SEROLOGIC AND MOLECULAR EVIDENCE OF EHRLICHIA SPP. IN COYOTES IN CALIFORNIA

Authors: Pusterla, Nicola, Chang, Chao-Chin, Chomel, Bruno B., Chae, Joon-Seok, Foley, Janet E., et al.

Source: Journal of Wildlife Diseases, 36(3) : 494-499

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-36.3.494

Journal of Wildlife Diseases, 36(3), 2000, pp. 494–499

SEROLOGIC AND MOLECULAR EVIDENCE OF *EHRLICHIA* SPP. IN COYOTES IN CALIFORNIA

Nicola Pusterla,[1,5] Chao-Chin Chang,[2] Bruno B. Chomel,[2] Joon-Seok Chae,[1] Janet E. Foley,[1] Elfriede DeRock,[1] Vicki L. Kramer,[3] Hans Lutz,[4] and John E. Madigan[1]

[1] Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California 95616, USA
[2] Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, California 95616, USA
[3] California Department of Health Services, Vector-Borne Disease Section, Sacramento, California 94234, USA
[4] Clinical Laboratory, Department of Veterinary Internal Medicine, University of Zurich, CH-8057 Zurich, Switzerland
[5] Corresponding author (e-mail: npusterla@ucdavis.edu)

ABSTRACT: In order to determine the role of coyotes in the epidemiology of granulocytic and monocytic ehrlichial agents in California (USA), we tested 149 serum samples for antibodies against *Ehrlichia equi, E. risticii,* and *E. canis,* using an indirect immunofluorescent antibody test. Polymerase chain reaction (PCR) assay was used to survey for the presence of members of the *E. phagocytophila* genogroup, *E. risticii* and *E. canis* in blood samples of 95 coyotes. Sixty-eight (46%) samples were seropositive for *E. equi,* two (1%) for *E. risticii* and none of the samples had antibodies reactive to *E. canis.* Two and one coyote were positive for *E. risticii* and members of the *E. phagocytophila* genogroup by PCR assay, respectively. In contrast, the 95 samples were negative for *E. canis* by PCR. Ninety-five percent of the 68 *E. equi* seropositive coyotes and the one coyote PCR positive for members of the *E. phagocytophila* genogroup originated from a coastal area. However, the two *E. risticii* seropositive coyotes and the two coyotes PCR positive for *E. risticii* were from northern California. Sequence analysis of the three amplified PCR products revealed the agent to be similar in two coyotes to the sequences of *E. risticii* from horses originating from northern California and identical in one coyote to the agent of human granulocytic ehrlichiosis and *E. equi* from California. Thus, coyotes are exposed to granulocytic ehrlichiae and *E. risticii* and may play a role in the epidemiology of these ehrlichial agents in California.

Key words: Coyote, *Canis latrans,* granulocytic ehrlichiae, *Ehrlichia risticii,* indirect immunofluorescence antibody test, polymerase chain reaction, reservoir hosts, survey.

INTRODUCTION

Ehrlichioses are rickettsial diseases of animals and humans caused by intracellular microorganisms of the genus *Ehrlichia.* Current classification places members of this genus into three genogroups, which are the *E. phagocytophila,* the *E. canis,* and the *E. sennetsu* groups, based on morphological, phylogenetic and antigenic analysis (Rikihisa, 1991). These bacteria infect leukocytes of mammalian hosts and are transmitted mostly by ticks. Recently, studies have shown members of the *E. sennetsu* genogroup to be transmitted by trematode vectors using freshwater snails as intermediate hosts (Barlough et al., 1998; Reubel et al., 1998). Although some ehrlichiae are strictly host specific in nature, some members of this genus infect or may cause disease in unusual hosts. In dogs and wild canids, *E. canis, E. ewingii,* and *E. platys* are thought to be host specific, while *E. chaffeensis,* the agent of human granulocytic ehrlichiosis (HGE), *E. equi* and *E. risticii* have been sporadically reported in these species (Greig et al., 1996; Kakoma et al., 1994; Murphy et al., 1998; Woody and Hoskins, 1991). In areas where ehrlichial diseases occur in humans and domestic animals, wild canids may serve as reservoir hosts for these agents (Amyx and Huxsoll, 1973; Pusterla et al., 1999). Since members of the *E. phagocytophila* genogroup and *E. risticii* are the most encountered ehrlichiae in California (USA), we investigated their occurrence in coyotes, together with *E. canis,* the worldwide most important ehrlichial species in canids.

MATERIALS AND METHODS

One hundred and ten serum samples, 39 uncoagulated blood samples, and 56 Nobuto filter

strips (Advantec/MSS Inc., Pleasanton, California, USA) from 205 coyotes (*Canis latrans*) were obtained from the California Department of Fish and Game (Rancho Cordova, California, USA) and the California Department of Health Services, Vector-Borne Disease Section (Sacramento, California, USA). Samples were collected from 1998 through 1999 and kept frozen at −20 C until tested. For the 39 uncoagulated blood samples, both serological testing and polymerase chain reaction (PCR) were performed. Serum and plasma samples for indirect immunofluorescence antibody (IFA) test were available from coyotes originating from Los Angeles (n = 18), Santa Clara (110) and Tehama (21) counties (California). Nobuto strips and uncoagulated blood for PCR were collected from coyotes from El Dorado (7), Kern (11), Los Angeles (18), Modoc (9), Plumas (7), San Luis Obispo (8), Santa Clara (11), Sierra (3) and Tehama counties (21) (California). One hundred and sixty-two coyotes were adults (>1-yr-old) and forty-three were juveniles (<1-yr-old). Forty-nine percent of the coyotes were males and 51% were females.

Serum and plasma samples were examined for antibodies to ehrlichiae via indirect immunofluorescent assays. The serological detection of antibodies to *E. canis* was performed according to Ristic et al. (1972). *Ehrlichia equi* and *E. risticii* antigen were prepared and used for the detection of antibodies as described previously (Madigan et al., 1990, 1995). The conjugate was fluorescein isothiocyanate-conjugated goat anti-dog IgG (Veterinary Diagnostic Technology, Inc., Wheat Ridge, Colorado, USA). Titers ≥1:10 were considered positive in accordance with the reference range established in our laboratory. Univariate statistical analysis of the prevalence of titers was performed using Fisher's exact test (Statistix program for Windows, Analytical Software, Tallahassee, Florida, USA), and $P \leq 0.05$ was considered statistically significant.

A 5 mm × 5 mm piece of each Nobuto filter strip, initially dipped in the animal blood and air dried, were cut with sterile scissors in a 1.5 ml tube and allowed to soak in phosphate-buffered saline overnight at 4 C. After centrifugation the paper strips were removed and the pellet was used for DNA purification. Genomic DNA extraction was performed using a QIAamp DNA Blood Kit (Qiagen, Santa Clarita, California, USA) according to the manufacturer's instructions. To avoid contamination the extraction steps were performed under a laminar flow and negative controls (Nobuto filter strips initially dipped in blood from a pathogen-free dog) were included. DNA was examined for the presence of *E. phagocytophila* genogroup agents, *E. risticii*, and *E. canis* genomic DNA by PCR. Nested PCR assays for members of the *E. phagocytophila* genogroup and *E. risticii*, which amplify a 928-bp and a 527-bp product of the 16S rRNA gene, respectively, were performed as described by Barlough et al. (1996, 1997a). A single-round PCR, which amplifies an *E. canis* specific amplicon, was constructed from 16S rRNA gene sequences previously published (Anderson et al., 1991). The primer pair used for *E. canis* included EC1 (5′CGGACAATTATTTATAGCCTC3′) and EC2 (5′CCATTACCTCACCAACTAG3′). The *E. canis* PCR assay generated a 191-bp product with a detection threshold of one cell infected in vitro with *E. canis* (data not shown). Cycling conditions involved an initial 2 min denaturation at 94 C, followed by 40 cycles, each consisting of a 30 sec denaturation at 94 C, a 1 min annealing at 53C, and a 1 min extension at 72 C. These cycles were followed by a final 5 min extension at 72 C. The PCR assays were performed on a automated DNA thermal cycler (MJ Research Inc., Watertown, Massachusetts, USA). DNA from an uninfected dog and horse, and from an *E. equi* and *E. risticii* infected horse, and an *E. canis* infected dog were used as negative and positive PCR control, respectively. In order to reduce the potential for contamination, DNA extraction, PCR setup, and gel electrophoresis were performed in three separate rooms, and the PCR mixtures were pipetted under laminar flow. Generated PCR products were resolved on 1.5% agarose gels, stained with ethidium bromide and examined under UV illumination. The resulting PCR products were extracted from the gel using a gel band purification kit (QIAquick Gel Extraction Kit, Qiagen). Sequencing of both DNA strands was performed with a fluorescence-based automated sequencing system (PE Biosystems, Foster City, California, USA).

RESULTS

Sixty-eight (46%) samples had antibodies reactive to *E. equi*, two (1%) to *E. risticii*, and none of the samples had antibodies reactive to *E. canis* (Table 1). Seroprevalence for *E. equi* and *E. risticii* varied among the counties. The prevalence for *E. equi* from Los Angeles and Santa Clara counties was similar ($P > 0.05$). The prevalence for *E. equi* from Tehama County was significantly lower than from Los Angeles County ($P = 0.002$) and from Santa Clara County ($P = 0.002$). There was no major difference in prevalence by

TABLE 1. Results of serological examination of 149 coyote samples for *Ehrlichia equi*, *E. risticii* and *E. canis* by indirect immunofluorescence antibody test.

Origin (county)	*E. equi* Reactive[a]/ total (%)	*E. equi* IFA titers (*n*)	*E. risticii* Reactive[a]/ total (%)	*E. risticii* IFA titers (*n*)	*E. canis* Reactive[a]
Los Angeles	9/18 (50)	10(3), 20(3), 40(2), 320(1)	0	<10(18)	0
Santa Clara	56/110 (51)	10(14), 20(5), 40(7), 80(6), 160(8), 320(12), 640(2), 1,280(2)	0	<10(110)	0
Tehama	3/21 (14)	10(2), 80(1)	2/21 (10)	10(1), 20(1)	0
Total	68/149 (46)		2/149 (1)		0

[a] IFA titer of ≥1:10.

sex; however, the prevalence was significantly higher in adult than in juvenile coyotes ($P = 0.01$). Both *E. risticii* seropositive coyotes were adults and found in Tehama County; compared to Los Angeles and Santa Clara counties, the difference was statistically significant ($P = 0.0004$).

Nested PCR yielded positive products for *E. risticii* DNA in one adult and one juvenile coyote originating from northern California (Tehama and Plumas counties) and for *E. phagocytophila* genogroup members in one juvenile coyote from central coastal California (Santa Clara County). The nucleotide sequence of the two *E. risticii*-positive coyotes differed in two nucleotide positions and was closely related to the sequences of *E. risticii* strains originating from northern California (Table 2; Barlough et al., 1998; Reubel et al., 1998). The nucleotide sequence of the coyote from Santa Clara County was 100% homologous to that of *E. equi* and the agent of HGE from California (Foley et al., 1999).

The 16S rRNA gene sequence of the coyote strains have been deposited in GenBank under the accession numbers AF170727 (coyote from Tehama County), AF170728 (coyote from Santa Clara County) and AF170729 (coyote from Pluma County).

DISCUSSION

Although ehrlichial diseases were once considered rare in the USA, this is no lon-

TABLE 2. Nucleotide differences at the 5′ end of the 16S rRNA genes of coyote, equine and snail *E. risticii* strains from northern California.

DNA source	Nucleotide at the following position[a]: 76	94	97	131	218	309	342	363
E. risticii Illinois	G	G	C	G	G	A	A	A
Coyote (Tehama County)	.	.	A	A	.	.	G	.
Coyote (Plumas County)	.	.	A	A	A.	.	G	G
Snail (*Juga* spp.)[b]	.	.	.	.	.	.	.	.
Snail (SHSN-1)[c]	.	.	.	.	.	.	.	.
Snail (SHSN-2)[c]	.	.	.	.	.	G	.	.
Horse (SRC)[d]	.	.	.	.	.	.	.	.
Horse (Dr Pepper)[b]	.	.	.	.	.	.	.	.
Horse (Ms Annie)[b]	.	.	.	.	.	.	.	.
Horse (Doc)[b]	A	A	A	A	.	.	.	.

[a] Differences in the nucleotide sequence are relative to the sequence of the type strain *E. risticii* Illinois (GenBank accession no. U21290); identical nucleotides are indicated by periods, and nucleotide differences are indicated by capital letters.
[b] According to Reubel et al. (1998).
[c] According to Barlough et al. (1998).
[d] According to Madigan et al. (1997).

ger true. Diagnoses of ehrlichial diseases are being made in the USA with increasing frequency in part because of improving diagnostic tests, and increasing clinical awareness. In California, the agents of HGE/*E. equi* have a broad host range including humans (Gewirtz et al., 1996; Foley et al., 1999), horses (Madigan and Gribble, 1987), dogs (Madewell and Gribble, 1982) and deer (Foley et al., 1998). *E. risticii* has as yet only been found in horses and in trematode stages collected from freshwater snails from northern California (Madigan et al., 1997; Barlough et al., 1998; Reubel et al., 1998) and *E. canis*, known to occur worldwide, has to the knowledge of the authors never been confirmed in California. The distribution of the ehrlichial agents is largely dependent on the distribution of the vectors. So far *Ixodes pacificus*, vector of the agent of HGE/*E. equi*, has been reported predominantly from the coastal and Sierra Nevada foothills, while *Rhipicephalus sanguineus*, vector of *E. canis* and almost exclusively a parasite of dogs in North America, is associated with the large population centers in California (Furman and Loomis, 1984). The vector of *E. risticii* is a helminth closely associated with aquatic habitats in northern California (Reubel et al., 1998). In our opinion, coyotes are ideal wild animals to study the importance and spread of ehrlichial diseases, because of their wide distribution, their usual fidelity to a specific territorial area, their contact with ehrlichial vectors, and their susceptibility to ehrlichial diseases (Ewing et al., 1964; Amyx and Huxsoll, 1973; Pusterla et al., 1999).

We are aware that the low cut off titer set for our serological study could artificially inflate the percentage of animals testing seropositive. However, since we tested three different ehrlichial agents, which belong to three different genogroups, the risk of cross-reaction was considered low (Dumler et al., 1995). The *E. equi* serology data demonstrate that exposure to members of the *E. phagocytophila* genogroup is common in coyotes. The high prevalence found in coyotes from Santa Clara and Los Angeles counties was influenced by age and might be related to increased exposure to the tick vector, or to a high prevalence of ehrlichial agent in the tick population in these areas. *Ixodes pacificus* ticks are predominantly spread in the coastal area and are reported to feed on coyotes (Furman and Loomis, 1984). Recent epidemiological studies have found members of the *E. phagocytophila* genogroup in 0.3–6.7% of adult *I. pacificus* from the coastal area and the Sierra Nevada foothills (Barlough et al., 1997b; Kramer et al., 1999). The significantly lower seroprevalence of coyotes from Tehama County may be attributable to differences in tick distribution among these areas. Sequencing of DNA revealed that the PCR positive-coyote from Santa Clara County was infected with the agent of HGE/*E. equi*. The low PCR prevalence in the coyote population suggests that this species is not a major reservoir for members of the *E. phagocytophila* genogroup. Most of the infected coyotes probably do not develop clinical signs, but develop antibody titers. This makes coyotes an ideal indicator species for granulocytic ehrlichiosis.

Efforts have been made in recent years to establish the host range of *E. risticii*. Dogs and cats are susceptible to infection with *E. risticii* and have been suggested as a reservoir in the field (Dawson et al., 1988; Ristic et al., 1988; Kakoma et al., 1994). All of the *E. risticii*-seropositive coyotes originated from northern California, where the disease occurs naturally in horses (Madigan et al. 1997). Since the vector and the life cycle of *E. risticii* are still unfolding, the role of wild canids is difficult to assess. Coyotes may be a reservoir host of *E. risticii* or an accidental host. DNA sequence of material collected from the coyotes was closely related to the sequences of *E. risticii* from northern California (Madigan et al., 1997; Barlough et al., 1998; Reubel et al., 1998). The small molecular discrepancy between the *E. ris-*

ticii coyote strains reported in this study has previously been shown between different horse strains and is probably part of the genetic variation of *E. risticii* (Reubel et al., 1998). The lack of knowledge of how *E. risticii* is transmitted in nature requires additional studies involving the coyote as a potential reservoir.

The only evidence for a susceptible *E. canis* host, other than dog, in the USA was supplied by Ewing et al. (1964) when they experimentally infected a coyote. The finding that none of the coyotes had antibodies reactive to *E. canis* or tested PCR positive was expected, because clinical cases have not been confirmed in dogs from California and *R. sanguineus* ticks are not widespread. However, dogs may introduce the agent in the non-infected tick population when imported from endemic areas.

ACKNOWLEDGEMENTS

The senior author is supported by the Schweizerische Stiftung für Medizinisch-Biologische Stipendien (Hoffmann-La Roche AG, Basel, Switzerland). The authors wish to thank D. Simpson for supplying blood samples from Santa Clara County.

LITERATURE CITED

AMYX, H., AND D. HUXSOLL. 1973. Red and gray foxes—potential reservoir hosts for *Ehrlichia canis.* Journal of Wildlife Diseases 9: 47–50.

ANDERSON, B., J. DAWSON, D. JONES, AND K. WILSON. 1991. *Ehrlichia chaffeensis,* a new species associated with human ehrlichiosis. Journal of Clinical Microbiology 29: 2838–2842.

BARLOUGH, J., J. MADIGAN, E. DEROCK, AND L. BIGORNIA. 1996. Nested polymerase chain reaction for detection of *Ehrlichia equi* genomic DNA in horses and ticks (*Ixodes pacificus*). Veterinary Parasitology 63: 319–329.

———, Y. RIKIHISA, AND J. MADIGAN. 1997a. Nested polymerase chain reaction for detection of *Ehrlichia risticii* genomic DNA in infected horses. Veterinary Parasitology 68: 367–373.

———, J. MADIGAN, V. KRAMER, J. CLOVER, L. HUI, J. WEBB, AND L. VREDEVOE. 1997b. *Ehrlichia phagocytophila* genogroup rickettsiae in ixodid ticks from California collected in 1995 and 1996. Journal of Clinical Microbiology 35: 2018–2021.

———, G. REUBEL, J. MADIGAN, L. VREDEVOE, P. MILLER, AND Y. RIKIHISA. 1998. Detection of *Ehrlichia risticii,* the agent of Potomac horse fever, in freshwater stream snails (Pleuroceridae: *Juga* spp.) from northern California. Applied and Environmental Microbiology 64: 2888–2893.

DAWSON, J., I. ABEYGUNAWARDENA, C. HOLLAND, M. BUESE, AND M. RISTIC. 1988. Susceptibility of cats to infection with *Ehrlichia risticii,* causative agent of equine monocytic ehrlichiosis. American Journal of Veterinary Research 49: 2096–2100.

DUMLER, J., K. ASANOVICH, J. BAKKEN, P. RICHTER, R. KIMSEY, AND J. MADIGAN. 1995. Serologic cross-reactions among *Ehrlichia equi, Ehrlichia phagocytophila,* and human granulocytic Ehrlichia. Journal of Clinical Microbiology 33: 1098–1103.

EWING, S., R. BUCKNER, AND B. STRINGER. 1964. The coyote, a potential host for *Babesia canis* and *Ehrlichia* sp. The Journal of Parasitology 50: 704.

FOLEY, J., J. BARLOUGH, R. KIMSEY, J. MADIGAN, E. DEROCK, AND A. POLAND. 1998. *Ehrlichia* spp. in cervids from California. Journal of Wildlife Diseases 34: 731–737.

———, L. CRAWFORD-MIKSZA, J. DUMLER, C. GLASER, J.-S. CHAE, E. YEH, D. SCHNURR, R. HOOD, W. HUNTER, AND J. MADIGAN. 1999. Human granulocytic ehrlichiosis in northern California: Two case descriptions with genetic analysis of the ehrlichiae. Clinical Infectious Diseases 29: 388–392.

FURMAN, D., AND E. LOOMIS. 1984. The ticks of California (Acari: Ixodida), Vol. 25. Bulletin of California Insect Survey. University of California Press, Berkeley, California, 239 pp.

GEWIRTZ, A., P. CORNBLEET, D. VUGIA, C. TRAVER, J. NIEDERHUBER, C. KOLBERT, AND D. PERSING. 1996. Human granulocytic ehrlichiosis: report of a case in northern California. Clinical Infectious Disease 23: 653–654.

GREIG, B., K. ASANOVICH, P. ARMSTRONG, AND J. DUMLER. 1996. Geographic, clinical, serologic, and molecular evidence of granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. Journal of Clinical Microbiology 34: 44–48.

KAKOMA, I., R. HANSEN, B. ANDERSON, T. HANLEY, K. SIMS, L. LIU, C. BELLAMY, M. LONG, AND B. BAEK. 1994. Cultural, molecular, and immunological characterization of the etiologic agent for atypical canine ehrlichiosis. Journal of Clinical Microbiology 32: 170–175.

KRAMER, V., M. RANDOLPH, L. HUI, W. IRWIN, A. GUTIERREZ, AND D. VUGIA. 1999. Detection of the agents of human ehrlichioses in ixodid ticks from California. American Journal of Tropical Medicine and Hygiene 60: 62–65.

MADEWELL, B., AND D. GRIBBLE. 1982. Infection in two dogs with an agent resembling *Ehrlichia equi.* Journal of the American Veterinary Medical Association 180: 512–514.

MADIGAN, J., AND D. GRIBBLE. 1987. Equine ehr-

lichiosis in northern California: 49 cases (1968-1981). Journal of the American Veterinary Medical Association 190: 445–448.

———, S. HIETALA, S. CHALMERS, AND E. DEROCK. 1990. Seroepidemiologic survey of antibodies to *Ehrlichia equi* in horses of northern California. Journal of the American Veterinary Medical Association 196: 1962–1964.

———, Y. RIKIHISA, J. PALMER, E. DEROCK, AND J. MOTT. 1995. Evidence for a high rate of false-positive results with the indirect fluorescent antibody test for *Ehrlichia risticii* antibody in horses. Journal of the American Veterinary Medical Association 207: 1448–1453.

———, J. BARLOUGH, Y. RIKIHISA, B. WEN, P. MILLER, AND T. SAMPSON. 1997. Identification of an enzootic diarrhea ("Shasta River Crud") in northern California as Potomac horse fever. Journal of Equine Veterinary Science 17: 270–272.

MURPHY, G., S. EWING, L. WHITWORTH, J. FOX, AND A. KOCAN. 1998. A molecular and serologic survey of *Ehrlichia canis, E. chaffeensis,* and *E. ewingii* in dogs and ticks from Oklahoma. Veterinary Parasitology 79: 325–339.

PUSTERLA, N., P. DEPLAZES, U. BRAUN, AND H. LUTZ. 1999. Serological evidence of infection with *Ehrlichia* spp. in red foxes (*Vulpes vulpes*) in Switzerland. Journal of Clinical Microbiology 37: 1168–1169.

REUBEL, G., J. BARLOUGH, AND J. MADIGAN. 1998. Production and characterization of *Ehrlichia risticii,* the agent of Potomac horse fever, from snails (Pleuroceridae: *Juga* spp.) in aquarium culture and genetic comparison to equine strains. Journal of Clinical Microbiology 36: 1501–1511.

RIKIHISA, Y. 1991. The tribe *Ehrlichieae* and ehrlichial diseases. Clinical Microbiology Reviews 4: 286–308.

RISTIC, M., D. HUXSOLL, R. WEISIGER, P. HILDEBRANDT, AND M. NYINDO. 1972. Serological diagnosis of tropical canine pancytopenia by indirect immunofluorescence. Infection and Immunity 6: 226–231.

———, J. DAWSON, C. HOLLAND, AND A. JENNY. 1988. Susceptibility of dogs to infection with *Ehrlichia risticii,* causative agent of equine monocytic ehrlichiosis (Potomac horse fever). American Journal of Veterinary Research 49: 1497–1500.

WOODY, B., AND J. HOSKINS. 1991. Ehrlichial diseases of dogs. Veterinary Clinics of North America Small Animal Practice 21: 75–98.

Received for publication 14 October 1999.