



## Expression of pro-and-anti-apoptotic antigens in the cerebellum of dogs naturally infected with canine distemper virus

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### Abstract

Canine distemper virus (CDV) may induce multifocal demyelination in the central nervous system of infected dogs. The present work investigated apoptosis in white and gray matter (granular layer) in the cerebellum of naturally infected dogs by the analysis of the expression of the pro-apoptotic antigens caspase – 2 and – 3 , b(terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL-staining) positivity, annexin-V immunodetection, and the presence of the anti-apoptotic antigens, BCL-2 and p53. Cerebellum specimens were obtained from the Laboratory of Animal Pathology, from 1995 to 2009, and the 5-µm thick fragments were stained both with hematoxylin-eosin and Shorr. All samples were diagnosed as positive for CDV genome by reverse transcriptase polymerase chain reaction targeting the nucleocapsid gene. The anti-apoptotic pathways evidenced in this study were BCL-2 and p53 proteins that were intensively detected in cerebellum of CDV positive slides (40-80% of labeled cells/mm<sup>2</sup>). In addition, the apoptosis markers annexin-V and TUNEL are directly correlated among the same samples (80 and 40% of labeled cells, respectively). This is the first description of p53 and annexin-V expression, characterized as anti-apoptotic and apoptotic proteins, involvement in canine natural cases of CDV infections.

**Key words:** CDV; programmed cell death; host-virus interaction.

### Introduction

Canine distemper virus (CDV) causes canine distemper (CD) in dogs and other carnivores and belongs to the genus *Morbillivirus* within the family *Paramyxoviridae* (12). The virus is a highly contagious pathogen that occurs worldwide and its infection in dogs generally induces a multi-systemic disease (3, 12). In the case of measles virus (MeV), a virus of the same family which infects only humans and certain nonhuman primates, fatal disease outcome is rare, while some animal morbilliviruses, especially those infecting carnivores, can approach 100% of mortality (12). In addition, CDV spreads at approximately 10 days post-infection, from the primary replication sites to epithelial tissues and reaches the central nervous system (CNS), resulting in a demyelinating disease

(12). In natural cases of CD, immunosuppression followed by secondary infections is the leading cause of death (1, 13). However, previous studies performed on CDV have demonstrated that despite effective clearance of the virus in inflammatory lesions in the CNS of infected dogs, the virus has the ability to spread to other areas, even eliciting new lesions (1, 11, 13).

To enhance cell viability and facilitate replication, viruses possess multiple mechanisms to inhibit the host response (8). Apoptosis serves as an innate cellular response to infection that limits both the time and cellular machinery available for viral replication (8). There are two major pathways of apoptosis: the death receptor-mediated external signal pathway-extrinsic pathway, and the mitochondrial-mediated internal signal pathway-intrinsic pathway (8,

11). According to this, the present investigation was conducted to elucidate some host-virus interactions using the cerebellum of naturally infected dogs with CDV. The TUNEL *in situ* and immunohistochemical assays (IHC) for the expression of anti-apoptotic (p53 and Bcl-2) antigens and pro- apoptotic caspase-2 and -3 antigens were performed. Viral antigens and RNA were also considered for analysis.

## Materials and Methods

### *Samples and CDV diagnosis*

Paraffin embedded samples, composed by the cerebellum of 43 positive dogs necropsied at the Veterinary School, Laboratory of Animal Pathology from 1995 to 2009, were used. Control animals (n= 20) were provided by veterinary clinics where they have died from causes other than infectious diseases. The paraffin-embedded samples were submitted to viral RNA extraction following procedures previously described (3, 4, 6). The conventional gel-based reverse transcription-polymerase chain reaction (RT-PCR) was applied targeting the nucleocapsid gene (3). In order to characterize the histological lesions and observe the inclusion bodies, hematoxylin eosin (HE) and Shorr staining were performed (10).

### *Immunohistochemistry assay (IHC)*

To perform the IHC assay, a standard avidin-biotin-peroxidase complex (ABC, DakoCytomation, CA, USA) method was used as previously described

(3, 6, 13). Unstained sections (4- $\mu$ m) were deparaffinized, rehydrated and washed in buffered saline with 0,1% Tween 80. The first step was to put the sections suspended in citrate buffer (pH 6.1) in the microwave for 15 min at 700 W in order to activate the viral antigen, which is normally damaged by formaldehyde fixation. Just before staining, the slides were treated three times with 50% hydrogen peroxide (30 V) for 30 min to inactivate the endogenous peroxidase commonly found in inflammatory reactions. The slides were then washed 5 times for 10 min each wash in buffered saline to remove residues. The next step was to block nonspecific binding by incubating in 15% reconstituted dry nonfat milk for 90 min. The optimum primary antibody dilutions used are described in Table 1. The slides were covered by 200 $\mu$ l of diluted antibody overnight at 4 °C in a humidified chamber. After 5 washes, 100  $\mu$ l streptavidin-peroxidase complex (Dako Cytomation) was added to each slide, which were incubated for 1 h at 37 °C. In addition, a substrate made fresh in the dark, by mixing equal volumes of 0.02% hydrogen peroxide and 0.6 mg DAB (3,3- diaminobenzidine tetrahydrochloride, Gibco BRL, cat # 15972-011), was added to the slides for 30 min at room temperature. The reaction was stopped by washing with tap water and the specific brown color was revealed after counterstaining with Meyer's haematoxylin. An intense brownish deposit was considered positive. Omission of the primary antibody was used as a negative control for the different antibodies.

Table 1: Details of the primary antibodies used in IHC.

	<b>Antibody</b>	<b>Dilution</b>	<b>Species</b>	<b>Supplier</b>
<b>annexin-V</b>	Monoclonal anti-annexin-V	1:4000	Mouse	Sigma-Aldrich® (cat # A8604)
<b>caspase-2</b>	Monoclonal anti-caspase-2 (ICH1 L)	1:400	Rabbit	Sigma-Aldrich® (cat # C7349)
<b>caspase-3</b>	Monoclonal anti-caspase-3, Active	1:1000	Rabbit	Sigma-Aldrich® (cat # C8487)
<b>Bcl-2</b>	Monoclonal anti-Bcl-2	1:500	Mouse	Sigma-Aldrich® (cat # B9804)
<b>p53</b>	Monoclonal anti-p53	1:100	Mouse	Sigma-Aldrich® (cat # P3737)

### *TUNEL assay*

TUNEL staining was performed following the manufacturer's instructions (A35126 APO-BrdU<sup>TM</sup> TUNEL Assay Kit, Invitrogen<sup>TM</sup>). Briefly, the slides were washed twice in wash buffer (ABO-BRDU Kit) and incubated at 4°C for 12 h in prepared DNA-labeling solution (ABO-BRDU Kit) containing TdT enzyme, BrdUTP, TdT reaction buffer and distilled water. After washing twice in rinse buffer (ABO-BRDU Kit), the sections were incubated in the dark for

30 min at room temperature with the antibody solution (fluorescein-labeled anti-BrdU monoclonal antibody and Rinse buffer). The slides were counterstained with propidium iodide. The positive control consisted of slides pre-incubated with 3U/ml of DNase for 1 h at 37°C. A negative control was incubated without the TdT enzyme.

### *Statistical analysis*

The intensities of IHC labeling were visualized under a light Axio Imager A.1 microscope connected to an AxioCam MRc (Carl Zeiss Oberkochen, Germany) and the number of positively labeled cells was counted (cell per mm<sup>2</sup>) using the Axiovision 4.7 software (Carl Zeiss) grid tool. For each slide, at least 7 microscopic fields covering all cerebellum area were randomly chosen (13). The SAS v 8.2 (SAS Institute Inc. Cary, NC, USA) and Origin software were used for data analysis. A *P* value less than 0.05 were statistically significant.

#### Ethical concerns

All animal handling and sample collection procedures were performed in accordance with the recommendations of the Brazilian College on Animal Experimentation (COBEA), and all experiments were approved by Institution Ethics and Animal Welfare Committee.

### Results

#### Histopathology findings

Microscopic cerebellar lesions were classified as purposed previously: (i) acute demyelinating lesion and (ii) chronic demyelinating lesion. Acute demyelinating lesion was characterized by absence of inflammatory infiltration, presence of white matter spongy vacuolation, astrocytic gliosis, the presence of gemistocytes, increased number of microglial cells, presence of Gitter cells, inclusion bodies and astrocytic syncytia (Fig. 1A). Chronic cerebellar lesions consisted of severe and extensive perivascular cuffs, severe influx of lymphoplasmacytic inflammatory cells, and proliferation of glial components (Fig 1B). These angiocentric lesions were formed by more than four layers of mononuclear inflammatory cells, whereas lymphocytes and plasma cells were the predominant cells as observed in HE-stained tissue sections (Fig. 1B). Several irregularly shaped astrocytic multinucleated giant cells were observed and some of these cells contained intra-nuclear and intra-cytoplasmic inclusions (Fig. 2). None of the negative samples had shown any lesion similar to CDV infection. Infectious agents were not identified in tissues by periodic acid-Schiff, Gomori's methanamine silver, Ziehl-Nielsen, and Giemsa staining (data not shown).

#### IHC results

We observed a positive correlation ( $r^2 = 0.8976$ ) among p53 (Fig. 3A) and BCl-2 (Fig. 3B) detected in all slides from the cerebellum. The annexin-V staining was observed diffusely in the molecular layer (Fig. 4). The TUNEL staining showed a very intense signal in the Purkinje cells (Fig 5A and B). Furthermore, no unspecific label could be visualized in the control group. None positive reaction could be documented for caspases-2 and-3 in all analyzed slides. In respect to viral RNA detection by RT-PCR all

positive samples were considered as being infected by CDV (data not shown). In addition, the number of TUNEL- and annexin-V-positive cells were positively correlated ( $r^2 = 0.9765$ ) in all slides analyzed (Fig 6).

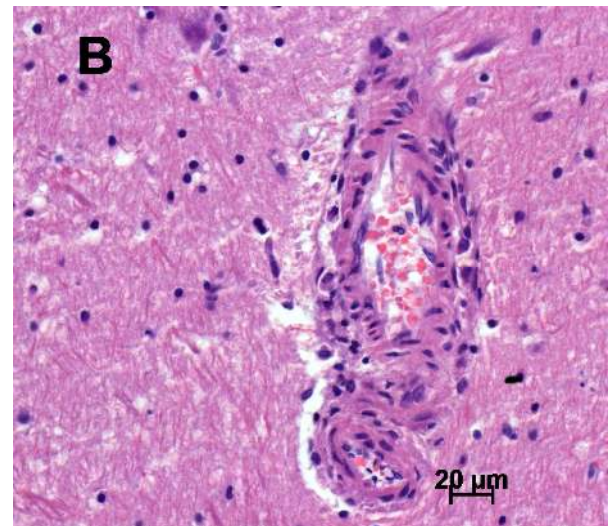
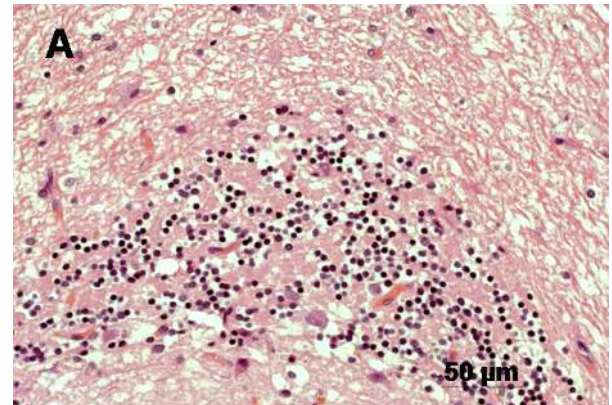


Fig. 1. Photomicrography of microscopic aspects of the cerebellum from CDV infected dogs. A) Inflammatory cells and proliferation of glial components; B) Severe influx of lymphoplasmatic inflammatory cells with more than four layers of mononuclear cells. Hematoxylin & Eosin stain; scale bar 50 μm.

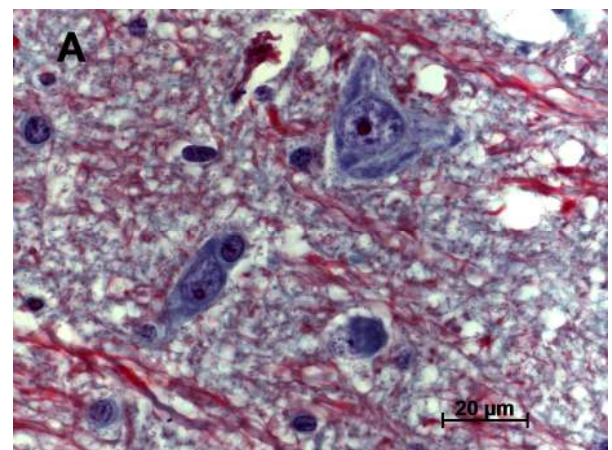


Fig. 2. Photomicrography of Shorr staining showing several irregular multinucleated cells (astrocytic syncytia) containing intra-nuclear inclusion bodies typical of CDV infection. (Scale bar 20 μm).

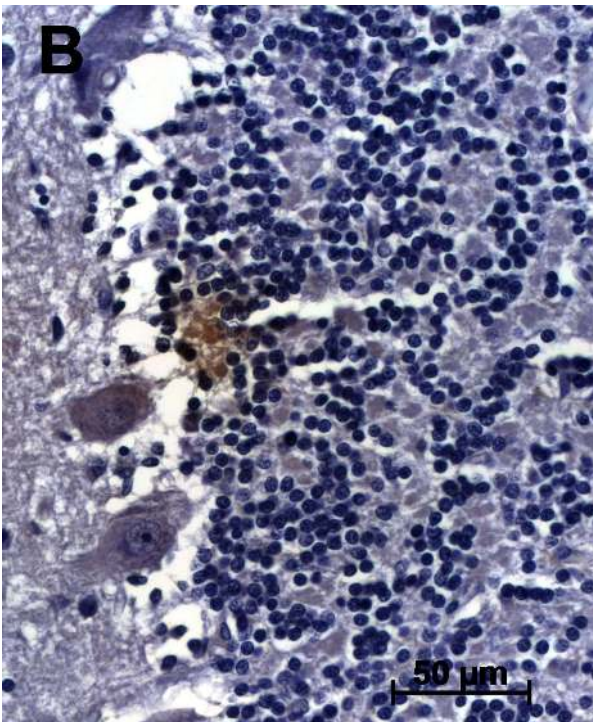
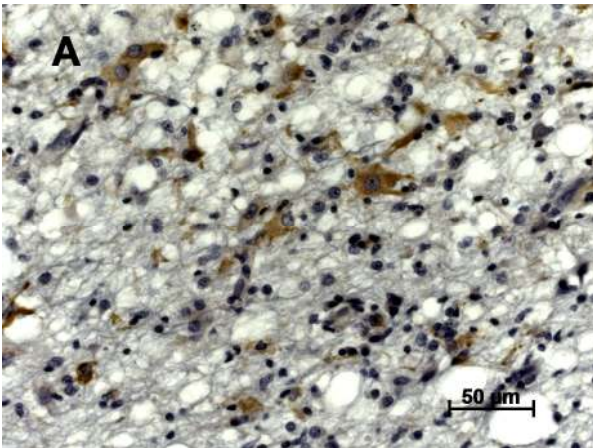


Fig. 3. Photomicrography of immunohistochemical assay for p53 (A) and BCl-2 (B) antigens detection in the cerebellum of infected dogs with CDV. The streptavidin-biotin peroxidase complex method was used (scale bar 50 μm).

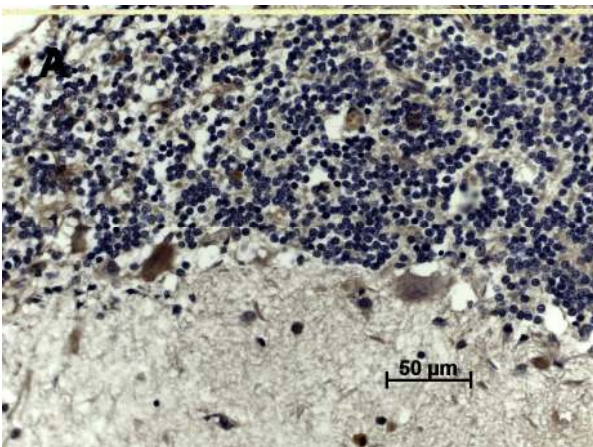


Fig. 4. Photomicrography of immunohistochemical assay for annexin-V antigen detection in the

cerebellum of infected dogs with CDV. The streptavidin-biotin peroxidase complex method was used (scale bar 50 μm).

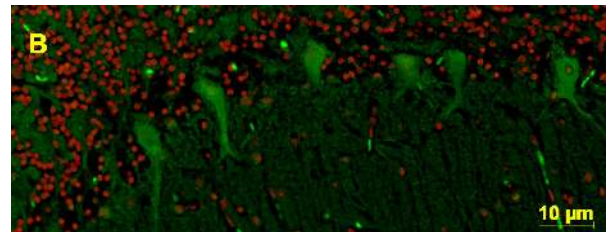
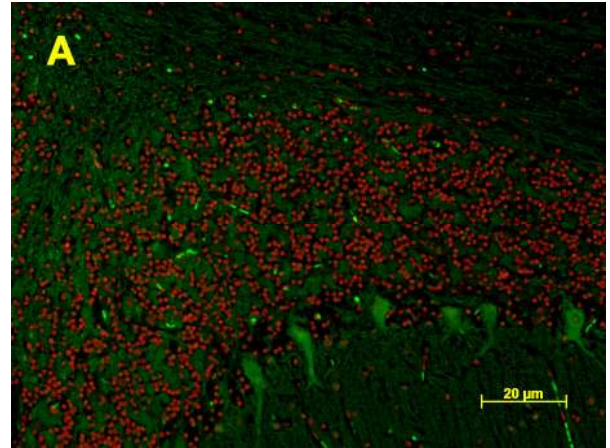


Fig. 5. Photomicrography of immunohistochemical assay for TUNEL *in situ* assay antigen in the cerebellum of infected dogs with CDV. A) Purkinje cells positively marked B) Higher magnification of A. The immunofluorescence method was used (scale bar 20 and 10 μm).

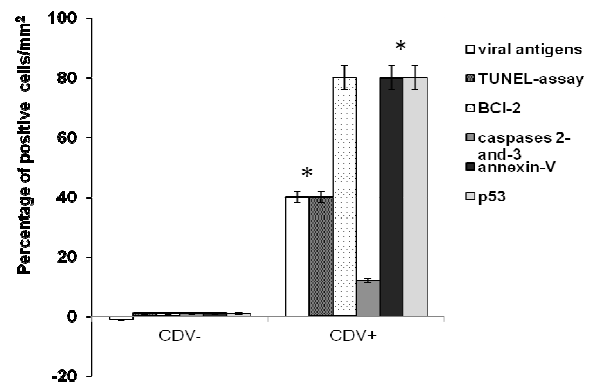


Fig. 6. Frequency of detection of TUNEL assay, annexin-V, caspases 2- and-3, BCl-2 and p53 in cerebellum of naturally infected dogs (CDV +) and a control group (CDV -) scored on a scale of 1-5 and plotted as positive cells per mm<sup>2</sup>. \* corresponded to statistically significant.

### Discussion

The cerebellar lesions observed in this study were similar to those described by other authors for chronic inflammatory demyelination (12). The

inflammatory phenomenon is related to virus elimination from the CNS and can increase lesion severity possibly due to factors released by the cellular response (1, 13). The present study showed by IHC that viral inclusion bodies were present mainly in the nuclei of white matter neurons and glial cells.

The pro-apoptotic pathways studied here were composed by TUNEL *in situ* detection and annexin-V expression. Additionally, TUNEL *in situ* reaction showed that Purkinje cells were the most labeled cells. It has been reported by other studies that gemistocytes were also labeled by TUNEL assay and many cells that underwent apoptosis in the granular cells layer could also be detected as TUNEL-positive cells (9, 10, 12). In our study, most of the annexin-V-positive cells were diffuse in the white matter. Typical features of apoptotic cells include a series of cellular, morphological and biochemical alterations such as chromatin condensation, phosphatidylserine (PS) exposure, cytoplasmic shrinkage, membrane blebbing, DNA fragmentation (7, 11). Annexin-V binding is reported to result from the loss of phospholipid asymmetry and PS. In addition, PS exposure on the outer surface of the plasma membrane occurs in the early stages of apoptosis (8). The first detection of annexin-V involvement in natural cases of CDV infections is reported in this study. Another study showed the annexin-V involvement in early stages of CDV infections however the B95a cell line was used as an *in vitro* model for the same purpose of this study (8). In resume, apoptosis could be directly correlated to annexin-V and TUNEL positive reaction in the cerebellum of naturally infected dogs.

The anti-apoptotic pathways evidenced in this study were Bcl-2 and p53 proteins. In a recent report, expression of Bcl-2 in peripheral blood and cerebellum were similar (2), in contrast to our results that revealed Bcl-2 intensively expressed in cells of the granular layer. However the respective blood was not included in the present investigation. It has been demonstrated that infection with CDV induces apoptosis in cerebellum and lymph nodes in different ways (1, 2, 9, 10, 12). Lymph node apoptosis apparently occurs via caspase-3 activation, through the caspase-8 pathway, and cerebellum apoptosis apparently occurs via caspase-3 activation, through caspase-8 and mitochondrial pathways (13). In fact, the real role of mitochondria in CDV infections was not elucidated. According to our results, Bcl-2 should be involved in CDV infection in spite of many studies which describe activation of caspases, both *in vivo* and *in vitro* CDV infections (2, 5, 8, 11), in this study neither caspase-2 nor caspase-3 antigens could be detected. As a result of the complex interplay between virus and host, infection of different cell types by the same virus does not necessarily activate the same cell death pathway (8).

The nuclear phosphoprotein, coded by p53, has important specific functions as a cell proliferation suppressor agent in DNA repair and plays an important role in the induction of programmed cell death (7, 8). This study represents the first description of p53 expression in natural cases of CDV. It is known that

some other viruses have the participation of p53 expression during infection, but the real function of this protein remains unclear in CDV disease (7). Thus, the complete mechanism used by CDV to induce (or not) apoptosis is not completely understood so far. The question whether apoptosis is a host defense against CDV infections or a virus strategy used to spread and infect other cells must be investigated in further studies.

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### References

1. BEINEKE, A., MARKUS, S., BORLAK, J., THUM, T., BAUMGÄRTNER, W. Increase of pro-inflammatory cytokine expression in non-demyelinating early cerebral lesions in nervous canine distemper. *Viral Immunol.*, 2008, 21, 401-410.
2. DEL PUERTO, H.L., MARTINS, A.S., MORO, L., MILSTED, A., ALVES, F., BRAZ, G.F., VASCONCELOS, A.C. Caspase-3/-8/-9, Bax and Bcl-2 expression in the cerebellum, lymph nodes and leukocytes of dogs naturally infected with canine distemper virus. *Gen. Mol. Res.*, 2010, 151-161.
3. HEADLEY, S.A., AMUDE, A.M., ALFIERI, F.A., BRACARENSE, A.P.F.R.L., ALFIERI, A.A., SUMMERS, B.A. Molecular detection of Canine distemper virus and immunohistochemical characterization of the neurologic lesions in naturally occurring old dog encephalitis. *J. Vet. Diagn. Invest.*, 2009, 21, 588-597.
4. HOYLAND, J.A., DIXON, J.A., BERRY, J.L., DAVIES, M., SELBY, P.L., MEE, A.P. A comparison of *in situ* hybridization reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ*-RT-PCR for the detection of canine distemper virus RNA in Paget's disease. *J. Virol. Meth.*, 2003, 109, 253-259.
5. KAJITA, M., KATAYAMA, H., MURATA, T., KAI, C., HORI, M., OZAKI, H. Canine distemper virus induces apoptosis through caspase-3 and -8 activation in Vero cells. *J. Vet. Med. B.*, 2003, 53, 273-277.
6. LAN, N.T., YAMAGUCHI, R., KIEN, T.T., HIRAI, T., HIDAKA, Y., NAM, N.H. First isolation and characterization of canine distemper virus in Vietnam with the immunohistochemical examination of the dog. *J. Vet. Med. Sci.*, 2009, 71, 155-162.

7. LI, F.Q., TAM, J.P., LIU, D.X.. Cell cycle arrest and apoptosis induced by the coronavirus infectious bronchitis virus in the absence of p53. *Virology* 2007, 365, 435-445.
8. MAJNO, G., JORIS, I. Apoptosis, oncosis and necrosis. An overview of cell death. *Am. J. Pathol.*, 1995, 146, 3-15.
9. MORO, L de SOUSA, M. A., de MORAES, A.C., de ARAUJO SANTOS J. E. DOS SANTOS NUNES, R. A. CARNEIRO, R. CARVALHO, A. C. VASCONCELOS. Apoptosis in canine distemper. *Arch. Virol.*, 2003a, 148, 153-164.
10. MORO, L., MARTINS, A. S., ALVES, C. M., SANTOS, F. G. A., DEL PUERTO, H. L., VASCONCELOS, A. C. Apoptosis in the cerebellum of dogs with distemper. *J. Vet. Med. B.*, 2003b, 50, 221-225.
11. PILLET, S., MESSLING, V.v. Canine distemper virus selectively inhibits apoptosis progression in infected immune cells. *J. Virol.*, 2009, 83, 6279-6287.
12. VANDEVELDE, M., ZURBRIGGEN, A. Demyelination in canine distemper virus infection: a review. *Acta Neuropathol.*, 2005, 109, 56-68.
13. WÜNSCHMANN A., KREMMER, E., BAUMGÄRTNER, W. Phenotypical characterization of T and B cell areas in lymphoid tissues of dogs with spontaneous distemper. *Vet. Immunol. Immunopathol.* , 2000, 73, 83-98.