Prevalence of Tick-Borne Pathogens in Northeast Missouri

by Deborah A. Hudman, MS & Neil J. Sargentini, PhD†

Future studies on management techniques and their influence on reducing either number of ticks or pathogen prevalence is desirable.

Deborah A. Hudman, MS, (above), and Neil J. Sargentini, PhD,†are in the Department of Microbiology/Immunology, A.T. Still University Kirksville College of Osteopathic Medicine
Contact: dhudman@atsu.edu

Abstract

We evaluated *Amblyomma americanum* (lone star tick) and *Dermacentor variabilis* (American dog tick) in northeast Missouri for the presence of *Borrelia*, *Ehrlichia*, and *Rickettsia* bacteria and Heartland virus. We screened 436 individual adult lone star ticks (86% of all ticks collected) and infection rates were 6% for *B. lonestari*, 19% for *E. chaffeensis*, 3% for *E. ewingii*, 36% for *R. amblyommatis,* and 1% for *R. montanensis*. In the 189 individual American dog ticks, infection rates were 19% for *E. chaffeensis*, 15% for *E. ewingii*, 4% for *R. amblyommatis*, and 5% for *R. montanensis*. In addition, we screened 20 pools of adults and 30 pools of nymphs for the Heartland virus which was not detected. Understanding the presence and epidemiology of these causative (*E. chaffeensis* and *E. ewingii*) and suspected (*B. lonestari, R. amblyommatis, and R. montanensis*) agents in Missouri should increase awareness of potential tick-borne disease in the medical community.

Introduction

Ticks are vectors for a variety of microorganisms, many of which are known agents of zoonotic disease. Missouri tick species are capable of transmitting pathogens such as Rocky Mountain spotted fever (RMSF), ehrlichiosis, tularemia, southern tick associated rash illness (STARI), Heartland virus, and Bourbon virus.[1] Tick populations and associated pathogens can vary greatly across landscapes so understanding local tick population dynamics could provide valuable insight into potential threats to public health. In 2014, we surveyed four state owned conservation areas in Adair County, Missouri for ticks and evaluated 308 pools for the presence/absence of *Borrelia*, *Ehrlichia*, and *Rickettsia* bacteria.[2] In 2015, we expanded on those initial findings and attained true infection rates in adult *Amblyomma americanum* (Linnaeus, 1758), and *Dermacentor variabilis* (Say, 1821) ticks, as opposed to estimates of pooled samples, and expanded our surveillance to include the Heartland virus. We also investigated how managed vs unmanaged sites affected both the presence of ticks and their pathogens. The data collected from this study provides insight to the potential effect of land management practices on associated tick populations and their pathogens and thus the risk of potential disease transmission.

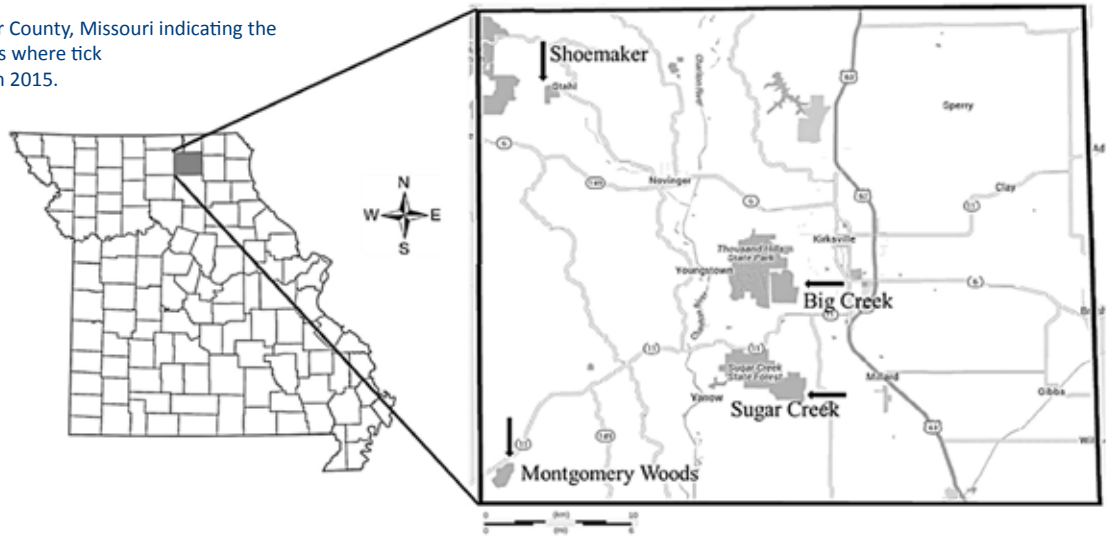

Figure 1. Map of Adair County, Missouri indicating the four conservation sites where tick collections occurred in 2015.

Materials and Methods

Tick Collections

In 2015, ticks were collected from April through September at four conservation areas in Adair County, Missouri with the exception of June, due to heavy rains (Figure 1). Each collection site was sampled once per month for 5 months for a total of 20 collection days. Questing ticks were collected using a cloth drag-flag method and by removing ticks from the collector's clothing.[3] Ticks were stored in 70% ethanol until analyses. All collected ticks were identified morphologically to the species using a dissecting microscope and appropriate taxonomic keys.[4-5] Ticks were pooled according to collection site, species, developmental stage, and sex if adults. Two of these conservation areas are managed sites (Big Creek and Sugar Creek). Big Creek is managed through food plots, burning, and mowing while Sugar Creek is managed by thin and release timber prescription and mowing. The other two sites (Shoemaker and Montgomery Woods) have been unmanaged for the last ten years (Y. Amerman and B. Kelso, pers. comm.).

RNA Extraction

Twenty adult pools (5 adults/pool, n=100) and 30 nymph pools (25 nymphs/pool, n=750) had their total RNA and DNA extracted via BlackPREP tick DNA/RNA kit (Analytikjena, Jena, Germany) according to the manufacturer's instructions. All RNA lysate was stored at -80°C until analysis by quantitative real-time polymerase chain reaction (qPCR), but DNA lysate was stored at -20°C until polymerase chain reaction (PCR) analysis.

DNA Extraction. Individual adult ticks (n=625) had only their DNA extracted. An entire tick was removed from ethanol, allowed to dry, and placed in a 1.5-ml RINO screw cap tube (Next Advance, Averill Park, NY) containing 125 μl of DNAzol Genomic DNA Isolation reagent (Molecular Research Center, Inc., Cincinnati, OH) and Proteinase K (Ambion, Austin, TX) at 0.1 mg/ml. Following overnight incubation at room temperature, 150 mg of 2.0 mm zirconium oxide beads (Glenn Mills, Inc., Clifton, NJ) were placed in each RINO tube and homogenized using a Bullet Blender Storm (Next Advance, Averill Park, NY) at speed 12 for 3 minutes. The tick homogenate was then incubated at room temperature for 3 h, heated at 95°C for 10 min, and centrifuged at 10,000 x g for 10 min at room temperature as described.[6] To verify the quantity and quality of DNA extraction, 260/280 and 260/230 nm absorbance ratios were determined using a spectrophotometer (NanoDrop®ND-2000c, ThermoScientific, Wilmington, DE) according to manufacturer's instructions.

Quantitative Real Time Polymerase Chain Reaction (qPCR)

Sequence information for HRTV primer-probe sets 1 and 4 which were used for virus detection and confirmation along with cycle conditions are as described.[7] Heartland virus strain MO4 RNA lysate

Table 1. Numbers and Life Stages of Tick Species Collected from Four Conservation Areas in Adair County, Missouri, 2015

Location	*Amblyomma americanum* (n=1,235)			*Dermacentor variabilis* (n=206)	
	Females	Males	Nymphs	Females	Males
Big Creek	33	36	78	19	25
Montgomery Woods	93	119	152	13	30
Sugar Creek	61	70	198	9	10
Shoemaker	75	34	286	39	61
Total	262	259	714	80	126

(positive control) and two negative controls, one tube with no RNA and the other with no reverse transcriptase was run concurrently with the 50 pooled samples.

Polymerase Chain Reaction (PCR)

PCR-based assays were used to amplify and identify bacterial DNA within individual and pooled tick samples. PCR reactions of 25 μl contained 1.0 μM of each primer (Fisher Scientific, Pittsburgh, PA), 10 mM of each dNTP, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 1.25 U of *Taq* DNA Polymerase (Fisher Scientific, Pittsburgh, PA), and 100 ng of extracted DNA from pooled ticks. With every PCR assay, a positive and negative control was included. Negative controls consisted of water in place of DNA and positive controls consisted of known DNA samples: *Borrelia* (KPL, Gaithesburg, MD), *Ehrlichia*, and *Rickettsia* (CDC, Atlanta, GA). PCR reactants were kept on ice until transferred to a thermal cycler (T100 BioRad, Hercules, CA).

All samples of extracted DNA were tested for the presence of *Borrelia* and *Ehrlichia* species by nested PCR. The initial genus-wide PCR assays were used to amplify a segment of the *flaB* gene for *Borrelia* spp. and the 16S rRNA fragment for *Ehrlichia* spp.[8-11] The nested PCR assays used unique primers to amplify a secondary target within the initial PCR product to ensure specificity. Samples still positive for *Borrelia* spp. after the initial nested PCR were subjected to a separate nested PCR for the 16S-23S rRNA intergenic spacer region.[12] The second nested PCR was done because it is still unclear to what extent the flagellin gene is able to differentiate between closely related *Borrelia* species when sequenced. Isolated DNA was examined for the presence of *Rickettsia* spp. by amplifying a segment of the citrate synthase gene.[13] All tick DNA samples that tested positive for presence of the *gltA* gene were also tested for presence of the *rOmpA* gene.[13] *Rickettsia* DNA samples that remained positive were digested with *PstI* (Promega, Madison, WI) for restriction fragment length polymorphism analysis as described.[14-15]

DNA extractions and PCR were performed in different rooms on different days. Certified DNA/RNase-free filter barrier tips were used to prevent aerosol contamination. Also, representative positive PCR products were purified and bidirectionally sequenced by an outside source (Macrogen, Rockville, MD) using both the forward and reverse primers to establish and confirm identity. Nucleotide sequences were analyzed using Nucleotide Basic Local Alignment Search Tool (BLASTN) available online at the National Center for Biotechnology Information (NCBI).

Infection rates in tick pools were calculated using the Maximum Likelihood Estimation (MLE) with 95% confidence interval (CI) for unequal pool sizes and expressed as the MLE of infection rate per 100 ticks. The MLE was calculated using the PooledInfRate, ver. 4.0 add-on to Microsoft Excel.[16]

Table 2. The presence of *Borrelia*, *Ehrlichia* and *Rickettsia* spp. in individual adult *Amblyomma americanum* and *Dermacentor variabilis* in Northeast Missouri

Amblyomma americanum (n=436)					
Site	*B. lonestari*	*E. chaffeensis*	*E. ewingii*	*R. amblyommatis*	*R. montanensis*
Big creek (n=49)	3 (6%)	18 (37%)	0	14 (29%)	4 (8%)
Mont. woods (n=192)	16 (8%)	38 (20%)	4 (2%)	73 (38%)	0
Sugar creek (n=111)	1 (1%)	17 (15%)	2 (2%)	47 (42%)	1 (1%)
Shoemaker (n=84)	6 (7%)	11 (13%)	5 (6%)	24 (29%)	1 (1%)
Total	26 (6%)	84 (19%)	11 (3%)	158 (36%)	6 (1%)
Dermacentor variablilis (n=189)					
Site	*B. lonestari*	*E. chaffeensis*	*E. ewingii*	*R. amblyommatis*	*R. montanensis*
Big creek (n=44)	0	8 (18%)	12 (27%)	2 (5%)	1 (2%)
Mont. woods (n=43)	0	14 (33%)	5 (12%)	4 (9%)	0
Sugar creek (n=19)	0	3 (16%)	0	1 (5%)	3 (16%)
Shoemaker (n=83)	0	10 (12%)	11 (13%)	2 (2%)	3 (4%)
Total	0	35 (19%)	28 (15%)	9 (5%)	7 (4%)

Results

Amblyomma americanum (lone star tick) comprised 86% of all collected ticks, while *Dermacentor variabilis* (American dog tick) comprised 14% (Table 1). *Ixodes scapularis*, the blacklegged tick, was present but in such low numbers that we did not analyze further. In the 436 individual adult lone star ticks screened, infection rates were 6% for *B. lonestari*, 19% for *E. chaffeensis*, 3% for *E. ewingii*, 36% for *R. amblyommatis,* and 1% for *R. montanensis* (Table 2). In the 16 adult lone star pools, infection rates were 1.3% (CI 0.1-6) for *E. chaffeensis,* 1.3% (CI 0.1-6) for *E. ewingii*, and 27% (CI 15.7-44.6) for *R. amblyommatis* (data not shown). In the 189 individual adult American dog ticks screened, infection rates were 19% for *E. chaffeensis*, 15% for *E. ewingii*, 5% for *R. amblyommatis*, and 4% for *R. montanensis* (Table 2). In the four American dog tick pools, infection rate was 5% (CI 0.3-23.5) for *E. chaffeensis* and no other bacterial species were detected (data not shown). Infection rates for the 30 nymph lone star pools were calculated as *B. lonestari* (0.3%, CI 0.1-0.9), *E. chaffeensis* (2.4%, CI 1.4-4.0), *E. ewingii* (0.3%, CI 0.1-0.9), and *R. amblyommatis* (3.5%, CI 2.2-5.6) (data not shown).

Both species of tick were most frequently encountered in unmanaged sites vs managed sites (Table 3). Unmanaged sites had similar prevalence of pathogens in ticks collected compared to managed sites with the exception of *B. lonestari*, which was more prevalent in unmanaged sites (Table 3). All four collection sites had *B. lonestari* infections present with the highest prevalence being detected at Montgomery Woods (Table 2). *Borrelia lonestari* infections were present in both adult and nymph lone star ticks, however, none were detected in

Table 3. Comparing the presence of *Borrelia*, *Ehrlichia* and *Rickettsia* spp. in managed vs unmanaged conservation areas in Northeast Missouri in individual adult ticks

Amblyomma americanum (n=436)					
Site	*B. lonestari*	*E. chaffeensis*	*E. ewingii*	*R. amblyommatis*	*R. montanensis*
Managed (n=160)	4 (3%)	35 (22%)	2 (1%)	61 (38%)	5 (3%)
Unmanaged (n=276)	22 (8%)	49 (18%)	9 (3%)	97 (35%)	1 (0.04%)
Dermacentor variablilis (n=189)					
Site	*B. lonestari*	*E. chaffeensis*	*E. ewingii*	*R. amblyommatis*	*R. montanensis*
Managed (n=63)	0	11 (17%)	12 (19%)	3 (5%)	4 (6%)
Unmanaged (n=126)	0	24 (19%)	16 (13%)	6 (5%)	3 (2%)
Total of both species (n=625)					
Site	*B. lonestari*	*E. chaffeensis*	*E. ewingii*	*R. amblyommatis*	*R. montanensis*
Managed (n=223)	4 (2%)	46 (21%)	14 (6%)	64 (29%)	9 (4%)
Unmanaged (n=402)	22 (5%)	73 (18%)	25 (6%)	103 (26%)	4 (1%)

the American dog ticks. Nine samples positive for *Borrelia lonestari* were sequenced and all nine samples were 99 - 100% identical to *B. lonestari* (GenBank accession number AY363709.1).

Ehrlichia infections were present at all four collection sites and all life stages of ticks collected. *E. chaffeensis* infection in adult lone star ticks was most prevalent in Big Creek (Table 2). In American dog ticks *E. chaffeensis* was the most prevalent bacteria species detected. Six positive samples for *E. chaffeensis* were sequenced and four were 99% identical while the other two were 100% identical to *E. chaffeensis* (strain West Paces; GenBank accession number CP007480.1). *Ehrlichia ewingii* was less prevalent in tick populations when compared to *E. chaffeensis*. Three samples positive for *E. ewingii* were sequenced and all three samples were 99% identical to *E. ewingii* Panola Mtn. (GenBank accession number DQ365880.1).

All four sites had *Rickettsia amblyommatis* present (Table 2). Prevalence of *R. amblyommatis* was higher than any other bacteria species detected in the lone star tick. Three *R. amblyommatis* positive samples were 100% identical to *R. amblyommatis* (GenBank accession number CP015012.1). *Rickettsia montanensis* was not detected in Montgomery Woods and was more commonly detected in managed sites. Two positive samples for *R. montanensis* were sequenced and found 99% identical to *R. montanensis* (strain Montana; GenBank accession number U55823.1).

Discussion

Screening for potential pathogens in Adair County has provided insight into the distribution of tick species and the pathogens they may carry. Because tick populations and associated pathogens can vary, a better understanding of the pathogens carried by ticks in a given geographic area can alert health care providers of specific health risks leading to better diagnosis and treatments.

Borrelia lonestari is a putative agent of STARI and we detected a 6% infection rate in adult lone star ticks. Other studies have reported a 1 - 3% prevalence of lone star ticks containing the spirochete *B. lonestari* which

is slightly lower than our findings.[9,17] It is important to note, that no *Borrelia* species in lone star ticks have been confirmed to be pathogenic to humans.[18] Lone star ticks have been shown repeatedly unable to vector *B. burgdorferi*, the etiological agent of Lyme disease, and is thus not considered a viable host to this well-known tick-borne disease.[19]

Ehrlichia chaffeensis and *E. ewingii* are causative agents for human ehrlichiosis and are the only known pathogens detected in our study. Lone star ticks are the primary vector of both these bacteria in the United States. Typical symptoms include fever, headache, fatigue, and muscle aches but this disease can be fatal. Previous reports of prevalence of *Ehrlichia* spp. in Southwest Missouri range from 3-10% infection rate.[20] Other endemic states have reported prevalences ranging from 2-18%.[17, 21-22] Our data reports on the high end of prevalence which may be a local phenomenon or an increase of infection over time. The overall rate of reported ehrlichiosis in the United States has increased 4-fold since 2000.[23] Missouri has the second highest reported incidence rate of *E. chaffeensis* following Oklahoma and tied for the highest incidence rate of *E. ewingii*.[23]

For both years, no *R. rickettsii*, the agent of Rocky Mountain Spotted Fever (RMSF), was detected. *Rickettsia amblyommatis* has been implicated as a cause of RMSF-like disease in humans and has been associated with a rash following a lone star tick bite.[24-25] Reports of RMSF disease occur in Adair County, so either we are just simply not collecting enough ticks to detect *R. rickettsii* or this data is further evidence that where lone star ticks are the predominant tick species perhaps *R. amblyommatis* can cause a RMSF-like disease.[26] *Rickettsia montanensis* is another bacteria within the spotted fever group (SFG) rickettsia that may also cause mild RMSF-like disease.[27] If *R. montanensis* can cause a rash then it is very likely that it could lead to misdiagnosis because rickettsial serologic assays are not able to distinguish between species of the SFG.[28-29] Further evidence is needed to confirm if either *R. amblyommatis* or *R. montanensis* is of any medical concern.

No Heartland virus was detected in our survey. We are not yet confident to state that the virus is not present in the area as it has been found at very low prevalence in northwestern Missouri.[7] Savage et al. report infection rates of one per 585 nymphs and 1 per 1,000 adults and we tested a total of 750 nymphs and 100 adults for the virus. More testing would need to be done to confirm absence or presence of virus.

In comparing managed sites (Big Creek and Sugar Creek) with unmanaged sites (Shoemaker and Montgomery Woods) the number of ticks was nearly double in the unmanaged sites. With the prevalence of tick-borne pathogens being more or less similar between managed vs unmanaged sites, the fact that tick numbers are nearly double in non-managed sites makes an argument that some form of management can reduce the risk of potential disease transmission. Future studies on management techniques and their influence on reducing either number of ticks or pathogen prevalence is desirable.

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Disclosure

None reported. **MM**