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American canine hepatozoonosis

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Abstract

American canine hepatozoonosis is an emerging, tick-transmitted infection of domestic dogs caused by a recently recognized species of apicomplexan parasite, *Hepatozoon americanum*. The known definitive host of the protozoan is the Gulf Coast tick, *Amblyomma maculatum*. Presently recognized intermediate hosts include the domestic dog and the coyote, *Canis latrans*. Laboratory-reared larval or nymphal *A. maculatum* can be infected readily by feeding to repletion on a parasitemic intermediate host; sporogony requires 35–40 days. Transmission of infection to the dog has been produced experimentally by oral administration of mature oocysts or oocyst-containing ticks. Canine disease follows experimental exposure in 4–6 weeks and is characterized by systemic illness, extreme neutrophilic leukocytosis, muscle and bone pain, and proliferation of periosteal bone. Histopathological findings include multifocal skeletal and cardiac myositis associated with escape of mature merozoites from within the host-cell environment. There is also rapid onset of periosteal activation and osteogenesis and, less frequently, glomerulopathy and amyloidosis. Sequential stages of development of *H. americanum* in both the dog and the tick have been elucidated. Gamonts potentially infectious to ticks have been observed in peripheral blood leukocytes of the dog in as few as 28 days after exposure to oocysts. Young coyotes experimentally exposed to a canine strain of *H. americanum* acquired disease indistinguishable from that of similarly exposed young dogs.

Keywords: American canine hepatozoonosis, hepatozoon, dogs, tick-transmitted diseases

Introduction

The genus *Hepatozoon* is a collection of more than 300 species of apicomplexan parasites that require two hosts to complete their life cycle. Syngamy and sporogony with the production of polysporocystic oocysts occurs in hematophagous invertebrates. Merogony occurs in vertebrate hosts. Transmission to vertebrates occurs exclusively by ingestion of invertebrates containing infective oocysts. Invertebrate hosts become infected by consumption of blood from infected vertebrate hosts (Smith, 1996).

Canine hepatozoonosis is a disease widely distributed in warm and temperate regions of the world and is caused by at least two species of *Hepatozoon*, *H. canis* and *H. americanum*. The disease was first recognized in India (Bentley, 1905; James, 1905). The causal agent, first named *Leukocytozoon canis* (James, 1905), later renamed *Hepatozoon canis* (Wenyon, 1926), undergoes sporogony in the brown dog tick, *Rhipicephalus sanguineus* (Christophers, 1907). Canine hepatozoonosis, presumed to be caused by *H. canis*, occurs widely in the Old World and has been reported to be hosted by several tick species (McCully *et al.*, 1975; Murata *et al.*, 1995; Craig, 1998; O'Dwyer *et al.*, 2001). Clinical disease caused by *H. canis*, though potentially severe, is generally mild (Baneth and Weigler, 1997).

Seven decades after its discovery in India, canine hepatozoonosis was recognized in domestic dogs in Texas (Craig *et al.*, 1978). Since that report, the geographical distribution of disease in the USA has expanded and its prevalence has increased greatly. Disease has now been recognized in all states along the

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Gulf Coast and hundreds of miles inland. Numerous cases from Louisiana, Alabama, Georgia, Florida and Tennessee are included in reports of the disease and its treatment (Gosset *et al.*, 1985; Macintire *et al.*, 2001). More than 50 cases have been confirmed in Oklahoma since the first case was recognized in 1995. In early reports it was assumed that the American disease was caused by *H. canis*. However, the disease in American dogs was more severe, often leading to debility and death. As incongruities in clinical and pathological features between the American and Old World diseases were increasingly recognized, specific etiology became questioned. Workers at Auburn University established that the etiological agent was a unique species different from *H. canis*; they designated the new species *H. americanum* (Vincent-Johnson *et al.*, 1997a). Variation in the 18S rRNA gene as well as differences in the definitive host, the shape of sporozoites, the number of sporozoites per sporocyst, and in signs and lesions of the disease serve to substantiate that *H. canis* and *H. americanum* are different species (Baneth *et al.*, 2000; Mathew *et al.*, 2000; Inokuma *et al.*, 2002). Further, it has been established that the Gulf Coast tick, *A. maculatum*, is a highly suitable definitive host of *H. americanum* (Mathew *et al.*, 1998; Ewing *et. al.*, 2002b). Herein we report the considerable progress made with respect to host relationships and developmental cycles of the parasite and in the diagnosis and management of the disease.

Clinical signs

The common clinical signs associated with naturally acquired American canine hepatozoonosis (ACH) are fever, stiffness, lameness, recumbency, mucopurulent ocular discharge, weight loss and muscle atrophy (Craig *et al.*, 1978; Barton *et al.*, 1985; Macintire *et al.*, 1997; Panciera *et al.*, 1998). The onset of illness in experimentally infected dogs is signaled by elevation of body temperature and mild depression beginning 3–5 weeks post-exposure (PE). These signs are followed shortly by painful anxious attitude, stiffness, conjunctival discharge, weakness, severe bone and muscle pain, reluctance to rise, weight loss, muscle atrophy and recumbency (Mathew *et al.*, 1998; Panciera *et al.*, 1999; Cummings, 2001). Neutrophilic leukocytosis, often extreme, is a consistent and obligatory feature of active disease (Gaunt *et al.*, 1983; Macintire *et al.*, 1997). Its onset coincides with or slightly precedes the initiation of clinical illness (Cummings, 2001). Limb edema and periosteal bone proliferation occur in severe cases. Periosteal bone proliferation is symmetrical and involves primarily the proximal long bones of the limbs, but can involve elements of the axial skeleton and flat bones as well. Distal bones of the limbs are usually spared (Panciera *et al.*, 2000). Periosteal lesions are demonstrable by radiography and/or scintigraphy as early as 5–6 weeks PE (Drost *et al.*, 2003). Serum chemistry aberrations include mild elevation of alkaline phosphatase, hypoalbuminemia and hyperglobulinemia. Artifactual hypoglycemia caused by increased post-collection metabolism of glucose by the extreme leukocytosis occurs (Macintire *et al.*, 1997; Vincent-Johnson *et al.*, 1997b). Longstanding infection may result in glomerulopathy and renal failure. Clinical signs may extend over weeks to several months with periods of remission or progression to severe debility and death. Some diseased animals spontaneously recover but remain infected for extended periods (Barton *et al.*, 1985; Vincent-Johnson *et al.*, 1997b; Ewing *et al.*, 2003).

Pathology

Gross lesions associated with ACH are limited. Often there are diffuse, streaky or pinpoint paleness and reduction in skeletal muscle mass, particularly noticeable in the temporal muscles. Body lymphoid organs may be moderately enlarged. Subcutaneous edema may be present in the distal segments of the limbs, especially in animals that have been recumbent. In such animals synovial fluid may be excessive, of reduced viscosity and slightly turbid. Dogs with longstanding disease may exhibit peritoneal effusion associated with protein-losing nephropathy or protozoal myocarditis. Most dogs with active disease have disseminated and symmetrical proliferation of periosteal bone (Fig. 1A and B). There is histological evidence of periosteal activation/proliferation in experimentally induced disease as soon as 5 weeks PE and radiographic evidence very shortly thereafter. Rather severe lesions may develop by 9–10 weeks PE (Cummings, 2001; Drost *et al.*, 2003). We suspect that younger animals may develop lesions more rapidly and to greater severity than older animals. The periosteal lesion is grossly and histologically very similar to hypertrophic osteopathy (HO) of dogs and other animals and hypertrophic osteoarthropathy (HOA) of humans (Panciera *et al.*, 2000). The pathogenesis of neither the *Hepatozoon*-associated lesion nor that of HO/HOA is well established. The osseous lesions of ACH, as well as those of HO and HOA, resolve when clinical ACH abates or when the lesion predisposing to HO/HOA is removed. Because very few diseases are characterized by disseminated periosteal bone proliferation, we suspect that the pathogenetic mechanisms for lesion development in these diseases, though not presently known, are similar.

Histologically, parasitic lesions of ACH have been observed in many organs but most commonly and in greatest concentration in skeletal and cardiac muscle (Fig. 2A–D). Lesions occur occasionally in adipose and loose connective tissues and even less frequently in other organs and tissues (lymph nodes, spleen, salivary

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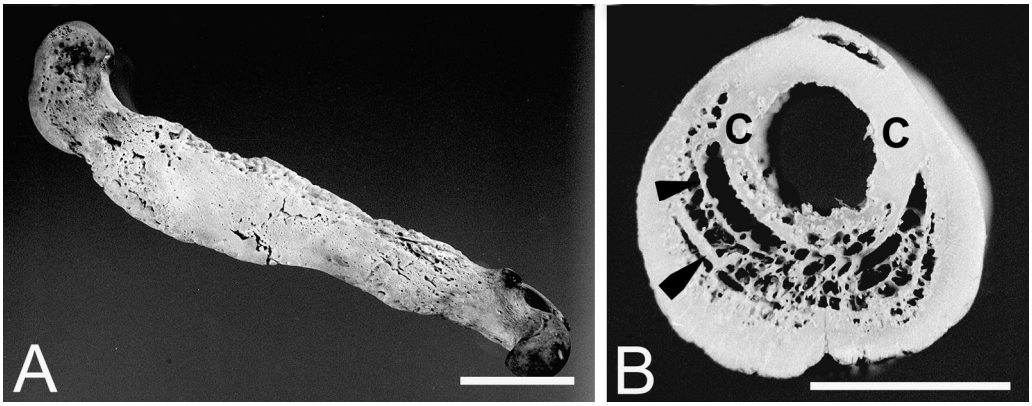

Fig. 1. (A) Femur of a young coyote 4 months after experimental exposure to sporulated oocysts of *H. americanum*. Note the irregular surface and markedly increased diameter of the femoral shaft. Bar = 2 cm. (B) Cross-section of midshaft of the humerus of a young infected coyote. Marked periosteal bone production is distributed eccentrically on the original cortex (C). Pseudocortices (arrowheads) are arranged parallel to the original cortex. Bar = 1 cm.

gland, liver, pancreas, the gastric, small and large intestinal walls, lung, subsynovial adipose tissue) (Panciera *et al*., 1998). Parasitic lesions have not been observed in organs of the central nervous system or periosteum. In addition to the parasitic lesions themselves, there are often areas of neutrophilic skeletal and cardiac myositis. Other salient histological lesions sometimes include membranous glomerulopathy, renal and other localizations of amyloid deposition, and lymphoplasmacytic inflammation of synovial membranes (Macintire *et al*., 1997; Vincent-Johnson *et al*., 1997b; Panciera *et al*., 1998; Cummings, 2001).

Diagnosis

Clinical signs combined with neutrophilic leukocytosis and symmetrical periosteal bone proliferation evoke a strong presumptive diagnosis of ACH (Panciera *et al*., 1997). Diagnosis can be confirmed sometimes by demonstration of gamonts in peripheral blood leukocytes; however, parasitemia in ACH is usually minimal and difficult to demonstrate. Although Romanowsky-type stains are adequate for demonstration of gamonts in peripheral blood leukocytes, a modified naphthol–ASD–chloracetate esterase stain provides enhanced staining (Mercer and Craig, 1988). An immunohistochemical procedure has been used successfully to demonstrate various stages of the organism in histological sections of canine tissues (Panciera *et al*., 2001). Its application to peripheral blood leukocytes has not been explored.

Confirmation of presumptive diagnosis is most reliably achieved by histopathological demonstration of one of the unique lesions of the infection in skeletal muscle biopsy (Craig *et al*., 1984; Panciera *et al*., 1998). The density of parasite lesions in various named skeletal muscles, though somewhat variable in individual animals, is not substantively different in individual muscles (Panciera *et al*., 1999). There are occasional instances when infected animals have negative biopsy results. We examined muscle biopsies semiannually from a naturally infected dog that recovered from clinical illness without antimicrobial medication. Three of the 15 biopsies (at the 36th, 54th and 66th months) were negative, yet *A. maculatum*, allowed to feed at these intervals, consistently became infected (Ewing *et al*., 2003). The concentration of lesions within muscle of this dog diminished with time.

An indirect enzyme-linked immunosorbent assay for the diagnosis of infection with *H. americanum* has been developed (Mathew *et al*., 2001). The procedure has relatively high sensitivity and specificity and not only offers advantage as a minimally invasive diagnostic aid for canine infection but, with modification, should also be a useful technique in epidemiological studies. As with many emerging diseases, initial impressions are that ACH is progressive and quite highly fatal in domestic dogs. Until means to identify dogs that have been infected and recovered are applied to a large population, morbidity and mortality rates will remain unknown.

Treatment

Relative success in the resolution of clinical ACH may be expected by following a protocol that includes administration of trimethoprim–sulfadiazine, clindamycin and pyrimethamine followed by long-term administration of decoquinate (Macintire *et al*., 2001). Though clinical illness may be held in abeyance for an extended period provided the treatment protocol is strictly adhered to, infection persists for a prolonged but unknown period (Macintire *et al*., 2001). Therapy with other antiprotozoal

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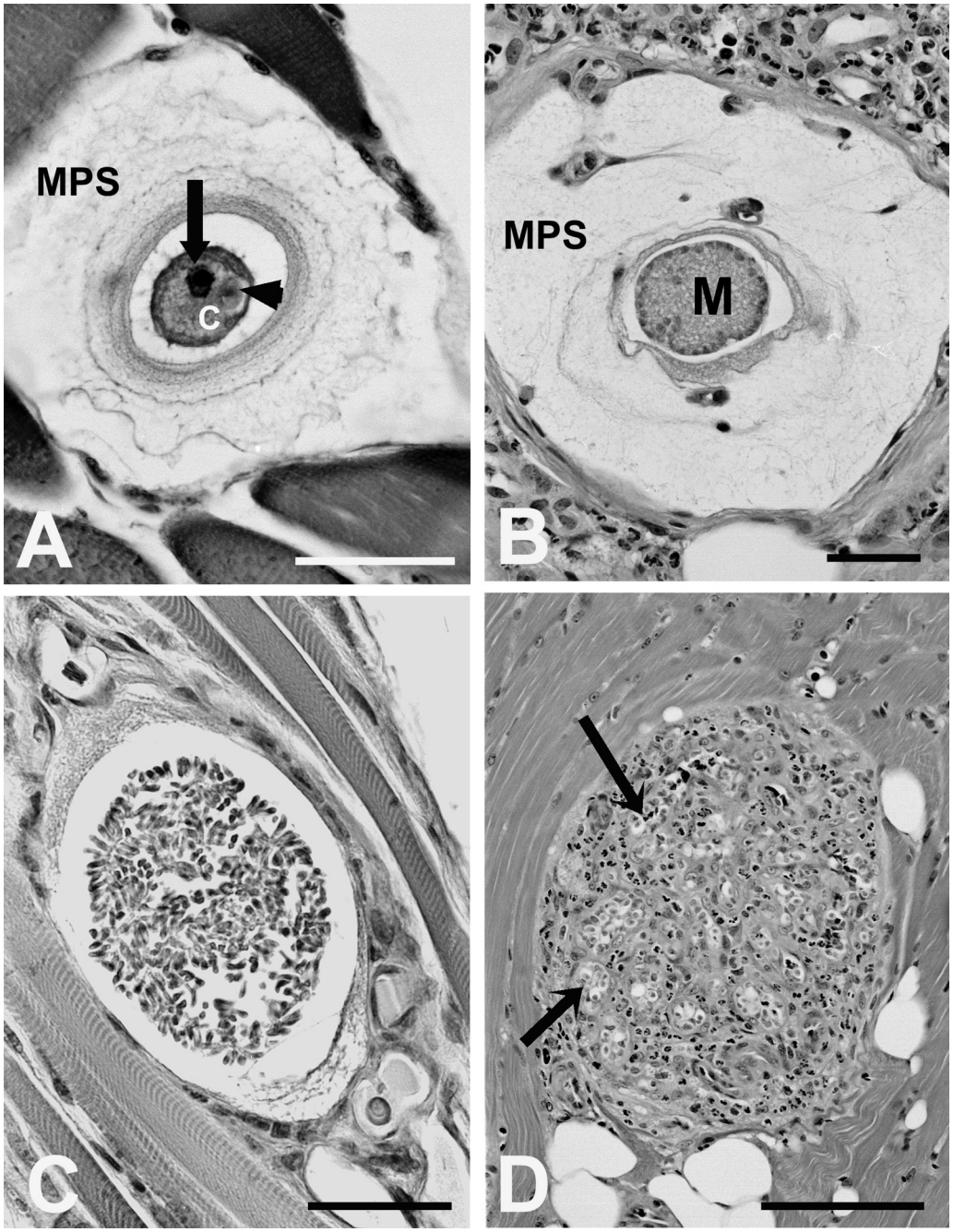

Fig. 2. (A) Early stage of merogony in skeletal muscle. So-called onionskin cyst composed of centrally located canine cell (C) with nucleus (arrow), intracytoplasmic parasite (sporozoite/trophozoite; arrowhead) and concentrically oriented mucopolysaccharide material (MPS). Hematoxylin and eosin stain. Bar = 50 µm. (B) Parasite (meront body; M) containing aggregated nucleoplasm at its margins. Mucopolysaccharide (MPS) surrounds the host cell occupied by the meront. Hematoxylin and eosin stain. Bar = 50 µm. (C) Masses of individual merozoites contained within a meront, which is surrounded by a narrow zone of mucopolysaccharide. Hematoxylin and eosin stain. Bar = 50 µm. (D) Parasitic granuloma. Many macrophages of the granuloma contain intracytoplasmic parasites, either gamonts or merozoites. Note the numerous, large-diameter vascular spaces (arrows). Hematoxylin and eosin stain. Bar = 100 µm.

drugs, for example imidocarb dipropionate, toltrazuril or diminazine aceturate, may be transiently effective but relapses usually occur (Macintire *et al.*, 1997, 2001). None of the treatment protocols thus far reported effectively eliminate infection. Treatment with non-steroidal anti-inflammatory drugs is palliative.

Developmental cycles

Infection of the canine intermediate host is acquired by ingestion of sporulated oocysts that develop in gut cells and are contained in the hemocele of the infected host tick. Oocysts are easily ruptured and presumably do so

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with the liberation of sporocysts in the upper alimentary tract. Sporozoites within sporocysts are activated by canine bile, become motile within minutes after exposure to bile (*in vitro*) and are liberated shortly when the sporocyst wall ruptures (Mathew *et al.*, 1998; Ewing *et al.*, 2000). The exact locus in the alimentary canal where sporozoites excyst is not known. It is known that, following administration of oocysts to young dogs or coyotes, large modified (activated) macrophages that contain the parasite appear in various organs and tissues of the body, especially between the fibers of striated (skeletal and cardiac) muscle (Panciera *et al.*, 1999; Cummings, 2001). We suspect that sporozoites enter host macrophages centrally in the gut wall, mesenteric lymph nodes or liver and are transported intracellularly to be deposited in various organs. However, the possibility that sporozoites liberated in the alimentary canal become disseminated hematogenously as free parasites and enter macrophages in peripheral tissues cannot be discounted. We have attempted without success to demonstrate sporozoites in the lamina propria and submucosa of the small intestine, the mesenteric nodes and liver.

The modified, parasite-containing host cells have been established to be monocytes/macrophages by fine structural (electron-microscopic) and immunohistochemical methods (Droleskey *et al.*, 1993; Cummings, 2001). Sporozoites within the modified host cells undergo trophic growth and merogony is initiated. Concurrently, the host cell cytoplasm becomes vacuolated and produces non-sulfated acid mucopolysaccharide, which it secretes into the adjacent pericellular space, producing the so-called onionskin cyst (Panciera *et al.*, 1998; Cummings, 2001). The host cell containing the parasite forms the central structure of the cyst and is surrounded by concentrically arranged, multilamellar, mucopolysaccharide material (Fig. 2A). Occasional collagen fibers and capillaries are distributed within the cyst and tissues at the margin of the cyst are compressed by this expanding structure. Cyst dimensions increase for about 20–30 weeks PE, reaching maximal dimensions of 200 × 300 μm or greater (Panciera *et al.*, 1999). Merogony proceeds with the formation of a meront, aggregation of nucleoplasm and the formation of merozoites (Fig. 2B, C). Mature merozoites are liberated as the cell membrane and mucopolysaccharide cyst material are breached. A severe local, sometimes spreading, inflammatory response follows. The neutrophil is the major reacting cell initially; the acute lesion rapidly progresses to one predominated by macrophages and granulomatous inflammation (Fig. 2D). The maturing lesion is virtually always localized with well-delimited margins (Panciera *et al.*, 1998, 1999).

Many macrophages within the granuloma harbor a single spherical or ovoid intracytoplasmic parasite (Panciera *et al.*, 1998, 2001). With light-microscopic observation these parasites appear to be of one morphological type, but electron-microscopically two types of organism have been observed (Cummings, 2001). One type had features characteristic of a gamont; the second had features of a merozoite. Parasite-containing macrophages are present within the stroma, vessel lumens and occasionally within the wall of vessels of the granulomas. The vasculature of granulomas is thin-walled, often of large diameter and lined by plump endothelial cells. Gamont-containing macrophages that are within vessel lumens are presumably the source of infection for feeding ticks (Panciera *et al.*, 1998).

It is unclear whether most trophozoites undergo merogony according to a consistent timetable. We have observed modified host cells containing parasites as soon as 20 days PE and numerous developing meronts 25 days PE. Granuloma formation has been observed as early as 32 days PE and the presence of parasite-containing leukocytes in peripheral blood leukocytes as early as 28 days PE (Mathew *et al.*, 1998; Panciera *et al.*, 1999; Cummings, 2001).

Electron micrographs of parasite-containing host cells in buffy-coat preparations revealed the cellular morphology of a macrophage (Cummings, 2001). Thus, parasite-containing cells in peripheral blood smears are macrophages rather than neutrophils, as has been commonly reported. The identification of merozoites contained in host cells within granulomas supports the unconfirmed hypothesis that merozoites recycle and initiate repeated merogonic cycles. That repetition of asexual generations occurs in ACH would provide a logical basis for prolonged infection and for the efficacy of decoquinate in the control of *H. americanum* infection (Lindsay *et al.*, 1997).

Syngamy and sporogonic development of *H. americanum* in its definitive host, *A. maculatum*, have been described by Matthew *et al.* (1999). Though differentiation of gamonts to gametes was not observed, micro- and macrogametes were recognized individually and stages of syngamy and sporogony were delineated. Reproduction occurred in unidentified cells of the tick gut, but as oocysts matured some became free in the hemocele of the tick (Fig. 3A, B). The interval from repletion of the tick on the canine host to oocyst maturity was as little as 42 days in newly molted adult ticks. The larvae of *A. maculatum* also support syngamy and sporogony of *H. americanum* (Ewing *et al.*, 2002a). Mature oocysts developed in newly molted nymphal ticks as early as 33 days after larvae had fed to repletion on an infected dog.

Experimental transmission

During the early years of canine hepatozoonosis in USA it was assumed that its causal agent was *H. canis* and that the tick definitive host was *R. sanguineus*. There is a single report of success in transmission of ACH to

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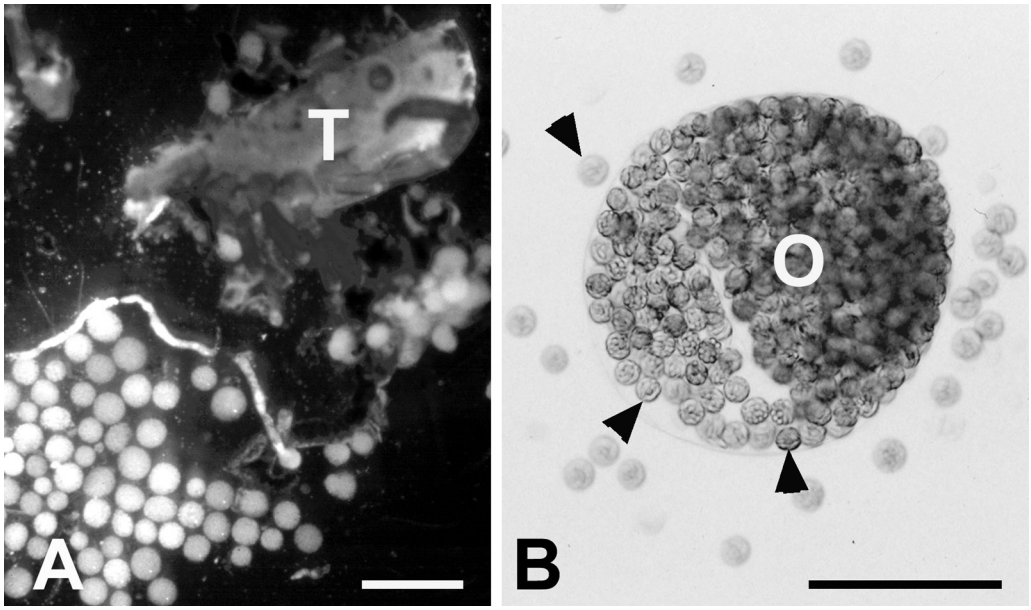

Fig. 3. (A) Numerous spherical oocysts of *H. americanum* released from a bisected *A. maculatum* (T). Bar = 75 µm. (B) Oocyst of *H. americanum* recovered from an adult *A. maculatum* experimentally infected as a nymph. Several sporocysts (arrowheads) have been released from the oocyst (O). Bar = 200 µm.

dogs by oral exposure of experimental dogs to adult *R. sanguineus* that had fed on *Hepatozoon*-infected dogs as nymphs (Nordgren and Craig, 1984). In that series of studies it was further reported that muscle tissue from infected dogs fed or injected subcutaneously into susceptible experimental dogs did not induce infection. Attempts by others to infect and transmit *H. americanum* with *R. sanguineus* have been unsuccessful (Macintire *et al.*, 1998; Mathew *et al.*, 1998; Ewing *et al.*, 2002b). Auburn University workers observed *Hepatozoon*-type oocysts in *A. maculatum*, the Gulf Coast tick, removed from a dog with naturally occurring confirmed hepatozoonosis (Macintire *et al.*, 1997). Workers at Oklahoma State University subsequently succeeded in experimentally infecting laboratory-reared nymphal Gulf Coast ticks and also in transmitting infection to dogs by oral administration of oocyst-containing *A. maculatum* (Mathew *et al.*, 1998). Three other nymphal ixodid ticks, *R. sanguineus*, *Dermacentor variabilis* and *Amblyomma americanum*, exposed to an infected dog according to the same protocol as that used with *A. maculatum*, did not become infected (Mathew *et al.*, 1998). Subsequent experiments conducted over several years involving numerous infected dogs showed that infection occurred in over 90% of more than 900 nymphal *A. maculatum* allowed to feed to repletion on parasitemic dogs. On the other hand, 225 *R. sanguineus*, 194 *D. variabilis* and 98 *A. americanum* failed to become infected (Ewing *et al.*, 2002b).

In another study it was established that larval Gulf Coast ticks, when fed on *Hepatozoon*-infected dogs, acquired *H. americanum* infection (Ewing *et al.*, 2002a). Infected larvae supported development and mature oocysts were found in newly molted nymphal ticks. Adult ticks that developed from nymphs that were infected as larvae, but not re-exposed when feeding as nymphs, also contained mature oocysts. Oocysts from both the nymphal and adult ticks were infectious for dogs. Thus transtadial transfer of infection occurs. We are not aware of evidence that transovarial transmission exists. Because larval *A. maculatum* have a different host range from nymphal ticks, the fact of larval infection and transtadial transmission with *H. americanum* has major epidemiological implications. It remains unclear where the ticks that infect dogs become infected.

Other intermediate hosts

Hepatozoonosis has been reported in the coyote, *Canis latrans*, in Texas (Davis *et al.*, 1978; Mercer *et al.*, 1988). Nine of 16 and eight of 20 free-ranging coyotes in Oklahoma were infected with *Hepatozoon* sp. (Kocan *et al.*, 1999, 2000). Histological lesions associated with the infection were quite similar to the lesions of *H. americanum* infection in domestic dogs. *H. americanum* oocysts derived from laboratory-reared ticks that fed on an infected dog induced infection and severe disease when fed to two young coyote pups (Kocan *et al.*, 2000; Panciera et *al.*, 2000). The disease and lesions, including severe periosteal bone proliferation, were indistinguishable from disease and lesions produced by experimental infection in young dogs. Periosteal bone proliferation

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was not observed in naturally infected, mature, free-ranging coyotes (Kocan *et al*., 2000). The presumptive identity of the coyote-derived parasite is *H. americanum* (Kocan *et al*., 2000). Naturally infected adult coyotes examined to date seem to be less severely parasitized than dogs and there generally appear to be fewer parasite-containing host cells in the granulomas of coyotes (Kocan *et al*., 1999). These observations suggest that the coyote is more resistant to severe infection than the dog, creating questions as to the importance of the coyote as a reservoir/vector of *H. americanum*. On the other hand, all feeding stages of the Gulf Coast tick have been found to feed on coyotes; thus, infected coyotes could be a source of infected larval and nymphal ticks. Nevertheless, we suspect that there are other feral hosts that serve as reservoirs of *H. americanum* and serve as a source of infection for larval and nymphal *A. maculatum*.

The prevalence of ACH has increased markedly in recent years and the trend will probably continue, especially if the range of *A. maculatum* spreads. Priority areas of future investigation should include the role of the coyote as a reservoir of infection, the identification of other potential reservoirs, the possibility of alternative methods of transmission, the prevalence of infection in asymptomatic dogs, and the perfection of less invasive diagnostic methods than are currently used. The frequent induction of periosteal bone proliferation in ACH simulating HO and HOA provides an opportunity for studies of the pathogenesis of symmetrical periosteal osteogenesis as an animal model of human disease.

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