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A serologic investigation of wild small mammal suitability as reservoir hosts for Heartland virus in Georgia

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By

Leah Aeschleman

B.S., University of Delaware, 2021
MPH, Emory University
2023

Thesis Committee Chair: Gonzalo Vazquez-Prokopec, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
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Master of Public Health
in Epidemiology
2023

Abstract

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By Leah Aeschleman

Heartland virus (HRTV) is a novel tick borne phleboviruses found relatively recently in the United States in 2009. Like all tick-borne disease, environmental changes in temperature, humidity, and landscape influence the geographic distribution of the pathogen. Additionally, understanding the scope of animal reservoirs can provide insight into the epidemiology of HRTV, specifically to identify pathways for pathogen spillover into humans. In the following study, small mammals from HRTV confirmed sites in Georgia were collected and sampled for serum and presence of ticks. Serum was used to determine wild mice as a candidate as a reservoir host for HRTV. Weather conditions were retrospectively documented to further our understanding of climatic influencing catch per unit effort (CPUE). Prior to analyzing field samples, a ELISA assay to detect HRTV exposure in mice was developed and validated. None of the 11 serum samples from the 2022 field season tested positive for neutralizing antibodies against HRTV through our in-house ELISA. Average temperature and relative humidity showed no statistically significant association in mice CPUE. This study provides the foundation for larger studies of the role of rodents in HRTV transmission ecology, as it developed the methods for future serum testing and provides limited information about small mammal prevalence to HRTV. Due to its small sample size, our study will help generate future information by testing

small mammals collected in future field seasons and further investigate their role in the HRTV transmission cycle.

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Abstract:

Heartland virus (HRTV) is a novel tick borne phleboviruses found relatively recently in the United States in 2009. Like all tick-borne disease, environmental changes in temperature, humidity, and landscape influence the geographic distribution of the pathogen. Additionally, understanding the scope of animal reservoirs can provide insight into the epidemiology of HRTV, specifically to identify pathways for pathogen spillover into humans. In the following study, small mammals from HRTV confirmed sites in Georgia were collected and sampled for serum and presence of ticks. Serum was used to determine wild mice as a candidate as a reservoir host for HRTV. Weather conditions were retrospectively documented to further our understanding of climatic influencing catch per unit effort (CPUE). Prior to analyzing field samples, a ELISA assay to detect HRTV exposure in mice was developed and validated. None of the 11 serum samples from the 2022 field season tested positive for neutralizing antibodies against HRTV through our in-house ELISA. Average temperature and relative humidity showed no statistically significant association in mice CPUE. This study provides the foundation for larger studies of the role of rodents in HRTV transmission ecology, as it developed the methods for future serum testing and provides limited information about small mammal prevalence to HRTV. Due to its small sample size, our study will help generate future information by testing small mammals collected in future field seasons and further investigate their role in the HRTV transmission cycle.

Introduction:

Emerging infectious diseases (EID) are a rising concern for public health. Factors contributing to the rise in EIDs include socio-economic, environmental and ecological factors[1]. EIDs are dominated by zoonotic pathogens where 60.3% are from a non-human animal source[1]. Of these emerging zoonotic diseases 71.8% originate from wildlife [1]. In addition to the rising concern for zoonotic pathogens, vector-borne diseases are emerging at higher rates due climate change and the increased geographical expansion of mosquitoes and ticks.

Throughout ? and ?, vector-borne diseases made up to 22.8 % of all detected EIDs [1]. In the North America, Europe and Asia ticks are responsible for 95% of locally acquired vector-borne diseases[2]. Environmental conditions determine tick geographic distribution [3]. Changes in habitat features can influence tick survival and the establishment of new tick populations [3]. Higher temperatures associated with climate change have accelerated the tick life cycle. This temperature change increases tick abundance in already established populations and enables ticks to spread to higher latitudes, increasing geographic dispersion. [3].

Environmental and anthropogenic changes such as rising temperatures and deforestation are forcing humans and wildlife to have increasing overlap [1]. Animals are frequently reservoirs for zoonotic and tick-borne pathogens [3]. Determining what species are compatible for spillover of disease is critical in understanding the role of wildlife in epidemiological transmission cycle of disease. When vectors feed on infected reservoirs hosts, the pathogen can be acquired and transmitted to other animals or humans. In the context of tick-borne diseases, mice often serve as amplification hosts [3]. The presence of a large population of infected

animals can provide ample opportunity for pathogen spillover events into humans. Identifying and monitoring reservoir populations of vector borne disease is crucial in controlling transmission [3].

An example of a novel emerging tick borne disease is heartland virus (HRTV). This phlebovirus was first discovered in 2009 when two men from Missouri presented febrile illness 5-7 days after being bitten by a tick. *Amblyomma americanum*, the lone star is the primary vector for HRTV [4]. Since its discovery HRTV has been detected in Arkansas, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Missouri, North Carolina, Oklahoma, and Tennessee. More than 60 cases have been reported nationally[5]. HRTV as a vector borne disease, relays heavily on environmental and wildlife factors. *Amblyomma americanum* has non-specific feeding patterns and feeds primarily on medium and large sized hosts [6] This is a concern for understanding reservoir hosts for HRTV virus because there is great ambiguity in potential host candidates.

Studying the ecology of HRTV and potential animal reservoirs can provide valuable insight into HRTV disease epidemiology. Seropositive animals can be used as sentinel indicators of disease presence in a geographic area. Small mammals are of particular interest due to their smaller home rangers (10-15 feet)[7] compared to the lone star ticks medium – large sized hosts with larger home ranges.

There have been preliminary investigations done to evaluate potential reservoir candidates for HRTV. Neutralizing antibodies have been detected in banked blood samples from deer, raccoons, coyotes, and moose [8]. Although these animals do not satisfy the role as the reservoir for the virus, they aid in HRTV horizontal transmission among co-feeding ticks.

Mice are suitable hosts for *A. americanum* during early life stages such as larva and nymphs [9]. HRTV can be passed transstadially [10]. This is an implication in understanding HRTV disease epidemiology since ticks in nymphal or larva life stages can feed from a infected mouse and carry the disease until adulthood, infecting other invertebrates and humans.

HRTV is genetically similar to severe fever with thrombocytopenia syndrome virus (SFTSV)[4]. This tick-borne disease in China commonly infects domestic farm animals such as goats, sheep, cattle, dogs, pigs and chickens[11]. Studies in China have detected SFTSV antibodies and RNA, suggesting rodents and shrew as potential hosts [12]. Due to the close genetic similarity of HRTV to SFTSV there is potential for the rodents to be hosts of HRTV. SFTSV is vectored by the Asian Longhorned tick. This tick is invasive to the United States and has recently been discovered in wildlife management areas in Georgia. This tick poses as an additional concern for future implications of HRTV due to its ability to reproduce asexually and amplify other tick-borne disease.

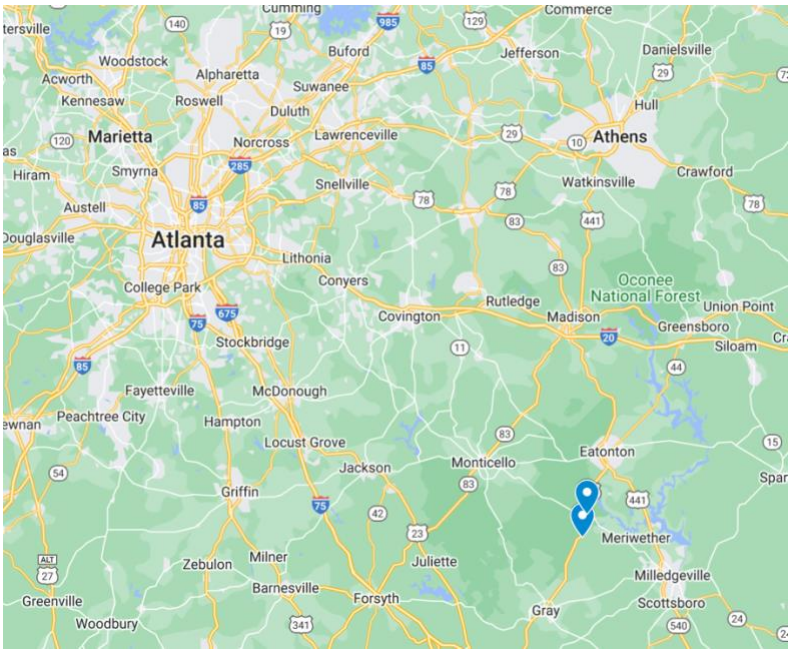
To further understand the prevalence of HRTV in wildlife we conducted a study evaluating mice as hosts for the HRTV. Blood samples were collected from mice living in regions confirmed with HRTV positive ticks. Serum samples were tested for neutralizing antibodies against HRTV using a enzyme-linked immunosorbent assay. The prevalence of antibodies will help in understanding the HRTV transmission cycle in greater detail.

Methods:

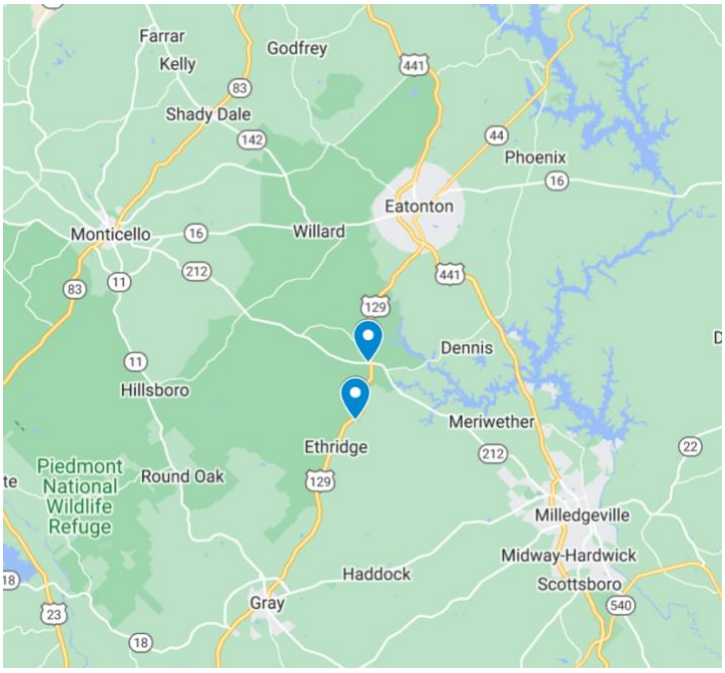
Study sites

Study sites were selected based off of previous findings of indicating high tick densities, the presence of (HRTV) positive ticks, and of proximity to seropositive white-tailed deer with antibodies to HRTV [13]. The two study sites were 130 km southeast of Atlanta and adjacent to

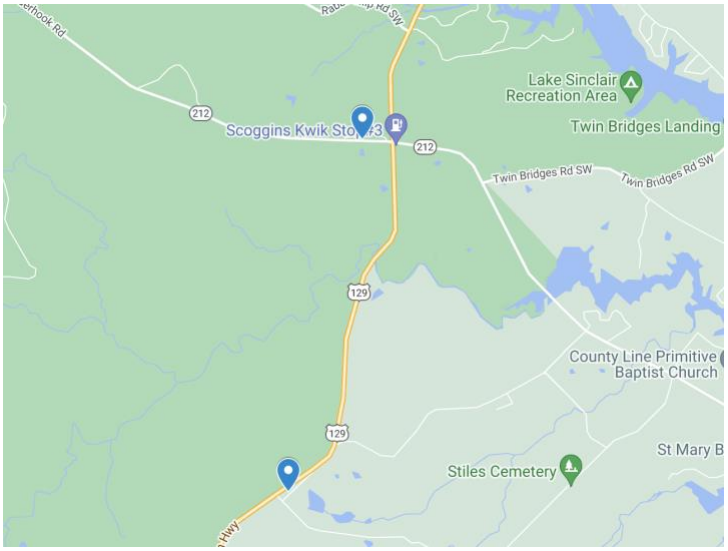
the county with the only reported human case of HRTV documented in 2005[13]. Tick collection and mammal trapping occurred between April 2022 and September 2022. The study sites fell within the Southern Outer Piedmont ecoregion of Georgia. This region has a lower elevation with less precipitation[14].



Map1: Depicting study sites with confirmed positive HRTV tick. These sites were selected as sampling locations for small mammals



Map 2: A magnified picture demonstrating sampling sites



Map 3: Demonstrating the proximity of study sampling sites used for the study that have confirmed HRTV ticks

Small mammal collection

Blood samples were collected from live mice captured via Sherman traps residing in the selected field sites. Sherman traps were set and baited with a combination of mixed nut butter,

banana extract and old-fashioned oats at dusk. Approximately 60 traps were set in transects opportunistically each night, specifically targeting the forest edge and microhabitats suitable for Peromyscus leucopus and Peromyscus maniculatus. Ideal trap locations include those with high plant coverage in line with a fallen tree or near a hole or hiding place in a tree. Each sherman trap's location was marked using flagging tape(see appendix E). To ensure trap sensitivity, we tested the trigger pad on rear end of the trap by lightly pressing a twig or finger to the back. If the front door did not snap shut after weight was applied to the trigger we adjusted the treadle to be more sensitive.

Traps were collected at dawn. If the trap door was not triggered, the trap was picked up and the bait was removed to clean for next use. If the trap door was closed, the trap was examined by carefully turning it so that the front door faced up to peer inside. Sometimes the traps were triggered from larger wildlife moving through the forest or weather. If there was an animal inside, the trap door was kept closed and the animal was brought back to the processing station until all traps were collected.

Collecting blood consisted of euthanizing the animal and collecting data on physical characteristics. The animal was first moved into a thick plastic bag to lower the risk of escape before transfer to the euthanasia chamber. We used carbon dioxide to euthanize the animal with a fill rate of 30-70% per minute. Once the animal was deceased, it was removed from the chamber where data was collected on species, weight, length, sex and presence of attached ticks. Blood was collected via cardiothoracic puncture and stored in a microtainer serum separating tube. Samples were transported in a cooler with an ice pack back to Emory University where the blood samples were centrifuged 10,000 g for 3 minutes. The serum was then pipetted out and stored in a PCR tube in a –80 Celsius freezer until we could process samples for antibodies against

HRTV. Weather was retrospectively recorded to document conditions associated with higher animal catch rates.

Tick collection and processing

Ticks in nymph and adult life stages were collected concurrently from study sites when traps were set to collect small mammals. Questing ticks were collected through standard flagging methodology [15]. Ticks were then transported back to the laboratory.

Ticks were pooled based on species, life stage, sex, and collection site. Pools consisted of ≤ 5 adults and ≤ 25 nymphs. Tick pools were prepared for molecular testing for HRTV by adding 1 mL of BA-1 dilutant (1× medium 199 with Hanks balanced salt solution, 0.05 mole/L Tris buffer [pH 7.6], 1% bovine serum albumin, 0.35 g sodium bicarbonate/L, 100 μg/L streptomycin, 1 μg/mL amphotericin B) to each pool and crushed using a 7-mL glass TenBroeck grinder [13]. The homogenate was then transferred to a 2 mL cryotube and stored at -80 degrees Celsius until ready for molecular testing.

The tick homogenates were tested for HRTV by extracting the RNA using a QIAGEN RNA Extraction kit. Quantitative real-time PCR was conducted by using a Quantitect Probe PCR kit with primers designed for small segments of HRTV genome as described by Savage et. al[16].

HRTV Enzyme-Linked Immunosorbent Assay (ELISA)

Serum samples were analyzed using a IgG sandwich enzyme-linked immunosorbent assay (ELISA). The ELISA was developed to detect neutralizing antibodies for HRTV. The initial ELISA trials were done using reagents for human samples. Developing a successful

ELISA protocol for humans could then be adapted for mouse samples collected during the summer 2022 field season.

Day Number	Steps
Day 1	1. 96 well plate was coated with 75uL monoclonal antibody (HRTV mAb 2BB5) diluted in coating buffer (Carbonate/ bicarbonate buffer pH 9.6) 2. Incubated plate overnight at 4 degrees Celsius
Day 2	3. Wash plate 3 times using the plate washer with wash buffer (Phosphate buffered saline (PBS), 0.05% Tween 20, pH 7.4) 4. Add 200 uL of blocking buffer (3% goat serum diluted in phosphate buffered saline (PBS), 0.1% Tween 20, pH 7.4) to each well then incubated at 37°C for 2 hours 5. Wash plate 5 times using the plate automatic washer 6. Add 50 uL HRTV+ antigen or control antigen diluted in blocking buffer to designated wells. 7. Incubate overnight at 4°C
Day 3	8. Wash plate 5 times using the automatic plate washer 9. Add 50 uL positive control and negative control serum diluted in blocking buffer to designated wells. 10. Incubate for 1 hour at 37°C 11. Wash plate 5 times using the automatic plate washer 12. Add 50 uL of conjugate antibody(alkaline phosphatase-conjugated goat anti-human IgG) diluted in blocking buffer to each well 13. Incubate for 1 hour at 37°C 14. Wash plate 5 times twice using the automatic plate washer 15. Add 100 uL of Sigma 104 phosphatase substrate (1-step PNPP) 16. Immediately place plate in plate reader where it will be read every 5 minutes for 30 minutes at 405 nm

Table 1: A condensed version of the final human sandwich ELISA assay used to adapt for the mouse serum samples. The table is broken into the section based on the corresponding day in the protocol and reagents used

To ensure validity and reduce waste of valuable reagents, a series of titration trials were conducted to determine the best dilution factors for each reagent. Per CDC recommended analysis, final dilution factors were chosen based on having an average optical density (OD) between 0.8 and 1 and validity test greater then 2. The validity test provides information

describing the discrimination between positive and negative controls. It is calculated by determining the average OD test specimen with HRTV antigen (P) / Mean OD negative control with HRTV antigen.

In the first trial the ELISA was run using the CDC-recommend dilutions except for the HRTV viral antigen. The CDC recommended diluting reagents in wash buffer (Phosphate buffered saline (PBS), 0.05% Tween 20, pH 7.4) and blocking for only 1 hour on day 2. The HRTV+ antigen was titrated first since it was the most limiting reagent. It was important to determine the minimum amount need to elicit a strong signal moving through future reagent titrations. The HRTV viral antigen was run in duplicates at 1:80 as the best guess from the CDC. Duplicates of HRTV viral antigen diluted to 1:10 were run on the same plate. The 1:10 dilution ensured the binding site were saturated and would generate results. Duplicates of negative antigen were run on same plate as a comparison.

The first test trial demonstrated high background in the negative controls. The average OD for these wells was greater than 1 for all time intervals indicating the presence of HTRV specific antibodies when they are not present. Cross reactivity or nonspecific binding could cause this reading. Trial 1 indicated the 1:10 dilution was too concentrated and exceeded the OD limit for the plate reader. The results from this trial indicated the 1:80 dilution for HRTV antigen was sufficient but the assay would benefit from a higher concentration of HRTV viral antigen and improved blocking of non-specific binding.

In trial 2, nonspecific binding was accounted for by increasing blocking time after coating the plate with monoclonal antibody from one hour to two hours and diluting all reagents in blocking buffer instead of wash buffer. In trial 2, the normal and viral antigen were diluted to 1:40 and 1:80 to determine a more appropriate HRTV+ antigen concentration.

Results of trial 2 indicated blocking for 2 hours and diluting in block buffer substantially reduced background signaling. The results showed the 1:40 HRTV positive antigen dilution factor worked slightly better than the 1:80. Since HRTV+ antigen was the most limiting reagent all plates moving forward were diluted at 1:60 in blocking buffer.

The remaining reagents were titrated one at a time in the following order: coating antibody, conjugate antibody, positive control. To titrate the coating antibody (mAb 2BB5) we began two-fold serial dilutions at 1:500 up until 1:25600. All other reagents were used at suggested concentrations from the CDC except for the HRTV viral antigen which was used at 1:60. Individual tubes were labeled per their corresponding dilution factor and wells were then coated with the corresponding appropriate concentration in duplicates. The ELISA was then run according to the protocol described above.

The results of the titration trial for the coating antibody indicated 1:1000 produced the best results. At 20 minutes the average OD was 1.97 and had a discrimination factor of 12.8. A average OD of 1.97 is greater than the 1-0.8 threshold. The average OD for 1:2000 dilution is less then 0.8. A higher signal is preferred over weak signal. A 1:1000 dilution factor for the coating antibody was used moving forward when to determine subsequent reagent titrations

For the next titration trial of the conjugate antibody, the plate was run per protocol using the coating antibody at 1:1000, HRTV+ antigen at 1:60 and positive and negative control at the CDC recommend dilutions. On day 3, serial dilutions were completed for the conjugate antibody. The initial stock for the serial dilution was 1:500. This concentration is then diluted in two-fold until 1:640000. The ELISA was then run according to protocol described above.

The results indicated that the 1:4000 dilution for the conjugate antibody produced the best results. Average optical density was 0.958 which is between the 0.8 and 1 and the validity test was 4.42, which is greater than 2 and can be considered valid.

The final titration trial was for the positive control reagents (HRTV antigen and HRTV positive serum). The ELISA coated with 1:1000 monoclonal antibody and run per protocol. On day 2 the HRTV+ antigen was diluted at 1:40, 1:60, 1:80, 1:100 and 1:200. The negative control wells had a 1:60 dilution as that dilution had been successful in previous trials. The ELISA was run per protocol. On day 3 the positive control sera was prepared to the appropriate dilutions consisting of 1:40, 1:80 and 1:100. Serial dilutions were prepared in 2-fold ranging from 1:100 to 1:1600. Wells were filled in duplicates with the appropriate dilutions. The ELISA was run according to protocol.

Results indicated that HRTV positive antigens dilutions prepared at 1:60 provide optimal results. The average OD was 1.03 which slightly above one. The Validity test was 7.08 which is greater than the cutoff of 2. The titrations of positive control serum indicated optimal results at 1:400. The average OD at 20 minutes was 1.02 which is slightly above cut off of one. The validity test had a result of 6.96 which is greater than the cutoff of 2.

Through systematically adjusting reagents for the human ELISA we were able to optimize results. This helps in saving valuable reagents and increasing the sensitivity of the assay, elevating the quality of results once testing patient samples. Understanding the reagent concentrations that elicit a robust signal help in gauging the dilutions factors to begin with when adapting the ELISA for mouse serum samples.

Mouse adapted HRTV Enzyme Linked Immunoassay

To determine small mammal competency as hosts for HRTV, serum samples collected from the summer 2022 field season were analyzed for neutralizing antibodies. This process involved adapting the human HRTV Enzyme-Linked Immunoassay (ELISA) (described above) for mice. The human ELISA protocol incorporates a mouse monoclonal antibody as the coating reagent. To prevent the potential issue of cross reactivity due to the monoclonal antibody being the same species as the test samples (and creating non-specific binding), rabbit serum with HRTV antibodies was used to coat the wells instead.

Trial one involved titrating both the HRTV antibody positive rabbit serum and the positive control serum on the same plate using separate rows. Human ELISA indicated coating wells using HRTV mAb 2BB5 at 1:1000 produced reliable results. To test around this recommended dilution factor, HRTV + rabbit serum was titrated using two-fold dilutions in duplicates starting at 1:500 to 1:4000 on dya one. Wells used for the negative control, negative antigen and positive control titration trial were coated with 1:500 rabbit serum. The plate was then incubated at 4 °C overnight. The plate was run per protocol (described above) until day 3 when serial dilutions were made for HRTV positive and negative control serum

Human ELISA indicated coating wells using HRTV mAb 2BB5 at 1:1000 produced reliable results. To test around this recommended dilution factor, HRTV + rabbit serum was titrated using two-fold dilutions in duplicates starting at 1:500 to 1:4000 on day one. Wells used for the negative control, negative antigen and positive control titration trial were coated with 1:500 rabbit serum. The plate was then incubated at 4 °C overnight. The plate was run per protocol (described above) until day 3 when serial dilutions were made for HRTV positive and negative control serum.

The negative control serum was prepared using a 1:60 dilution factor and added to designated wells. The positive control serum was prepared at 1:40, 1:80, 1:100, 1:200,1:400, 1:800 and 1:1600. Replicates used for determining appropriate dilution factor for HRTV+ rabbit serum were coated with 1:80 positive serum. Remaining HRTV+ control serum was added to designated wells to test signal strength.

Results from the first trial indicated high background and demonstrated the HRTV+ control serum had low reactivity. The wells coated using positive control serum had consistently low optical densities below 0.8 at all time intervals and dilution factors ranging from 1:500 through 1:1600. The results indicated coating using the HRTV+ rabbit serum at 1:2000 produced the best signal. This dilution factor was in the middle of the serial dilution and not consistent with expected dilution curves.

To improve blocking and reduce background the second trial compared blocking with 3% goat serum and 10% fetal bovine serum (FBS) in phosphate buffered saline (PBS), 0.1% Tween 20, pH 7.4 . The wells were coated in duplicates of HRTV+ rabbit serum at 1:1500, 1:2000,1:2500 and 1:3000. This was done to further test around the 1:2000 dilution factor. Rows B and C were blocked with blocking buffer containing 3% goat serum. Rows D and E were blocked with a blocking buffer containing 10% FBS. The ELISA was run according to protocol using viral and normal HRTV antigen at 1:40 and positive and negative control serum at 1:40 and the conjugate antibody at 1:1000.

The second trial demonstrated that using 10% FBS blocking buffer worked better than the 3% goat serum. Overall average ODs for both blocking serums were still low (<0.7). Ideally average ODs should be between 0.8 and 1. The validity test for positive controls and negative antigen using 10 % FBS generated values all greater then 2, indicating sufficient discrimination.

Results indicated coating wells using HRTV+ rabbit serum at 1:3000 elicited the strongest at the 20 minute time interval. The OD signal increased in a linear ratio as the concentration of rabbit serum decreased. This could indicate the 1:3000 is too dilute to get a robust signal or is too saturated to generate a robust signal.

In the third trial, the goal was to increase signaling by using higher concentrations of HRTV+ control serum and blocking using 10% FBS. The wells were coated using 1:3000 HRTV+ rabbit serum on day 1, except for one set of duplicates coated using 1:150 HRTV+ rabbit serum. Positive control concentrations were increased to 1:5, 1:10, and 1:20. The wells coated using the 1:150 rabbit serum used a positive control concentration of 1:40 as a comparison to previous trials. The ELISA was run per protocol.

Results from trial 3 demonstrated a 1:5 concentration of HRTV+ control serum indicated using the HRTV+ control serum at 1:5 satisfied the validity test at all time intervals greater than 15 minutes. This result demonstrates enough discrimination between negative and positive samples; however, OD signal was still weak and did not surpass 0.6. This indicates there is still low signaling in the assy. The wells coated using rabbit serum concentration at 1:150 passed the validity test for time intervals greater then 20 despite using the positive control serum at a lower concentration of 1:40.

Results from trial 3 demonstrated coating with HRTV+ rabbit serum at 1:150 on day 1 and coating with HRTV+ control serum on day 3 improved discrimination between positive and negative samples. A final trial was run with a single set of positive and negative control duplicates. The protocol combined findings from trial 3. Wells were coated using 1:150 HRTV+

rabbit serum on day 1 and 1:5 HRTV+ control serum as these concentrations separately had the highest average ODs seen in previous trials.

Results from the last titration trial satisfied both criteria of having an average OD greater then 0.8 and had a validity test value greater then 2. The following protocol was used to test the summer 2022 field samples.

Day Number	Steps
Day 1	1. 96 well plate was coated with 75uL HRTV+ rabbit serum diluted to 1:150 in coating buffer (Carbonate/ bicarbonate buffer pH 9.6) 2. Incubated plate overnight at 4 degrees Celsius
Day 2	3. Wash plate 3 times using the plate washer with wash buffer (Phosphate buffered saline (PBS), 0.05% Tween 20, pH 7.4) 4. Add 200 uL of blocking buffer (10% FBS diluted in phosphate buffered saline (PBS), 0.1% Tween 20, pH 7.4) to each well then incubated at 37°C for 2 hours 5. Wash plate 5 times using the plate automatic washer 6. Add 50 uL HRTV+ antigen (1:40 in FBS blocking buffer) or control antigen (1:60 in FBS blocking buffer) to designated wells. 7. Incubate overnight at 4°C
Day 3	8. Wash plate 5 times using the automatic plate washer 9. Add 50 uL mouse positive control(1:5) and mouse negative control serum (1:40) diluted in FBS blocking buffer to designated wells. 10. Incubate for 1 hour at 37°C 11. Wash plate 5 times using the automatic plate washer 12. Add 50 uL of conjugate antibody(Alkaline phosphatase-conjugated goat anti-mouse IgG) diluted in blocking buffer to each well 13. Incubate for 1 hour at 37°C 14. Wash plate 5 times twice using the automatic plate washer 15. Add 100 uL of Sigma 104 phosphatase substrate (1-step PNPP) 16. Immediately place plate in plate reader where it will be read every 5 minutes for 30 minutes at 405 nm

Table 2: This table is representing the final protocol used to test the serum samples from the summer 20222 field season. This protocol was adapted to optimize the reactivity of reagents specific for mice. It is broken down by day and corresponding step.

	2	3	4	5	6	7	8	9	10	11
A										
B	Pos Ctl, HRTV Ant	Pos Ctl, norm Ant	Neg Ctl, HRTV Ant	Neg Ctl, norm Ant	ST002, HRTV ant	ST002, norm ant	ST006, HRTV ant	ST006, norm ant	ST007, HRTV ant	ST007, norm ant
C	Pos Ctl, HRTV Ant	Pos Ctl, norm Ant	Neg Ctl, HRTV Ant	Neg Ctl, norm Ant	ST002, HRTV ant	ST002, norm ant	ST006, HRTV ant	ST006, norm ant	ST007, HRTV ant	ST007, norm ant
D	ST008, HRTV ant	ST008, norm ant			KH001, HRTV ant	KH001, norm ant	KH002, HRTV ant	KH002, norm ant	ST011, HRTV ant	ST011, norm ant
E	ST008, HRTV ant	ST008, norm ant			KH001, HRTV ant	KH001, norm ant	KH002, HRTV ant	KH002, norm ant	ST011, HRTV ant	ST011, norm ant
F	ST012, HRTV ant	ST012, norm ant	ST013, HRTV ant	ST013, norm ant	ST014, HRTV ant	ST014, norm ant	ST018, HRTV ant	ST018, norm ant		
G	ST012, HRTV ant	ST012, norm ant	ST013, HRTV ant	ST013, norm ant	ST014, HRTV ant	ST014, norm ant	ST018, HRTV ant	ST018, norm ant		

Figure 1: This figure is representing the plate map used for the summer 2022 field season. The chart demonstrates where each sample was placed on the plate and the controls used to validate the assay.

Results:

We collected a total of 20 live mammals during the summer field season. Blood was drawn from 11 mice and 5 shrews. Blood was not able to be collected from one mouse. This is likely due to dehydration. Information about species, length, weight, and tick presence were documented for each animal.

ID #	Date	Common Name	Scientific Name	Location	Weight (grams)	Length cm	Ticks	Notes
ST004	5/3/2022	Southern short-tailed shrew	Blaina carolinensis	Starling Road	-	-	-	Found deceased
ST005	5/3/2022	Southern short-tailed shrew	Blaina carolinensis	Starling Road	10	10	0	
ST002	5/17/2022	White-footed mouse	Peromyscus Leucopus	Starling Road	25	17	1	
ST006	6/3/2022	Deer Mouse	peromyscus maniculatus	Starling Road	15	14.5	0	

ST007	6/3/2022	White-footed mouse	Peromyscus Leucopus	Starling Road	21	16	0	
ST008	6/21/2022	White-footed mouse	Peromyscus Leucopus	Starling Road	17	14.5	0	
ST009	6/21/2022	Deer Mouse	Peromyscus maniculatus	Starling Road	19	14	0	No blood collected
ST0019	6/21/2022	White-footed mouse	Peromyscus Leucopus	Starling road	-	-	-	Escaped
ST010	6/27/2022	Southern short-tailed shrew	Blaina carolinensis	Starling Road	5	8	0	
ST020	7/14/2022	Southern short-tailed shrew	Blaina carolinensis	Starling Road	-	-	-	Found deceased
KH001	8/4/2022	White-footed mouse	Peromyscus Leucopus	Kinderhook Road	27	16	0	
KH002	8/4/2022	White-footed mouse	Peromyscus Leucopus	Kinderhook Road	34	18	0	
ST011	8/4/2022	White-footed mouse	Peromyscus Leucopus	Starling Road	20	15	5	
ST012	9/3/2022	White-footed mouse	Peromyscus Leucopus	Starling Road	24	15	0	
ST013	9/3/2022	White-footed mouse	Peromyscus Leucopus	Starling Road	30	17	0	
ST014	9/3/2022	White-footed mouse	Peromyscus Leucopus	Starling Road	37	17	0	
ST015	9/3/2022	Southern short-tailed shrew	Blaina carolinensis	Starling Road	7	9	0	
ST016	9/30/2022	Southern short-tailed shrew	Blaina carolinensis	Starling Road	5	7.8	0	
ST017	9/30/2022	Southern short-tailed shrew	Blaina carolinensis	Starling Road	6	8.8	0	
ST018	9/30/2022	Southern short-tailed shrew	Peromyscus Leucopus	Starling Road	23	15	0	

Table 3: This table is representing the characteristics of each animal caught during the summer 2022 field season. Each ID corresponds to a unique animal. Information was documented for length, species, weight, presence of ticks, location caught and date. Additional notes were documented accordingly.

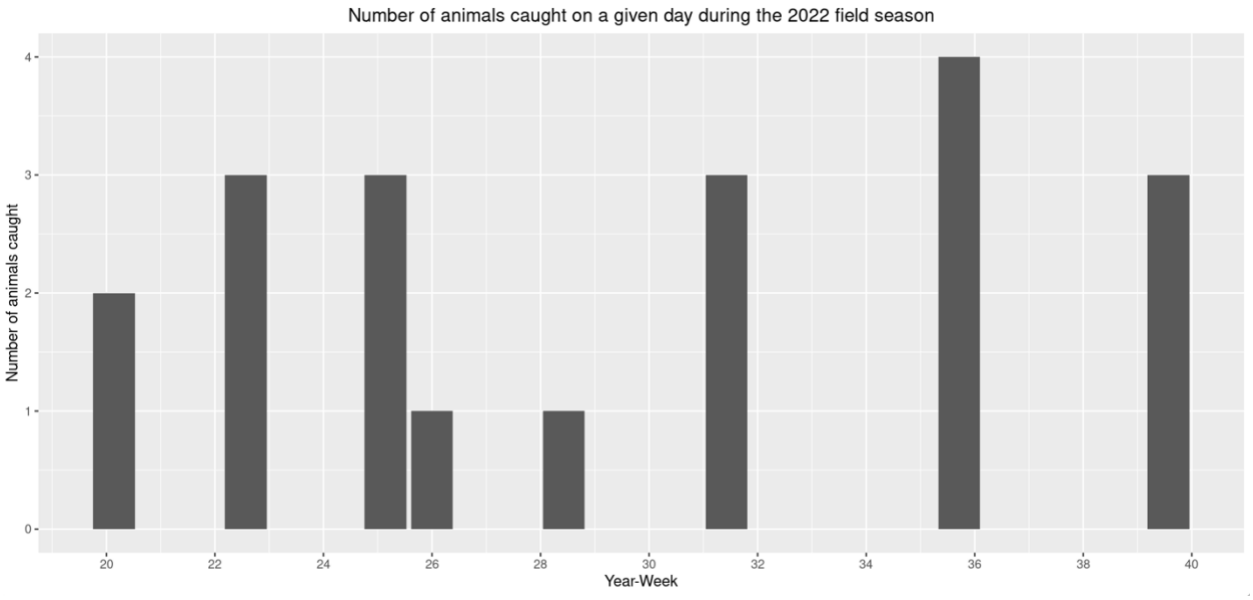


Figure 2: This figure is representing the total number of animals caught during the summer 2022 field season. The graph is broken up by the number of animals collected on a given day and the week of the year trapping was done.

Temperature and precipitation were retrospectively recorded to document the conditions associated with the highest catch rate. Weather conditions and moon phases impact small mammal behavior. Small mammals adjust their behavior to reduce predation risk. Cloudy, warm, and damp nights with new moons are associated with increased mammal foraging[17]. Data from Haddock County weather station was used to identify the weather conditions associated with higher catch rates. A Poisson regression analysis was run in R to determine if average temperature and relative humidity were associated with the number of animals captured.

Date 2022	Number Of Animals	Weather Conditions	Average Overnight Temp	Relative Humidity	Moon Phase
May 16th/17th	1	Clear	24.4	71.5	Waxing Crescent
June 2nd/3rd	2	Clear/passing storms	24.7	83	Waxing Crescent
June 20th/ 21st	3	Clear	25.8	61	Last Quarter Phase
June 26th/27th	1	Light Rain	23.0	60.8	Waning Crescent

July 13th/14th	1	Passing light storms	25.5	90.5	Full moon
August 3rd/4th	3	Thunderstorms	28.0	87.5	Waning Crescent
September 2nd/3rd	4	Cloudy/fog/light rain	25.5	78	First Quarter
September 29th/30th	3	Clear	18.3	65	Waxing Crescent

Table 4: Breakdown of the weather conditions associated with dates animals were collected. The results are not stratified by location since the proximity of site were very close. The total number of animals we documented from total trip.

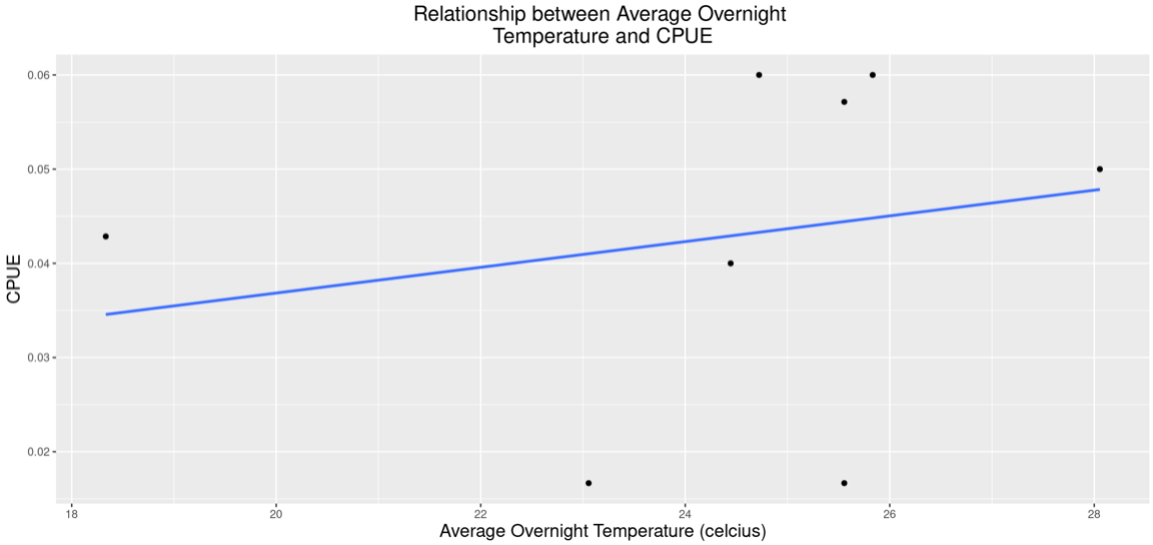


Figure 3: A demonstration of average temperature's effect on CPUE.

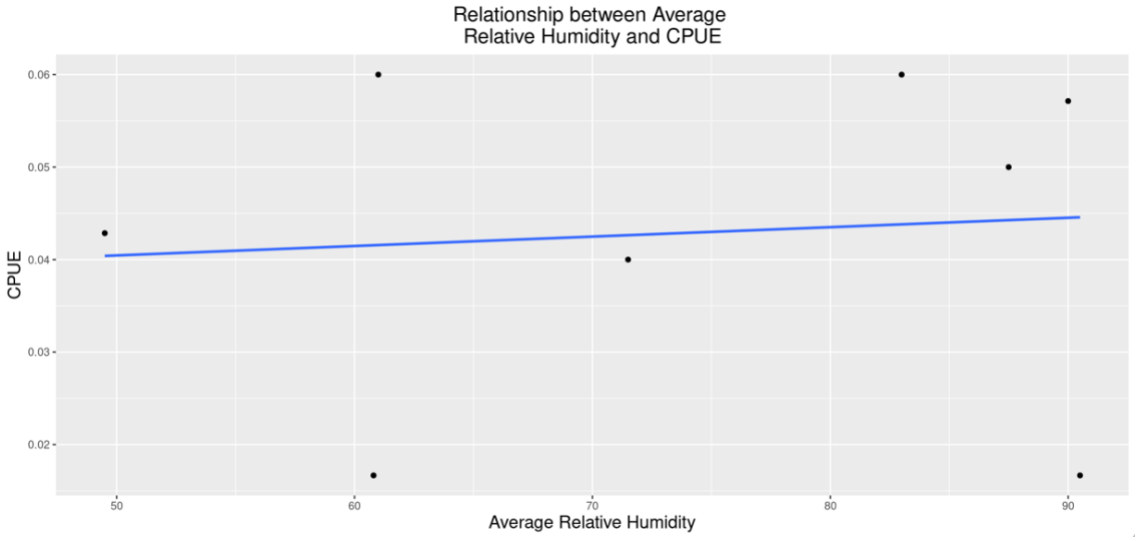


Figure 4: A demonstration of relative humidity's effect on CPUE.

Predictor	Coefficient	Standard Error	Z Score	P-Value
Intercept	-24.3	3.926 x 10^5	0	1
Average Temp	-2.35E-16	2.324 x 10^4	0	1
Rel Humidity	2.93E-16	4.197 x 10^3	0	1

Table 5: Summary of Poisson regression model for CPUE with average temperature and relative humidity as predictors.

Results of the regression analysis demonstrated no statistically significant association between average temperature and relative humidity. The correlation coefficient for both average temperature and relative humidity were both very close to zero. The standard error for average temperature and relative humidity were quite large, indicating high levels of uncertainty for their estimates. The residual deviance of models had low values, indicating the fitted model is a good fit of the data.

Tick samples were analyzed from all trips to study sites. We collected a total of 2645 ticks, comprising of 1054 nymphs, 503 adult *A. americanum*, 18 *Dermacentor variabilis*, 47 I*xodes scapularis*, 6 *A. maculatum* between March 5th to August 8Th. Two pools of *ambyloma americanium* nymphs tested positive for HRTV virus through PCR. Positive nymph pools were collected during peak tick season on May 12th 2022, and June 10th 2022. Of the 6 engorged blood-feeding larva collected from mice, none tested positive for HTRV.

No. Pools	*Collection Date*	*Site*	*No. Specimens in pool*	*HRTV real-time PCR result for homogenate*
6	5/12/2022	ST	27	+
13	6/10/2022	ST	28	+

Figure 5: A breakdown of the two HRTV positive tick pools collected during the 2022 filed season. Both positive pools were from nymphs residing on the site along Starling road.

The 11 mouse serum samples collected from the 2022 field season all tested negative for HRTV neutralizing antibodies using this ELISA protocol. Due to lack of reagents, serum samples from shrews were not tested using the ELISA. Results were analyzed at the 25-minute mark. This is when the signal was the highest and background was at a suitable level. Discrimination between the negative and positive control samples met the acceptable criteria with a value of 2.938.

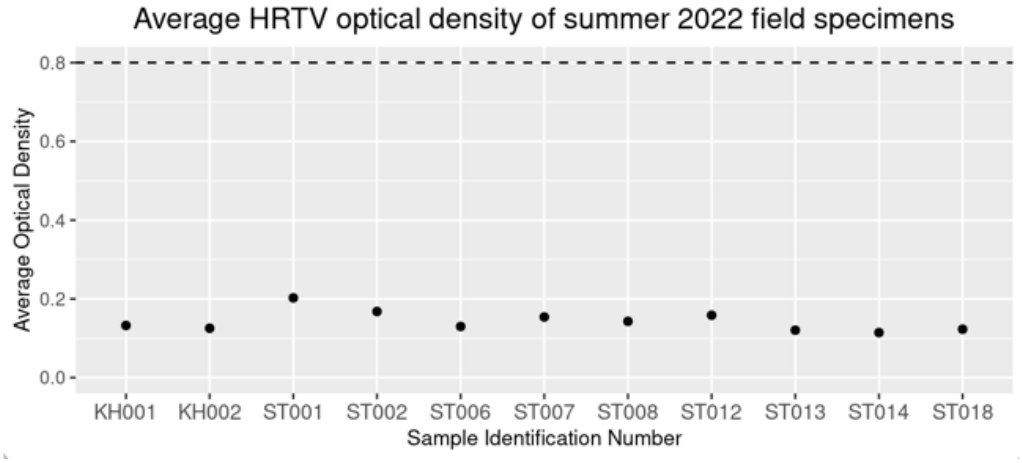


Figure 5: Graph showing levels of signal for each animal after testing using the in house ELISA. Samples were analyzed at the 25 minute mark. A sample is considered positive when the average optical density reaches above 0.8. All serum samples collected over the summer did not reach this threshold.

When testing the serum samples from the summer 2022 field season, 8 wells were used to determine the sensitivity between the positive and negative controls and the HRTV antigen and control antigen (see appendix for plate map). After running the final plate, the wells coated with control antigen and positive control serum demonstrated high signal. This is important to consider when interpreting results since it is possible the positive control cross reacts with the control antigen.

Discussion

This study provide insight for future research regarding small mammals as hosts for HRTV. Results from this study indicated the animal captured from sites with HRTV confirmed ticks did not have neutralizing antibodies against HRTV. Although no immune response was observed it is important to continue researching the role of wild small mammals in the HRTV transmission cycle due to their capacity as hosts for the lone star tick during larva and nymphal life stages.

Previous research evaluating mice as hosts for HRTV have only been done in a controlled environment where C57BL/6 mice and Ag129 mice were experimentally inoculated. Both knockout mice showed immune response and neutralizing antibodies against HRTV after inoculation [18]. Research has shown the immune system of wild and laboratory mice differ in activation of myeloid cells and cytokine responses to pathogens[19]. For this reason it is important to continue investigating wild mice as host for HRTV despite the samples testing negative.

Of the 2,645 tick collected only 2 pools of nymphs tested positive for active HRTV virus infection. This is consistent to previous years where there are low levels of virus circulation within the tick population. The likelihood of a susceptible mouse coming in contact with a infected tick is limited. Since the prevalence of HTRV is already low, It is important to further investigate what animal species will accelerate the transmission cycle. Through collection of more serum samples from mice and small mammals, additional conclusion can be made indicating which species aid in the disease epidemiology of HRTV.

A limitation of the ELISA used to test serum samples is the cross reactivity exhibited between the positive control serum and control antigen. The signal between these two reagents was greater then an acceptable threshold indicating the need to further investigation. Moving forward the there is a need for a positive control with higher reactivity with the positive antigen. This could be a challenge due to limited studies indicting elevated levels of infection in laboratory mice.

Results from the Poisson regression analysis for weather conditions effect on catch per unit effort should be interpreted lightly due to the limited sample size and lack of longitudinal data collected. This study determined average temperature and relative humidity did not have a significant association with CPUE. Previous studies have provided evidence that temperature and humidity do effect small mammal activity due to difficulty in regulating body temperature in lower damper environments.

Limitations within this study include the small samples size. Only 11 serum samples from the summer 2022 field season were collected and processed using the ELISA. This limited sample size results in low statistical power and generalizability. Next steps should include collecting additional serum samples from mice living in regions with HRTV confirmed ticks.

Conclusion and Recommendations

Tick borne diseases pose a significant threat to public health due to their increasing geographic landscape, caused by climate change. There is an elevated level of caution required as new emerging vector borne disease are introduced globally though habitat loss and increased wildlife and human overlap. A better understanding of animal species serving as revisors, or hosts of vector borne disease can help in implementing initiatives that reduce human to animal

exposure. In the context of this study, wild mice were evaluated as hosts for HRTV, through serologic investigation of neutralizing antibodies against HRTV. Although our research found no immune response among these animals, further investigation and increased sample collection is required to validate these results.

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Appendix:

Plate map A:
Used as a guide for the first titration trial completed for mice and to determine appropriate concentrations of rabbit serum used to coat the wells on day 1 and the mouse positive control serum.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		R1: RAB+ 1:500 (best guess)	R2: RAB+ 1:1000	R3: RAB+ 1:2000	R4: RAB+ 1:4000	R5: -CTL 1:500	R6: Antigen neg ctl					
C		R1: RAB+ 1:500 (best guess)	R2: RAB+ 1:1000	R3: RAB+ 1:2000	R4: RAB+ 1:4000	R5: -CTL 1:500	R6: Antigen neg ctl					
D		R1: + CTL 1:40	R2: + CTL 1:80	R3: + CTL 1:100	R4: + CTL 1:200	R5: + CTL 1:400	R6: + CTL 1:800	R7: + CTL 1:1600				
E		R1: + CTL 1:40	R2: + CTL 1:80	R3: + CTL 1:100	R4: + CTL 1:200	R5: + CTL 1:400	R6: + CTL 1:800	R7: + CTL 1:1600				
F												

G												
H												

Plate Map B:

Plate map detailing placement of each reagent in the trail used to determine if goat serum or FBS serum worked better as a blocking reagent

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B Goat		R1: RAB+ 1:1500	R2: RAB+ 1:2000	R3: RAB+ 1:2500	R4: RAB+ 1:3000	R5: -CTL 1:2000	R6: Antigen neg ctl					
C Goat		R1: RAB+ 1:1500	R2: RAB+ 1:2000	R3: RAB+ 1:2500	R4: RAB+ 1:3000	R5: -CTL 1:2000	R6: Antigen neg ctl					
D FBS		R1: RAB+ 1:1500	R2: RAB+ 1:2000	R3: RAB+ 1:2500	R4: RAB+ 1:3000	R5: -CTL 1:2000	R6: Antigen neg ctl					
E FBS		R1: RAB+ 1:1500	R2: RAB+ 1:2000	R3: RAB+ 1:2500	R4: RAB+ 1:3000	R5: -CTL 1:2000	R6: Antigen neg ctl					
F												
G												
H												

Plate Map C:
Plate map used when determining the final dilution factors for positive control and rabbit serum.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		R1: 1:5 +CTL	R2: 1:10 +CTL	R3: 1:20 +CTL	R4: 1:3000 -CTL							
C		R1: 1:5 +CTL	R2: 1:10 +CTL	R3: 1:20 +CTL	R4: 1:3000 -CTL							
D		R6: 1:150 +RAB	R7: 1:150 RAB, -CTL									
E		R6: 1:150 +RAB	R7: 1:150 RAB, -CTL									
F												
G												
H												

Appendix D:
A breakdown of signal produced by each serum sample and well at the 20, 25 and 30 minute mark. As time progresses signal increases and so does background. This is important to identify when interpreting results.

	2	3	4	5	6	7	8	9	10	11
A	0.049	0.054	0.048	0.049	0.052	0.047	0.046	0.045	0.046	0.043	
	0.047	0.057	0.048	0.046	0.052	0.046	0.047	0.048	0.046	0.043	
	0.047	0.055	0.046	0.049	0.051	0.047	0.047	0.045	0.046	0.042	
B	0.483	0.451	0.18	0.176	0.151	0.156	0.131	0.112	0.138	0.155	20 min
	0.594	0.555	0.213	0.205	0.173	0.179	0.142	0.122	0.156	0.179	25 min
	0.713	0.659	0.245	0.234	0.195	0.203	0.152	0.132	0.173	0.204	30 min
C	0.484	0.431	0.167	0.177	0.145	0.137	0.109	0.109	0.136	0.151	20 min
	0.602	0.528	0.194	0.202	0.163	0.155	0.118	0.118	0.152	0.175	25 min
	0.717	0.626	0.221	0.229	0.181	0.172	0.127	0.128	0.169	0.197	30 min
D	0.126	0.125	0.052	0.051	0.116	0.113	0.115	0.109	0.163	0.171	20 min
	0.14	0.136	0.052	0.05	0.128	0.124	0.126	0.12	0.191	0.198	25 min
	0.152	0.147	0.052	0.05	0.14	0.134	0.136	0.13	0.217	0.226	30 min
E	0.131	0.117	0.049	0.049	0.125	0.121	0.115	0.11	0.185	0.151	20 min
	0.146	0.127	0.049	0.051	0.137	0.133	0.125	0.118	0.214	0.172	25 min
	0.158	0.137	0.048	0.051	0.148	0.144	0.134	0.127	0.243	0.193	30 min
F	0.145	0.124	0.105	0.109	0.107	0.106	0.109	0.107	0.079	0.079	20 min
	0.159	0.135	0.112	0.117	0.115	0.113	0.117	0.115	0.079	0.079	25 min
	0.172	0.146	0.119	0.124	0.122	0.12	0.125	0.122	0.079	0.079	30 min
G	0.143	0.137	0.119	0.117	0.106	0.104	0.118	0.119	0.047	0.047	20 min
	0.158	0.151	0.129	0.127	0.114	0.111	0.129	0.13	0.047	0.046	25 min
	0.173	0.166	0.14	0.137	0.121	0.118	0.138	0.142	0.047	0.048	30 min
H	0.047	0.047	0.048	0.046	0.047	0.046	0.047	0.045	0.045	0.046	
	0.047	0.047	0.048	0.047	0.047	0.046	0.049	0.048	0.046	0.046	
	0.048	0.048	0.047	0.045	0.048	0.047	0.044	0.046	0.048	0.046	

Appendix E: Demonstration of ideal trap placement and flagging.

Appendix F: A photo demonstrating moving the animal from Sherman trap to plastic bag to euthanasia chamber.

Appendix G: Photo describing processing station set up with materials in proper location

Appendix H: Photo of specimen ST010