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Molecular diagnosis and clinicopathological characteristics of canine distemper neurologic disease

I.S. Jesus[1], A.V. Cruz[1], C.M.P. Santos[1], E.C.M. Barra[1], J.C. Costa[1], T.R.M. Monteiro[1], C.C. Santos[1], K.F. Valente[1], B.W.L. Silva[1], E.D.L. Rodrigues[1], J.C.F. Almeida[1], E.Silva Filho[1], M.J.F.M. Figueiredo[2], S.K.S.A. Sousa[2], S.P. Silva[3], L.M.N. Casseb[3] A.M.G. Negrão[1] and A.R. Casseb[1]

[1] Laboratório de Sorologia e Biologia Molecular, Universidade Federal Rural da Amazônia, Belém, PA, Brasil
[2] Hospital Veterinário da Universidade Federal Rural da Amazônia, Belém, PA, Brasil
[3] Departamento de Arbovirologia e Febres Hemorrágicas, Instituto Evandro Chagas, Ananindeua, PA, Brasil

Corresponding author: A.V. Cruz
E-mail: andrea.vianacruz@gmail.com

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ABSTRACT. Canine distemper is a highly infectious disease, distributed throughout the world. It is characterized by lymphotropism, neurotropism, and epitheliotropism, resulting in severe clinical changes and death. We report on the clinical and hematological findings of dogs with neurological distemper. Thirty-two mixed breed dogs of both sexes and of various age groups with clinical presentation suggestive of distemper with neurological involvement were evaluated. Blood and urine samples were collected for hematological and PCR analysis. Of the 32 animals evaluated by the RT-PCR technique, 22 were positive for the distemper virus. In the clinical presentation of distemper positive animals, neurological disorders stood out, with myoclonus being the most prevalent (18/22). In the hematological evaluation, erythrocytes and leukocytes were within the reference range; thrombocytopenia and lymphopenia were the most relevant findings in dogs with neurological involvement and could be used by veterinary clinicians as auxiliary diagnostic parameters.

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Key words: RNA virus infections; Neurological disorders; Domestic dogs

INTRODUCTION

Canine distemper is an infectious and febrile disease that affects canids, especially domestic dogs, caused by the Canine distemper virus (CDV) of the genus Morbillivirus, Canine *morbillivirus* species. This virus has a negative-strand helical RNA and a lipoprotein envelope; these characteristics of the virus induce cell fusion and immune-mediated cytolysis of infected cells. One of its characteristics is tropism for lymphocytes, leading to immunosuppression and predisposition to opportunistic infections (Scobesberger et al., 2005; Nelson and Couto, 2015; Pratakpiriya et al., 2017; ICTV, 2020).

Depending on the viral strain and the host's immunological conditions, variations in the clinical signs of the disease may occur (Lempp et al., 2014). Dogs with CDV infection can develop progressive nervous system disturbances, respiratory and gastrointestinal tract disorders (Beineke et al., 2008; von Rüden et al., 2012). According to Brito et al. (2016) the highest incidence occurs when there is a decrease in the rate of maternal antibodies, usually in animals aged between 60 and 90 days of age. Knowledge of the laboratory parameters of distemper in dogs can guide the diagnosis and prognosis of the disease. Clinical history, symptomatology and hematological findings help the diagnosis in routine, where anemia, lymphopenia and thrombocytopenia are consistent hematological changes (Buragohain et al., 2017; Silva et al., 2017). For the ante-mortem laboratory diagnosis, several methods were developed, highlighting the search for inclusion corpuscles in cells present in body secretions and circulating neutrophils, direct immunofluorescence, immunohistochemistry, isolation of distemper virus in cell culture and the Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR) (Willi et al., 2015).

The control of DCV occurs through the vaccination of animals, however due to the wide variety of susceptible hosts, in Brazil there are 54.2 million dogs (IBGE, 2018), and the great severity of this viral disease, eradication is considered impossible (Beineke et al., 2008; Amude et al., 2010; Temilade et al., 2015). Therefore, accurate and rapid diagnosis is main important, so that better treatment of such disease can be carried out. It presents a difficult clinical diagnosis, with regard to symptomatology in the different phases, which can be confused with other diseases. We describe the clinical and hematological findings of dogs with neurological distemper at the Veterinary Hospital of the Universidade Rural da Amazônia, from January 2017 to January 2018.

MATERIAL AND METHODS

This study was submitted and approved by the Ethics Committee on the Use of Animals (CEUA) of the Universidade Federal Rural da Amazônia (UFRA) with protocol number 001/2016.

Collecting of samples

For the study, 32 mixed breed dogs were evaluated, of both sexes and of different age groups, coming from the clinical care of Veterinary Hospital Prof. Mário Dias Teixeira of the Universidade Federal Rural da Amazônia (HOVET- UFRA), Belém, PA, from January 2017 to January 2018, with clinical conditions suggestive of neurological canine

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distemper. For complete blood count and PCR assay, blood and urine samples were collected. The collection of biological material from each dog was performed by cephalic or jugular venipuncture, collecting 5 mL of blood, with the aid of a 5 mL BD syringe and 25 X 8 mm BD needles (BD Vacutainer®). Blood samples were immediately placed in two plastic test tubes containing the anticoagulant ethylenediaminetetraacetic acid, one to perform the complete blood count (erythrogram, white blood cell, and platelets), according to the methodology described by Coles (1986) and the other for nPCR, which were kept at -86ºC.

The urine was collection performed by cystocentesis, according to Rubin (2002), with 5 mL of urine obtained, placed in sterile plastic tubes, centrifuged at 4°C at a speed of 12000 rpm for 15 min. After, the supernatant was discarded, and the sediment stored together with the blood in a freezer at -80°C until the moment of analysis.

In addition to whole blood for PCR, they were collected through cystocentesis, according to Rubin (2002). 5 mL of urine disposed in sterile plastic tubes, centrifuged at 4°C at a speed of 12000 rpm for 15 min. The supernatant was discarded and the sediment was stored together with the blood in a freezer at -80°C.

Hematological parameters

The erythrogram, leukogram and platelet analyses were performed using the semi-automatic method, using the BCVet 2800 device (Auto Hematology Analyzer, Mindray® Bio-Medical Electronics Co. Ltda, Shenzhen - Guangdong) with configuration for the canine species. The differential leukocyte count was performed on a blood smear slide stained by the rapid panoptic method and then taken to the optical microscope for observation (Lopes et al., 2007).

RNA extraction and RT-PCR

To confirm suspected cases of canine distemper, RNA from whole blood and urine samples was extracted using Trizol® Reagents (Invitrogen -Life Technologies), according to Chomczynski and Sacchi (1987). The RNA was eluted in 35µL of elution buffer (UltraPure™ DNase/RNase-Free Distilled Water) and submitted to RT- PCR.

In reverse transcription (RT), the enzyme SuperScript III Reverse Transcriptase was used (200 U/µL- Invitrogen ™) and the primer pair CDV_Rev1 (5′CCC ATG GAG TTT TCA AGT TC -3′/ IDTDNA®) and CDV_For1 (5′- TCC CAA GCA TCA ACT CTG -3′/ IDTDNA®). In the first stage, were added in a sterile 200 µL microtube, 12 µL of distilled water, 2.0 µL of dNTP and 1.0 µL of primers (CDV R1 and CDV F1). Then, all reagents were vortexed for 15 ss and heated at 65°C for 5 min. In the s stage, 4.0 µL of buffer, 1.0 µL of DTT, 0.3 µL of SuperScript III Reverse Transcriptase (200 U / µL- Invitrogen TM) and 3.0 µL of RNA were added to the first stage tube. After adding the reagents, the samples remained at room temperature of 20ºC for 5 min. All reactions were prepared on ice and transferred to amplification in the automatic thermo cycler (CFX96 Touch™), followed by denaturation at 94°C for 1 min and 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 59.5°C for 2 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min (Frisk et al., 1999).

PCR was performed in a final volume of 25 µL, containing 5.0 µL of RT sample, 2.5 µL of Buffer, 2.0 µL of dNTP, 1.0 µL of MgCl2, 1.5 µL of the primers (CDV-R1 and CDV -F1), 0.3 µL of Platinum TAQ DNA Polymerase (Invitrogen TM) and 11, 2 µL of ultrapure water. The reaction was transferred to the thermal cycler, with the following steps: an initial denaturation at 94°C for 1 min, 2 cycle of denaturation 94°C for 30 s, annealing at 55°C for 30 s, polymerization at 75°C for 45 s and final extension at 72°C for 2 min. A positive and a negative control were added to all reactions. The RT-PCR products were analyzed by electrophoresis on a 1% agarose gel in TAE Buffer 50x ultrapure buffer, stained for DNA tracking with Blue Juice ™ (Invitrogen TM), examined comparatively with DNA markers up to 1500bp and visualized with the aid of UV transilluminator. Samples with fragments of 730bp were considered positive (Pozza et al. 2007).

Data analysis

The data were tabulated and statistically treated by simple percentage and the differences between the percentages were compared with each other using the Chi-square test (X^2), assuming statistical significance for $P < 0.05$ and, for values less than 5, the G test. Hematological variations were treated using descriptive statistics. To assess the existence of a correlation between the PCR result and the hematological values obtained, the logistic regression test was applied, implemented in the Microsoft Excel and BioEstat 5.3 programs.

RESULTS

For the total of animals evaluated by the RT-PCR technique, 22/32 were positive for canine distemper virus, represented by 20/28 and 2/4 in males and females, respectively, with a significant difference between the positivity of males and females ($X^2 = 84.375$, $P < 0.0001$). Among dogs with infection, four were up to one year old, nine were aged between 2 to 3 years and nine animals were aged between 4 to 8 years, indicating that the disease affects dogs of different age groups. Regarding the clinical presentation of positive animals, neurological disorders stood out, with myoclonus being the most prevalent, followed by vocalization. Other signs included cervical stiffness, nystagmus, motor incoordination, seizures and paresis, as described in Table 1.

Table 1. Frequency of neurological signs observed alone or in association, according to the age range of the 22 dogs positive for the distemper virus evaluated by the RT-PCR.

	Age range (years)			
clinical sign	≤ 1 (N=4)	2-3 (N=9)	4-8 (N=9)	Total (N=22)
Myoclonus	3	7	8	18
Neck stiffness	0	2	3	5
Nystagmus	0	0	3	3
Motor incoordination	1	4	6	10
Convulsions	4	3	1	8
Vocalization	2	7	7	16
Paresis	3	5	7	15

In the hematological evaluation, the minimum, maximum, standard deviation and mean values of the erythrogram and leukogram plus the reference values of negative and

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positive dogs for distemper virus are shown in Table 2. In 75% (24/32) of the analyzed samples, the erythrocytes were lower than the reference range, showing a statistically significant difference (X^2= 25, P < 0.0001) between the reference values, and 8/32 of the animals showed normal values among these, 6/8 animals positive for RT-PCR. Regarding hemoglobin and hematocrit counts, it was observed that both infected and negative dogs had an average below the reference values, being characterized by normochromic normocytic anemia in 24/32 of the animals in the study.

Table 2. Hematological values (minimum, maximum, standard deviation and mean), according to the result of the PCR-RT for distemper, plus the n and reference values.

Parameters	Reference intervals	negative (n=10)	positive (n=22)
Erythrocytes (10^6/mm³)	5.5 – 8.5	1.13 – 6.59 (±1.67) mean 4.475	1.13 – 6.59 (±1.67) mean 4.47
Hemoglobin (g%)	12 – 18	2.1 - 14.6 (±4.08) mean 10.37	6.1 - 17.1 (±2.76) mean 10.87
Hematocrit (%)	37 – 55	8.5 - 46.3 (±12.03) mean 32.63	10 - 53.8 (±9.14) mean 33.29
Platelets (10^3/mm³)	200000 – 500000	20000 – 358000 (±114827) mean 229600	9000 – 770000 (±191205) mean 218090
Leukocytes (cells/mm³)	6 – 17	3.4 – 800 (±249.31) mean 90.75	0 – 29 (±7.61) mean 12.15
Rod neutrophils (cells/mm³)	0 – 300	0 – 204 (±64.44) mean 30	0 – 2030 (±431.96) mean 97.91
Segmented (cells/mm³)	3000 – 11500	0 – 21663 (±7414.46) mean 9308	0 – 24360 (±7491.78) mean 9160.59
Eosinophils (cells/mm³)	100 – 1250	0 – 996 (±321.6) mean 267	0 – 1918 (±460.8) mean 229.1
Lymphocytes (cells/mm³)	1000 – 4800	0 – 1568 (±584.1) mean 516.9	0 – 2310 (±725.2) mean 822.6
Monocytes (cells/mm³)	150 – 1350	0 – 1245 (±437.47) mean 398.9	0 – 1450 (±489.74) mean 527.27

Regarding the number of platelets, 15 of 32 the samples presented values within the reference standard, 15 of 32 presented thrombocytopenia, while 2 of 32 presented thrombocytosis (X^2 =33.008, P < 0.0001). Among animals with normal platelet values, 53.33% (8/15) were positive on RT-PCR, while 7 of 15 were negative (X^2 =0.444, P = 0.5050), with no statistically significant difference between the results. Animals with thrombocytopenia, 80% (12/15) were positive on RT-CRP, while 3 of 15 were negative, a significant difference (X^2 =36, P < 0.0001). Of the samples with thrombocytosis, 100% (2/2) were positive (X^2 =100, P < 0.0001) as described in Table 3.

Regarding the leukogram, 15 of 32 of the samples showed normal leukocytes values. Leukopenia was observed in 7 samples and leukocytosis in 10 in the analyzed samples. Among animals with normal leukocyte values, 11/15 were positive on RT-CRP, while 4 were negative (X^2 =21.772, P < 0.0001). Of the animals with leukopenia, 4/7 were positive on RT-PCR, compared to 3 of 7 negative, with no statistically significant difference (X^2=18.37, P < 0.0001). Of the samples with leukocytosis, 2 of 10 were positive (X^2=4, P = 0.0455). When analyzing eosinophils, it was observed that 12 of 32 of the samples showed normal values. 9 presented eosinophilia, and 11 eosinopenia.

Among animals with eosinophils within the reference standard, 6/12 were positive in RT-PCR. Regarding eosinophilia, 5/9 were positive (X^2=100, P < 0.0001). Of those that were below the values, 9/11 of the animals were positive (X^2=33,524, P < 0.0001). In the lymphocyte count, 13 of 32 presented normal values and 19/32 were below the reference values (X^2 =53.54, P < 0.0001). Among animals with normal lymphocyte values, 7/13 were positive on RT-PCR, while 6 of 13 were negative (X^2 =32.769, P < 0.0001).

Table 3. Frequency of erythrogram and leukogram findings in 22 dogs positive for distemper from January 2017 to January 2018.

Parameters	No. dogs	Positives
Erythrocytes Normal	08/32	6/8
Erythropenia	-	-
	24/32	-
Platelets Normal	15/32	8/15
Thrombocytosis	2/32	02/02
Thrombocytopenia	15/32	12/15
Leukocytes Normal	15/32	11/15
Leukocytosis	10/32	2/10
Leukopenia	7/32	4/7
Eosinophils Normal	12/32	6/12
Eosinophilia	9/32	5/9
Eosinopenia	11/32	9/11
Lymphocytes Normal	13/32	7/13
Lymphopenia	-	-
	19/32	12/19
Monocytes Normal	21/32	13/21
Monocytosis	2/32	2/2
Monocytopenia	9/32	5/9

DISCUSSION

Among dogs with results confirmed with distemper virus infection, all age groups showed positive results, with emphasis on adult dogs, the same was being observed by Bastos (2018) and Freire et al. (2019) in studies carried out on dogs naturally infected with canine distemper virus (CDV) of different age groups, in which adult dogs were more affected than puppies. Similar results were found by Ogbu et al. (2017) which observed this disease mainly in puppies confirming that the prevalence of distemper in dogs has no age predilection. However, Headley and Graça (2000) observed young dogs, between zero and 1.5 year, were more affected, and contributed to 62.8% (157/250) of all CDV diagnosed cases. All these results are important as they demonstrate a variation in the age groups of animals with canine distemper.

In the study, myoclonus stood out among the clinical signs observed. The clinical diagnosis of distemper has often been made when systemic signs precede or accompany neurological disease. And myoclonus is often seen in infections with CDV (Elia et al., 2006). As in the Vandevelde et al. (1982) study that analyzed nine animals and observed the

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presence of myoclonus in 3 dogs. However, such clinical criteria for the diagnosis of distemper must be carefully evaluated, because although most cases of distemper nerve have conventional presentations, neurological disease can be observed in the absence of systemic disease and myoclonus (Amude et al., 2012).

Besides that, systemic involvement may be present in other CNS infectious conditions and myoclonus, as they have also been described in diseases of the nervous system other than distemper (Frade et al., 2018). In the hematological analysis of the affected dogs, it was observed that the total hemoglobin and hematocrit counts show indexes below the reference values in animals with the infection, with hematocrit ranging from 10 - 53.8% (mean 33.29%); similar data were found by Ezeibe et al., (2008) who demonstrated the prevalence of anemia in dogs with distemper due to the persistence of the virus in the bone marrow, resulting in consistent hematological changes. It is suggested that CDV infection causes the release of interleukin-1 by macrophages, making iron little available for the production of red blood cells. There is also a decrease in the response to anemia due to the low production of erythropoietin, a decrease in the response of the bone marrow to erythropoietin and lack of iron, leading to the limitation of erythropoiesis (Waner and Harrus, 2007). Among dogs that presented thrombocytopenia, 80% had the infection and 53.33% had platelet values within the reference standards. Such data reveal that many dogs diagnosed with the disease in the neurological phase may or may not have platelet alterations. Silva et al., (2017) when evaluating the hematological characteristics in dogs with the disease, they also observed thrombocytopenia as a remarkable data in animals.

Regarding the leukocyte count of the evaluated dogs, 73.33% were within the reference values, 20% had leukocytosis while leukopenia was observed in 57.14% of the animals. Ezeibe et al., (2008) also correlated variations in the total leukocyte count, highlighting the association between leukopenia and early lymphopenia, followed by late lymphocytosis and leukocytosis. Generalized viral multiplication, in four to six days, may be responsible for the initial lymphopenia and consequently leukopenia (Freire et al., 2019). Lymphotropism is considered a characteristic of *Morbillivirus* infection. Thus causing the loss of the capacity of lymphocyte proliferation with consequent immunosuppression and increased susceptibility of the host to opportunistic infections, although it may be absent in some cases because it is not a specific clinical finding (Tatsuo et al., 2001). In the present study, lymphopenia was a predominant finding, of the dogs affected, , with values ranging from 0 - 2310% (Mean 822%). The same was observed by Barbosa et al., (2011), researching hematological laboratory changes in dogs positive for distemper; they observed lymphopenia in 76% of the animals.

In this case, the research presents a scientific contribution when it describes the main hematological findings only in dogs with neurological system involvement. All parameters were evaluated in dogs that presented one or more signs suggestive of central nervous system involvement, confirmed by PCR, both in urine and in whole blood.

CONCLUSIONS

It is concluded that myoclonus followed by paresis stand out among the clinical findings in studied dogs. In hematological findings, erythrocytes and leukocytes within the reference range, thrombocytopenia and lymphopenia were the most relevant findings in dogs with neurological involvement. Our results also indicate that in dogs with signs of

neurological distemper, urine and whole blood analysis can help in the definitive diagnosis of the disease.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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