Case Report

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Detection of *Ehrlichia chaffeensis* in a naturally infected elk (*Cervus elaphus*) from Missouri, USA

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Introduction: *Ehrlichia chaffeensis* is an emerging zoonotic tick-borne rickettsial pathogen that has been detected in a wide range of vertebrate hosts, including ruminants, canids and primates. Although white-tailed deer (*Odocoileus virginianus*) are considered the primary reservoir of *E. chaffeensis*, this pathogen was also reported in other naturally infected cervids, including Korean spotted or sika deer (*Cervus nippon*) and Brazilian marsh deer (*Blastocerus dichotomus*).

Case presentation: A captive adult bull elk (*Cervus elaphus*) from east-central Missouri was submitted for post-mortem analysis. The elk was in poor body condition with easily palpable ribs and vertebral spinal processes. Excoriations were noted on the occipital region of the head and on the left scapula, which had moderate amounts of maggots within the lesions. Large numbers of ticks were scattered over the body. Novel and established PCR assays were used to detect *E. chaffeensis* in blood and spleen samples from this elk, but the pathogen was not detected in *Dermacentor albipictus* ticks collected at necropsy. Portions of several gene sequences were analysed from the infecting agent.

Conclusion To the best of our knowledge this is the first report of *E. chaffeensis* infection in an elk. It was not determined whether the pathogen contributed to cause of death. Notably, the pathogen was not detected in *D. albipictus* ticks collected from the elk.

Keywords: *Dermacentor albipictus*; *Ehrlichia chaffeensis*; elk; PCR-based diagnostic tests; tick-borne pathogen.

Received 5 December 2014
Accepted 9 January 2015

Introduction

Ehrlichia chaffeensis is an emerging tick-borne rickettsial pathogen and the zoonotic aetiologic agent of human monocytic ehrlichiosis (Paddock & Childs, 2003). White-tailed deer (*Odocoileus virginianus*) are recognized as the primary mammalian reservoir, and *Amblyomma americanum* ticks as the primary vector of this pathogen. However, like many zoonotic vector-borne pathogens, *E. chaffeensis* is infectious to a divergent range of mammals. PCR assays were used to detect this pathogen in coyotes, dogs, goats, lemurs (Williams *et al.*, 2002; Yabsley *et al.*, 2004), sika deer (Kawahara *et al.*, 2009), Brazilian marsh deer (Machado *et al.*, 2006) and Korean spotted deer (Lee *et al.*, 2009). Serologic evidence of *E. chaffeensis* infections was reported for red foxes, grey foxes, raccoons, opossums, rabbits and mule deer (Yabsley *et al.*, 2005). Unlike many other tick-borne pathogens, *E. chaffeensis* is also reported to infect an unusually divergent range of ticks, including

members of the ixodid tribes Ixodinae, Amblyomminae, Haemophysalinae and Rhipicephalinae.

In this report, blood from an elk carcass tested PCR-positive with a generic assay for *Ehrlichia*, which was followed by several different *E. chaffeensis*-specific PCR assays of different blood, spleen and *Dermacentor albipictus* tick samples collected from the same carcass. Although *E. chaffeensis* was detected in blood and spleen samples, this pathogen was not detected in the one-host ticks, *D. albipictus*, parasitizing the animal.

Case report

An adult bull elk (*Cervus elaphus*) was submitted to the University of Missouri Veterinary Medical Diagnostic Laboratory (MU VMDL) for post-mortem analysis. The bull elk was in poor body condition with easily palpable ribs and vertebral spinal processes. Excoriations were noted on the occipital region of the head and on the left scapula, which had moderate amounts of maggots within

Five supplementary figures are available with the online Supplementary Material.

the lesions. It was also noted that large numbers of ticks were scattered over the body. No other gross lesions were noted. On microscopic examination, the skin had a severe ulcerative dermatitis with large numbers of eosinophils, neutrophils, macrophages and mast cells in the superficial and mid-dermis, especially around blood vessels and adnexal structures. A thick serocellular crust with bacterial colonies, often cocci, was also noted. No apparent aetiology was present in the skin sections examined. No significant histopathologic changes were noted in the liver, kidney, lung, heart, spleen or testicle.

The elk was a captive animal from east-central Missouri, where *E. chaffeensis* is endemic. As ticks were found parasitizing the animal, blood was tested for ehrlichial infection, and blood, spleen and ticks collected from the elk were subsequently sent to our laboratory for tick identification and for more specific PCR-based assays for *Ehrlichia* spp.

Investigations

Tick identification

To substantiate speciation based on external morphology, molecular techniques were used to confirm the identity of the ticks collected from the elk. The 18S rRNA internal transcribed spacer-2 (ITS-2) sequence has been used to characterize tick species, including those of the genus *Dermacentor* (Dergousoff & Chilton, 2007; Shone *et al.*, 2006). Tick samples collected from the elk and *Dermacentor variabilis* controls were amplified with PCR using primers DermITS2-F (GTGCGTCCGTCGACTCGTT) and DermITS2-R (TC-GCCCAACACGGCGCTACT) as previously described (Dergousoff & Chilton, 2007; Shone *et al.*, 2006). These PCR products were subjected to agarose electrophoresis and sequence analysis.

PCR assays for *E. chaffeensis*

Heparinized elk blood (1.0 ml) was centrifuged at 1200 g for 30 min at 4 °C, and buffy coats (approximately 200 µl) were manually transferred to 1.5 ml microcentrifuge tubes for nucleic acid isolation and PCR analysis. Spleen samples were stored at -80 °C prior to DNA extraction. Ticks were stored in a humidity chamber for 112 days at room temperature and 95 to 100 % relative humidity, with a 12 h:12 h (light:dark) photoperiod. A dichotomous key was used to identify four adult male and two adult female ticks, which were then bisected along the sagittal plane, pooled and placed on dry ice for template isolation. Nucleic acids were extracted from buffy coat and tick samples with the High Pure Viral Nucleic Acid kit (Roche) as previously described (McClure *et al.*, 2009). Spleen samples were thawed on ice and nucleic acids were isolated from 10 mg portions with the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. Six PCR assays were used to test these samples for the presence

of *E. chaffeensis*, and each sample was tested a minimum of two times with each PCR assay.

The first assay was based on a hypothetical outer membrane protein gene (*homp*) sequence that is unique to the *E. chaffeensis* genome sequence, which was used to design and optimize a novel fluorogenic real-time PCR assay (Stoffel *et al.*, 2014). This *E. chaffeensis*-specific assay used the forward primer ECH144762S, reverse primer ECH144994A and the fluorogenic probe ECH144816S (Table 1). Templates (2.5 µl) were assayed in 25 µl reactions containing 1 × PCR buffer, 2 mM MgCl₂, 0.06 U µl⁻¹ Platinum *Taq* DNA polymerase, 200 µM dNTP mix (Invitrogen), 1 % (v/v) DMSO, 1.8 µM each of primers ECH144762S and ECH144994A and 0.25 µM of ECH144816S probe. These assays were run in an ABI 7300 real-time thermocycler (Applied Biosystems) at 95 °C for 2 min followed by 60 cycles of 95 °C for 15 s and 61 °C for 1 min. PCR-positive samples were also subjected to 1.5 % agarose electrophoresis with 1 × TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and ethidium bromide (0.5 µg ml⁻¹).

A conventional PCR assay based on the P28 outer membrane protein gene of *E. chaffeensis* was also used to assay the same samples (Wagner *et al.*, 2004). Templates (2.5 µl) were assayed in 25 µl reactions containing 1 × PCR buffer, 2 mM MgCl₂, and 200 µM dNTP mix, 0.06 U µl⁻¹ Platinum *Taq* DNA polymerase, 8 % DMSO, and 1 µM each of primers ECH81-101 and ECH360-341. These assays were run in an ABI 2720 thermocycler at 93 °C for 2 min, followed by 95 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s, followed by a final extension step of 72 °C for 7 min. These PCR products were electrophoresed as described above.

A 16S rRNA gene (16S rDNA)-based real-time PCR assay for *E. chaffeensis* was also used to assay buffy coat and spleen samples (Loftis *et al.*, 2003). Templates (2.5 µl) were assayed in 25 µl reactions containing 1 × PCR buffer, 2 mM MgCl₂, 0.06 U µl⁻¹ Platinum *Taq* DNA polymerase, 200 µM dNTP mix (Invitrogen), 0.2 µM forward primer ECH16S-17, 0.8 µM reverse primer ECH16S-97 and 0.1 µM fluorogenic probe, ECH16S-38 (Table 1). These reactions were run in an ABI 7300 real-time thermocycler at 95 °C for 2 min followed by 60 cycles of 95 °C for 15 s and 61 °C for 1 min.

A *trp32*-based PCR was also used with nested primer sets FB5A and FB3A or FB5 and FB3 (Table 1) (Paddock & Childs, 2003). Templates (2.5 µl) were assayed in 25 µl reactions containing 1 × PCR buffer, 2 mM MgCl₂, 0.06 U µl⁻¹ Platinum *Taq* DNA polymerase, 200 µM dNTP mix (Invitrogen) and 0.5 µM of each primer. PCR reactions were run in an ABI 2720 thermocycler with 3 cycles of 94 °C for 1 min, 52 °C for 2 min and 70 °C for 1.5 min, followed by 37 cycles of 88 °C for 1 min, 55 °C for 1.5 min and 70 °C for 1.5 min, with a final extension step of 68 °C for 5 min. These PCR products were also electrophoresed as described above.

Table 1. Sequence analyses of amplicons generated from different *E. chaffeensis*-specific PCR assays

Gene targeted	Primer and probe sequences	Amplicon size (bp)	Genbank accession no.	Closest matching strain	Identity (%)
<i>homp</i>	ECH144762S (ACCCCTGCAACACCTACAAAC), ECH144994A (CCACCTACAGCACCTAAAGCA) and the fluorogenic probe ECH144816S (FAM-TCTGTAACACCTATAGCATCTGCACGCG-BHQ)	233	KP236455	Ark	99.48
<i>p28</i>	ECH81-101 (AGCAGGTAGTGGTATTAAACGG) and ECH360-341 (AGATACCTCAAGCTCTATTC)	279	KP236456	V2	86.98
<i>trp32</i>	FB5A (GTGACATCTTAGTTTAAATAGAAC) and FB3A (AAGACTGAAACGTTATAGAG) or FB5 (AAATAGGGTATAAATATGTAC) and FB3 (GCCTAATTGAGATAAACTAAC)	849	KP236457	Ark	99.75
16S rDNA	ECH16S-17 (GCGGCAAGCCTAACACATG), ECH16S-97 (CCCGTCTGCCACTAACCAATTATT) and the fluorogenic probe ECH16S-38 (FAM-AGTCGAACGGACAATTGCTTATAACCTTTTGGT-BHQ1)	81	NA	NA	NA

NA, Not applicable.

Amplicon sequence analysis

Amplicons were excised from agarose gels and DNA extracted using the Wizard SV Gel and PCR Clean-Up System (Promega). Nucleotide sequences were determined in both directions with the same forward and reverse primers used for the respective PCR assays, with a 3730 96-capillary DNA Analyzer (Applied Biosystems), at the University of Missouri DNA Core Facility. Regions of local similarity between nucleotide sequences were assessed with the BLAST program (National Center for Biotechnology Information, Bethesda, MD) against nucleotide collections using default parameters. Sequence alignments were produced with CLUSTAL W2 using default nucleotide parameters (Chenna *et al.*, 2003).

Diagnosis

Tick identification

Using morphologic characteristics of individual ticks, a dichotomous key was used to identify the ticks removed from the elk (Fig. S1, available in the online Supplementary Material). For each tick examined, the basis capitulum was visible from the dorsal surface, and the palps were approximately the same length as the basis capitulum, with second palpal segments of equal width and length. The spiracular plates displayed morphologic variation ranging from round to showing a dorsal prolongation. Ornate scuta were present on all of the ticks. Based on these characteristics, the four male and two female ticks were tentatively identified as *D. albipictus*. The ITS-2 sequences amplified from these ticks with primers DermITS-F and DermITS-R (Fig. S2; Genbank accession no. KP236454) were 100 % identical to the nucleotide sequence reported for *D. albipictus* (Dergousoff & Chilton, 2007; Shone *et al.*, 2006).

PCR assays for *E. chaffeensis*

Blood samples tested at the MU VMDL with a generic *Ehrlichia* assay were PCR-positive, but the species of pathogen was not determined. Therefore, a novel *homp*-based PCR assay designed for detection of *E. chaffeensis* was used to test buffy coat samples from the elk. Elk buffy coat samples tested PCR-positive for *E. chaffeensis*, which was confirmed by approximately 99.5 % identity between the elk-derived sequence and the target sequence from the *E. chaffeensis* (Arkansas strain) genome (Fig. S3).

Genetic diversity of *p28* has been used to differentiate human isolates (Wagner *et al.*, 2004; Yu *et al.*, 1999). Therefore elk buffy coat samples were assayed with an *E. chaffeensis p28*-based PCR assay to further characterize the *E. chaffeensis* strain present. In two trials, one of two and one of four replicates, respectively, were amplified with this assay. There was a substantial difference (87 % identity) between the two *p28* sequences generated from the elk

Fig. 1. PCR products from elk tissue and tick samples amplified with the *E. chaffeensis* *trp32*-based assays. Elk buffy coat biological replicates (BC1–6), spleen biological replicates (S1–2), pooled tick sample (T), no-template controls (N), and *E. chaffeensis* Arkansas strain-infected DH82 samples were assayed and electrophoresed with a 100 bp molecular mass marker (M). (a) Trials 1 and 2 utilizing primers FB5A and FB3A. (b) Trials 1 and 2 utilizing primers FB5 and FB3.

buffy coat samples compared with the closest match, the V2 strain of *E. chaffeensis*. Interestingly, there were also mismatched nucleotides at the 5' end of the forward primer, which partially explained the relatively low efficiency of this assay and the lack of amplification from all of the replicates tested (Fig. S4).

The 16S rDNA sequence is 100 % identical among known US isolates of *E. chaffeensis* (Yu *et al.*, 2006); so these elk

samples were further examined with a 16S rDNA-based real-time PCR assay for *E. chaffeensis* (Loftis *et al.*, 2003). This assay uses a fluorogenic probe that is specific for *E. chaffeensis* and that is not expected to detect *Ehrlichia muris*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Anaplasma phagocytophilum*, *Neorickettsia sennetsu* or *Rickettsia prowazekii* DNA. All buffy coat samples and one of two spleen samples were PCR-positive with the 16S rDNA-based assay (Fig. S5).


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FB5A  GTGACATCTTAGTTTAATAGAAC >
Ark   -----TAATAGAACTTTATATTTATATATGATTATATATA 49
Elk   -----TAATAGAACTTTATATTTATATATGATTA-ATATA
                                           *

Ark   TAATGATAATGGTATGTGGTTATAACTGCTTATTAGTTGATCATGTACCTGTGTGTTATG 109
Elk   TAATGATAATGGTATGTGGTTATAACTGCTTATTAGTTGATCATGTACCTGTGTGTTATG

Ark   TTAAATAGGGTATAAATATGTCAACAATTCTCTGAAGATAATATGGGTAATATACAAATGC 169
Elk   TTAAATAGGGTATAAATATGTCAACAATTCTCTGAAGATAATATGGGTAATATACAAATGC
      FB5 >

Ark   CTTTTGATTCTGATTCACATGAGCCTTCTCATCTTGAGCTACCTAGTCTTTCTGAAGAAG 229
Elk   CTTTTGATTCTGATTCACATGAGCCTTCTCATCTTGAGCTACCTAGTCTTTCTGAAGAAG

Ark   TGATTCAATTAGAGAGTGATCTACAACAATCTTCTAATTCTGATTTACACGGGTCTTTTT 289
Elk   TGATTCAATTAGAGAGTGATCTACAACAATCTTCTAATTCTGATTTACACGGGTCTTTTT

Ark   CTGTTGAGTTATTTGATCCTTTTAAAGAAGCAGTTCAATTGGGGAATGATCTACAACAAT 349
Elk   CTGTTGAGTTATTTGATCCTTTTAAAGAAGCAGTTCAATTGGGGAATGATCTACAACAAT

Ark   CTTCTGATTCTGATTTACACGGGTCTTTTTCTGTTGAGTTATTTGATCCTTCTAAAGAAG 409
Elk   CTTCTGATTCTGATTTACACGGGTCTTTTTCTGTTGAGTTATTTGATCCTTCTAAAGAAG

Ark   AAGTTCAATTGGAGAGTGATCTACAACAATCTTCTAATTCTGATTTACACGAGTCTTCTT 469
Elk   AAGTTCAATTGGAGAGTGATCTACAACAATCTTCTAATTCTGATTTACACGAGTCTTCTT

Ark   TTGTTGAGTTACCTGGTCCTTCCAAAGAAGAAGTTCAATTGGAAGATGATGCTAAAAATG 529
Elk   TTGTTGAGTTACCTGGTCCTTCCAAAGAAGAAGTTCAATTGGAAGATGATGCTAAAAATG

Ark   TAGTATATGGACAAGACCATGTTAGTTTATCTGAATTAGGCTTATTGTTAGGTGGTGTGTT 589
Elk   TAGTATATGGACAAGACCATGTTAGTTTATCTGAATTAGGCTTATTGTTAGGTGGTGTGTT
      < FB3

Ark   TTAGTACAATGAATTATTTGTCTGGTTATACACCGTATTATTATCATCATTATTGTTGTT 649
Elk   TTAGTACAATGAATTATTTGTCTAGTTATACACCGTATTATTATCATCATTATTGTTGTT
      *

Ark   ATAATCCTTATTATTATTTTGATTATGTTACTCCAGATTATTGTCATCACTGTAGTGAAA 709
Elk   ATAATCCTTATTATTATTTTGATTATGTTACTCCAGATTATTGTCATCACTGTAGTGAAA

Ark   GTAGTTTAGAGTAGGATATTTAGAAATATAAATGGTTGTTGACTTCACAAAAGGTGTAGT 769
Elk   GTAGTTTAGAGTAGGATATTTAGAAATATAAATGGTTGTTGACTTCACAAAAGGTGTAGT

Ark   TTTATATGTTTTATGCTGTTTTATAGTGTTATAAGGATATGAGTTGTTTTTACTATTTTT 829
Elk   TTTATATGTTTTATGCTGTTTTATAGTGTTATAAGGATATGAGTTGTTTTTACTATTTTT

Ark   CTCTAT----- 849
Elk   CTCTAT-----
      < CTCTATAACGTTTCAGTCTT FB3A

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Fig. 2. Alignment of elk buffy coat-derived (Elk) and *E. chaffeensis* Arkansas strain-infected DH82 cell (Ark) amplicon sequences generated with *E. chaffeensis* *trp32* primer sets. Nucleotide discrepancies between the sequences are denoted by asterisks.

E. chaffeensis *trp32* was also shown to exhibit inter-strain variation; so nested *trp32*-based primer sets were used to assay the elk- and tick-derived templates. These primer sets are commonly used for nested PCR, but the analytical

sensitivity was sufficient to use these assays individually in this study. Templates were assayed from six different elk buffy coats, two spleen samples and the pooled tick sample. In the first trial, primers FB5A and FB3A amplified target

sequences from three of the six buffy coats and one of the two spleen samples, but the pooled tick sample was PCR-negative (Fig. 1). Similar results were observed in the second trial, except an additional buffy coat sample tested PCR-positive. Primers FB5 and FB3 amplified all six buffy coats and both spleen replicates in the first trial, all but one spleen sample in the second trial, and none of the pooled tick samples in either trial. With the exception of a single nucleotide, the amplicon sequence derived from the elk samples was nearly 100 % identical to that of the *E. chaffeensis* Arkansas strain (Fig. 2).

Discussion

Multiple locus sequence analysis was used to confirm that *E. chaffeensis* was consistently detected in elk buffy coat and spleen samples through PCR assays based on four different gene sequences (Table 1). *E. chaffeensis* has been detected in tissues from other cervids, including blood, bone marrow, inguinal lymph nodes, scapular lymph nodes and spleen from white-tailed deer (Arens *et al.*, 2003; Paddock & Childs, 2003), and blood from Korean spotted deer, also known as sika deer, and Brazilian marsh deer (Kawahara *et al.*, 2009; Lee *et al.*, 2009; Machado *et al.*, 2006).

A definitive cause of death was not determined for the elk, but *E. chaffeensis* is not thought to cause clinical disease in white-tailed deer (Paddock & Childs, 2003; Varela-Stokes, 2007). Anorexia and depression, signs of ehrlichiosis in other species (Goodman *et al.*, 2003; Harrus & Waner, 2011; Stich *et al.*, 2008), were observed in sika deer in which *E. chaffeensis* was detected; however, these deer were co-infected with *Anaplasma bovis*.

Studies examining elk for the presence of ehrlichial pathogens are limited. Elk from California tested PCR-positive for an *Ehrlichia*-like species that was not *E. chaffeensis* (Foley *et al.*, 1998). However, human monocytic ehrlichiosis is considered relatively rare in California, and *A. americanum*, the primary vector of *E. chaffeensis* in the USA, is not established in California. Therefore it is not surprising that *E. chaffeensis* was not highly prevalent among wildlife hosts in that state (Paddock & Childs, 2003). Conversely, human ehrlichiosis is highly prevalent in Missouri, where *A. americanum* and white-tailed deer are indigenous and where *E. chaffeensis* has been detected in both of these reservoir populations.

Genetic diversity has been demonstrated among *p28* and *trp32* sequences from different *E. chaffeensis* strains. In the present study, the regions of *p28* and *trp32* amplified from elk tissues varied from those of reported strains. The *trp32* sequence was similar to that of the Arkansas strain sequence with one mismatch. Conversely, considerable variation was observed between the *p28* amplicon and sequences reported from other *E. chaffeensis* strains, with most similarity to the *E. chaffeensis* V2 strain.

Ticks collected from the elk were identified using morphologic characteristics and molecular techniques. As previously reported, the spiracular plates of *D. albipictus* displayed some morphologic variation ranging from round to dorsally elongated (Leo *et al.*, 2010). Interestingly, *E. chaffeensis* was not detected in *D. albipictus* collected from the infected elk described in this report, even though this tick is a member of the same ixodid tribe (i.e. the Rhipicephalinae) as other tick species known to acquire *E. chaffeensis* under natural and experimental conditions (delos Santos *et al.*, 2007; Roland *et al.*, 1998; Stoffel *et al.*, 2014). Furthermore, the atypical one-host feeding behaviour of *D. albipictus* means that it is almost certain that these adult-stage ticks fed on the elk, both as larvae and as nymphs.

We speculate that a different tick species transmitted *E. chaffeensis* to the elk, probably *A. americanum*. It seems unlikely that *D. albipictus* would be an important vector or reservoir for *E. chaffeensis*. Nevertheless, our study indicates that elk are susceptible to the infection and suggests that it would be prudent to investigate possible epidemiologic ramifications of this observation.

In conclusion, to the best of our knowledge, this is the first demonstration of a natural *E. chaffeensis* infection of an elk. We do not claim that this infection contributed to death because a definitive cause of death was not ascertained. Moreover, testing of a single elk at necropsy does not demonstrate susceptibility of this host species to the infection or the disease; further studies are required in order to investigate elk populations for infection and exposure to *E. chaffeensis* as well as to evaluate the pathogenicity of *E. chaffeensis* in elk.

Acknowledgements

This work was supported by NIH T32 RR007004 (RTS), R01 AI47932 (RWS), the MU Department of Veterinary Pathobiology and the MU College of Veterinary Medicine Council on Research.

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