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EHRLICHIA SPP. IN CERVIDS FROM CALIFORNIA

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ABSTRACT: Blood samples from six mule deer (*Odocoileus hemionus hemionus*), 15 black-tailed deer (*O. hemionus columbianus*), and 29 elk (*Cervus elaphus nannodes*) were assayed for human monocytic and human granulocytic ehrlichiosis (HGE) by polymerase chain reaction (PCR), DNA sequencing, and serology to determine whether or not cervids are involved in the maintenance of these potential human pathogens in California (USA). The deer were sampled in August to October 1992–95. The 29 tule elk from Point Reyes National Seashore were sampled in August 1997. All deer were seronegative for antibodies to HGE/*Ehrlichia equi*, while the *E. equi* seroprevalence among elk was 17%. The 16S rDNA PCR prevalence in deer was 38% (in mule deer and black-tailed deer) for *Ehrlichia*-like sp. of white-tailed deer, 5% (one black-tailed deer only) for *E. equi*, and 0% for *E. chaffeensis*. The PCR prevalence in elk was 0% for *Ehrlichia*-like sp. of white-tailed deer, 31% for *E. equi*, and 0% for *E. chaffeensis*. The *E. equi* from two positive elk samples was successfully propagated in HL-60 cell cultures. DNA sequencing confirmed that the *Ehrlichia*-like sp. sequences from deer in California were closely related to sequences reported from white-tailed deer from Oklahoma and Georgia. The *E. equi* strain from deer and elk resembled other *E. equi* strains from California. These results suggest that cervids may be important in the natural maintenance of *E. equi* in California.

Key words: *Cervus elaphus nannodes*, *Ehrlichia equi*, human granulocytic ehrlichiosis, *Odocoileus hemionus columbianus*, *Odocoileus hemionus hemionus*, survey.

INTRODUCTION

Two potentially fatal diseases of humans caused by bacteria in the genus *Ehrlichia* (Rickettsiales: Ehrlichieae) have recently been described in the eastern USA and in California (USA) (Chen et al., 1994; Maeda et al., 1987). Human monocytic ehrlichiosis is caused by *E. chaffeensis* (Dawson et al., 1991) and appears to be transmitted by the tick *Amblyomma americanum* (Anderson et al., 1993; Lockhart et al., 1995). Human granulocytic ehrlichiosis (HGE) may be caused by *E. equi* or the unnamed genetic and serologic relative "HGE agent" (Dumler et al., 1995; Madigan et al., 1996). *Ixodes scapularis* transmits the HGE agent in the eastern USA (Telford et al., 1996) and *I. pacificus* appears to transmit HGE in California (Barlough et al., 1997; Richter et al., 1996).

Possible reservoir hosts of both *Ehrlichia* spp. have been identified, although the entire natural history has yet to be elucidated. White-tailed deer (*Odocoileus virginianus*) are a competent host for *E. chaffeensis* (Dawson et al., 1994a; Ewing et al., 1995) and *E. chaffeensis* has been identified by polymerase chain reaction (PCR) and culture in the blood of naturally infected white-tailed deer (Lockhart et al., 1997). In white-tailed deer of the eastern USA, there was a high prevalence of *E. chaffeensis* antibodies (Dawson et al., 1994a; Lockhart et al., 1995). White-tailed deer as well as mule and black-tailed deer (*Odocoileus hemionus hemionus*, and *O. hemionus columbianus*, respectively) seem to be possible reservoirs of HGE as well, despite reports that the HGE agent is prevalent in *Peromyscus leucopus* (Telford et al., 1996) and other small mammal species (Walls et al., 1997) in the eastern USA. The ticks *I. scapularis* and *I. pacificus* feed on deer as nymphs and adults (Furman and Loomis, 1984; Patrick, 1976; Westrom et al., 1985). Human exposure to deer blood may be a risk factor for acqui-

sition of HGE (Bakken et al., 1996). Deer in the eastern USA had a prevalence of HGE antibodies of 8% (Belongia et al., 1997) to 47% (Walls et al., 1996), and prevalence of HGE DNA (16S rDNA PCR) of 15% (Bakken et al., 1996; Belongia et al., 1997). However, DNA sequencing of the ehrlichiae in white-tailed deer revealed a previously undescribed species within the *E. phagocytophila*-genogroup (Dawson et al., 1996), designated *Ehrlichia*-like sp. of white-tailed deer.

This paper reports the finding of the *Ehrlichia*-like sp. similar to that reported in white-tailed deer by (Dawson et al., 1996) in mule deer and black-tailed deer in California and *E. equi* in tule elk and a black-tailed deer. This discovery was unexpected because cervid populations in California are geographically distant from those of eastern white-tailed deer. Moreover, distinct ecological cycles are likely to be involved in the maintenance of *Ehrlichia* spp. in California versus the eastern USA because of different tick vectors.

MATERIALS AND METHODS

Blood was collected from 21 deer in August to October 1992–95 from four sites in California and 29 tule elk in August 1997 from Point Reyes National Seashore, California, USA (38°08′N, 123°12′W). Six mule deer were from Coleville (California, USA; 38°34′N, 119°26′W). The remainder were black-tailed deer: one from Solano County (California, USA; 38°17′N, 122°9′W), 12 from Hopland (California, USA; 38°58′N, 123°7′W), and two from Philo (California, USA; 39°4′N, 123°25′W). The blood was collected in three different manners: blood from deer from Philo was collected in acid citrate dextrose (ACD), blood from elk was collected in EDTA, and blood from all other deer was collected without anticoagulant. Ticks were collected from one elk calf during restraint, placed in 70% ethanol, and identified by means of a key (Furman and Loomis, 1984).

All blood samples were kept cool and centrifuged within 10 hr at 2,500 *g* for 10 min and separated into buffy coat (ACD and EDTA tubes only) and serum or plasma. Plasma and serum were serially diluted in 0.01 M phosphate-buffered saline to end-point, with 1:10 as the starting dilution. The immunofluorescent assay for anti-HGE IgG was performed as described by Madigan et al. (1990) using HGE-infected horse neutrophils as substrate and fluorescein isothiocyanate-labeled rabbit anti-deer IgG (Kirkegaard and Perry, Gaithersburg, Maryland, USA). The cutoff for a positive titer was 1:80.

Buffy coats were cultured on HL-60 cells as described by Goodman et al. (1996), with the following modifications. Cell culture at a density of 2×10^6 cells/5 ml RPMI 1640 (Life Technologies, Gaithersburg, Maryland, USA), containing 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine was inoculated with 200 µl buffy coat and maintained at 37 C and 5% CO_2. After 2 days, the cell culture was subdivided by removing 2 ml and replacing with 2 ml fresh medium. After two additional days, 2 ml of culture were replaced with 1×10^6 uninfected HL-60 cells in 2 ml fresh RPMI. Infection of HL-60 cells was assessed directly by Wright staining and PCR.

Extraction of DNA from buffy coat, serum, plasma, and cell culture was performed by salt lysis (Barlough et al., 1996). Nested PCR for *E. equi* 16S rDNA subunit was performed as described (Barlough et al., 1996), using external primers from conserved eubacterial 16S rDNA and internal primers specific to *E. equi*/HGE. A nested PCR that should amplify most known *Ehrlichia* spp. was performed to screen for *E. equi, E. chaffeensis,* and *Ehrlichia*-like sp. of white-tailed deer, as described by Warner and Dawson (1996). A nested PCR for *Ehrlichia*-like sp. of white-tailed deer was performed according to a published method (Little et al., 1997). A single-round PCR to obtain 16S DNA for sequencing was performed using conserved primers at the 8 and 1492 positions of all known eubacteria (Foley et al., 1998). Amplification was performed in a thermal cycler (MJ Research, Watertown, Massachusetts, USA) and PCR products visualized in 1.5% agarose gels stained with ethidium bromide.

Products from the PCR reaction were cloned into competent *E. coli* cells using the TA-cloning kit (Invitrogen, San Diego, California, USA), according to manufacturer's instructions. Plasmid inserts were verified by restriction endonuclease digestion of plasmids followed by agarose gel electrophoresis to determine insert size, and by PCR on plasmid DNA using specific primers. Products from deer AC28, "Jeff," and AC40 and elk GOB and OGR were sequenced directly, i.e., without cloning, following preparation in Microcon spin columns (Amicon, Beverly, Massachusetts, USA). Dye terminator cycle DNA sequencing was performed with the Ready Reaction Kit with AmpliTaq DNA polymerase FS (ABI Prism, Foster City, California, USA). Reactions were

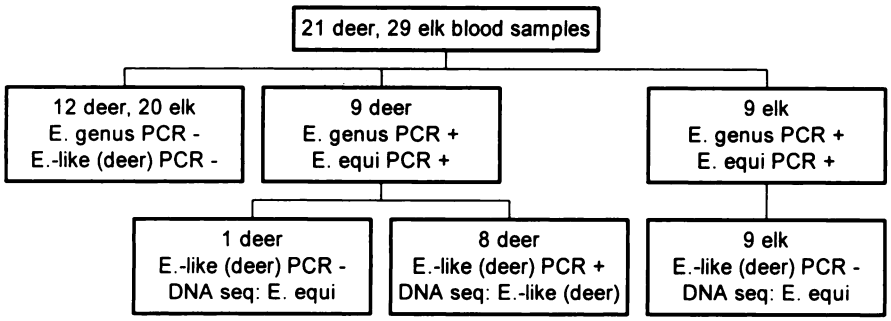

FIGURE 1. Flow chart of 16S rDNA sample results of *Ehrlichia* PCR from deer and tule elk in California.

run on a 4.25% acrylamide/bisacrylamide gel using an ABI Prism 377 DNA Sequencer. Products were analyzed with the ABI Prism Sequencing 2.1.1 software. The DNA sequences from deer and elk (excluding directly sequenced isolates) were compared to sequences maintained in Genbank using the BLAST algorithm (Altschul et al., 1990) from the National Center for Biotechnology Information. A dendrogram was generated by sequence comparison using the Cantor-Jukes correction, followed by distance calculations using the UPGMA algorithm. Sequences included for comparison were: *E. equi* (U72879), *Ehrlichia*-like sp. of white-tailed deer from Oklahoma and Georgia (U27101, U27102, U27103, U27104), HGE agent (U23039 and U23038), *E. phagocytophila* (M73224), *E. canis* (U73226), *E. chaffeensis* (U73222), and *Rickettsia rickettsii* (U11021).

RESULTS

All plasma and serum samples from deer tested negative for antibodies to the HGE/*E. equi* organism. Results of PCR using the *E. equi*-specific protocol and the pan-*Ehrlichia* protocol on buffy coat samples were negative as well (Fig. 1). In contrast, 9/21 (43%) deer were weakly PCR-positive in serum or plasma using the pan-*Ehrlichia* PCR, including four black-tailed deer and five mule deer. These same deer were PCR-positive using the *E. equi*-specific protocol. The *Ehrlichia*-like sp. of white-tailed deer-specific PCR resulted in negative test results in the 12 deer that were negative by *E. equi* PCR. Of the nine *E. equi*-positive deer, eight of the 21 deer (38%) also had *Ehrlichia*-like sp.

Of 23 plasma samples tested from tule elk, four (17%) contained IgG antibodies reactive to HGE antigen. Nine of 29 (31%) elk plasma samples were PCR-positive for *Ehrlichia*-group DNA using the pan-*Ehrlichia* protocol; only one of these (elk GOB) was among the seropositive group. Only one (elk OOO) of the nine PCR-positive samples in plasma was PCR-positive in buffy coat. PCR specific for *E. equi* amplified all nine of the pan-*Ehrlichia* positive samples, while *Ehrlichia*-like sp. of deer PCR amplified three of the pan-*Ehrlichia* positive plasma samples.

Two of four HL60 cell cultures containing elk buffy coat became *Ehrlichia* positive on day 11 after inoculation. Infection was confirmed by cell appearance, which became fragmented and ragged, presence of dense grayish morulae within cell cytoplasm, and positive nested PCR results from cell culture precipitate using the pan-*Ehrlichia* protocol. The DNA sequences of the PCR products (pan-*Ehrlichia*) from the culture were found to be identical with the sequences of the PCR products from the original blood sample.

Sequencing of DNA and construction of a dendrogram revealed that the PCR-positive deer were infected with *Ehrlichia* spp. from two different clades: deer AC32 (a black-tailed deer) was infected with an *E. equi*, while the other deer (black-tailed and mule) were infected with an *Ehrlichia*-like sp. that was in the same clade as previously reported by (Dawson et al., 1996) (Fig. 2). Sequencing of the elk frag-

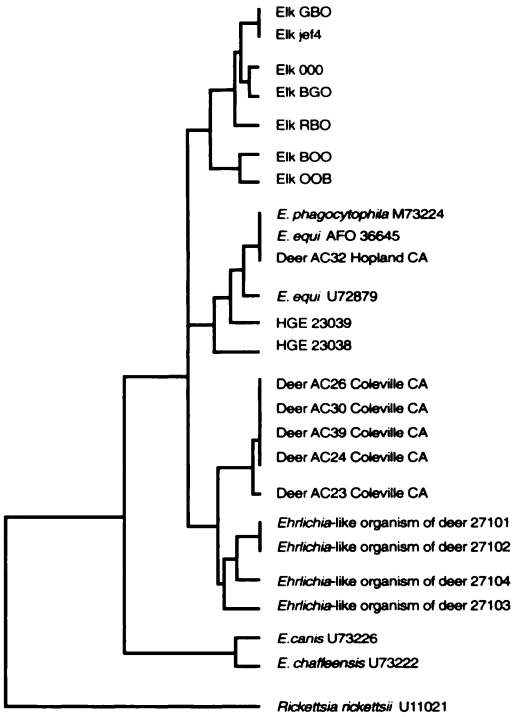

FIGURE 2. Phylogram of ehrlichiae and rickettsiae of humans and other animals with *Ehrlichia* spp. isolates from deer and elk in California. See text for accession numbers and origin of isolates.

ments confirmed infection with *E. equi*. The DNA sequence of the isolate from deer AC32 was identical over the region sequenced to Genbank accession #AF036645, which was isolated from a horse in California.

The ticks removed from the elk calf were the winter tick, *Dermacentor albipictus*. These included three larvae and five nymphs.

DISCUSSION

Wild cervid populations in California are infected with *E. equi*, an important cause of morbidity in horses, dogs, and possibly humans, as well as with a new species of *Ehrlichia* designated *Ehrlichia*-like sp. of deer. California cervids have a similar or identical *Ehrlichia*-like sp. as white-tailed deer from the eastern USA, which was unexpected given the presence of different tick vectors with unique ecologies. No cervids had positive PCR results for *E. chaffeensis*, despite the occurrence of human monocytic ehrlichiosis in California and the report that white-tailed deer may be the primary reservoir of *E. chaffeensis* in the eastern USA (Lockhart et al., 1997).

Detection of ehrlichiae in deer and elk was facilitated by PCR, using separate specific protocols for *E. equi* (Barlough et al., 1996) and *Ehrlichia*-like sp. of deer (Little et al., 1997) in addition to a generic pan-*Ehrlichia* protocol (Warner and Dawson, 1996). The pan-*Ehrlichia* nested primer set was designed to be specific and sensitive for all species in the *Ehrlichia* genus, and has been shown to amplify *E. equi*, *E. chaffeensis*, *Ehrlichia*-like sp. of deer, *E. canis*, and *E. phagocytophila*. It does not amplify the agent of Potomac horse fever, *E. risticii*, but this is probably because that agent is inappropriately included in the genus *Ehrlichia* (Wen et al., 1995). Unfortunately, the pan-*Ehrlichia* protocol does amplify some bacteria well outside the rickettsial clade including *Pseudomonas* spp. and *Psychrobacter* spp. (J. Foley, unpubl. data), requiring all products obtained to be further confirmed as *Ehrlichia* by DNA probes or sequencing. Nevertheless, we used the generic protocol in order to screen for *E. canis*, *E. chaffeensis*, *E. equi*, HGE, and *Ehrlichia*-like sp. of deer. We found that many of the samples containing *Ehrlichia*-like sp. were weakly PCR-positive using the *E. equi*-specific protocol. PCR positive results using the *Ehrlichia*-like sp. protocol occurred in samples actually infected with *Ehrlichia*-like sp., as well as three elk infected with *E. equi*. Because of the variable specificity of these protocols, final identification of *Ehrlichia* spp. was made by DNA sequencing.

The finding that PCR signals from sera or plasma of both deer and elk were stronger than those from buffy coat was unexpected, given that granulocytes are the target cells for the *E. phagocytophila* clade ehrlichiae. The handling of deer samples

had not been optimal for PCR, making it possible that blood cells might have lysed and released *Ehrlichia* DNA into the serum, as reported for infected cell cultures (Goodman et al., 1996). In contrast, the elk samples were promptly cooled and carefully handled, yet they also yielded more *Ehrlichia* DNA in serum and plasma than in buffy coat. It is possible that PCR inhibitors such as hemoglobin were not fully eliminated during DNA extraction from buffy coat, resulting in falsely negative PCR tests. Further investigation of this possibility could involve comparison of different extraction techniques for deer samples.

The clinical significance of *Ehrlichia*-like sp. and *E. equi* in deer and elk is unknown. Deer have been reported to be infected with *E. chaffeensis* both in nature (Lockhart et al., 1997) and by experimental inoculation (Dawson et al., 1994b; Ewing et al., 1995), largely without detectable clinical signs. It is not clear why the anti-HGE antibody prevalence was so low in deer, suggesting either that the IFA assay was insensitive or that deer failed to develop antibody titers comparable to horses, dogs, cats, and other laboratory animal models of HGE. Our findings are similar to those of Belongia et al. (1997), who found little relationship between PCR test results and IFA results in HGE-infected white-tailed deer, although they did not confirm all PCR results with DNA sequencing. In contrast, Walls et al. (1996) reported that 47% of white-tailed deer sera from Wisconsin had antibodies to *E. equi* which were confirmed by western blot to have a 42 kDa band specific to *E. phagocytophila*-group ehrlichiae. It is not known whether infection with *Ehrlichia*-like sp. of deer also produces this 42 kDa band.

The PCR-prevalence of *Ehrlichia*-like sp. was previously reported to be from 65 to 100% in Georgia and Oklahoma (Dawson et al., 1996; Little et al., 1997). Our finding of 38% for deer is comparable. Little is known about the ecology of *Ehrlichia*-like sp. of deer, except that its host range includes several cervid species and it has not been found in non-cervid hosts. The vector is unknown, although Little et al. (1997) associated infestations of *A. americanum* with PCR-positive deer in the eastern USA and Lockhart et al. (1997) found the *Ehrlichia*-like organism of deer by PCR in *A. americanum* pools collected from white-tailed deer in Georgia. However, *A. americanum* occurs rarely in California, typically during livestock shipping (Furman and Loomis, 1984). Ticks of cervids at Point Reyes include *D. albipictus, D. occidentalis,* and *I. pacificus* (Lane and Burgdorfer, 1986). *D. albipictus* is unlikely to vector the *Ehrlichia*-like sp. because it is a one-host tick and would not spread infection among host individuals unless the *Ehrlichia*-like sp. is maintained transovarially in the ticks.

The finding of *E. equi* in deer and elk confirms that these species contribute to the persistence of *E. equi* in nature, but does not confirm cervids as the sole reservoir hosts. The high PCR prevalence (31% for elk) despite low seroprevalence is intriguing, because it suggests that cervids are either often infected or are infected for a longer duration and with less seroconversion than has been observed in other animal species (or that presently used assays are insensitive for deer). The seasonal distribution of blood samples in this study was predominantly summer, a season during which nymphal ticks are more common on deer than adult ticks, and during which equine and canine granulocytic ehrlichiosis are infrequent. If cervids are persistently bacteremic, particularly during the summer, it is likely that they contribute to ehrlichial persistence in California.

DNA sequencing of cervid ehrlichiae from California confirmed that there are two ehrlichial clades represented: the granulocytic ehrlichia clade and the clade containing *Ehrlichia*-like sp. from Oklahoma, Georgia, and California. The west coast *Ehrlichia*-like sp. were 99 to 100%

similar to eastern *Ehrlichia*-like sp. from Oklahoma and Georgia. Deer and elk are sympatric over much of the elk range in California; however, it is not known whether the *Ehrlichia*-like organism occurs in cervids between California and the previously described Oklahoma cases. The lack of genetic variability among the *Ehrlichia*-like sp. isolates may reflect frequent mixing of isolates, a recent introduction of these isolates into all host populations, or selection against mutants that might have arisen.

The DNA sequences of *E. equi* from deer AC32 and the elk were more than 99% similar to other granulocytic ehrlichiae of humans and animals and identical to isolates recently reported from horse cases in California. The fact that the elk appear to represent a separate clade from the HGE and *E. equi* isolates is likely due to the fact that most granulocytic ehrlichial sequences in Genbank are from the eastern USA. Further research is necessary to compare the cervid isolates genetically with horse, canine, and human isolates from the same geographic origin.

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