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Experimental Infection of Adult and Juvenile Coyotes with Domestic Dog and Wild Coyote Isolates of *Hepatozoon americanum* (Apicomplexa: Adeleorina)

Jennifer Jane Garrett,[1] A. Alan Kocan,[1] Mason V. Reichard,[1,3] Roger J. Panciera,[1] Robert J. Bahr,[2] and Sidney A. Ewing[1] [1] Department of Veterinary Pathobiology, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078, USA; [2] Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078, USA; [3] Corresponding author (email: rmason@okstate.edu).

ABSTRACT: Each of five adult and four juvenile coyotes (*Canis latrans*) was exposed to an oral dose of 50 *Hepatozoon americanum* oocysts recovered from *Amblyomma maculatum* ticks that previously fed on either naturally infected domestic dogs (*Canis familiaris*) or naturally infected wild coyotes. All coyotes exposed to *H. americanum* became infected, regardless of isolate source, and all exhibited mild to moderate clinical disease that simulated American canine hepatozoonosis in naturally infected dogs. At 100 days postexposure, parasitemia was greater in juvenile than adult coyotes (0.9% and 0.3%, respectively); radiographic imaging of femurs revealed moderate exostosis in all juveniles and mild to moderate new bone growth in four of five (80%) adult coyotes. Gross postmortem analysis of bone lesions demonstrated variation between age groups of coyotes but not between isolates of *H. americanum.* Microscopic evaluation of skeletal muscle revealed that parasite-induced lesions were significantly more numerous (t=5.0, df=7, P=0.001) in juvenile than adult coyotes. Results of this study indicate that juvenile and adult coyotes are equally susceptible to experimental infection with *H. americanum* isolated from domestic dog and wild coyote sources. The age of coyotes at the time of exposure, and possibly the number of *H. americanum* oocysts ingested, might influence morbidity and mortality, but it appears that both adult and juvenile coyotes could be reservoirs of *H. americanum.*

Key words: Amblyomma maculatum, American canine hepatozoonosis, *Canis latrans,* coyote, emerging tickborne disease, *Hepatozoon americanum.*

American canine hepatozoonosis (ACH), caused by the protozoan parasite *Hepatozoon americanum,* is an emerging tickborne disease of domestic dogs (*Canis familiaris*) throughout the south central and southeastern United States (Vincent-Johnson et al., 1997). Cases of ACH (Macintire et al., 1997; Panciera et al., 1998) correlate with the geographic distribution of *Amblyomma maculatum* (Semtner and Hair, 1973), the only known vector (Mathew et al., 1998). Dogs with ACH exhibit ocular discharge, wasting, gait abnormalities, and marked mature neutrophilia (Macintire et al., 2001). Radiographs, particularly of the long bones from dogs infected with *H. americanum,* reveal periosteal bone proliferation (Panciera et al., 2000). Diagnosis of infection with *H. americanum* is dependent on microscopic observation of gamonts within circulating leukocytes or distinctive parasitic lesions in histologic sections of excised striated muscle (Ewing et al., 2000).

Amblyomma maculatum can acquire, maintain, and transmit *H. americanum* through each instar molt (Ewing et al., 2002a, b). Sexual development of *H. americanum* occurs within *A. maculatum,* with subsequent production of infective sporozoites within oocysts (Mathew et al., 1999). Dogs become infected on ingestion of ticks containing sporozoites, presumably through either grooming activities or consumption of tick-infested prey (Mathew et al., 1998).

Although the role of coyotes (*Canis latrans*) in the maintenance and transmission of *H. americanum* to domestic dogs has been speculated upon, it is presently unclear. Previous studies have demonstrated that greater than half (56%) of adult coyotes sampled from Oklahoma (USA) and Texas (USA) are naturally infected with *H. americanum,* although they exhibit no signs of disease or bone lesions (Kocan et al., 1999, 2000).

In addition, field studies in Oklahoma have reported that adult coyotes can be infested with one or more of larval, nymphal, or adult *A. maculatum* (Semtner and Hair, 1973; Kocan et al., 1999).

Experimental infection of juvenile coyotes exposed to an oral dose of 100 *H. americanum* oocysts obtained from ticks fed on a naturally infected dog resulted in disease similar to that observed in naturally infected dogs, including bone lesions (Panciera et al., 1998; Kocan et al., 2000). Disease in those juvenile coyotes was severe; euthanasia was performed approximately 50 days postexposure (Kocan et al., 2000). Experimental exposure of young dogs to doses of >100 *H. americanum* oocysts also resulted in severe disease and the need for euthanasia (Drost et al., 2003).

The objective of this study was to ascertain susceptibility of adult and juvenile coyotes to experimental infection with a dose of 50 *H. americanum* oocysts obtained from *A. maculatum* ticks acquisition-fed on either naturally infected domestic dogs or naturally infected wild coyotes. In an effort to further define the interrelationship of wild coyotes, domestic dogs, and ACH, qualitative and quantitative comparisons were made of severity of clinical disease, number and type of parasitic muscle lesions, levels of parasitemia, radiographic and gross analyses of bone lesions between adult and juvenile coyotes, and source of *H. americanum* isolate.

Coyotes were obtained as pups from central and north-central Oklahoma (35°50′N to 36°50′N, 97°0′W to 98°0′W) in February and March 2001 from the Predator Damage Management Unit, United States Department of Agriculture. Before experimental exposure, all coyotes were determined free of infection with *H. americanum* through histologic examination of muscle from the biceps femoris. Coyote pups were hand-raised at the Wild Animal Research Facility of Oklahoma State University (OSU) in accordance with standards of OSU Institutional Animal Care and Use Committee. Five sexually immature coyotes <6 mo of age and six adult coyotes between 1 and 2 yr of age served as experimental animals. In addition, one adult and one juvenile coyote served as uninfected controls. Monthly application of fipronil (Frontline Plus®, Merial, France) was used as flea and tick control for all experimental animals.

Amblyomma maculatum (Acarina: Ixodidae) were removed from naturally infected wild coyotes as replete nymphs or purchased as newly molted nymphs from the tick-rearing facility of the Oklahoma Agricultural Experiment Station, Stillwater, Oklahoma, and acquisition-fed on naturally infected domestic dogs or wild coyotes. Procedures for acquisition feeding of *A. maculation* followed those reported by Mathew et al. (1998) and Kocan et al. (2000).

Because of limitations in the availability of experimental animals, this study was chronologically divided into three 100-day trails from March to December 2002. Each coyote was exposed to 50 *H. americanum* oocysts by hand-feeding a mixture of oocysts with commercially prepared canned dog food or by directly exposing sedated coyotes to oocysts through oral intubation. Three adult coyotes received oocysts of *H. americanum* derived from ticks that had fed on a domestic dog, whereas two adult coyotes received oocyts of *H. americanum* from ticks that parasitized wild coyotes. Two juvenile coyotes received oocysts of *H. americanum* of domestic dog origin and two juvenile coyotes received oocysts of wild coyote origin. Control coyotes (one adult and one pup) received only saline.

In all three trials, adult and juvenile coyotes were observed daily for signs of clinical disease and gait disturbance. In addition, during the first trial, three exposed adult coyotes and the adult control were sedated weekly, and general physical examinations were performed. Gross and histopathologic evaluation of selected tissues obtained at necropsy was also per-

formed to characterize disease in coyotes from the three trials. Levels of parasitemia were calculated as the number of *H. americanum* gamonts per 1,000 leukocytes observed in Giemsa-stained blood films observed microscopically (1,000×) in blood films collected at 100 days postexposure from each coyote.

Samples of biceps femoris, triceps brachii, temporalis, and longissimus dorsi muscles were obtained from sedated coyotes before experimental exposure and again at postmortem. Tissues were fixed in 10% neutral buffered formalin and processed for routine histologic examination. Hematoxylin and eosin–stained sections of skeletal muscle ($0.5\times2\times2$ cm^3) from each coyote were examined by light microscopy (1,000×) for the presence of *H. americanum.* Parasitic lesions observed within the 2-cm^2 area of muscle were classified morphologically as either "onion-skin" cysts or granulomas and were enumerated (Panciera et al., 1998).

Radiographic surveys of the right hind leg were accomplished on sedated coyotes before experimental exposure and again after euthanasia. New bone growth observed in postmortem craniocaudal and mediolateral radiographic views were subjectively graded according to the presence or absence of new bone growth, the amount of new bone growth that had occurred, and the number of bones visible in the radiographic field that were affected.

Femurs from each coyote were collected after necropsy and cleaned according to procedures described by Panciera et al. (2000). The volume of each femur was determined through the average of eight circumferential measurements of the diaphysis at 0.25-cm longitudinal increments. Averages from all incremental points were incorporated into the equation for Simpson's Rule for uneven area and volume (Oberg et al., 1976). Femurs with new bone growth had greater volume and the difference after subtraction of control femur volume represented an approximation of the amount of periosteal deposition that had occurred on the bone.

The number of cysts and granulomas, amount of gross bone proliferation, and amount of new bone growth observed in radiographs were compared by *t*-test evaluation after analysis for normality by the Kolmogorov-Smirnov test (Sokol and Rohlf, 1997). Values for muscle and bone lesions obtained through quantitative methods of analyses described above were compared between age groups of coyotes and between isolate sources of *H. americanum.* The Bonferroni adjustment for multiple comparisons was used as the criterion of statistical significance (Sokol and Rohlf, 1997).

All exposed animals, regardless of age or source of isolate, became infected and exhibited clinical disease consistent with that reported from domestic dogs with naturally acquired ACH (Ewing et al., 2000). However, no gait abnormalities (0%) were observed in any of the coyotes. Among adult coyotes, two of five (40%) exhibited minimal disease and two of five (40%) exhibited mild disease. One adult (20%) and each of the four (100%) juvenile coyotes exhibited moderate clinical disease. However, no mortality (0%) resulted from the experimental dose of 50 *H. americanum* oocysts, the smallest dose heretofore recorded to produce experimental infection, suggesting that the severity of disease could be related to the number of oocysts ingested.

Gamonts of *H. americanum* were detected in stained blood films collected 100 days postexposure from all but one (80%) adult coyote. Overall parasitemia, regardless of age or isolate, was consistent with levels (<1%) reported from dogs infected with ACH. However, juvenile coyotes had a slightly higher parasitemia (0.9%) than did adult coyotes (0.3%), regardless of source isolate.

Histologic evaluation of striated muscle obtained 100 days postexposure from experimentally infected adult and juvenile coyotes revealed histologic lesions com-

parable to those previously reported from dogs and coyotes infected with *H. americanum.* In all infected coyotes, the average number of onion-skin cysts (±SE) per 2 cm^2 of skeletal muscle (29.3±3.6) were significantly greater (t=6.8, df=16, P<0.001) than the average number of granulomas (3.3±1.1). No difference was found in the number of onion-skin cysts observed in skeletal muscle tissue in adults compared with juveniles. However, a significant difference (t=5.0, df=7, P=0.001) in the average number (±SE) of granulomas was observed in 2 cm^2 of skeletal muscle from juvenile coyotes (7.0±1.1) compared with adult coyotes (1.4±1.1).

Postmortem radiographic images revealed that juvenile coyotes were apparently slightly more likely to develop proliferation of periosteal bone because each juvenile (100%) exhibited new bone growth moderate in severity, whereas new bone growth was detected in all but one (89%) adult coyote. Periosteal bone proliferation observed in radiographs of the four adult coyotes affected with exostosis ranged in severity from mild (three of four adults: 75%) to moderate (one of four adult coyotes: 25%). Statistically significant differences were not detected in the severity or amount of new bone growth observed in radiographs or measured grossly. However, when measured grossly, juvenile femurs had more bone surface covered with periosteal deposits (median 71.85, range 49–87%) than did femurs from adults (median 30.94, range 1–67%).

New bone growth of the endosteum was also detected by radiography in eight of the nine (89%) infected coyotes, and mineralizations were observed within medullary cavities of the femur and tibia in seven of nine (78%) radiographs from infected coyotes. Endosteal bone growth and medullary opacities are findings noted for the first time in association with experimental infection of coyotes with *H. americanum,* but the significance of these unique radiographic observations has not been determined.

The variation in parasitemia, new bone growth, clinical disease, and number of granulomas detected in juvenile coyotes in this study might indicate that the age of the animal at the time of exposure can influence the resultant disease. Additionally, infection in all experimentally exposed coyotes, regardless of *H. americanum* oocyst source (domestic dog or wild coyote), supports the presumption that adult and juvenile coyotes might be suitable reservoirs of *H. americanum* and could contribute to the occurrence of ACH in domestic dogs.

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