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Short communication

Detection molecular of *Rangelia vitalii* in dogs from Parana State, Southern Brazil

Detecção molecular de *Rangelia vitalii* em cães do Estado do Paraná, Sul do Brasil

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Abstract

Rangelia vitalii infects erythrocytes, leukocytes and endothelial cells of dogs. The present study aimed to report the molecular detection confirmed by sequencing of *R. vitalii* in the state of Paraná, as well as describe the clinical, hematological and biochemical alterations of the infected dogs. Three sick dogs from the metropolitan area of Curitiba, PR, Brazil, underwent a physical exam, and laboratory tests included hematology, biochemistry, polymerase chain reaction (PCR), and gene sequencing. Clinical signs included apathy, anorexia, and hemorrhage. Intra-erythrocytic and extracellular piroplasms were found on peripheral blood smears from all three dogs. Blood samples from these animals were positive for *Babesia* sp. by PCR targeting 18S rRNA. PCR products from all three dogs were sequenced, and BLAST analysis showed that the PCR-generated sequences were highly homologous with those of *R. vitalii* previously reported. Hematologic findings included severe anemia, shift of neutrophils to the regenerative left, and thrombocytopenia. Serum urea levels were increased in all three dogs, and direct bilirubin levels were elevated in one dog.

Keywords: Dog, hemoparasite, *nambiuvú*, rangeliosis, tick-borne disease.

Resumo

Rangelia vitalii infecta eritrócitos, leucócitos e células endoteliais de cães. O presente estudo objetivou relatar a detecção molecular confirmada por sequenciamento de *R. vitalii* no estado do Paraná e descrever as alterações clínicas, hematológicas e bioquímicas dos cães infectados. Três cães doentes da região metropolitana de Curitiba, PR, Brasil, foram submetidos a exame físico e exames laboratoriais que incluíram hematologia, bioquímica, reação em cadeia da polimerase (PCR) e sequenciamento genético. Os sinais clínicos incluíram apatia, anorexia e hemorragia. Piroplasmas intra-eritrocíticos e extracelulares foram encontrados em esfregaços de sangue periférico dos três cães. As amostras de sangue destes animais foram positivas para *Babesia* sp. pela PCR baseada no gene 18S rRNA. Os produtos de PCR dos três cães foram sequenciados e a análise de BLAST mostrou que as seqüências geradas por PCR eram altamente homólogas com as de *R. vitalii* previamente relatadas. Os achados hematológicos incluíram anemia grave, desvio de neutrófilos à esquerda regenerativo e trombocitopenia. Os níveis de uréia no soro aumentaram nos três cães, e os níveis de bilirrubina direta foram elevados em um cão.

Palavras-chave: Cão, hemoparasita, *nambiuvú*, rangeliose, doença transmitida por carrapato.

Canine rangeliosis is a hemorrhagic and hemolytic disease transmitted by the ixodid tick *Amblyomma aureolatum* (SOARES et al., 2018). It is caused by the protozoan parasite *R. vitalii* belonging to the order Piroplasmida that infects erythrocytes, leukocytes and endothelial cells (LORETTI & BARROS, 2005). In Brazil, *R. vitalii* has been reported in domestic dogs in southern and southeastern regions (GOTTLIEB et al., 2016; LEMOS et al., 2017). Few studies report the biochemical abnormalities observed in the serum of dogs infected with *R. vitalii* (COSTA et al., 2012). The present study aimed to report the molecular diagnosis of *R. vitalii* in dogs naturally infected from the metropolitan area of the city of Curitiba, state of Paraná, south Brazil, and to

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describe the clinical, hematological and biochemical findings of the infected dogs.

This study was approved by the Ethics Committee of the Federal University of Paraná (UFPR) (approval no. 035/2015). The clinical records of three male, mixed-breed dogs (dogs 1-3), presented between November 2015 and February 2016 to the Veterinary Hospital from the UFPR, were reviewed. Veterinarians performed the physical examination, and blood samples were collected (3 mL) by venipuncture into EDTA tubes, and into tubes without anticoagulant. A small amount of blood was sampled from the capillary beds of the ear tip for peripheral blood smears.

A Mindray BC 2800 Vet® automatic hematology analyzer was used for total erythrocyte and leukocyte counts and for hemoglobin concentration measurement. The microhematocrit technique was used to obtain the hematocrit. Total plasma protein (PPT) concentration was determined by refractometry. Differential leukocyte count and screening for hemoparasites were performed on Romanovsky-stained blood smears examined by light microscopy. The tubes without anticoagulant were centrifuged at 5000 rpm for 5 minutes to obtain serum. Biochemical parameters were determined in serum using commercial kits (Labtest®) in a Mindray BS-200® automated biochemical analyzer. Hematology and biochemistry results were interpreted based on canine reference ranges (KANEKO et al., 1997; MEINKOTH & CLINKENBEARD, 2000).

Genomic DNA for PCR was extracted from blood samples collected in EDTA tubes via a PureLink™ Genomic DNA Mini Kit (Invitrogen®), according to the manufacturer's instructions. Primers used were BAB143-16 and BAB694-667 previously described (ALMEIDA et al., 2013), and a 551-bp fragment of the *Babesia* sp. 18S rRNA gene was obtained after PCR DNA amplification. PCR products were analyzed on a 1.5% agarose gel stained with GelRed® and examined under UV transillumination. PCR products were sequenced by Sanger method (ABI PRISM® 310 Genetic Analyser, Applied Biosystems), and the sequences obtained were submitted to BLAST analysis and compared with those in the GenBank to confirm the identity of the detected sequences.

Table 1 presents information on the three sick dogs including signalment, history, and clinical signs. One dog (dog 1) died. The hematological abnormalities in these animals are summarized in Table 2. The main laboratory abnormalities in all three dogs were severe anemia, shift of neutrophils to the regenerative left, presence of toxic neutrophils, and thrombocytopenia. Neutrophilia was observed in two dogs (dos 1 and 2). Polychromatophilic erythrocytes were not observed. Intraerythrocytic and extracellular forms of the piroplasm were found in the peripheral blood smears of all three patients (Figure 1). Serum biochemistry results are shown in Table 3.

Table 1. Signalment, history and clinical signs in dogs naturally infected with *R. vitalii,* from Curitiba, Paraná, Brazil.

Dog	City	Age	History	Clinical signs
1	Curitiba	9 years	Apathy, anorexia, tick infestation	Apathy, anorexia, dehydration, weak pulse, difficulty breathing, fever, jaundice, pinnal bledding
2	São José dos Pinhais	6 months	Apathy, anorexia	Apathy, anorexia, mucosal pallor, dehydration, hematochezia, melena, body condition score: thin
3	Curitiba	6 years	Apathy, anorexia, tick infestation	Apathy, anorexia, mucosal pallor, dehydration, hematochezia, melena, tachypnea, tachycardia

Table 2. Hematological findings and PPT levels of dogs naturally infected with *R. vitalii,* from Curitiba, Paraná, Brazil.

	Dog 1	Dog 2	Dog 3	RV (adults; 3-6 months)
Eryt. (millions/µL)	2.2	0.7	1.4	5.5-8.5; 5.5-7.0
Hb (g/dL)	5.1	1.7	3.3	12.0-18.0; 11.0-15.5
Ht (%)	17	6	11	37-55; 34-40
VGM	76	86	80	60-77; 65-78
CHGM	30	28	30	32-36; 30-35
Total Leuk. (/µL)	33,700	34,300	9,400	6,000-17,000; 8,000-16,000
Seg. Neut. (/µL)	28,645	24,696	7,426	3,000-11,500; 3,700-11,100
Band cells (/µL)	2,359	686	846	0-300; 0-160
Lymphocytes (/µL)	1,685	8,500	1,128	1,000-4,800; 2,250-7,200
Toxic Neutrophils	Rare, with mild cytoplasmic basophilia	Rare, with moderate cytoplasmic basophilia	> 30%, with cytoplasmic basophilia, cytoplasmic vacuolization, toxic granulation and Dohle corpuscles.	Absent
Platelets (/µL)	180,000	92,000	48,000	200,000-500,000
PPT (g/dL)	5.2	5.8	5.8	6.0-8.0; 5.0-6.5

Eryt.: erythrocytes; Hb: hemoglobin; Ht: hematocrit; VGM: mean globular volume; CHGM: mean globular hemoglobin concentration; Leuk.: leukocytes; Seg.: segmented; Neut.: neutrophils; RV: reference values; PPT: total plasma protein.

Blood samples from all three dogs were positive for *Babesia* sp. by PCR (ALMEIDA et al., 2013). However, sequence analysis of the PCR products showed that these were *R. vitalii*. Sequences were deposited in GenBank (Accession numbers: MG027583.1, MG027584.1 and MG027585.1). The DNA sequences were compared with those from other piroplasms of dogs deposited in GenBank. Our sequences showed high degree of homology (100% for dogs 1 and 2, and 99% for dog 3) with those of *R. vitalii* reported in the State of Rio Grande do Sul, south Brazil (KT323930.1), in the State of Rio de Janeiro, southeast Brazil (KU710789.1), and in Uruguay (KP202861.1) (Figure 2).

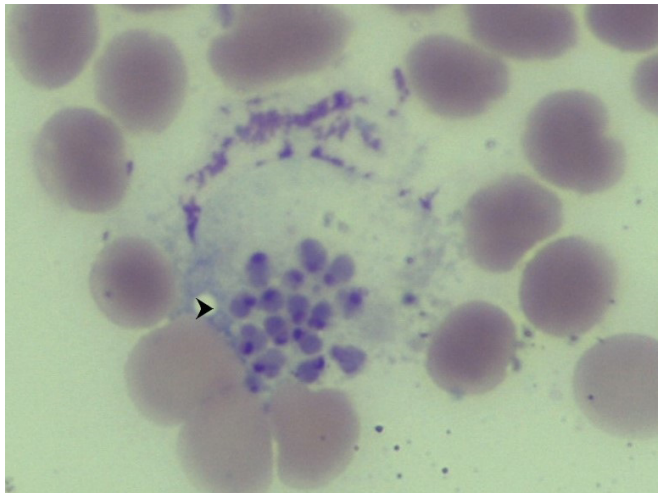


Figure 1. Photomicrograph of peripheral blood smear of dog (dog 2) naturally infected with *R. vitalii*. Presence of extracellular piroplasms, oil immersion objective, 1000x. Romanovsky stain.

In this study, intraerythrocytic and extracellular forms of *R. vitalii* were seen in peripheral blood smears from all three dogs. *R. vitalii* is morphologically similar to *B. vogeli* on blood smears, but genetically distinct in molecular phylogeny (LEMOS et al., 2017). In the State of Paraná, south Brazil, only one study detected *R. vitalii* in a dog by molecular diagnosis (MONGRUEL et al., 2017).

In the present report, the clinical signs observed in all three dogs included apathy, anorexia, dehydration, pale or icteric mucous membranes, and hemorrhages. These findings corroborate previous studies (LORETTI & BARROS, 2005; FIGHERA et al., 2010; GOTTLIEB et al., 2016; FREDO et al., 2017). One dog with jaundice (dog 1) succumbed to rangeliosis. Icterus is a common necropsy finding in dogs with rangeliosis (FIGHERA et al., 2010).

In canine rangeliosis, coagulation disorders affecting primary hemostasis cause a hemorrhagic disease (PAIM et al., 2012). Two dogs from our study (dogs 2 and 3) had hematochezia,

Table 3. Biochemical profile of dogs naturally infected with *R. vitalii*.

	Dog 1	Dog 2	Dog 3	RV
Urea (mg/dL)	129.1	97.8	139.5	21.0-60.0
Creatinine (mg/dL)	1.2	0.6	1.3	0.5-1.5
Alanine aminotransferase (U/L)	NP	40.3	30.9	21.0-102.0
Aspartate aminotransferase - AST (U/L)	88.0	NP	NP	23.0-66.0
Alkaline Phosphatase (U/L)	133.6	85.4	83.6	20.0-156.0
Gamma-glutamyltransferase - GGT (U/L)	8.0	NP	NP	1.2-6.4
Direct bilirubin (mg/dL)	3.80	NP	NP	0.06-0.12
Indirect bilirubin (mg/dL)	0.10	NP	NP	0.04-0.40
Total bilirubin (mg/dL)	3.9	NP	NP	0.10-0.50
Total protein - PT (g/dL)	4.7	4.9	NP	5.4-7.1
Albumin (g/dL)	1.2	1.4	NP	2.6-3.3
Globulin (mg/dL)	3.5	3.5	NP	2.7-4.4

RV: reference values; NP: not performed.



Figure 2. Distance tree (Neighbor-Joining method), conducted using MEGA software (Molecular Evolutionary Genetics For Review Only Analysis) version 6.0 (TAMURA et al., 2013). *Hepatozoon canis* was used as outgroup control.

which is a clinical sign that has been observed by other authors (FIGHERA et al., 2010; FREDO et al., 2017). Hemorrhagic diathesis is a common and striking clinical presentation of cases of *R. vitalii* infection in dogs (LORETTI & BARROS, 2005; PAIM et al., 2012). The tips of the ears from one dog (dog 1) were bleeding when he was presented for veterinary care. Severe, bilateral pinnal hemorrhage is a typical clinical feature of canine rangeliosis, and occurs in 14.3% of the cases according to previous studies (FIGHERA et al., 2010).

Anemia and thrombocytopenia are the most common hematological findings in cases of canine rangeliosis (FIGHERA et al., 2010; PAIM et al., 2012). The absence of polychromatophilia in our study differs from previous studies where most dogs with rangeliosis presented regenerative anemia (FIGHERA et al., 2010). In our study, hematologic abnormalities included leukocytosis and neutrophilia in two dogs (dogs 1 and 2) and regenerative left shift in all three dogs. These changes in the leukogram are associated with inflammation as the inflammatory response accelerates the production and release of neutrophils from the bone marrow resulting in neutrophilia with the presence of immature neutrophils in the circulation. Increased neutropoiesis induced by inflammation results in the release of toxic neutrophils into the bloodstream (SCHULTZE, 2010). The findings of regenerative left shift in all three dogs and lymphocytosis in dog 2 are consistent with findings from a recently published study about natural cases of canine rangeliosis (FREDO et al., 2017). In the present study, all 3 dogs were thrombocytopenic, corroborating previous studies (LORETTI & BARROS, 2005; FIGHERA et al., 2010; FREDO et al., 2017).

Hemorrhage and anorexia are the most likely explanations for the hypoproteinemia in three dogs (TVEDTEN, 2010). We suggest that hypoalbuminemia in dogs 1 and 2 occurred due to albumin is a negative acute-phase inflammatory protein (CERON et al., 2005). Previous studies evaluating the biochemical parameters of dogs experimentally infected with *R. vitalii* didn't find any changes in the creatinine and urea serum levels (COSTA et al., 2012). In our study, there were also no abnormalities in the creatinine dosages of all three dogs with rangeliosis. However, urea levels were increased in all three dogs, most probably due to anorexia and degradation of endogenous proteins (KANEKO et al., 1997). AST and GGT enzymes, and total and direct bilirubin levels were increased in dog 1. In this report, elevated AST levels indicate hepatic injury. Elevated GGT levels and increased serum direct bilirubin levels are indicators of cholestasis and hepatic jaundice (STOCKHAM & SCOTT, 2011).

The present study highlights the importance of the occurrence of the canine rangeliosis in the region and the need to include it as a differential diagnosis for other hemoparasites.

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