ANATOMOPATHOLOGICAL CHANGES IN CANINE DISTEMPER SEROPOSITIVE DOGS AND VIRUS DETECTION IN SINOATRIAL NODES

ALTERAÇÕES ANATOMOPATOLÓGICAS EM CÃES SOROPOSITIVOS PARA CINOMOSE E DETECÇÃO DO VÍRUS EM NÓ SINOATRIAL

José Eugenio Diniz BASTOS[1]; Neide Maria SILVA[2]; Marisol Patrícia Pallete BRICEÑO[3]; Tais Meziara WILSON[4]; Alessandra Aparecida MEDEIROS-RONCHI[1]

1. Associate Professor of Faculty of Veterinary Medicine, Federal University of Uberlândia - UFU, Uberlândia, MG, Brazil;
2. Associate Professor of Institute of Biomedical Sciences - UFU, Uberlândia, MG, Brazil; 3. PhD, UFU, Uberlândia, MG, Brazil;
4. Doctorate in Animal Science, University of Brasília, Brasília, DF, Brazil.

ABSTRACT: Canine distemper is a viral disease that affects several systems on dogs, among them, the cardiovascular system. The aim of this study was to identify canine distemper virus (CDV) in the sinoatrial node (SAN) of dogs serologically positive for distemper by Polymerase Chain Reaction preceded by reverse transcription (RT-PCR), and to analyze gross and microscopic changes of distemper in the heart and other tissues. SAN and tissue fragments were collected from 17 serologically positive dead animals, necropsied from October 2015 to December 2016. In the heart, right heart dilatation was observed in 13 dogs (76.47%) and left concentric hypertrophy in two dogs (11.76%). Microscopically, lymphocytic myocarditis was observed in four (23.53%) dogs and 41.18% presented viral inclusion corpuscles of CDV in the bladder epithelium. Only one (5.88%) dog presented a 319 bp target fragment for distemper virus using primers CDV 1 and CDV 2 at the sinoatrial node. In conclusion, CDV can be located in the sinoatrial node of naturally infected dogs, as demonstrated in this study by the RT-PCR technique, reinforcing the hypothesis that CDV is capable of causing inflammatory lesions in the sinoatrial node of this species. Macroscopic and microscopic cardiac changes are frequently observed in dogs with distemper, mainly cardiac dilatation and myocarditis. Viral inclusions of CDV in bladder epithelial cells are an important microscopic finding for the diagnosis of distemper.

KEYWORDS: Cardiac Conduction System. Canine Distemper Virus. Cardiomyopathy.

INTRODUCTION

The sinoatrial node (SAN) is responsible for the heart rhythm and is essential for the normal physiology of the heart (JOUNG et al., 2011). His bundle together with the atrioventricular node and the Purkinge fibers are the specialized conduction system of the heart (CUNNINGHAM; KLEIN, 2008).

SAN dysfunction has multifactorial etiologies. Monfredi et al. (2010) and Andrade et al. (1988) reported that the SAN also shows an increasing density as the age progresses and that sinoatrial node dysfunction can be considered an aging phenomenon.

Information about microorganisms that affect the canine SAN are rare. The possibility of canine distemper virus (CDV) causing injury specifically in this area as suggested by Mendonça and Coelho (2006) raised interest about the presence of the virus in this cardiac structure. Nelson and Couto (2015) reported that cardiotropic viruses play an important role in the pathogenesis of myocarditis in several species.

Canine distemper virus is the most frequent pathogen for dogs and wild carnivores and its infection is relevant in all continents and is associated with high mortality and morbidity. In the dog, it develops respiratory, gastrointestinal, dermatological, ophthalmic, neurological manifestations and may cause myocarditis (BUDASZEWSKI et al., 2014).

Higgins et al. (1981) inoculated the CDV strain in dogs and 36% presented cardiac lesions. Resende et al. (2009) analyzed the myocardium of left ventricle of seropositive dogs for distemper and verified myocarditis in 42.8% of the animals.

Araújo (2017) evaluated the influence of the CDV on the cardiovascular system and verified that there is virus activity in the excitatory system and specialized heart conductors. Mendonça and Coelho (2006) studied SAN lesions on dogs serologically positive for distemper and observed cardiac dilatation in 97.14% of the animals in addition to inflammatory and degenerative alterations. Bastos et al. (2007) also verified degenerative, vascular and inflammatory microscopic lesions in the SAN in dilated hearts of dogs with clinical suspicion of distemper. CDV belongs to the genus *Morbillivirus*

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of the family *Paramixoviridae* (VAN REGENMORTEL et al., 2000) and is a single-stranded RNA virus of negative polarity. Polymerase chain reaction (PCR) has been used to diagnose canine distemper and the main advantages of this technique preceded by a reverse transcription step (RT-PCR) for RNA viruses include the speed of obtaining results, no need of viral particle infectivity and it's high levels of sensitivity and specificity (FRISK et al., 1999; GEBARA et al., 2004).

The aim of this study was to identify CDV in the SAN of dogs serologically positive for distemper using RT-PCR and to analyze gross and microscopic changes of distemper in the heart and other tissues.

MATERIAL AND METHODS

Animals and clinical data

Cases of necropsied dogs were selected at the Animal Pathology Laboratory of the Veterinary Hospital of the Faculty of Veterinary Medicine of the Federal University of Uberlândia from October 2015 to December 2016. The animals had received the clinical and serological diagnosis of canine distemper but did not respond to the conventional therapy for the disease and came to natural death.

The clinical diagnosis was made based on laboratory exams and signs: anorexia, hyperthermia, conjunctivitis, optic neuritis, uveitis, chorioretinitis, dry keratoconjunctivitis, upper respiratory tract inflammation with mucopurulent nasal discharge, pneumonia, hemorrhagic gastroenteritis or the lack of it, cervical stiffness, convulsions, myoclonus, ataxia of cerebellar or vestibular origin, paresis, tetraparesis, vesicular pustules, nasal and/or pads hyperkeratosis, lymphocytopenia, thrombocytopenia and anemia (GREENE et al., 2012; DEEM et al., 2000). The serological diagnosis of distemper was performed using immunochromatographic immunoassay for the qualitative detection of antibodies (IgG and IgM) for distemper (Alere Cinomose AgTest®) according to manufacturer`s protocol.

The animals were divided into three groups by age: young (up to one year old), adults (one to nine years of age) and the elderly (ten years of age or older), according to (FIGHERA et al., 2008). Data such as breed, age, sex and macroscopic lesions observed during necropsy were recorded.

The necropsies were performed within 30 minutes after death and in the impossibility of performing them this way, the body was kept under refrigeration in a cold room for a maximum of three hours. At the beginning of the necroscopic procedure, the costochondral joints were opened and the thoracic cavity was exposed. The heart was removed and, using sterile material, a 2 cm fragment was collected from the initial portion of the caudal cranial cava, near the end sulcus region, where the SAN is located. The SAN fragment was immediately packed into sterile cryotubes and kept in a container with crushed ice until they were sent to ultra-freezer storage at -80°C.

The necropsy technique was performed following the organ inspection sequence of the necropsy specimen procedures of the Laboratory of Animal Pathology. Fragments of myocardium, lung, pancreas, liver, bladder (for research of inclusion corpuscles of the distemper virus), kidney and other tissues that presented macroscopic alterations were collected. The fragments were fixed in 10% buffered formalin for 48 hours and routinely processed for the preparation of histological slides stained with Hematoxylin and Eosin (HE) (TOLOSA et al., 2003).

Detection of distemper virus by RT-PCR

The fragments containing the sinoatrial node maintained in ultra-freezer -80ºC were used to extract viral RNA using Trizol reagent (Life Technologies®), according to the manufacturer's instructions, at the Laboratory of Immunopathology of the Federal University of Uberlândia.

The first complementary DNA strand (cDNA) was synthesized from 2 µg of total RNA. Initially 25 pmol of the reverse primer CDV strain Onderstepoort, CDV 02 (5'-GATTGCTTAGGACCAGTAGC-3') were added to 2 µg of RNA and denaturation was done at 70° C for 10 minutes. Immediately after the addition of Impron II reverse transcriptase (Promega®, Madison, WI, USA), 2µl; 250 µM dNTP mix, 8 µl of RT buffer, 3 mM magnesium chloride, 2 µl Dithiothreitol (DTT) at 20 mM and RNAse-free water to complete the final volume of 40 µl. The reaction was incubated at 42˚C for 1 hour, then cooled at 4˚C and maintained at -80˚C until use. The RNA virus from the distemper vaccine (Duramune D®, Fort DODGE) was used as a positive control of the reaction. Specific primers that amplified the CDV nucleocapsid gene were used, according to (DEL PUERTO et al., 2010), CDV01, forward, (5'-CAGCACCGTACATGGTTATC-3') and reverse CDV 02 (5' GATTGCTTAGGACCAGTAGC-3') which amplifies a 319bp fragment; and canine housekeeping gene □-actin-specific primers: F (5'-CACCTTCTACAACGAGCTGCG-3') and R (5'-ATCTTCTCACGGTTGGCCT-3') which amplifies

a 93bp fragment. 5µl of the cDNA from each sample were used; 25 pmol of each primer; 250 µM dNTP mix (Promega®, Madison, WI, USA); 2 mM MgCl 2; 1.25 U GoTaq® Flexi DNA Polymerase (Promega®, Madison, WI, USA) or 0.25µl; Taq buffer (10µl) and ultrapure water to make up the final volume of 50µL. PCR conditions were: initial denaturation at 93˚C for 2 minutes, 1 cycle followed by 40 cycles of denaturation at 93˚C for 1 minute, annealing at 56˚C for 1 minute, and extension at 72˚C for 1 minute. Final extension at 72°C for 5 minutes.

The amplified products were stained with SYBR Safe DNA gen stain (Invitrogen®) and were separated by a horizontal electrophoresis system (Loccus Biotechnology LCH®) in 1.5% agarose gel and visualized with the gel documentation system MiniBIS – PRO (DNR Bio-Imaging Systems).

RESULTS

Seventeen canine distemper seropositive dogs with clinical symptomatology compatible with distemper were used. Four were males and 13 females, with respect to breed 15 were mixed-breed dogs, one Poodle and one Cocker Spaniel. As to age seven (41.18%) were young, three (17.64%) adults and seven (41.18%) were elderly.

Gross and microscopic lesions

With regard to the macroscopic aspect of the heart the alterations evidenced were: right heart dilation in 13 dogs (76.47%) (Figure 1) and left concentric hypertrophy in two dogs (11.76%). Microscopically, lymphocytic myocarditis was observed in four (23.53%) dogs (Table1).

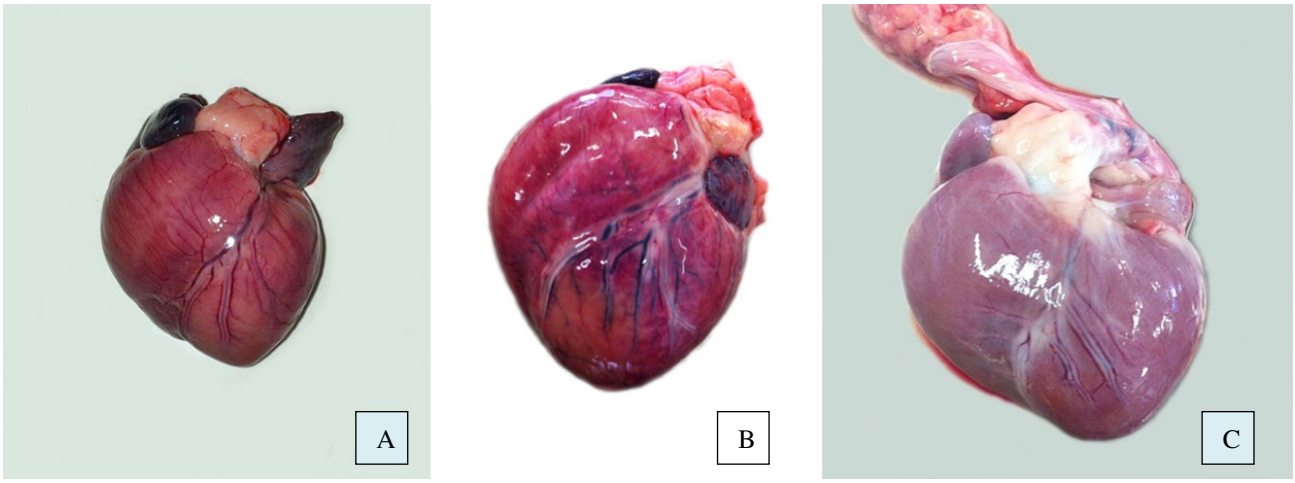

Figure 1. Heart gross lesions of dogs serologically positive for distemper. (A) the heart was enlarged and remarkably globoid; (B) left concentric hypertrophy and congestive blood vessels; (C) right heart dilatation.

Macroscopically, splenomegaly was observed in 35.29% and hepatomegaly was present in 17.64% of the seropositive dogs for distemper. Microscopically, interstitial nephritis was observed in 70.58% of dogs, and in 41.18% there were viral inclusion bodies in the bladder epithelium (Figure 2). In the digestive system the main changes were hepatic lipidosis and enteritis in 47.05% of dogs and in 5.88% of the dogs there were viral inclusion bodies in the hepatocytes. Pneumonia was observed in 52.94% dogs. In the spleen, lymphoid atrophy was observed in 29.41% dogs (Table 1).

RT-PCR

Of the 17 seropositive dogs for distemper, 5.88% presented a 319 bp target fragment for distemper using the CDV1 and CDV2 primers at the sinoatrial node using the RT-PCR technique. This animal was a four-month-old female, mixed-breed, and with incomplete vaccination scheme. Clinically presented myoclonus, abdominal pustule, parakeratosis in the pads and nasal plane. Gross lesions were right heart dilation, histoplasmacytic pancreatitis, lymphoplasmacytic pneumonia, lymphoid atrophy in the spleen and platelet disease (12,000 platelets/µL of blood).

Table 1. Microscopic lesions of 17 canine distemper virus seropositive dogs, Uberlândia, 2017.

MICROSCOPIC LESIONS	N . ABSOLUTE (%)
Cardiovascular system	
Lymphocytic focal myocarditis	4 (23.52%)
Urinary system	
Lymphoplasmacytic interstitial nephritis	12 (70.58%)
Intranuclear inclusion of bladder epithelium	7 (41.17%)
Lymphocytic cystitis	2 (11.76%)
Digestive system	
Hepatic Lipidosis	8 (47.05%)
Enteritis	8 (47.05%)
Lymphoplasmacytic hepatitis	7 (41.16%)
Lymphoplasmacytic pancreatitis	3 (17.64%)
Hepatic necrosis	1 (5.88%)
Intranuclear inclusion in hepatocytes	1 (5.88%)
Pancreatic hemorrhage	1 (5.88%)
Respiratory system	
Lymphoplasmacytic pneumonia	7 (41.16%)
Purulent pneumonia	2 (11.76%)
Hematopoietic system	
Splenic lymphoid atrophy	5 (29.4%)
Splenic hemosiderosis	2 (11.76%)

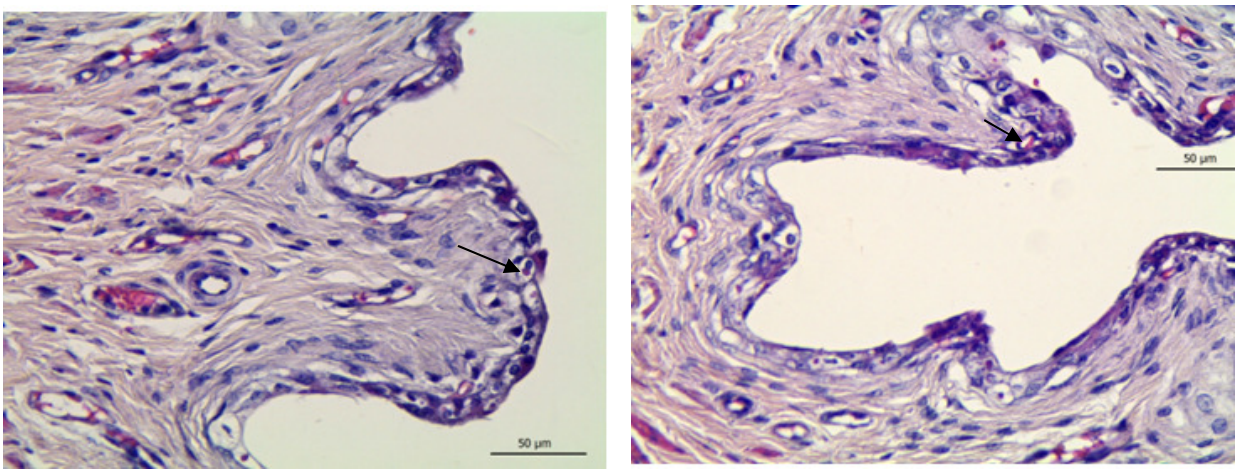

Figure 2. Intranuclear inclusion bodies (black arrows) of canine distemper virus in bladder epithelium of naturally infected dog. Hematoxylin and eosin stain, 40x.

DISCUSSION

In the present study most dogs with distemper were adults or elderly. Silva et al. (2009) and Santos et al. (2012) reported that distemper is more frequent in dogs over one year of age, as observed in our results. However, the disease can reach dogs of different ages and the incidence of major distemper cases occurs between 60 and 90 days of age (CORRÊA; CORRÊA, 1992). Even so, these same authors reported that the disease may develop in animals from 7 to 9 years old, the age observed in the present study, but the lethality is higher in young dogs especially when the virus acts on the central nervous system (CORRÊA; CORRÊA, 1992).

In the present study, 76.47% of the dogs presented cardiac dilatation. Several agents are capable of cause cardiac dilatation. Janus et al. (2014) studied dogs with heart disease and observed 63.63% with cardiac dilation identifying the

presence of *Borrelia burgdorferi in* 54.54% of the dogs. These authors also mentioned distemper and ehrlichiosis as possible agents responsible for myocarditis and cardiac dilatation.

The distemper virus is pantropic and can affect the cardiac musculature and its association with heart lesions was already mentioned by several authors (MENDONÇA; COELHO, 2006; RESENDE et al., 2009) Mendonça and Coelho (2006) evaluated seropositive dogs for distemper and identified 97.14% of hearts with cardiac dilatation and Resende et al. (2009) observed 88.57%. Higgins et al. (1981) studied dogs inoculated with CDV and found cardiac dilation in only one dog (9.09%).

Lymphocytic myocarditis, observed in the present study, may be associated with the presence of the virus in the tissue. The immune response of the host contributes to the installation of the inflammatory process and consequently myocardial injury (NELSON; COUTO, 2015; MENDONÇA; COELHO, 2006).

Lymphocytic myocarditis is reported by several authors. (HIGGINS et al., 1981; SONNE et al., 2009; MENDONÇA; COELHO, 2006; WOOLLEY et al., 2007; KANESHIGE et al., 2007; RESENDE et al., 2009) as a lesion associated with the distemper virus. Resende et al. (2009) studying dogs with CDV found 42.8% with lymphocytic myocarditis, 31.4% with hyaline degeneration, 14.3% with hyperemia and 11.4% with hemorrhage. However, Sonne et al. (2009) did not observe significant microscopic changes in the heart of dogs with distemper.

Kaneshige et al. (2007) related distemper and parvovirus as possible viruses associated with myocarditis, as well as bacteria and protozoa such as *Trypanosoma cruzi*. Scalabrini et al. (1996) studied dog infected with *Trypanosoma cruzi* and observed myocarditis and inflammation in the sinoatrial node. Woolley et al. (2007) reported the canine distemper virus as responsible for myocarditis in addition to parvovirus, bacteria, *Borrelia burgdorferi* and leishmaniasis.

In the present study it was not possible to evaluate microscopically the sinoatrial node region since it was harvested for CDV identification by RT-PCR. However, Bastos et al. (2007) observed microscopic lesions in the sinoatrial node of dogs with cardiac dilation such as lymphocytic inflammation (45.3%), hyaline degeneration (26.2%), fatty degeneration (9.5%) and hemorrhage (7.1 %).

Mendonça and Coelho (2006) also studied lesions in the sinoatrial node of dogs serologically positive for distemper and observed lymphocytic infiltration in 42.86%; fat infiltration in 28.57%; hyaline degeneration at 8.57%; hemorrhage in 18.57% and absence of lesions in 25.7%.

Araújo (2017) evaluated the influence of the distemper virus on the cardiovascular system and verified that there is virus activity in the excitatory and specialized heart conduction system. Dogs positive for the distemper virus had decreased heart rate variability and the negativity of arrhythmias to holter.

Several agents have been described as causing damage to the sinoatrial node but there are no reports of agent identification in this anatomical structure. In the present study it was possible to detect the distemper virus in the SAN by the RT-PCR technique proving that this virus can be located in the dog sinoatrial node. Studies performed so far cite inflammatory process in this cardiac structure, but without identification of the etiological agent responsible for the inflammation. Likewise, to date there are reports of the presence of CDV detected by RT-PCR in several types of biological samples and tissues, but not specifically in the sinoatrial node.

CDV was detected at low levels (2.79×10^4 copies of RNA /μL) in a dog heart seropositive for distemper and at high levels in other tissues, such as skin, muscle and oral mucosa, with viral load varying from 2, 10×10^6 to 4.25×10^8 μL (ELIA et al., 2006).

Previous studies using the RT-PCR technique had demonstrated CDV RNA in other tissues and fluids. CDV was detected by RT-PCR in 86% serum samples and 88% of whole blood and cerebrospinal fluid samples from immunohistochemically confirmed dogs with distemper (FRISK et al., 1999). Gebara et al. (2004) obtained amplification of the CDV nucleoprotein gene in 47.1% of urine samples from dogs with clinical signs suggestive of distemper.

Depending on the clinical presentation and evolution of canine distemper CDV may be present in a variety of biological samples. At different stages of infection urine, whole blood, leukocytes, feces, saliva, respiratory secretion and CSF may present the virus in various titres (FRISK et al., 1999; ELIA et al., 2006; SANTOS et al. 2012). Negrão et al. (2007) obtained the amplification of a CDV fragment from the urine or leukocytes of 66.5% of the animals with canine distemper and stated that the different forms of clinical presentation and evolution of canine distemper can make it difficult to choose only a type of

biological material to perform the ante-mortem etiological diagnosis.

However, RT-PCR has limitation and may present false negative results. Several factors may interfere with the results such as selection of the nucleotide target sequence to be amplified, method of RNA extraction, standardization of reagents used in the technique, selection of biological material to be used, conservation form and storage time since it is a virus whose genome consists of single stranded RNA which can easily undergo degradation (FRISK et al, 1999; GEBARA et al., 2004; KIM et al., 2001).

Virus inclusions bodies were identified in the bladder epithelial cells in 41.18% of the animals, and it may be considered an important microscopic finding for the diagnosis of distemper. Kubo et al. (2007) observed viral inclusions bodies in the bladder of canines infected with the distemper virus in 73.0% of the cases. Sonne et al. (2009) in 27.8% of the dogs and these authors associated the low frequency of inclusions in the bladder with the desquamation observed in the epithelium of the same making it impossible to visualize the inclusion bodies.

Regarding the absence of inclusions bodies in other organs Sonne et al. (2009) consider that these corpuscles can be identified in other organs or not and is therefore not mandatory. Batista, Moura and Reis (2000) and Aleman et al. (1992) also reported the low frequency of inclusion bodies in dogs with distemper.

The digestive system was the most affected in dogs with distemper in this study, especially liver and pancreas. (KANESHIGE et al., 2007) observed hepatic congestion and necrosis of hepatocytes in dogs with distemper and cite that CDV may cause liver injury.

Considering that CDV is a multisystemic agent, it may be capable of causing hepatitis (KANESHIGE et al., 2007), observed in 41.16% of the dogs in this study. Hepatic lipidosis was frequent and may be associated with heart failure (ANDERSON, 1992; TAMS, 2005), since most of the animals presented macroscopic or microscopic cardiac alterations. However, differently from these studies Sonne et al. (2009) did not identify significant microscopic changes in the liver.

Pancreatic lesions were frequent in this study and Tams (2005) cites that the canine distemper virus can cause pancreatitis, reinforcing the findings of this work. Lymphoid atrophy was observed in 29.4% of dogs as reported by Kubo et al. (2007).

Deem et al. (2000) cited pneumonia in dogs with distemper as one of the important postmortem findings and in their study 52.92% of the dogs had pneumonia. Sonne et al. (2009) verified 44.4% of the animals with pneumonia and Kubo et al. (2007) reported that the main finding in 100 dogs with distemper was pneumonia. Also Tudury et al. (1997) reported 12.34% of dogs with distemper showing bronchopneumonia.

CONCLUSIONS

Gross and microscopic cardiac changes are frequently observed in dogs with distemper mainly cardiac dilation and myocarditis. Viral inclusions bodies in bladder epithelial cells are an important microscopic finding for the diagnosis of distemper, and lymphoplasmacytic interstitial nephritis was also a frequent lesion.

CDV can be located in the sinoatrial node of naturally infected dogs as demonstrated in this study by the RT-PCR technique reinforcing the hypothesis that CDV is capable of causing inflammatory lesions in the sinoatrial node of this species and disorders in the cardiac dynamics, compromising its normal physiology and leading the dog to death.

Investigative studies of sinoatrial node lesions should be continued not only in dogs but in other domestic and wild species.

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RESUMO: A cinomose canina é uma doença viral que afeta vários sistemas, dentre eles o cardiovascular. Objetivou-se identificar o vírus da cinomose canina no nó sinoatrial (NSA) de cães sorologicamente positivos para cinomose, através da reação em cadeia da polimerase, precedida de transcrição reversa (RT-PCR), além de analisar os achados macroscópicos e histológicos da cinomose no coração e outros tecidos. Foram coletados fragmentos de tecidos e do NSA de 17 cães sorologicamente positivos para cinomose que vieram a óbito e foram necropsiados no período de outubro de 2015 a dezembro de 2016. No coração

observou-se dilatação cardíaca direita em 76,47% dos cães e hipertrofia concêntrica esquerda em 11,76% dos cães. Microscopicamente observou-se miocardite linfocítica em 23,53% dos cães e 41,18% apresentou corpúsculos de inclusão viral no epitélio vesical. Somente um (5,88%) cão apresentou fragmento alvo de 319 bp para cinomose utilizando os primers VCC1 e VCC2, no nó sinoatrial. Conclui-se que o VCC pode localizar-se no nó sinoatrial de cães naturalmente infectados, como demonstrados neste estudo pela técnica de RT-PCR, reforçando a hipótese de que o VCC é capaz de provocar lesões inflamatórias no nó sinoatrial dessa espécie. Alterações cardíacas macroscópicas e microscópicas, principalmente dilatação cardíaca e miocardite, são frequentemente observadas em cães com cinomose. Inclusões virais nas células epiteliais da bexiga são importantes achados microscópicos para diagnóstico da cinomose.

PALAVRAS-CHAVE: Sistema de Condução Cardíaco. Vírus da Cinomose Canina. Cardiomiopatias.

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