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Comunicação

[*Communication*]

Abortion and fetal death in bitches due anemia caused by vector-borne diseases

[*Abortamento e morte fetal em cadelas devido anemia causada por doenças transmitidas por vetores*]

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Many authors consider male infertility and mistimed breeding as the major causes of reproductive failure (RF) in bitches. However, once these causes are excluded, the role of infectious diseases in pregnancy losses should be addressed. The impact of infectious diseases on the normal progression of pregnancy can be associated with the direct action of the infectious agents or their toxins, inducing placentitis that leads to fetal death (Givens and Marley, 2008). In dogs, the viral action of the canid herpesvirus 1 (CaHV-1) and the bacterium *Brucella canis* have been found to be the main causes of reproductive problems. In Brazil, leishmaniasis, parvoviruses, and tick-borne diseases (*Anaplasma platys* and *Ehrlichia canis*) are endemic, particularly in the North-East region, and all these pathogens can cause anemia. Apart from these agents, previous studies on reproductive disorders in dogs have neglected other pathogens such as *Neospora caninum* (Greene, 2012). Due to the lack of research concerning potential causes of RF, this study was designed to investigate the association between infectious agents, unexplained RF (abortion, stillbirth, or fetal death), and anemia in bitches.

Samples from 36 pregnant dogs (20 with RF and 16 without RF) treated at the Veterinary Hospital Jerônimo *Dix-Huit* Rosado Maia-HOVET in the Federal Rural University of the Semi-Arid (UFERSA) in Mossoró/RN, North-East Brazil, were obtained according to the Ethics Committee on Animal Use (CEUA) under license number 23091.006326/2014-88 (date of approval March 3rd, 2015). Depending on the clinical condition: RF (unhealthy) or without RF (healthy), tissue samples were collected through ovariosalpingohisterectomy, Caesarean, parturition, and through the *post-mortem* examination of the uterus, placenta, and fetus. Blood samples were collected after the RF episodes or after parturition in healthy bitches, and a complete blood count (CBC) was performed in both groups. In both groups, using a STRATEC Molecular kit (Invitek), DNA was extracted from the female dogs (blood/serum for all bitches, and uterus/placenta in unhealthy group) and from fetus (amniotic fluid, fetal abdominal fluid, and pool of liver/spleen and kidney/lung) of the bitches with RF. The samples were subjected to molecular/serological immunodiagnostic tests, both for detection of pathogens related to RF and for anemia (hemoglobin, erythrocytes, and hematocrit below the reference values, Table 1). A Chi-square test was used to determine if there was a relation between the presence/absence of the infectious pathogens and RF. In addition, an analysis of variance (ANOVA) was conducted to determine if RF was related to the blood biochemistry parameters measured (anemia).

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None of the animals tested positive for the main infectious causes of RF in bitches in either group (Table 2). Canine parvovirus (CPV) was detected in the maternal blood without hematological alterations, and fetal tissues had signs of autolysis, which probably occurred after the infection (Table 3). For leishmaniasis, five females (C2, C4, C6, C9, C16; RF group) were serologically positive (Table 2). Excepting C16, the other four animals also had co-infection with *A. platys*; C6, C9 and C16 also had anemia with CBC with characteristics of infection (leukocytosis with neutrophilia), and C9 was positive for leishmaniasis in PCR (Table 2). Some bitches (C1, C3, C7, C13, Tab.2) presented co-infection of *E. canis/A. platys*, demonstrating anemia and thrombocytopenia (except female C3 which showed only anemia and CBC with characteristics of infection).

Table 1. Molecular and serologic tests used for detection of pathogens in bitches

Molecular Test	Microorganism	Analyzed material	Sequence 5´ - 3´
qPCR*	Canid herpesvirus1	Blood, fetal fluid, pool of spleen+kidneys, pool of liver+lungs	ACAGAGTTGATTGATAGAAGAGGTATG CTGGTGTATTAAACT TTGAAGGCTTTA
			6-FAM- TCTCTGGGGTCTTCATCCTTAT CAAATGCG- BHQ1
PCR	*Brucella* spp.	Blood and placenta	ACATAGATCGCAGGCCAGTCA AGATACCGACGCAAACGCTAC
HRM-qPCR*	*Ehrlichia* spp. *E. canis*	Blood and pool of spleen+kidneys	CTCAGAACGAACGCTGG
			ACCATTTCTARTGCTATYCCRTACTA
qPCR	*Anaplasma platys*	Blood	TTTTTGTCGTAGCTTGCTATGATA TGTGGGTACCGTCATTATCTTCCCCA
qPCR	*Neospora caninum*	Blood and pool of spleen+kidneys	GTGAGAGGTGGGATACG
			GTCCGCTTGCTCCCTA
qPCR	Canine parvovirus	Blood and pool of spleen+kidneys	CAT TGG GCT TAC CAC CAT TT
			CCA ACC TCA GCT GGT CTC AT
PCR	*Leishmania* spp.	Blood	GGGTTGGTGTAAAATAGGG CAGAACGCCCCTACCCG

Serologic test	Microorganism	Analyzed material	Kits, antigen, and antibody
AGID*	*Brucella canis*	Serum	*B. ovis* Reo 198 surface antigen test kit (Tec-Par)
RBT*	*Brucella abortus*	Serum	*B. abortus* 1119-3 antigen (Biological Institute)
2ME-SAT*	*Brucella abortus*	Serum	*B. abortus* antigen (Tec-Par)
SAT*	*Brucella abortus*	Serum	*B. abortus* antigen (Tec-Par)
IFAT*	*Leishmania major*	Serum	anti IgG *Leishmania major* (Center of Zoonosis Control)

*qPCR, Real Time PCR for Canid herpesvirus1; HRM, High Resolution Melting for *Ehrlichia spp. E. canis*; AGID, Immunodiffusion agar gel for *B. canis*; RBT, Rose Bengal acidification test for *B. abortus*; SAT, Serum agglutination Test for *B. abortus*; 2ME, 2-mercaptoethanol for *B. abortus*; IFAT, Immunofluorescence Assay for *L. major.*

The other females with ehrlichiosis/anaplasmosis had at least one alteration in the CBC and alterations in the fetuses that suggest an infectious pathogen (Table 3). The presence or absence of the pathogen did not significantly explain the occurrence of RF ($\chi 2 = 0.02$, $P>0.05$, $n = 20$). Regarding the hematological data, a relation between anemia and RF in the animals was observed when considering the erythrocytes (F 32.2, $DF= 1.33$, $P<0.05$, Figure 1a), hemoglobin levels ($F = 32.8$, $DF = 1.33$, $P<0.05$, Figure 1b), and the haematocrit ($F = 35$, $DF = 1.33$, $P<0.05$, Figure 1c). This indicates a significant difference between animals who

suffered RF (unhealthy group), and those who did not experience RF (healthy group). About the data in Tab. 2 and 3, CaHV-1, brucellosis, and neosporosis may cause abortion of fetuses in late pregnancy and foetal autolysis (Greene, 2012). A previous work also did not find *B. canis* or CaHV-1 in maternal tissues of bitches with RF (Mir *et al.*, 2013). Since dogs are vaccinated for CPV, RF associated with this disease is unlikely to occur due to mass population immunity (Givens and Marley, 2008). Leishmaniasis is zoonotic and endemic in North-Eastern Brazil (Costa *et al.*, 2014); it is a systemic disease, which can lead to anemia and placentitis (Oliveira *et al.*, 2015).

Table 2. Results of molecular/serological tests, CBC and microscopy of bitches with reproductive problems

Bitches	AGID *B.*canis	RBT *B.abortus*	2ME-SAT *B.abortus*	SAT *B.abortus*	IFAT *L.major*[a]	qPCR CaHV-1	PCR *B.canis*	PCR Parvovirus	PCR *N.caninum*	qPCR *Leishmania* spp.	HRM-qPCR *E.canis*	qPCR *A.platys*	Smear *A.platys*	A	T	CBC
B1	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-
B2	-	-	-	-	80	-	-	-	-	-	-	-	+	-	-	+
B3	-	+	-	-	-	-	-	-	-	-	+	-	+	+	-	+
B4	-	-	-	-	40	-	-	-	-	-	-	-	+	-	-	+
B5	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
B6	-	-	-	-	40	-	-	-	-	-	-	-	+	+	+	+
B7	-	+	-	-	-	-	-	-	-	-	+	-	+	+	+	-
B8	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
B9	-	-	-	-	160	-	-	-	-	+	-	-	+	+	-	+
B10	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B11	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+
B12	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
B13	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-
B14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B15	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
B16	-	+	-	-	80	-	-	-	-	-	-	-	-	+	-	+
B17	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+
B18	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+
B19	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
B20	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-

* AGID, Immunodiffusion agar gel for *B. canis*; RBT, Rose bengal acidification test for *B. abortus*; SAT, Serum agglutination test for *B. abortus*; 2ME, 2-mercaptoethanol for *B. abortus*; IFAT, Immunofluorescence assay for *L. major*, [a] Titration of antibodies; qPCR, Real Time PCR for CaHV-1, *Leishmania spp.*, *A.platys*; HRM, High Resolution Melting for *E.canis* ; CBC, Complete blood count consistent with infection; CaHV-1, Canid herpesvirus 1; A, Anaemia; T, Thrombocytopenia

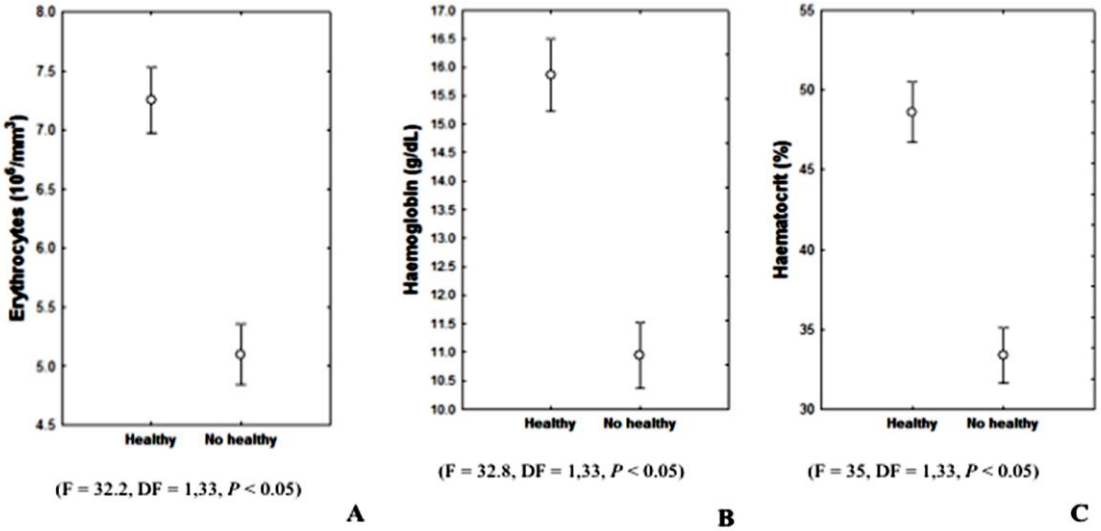

Figure 1. Reproductive failures (RF) in bitches due to anemia. Blood analysis comparing females that suffered of RF (unhealthy) and females that did not suffer of RF (healthy). A, Quantification of erythrocytes (mean ± standard error; in 1x106/mm3); B, Number for hemoglobin (mean ± standard error; in g/dL); C, Quantification of haematocrit (mean ± standard error; in %).

Table 3. *Post mortem* exam of maternal/fetal tissues, fetuses, and gestational age of each female with reproductive disorders

Animal[a]	Fetuses	Uterus	Placenta	Fetal description	GA
C1	F1	Diffusely red mucosa	Blackened and green-yellow areas	Well-developed fetus	61
C2	F1/F2	Diffusely red mucosa	Blackened and green-yellow linear area	Autolysis	54
C3	F1/F2	Diffusely red mucosa	Autolysed	Autolysis	58
C4	F1	No alterations	Blackened placenta	No alterations	59
	F2		Blackened placenta	No alterations	
	F3		Blackened placenta	Kidneys red with black dots	
	F4		Blackened placenta	Distended gall bladder	
	F5		Blackened placenta	No alterations	
C6	F1	Diffusely red mucosa with blackened areas	Autolysed	Autolysis	60
C11	F1	No alterations	Autolysed	Heart with severe congestion	59
	F2		Autolysed	Heart with severe congestion	
C12	F1	No alterations	Thickened placenta	No alterations	47
	F2		Thickened placenta	No alterations	
	F3			Acute and diffuse liver pallor	
	F4		Thickened placenta	Diffuse purplish spots located in the anterior and posterior limbs, and in the left cervical region	
C14	F1	Mucosa thickened and diffusely red	Autolysed	Autolysis	60
C15	F1	No alterations	Autolysed	Autolysis	60
	F2			Autolysis	
C16	F1	Diffusely red mucosa with blackened areas	Autolysed	Undergoing autolysis	55
C20	F1	Diffusely red mucosa with areas of greenish colour	Undergoing autolysis	Undergoing autolysis	54
	F2		Autolysis	Undergoing autolysis	
	F3		Autolysis	Undergoing autolysis	

[a]Female dogs (C5, C7, C8, C9, C10, C13, C17, C18, C19) had abortion episodes at home and the owners did not bring the fetuses to the Veterinary Hospital. GA: Gestational age in days

The other four animals also had co-infection with *A. platys*, so we cannot infer which disease caused the anemia. Their features in fetuses (Table 3) suggest a pathogenesis of infectious origin. Ehrlichiosis and anaplasmosis are related as emerging zoonotic diseases (Arraga-Alvarado *et al.*, 2014), which are not associated with RF in animals. These pathogens may have caused weakness and consequently RF. Thrombocytopenia in dogs with leishmaniasis (C6), ehrlichiosis (C1, C7, C13), and anaplasmosis (C1, C7, C13, C19) is expected; however, RF was not previously described as a clinical sign (Borin *et al.*, 2009). The presence of these pathogens induces anemia, which can increase the likelihood of RF. Anemia has been associated with animals chronically infected with multisystemic diseases (anaplasmosis, ehrlichiosis, or leishmaniasis), and clinical signs vary with the severity of the

disease and the presence of co-infections (Borin *et al.*, 2009; Ferreira *et al.*, 2014). Genetic diversity of *E. canis* strains can express different forms of hematological and clinical manifestations of the disease. However, previous studies on clinical signs of anaplasmosis, ehrlichiosis, or leishmaniasis were conducted in nonpregnant animals. The co-infection of *E. canis* and *A. platys* in nonpregnant dogs resulted in more pronounced anemia and thrombocytopenia (Gaunt *et al.*, 2010). Maternal and perinatal mortality is significantly higher in anemic women (Bencaiova *et al*., 2012). Maternal systemic diseases can result in fever, anemia, or endotoxemia, which in turn can result in RF (Givens and Marley, 2008). The endotheliochorial placenta of dogs allows high blood supply to the fetus. Maternal infections by intracellular parasites can lower oxygen levels in the blood transported to the fetus, leading to RF. The principal reproductive pathogens were not found in this study, which suggests that the systemic disease diagnosed in the maternal blood is related to RF. Hence, bitches with anemia caused by vector-borne diseases are significantly more likely to experience RF than those with normal hematological values (Figure 1).

Keywords: *Anaplasma, Ehrlichia, Leishmania*, reproductive failures

RESUMO

Doenças infecciosas são as maiores responsáveis por falhas reprodutivas (FR) em cadelas, causando aborto, morte fetal e natimortalidade. Este estudo teve como objetivo investigar a associação entre agentes infecciosos, FR inexplicáveis e anemia em cadelas. Todas as amostras maternas e fetais foram negativas para a presença dos principais agentes infecciosos causadores de FR: herpes vírus canino 1, Neospora caninum, Brucella *spp. e* B. canis, *enquanto agentes como o de* Leishmania *spp., parvovírus canino,* Ehrlichia canis *e* Anaplasma platys *foram encontrados em sangue materno. Coinfecções de* A. platys/E. canis *e* A. platys/Leishmania *spp. foram diagnosticadas. Os resultados indicam que os animais com anemia causadas por doenças transmitidas por vetores podem ser mais suscetíveis a sofrerem FR do que animais com valores hematológicos normais.*

Palavras-chave: Anaplasma, Ehrlichia, Leishmania, *falhas reprodutivas*

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