alignment of 4 (L) or 5 (M and S) complete reference HRTV
391 sequences.(50) Primers that had high annealing temperatures (65°C or greater) or had SNPs
392 expected to affect primer binding, as determined by comparison to additional partial genome
393 sequences, were redesigned in Geneious Prime (v2022.2). A set of 36 L, 18 M, and 10 S primer
394 pairs were created to cover the entire genome, with each primer pair amplifying an
395 approximately 250bp region (Supplemental Table S4). The HRTV-specific primer sequences
396 were concatenated with a universal Nextera adapter sequence to allow Nextera XT indexing and
397 amplification.(51, 52) Primers were synthesized by IDT, pooled, and tested using a previously-
398 sequenced HRTV-positive sample.(15) Primer concentrations were optimized to ensure
399 approximately equal coverage across the entire genome.

400

401 HRTV full genome multiplex amplicon sequencing

402 RNA samples that tested positive for HRTV via RT-qPCR underwent heat-labile dsDNase
403 treatment (ArticZymes), single-stranded cDNA synthesis (Fisher/Invitrogen) and multiplex PCR
404 using Q5 Hot Start High-Fidelity Polymerase (New England Biolabs) using the custom-designed
405 primers. The resulting amplicons were indexed using Nextera XT (Illumina) and quantified using
406 the KAPA Universal Complete Kit (Roche). Libraries were pooled to equimolar concentration
407 and sequenced with 300bp paired-end reads on an Illumina MiSeq. Reads were filtered for
408 quality, and adaptor sequences were removed, before undergoing reference-based assembly
409 using ViralRecon v. 2.6.0 with reference sequences L: NC_024495, M: NC_024494, and S:

410 NC_024496. A more complete protocol for this sequencing method can be found in
411 Supplemental Text S1.
412
413 Phylogenetic analysis
414 Twelve reference sequences of Heartland virus L, M, and S segments present in GenBank as of
415 March 1st, 2024 were downloaded for analysis. Selection criteria included: related to a field
416 collected case (tick or human), no identical isolates (if an isolate was sequenced more than once,
417 the earliest was selected), and >95% genome coverage. Newly-generated sequences and
418 reference sequences were aligned by segment in Geneious Prime (v2022.2.2) using MAFFT
419 (v7.450) with default parameters.(53, 54) Maximum-likelihood phylogenetic trees were
420 generated for each segment using PhyML 3.0,(55, 56) with best fit substitution models: GTR+G
421 for the L segment, HKY85+G for the M segment, and K80+I for the S segment. Trees were
422 subsequently visualized and annotated using iTOL(57). Reassortment was assessed through two
423 methods 1) by visually comparing segment relationships across different clades within the trees
424 and 2) by testing aligned concatenated coding regions with the recombination detection program,
425 RDP4.(58)
426
427 Bayesian time-scaled phylogenetic analysis
428 To construct time-scaled phylogenies, the temporal signal of each segment's coding region was
429 evaluated using TempEST(59), and one outlier was removed using a threshold of 0.03 with
430 unbalanced residuals on the residual histogram distribution (reference sequence OK480062.1,
431 Supplemental Figure S1). There was evidence for moderate temporal signal that varied by
432 segment, with root-to-tip correlation coefficients of 0.59 for the L segment, 0.55 for the M

433 segment, and 0.33 for the S segment. Time-scaled phylogenies were therefore constructed only
434 for the L and M segments, using BEAST 2.7.6 with a chain length of 600 million.(60) We used a
435 GTR+gamma nucleotide substitution model, a constant coalescent population, and a strict
436 molecular clock with a prior of 2E-4,(25, 26, 28) with a lognormal distribution. The log files
437 were examined with Tracer v1.7.2 and confirmed to have ESS values >200 for all
438 parameters.(61) Maximum clade credibility trees were generated with 10% burn-in using
439 TreeAnnotator v2.7.6.(60) More complex models, including a relaxed molecular clock and
440 skyline models of population growth, did not converge, likely as a result of the limited HRTV
441 data available.
442
443 Geographic clustering analysis
444 The Mantel test(62) was used to assess the relationship between geographic distance and genetic
445 divergence. Because exact coordinates were not known for the reference sequences, the location
446 was estimated using the finest resolution information present. For samples with only a state
447 recorded for the source location in GenBank, the center point of the most central county in the
448 state was assigned, using USGS(63) and Latitude.to (v1.64-im). For samples with a county and a
449 state recorded for the source location in GenBank, the center of the county listed for that
450 sequence was assigned. For sequences associated with publications, the smallest resolution
451 estimate was made based on recorded location information.(9, 11, 17) Lastly, for the two initial
452 Missouri human case sequences, Andrews county and Nodaway county centers were used based
453 on Savage et al.(17) The Mantel test was run using the vegan package (v2.5-7) in R (v4.0.4)
454 using 1000 permutations.
455

456 Host bloodmeal analysis

457 Nucleic acid extracted from HRTV-positive *A. americanum* pools and matched HRTV-negative
458 *A. americanum* pools – containing a similar number of ticks, and the same life stage, collection
459 site, and collection date – were tested by multiplex qPCR targeting retrotransposons of potential
460 hosts, according to established protocols.(64, 65) Species included in the assay were: mice,
461 voles, rabbits, birds, shrews, squirrels/chipmunks, deer, skunks and opossums.

462
463

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653 Author Contributions

654 SB, GVP, and AP conceived of the study. Field collections were conducted by SB, NS, AL, LA,

655 EF, TW, SC, IR, and MS. Lab processing, amplicon sequencing design and sequencing was

656 completed by SB, NS, EO, MS and HD. Phylogenetic analyses were conducted by SB, TF, and

657 AP. Host bloodmeal analysis was conducted by HKG and SRT. All authors read, edited, and

658 approved the final manuscript.

659

660 Competing Interests Statement

661 The authors declare they have no competing interests