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The diagnosis of ehrlichiosis in Americas and the impact on public health

El diagnóstico de ehrliquiosis y su impacto en la salud pública de las Américas

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Introduction and etiology

The genus *Ehrlichia* consists of tick-transmitted bacteria that infect leukocytes and endothelial cells in mammals, and different tissues of its vector. Ehrlichiosis is considered an emerging infectious disease in both humans and animals. The infection has a worldwide distribution, but in the American continent only the recognized species *Ehrlichia canis*, *E. chaffeensis*, *E. ewingii* and the recent named *E. minasensis* and *E. muris* subsp. *eauclairensis* occurs (Dumler *et al.,* 2001; Cabezas-Cruz *et al.,* 2016; Pritt *et al.,* 2017). The DNA from the *Ehrlichia* genotype known as Panola Mountain *Ehrlichia* from the USA and different native genotypes of *Ehrlichia* sp from Nicaragua, Argentina, and Brazil has been reported associated with infections in dogs, goats, humans, horses, mice, ticks, and wild animals (Loftis *et al.,* 2008; Reeves *et al.,* 2008; Widmer *et al.,* 2011; Almeida *et al.,* 2013; Qurollo *et al.,* 2013; Vieira *et al.,* 2016; Cicuttin *et al.,* 2017; Soares *et al.,* 2017).

Ehrlichia canis, the etiologic agent of canine monocytotropic ehrlichiosis (CME), occurring in the whole America continent, especially between the subtropical zone (located at approximately 40° North and South latitude, respectively). The bacteria infect primarily monocytic lineage of dogs. The arthropod vector of *E. canis* is the brown dog tick *Rhipicephalus sanguineus* (Stich *et al.,* 2008). *E. chaffeensis*, the etiologic agent of human monocytotropic ehrlichiosis (HME) occurring specially in North America. Persistently infected white-tailed deer (*Odocoileus virginianis*) and possibly dogs or other carnivores serve as reservoir hosts. An *E. canis* closely related organism, named *E. minasensis* (Cabezas-Cruz *et al.,* 2016) was reported in dairy cattle and mule deer (*Odocoileus hemionus*) in Canada and in dairy and beef cattle in mid-western Brazil (Gajadhar *et al.,* 2010; Lobanov *et al.,* 2012; Aguiar *et al.,* 2014). Clinical signs of ehrlichiosis was observed in experimentally infected calves (Aguiar *et al.,* 2014). The arthropod vector in Brazil is the *Rhipicephalus microplus* tick which bacterium first isolated from the salivary secretion. Possible vectors in North America remain undefined despite the parasitism of *Dermacentor albipictus*, *Dermacentor andersoni*, and different *Ixodes* species reported in cattle (Gregson, 1956; Gajadhar *et al.,* 2010).

Ehrlichia are small, Gram negative, tick-transmitted obligate intracellular bacteria that form microcolonies within membrane-bound cytoplasmic vacuoles, called morulae (Latin *morum* = mulberry; Popov *et al.,* 1998). *Ehrlichia* infects primarily leukocytes (monocytes, macrophages, granulocytes) and endothelial cells in mammals, and salivary glands, intestinal epithelium, and hemolymph cells in ticks (Groves *et al.,* 1975). It has been usually isolated and maintained *in vitro* in dog histiocytic derivate (DH82) cell line (Dawson *et al.,* 1991; Aguiar *et al.,* 2013; Cabezas-Cruz *et al.,* 2016).

Molecular pathogenesis

In view of its close relationship with *E. canis, E. chaffeensis* and *E. minasensis,* the similarities observed between canine, human and bovine ehrlichiosis, this section describes the genera pathogenesis of *Ehrlichia*, which suggests that they may present various similarities. Of the three organisms, *E. chaffeensis* has been more extensively studied because of its human health importance. Because *Ehrlichia* spp lack capsules, common pili, and LPS, the envelope proteins provide a critical interface between these bacteria and their hosts. The surface-exposed proteins in *E. chaffeensis* are OMP-1/P28 proteins and TRP47, TRP32, and TRP120, and the ortholog *E. canis* surface-exposed proteins OMP1B/P30 and TRP36, TRP32 and TRP140, respectively. They are highly immunogenic in infected patients and animals; they have been the primary focus as candidates for the development of differential diagnostic antigens and vaccines (Rikihisa *et al.,* 2015). The immunoreactive surface proteins TRP47 and TRP36 of *E. chaffeensi*s and *E. canis* are suspected to be adhesins involved in the ehrlichial attachment and entry into the host cell. In addition, these proteins contain a major antibody epitope in the tandem repeat region (Doyle *et al.,* 2006) that can be used as antigens for serological diagnosis (Cárdenas *et al.,* 2007; Aguiar and Melo, 2015). These proteins have been also associated with immune evasion (McBride and Walker, 2011).

The incubation period of CME and cattle ehrlichiosis is 8 to 20 d. The organisms multiply in macrophages of the mononuclear phagocyte system by binary fission and spread throughout the body. Infection is thought to spread between cells through exit and uptake via adjacent cytoplasmic projections. Replication in the host takes place in secluded membrane-bound vacuoles protected from the host immune surveillance system, lysosomes, and oxygen reactive intermediates. A mechanism for adaptation that allows ehrlichiae to reside within vacuoles and communicate with the host cell through the endoplasmic reticulum has been identified in a group of ankyrin genes encoding proteins that are suggested to mediate specific protein-protein interactions. Ankyrin proteins also affect proinflammatory cytokine expression and the downregulation of cell cycle regulators. *Ehrlichiae* can be released to infect new cells, by host cell membrane rupture at a late stage of morulae formation (Harrus *et al.,* 2012).

Anti-*E. canis* IgG antibodies generally appear about 15 d after experimental infection. IgG2 antibody reaction to *E. canis* is the principal response in all phases of the CME. It has been proposed that isotype switching to IgG2 subclass antibodies in dogs is associated with a T-helper type 1 response and a corresponding production of interferon (IFN)-γ. This proposition has been strengthened by the finding of persistent expression of IFN-γ and tumor necrosis factor (TNF)-α messenger RNA (mRNA) from d 2 to 8 after infection of dogs with the Oklahoma strain of *E. canis* and continuing to d 56 post- inoculation (PI). Furthermore, IFN-γ and TNF-α exert an anti-rickettsial effect via the induction of nitric acid synthesis. Apparently, T-cell-induced immunity and IFN-γ secretion play a predominant role in recovery from ehrlichial infections. Persistence of *E. canis* is achieved by evasion of the host immune system. This occurs through constant alterations of the organism's surface antigens and the expression of different protein variants. In this regard, proteins with tandem repeats play an important role in the pathogenicity and pathogen-host cell interaction (Harrus *et al.,* 2012; Rikihisa, 2015).

Clinical signs of CME and cattle ehrlichiosis

The CME is a multisystemic disorder. *E. canis* infections can be acute, subclinical, or chronic in dogs. Common clinical signs include depression, lethargy, anorexia, weight loss, and hemorrhagic tendencies. The bleeding is usually exhibited by dermal petechiae or ecchymoses or both. Epistaxis is frequently noticed in CME. Detectable *E. canis* DNA and morulae structure in peripheral blood smears can be observed 10 to 14 d post-infection (dpi; Harrus and Waner, 2011). Despite the fact that reports of bovine ehrlichiosis in Brazil date back to the 1980s, several aspects of its pathogeny remain unclear. However, in view of its close relationship with *E. canis* and the similarities observed between canine and bovine ehrlichiosis, clinical signs observed in experimental infection suggests that they may present various similarities. Calves experimentally infected with *E. minasensis* showed positive PCR results beginning 12 to 23 dpi and ehrlichial morulae were observed in the

cytoplasm of monocytes in peripheral blood smears after 28 dpi (Gajadhar *et al.,* 2010; Aguiar *et al.,* 2014).

Diagnosis

The diagnosis of ehrlichiosis is made, based on a combination of the animal's history (i.e. living in an endemic area, tick infestation, age), clinical and hematological indicators, serologic evidence, and molecular confirmation.

Cytology

Blood smear examination is not an effective diagnostic method as morulae are visualized only during the acute phase and the percentage of infected cells is usually less than 1% (Cadman *et al.,* 1994). Diagnostic sensitivity between cytological methods was assessed in 50 dogs naturally infected by *E. canis*. During the acute phase of the disease, the highest sensitivities were found in buffy coats (66%) and lymph nodes (60.4%) compared to peripheral blood (8%) examinations (Mylonakis *et al.,* 2003). The demonstration of typical cytoplasmic *Ehrlichia* morulae in monocytes in blood smears by light microscopy strongly supports a diagnosis of ehrlichiosis in dogs and cattle.

Serologic testing

Traditionally, indirect immunofluorescence (IFA) has been the serological test of choice for ehrlichiosis. The interpretation of indirect FA results must take into account the history, clinical signs, and laboratory findings. A positive result must be interpreted with caution, as it may represent current infection, resolved infection, or merely exposure. In this case, a second evaluation after 15 d should be considered and may be helpful in the interpretation of serologic results in these circumstances (Aguiar, 2016). Titers originated by previous exposure should be a limiting factor to be considered in endemic regions. In Brazil, serological results must be taking in account to determine clinical diagnosis. The prevalence of antibodies anti-*Ehrlichia* spp infection in healthy dogs and from selected hospital populations around the country ranged from 4.3% (Saito *et al.,* 2008) to 77.0% (Witter *et al.,* 2013). In Cuiabá, Midwestern Brazil, the last serologic enquiry reported the prevalence ranging from 38-48% among healthy dogs, in this sense, half of the city's canine population has antibodies against *Ehrlichia,* which may make in some circumstances difficult the definitive diagnosis of the disease on a single evaluation.

In addition to IFA, several other serological tests are commercially available to diagnose ehrlichiosis e.g. Enzyme Linked Immunosorbent Assay (ELISA), immunoblot, competitive Enzyme Linked Immunosorbent Assay (cELISA; Vieira *et al.,* 2011). Also called "point-of-care tests" to detect anti-*E. canis* antibodies, the results obtained from these kits are qualitative and semi quantitative in some; however, they can be rapidly obtained in the clinic setting. The tests used are sensitive and specific, especially when the indirect FA titers are greater than 320. The kits have the advantages of a relative low cost and provide evidence for exposure to *E. canis*, which then assists with an early diagnosis with minimal equipment and personnel (Harrus *et al.,* 2012).

Although this technique is still widely used, a significant number of false positives may occur due to cross-reactivity with other organisms from the genera *Ehrlichia, Anaplasma* and *Neorickettsia* (Harrus *et al.,* 2012). In order to detect and distinguish *E. canis* antibodies from related organisms, ELISA-based on recombinant proteins or peptide assay has been evaluated. ELISA using synthetic peptides to serologically distinguish *E. canis* and *E. chaffeensis* infections have been previously reported (Doyle *et al.,* 2006; McBride *et al.,* 2007; Luo *et al.,* 2008). Similarly, based on the molecular identification and characterization of the *E. canis* TRP36 genotypes in Brazil (Aguiar *et al.,* 2013), an ELISA capable of serologically distinguishing antibodies against two different *E. canis* genotypes using synthetic peptides was developed and proved useful for understanding the epidemiology of canine ehrlichiosis in Brazil (Aguiar *et al.,* 2016).

Molecular genetic detection

Molecular detection of the *Ehrlichia* genus by polymerase chain reaction (PCR), nested-PCR and real-time PCR has been used to identify individuals infected either experimentally or naturally in both acute and chronic phase. Several assays are based on different targets genes, but the most commonly used are *rrs*, *p30* and *dsb.* This technique is a sensitive and specific test compared to other methods, although false positive results can still occur with relative low annealing temperatures, when contaminants are present or non-specific amplifications occur. Negative PCR result denotes that no target DNA was detected, but does

not necessarily prove that no DNA was present in the sample (Harrus and Waner, 2012; Vieira *et al.,* 2011).

Due to the cyclical nature of ehrlichiosis, PCR may have unsatisfactory performance depending on the stage of infection. Although the prevalence of antibodies in some tropical regions is high, the occurrence of positive PCR in dogs might be low. In the Virology and Rickettsiosis Laboratory of the Veterinary College of the Federal University of Mato Grosso, from the 981 PCR tests performed since 2014 for the diagnosis of CME, 30% (284 samples) presented positive results, although the seroprevalence of antibodies in the region is around 50-60%. In this scenario, PCR may make it unfeasible to be used in non-endemic areas, where serology may be useful for the final diagnostic. In endemic regions, therefore, the use of PCR should be recommended, since the frequency of seropositive dogs is high. In order to avoid unspecific results, some PCR reactions must be confirmed by sequencing reaction mainly when generic targets are used in different assays.

Public health

The *E. chaffeensis*, *E. canis, E. muris* subsp. *eauclairensis* and the Panola Mountain *Ehrlichia* genotype have been implicated in the etiology of human monocytic ehrlichiosis (HME). In the USA, *E. chaffeensis* infection in humans is well established. Acute fever, headache, myalgia, anorexia, and chills generally characterize the HME, and it is frequently accompanied by leukopenia, thrombocytopenia, anemia, and elevation of serum hepatic aminotransferases. The severity of the disease varies from asymptomatic seroconversion to death, and severe morbidity is frequently documented (Paddock and Childs, 2003).

Human infection with *E. muris* subsp. *eauclairensis* causes an illness quite similar to HME characterized by fever, headache, myalgia, lymphopenia and thrombocytopenia (Pritt *et al.,* 2011; Johnson *et al.,* 2015). This *Ehrlichia* subspecies is also serologically cross-reactive with *E. chaffeensis* as determined by IFA (Pritt *et al.,* 2011) which can make difficult serological investigation. The target cell in naturally infected vertebrate hosts is unknown; however, ehrlichiae can be found in mononuclear and endothelial cells of various organs and tissues in mice experimentally infected with this organism (Saito *et al., 2015*). Human infection seemed to be associated with *Ixodes scapularis* removed from soldiers in the Midwestern and the northeastern United States (Stromdahl *et al.,* 2014).

E. canis a recognized dog pathogen was isolated and molecularly characterized from an asymptomatic human in Venezuela (Pérez *et al.,* 1996) but only in 2006 it was associated to a clinically compatible case of human ehrlichiosis (Pérez *et al.,* 2006). Despite confirmatory diagnostic methods performed in these previous reports, human ehrlichiosis caused by *E. canis* was never fully understood and totally accepted. The main route of infection still needs clarification since *R. sanguineus* ticks which is naturally adapted to *E. canis* usually feeds on dogs, their natural host in the environment (Stich *et al.,* 2008). This issue recently won new data when 3.6% and 35% of blood and serum samples from a human blood bank donors in Cost Rica shown positive results for PCR and IFAT assays. Curiously, DNA sequence of *dsb* and *TRP36* genes revealed to be a new genotype of *E. canis* (Bouza-Mora *et al.,* 2017). In Brazil, where the presence of *E. canis* infected dogs is endemic, few studies involving human ehrlichiosis have been carried out. Two serological inquiries showed prevalence of anti-*Ehrlichia* spp. antibodies not greater than 5%, where in one of them, no antibodies against *E. chaffensis* or *E. canis* were observed when specific antigens were used for these agents (Vieira *et al.,* 2013; Bezerra *et al.,* 2017). These findings suggest that in Brazil, species of *Ehrlichia* that stimulate antibody response in humans remain undefined.

Conclusion

Different species of *Ehrlichia* are important under the context of public health and require differential diagnostic methods. A better understanding of the natural history of these infections in America, is also required in order to considered to aid with the treatment of these pathologies. Implementation of definitive diagnosis of ehrlichiosis must be evaluated according to regional characteristics. Antibodies research for clinical cases has proved to be useful when employed in non-endemic areas while PCR has proven useful to differentiate patients with bacteremia in areas where seroprevalence is high.

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