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Detection of *Borrelia*, *Ehrlichia*, and *Rickettsia* spp. in ticks in northeast Missouri

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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. We collected actively questing ticks from four sites within Adair County, Missouri. A total of 15,162 ticks were collected, of which 13,980 were grouped in 308 pools (lone star ticks, 288 pools; American dog ticks, 20 pools) and tested for presence/absence of bacteria using polymerase chain reaction. Infection rates were calculated as the maximum likelihood estimation (MLE) with 95% confidence intervals (CI). Of the 308 pools tested, 229 (74.4%) were infected with bacteria and the overall MLE of the infection rate per 100 ticks was calculated as 2.9% (CI 2.61–3.21). Infection rates varied among life stages, 28.6% (CI 23.89–33.97) in adults, 7.0% (CI 5.10–9.86) in nymphs, and 1.0% (CI 0.75–1.20) in larvae. In the 116 adult lone star pools, infection rates were calculated for *Borrelia lonestari* (1.4%), *Borrelia* spp. (2.7%), *Ehrlichia chaffeensis* (6.1%), *Ehrlichia ewingii* (3.3%), *Rickettsia amblyommii* (18.3%), and *Rickettsia montanensis* (0.4%). Infection rates for the 52 nymphal lone star pools were calculated as *B. lonestari* (1.03%), *Borrelia* spp. (0.40%), *E. chaffeensis* (2.02%), *E. ewingii* (0.24%), and *R. amblyommii* (2.70%). In the 20 adult American dog tick pools, infection rates were determined as *E. chaffeensis* (9.47%), *E. ewingii* (5.47%), and *R. montanensis* (8.06%). Eight *Borrelia* samples were sequenced with five 99–100% identical to *B. burgdorferi* (s.l.) and three 99% identical to *B. lonestari*. Eight samples were sequenced for *E. chaffeensis* (all 99–100% identical) and one sample was sequenced for *E. ewingii* (99% identical). Seven samples were sequenced for *Rickettsia* and three were 99% identical to *R. montanensis* and four were 100% identical to *R. amblyommii*. This study demonstrates *B. lonestari*, *E. chaffeensis*, *E. ewingii*, *R. amblyommii*, and *R. montanensis* in northeast Missouri ticks for the first time. Understanding the presence and epidemiology of these causative (*E. chaffeensis* and *E. ewingii*) and suspected (*B. lonestari* and *R. amblyommii*) agents in Missouri should increase awareness of potential tick-borne disease in the medical community.

1. Introduction

There are several species of ticks in Missouri capable of transmitting tick-borne pathogens to humans. The most common ticks in Missouri associated with pathogen transmission (in descending order of abundance) are the lone star tick, *Amblyomma americanum*, (Linnaeus), American dog tick, *Dermacentor variabilis* (Say), and the blacklegged tick, *Ixodes scapularis* (Say). All of these tick species are capable of transmitting one or more zoonotic pathogens (CDC, 2015). Missouri is one of five states that accounts for over 60% of all Rocky Mountain spotted fever (RMSF) cases and one of three states that accounts for 30% of all reported *Ehrlichia chaffeensis* infections (CDC, 2013a,b). While the vectors of most tick-borne pathogens are generally known and accepted, there are some cases in which the causative agent of a particular disease is unclear and/or the capability of alternative tick vector species to maintain and transmit a pathogen is poorly understood (Ostfeld, 2011).

Because tick populations and associated pathogens can vary, there is a need to understand tick population dynamics within a particular region (Ogden et al., 2013). Such knowledge provides valuable insight into potential threats to public health that could not necessarily be inferred by knowledge based on data from other regions. For example, there is still great uncertainty regarding the occurrence of Lyme disease and the newly emerging STARI (southern tick-associated rash illness) in Missouri. *Borrelia burgdorferi* sensu lato (s.l.) was first reported to be isolated and cultivated from Missouri in 1995 from ticks that had been feeding on cottontail

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rabbits (*Sylvilagus floridanus*) (Oliver et al., 1998). Even though possibly present in the tick population, *B. burgdorferi* (s.l.) has not yet been isolated from any patient in Missouri showing symptoms consistent with Lyme disease (DHSS, 2009). Similarly, physicians in the south central and southeastern United States have observed Lyme disease-like rashes (STARI) on patients with a recent history of tick bite (Masters et al., 1998). *A. americanum* is associated with STARI, but causation has not been proven. While there is evidence that *Borrelia lonestari* or *Rickettsia amblyommii* may be a causative agent of STARI, the evidence is not definitive. Specifically, James et al. (James et al., 2001) detected *B. lonestari* DNA in an attached tick and skin biopsy of a presumed STARI patient. However, subsequent studies have not been able to detect *B. lonestari* in STARI patients (Wormser et al., 2005; Masters et al., 2008). More recently, serologic evidence of *R. amblyommii* has been detected in some STARI patients, but definitive evidence is lacking (Billeter et al., 2007; Apperson et al., 2008; Nicholson et al., 2009; Parola et al., 2009). Therefore, to manage tick-borne diseases and decrease infection risk, improved understanding of tick populations and tick-borne pathogen presence is needed.

Adair County is a rural landscape encompassing 567 square miles with a 2012 population estimate of 26,000 residents. Many people spend significant time outdoors in the rural landscape due to farming and recreation activities, and because of its rural nature, many homes are in direct contact with wooded areas and grasslands. For these reasons, direct contact with ticks is common in Adair County, which could lead to increased risk of tick-borne disease. Many tick-borne diseases are under-reported due to patients not seeking medical attention or their symptoms being incorrectly diagnosed (Dumler, 2011). In the state of Missouri from 2009 to 2011, 503 cases of ehrlichiosis, 801 cases of RMSF, and 23 cases of Lyme disease were reported (DHSS, 2012). However, these reports were typically not supported by isolation and identification of the agent, and never in the case of Lyme disease. Since ticks are abundant in Adair County, suggesting a high risk of tick-borne disease, we determined the presence/absence of human pathogens, i.e., *Borrelia*, *Ehrlichia*, and *Rickettsia*, in the tick population of Adair County, northeastern Missouri.

2. Materials and methods

2.1. Tick collections

In 2014, ticks were collected from April through September at four conservation sites in Adair County (Fig. 1). Collections were conducted between 1000 and 1800 h. Each collection site was sampled once per month for 6 months for a total of 24 collection days. On rainy days or when vegetation was wet, alternate days were used for tick collection. Sites were selected to represent the most common distinct plant community types in Adair County including oak-hickory forests, old fields and cropland, riparian, and native grasslands. Big Creek (1064 acres) and Sugar Creek Conservation area (2600 acres) are managed on a routine basis. Big Creek tick collections occurred in edge habitat along forest and old field or food plot habitats where both burns and disking were conducted. Sugar Creek Conservation Area is primarily a younger managed forest and ticks were collected in a thin and release timber stand. Montgomery Woods (348 acres) and Shoemaker Conservation areas (259 acres) are much smaller in size and no habitat manipulations were conducted during 2014. Tick sampling in Montgomery Woods occurred in mature oak-hickory hardwood forest and for Shoemaker Conservation area in edge habitat of oak-hickory stands bordered with native grasslands.

Questing ticks were collected using a cloth drag-flag method (Carroll and Schmidtmann, 1992), carbon dioxide-baited traps, and by removing ticks from the collector's clothing. Ticks were stored in 70% ethanol until subjected to DNA extraction. All collected ticks were identified to species using a dissecting microscope and with reference to standard taxonomic keys (Brinton et al., 1965; Keirans and Litwak, 1989; Durden and Keirans, 1996; Keirans and Durden, 1998). Ticks were grouped into pools by site, collection date, species, sex, and life stage. Adult ticks were bisected laterally using aseptic technique and sterile scalpel blades, and half of each adult tick was stored in 100% ethanol at −80 °C for future analysis, if needed. Tick pools contained 100 larvae, 25 nymphs, or the halves of 5 adult ticks.

2.2. DNA extraction

An entire tick pool (as described above), i.e., a tick sample, was removed from ethanol, allowed to dry, and placed in a 1.5-ml microfuge tube containing 120 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, each tick sample was crushed using a motorized pestle mixer with sterile pestles. The resulting tick homogenates were incubated at room temperature for 4–6 h, heated at 95 °C for 10 min, and centrifuged at 10,000*g* for 10 min at room temperature as described (Schulze et al., 2011). An 80-μl volume of each supernatant was transferred to a 1.5-ml microfuge tube for DNA isolation. DNA was precipitated by the addition of 40 μl of 100% ethanol, followed by gentle inversion and incubation for 10 min at room temperature. DNA precipitates were pelleted by centrifugation at 16,000*g* for 10 min. Each DNA pellet was washed twice with 75% ethanol and dissolved in 35 μl of Tris-low-EDTA buffer (10 mM Tris-HCl pH 8.0 and 0.1 mM EDTA) before storage at 4 °C. Two samples, which contained no DNA, were added to each extraction line, one at the beginning and one at the end, as a quality control. This procedure indicated that no contamination occurred during our extraction process. To verify the quantity and quality of DNA extraction, 260/280 and 260/230 nm absorbance ratios were detemined in samples using a spectrophotometer (NanoDrop® ND-2000c, ThermoScientific, Wilmington, DE) according to manufacturer's instructions.

2.3. Polymerase chain reaction (PCR)

PCR-based assays were used to amplify and identify bacterial DNA within pooled tick samples. PCR reactions of 50 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 (0.3–9 μl). PCR reactants were kept on ice until transferred to a thermal cycler (PTC-100, MJ Research Inc.). Analysis of each DNA sample was dependent upon two screens using PCR amplification. Screen-I PCR used pairs of oligonucleotide primers designed to amplify group-specific DNA targets, i.e., for *Borrelia* spp., the flagellin gene (Barbour et al., 1996); for *Ehrlichia* spp., the 16S rRNA gene (Schulze et al., 2011); for *Rickettsia* species the *gltA* gene (Parola et al., 2005) (Table 1). Thermal cycling parameters for Screen-I PCRs of bacterial genes included an initial 15-min 95 °C denaturation step, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and a single, final 72 °C extension step for 5 min.

Screen-I PCR product samples were subjected to 1% agarose gel electrophoresis along with a standardized DNA-Ladder, and both positive and negative controls. Negative controls consisted of water in place of DNA. Positive controls consisted of known DNA samples: *Borrelia* (KPL, Gaithesburg, MD), *Ehrlichia*, and *Rickettsia* (CDC, Atlanta, GA). All positive samples from Screen-I PCRs

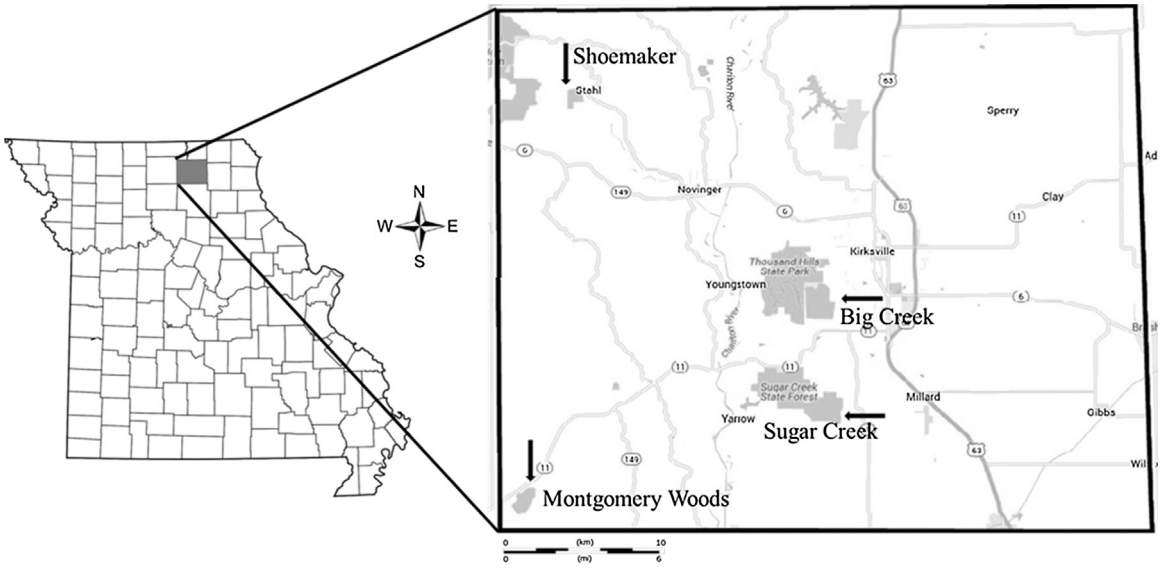

Fig. 1. Map of Adair County, Missouri indicating the four conservation sites where tick collections occurred from April to September 2014.

Table 1
Nucleotide sequence of primers used for pcr analysis of dna extracted from pooled tick specimens.

Primer name	Gene	Sequence (5′ → 3′)	Screen	Amplicon size (bp)	Reference
Borrelia spp.					
FLaLL	*flagellin*	ACA TAT TCA GAT GCA GAC AGA GGT	Primary		
FlaRL	*flagellin*	GCA ATC ATA GCC ATT GCA GAT TGT	Primary	641	Barbour et al. (1996)
FlaLS	*flagellin*	AAC AGC TGA AGA GCT TGG AAT G	Nested		
FlaRS	*flagellin*	CTT TGA TCA CTT ATC ATT CTA ATA GC	Nested	330	Barbour et al. (1996)
Fla522F	*flagellin*	GGT ACA TAT TCA GAT GCA GAC AGA GGG	Primary for *B. lonestari*		
Fla1182R	*flagellin*	GCA CTT GAT TTG CTT GTG CAA TCA TAG CC	Primary for *B. lonestari*	661	Williamson et al. (2010)
Ehrlichia spp.					
ECC	16S	AGA ACG AAC GCT GGC AAG CC	Primary		
ECB	16S	CGT ATT ACC GCG GCT GGC A	Primary	490	Schulze et al. (2011)
HE1	16S	CAA TTG CTT ATA ACC TTT TGG TTA TAA AT	Nested		
HE3	16S	TAT AGG TAC CGT CAT TAT CTT CCC TAT	Nested	389	Schulze et al. (2011)
EWF1	16S	TCG AAC GAA CAA TTC CTA AA	Nested		
HE3	16S	TAT AGG TAC CGT CAT TAT CTT CCC TAT	Nested	403	Schulze et al. (2011)
Rickettsia spp.					
RpCS.877P	*gltA*	GGG GGC CTG CTC ACG GCG G	Primary		
RpCS.1258n	*gltA*	ATT GCA AAA AGT ACA GTG AAC A	Primary	381	Parola et al. (2005)
	ompA	ATG GCG AAT ATT TCT CCA AAA	Secondary		
Rr190.602n	*ompA*	AGT GCA TTC GCT CCC CCT	Secondary	532	Regnery et al. (1991)

(appropriately-sized bands visualized on a gel) were tested further in Screen-II using additional PCR amplifications.

For Screen-II, nested PCR was conducted for *Borrelia* and *Ehrlichia* to amplify a secondary target within the Screen-I product to ensure specificity. For the nested PCR, 5 μl of the primary product was used as template in a 50-μl reaction containing the same PCR components with the exception of the primers. Thermal cycler parameters for nested-stage PCR were: an initial 15-min 95 °C denaturation step, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and a single, final 72 °C extension for 5 min *Borrelia* nested primers were designed to amplify a ~330 bp fragment of the *flagellin* gene (*flaB*). Nested primers for *E. chaffeensis* and *E. ewingii* were designed to amplify a 389 bp and a 403 bp fragment, respectively, within the 16S rDNA fragment.

Rickettsia Screen-II primers were not nested within the *gltA* gene, but targeted the *ompA* gene in the original tick DNA extract (Table 1) and underwent the same PCR conditions as outlined in Screen-I. Thus, all tick DNA samples that tested positive for presence of the *gltA* gene were also tested for presence of the *ompA* gene (Regnery et al., 1991).

All Screen-II PCR product samples were subjected to 1% agarose gel electrophoresis (TAE Buffer) as described for small product lengths (Sambrook et al., 1989). Samples still positive for *Borrelia* spp. after Screen-II were subjected to a primer set specific to *B. lonestari* (*flaB*). *Rickettsia* DNA samples that remained positive for the second amplification (532-bp sequence) were digested with *PstI* (Promega, Madison, WI) for restriction fragment length polymorphism (RFLP) analysis. Enzymatic digestion was performed by incubating 2.0 μl of the amplification product with 1.0 μl of enzyme buffer and 5 U of *PstI* endonuclease for 2 h at 37 °C before sizing products with 2% agarose gel electrophoresis (Eremeeva et al., 1994; Stromdahl et al., 2008).

For quality control, PCR was performed on tick material spiked with known *Borrelia* (KPL, Gaithesburg, MD), *Ehrlichia*, and *Rickettsia* (CDC, Atlanta, GA) DNA to determine the accuracy of our techniques. Quantities of DNA spiked ranged from 10 ng – 0.625 ng per 50 μl reaction and all quantities were successfully detected through the use of agarose gel electrophoresis. 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 (*B. lonestari*,

Table 2
Numbers and life stages of tick species collected at four conservation areas in adair county, Nissouri, Npril to September 2014.

Tick Species	Number collected (% of total collected within each species)				
	Females	Males	Nymphs	Larvae	Total
A. americanum	378 (2.5)	323 (2.2)	1622 (10.8)	12,644 (84.5)	14,967
D. variabilis	79 (42.2)	107 (57.2)	1 (0.6)	0	187
I. scapularis	3 (37.5)	4 (50)	1 (12.5)	0	8

Table 3
Numbers and life stages of tick species from four conservation areas in adair county, Missouri, April to September 2014.

Location	*Amblyomma americanum*				*Dermacentor variabilis*	
	Females	Males	Nymphs	Larvae	Females	Males
Big Creek	65	53	103	3415	37	61
Mont. Woods	104	74	426	2400	4	5
Sugar Creek	87	72	389	3528	9	4
Shoemaker	122	124	704	3301	29	37

Borrelia spp., *E. chaffeensis*, *E. ewingii*, *R. amblyommii*, and *Rickettsia montanensis montanensis*) were purified with QIAquick-Spin PCR columns (QIAGEN, Valencia, CA), and verified by commercial DNA sequencing (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). In all BLASTN (2.3.0+) searches, the targeted organism always had the lowest E (Expect) value, i.e., E values ranged from 9e-159 to 2e-175, the lower the E-value the more "significant" the match (Zhang et al., 2000).

The minimum infection rate (MIR) for pooled ticks is the number of positive pools per total number of pools and assumes that each positive pool of ticks contains only one infected tick. Infection rates in tick pools were also 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 (Biggerstaff, 2005). MLE calculations are based on the number of pools, pool sizes (number of ticks per pool), and number of positive pools.

3. Results

A total of 15,162 ticks were collected (Table 2). Lone star ticks (*A. americanum*) comprised 99% of all collected ticks, while American dog ticks (*D. variabilis*) comprised 1% (*I. scapularis* ticks were a rare presence and not analyzed further). Ticks were collected at four conservation areas and the species and life stage composition, according to collection site, are shown in Table 3.

For further analysis, ticks were pooled by collection site following a formula of 100 larvae, 25 nymphs, or the halves of 5 adult ticks per pool, such that a total of 13,980 ticks were grouped in a total of 308 pools. *A. americanum* yielded a total of 288 pools, i.e., 116 (40%) pools of adults, 52 (18%) pools of nymphs, and 120 (42%) pools of larvae, while *D. variabilis* yielded only 20 pools of adults (Table 4).

Of those 308 pooled samples, screen-I produced 67 *Borrelia* spp., 128 *Ehrlichia* spp., and 234 *Rickettsia* spp. positive samples. Forty samples (60%) remained positive for *Borrelia* spp., 101 samples (79%) remained positive for *Ehrlichia* spp., and 178 samples (76%) remained positive for *Rickettsia* spp. post Screen-II. Of 308 total tick pools, 229 (74% pools positive) contained one or more target bacteria. Of the 229 pools containing bacteria, the descending order of abundance was: *R. amblyommii* (74% pools positive), *E. chaffeensis* (32% pools positive), *E. ewingii* (12% pools positive), *B. lonestari* (9% pools positive), and *R. montanensis* (4% pools positive) (Table 4).

All four collection sites had *Borrelia* spp. infections present. However, *B. lonestari* was only confirmed in Shoemaker and Montgomery Woods (Table 5). *Borrelia* infections were present in both adult and nymphal lone star ticks, however, none were detected in the larvae pools (Table 4). Eight samples positive for *Borrelia* spp. were sequenced, all collection sites were represented. Four samples were 99% identical to "*B. burgdorferi*" (strains B31, CA382, and M11p; GenBank accession numbers CP009656.1, CP005925.1 and KM269447.1) and one sample was 100% identical to "*B. burgdorferi*" (strain N40; GenBank accession number CP002228.1). The remaining three samples were 99% identical to "*B. lonestari*" V-2002 (GenBank accession number AY850064.1). The NCBI defines sequences that show 99% identity or greater as typically the same species.

Ehrlichia infections were present at all four collection sites and all life stages of ticks collected. Sugar Creek had the highest prevalence of *E. chaffeensis,* and Montgomery Woods had the highest prevalence of *E. ewingii* (Table 5). Eight samples positive for *E. chaffeensis* were sequenced, all collection sites were represented. Six samples were 99% identical and two were 100% identical to *E. chaffeensis* (strain West Paces; GenBank accession number CP007480.1). We sent one positive *E. ewingii* sample to be sequenced, the sample was 99% identical to *E. ewingii* Panola Mtn. (GenBank accession number DQ365880.1). Five of our 120 larval pools tested positive for *Ehrlichia*, four for *E. chaffeensis* and one for *E. ewingii*. Montgomery Woods, Big Creek, and Sugar Creek all had one sample positive, while Shoemaker had two samples positive. It is important to note that none of the collected larvae displayed any engorgement. Three of the *E. chaffeensis* positive pooled larvae DNA samples were sent for sequence verification and all three were confirmed for *E. chaffeensis* (West Paces 99% identity, GenBank accession number CP007480.1).

All four sites had *R. amblyommii* present and Big Creek had the highest prevalence (Table 5). Seven positive *Rickettsia* samples were sequenced, all collection sites were represented. Three samples positive for *R. montanensis* were 99% identical to *R. montanensis* (strain Montana; GenBank accession number U55823.1) and the remaining four *R. amblyommii* positive samples were 100% identical to *R. amblyommii* (GenBank accession numbers EF063690.1 and EF689733.1).

4. Discussion

B. lonestari is present in lone star ticks in Adair County. In addition, it appears that another species of *Borrelia* is also present and we are currently investigating this finding. Only the *flagellin* gene was analyzed and due to the recent findings of Stromdahl et al. (2015), we are not yet confident to state any specific species. It is now understood that these nested primers may result in non-specific amplification and thus are unable to differentiate between closely-related *Borrelia* species. There has been some evidence that lone star ticks can transovarially transmit *B. lonestari* (Stromdahl et al., 2008; Killmaster et al., 2014), however, we found no evidence of transovarial transmission in Adair County as no *Borrelia* species were detected in any of the larvae pools (Table 4). Our data on the presence of *B. lonestari* in lone star ticks of Adair County add to the limited number of studies conducted in Missouri to detect *Borrelia* in ticks (Feir et al., 1994; Bacon et al., 2003). It is important to note, that no *Borrelia* species in lone star ticks have yet been confirmed to be pathogenic to humans (Stromdahl et al., 2015).

In southwest Missouri, *E. chaffeensis* and *E. ewingii* have been reported in 9.8% and 5.4% of adult lone star ticks, and in 6.7% and 3.3% of American dog ticks, respectively (Steiert and Gilfoy,

Table 4
Numbers of tick pools demonstrating bacterial pathogens in tick species in adair county, Missouri, April to September 2014.

Total no. of pools	*Amblyomma americanum* (288 pools[a])				*Dermacentor variabilis* (20 pools[a])	
	Females	Males	Nymphs	Larvae	Females	Males
	63	53	52	120	7	13
Borrelia spp.	8	7	5	0	0	0
B. lonestari	6	2	12	0	0	0
E. chaffeensis	32	9	21	4	3	5
E. ewingii	16	2	3	1	1	4
R. amblyommii	43	31	26	69	0	0
R. montanensis	2	0	0	0	3	4
No. of pools with bacteria (% of total)	59 (94%)	36 (68%)	44 (85%)	74 (58%)	5 (71%)	11 (85%)

[a] Tick pools contained 100 larvae, 25 nymphs, or the halves of 5 adult ticks (total pools = 308).

Table 5
The presence of *Borrelia*, *Ehrlichia* and *Rickettsia* spp. in adult *Amblyomma americanum* in northeast Missouri.

	Borrelia lonestari			*Rickettsia amblyommii*		
	MIR[a] % Pos.	MLE[b]		MIR[a] % Pos.	MLE[b]	
Site		% Pos.	(95% CI)		% Pos.	(95% CI)
Big Creek	0.00	0.00	(0.00–3.79)	18.89	39.66	(24.91–68.25)
Montgomery Woods	3.40	3.66	(1.38–7.96)	13.10	18.76	(12.01–28.15)
Sugar Creek	0.00	0.00	(0.00–2.63)	11.11	14.66	(8.80–23.09)
Shoemaker	1.40	1.46	(0.39–3.90)	10.95	14.47	(9.63–20.96)
	Ehrlichia chaffeensis			*Ehrlichia ewingii*		
	MIR[a] % Pos.	MLE[b]		MIR[a] % Pos.	MLE[b]	
Site		% Pos.	(95% CI)		% Pos.	(95% CI)
Big Creek	3.33	3.50	(0.94–9.28)	2.22	2.27	(0.41–7.32)
Montgomery Woods	4.83	5.29	(2.38–10.27)	6.90	7.98	(4.16–13.92)
Sugar Creek	10.37	13.32	(7.83–21.32)	0.74	0.74	(0.04–3.55)
Shoemaker	8.10	9.74	(5.99–15.00)	2.38	2.48	(0.93–5.41)

[a] MIR (minimum infection rate) for pooled ticks assumes that only one tick per pool is infected.
[b] MLE (maximum likelihood estimation; from Pooled Infection Rate calculator, Excel add-In) estimates the maximum likely infection rate (%) based on the number of pools, number of ticks per pool, and number of positive pools and provides a 95% confidence interval (CI) on the estimate (Biggerstaff, 2005).

2002). In Macon County, just south of Adair County in northeast Missouri, two of the four adult ticks collected were positive for *E. chaffeensis* (Roland et al., 1998). In our study for Adair County, we report *E. chaffeensis* and *E. ewingii* infection rates of 8.32% (CI 6.12–11.06) and 3.30% (CI 2.04–5.08) in adult lone star ticks, and 9.47% (CI 4.56–17.53) and 5.47% (CI 2.08–11.83) in American dog ticks, respectively. Lone star nymphs had an infection rate of 2.15% (CI 1.39–3.22) for *E. chaffeensis* and 0.24% (CI 0.06–0.64) for *E. ewingii*. A study conducted in southeastern Virginia reported *E. chaffeensis* and *E. ewingii* in 7.8% and 18.3% of adult lone star ticks, respectively, 0% of either *Ehrlichia* species in American dog ticks, and 2.1% and 3.2% for *E. chaffeensis* and *E. ewingii*, respectively, in lone star nymphs (Wright et al., 2014). Nebraska reported lower infection rates for both *E. chaffeensis* and *E. ewingii* at 1.6% (Maegli et al., 2016) as well as northeastern Georgia with a 2.0% infection rate for *E. chaffeensis* and 4.8% infection rate for *E. ewingii* in adult lone star ticks (Varela et al., 2004). Mixson et al. (2006) surveyed lone star ticks in nine states and found the overall prevalence of 4.7% for *E. chaffeensis* and 3.5% for *E. ewingii*. Overall, the prevalence of infection of lone star ticks in our study was consistent with previous findings where the disease is endemic (Childs and Paddock, 2003). In addition, we have evidence of *Ehrlichia* being in five of our 120 larval pools, in contrast to earlier findings (Long et al., 2003; Castellaw et al., 2010). It is important to note that these five samples were collected at four different locations, in three separate months, and DNA was isolated on three separate dates. We are in the process of investigating this finding further.

In stark contrast, Missouri is endemic for RMSF, however no *R. rickettsii*, the agent of RMSF, was detected in any of our tick pools. The American dog tick (1% of our collected ticks) is considered an epidemiologically important vector of *R. rickettsii,* but it can be found occasionally in lone star ticks as well (Parola et al., 2005). It has been demonstrated that ticks infected with *Rickettsia peacockii* were refractory to infection with *R. rickettsii* (Burgdorfer et al., 1981). Interestingly, seven of the nine pools of American dog ticks contained *R. montanensis*, a nonpathogenic rickettsial species. Perhaps *R. montanensis* also impedes infection by *R. rickettsii* in the American dog tick. While human RMSF cases have increased dramatically since 2000 (Openshaw et al., 2010), overall tick surveys indicate that *R. rickettsii* is less prevalent in vector ticks, and, in most cases, the positive ticks harbor nonpathogenic rickettsia or at least species implicated in less severe illness (Azad and Beard, 1998). In 2015, Missouri reported RMSF to occur at a rate of 5.68 per 100,000 population (318 cases as of week 45), and two cases were confirmed in Adair County in 2012 (DHSS, 2012). Either we simply missed collecting any ticks in our study that were infected with *R. rickettsii*, or perhaps *R. amblyommii* can cause a RMSF-like disease. In support of the latter hypothesis, it has been proposed that some cases of RMSF in North Carolina may be due to *R. amblyommii* instead of *R. rickettsii* (Apperson et al., 2008; Smith et al., 2010). To date, no definitive role has been defined for *R. amblyommii* in human pathogenesis, but a recent study has shown that lone star ticks parasitizing humans were frequently infected with *R. amblyommii* (Jiang et al., 2010). Lone star ticks collected from Missouri and Kansas had a greater than 90% infection rate of *R. amblyommii* DNA (Berrada et al., 2011). *R. amblyommii* was the most prevalent bacterium found in this study and perhaps is also playing a role in RMSF-like disease in Adair County.

Screening ticks for a range of bacterial agents has given some insight regarding distributions of tick species and potentially

pathogenic agents present in those tick species. The most frequently encountered tick in our study was the lone star tick. Lone star ticks are three-host ticks that obtain their blood meals from various vertebrate hosts. All stages of these aggressive, generalist feeders are commonly found attached to humans (Merten and Durden, 2000). Lone star ticks were most prevalent in the Shoemaker conservation area, the smallest area in our study (259 acres). Tick sampling in the Shoemaker conservation area occurred in the edge habitat of native grasslands and oak-hickory wooded hills. Perhaps this edge habitat, where habitat management did not occur, invites increased numbers of potential hosts thus explaining the highest prevalence of lone star ticks. In contrast, 53% of all American dog ticks were collected at Big Creek conservation area with an *Ehrlichia* infection rate of 17.77% (CI 9.13–31.80). This habitat is similar to Shoemaker with the exception that it is highly managed with both controlled burns and feed plots.

The distribution of pathogens at our four collection sites was not homogeneous, indicating several hot spots where intervention may be warranted. For instance, the Montgomery Woods conservation area is the second to smallest area in our study (348 acres) and yet had the highest prevalence of both *B. lonestari* and *E. ewingii*. In Montgomery Woods, no male lone star ticks were infected with *E. ewingii,* but nearly 59% of the female tick pools were positive.

Continued study and monitoring is warranted to establish the "normal" prevalence of human pathogens, because yearly variations among tick populations and infection rates are likely to occur due to climatic conditions, host density, and management of habitat. By screening ticks in Adair County for the presence/absence of these bacterial pathogens, we have not only determined the presence of all three pathogens, but have also implicated high prevalence.

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References

Apperson, C.S., Engber, B., Nicholson, W.L., Mead, D.G., Engel, J., Yabsley, M.J., Dail, K., Johnson, J., Watson, D.W., 2008. Tick-borne diseases in North Carolina: is *Rickettsia amblyommii* a possible cause of rickettsiosis reported as Rocky Mountain spotted fever? Vector Borne Zoonotic Dis. 8, 597–606.

Azad, A.F., Beard, C.B., 1998. Rickettsial pathogens and their arthropod vectors. Emerg. Infect. Dis. 4, 179–186.

Bacon, R.M., Gilmore Jr., R.D., Quintana, M., Piesman, J., Johnson, B.J., 2003. DNA evidence of *Borrelia lonestari* in *Amblyomma americanum* (Acari: ixodidae) in southeast Missouri. J. Med. Entomol. 40, 590–592.

Barbour, A.G., Maupin, G.O., Teltwo, G.J., Carter, C.J., Piesman, J., 1996. Identification of an uncultivable *Borrelia* species in the hard tick *Amblyomma americanum*: possible agent of a Lyme disease-like illness. J. Infect. Dis. 173, 403–409.

Berrada, Z.L., Goethert, H.K., Cunningham, J., Telford 3rd, S.R., 2011. *Rickettsia rickettsii* (Rickettsiales: rickettsiaceae) in *Amblyomma americanum* (Acari: ixodidae) from kansas. J. Med. Entomol. 48, 461–467.

Biggerstaff, B., 2005. PooledInfRate software. Vector Borne Zoonotic Dis. 5, 420–421.

Billeter, S.A., Blanton, H.L., Little, S.E., Levy, M.G., Breitschwerdt, E.B., 2007. Detection of *Rickettsia amblyommii* in association with a tick bite rash. Vector Borne Zoonotic Dis. 7, 607–610.

Brinton, E.P., Beck, D.E., Allred, D.M., 1965. Identification of the Adults, Nymphs, and Larvae of Tick of the Genus *Dermacentor Koch* (Ixodidae) in the Western United States, vol. 5. Brigham Young University, Provo, UT.

Burgdorfer, W., Hayes, S.F., Mavros, A.J., 1981. Nonpathogenic rickettsiae in *Dermacentor andersoni*: a limiting factor for the distribution of *Rickettsia rickettsia*. In: Burgdorfer, W., Anacker, R.L. (Eds.), *Rickettsia* and *Rickettsial* Diseases. Academic Press, New York, N.Y.

CDC, 2013a. Statistics and Epidemiology, Rocky Mountain Spotted Fever (RMSF), http://www.cdc.gov/rmsf/stats (accessed 18.11.15).

CDC, 2013b. Annual Cases of Ehrlichiosis in the United States Centers for Disease Control and Prevention, http://www.cdc.gov/ehrlichiosis/stats (accessed 18.11.15).

CDC, 2015. Tickborne Diseases of the United States, http://www.cdc.gov/lyme/resources/tickborne.diseases.pdf (accessed 18.11.15).

Carroll, J.F., Schmidtmann, E.T., 1992. Tick sweep: modification of the tick drag-flag method for sampling nymphs of the deer tick (Acari: ixodidae). J. Med. Entomol. 29, 352–355.

Castellaw, A.H., Showers, J., Goddard, J., Chenney, E.F., Varela-Stokes, A.S., 2010. Detection of vector-borne agents in lone star ticks, *Amblyomma americanum* (Acari: ixodidae), from Mississippi. J. Med. Entomol. 47, 473–476.

Childs, J.E., Paddock, C.D., 2003. The ascendancy of *Amblyomma americanum* as a vector of pathogens affecting humans in the United States. Annu. Rev. Entomol 48, 307–337.

DHSS, 2009. Lyme Disease Position Paper, http://health.mo.gov/living/healthcondiseases/communicable/tickscarrydisease/ldpositionpaper.php (accessed 12.10.15).

DHSS, 2012. Data & Statistical Reports, Case Counts of Selected Communicable Diseases, 15-Year Report, http://health.mo.gov/living/healthcondiseases/communicable/communicabledisease/pdf/15yr.pdf (accessed 12.10.15).

Dumler, J.S., 2011. *Ehrlichia* and *Anaplasma*: Surveillance, Coinfection, and Research Needs. In: An Overview of Tick-Borne Disease, http://www.ncbi.nlm.nih.gov/books/NBK57013/ (accessed 12.10.15).

Durden, L.A., Keirans, J.E., 1996. Nymphs of the Genus *Ixodes* (Acari: Ixodidae) in the United States: Taxonomy, Identification Key, Distribution, Hosts, and Medical/veterinary Importance. Entomological Society of America, Lanham, MD.

Eremeeva, M., Yu, X., Raoult, D., 1994. Differentiation among spotted fever group rickettsiae species by analysis of restriction fragment length polymorphism of PCR-amplified DNA. J. Clin. Microbiol. 32, 803–810.

Feir, D., Santanello, C.R., Li, B.W., Xie, S., Masters, E., Marconi, R., Weil, G., 1994. Evidence supporting the presence of *Borrelia burgdorferi* in Missouri. Am. J. Trop. Med. Hyg. 51, 475–482.

James, A.M., Liveris, D., Wormser, G.P., Schwartz, I., Montecalvo, M.A., Johnson, B.J., 2001. *Borrelia lonestari* infection after a bite by an *Amblyomma americanum* tick. J. Infect. Dis. 183, 1810–1814.

Jiang, J., Yarina, T., Miller, M.K., Stromdahl, E.Y., Richards, A.L., 2010. Molecular detection of *Rickettsia amblyommii* in *Amblyomma americanum* parasitizing humans. Vector Borne Zoonotic Dis. 10, 329–340.

Keirans, J.E., Durden, L.A., 1998. Illustrated key to nymphs of the tick genus *Amblyomma* (Acari: ixodidae) found in the United States. J. Med. Entomol. 35, 489–495.

Keirans, J.E., Litwak, T.R., 1989. Pictorial key to the adults of hard ticks, family Ixodidae (Ixodida: ixodoidea), east of the Mississippi River. J. Med. Entomol. 26, 435–448.

Killmaster, L.F., Loftis, A.D., Zemtsova, G.E., Levin, M.L., 2014. Detection of bacterial agents in *Amblyomma americanum* (Acari: ixodidae) from Georgia, USA, and the use of a multiplex assay to differentiate *Ehrlichia chaffeensis* and *Ehrlichia ewingii*. J. Med. Entomol. 51, 868–872.

Long, S.W., Zhang, X., Zhang, J., Ruble, R.P., Teel, P., Yu, X.J., 2003. Evaluation of transovarial transmission and transmissibility of *Ehrlichia chaffeensis* (Rickettsiales: anaplasmataceae) in *Amblyomma americanum* (Acari: ixodidae). J. Med. Entomol. 40, 1000–1004.

Maegli, A., Loy, J.D., Cortinas, R., 2016. Note on *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *Borrelia lonestari* infection in lone star ticks (Acari Ixodidae), Nebraska, USA. Ticks Tick Borne Dis. 7, 154–158.

Masters, E., Granter, S., Duray, P., Cordes, P., 1998. Physician-diagnosed erythema migrans and erythema migrans-like rashes following Lone Star tick bites. Arch. Dermatol. 134, 955–960.

Masters, E.J., Grigery, C.N., Masters, R.W., 2008. STARI, or masters disease: lone star tick-vectored lyme-like illness. Infect. Dis. Clin. North Am. 22, 361–376.

Merten, H.A., Durden, L.A., 2000. A state-by-state survey of ticks recorded from humans in the United States. J. Vector Ecol. 25, 102–113.

Mixson, T.R., Campbell, S.R., Gill, J.S., Ginsberg, H.S., Reichard, M.V., Schulze, T.L., Dasch, G.A., 2006. Prevalence of *Ehrlichia*, *Borrelia*, and *Rickettsial* agents in *Amblyomma americanum* (Acari: ixodidae) collected from nine states. J. Med. Entomol. 43, 1261–1268.

Nicholson, W.L., Masters, E., Wormser, G.P., 2009. Preliminary serologic investigation of *Rickettsia amblyommii* in the aetiology of Southern tick associated rash illness (STARI). Clin. Microbiol. Infect. 15 (Suppl 2), 235–236.

Ogden, N.H., Mechai, S., Margos, G., 2013. Changing geographic ranges of ticks and tick-borne pathogens: drivers, mechanisms and consequences for pathogen diversity. Front. Cell Infect. Microbiol. 3, 46.

Oliver Jr., J.H., Kollars Jr., T.M., Chandler Jr., F.W., James, A.M., Masters, E.J., Lane, R.S., Huey, L.O., 1998. First isolation and cultivation of *Borrelia burgdorferi* sensu lato from Missouri. J. Clin. Microbiol. 36, 1–5.

Openshaw, J.J., Swerdlow, D.L., Krebs, J.W., Holman, R.C., Mandel, E., Harvey, A., Haberling, D., Massung, R.F., McQuiston, J.H., 2010. Rocky mountain spotted fever in the United States, 2000–2007: interpreting contemporary increases in incidence. Am. J. Trop. Med. Hyg. 83, 174–182.

Ostfeld, R.S., 2011. A Systems Approach to Understanding Tick-Borne Diseases: People, Animals, and Ecosystems In: An Overview of Tick-Borne Diseases, http://www.ncbi.nlm.nih.gov/books/NBK57013/ (accessed 12.10.15.).

Parola, P., Paddock, C.D., Raoult, D., 2005. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin. Microbiol. Rev. 18, 719–756.

Parola, P., Labruna, M.B., Raoult, D., 2009. Tick-borne rickettsioses in America: unanswered questions and emerging diseases. Curr. Infect. Dis. Rep. 11, 40–50.
Regnery, R.L., Spruill, C.L., Plikaytis, B.D., 1991. Genotypic identification of *rickettsiae* and estimation of intraspecies sequence divergence for portions of two *rickettsial* genes. J. Bacteriol. 173, 1576–1589.
Roland, W.E., Everett, E.D., Cyr, T.L., Hasan, S.Z., Dommaraju, C.B., McDonald, G.A., 1998. *Ehrlichia chaffeensis* in Missouri ticks. Am. J. Trop. Med. Hyg. 59, 641–643.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
Schulze, T.L., Jordan, R.A., White, J.C., Roegner, V.E., Healy, S.P., 2011. Geographical distribution and prevalence of selected *Borrelia*, *Ehrlichia*, and *Rickettsia* infections in *Amblyomma americanum* (Acari: ixodidae) in New Jersey. J. Am. Mosq. Control Assoc. 27, 236–244.
Smith, M.P., Ponnusamy, L., Jiang, J., Ayyash, L.A., Richards, A.L., Apperson, C.S., 2010. Bacterial pathogens in ixodid ticks from a Piedmont County in North Carolina: prevalence of rickettsial organisms. Vector Borne Zoonotic Dis. 10, 939–952.
Steiert, J.G., Gilfoy, F., 2002. Infection rates of *Amblyomma americanum* and *Dermacentor variabilis* by *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in southwest Missouri. Vector Borne Zoonotic Dis. 2, 53–60.
Stromdahl, E.Y., Vince, M.A., Billingsley, P.M., Dobbs, N.A., Williamson, P.C., 2008. *Rickettsia amblyommii* infecting *Amblyomma americanum* larvae. Vector Borne Zoonotic Dis. 8, 15–24.
Stromdahl, E.Y., Nadolny, R.M., Gibbons, J.A., Auckland, L.D., Vince, M.A., Elkins, C.E., Murphy, M.P., Hickling, G.J., Eshoo, M.W., Carolan, H.E., Crowder, C.D., Pilgard, M.A., Hamer, S.A., 2015. Borrelia burgdorferi not confirmed in human-biting *Amblyomma americanum* ticks from the southeastern United States. J. Clin. Microbiol. 53, 1697–1704.
Varela, A.S., Moore, V.A., Little, S.E., 2004. Disease agents in *Amblyomma americanum* from northeastern Georgia. J. Med. Entomol. 41, 753–759.
Williamson, P.C., Billingsley, P.M., Teltow, G.J., Seals, J.P., Turnbough, M.A., Atkinson, S.F., 2010. Borrelia, *Ehrlichia*, and *Rickettsia* spp. in ticks removed from persons, Texas, USA. Emerg Infect. Dis. 16, 441–446.
Wormser, G.P., Masters, E., Liveris, D., Nowakowski, J., Nadelman, R.B., Holmgren, D., Bittker, S., Cooper, D., Wang, G., Schwartz, I., 2005. Microbiologic evaluation of patients from Missouri with erythema migrans. Clin. Infect. Dis. 40, 423–428.
Wright, C.L., Gaff, H.D., Hynes, W.L., 2014. Prevalence of *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in *Amblyomma americanum* and *Dermacentor variabilis* collected from southeastern Virginia, 2010–2011. Ticks Tick Borne Dis. 5, 978–982.
Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 7, 203–214.