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Note on *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and "*Borrelia lonestari*" Infection in Lone Star Ticks (Acari: Ixodidae), Nebraska, USA

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Note on *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and "*Borrelia lonestari*" infection in lone star ticks (Acari: Ixodidae), Nebraska, USA

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Abstract

The lone star tick, *Amblyomma americanum* (L.) (Acari: Ixodidae), is established in southeastern Nebraska, yet the prevalence of tick-associated microorganisms is not known. An initial PCR-based analysis for *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *Borrelia* infection in host-seeking adult ticks collected in southeast Nebraska was conducted. A total of 251 adult ticks collected in six sites in southeast Nebraska were tested. *E. chaffeensis*, *E. ewingii*, and *Borrelia* spp. were present, and the prevalence of each was approximately 1.6%. This study demonstrates that *Ehrlichia* spp. are present in Nebraska lone star tick populations.

Keywords: ticks, tick-borne microorganisms, *Ehrlichia*, Nebraska

1. Introduction

The lone star tick, *Amblyomma americanum* (L.), is an aggressive ectoparasite and vector of various human and animal pathogens, including *Ehrlichia chaffeensis* (human monocytic

ehrlichiosis) (Anderson et al., 1993; Ewing et al., 1995), *Ehrlichia ewingii* (canine granulocytic ehrlichiosis) (Anziani et al., 1990; Mixson et al., 2006; Heise et al., 2010), *Rickettsia parkeri* (Tidewater spotted fever) (Macaluso and Azad, 2005; Cohen et al., 2009; Jiang et al., 2012), *Francisella tularensis* (tularemia) (Hopla and Downs, 1953), and the recently described phlebovirus, Heartland virus (Bunyaviridae) (Savage et al., 2013; McMullan et al., 2012). Other microorganisms associated with lone star ticks include *Rickettsia amblyommii* (Apperson et al., 2008; Burgdorfer et al., 1981) and *Borrelia* spp.(Barbour et al., 1996; Stromdahl et al., 2003).

Lone star ticks became established in southeastern Nebraska during the 1980s; presently, populations occur from the south-central to east-central portion of the state, including the Omaha-Lincoln and Platte River corridors (Cortinas and Spomer, 2013). In southeastern collection sites, lone star ticks were more prevalent compared to American dog ticks (*Dermacentor variabilis* (Say)) (Cortinas and Spomer, 2013), and the lone star tick's high densities and wide host range in all life stages likely increase the possibility of encounter with human and animal hosts. Yet, the prevalence of lone star tick-borne pathogens in the state is not known.

Human epidemiological data suggests that lone star tick-borne pathogen transmission may be occurring in the state. There were 17 probable and one confirmed case of human monocytic ehrlichiosis (HME) between 1986 and 1997. These cases were diagnosed by indirect immunofluorescence assay (IFA) at the Centers for Disease Control and Prevention (CDC); at the time, ehrlichiosis cases were not reportable in the state (McQuiston et al., 1999). However, there may have been confounding factors—a lack of tick-borne disease awareness among doctors and the public may have led to under-reporting of locally acquired cases, some cases may have not been locally acquired (McQuiston et al., 1999), and cross reactivity of *E. chaffeensis* serological tests with *E. ewingii* and *Anaplasma phagocytophilia* was possible (Paddock and Childs, 2003). Since 1999, ehrlichiosis cases have been reportable, and prior to 2007, no cases were reported. However, between 2007 and 2012, ten cases have been reported and confirmed (Adams et al., 2014; Nieves et al., 2009). These data, coupled with anecdotes from the public about the occurrence of bull's eye rashes, encouraged us to evaluate the prevalence of *E. chaffeensis*, *E. ewingii*, and *Borrelia* spp. in lone star tick adults collected in southeastern Nebraska.

2. Material and methods

Ticks were collected from May to August 2012, during the time when lone star tick adults and nymphs are actively questing in the region (Kollars et al., 2000; Cortinas and Spomer, 2013). Six sampling sites where lone star ticks are present were selected (Cortinas and Spomer, 2013). Indian Cave State Park (SP), Kinters Ford State Wildlife Management Area (SWMA), and Table Rock State Wildlife Management Area (SWMA) are located in extreme southeast Nebraska near the Missouri and Kansas borders (Fig. 1).These sites had the highest lone star tick densities in previous collections (Cortinas and Spomer, 2013). Prairie Pines, Schramm Park State Recreation Area (SRA), and Wilderness Park are sites located in the Lincoln-Omaha corridor (Fig. 1).

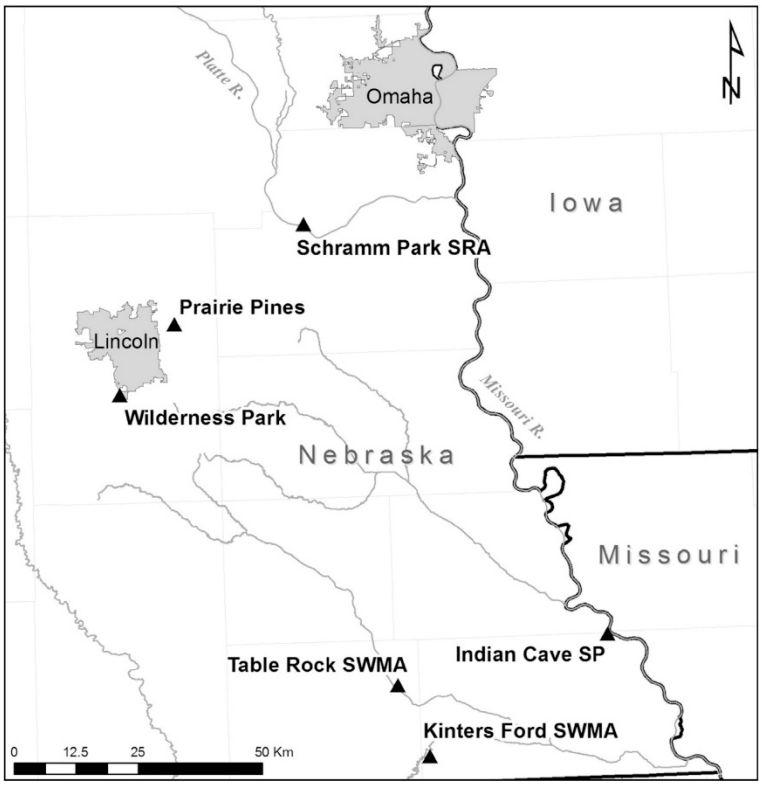

Figure 1. Map of southeast Nebraska indicating the location of the six collection sites.

Indian Cave SP, Prairie Pines, Schramm Park SRA, and Wilderness Park were visited six times every other week, whereas Table Rock and Kinters Ford were visited three times on visits that were at least four weeks apart due to poor weather conditions for sampling. Ticks were collected using carbon dioxide (CO_2) baited traps, as previously described (Lockhart et al., 1997). At least 20 traps were used per site visit and traps were placed approximately 20 m apart in forested areas. Traps operated for at least two hours from mid-morning to late-morning, after which the traps were gathered and transported to the laboratory. Ticks were removed from the traps, identified to life stage, sex, and species (Cooley and Kohls, 1944; Keirans and Litwak, 1989; Keirans and Durden, 1998), surface sterilized by being placed in a 1.7 ml centrifuge tube containing 100% ethyl alcohol and vortexed for 5 min, transferred into vials containing 95% ethyl alcohol to maintain tick surface sterility, and kept at −20°C prior to DNA extraction. Adult ticks were individually stored and nymphs were stored in groups of 10 based on collection site, date, and trap number. Voucher tick specimens were submitted to the Harold W. Manter Laboratory of Parasitology (University of Nebraska State Museum, Lincoln, NE) (Collection numbers 64578, 64579, 64580, 64581).

Individual adult ticks in autoclaved 1.7 mL microcentrifuge tubes were snap frozen in liquid nitrogen and mechanically crushed using two-inch disposable pestles (Fisher Scien-

tific, Pittsburgh, Pennsylvania). Enzymatic digestion was performed with 20 μL of proteinase K and 4 μL polyacryl carrier (Molecular Research Center, Inc., Cincinnati, Ohio) to isolate small amounts of extracted DNA. Tick DNA was then extracted using the Animal Tissues Spin-Column protocol from DNeasy Blood and Tissue Kit (Qiagen, Valencia, California). Extraction material from each tick was stored at −20°C.

Amplification of a ~389 bp fragment of the 16S rDNA fragment for *E. chaffeensis* (Anderson et al., 1992) was performed by combining individual tick extractions into pools of three using a nested PCR protocol. The initial amplification utilized general primers ECB and ECC for *Ehrlichia* spp. (Heise et al., 2010) in a 50 μL reaction. Individual samples contained 25 μL *Taq* PCR Master Mix Kit (Qiagen), 15 μL PCR Grade Water (Roche, Mannheim, Germany), 4 μL of each primer (10 μM) (Integrated DNA Technologies, Coralville, Iowa), and 2 μL of sample DNA (0.04–55.26 ng/μL). Alternatively, equal amounts of DNA extracted from three ticks (2 μL of sample DNA from each tick resulting in 6 μL of pooled sample DNA) were pooled and evaluated using the same conditions described above, reducing PCR Grade Water to 11 μL, and resulting in 50 μL total volume. Cycling conditions as described by Anderson and colleagues (1992)were used.

For the secondary nested reaction, 2 μL (individual samples) or 6 μL (pools) of primary product was used as template in a 50 μL total volume reaction containing the same PCR components with the exception of the primers HE3 and HE1 (Anderson et al., 1992; Heise et al., 2010). Cycling conditions as described in Anderson et al.(1992) were utilized.

Reaction products were analyzed using gel capillary electrophoresis (QIAxel Advanced System, Qiagen). A negative control containing PCR grade water and an *E. chaffeensis* positive control was run on each gel (provided by Dr. Susan Little and Jeff Gruntmeir, Oklahoma State University Center for Veterinary Health Sciences, Stillwater, Oklahoma).

Amplification of a ~407 bp fragment of the 16S rDNA fragment for *E. ewingii* (Steiert and Gilfoy, 2002) for individual and pooled samples was achieved using a nested PCR protocol using external primers ECB and ECC (Heise et al., 2010) as described for *E. chaffeensis*. The cycling conditions used are described in Steiert and Gilfoy (2002).

For the secondary nested reaction, 2 μL (individual samples) or 6 μL (pools) of the primary product was used as template in a 50 μL reaction containing the same PCR components with the exception of the primers HE3 and EE72. Cycling conditions as described in Steiert and Gilfoy (2002) were utilized. Reaction products were analyzed using gel capillary electrophoresis. A negative control containing PCR grade water and an *E. ewingii* control was run on each gel (provided by Dr. Susan Little and Jeff Gruntmeir, Oklahoma State University Center for Veterinary Health Sciences, Stillwater, Oklahoma).

Borrelia specific PCR analysis targeted the *Borrelia* flagellin gene (flaB) (~350 bp) using a nested protocol (Barbour et al., 1996; Stromdahl et al., 2003). Primer pairs FlaLL and FlaRL were used in the first reaction. Reactions were prepared using 25 μL Taq PCR Master Mix (Qiagen) that contained 2.5 U of *Taq* DNA polymerase, 1 × QIAGEN PCR buffer, 1.5 mM $MgCl_2$, and 200 μM of each dNTP, 5 μl of each primer (10 μM), 9 μl of nuclease-free water, and 6 μl of sample DNA. For the secondary nested reaction, 2 μL (individual samples) or 6 μL (pools) of primary product was used as template in a 50 μL total volume reaction containing the same PCR components with the exception of the primer pairs FlaLS and FlaRS in place of the initial primers. Final concentrations of the reaction are 2.5 U of

Taq, 1.5 μM $MgCl_2$, 200 μM of each dNTP, and 1 μM of each primer. Cycling conditions described in Barbour et al. (1996) were used for both reactions. Reaction products were analyzed using gel capillary electrophoresis. A negative control containing PCR grade water and a "*Borrelia lonestari*" positive control sample was run on each gel. The positive control was provided by Dr. Susan Little and Jeff Gruntmeir (Oklahoma State University Center for Veterinary Health Sciences, Stillwater, Oklahoma).

Ehrlichia positive controls and randomly selected positive samples amplicons were sent to Eurofins MWG Operon (Huntsville, Alabama) for DNA sequencing. DNA sequences were trimmed and aligned using align sequences function of the Nucleotide Basic Local Alignment Search Tool (BLASTn) (NCBI) and then inputted into Standard Nucleotide BLAST (NCBI) program to compare the samples nucleotide sequences to the databases.

FlaLL and FlaRL amplicons from all *Borrelia* positive samples were sent to the Iowa State University DNA Facility for sequence analysis. Prior to sequencing, PCR products were purified with deoxynucleotide triphosphates (dNTPs) and primers removed using ExoSAP-IT per manufacturer's instructions (Affymetrix, Santa Clara, California). Sequence trimming, assembly, alignment, and phylogenetic analysis were conducted using CLC Main Workbench 7.5 (Qiagen).

3. Results

A total of 251 (140 females, 111 males) out of 747 adult *A. americanum* was randomly selected among sample sets blocked by site and date of sampling for PCR analysis (Table 1). Eighty-five pools consisting of a maximum of three ticks based on site and collection date were initially tested. Individual tick extractions of positive pools were subsequently processed.

Across all sites, four of 251 (1.6%) ticks were positive for *E. chaffeensis* (Table 1). All positive ticks originated from one site, Table Rock SWMA, in which 8.0% (4/50) of collected ticks were positive. Four ticks from three sites tested positive for *E. ewingii* (Table 1) (Table Rock SWMA, Indian Cave SP, and Schramm Park SRA).

Table 1. Number of adult *Amblyomma americanum* analyzed and infected with selected bacterial organisms by collection site

Site	Ticks analyzed	*E. chaffeensis*	*E. ewingii*	*"B. lonestari"*
Indian Cave SP	76	0	2 (0.03)	1 (0.01)
Kinters Ford SWMA	53	0	0	1 (0.02)
Prairie Pines	23	0	0	0
Schramm Park SRA	35	0	1 (0.03)	2 (0.06)
Table Rock SWMA	50	4 (0.08)	1 (0.02)	0
Wilderness Park	14	0	0	0
Total	251	4 (0.02)	4 (0.02)	4 (0.02)

Four ticks from three sites were positive for *Borrelia* spp. (Kinters Ford SWMA, Indian Cave SP, and Schramm Park SRA). The *E. ewingii*–infected tick collected at Schramm Park SRA was co-infected with *Borrelia* spp.

Positive controls, as well as two randomly selected *E. chaffeensis* and three *E. ewingii* positive samples, were sequenced to verify PCR results. As expected, sequences for *E. chaffeensis* positive control were 100% identical to *E. chaffeensis* 16S ribosomal RNA gene (GenBank accession number KF034786.1) and *E. chaffeensis* Arkansas strain 16S ribosomal RNA (GenBank accession number NR_074500.1), and sequence for *E. ewingii* positive control was 100% identical to *E. ewingii* genotype Panola Mountain 16S ribosomal RNA gene (GenBank accession number DQ365880.1). *E. chaffeensis* positive samples were 100% identical to the *E. chaffeensis* control sequence, and sequences for positive samples for *E. ewingii* were 100% identical to the *E. ewingii* control.

All samples positive for *Borrelia* spp. were also sequenced. Consensus sequence demonstrated 100% identity to "*B. lonestari*" MOV-2002 (GenBank accession number AY850064.1).

4. Discussion

The only other Nebraska study that has assessed lone star tick pathogen prevalence did not demonstrate any *E. chaffeensis*–infected ticks, though only 40 ticks in pools of ten were tested (Anderson et al., 1993). The overall *E. chaffeensis* prevalence (1.6%) is in the low portion of the range observed in other studies in which prevalence varies between 0.4 and 23% (Burket et al., 1998; Roland et al., 1998; Steiert and Gilfoy, 2002). Compared to *E. chaffeensis*, *E. ewingii*–infected ticks were more widespread in southeastern Nebraska, found in three sites (Indian Cave SP, Schramm Park SRA, and Table Rock SWMA). Tick infection rates between 2 and 2.9% were within the boundaries of other reported ranges (Wolf et al., 2000; Steiert and Gilfoy, 2002; Mixson et al., 2006; Cohen et al., 2010; Killmaster et al., 2014). "*B. lonestari*"–positive ticks were found in three sites (Indian Cave SP, Kinters Ford SWMA, and Schramm Park SRA) and infection rates within positive sites ranged between 1 and 6%. Other studies have demonstrated prevalence similar to the one observed at Schramm Park SRA (6%) (Barbour et al., 1996; Bacon et al., 2003; Taft et al., 2005; Stromdahl et al., 2015).

The genus-wide *Borrelia* primers from Barbour et al. (1996) provide evidence of the absence of other *Borrelia* species other than "*B. lonestari*." A recent publication (Stromdahl et al., 2015) describes the ability of these primers to amplify novel *Borrelia* strains potentially associated with human illness (*B. americana*, *B. andersoni*, *B. bissettii*) as well as *B. burgdorferi*. The lack of pathogenic *Borrelia* in *A. americanum* from Nebraska is important evidence because the role of these ticks in the transmission of Lyme group *Borrelia* is somewhat controversial.

Sites located close to Lincoln (Prairie Pines and Wilderness Park),which we hypothesize have been more recently invaded by lone star ticks (Cortinas and Spomer, 2013), did not yield any positive ticks, though the number of ticks tested may have been insufficient to detect low infection rates.

In conclusion, our results demonstrate that lone star ticks in Nebraska are infected with *E. chaffeensis*, *E. ewingii*, and "*B. lonestari*." As we continue to assess the status of the lone

star tick in Nebraska, we will incorporate tick-borne disease pathogen testing of all tick life stages and tick hosts, including potential natural hosts, as these organisms are of veterinary and public health importance.

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References

Adams, D.A., Jajosky, R.A., Ajani, U., Kriseman, J., Sharp, P., Onwen, D.H., Schley, A.W., Anderson, W.J., Grigoryan, A., Aranas, A.E., Wodajo, M.S., Abellera, J.P., Centers for Disease Control and Prevention (CDC), 2014. Summary of notifiable diseases—United States, 2012. MMWR Morb. Mortal. Wkly. Rep 61, 1–121.

Anderson, B.E., Sims, K.G., Olson, J.G., Childs, J.E., Piesman, J.F., Happ, C.M., Maupin, G.O., Johnson, B.J., 1993. Amblyomma americanum: a potential vector of human ehrlichiosis. Am. J. Trop. Med. Hyg. 49, 239–244.

Anderson, B.E., Sumner, J.W., Dawson, J.E., Tzianabos, T., Greene, C.R., Olson, J.G., Fishbein, D.B., Olsen-Rasmussen, M., Holloway, B.P., George, E.H., 1992. Detection of the etiologic agent of human ehrlichiosis by polymerase chain reaction. J. Clin. Microbiol. 30, 775–780.

Anziani, O.S., Ewing, S.A., Barker, R.W., 1990. Experimental transmission of agranulocytic form of the tribe Ehrlichieae by *Dermacentor variabilis* and *Amblyomma americanum* to dogs. Am. J. Vet. Res. 51, 929–931.

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.

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., Teltow, 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.

Burgdorfer, W., Hayes, S.F., Thomas, L.H., Lancaster, J.L., 1981. A new spotted fever–group rickettsia from the lone star tick, *Amblyomma americanum*. In: Burgdorfer, W., Anacker, R.L. (Eds.), Rickettsia and Rickettsial Diseases. Academic Press, New York, pp. 595–602.

Burket, C.T., Vann, C.N., Pinger, R.R., Chatot, C.L., Steiner, F.E., 1998. Minimum infection rate of *Amblyomma americanum* (Acari: Ixodidae) by *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) in southern Indiana. J. Med. Entomol. 35, 653–659.

Cohen, S.B., Yabsley, M.J., Freye, J.D., Dunlap, B.G., Rowland, M.E., Huang, J., Dunn, J.R., Jones, T.F., Moncayo, A.C., 2010. Prevalence of *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in ticks from Tennessee. Vector Borne Zoonotic Dis. 10, 435–440.

Cohen, S.B., Yabsley, M.J., Garrison, L.E., Freye, J.D., Dunlap, B.G., Dunn, J.R., Mead, D.G., Jones, T.F., Moncayo, A.C., 2009. *Rickettsia parkeri* in *Amblyomma americanum* ticks, Tennessee and Georgia, USA. Emerg. Infect. Dis. 15, 1471–1473.

Cooley, R.M., Kohls, G.M., 1944. The genus *Amblyomma* (Ixodidae) in the United States. J. Parasitol. 30, 77–111.

Cortinas, R., Spomer, S., 2013. Lone star tick (Acari: Ixodidae) occurrence in Nebraska: historical and current perspectives. J. Med. Entomol. 50, 244–251.

Ewing, S.A., Dawson, J.E., Kocan, A.A., Barker, R.W., Warner, C.K., Panciera, R.J., Fox, J.C., Kocan, K.M., Blouin, E.F., 1995. Experimental transmission of *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) among white-tailed deer by *Amblyomma americanum* (Acari: Ixodidae). J. Med. Entomol. 32, 368–374.

Heise, S.R., Elshahed, M.S., Little, S.E., 2010. Bacterial diversity in *Amblyomma americanum* (Acari: Ixodidae) with a focus on members of the genus *Rickettsia*. J. Med. Entomol. 47, 258–268.

Hopla, C.E., Downs, C.M., 1953. The isolation of *Bacterium tularense* from the tick *Amblyomma americanum*. J. Kans. Entomol. Soc. 26, 71–72.

Jiang, J., Stromdahl, E.Y., Richards, A.L., 2012. Detection of *Rickettsia parkeri* and Candidatus *Rickettsia andeanae* in *Amblyomma maculatum* Gulf Coast ticks collected from humans in the United States. Vector Borne Zoonotic Dis. 12, 175–182.

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.

Kollars Jr., T.M., Oliver Jr., J.H., Durden, L.A., Kollars, P.G., 2000. Host association and seasonal activity of *Amblyomma americanum* (Acari: Ixodidae) in Missouri. J. Parasitol. 86, 1156–1159.

Lockhart, J.M., Davidson, W.R., Stallknecht, D.E., Dawson, J.E., Little, S.E., 1997. Natural history of *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) in the piedmont physiographic province of Georgia. J. Parasitol. 83, 887–894.

Macaluso, K.R., Azad, A.F., 2005. Rocky Mountain spotted fever and other spotted fever group rickettsioses. In: Goodman, J.L., Dennis, D.T., Sonenshine, D.E.(Eds.), Tick-Borne Diseases of Humans. ASM Press, Washington, DC, pp. 292–301.

McMullan, L.K., Folk, S.M., Kelly, A.J., MacNeil, A., Goldsmith, C.S., Metcalfe, M.G., Batten, B.C., Albarino, C.G., Zaki, S.R., Rollin, P.E., Nicholson, W.L., Nichol, S.T., 2012. A new phlebovirus associated with severe febrile illness in Missouri. N. Engl. J. Med. 367, 834–841.

McQuiston, J.H., Paddock, C.D., Holman, R.C., Childs, J.E., 1999. The human ehrlichioses in the United States. Emerg. Infect. Dis. 5, 635–642.

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.

Nieves, E., Jajosky, R.A., Adams, D.A., Sharp, P., Anderson, W.J., Aponte, J.J., 2009. Summary of notifiable diseases—United States, 2007. MMWR Morb. Mortal. Wkly. Rep. 56, 1–94.

Paddock, C.D., Childs, J.E., 2003. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. Clin. Microbiol. Rev. 16, 37–64.

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.

Savage, H.M., Godsey Jr., M.S., Lambert, A., Panella, N.A., Burkhalter, K.L., Harmon, J.R., Lash, R.R., Ashley, D.C., Nicholson, W.L., 2013. First detection of heartland virus (Bunyaviridae: Phlebovirus) from field collected arthropods. Am. J. Trop. Med. Hyg. 89, 445–452.

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., 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.

Stromdahl, E.Y., Williamson, P.C., Kollars Jr., T.M., Evans, S.R., Barry, R.K., Vince, M.A., Dobbs, N.A., 2003. Evidence of *Borrelia lonestari* DNA in *Amblyomma americanum* (Acari: Ixodidae) removed from humans. J. Clin. Microbiol. 41, 5557–5562.

Taft, S.C., Miller, M.K., Wright, S.M., 2005. Distribution of borreliae among ticks collected from eastern states. Vector Borne Zoonotic Dis. 5, 383–389.

Wolf, L., McPherson, T., Harrison, B., Engber, B., Anderson, A., Whitt, P., 2000. Prevalence of *Ehrlichia ewingii* in *Amblyomma americanum* in North Carolina. J. Clin. Microbiol. 38, 2795.