Canine monocytic ehrlichiosis in Buenos Aires, Argentina: comparison of serological and molecular assays[1]

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ABSTRACT.- Martin P.L., De Salvo M.N., Cicuttin G.L. & Arauz M.S. 2019. **Canine monocytic ehrlichiosis in Buenos Aires, Argentina: comparison of serological and molecular assays.** *Pesquisa Veterinária Brasileira 39(8):649-654.* Laboratorio de Zoonosis Bacterianas y Parasitarias Transmitidas por Vectores, Instituto de Zoonosis Luis Pasteur, Av. Díaz Velez 4821, CABA, CP C1405DCD, Argentina. E-mail: gcicuttin@gmail.com

Canine monocytic ehrlichiosis (CME) is an infectious disease caused by the bacterium *Ehrlichia canis* and transmitted by *Rhipicephalus sanguineus* sensu lato, a tick with worldwide distribution. When not diagnosed and treated early, disease can be severe. Currently, the disease is confirmed by serological or molecular assays. The objective of this study was to compare a serological assay based on immunochromatography (SPEED® EHRLI immunochromatographic test; BVT, France) and a molecular assay (a screening PCR followed by a nested PCR specific for *E. canis*) for the diagnosis of *E. canis* in suspected dogs from Buenos Aires city and southern Greater Buenos Aires, Argentina. Blood samples from 20 clinically healthy dogs (Control Group) and from 80 sick dogs suspected of having CME (Groups 1 to 4) were tested in parallel. Neither the immunochromatographic test nor the PCR assay was able to detect the presence of *E. canis* in the Control Group. In the group which had been previously tested by serology, the agreement between the tests was low (kappa: 0.200), whereas in the group which had been previously tested by PCR, the concordance between the tests was adequate (kappa: 0.650). The concordance between the tests evaluated in the total population studied was moderate (kappa: 0.496). The results of our study suggest that the use of rapid serological tests as a first approach, together with subsequent confirmation by PCR, will improve the diagnosis of CME.

INDEX TERMS: Canine, monocytic ehrlichiosis, Buenos Aires, Argentina, serology, molecular assay, dogs, diagnosis, ehrlichiosis, PCR, bacterioses.

RESUMO.- [**Ehrlichiose monocítica canina em Buenos Aires: comparação de testes serológicos e moleculares.**] A ehrlichiose monocítica canina (CME) é uma doença infecciosa transmitida pelo carrapato *Rhipicephalus sanguineus* sensu lato com distribuição mundial causada por *Ehrlichia canis*, que pode produzir uma doença grave se não foi diagnosticada e tratada precocemente. A confirmação da doença é feita diretamente pela detecção do DNA fazendo a reação em cadeia da polimerase (PCR) ou indiretamente por métodos sorológicos.

O objetivo deste estudo foi comparar o método sorológico baseado na imunocromatografia e a técnica de PCR para o diagnóstico de *E. canis* em cães suspeitos da Cidade de Buenos Aires e da região sul da Grande Buenos Aires. As amostras de sangue de 20 cães clinicamente saudáveis (Grupo Controle) e de 80 cães com suspeita clínica de CME (Grupo 1-4) foram avaliadas em paralelo. O diagnóstico serológico foi feito pelo teste imunocromatográfico SPEED® EHRLI (BVT, França). Para a detecção molecular, foi utilizada uma PCR de triagem para amplificar um fragmento de 345 pb do gene que codifica a subunidade 16S do rRNA da família Anaplasmataceae. As amostras positivas depois foram processadas pela PCR aninhada específica para *E. canis*. No Grupo Controle, a presença de *E. canis* não foi detectada por PCR ou anticorpos específicos com o teste imunocromatográfico. No grupo em que a sorologia foi solicitada inicialmente (1 e 2), a concordância entre os testes foi baixo (kappa: 0,200) enquanto que no grupo onde o teste inicialmente solicitado foi a PCR, a concordância

entre os testes era adequado (kappa: 0,650). A concordância entre os testes avaliados na população total estudada foi moderada (kappa: 0,496). Em conclusão, os resultados do nosso estudo sugerem que o uso de testes serológicos rápidos inicialmente, juntamente com a confirmação subsequente por PCR, permitirá melhorar o diagnóstico de CME.

TERMOS DE INDEXAÇÃO: Ehrlichiose monocítica, caninos, Buenos Aires, teste serológico, teste molecular, cães, diagnóstico, ehrlichiose, PCR, bacterioses.

INTRODUCTION

Canine monocytic ehrlichiosis (CME) is a tick-borne disease caused by *Ehrlichia canis*, an obligate intracellular bacterium of the family Anaplasmataceae. This bacterium is mainly transmitted by the tick *Rhipicephalus sanguineus* sensu lato (Dumler et al. 2001, Bremer et al. 2005). In dogs, the clinical presentation of CME can range from mild to severe depending on the immune status of the host, virulence of the strain and co-infection with other microorganisms (Klag et al. 1991, Unver et al. 2009, Rotondano et al. 2015). The course of CME can be divided into three phases: acute, subclinical and chronic. The acute phase is characterized by high fever, depression, lethargy, anorexia, lymphadenomegaly, splenomegaly, hemorrhagic tendencies and ophthalmological signs (Neer et al. 2002, Leiva et al. 2005, Harrus et al. 2012). Untreated dogs and those treated inappropriately may recover clinically but then enter the subclinical phase. In this phase, dogs show no clinical signs but may remain persistent carriers of *E. canis*. In the chronic phase, signs are more severe and infected dogs may be less responsive to therapy (Neer et al. 2002, Harrus et al. 2012). Therefore, early and accurate diagnosis of suspected cases in dogs is essential to alter the course of this disease (Neer et al. 2002).

Currently, diagnosis of CME includes direct (e.g. blood smears and PCR) and indirect (serology) methods (Harrus & Waner 2011). The evaluation of blood smears has low sensitivity and specificity (Mylonakis et al. 2003, Ramos et al. 2009, Harrus et al. 2012). Although this method may be optimized by the examination of multiple buffy coat smears, the presence of platelets, lymphocytic azurophilic granules, and phagocytosed nuclear material may all be confused with ehrlichial inclusions (Harrus & Waner 2011). In contrast, the PCR assay is a sensitive method to detect *E. canis* DNA. Thus, several assays based on different target genes, such as 16S rRNA, *p28*, *p30*, *dsb*, *VirB9*, and *groESL*, have been published (Stich et al. 2002, Labruna et al. 2007, Baneth et al. 2009, Cicuttin et al. 2016). Both conventional and real time PCR have the advantage over serology that they can detect active infection in a single sample (Harrus & Waner 2011, Maggi et al. 2014). However, in animals with subclinical infection, in which *E. canis* persists in the bone marrow or spleen but is below the limit of PCR detection in peripheral blood, these assays could give negative results (Harrus et al. 1998, Otranto et al. 2010). Regarding indirect methods, the indirect immunofluorescence antibody assay is considered the 'gold standard' for detection and titration of *E. canis* antibodies (Waner et al. 2001, Harrus et al. 2002). However, this assay usually presents cross-reactivity between *E. canis* and other ehrlichiae and to confirm recent infection, it should be repeated in 2-3 weeks to demonstrate seroconversion

(i.e. a four-fold change in the patient's antibody titer between acute and convalescent serum samples). There are also several commercial serological assays, such as rMAP2 ELISA, InDx Canine Multiassay Dip-S-Ticks (PanBio, InDx), Snap Combo, Snap3Dx (IDEXX Laboratories), Snap 4Dx (IDEXX Laboratories), and SPEED® EHRLI (BVT, France), which have been designed for in-clinic use. However, serological assays are not appropriate to confirm active infection because antibodies can persist for variable intervals after a pathogen is eliminated (Harrus et al. 1998). For a suitable diagnosis of CME and to detect acutely infected dogs before seroconversion and/or sub clinically infected dogs with blood negative PCR, it is thus recommended to use serology in conjunction with molecular detection methods (Çetinkaya et al. 2016).

In Argentina, *E. canis* has only been confirmed in dogs with compatible signs of EMC from Buenos Aires (Eiras et al. 2013, Cicuttin et al. 2016) and *R. sanguineus* ticks from Formosa and Buenos Aires (Cicuttin et al. 2015, Cicuttin et al. 2017). Using molecular assays, Eiras et al. (2013) have detected *E. canis* in 6/86 (7.0%) dogs with suspected blood smear evidence and/or thrombocytopenia from southern Greater Buenos Aires. Cicuttin et al. (2016) in samples of canines with symptoms compatible with EMC of the Metropolitan Area of Buenos Aires described a prevalence of 6.7% (15/223) to *E. canis*. On the other hand, in clinical healthy dogs from Buenos Aires, San Luis, Córdoba and Santa Fe, only the presence of *Anaplasma platys* DNA, but not *E. canis*, was demonstrated (Cicuttin et al. 2011, 2014a, 2014b, 2017a, Mascarelli et al. 2016). Serological studies published in our country are scarce. Mera y Sierra & Neira (2014) found that 46.6% of canines from Mendoza with symptoms compatible were seropositive for *E. canis* with Speed-Ehrli serological assay.

Since comparison between these direct and indirect assays may provide valuable information for the diagnosis of CME in clinical practice, the objective of this study was to compare a serological assay based on immunochromatography with a molecular assay for diagnosis of CME by using samples from healthy dogs and dogs suspected of being infected with CME from Buenos Aires, Argentina.

MATERIALS AND METHODS

Sample collection. Whole blood samples with EDTA anticoagulant received for the diagnosis of CME were kindly provided by two diagnostic centers: "Laboratorio Diagnóstico Veterinario Sur", Quilmes, southern Buenos Aires (a center where dogs are diagnosed by means of a serological assay), and "Instituto de Zoonosis Luis Pasteur", Buenos Aires, Argentina (a center where dogs are diagnosed by means of a molecular assay). Only samples from animals with presumptive clinical diagnosis of CME were selected. In all cases, the samples were stored at -20°C until their processing. In addition, whole blood samples with EDTA from clinically healthy dogs from the same locations were included to establish background exposure or infection (Control Group). The samples from sick dogs were divided into four groups taking into account the result of the serology or PCR assay previously performed at the diagnostic center: Group 1, which consisted of 20 samples in which the serological SPEED® EHRLI assay had been negative for *Ehrlichia canis*; Group 2, which consisted of 20 samples in which the serological SPEED® EHRLI assay had been positive for *E. canis*; Group 3, which consisted of 20 samples in which the PCR assay (screening PCR targeted to the 16S ribosomal RNA and nested PCR targeted to a specific fragment

of the 16S rRNA gene from *E. canis* assays) had been negative for *E. canis*; and Group 4, which consisted of 20 samples in which the PCR assay had been positive for *E. canis.*

Serological assay. The serological diagnosis of the samples obtained from the two diagnostic centers was also performed by the SPEED® EHRLI assay. This commercial assay is based on the detection of anti-*E. canis* antibodies by using an immunochromatographic membrane. The cut-off value from which the assay detects specific antibodies is not specified by the manufacturer. The sensitivity and specificity of the assay is 87% and 95% respectively (Martin 2004).

Whole blood was used according to the manufacturer's instructions. This method was used for the detection of anti-*E. canis* antibodies in the 60 samples corresponding to Groups 3 and 4 and the controls.

Molecular assay. For the molecular assays, DNA was extracted from 200μL of whole blood samples with EDTA anticoagulant, using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. Initial screening for the family Anaplasmataceae was performed with a screening PCR assay targeted to the 16S ribosomal RNA (16S rRNA) gene (Table 1). Reactions were performed in a final volume of 25μL, containing 12.5pmol of each primer. The thermocycling conditions for the reactions were 95°C for 5min, followed by 34 cycles at 95°C for 30 seconds, with annealing at 55°C for 30s, and extension at 72°C for 90s; a final extension step at 72°C for 5min was used. In each PCR reaction, an endogenous control was included to amplify a 289bp region of exon III of the beta-actin gene to evaluate the presence of inhibitors. *Anaplasma centrale* was used as a positive control and nuclease-free water was used as a negative control.

Positive DNAs by screening PCR assay were then analyzed by nested PCR assay to amplify a specific fragment of the 16S rRNA gene from *E. canis* described by Breitschwerdt et al. (1998). Moreover, another nested PCR assay was performed to amplify a fragment of the 16S rRNA gene from *Anaplasma platys* (Kordick et al. 1999). The sequences of these primers are shown in Table 1. These methods were used for the detection of *E. canis* or *A. platys* DNA in the 60 samples corresponding to Groups 1 and 2 and the Controls.

Statistical analysis. Statistical analysis was carried out using Epi Info 7.1.2.0 (Centers for Disease Control and Prevention, Atlanta, USA) and WinEpi (Facultad de Veterinaria, Universidad de Zaragoza, Spain). The concordance between the SPEED®EHRLI serological assay and the PCR was determined by calculating kappa values with 95% confidence intervals. According to the kappa values, the concordance between assays was classified as follows: 0-0.20 indicated poor agreement, 0.21-0.40 indicated fair agreement, 0.41-0.60 indicated moderate agreement, 0.61-0.80 indicated strong agreement, and 0.81-1 indicated almost perfect agreement (Landis & Koch 1977).

RESULTS

The results of the SPEED®EHRLI serological assay versus the PCR-positive samples within each group and the agreement between both assays are summarized in Table 2. The internal control used in the screening PCR assay was positive in all reactions.

The 20 healthy dog blood samples (Control Group) were negative both for the detection of antibodies by the SPEED®EHRLI serological assay and for the detection of DNA by the screening-PCR assay.

In Groups 1 and 2 (patients with presumptive diagnosis of CME in which the assay initially performed was the serology and the PCR assay was retrospectively performed), the concordance between the assays was poor (kappa value: 0.200, 95%, CI -0.021-0.421). In Group 1, the nested PCR specific to *Ehrlichia canis* showed one positive result, whereas one of the samples was positive by the nested PCR specific to *Anaplasma platys*. Five out of the 20 dogs in Group 2 were PCR-positive to *E. canis*.

In Groups 3 and 4 (patients with presumptive diagnosis of CME in which the assay initially performed was the PCR assay and then the serological assay was retrospectively performed), the agreement between the assays was strong (kappa value: 0.650, 95%, CI 0.340-0.960).

Table 1. Primer pairs used in the present study to detect *Ehrlichia canis* and *Anaplasma platys*

Primer pairs	Primer	Sequence (5´3´)	Reference
Family Anaplasmataceae	EHR16S-D	GGTACCYACAGAAGAAGTCC	Parola et al. (2000)
	EHR16S-R	TAGCACTCATCGTTTACAGC	
Outside primers for family Anaplasmataceae	EHR-OUT1	CTGGCGGCAAGCYTAACACATGCCAACATCTCACGAC	Breitschwerdt et al. (1998)
	EHR-OUT2	GCTCGTTGCGGGACTTAACCCAACATCTCACGAC	
Inner primers for *E. canis*	HE3-R	CTTCTATAGGTACCGTCATTATCTTCCCTAT	Breitschwerdt et al. (1998)
	E. canis	CAATTATTTATAGCCTCTGGCTATAGGAA	
Inner primers for *A. platys*	*E. platys*	GAT TTTTGTCGTAGCTTGCTA	Kordick et al. (1999)
	Ehrl3-IP2	TCATCTAATAGCGATAAATC	

Table 2. Results of serology and PCR assays

Groups	No. of dogs	CI positive (%)	PCR positive (%)	Concordance (%)
Control	20	0(0%)	0(0%)	-
1	20	0 (0)	1(5%)	0/1 (0)
2	20	20(100%)	5(25%)	5/20 (25)
3	20	4(20%)	0(0%)	0/4 (0)
4	20	17(85%)	20(100%)	17/20 (85)

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