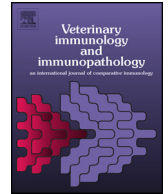




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Short communication

## Zymographic patterns of MMP-2 and MMP-9 in the CSF and cerebellum of dogs with subacute distemper leukoencephalitis



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### ARTICLE INFO

#### Article history:

Received 22 September 2012

Received in revised form 31 March 2013

Accepted 7 April 2013

#### Keywords:

Canine distemper virus

Central nervous system

Inflammation

Matrix metalloproteinases

Zymography

### ABSTRACT

Distemper leukoencephalitis is a disease caused by the canine distemper virus (CDV) infection. It is a demyelinating disease affecting mainly the white matter of the cerebellum and areas adjacent to the fourth ventricle; the enzymes of the matrix metalloproteinases (MMPs) group, especially MMP-2 and MMP-9 have a key role in the myelin basic protein fragmentation and in demyelination, as well as in leukocyte traffic into the nervous milieu. To evaluate the involvement of MMPs during subacute distemper leukoencephalitis, we measured the levels of MMP-2 and MMP-9 by zymography in the cerebrospinal fluid (CSF) and in the cerebellum of 14 dogs naturally infected with CDV and 10 uninfected dogs. The infected dogs presented high levels of pro-MMP-2 in the CSF and elevated levels of pro-MMP-2 and pro-MMP-9 in the cerebellar tissue. Active MMP-2 was detected in the CSF of some infected dogs. As active MMP-2 and MMP-9 are required for cellular migration across the blood–brain barrier and any interference between MMPs and their inhibitors may result in an amplification of demyelination, this study gives additional support to the involvement of MMPs during subacute distemper leukoencephalitis and suggests that MMP-2 and MMP-9 may take part in the brain inflammatory changes of this disease.

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## 1. Introduction

Canine distemper is a viral, highly contagious and severe canine disease. The etiological agent belongs to the

*Morbillivirus* genus, *Paramyxoviridae* family. The canine distemper virus (CDV) is a simple strand RNA microorganism with negative polarity (Beineke et al., 2009). The virus initially infects the lymphoid tissue and reaches the central nervous system (CNS) by means of meningeal macrophages and infected lymphocytes present in blood or in cerebrospinal fluid (CSF), through the ependyma-choroid plexus route (Greene and Appel, 2006; Beineke et al., 2009).

Due to the diversity of clinical manifestations, the brain lesions of dogs with distemper leukoencephalitis are classified as acute, subacute and chronic, depending on the

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amount of viral particles, the extension of the myelin loss and the composition and severity of the inflammatory infiltrate (Alldinger et al., 1993; Wünschmann et al., 1999; Silva et al., 2009). Nevertheless, Beineke et al. (2009) reported that lesions with different ages can be seen in the brain of the same dog.

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes involved in remodeling extracellular matrix and cell-matrix interactions. An imbalance in MMPs and their inhibitors (TIMPs) has been suggested as a cause of lesion initiation and progression during canine distemper virus infection (Miao et al., 2003; Gröters et al., 2005). Green et al. (2011), working with human CSF, demonstrated that the expression of MMPs/TIMPs in the CSF during CNS infections is determined by the etiologic agent, and that MMP-2 and MMP-9 are especially involved in inflammatory cell migration into the neuropil due to their ability to digest type-IV collagen, which is the major component of the basal membrane that involves the capillaries (Rosemberg, 2002). Further, MMPs and TIMPs, together with other classical biomarkers, have been used to differentiate Alzheimer's disease from vascular dementia (Bjerke et al., 2011), but biomarkers do not usually have relevance in clinical animal diagnosis and prognosis. Therefore, with this study, we aimed to detect MMP-2 and MMP-9 in the CSF and in cerebellar tissue by gelatin-zymography, as well as to identify these MMPs and inflammatory cells in the cerebellum using immunohistochemistry.

## 2. Materials and methods

### 2.1. Animals and experimental design

A total of 14 dogs that died from canine distemper diagnosis were included as infected dogs. Ten dogs that died with a negative diagnosis for canine distemper and without any brain alterations were included in the uninfected control group. The animals were provided from the Veterinary Teaching Hospital of UNESP – São Paulo State University and from the Zoonosis Control Center, both in the municipality of Araçatuba, São Paulo State, Brazil. Samples of CSF and the whole brain were collected. The brain was sagittally sliced after removal; one hemisphere was fixed in 10% neutral buffered formalin and the other hemisphere was frozen at  $-80^{\circ}\text{C}$ .

### 2.2. Canine distemper confirmative diagnosis

Confirmative diagnosis was achieved by direct immunofluorescence test as per Silva et al. (2004), with a monoclonal anti-CDV antibody (VMRD, 210-06-CDV) (Fig. 1A), and by RT-PCR with specific primers (sense: 5'-GCTCTGGGTTGCATGAGTT-3'; antisense: 5'-GCTGTTTACCCATCTGTTG-3'; GenBank accession number AF378705) (Fig. 1B). The staging of the CDV infection as subacute (demyelination, astrogliosis and mild-to-moderate inflammatory infiltrate) was achieved by analyzing Hematoxylin and Eosin (HE)-stained tissues by light microscopy (Fig. 1C and D), according to the

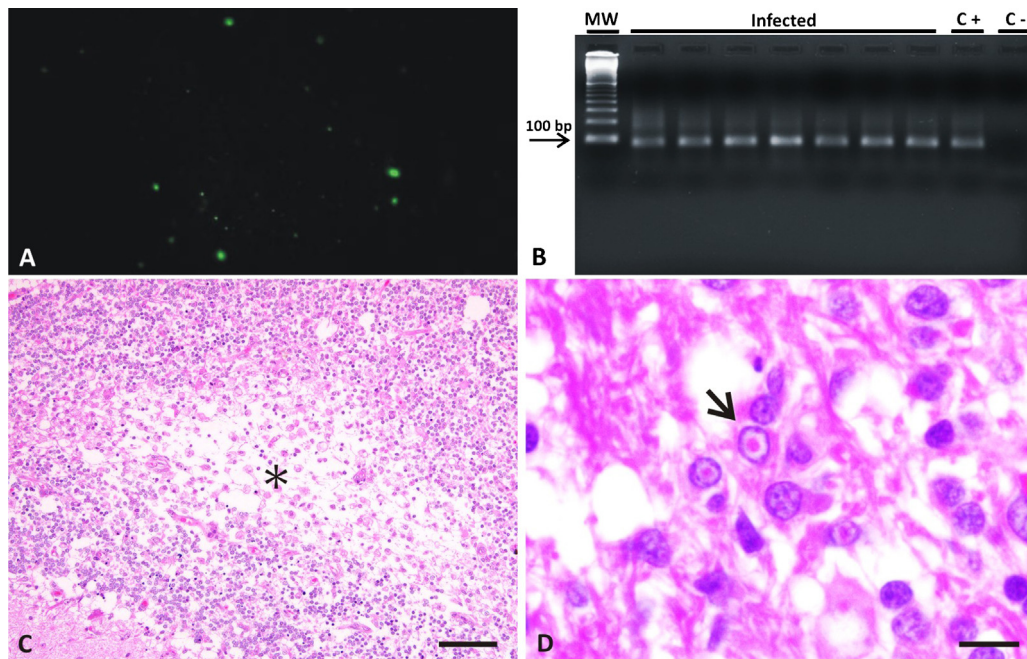
parameters described by Beineke et al. (2009) and Silva et al. (2009).

### 2.3. Gelatin zymography

One of the hemispheres of each brain was frozen at  $-80^{\circ}\text{C}$  immediately after removal. Fragments from the cerebellum and cerebellar peduncles were macerated at  $4^{\circ}\text{C}$  in the extraction buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, 5 mM  $\text{CaCl}_2$ , 0.05% (w/v) Brij-35, plus a protease inhibitor cocktail (Complete, EDTA-free 11 873 580 001, Roche). Samples were then centrifuged at  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ , and the supernatants were collected. For CSF zymography, there was no pre-treatment of the samples, but centrifugation was performed. The total protein content was measured using the bicinchoninic acid (BCA) method (23225, Pierce Biotechnology). The gelatinolytic activity of these samples was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gelatin zymography, according to the method previously described by Machado et al. (2010) and by Marangoni et al. (2011). MMP identity and normalization between gels was achieved with a sample of canine mammary adenocarcinoma previously standardized with human recombinant MMP-2 (PF037, Calbiochem) and MMP-9 (PF038, Calbiochem). The gels were digitalized and the integrated density of the bands, expressed as arbitrary units, was calculated using the open-access software ImageJ 1.46r (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij>).

### 2.4. Immunohistochemistry (IHC)

For IHC, after dewaxing, endogenous peroxidase activity was blocked by incubating sections in 2% (v/v) hydrogen peroxide 30 vol. diluted in 50% (v/v) methanol for 30 min. Pre-treatments for antigen retrieval were done according to the primary antibody specification (Table 1). Non-specific binding was blocked with 3% (w/v) non-fat dry milk in PBS (phosphate-buffered saline) pH 7.2 for 30 min. Sections were incubated with the primary antibodies (Table 1) for 18–22 h at  $4^{\circ}\text{C}$  in a humidified chamber. Slides were washed in PBS, incubated with a biotinylated secondary antibody and with streptavidin-HRP complex (LSAB+ Kit, Dako, K0690) according to the manufacturer's instructions. The reaction was developed with 3,3'-diaminobenzidine (Dako, K3468). The slides were then counterstained with Harris's hematoxylin, dehydrated, cleared, and mounted with cover-slips. Negative control sections were performed by replacing the primary antibody with PBS. Lymph node tissue was used as a positive control for inflammatory cells. Tissue samples were examined by light microscopy and the positive-stained area was measured by computerized image-analysis software (Image-Pro Plus 6.1, Media Cybernetics) as per Melo and Machado (2011). The intensity of the positive staining was evaluated in the cerebellum and cerebellar peduncles, in a total area of  $271,518.12 \mu\text{m}^2$ . The results are expressed as the percentage of the tissue's positive-stained area. All analyses were done "blind", without knowledge of the experimental groups.



**Fig. 1.** Diagnosis of canine distemper virus (CDV) infection. (A) Positive direct immunofluorescence test on acetone-fixed cerebellum smear, evidenced by the fluorescent green spots. FITC. (B) Representative RT-PCR. MW: molecular weight of 100 base pairs (bp). Infected: dogs showing a positive 83 bp band. C+: positive control. C-: negative control. (C) Subacute lesion (\*) in the cerebellum, evidenced by vacuolization of the white matter and presence of macrophages with foamy cytoplasm (*Gitter* cells). HE, bar = 100 µm. (D) Eosinophilic intranuclear inclusion body (Lentz body; arrow) in the cerebellum. HE, bar = 15 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

### 2.5. Statistical analysis

The differences between groups were determined by the Mann–Whitney test or by the Wilcoxon signed-rank test, depending on the analyzed data. A value of  $P < 0.05$  was considered statistically significant. Data are expressed as the median (interquartile range). All statistical analyses were performed using Prism 5 software (GraphPad).

### 2.6. Ethical issue

This study was approved by the Institutional Ethics and Animal Welfare Committee (CEEA – Comissão de

Ética e Experimentação Animal, UNESP, process number 2008/005733).

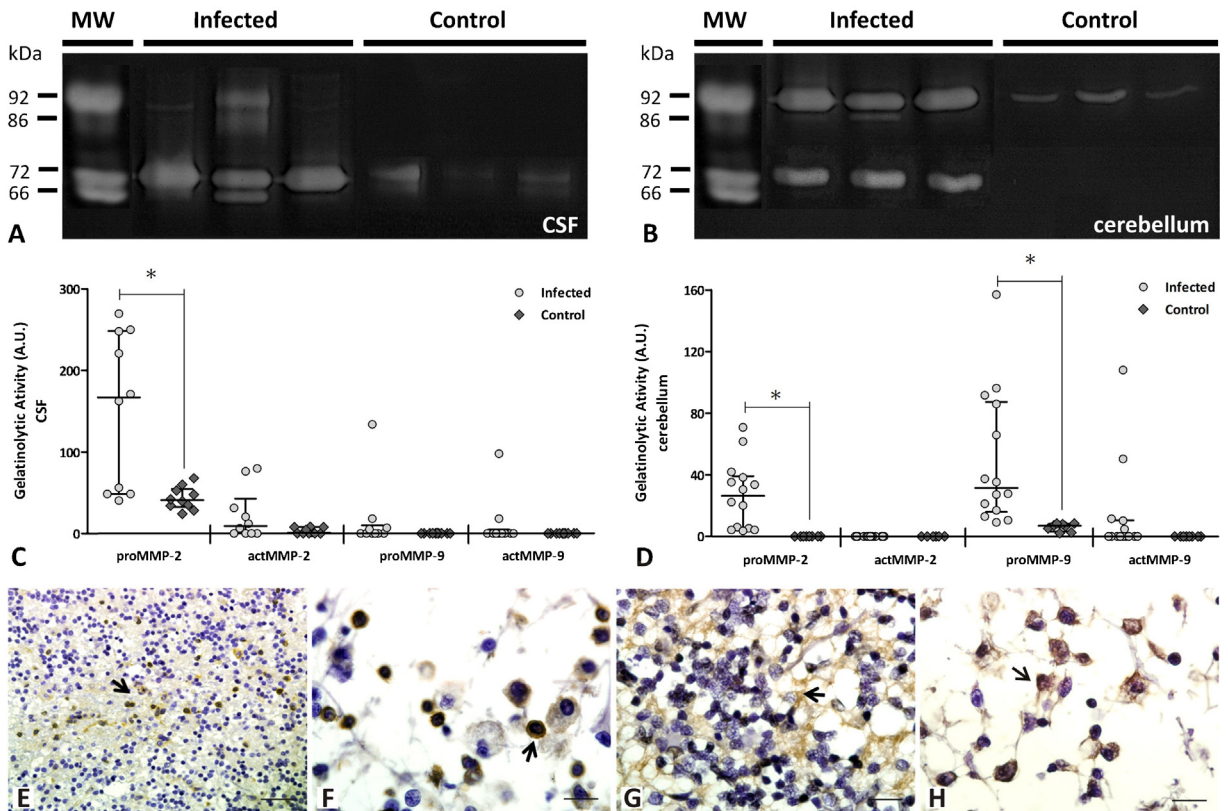
## 3. Results and discussion

Representative zymograms from the CSF and from the cerebellum/cerebellar peduncles of dogs are shown in Fig. 2A and B. Zymographic assays revealed gelatinolytic activity related to enzymes with molecular weights of 92 kDa (pro-MMP-9), 86 kDa (active MMP-9), 72 kDa (pro-MMP-2), and 66 kDa (active MMP-2). In the CSF, the levels of pro-MMP-2 in the infected dogs were significantly higher than in the control dogs ( $P = 0.0039$ ). For active MMP-2, even though there was no difference between

**Table 1**

Panel of antibodies used in this study to characterize astrocytes, T and B lymphocytes, macrophages, and matrix metalloproteinases 2 and 9 in the cerebellum of dogs with subacute distemper leukoencephalitis.

Antibody	Specificity	Dilution	Pre-treatment	Source
Polyclonal rabbit anti-human GFAP (glial fibrillary acidic protein)	Astrocytes	1:500	Trypsin 37 °C for 30 min	Sigma–Aldrich, G9269
Polyclonal rabbit anti-human CD3	T lymphocytes	1:200	Citrate pH 6.0 in steamer for 30 min	Dako, A0452
Monoclonal mouse anti-human CD79α, clone HM57	B lymphocytes	1:50	Tris–EDTA–Tween pH 9.0 in steamer for 30 min	Dako, M7051
Monoclonal mouse anti-human Myeloid/Histiocyte antigen, clone MAC387	Macrophages/microglial cells	1:100	Citrate pH 6.0 in steamer for 30 min	Dako, M0747
Monoclonal mouse anti-MMP-2	MMP-2 (active and pro-forms)	1:100	Citrate pH 6.0 in steamer for 30 min	Calbiochem, IM51
Polyclonal rabbit anti-human MMP-9	MMP-9 (active and pro-forms)	Ready-to-use	Tris–EDTA–Tween pH 9.0 in steamer for 30 min	NeoMarkers, RB-9234-R7



**Fig. 2.** Matrix metalloproteinases in the brain of dogs with subacute distemper leukoencephalitis. (A and B) Representative zymograms from the cerebrospinal fluid (CSF) (A) and cerebellum/cerebellar peduncles (B). Note the presence of gelatinolytic bands corresponding to pro-MMP-9 (92 kDa), active MMP-9 (86 kDa), pro-MMP-2 (72 kDa), and active MMP-2 (66 kDa). MW: Molecular weight (canine mammary adenocarcinoma). (C and D) Quantification of active and pro-forms of MMP-2 and MMP-9 in the CSF (C) and cerebellum/cerebellar peduncles (D). Horizontal lines represent the median and the interquartile range, regarding the intensity of the inverse optical density units (arbitrary units – A.U.) from the gelatinolytic bands. \* indicates  $P$  values < 0.05. (E) MMP-2 positive cells in the cerebellar white matter with microglial morphology (arrow). (F) Mononuclear cells presenting intense cytoplasmic staining for MMP-2 (arrow) in a focus of malacia. (G) Diffuse MMP-9 staining (arrow) in a vacuolated area. (H) Mononuclear cells with foamy macrophage morphology presenting positive cytoplasmic staining for MMP-9 (arrow) in an area of malacia. LSAB+ method, bars = 100  $\mu$ m (E), 50  $\mu$ m (G), 20  $\mu$ m (F, H).

groups ( $P=0.1910$ ), the levels of this enzyme were elevated in some infected dogs. Regarding pro- and active MMP-9, these enzymes were not detected or detected in an inconsistent way in a few infected samples. Pro-MMP-9 presented no difference between infected and control dogs ( $P=0.2838$ ), and active MMP-9, although not present in the control group, showed no difference between groups ( $P=0.2642$ ) (Fig. 2C).

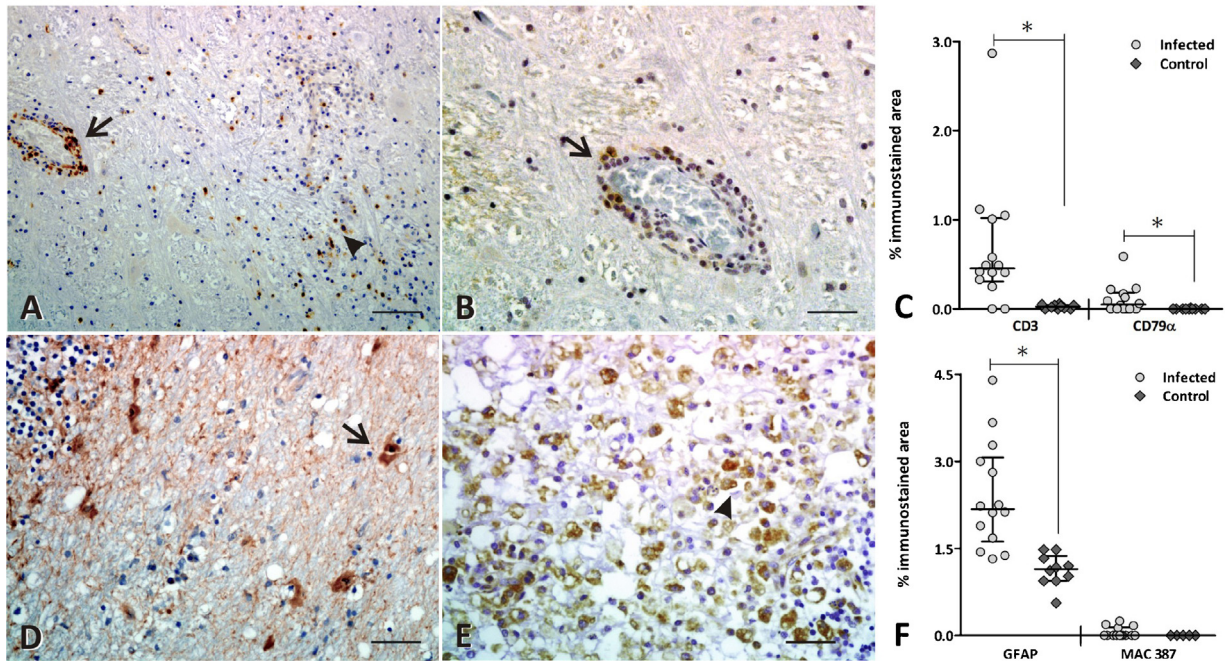
In the cerebellum/cerebellar peduncles, the control group presented only faint 92 kDa bands corresponding to pro-MMP-9. The infected group showed gelatinolytic bands with molecular weights consistent with pro-MMP-9, active MMP-9, and pro-MMP-2. Comparing the groups, the infected dogs presented higher levels of pro-MMP-9 ( $P=0.0002$ ) and pro-MMP-2 ( $P=0.0005$ ) than the control dogs. For active MMP-9, even if there was detection in infected animals, its levels were statistically similar to those in the control animals ( $P=0.1282$ ; Fig. 2D).

This study, according to the authors' knowledge, is the first to use zymography to evaluate MMP-2 and MMP-9 during subacute distemper leukoencephalitis. Zymographic analysis of MMP-2 and MMP-9 allowed the identification of these enzymes according to their molecular

weights. In fact, this method was sensitive enough to allow the distinction between the active and the pro-forms of MMP-2 and MMP-9 (Kleiner and Stetler-Stevenson, 1994).

In agreement with Bergman et al. (2002), only the pro-form of MMP-2 was detected in the CSF of the clinically healthy dogs, whereas MMP-9 was not detectable in any form. During distemper leukoencephalitis, in the CSF, increased activity of pro-MMP-2 was predominantly noticed; on the other hand, pro-MMP-2 and pro-MMP-9 were highly detected in the cerebellum/cerebellar peduncles, although active MMP-9 was also detected in some samples. MMPs are involved in the pathogenesis of several viral infections in the CNS and the imbalance between MMPs and TIMPs has been reported not only in humans with immunodeficiency virus (HIV) infections (Conant et al., 1999), but also in experimental murine infection with Japanese encephalitis virus (Shukla et al., 2012), coronavirus (Marten and Zhou, 2005) and West Nile virus (Wang et al., 2008).

The detection of pro-MMP-2 and pro-MMP-9 in the cerebellum/cerebellar peduncles in this study is in agreement with Miao et al. (2003), that, by immunohistochemistry, detected an up-regulated expression of



**Fig. 3.** Inflammatory changes in the cerebellum/cerebellar peduncles of dogs with subacute distemper leukoencephalitis. (A) CD3<sup>+</sup> T lymphocytes constituting a perivascular cuff (arrow) and diffusely distributed within an area of gliosis (arrowhead), bar = 100  $\mu$ m. (B) Few CD79 $\alpha$ <sup>+</sup> B lymphocytes within a perivascular cuff, around the same blood vessel as in A. Note also that the perivascular cuff is three cell layers thick, bar = 50  $\mu$ m. (C) Quantification of CD3 and CD79 $\alpha$  staining in the cerebellum/cerebellar peduncles of dogs. Horizontal lines indicate the median and the interquartile range. \* indicates *P* values <0.05. (D) Fibrous astrocytes (arrow) presenting very intense staining for GFAP, bar = 50  $\mu$ m. (E) Gitter cells presenting cytoplasmic staining for the MAC 387 antibody (arrowhead), with different intensities, in a focus of malacia, bar = 50  $\mu$ m. (F) Quantification of GFAP and MAC387 staining in the cerebellum/cerebellar peduncles of dogs. Horizontal lines indicate the median and the interquartile range. \* indicates *P* values <0.05.

MMPs-1, -2, -3, -7, -9, -12, -13 and -14 in the brain of dogs with distemper, in a phase-dependent manner. However, IHC detects both active and pro-forms of MMPs together, whereas zymography is clearly able to differentially detect them.

By means of IHC, it was possible to detect in which cell population the MMPs were present. Faint cytoplasmic MMP-2-staining was noticed in mononuclear cells, ependymal cells and in rod cells with microglial morphology (Fig. 2E). However, the main MMP-2-positive cell population was composed of mononuclear cells in the white and gray matter and in the leptomeninges, with more intense staining in areas of malacia and demyelination (Fig. 2F). MMP-9 presented diffuse staining in areas of malacia (Fig. 2G) and cytoplasmic staining in inflammatory cells inside blood vessels, in Gitter cells within the demyelination plaques, and in some neurons, ependymal cells and perivascular cells (Fig. 2H).

IHC also facilitated the identification and quantification of inflammatory cells trafficking through the cerebellum. An important component of the inflammatory infiltrate at the injured tissue was the CD3<sup>+</sup> T lymphocytes, which were detected mainly in the perivascular cuffs and diffusely distributed along the demyelination plaques. The perivascular infiltration was two to three cell layers thick (Fig. 3A). However, in some dogs, with the progression of demyelination, the vacuoles seemed to push the cells around them, and T lymphocytes were distributed in

clusters in these cases. These cells comprised 0.46% of the immunostained area in the infected group, while the control group presented only 0.03%, representing a significant difference (*P* = 0.0020; Fig. 3C). B lymphocytes were mainly observed in the leptomeninges and within the perivascular cuffs, also two to three cell layers thick (Fig. 3B), and even if in smaller number than T cells, CD79 $\alpha$ <sup>+</sup> B lymphocytes were detected at higher levels in the infected group than the control group (*P* = 0.0140; Fig. 3C).

With regards to astrocytes, the main cerebellar alteration was astrogliosis. The presence of intranuclear inclusions (Fig. 1D) and gemistocytic cells was also a frequent finding. The astrocytes in the infected dogs presented an increase in the cytoplasm volume and in the length of the cytoplasmic processes, and also a more intense cytoplasmic staining (Fig. 3D). The GFAP immunoreactivity in the astrocytes of the infected dogs comprised an area of 2.18%, while the area of the control dogs consisted of 1.15%, characterizing a significant difference (*P* = 0.0003; Fig. 3F). Regarding macrophages/microglial cells, there was positive detection in only 4 out of 14 infected dogs, within and around the demyelination plaques (as Gitter cells) (Fig. 3E). No positive staining was noticed in the control group.

Since the pathogenesis of distemper leukoencephalitis is phase-dependent, the source of MMPs within the CNS may vary according to the time of infection. MMP-9, MMP-14 and TIMP-1 are expressed mostly in astrocytes and in macrophages/microglia in initial lesions, while infiltrating

lymphocytes as well as activated macrophages/microglia are the main source of these components in advanced distemper leukoencephalitis (Gröters et al., 2005). The production of active MMP-2 and MMP-9 by T cells, monocytes and dendritic cells is required for their migration across the blood–brain barrier (BBB) and their subsequent invasion of the CNS compartment (Rosemberg, 2002; Stamatovic et al., 2008). By immunohistochemistry, we detected MMP-2 and MMP-9 preferentially in mononuclear cells. Nevertheless, the role of MMPs within the CNS may start prior to leukocyte infiltration. In canine inflammatory brain lesions, microglial cells expressed a variety of MMPs and TIMPs mRNAs, including MMP-2 and MMP-9; and some dogs also presented MMP-2 activity in brain-zymography (Stein et al., 2011). Further, in Theiler's murine encephalomyelitis (TME), a virus-induced model of multiple sclerosis, after stereotaxic injection of activated MMP-3, -9, and -12 into the caudal cerebellar peduncle of adult mice, the authors noticed a focally extensive primary demyelination, before the infiltration of inflammatory cells, as well as a reduction in the number of oligodendrocytes (Hansmann et al., 2012). In demyelination, MMPs also act in myelin basic protein fragmentation (Shiryayev et al., 2009).

Due to the morphological similarities between canine distemper demyelinating leukoencephalitis and human demyelinating diseases, such as multiple sclerosis, canine distemper represents one of the few animal models for spontaneous demyelinating diseases to study the pathogenesis of myelin loss associated with immunomediated mechanisms (Baumgärtner and Alldinger, 2005). In multiple sclerosis, MMP-9 seems to play a critical role in early lesions (Cossins et al., 1997), whereas MMP-2 seems to have an ambiguous role in initial and advanced lesions (Lindberg et al., 2001). Further, blood monocytes from patients with multiple sclerosis showed an elevated level of several MMPs, including MMP-1, -3, -7 and -9 (Kouwenhoven et al., 2001).

The characterization of separate roles for individual MMPs is complicated by the wide variation of expression patterns, kinetics, substrate, and cell specificity in different inflammatory settings (Savarin et al., 2011). By zymography, there was no way of detecting a constant profile of MMPs in the CSF and the cerebellar tissue of the dogs and establishing a secure biomarker for the subacute inflammatory phase of the CDV infection; however, pro-MMP-2 was present in most of the CSF samples and pro-MMP-2 and pro-MMP-9 were increased in the cerebellum. The MMPs/TIMPs imbalance may contribute to the complex mechanisms enrolled in distemper leukoencephalitis and therapies based on metalloproteinase inhibitors for dogs may be an additional aid to help in the recovery or stop the progression of the lesions in animals in the acute phase of disease.

#### Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

#### Acknowledgments

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). M.S. Souza was supported by a FAPESP scholarship (grant number 2008/57637-2), as well as A.A. Machado (grant number 2009/51383-1) and D.S. Migliolo (grant number 2009/51382-5).

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