ALEXANDRE MENDES AMUDE

AVALIAÇÃO NEUROLÓGICA E LABORATORIAL DE CASOS DE ENCEFALOMIELITE PELO VÍRUS DA CINOMOSE CANINA NA AUSÊNCIA DE SINAIS SISTÊMICOS E MIOCLONIA

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal (área de concentração: Sanidade Animal) da Universidade Estadual de Londrina, como requisito parcial para a obtenção do título de Mestre em Ciência Animal.

Orientação: Profa. Dra. Alice F. Alfieri
Co-Orientação: Prof. Dr. Amauri A. Alfieri

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Londrina, 29 de julho de 2005.

O presente trabalho foi realizado no Laboratório de Virologia Animal, Departamento de Medicina Veterinária Preventiva, Centro de Ciências Agrárias, Universidade Estadual de Londrina, em parceria com o Hospital Veterinário.

Os recursos financeiros para o desenvolvimento do projeto foram obtidos junto às agências e órgãos de fomento à pesquisa abaixo relacionados:

1. **CAPES: Conselho de Aperfeiçoamento de Pessoal de Ensino Superior.**

2. **CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico.**

3. **Fundação Araucária, (Fundação de Amparo a Pesquisa do Estado do Paraná).**

AGRADECIMENTOS

Agradeço em primeiro lugar a Deus por ter me dado o ***Dom da vida***, por ter inundado minha mente com idéias brilhantes, por ter me dado inteligência, raciocínio, sabedoria, discernimento, humildade, me capacitado para as lutas e me fortalecido nas dificuldades. Ao Espírito Santo por ter frutificado em minha vida com amor, alegria, paz, generosidade, beniginidade, bondade, fidelidade, mansidão e domínio próprio.

Agradeço aos meus pais, Armando Charifi Amude e Maida Maria de Oliveira Mendes Amude, pelo amor, dedicação, educação dada, princípios passados, incentivos, força, encorajamento e investimento. A minha mãe pelo empenho, esforço, por nunca ter me deixado desistir de acreditar que os últimos seriam os primeiros, pelas noites de sono "perdidas", pelas varadas e puxões de orelha. Ao meu pai pela força, por acreditar em mim, pelo que ele foi, é e sempre será em minha vida. Homem esforçado, batalhador, de caráter, íntegro, de reputação, exemplo de pai, de profissional, de marido, de vencedor. Meu pai, meu Herói!

Aos meus irmãos Fábio e Amanda pelo amor, companhia, amizade e bons e inesquecíveis momentos que vivemos e sei que iremos viver juntos ainda. Fábio, talvez você não saiba, mas a sua presença foi e é de fundamental importância na minha vida. A Amanda pelo apoio, incentivo, atenção, preocupação e dedicação.

A minha vó e madrinha Amélia pelo carinho, amor, zelo, atenção, dedicação e pelas madrugadas acordadas ao meu lado para esperar o garninzé cantar. Aos meus avós Armando Amude (*in memoriam*), Maria Eugênia Amude (*in memoriam*) e Delcides Borges Mendes (*in memoriam*).

A minha noiva Andréa pelo amor, companheirismo, apoio, atenção, preocupação, incentivos, e pelos momentos felizes e inesquecíveis que já vivemos e que hoje tenho convicção

que ainda iremos viver juntos. Pelos planos e sonhos já concretizados e aos ideais e objetivos que com a graça de Deus ainda iremos alcançar.

Aos meus tios Gilberto e Amélia, Roberto e Stela, Antônio e Adegmar, José Antônio e Leoni (tios e padrinhos), Rosângela, Mauro e Zaine, Mohamed e Tereza (*in meoriam*), e Stephânia, pelo amor, presença, exemplos, incentivos, atenção, carinho e dedicação.

Aos meus primos Regina, Mirian, Mauro Sérgio, Samara, Luciano, Fabiano, Gustavo, Thiago, Ana Carolina, Gabriel, Mariam, Caio Victor e Daniel, pela amizade, companhia e presença. Ao Gustavo, Thiago, Ana Carolina e Gabriel pelos nossos preciosos e inesquecíveis momentos vividos juntos na infância. Ao Caio Victor pela atenção e notória admiração e interesse pelo que eu faço. Caio fico feliz de saber que embora poucos os momentos que passamos juntos, eles foram preciosos, importantes e simbolizam tanto em sua vida. Ao Danielzinho pelas molecagens, brincadeiras e traquinagens que me alegram e me distraem nos finais de semana. A minha afilhada Maria Luiza, por ter entrado na minha vida.

A minha cachorra Brisa pelo "amor" incondicional, fidelidade, lealdade, e obediência.

A Kelly, Paloma e Élida, pela companhia, carinho, caminhada, preocupação, força, brincadeiras, ajuda e incentivo durante a graduação.

Aos professores Milton Yamamura e Nilva Mascarenhas, pela amizade, incentivo e apoio. Por terem sido exemplo, por terem me ajudado na formação profissional, intelectual, pessoal e científica.

Aos professores Alice e Amauri Alfieri por terem me aceitado, mesmo caindo de pára-quedas no laboratório de Virologia Animal. Por terem acreditado em mim e dado um voto de confiança. Pelo exemplo de vida, de profissionais, de cientistas, de pais e professores. Pela orientação, amizade, preocupação e apoio.

A professora Mônica Vicky Bahr Arias pelo incentivo e apoio, por ter me ajudado a desmistificar os ‘‘enigmas’’ da neurologia.

A professora Luciane Biazzono Nabut, que tive o prazer de conhecer no último ano de graduação e que também me ajudou bastante na neurologia veterinária.

A professora Janis Regian Messias Gonzales pelos conselhos dados em 2003, por me ajudar a ver que o “não” de hoje pode ser o melhor para o amanhã.

Aos professores Antônio Carlos Faria dos Reis e Mara Balarin pela colaboração na execução desse trabalho.

A Glei pela amizade, atenção, companhia, preocupação, apoio, incentivo, conselhos, almoços, jantas, bolos e arranca rabos.

Aos meus colegas e amigos de pós-graduação, Vanessa (Japa), Bruna, Vanessa Blaschi, Eleine, Letícia, Juliana Galhardo, Lú Takemura, Frango, Fabiana, Jú Dias, Fábio Galli, Wanderlei, Dengoso, Salmazo, Dunga, Andréia, Rosa, Graziela, Mara, Fábio Negrão, Sheila, Fran, Michele Igarashi, Paula Kawasaki e Kátia Tamekuni. A Vanessa (Japa) pelo apoio, confiança, atenção, amizade e preocupação. A Eleine pela amizade, confiança e pelos “papos cabeças” e “viagem” na psicologia na busca do auto-entendimento. A Fabiana e a Jú Dias pela companhia, amizade, atenção, “fofocas”, e pelos momentos de descontração. A Fabiana, Flávia, Juliana e Vanessa Blaschi pela amizade e por ter cedido a casa para as nossas “festinhas gastronômicas”. A Michele, Paula e Kátia pela amizade, companhia, incentivo, brincadeiras, risadas e pela caminhada desde o tempo em que eu era da parasito (nossa faz tempo, hein!). Vocês não me esperaram, mas a vida nos colocou juntos novamente.

A Betinha, Flora e Marlise pela amizade, ensinamentos, companheirismo, incentivos, conselhos, sugestões, apoio, dedicação, paciência e amor. Por terem aberto a porta de suas casas

para me acolher nos momentos mais difíceis e de extrema confusão. Pelo exemplo de profissionais e de pessoas que são.

Aos pastores João Victor e José Antônio pelo sustento e apoio espiritual. Ao pastor José Antônio pela amizade, preocupação, conselhos, atenção, orações, apoio e compreensão. Pelo exemplo de marido amoroso, paciente e tolerante, homem batalhador, de oração e fiel. Ao pastor Dauro e Henrique pelas orações e conselhos.

Aos residentes do HV (muitos já ausentes): Mônica, Alessandra, Bruna, Danilo (Puff), Kelly, Shigueru, Carlos Alexandre, Carol, Adalton, Maria Carolina, Maíra, Viviane, Daniela, Bernardo e Lígia pela amizade, envio de material e notificação dos casos. Aos enfermeiros do HV: Nélio, Rose, Vilma, Juju, Luiz, Cida, Lourdes, Osvaldir e Nelza pela amizade e colaboração.

A Kerlei, Dalíria, e Maria pela amizade, ensinamentos, paciência e companhia. A Dalíria pela atenção, preocupação e zelo.

Aos professores Italmar, Janis e Mônica pela presença e sugestões no exame de qualificação.

Aos estagiários do Laboratório de Virologia Animal Michele, Gustavo, Taine, Rebeca, e Guilherme pela amizade e momentos de descontração.

Ao Zé, Joãozinho, Inês e Claudia pela amizade e colaboração; a Maria José e Neuza pela amizade, pelos cafezinhos e doces de leite; ao Nelson Alexandre e aos funcionáros do DMVP; e a CAPES pela bolsa de estudo.

“...corramos com perseverança a carreira que nos está proposta, olhando firmemente para o Autor e Consumador da fé, Jesus, o qual em troca da alegria que lhe estava proposta, suportou a cruz, não fazendo caso da ignomínia, e está assentado á destra do trono de Deus.”

(Hebreus 12: 1-2)

“Bem-aventurados os humildes de espírito, porque deles é o reino dos céus.
Bem-aventurados os que choram, porque serão consolados.
Bem-aventurados os mansos, porque herdarão a terra.
Bem-aventurados os que tem sede e fome de justiça, porque serão fartos.
Bem-aventurados os misericordiosos porque receberão misericórdia.
Bem-aventurado os limpos de coração porque verão a Deus.
Bem-aventurado os pacificadores, porque serão chamados filhos de Deus.
Bem-aventurados os perseguidos por causa da justiça, porque deles é o reino dos céus.”

(Mateus 05)

The nervous system is almost entirely inaccessible to direct observation. As a rule, the state of the nervous system can be ascertained only by the manner in which its work is done, and morbid states reveal their presence by the derangement of funtion which they cause.

Sir William Gomers **(1845 – 1915)**

Modern radiographic imagins techniques and eletrodiagnostic methods are changing the scope of neurological diagnosis, but the fundamental wisdom of Gower' words is not diminished. The signs of disease remain the most meaningful evidence of lesions of the nervous system.

Sheldon A. Steinberg, **VMD**

The neurological examination is the basic and the most important tool of clinical neurology.

Kyle G. Braund
BVSc, MVSc, PHD, FRCVS, Dip. ACVIM (Neurology)

AMUDE, A. M. **Avaliação neurológica e laboratorial de casos de encefalomielite pelo vírus da cinomose canina na ausência de sinais sistêmicos e mioclonia.** 2005. 111f. Dissertação (Mestrado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2005.

RESUMO

O diagnóstico clínico da encefalomielite pela cinomose em cães é difícil nos casos em que os sinais sistêmicos e a mioclonia estão ausentes. Nessa situação, o apoio laboratorial é necessário para confirmar a doença, no entanto os métodos disponíveis para o diagnóstico *ante mortem* da cinomose têm valor limitado. O conhecimento sobre as possíveis apresentações clinico-patológicas, assim como a evolução das síndromes clínicas causadas pelo vírus da cinomose canina (CDV), é importante para o reconhecimento clínico e diagnóstico etiológico específico *ante* e *post mortem*. O objetivo desse estudo foi reconhecer e apresentar as síndromes clínicas da cinomose canina, avaliando os exames hematológicos e liquóricos, as secções histopatológicas, e o resultado do exame neurológico de cães com encefalomielite pelo CDV apresentados exclusivamente com doença neurológica sem mioclonia, e verificar se esses dados laboratoriais e clínicos contribuem ou não para um diagnóstico etiológico. Foram prospectivamente investigados 20 cães apresentados com déficits neurológicos sem os sinais comuns da cinomose (sinais sistêmicos e mioclonia) no momento da admissão hospitalar, nos quais o hemograma e a avaliação do líquor foram realizados *ante mortem*, e no *post mortem* amostras de SNC foram obtidas na necropsia. Oito dos 20 cães foram diagnosticados, por RT-PCR e histopatolologia, com encefalomielite pelo CDV. De acordo com os sinais neurológicos e curso clinico, esses 8 cães com encefalomielite pela cinomose foram agrupados em 3 síndromes clínicas: Encefalite do cão velho (ODE) (1), encefalomielite do cão jovem (CDEID) (1), e encefalomielite multifocal do cão adulto (MDEMD) (6). Alterações no exame hematológico, quando verificadas, foram consideradas não-específicas, no entanto a avaliação do líquor sugeriu a infecção viral devido a uma pleocitose linfocítica. No *post mortem*, lesões desmielinizantes foram uma constante no SNC e a encefalomielite crônica foi predominante nos casos de encefalomielite pelo CDV.

Palavras chaves*:* Cães. Vírus da cinomose canina. Encefalomielitie.

AMUDE, A.M. **Neurological and laboratorial evaluation of canine distemper virus encephalomyelitis cases in the absence of systemic signs and myoclonus.** 2005. 111f. Dissertação (Mestrado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2005.

ABSTRACT

The clinical diagnosis of distemper encephalomyelitis in dogs is difficult in cases which systemic signs and/or myoclonus are absent. In such cases a laboratory assay is required to confirm this disease, however the methods available for an *ante mortem* diagnosis of distemper to date are of limited value. The knowledge about canine distemper virus (CDV) encephalomyelitis possible clinicopathological features, as well the course of the CDV clinical syndromes are important for clinical recognizing and specific etiological diagnosis *ante* and *post mortem*. The aim of this study was to recognize and present the clinical syndromes of canine distemper, and to evaluate the hematological parameters, CSF evaluation, and CNS histopathological section as well the results of the neurological examination, from dogs with CDV encephalomyelitis presented exclusively with neurological disease without myoclonus, and verify if and how these parameters contribute to an etiologic diagnosis. Were prospectively investigated 20 dogs presented with neurological deficits without the common CDV signs (systemic signs and myoclonus) at the time of hospital admission, in which the hemogram and CSF evaluation were performed *ante mortem*, and at *post mortem* were obtained CNS samples at necropsy. Eight out of 20 dogs were diagnosed, by RT-PCR and histopathology, as suffering from CDV encephalomyelitis. According to the neurological signs the 8 dogs suffering from distemper encephalomyelitis were grouped in three clinical syndromes: Old dog encephalitis (ODE) (1), canine distemper encephalitis in immature dogs (CDEID) (1), and multifocal distemper encephalomyelitis in mature dogs (MDEMD) (6). Changes in hematological parameters, when observed, were considered non-specific, nevertheless the CSF evaluation could suggest CDV infection in dogs suffering from CDV encephalomyelitis, by a lymphocytic pleocytosis. At *post mortem*, demyelinating lesions was a constant finding in CNS sections and chronic CDV encephalomyelitis was predominant in the distemper dogs.

Keywords*:* Dogs. Canine distemper virus. Encephalomyelitis.

SUMÁRIO

LITERATURE REVIEW

"The Nervous Form of Distemper"

Abstract

Canine distemper is a systemic infection, frequently lethal in dogs. The canine distemper virus (CDV) causes a persistent infection of the central nervous system resulting in a progressive, multifocal demyelinating disease. In dogs, CDV infection can result in gastrointestinal and/or respiratory signs, frequently with central nervous system involvement. Myoclonus has been a common and characteristic sign observed in dogs with distemper encephalomyelitis. However, the nervous deficits may occur in the absence of myoclonus and systemic involvement. The clinical diagnose of distemper is often difficult when systemic signs preceding or accompanying the neurological disease is absent. This review will point the clinical course and the neurological signs of nervous distemper, as well the clinical syndromes of CDV infection, neuropathology of acute and chronic demyelination, and diagnostic aids of CDV encephalomyelitis.

Keywords: Nervous distemper. Canine distemper virus. Encephalomyelitis.

INTRODUCTION

Different causes such as degenerative, metabolic, autoimmune, nutritional, inflammatory, infectious, toxic and vascular may lead to a neurological disease. In dogs, among infectious causes, viral disease such as canine distemper can be involved on the pathogenesis of lesions in the central nervous system (CNS) (Braund, 2001).

Canine distemper is a systemic disease caused by canine distemper virus (CDV), frequently lethal in dogs, other non-domestic carnivores and some marine mammals (Summer and Appel, 1994). The CDV is a non-segmented, negative single-stranded RNA virus, closely related to measles virus and rinderpest virus, two other members of the genus *Morbillivirus* of the *Paramyxoviridae* family. The virion is relatively large (150-240 nm), surrounded by a lipoprotein envelope derived from virus glycoproteins incorporated into the cell membrane (Fauquet et al., 2004). The virus leads a multisystemic infection, which is often associated with viral spread to the CNS, resulting in a progressive, multifocal demyelinating disease (Muller et al., 1995; Meertens et al., 2003).

CDV is generally transmitted as an aerosol infection to the upper respiratory tract. The primary virus replication takes place in the lymphoid tissues. Infection of these tissues is associated with severe long lasting immunossupression. At about 10 days post infection (p.i.), CDV starts to spread from the sites of primary replication to various epithelial tissues and the CNS (Vandevelde and Zurbriggen, 1995). During viraemia CDV infects the brain in the majority, if not all cases, regardless of the clinical signs (Summer et al., 1995).

In dogs, CDV infection can result in subclinical infection, or clinical disease characterized by gastrointestinal and/or respiratory signs, known as epithelial or systemic signs of distemper, frequently with CNS involvement. The virulence of the virus strain, the age and the immunocompetence of the dog determine the course and outcome of the disease (Tipold et al.,

1992; Greene and Appel, 1998). In clinical studies epithelial signs of the disease such as vomiting, diarrhea, and respiratory signs are common clinical findings in dogs with CDV encephalomyelitis (Okita et al., 1997; Moritz et al., 2000; Koutinas et al., 2002; Moro et al., 2003; Gebara et al., 2004 a/b). When systemic signs are preceding or accompanying the neurological disease, distemper is often a probable suspected as diagnosis (Moritz et al., 2000). However, such classical presentation is not the rule. Nervous signs may also occur without any other systemic signs (Baumgärtner et al., 1989; Tipold, 1992, Tipold et al., 1996). Myoclonus, a high suggestive sign of nervous distemper, may be also absent (Tipold et al., 1992; Vandevelde and Cachin, 1993). According to Tipold et al. (1994) in patients with only neurological signs, the CDV infection is only one possible differential diagnosis. Nevertheless, in areas where CDV is endemic, this virus should be always considerate as an important differential diagnose in dogs with progressive and multfocal neurological disease, even when the typical CDV systemic signs (vomiting, diarrhea, and respiratory signs) and myoclonus are not present (Amude et al., 2005 c). When the neurological examination suggests a progressive and multifocal neurological disease the inflammatory and infectious causes should be always considerate.

It had been claimed that neurological signs of CDV might occur much later (months or years) after the systemic infection, as a “post infectious encephalitis”. This concept had been used to justify some situations where the clinical course was restricted to the neurological disease. In spite of this there is no experimental evidence at all to support this notion (Tipold et al, 1992; Vandevelde and Zurbriggen, 1995). We were able to detect the CDV in different biological samples (urine, whole blood, cerebrospinal fluid, CNS fragments) from distemper dogs with neurological presentation in which the typical systemic signs of the disease were absent during the clinical evolution. In most of these cases (4/5) the virus was detected in urine (Amude et al., 2005 a). This result in spontaneous cases of nervous distemper suggest that at the moment of the

clinical presentation, even in cases which there are only neurological signs, the animals are undergoing a systemic infection or the infection had just been a recent event, since the virus still was identified in the urine of the animal with this clinical presentation restricted to the nervous fase.

Despite the relative large literature about neuropathology, immunology and laboratorial findings on nervous distemper, there are only a few indepth studies on the clinical neurology of distemper involving the neurological signs (Tipold et al, 1992).

CLINICAL COURSE AND NEUROLOGICAL SIGNS

The neurological manifestation of distemper may occur simultaneously to the systemic signs or begin 1 to 3 weeks after recovery from systemic illness (Greene and Appel, 1998). The systemic signs include decreased appetite, fever, serous oculonasal discharge, coughing, dyspnea, vomiting and diarrhea. These signs can occur in various combinations. Nervous signs may also occur without any other signs. The clinical signs, course of the disease, and the type of nervous system involvement vary depending on virulence of the virus strain, environmental conditions, and the host age and immune status (Shell, 1990; Tipold et al., 1992; Greene and Appel, 1998). Neonatal pups and immunodeficient dogs are more prone to develop neuronal necrosis, while older or more immunocompetent dogs develop demyelination (Shell, 1990).

On clinical examination about 2/3 of the animals are presented with extraneural signs, including conjunctivitis and fever, respiratory signs, gastrointestinal involvement, tonsillitis, and cachexia. In a 1/3 of the dogs no extraneural signs are found on the physical examination (Tipold et al., 1992).

The type of the neurological signs depends on the CDV distribution in the CNS and localization of the lesions, nevertheless a clinicopathological correlation is often lacking

(Vandevelde and Cachin, 1993, Koutinas et al., 2002). CDV affects both white and gray matter into the CNS. Thus a variety of neurological signs may be observed, including behavioral changes, seizures, cerebellar (head and body ataxia, intentional tremor, hypermetria) and vestibular signs (head tilt, falling, circling, nystagmus), visual deficits, paresis, paralysis, limb weakness, and myoclonus (Tipold et al., 1992; Vandevelde and Cachin, 1993; Tipold et al., 1996). Although the clinical signs of seizures depend on which part of the cerebrum is affected, many seizures are described as "chewing-gum fits" (Shell, 1990). Signs of leptomeningitis, such as cervical rigidity and generalized hyperesthesia, may also occur (Greene and Appel, 1998; Koutinas et al., 2002). Neurological signs may be acute or chronic, however are typically progressive (Greene and Appel, 1998; Vandevelde and Cachin, 1993).

Generalized or localized myoclonus has been shown a common and characteristic sign observed in dogs with distemper encephalitis (Moritz et al., 2000; Frisk et al., 1999; Braund, 2001; Koutinas et al, 2002; Moro et al, 2003). It is characterized as a rhythmic jerking of single muscles or muscle groups. Although myoclonus is highly suggestive of nervous distemper, it is absent in more than half of the cases (Tipold et al., 1992). Experimental studies have shown that focal spinal cord lesion may be responsible for this sign. It is speculated that the site of damage is the lower motor neurons of the spinal cord or the cranial nerve nuclei. It is also possible that a basal nuclei lesion may initiate myoclonus by establishing a "pacemarker" in the cord or brainstem (de Lahunta, 1983). The mechanism of myoclonus in distemper is not well understood. In a clinicopathologic study 5 out of 13 spontaneous distemper cases with myoclonus, no lesions were found in the neural gray column of the relevant spinal cord segments to account for the presence of myoclonus (Koutinas et al, 2002).

In several studies involving natural-occurring nervous distemper, systemic signs simultaneously to the neurological disease have been the most common clinical course, as well

seizures and myoclonus have been considered the most frequent neurological findings (Frisk et al., 1999; Moritz et al., 2000; Headley and Graça, 2000; Koutinas et al., 2002; Moro et al., 2003; Gebara et al., 2004 a/b; Saito et al., 2005 a/b). Nevertheless these clinical findings may result from a bias of selection, since the authors considered as inclusion criteria classical clinical findings of distemper. The classical distemper presentation is not the rule (Tipold et al, 1992). Authors about nervous distemper had been claiming that the typical systemic findings are absence in about half of the cases (Vandevelde and Cachin, 1993), and myoclonus may be absent in more than half of the cases (Tipold et al., 1992). Seizure is cortical and subcortical signs often observed in immature dogs infected with CDV, however it is not an usual finding of mature dogs suffering from CDV encephalomyelitis (Shell, 1990; Braund, 1994; Braund, 2001). We were able to recognize distemper in animals with neurological presentation without myoclonus and in the absence of previous or concomitant systemic disease (Amude et al., 2005 a/b/c).

The distemper encephalomyelitis usually is a multifocal neurological disease, and the clinical findings reflect the wide virus and lesions dissemination in the CNS (Shell, 1990; Koutinas et al., 2002). Sometimes neurological signs in distemper dogs, suggest a restricted localization of the lesion within the CNS (Tipold et al., 1992; Koutinas et al., 2002) with a single distinct neurological syndrome (Braund, 1994).

Several clinical syndromes associated with distemper have been recognized in dogs (Shell, 1990; Braund, 1994, Braund, 2001), like as: canine distemper encephalomyelitis in immature dogs; multifocal distemper encephalomyelitis in mature dogs; old dog encephalitis and, post-vaccinal canine distemper encephalitis.

Canine Distemper Encephalomyelitis in Immature Dogs

This is the most common form of CDV infection and is often initially characterized by systemic evidence of gastrointestinal and respiratory disturbances (Braund, 1994), but in some situation the nervous disease may take place in the absence of the systemic involvement (Braund, 2001). Hyperkeratosis of the footpad may be seen. Additionally many animals have conjunctivitis and chorioretinitis. Neurological signs are quite varied, often asymmetrical, and usually suggest a multifocal distribuition of lesions (Shell, 1990; Braund, 2001). Signs of localization in cortical and subcortical (seizures, personality changes) areas, brainstem (cranial nerves deficits) and cerebellum (hypermetria) are often observed, while spinal cord (paresis/paraplegia) signs are observed occasionally (Shell, 1990). Myoclonus may be a characteristic sign. CDV is probably the most common cause of seizures in dogs younger than 6 month of age (Braund, 1994).

Multifocal Distemper Encephalomyelitis in Mature Dogs

In mature dogs between the age of 4 and 8 years, CDV can produce a type of multifocal encephalitis that is characterized by a chronic course (Braund, 2001). Vaccinated animals may be affected. This neurological manifestation is not preceded by, nor is it coincident with, the systemic signs (Shell, 1990). The initial neurological presentation consists of signs of weakness, generalized incoordenation, and occasional falling. Usually these signs progress to tetraplegia. During the course of the disease signs of localization in cerebellum, brainstem, and spinal cord are common. Cortical and subcortical signs are not features of this disease and affected animals maintain a normal mental status (Braund, 1994).

Old Dog Encephalitis

Old dog encephalitis (ODE) is a rare subacute or chronic progressive panencephalitis with very low incidence around the world that is believed to be caused by CDV infection (Shell, 1990; Braund, 1994). There was some speculations that this form of distemper no longer existed since no spontaneous cases had been observed at several institutes over the past decade (Braund, 2001). Affected dogs are usually older than six years of age and there are no related systemic signs (Braund, 2001). The only clinical signs are related to the cortical and subcortical lesions (Vandevelde et al., 1980) such as visual deficits, depression, compulsive circling, and head-pressing against objects (Shell, 1990). In contrast to the signs associated with acute or chronic encephalomyelitis in immature dogs, signs of brainstem, and spinal cord disease are usually absent in cases of ODE (Shell, 1990; Braund, 2001). There is also a relative sparing of the cerebellar signs (Vandevelde et al., 1980). Histopathological changes are similar to those seen in multiple sclerosis, a demyelinating disease that takes place in human being (Adams et al., 1975; Shell, 1990).

Post-vaccinal Canine Distemper Encephalitis

Post-vaccinal canine distemper encephalitis is a rare condition that occurs in young animals, especially those less than six months of age (Shell, 1990; Braund, 2001). It is believed to be associated with vaccination using live CDV strain. It can occur one to two weeks after the animal vaccination. The pathogenesis of this disease is unclear. It may result from: i) insufficient attenuation of the vaccine virus which causes subsequent infection of the CNS; ii) the triggering of a latent distemper infection by vaccination; iii) other vaccine components; or iv) an enhanced susceptibility of the animal (Bestetti et al., 1978). According an experienced virologist pos-vaccination disease no longer exist as a disease identity, and it probably may occur in cases

which the animal already was incubating the field virus at the moment of vaccination, since the disease incubation period range about 14 days (Amauri Alfieri, 2004 – personal communication).

NEUROPATHOLOGY OF CDV INFECTION

The exact mechanism of CDV entry into the CNS has not been entirely clarified. The frequent occurrence of periventricular and subpial lesions and the fact that CDV can easily be found in chroid plexus cells and ependyma suggest entry of the virus into the brain tissue by cerebrospinal fluid (CSF) pathways, presumably by infected immune cells (Vandevelde and Zurbriggen, 1995).

The spectrum of the lesions appears to be wide, however the neuropathology of spontaneous distemper is remarkably constant. The variability of the neuropathology is largely due to the lesions evolution when the disease progress (Vandevelde and Zurbriggen, 1995; Schobesberger et al., 2002). Some variability may be due to virus strain differences, although there is little concrete evidence that these play a role in natural disease in dogs (Vandevelde and Zurbriggen, 1995). In the gray matter, CDV infects neurons which can lead to neuronal necrosis and even polioencephalomalacia. Nevertheless there are possibilities that the malacia lesions are relationship to seizure-induced hypoxia-ischemia (Braund, 2001). It has been known that the white matter lesions in distemper are characterized by selective loss of myelin sheaths. The demyelinating lesions are not the only responsible for severe neurological signs but are also thought to be a model for human demyelinating conditions such as multiple sclerosis. Because of these features the white matter pathology has been the focus of several recent studies (Zurbriggen et al., 1998; Schobesberger et al., 1999; Tipold et al., 1999; Schobesberger et al., 2002; Moro et al., 2003; Veronika et al., 2004).

The histopathological findings of distemper encephalomyelitis usually reflect a multifocal disease with the wide virus dissemination in the CNS (Shell, 1990; Koutinas et al, 2002). Experimental studies have shown a very high incidence of subclinical lesions with the absence of clinicopathological correlation in neurological distemper (Vandevelde and Cachin, 1993).

Studies have to consider an acute and a chronic stage in the development of CDV-induced demyelination. Different lesion stages can occur within the CNS of the same animal (Vandevelde et al., 1985). The temporal course of the lesions development after CDV infection is reflected by grouping distemper lesions into three different categories (acute, subacute and chronic) according to the degree of demyelination and inflammation within the white matter (Vandevelde et al., 1985; Tipold et al., 1999; Alldinger et al., 2000). In addition to these three categories, there are very early findings in which no demyelination is noted, although the CDV infection may be identified. Recently Schobesberger et al. (2002) introduced the term of peracute lesions to consider this last category.

The pathogenesis of acute demyelination

The initial myelin lesions develop during a period of severe immunosuppression and are not inflammatory (Vandevelde and Zurbriggen, 1995). This mechanism of demyelination have been examined and it was shown that the initial lesions is directly virus-induced, since there is a clear correlation between the occurrence of demyelination and the CDV replication in the cells of the white matter (Vandevelde et al., 1985).

The obvious explanation for the phenomenon of demyelination would be the oligodendroglial infection. Segmental demyelination (Higgins et al., 1982) and degenerative oligodendroglial changes in acute foci (Summer and Appel, 1987) are strongly suggestive of a primary oligodendroglial lesion. The effect of CDV on oligodendrocytes and other glial cells was studied

extensively in primary dog brain cell cultures (DBCC) (Zurbriggen and Vandevelde, 1993). DBCC contain numerous astrocytes and oligodendrocytes, which can be unequivocally identified with antibodies against cell specific markers. Despite considerable efforts using immunocytochemical and ultrastructural techniques, CDV proteins or viral nucleocapsids were only very rarely found in oligodendrocytes, in contrast to astrocytes and microglial cells which easily support CDV infection (Zurbriggen et al., 1986, 1987; Vandevelde et al., 1985). Recently, using *in situ* hybridization techniques, was found that oligodendrocytes in CDV-infected brain cultures contain CDV mRNA corresponding to all viral genes, despite the fact that these cells do not produce viral proteins (Zurbriggen et al., 1993). This *in vitro* found suggest that the oligodendrocytes degeneration may be probably result from a restricted CDV infection without viral protein production. Using a combination of immunocytochemistry and *in situ* hybridization Graber et al. (1995), demonstrate the transcription of the entire virus genome in oligodendrocytes in CDV-infected brain cell cultures. Graber et al. (1995) also shown that a restricted infection of oligodendrocytes with CDV down-regulates the transcription of the major myelin genes coding for proteolipid protein, myelin basic protein and myelin-associated glycoprotein in similar way. Consequently, the infected cells are no longer able to synthesize all the membrane compounds which are necessary for maintaining their structure integrity. Zurbriggen et al. (1998) studying the oligodendroglial pathology in canine distemper, confirmed that restriction infection of oligodendrocytes also occurs *in vivo*, as already described for culture cells, and that the CDV infection leads to massive down-regulation of myelin gene expression in demyelination lesions and that this effect correlates in part with restricted infection of oligodendrocytes. However the number of CDV mRNA-expressing oligodendrocytes found in infected but not yet demyelinated areas seems - with 8% of all oligodendrocytes in the infected area - rather small to explain ensuing demyelination (Zurbriggen et al., 1998). The majority of infected cells in the CNS are

astrocytes (Mutinelli et al., 1989; Summers and Appel, 1987; Schobesberger et al., 2002), which are important in maintaining the tissue homeostasis. The alteration of the astrocytes population may contribute to the development of demyelination, and an indirect mechanism could play a role in oligondendrocytes dysfunction.

Schobesberger et al. (1999) have addressed the question of apoptosis or necrosis importance in the pathogenesis of oligodendroglial degeneration in distemper. However, in dog brain tissue sections, was found no obvious morphological or biochemical evidence for oligodendroglial apoptosis in the initial demyelinating lesions (Schobesberger et al., 1999, 2002). However, apoptosis can not be totally excluded from playing a role in the disappearance of oligodendrocytes from CDV-induced demyelinating plaques. Lesion development in distemper is a slow process lasting over weeks. Apoptosis on the other hand is a fast event and apoptotic cell fragments are quickly phagocytosed (Majno and Joris, 1995).

The contribution of the immune response to early lesions development is not clear. Despite severe immunosupression and lack of perivascular cuffing, numerous $CD8^+$ cells are found in acute demyelinating lesions and also diffusely distributed in the brain parenchyma, roughly corresponding with areas of viral infection (Tipold et al., 1999).

Findings in acute non-inflammatory demyelination included viral replication in astrocytes (Vandevelde et al., 1982), restricted infection of low numbers of oligogendrocytes (Zurbriggen et al., 1998) and diffuse invasion with $CD8^+$ cells (Tipold et al., 2001), none of which have by themselves provided a satisfactory explanation for myelin destruction in distemper. Diffuse upregulation of MHC II in the white matter in the early state of distemper (Alldinger et al., 1996), upregulation of metallo-proteinases (Maio et al., 2003) and immunocytochemical evidence of microglial hyperplasia in initial demyelinating lesions (Tipold et al., 1999) suggest that microglia could be involved in the pathogenesis of early demyelination. Stein et al. (2004) showed a clear

correlation between upregulation of various microglial functions and the presence of demyelination.

The mechanism of acute demyelination in distemper is not yet completely understood, despite the extensive studies.

The pathogenesis of chronic demyelination

The chronic lesions are characterized by influx of inflammatory cells, mostly mononuclear, and coincide with the recovery of the immune system (Vandevelde and Zurbriggen, 1995). The inflammatory cells accumulate around blood vessels forming partly multi-layered perivascular cuffs, and also invade the parenchyma (Schobesberger et al., 1999, Schobesberger et al., 2002). The inflamatory reaction in the demyelinating lesions can lead to progression of the tissue damage. There is often necrosis of the tissue in such lesions (Schobesberger et al., 2002; Gebara et al., 2004 a).

Evidence of autoimmunity is not unusual in virus infection in different organs system, including the brain. Anti-myelin antibodies in serum have been known for a long time to occur in distemper (Krakowka et al., 1973). Vandevelde et al. (1986) found such antibodies also in the CSF of dogs with distemper and that these antibodies were locally produced in the inflammatory brain lesions. One mechanism by which anti-myelin antibodies could induce demyelination would be antibody-depending cytotoxicity. However the autoimmune reaction in distemper are probably epiphenomena which are not primary involved in the chronic demyelinating process (Vandevelde and Zurbriggen, 1995).

The chronic stage of the disease is characterized by immunopathological complication. Tissue damage and demyelination may result from the innocent bystander effects of infiltrating virus-specific T and B cells and their cytokine products (Vandevelde and Zurbriggen, 1995). The

inflammation is also associated with intrathecal immunoglobulin synthesis. It has been known for a long time that antiviral antibodies play a dominant role in immunity against CDV (Vandevelde and Zurbriggen, 1995). Bollo et al. (1986) found that the occurrence of anti-CDV antibodies in the CSF coincided with clearance of CDV and CDV containing cells from the inflammatory lesions. Since oligodendrocytes do not express viral proteins, progression of demyelination could hardly be explained by an antiviral cytotoxic reaction killing infected oligodendrocytes. Other types of antiviral immune response could be responsible for the inflammatory tissue damage seen in distemper. Macrophages, which are very numerous in distemper lesions, would play an important role. It was shown that antiviral antibodies bound to the surface of CDV infected cells interacted with the Fc receptors of neighbouring macrophages by way of their Fc portions (Burge et al., 1989; Griot et al., 1989 a,b). This interaction resulted in a respiratory burst of the macrophages with release of reactive oxygen radicals, which can be harmful to oligodendrocytes. So the humoral antiviral immune response could also leads to destruction of oligodendrocytes as innocent bystander cells.

The inflammatory phenomenon is related to virus clearance from CNS lesions. However it has been shown that CDV can persist in white matter areas outside of the inflammatory demyelinating lesions or over even in the immediate periphery of such lesions (Vandevelde and Zurbriggen, 1995; Schobesberger et al., 1999; Schobesberger et al., 2002). The virus persistence is the key to the pathogenesis of the chronic lesions, and seems to contribute to lesion maintenance and progression. The mechanism of persistence of CDV is not yet understood. Have been demonstrated, by *in vitro* assay, that CDV virulent strains which are able to persist in the CNS have a noncytolytic selective virus spread and a different way of virus release so that very little virus is released outside the nervous cells (Zurbriggen et al., 1995; Meertens et al., 2003). It also has been shown that these virus strains have an impaired and limited budding (Stettler et al.,

1997). It leads a very limited release of cell debris and virus particles in the extracellular space. As a result, macrophages stimulation attracting the antiviral immune response in the areas of active viral replication is avoided. This particular type of spread is related to differences in viral assembly as compared to attenuated distemper viruses. Sequencing studies have shown differences between virulent and attenuated CDV at the level of the nucleoprotein gene (Frisk et al., 1999; Scagliarine et al., 2003). Its difference has been incriminated as a molecular determinant of persistence (Stettler and Zurbriggen, 1995).

DIAGNOSIS AIDS

When the animal are presented with the characteristic signs of the CDV infection, such as myoclonus and systemic signs preceding or accompanying the neurological deficits, the clinical diagnosis of distemper is suggested. However the clinical diagnosis is often hard when this typical systemic involvement of the disease is absent. Likewise, myoclonus, which is highly suggestive of nervous distemper, is absent in more than half of the cases. Distemper should be always considered as a differential diagnosis, when the neurological signs and clinical history suggest a multifocal and progressive disease. Nevertheless, focal neurological disease may be found. In such cases of distemper with focal nervous signs and in which other typical findings such as extraneural signs or myoclonus are lacking, are a diagnostic challenge for the veterinary (Tipold et al., 1992). CSF examination in such animals may reveal the presence of inflammatory involvement suggesting the possibility of CDV infection. CSF analysis may support the diagnosis of CDV infection if a lymphocytic pleocytosis (greater than 5 leukocytes/μl) is present (Shell, 1990). A CSF pleocytosis that is more than 60% lymphocytes has been typically associated with viral encephalitis, but has also been reported with granulomatous meningoencephalitis (GME)

and bacterial infection especially after antibiotic therapy (Chrisman, 1992). In the inflammatory distemper the cell count rarely exceeds 30 cell/μL (Vandevelde and Cachin, 1993). However, Amude et al. (2005b) reported a severe lymphocytic pleocytosis (> 500 cell/ μL) in a distemper non-inflammatory nervous case. Sometimes there are no abnormalities in the CSF from distemper dogs with neurological disease (Shell, 1990). In 8 out of 19 dogs with distemper encephalomyelitis the CSF was normal (Koutinas et al., 2000).

In experimental infected animals frequent hematological findings is lymphopenia, sometimes combined with leucopenia or leucocytosis with left shift, anemia, and rarely thrombocytopenia. Several hematological abnormalities have been reported with distemper in natural occurring disease (Moritz et al., 2000). Tudury et al. (1997), studding the nervous form of distemper, reported that anemia and lymphopenia were frequent hematological changes found in distemper dogs. However the previous studies were descriptive studies, and a cause-consequence relationship may be not secured. According Shell (1990), the hematological changes are frequently absent or non-specific in CDV naturally infected dogs. In a study, when the hematological findings from distemper dogs were compared with data from non-distemper dogs, both from the same hospital population and sharing resembling clinical conditions, no significant statistic difference was found (Gebara et al., 2004b).

Serological examination is not very useful in the diagnosis of distemper, because a high titers of anti-CDV antibodies may be a result of prior vaccination, as well as of previous subclinical or clinical infection. On the other hand, during severe distemper, the antibody titers may be low because of the strong immunosuppressive properties of CDV. Detection of neutralizing antibodies did not correlate with the form of distemper, antigen distribution, or RT-PCR results, indicating the noncontributory role of neutralizing antibody titers for the etiological diagnosis of distemper (Frisk et al., 1999). The anti-CDV antibodies detection in CSF could be a good option

to diagnose distemper in dogs with neurological disease. However dogs can die from distemper without detectionable anti-CDV neutralizing antibodies titers in the CSF in the acute state of the disease (Shell, 1990). The virus neutralizing antibody titers in CSF samples was detected in only 2 dogs out of 10 dogs with confirmed CDV infection (Frisk et al., 1999).

A final diagnosis is based on the demonstration of viral antigens in scrapings and body fluids such as conjunctival smears, tracheal washing, and urine sediment (Tipold et al., 1992). Direct immunofluorescence test are routinely and widely used with this propose. However, in the subacute or chronic forms of the disease this test gives false-negative results. In 12 dogs suffering from natural distemper confirmed by N-PCR, Jóźwik and Frymus (2005) diagnosed CDV infection by direct immunofluorescent test in only 6 dogs. No CDV antigen could be detected in the peripheral blood of 6 out of 7 distemper dogs with only systemic signs, 5 out of 13 distemper dogs with systemic and neurological signs, and all (7/7) distemper dogs with only nervous signs (Moritz et al., 2000). Viral antigen can be also hard to find in the extraneural tissues in cases with neurological distemper without systemic involvement (Tipold et al., 1992). The methods available for *ante mortem* distemper etiologically diagnosis to date are of limited value, and in the majority of cases a definitive diagnosis is only possible at *post mortem* (Baumgärtner, 1993).

Recently the RT-PCR has been introduced as a usefulness, fast, sensitive, and specific method to diagnose CDV infection in dogs (Frisk et al., 1999; Shin et al., 2004; Jóźwik and Frymus, 2005; Amude et al., 2005a). Urine, serum, whole blood, and CSF are body fluids that have been used for CDV detection by RT-PCR in dogs with characteristics and commons signs of the disease (Frisk et al., 1999; Shin et al., 1995; Kim et al., 2001; Moritz et al., 2000; Gebara et al., 2004 a/b; Saito et al., 2005 a/b). The sensitivity of the RT-PCR varies with selected primers, RNA extraction methods, and clinical sample analyzed. Frisk et al. (1999) had a sensitivity of 86% (25/29) and 88% (14/16) when serum and whole blood respectively were used as biological

sample in RT-PCR for distemper diagnosis in naturally-occurring classical disease. Gebara et al. (2004 a/b) and Saito et al. (2005 a) used urine, from dogs with characteristics clinical findings of distemper, in order to detect CDV by RT-PCR in naturally-occurring disease. We recently observed that urine might also be a good biological sample for *ante mortem* CDV detection by RT-PCR in dogs with distemper encephalomyelitis without the typical clinical presentation that is characterized by nervous disease with myoclonus and systemic findings (gastrointestinal and/or respiratory signs), being serum and whole blood not sensitive samples for this propose (Amude et al., 2005a). By RT-PCR CDV could be detected in 4 out of 5 urine samples from dogs with distemper encephalomyelitis, however the virus only could be detected in one blood sample and none of the serum samples from the same distemper dogs. Curiously the CDV only could be detected by RT-PCR in only 2 out of 5 CSF samples, being the urine the biological sample more sensitive (4/5). The CSF RT-PCR results probably were false negative results that might be due to a complete lack or the presence of only low levels of CDV RNA in the CSF. The low cellularity of the CSF, as well the pathogenesis of the virulent CDV strains might contribute to RT-PCR false negative results found in the CSF sample (Amude et al., 2005a). The dog with distemper encephalomyelitis in which the virus could not be detected in urine could be diagnosed ante mortem as distemper dog by RT-PCR using CSF. Thus, the use of RT-PCR with two differents body fluid (urine and CSF) can increase the technique sensitivity for *ante mortem* diagnosis of distemper in dogs with the clinical presentation restricted to neurological disease without myoclonus (Amude et al., 2005a).

REFERENCES

1. Adams, J.M., Brown, W.J. and Snow, H.D., 1975. Old dog encephalitis and demyelinating diseases in man. *Veterinary Pathology,*12, 220-226.
2. Alldinger, S., Fonfara, S., Kremmer, E. and Baumgärtner, W., 2000. Up-regulation of the hyaluronate receptor CD44 in canine distemper demyelinated plaques. *Acta Neuropathologica*, 99, 138-146.
3. Alldinger, S., Wunschmann, A., Baumgärtner, W., Voss, C. and Kremmer, E., 1996. Up-regulation of major histocompatibility complex class II antigen expression in the central nervous system of dogs with spontaneous canine distemper virus encephalitis. *Acta Neuropathologica*, 92, 273-280.
4. Alfieri, A.A., 2005. Laboratorio de Virologia Animal, Centro de Ciencias Agrarias, Departamento de Medicina Veterinaria Preventiva, Universidade Estadual de Londrina, personal communication.
5. Amude, A.M., Alfieri, A.A. and Alfieri A.F., 2005a. *ante mortem* Diagnosis of CDV Infection by RT-PCR in Distemper Dogs with Neurological Deficits without the Typical Clinical Presentation. *Veterinary Research Communication,* "*in press*".
6. Amude, A.M., Alfieri, A.A., Balarin, M.R.S., dos Reis, A.C.F. and Alfieri, A.F., 2005b. Severe lymphocytic pleocytosis in cerebrospinal fluid from a dog with neurological deficits. *Journal of Veterinary Clinical Pathology*, "*in press*".
7. Amude, A.M., Carvalho, G.A., Balarin, M.R.S., Arias, M.V.B., Reis, A.C.F., Alfieri, A.A. and Alfieri, A.F., 2005c. Encefalomielite pelo vírus da cinomose canina em cães sem sinais epiteliais da doença – relato de três casos. *Revista Clínica Veterinária*, submetido.

8. Bestetti, G., Fatzer, R. and Fankhauser, R., 1978. Encephalitis following vaccination against distemper and infectious hepatitis in a dog. *Acta Neuropathologica*, 43, 69-75.
9. Baumgärtner, W., 1993. Virale Infektionskrankrankheiten bei Junghunden. *Praktischer Tierarzi*, 1, 26-32.
10. Baumgärtner, W., Örrvell, C. and Reinacher, M., 1989. Naturally occurring canine distemper virus encephalitis: distribuition and expression of viral polypeptides in nervous tissues. *Acta Neuropathologica*, 78, 504-512.
11. Bollo, E., Zurbriggen, A., Vandevelde, M. and Faukhauser, R., 1986. Canine distemper virus clearance in chronic inflammatory demyelination. *Acta Neuropathologica*, 72, 69-73.
12. Braund, K.G., 2001. *Inflammatory Diseases of the Central Nervous System*. Available at: www.ivis.org. Accessed Nov 11, 2001.
13. Braund, K.G., 1994. *Clinical Syndromes in Veterinary Neurology*. 2nd ed. St Louis, MO: Mosby.
14. Bürge, T., Griot, C., Vandevelde, M. and Peterhans, E., 1989. Antiviral antibodies stimulate production of reactive oxygen species in cultured canine brain cells infected with canine distemper virus. *Journal of Virology*, 63, 2790-2797.
15. De Lahunta, A., 1983. *Veterinary Neuroanatomy and Clinical Neurology*, 2nd ed. Philadelphia: W.B. Saunders.
16. Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, V. and Ball, L.A., 2004. *Virus Taxonomy*. The light report. San Diego: Academic Press.
17. Frisk, A.L., Konig, M., Moritz, A. and Baumgärtner, W., 1999. Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole

blood, and cerebrospinal fluid from dogs with distemper. *Journal of Clinical Microbiology*, 37, 3634-3643.

18. Gebara, C.M.S., Wosiacki, S.R., Negrão, F.J., Alfieri, A.A. and Alfieri, A.F., 2004a. Lesões histopatológicas no sistema nervoso central de cães com encefalite e diagnóstico molecular da infecção pelo vírus da cinomose canina. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia,* 56, 168-174.
19. Gebara, C.M.S., Wosiacki, S.R., Negrão, F.J., de Oliveira, D.B., Beloni, S.N.B., Alfieri, A.A. and Alfieri, A.F., 2004b. Detecção do gene da nucleoproteína do vírus da cinomose canina por RT-PCR em urina de cães com sinais clínicos de cinomose. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 56, 480-487.
20. Graber, H., Müller, C., Vandevelde, M. And Zurbriggen, A., 1995. Restricted infection with canine distemper virus leads to down-regulation of myelin gene transcription in cultured oligodendrocytes. *Acta Neuropathologica*, 90, 312-318.
21. Greene, G.E. and Appel, M.J., 1998. Canine distemper. In: G.E. Greene (eds), *Infectious Diseases of dog and the cat*. 2nd ed. Philadelphia: W.B.Saunders, 9-22.
22. Griot, C., Bürge, T., Vandevelde, M. and Peterhans, E., 1989a. Antibody-induced generation of reactive oxygen radicals by brain macrophages in canine distemper encephalitis: a mechanism for bystander demyelination. *Acta Neuropathologica*, 78, 396-403.
23. Griot, C., Bürge, T., Vandevelde, M. and Peterhans, E., 1989b. Bystander demyelination through antibody induced macrophage activation in canine distemper virus infection. *Archives of Neurology and Psychiatry*, 140, 39-41.
24. Headley, S.A. and Graça, D.L., 2000. Canine distemper: epidemiological findings of 250 cases. *Brazilian Journal of Veterinary Research and Animal Science*, 37, 136-140.

25. Headley, S.A., Soares, I.C. and Graça, D.L., 2001. Glial Fibrillary Acidic Protein (GFAP) – immunoreactive Astrocytes in Dogs Infected with Canine Distemper Virus. *Journal of Comparative Pathology*, 125, 90-97.

26. Higgins, R., Krakowka, S., Metzler, A. and Koestner, A., 1982. Primary demyelination in experimental canine distemper virus induced encephalomyelitis in gnotobiotic dogs. *Acta Neuropathologica*, 58, 1-8.

27. Jóźwik, A. and Frymus, T., 2005. Comparison of the Immunofluorescence Assay with RT-PCR and Nested PCR in the Diagnosis of Canine Distemper. *Veterinary Research Communications*, 29, 347-359.

28. Kim, Y.H., Cho, K.W., Youn, H.Y., Yoo, H.S. and Han, H.R., 2001. Detection of canine distemper virus through one step RT-PCR combined with nested PCR. *Journal of Veterinary Science*, 2, 59-63.

29. Koutinas, A.F., Polizopoulou, Z.S., Baumgärtner, W., Lekkas, S. and Kontos, V., 2002. Relation of clinical sings to pathological changes in 19 cases of canine distemper encephalomyelitis. *Journal of the Comparative Pathology*, 126, 47-56.

30. Krakowaka, S., McCullough, B., Koestner, A. and olsen, R., 1973. Myelin-specific autoantibodies associated with central nervous system demyelination in canine distemper virus infection. *Infection and Immunology*, 8, 819-827.

31. Maio, Q., Baumgärtner, W., Failing, K. and Alldinger, S., 2003. Phase-dependent expression of matrix metalloproteinases and their inhibitions in demyelinating canine distemper encephalitis. *Acta Neuropathologica*, 106, 486-494.

32. Majno, G. and Joris, I., 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. *American Journal of Pathology*, 176, 3-15.

33. Meertens, N., Stoffel, M.H., Cherpillod, P., Wittek, R., Vandevelde, M. and Zurbriggen, A., 2003. Mechanism of reduction of virus release and cell-cell fusion in persistent canine distemper virus infection. *Acta Neuropathologica*, 106, 303-310.

34. Moritz, A., Frisk, A.L. and Baumgärtner, W., 2000. The evaluation of diagnostic procedures for the detection of canine distemper virus infection. *European Journal of Companion Animal Practice*, 10, 37-47.

35. Moro, L., Martins, A.S., Alves, C.M., Santos, F.G.A., Del Puerto, H.L. and Vasconcelos, A.C., 2003. Apoptosis in the Cerebellum of Dogs with Distemper. *Journal of Veterinary Medicine Series B*, 50, 221-225.

36. Muetinelli, F., Vandevelde, M., Griot, C. And Richard, A., 1989. Astrocytes infection in canine distemper virus-induced demyelination. *Acta Neuropathologica*, 77, 333-335.

37. Muller, C.F., Fatzer, R.S., Beck, K., Vandevelde, M. and Zurbriggen, A., 1995. Studies on canine distemper virus persistence in the central nervous system. *Acta Neuropathologica*, 89, 438-445.

38. Okita, M., Yanai, T., Ochikubo, F., Gemma, T., Mori, T., Maseki, T., Yamanouchi, K., Mikami, T. and Kai, C., 1997. Histopathological features of canine distemper recently observed in Japan. *Journal of the Comparative Pathology*, 116, 403-408.

39. Saito, T.B., Alfieri, A.A., Wosiacki, S.R., Negrão, F.J., Morais, H.S.A. and Alfieri, A.F., 2005a. Detection of canine distemper virus by reverse transcription-polimerase chain reaction in the urine of dogs with clinical signs of distemper encephalitis. *Research in Veterinary Science*, 79, "in press".

40. Saito, T.B., Alfieri, A.A., Wosiacki, S.R., Negrão, F.J., Morais, H.S.A. and Alfieri, A.F., 2005b. *Ante* and *Post mortem* detection of canine distemper virus by RT-PCR in dogs with distemper. *Archivos de Medicina Veterinaria*, 37, "in press".

41. Scagliarini, A., Battilani, M., Ciulli, S., Prosperi, S. and Morganti, L., 2003. Molecular Analysis of the NP Gene of Italian CDV Isolates. *Veterinary Research Communications*, 27, 355-357.

42. Schobesberger, M., Zurbriggen, A., Doherr, M.G., Weissenböck, H., Vandevelde, M., Lassman, H. and Griot, C., 2002. Demyelination precedes oligodendrocyte loss in canine distemper virus-induced encephalitis. *Acta Neuropathologica*, 103, 11-19.

43. Schobesberger, M., Zurbriggen, A., Summerfield, M., Vandevelde, M. and Griot, C., 1999. Oligodendroglial degeneration in distemper: apoptosis or necrosis? *Acta Neuropathologica*, 97, 279-287.

44. Shell, L.G., 1990. Canine Distemper. *Compendiun on Continuing Education for the Practicing Veterinarian*, 12, 173-179.

45. Shin, Y., Mori, T., Okita, M., Gemma, T., Kai, C. and Mikami, T., 1995. Detection of canine distemper virus nucleocapsid protein gene in canine peripheral blood mononuclear cells by RT-PCR. *Journal of Veterinary Medical Science*, 57, 439-445.

46. Stein, V.M., Czub, M., Hansen, R., Leibold, W., Moore, P.F., Zurbriggen, A. and Tipold, A., 2004. Characterization of canine microglial cells isolated in vivo. *Veterinary Immunology and Immunopathology*, 99, 73-85.

47. Stein, V.M., Czub, M., Schreiner, N., Moore, P.F., Vandevelde, M., Zurbriggen, A. and Tipold, A., 2004. Microglial activation in demyelinating canine distemper lesions. *Journal of Neuroimmunology*, 153, 122-131.

48. Stettler, M., Beck, K., Wagner, A., Vandevelde, M. and Zurbriggen, A., 1997. Determinants of persistence in canine distemper viruses. *Veterinary Microbiology*, 57, 83-93.

49. Stettler, M. and Zurbriggen, A., 1995. Nucleotide and deduced aminoacid sequences of the nucleocapsid protein of the virulent A75/17-CDV strain of canine distemper virus. *Veterinary Microbiology*, 44, 211-217.

50. Summers, B.A. and Appel, M.J., 1994. Aspects of canine distemper virus and measles virus encephalomyelitis, *Neuropathology and Applied Neurobiology*, 20, 525-534.

51. Summers, B.A. and Appel, M.J., 1987. Demyelination in canine distemper encephalomyelitis: an ultrastructural analysis. *Journal of Neurocytology*, 16, 871-881.

52. Summers, B.A., Cummings, J.F. and de Lahunta, A., 1995. *Veterinary Neuropatology*. St Louis: Mosby.

53. Tipold, A., Jaggy, A., Zurbriggen, A. and Vandevelde, M., 1996. Neurological signs in canine distemper encephalomyelitis – a clinical study. *European Journal of Companion Animal Practice*, 7, 33-38.

54. Tipold, A., Jaggy, A., and Zurbriggen, A., 1994. Neurologische Symptome bei hundestaupe – eine klinische Studie. *Wiener Tierärztliche Monatsschrift*, 81, 274-279.

55. Tipold, A., Moore, P., Zurbriggen, A., Burgener, I., Barben, G. and Vandevelde, M., 1999. Early T cell response in the central nervous system in canine distemper virus infection. *Acta Neuropathologica*, 97, 45-56.

56. Tipold, A., Vandevelde, M. and Jaggy, A., 1992. Neurological manifestation of canine distemper virus infection. *Journal of Small Animal Practice*, 33, 466-470.

57. Tipold, A., Vandevelde, M., Wittek, R., Moore, p., Summerfield, A. and Zurbriggen, A., 2001. Partial protection and intrathecal invasion of CD8+ T cells in acute canine distemper virus infection. *Veterinary Microbiology*, 83, 189-203.

58. Tudury, A.E., Arias, M.V.B., Bracarense, A.P.F.L., Megid, J., and Dias, R.F. Júnior, 1997. Observações Clínicas e Laboratoriais em Cães com Cinomose Nervosa. *Ciência Rura*l, 27, 229-235.

59. Vandevelde, M. and Cachin, M., 1993. The Neurologic Form of Canine Distemper. In: J.D. Bonagura and R.W. Kirk (eds), *Kirk's Current Veterinary Therapy XI, Small Animal Practice*. Philadelphia: W.B. Saunders, 1003-1007.

60. Vandevelde, M., Higgins, R.J., Kristensen, B., Kristensen, F., Steck, A.J. and Kihm, U., 1982. Demyelination in experimental canine distemper virus infection: immunological, pathologic, and immunohistological studies. *Acta Neuropathologica*, 56, 285-293.

61. Vandevelde, M., Kristensen, B., Braund, K. G., Greene, G. E., Swango, L. T. and Hoerlein, B. F., 1980. Chronic Canine distemper virus encephalitis in mature dogs. *Veterinary Pathology*, 17, 17-28.

62. Vandevelde M, and Zurbriggen A., 1995. The neurobiology of canine distemper virus infection. *Veterinary Microbiology*, 44, 271-280.

63. Vandevelde, M., Zurbriggen, A., Dumas, M. and Palmer, D.G., 1985. Canine distemper virus does not infect oligodendrocytes in vitro. *Journal of the Neurological Sciences*, 69, 133-137.

64. Vandevelde, M., Zurbriggen, A., Steck, A. and Bichsel, P., 1986. Studies on the intrathecal humoral immune response in canine distemper encephalitis. *Journal of Neuroimmunology*, 11, 41-51.

65. Zurbriggen, A., Schmid, I., Graber, H.U. and Vandevelde, M., 1998. Oligodendroglial pathology in canine distemper. *Acta Neuropathologica*, 95, 71-77.

66. Zurbriggen, A., and Vandevelde, M., 1993. Canine distemper virus-induced glial cell changes in vitro. *Acta Neuropathologica*, 62, 51-58.

67. Zurbriggen, A., Vandevelde, M. and Dumas, M., 1986. Secondary degeneration of oligodendrocytes in canine distemper virus infection in vitro. *Laboratory Investigation*, 54, 424-431.

68. Zurbriggen, A., Vandevelde, M., Dumas, M., Griot, C. and Bollo, E., 1987. Oligodendroglial pathology in canine distemper virus infection in vitro. *Ata Neuropathologica*, 74, 366-373.

69. Zurbriggen, A., Yamawaki, M. and Vandevelde, M., 1993. Restricted canine distemper virus infection of oligodendrocytes. *Laboratory Investigation*, 68, 277-284.

OBJETIVOS

Objetivo Geral:

- Avaliar a presença do vírus da cinomose canina (CDV) em cães com síndromes neurológicas centrais (cerebrais e medulares), sem sinais clínicos sistêmicos e mioclonia no momento da apresentação hospitalar, utilizando a técnica de Transcrição Reversa seguida pela Reação em Cadeia pela Polimerase (RT-PCR).

Objetivos Específicos:

- Identificar e caracterizar as síndromes clínicas causadas pela infecção do CDV, nas quais a mioclonia e os sinais sistêmicos estejam ausentes no momento da admissão hospitalar.
- Avaliar as alterações hematológicas e liquóricos; as lesões histopatológicas no sistema nervoso central; assim como o resultado do exame neurológico proveniente de cães com encefalomielite por infecção natural pelo CDV, apresentados exclusivamente com sinais neurológicos e sem mioclonia no momento da admissão hospitalar.
- Verificar de que forma esses achados clínicos e laboratoriais podem ou não contribuir para o diagnóstico etiológico.

ARTIGOS PARA PUBLICAÇÃO

“Clinical syndromes of canine distemper virus encephalomyelitis in absence of distemper usual clinical findings”

Artigo a ser encaminhado para apreciação pelo periódico *Australian Veterinary Journal*

“Distemper encephalomyelitis-clinicopathological findings in eight distemper dogs presented exclusively with the neurological disease.”

Artigo a ser encaminhado para apreciação pelo periódico *Journal of American Veterinary Medical Association*

Clinical syndromes of canine distemper virus encephalomyelitis in absence of distemper usual clinical findings

Abstract

The aim of this study was to recognize and present the canine distemper virus (CDV) clinical syndromes that take place in absence of distemper usual clinical findings. For the study were prospectively investigated dogs presented with neurological deficits and absence of systemic signs and myoclonus at the time of hospital admission. Twenty dogs clinically followed up which were obtained central nervous system (CNS) samples at necropsy were included in this study. The distemper diagnosis was performed at *post mortem* by the CDV detection in the CNS using reverse transcription - polymerase chain reaction (RT-PCR), and histopathology. Eight (40%) out of 20 dogs were diagnosed as suffering from CDV encephalomyelitis. According to the nervous signs the distemper dogs were grouped in three clinical syndromes: Old dog encephalitis (ODE) (1 dog), canine distemper encephalitis in immature dogs (CDEID) (1 dog), and multifocal distemper encephalomyelitis in mature dogs (MDEMD) (6 dogs). CDV encephalomyelitis could be identified at *post mortem* in 40% of the cases, even with the failure of distemper clinical diagnosis at the time of hospital admission due to the absence of common and characteristic signs of distemper. As distemper encephalomyelitis clinical diagnosis is often difficult when myoclonus and extra-neural signs are absent, and considering that it is an infectious disease with guarded prognosis, the knowledge about these clinical syndromes may facility the clinical diagnosis in such cases in which the common distemper signs are not present.

Key words: dog; canine distemper virus; distemper; encephalomyelitis, RT-PCR.

INTRODUCTION

Canine distemper is a systemic disease caused by canine distemper virus (CDV), frequently lethal in dogs, other non-domestic carnivores and some marine mammals (Summer and Appel, 1994). The CDV is a non-segmented, negative single-stranded RNA virus, closely related to measles virus and rinderpest virus, two other members of the genus *Morbillivirus* of the *Paramyxoviridae* family. The virion is relative large (150-240 nm), surrounded by a lipoprotein envelope derived from virus glycoproteins incorporated into the cell membrane (Fauquet et al., 2004).

CDV is generally transmitted as an aerosol infection to the upper respiratory tract. The primary virus replication takes place in the lymphoid tissues. Infection of these tissues is associated with severe long lasting immunossupression. At about 10 days post infection (p.i.), CDV starts to spread from the sites of primary replication to various epithelial tissues and the central nervous system (CNS) (Vandevelde and Zurbriggen, 1995). The virus leads a multisystemic infection, which is often associated with viral spread to the CNS, resulting in a progressive, multifocal demyelinating disease (Muller et al., 1995; Meertens et al., 2003). During viraemia CDV infects brain in the majority, if not all cases, regardless of the clinical signs (Summer et al., 1995).

In dogs, CDV infection can result in subclinical infection, or clinical disease characterized by gastrointestinal and/or respiratory signs, frequently with CNS involvement. Gastrointestinal and respiratory signs are known as epithelial or systemic signs of distemper. The virulence of the virus strain, the age and the immunocompetence of the dog determine the course and outcome of the disease (Tipold et al., 1992; Greene and Appel, 1998). The type of the neurological signs depends on the virus distribution in the CNS and localization of the lesions, nevertheless a

clinicopathological correlation is often lacking (Vandevelde and Cachin, 1993, Koutinas et al., 2002). CDV affects both white and gray matter in the CNS (Summers et al., 1995). Thus, a variety of neurological signs may be observed. Despite the relative large literature about neuropathology, immunology and laboratorial findings on nervous distemper, studies on the clinical neurology involving the nervous signs of distemper are still uncommon.

Several clinical syndromes associated with distemper encephalomyelitis have been recognized in dogs like as: Canine distemper encephalomyelitis in immature dogs (CDEID), multifocal distemper encephalomyelitis in mature dogs (MDEMD), old dog encephalitis (ODE), and post-vaccinal canine distemper encephalitis (Shell, 1990; Braund, 1994, Braund, 2001). When systemic signs are preceding or accompanying the neurological disease, distemper encephalomyelitis is often a probable suspected as diagnosis (Moritz et al., 2000). However, such "classical" presentation is not the rule. Nervous signs may also occur without any other signs (Baumgärtner et al., 1989; Tipold et al., 1992, Tipold et al., 1996). The clinical diagnosis of distemper encephalomyelitis is difficult when the systemic signs are absent at the time of hospital admission (Tipold et al., 1992; Vandevelde and Cachin, 1993). Myoclonus, a highly suggestive of nervous distemper, may be also absent. These cases, which are presented without systemic signs and myoclonus, are a diagnostic challenge for the veterinary (Tipold et al., 1992). So the knowledge about the clinical syndromes caused by CDV infection in which the typical distemper clinical findings are not present is important for clinical recognition of distemper encephalomyelitis in such cases. The aim of this study was to recognize and present the CDV encephalomyelitis clinical syndromes in naturally infected dogs, in which the systemic signs and myoclonus were not present at the time of hospital admission.

MATERIAL AND METHODS

Inclusion criteria

Were investigated prospectively dogs presented to the Medical and Surgical Division from Veterinary Teaching Hospital of Universidade Estadual de Londrina, Londrina, Paraná, Brazil, from november 2003 to november 2004, with neurological deficits and absence of systemic signs and myoclonus at the time of hospital admission. As systemic signs were considered fever, gastrintestinal and respiratory signs. Ophthalmic evaluation was not considerate. The animals that showed systemic signs and/ or myoclonus after the hospital admission were not excluded from the study. Dogs in which the myelography evaluation suggested a compressive lesion and that a traumatic or toxic event could be recognized as the causative of the neurological presentation were excluded from this investigation. Dogs clinically followed up, in which were possible to obtain CNS samples corresponding to the neurological signs at necropsy, were evaluated in this clinical study.

Animals and clinical samples

The dogs were followed up from the hospital admission until the clinical outcome (euthanasia or natural death, despite treatment), and all the data about neurological evolution were recorded. Euthanasia when elicited, was performed by intravenous infusion of 10% KCl, under general anesthesia preceded by tranquilization. The owner's consent was obtained before both, clinical follow up and clinical samples collection.

The dogs included in this clinical trial were not submitted to any stressing or suffering proceeding. The investigator did the neurological examinations during the hospital routine procedure. The clinical decision, medical treatment, or other diagnostic procedures were performed by the attending veterinary. CNS fragments corresponding to the neurological signs, collected at necropsy, were submitted for histopathological examination. A second fresh CNS fragment was used for CDV detection by reverse transcription – polymerase chain reaction (RT-PCR).

Diagnostic criteria

The distemper diagnosis was carried out at *post mortem*, by RT-PCR for CDV detection in the fresh CNS fragment and CNS histopathology. The histopathological findings considerate as suggestive of CDV infection in the CNS were focal to multifocal vacuoles in the white matter (demyelination) with or without inflammatory involvement.

RT-PCR

For CDV detection by RT-PCR, immediately after the CNS collection the RNA was extracted from an aliquot of 300 μL of suspension (10% w/v) of fresh CNS fragments in phosphate buffered saline (PBS), according to the silica/guanidine isothiocyanate method, described by Boom et al. (1990). Aliquots of ultrapure (MilliQ®) sterile water were included as negative control in all the RNA extractions.

RT-PCR was performed using the oligonucleotides primers (Invitrogen™ Life Technologies, USA) CDV 1 (sense) [5'-aca gga ttg ctg agg acc tat-3', nt 769-789] and CDV2 (anti-sense) [5'-caa gat aac cat gta cgg tgc-3', nt.1055-1035], designed to amplify an amplicon of 287 bp size of the CDV nucleoprotein gene (Frisk et al., 1999).

The reverse transcription was performed with 9 μL of extracted RNA and 2.0 pmol of CDV1 that was denaturated at 70°C for 10 min and immediately transferred to ice for 5 min. Following this step was added the RT-MIX solution, made of 0.2 mM of each dNTP (Invitrogen™ Life Technologies, USA), 1x PCR-buffer (Invitrogen™ Life Technologies, USA) (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 1.5 mM $MgCl_2$, 100 units of reverse transcriptase enzyme M-MLV (Invitrogen™ Life Technologies, USA) and ultrapure sterile water to a final volume of 20 μl. After homogenization, the solution was incubed at 42°C for 30 min followed at 70°C for 10 min for the enzyme inactivation.

For the PCR reaction were used 5 μL of cDNA, 0.4 pmol of each primers (CDV1 and CDV2), 0.2 mM of each dNTP, 1x PCR-buffer (20 mM Tris-HCl pH 8,4 e 50 mM KCl), 1,5 mM $MgCl_2$, 2,5 units of Platinum® Taq DNA Polymerase (Invitrogen™Life Technologies, USA) and ultrapure sterile water to a final volume of 50 μL. The reaction was realized in a thermocycler (PTC 200, MJ Research Co. Water Town, Ma, USA), using the following time and temperature conditions: i) a initial denaturation step at 94 °C for 1 min; ii) 40 cycles at 94ºC for 1 min, 59ºC for 2 min and 72^{o}C for 1 min; iii) a final extension step at 72^{o}C for 7 min. A CDV Rockborn strain infected Madin Darby canine kidney (MDCK) cell was used as CDV positive control.

The amplified products were analyzed by electrophoresis in 2% agarose gel with ethidium bromide (0,5 μg/mL) in TBE buffer pH 8.4 (89 mM Tris-HCl; 89 mM boric acid; 2 mM EDTA) in constant voltage (90 V) for approximately 45 min and visualized under UV light.

The identities of the RT-PCR products were confirmed by RFLP (restriction fragment length polymorphism) with *Hinf* I (Invitrogen™Life Technologies, USA) enzyme digestion. The digestion was performed with 18 μL of RT-PCR product, according manufacture's instructions.

Histopathology

CNS tissue sections were examined for CDV-induced lesions. For this, CNS samples were fixed in 10% buffered neutral formalin, embebed in histological paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (HE), following routine procedures.

RESULTS

Twenty dogs clinically followed up that filled the inclusion criteria and in which were possible to obtain CNS samples corresponding to the neurological signs at necropsy, were evaluated in this study. In 17 dogs the owner elicited euthanasia, and the other 3 dogs died during the hospitalization period. According to the diagnostic criteria 8 out of 20 dogs were considered as suffering from distemper encephalomyelitis.

The onset of neurological signs was acute in two distemper dogs (cases nº 18 and 22) and gradual with a progressive course in the remaining 6 distemper dogs. Four animals had received 3 or more vaccination against distemper with life modified virus (nº 15, 16, 22, and 30), two were unvaccinated dogs (nº 05 and 13), while the remaining two distemper dogs had an unknown vaccination status (nº 17 and 18). The age, breed, sex, vaccination records, survive time after the beginning of neurological deficits from the dogs with distemper encephalomyelitis are presented

in the table 1. One distemper dog (nº 15) displayed myoclonus 22 days after the beginning of neurological presentation. Two distemper dogs showed systemic signs after the admission during the neurological disease evolution, one (nº 22) on the fourth day (vomiting and diarrhea), and the other (nº 30) on the eightieth day (diarrhea) of clinical evolution. According to the neurological signs the distemper dogs were grouped in three CDV clinical syndromes: ODE (n° 13), CDEID (n° 16), and MDEMD (n° 05, 15, 17, 18, 22, 30). The clinical syndromes and the respective neurological deficits observed in the dogs with distemper encephalomyelitis during the clinical follow up are presented in the table 2.

The RT-PCR in fresh CNS fragments from 8 dogs (nº 05, 13, 15, 16, 17, 18, 22, 30) amplified a fragment of 287 bp size that was cleaved by *Hinf* I as expected to the CDV, and yielded fragments of 227 and 60 bp size. All the dogs in which CDV was detected by RT-PCR showed histopathological findings compatible with CDV encephalomyelitis, and none of them showed cysts of *Toxoplasma gondii* or *Neospora caninum* in the CNS fragments examined. In one dog (nº 22) the histopathological examination was not possible because the fixed CNS fragments were lost during the procedure, however the CDV was identified in fresh CNS fragments by RT-PCR. The frequent histopathological findings in the CNS of distemper dogs consisted of focal to multifocal vacuoles in the white matter (demyelination), perivascular mononuclear cell infiltration, mononuclear cells infiltrated diffusely into the parenchyma, malacia, glial reaction, and perimeningeal mononuclear infiltrate. In none of the other 12 dogs were observed lesions compatible with CDV infection.

DISCUSSION

The RT-PCR has been a usefulness, fast, sensitive, and specific method for detection of CDV infection in dogs (Frisk et al., 1999; Moritz et al., 2000; Kim et al., 2001; Rzeżutka and Mizak, 2002; Gebara et al., 2004a/b; Shin et al., 2004; Jóźwik and Frymus, 2005; Amude et al., 2005). The sensitivity of this technique varies with selected primers, RNA extraction methods, and clinical sample analyzed. The set of NP gene specific oligonucleotide primers included in the present study was previously used, for CDV detection, by different authors (Frisk et al., 1999; Moritz et al., 2000; Gebara et al., 2004 a/b; Jóźwik and Frysmus, 2005; Saito et al., 2005 a/b). Since the NP is required for virus replication, the mRNA of the NP gene is transcribed most extensively in infected cells (Shin et al., 1995) and seems to be a good marker of infection. The specificity of RT-PCR products obtained by the set of primers used in our study had already been tested by Frisk et al. (1999) by sequencing of obtained amplification. Besides this, the set of primers are valuable in the diagnosis of distemper because they could detect the nucleoprotein gene of different CDV strains (Onderstepoort; Snyder Hill; Rockborn; Lederle) (Jóźwik and Frysmus, 2005). The silica/guanidine isothiocyanate method used for CDV RNA extraction in this study, was accessed by Saito et al. (2005b) as the best method for CDV RNA extraction. Fresh and frozen organs fragment were also good choice as clinical samples to be submitted to RT-PCR for CDV detection (Rzeżutka and Mizak, 2002; Saito et al., 2005b). These considerations make our RT-PCR in fresh CNS fragments a good and available criterion for CDV diagnosis. The RT-PCR and RFLP results, associated with the histopathological findings compatible with CDV encephalomyelitis (Vandeveld and Zurbriggen, 1995; Summer et al., 1995; Schobesberger et al., 2002; Gebara et al., 2004a), support that the CDV was the etiological agent causative of the neurological signs in 8 out of 20 dogs included in this clinical study. According

to the neurological presentation one case was recognized as ODE (dog nº 13), one as CDEID (dog nº 16), and 6 cases as MDEMD (dog nº 05, 15, 17, 18, 22, 30).

Neurological findings showed by one 11-years-old dog (nº 13) such as behavioral change (aggressiveness), inappropriate consciousness content, head pressing against objects, circling and compulsive walking are ODE usual neurological clinical manifestation, which reflect cortical and subcortical involvement. Seizure-like activity reported by the owner is an unusual clinical finding for naturally occuring ODE that likely reflects localization of the virus in the cerebral cortical neurons, however it was already reported in an experimental ODE case (Axthelm and Krakowka, 1998). However during the clinical follow up no seizure-like activity was observed. The absence of cranial nervous deficits and normal mental level (alert), suggest no clinical alteration related with brainstem on the ODE-like discussed case. Ataxia, weakness, dullness and hyperkinesis, displayed by the presented dog, also were reported in an experimental ODE case (Axthelm and Krakowka, 1998). Hyperkinesis was manifested as exaggerated and uncoordinated hind limb placement. This kind of hyperkinesis is known as dysmetria/hypermetria. ODE is a rare subacute or chronic progressive panencephalitis with very low incidence around the world that is believed to be caused by CDV infection (Shell, 1990; Braund, 2001). There is some speculations that this form of distemper no longer exists since no spontaneous cases have been observed at several institutes over the past decade (Braund, 2001). Affected dogs are usually older than six years of age and there are no related systemic signs (Braund, 2001). The only clinical signs are related to the cortical and subcortical lesions (Vandevelde et al, 1980; Shell, 1990). The pathological findings suggest that the infection is restricted to the CNS (Braund, 2001). In contrast to the signs associated with acute or chronic encephalomyelitis in immature dogs, signs of brainsteam, and spinal cord disease are usually absent in cases of ODE (Shell, 1990; Braund, 2001). There is also a relative sparing of the cerebellar signs (Vandevelde et al, 1980). Nevertheless, in our ODE case

some cerebellar signs could be observed, such as dysmetria (hyperkicesia) and intentional tremor of the head.

Partial seizures characterized as “chewing-gum fits” with secondary generalization, vertical positional nystagmus and tetraparesis were respectively cortical and brainstem deficits showed by one 7-month-old dog (dog nº 16) that characterizes the neurological presentation of CDEID. CDEID is the most common form of CDV infection and is often initially characterized by systemic evidence of gastrointestinal and respiratory disturbances (Braund, 1994; Braund, 2001). In our study this clinical syndrome was not the most common due to a bias of selection designed for this study. Neurological signs of CDEID are quite varied, often asymmetrical, and usually suggest a multifocal distribuition of lesions (Shell, 1990; Braund, 2001). Signs of localization in cortical and subcortical (seizures, personality changes) areas, brainstem (cranial nerves deficits) and cerebellum (hypermetria) are often observed, while spinal cord (paresis/paraplegia) signs are observed occasionally. Seizure likely reflects localization of the virus within cerebral cortical neurons. CDV is probably the most common cause of convulsions in young dogs (Braund, 1994). Although the clinical signs of seizures depend on which part of the cerebrum is affected, many seizures observed in distemper dogs are described as “chewing-gum fits” (Shell, 1990). Although systemic disturbances are often observed with CDEID, they may be absent (Braund, 2001), as well showed by the present CDEID case. Myoclonus, a rhythmic jerking of single muscles or muscle groups, is a characteristic sign of distemper encephalomyelitis (Braund, 1994; Braund, 2001), however it was lacking in the CDEID presented. No previous gastrointestinal disturb was reported by the owner. Coughing was related almost one month before the neurological presentation. Nevertheless as no diagnostic procedure was performed at that time, it is difficult to affirm that the CDV was the coughing causative, but this possibility cannot be ruled out.

The owner commonly related an initial history of weakness and ataxia of the hind limbs, and sometimes falling in 4 (cases nº 05, 15, 17, and 30) out of 6 cases recognized as suffering from MDEMD. At the moment of hospital admission or during the clinical follow up, signs of localization in cerebellum (intentional tremor, head and trunk ataxia, and dysmetria with hypermetria) brainstem (vertical positional nystagmus, rotatory spontaneous nystagmus, positional ventrolateral strabismus, head tilt), and spinal cord (paraplegia) were easily recognized in single or various combinations in all the six dogs suffering from this distemper clinical syndrome. Behavioral changes, compulsive walking, and seizures are cortical and subcortical signs that were not observed during the clinical evolution of these animals. In 5 MDEMD cases (nº 05, 15, 17, 18 and 30) the neurological condition progress to tetraparesis/plegia in a recumbent form. In one case (dog nº 15) myoclonus could be verified during the clinical evolution 22 days after the beginning of the neurological deficits. According to the literature myoclonic movements are usually not observed on MDEMD, although head tremors may be seen (Braund, 2001). MDEMD is a type of CDV multifocal encephalomyelitis in mature dogs with relatively low incidence that is characterized by a chronic course and does not appear to be related to breed or sex (Braund, 2001). In our study it was a representative group due to a bias of selection designed for this study. Vaccinated animals may be affected. This neurological manifestation is not preceded by, nor is it coincident with, the systemic signs (Shell, 1990). The initial neurological presentation consists of signs of weakness on the hind limbs, generalized incoordenation, and occasional falling. Usually these signs progress to tetraplegia. During the course of the disease signs of localization in cerebellum, brainstem, and spinal cord are common (Shell, 1990; Braund, 1994). Some animals will have signs of facial paralysis, head tilt, and nystagmus (Braund, 2001). Cortical and subcortical signs such as generalized seizures and

behavioral change are not features of this disease and affected animals maintain a normal mental status (Braund, 1994).

Although MDEMD is a type of CDV multifocal encephalomyelitis (Braund, 2001), two dogs suffering from this CDV encephalomyelitis form were presented with signs suggesting a focal CNS lesion. One dog (nº 22) was presented with focal spinal cord signs (spastic paraplegia). The other one (n° 18) was presented with typical focal cerebellar involvement (truncal and head ataxia, intentional tremor of the head, and dysmetria with severe hypermetria). In the first case the myelography was performed and no compressive lesion was recognized. This dog remained hospitalized and during the clinical follow up, was verified that the signs progress, making possible the recognition of a progressive spinal cord disease. Such cases which are presented with focal spinal cords signs and in which other typical findings such as extraneural signs or myoclonus are absent at the time of presentation, are a diagnostic challenge for the veterinary (Tipold et al, 1992). The literature claims that MDEMD not coincide with systemic signs that are often seen in young dogs. Nevertheless, in our study two dogs with MDEMD showed gastrointestinal signs after the manifestation of the neurological deficits. One of the two dogs (n° 22) also displayed respiratory signs and thorax radiography revealed an interstitial pulmonary density. Nevertheless the etiology remains unclear since no diagnostic procedure was performed to rule out other causes of these systemic signs.

In cases of distemper encephalomyelitis presented without myoclonus and extra-neural signs at the moment of hospital admission, such as the dogs evaluated in this study, the clinical diagnosis of distemper is often difficult (Vandevelde and Cachin, 1993; Tipold et al, 1992). In such cases a laboratory assay may be required to confirm the disease. A final *ante mortem* diagnosis of distemper is based on the demonstration of viral antigens in scrapings and body

fluids such as conjunctival and vaginal smears, tracheal washing, and urine sediment (Tipold et al., 1992). For this, direct immunofluorescence test are routinely and widely used. Unfortunately in the subacute or chronic forms of the disease, this test gives false-negative results (Jóźwik and Frymus, 2005). According Tipold et al. (1992) viral antigen may also be hard to find in the extraneural tissues in cases with neurological distemper without systemic signs. Serological examination has not been very useful in the diagnosis of distemper, because a high titre of anti-CDV antibodies may be a result of prior vaccination, as well as of previous subclinical or clinical infection. On the other hand, during severe distemper, the antibody titre may be low because of the strong immunosuppressive properties of CDV. The methods available for *ante mortem* distemper diagnosis to date are of limited value, and in the majority of cases a definitive diagnosis is only possible at *post mortem* (Baumgärtner, 1993). Recently we observed that urine is a sensitive biological sample for *ante mortem* CDV detection by RT-PCR in dogs with CDV encephalomyelitis in which the distemper clinical diagnosis were not possible to be ideally performed due to absence of common and characteristics findings of the disease (myoclonus and systemic involvement concomitant to neurological disease). In 4 out of 5 dogs with CDV encephalomyelitis the virus could be detected in urine by RT-PCR (Amude et al., 2005).

Although extraneural signs and myoclonus may be frequent in CDV infection, a neurological presentation in the absence of these signs can potentially be a canine distemper clinical presentation, such as ODE, CDEID, or MDEMD. As distemper is an infectious disease with poor prognosis in which there is no effective antiviral treatment, early and secure diagnosis of CDV infection is important, and the knowledge about clinical syndromes in which the common and typical signs may be lacking may facility the clinical diagnosis of distemper encephalomyelitis in such cases.

Table 1 – Dogs with distemper (N = 08) attending at Veterinary Hospital of Universidade Estadual de Londrina from november 2003 to november 2004, and their respective data: (age, breed, sex, survive, and vaccination records).

dog	age (month)	breed	Sex	Survive (days)	Vaccination records
05	36	Mixed breed	F	12	Unvaccinated
13	132	Mixed breed	F	31	Unvaccinated
15	62	Collie	F	32	Vaccinated
16	7	Mixed breed	M	15	Vaccinated
17	18	German shepherd	M	33	NA
18	21	Cocker spaniel	M	7	NA
22	145	Cocker spaniel	F	10	Vaccinated
30	55	Boxer	M	22	Vaccinated

NA: not available

Table 2 – Clinical syndromes and respective neurological deficits during the clinical evolution showed by the dogs with distemper (N = 08) attending at Veterinary Hospital of Universidade Estadual de Londrina from november 2003 to november 2004, diagnosed at *post mortem* by RT-PCR and histopathological examination.

Clinical syndrome	dog	Neurological deficits
Old dog encephalitis (ODE)	13	Seizure-like activity reported by the owner, personality change (aggressiveness), alert but with an inappropriate consciousness contend, compulsive walking, circling to the right and to the left, truncal and head ataxia[#], head pressing, postural reactions deficits, mild hypermetria (hyperkinesia), tetraparesis.
Canine distemper encephalomyelitis in immature dogs (CDEID)	16	Compulsive walking, focal seizure with secondary generalization, inappropriate consciousness contend, positional vertical nystagmus, menace deficit, spastic tetraparesis, postural reactions deficits, decerebelate rigity[&].
Multifocal distemper encephalomyelitis in mature dogs (MDEMD)	05	Weakness of hind limbs, spastic tetraparesis/plegia, postural reactions absence, truncal ataxia, head ataxia, rotatory spontaneous bilateral nystagmus, vestibular strabismus[*].
	15	Weakness of the hind limbs, truncal ataxia, head ataxia, vertical positional nystagmus, postural reactions deficits, spastic tetraparesis/plegia, myoclonus in the left hind limb and mastigatory muscle.
	17	Weakness of the hind limbs, truncal ataxia, head ataxia, spastic tetraparesis/plegia, postural reactions deficits, pain to spinal cord palpation.
	18	Truncal ataxia, head ataxia, spastic tetraparesis, positional vertical nystagmus with change to rotatory with change in the head positions, severe hypermetria, postural reactions deficits, decerebelate rigity.
	22	Spastic paraplegia following to flaccid paraplegia, postural reactions absent in the hind limbs.
	30	Ataxia, head tilt to the right, spastic tetraparesis/plegia, positional horizontal nystagmus, postural reactions deficits.

Head ataxia: intentional tremor of the head

[&] Decerebelate rigity: opisthotonos with spasticity and rigidity of thoracic limb

*Vestibular strabismus: positional ventrolateral strabismus when the head was dorsally extended

REFERENCES

1. Amude, A.M., Alfieri, A.A. and Alfieri A.F., 2005. *ante mortem* Diagnosis of CDV Infection by RT-PCR in Distemper Dogs with Neurological Deficits without the Typical Clinical Presentation. *Veterinary Research Communication* "in press".
2. Axthelm, M.K. and Krakowka, S., 1998. Experimental Old Dog Encephalitis (ODE) in a Gnotobiotic Dog. *Veterinary Pathology*, 35, 527-535.
3. Baumgärtner, W., 1993. Virale Infektionskrankrankheiten bei Junghunden. *Praktischer Tierarzi*, 1, 26-32.
4. Baumgärtner, W., Örrvell, C. and Reinacher, M., 1989. Naturally occurring canine distemper virus encephalitis: distribuition and expression of viral polypeptides in nervous tissues. *Acta Neuropathologica,* 78, 504-512.
5. Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, P.M.E., Werthein-van Dillen and Noordaa-Vader, J., 1990. Rapid and simple method for purification of nucleic acids, *Journal of Clinical Microbiology*, 28, 495-503.
6. Braund, K.G., 2001. *Inflammatory Diseases of the Central Nervous System*. Available at: www.ivis.org. Accessed Nov 11, 2001.
7. Braund, K.G., 1994. *Clinical Syndromes in Veterinary Neurology*. 2nd ed. St Louis, MO: Mosby.
8. Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, V. and Ball, L.A., 2004. *Virus Taxonomy*. The light report. San Diego: Academic Press.
9. Frisk, A.L., Konig, M., Moritz, A. and Baumgärtner, W., 1999. Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. *Journal of Clinical Microbiology*, 37, 3634-3643.

10. Gebara, C.M.S., Wosiacki, S.R., Negrão, F.J., Alfieri, A.A. and Alfieri, A.F., 2004a. Lesões histopatológicas no sistema nervoso central de cães com encefalite e diagnóstico molecular da infecção pelo vírus da cinomose canina. *Arquivos Brasileiros de Medicina Veterinária e Zootecnia,* 56, 168-174.
11. Gebara, C.M.S., Wosiacki, S.R., Negrão, F.J., de Oliveira, D.B., Beloni, S.N.B., Alfieri, A.A. and Alfieri, A.F., 2004b. Detecção do gene da nucleoproteína do vírus da cinomose canina por RT-PCR em urina de cães com sinais clínicos de cinomose. *Arquivos Brasileiros de Medicina Veterinária e Zootecnia*, 56, 480-487.
12. Greene, G.E. and Appel, M.J., 1998. Canine distemper. In: G.E. Greene (eds), *Infectious Diseases of dog and the cat*, 2nd edn, (W.B.Saunders, Philadelphia), 9-22.
13. Jóźwik, A. and Frymus, T., 2005. Comparison of the Immunofluorescence Assay with RT-PCR and Nested PCR in the Diagnosis of Canine Distemper. *Veterinary Research Communications*, 29, 347-359.
14. Kim, Y.H., Cho, K.W., Youn, H.Y., Yoo, H.S. and Han, H.R., 2001. Detection of canine distemper virus through one step RT-PCR combined with nested PCR. *Journal of Veterinary Science*, 2, 59-63.
15. Koutinas, A.F., Polizopoulou, Z.S., Baumgärtner, W., Lekkas, S. and Kontos, V., 2002. Relation of clinical sings to pathological changes in 19 cases of canine distemper encephalomyelitis. *Journal of the Comparative Pathology*, 126, 47-56.
16. Meertens, N., Stoffel, M.H., Cherpillod, P., Wittek, R., Vandevelde, M. and Zurbriggen, A., 2003. Mechanism of reduction of virus release and cell-cell fusion in persistent canine distemper virus infection. *Acta Neuropathologica*, 106, 303-310.

17. Moritz, A., Frisk, A.L. and Baumgärtner, W., 2000. The evaluation of diagnostic procedures for the detection of canine distemper virus infection. *European Journal of Companion Animal Practice*, 10, 37-47.

18. Moro, L., Martins, A.S., Alves, C.M., Santos, F.G.A., Del Puerto, H.L. and Vasconcelos, A.C., 2003. Apoptosis in the Cerebellum of Dogs with Distemper. *Journal of Veterinary Medicine Series B*, 50, 221-225.

19. Muller, C.F., Fatzer, R.S., Beck, K., Vandevelde, M. and Zurbriggen, A., 1995. Studies on canine distemper virus persistence in the central nervous system. *Acta Neuropathologica*, 89, 438-445.

20. Rzeżutka, A. and Mizak, B., 2002. Application of N-PCR for diagnosis of distemper in dogs and fur animals. *Veterinary Microbiology*, 88, 95-103.

21. Saito, T.B., Alfieri, A.A., Wosiacki, S.R., Negrão, F.J., Morais, H.S.A. and Alfieri, A.F., 2005a. Detection of canine distemper virus by reverse transcription-polimerase chain reaction in the urine of dogs with clinical signs of distemper encephalitis. *Research in Veterinary Science*, 79, "in press".

22. Saito, T.B., Alfieri, A.A., Wosiacki, S.R., Negrão, F.J., Morais, H.S.A. and Alfieri, A.F., 2005b. *Ante* and *Post mortem* detection of canine distemper virus by RT-PCR in dogs with distemper. *Archivos de Medicina Veterinaria*, 37, "in press".

23. Shin, Y. J., Cho, K. O., Cho, H. S., Kang, S. K., Kim, H. J., Kim, Y. H., Park, H. S. and Park, N. Y., 2004. Comparison of one-step RT-PCR and nested PCR for the detection of canine distemper virus in clinical samples. *Australian Veterinary Journal*, 82, 83-86.

24. Shin, Y., Mori, T., Okita, M., Gemma, T., Kai, C. and Mikami, T., 1995. Detection of canine distemper virus nucleocapsid protein gene in canine peripheral blood mononuclear cells by RT-PCR. *Journal of Veterinary Medical Science*, 57, 439-445.

25. Summers, B.A. and Appel, M.J., 1994. Aspects of canine distemper virus and measles virus encephalomyelitis, *Neuropathology and Applied Neurobiology*, 20, 525-534.

26. Summer, B.A., Cummings, J.F. and de Lahunta, A., 1995. *Veterinary Neuropatology*. St Louis: Mosby.

27. Schobesberger, M., Zurbriggen, A., Doherr, M.G., Weissenböck, H., Vandevelde, M., Lassman, H. and Griot, C., 2002. Demyelination precedes oligodendrocyte loss in canine distemper virus-induced encephalitis. *Acta Neuropathologica*, 103, 11-19.

28. Shell, L.G., 1990. Canine Distemper. *Compendiun on Continuing Education for the Practicing Veterinarian*, 12, 173-179.

29. Tipold, A., Vandevelde, M. and Jaggy, A., 1992. Neurological manifestation of canine distemper virus infection. *Journal of Small Animal Practice*, 33, 466-470.

30. Tipold, A., Jaggy, A., Zurbriggen, A. and Vandevelde, M., 1996. Neurological signs in canine distemper encephalomyelitis – a clinical study. *European Journal of Companion Animal Practice*, 7, 33-38.

31. Vandevelde, M. and Cachin, M., 1993. The Neurologic Form of Canine Distemper. In: J.D. Bonagura and R.W. Kirk (eds), *Kirk's Current Veterinary Therapy XI, Small Animal Practice*. Philadelphia: W.B. Saunders, 1003-1007.

32. Vandevelde M, and Zurbriggen A., 1995. The neurobiology of canine distemper virus infection. *Veterinary Microbiology*, 44, 271-280.

33. Vandevelde, M., Kristensen, B., Braund, K. G., Greene, G. E., Swango, L. T. and Hoerlein, B. F., 1980. Chronic Canine distemper virus encephalitis in mature dogs. *Veterinary Pathology*, 17, 17-28.

Distemper encephalomyelitis-clinicopathological findings in eight distemper dogs presented exclusively with the neurological disease

Abstract

The aim of this study was to evaluate the hematological parameters, the results of cerebrospinal fluid (CSF) examination, the findings on central nervous system (CNS) histopathological section, as well the results of the neurological examination, from dogs with CDV encephalomyelitis in which the distemper clinical diagnosis were not possible to be accomplished at the time of presentation due to absence of the typical distemper clinical findings, and verify if and how these laboratorial and clinical findings contribute to an etiologic diagnosis. It was prospectively investigated 20 necropsied dogs which had been presented with neurological deficits without myoclonus and in absence of systemic signs at the time of hospital presentation, in which the hemogram, CSF evaluation, and neurological follow up were performed *ante mortem*. Eight out of 20 dogs were diagnosed with distemper encephalomyelitis at *post mortem* by reverse transcription – polymerase chain reaction (RT-PCR) and histothological examination. Cerebellar and/or vestibular signs progressing to tetraparesis/plegia was a neurological evolution frequently observed in the dogs with distemper encephalomyelitis. Changes in hematological parameters were frequently absent or non-specific, nevertheless the CSF evaluation could suggest CDV infection by a lymphocytic pleocytosis. At *post mortem*, demyelinating lesions was a constant finding in CNS sections and chronic CDV encephalomyelitis was predominant among distemper dogs.

Key words: dog; canine distemper virus; distemper, encephalomyelitis, hemogram, CSF, histopathology, RT-PCR.

INTRODUCTION

Different causes such as, degenerative, metabolic, autoimmune, nutritional, inflammatory, infectious, toxic and vascular, are able to lead to neurological disease. In dogs, among infectious etiologies, virus infection such as canine distemper virus (CDV) may be involved on the pathogenesis of lesions in the central nervous system (CNS) (Braund, 2001).

The CDV is a non-segmented, negative single-stranded RNA virus, closely related to measles virus and rinderpest virus, two other members of the genus *Morbillivirus* of the *Paramyxoviridae* family (Fauquet et al., 2004). Gastrointestinal and/or respiratory signs, frequently with CNS involvement, characterize the classical clinical presentation of distemper. However this classical presentation is not the rule. Nervous signs can occur without any other systemic sign (Baumgärtner et al., 1989). Authors about nervous distemper have been claiming that the typical systemic findings, such as respiratory and gastrointestinal involvement, may be absent (Vandevelde and Cachin, 1993), and myoclonus, a common and characteristic sign, may be not present (Tipold et al, 1992). In such cases presented without myoclonus and extra-neural signs, the clinical diagnosis of distemper is often difficult and may be a challenge for the veterinary (Vandevelde and Cachin, 1993; Tipold et al, 1992).

A final *ante mortem* diagnosis of distemper is based on the demonstration of viral antigens in scrapings and body fluids such as conjunctival and vaginal smears, tracheal washing, and urine sediment (Tipold et al., 1992). For this, direct immunofluorescence test are routinely and widely used. Unfortunately, this assay can confirm distemper only within 3 weeks after infection, because after this time the virus disappears from the epithelial cells. Thus, in the subacute or chronic forms of the disease, this test gives false-negative results (Jóźwik and Frymus, 2005). According to Tipold et al. (1992) viral antigen may also be hard to find in the extraneural tissues

in cases of nervous distemper without systemic signs. Serological examination has not been very useful in the diagnosis of distemper, because a high titre of anti-CDV antibodies may be a result of prior vaccination, as well as of previous subclinical or clinical infection. On the other hand, during severe distemper, the antibody titre may be low because of the strong immunosuppressive properties of CDV. The methods available for *ante mortem* diagnosis of distemper to date are of limited value, and in the majority of cases a definitive diagnosis is only possible at *post mortem* (Baumgärtner, 1993). For *post mortem* diagnosis of CDV encephalomyelitis, detection of CDV antigen in CNS sections by immunohistochemistry has been a specific method. However in chronic CDV encephalomyelitis lesions, viral antigen may disappear from the inflammatory demyelinating lesions due to the antiviral immune responses (Mueller et al., 1995). Koutinas et al. (2002) found negative immunohistochemistry in a CNS from a dog with chronic CDV encephalomyelitis.

Considering the infectious potential of the disease, the suspicion of distemper may be very important in order to not hospitalizing the animal. Besides this, the diagnosis of CDV infection is essential for the prognostic evaluation of affected individuals, and for ruling-out of differential diagnosis, especially in animals with neurological disease. The knowledge about possible clinicopathological presentation of CDV encephalomyelitis, particularly in cases in which the clinical diagnosis is difficult due to absence of myoclonus and systemic signs simultaneously with the neurological disease at the time of clinical presentation, is important for distemper clinical recognizing and *ante* and *post mortem* diagnosis. The aim of this study was to evaluate the hematological parameters, the result of cerebrospinal fluid (CSF) examination, the findings on CNS histopathological section, as well the results of the neurological examination, from dogs with CDV encephalomyelitis diagnosed at *post mortem* by RT-PCR and histopathology, in which the distemper clinical diagnosis were not possible to be accomplished at the time of presentation

due to absence of the distemper typical clinical findings such as myoclonus and extra-neural signs, and verify if and how these parameters contribute to an etiologic diagnosis.

MATERIAL AND METHODS

Inclusion criteria

Dogs presented to the Medical and Surgical division from Veterinary Teaching Hospital of Universidade Estadual de Londrina, Paraná, Brazil, from november 2003 to november 2004, with neurological deficits without myoclonus and absence of systemic signs at the time of hospital admission, in which the hemogram and CSF evaluation were performed *ante mortem*, were prospectively follow up during the clinical evolution. As systemic signs were considered fever, gastrointestinal and respiratory signs. Ophthalmic evaluation was not considered. Dogs in which systemic signs and/or myoclonus was observed after the hospital admission were not excluded from this investigation. We excluded the animals in which the myelography evaluation suggested compressive lesion, and that a traumatic or toxic event could be recognized as the cause of the neurological presentation. For this study were included dogs in which were possible to obtain CNS samples corresponding to the neurological signs at necropsy.

Animals and clinical samples.

The dogs were accompanied from the hospital admission until the clinical outcome (euthanasia or natural death, despite treatment), and all the data about neurological evolution were recorded. Euthanasia, when elicited, was performed by intravenous infusion of 10% KCl, under general anesthesia preceded by tranquilization. The owner's consent was obtained before both, investigation inclusion and clinical samples collection.

The dogs included in this clinicopathological study were not submitted to any stressing or suffering proceeding. The clinical decision, medical treatment, or other diagnostic procedures were performed by the attending veterinary. The collections of samples at *ante mortem* (blood and CSF) were performed by attending veterinary during the hospital routine procedure, while the collections of samples at *post mortem* (CNS fragments corresponding to the neurological signs) were done during the necropsy.

Diagnostic criteria

The distemper encephalomyelitis diagnosis was carried out at *post mortem*, through RT-PCR for CDV detection in the fresh CNS fragment and CNS histopathology. The histopathological findings considerate as suggestive of CDV infection in the CNS was focal to multifocal vacuoles in the white matter (demyelination) with or without inflammatory involvement.

The dogs, which CDV could be excluded as causative of the neurological signs at *post mortem* were grouped and used for comparative propose, since they were from the same hospital population and shared the same inclusion criteria.

RT-PCR

For CDV detection by RT-PCR, RNA was extracted from an aliquot of 300 μL of suspension (10% w/v) of fresh CNS fragments in PBS (phosphate buffered saline), according to the silica/guanidine isothiocyanate method, described by Boom et al. (1990). Aliquots of ultrapure (MilliQ®) sterile water were included as negative control in all the RNA extractions. The RNA extraction was performed immediately after the CNS collection.

RT-PCR was performed using the oligonucleotides primers (Invitrogen™Life Technologies, USA) CDV 1 (sense) [5'-aca gga ttg ctg agg acc tat-3', nt 769-789] and CDV2 (anti-sense) [5'-caa gat aac cat gta cgg tgc-3', nt.1055-1035], designed to amplify an amplicon of 287 bp size of the CDV nucleoprotein gene (Frisk et al., 1999).

The reverse transcription was performed with 9 μL of RNA extract and 2.0 pmol of CDV1 that was denaturated at 70°C for 10 min and immediately transferred to ice for 5 min. Following this step was added the RT-MIX solution, made of 0.2 mM of each dNTP (Invitrogeies™ Life Technology, USA), 1x PCR-buffer (Invitrogeies™ Life Technology, USA) (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 1.5 mM $MgCl_2$, 100 units of reverse transcriptase enzyme M-MLV (Invitrogeies™ Life Technology, USA) and ultrapure sterile water to a final volume of 20 μl. After homogenization, the solution was incubed at 42°C for 30 min followed by the enzyme inactivation at 70°C for 10 min.

For the PCR reaction were used 5 μL of cDNA, 0.4 pmol of each primers (CDV1 and CDV2), 0.2 mM of each dNTP, 1x PCR-buffer (20 mM Tris-HCl pH 8,4 e 50 mM KCl), 1,5 mM $MgCl_2$, 2,5 units of Platinum® Taq DNA Polymerase (Invitrogen™ Life Technologies, USA) and ultrapure sterile water to a final volume of 50 μL. The reaction was realized in a thermocycler (PTC 200, MJ Research Co. Water Town, Ma, USA), using the following time and temperature

conditions: i) a initial denaturation step at 94 °C for 1 min; ii) 40 cycles at 94ºC for 1 min, 59ºC for 2 min and 72°C for 1 min; iii) a final extension step at 72°C for 7 min. A CDV Rockborn strain infected Madin Darby canine kidney (MDCK) cell was used as CDV positive control.

The amplified products were analyzed by electrophoresis in 2% agarose gel with ethidium bromide (0,5 μg/mL) in TBE buffer pH 8.4 (89 mM Tris-HCl; 89 mM boric acid; 2 mM EDTA) in constant voltage (90 V) for approximately 45 min and visualized under UV light.

The identities of the RT-PCR products were confirmed by RFLP (Restriction fragment length polymorphism) with *Hinf* I (Invitrogen™Life Technology, USA) enzyme digestion. The digestion was performed with 18 μL of RT-PCR sample, according manufacture's instructions.

Histopathology

CNS tissue sections were examined for CDV-induced lesions. The CNS fragments corresponding to the signs were fixed in 10% buffered neutral formalin, embebed in histological paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (HE), following routine procedures.

Hematological examination and CSF analysis

The blood obtained was treated with 10 % EDTA as an anticoagulant. The hematological parameters [hematocrit (Hct), red blood cell count (RBC), white blood cell count (WBC), and hemoglobin contend] were determined by an automated hematology instrument (computer analyseur d' hematologie MS4, Melet Schloesing Laboratories, France). The differential leukocyte count was performed by manual method under light microscopy using blood smear stained with Diff-Quik (Laborclin, Pinhais, PR, BR).

The CSF was collected from cerebellomedulary cistern under general anesthesia. Biochemical and cytological evaluation were performed. The CSF cells total number was count in a hemocytometer. Biochemical evaluation consisted of protein quantification that was performed by colorimetric method. One milliliter of CSF was centrifuged at low speed for 5 min. The supernatant was withdrawn with a pipette and subsequently used for protein quantification. A drop of serum was added to the cell pellet, and this mixture was then gently agitated. One drop was gently smeared on a glass slide and stained with Diff-Quik and observed under light microscopy.

RESULTS

Twenty dogs clinically followed up, in which were possible to obtain CNS samples corresponding to the neurological signs at necropsy, were evaluated in this study. In 17 dogs the owner elicited euthanasia, and the other 3 dogs died during the hospitalization period. According to the diagnostic criteria 8 out of 20 dogs were considered as suffering from distemper encephalomyelitis. The results of the non-distemper dogs can be found in the tables (tables 2 and 4).

Neurological signs and clinical history

The neurological signs observed in distemper dogs, either singly or in various combinations, were behavioral changes, compulsive walking, circling, vertical positional nystagmus, horizontal positional nystagmus, rotatory spontaneous nystagmus, positional ventrolateral strabismus (when the head was dorsally extended), head tilt, seizure, intentional tremor of the head, head and trunk ataxia, tetraparesis/plegia, paraplegia, opisthotonos with spasticity and rigidity of thoracic limb, myoclonus, spinal hiperesthesia, and dysmetria with hypermetria (table 1). Although myoclonus was absent at the time of admission, one dog (case 15) manifests it 22 days after hospital presentation, during the clinical evolution. The onset of neurological signs was acute in two distemper dogs (cases 18 and 22) and gradual with a progressive course in the remaining 6 distemper dogs (cases 05, 13, 15, 16, 17, 30), however the nervous signs were progressive and multifocal in all eight dogs. Distemper dogs were 59.4 month old on average (the age range from 7 month to 13 years old). Four animals had received 3 or more vaccination against distemper with life modified virus (nº 15, 16, 22, and 30), two were unvaccinated dogs (nº 05 and 13), while the other two dogs had an unknown vaccination status (nº 17 and 18).

CDV detection by RT-PCR

The RT-PCR in fresh CNS samples from 8 (40%) out of 20 dogs (n° 05, 13, 15, 16, 17, 18, 22, 30) amplified a fragment of 287 bp size that was cleaved by *Hinf* I as expected to the CDV, and yielded fragments of 227 and 60 bp size.

Histopathology

All the dogs which CDV could be detected in fresh CNS samples by RT-PCR showed histopathological lesions compatible with CDV encephalomyelitis, and none of them showed cysts of *Toxoplasma gondii* or *Neospora caninum* in the CNS fragments examined. In one dog (n° 22) the histopathological examination could not be performed because the fixed samples were lost during the procedure, nevertheless the CDV could be identified by RT-PCR in the CNS. None of the dogs (the remaining 12 dogs) in which the CDV was not detected in the CNS by RT-PCR assay showed lesions compatible to CDV infection. The frequent histopathological findings in the CNS of the distemper dogs consisted of focal to multifocal vacuoles in the white matter, perivascular mononuclear cell infiltration, mononuclear cells infiltrated diffusely into the parenchyma, malacia, glial reaction, and perimeningeal mononuclear infiltrate. These findings were observed in the CNS fragment corresponding to the neurological signs in various combinations. According to signs of demyelination and inflammation affected areas were grouped in acute or chronic CDV encephalomyelitis. These foci were located in the white matter of the sections. Two dogs were suffering from the acute non-inflammatory CDV encephalomyelitis (nº 13, 16), while the other was suffering from chronic inflammatory CDV encephalomyelitis (nº 05, 15, 17, 18, 30) (table 3). Acute lesions were characterized by demyelination with focal to multifocal vacuolation of the white matter. Mononuclear cells infiltrated into the parenchyma and perivascular cell infiltrates were absent. There were an increase number of reactive astrocytes, macrophages and microglial cells. Occasionally, scattered lymphoid cells were found around blood vessels. In chronic lesions the invasion of inflammatory cells was prominent. Lymphocytes accumulated around blood vessel forming partly multi-layered perivascular cuffs. Immune cells also diffusely invaded the parenchyma. The demyelinating lesions could be observed as either a marked spongy state with relative small vacuoles or more

diffuse with large vacuoles, probably due to tissue edema. Severe destruction of the white matter with large areas of malacia could be also observed.

Hemogram and CSF evaluation

Anemia, neutropenia, neutrophilia, and lymphopenia were hematological abnormalities found singly or in combination on the hemogram of 5 out of 8 dogs with distemper encephalomyelitis. In three out of 8 distemper dogs, no hematological abnormalities were observed. Lymphocytic pleocytosis characterized the abnormality in the CSF from all (8/8) distemper dogs. The hematological examination and CSF evaluation from distemper dogs are presented in the table 3.

DISCUSSION

The RT-PCR is a usefulness, fast, sensitive, and specific method for detection of CDV infection in dogs (Frisk et al., 1999; Moritz et al., 2000; Kim et al., 2001; Rzeżutka and Mizak, 2002; Gebara et al., 2004a/b; Shin et al., 2004; Jóźwik and Frysmus, 2005; Amude et al., 2005). The set of NP gene specific primers included in the present study was previously used, for CDV detection, by different authors (Frisk et al., 1999; Moritz et al., 2000; Jóźwik and Frysmus, 2005). Since the NP is required for virus replication, the mRNA of the NP gene is transcribed most extensively in infected cells (Shin et al., 1995) and seems to be a good marker of infection. The histopathological findings in the CNS of dogs with distemper molecular diagnosis were compatible with CDV encephalomyelitis (Vandeveld and Zurbriggen, 1995; Summer et al., 1995; Schobesberger et al., 2002; Gebara et al., 2004a). The RT-PCR and RFLP results, associated with the histopathological findings, support that the CDV was the etiological agent causative of the neurological signs in 8 (40%) out of 20 dogs included in this study.

As the CDV may affects both white and gray matter within the CNS (Summer et al., 1995) a variety of neurological signs may be observed, including behavioral changes, seizures, cerebellar and vestibular signs, visual deficits, paresis, paralysis, and myoclonus (Shell, 1990; Tipold et al, 1992; Vandevelde and Cachin, 1993; Tipold et al, 1996). Considering the neurological signs observed during the neurological examination, at the time of admission and through the clinical follow up, of the dogs with distemper encephalomyelitis of this study, vertical positional nystagmus, horizontal positional nystagmus, rotatory spontaneous nystagmus, and positional ventrolateral strabismus when the head was dorsally extended, were vestibular signs. Truncal ataxia, head ataxia such as intentional tremor, and hypermetria were common cerebellar sings. Opisthotonos with spasticity and rigidity of thoracic limb was also verified in 2 distemper dogs and is known as decerebelate rigidity. This signs take place with rostral cerebellar lesion (Braund, 1994), and has been a common sign typically observed with acute cranial trauma or during the neurological progression of brain edema after the cranial trauma (Dewey et al., 1992). In our clinical study the decerebelate rigidity was manifested mainly after clinical manipulation. Tudury et al. (1997), studding distemper in dogs with systemic and neurological involvement suggestive of CDV infection, verified that vestibular and cerebellar signs, had very low frequency in dogs with CDV encephalomyelitis presented with the classical clinical findings of distemper. Similar results also were mentioned by Parker (1978). In our investigation, studing the cases of distemper encephalomyelitis presented without myoclonus and systemic disturbs, cerebellar and vestibular signs were frequents. Truncal ataxia and head ataxia such as intentional tremor of the head, were not only frequent cerebellar signs, like as a neurological parameter that suggested the distemper encephalomyelitis clinical diagnosis in our opinion. These signs were found in 62.5% (5/8) of distemper dogs, while in the group used for comparative purpose it had a low frequency [16.66% (2/12)]. Greene and Appel (1998) and Shell (1990) also state that cerebellar and

vestibullar signs are common with CDV infection. According to the literature, there is a CDV lesion apparent predilection for the central white matter of the cerebellum, and cerebellar peduncles (Summer et al., 1995). Vestibular signs may take place with lesions in cerebellar peduncles (Braund, 1994). Nystagmus, a vestibular sign, was also frequent [62% (5/8)] in our dogs with distemper encephalomyelitis. Nevertheless the frequency of other typical vestibular signs, such as head tilt [12.5% (1/8)], not matched with nystagmus frequency. In the group used for comparative purpose the nystagmus frequency was not so high, however both, nystagmus and head tilt had a similar frequency [33.33% (4/12)]. Cerebellar and/or vestibular signs progressing to tetraparesis/plegia were also a neurological evolution that suggested the clinical diagnosis of distemper encephalomyelitis in our study, since this clinical evolution took place in 87.5% (7/8) of distemper dogs, while it was only observed in 25% (3/12) of non distemper dogs (data not showed).

In our study it was possible to recognize histopathological findings compatible with acute CDV demyelination in two cases, while the others were compatible with the chronic CDV encephalomyelitis. The acute CDV encephalomyelitis develops during a period of severe immunosuppression and is not inflammatory (Vandevelde and Zurbriggen, 1995). This mechanism of demyelination have been examined and it was shown that the initial lesions is directly virus-induced, since there is a clear correlation between the occurrence of demyelination and the CDV replication in the cells of the white matter (Vandevelde et al, 1985). Although the literature claim that this encephalomyelitis form develops during severe immunosuppression (Vandevelde and Zurbriggen, 1995), the dogs suffering from acute demyelination were not considered as undergoing an immunosuppression period since they were not lymphopenic in none of the hematological examination performed. The chronic CDV lesions are characterized by influx of inflammatory cells, mostly mononuclear, and coincide with the recovery of the immune

system (Vandevelde and Zurbriggen, 1995). The inflammatory cells accumulate around blood vessels forming partly multi-layered perivascular cuffs, and also invaded the parenchyma (Schobesberger et al, 1999; Schobesberger et al, 2002). The inflammatory reaction in the demyelinating lesions can lead to progression of the tissue damage. Although chronic CDV lesions coincide with recovery of the immune system (Vandevelde and Zurbriggen, 1995), 3 out of 5 dogs suffering from chronic encephalomyelitis were lymphopenic, even in the last examination immediately before the euthanasia. The predominance of early (acute) distemper encephalitis over late (chronic) lesions, has been observed by some author (Vandevelde et al., 1981; Palmer et al., 1990). Headley et al (2001) also observed this pathological finding and suggested that early lesions are probably the most frequent occurring forms of distemper encephalitis under natural conditions. However, in our study with naturally infected dogs, the late lesions compatible with the chronic CDV encephalomyelitis were predominant, probably due to a bias of selection. The spectrum of the CDV lesions appears to be wide, nevertheless the neuropathology of spontaneous distemper is remarkably constant. The variability of the neuropathology is largely due to the lesions evolution when the disease progress (Vandevelde and Zurbriggen, 1995; Schobesberger et al., 2002). Headley et al. (2001), as well as several authors, had been studying naturally occurring distemper mainly in immature dogs, since the prevalence of the disease is high in this population and it's the most common form of CDV infection (Braund, 1994). Immature dogs infected with CDV are more prone do develop seizure. This cortical and subcortical sign in distemper is of difficult control and may lead to death (Shell, 1990). Because of this clinical condition, the main of the distemper dogs studied by Headley et al. (2001), Vandevelde et al. (1981), and Palmer et al. (1990), died or were euthanized early during the disease progression, and it is possible that the late lesions compatible with chronic CDV encephalomyelitis did not have time to occur. In addition to acute or chronic CDV demyelination,

there are very early findings in which no demyelination is noted, although the CDV infection may be identified. Recently Schobesberger et al (2002) introduced the term of peracute lesions to consider this category. In our study peracute lesions were not verified through histopathological examination on the CNS fragments collected, being the demyelinating lesions a constant parameter.

Different abnormalities were found in the hematological examination of dogs with distemper encephalomyelitis. However these abnormalities could not be used as parameters for CDV infection since they were also frequently present in the hemogram of the group used for comparative purpose. Nevertheless in 3 out of 8 distemper dogs the hematological parameters were within normal limits. In experimental infected animals, frequent hematological findings are lymphopenia, sometimes combined with leukopenia or leukocytosis with left shift, anemia, and rarely thrombocytopenia. Several hematological abnormalities have been reported with distemper in natural occurring disease (Moritz et al, 2000). Tudury et al (1997), studding the nervous form of distemper, reported that anemia and lymphopenia were frequent hematological changes found in cases of distemper encephalomyelitis. However the previous cited studies were descriptive studies and a cause-consequence relationship could not be secured. According Shell (1990), the hematological parameters are frequently absent or nonspecific in dogs with CDV naturally infected. In a study, when the hematological findings from distemper dogs were compared with data from non-distemper dogs, no significant statistic difference was found (Gebara et al, 2004b). Lymphopenia has been widely used in the veterinary hospital routine to incriminate distemper as a potential diagnosis in dogs suffering from neurological disturbs. According our results, this hematological finding interpretation should be done with caution, since lymphopenia had a similar frequency in distemper (3/8) and no-distemper dogs (4/12), both suffering from neurological deficits. The practicing veterinary need to take care with this hematological finding

and attend to the fact that the mainly ill dogs are undergoing to a stressing period. Stress is a potential cause of lymphopenia in dogs.

The abnormalities in the CSF from all dogs with CDV encephalomyelitis consisted of pleocytosis with lymphocytes predominance. The total protein was within normal limits. When the typical clinical findings, such as systemic signs and myoclonus are lacking at the time of hospital admission, the clinical diagnosis of distemper encephalomyelitis is difficult (Tipold et al, 1992; Vandevelde and Cachin, 1993), and laboratorial procedures are required to suspect CDV infection. Examination of the CSF can be a very useful diagnostic procedure in distemper encephalomyelitis. CSF analysis may support the diagnosis of CDV infection if a lymphocytic pleocytosis (greater than 5 leukocytes/µl) is present (Shell, 1990). A CSF pleocytosis that is more than 60% lymphocytes has been typically associated with viral encephalitis, but has also been reported with granulomatous meningoencephalitis (GME) and bacterial infection especially after antibiotic therapy (Chrisman, 1992). It has been claimed that a mild to moderate pleocytosis (5-100 WBC/µl) is frequently associated with CDV infection (Braund, 1994) and CSF analysis in distemper often yields less dramatic pleocytosis (less than 25 cells/µl) than GME (Bailey and Higgins, 1986). Mild pleocytosis (10 - 45 cells/µl) were found in 6 (75%) (n° 05, 13, 15, 18, 22, 30) out of 8 cases of distemper encephalomyelitis, and moderate pleocytosis (61 cells/µl) in 1 (12.5%) (n° 17) case. The remaining case (n° 16) revealed a severe pleocytosis (544 cells/µl). This last CSF finding is an unusual result for CDV infection. Severe pleocytosis with lymphocytes predominance has been often associated with GME (Chrisman, 1992; Ryan et al., 2001). Not all dogs infected with CDV, however, will have changes in CSF (Shell, 1990). Tipold et al. (1996) reported that in 3 out of 40 CDV encephalomyelitis cases the CSF was normal. Koutinas et al. (2002) also reported normal CSF in 8 out of 19 distemper encephalomyelitis dogs.

The CSF results from the distemper dogs were abnormal in all the studied cases of the present study. The CSF pleocytosis showed by all distemper dogs might suggest an inflammatory involvement, however 2 cases were suffering from the non-inflammatory CDV encephalomyelitis. The dog which showed the greater lymphocytic pleocytosis (544 cells/μl), contradictorily, was one which was suffering from the acute non-inflammatory encephalomyelitis. Other authors also had reported a lack of correlation between the CSF and CNS histopathological findings. CSF abnormalities were seen in two dogs with the non-inflammatory form of the CDV encephalomyelitis (Koutinas et al., 2002). Tipold et al. (1996) reported that in 17 out of 32 cases with the non-inflammatory encephalomyelitis form of the disease, the CSF was abnormal. In our investigation CSF evaluation suggested the diagnosis of CDV encephalomyelitis, since 100% (8/8) of the case with CDV encephalomyelitis were presented with lymphocytic pleocytosis, while this finding was present in only 33.33% (4/12) of the dogs from the group used for comparative purpose.

In several studies involving natural-occurring nervous distemper, systemic signs simultaneously to the neurological disease have been the most common clinical course, as well seizures and myoclonus have been considered the most frequent neurological findings (Shin et al, 1995; Tudury et al., 1997; Frisk et al, 1999; Moritz et al, 2000; Koutinas et al, 2002; Moro et al 2003; Gebara et al, 2004 a/b; Saito et al., 2005 a/b). Nevertheless these clinical findings may result from a bias of selection, since the authors considered as inclusion criteria classical clinical findings of distemper. The “classical” distemper presentation is not the rule (Tipold et al, 1992). Researchers about nervous distemper had been claiming that the typical systemic findings are absence in about half of the cases (Vandevelde and Cachin, 1993), and myoclonus may be lacking in more than half of the cases (Tipold et al, 1992). Our study was able to recognize

distemper in 40% (8/20) of dogs with neurological presentation without myoclonus and in the absence of systemic disease at the time of hospital admission. Seizure was not an often neurological sign because this cortical/subcortical sign has been typically found in young dogs. This CDV encephalomyelitis clinical form is frequently associated with systemic involvement, and this clinical presentation was not included in our study.

It had been claimed that neurological signs of CDV might occur much later (months or years) after the systemic infection, as a "post infectious encephalitis". This concept had been used to justify the situations where the clinical course was restricted to the neurological disease. In spite of this there is no experimental evidence at all to support this notion (Tipold et al, 1992; Vandevelde and Zurbriggen, 1995). In previous study we were able to detect the CDV in urine from distemper dogs with neurological presentation without the typical systemic signs of the disease (Amude et al., 2005). This results in spontaneous cases of nervous distemper suggest that at the moment of the clinical presentation, even in cases which there are only the neurological signs, the animals are undergoing a systemic infection or the infection had just been a recent event, since the virus still was identified in urine from animals with the clinical condition restricted to the neurological presentation.

Although the clinical diagnosis of distemper is difficult when systemic signs and myoclonus are lacking at the time of hospital admission, cerebellar and/or vestibular signs progressing to tetraparesis, is a neurological presentation that may suggest CDV encephalomyelitis as diagnosis. In dogs with distemper encephalomyelitis, changes in hematological parameters are frequently absent or non-specific, nevertheless the CSF evaluation may suggest CDV infection by a lymphocytic pleocytosis. At *post mortem*, demyelinating lesions may be a constant finding in CNS sections from distemper dogs presented exclusively neurological disease, and chronic encephalomyelitis is predominant.

Table 1 - Frequency of neurological deficits verified during the clinical evolution from the dogs with distemper encephalomyelitis (N = 08) attending at Veterinary Hospital of Universidade Estadual de Londrina from november 2003 to november 2004, diagnosed at *post mortem* by RT-PCR and histopathological examination.

Neurological deficit	f	%
Postural reactions deficits	8	100.0
Tetraparesis/plegia	6	75.0
Nystagmus	5	62.5
Truncal ataxia/ head ataxia (intentional tremor of the head)	5	62.5
Inappropriate consciousness content/ compulsive walking/ seizure/ dysmetria (hypermetria)/ decerebelate rigity[a].	2	25.0
Head tilt/ vestibular strabismus[b]/ personality changes/ walking in circles/ head pressing/ myoclonus/ menace deficit/ paraplegia/ spinal hiperesthesy.	1	12.5

[a] opisthotonos with spasticity and rigidity of thoracic limb

[b] positional ventrolateral strabismus when the head was dorsally extended

Table 2 - Frequency of the neurological deficits verified during the clinical evolution from the non distemper dogs (N = 12) attending at Veterinary Hospital of Universidade Estadual de Londrina from november 2003 to november 2004.

Neurological deficit	f	%
Postural reactions deficits	10	83.3
Tetraparesis/plegia	6	50.0
Ataxia of the limbs/ depression	5	41.7
Nystagmus/ head tilt/ vestibular strabismus[a]	4	33.3
Seizure/ menace deficit	3	25.0
Head ataxia (intentional tremor of the head)/ inappropriate consciousness content/ walking in tight circles/ decerebelate rigity[b]/ paraplegia/ spontaneous strabismus/ midriase.	2	16.7
Personality changes/ walking in open circles/ myoclonus/ spinal hiperesthesy/ hemiparesis/ facial paralysis/ cervical pain/ absence of oculocephalic reflex/ impaired retraction of eyeball.	1	8.3

[a] positional ventrolateral strabismus when the head was dorsally extended

[b] opisthotonos with spasticity and rigidity of thoracic limb

Table 3 – Encephalomyelitis type and respective abnormalities in the hematological parameters and cerebrospinal fluid evaluation from the dogs with distemper encephalomyelitis (N = 08) attending at Veterinary Hospital of Universidade Estadual de Londrina from november 2003 to november 2004, diagnosed at *post mortem* by RT-PCR and histopathological examination.

Histopathological features	dog	hemogram	CSF evaluation
Acute non-inflammatory CDV encephalomyelitis	13	Anemia ($4,03x10^6$ RBC/µl, Hct 23,8%, Hgb 7,6 g/dl)	Mild pleocytosis (18 WBC/µl) with 68% lymphocytes and 32% neutrophils
	16	Neutrophilia with left shift ($18x10^3$ neutrophils/µl, $3,24x10^3$ band cells/µl)	Severe pleocytosis (544 WBC/µl) with 75% lymphocytes, 15% neutrophils, and 5% monocytes
Chronic inflammatory CDV encephalomyelitis	05	Lymphopenia (846/µl)	Mild pleocytosis (10 WBC/µl) with 98% lymphocytes and 2% neutrophils)
	15	Neutropenia (2865/µl), limphopenia (764/µl)	Mild pleocytosis (23 WBC/µl) with 85% lymphocytes and 15% neutrophils
	17	Within normal limits	Moderate pleocytosis (61 WBC/µl) with 98% lymphocytes and 2% neutrophils
	18	Within normal limitis	Mild pleocytosis (45 WBC/µl) with 94% lymphocytes and 6% neutrophils
	30	Lymphopenia (360/µl)	Mild pleocytosis (26 WBC/ µl) with 64% lymphocytes and 36% neutroplis
Not available	22	Within normal litmits	Mild pleocytosis (43 WBC/µl) with 97% lymphocytes and 3% neutrophils

Table 4 - Abnormalities in the hematological parameters, and findings in the cerebrospinal fluid evaluation of the non distemper dogs (N = 12) attending at Veterinary Hospital of Universidade Estadual de Londrina from november 2003 to november 2004.

case	hemogram	CSF evaluation
01	Lymphopenia (664/µl), anemia (3,20x10^6 RBC/µl, Hct 22,1%, Hgb 5,7 g/dl, 01% eritroblastos)	Moderate protein increasing (78 mg/dl)
04	Within normal limits	Within normal limits
06	lymphopenia (284/µl)	Mild protein increasing (44,2 mg/dl), severe lymphocytic pleocytosis (1579 WBC/µl) with 99% lymphocytes and 1% neutrophils
07	Neutropenia (84/µl), lymphopenia (196/µl), anemia (2,83x106 RBC/µl, Hct 18%, Hgb 5,3g/dl), trombocytopenia (25x10^3/µl)	Mild lymphocytic pleocytosis (33 WBC/µl) with 90% lymphocytes and 10% neutrophils
08	Lymphopenia (119/µl), anemia (4,49x10^6 RBC/µl, Hct 29,1%, Hgb 8,2 g/dl)	Mild lymphocytic pleocytosis (13 WBC/µl) with 94% lymphocytes and 6% neutrophils
09	Within normal limits	Mild mixed cell pleocytosis (9 WBC/µl) with 58% lymphocytes and 42 % neutrophils
10	Trombocytopenia (42x10^3/µl)	Not available
14	Within normal limits	Moderate mixed cell pleocytosis (98 WBC/µl) with 52% lymphocytes and 58% neutrophils
21	Within normal limits	Mild protein increasing (33,8 mg/dl), severe mixed cell pleocytosis (444 WBC/µl) with 43% lymphocytes and 57% neutrophils
27	Within normal limits	Within normal limits
28	Anemia (3,10x10^6 RBC/ µl, Hct 19,6%, Hgb 6,0 g/dl)	Within normal limits
31	Within normal limits	Moderate lymphocytic pleocytosis (59 WBC/ µl) with 87% lymphocytes, 12% neutroplis and 1% monocytes

REFERENCES

1. Amude, A.M., Alfieri, A.A. and Alfieri A.F., 2005. *ante mortem* Diagnosis of CDV Infection by RT-PCR in Distemper Dogs with Neurological Deficits without the Typical Clinical Presentation. *Veterinary Research Communication* "in press".
2. Bailey, C. and Higgins, R., 1986. Characteristics of cerebrospinal fluid associated with canine granulomnatous meningoencephalomyelitis: A retrospective study. *Journal of American Veterinary Medical Association*, 188, 418-421.
3. Baumgärtner, W., 1993. Virale Infektionskrankrankheiten bei Junghunden. *Praktischer Tierarzi*, 1, 26-32.
4. Baumgärtner, W., Örrvell, C. and Reinacher, M., 1989. Naturally occurring canine distemper virus encephalitis: distribuition and expression of viral polypeptides in nervous tissues. *Acta Neuropathologica,* 78, 504-512.
5. Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, P.M.E., Werthein-van Dillen and Noordaa-Vader, J., 1990. Rapid and simple method for purification of nucleic acids, *Journal of Clinical Microbiology*, 28, 495-503.
6. Braund, K.G., 2001. *Inflammatory Diseases of the Central Nervous System*. Available at: www.ivis.org. Accessed Nov 11, 2001.
7. Braund, K.G., 1994. *Clinical Syndromes in Veterinary Neurology*. 2nd ed. St Louis, MO: Mosby.
8. Chrisman, C.L., 1992. Cerebrospinal Fluid Analysis. *Veterinary Clinics of North America:Small Animal Practice*,22, 781-807.

9. Dewey, C.W., Budsberg, S.C. and Oliver, J.E., 1992. Principles of Head Trauma Management in Dogs and Cats – Part I. Compedium an continuing Education for the Practicing Veterinarian, 14, 199-207.

10. Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, V. and Ball, L.A., 2004. *Virus Taxonomy*. The light report. San Diego: Academic Press.

11. Frisk, A.L., Konig, M., Moritz, A. and Baumgärtner, W., 1999. Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. *Journal of Clinical Microbiology*, 37, 3634-3643.

12. Gebara, C.M.S., Wosiacki, S.R., Negrão, F.J., Alfieri, A.A. and Alfieri, A.F., 2004a. Lesões histopatológicas no sistema nervoso central de cães com encefalite e diagnóstico molecular da infecção pelo vírus da cinomose canina. *Arquivos Brasileiros de Medicina Veterinária e Zootecnia,* 56, 168-174.

13. Gebara, C.M.S., Wosiacki, S.R., Negrão, F.J., de Oliveira, D.B., Beloni, S.N.B., Alfieri, A.A. and Alfieri, A.F., 2004b. Detecção do gene da nucleoproteína do vírus da cinomose canina por RT-PCR em urina de cães com sinais clínicos de cinomose. *Arquivos Brasileiros de Medicina Veterinária e Zootecnia*, 56, 480-487.

14. Headley, S.A., Soares, I.C. and Graça, D.L., 2001. Glial Fibrillary Acidic Protein (GFAP) – immunoreactive Astrocytes in Dogs Infected with Canine Distemper Virus. *Journal of Comparative Pathology*, 125: 90-97.

15. Jóźwik, A. and Frymus, T., 2005. Comparison of the Immunofluorescence Assay with RT-PCR and Nested PCR in the Diagnosis of Canine Distemper. *Veterinary Research Communications*, 29, 347-359.

16. Koutinas, A.F., Polizopoulou, Z.S., Baumgärtner, W., Lekkas, S. and Kontos, V., 2002. Relation of clinical sings to pathological changes in 19 cases of canine distemper encephalomyelitis. *Journal of the Comparative Pathology*, 126, 47-56.

17. Kim, Y.H., Cho, K.W., Youn, H.Y., Yoo, H.S. and Han, H.R., 2001. Detection of canine distemper virus through one step RT-PCR combined with nested PCR. *Journal of Veterinary Science*, 2, 59-63.

18. Moritz, A., Frisk, A.L. and Baumgärtner, W., 2000. The evaluation of diagnostic procedures for the detection of canine distemper virus infection. *European Journal of Companion Animal Practice*, 10, 37-47.

19. Moro, L., Martins, A.S., Alves, C.M., Santos, F.G.A., Del Puerto, H.L. and Vasconcelos, A.C., 2003. Apoptosis in the Cerebellum of Dogs with Distemper. *Journal of Veterinary Medicine Series B*, 50, 221-225.

20. Muller, C.F., Fatzer, R.S., Beck, K., Vandevelde, M. and Zurbriggen, A., 1995. Studies on canine distemper virus persistence in the central nervous system. *Acta Neuropathologica*, 89, 438-445.

21. Parker, A.J., 1978. The many faces of canine distemper. *Canine Practice*, 5, 25-28.

22. Palmer, D.G., Huxtable, C.R.R., and Thomas, J.B., 1990. Immuhistochemical demonstration of canine distemper virus antigen as an aid in the diagnosis of distemper encephalomyelitis. *Research in Veterinary Science*, 49, 177-181.

23. Ryan, K., Marks, S.L. and Kerwin, S.C., 2001. Granulomatous Meningoencephalitis in Dogs. *Compendium on Continuing Education for the Practicing Veterinarian*, 23, 644-650.

24. Rzeżutka, A. and Mizak, B., 2002. Application of N-PCR for diagnosis of distemper in dogs and fur animals. *Veterinary Microbiology*, 88, 95-103.

25. Saito, T.B., Alfieri, A.A., Wosiacki, S.R., Negrão, F.J., Morais, H.S.A. and Alfieri, A.F., 2005a. Detection of canine distemper virus by reverse transcription-polimerase chain reaction in the urine of dogs with clinical signs of distemper encephalitis. *Research in Veterinary Science*, 79, "in press".

26. Saito, T.B., Alfieri, A.A., Wosiacki, S.R., Negrão, F.J., Morais, H.S.A. and Alfieri, A.F., 2005b. *Ante* and *Post mortem* detection of canine distemper virus by RT-PCR in dogs with distemper. *Archivos de Medicina Veterinaria*, 37, "in press".

27. Shin, Y. J., Cho, K. O., Cho, H. S., Kang, S. K., Kim, H. J., Kim, Y. H., Park, H. S. and Park, N. Y., 2004. Comparison of one-step RT-PCR and nested PCR for the detection of canine distemper virus in clinical samples. *Australian Veterinary Journal*, 82, 83-86.

28. Shin, Y., Mori, T., Okita, M., Gemma, T., Kai, C. and Mikami, T., 1995. Detection of canine distemper virus nucleocapsid protein gene in canine peripheral blood mononuclear cells by RT-PCR. *Journal of Veterinary Medical Science*, 57, 439-445.

29. Summer, B.A., Cummings, J.F. and de Lahunta, A., 1995. *Veterinary Neuropatology*. St Louis: Mosby.

30. Schobesberger, M., Zurbriggen, A., Doherr, M.G., Weissenböck, H., Vandevelde, M., Lassman, H. and Griot, C., 2002. Demyelination precedes oligodendrocyte loss in canine distemper virus-induced encephalitis. *Acta Neuropathologica*, 103, 11-19.

31. Schobesberger, M., Zurbriggen, A., Summerfield, M., Vandevelde, M. and Griot, C., 1999. Oligodendroglial degeneration in distemper: apoptosis or necrosis? *Acta Neuropathologica*, 97, 279-287.

32. Shell, L.G., 1990. Canine Distemper. *Compendiun on Continuing Education for the Practicing Veterinarian*, 12, 173-179.

33. Tipold, A., Vandevelde, M. and Jaggy, A., 1992. Neurological manifestation of canine distemper virus infection. *Journal of Small Animal Practice*, 33, 466-470.

34. Tipold, A., Jaggy, A., Zurbriggen, A. and Vandevelde, M., 1996. Neurological signs in canine distemper encephalomyelitis – a clinical study. *European Journal of Companion Animal Practice*, 7, 33-38.

35. Tudury, A.E., Arias, M.V.B., Bracarense, A.P.F.L., Megid, J., and Dias, R.F. Júnior, 1997. Observações Clínicas e Labotaroriais em Cães com Cinomose Nervosa. *Ciência Rura*l, 27, 229-235.

36. Vandevelde, M. and Cachin, M., 1993. The Neurologic Form of Canine Distemper. In: J.D. Bonagura and R.W. Kirk (eds), *Kirk's Current Veterinary Therapy XI, Small Animal Practice*. Philadelphia: W.B. Saunders, 1003-1007.

37. Vandevelde, M., Fankhauser, R., Kristensen, F. and Kristensen, B., 1981. Immunoglobulins in demyelinating lesions in canine distemper encephalolyelitis – an immunohistological study. *Acta Neuropathologica*, 54, 31-41.

38. Vandevelde M, and Zurbriggen A., 1995. The neurobiology of canine distemper virus infection. *Veterinary Microbiology*, 44, 271-280.

39. Vandevelde, M., Zurbriggen, A., Dumas, M. and Palmer, D.G., 1985. Canine distemper virus does not infect oligodendrocytes in vitro. *Journal of the Neurological Sciences*, 69, 133-137.

CONCLUSÕES

- Encefalite do cão velho, encefalomielite do cão jovem e encefalomielite do cão adulto são síndromes clínicas determinadas pela infecção do vírus da cinomose canina, onde a mioclonia e os sinais sistêmicos podem estar ausentes no momento da manifestação dos déficits neurológicos.
- Os parâmetros hematológicos podem estar normais, e quando alterados não são considerados específicos no curso da encefalomielite pelo CDV.
- A análise do líquor pode sugerir infecção pelo CDV quando pleocitose linfocitária é verificada.
- Lesões desmielinizantes foram uma constante nos cortes histopatológicos, sendo a encefalomielite crônica predominante.
- Sinais cerebelares e/ou vestibulares progredindo para tetraparesia, foi uma apresentação neurológica que sugeriu a encefalomielite pelo CDV como diagnóstico clínico.

ANEXOS

ANEXO 1

Lista de reagentes

ANEXO 1 – Lista de reagentes

1. Ácido bórico (PM 61,83)
2. Agarose (Gibco BRL®)
3. Acetona PA (PM 78,13) (Dinâmica®)
4. Álcool etílico hidratado 92,8º INPM – 95,0º GL (C_2H_2OH) 35,71 ml
5. Brometo de etídio ($C_{21}H_{20}N_3Br$ - PM 394,3) (SIGMA®)
6. Cloreto de Magnésio ($MgCl_2$) (Invitrogen™ Life Technologies, EUA)
7. dNTP (100 mM, 4 x 250 µL; 25 µmol cada - 100 mM dATP, 100 mM dCTP, 100 mM dGTP, 100 mM dTTP) (Invitrogen™ Life Technologies)
8. EDTA dissodium (PM 372,24)
9. Isotiocianato de guanidina (PM 118,16) (Gibco BRL®)
10. Oligonucleotídeos iniciadores (Invitrogen™ Life Technologies, EUA)
11. Padrão de tamanho molecular 123 pb (Invitrogen™ Life Technologies, EUA)
12. PCR Buffer 10x (200 mM Tris-HCl, pH 8.4; 500 mM KCl - Invitrogen™ Life Technologies, EUA)
13. Taq DNA polymerase recombinant – 5U/µl (Invitrogen™ Life Technologies, EUA)
14. Triton X-100 (Gibco®)
15. Heparina (LIQUEMINE, ROCHE, BRASIL)

ANEXO 2

Lista de soluções e tampões

ANEXO 2 – Lista de soluções e tampões

Preparo de soluções e tampões de biologia molecular

Álcool Etílico 70%

- Álcool etílico hidratado 92,8^{o} INPM – 95,0^{o} GL (C_2H_2OH) 35,71 mL
- 100 mL de H_2O miliQ q.s.p.

Sílica

- 60 g de sílica (Dióxido de sílica - SiO_2)
- 500 mL de H_2O milliQ autoclavada q.s.p.
- agitar lentamente e manter em repouso durante 24 horas
- desprezar 430 mL do sobrenadante por sucção
- ressuspender 500 mL da sílica em H_2O miliQ autoclavada q.s.p.
- permanecer em repouso por 5 horas
- desprezar 440 mL do sobrenadante
- adicionar 600μL de ácido clorídrico (HCl) - ajustar o pH (2,0)
- aliqüotar e autoclavar

Tampão L6

- 120g de isotiocianato de guanidina
- 100 mL de Tris-HCl 0,1M (pH 6,4)
- 22 mL de EDTA (ácido etilenodiaminotetraacético) 0,2M (pH 8,0)
- 2,6 g de Triton X-100

Fonte: BOOM *et al*. (1998)

Tampão L2

- 120 g de isotiocianato de guanidina
- 100 mL de Tris-HCl 0,1M (pH 6,4)

Fonte: BOOM *et al*. (1998)

Tampão Fostato Salina - PBS 1x [ ]

- Cloreto de sodio, P.A. (NaCl) 8,0 g (137 mM)
- Cloreto de potássio, P.A. (KCl) 0,2 g (3 mM)
- Sódio fosfato dibásico anidro, P.A. (Na_2HPO_4) 1,2 g (8 mM)
- Potássio fosfato monobásico, P.A. (KH_2PO_4) 0,2 g (15 mM)
- H_2O bd q.s.p. 1.000 mL

Tampão de corrida para eletroforese em gel de agarose - TEB (Tris – ácido bórico – EDTA) 10x[ ]

- 10,778 g de Tris (Hidroximetil amino metano) - 89 mM
- 5,503 g de ácido bórico (H_3BO_3) - 89 mM
- 0,747 g de EDTA - 2 mM
- H_2O bd q.s.p. 1.000 mL
- pH 8,4

Tampão de amostra para Agarose

- Azul de bromofenol 0,25%
- Sacarose, P.A. – sucrose ($C_{12}H_{22}O_{11}$) 45%

ANEXO 3

Protocolos de técnicas moleculares

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Protocolos de técnicas moleculares

Extração do Ácido nucleico pela técnica de sílica / tiocianato de guanidina

-Adicionar 300μL de suspensão 10% p/v em PBS de macerado do sistema nervoso central
- Adicionar 25 μL de sílica hidratada
- Adicionar 900 μL de solução L6
- Homogeneizar em vortex
- Agitar durante 30 minutos em temperatura ambiente
- Centrifugar a 10.000 x g durante 30 segundos
- Desprezar o sobrenadante em solução contendo NaOH 10 M
- Adicionar 500 μL de solução L2
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g durante 30 segundos
- Desprezar o sobrenadante em solução contendo NaOH 10M
- Adicionar 500 μL de solução L2
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g durante 30 segundos
- Desprezar o sobrenadante em solução contendo NaOH 10M
- Adicionar 1000 μL de etanol 70% gelado
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g durante 30 segundos
- Desprezar o sobrenadante
- Adicionar 1000 μL de etanol 70% gelado
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g durante 30 segundos
- Desprezar o sobrenadante
- Adicionar 1000 μL de acetona PA gelada

- Homogeneizar em vórtex
- Desprezar o sobrenadante
- Secar o pellet em banho-maria a 56^{o}C durante 15 minutos
- Adicionar 50 μL de água ultrapura autoclavada
- Homogeneizar em vórtex
- Deixar em banho-maria à 56^{o}C durante 15 minutos
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g durante 2 minutos
- Recolher o sobrenadante (40μL)
- Estocar a –20^{o}C

Gel de Agarose a 2%

- Agarose 1,0 g
- TEB 1x[ ] 50 mL
- Ethidium bromide ($C_{21}H_{20}N_3Br$) 15 μL
- aplicar 8 μL do produto com 2 μL do tampão de amostra para agarose e submeter à eletroforese no tampão de corrida
- visualizar em luz ultravioleta

APÊNDICES

Documentação fotográfica

Quadro 1

Documentação fotográfica

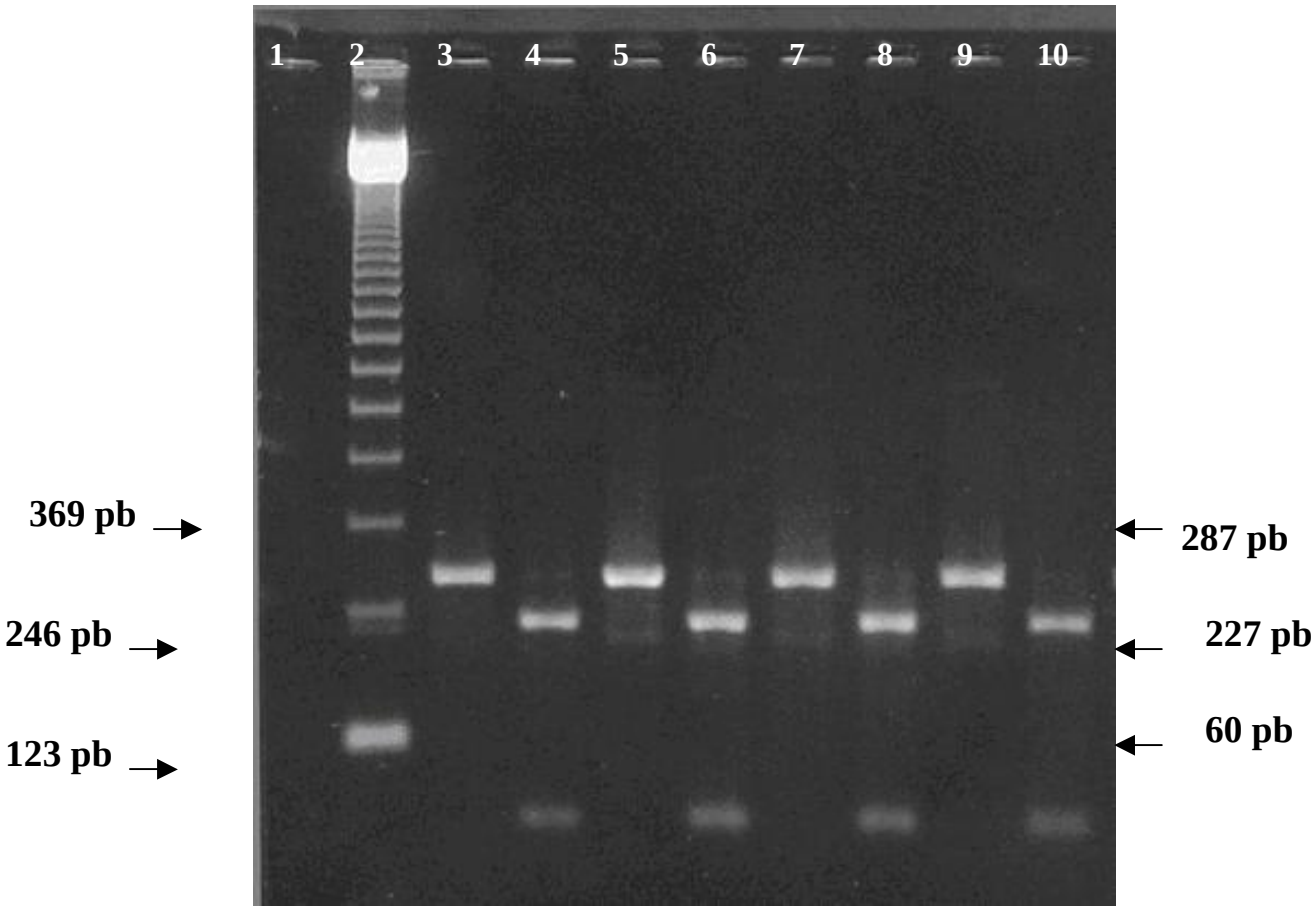

Figura 1. Eletroforese em gel de agarose 2%, corado com brometo de etídio, dos produtos de amplificação do gene da nucleoproteína do vírus da cinomose canina, obtidos pela técnica da RT-PCR realizada em fragmentos de sistema nervoso central (SNC) de cães com sinais clínico neurológicos, e dos fragmentos do cDNA clivados com a enzima de restrição *Hinf* I. Canaleta 1. Controle negativo da extração do ácido nucléico (água ultrapura autoclavada); Canaleta 2: Padrão de tamanho molecular com 123 pb (Invitrogen Life Technology, EUA); Canaletas 3, 5, 7 e 9: Estirpe Rockborn do vírus da cinomose canina (controle positivo) (3), amostras do SNC do cão 13 (5), cão 16 (7) e do cão 30 (9); Canaletas 4, 6, 8 e 10: Amostras do SNC referente às canaletas 3, 5, 7 e 9, clivadas com a enzima *Hinf* I.

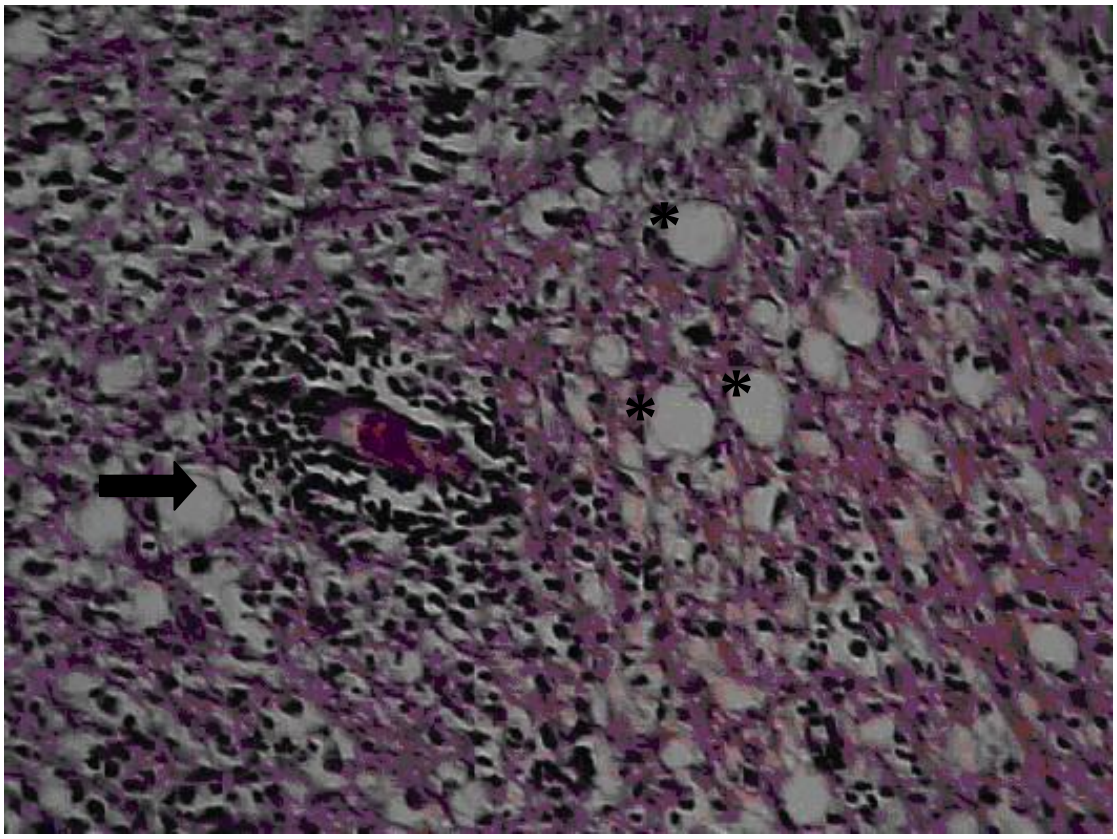

Figura 2: Fotomicrografia do cerebelo (substância branca) do cão 05 que apresentou sinal clínico neurológico e diagnóstico etiológico positivo para o vírus da cinomose canina realizado pela técnica da RT-PCR, evidenciando achados histopatológicos compatíveis com a encefalomielite crônica da cinomose: infiltrado inflamatório mononuclear perivascular (➡), vacuolização da substância branca [desmielinização (*)], e infiltrado inflamatório mononuclear no parênquima. (coloração HE; objetiva de 20X).

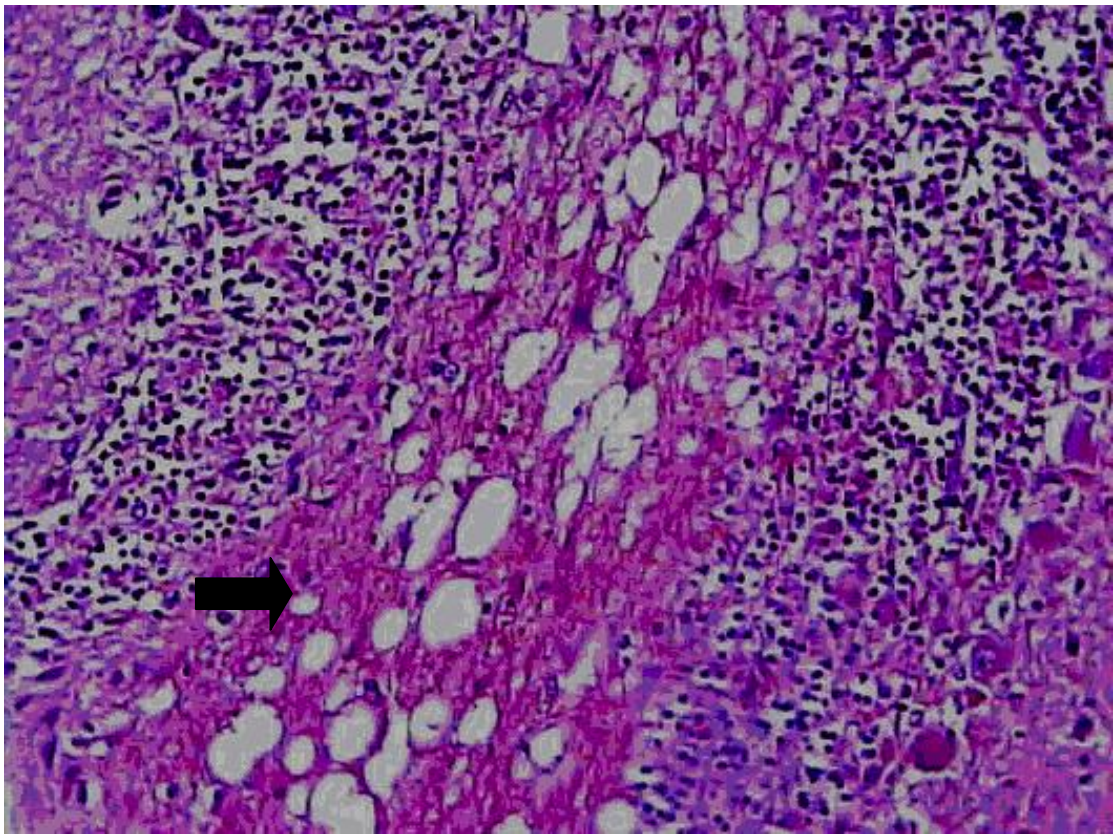

Figura 3: Fotomicrografia do cerebelo do cão 16 que apresentou sinal clínico neurológico e diagnóstico etiológico positivo para o vírus da cinomose canina realizado pela técnica da RT-PCR, evidenciando achados histopatológicos compatíveis com a encefalomielite aguda da cinomose: vacuolização multifocal da substância branca [desmielinização (➡)] e ausência de resposta inflamatória (coloração HE; objetiva de 10X).

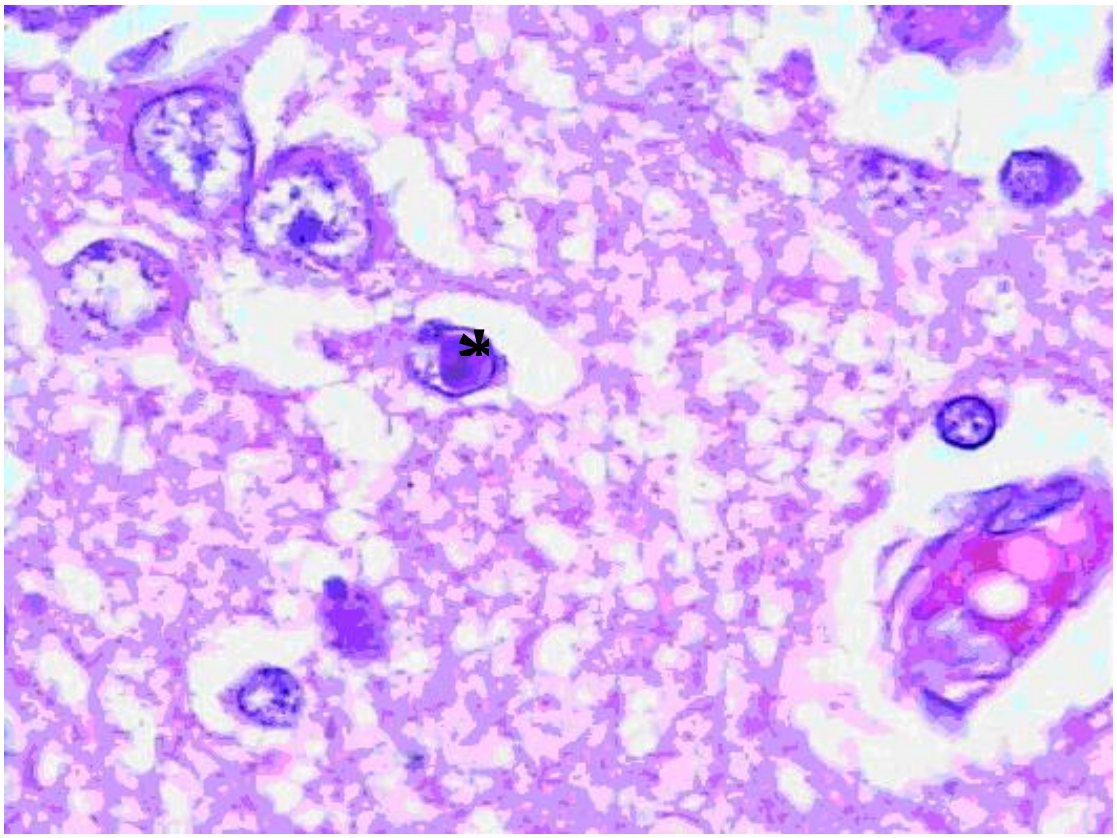

Figura 4. Fotomicrografia do corte histológico do SNC do cão 16 com corpúsculo de inclusão viral (*) (coloração HE; imersão).

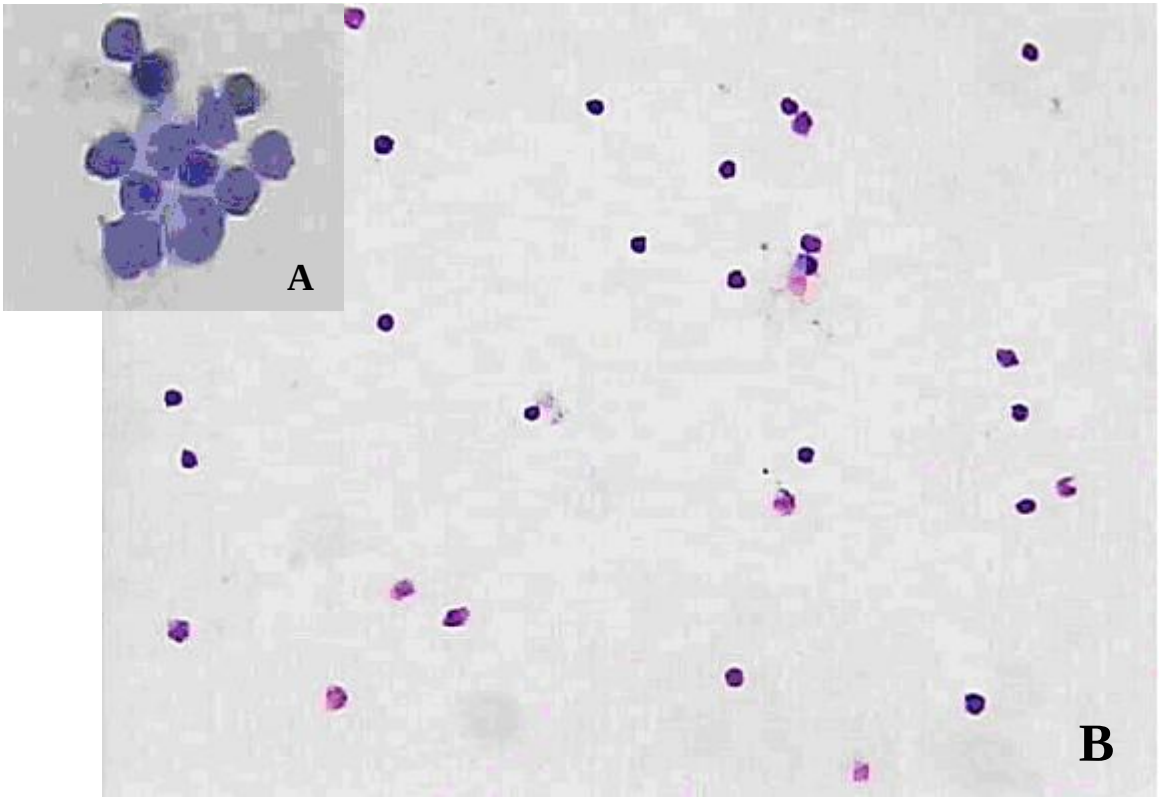

Figura 5. Fotomicrografia de esfregaço de líquor com pleocitose linfocítica (Diff-Quik). A: objetiva de 40X; B: objetiva de 20 X.

Quadro 1 – Distribuição dos 20 animais incluídos nessa pesquisa com relação à idade, raça, sexo, sobrevida, dados de vacinação, déficits neurológicos observados durante a evolução clínica e respectivas síndromes neurológicas.

	Cão/idade (meses)	Raça/sexo	Sobrevida (dias)	vacina	Déficits neurológicos observados durante a evolução neurológica	Síndrome neurológica
Grupo I	13/36	SRD/F	12	Não	Atividade semelhante a convulsão (relatada pelo proprietário), alteração de comportamento (agressividade), conteúdo de consciência inapropriado, andar compulsivo, andar em círculos, ataxia do tronco e cabeça[#], compressão de cabeça, déficits de reações posturais, hipermetria leve, tetraparesia.	Cerebral e cerebelar
	16/132	SRD/F	31	Não	Andar compulsivo, convulsão focal com generalização secundária, conteúdo de consciência inapropriado, nistagmo posicional vertical, déficit de reação de ameaça, tetraparesia espástica, déficits de reações posturais, rigidez de decerebelação[&].	Cerebral, vestibular e cerebelar
	05/62	Collie/F	32	Sim	Fraqueza dos membros posteriores, tetraparsi/plegia espástica, ausência de reações posturais, ataxia do tronco e cabeça, nistagmo espontâneo rotatório bilateral, estrabismo vestibular [*].	Cerebelar e vestibular
	15/7	SRD/M	15	Sim	Fraqueza dos membros posteriors, ataxia do tronco e cabeça, nistagmo posicional vertical, déficits de reações posturais, tetraparesia/plegia espástica, mioclonia no membro posterior esquerdo e nos músculos mastigatórios.	Cerebelar e vestibular
	17/18	Pastor Alemão/M	33	ND	Fraqueza dos membros posteriores, ataxia do tronco e cabeça, nistagmo vertical posicional, tetraparesia/plegia espástica, déficits de reações posturais, dor a palpação da coluna.	Cerebelar e vestibular
	18/21	Cooker spaniel/M	7	ND	Ataxia do tronco e cabeça, teraparesia espástica, nistagmo vertical posicional com mudança para rotatório com alterações da posição da cabeça, severa hipermetria, déficits de reaçoes posturais rigidez de decerebelação.	Cerebelar e vestibular
	22/145	Cooker spaniel/F	10	Sim	Paraplegia espástica evoluindo para paraplegia flácida, ausência de reações posturais nos membros posteriores.	Toracolombar e lombosacra
	30/55	Boxer/M	22	Sim	Ataxia do tronco e cabeça, torção de cabeça para direita, tetraparesia/plegia espástica, nistagmo posicional horizontal, déficits de reações posturais.	Vestibular e cerebelar

Grupo II	01/48	Fila Brasilero/M	68	Não	Ataxia nos 4 membros, tetraparesia espástica, ausência de reações posturais, nível de consciência deprimido, nistagmo vertical (OD), paralisia facial (D).	pontobulbar
	04/15	Lhasa Apso/M	43	Sim	Estrabismo lateral bilateral espontâneo, estrabismo vestibular (OE), midríase bilateral, ausência da reação de ameaça, cegueira, andar em círculos pequenos para a esquerda, torção de cabeça para esquerda, paraplegia espástica, reações posturais ausentes nos posteriores e normal nos anteriores, ausência de dor superficial nos MPs e diminuição da dor profunda nos MPs.	Mesencefálica, vestibular e toracolombar
	06/131	Pincher/M	240	Sim	Histórico passado de caminhar em círculos abertos à esquerda de evolução crônica. Convulsão há 8 meses. Evoluiu para convulsões em cluster de difícil controle. Convulsões focais (movimentos de mascar) com generalização secundária.	Cerebral
	07/130	SRD/M	7	Sim	Ataxia dos 4 membros, ataxia de cabeça, torção de cabeça, tetraparesia espástica, ausência das reações posturais, nível de consciência deprimido com evolução para coma, estrabismo ventrolateral espontâneo em OD, nistagmo horizontal OD, midríase bilateral, ausência da reação de ameaça e reflexo oculovestibular, postura de decerebelação.	Mesensefálica, vestibular e cerebelar
	08/ND	Collie/F	24	Não	Nível de consciência deprimido, estrabismo vestibular bilateral, tetraparesia espástica, reações posturais diminuídas, torção de cabeça para a direita, ataxia de cabeça.	vestibular e cerebelar
	09/84	Pastor Alemão/M	31	Sim	Alteração de personalidade (agressividade), conteúdo de consciência inapropriado com nível alerta, fraqueza episódica dos membros. Reações posturais ausentes	Cerebral
	10/46	Pincher/F	4	Sim	Andar em círculos e quedas (rolava) para o lado direito, tetraparesia espástica, ausência de reações posturais, convulsões, nível de consciência deprimido com conteúdo inapropriado, nistagmo posicional horizontal, estrabismo vestibular (OD), torção de cabeça à esquerda, diminuição da rima palpebral esquerda, movimentos espasmódicos rítmicos (mioclonia) na face e no membro anterior esquerdo e posterior esquerdo.	Cerebral e vestibular
Grupo II	14/7	SRD/F	2	Sim	Ataxia dos 4 membros, tetraparesia espástica, nível de consciência deprimido, nistagmo posisional vertical, diminuição da reação de ameaça, diminuição do reflexo de retração do globo ocular, reações posturais ausentes, rigidez de decerebelação.	Pontobulbar e cerebelar
	21/32	Labrador/F	70	Sim	Ataxia dos membros posteriores, paraplegia espástica, reações posturais ausentes nos membros posteriores, dor a palpação da coluna toracolombar.	Toracolombar
	27/23	SRD/F	ND	ND	Convulsão	Cerebral

	28/36	Rotweiller/M	ND	ND	Ataxia dos 4 membros com hiperreflexia e estrabismo medial	Pontobulbar
	31/106	SRD/F	51	Não	Iniciou com convulsão há 3 meses, evoluiu para hemiparesia (direita) e apresentou estrabismo vestibular, ausência de reações posturais do lado direito.	Cerebral e vestibular

Grupo I: Animais diagnosticados com encefalomielite pelo CDV
Grupo II: Animais onde o CDV foi excluído como causador dos sinais neurológicos
SRD: sem raça definida
ND: não disponível
Ataxia da cabeça: tremor de intenção da cabeça.
[&] Rigidez de decerebelação: opstótono com espasticidade e rigidez dos membros torácicos.
* Estrabismo vestibular: estrabismo posicional ventrolateral quando a cabeça é dorsalmente estendida