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Seroprevalence of Heartland Virus in Georgia

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Seroprevalence of Heartland Virus in Georgia

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Abstract

Seroprevalence of Heartland Virus in Georgia
By Erick Ojeda

Introduction: Heartland Virus (HRTV) is an emerging tick-borne arbovirus that had been detected around the Southeast, Midwest, and Northeast regions. There have been over 60 cases of HRTV present in the United States up to date. HRTV is described as a single-stranded negative – sense RNA Bandavirus. HRTV is transmitted by the Lone Star Tick (*A. americanum*) and is known to feed on a wide variety of medium- to large-size organisms. In the southeastern United States, *A. americanum* is the tick most frequently associated with human bites Our hypothesis is that the prevalence of HRTV has been underreported due to nonspecific illness and lack of awareness.

Objective: The main objective of the study was to analyze the seroprevalence of HRTV among Georgia residents.

Methods: We developed a sandwich Enzyme- Linked Immunosorbent Assay (ELISA) for HRTV. Because this was a new assay, we performed detailed titrations of each reagent to determine the best concentration. We tested 201 serum samples that were collected from patients registered through the Emory Healthcare System. We collected data elements including the specimen collection date, specimen type, county of residency, patient demographics, and laboratory testing diagnostic results.

Results: A darker optical density background indicates the presence of HRTV IgG antibodies present in the sample. We successfully optimized the new HRTV IgG ELISA using positive and negative controls. There were no HRTV positive patients' samples detected in the study.

Discussion: Out of the 201 serum samples analyzed in the study, zero samples were positive for HRTV, indicating a low seroprevalence. Future work will test more samples from a broader range throughout Georgia and will include patients with known outdoor exposure.

Conclusion: There were no positive HRTV antibodies present on the 201 serum samples. Even though samples could test negative there can be more exposures of HRTV that is unknown and more samples from various health systems must be tested.

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Background

Heartland Virus (HRTV) is an emerging tick-borne arbovirus that had been identified in 2009 in Missouri from two severely ill farmers[1-3]. There had been one case of HRTV reported by the Centers for Disease Control and Prevention in Georgia[4]. There also had been studies showing seroprevalence in white-tailed deer. We found HRTV in Georgia which is what prompted this study. We are studying HRTV to estimate the seroprevalence of IgG antibodies against Heartland Virus and identify the risk of infection in the state of Georgia.

Virology

HRTV is a segmented single-stranded negative-sense RNA virus that was first classified as a Phlebovirus. It was reclassified as a Bandavirus related to Severe Fever with thrombocytopenia syndrome virus (SFRTV) or Dabie Bandavirus[5]. The Heartland viral sequences of the S, M, and L segments are similar to that of the SFRTV clade[6-9]. The small (S) segment is 1.7 kb in length which encodes a nucleoprotein that encapsidates the genomic RNA and a nonstructural (NSs) protein in a coding strategy that is considered ambisense[1, 7]. The L segment is 6.4 kb in length and encodes a large RNA-dependent RNA polymerase[1]. The L protein is responsible for enzymatic activities affiliated with viral replication and transcription which is implicated to viral pathogenesis. Another segment is M, which is 3.4 kb in length and encodes a polyprotein processed into the viral glycoproteins Gc and Gn, which were used for virion entry assembly[1]. HRTV NSs inhibits the virus-triggered activation of IFN-β promoter by targeting the IFN stimulated response element, plus the IFN-β promoter modulates the secretion of cytokines which regulates inflammation[7].

Epidemiology

HRTV was first detected 2009 and is transmitted by the American Lone Star Tick (*Amblyomma americanum*). As of November 2022, more than 60 HRTV cases had been reported around the Southeast, Midwest, and Northeast regions such as Pennsylvania and New York[4, 10]. The median age of individuals who contracted HRTV was 66 years (range: 29 – 80 years)[11].

Amblyomma americanum, a putative vector, is known to feed a wide range of medium- to large-sized vertebrate hosts[12, 13]. Serologic assessment of domestic and wildtype populations shows that seroprevalence rates have been reported from horses, northern racoons, and white-tailed deer, but not in birds[11, 14-16]. *A. americanum* saliva can be irritating, nymphs and adult females most frequently bite humans and transmits the disease[17]. Assessments of white-tailed deer and other large mammals have reported seropositivity cases across a wider spectrum than humans infected with HRTV in multiple locations[18, 19]. In the southeastern United States, *A. americanum* ticks were most frequently associated with human bites, there was evaluation of seroprevalence of white-tailed deer for HRTV antibodies[4, 20]. There can be seropositive coyotes and moose, which indicates they were exposed to HRTV in certain situations and can be beneficial as targets for serosurveillance[21]. Information on the enzootic cycle of HRTV from ticks to humans is limited, and there are gaps regarding transmission ecology and evolution.

Tick infection rates in northwestern Missouri *A. americanum* during the April to July 2013 study periods showed an infection rate of 1.13 per 1,000 in adults (0.33 on females, 1.90 on males), and 1.79 per 1,000 for nymphs. The infection rate among nymphs, which compromise the largest data set, corresponded with 1/559 infections[16]. Another study in eastern Kansas analyzed the presence of HRTV in 964 pools, and five HRTV positive ticks were detected and confirmed using real-time reverse transcription PCR (rRT-PCR)[22]. Based on the Newman et

al. study, *A. americanum* were collected from the William B. Bankhead National Forest during 2018 and the minimum infection rates was 0.57 per 100 ticks screened of the 235 nymph pools tested[23]. Based on the Tuten et al. study, one year after two human cases were detected in 2018 in Illinois, HRTV was detected from *A. americanum* ticks collected[24]. Evidence of widespread HRTV transmission was demonstrated throughout Suffolk County, New York, and tick minimal infection rates ranged from 0% to 1.1%[25]. The minimum infection rate (MIR) for the study area in Georgia for *A. americanum* was 0.46 / 1000 ticks during 2019 overall[4].

There had been human cases present in Indiana and southern Illinois[26]. There has only been one confirmed seropositivity case in Illinois (4%, 95% CI 1 – 20) and two cases in Indiana (3%, 95% CI 1 – 11)[21]. A Northwest Missouri blood bank survey estimated a seroprevalence rate of 0.9% (95% CI 0.4 – 4.2%) for HRTV, a possibility for human exposure and infection[27].

Clinical HRTV symptoms include fatigue, fever, headache, nausea, diarrhea, and myalgia[1]. Laboratory features associated with HRTV, include thrombocytopenia, elevated liver enzyme studies, and leukopenia[1, 28-31]. HRTV can cause fatal, widely disseminated infections in patients without substantial preexisting comorbidities[32]. Clinicians should be aware of this pathogen, along with the potential for overlapping symptomologies with other tickborne infections. Exposures had occurred in locations where outdoor activities are common such as hunting, hiking/camping, yard work/gardening, and walking in forested areas[3]. Testing should be conducted with patients who resided or travelled in areas with previous HRTV infection or *A. americanum* exposure[3, 10, 21].

Aim

I hypothesize that HRTV infection was underreported due to nonspecific symptoms, along with lack of awareness of the disease. The aim of this study is to assess the seroprevalence

of HRTV infection throughout the state of Georgia based on IgG seropositivity. Analyzing IgG seropositivity will detect prior infections, as IgG forms weeks after initial infection and can persist for years in some cases[33]. Secondary objectives include identifying regions with an increased risk of HRTV infection in Georgia and identifying demographic and clinical characteristics associated with HRTV infection. Findings will assist researchers to identify HRTV infection risks in the state of Georgia based on county of origin and potential hotspots for HRTV.

Design & Methods

Study Design

The population for the study was patients visiting Emory Healthcare facilities who have had their serum drawn for routine clinical care (n = 201). The patients were not enrolled and there were no inclusion/exclusion criteria based on age or status as a vulnerable population. In this study, the main outcome was the seroprevalence of HRTV among Georgia residents to help understand the scope of exposure to this emerging virus. Collection of discarded serum specimens were used in the analysis, along with associated clinical and epidemiological data from patients in the Emory Healthcare System. Data elements included specimen type, specimen collection date, county of residence, laboratory diagnostic testing results including bacterial and viral infections, and patient demographics (age, gender, race, ethnicity, zip code).

General Assay Set-up

The Enzyme-Linked Absorbent Immunosorbent Assay (ELISA) is a test that can be used to detect Immunoglobulin G (IgG) antibodies, which form a basis of long-term protection. Serum antibodies are considered essential in the adaptive and innate immune response and immunological memory. The survival of antibody secreting cells determines their contribution to

the immune response and to long-lasting immunity, and their composition results from tightly regulated differentiation of B lymphocytes. In this study, a Sandwich ELISA was used where a target antigen contains two antigenic sites that can bind to antibodies. The captured antibody was immobilized on the surface, while the detection antibody was applied at the last step before quantification (Figure 1)[34]. Immulon II HB flat-bottomed 96 well plates were used for performing HRTV ELISA. Each sample was run in duplicates, the slots highlighted in light green are the samples that were ran in duplicated with both the HRTV antigen, while the slots in dark green represent the samples with control antigen (Figure 2). The slots highlighted in blue were duplicates of the positive control serum that contained the HRTV antigen and the control antigen (Figure 2). The slots highlighted in red were the duplicates of negative control serum associated with HRTV antigens and control antigen (Figure 2).

As a general description of the assay, first the inner 60 well of the 96 well plates were coated with the appropriate group-reactive coating-monoclonal antibody diluted in coating buffer. HRTV antigen was added to the even wells and the control antigen was added to the odd wells. The control antigen is characterized by the absence of HRTV and necessary for successful analyte detection. The serum is added and if HRTV antibodies are present in the serum, they bind with the antigens. The conjugate antibody was added, which binds to human IgG, and then a detector was added which changes its color. Conjugated streptavidin binds with biotin on the conjugate antibody on the assay which increases signal amplification. The para-Nitrophenyl-Phosphate (pNPP), also referred to as Alkaline Phosphate Yellow, was used in ELISA applications to analyze alkaline phosphatase, and acts as a substrate that reacts with the conjugate antibody. When alkaline phosphate and pNPP are reacted, a yellow-water soluble reaction product was formed in the ELISA. The reaction product absorbs light at around 405

nm[35]. PNPP is defined as a chromogenic substrate which determines enzymatic activity for multiple assays, including ELISA.

As a specific description of the procedure, on the first day, the inner wells were coated in Heartland coating antibody diluted with coating buffer. The Heartland coating antibody 2BB5 working dilution was made manually. HRTV coating antibody 2BB5 have a monovalent affinity, binding to the same epitope on the nucleocapsid protein of HRTV[36]. The Heartland coating antibody 2BB5 working dilution was coated into each well at 75 µl, then incubated at 4˚C overnight.

On the second day, the wells were washed 3x with wash buffer in the plate washer. The plates were blocked with 200 µl of blocking buffer per well. Blocking buffer is needed for improving sensitivity and reducing background signal in order to improve stability for the assay. After the wells were blocked, the wells were incubated at room temperature with humidity for one hour. Within 50 minutes of the incubation, both the HRTV+ and control antigen were prepared. The wells were washed during five cycles with washing buffer through the automatic plate washer. Wells had to be filled to the top during each cycle. There was 50 µl of HRTV+ antigen added per well to the left replicate wells of each serum block (H+). To the right replicate wells of each block, 50 µl were added per well of control antigen diluted in wash buffer with the same concentrations at the HRTV antigen (H-). The samples were then incubated overnight at 4˚C in a humidified chamber.

On the third day, the plates were washed 5 times. 50 µl of the positive control serum, negative control serum, or test serum was added to each well, following the plate layout. The plates were then incubated for an hour at 37˚C in a humidified incubator. The plates were then

washed 5 times with wash buffer. To all wells, 50 µl of conjugate antibody was added and incubated at 37˚C in a humid chamber for an hour.

The plates were washed five times with wash buffer, then the plates were prepared for the second wash series. After the PNPP was mixed, 100 µl were added into each well. The PNPP produced a yellow-water soluble reaction product that identified the enzymatic activity to analyze the presence or absence of HRTV antibodies. The plate was placed in the plate reader and the sequential reads protocol was activated with reads at 405 nm every 5 minutes until it reaches 30 minutes. The plate was placed on a plate reader to analyze color change via optical density to determine the presence or absence of HRTV antibodies.

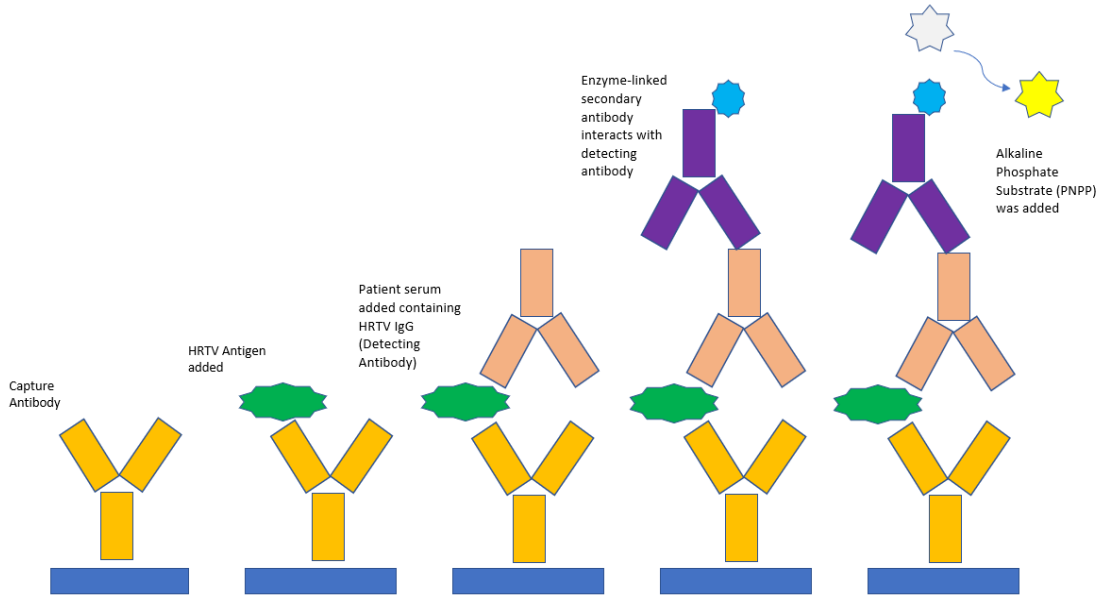

Figure 1: ***Structure of the Sandwich ELISA:*** *Coat the surface with the capture antibody. Add the target antigen which binds with the immobilized capture antibodies. The serum was added which measures a wide array of analytes to recognize the HRTV antibodies which bind to antigen. Adding the detection labeled antibody conjugate with biotin helps ensure it binds to the antibody present in the serum. Alkaline Phosphate Yellow (pNPP) acts as a soluble substrate to yield a colorimetric reaction. Adding the TMB substrate assists in measuring the chromogenic detection of the enzyme-linked interactions[34].*

Plate	1	2	3	4	5	6	7	8	9	10	11	12

A												
B		Pos Ctl, HRTV Ant	Pos Ctl, ctrl ant	Neg Ctl, HRTV Ant	Neg Ctl, ctrl ant	Sample, HRTV ant	Sample, ctrl ant	Sample, HRTV ant	Sample, ctrl ant	Sample, HRTV ant	Sample, ctrl ant	
C		Pos Ctl, HRTV ant	Pos Ctl, ctrl Ant	Neg Ctl, HRTV ant	Neg Ctl, ctrl Ant	Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	
D		Sample, HRTV ant	Sample, ctrl ant	Sample, HRTV ant	Sample, ctrl ant	Sample, HRTV ant	Sample, ctrl ant	Sample, HRTV ant	Sample, ctrl ant	Sample, HRTV ant	Sample, ctrl ant	
E		Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	
F		Sample, HRTV ant	Sample, ctrl ant	Sample, HRTV ant	Sample, ctrl ant	Sample, HRTV ant	S11, ctrl ant	Sample, HRTV ant	Sample, ctrl ant	Sample, HRTV ant	Sample, ctrl ant	
G		Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	Sample (duplicate), HRTV ant	Sample (duplicate), ctrl ant	
H												

Figure 2: Plate template for the Enzyme Linked Immunosorbent Assay (ELISA) based on previous plate analysis used by the Centers for Disease Control and Prevention

Results

Optimizing Concentrations of the Reagents

Because this was a new assay we developed, we had to optimize the concentration of the coating antibody, the antigen, and the binding antibody. For all optimization steps, we targeted the optical density of ~1.0 for the positive control serum and ~0.2 for the negative control serum with the HRTV antigen. The plates must be valid to interpret the results. Validity was determined by dividing the mean optical density of the positive control serum with the HRTV antigen by the mean optical density negative control serum with the HRTV antigen. The ratio

must be equal or greater than 2 for it to be valid. After performing the ELISA, each plate was read at 5-minute intervals from 0 to 30 minutes to determine the optimal time of plate reading. The control antigen was used when looking at an individual sample. If the sample optical density with the HRTV antigen is greater than 2 times the negative control serum optical density with HRTV antigen, we look to the sample optical density with the control antigen. If the sample optical density with HRTV antigen or the one with negative antigen greater than 2, we consider that to be a potential positive. Without running the negative, we would not know if the samples were true or false positives.

Coating Antibody Titration Experiment: We tested concentrations of coating antibody ranging from 1 to 500, 1 to 1,000, 1 to 2,000, 1 to 4,000, 1 to 8,000, 1 to 16,000, 1 to 32,000, 1 to 64,000, and 1 to 128,000. We selected the most dilute coating antibody that gave an optical density above 1 which was 1:1000 (Figure 3, Figure S1). Going forward we used the dilution of 1 to 1000 for the coating antibody in future experiments.

HRTV Coating Antibody 2BB5		
	20 min OD	P/N
1 to 500	1.59	10.42
1 to 1000	1.97	12.87
1 to 2000	0.71	4.66
1 to 4000	0.3	1.94
1 to 8000	0.16	1.02
1 to 16000	0.13	0.82
1 to 32000	0.11	0.70
1 to 64000	0.15	0.96
1 to 128000	0.09	0.59
neg ctl	0.15	

Figure 3: Titrations presented for HRTV Coating Antibody 2BB5, demonstrating that the optimal dilution factor of 1:1000.

HRTV and Control Antigen Titration Experiment: We tested concentrations of coating antibody ranging from 1 to 40, 1 to 60, 1 to 80, 1 to 100, and 1 to 200. We selected the most dilute control antigen that gave an optical density of ~1.0 which was 1:60 (Figure 4, Figure S2). Going forward, we used the dilution of 1 to 1000 for the coating antibody in future experiments.

HRTV Antigen		
	20 min avg OD	P/N
1 to 40	1.79	12.23
1 to 60	1.04	7.08
1 to 80	0.77	5.26
1 to 100	0.6	4.07
1 to 200	0.31	2.1
neg ctl	0.15	

Figure 4: Titrations presented for HRTV and control antigen, demonstrating that the optimal dilution factor was 1:60.

Positive Control Serum Titration Experiment: We tested concentrations of the positive control serum ranging from Results from 1 to 40, 1 to 60, 1 to 80, 1 to 100, 1 to 200, 1 to 400, 1 to 800, and 1 to 1600. We selected the most dilute positive control serum that has an optical density of ~1.0 at 1:400 (Figure 5, Figure S3). Going forward, we used the dilution of 1 to 400 for the coating antibody in future experiments.

Positive Control Serum		
	20 min avg OD	P/N
1 to 40	1.44	9.82
1 to 80	1.41	9.65
1 to 100	1.53	10.42
1 to 200	1.15	7.85
1 to 400	1.02	6.97
1 to 800	0.67	4.57
1 to 1600	0.64	4.36
Neg ctl	0.15	

Figure 5: Titrations presented for the positive control serum demonstrating that the optimal dilution factor is 1:400.

Negative Control Serum Titration Experiment: We tested concentrations of excess serum from another study to be used as negative control serum at 1 to 20 and 1 to 80. We selected the most dilute negative control serum that has an optical density of ~0.2 with a final dilution factor of 1:80 (Figure 6, Figure S4). Going forward, we used the dilution of 1 to 80 for the coating antibody in future experiments.

Negative Control Serum				
20 min	Sample	Dilution	OD	pos/neg OD
1.32	Pos Ctl	1:80	1.32	
	18	1:20	0.34	3.90
	18	1:80	0.16	8.51

	23	1:20	0.27	4.96
	23	1:80	0.2	6.63
	26	1:20	0.23	5.71
	26	1:80	0.16	8.35
	27	1:20	0.2	6.66
	27	1:80	0.14	9.16
	28	1:20	0.21	6.37
	28	1:80	0.17	7.80
	29	1:20	X	
	29	1:80	0.16	8.14
	31	1:20	0.22	5.97
	31	1:80	0.16	8.3
	34	1:20	0.68	1.95
	34	1:80	0.3	4.47
	36	1:20	0.27	4.96
	36	1:80	0.16	8.09
	38	1:20	0.29	4.52
	38	1:80	0.25	5.3
	39	1:20	0.41	3.26
	39	1:80	0.22	5.97
	40	1:20	0.28	4.69
	40	1:80	0.16	8.19
	41	1:20	0.28	4.71
	41	1:80	0.17	7.99
	49	1:20	0.19	6.83
	49	1:80	0.13	10.07
	54	1:20	0.25	5.34
	54	1:80	0.16	8.19
	55	1:20	0.28	4.76
	55	1:80	0.18	7.25
	61	1:20	0.29	4.6
	61	1:80	0.19	7.09
	67	1:20	0.22	5.94
	67	1:80	0.15	9.03
	68	1:20	0.26	5.15
	68	1:80	0.17	7.9
	69	1:20	0.70	1.88
	69	1:80	0.30	4.37
	76	1:20	0.46	2.89
	76	1:80	0.26	5.11
	77	1:20	0.49	2.70
	77	1:80	0.29	4.56
	78	1:20	0.52	2.56
	78	1:80	0.31	4.28
	82	1:20	0.32	4.13
	82	1:80	0.15	8.74

Figure 6: Titrations presented for the negative control serum, demonstrating that the optimal dilution factor is 1:80.

Conjugate Antibody Titration Experiment: We tested concentrations for the conjugate antibody at 1 to 500, 1 to 1000, 1 to 2000, 1 to 4000, 1 to 8000, 1 to 16000, 1 to 32000, and 1 to

64000. We selected the most dilute conjugate antibody with an optical density of 1 with the final dilution factor at 1:4000 (Figure 3, Figure S5). Going forward, we used the dilution of 1 to 4000 for the coating antibody in future experiments.

Conjugate Antibody		
	avg OD@ 20 min	P/N
1 to 500	1.46	6.72
1 to 1000	1.52	7.01
1 to 2000	1.22	5.62
1 to 4000	0.96	4.42
1 to 8000	0.63	2.92
1 to 16000	0.33	1.5
1 to 32000	0.2	0.93
1 to 64000	0.13	0.61
neg control	0.22	

Figure 7: Titrations presented for the conjugate antibody, demonstrating that the optimal dilution factor is 1:4000.

Sample Testing

We obtained 201 randomly selected serum specimens that were originally sent for Vitamin D testing at Emory Medical Laboratories and used our established protocol to test these samples. We tested these samples across 16 plates, each of which contained 13 samples along with a positive and a negative control.

Sample Plate Results

The format of the plate describes how the samples were analyzed (Figure 8). A darker optical density background indicates that there are HRTV antibodies present in the sample. Based on the first week with samples 1 – 51, there were no samples with positive HRTV antibodies, and all the plates were valid for analysis, based on dividing the mean optical density of the positive control serum with the HRTV antigen by the mean optical density negative control serum with the HRTV antigen to analyze a validity rate of 2 or higher. From the second week with samples 52 to 101, there were not any positive results, and all the plates were valid for analysis. On week three, based on samples 102 to 151, there were not any positive results, and all

the plates were valid for analysis. On week four, based on samples 152 to 201, there were not any positive results, and all the plates were valid for analysis.

	2	3	4	5	6	7	8	9	10	11	
	0.259	0.094	0.094	0.095	0.087	0.085	0.079	0.081	0.081	0.077	0 min
	0.471	0.098	0.11	0.112	0.091	0.084	0.082	0.087	0.086	0.088	5 min
	0.627	0.128	0.157	0.143	0.121	0.116	0.106	0.114	0.102	0.112	10 min
	1.071	0.161	0.216	0.196	0.157	0.147	0.136	0.15	0.123	0.145	15 min
	1.25	0.195	0.277	0.253	0.194	0.177	0.166	0.18	0.147	0.177	20 min
	1.536	0.233	0.344	0.311	0.234	0.203	0.197	0.218	0.172	0.215	25 min
B	1.842	0.273	0.412	0.371	0.276	0.239	0.229	0.256	0.2	0.252	30 min
	0.254	0.088	0.094	0.093	0.084	0.082	0.08	0.081	0.08	0.08	0 min
	0.441	0.092	0.099	0.102	0.091	0.088	0.085	0.087	0.095	0.088	5 min
	0.606	0.113	0.151	0.15	0.12	0.108	0.109	0.107	0.113	0.125	10 min
	0.912	0.143	0.192	0.194	0.14	0.133	0.133	0.138	0.147	0.156	15 min
	1.153	0.173	0.239	0.236	0.157	0.16	0.16	0.167	0.181	0.189	20 min
	1.381	0.206	0.291	0.281	0.183	0.19	0.189	0.199	0.219	0.219	25 min
C	1.638	0.239	0.343	0.333	0.208	0.22	0.22	0.23	0.259	0.257	30 min
	0.091	0.085	0.09	0.084	0.082	0.08	0.082	0.077	0.077	0.077	0 min
	0.102	0.096	0.095	0.092	0.086	0.085	0.083	0.08	0.084	0.082	5 min
	0.131	0.117	0.13	0.115	0.105	0.109	0.103	0.096	0.105	0.105	10 min
	0.166	0.144	0.157	0.137	0.122	0.128	0.122	0.112	0.125	0.129	15 min
	0.199	0.169	0.186	0.159	0.141	0.147	0.142	0.129	0.146	0.152	20 min
	0.238	0.197	0.218	0.184	0.162	0.167	0.165	0.148	0.17	0.173	25 min
D	0.276	0.227	0.252	0.212	0.185	0.188	0.189	0.167	0.195	0.197	30 min
	0.084	0.083	0.083	0.087	0.085	0.081	0.079	0.079	0.077	0.077	0 min
	0.089	0.089	0.089	0.095	0.092	0.088	0.084	0.086	0.083	0.084	5 min
	0.107	0.106	0.111	0.124	0.11	0.111	0.099	0.102	0.101	0.101	10 min
	0.127	0.127	0.128	0.146	0.137	0.132	0.117	0.117	0.122	0.134	15 min
	0.148	0.145	0.143	0.168	0.156	0.151	0.131	0.134	0.141	0.154	20 min
	0.172	0.168	0.163	0.195	0.18	0.174	0.15	0.15	0.162	0.18	25 min
E	0.195	0.189	0.182	0.223	0.204	0.197	0.168	0.168	0.184	0.211	30 min
	0.086	0.087	0.084	0.087	0.083	0.087	0.08	0.08	0.079	0.077	0 min
	0.097	0.097	0.093	0.094	0.088	0.096	0.086	0.086	0.086	0.083	5 min
	0.129	0.123	0.114	0.122	0.111	0.114	0.105	0.099	0.102	0.106	10 min
	0.152	0.145	0.134	0.145	0.128	0.137	0.122	0.11	0.121	0.126	15 min
	0.18	0.168	0.153	0.167	0.146	0.158	0.137	0.122	0.139	0.139	20 min
	0.208	0.194	0.175	0.192	0.166	0.183	0.155	0.135	0.157	0.163	25 min
F	0.237	0.219	0.197	0.218	0.186	0.208	0.175	0.149	0.177	0.185	30 min
	0.089	0.087	0.087	0.082	0.082	0.086	0.08	0.08	0.077	0.078	0 min
	0.105	0.105	0.095	0.097	0.095	0.099	0.089	0.09	0.083	0.086	5 min
	0.134	0.138	0.124	0.123	0.115	0.119	0.106	0.106	0.103	0.115	10 min
	0.162	0.167	0.148	0.146	0.133	0.142	0.122	0.123	0.116	0.136	15 min
G	0.191	0.2	0.175	0.171	0.155	0.164	0.14	0.142	0.131	0.158	20 min

0.222	0.23	0.203	0.198	0.177	0.187	0.159	0.161	0.146	0.18	25 min
0.255	0.262	0.234	0.226	0.2	0.213	0.18	0.183	0.161	0.204	30 min

Figure 8: Reference of the plate results to demonstrate then optical background for positivity of HRTV antibodies.

Discussion

We successfully created a standardized working protocol for a HRTV IgG sandwich ELISA. In collaboration with the CDC, we identified the optimal concentrations of the ELISA reagents to perform well for identification of HRTV IgG. We accomplished our goal of sample collection in our partnership with the Emory Healthcare system. In a pilot screen of 201 serum samples from Emory patients, zero samples were positive for HRTV (n = 201). Although future work will allow a more comprehensive seroprevalence study, our preliminary findings suggest that HRTV is rare, similar to other seroprevalence studies performed[4, 16, 22-27].

The study had notable strengths. Because of the titrations performed, our assay was able to provide a sensitivity-specificity reaction to analyze antibodies. Even though there were no HRTV antibodies present in 201 samples, there were no flaws in the designing of the experiment and all samples were read appropriately. The study also had some limitations. There were zero samples tested for the virus, however, because of case counts in Georgia and surrounding states, is not surprising due to our limiting sample size. There were smaller sample sizes which can increase the risk of bias and statistical power. Further sample collecting is necessary from multiple hospital systems in future studies.

A critical part of arboviral diagnosis pertains to the serological testing for related viruses[37]. Vaccines for these viruses are currently unavailable, making an understanding of how many people and promoting awareness of the disease critical to avoid further disease spread. Clinical presentations can be ambiguous and can be difficult to diagnose based on the

symptoms. Advantages for using the ELISA for HRTV IgG analysis would be highly specific and sensitive results, cost-effective processing, and simultaneous analyses could be performed without complicated sample pre-treatment. Disadvantages of the ELISA would be false negative and positive results. False results can occur from insufficient blocking of immobilized antigen and have cross-reactivity[38, 39].

Conclusion

There has been no recent positivity in HRTV cases based on our data. There were not enough samples in the study that would provide a high amount of HRTV antibody presence. Despite these null findings, that does not mean that HRTV virus is not present within Georgia. In the future, it would be necessary to have more samples from multiple health centers outside of the Emory Healthcare system to make the study more reliable and reduce the risk of bias. In the future, analyzing more serum samples could assist on how infectious rates in Georgia are compared to other states and if it is higher than the expected prevalence of cases. Identifying HRTV can assist in creating new vaccinations to build protection by creating antibodies and eradicating HRTV infectious levels and promote safety when being exposed to *A. americanum.*

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Supplementary Figures

HRTV Coating Antibody 2BB5 Titration

		2	3	4	5	6	7	8	9	10	11
0 min		0.356	0.332	0.176	0.103	0.089	0.079	0.082	0.079	0.079	0.079
5 min		0.848	0.993	0.466	0.192	0.126	0.098	0.107	0.09	0.09	0.084
10 min		1.485	1.754	0.849	0.309	0.173	0.123	0.142	0.103	0.104	0.088
15 min		1.853	2.171	0.886	0.344	0.174	0.147	0.164	0.099	0.097	0.084
20 min		1.728	2.145	0.823	0.34	0.168	0.133	0.151	0.109	0.11	0.09
25 min		2.249	2.711	0.993	0.392	0.208	0.157	0.18	0.119	0.123	0.095
30 min	B	2.693	3.116	1.203	0.444	0.232	0.175	0.205	0.129	0.134	0.1
0 min		0.198	0.243	0.156	0.096	0.085	0.079	0.082	0.079	0.083	0.08
5 min		0.655	0.872	0.434	0.179	0.115	0.096	0.104	0.093	0.115	0.085
10 min		1.279	1.603	0.694	0.283	0.151	0.117	0.136	0.101	0.169	0.093
15 min		1.405	1.26	0.456	0.197	0.118	0.102	0.117	0.097	0.206	0.087
20 min		1.461	1.792	0.603	0.253	0.143	0.119	0.136	0.106	0.184	0.091

25 min		1.927	2.243	0.763	0.298	0.163	0.134	0.157	0.118	0.22	0.096
30 min	C	2.358	2.678	0.92	0.35	0.182	0.147	0.178	0.126	0.253	0.1
0 min		0.08									
5 min		0.093									
10 min		0.113									
15 min		0.151									
20 min		0.169									
25 min		0.163									
30 min	D	0.186									
0 min		0.079									
5 min		0.092									
10 min		0.111									
15 min		0.149									
20 min		0.137									
25 min		0.156									
30 min	E	0.18									

negative ctl	
mean	0.153
st dev	0.022627417
3 SD	0.220882251

Figure S1: Titration of the HRTV Coating Antibody 2BB5. Based on the results, the optical density at 1:1000 will be used moving forward for the ELISA

HRTV Antigen Titration

	1 to 40 2	1 to 60 3	1 to 80 4	1 to 100 5	1 to 200 6	Neg control 7	
B	0.138	0.121	0.103	0.143	0.085	0.077	0 min
	0.774	0.525	0.386	0.32	0.171	0.09	5 min
	0.834	0.628	0.486	0.374	0.232	0.11	10 min
	0.992	0.686	0.48	0.395	0.21	0.136	15 min
	1.7	1.117	0.767	0.646	0.298	0.147	20 min
	2.295	1.527	1.07	0.857	0.413	0.163	25 min
	2.771	1.874	1.329	1.048	0.503	0.197	30 min
C	0.255	0.149	0.132	0.122	0.1	0.081	0 min
	0.775	0.383	0.34	0.292	0.182	0.1	5 min
	1.329	0.645	0.597	0.501	0.282	0.124	10 min
	1.925	0.954	0.86	0.682	0.383	0.148	15 min
	1.883	0.958	0.775	0.546	0.317	0.146	20 min
	1.977	0.925	0.885	0.706	0.398	0.175	25 min
	2.632	1.271	1.188	0.966	0.539	0.225	30 min

Figure S2: Titration results of the HRTV Antigen. As suspected, 1:60 is best dilution for HRTV and control antigen.

Positive Control Serum Titration

	1 to 40 2	1 to 80 3	1 to 100 4	1 to 200 5	1 to 400 6	1 to 800 7	1 to 1600 8	Neg ctl 9	
D		0.161	0.184	0.175	0.154	0.145	0.147	0.077	0 min
	0.505	0.413	0.45	0.501	0.358	0.36	0.287	0.09	5 min
	0.867	0.732	0.77	0.873	0.575	0.629	0.485	0.11	10 min
	1.34	1.131	1.186	1.076	0.848	0.834	0.733	0.136	15 min
	1.332	1.095	1.455	0.887	1.219	0.652	0.616	0.147	20 min
	1.388	1.237	1.253	1.179	1.095	0.874	0.705	0.163	25 min
	1.81	1.627	1.657	1.546	1.144	1.123	0.88	0.197	30 min
E	0.206	0.205	0.202	0.188	0.171	0.155	0.139	0.081	0 min
	0.196	0.475	0.478	0.443	0.393	0.394	0.282	0.1	5 min
	0.723	0.771	0.798	0.751	0.698	0.718	0.483	0.124	10 min
	1.079	1.164	1.206	1.129	1.097	0.755	0.768	0.148	15 min
	1.544	1.733	1.597	1.414	0.822	0.688	0.661	0.146	20 min
	1.439	1.54	1.492	1.255	0.996	0.933	0.722	0.175	25 min
	1.577	1.897	1.69	1.494	1.299	1.145	0.9	0.225	30 min

Figure S3: Titration for the positive control serum. We can start to use the positive serum at 1:400.

Negative Control Serum Titration

	3	5	7	9	11	
B	0.155	0.199	0.158	0.144	0.169	20 min
C	0.162	0.159	0.295	0.163	0.249	20 min
D	0.221	0.161	0.165	0.131	0.161	20 min
E	0.182	0.186	0.146	0.167	0.302	20 min
F	0.258	0.289	0.308	0.151		20 min

mean	0.18724
SD	0.05632397
Pos cut pt	0.35621191

Figure S4: Titration for the negative control serum. The outlier would be >0.35 OD.

Conjugate Antibody Titration

	2	3	4	5	6	7	8	9	10	
	0.175	0.171	0.158	0.133	0.109	0.096	0.085	0.08	0.078	0 min
B	0.631	0.754	0.622	0.453	0.331	0.221	0.14	0.105	0.112	5 min

	1.21	1.304	0.974	0.843	0.493	0.284	0.188	0.122	0.174	10 min
	1.426	1.053	0.794	0.763	0.406	0.232	0.15	0.109	0.19	15 min
	1.408	1.598	1.268	0.933	0.59	0.341	0.205	0.133	0.222	20 min
	2.064	2.038	1.621	1.209	0.764	0.424	0.247	0.151	0.282	25 min
	2.412	2.443	1.915	1.454	0.916	0.503	0.287	0.169	0.332	30 min
	0.189	0.152	0.147	0.134	0.11	0.085	0.084	0.078	0.08	0 min
	0.743	0.648	0.578	0.47	0.253	0.176	0.126	0.101	0.119	5 min
	1.359	1.286	1.084	0.905	0.441	0.302	0.199	0.138	0.179	10 min
	1.141	1.253	0.861	0.703	0.677	0.225	0.179	0.11	0.159	15 min
	1.502	1.439	1.166	0.983	0.673	0.309	0.196	0.134	0.211	20 min
	2.1	1.921	1.542	1.227	0.671	0.382	0.237	0.153	0.254	25 min
C	2.483	2.299	1.816	1.49	0.829	0.46	0.272	0.169	0.297	30 min

Figure S5: Titration for the conjugate antibody. Based off of the results, we will use the conjugate antibody at 1:4000 because the OD was less than 1 and greater than 0.8, the validity is greater than 2.