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Review

Diagnosis of canine monocytotropic ehrlichiosis (*Ehrlichia canis*): An overview

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ABSTRACT

Canine monocytotropic ehrlichiosis (CME), caused by the rickettsia *Ehrlichia canis*, an important canine disease with a worldwide distribution. Diagnosis of the disease can be challenging due to its different phases and multiple clinical manifestations. CME should be suspected when a compatible history (living in or traveling to an endemic region, previous tick exposure), typical clinical signs and characteristic hematological and biochemical abnormalities are present. Traditional diagnostic techniques including hematology, cytology, serology and isolation are valuable diagnostic tools for CME, however a definitive diagnosis of *E. canis* infection requires molecular techniques. This article reviews the current literature covering the diagnosis of infection caused by *E. canis*.

Introduction

Canine monocytotropic ehrlichiosis (CME) is an important canine disease with a worldwide distribution. The disease was initially identified by Donatien and Lestoquard in Algeria in 1935 (Donatien and Lestoquard, 1937). Later in the 1970s the disease gained extensive attention when a large number of American military dogs, mainly German shepherds, died from the disease during the Vietnam War. The etiological agent of CME is the obligate intracellular rickettsia *Ehrlichia canis*, a tick (*Rhipicephalus sanguineus*)-borne transmitted organism (Groves et al., 1975).

E. canis is a Gram negative, highly pleomorphic bacterium, which is enveloped with a rippled thin outer membrane (Rikihisa et al., 1985, 1997). It consists of a single circular chromosome containing 1,315,030 nucleotides (Mavromatis et al., 2006). Infection with *E. canis* occurs mainly during the warm season when the vector tick is active. Most dogs recover from the acute and subclinical phases when treated with doxycycline or other tetracyclines at appropriate dosages and for adequate periods of time (Harrus et al., 1998b, 2004). Some dogs enter the chronic phase of the disease for which the prognosis is grave (Mylonakis et al., 2004).

Diagnosis of the disease can be challenging due to its different phases and multiple clinical manifestations. CME should be suspected when a compatible history (living in or travel to an endemic region, previous tick exposure), typical clinical signs and characteristic hematological and biochemical abnormalities are present. Traditional diagnostic techniques (hematology, cytology, serology and isolation) are valuable diagnostic tools for CME, however a definitive diagnosis of *E. canis* infection requires molecular techniques. This article reviews the current literature covering the diagnosis of infection caused by *E. canis*.

Clinical signs

CME is a multisystemic disease manifesting in acute, subclinical or chronic forms. *E. canis* can infect all breeds of dogs but the German shepherd dog appears to be more susceptible, showing the more severe form of the disease with a higher morbidity and mortality compared to other breeds (Nyindo et al., 1980). No age or gender predilection has been established. Disease manifestations may be affected by the pathogenicity of different *E. canis* strains and co-infections with other arthropod-borne pathogens such as *Babesia canis vogeli* and *Hepatozoon canis* transmitted by the same vector (Gal et al., 2007).

The acute disease is characterized by a high fever, depression, lethargy, anorexia, lymphadenomegaly, splenomegaly and hemorrhagic tendencies. The latter are usually exhibited by dermal petechiae and ecchymoses, and epistaxis. Ophthalmological lesions are frequent and include anterior uveitis, chorioretinitis, papilledema, retinal hemorrhage, presence of retinal perivascular infiltrates, and bullous retinal detachment (Komnenou et al., 2007). Blindness may occur as a result of blood hyperviscosity leading to subretinal hemorrhage and retinal detachment (Harrus et al., 1998a). Neurological manifestations as a result of meningitis and/or meningeal bleeding can manifest in a wide range of neurological signs.

During the subclinical phase no clinical signs are evident (Waner et al., 1997). For reasons still unclear, certain dogs will progress to the chronic phase of CME. During the chronic phase, symptoms similar to those seen in the acute phase may occur but with a

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greater severity. Pale mucous membranes and weakness, bleeding and significant weight loss are common findings in this phase (Harrus et al., 1997).

Hematological findings

A complete blood count (CBC) is an essential constituent in the diagnosis of CME. During the acute stage, moderate to severe thrombocytopenia is a distinctive hematological finding. Blood smear evaluation of platelet numbers is mandatory in order to confirm the presence of a true thrombocytopenia rather than *in vitro* pseudo-thrombocytopenia. Significant thrombocytopenia develops in experimentally infected dogs on approximately day 10 reaching a nadir on the third week post-infection, with platelet counts ranging from 20,000 to 52,000/μL. The mean platelet volume (MPV) follows an inverse pattern increasing from day 6 post-infection onwards. The thrombocytopenia in the acute phase is generally accompanied by mild anemia and mildly reduced white blood cell counts compared to pre-infection values.

During the subclinical phase, a mild thrombocytopenia may be present in the absence of clinical findings. In experimentally infected dogs, reduced platelet counts by up to 42% have been observed with counts as low as 140,000/μL (Harrus et al., 1998b; Waner et al., 1997). Leukocyte and erythrocyte counts may also be reduced, although these changes may be relatively mild and difficult to notice in a clinical setting (Waner et al., 1997). In the chronic phase, thrombocytopenia is usually severe and accompanied by marked anemia and leukopenia. Marked pancytopenia due to bone marrow hypoplasia is a hallmark of the chronic severe form (Harrus et al., 1997).

Platelet counts and their magnitude have been suggested and are being used as a screening test for CME in endemic regions. While only 1/71 (1.4%) non-thrombocytopenic dogs (platelet counts >200,000/μL) was positive for *E. canis* DNA (*16S rRNA*) by polymerase chain reaction (PCR), 13/62 (21%) dogs with platelet counts of 100,000–200,000/μL, and 53/84 (63.1%) dogs with platelet counts <100,000/μL were PCR-positive for *E. canis* (Bulla et al., 2004). This highlights the importance of evaluating true platelet counts in suspected dogs.

Blood smear evaluation

Demonstration of typical cytoplasmic *E. canis*-morulae in monocytes in blood smears by light microscopy strongly supports a diagnosis of CME. Morulae are membrane bound vacuoles which are usually densely packed with bacteria, as seen by electron microscopy (Hildebrandt et al., 1973). Unfortunately the search for morulae is difficult and time consuming and has been estimated to be successful in only about 4% of cases (Woody and Hoskins, 1991). It may be optimized by the examination of multiple buffy coat smears which significantly increases the chances of detecting *E. canis*-morulae. Sixty-six percent sensitivity was achieved after evaluating 1000 oil immersion fields (magnification ×1000) of buffy coat smears. The sensitivity of evaluating 1000 oil immersion fields of bone marrow was 34%. The time required to screen 1000 oil immersion fields ranged from 50 to 60 min (Mylonakis et al., 2003).

Platelets, lymphocytic azurophilic granules, and phagocytosed nuclear material can all be confused with ehrlichial inclusions. Furthermore, other ehrlichial organisms belonging to the *Anaplasmataceae* family (*Ehrlichia chaffeensis, Neorickettsia risticii* and *Ehrlichia ruminantium*) may also infect canine monocytes (Breitschwerdt et al., 1998; Kakoma et al., 1994; Kelly et al., 1994). Epidemiological data on the prevalence of ehrlichial pathogens and their vectors for a particular geographic region will dictate which differential diagnoses may be relevant and should be considered for the particular area. Molecular characterization by PCR and sequencing may be required in order to specify the organism involved.

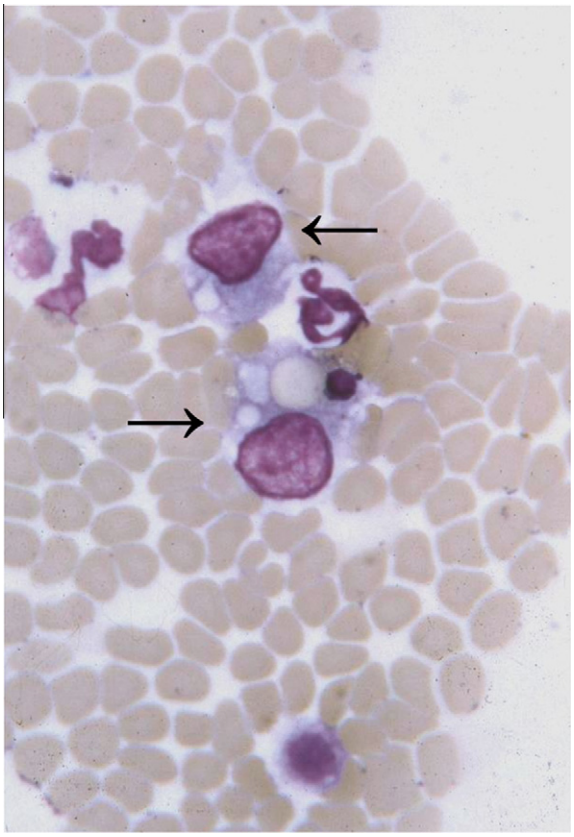

Fig. 1. Blood smear of a dog infected with *Ehrlichia canis*. Note the reactive monocytes (arrows), erythrophagocytosis, phagocytosis of nuclear material and megaplatelets. Courtesy of Dr. Itamar Aroch, Koret School of Veterinary Medicine, The Hebrew University of Jerusalem.

Blood smears of infected dogs may contain reactive monocytes, erythrophagocytosis, thrombophagocytosis, phagocytosis of nuclear material and megaplatelets (Fig. 1). However, red blood cell morphology is unremarkable in most cases (Harrus et al., 1997). Large granular lymphocytosis may be present in advanced stages. A previous report has documented lymphocytosis of 13.0×10^3/μL (reference range: $1.5–5.0 \times 10^3$/μL). Approximately 50% of 200 lymphocytes evaluated in Wright's stained blood smears had prominent azurophilic granules and the cell nuclei were often cleaved or indented with mature nuclear chromatin, consistent with large granular lymphocytes (Heeb et al., 2003). Clinicians should be aware of this phenomenon as these cases may be erroneously diagnosed as lymphocytic leukemia.

Blood smear evaluation is also necessary to evaluate co-infections with other tick-borne pathogens (e.g. *Babesia canis* and *Hepatozoon canis*) which influence disease manifestation, severity and treatment outcome (Gal et al., 2007).

Biochemistry

Hypoalbuminemia, hyperglobulinemia and hypergammaglobulinemia are common serum biochemical alterations in CME. Serum protein electrophoresis may reveal polyclonal hypergammaglobulinemia in most cases, but monoclonal gammopathies may also occur (Harrus et al., 1996). Mild increases in alanine aminotransferase (ALT) and alkaline phosphatase (ALP) are frequent in dogs during the acute phase.

Acute phase protein concentrations may increase during clinical CME. Plasma C-reactive protein (CRP) concentrations have been shown to increase between 4 and 16 days and peaked between 15 and 42 days after inoculation with *E. canis* in five dogs (Shimada et al., 2002). In another study, CRP and α1-acid glycoprotein (AAG) levels were measured in the plasma of five dogs experimentally inoculated with *E. canis* causing a mild disease, in 12 naturally infected dogs with severe clinical signs and in 10 control dogs. In all *E. canis*-experimentally inoculated dogs, a 2–9 fold increase in CRP and AAG concentrations occurred 4–6 days post-inoculation. Despite the persistence of *E. canis* in the experimental dogs, CRP and AAG concentrations gradually declined to pre-exposure levels by day 34 post-exposure. The majority of naturally infected dogs with severe clinical illness also demonstrated increased levels of CRP and AAG (Rikihisa et al., 1994).

Serology

Several serological methods have been developed for the diagnosis of CME and are considered valuable screening and/or diagnostic tools. The indirect immunofluorescence antibody (IFA) test for anti-*E. canis* IgG antibodies is considered the serological 'gold standard', indicating exposure to *E. canis*. IgM is not considered a reliable indicator of *E. canis* exposure due to the inconsistent development of IgM antibodies in the course of the disease (McBride et al., 2003). In contrast, IgG titers $\geqslant$1:40 are considered positive for *E. canis* exposure. For acute infections, two consecutive IFA tests, 7–14 days apart, are recommended, and a 4-fold increase in antibody titers is suggestive of an active infection. Anti-ehrlichial IgG antibodies persist for several months to years after treatment and elimination of the rickettsia (Bartsch and Greene, 1996).

In addition to the IFA test, enzyme-linked immunosorbent assays (ELISA) have been developed and found useful in the diagnosis of the disease (Harrus et al., 2002). Dot-ELISA kits for the detection of *E. canis* IgG antibodies are commercially available and are commonly used as 'point-of-care tests'. Three different ELISA kits were compared with the IFA test in detecting anti-*E. canis* IgG antibodies: (1) an ELISA kit based on the recombinant major antigenic protein 2 (rMAP2); (2) the Immunocomb dot-ELISA test, using a crude ehrlichial extract (Biogal, Galed Laboratories), and (3) the SNAP 3Dx assay utilizing synthetic peptides derived from the major immunodominant *E. canis* proteins P30 and P30-1 (IDEXX Laboratories) (Harrus et al., 2002). Samples tested were collected from Israeli dogs suspected to be naturally infected with *E. canis* and from experimentally infected dogs. When qualitative results (positive/negative) were compared, there was an overall agreement of 74.6% (50/67 samples) between the IFA test and the three ELISA tests. Sixteen of the 17 samples with disagreement (false negative results) had IFA titers $\leqslant$1:320. The specificity of the Immunocomb and the IDEXX 3Dx SNAP tests was very high (0.98 and 1.00, respectively). The authors concluded that the dot-ELISA kits were specific and sensitive especially for IFA titers $\geqslant$1:320. It was recommended that the ELISA test should be repeated 1–2 weeks after the first antibody assay to overcome this sensitivity problem (Harrus et al., 2002).

The 3Dx SNAP test was used to determine the clinical relevance of annual *E. canis* screening. This test alone was not considered adequate for interpretation of the clinical relevance, and its results should therefore be used in combination with platelet counts and molecular results (Hegarty et al., 2009).

Antibodies against several other ehrlichial organisms that cross-react with *E. canis* complicate the serological diagnosis of CME (Waner et al., 2001). Exposure to such species should be considered in the light of the geographic residential area and travel history of the suspected dog. Antibodies against *E. canis* are known to cross-react with antigens of *N. helminthoeca* and *N. risticii*, and conversely, canine antibodies to *N. helminthoeca* cross-react with *E. canis* antigens (Rikihisa, 1991). IFA cannot discriminate between *E. canis, Ehrlichia ewingii, E. chaffeensis* and *E. ruminantium* antibodies (Cardenas et al., 2007). Cross-reaction between *E. canis* antibodies and *Anaplasma phagocytophilum* antigen has also been documented to occur over time. Antibodies from six dogs artificially infected with *E. canis* failed to cross-react with *A. phagocytophilum* antigens during the acute phase of the disease. However, 55 days post-infection, IgG antibodies cross-reacting with *A. phagocytophilum* were first detected in two dogs. Cross-reacting antibodies were detected in two additional dogs on day 77 post-infection, and in the last two dogs by day 150 post-infection (Waner et al., 1998).

There is no serological cross reactivity between *E. canis* and *Anaplasma platys*, the etiological agent of canine thrombocytotropic anaplasmosis (French and Harvey, 1983). Little, if any, cross-reactivity occurs between *E. canis* and *Rickettsia rickettsii*, the etiologic agent of Rocky Mountain Spotted Fever (RMSF). Due to the similar clinical presentation of these two diseases, dogs with clinical signs of ehrlichiosis which are seronegative for *E. canis* should be tested for RMSF (Greene et al., 1985). Interpretation of serological results must take into account the anamnesis and presenting clinical signs, the possibility of multiple tick-borne infections which may affect the clinical presentation, and cross-reactivities with related ehrlichial organisms.

Western immunoblot is a more specific serological technique which can assist in characterizing the infecting agent by giving 'fingerprints' of its immunogenic protein profile. Usually a homogeneous pattern is retrieved among different strains of *E. canis*. However, antigenic diversity among different strains in various regions of the world has been suggested (Hegarty et al., 1997). Western immunoblotting has been used to characterize and distinguish between infection with different organisms causing ehrlichiosis, anaplasmosis, or neorickettsiosis and has the potential to solve dilemmas involving serologic cross-reactivities.

In a study aimed to identify all major immunoreactive antigens of *E. canis* and to determine the kinetics of the antibody response to *E. canis*, dogs were experimentally infected by intradermal and subcutaneous routes. The earliest antibody response was detected on day 21 post-infection. At this stage the antibody response was directed at the 37- and 19-kDa proteins. From days 21 to 28 the major immunoreactive antigens recognized were the 19-, 37-, 75-, and 140-KDa proteins. The minor antigens were 72- and 79-KDa proteins. Later reactive proteins recognized from days 28 to 42 were the 28-, 42-, 72-, 95- and 200-kDa proteins (McBride et al., 2003).

Western immunoblotting has proved useful in distinguishing between infections with *E. canis* and *E. ewingii*. This characteristic is important because most dogs with *E. ewingii* infection will have positive IFA titers to *E. canis*. No IFA test is available for *E. ewingii* as the organism cannot be cultivated *in vitro*. Sera of dogs infected with different *Ehrlichia* species have been found to demonstrate a number of possible reaction patterns to the 27–30-kDa proteins when evaluated by Western immunoblot using native *E. canis* antigens. Sera from *E. canis* infected dogs invariably reacted with the 28- and 30-kDa proteins, whereas *E. ewingii*-infected dogs did not display any reaction. Dogs infected with *E. chaffeensis* have been shown to display a variable reaction to these outer membrane proteins (O'Connor et al., 2006). The 28-kDa/Map-1 outer membrane protein genes are highly diversified among strains of *E. chaffeensis* and *E. ruminantium*, but are highly conserved among *E. canis* (Yu et al., 2007).

Isolation

A continuous canine macrophage cell line (DH82) has been used successfully to culture *E. canis*. This has led the way to the

development of the IFA test for CME in dogs, a step that enhanced research and diagnosis of CME (Lovering et al., 1980). The isolation and growth of *Ehrlichia* species is time and labor consuming. Isolation of *E. canis* is used more in research laboratories and less as a diagnostic tool. The isolation requires specific laboratory equipment and trained staff. Initial growth of the organism is a prolonged process, taking up to 10 weeks after inoculation. *E. canis* has also been adapted to grow in a continuous mouse macrophage cell line (J774.A1), which has been suggested as an alternative to the DH82 cell line (Keysary et al., 2001). Furthermore, a human microvascular endothelial immortal cell line (CDC/EU.HMEC-1) was also found useful for the isolation of *E. canis* (Dawson et al., 1993).

Molecular detection

PCR and sequencing are sensitive methods for detecting and characterizing *E. canis* DNA. False positive results may occur with relatively low annealing temperatures, when contaminants are present or when non-specific amplification occurs. A negative PCR result denotes that no target DNA was detected, but does not necessarily prove that no DNA was present in the sample (false negative result). Detection of *E. canis* DNA can be achieved as early as 4–10 days post-inoculation (Iqbal et al., 1994). Several assays are based on different target genes (e.g. *16S rRNA*, *p28*, *p30*, *dsb*, *VirB9*), however the *16S rRNA* and the *p30*-based PCR assays are most commonly used. The *p30*-based nested PCR assay has been shown to be more sensitive than the *16S rRNA*-based nested PCR assay (Stich et al., 2002). The fact that *E. canis* contains multiple copies of the *p30* gene, but only one copy of the *16S rRNA* gene, increases the chances for detection when *p30* is targeted.

PCR performed on spleen samples is considered more sensitive for the evaluation of ehrlichial elimination when compared to blood and bone marrow samples (Harrus et al., 2004, 1998b). PCR using serum samples may be useful when no blood samples are available. In serum samples analyzed in triplicate (*16S rRNA*-based nested PCR), *E. canis* DNA was amplified in 24/38 (63.1%) affected dogs (Mylonakis et al., 2009).

Quantitative real-time PCR (qPCR) is more sensitive than conventional PCR and allows for quantification of bacterial load. It has been used for the quantification of ehrlichial load in naturally and experimentally infected dogs (Baneth et al., 2009). It is less prone to contaminations than conventional PCR, and when contamination is present, it can be easily detected by using melting curve analyses. Therefore real-time PCR is rapidly becoming the preferred method for diagnosis of *E. canis*.

DNA/RNA chimeric primers have recently been constructed for *E. canis 16S rRNA*. Several RNA bases were incorporated into specific positions in the DNA primers (at the 5′ nearest neighbor to each predicted position for primer-dimer initiation) while no RNA stretches were allowed. The reactions were carried out without pre-amplification steps. The use of DNA/RNA chimeric primers resulted in a decrease in undesirable byproduct formation and a 10-fold increase in the assay sensitivity (Peleg et al., 2009).

The capabilities of real-time PCR instruments allow for the construction of multiplex assays for the simultaneous detection of several pathogens in one sample. Several multiplex qPCR assays have been developed for the simultaneous detection of *E. canis* and other organisms. A multiplex qPCR has been developed for simultaneous detection of *E. chaffeensis*, *E. canis*, *E. ewingii*, *A. phagocytophilum*, and *A. platys* (Sirigireddy and Ganta, 2005). Decreased assay sensitivity may be expected in the presence of additional reactions in multiplex assays.

A new molecular approach using real-time PCR is the high resolution melt (HRM) analysis. HRM real-time PCR is a molecular approach originally developed for single nucleotide polymorphism (SNP) genotyping (Wittwer et al., 2003). This technique may be useful for the discrimination between different ehrlichial species and strains.

There are several advantages for PCR (either conventional or real time) over serology, namely, (1) the early detection of DNA before seroconversion occurs, (2) its higher sensitivity, and (3) the detection of ehrlichial DNA rather than anti-*E. canis* antibodies, probably indicating active infection rather than exposure. The latter characteristic may allow clinicians to better monitor treatment.

Conclusions

Diagnosis of CME must be made in light of anamnesis, clinical signs and laboratory test results. Co-infections with other tick-borne pathogens may influence clinical signs and laboratory changes, and thus complicate the diagnosis. Platelet counts and serology are good screening laboratory tests; however, PCR techniques and sequencing are the definitive confirmatory tests for *E. canis* infection. Sequencing is important as to prevent misinterpretation of false positive results. Negative PCR results should be interpreted with caution, especially when multiplex PCR is used, since they cannot completely rule out the presence of DNA in the sample.

Conflict of interest statement

Neither of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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