UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

Walter Vicente Cardozo Areco

LESÕES NO SISTEMA NERVOSO CENTRAL E NA PELE DE CÃES COM CINOMOSE SUBMETIDOS À NECROPSIA NA REGIÃO CENTRAL DO RIO GRANDE DO SUL, BRASIL

Santa Maria, RS
2021

Walter Vicente Cardozo Areco

LESÕES NO SISTEMA NERVOSO CENTRAL E NA PELE DE CÃES COM CINOMOSE SUBMETIDOS À NECROPSIA NA REGIÃO CENTRAL DO RIO GRANDE DO SUL, BRASIL

Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Medicina Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Medicina Veterinária**

Orientador: Prof Dr. Eduardo Furtado Flores

Santa Maria, RS
2021

This study was financied in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001

Areco, Walter
LESÕES NO SISTEMA NERVOSO CENTRAL E NA PELE DE CÃES COM CINOMOSE SUBMETIDOS À NECROPSIA NA REGIÃO CENTRAL DO RIO GRANDE DO SUL, BRASIL / Walter Areco.- 2021.
76 p.; 30 cm

Orientador: Eduardo Flores
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós Graduação em Medicina Veterinária, RS, 2021

1. medula espinhal 2. desmielinização 3. hiperceratose 4. cutânea I. Flores, Eduardo II. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

Declaro, WALTER ARECO, para os devidos fins e sob as penas da lei, que a pesquisa constante neste trabalho de conclusão de curso (Tese) foi por mim elaborada e que as informações necessárias objeto de consulta em literatura e outras fontes estão devidamente referenciadas. Declaro, ainda, que este trabalho ou parte dele não foi apresentado anteriormente para obtenção de qualquer outro grau acadêmico, estando ciente de que a inveracidade da presente declaração poderá resultar na anulação da titulação pela Universidade, entre outras consequências legais.

UNIVERSIDADE FEDERAL DE SANTA MARIA
Walter Vicente Cardozo Areco

LESÕES NO SISTEMA NERVOSO CENTRAL E NA PELE DE CÃES COM CINOMOSE SUBMETIDOS À NECROPSIA NA REGIÃO CENTRAL DO RIO GRANDE DO SUL, BRASIL

Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Medicina Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Medicina Veterinária.**

Aprovado em 10 de dezembro de 2021:

Eduardo Furtado Flores, PhD (UFSM)
(Presidente/Orientador)

Cristina Gevehr Fernandes, Dr. (UFPel)

Eduardo Kenji Masuda, Dr. (Laboratório Axys)

Felipe Pierezan, Dr. (UFMG)

Paula Roberta Giaretta, PhD (UFMG)

Santa Maria, RS

2021

AGRADECIMENTOS

Agradeço a meus pais pelo apoio e compreensão, especialmente pelo carinho e delicada atenção durante estes anos.

A minha esposa, Pamela Mauger e minha filha Valéria pelo carinho e apoio nas importantes etapas da minha vida.

Ao meu orientador, professor Eduardo Furtado Flores, pela oportunidade, por todo o apoio, correções e sugestões e exemplo profissional.

A minha co-orientadora, professora Mariana Martins Flores, pela amizade, dedicação, paciência, orientação e por estar sempre disposta a ensinar, você foi, sem dúvida, agente de inspiração e motivação para construção desse trabalho.

Aos professores Glaucia Denise Kommers, Rafael Almeida Fighera e Luiz Francisco Irigoyen pelos ensinamentos, amizade, convivência e tempo dedicado a minha formação como patologista.

Aos meus amigos e colegas Harlan, Alex, Marcella, Taiara, Stella, Tatiana, Vanessa, Luiz, Nicolas, Eryca, Betina, Douglas, Renata, Amanda e Morgana agradeço pela ajuda e cada ensinamento tanto profissional como pessoal.

E a todos que de alguma forma contribuíram para a minha formação, seja pessoal ou profissional.

RESUMO

LESÕES NO SISTEMA NERVOSO CENTRAL E NA PELE DE CÃES COM CINOMOSE SUBMETIDOS À NECROPSIA NA REGIÃO CENTRAL DO RIO GRANDE DO SUL, BRASIL

AUTOR: Walter Vicente Cardozo Areco
ORIENTADOR: Eduardo Furtado Flores
CO-ORIENTADORA: Mariana Martins Flores

A cinomose é uma doença viral frequentemente fatal em cães, causada pelo vírus da cinomose canina (CDV). Muitos aspectos da cinomose permanecem pouco elucidados. O objetivo desse trabalho foi explorar formas de apresentação incomuns ou pouco exploradas na literatura. O primeiro artigo da tese teve o objetivo de caracterizar a distribuição e aspectos histopatológicos das lesões de medula espinhal em cães com a forma espontânea de leucoencefalomielite desmielinizante (LEMD). Foram estudados 17 cães com LEMD submetidos à necropsia no Laboratório de Patologia Veterinária da UFSM (2006-2008). Secções de medula foram submetidas a Hematoxilina e Eosina (H&E), Luxol Fast Blue e imuno-histoquímica para CDV. Setenta e duas das 231 secções de medula espinhal tinham lesões, que afetavam consistentemente a substância branca. A região lombossacra foi mais afetada (13/17), seguida de toracolombar (11/17), cervical (9/17) e cervicotorácica (9/17). As áreas mais afetadas foram funículos lateral (42/72) e dorsal (31/72). Desmielinização (17/17), astrocitose (17/17), microglioses (17/17), gemistócitos (11/17) e inflamação não supurativa (10/17) foram os achados mais comuns na substância branca. Alterações de substância cinzenta incluíram gliose (8/17), inflamação não supurativa (7/17) e malácia (5/17). Lesões agudas foram mais prevalentes (13/17), no entanto, era comum o mesmo cão apresentar lesões em diferentes estágios de evolução em regiões distintas da medula. As lesões de medula nem sempre se correlacionaram com os sinais clínicos apresentados, e alguns cães não apresentaram sinais indicativos de lesão em medula espinhal. O segundo estudo teve o objetivo de caracterizar lesões de hiperceratose em coxim, plano nasal e outras regiões de pele em cães com cinomose. Foram investigados 12 cães com cinomose e hiperceratose cutânea concomitante, submetidos à necropsia no Laboratório de Patologia Veterinária da UFSM (2006-2018). Foi realizada reavaliação histológica das lesões de pele e imuno-histoquímica para antígenos do CDV. Os 23 focos de hiperceratose cutânea afetaram coxins (11/12), plano nasal (4/12), região periocular (2/12), abdômen ventral (2/12), pele hirsuta do focinho (2/12), escroto (1/12) e vulva (1/12). Das 22 amostras de pele, 17 foram submetidas à reavaliação histológica. A análise histopatológica revelou hiperceratose ortoceratótica em todos os casos, por vezes acompanhada de outras lesões, dentre as quais: inclusões intracitoplasmáticas (14/17), acantose (9/17) e degeneração hidrópica (6/17). Quinze secções histológicas de 11 cães foram positivas na imuno-histoquímica. A marcação foi mais comum na epiderme, seguida por glândulas sudoríparas e células endoteliais/pericitos dos vasos sanguíneos. Não houve diferenças nos achados histopatológicos e imuno-histoquímicos entre pele da região naso-digital e das demais regiões. Ressalta-se a importância do reconhecimento de lesões cutâneas, contribuindo para o diagnóstico precoce. Esse trabalho caracterizou lesões pouco exploradas no sistema nervoso central e pele de cães com cinomose. Espera-se que os presentes resultados possam auxiliar no diagnóstico clinicopatológico de casos de cinomose com lesões de pele e medula espinhal em cães, além de contribuir para futuros estudos de patogênese da doença.

Palavras-chave: medula espinhal. Desmielinização. Hiperceratose. Cutânea.

ABSTRACT

CENTRAL NERVOUS SYSTEM AND SKIN LESIONS IN DOGS WITH CANINE DISTEMPER SUBMITTED TO NECROPSY IN THE CENTRAL REGION OF RIO GRANDE DO SUL, BRAZIL

AUTHOR: Walter Vicente Cardozo Areco
ADVISOR: Eduardo Furtado Flores
CO-ADVISOR: Mariana Martins Flores

Canine distemper is a frequently fatal viral disease of dogs caused by canine distemper virus (CDV). Many aspects of canine distemper remain poorly understood. The aim of this study was to explore unusual or poorly described presentations of distemper in the literature. The first study of this thesis aimed to characterize the distribution and histopathological aspects of spinal cord injuries in dogs with spontaneous demyelinating leucoencephalomyelitis (DLEM) induced by CDV. Seventeen dogs with DLEM submitted to necropsy at the Laboratory of Veterinary Pathology at UFSM (2006-2008) were studied. Spinal cord sections were subjected to hematoxylin and eosin (H&E), Luxol Fast Blue and immunohistochemistry for CDV. Seventy-two of 231 spinal cord sections from these animals had histological lesions, which consistently affected the white matter. The lumbosacral region was mostly affected (13/17), followed by the thoracolumbar (11/17), cervical (9/17) and cervicothoracic (9/17). Lesions were most common in the lateral (42/72) and dorsal funiculi (31/72). Demyelination (17/17), astrocytosis (17/17), microgliosis (17/17), gemistocytes (11/17) and non-suppurative inflammation (10/17) were frequent in the white matter. Grey matter changes were less common, and included gliosis (8/17), non-suppurative inflammation (7/17) and malacia (5/17). Acute lesions were most prevalent (13/17) but it was common (10/17) for the same dog to have lesions at different stages of evolution in distinct spinal cord regions. Spinal cord lesions did not always correlate with the reported clinical signs and some dogs did not present spinal cord-related clinical signs. The second study aimed to characterize hyperkeratosis in the footpads, nasal planum and other skin regions in dogs naturally infected with CDV. Twelve dogs with canine distemper and concomitant cutaneous hyperkeratosis submitted to necropsy at the Laboratory of Veterinary Pathology at UFSM (2006-2018) were retrospectively investigated. Paraffin blocks were retrieved and new skin sections were stained with H&E and submitted to immunohistochemistry for CDV antigens. Twenty-three foci of hyperkeratosis were described, affecting footpads (11/12), nasal planum (4/12), periocular region (2/12), ventral abdomen (2/12), haired skin from the snout (2/12), scrotum (1/12) and vulva (1/12). Histopathological analysis was performed in 17 skins and revealed orthokeratotic hyperkeratosis in all cases, sometimes accompanied by other lesions, including: intracytoplasmic inclusions (14/17 skins analyzed), acanthosis (9/17), hydropic degeneration (6/17) and parakeratotic hyperkeratosis (4/17). Fifteen histological sections from 11 dogs were positive by immunohistochemistry. Labelling was most common in the epidermis, followed by sweat glands and endothelial cells/pericytes of dermal blood vessels. No differences in histopathological and immunohistochemical findings between skin from the nasodigital region and non-nasodigital areas were evidenced. The importance of recognizing cutaneous lesions in distemper is highlighted, thus contributing to the early diagnosis. This investigation has characterized poorly explored cutaneous and spinal cord lesions in dogs with distemper. We hope our findings will facilitate the clinicopathologic diagnosis of future cases of distemper and contribute to future pathogenesis studies.

Key-words: spinal cord; Demyelination. Hyperkeratosis. Cutaneous.

LISTA DE ILUSTRAÇÕES

CAPÍTULO 3: MANUSCRITO 1

CAPÍTULO 4: MANUSCRITO 2

LISTA DE TABELAS

CAPÍTULO 3: MANUSCRITO 1

CAPÍTULO 4: MANUSCRITO 2

SUMÁRIO

1 INTRODUÇÃO

A cinomose é uma doença viral causada por um morbillivírus da família *Paramixoviridae* que afeta cães e outros carnívoros, tais como raposas, lobos, furões, guaxinins, quatis e felinos (GREENE, 2012). A doença é caracterizada por lesões em vários órgãos, causando sinais clínicos que envolvem os sistemas nervoso central, ocular, respiratório, gastrointestinal e a pele (BEINEKE et al., 2009).

A leucoencefalomielite desmielinizante induzida pela cinomose (LEMC) se caracteriza principalmente por desmielinização e inflamação não supurativa na substância branca do sistema nervoso central (SNC) (LEMPP et al., 2014). Frequentemente, essa forma de doença cursa com lesões na medula espinhal (VANDEVELDE; ZURBRIGGEN, 2005). Apesar disso, descrições referentes à distribuição das lesões neste órgão são pouco exploradas (HIGGINS et al., 1982a; HIGGINS; CHILD; VANDEVELDE, 1989; KOUTINAS et al., 2002; RAINE, 1976; SCHONING; LAYTON, 1992; SILVA et al., 2009; VANDEVELDE; KRISTENSEN, 1977).

A doença do coxim duro, ou *hard pad disease*, representa uma manifestação cutânea caracterizada por hiperceratose das almofadas plantares e do plano nasal (BEINEKE et al., 2008; FRISK et al., 1999; GRONE; DOHERR; ZURBRIGGEN, 2004a), sendo que raramente essas lesões podem ser observadas em outras regiões de pele (CASWELL; WILLIAMS, 2016). Estudos caracterizando a distribuição macroscópica e os aspectos histológicos das lesões de pele observadas nessa forma de cinomose são pouco frequentes.

Dessa forma, os objetivos deste trabalho foram caracterizar os aspectos morfológicos e imuno-histoquímicos das lesões desmielinizantes de medula espinhal e das lesões observadas na "doença do coxim duro" em cães com a forma espontânea de cinomose, ambas apresentações consideradas pouco exploradas na literatura.

2 REVISÃO DE LITERATURA

2.1. CARACTERÍSTICAS GERAIS DA INFECÇÃO PELO VÍRUS DA CINOMOSE

O vírus da cinomose (*canine distemper virus*, CDV) pertence ao gênero *Morbillivirus*, família *Paramyxoviridae*, junto com os vírus do sarampo, da peste bovina, da *peste-des-petits-ruminants* e morbillivírus de cetáceos. O CDV produz infecção e doença sistêmica grave em

cães, caracterizada por uma variedade de sinais clínicos, incluindo febre, sinais respiratórios e entéricos e distúrbios neurológicos. A doença causada pelo CDV é conhecida há séculos e foi descrita de forma inequívoca em livros do século XVII, relatando grandes epidemias na Europa (BLANCOU, 2004).

A introdução das vacinas com CDV vivo modificado (ML) na década de 1950 e seu uso extensivo ajudaram muito a controlar a doença (APPEL, 1987; APPEL; SUMMERS, 1995). Não obstante, a incidência de doenças relacionadas ao CDV em populações caninas em todo o mundo parece ter aumentado nas últimas décadas, e vários episódios de doença por CDV em animais vacinados foram relatados (BLIXENKRONE-MÖLLER et al., 1992; DECARO et al., 2004).

O CDV possui diâmetro variável (entre 150 a 250 nm), contém uma fita simples de RNA de polaridade negativa e é envolto por um nucleocapsídeo de simetria helicoidal. Além disso, é circundado por um envelope de lipoproteína derivado da membrana plasmática (GREENE, 2012). O CDV é considerado um vírus pancitotrópico, infectando uma grande variedade de células do sistema respiratório, digestivo, urinário, linfoide, reprodutivo, endócrino, nervoso, tegumentar e vascular (GRÖNE et al., 2003; GRONE; DOHERR; ZURBRIGGEN, 2004; KOUTINAS et al., 2004; KRAKOWKA; AXTHELM; GORHAM, 1987; LEMPP et al., 2014).

O CDV é um vírus altamente sensível, sendo suscetível à luz ultravioleta, ao calor e à dessecação. O vírus é inativado e é destruído em temperaturas de 50 a 60°C por 30 min. Em temperaturas de 0 a 4°C, no entanto, o vírus permanece viável por várias semanas no ambiente. Em temperaturas muito baixas (65°C), o vírus pode permanecer viável por até sete anos (GREENE, 2012).

A infecção pelo CDV é endêmica em vários países, com maior frequência de ocorrência em filhotes e cães jovens não vacinados (DEEM et al., 2000). Muitos países desenvolvidos reduziram a incidência da doença pela vacinação massiva de suas populações de cães. No entanto, nas últimas décadas, surtos esporádicos foram descritos na Alemanha, Japão e Finlândia (BEINEKE et al., 2009; EK-KOMMONEN et al., 1997; GEMMA et al., 1996; MORI et al., 1994). No Laboratório de Patologia Veterinária da Universidade Federal de Santa Maria, a cinomose foi considerada a causa mais comum de morte em cães submetidos à necropsia (FIGHERA et al., 2008).

A cinomose com sinais sistêmicos é a forma comumente reconhecida da doença e pode ocorrer em cães de qualquer idade. No entanto, é mais comum em cães não vacinados e em filhotes de 12 a 16 semanas de idade que já tenham perdido seus anticorpos maternos, ou

naqueles mais jovens que tenham recebido concentrações inadequadas desses anticorpos (GREENE, 2012).

A transmissão do vírus ocorre por aerossóis ou por contato direto, sendo o período de incubação de aproximadamente sete dias. Os cães se infectam principalmente pela via oronasal (APPEL; SUMMERS, 1995). Muito do que se conhece da fase inicial da infecção pelo CDV foi baseado em experimentos com cães. Entretanto, recentemente, os furões (*ferrets)* foram incorporados como modelos de estudo da doença, principalmente no que se refere às rotas de infecção e à interação do vírus com as células do hospedeiro no sistema nervoso central (SNC) (APPEL, 1969; LEMPP et al., 2014; LUDLOW et al., 2014; RUDD; CATTANEO; VON MESSLING, 2006).

A infecção pelo CDV é seguida da replicação viral que ocorre inicialmente nas tonsilas e nos linfonodos brônquicos (APPEL, 1969; BEINEKE et al., 2009; GREENE, 2012). Posteriormente, ocorre a primeira viremia, em que os vírus infectam células mononucleares circulantes (macrófagos e linfócitos) e se disseminam para outros órgãos linfoides e hematopoiéticos (tais como baço, timo, linfonodos e medula óssea), resultando em linfopenia e imunossupressão grave de longa duração (BEINEKE et al., 2009). Também pode haver infecção nos tecidos linfoides associados à mucosa (MALT) e nos macrófagos da lâmina própria do trato gastrointestinal (BEINEKE et al., 2009). A falha ou insuficiência da resposta humoral durante este período de infecção pode culminar em uma viremia secundária, enquanto uma boa resposta imune pode eliminar o vírus, resultando em recuperação (KRAKOWKA; COCKERELL; KOESTNER, 1975; MIELE; KRAKOWKA, 1983; VANDEVELDE; ZURBRIGGEN, 2005a). A viremia secundária pode resultar na disseminação do vírus para vários tecidos epiteliais, mesenquimais e SNC (APPEL, 1969).

Diferentes rotas de infecção do SNC pelo CDV já foram propostas, especialmente para o encéfalo (AXTHELM; KRAKOWKA, 1987; HIGGINS et al., 1982a, 1982b; KRAKOWKA; AXTHELM; GORHAM, 1987; LEMPP et al., 2014; VANDEVELDE et al., 1985; VANDEVELDE; ZURBRIGGEN, 2005a). Sabe-se que o CDV pode penetrar no tecido nervoso por via hematógena, atravessando as barreiras hematoencefálica e hematoliquórica (BEINEKE et al., 2009; VANDEVELDE; ZURBRIGGEN, 2005a). Além disso, a invasão pelo líquido cefalorraquidiano (LCR) já foi sugerida por estudos que demonstraram a presença do vírus no epitélio do plexo coroide, em linfócitos do LCR e no epêndima (APPEL, 1969; HIGGINS et al., 1982a, 1982b; SUMMERS; GREISEN; APPEL, 1979; TIPOLD et al., 2001a; VANDEVELDE et al., 1985; VANDEVELDE; ZURBRIGGEN, 2005a). Quanto à invasão por via hematógena, pesquisadores acreditam que o vírus invade os espaços de Virchow-Robin

através de linfócitos e monócitos infectados provenientes da circulação sistêmica (SUMMERS; GREISEN; APPEL, 1979; VANDEVELDE; ZURBRIGGEN, 2005). Essa ideia é sustentada pela presença do CDV em células mononucleares nas leptomeninges no nono dia pós-infecção, e posteriormente, nos espaços de Virchow-Robin (APPEL, 1969; HIGGINS et al., 1982). Recentemente, foi demonstrado que o CDV pode penetrar o SNC pelo nervo olfatório por meio de um estudo experimental em furões (RUDD; CATTANEO; VON MESSLING, 2006). Embora essa via de infecção não tenha sido comprovada em cães, a semelhança entre o curso da doença em furões e cães sugere que esse tipo de invasão também possa ocorrer nessa última espécie (BEINEKE et al., 2009; RUDD; CATTANEO; VON MESSLING, 2006). Os axônios dos neurônios olfatórios estão em contato muito próximo com as células epiteliais respiratórias, facilitando a infecção dos mesmos e migração do vírus por via axonal. Esses axônios fazem sinapses no bulbo olfatório, permitindo a disseminação do vírus em diferentes regiões encéfalo, incluindo lobo piriforme, hipocampo e hipotálamo (RUDD; CATTANEO; VON MESSLING, 2006).

Estudos sugerem disseminação do CDV por meio das células meningoteliais, da pia-máter (BAUMGÄRTNER; ÖRVELL; REINACHER, 1989; BEINEKE et al., 2009). Essa hipótese é fortalecida pela presença de antígeno viral nas células meningoteliais da pia-máter do cerebelo e na substância cinzenta subjacente a essas áreas, além da ocorrência de lesões subpiais (BAUMGÄRTNER; ÖRVELL; REINACHER, 1989; SUMMERS; GREISEN; APPEL, 1984; VANDEVELDE; KRISTENSEN, 1977).

Os animais infectados pelo CDV podem apresentar sinais sistêmicos variados, incluindo anorexia, vômito, diarreia, febre, secreções oculares e nasais mucopurulentas, tosse, dispneia e sinais neurológicos (GREENE, 2012). Os sinais sistêmicos da cinomose variam dependendo da virulência da cepa viral, das condições ambientais e da idade e imunidade do hospedeiro. Mais de 50% das infecções pelo CDV são subclínicas (GREENE, 2012). Um dos primeiros sinais de infecção é uma conjuntivite leve, serosa a mucopurulenta, seguida de uma tosse seca que rapidamente se torna úmida. A depressão e a anorexia são seguidas de vômitos e diarreia, cuja consistência varia de líquida a levemente sanguinolenta e mucosa (GREENE, 2012).

Os sinais neurológicos podem aparecer na ausência de qualquer outro sinal sistêmico, concomitantemente aos demais sinais ou aproximadamente 21 dias após a recuperação da clínica da doença sistêmica. Os sinais neurológicos variam entre convulsões, alterações comportamentais, astenia, ataxia e mioclonias (TIPOLD; VANDEVELDE; JAGGY, 1992), sendo os mais frequentes as mioclonias, convulsões e ataxia ((HIGGINS et al., 1982a, 1982b; KOUTINAS et al., 2002; SILVA et al., 2007). São descritas seis síndromes clínicas neurológicas

na cinomose: encefalomielite dos cães jovens, encefalomielite multifocal dos cães adultos, encefalite dos cães velhos (GREENE, 2012), encefalite pós-vacinal (GREENE, 2012; HARTLEY, 1974), encefalomielite desmielinizante crônica recidivante dos cães adultos (HIGGINS; CHILD; VANDEVELDE, 1989) e polioencefalite do corpúsculo de inclusão (NESSELER et al., 1997, 1999).

Na prática, o diagnóstico da cinomose pode se basear nos sinais clínicos, achados hematológicos (incluindo linfopenia causada pela depleção linfoide), achados patológicos e confirmação virológica (KRAKOWKA; KOESTNER, 1977). O isolamento viral é possível a partir do cultivo em macrófagos alveolares e linfócitos em até 24 a 48 horas (GREENE, 2012). As principais amostras para o diagnóstico virológico ante mortem são sangue, suabe nasal, conjuntival, vaginal ou LCR. Amostras post mortem que podem ser enviadas à virologia são pulmão, cérebro, tecido linfático e bexiga (GREENE, 2012). A técnica de imuno-histoquímica (IHQ) tem sido empregada para a detecção de antígenos da cinomose canina (MASUDA et al., 2006). As amostras principais para o diagnóstico post mortem a partir de IHQ são o epitélio dos coxins, bexiga, estômago, pulmão, encéfalo, baço, rins, tonsilas e intestino (DUCATELLE; COUSSEMENT; HOORENS, 1980; KOUTINAS et al., 2004; LIANG et al., 2007).

A maioria dos trabalhos sobre detecção molecular de cinomose envolve estudos prospectivos comparando sinais clínicos com amostras de sangue, soro, suabe conjuntival, urina, LCR e fragmentos de órgãos (ELIA et al., 2006; FRISK et al., 1999; GEBARA et al., 2004a, 2004b; NEGRÃO; ALFIERI; ALFIERI, 2007; SHIN et al., 1995). Foi realizada uma análise prospectiva na cidade de Londrina, estado do Paraná (PR) que investigou a detecção do gene da nucleoproteína do vírus da cinomose canina por reação da transcriptase reversa, seguida de uma reação em cadeia da polimerase (RT-PCR) em amostras de urina de cães com sinais clínicos de cinomose. De 87 amostras (oriundas de três grupos diferentes A, B e C), 41 (47%) foram positivas para o CDV. O grupo A, que teve cães com alterações clínicas sistêmicas, constatou 21 (51,2%) amostras positivas de 41 analisadas. O grupo B, com alterações clínicas neurológicas, constatou 11 (29,7%) amostras positivas de 37 testadas. Finalmente, no grupo C, que incluía cães que apresentaram simultaneamente alterações clínicas sistêmicas e neurológicas, todas as amostras foram positivas (9/9 [100%]) (GEBARA et al., 2004a). Negrão et al. (2006) realizou um estudo de avaliação da urina e de leucócitos como amostras biológicas para a detecção *ante-mortem* do vírus da cinomose canina por RT-PCR em cães naturalmente infectados. Entre maio e novembro de 2004, foram selecionados 188 cães com suspeita clínica de cinomose atendidos em um Hospital Veterinário. Independente da apresentação dos sinais clínicos foram observados 125 cães positivos (66,5%), sendo possível a amplificação do CDV

a partir da urina em 113 (90,4%) animais, e a partir dos leucócitos, em 88 (70,4%). Se os leucócitos fossem o único material biológico utilizado para o diagnóstico do CDV, teria havido 37 (29,6%) resultados falso-negativos independente da apresentação clínica. Se a urina fosse encaminhada para a realização do diagnóstico, haveria 12 (9,6%) resultados falso-negativos, um número menor, mas ainda significativo. Em um estudo no Japão, foi utilizada RT-PCR para detecção de RNA de CDV em células mononucleares do sangue periférico de cães com suspeita de cinomose, onde apenas 53% dos animais foram positivos por RT-PCR (SHIN et al., 1995), um número menor do que aquele apresentado por Negrão et al. (2006), que testou leucócitos em geral. Em um estudo na Alemanha, foi utilizado RT-PCR para a detecção de RNA de CDV em amostras de soro, sangue total e LCR de cães com infecção espontânea por CDV. Os resultados foram correlacionados com achados clínicos, patológicos, sorológicos e imuno-histoquímicos. O RNA do CDV foi detectado por RT-PCR em 86% das amostras de soro e 88% das amostras de sangue total e de LCR de cães com doença confirmada por imuno-histoquímica. No entanto, a sensibilidade do RT-PCR variou entre os primers selecionados, dependendo de sua posição no gene (FRISK et al., 1999, p. 4). O estudo de Gebara (2004b) em Londrina PR, avaliou histologicamente fragmentos de encéfalo e bexiga de cães com diagnóstico laboratorial de cinomose canina realizado pela técnica da RT-PCR, constatando que todos os cães (9/10) positivos em RT-PCR apresentaram alterações histológicas no cérebro e cerebelo característicos de encefalite aguda (5/9) ou crônica (4/9). Nas amostras de bexiga não foram observadas lesões histológicas.

Outro método de diagnóstico é pela visualização de corpúsculos de inclusão observados na histologia. Trata-se de agregados de vírions e proteínas virais, tendo grande valor diagnóstico (GREENE, 2012). Os corpúsculos de inclusão da cinomose podem ser intranucleares ou intracitoplasmáticos e são vistos principalmente no estômago, bexiga, pelve renal, epitélio conjuntival, epitélio dos brônquios, pele e sistema nervoso central (BEINEKE et al., 2009; GREENE, 2012; LEMPP et al., 2014; SILVA et al., 2009; SUMMERS; CUMMINGS; LAHUNTA, 1995)

2.2. ENCEFALOMIELITE DESMIELINIZANTE NA CINOMOSE

A infecção do SNC pelo CDV ainda dá origem a muitos estudos atualmente, principalmente porque muitos aspectos da patogênese ainda não são totalmente elucidados. O tipo de lesão que ocorre e a evolução da infecção no SNC dependem de numerosos fatores, incluindo a idade e a imunocompetência do hospedeiro no momento da exposição, as

propriedades neurotrópicas do vírus e o momento em que as lesões são examinadas (GREENE, 2012).

A encefalite dos cães velhos é um dos subtipos da doença neurológica induzida pela infecção por CDV. É uma doença inflamatória progressiva e crônica extremamente rara, que afeta principalmente a substância cinzenta dos lobos cerebrais e do tronco cerebral, sendo associada à infecção pelo CDV (HEADLEY et al., 2009a, 2009b). Esse tipo de encefalite ocorre em animais imunocomprometidos, ocorrendo persistência do vírus nos neurônios (AXTHELM; KRAKOWKA, 1998). Outra variante da doença neurológica é a "encefalite pós-vacinal", que cursa com poliencefalite com corpúsculo de inclusão. Como o próprio nome diz, ela ocorre, na maior parte das vezes, após a vacinação (NESSELER et al., 1997). As lesões são principalmente de encefalite desmielinizante no ângulo cerebelo-pontino com a presença de muitas inclusões neuronais e malácia ventral generalizada na ponte. Geralmente, essa forma ocorre sem inclusões virais nos órgãos viscerais (HARTLEY, 1974).

A leucoencefalomilite desmielinizante da cinomose (LEMC) é uma das lesões mais comuns na doença e, como o próprio nome diz, geralmente começa afetando a substância branca (BEINEKE et al., 2009; LEMPP et al., 2014). Estudos detalhados acerca da disseminação do vírus no SNC indicam que em uma fase rara e breve da doença, a substância cinzenta pode ser acometida antes do desenvolvimento de LEMC (BEINEKE et al., 2009; SUMMERS; GREISEN; APPEL, 1984; VANDEVELDE; KRISTENSEN, 1977).

A LEMC segue um determinado curso de tempo que se correlaciona com alterações neuropatológicas observadas. Um esquema de classificação histológica bem descrito tem sido utilizado por vários autores para categorizar a evolução temporal das lesões de substância branca nesta condição (SEEHUSEN et al., 2007; SEEHUSEN; BAUMGÄRTNER, 2010; ULRICH et al., 2014; VANDEVELDE et al., 1982). Com base nisso, destacam-se as lesões agudas, subagudas e crônicas. Esses estágios histológicos foram associados a quadros de tempo após a infecção experimental pelo CDV, que mostram alterações morfológicas semelhantes para cada tempo de evolução estudado (SEEHUSEN et al., 2007; SEEHUSEN; BAUMGÄRTNER, 2010; ULRICH et al., 2014; VANDEVELDE et al., 1982).

Histologicamente, as lesões desmielinizantes caracterizam-se por vacuolização e perda multifocal de mielina (ZURBRIGGEN; YAMAWAKI; VANDEVELDE, 1993) que, no SNC, podem apresentar-se sob duas formas distintas: uma aguda, inicial, não acompanhada por processo inflamatório, e uma crônica, posterior, na qual a inflamação torna-se presente (VANDEVELDE; ZURBRIGGEN, 2005; WÜNSCHMANN et al., 1999; ZURBRIGGEN; YAMAWAKI; VANDEVELDE, 1993). A forma aguda se correlaciona com a imunossupressão

inicial gerada pelo vírus ao acometer os linfócitos da circulação periférica, e a crônica, com o restabelecimento do número de linfócitos na circulação (VANDEVELDE; ZURBRIGGEN, 2005; WÜNSCHMANN et al., 1999). A desmielinização aguda é caracterizada pelo desenvolvimento de lesões na ausência de infiltração mononuclear (VANDEVELDE; ZURBRIGGEN, 2005). Cursa com vacuolização da mielina e uma extensa proliferação e hipertrofia de astrócitos, nos quais se observam inclusões virais no núcleo e no citoplasma (BEINEKE et al., 2009; VANDEVELDE; ZURBRIGGEN, 2005). Na forma crônica, além da desmielinização severa e da reação astrocitária intensa, observam-se dispersão de células inflamatórias pelo parênquima nervoso e presença de manguitos perivasculares, constituídos por plasmócitos, macrófagos e linfócitos (BEINEKE et al., 2009). Observa-se também diminuição na expressão de proteínas virais pelas células infectadas, o que pode estar relacionada a remoção do vírus pela resposta imunológica do hospedeiro (ZURBRIGGEN; YAMAWAKI; VANDEVELDE, 1993). Entre as lesões agudas e as crônicas, está localizado um grupo de lesões subagudas. Estas são divididas em “sem inflamação" e “com inflamação” (LEMPP et al., 2014). Tem sido proposto que lesões agudas podem corresponder aos dias 16-24, lesões subagudas aos dias 24-32 e lesões crônicas aos dias 29-63 após a infecção experimental (BEINEKE et al., 2009). No entanto, diferentes estágios da lesão podem ser observados simultaneamente em um mesmo indivíduo (BEINEKE et al., 2009), o que pode dificultar a determinação do tempo de evolução da lesão.

Estudos sobre a localização das lesões de SNC em cães com cinomose tem sido realizados por vários pesquisadores, sendo as áreas mais acometidas a substância branca do cerebelo, regiões periventriculares (especialmente o quarto ventrículo), pedúnculos cerebelares, hipocampo, tratos ópticos e medula espinhal (SUMMERS; CUMMINGS; LAHUNTA, 1995; VANDEVELDE; ZURBRIGGEN, 2005). No entanto, poucos estudos descrevem a distribuição das lesões na medula espinhal (HIGGINS et al., 1982a, 1982b; KOUTINAS et al., 2002; LEMPP et al., 2014; RAINE, 1976; SCHONING; LAYTON, 1992; SILVA et al., 2009; SUMMERS; GREISEN; APPEL, 1979). A maioria dos trabalhos sobre a detecção do vírus e das lesões de medula espinhal de cães com LEMC são relatos de caso ou estudos experimentais. Dessa forma, a distribuição neuroanatômica das lesões em casos espontâneos de LEMC são pouco exploradas, à diferença do encéfalo (HIGGINS et al., 1982a, 1982b; LEMPP et al., 2014; SUMMERS; CUMMINGS; LAHUNTA, 1995; VANDEVELDE; ZURBRIGGEN, 2005)

Um trabalho experimental abordando aspectos temporais da entrada viral, inflamação e desmielinização no SNC de cães constatou que no dia 24 pós-inoculação (P.I.), as lesões de desmielinização eram escassas na medula espinhal, contrastando com aquelas encontradas no

cérebro e tronco (SUMMERS; GREISEN; APPEL, 1979). Aparentemente, as mesmas se tornaram mais marcadas no dia 30 P.I. (SUMMERS; GREISEN; APPEL, 1979). Observou-se também, que aos 24 dias P.I., a desmielinização não era acompanhada de inflamação, mas raramente, foram encontrados manguitos perivasculares associados a pequenas áreas focais de gliose (SUMMERS; GREISEN; APPEL, 1979). Em outro estudo acerca de lesões agudas na substância branca da medula espinhal (RAINE, 1976), foram investigados três cães com encefalomielite por cinomose por meio de microscopia eletrônica. Nesses casos, a substância branca da medula espinhal de todos os animais revelou abundante desmielinização, gliose e ausência de inflamação. Dentro dessas lesões agudas, inclusões virais intranucleares foram frequentemente encontradas nos astrócitos. Raízes de nervos espinhais não demonstraram alterações nesse estudo (RAINE, 1976).

Foi realizado no LPV-UFSM um estudo prospectivo a fim de descrever a distribuição e evolução das lesões de encéfalo e medula espinhal em cães com cinomose (SILVA et al., 2009). Foram incluídos 54 cães com lesão na medula espinhal. A lesão mais prevalente foi a desmielinização, sendo o segmento mais acometido o cervical cranial (54%). Inflamação não supurativa e astrogliose também ocorreram, semelhante ao que é descrito no encéfalo. Esferoides axonais e câmaras de digestão foram lesões comumente associadas à desmielinização. Malacia e hipertrofia das células endoteliais dos vasos também puderam ser observadas, sendo que o segmento cervical cranial foi o mais afetado por malacia (SILVA et al., 2009). Um estudo realizado na Europa constatou que 11 de 17 cães acometidos por cinomose apresentaram lesões de medula. Quatro desses casos apresentavam lesões cervicais, seguidas de lesões nas regiões lombossacra e cervicotorácica. As lesões mais comuns na medula acometeram a substância branca e foram: desmielinização seguida de inflamação não supurativa (KOUTINAS et al., 2002).

Pesquisas indicam que o início da doença é diretamente mediado por vírus, no entanto, a progressão das placas desmielinizantes em estágios posteriores é reconhecida como um processo inflamatório imunomediado primário (ALLDINGER et al., 1996b; BAUMGÄRTNER; ÖRVELL; REINACHER, 1989; BEINEKE et al., 2009; VANDEVELDE; ZURBRIGGEN, 2005). Embora a inflamação na fase inicial da doença do LD seja mínima, a resposta imune celular aumenta drasticamente no estágio crônico (VANDEVELDE et al., 1981; WÜNSCHMANN et al., 1999). Apesar da falta de infiltrados perivasculares, a imunofenotipagem de infiltrados parenquimatosos revela que as células T CD8 + são o principal tipo de célula inflamatória durante o início precoce da doença e são encontradas principalmente em áreas de desmielinização aguda (TIPOLD et al., 1999a; WÜNSCHMANN et al., 1999).

Além disso, eles se correlacionam amplamente com áreas de infecção viral (TIPOLD et al., 2001b; WÜNSCHMANN et al., 1999). As células T CD8 + parecem estar particularmente protegidas da infecção pelo vírus e, portanto, são capazes de invadir significativamente o neuroparênquima que está agudamente infectado (TIPOLD et al., 2001).

A maioria dos linfócitos detectados nos espaços perivasculares são células T CD4 +, enquanto os focos de lesões desmielinizadas são predominantemente células T CD8 + (ALLDINGER et al., 1996; TIPOLD et al., 1999). Além disso, aproximadamente sete semanas após a infecção, numerosas células B começam a ocorrer, possivelmente contribuindo para a destruição da mielina na DL crônica, sugerindo um papel deletério da resposta imune humoral (ALLDINGER et al., 1996; VANDEVELDE et al., 1981, 1982).

Os resultados da imunofenotipagem são indicativos de um processo imunomediado do tipo tardio com citotoxicidade de células T dependente de anticorpos na fase crônica da DL (ALLDINGER et al., 1996; VANDEVELDE et al., 1982; WÜNSCHMANN et al., 1999). Além de uma infiltração de células imunes periféricas, as lesões inflamatórias mencionadas são paralelas a uma ativação de microglia / macrófagos com uma impressionante alta regulação de antígenos MHC classe II na microglia, células endoteliais, meníngeas e ependimárias que se correlacionam amplamente com o grau da expressão do antígeno viral (ALLDINGER et al., 1996; BEINEKE et al., 2008, 2009).

Além disso, a ativação microgial e desmielinização parecem estar claramente associadas (STEIN et al., 2004). Assim, a ativação de células microgliais induzida por vírus provavelmente desempenha um papel essencial na subsequente desmielinização do tipo *bystander* (ALLDINGER et al., 1996; TIPOLD et al., 1999; VANDEVELDE; ZURBRIGGEN, 2005). Acredita-se, assim, que a própria resposta imune antiviral local contribua para os danos à mielina, em parte pela produção de citocinas pró-inflamatórias (TIPOLD et al., 1999).

Foi demonstrado recentemente que o dano axonal ocorre em lesões precoces, mas ainda mielinizadas, além de placas desmielinizadas crônicas em cães que sofrem de leucoencefalomielite. Adicionalmente, uma densidade axonal progressivamente decrescente durante a doença foi observada. A axônios foram positivos para um marcador altamente sensível para dano axonal precoce que e a proteína precursora de b-amilóide (b-APP) em áreas que expressam o antígeno CDV, e ainda sem lesões visíveis (SEEHUSEN; BAUMGÄRTNER, 2010). Morfologicamente os axônios danificados são caracterizados por estruturas tumefeitas em bainhas de mielina dilatadas (COLEMAN, 2005; POVLISHOCK, 1992; POVLISHOCK; ERB; ASTRUC, 1992), que contêm organelas axoplasmáticas densamente compactadas (“corpos densos”). No SNC, as lesões axonais compartilham as características da degeneração

Walleriana, em que o segmento distal degenera após lesão axonal focal. Posteriormente, o segmento proximal pode sofrer degeneração retrógrada até o pericário ("dorso moribundo") (COLEMAN, 2005).

2.3. HIPERCERATOSE CUTÂNEA NA CINOMOSE

A cinomose pode ser caracterizada por diferentes tipos de lesão de pele. Em cães naturalmente infectados, podem ser encontradas lesões de pele referidas por alguns autores como impetigo (MILLER et al., 2013) ou dermatite pustular (CASWELL; WILLIAMS, 2016; GREENE, 2012; NELSON; COUTO, 2014). A inclusão da dermatite pustular como uma das lesões associadas à cinomose é muitas vezes polêmica, já que nem sempre é possível induzir pústulas cutâneas com infecções experimentais. Alguns pesquisadores (LAIDLAW e DUNKIN, 1926) acreditam que as mesmas sejam secundárias à infecção bacteriana, e não diretamente induzidas pelo vírus (APPEL; GILLESPIE; SIEGERT, 1972).

A hiperceratose dos coxins em associação com uma encefalite posteriormente identificada como cinomose foi descrita pela primeira vez por MacIntyre et al. (1948), e este complexo de doença foi denominado 'doença do coxim duro'. Lesões semelhantes no coxim plantar são vistas em outras doenças, como pênfigo foliáceo ou dermatose responsiva ao zinco, e este pode ser um dos motivos pelos quais o termo 'doença do coxim duro' atualmente não é usado com frequência (GROSS et al., 2008).

A doença do coxim duro representa uma manifestação cutânea incomum de cinomose e é caracterizada por hiperceratose principalmente dos coxins e do plano nasal (BEINEKE et al., 2008; FRISK et al., 1999; GRONE; DOHERR; ZURBRIGGEN, 2004a). Embora seja raramente mencionada, a lesão pode também afetar pele com pelos (CASWELL; WILLIAMS, 2016, HAINES et al., 1999).

Embora a patogênese dessa manifestação permaneça indeterminada, acredita-se que o CDV causa um distúrbio na diferenciação dos queratinócitos (GRONE; DOHERR; ZURBRIGGEN, 2004a; GRÖNE; ENGELHARDT; ZURBRIGGEN, 2003). O vírus penetra no epitélio dos coxins e plano nasal durante o segundo período virêmico e causa a proliferação de queratinócitos basais, além de afetar a diferenciação dos mesmos, resultando em acantose e hiperqueratose (ENGELHARDT et al., 2005; GRÖNE et al., 2003; GRÖNE; ENGELHARDT; ZURBRIGGEN, 2003). A hiperceratose é um achado constante nessas peles, enquanto alterações degenerativas e inflamatórias nem sempre estão presentes (APPEL, 1969; GRÖNE et al., 2003). Alguns autores acreditam na possibilidade de disseminação hematogênica do vírus para as almofadas plantares. Essa possibilidade se sustenta pela presença do antígeno do

CDV nos pericitos e células endoteliais dos vasos sanguíneos dérmicos em cães com cinomose, mesmo naqueles que não apresentam lesões no epitélio dos coxins (KOUTINAS et al., 2004).

Entre os dias 9 a 14 PI, cães com baixa resposta imunológica sofrem disseminação viral para muitos tecidos, incluindo pele, tratos gastrointestinal (GI), respiratório e geniturinário. Os sinais clínicos da doença nesses cães são geralmente dramáticos e graves, e o vírus geralmente persiste em seus tecidos até a morte (SUMMERS; GREISEN; APPEL, 1984). Aproximadamente entre 1 a 10 semanas após o início dos sinais clínicos ou exposição, alguns cães desenvolvem a hiperqueratose do coxim plantar (GRÖNE; ENGELHARDT; ZURBRIGGEN, 2003). A sequência de eventos patogênicos depende da cepa do vírus e pode ser atrasada em 1 a 2 semanas.(SUMMERS; GREISEN; APPEL, 1984). A dermatite vesicular e pustular em filhotes raramente está associada à doença do SNC, no entanto, observa-se uma forte associação entre a doença neurológica e o desenvolvimento de hiperceratose nasal e digital. A infecção da epiderme pelo vírus foi associada a certas cepas de CDV de tipo selvagem que produzem infecção não citocida em queratinócitos plantares da pata in vitro e em cães infectados experimentalmente. (ENGELHARDT et al., 2005; GRÖNE et al., 2003; GRÖNE; ENGELHARDT; ZURBRIGGEN, 2003) Alterações na sequência do gene que traduz a proteína H viral foram associadas à adaptação do vírus ao epitélio dos coxins, aumentando sua capacidade de replicação (RIVALS et al., 2007). Em cães com infecção crônica, ainda é questionável se o CDV pode persistir nas almofadas plantares ao longo da evolução da doença ou simplesmente representar um evento tardio (KOUTINAS et al., 2004) Outros investigadores acreditam que o CDV pode persistir por pelo menos 60 dias após a infecção no epitélio do coxim (GREENE, 2012).

Macroscopicamente, os coxins afetados são espessos, firmes e apresentam fissuras. As lesões são mais graves no centro e pouco flexíveis quando pressionadas (KOUTINAS et al., 2004, MACINTYRE; TREVAN; MONTGOMERIE, 1948). Histologicamente, são descritas hiperqueratose ortoceratótica, hiperplasia epidérmica, vacuolização difusa de queratinócitos (GRONE; DOHERR; ZURBRIGGEN, 2004a) e sincícios (GRONE; DOHERR; ZURBRIGGEN, 2004a). Pode haver leve infiltrado inflamatório constituído predominantemente de histiócitos, com alguns linfócitos e plasmócitos (KOUTINAS et al., 2004). Ceratinócitos degenerados ou necróticos podem estar presentes na lesão (GRONE; DOHERR; ZURBRIGGEN, 2004a). Corpúsculos de inclusão viral eosinofílicos, intracitoplasmáticos e/ou nucleares são frequentes na epiderme (GRÖNE; ENGELHARDT; ZURBRIGGEN, 2003, MILLER et al., 2013). Raramente, as inclusões afetam glândulas sebáceas e sudoríparas (MAEDA et al., 1994, GREENE, 2012).

A imunomarcação para antígenos do CDV é mais frequente na epiderme em lesões de hiperceratose. Aparentemente, há uma diferença entre cães com cinomose com e sem a doença do coxim duro, sendo que os últimos apresentam o antígeno viral predominantemente na derme (KOUTINAS et al., 2004). O antígeno do CDV tem localização intracitoplasmática e intranuclear nas células epidérmicas e dérmicas (HAINES et al., 1999). A distribuição do antígeno viral varia de pequenas áreas focais compostas por algumas células epiteliais até a maioria das células epiteliais de pele. A expressão do antígeno viral ocorre principalmente no estrato espinhoso e granuloso, e raramente no estrato basal (KOUTINAS et. al., 2004). Outros locais frequentes são fibroblastos, pericitos e células endoteliais da derme superficial, glândulas sudoríparas écrinas, e em menor extensão, em seus ductos. Na pele com pelos, o antígeno viral é mais frequentemente encontrado no epitélio do folículo piloso (HAINES et al., 1999; KOUTINAS et al., 2004). Queratinócitos do coxim são infectados com tanta frequência que a demonstração de antígeno viral em biópsias dessas regiões foi sugerida como um meio de diagnóstico *antemortem* de CDV (HAINES et al., 1999).

3 MANUSCRITO 1 –

Artigo aceito para publicação no *Journal of Comparative Pathology* em 18 de outubro de 2021.

INFECTIOUS DISEASE

Short title: Spinal Cord Lesions in Canine Distemper

Running head: W V C Areco *et al*

Histopathological Features of Spinal Cord Lesions in Dogs with Distemper-Associated Demyelinating Leucoencephalomyelitis

Walter Vicente Cardozo Areco[*,‡], Luis Antonio Scalabrin Tondo[*], Nicolas Carmo de Avila[*], Márcia Silva[*,§], Rafael Almeida Fighera[*], Glaucia Kommers[*], Mariana Martins Flores[*] and Eduardo Furtado Flores[†]

[*]Department of Pathology and [†]Departament of Veterinary Preventive Medicine, Universidade Federal de Santa Maria, Santa Maria, Rio Grande do Sul, Brazil

[‡]Present address: Nantes Hospital Veterinario, Assunción, Paraguay.

[§]Present address: Qualem Laboratório Veterinário, Santa Maria, Rio Grande do Sul, Brazil.

Correspondence to: M M Flores (e-mail: marianamflores@yahoo.com.br).

Summary

Demyelinating leucoencephalomyelitis (DLEM) is one of the pathological presentations of canine distemper, but its histological characteristics and topographical distribution of spinal cord injuries have been poorly explored. Seventeen dogs submitted for necropsy to a veterinary

pathology service (2006–2008) and diagnosed with distemper-associated DLEM were investigated. Seventy-two of 231 spinal cord sections from these animals had histological lesions, which consistently affected the white matter. The lumbosacral region was mostly affected (13/17), followed by the thoracolumbar (11/17), cervical (9/17) and cervicothoracic (9/17) regions. Among the 72 affected cord sections, lesions in the lateral (42/72) and dorsal funiculi (31/72) were the most common. Demyelination (17/17), astrocytosis (17/17), microgliosis (17/17), gemistocytes (11/17) and non-suppurative inflammation (10/17) were frequently seen in the white matter. Grey matter changes were less common, and included gliosis (8/17), non-suppurative inflammation (7/17) and malacia (5/17). Acute lesions were most prevalent (13/17) but it was common (10/17) for the same dog to have lesions at different stages of evolution in distinct spinal cord regions. Pathological changes in several spinal cords did not correlate with the reported clinical signs and some dogs did not present with spinal cord-related clinical signs. Our results highlight important aspects of the distribution and morphology of spinal cord lesions in dogs with distemper-associated DLEM, and may assist clinicians and pathologists in the diagnosis of spontaneous cases of this important infectious disease and contribute to further studies concerning distemper pathogenesis.

Keywords: central nervous system, demyelination, histopathology, leucoencephalomyelitis

Introduction

Canine distemper is a highly contagious viral disease, distributed worldwide and caused by canine distemper virus (CDV), a *Morbillivirus* belonging to the family *Paramyxoviridae* (Beineke *et al*, 2009). Distemper-associated demyelinating leucoencephalomyelitis (DLEM) is characterized by demyelination and non-suppurative inflammation of the white matter of the central nervous system (CNS) (Appel, 1969; Lempp *et al*, 2014). Although this presentation often affects the spinal cord

(Vandevelde *et al*, 2005), the distribution and histopathological aspects of DLEM lesions have not been described in detail (Vandevelde and Kristensen, 1977; Summers and Appel, 1987; Raw *et al*, 1992; Koutinas *et al*, 2002). Moreover, most histopathological studies concerning this subject are historical, and have included a small number of dogs (Perdrau and Pugh, 1930; Raine, 1976; Higgins *et al*, 1989), investigated a single spinal cord region (Summers and Appel, 1987) or have few details of lesion distribution (Vandevelde and Kristensen, 1977; Summers *et al*, 1979; Koutinas *et al*, 2002). A detailed investigation of the distribution and morphology of spinal cord lesions in dogs with distemper-associated DLEM may provide pathologists and clinicians with valuable information, facilitating clinical diagnosis and contributing to further research on distemper pathogenesis (Bathen-Noethen *et al*, 2008; Griffin *et al*, 2009). The aim of this study was to characterize the distribution and morphological aspects of spinal cord lesions in dogs with distemper-associated DLEM.

Materials and Methods

Dogs with distemper and presenting with signs of DLEM, submitted for necropsy to the Laboratório de Patologia Veterinária (LPV) of the Universidade Federal de Santa Maria (UFSM), Brazil, from 2006 to 2008, were investigated retrospectively with an emphasis on spinal cord lesions. The 17 cases included in the study had (1) a diagnosis of canine distemper confirmed by histopathological identification of viral inclusion bodies, (2) confirmed lesions of DLEM, (3) availability of multiple paraffin-embedded brain sections and of at least one paraffin-embedded cord section from each of the four regions (C1–C5 [cervical], C6–T2 [cervicothoracic], T3–L3 [thoracolumbar] and L4–S3 [lumbosacral]), and (5) confirmation by immunohistochemistry (IHC) of the presence of CDV antigen in at least one spinal cord section.

The dogs had been submitted by clinicians from the UFSM teaching hospital or from private practices. Data on age and clinical history supplied by the clinician were collected from the necropsy reports. The spinal cords and brains of all dogs were trimmed by two co-authors of this study (MS and RF) in a standardized protocol. Generally, more than one section at different levels of each spinal cord region (C1–C5, C6–T2, T3–L3, L4–S3) was available for histological evaluation. The following regions

of the brain were trimmed and routinely processed in all cases: (1) frontal lobe (including basal nuclei); (2) parietal lobe; (3) temporal lobe; (4) hippocampus; (5) piriform lobe; (6) thalamus; (7) occipital lobe; (8) mesencephalon (on the rostral colliculus region); (9) pons (with cerebellar peduncles); and (10) cerebellum. These dogs were included in a previous publication (Silva *et al*, 2009).

At least four histological sections of spinal cord (one from each spinal cord region) were re-evaluated in each dog. However, more than one section at different levels of the same cord region were available for histological examination from each dog. In total, 231 sections of spinal cord were examined, comprising 45 cervical, 31 cervicothoracic, 74 thoracolumbar and 81 lumbosacral transverse sections (Supplementary Table 1).

For spinal cord histological re-evaluation, the following criteria were analysed: (1) lesion distribution per cord region; (2) anatomical location of these lesions within transverse histological sections; and (3) the morphology of the lesions. For lesion location within transverse histological sections, medullary white matter was divided into the dorsal, lateral and ventral funiculi. Grey matter was divided into the dorsal and ventral horns and the region surrounding the central canal. In addition to a histological description, spinal cord and brain white matter lesions were classified according to their morphological features and stage of development following a system adapted from other studies (Gröne *et al*, 2000; Gröters *et al*, 2005; Seehusen and Baumgärtner, 2010) (Table 1).

All cord sections were stained with Luxol fast blue and areas of white matter with loss of the strong blue staining of normal tissue were interpreted as demyelination. All cord sections were submitted to IHC using a monoclonal antibody against the nucleoprotein of CDV (CDV-NP; VMRD, Pullman, Washington, USA). Antigen retrieval was performed by microwaving (10 min at full power) in citrate buffer pH6.0. Sections were incubated with the primary antibody diluted in phosphate-buffered saline with Tween 20 (1:800) for 1 h at 37°C. A polymer-HRP system (Easypath, São Paulo, Brazil) was used, followed by substrate development with 3,3'diaminobenzidine (DAB; Easypath). Spinal cord sections of four healthy dogs were included as negative controls for the histochemical and immunohistochemical techniques. Slides stained with haematoxylin and eosin (HE) and Luxol fast blue and subjected to IHC were analysed by two examiners (MF and WC).

Results

Histopathology

Among the 17 investigated dogs, the lumbosacral (13/17 [76.4%]) and thoracolumbar (11/17 [64.7%]) regions were most frequently affected by histological changes. All four cord regions were affected in only two dogs (2/17) and six dogs (6/17) had changes in only one region. The remaining dogs had lesions in three (5/17) and two (3/17) regions. In several animals (7/17), the damaged cord regions were invariably adjacent to each other. However, in some dogs (4/17), affected regions were occasionally interspersed with non-affected areas. The age, clinical signs and location of brain and spinal cord lesions in each dog are presented in Table 2.

Among all cord sections with histological lesions, white matter changes were most common in the lateral (42/72) (Fig. 1A) and dorsal (31/72) funiculi; the ventral funiculus was less frequently affected. Grey matter changes were most common in the dorsal horn (21/72). Some grey matter lesions involved the region surrounding the central canal (15/72) (Fig. 1B). Lesions were often multifocal within the same section and within different sections from the same dog. For example, it was common for a particular cord section to have changes restricted to the lateral funiculus, while the adjacent section had lesions confined to the dorsal funiculus. In four dogs (dogs 3, 12, 14 and 15), however, adjacent sections had lesions in the same anatomical location, which could indicate that these changes were continuous. These possibly contiguous changes were present in the dorsal funiculi (two proximate sections within the T3–L3 region and two within L4–S3), the lateral funiculi (three proximate sections within the C1–C5 region and two within L4–S3), the central canal (four proximate sections at the level of T3–L3), the dorsal horn (two proximate sections at the level of C1–C5 and two at C6–T2) and the ventral horn (two proximate sections at the level of C6–T3). In four histological spinal cord sections from three dogs (dogs 10, 11 and 15), the white matter changes were focally extensive, involving almost an entire half of the transverse section, with sparing of the other half. In one of these cases (dog 11), the grey matter of the dorsal and ventral horns was also affected.

The white matter lesions seen in the 17 dogs were: demyelination (17), astrocytosis (17), microgliosis (17), gemistocytes (11/17) (Fig. 2A), non-suppurative inflammation (10/17) (Fig. 2B), malacia (1/17) (Fig. 2C) and intranuclear inclusion bodies in astrocytes (1/17) (Fig. 2D). Grey matter

changes were less common, and consisted of gliosis (8/17), non-suppurative inflammation (7/17), malacia (5/17), presence of gemistocytes (3/17), which occasionally were binucleated (1/17), central chromatolysis (3/17), neuronal necrosis (3/17) and intranuclear inclusion bodies in astrocytes (1/17) or neurons (1/17). When lesions were subdivided into groups according to their morphological features and stage of development, acute lesions (focal vacuolation, mild gliosis with activated astrocytes and microglia) were most common (13/17 dogs). Five dogs had lesions classified as chronic (demyelination, gliosis, gemistocytosis, microgliosis and perivascular lymphohistiocytic infiltrates with more than three cell layers). It was common for the same dog to have lesions in different stages of development in distinct spinal cord regions (Table 3). In some dogs, differences in lesion evolution were also observed between spinal cord and brain (Table 2).

Details of the brain lesions in these dogs have been reported (Silva *et al*, 2009). Briefly, brain lesions were more frequent in the cerebellum (10), mesencephalon (8) and pons (6) and subacute and chronic lesions were more common than acute changes (Table 2). Demyelination was the most prevalent lesion, being seen in all dogs, mainly in the cerebellum, pons and diencephalon, and usually associated with astrogliosis and non-suppurative inflammation. Gemistocytes were present in most cases with astrogliosis. Non-suppurative leptomeningitis, malacia and cortical neuronal necrosis were moderately frequent in the brain. Inclusion bodies were frequent and seen mainly in astrocytes and less frequently in neurons, mainly in the nucleus.

Histochemistry

White matter demyelination was observed in the spinal cord of all 17 dogs and in 58 of the 72 spinal cord sections with lesions (11 cervical, 8 cervicothoracic, 19 thoracolumbar, 20 lumbosacral). Demyelinated areas stained light blue with Luxol fast blue in contrast to the dark blue of unaffected white matter (Fig. 3).

Immunohistochemistry

A total of thirty-eight spinal cord sections were CDV positive on IHC (Table 3). Cytoplasmic and nuclear immunolabelling with anti-CDV antibody was mostly restricted to cord sections with lesions (29/38),

but was also seen in some sections without histological lesions (9/38). Immunolabelling was observed mostly in astrocytes (Fig. 4) and neurons but also occasionally in other cells, such as oligodendrocytes, ependymal cells, endothelial cells of small capillaries and possibly microglia. The intensity of labelling was highly variable, being commonly strong to moderate, but weak in some other cases.

Discussion

It has been well established that distemper-associated DLEM may affect the spinal cord white matter (Summers *et al*, 1979; Raw *et al*, 1992; Vandevelde and Zubriggen, 2005). However, lesion distribution within the different anatomical locations of the spinal cord has not been adequately explored. The present study aimed to characterize spinal cord lesions in 17 dogs with spontaneous distemper-associated DLEM.

Lesion distribution in the various spinal cord regions and anatomical sites within transverse sections had an often random, asymmetrical and multifocal pattern. For example, in four of the 11 dogs with more than one damaged region, the histological changes involved a particular spinal cord region but spared the adjacent region. Only one spinal cord region was affected in six dogs. Furthermore, it was common for a particular histological section to have lesions restricted to the lateral funiculus, while the adjacent section had changes confined to the dorsal funiculus. Only a few lesions were considered bilateral. This random and multifocal lesion distribution was expected, since it is characteristic of infectious diseases such as canine distemper (Summers *et al*, 1979; Higgins *et al*, 1982a, b; Lempp *et al*, 2014). These findings emphasize the importance of adequate sampling and trimming of spinal cords in dogs dying of distemper-associated DLEM. Trimming multiple sections at different levels of the same spinal cord region, as performed in this investigation, may also increase the chances of detecting histological lesions. In contrast with the typically multifocal, asymmetrical and random distribution, in four cord sections from three dogs the white matter changes were focally

extensive, affecting almost an entire half of the transverse section and sparing the other half. In one of these cases, the grey matter (dorsal and ventral horn) was also affected. Two of the three dogs had cord-associated clinical signs. These extensive lesions may have resulted from the fusion of multiple small lesions or, alternatively, from the expansion of a single lesion. These two possibilities have been suggested for demyelinating lesions in humans with multiple sclerosis (Li *et al*, 2006).

Lesion location within the spinal cord did not always correlate with clinical signs. Three dogs from our study did not have any spinal cord-associated clinical signs described in their clinical history, although they had three, two and one spinal cord regions affected by histological lesions, respectively. A previous study found similar results, where three of 17 dogs did not have any association between neuroanatomical lesion location and clinical manifestation (Koutinas *et al*, 2002). In addition, it was not always possible in this study to define the origin of the neurological signs described in the clinical history. For instance, ataxia and myoclonus can correlate with either encephalic or spinal injuries (Tipold *et al*, 1992). Classic spinal cord-associated clinical signs, such as paresis, paralysis, difficulty/inability to stand and decreased limb proprioception, were common, being observed in eight of the 17 dogs investigated. The lateral funiculus was the most frequently lesioned anatomical site. This funiculus consists mainly of corticospinal tracts and damage is often associated with hindlimb paresis and paralysis (Lorenz *et al*, 2011), which were commonly described in our study. The second most frequently affected site was the dorsal funiculus, which mainly comprises sensory nerve fibres (Lorenz *et al*, 2011). However, lack of limb sensory response had not been documented in these dogs. It is important to emphasize that although distemper is characterized by multifocal histological changes, there is usually a dominant lesion, which is generally related to the clinical signs (Braund, 1994; de Lahunta *et al*, 2009). Myoclonus was the most prevalent neurological clinical sign, which agrees with other reports (Tipold *et al*, 1992; Koutinas *et al*,

2002). Despite being an extremely common clinical sign, the pathogenesis of myoclonus in canine distemper is not well understood (Tipold *et al*, 1992; Greene and Vandevelde, 2012). Experimental studies suggest that focal lesions in the spinal grey matter, particularly affecting cranial nerve nuclei and lower motor neurons, may be responsible for this important clinical sign (Summers *et al*, 1995; Greene and Vandevelde, 2011). In five of the seven cases of myoclonus in this study, lesions were detected in the spinal cord grey matter. However, in only one of these dogs was the lower motor neuron region affected, which could explain the presence of myoclonus due to spinal cord injuries according to the above hypothesis. Although clinical correlation with lesion location was beyond the scope of this study, it was possible to conclude that spinal cord lesions can be clinically silent in some cases of distemper-associated DLEM, which may lead to a lack of post-mortem spinal cord examination.

The histological appearance of CNS lesions in distemper-associated DLEM has been correlated with the chronological order of development of these changes, giving rise to a classification system (Vandevelde *et al*, 1981, 1982; Lempp *et al*, 2014). It is possible to infer that the various lesion groups included in this classification system represent the same process at different stages of chronological development (Vandevelde *et al*, 1981, 1982; Lempp *et al*, 2014). Accordingly, the fact that brain and spinal cord – and even different spinal cord regions – from the same dog may present with different lesion types is an interesting finding, and may contribute to future pathogenesis investigations. It was not possible to correlate lesion evolution with the chronological stage of viral infection in our study, in contrast to experimental investigations. In spontaneous CDV infections such as those investigated in this study, this type of correlation is not possible because several variables may influence viral propagation and lesion development in the CNS (Lempp *et al*, 2014).

Funding

This work was supported financially by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil. ED Flores has a fellowship from CNPq and WV Cardozo Areco has a scholarship from CAPES.

Conflict of Interest Statement

The authors declared no potential conflicts of interest with respect to the research, authorship or publication of this article.

References

Appel MJG (1969) Pathogenesis of canine distemper. *American Journal of Veterinary Research*, **30**, 1167–1182.

Bathen-Noethen A, Stein VM, Puff C, Baumgärtner W, Tipold A (2008). Magnetic resonance imaging findings in acute canine distemper virus. *Journal of Small Animal Practice*, **49**, 460–467**.**

Beineke A, Puff C, Seehusen F, Baumgärtner W (2009) Pathogenesis and immunopathology of systemic and nervous canine distemper. *Veterinary Immunology and Immunopathology*, **127**, 1–18.

Braund KG (1994) *Clinical Syndromes in Veterinary Neurology*, 2nd Edit, Mosby, St. Louis, p 477.

de Lahunta G, Glass E, Kent M (2009) General sensory systems: general proprioception and general somatic afferent. In: *Veterinary Neuroanatomy and Clinical Neurology*, 3rd Edit, Elsevier Saunders, St. Louis, pp 192–242.

Griffin JF, Young BD, Levine JM (2009) Imaging diagnosis – chronic canine distemper meningoencephalitis. *Veterinary Radiology & Ultrasound*, **50**, 182–184.

Greene CE, Vandevelde M (2012) Canine distemper. In: *Infectious Diseases of the Dog and Cat*, 4th Edit, CE Greene, Ed, Elsevier, St. Louis, pp 26–43.

Gröne A, Alldinger S, Baumgärtner W (2000) Interleukin-1b, -6, -12 and tumor necrosis factor-alpha expression in brains of dogs with canine distemper virus infection. *Journal of Neuroimmunology*, **110**, 20–30.

Gröters S, Alldinger S, Baumgärtner W (2005) Up-regulation of mRNA for matrix metalloproteinases-9 and -14 in advanced lesions of demyelinating canine distemper leukoencephalitis. *Acta Neuropathologica*, **110**, 369–382.

Higgins RJ, Child G, Vandevelde M (1989) Chronic relapsing demyelinating encephalomyelitis associated with persistent spontaneous canine distemper virus infection. *Acta Neuropathologica*, **77**, 441–444.

Higgins RJ, Krakowka SG, Metzler AE, Koestner A (1982a) Experimental canine distemper encephalomyelitis in neonatal gnotobiotic dogs. A sequential ultrastructural study. *Acta Neuropathologica*, **57**, 287–295.

Higgins RJ, Krakowka SG, Metzler AE, Koestner A (1982b) Primary demyelination in experimental canine distemper virus induced encephalomyelitis in gnotobiotic dogs. Sequential immunologic and morphologic findings. *Acta Neuropathologica*, **58**, 1–8.

Koutinas AF, Polizopoulou ZS, Baumgärtner W, Lekkas S, Kontos V (2002) Relation of clinical signs to pathological changes in 19 cases of canine distemper encephalomyelitis. *Journal of Comparative Pathology*, **126**, 47–56.

Lempp C, Spitzbarth I, Puff C, Cana A, Kegler K *et al* (2014) New aspects of the pathogenesis of canine distemper leukoencephalitis. *Viruses*, **6**, 2571–2601.

Li Z, Lu C, Wang Y, Hashizume Y, Yoshid M (2006) Heterogeneity of spinal cord pathology in multiple sclerosis and variants: a study of postmortem specimen from 13 Asian patients. *Neurology Asia*, **11**, 111–121.

Lorenz MD, Coates JR, Kent M (2011) Localization of lesions in the nervous system. In: *Handbook of Veterinary Neurology*, Elsevier Saunders, St Louis, pp 37–57.

Perdrau JR, Pugh LP (1930) The pathology of disseminated encephalomyelitis of the dog (the (nervous form of canine distemper). *The Journal of Pathology*, **33**, 79–91.

Raine CS (1976) On the development of CNS lesions in natural canine distemper encephalomyelitis. *Journal of the Neurological Sciences*, **30**, 13–28.

Raw ME, Pearson GR, Brown PJ, Baumgärtner W (1992) Canine distemper infection associated with acute nervous signs in dogs. *Veterinary Record*, **130**, 291–293.

Seehusen F, Baumgärtner W (2010) Axonal pathology and loss precede demyelination and accompany chronic lesions in a spontaneously occurring animal model of multiple sclerosis. *Brain Pathology,* **20**, 551–559.

Silva M, Fighera RA, Mazzanti A, Brum JS, Pierezan FP (2009) Neuropatologia da cinomose canina: 70 casos (2005–2008). *Pesquisa Veterinaria Brasileira*, **29**, 643–652.

Summers B, Appel MJG (1987) Demyelination in canine distemper encephalomyelitis: an ultrastructural analysis. *Journal of Neurocytology*, **16**, 871–881.

Summers BA, Cummings JF, de Lahunta A (1995) Degenerative diseases of the central nervous system. In: *Veterinary Neuropathology*, Mosby, St. Louis, pp 284–285.

Summers BA, Greisen HA, Appel MJ (1979) Early events in canine distemper demyelinating encephalomyelitis. *Acta Neuropathologica*, **46**, 1–10.

Tipold A, Vandevelde M, Jaggy A (1992) Neurological manifestations of canine distemper virus infections. *Journal of Small Animal Practice*, **33**, 466–470.

Vandevelde M, Fankhauser R, Kristensen F, Kristensen B (1981) Immunoglobulins in demyelinating lesions in canine distemper encephalitis. An immunohistological study. *Acta Neuropathologica*, **54**, 31–41.

Vandevelde M, Higgins RJ, Kristensen B, Kristensen F, Steck AJ *et al* (1982) Demyelination in experimental canine distemper virus infection: immunological, pathologic, and immunohistological studies. *Acta Neuropathologica*, **56**, 285–293.

Vandevelde M, Kristensen B (1977) Observations on the distribution of canine distemper virus in the central nervous system of dogs with demyelinating encephalitis. *Acta Neuropathologica*, **40**, 233–236.

Vandevelde M, Zurbriggen A (2005) Demyelination in canine distemper virus infection: a review. *Acta Neuropathologica*, **109**, 56–68.

Figure legends

Fig. 1. Leucomyelitis, distemper, spinal cord, dogs. (A) Lateral funiculus with focally extensive area of increased cellularity due to gliosis and perivascular inflammation (asterisk), classified as subacute with inflammation. No eosinophilia suggestive of demyelination. (B) Grey matter with focally extensive chronic lesion of non-suppurative perivascular inflammation (arrow) ventral to central canal. HE.

Fig. 2. Leucomyelitis, distemper, spinal cord, dogs. (A) Vacuolation and gliosis with gemistocytes (arrow) in white matter. (B) Extensive gliosis and perivascular lymphohistiocytic infiltration (arrow) in white matter. (C) Extensive malacia associated with numerous gitter cells (arrows) in white matter. (D) Eosinophilic intranuclear viral inclusion bodies within reactive astrocytes (arrows). HE.

Fig. 3. Leucomyelitis, distemper, spinal cord, dog. Focal area of demyelination indicated by less intense staining (asterisk). Luxol fast blue.

Fig. 4. Leucomyelitis, distemper, spinal cord, dog. Multiple CDV-positive cells, mostly astrocytes, in white matter. Inset: astrocyte with intense cytoplasmic and nuclear immunolabelling. IHC.

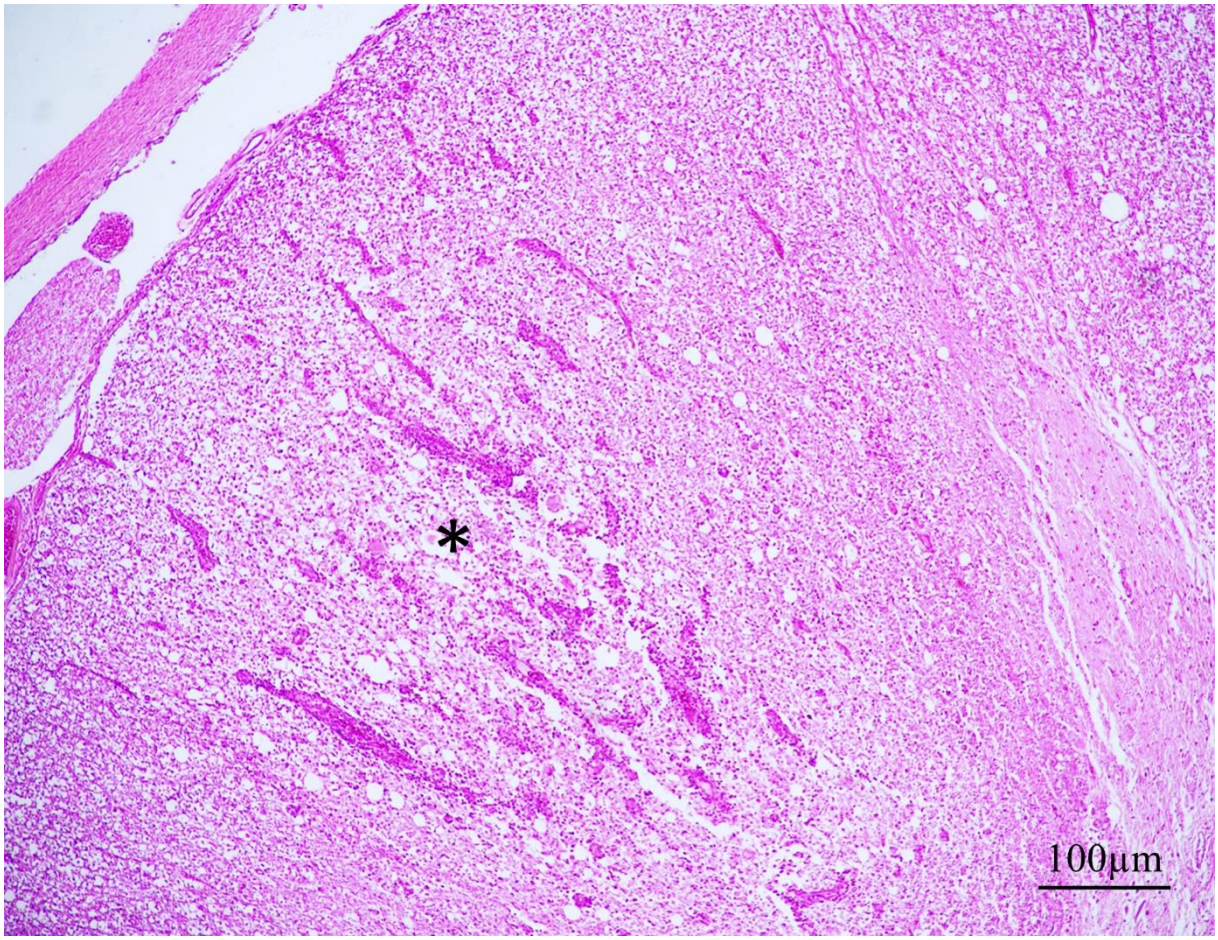


Figure 1A

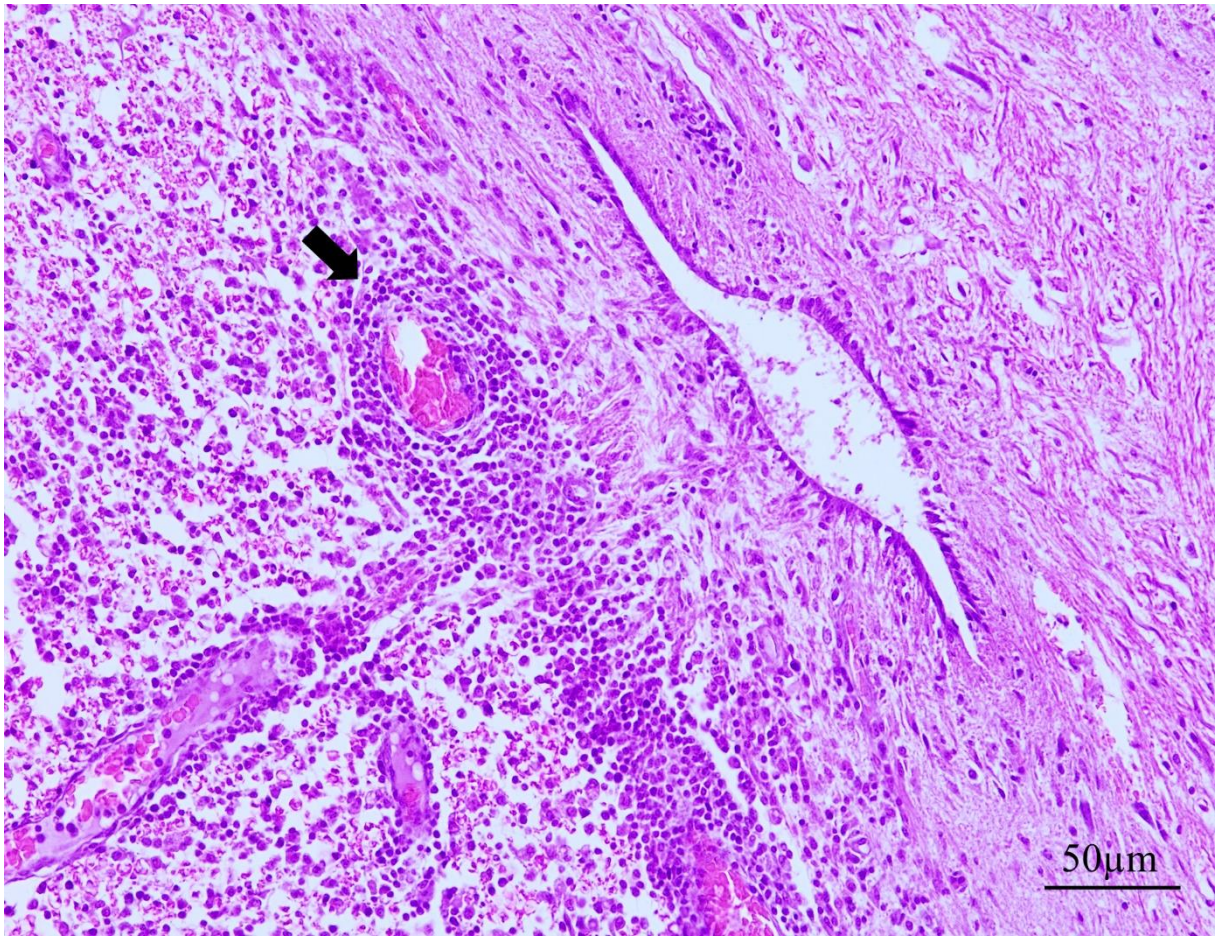


Figure 1B

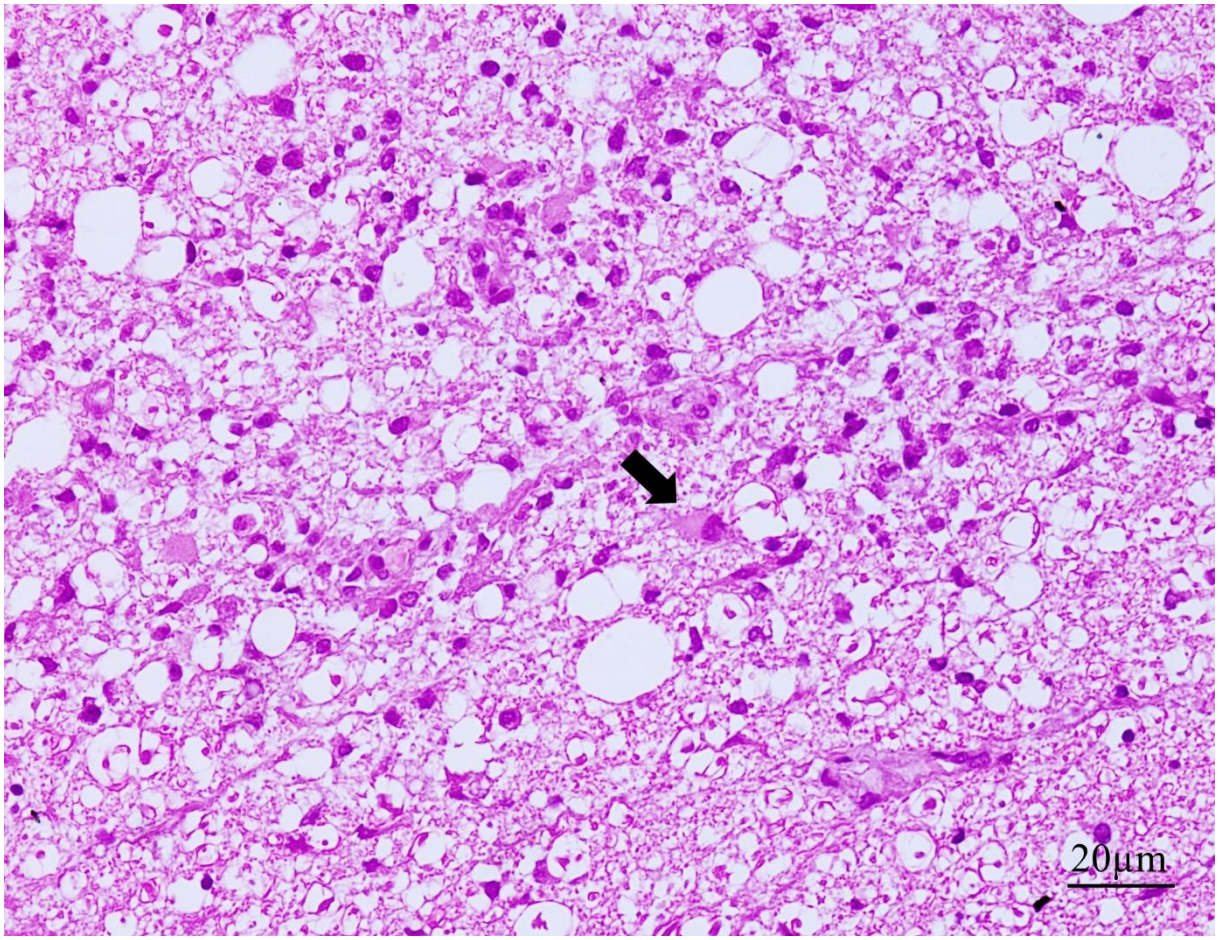


Figure 2A

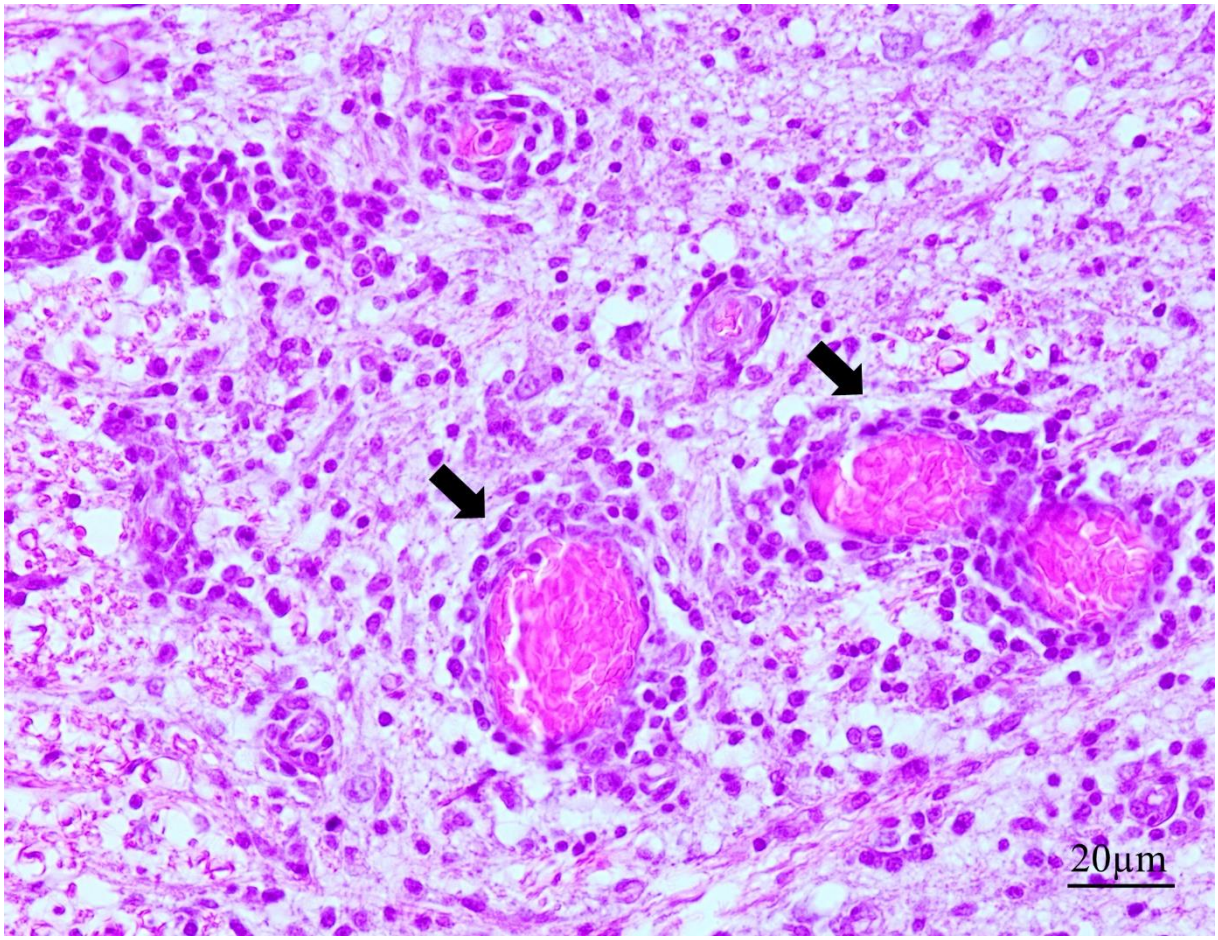


Figure 2B

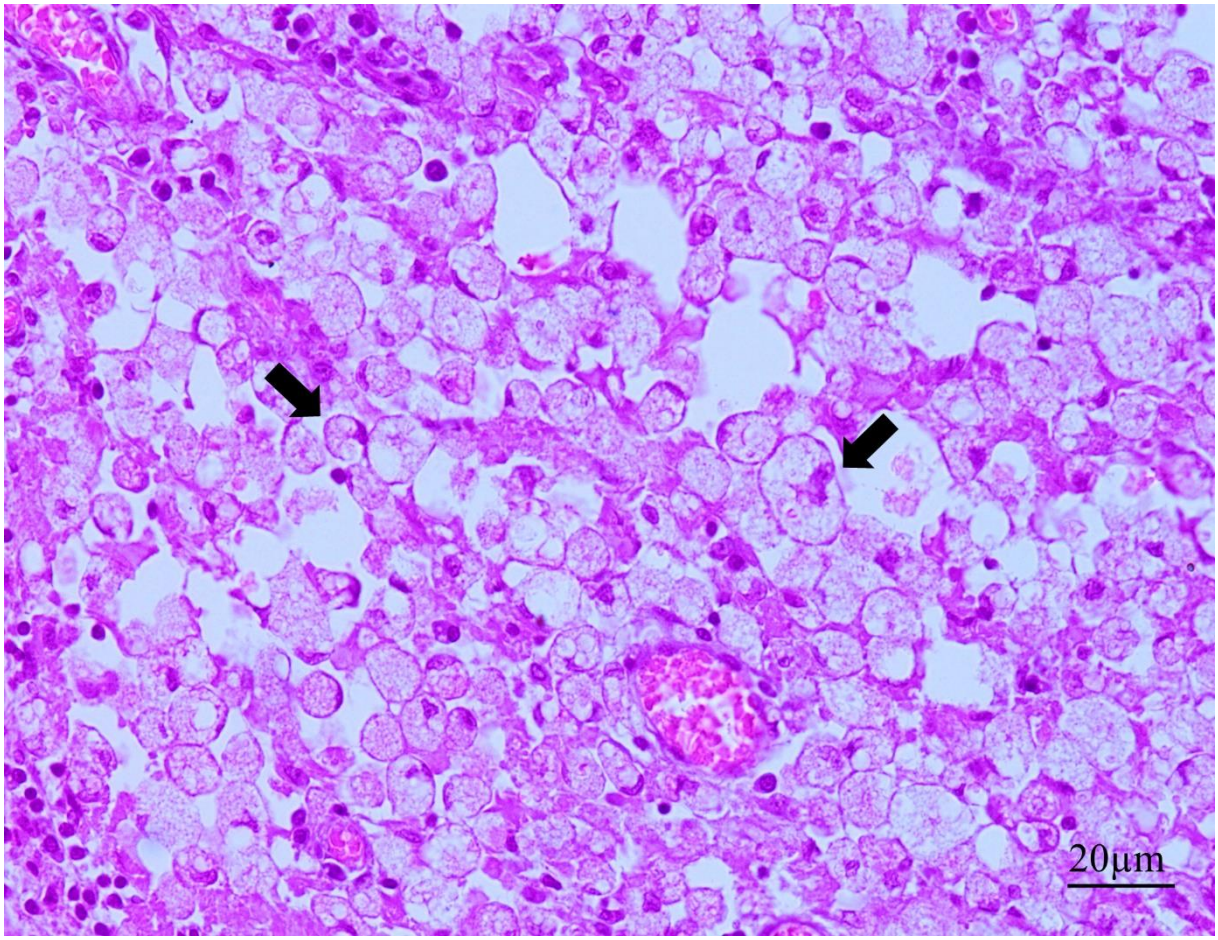


Figure 2C

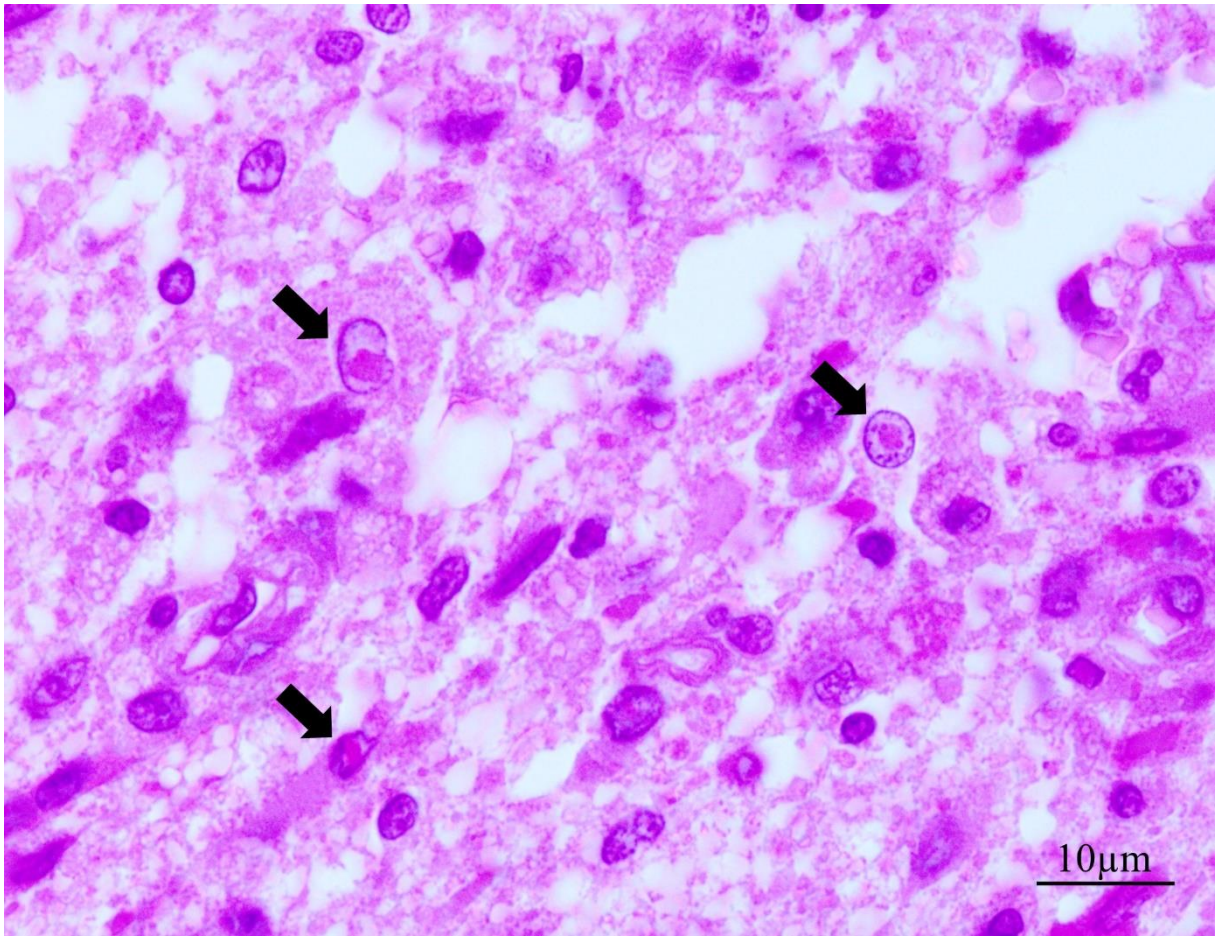


Figure 2D

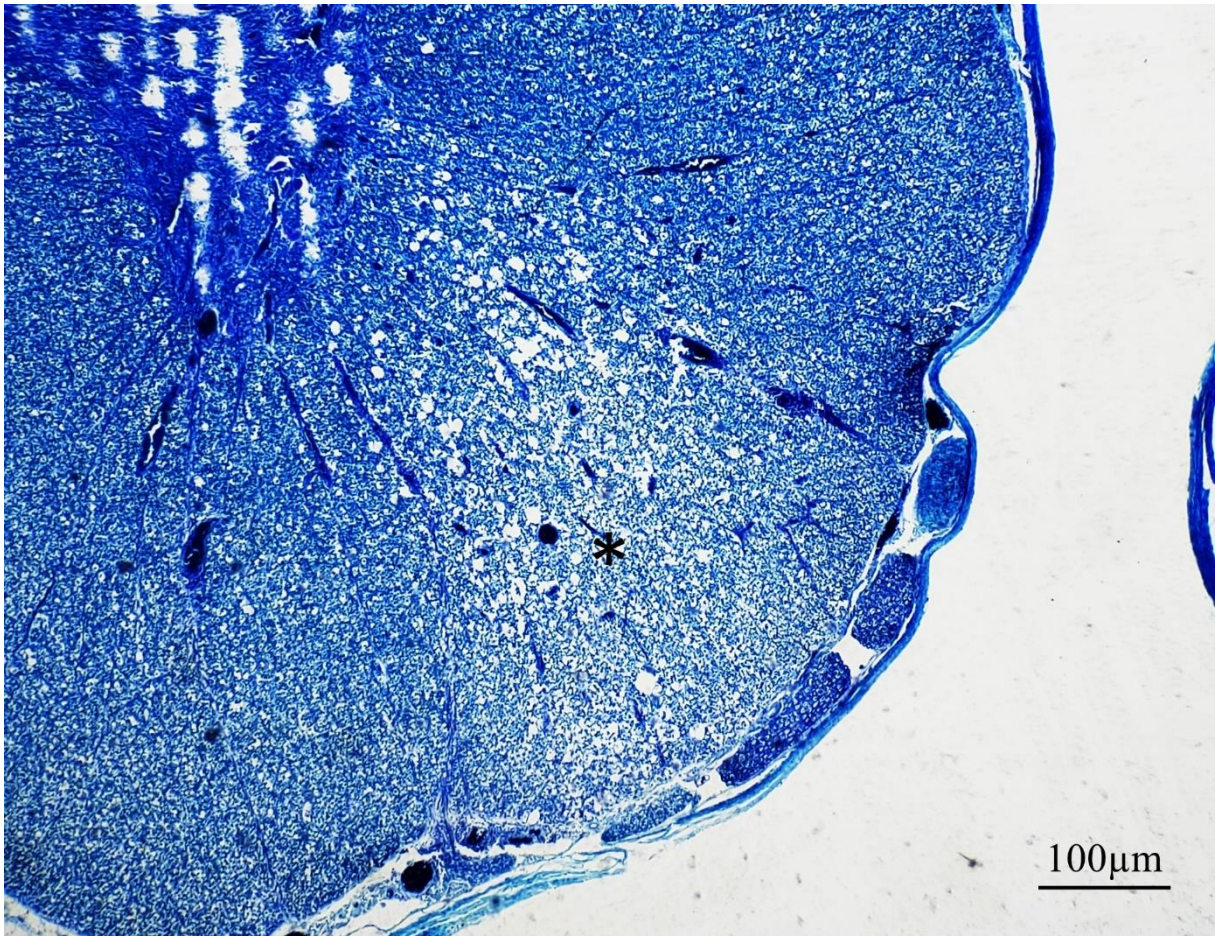


Figure 3

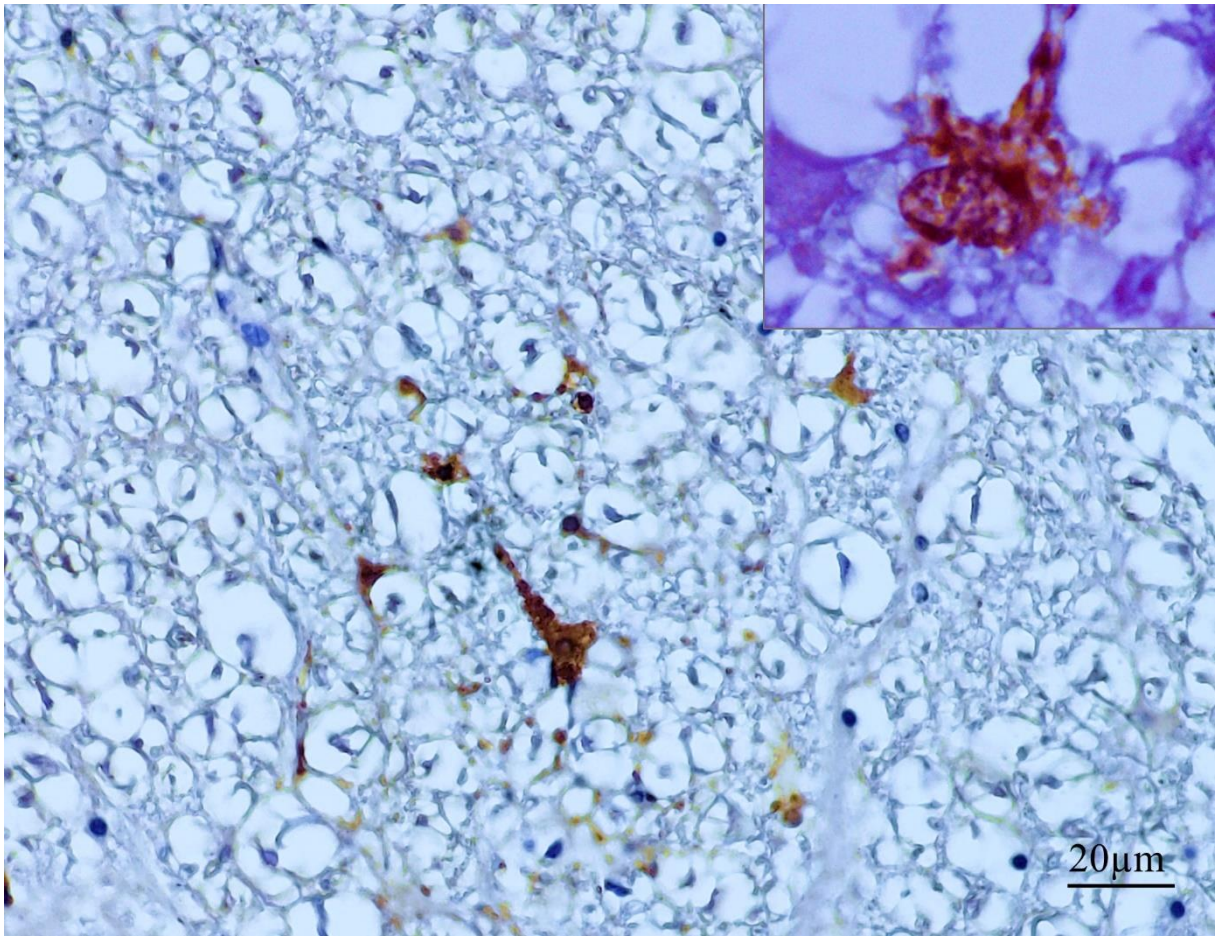


Figure 4

Table 1

Histopathological classification of white matter lesions in distemper-associated demyelinating leucomyelitis

Stage of Development	*Histopathological findings*
Acute	Focal vacuolation, mild gliosis with activated astrocytes and microglia
Subacute without inflammation (S)	Demyelination, moderate gliosis with gemistocytes and microglia
Subacute with inflammation (SW)	Similar to S, with additional perivascular lymphohistiocytic infiltrates (two to three cell layers deep)
Chronic	Similar to SW, but with perivascular lymphohistiocytic infiltrates containing more than three cell layers

Table 2
Brain and spinal cord lesion distribution and classification in 17 dogs with natural canine distemper demyelinating leucoencephalomyelitis

Dog no.	*D*	*Age* *	*Brain*										*Spinal cord*				*Neurological evolution***	*Neurological clinical signs*	*Clinical evolution***	*Non-neurological clinical signs*
			FL	PL	TL	OL	PI	HI	DI	ME	C	PO	CE	CT	TH	LS				
1	E	6m	-	-	SW	-	-	-	S	-	S	S	-	AC	-	-	1	Myoclonus, hindlimb paralysis, seizures	15	Lower respiratory tract signs
2	U	4y	AC	AC	AC	AC	-	-	-	AC	AC		-	AC	AC	S	11	Myoclonus, seizures, teeth grinding	11	Anorexia, vomit, lower respiratory tract signs, ocular and nasal discharge, footpad hyperkeratosis
3	E	1y	CH	SW		S	-	-	S	CH	CH	AC	-	-	CH	-	1	Difficulty standing up, myoclonus, seizures, vocalization	21	Ocular and nasal discharge, lower respiratory tract signs
4	E	3m	SW	-	-	-	SW	-	-	S	SW	SW	-	AC	AC	CH	10	Difficulty standing up, myoclonus, vocalization	20	Lower respiratory tract signs
5	E	7y	AC	-	S	S	-	-	S	S	S	S	S	-	-	AC	U	Seizures, vocalization	U	Ocular discharge
6	E	7y	-	AC	-	AC	-	-	-	-	AC		-	AC	AC	AC	1	Myoclonus	7	Diarrhoea, lower respiratory tract signs
7	E	3y	-	-	-	-	-	-	AC	AC	S		-	AC	AC	AC	U	U	U	U
8	E	5m	-	-	-	-	-	-	-	AC	AC		AC	-	-	-	U	Ataxia, vocalization	30	Diarrhoea, ocular discharge
9	E	2y	CH	-	-	SW	-	-	AC	CH	CH	AC	AC	SW	SW	AC	U	Seizures	-	None
10	E	2y	SW	SW	-	-	-	-	-	CH	SW	SW	SW	CH	SW	AC	U	Absent threat response and facial sensation, ataxia, decreased hindlimb proprioception	-	None
11	E	2y	-	-	-	-	-	-	-	-	S		CH	-	AC	SW	7	Paresis and falls	-	None
12	E	2y	-	-	-	-	-	-	-	-	CH		-	-	SW	SW	U	U	U	U
13	N	2m	-	-	-	-	-	-	-	-	SW		-	-	-	SW	-	Absent	15	Diarrhoea, dyspnoea, nasal discharge, footpad hyperkeratosis
14	E	6y	CH	-	-	-	-	-	CH	CH	-	CH	SW	-	-	-	U	Absent threat response and nasal sensation, ambulatory tetraparesis	-	None
15	E	8m	SW	SW	SW	SW	-	-	-	-	CH		-	AC	-	SW	10	Ataxia, hind limb paralysis and myoclonus	50	Diarrhoea, nasal and ocular discharge
16	E	10m	-	-	-		-	-	-	-	SW	SW	CH	AC	-	SW	60	Ataxia, myoclonus	60	Anorexia
17	N	7m	-	-	-	AC	-	-	AC	S	AC	AC	AC	-	-	-	7	Hind limb paresis	30	Anorexia, ocular discharge

*, age in years (y) and months (m); **, in days; - , no lesions observed; D, form of death; E, euthanasia; N, natural; U, unknown; FL, frontal lobe (including basal nuclei); PL, parietal lobe; TL, temporal lobe; OL, occipital lobe; PI, piriform lobe; HI, hippocampus; DI, diencephalon (with mamillary body); ME, mesencephalon (rostral colliculus region); C, cerebellum; PO, pons (with cerebellar peduncles); CE, cervical; CT, cervicothoracic; TH, thoracolumbar; LS, lumbosacral; S, subacute without inflammation; SW, subacute with inflammation; AC, acute; CH, chronic.

Table 3
Spinal cord lesion distribution and evolution in 17 dogs with natural canine distemper demyelinating leucoencephalomyelitis

Dog no.	*Cervical*				*Cervicothoracic*				*Thoracolumbar*				*Lumbosacral*				*Neurological clinical signs*
	LF	*DF*	*VF*	*GM*	*LF*	*DF*	*VF*	*GM*	*LF*	*DF*	*VF*	*GM*	*LF*	*DF*	*VF*	*GM*	
1	-	-	-	-	**AC**	-	-	-	-	-	-	-	-	-	-	-	Myoclonus, hindlimb paralysis, seizures
2	-	-	-	-	-	**AC**	-	-	-	**AC**	-	-	**S**	-	-	-	Myoclonus, seizures, teeth grinding
3	-	-	-	-	-	-	-	-	**CH**	**SW**	-	**DH VH**	-	-	-	**DH**	Difficulty standing up, myoclonus, seizures, vocalization
4	-	-	-	-	-	**AC**	-	-	**AC**	-	-	**DH CC**	**CH**	-	-	**DH**	Difficulty standing up, myoclonus, vocalization
5	**S**	-	-	-	-	-	-	-	-	-	-	-	**AC**	**AC**	-	**DH**	Seizures, vocalization
6	-	-	-	-	-	**AC**	-	-	-	**AC**	-	-	AC	-	AC	-	Myoclonus
7	-	-	-	-	AC	-	-	-	-	**AC**	**AC**	-	-	**AC**	-	-	U
8	**AC**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Ataxia, vocalization
9	AC	AC	AC	-	AC	SW	-	-	**AC**	**SW**	**AC**	-	-	**AC**	**AC**	-	Seizures
10	**SW**	**SW**	-	-	**AC**	**CH**	**AC**	**VH**	**SW**	**AC**	**SW**	**VH**	AC	AC	-	-	Absent threat response/facial sensation, ataxia, decreased hindlimb proprioception
11	**CH**	CH	**CH**	**DH VH CC**	AC	-	-	-	-	-	-	-	SW	-	-	VH	Paresis and falls
12	-	-	-	DH CC	-	-	-	**DH VH** CC	-	**SW**	-	DH VH CC	SW	AC	-	CC	U
13	-	-	-	-	-	-	-	-	-	-	-	-	-	**SW**	-	-	None
14	SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Absent threat response/nasal sensation, ambulatory tetraparesis
15	-	-	-	-	**AC**	**AC**	**AC**	**CC**	**AC**	**SW**	**SW**	**DH CC**	-	-	-	-	Ataxia, hindlimb paralysis and myoclonus
16	**CH**	-	**CH**	**VH**	**AC**	-	-	**DH VH**	-	-	-	DH CC	SW	-	AC	DH VH CC	Ataxia, myoclonus
17	AC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Hindlimb paresis

-, no lesions observed; LF, lateral funiculus; DF, dorsal funiculus; VF, ventral funiculus; GM, grey matter; AC, acute; S, subacute without inflammation; SW, subacute with inflammation; CH, chronic; DH, dorsal horn; VH, ventral horn; CC, central canal; U, unknown. Bold text represents CDV-positive tissue on immunohistochemistry.

4 MANUSCRITO 2 –

Artigo submetido ao periódico Journal of Comparative Pathology.

INFECTIOUS DISEASE

Short title: Virus-induced Hyperkeratosis in Canine Distemper

Running head: W V C Areco *et al*

Canine Distemper Virus-induced Hyperkeratosis in Nasodigital and Non-nasodigital Areas: Macroscopic Distribution, Histopathology and Viral Antigen Expression in 12 Dogs

Walter Vicente Cardozo Areco[*,‡], Ariel Aguiar[*], Vanessa Barraza[*], Rafael Almeida Fighera[*], Glaucia Kommers[*], Mariana Martins Flores[*] and Eduardo Furtado Flores[†]

[*]Department of Pathology and [†]Departament of Veterinary Preventive Medicine, Universidade Federal de Santa Maria, Santa Maria, Rio Grande do Sul, Brazil

[‡]Present address: Nantes Hospital Veterinario, Assunción, Paraguay.

Correspondence to: M M Flores (e-mail: marianamflores@yahoo.com.br).

Summary

Cutaneous hyperkeratosis is one of the many clinicopathological manifestations of canine distemper and is characterized by thickening and hardening of the skin, predominantly in nasodigital areas. Although this lesion may rarely affect other regions, this has been poorly described. The aim of this study was to describe the macroscopic distribution, histologic aspects and viral antigen expression in canine distemper virus-induced hyperkeratotic lesions in dogs,

with emphasis in non-nasodigital skin areas. Twelve dogs with canine distemper and cutaneous hyperkeratosis submitted to necropsy at an anatomic pathology service were investigated. Twenty-two cutaneous hyperkeratotic foci were observed, and affected footpads (11/22), nasal planum (3/22), haired skin from the snout (2/22), periocular area (2/22), ventral abdomen (2/22), scrotum (1/22) and vulva (1/22). The dogs had one (5/12), two (4/12) or three (3/12) regions concomitantly affected. Seventeen hyperkeratotic lesions were histologically evaluated. Orthokeratotic hyperkeratosis was a predominant feature, occasionally accompanied by other findings, including inclusion bodies (14/17), epidermal hyperplasia (9/17), keratinocyte hydropic degeneration (6/17). Ten dogs had antigen expression in at least one skin lesion. Fourteen (14/17) different hyperkeratotic foci were positive on immunohistochemistry, while three (3/17) were negative**.** Viral antigen expression was most common in the sweat glands (13/17), epidermis (11/17), and endothelial cells/pericytes from blood vessels (8/17). Histologic findings and antigen detection were similar among nasodigital and non-nasodigital areas. We emphasize the importance of clinicopathologic recognition of these lesions on the initial suspicion of canine distemper, allowing for an early treatment.

Keywords: footpad, nasal planum, hard pad disease, skin, cutaneous, immunohistochemistry, CDV

Introduction

Infection with canine distemper virus (CDV) induces a variety of clinical manifestations that may occur simultaneously or sequentially (Greene and Vandevelde, 2012; Caswell and Williams, 2016). Skin changes in dogs with distemper include the so called “hard pad disease”, characterized by footpad and nasal planum hyperkeratosis (Gröne *et al*, 2004a; Martella *et al*, 2008; Greene and Vandevelde, 2012; Mauldin and Peters-Kennedy, 2016; Caswell and Williams, 2016). The lesions are grossly characterized by skin thickening and hardening (Mauldin and Peters-Kennedy, 2016). Histologically, hyperkeratosis

may be accompanied by epidermal hyperplasia, degeneration and intraepithelial inclusion bodies, among other findings (Haines *et al,* 1999; Gröne *et al*, 2003b; Koutinas *et al*, 2004).

Although distemper-associated cutaneous hyperkeratosis has been known for a long time, investigations on gross distribution, histologic changes and antigen detection in these lesions are uncommon (Haines *et al,* 1999; Gröne *et al*, 2003a; Gröne *et al*, 2004b; Koutinas *et al*, 2004). The available studies are experimental, have examined small sized punch biopsy samples, describe single cases or do not detail histopathologic findings (Blixenkrone-Molled *et al,* 1993; Maeda *et al*, 1994; Haines *et al,* 1999; Gröne *et al*, 2003b; Koutinas *et al*, 2004; Engelhardt *et al,* 2005). In addition, they mostly describe changes in nasodigital epithelium, with descriptions of haired skin hyperkeratosis in dogs being rare (Maeda *et al*, 1994). The recognition of cutaneous hyperkeratotic changes by clinicians may facilitate the initial suspicion of canine distemper, allowing for an early diagnosis and treatment which, in turn, can improve the prognosis (Greene and Vandevelde, 2012). In addition, characterization studies in the subject may facilitate the anatomopathological diagnosis in biopsy and necropsy samples (Haines *et al,* 1999). They can also contribute to future pathogenesis studies on this important infectious disease. Based on this background, the aim of this study was to characterize the macroscopic distribution, histologic aspects, and viral antigen expression in CDV-induced cutaneous hyperkeratotic lesions in nasodigital and non-nasodigital areas in dogs.

Materials and Methods

A total of 38 dogs submitted to necropsy (2006-2018) in the *Laboratório de Patologia Veterinária* of the *Universidade Federal de Santa Maria*, Brazil, diagnosed with canine distemper and concomitant cutaneous hyperkeratosis were investigated in a retrospective approach. Skins with secondary infections (including lesions with *Candida* sp.*, Malassezia* sp.*, Demodex* sp.*, Dermatophilus* sp., dermatophytosis fungi, and bacterial folliculitis) and cases with incomplete necropsy data were excluded, resulting in a final number of 12 dogs. Age, breed, clinical history, macroscopic and histologic changes were collected from necropsy reports.

Paraffin-embedded skin samples, when available, were retrieved, and new 3 μm-thick skin sections were submitted to Hematoxylin and Eosin (HE) and immunohistochemistry (IHC) with an anti-CDV antibody. Cutaneous HE slides were re-evaluated for a detailed characterization of histologic changes. Two additional organs from each dog containing distemper-associated inflammation and/or inclusion bodies were submitted to IHC. IHC was performed with an anti-mouse CDV monoclonal antibody (CDV-NP, VMRD, Pullman, WA, USA). Antigen retrieval was by microwaving (10 min at full power) in citrate buffer pH6.0. Sections were incubated with the primary antibody diluted in PBST (CDV 1: 400) for one hour at 37°C. A polymer-HRP system (Easypath, SP, Brazil) was used, followed by substrate development with 3,3'diaminobenzidine (DAB; Easypath, SP, Brazil). IHC slides were evaluated to determine antigen distribution and labelling intensity.

Results

Most dogs were over one year old (Table 1). Eleven dogs had an unknown history of vaccination while one (case 2) was unvaccinated. The clinical evolution ranged from 7 to 40 days (median 15 days). Dog from case 3 had been submitted to a cutaneous biopsy due to the severe hyperkeratotic lesions in the snout.

Gross changes

The 12 dogs presented 22 skin foci of hyperkeratosis, including: footpads (11/22), nasal planum (3/22), haired skin from the snout (2/22), periocular area (2/22), ventral abdomen (2/22), scrotum (1/22) and vulva (1/22) (Table 1). The footpad was the only skin region singly affected (5/12). There were also animals with two (4/12) and three (3/12) regions affected concomitantly.

The footpads were grossly affected in 11 dogs. These were described as thickened (8/11), firm or hard (3/11), with peripheral fissures (1/11), all features being more pronounced in the center (Fig. 1). The lesion was seen in the footpads of all limbs (10/11), or occasionally, restricted to

the forelimbs (1/11). All lesions were diffuse and always seen in the metacarpal, metatarsal and digital footpads, with the exception of one dog (case 6), where the hindlimb lesion was focal and restricted to the metacarpal footpad. The nasal planum was affected in three dogs (3/12), and macroscopic features included crusts (2/3), fissures (1/3), thickening (1/3) (Fig. 2) and areas of skin detachment (1/3). Other skin regions were affected by hyperkeratosis in six dogs (6/12), with a total of eight different foci. The haired skin from the snout (2/12) included one lesion in the perinasal and perilabial areas and a second lesion in the haired skin from the dorsal snout. Ventral abdominal skin was thickened (1/2), with scaling (1/2) and crusts (1/2) (Fig. 3). Skins from the snout and/or periocular areas were thickened (3/3), with crusts (3), hypotrichosis (2/3), erosions (1/3), hyperpigmentation (1/3) and scaling (1/3) (Fig. 4). The scrotum had multifocal areas of thickening (Fig. 5), and the external vulvar surface was diffusely thickened and covered by multiple yellow-brown crusts (Fig. 6).

Histopathology

Of the 22 cutaneous hyperkeratotic foci, paraffin-embedded tissues for histologic evaluation and IHC were available in 17 samples (Table 2). Orthokeratotic hyperkeratosis was a prominent histologic feature in all cases (Figs. 7, 8), occasionally accompanied by epidermal hyperplasia (9/17) (Fig. 7), keratinocyte hydropic degeneration (6/17) (Fig. 8) and parakeratotic hyperkeratosis (4/17). Hydropic degeneration predominated in the epidermal stratum spinosum and granulosum, and it was classified as severe (ballooning degeneration) (n=3) or mild (n=3). Fourteen skin sections (14/17) from nine dogs had intracytoplasmic viral inclusions (Fig. 9), varying from abundant (3/14) to moderate (4/14) and scarce (7/14). Three lesions from three dogs (cases 2, 6 and 12) lacked viral inclusions. Syncytial cells were seen within the epidermis (Fig. 10) and sebaceous cells of a haired skin from the periocular region (case 3), and in the sweat glands of two footpads (cases 3 and 5).

Immunohistochemistry

Ten dogs had antigen expression in at least one skin lesion. Fourteen lesions (14/17) were IHC-positive, while three (3/17) (cases 5, 6 and 9) were negative (Table 4). Of these latter, two had viral inclusions on histology. CDV expression was detected in cells with and without viral inclusions. It was variably observed in the cytoplasm and/or nucleus of epidermal and follicular keratinocytes (Fig. 11), sweat glands, sebaceous glands (Fig. 12), endothelium and pericytes (Figs. 12), dermal mesenchymal cells, and dermal leucocytes (Fig. 12). One of the dogs with sweat gland necrosis (case 3) had a strong labeling of the necrotic gland. In the epidermis, labelling was most common in the stratum spinosum and granulosum, uncommon in the stratum corneum and lucidum and rare in the stratum basale. These epidermal layers had a diffuse or multifocal antigen expression. Labelling intensity was variable among sections and cell types (Table 4).

Discussion

Although CDV-induced cutaneous hyperkeratosis in dogs is traditionally described as being mainly restricted to nasodigital epithelium, it may also affect other cutaneous regions as well (Caswell and Williams, 2016). Among the rare references reinforcing this finding, one case report describes cutaneous hyperkeratosis around the mouth, nose and eyes (Maeda *et al*, 1994). It is also worth mentioning a second study, that has analyzed cutaneous punch biopsies from the dorsal neck of dogs with distemper, confirming hyperkeratosis in some samples, although no gross lesions were present (Haines *et al,* 1999). In addition to being rare, these studies are not very detailed, sometimes not thoroughly describing the gross or histopathologic findings in these skins (Maeda *et al*, 1994; Haines *et al,* 1999). The present investigation aimed to characterize gross distribution, histologic features and viral antigen expression in skins with CDV-induced hyperkeratosis, since morphologic details of these lesions are scarce (Gröne *et al*, 2003b; Koutinas *et al*, 2004). One of the most relevant findings was the presence of grossly visible hyperkeratosis in non-nasodigital areas in half of the dogs. Some affected areas, as the

skin from the snout, periocular and peri labial regions, had been already described by others as affected by CDV-induced hyperkeratosis (Maeda *et al*, 1994). In the other hand, scrotum, vulva and ventral abdomen were considered even more unusual sites for this lesion, not being mentioned by the previous studies in dogs. In addition to footpads, ferrets infected with CDV seem to develop skin lesions in other areas, including face, chin and lips (Kelleher, 2001; Perpiñán *et al*, 2008). Phocine distemper virus has been associated with cutaneous hyperkeratosis in seals, affecting the flippers and abdominal area (Lipscomb *et al*, 2001). Measles virus can induce similar cutaneous lesions in humans and non-human primates in different skin areas, including face, abdominal skin, thorax, abdomen, thighs and groin (Hall *et al*, 1971; Choi *et al*, 1999; Magdaleno-Tapial *et al*, 2019). Although these lesions are often described as "erythematous rash", most of them are macroscopically and histologically similar to the morphologic changes observed in distemper (Hall *et al*, 1971; Choi *et al*, 1999; Magdaleno-Tapial *et al*, 2019). On the other hand, they often spare soles and palms, which appears to differ from the gross distribution observed in this study, where footpads and/or nasal planum were always affected concomitantly with other cutaneous areas (Hall *et al*, 1971; Choi *et al*, 1999; Magdaleno-Tapial *et al*, 2019). This predominance of nasodigital lesions in our dogs probably facilitated the clinical suspicion of distemper. We emphasize the importance of clinical recognition of canine cutaneous hyperkeratosis on the initial suspicion of canine distemper, allowing for an early therapeutic intervention. Similarly, the cutaneous rash observed in humans with measles often facilitates the clinical diagnosis of this disease (Magdaleno-Tapial *et al*, 2019).

Histologic findings of nasodigital and non-nasodigital skins from this study were similar to what is described for classic CDV-induced hard pad disease, consistently characterized by orthokeratotic hyperkeratosis, with occasional additional changes, mostly viral inclusion bodies, epidermal hyperplasia and hydropic degeneration (Gröne *et al*, 2003b; Koutinas *et al*, 2004). These changes are also expected in other morbillivirus infections (Hall *et al*, 1971; Choi *et al*, 1999; Lipscomb *et al*, 2001; Magdaleno-Tapial *et al*, 2019) It is important to point out that no significant histologic differences were observed between nasodigital and non-nasodigital areas evaluated in this study. This indicates that lesions affecting cutaneous areas

other than footpads/nasal planum share the same pathogenesis observed in hardpad disease, being indeed part of this entity. The prevalence of viral inclusions in skin regions with CDV-induced hyperkeratosis varies greatly among different studies, probably due to variations of time between infection and death (Gröne *et al*, 2003a; Gröne *et al*, 2003b; Gröne *et al*, 2004b; Koutinas *et al*, 2004). Inclusion bodies are most numerous at days 10-14 post infection, reducing by 5-6 weeks (Caswell and Williams, 2016). In the present study, the period between infection and death was unknown, however, clinical evolution was available in some dogs, with a median of 15 days, which explains why inclusion bodies were frequent, similar to what was demonstrated by others (Gröne *et al*, 2003b).

Epidermal hyperplasia has been shown to make part of the pathogenesis of cutaneous hyperkeratosis in canine distemper and Measles in primates, however, it is not always detected in these morbillivirus-associated skin lesions (Choi *et al*, 1999; Gröne *et al*, 2003a; Gröne *et al*, 2003b). It has been suggested that hyperplasia may be a transient and early process in distemper (Gröne *et al*, 2003b). This would explain why studies with a shorter time post infection describe epidermal proliferation, while others, with a longer time post infection, do not (Gröne *et al*, 2003b; Gröne *et al*, 2004b; Koutinas *et al*, 2004). This lesion was observed in half of the dogs in this study, and the median clinical evolution of these dogs was 9 days, while dogs without hyperplasia had a median of 15 days. Unfortunately, the low number of investigated dogs does not allow us to make any statistical correlations between epidermal hyperplasia and clinical evolution time. A future study correlating these variables would contribute to characterizing the pathogenesis of skin lesions in distemper. Syncytial cell formation was uncommonly observed in this study, being seen in the epidermis and sebaceous cells of a haired skin, and in the sweat glands of two footpads. Despite of their low prevalence, syncytial cells have been occasionally described in hyperkeratotic skins of dogs and other mammals with distemper, and are characteristic of Morbillivirus infections (Hall *et al*, 1971; Choi *et al*, 1999; Lipscomb *et al*,

2001; Caswell and Williams, 2016; Magdaleno-Tapial *et al*, 2019). Interestingly, a former case report in a dog has mentioned syncytial cells being more numerous in the sweat glands when compared to epidermis, which agrees with our results (Maeda *et al*, 1994). Syncytial cells in the sebaceous glands and follicles, asides from epidermis, are a characteristic finding in humans with Measles (Magdaleno-Tapial *et al*, 2019).

IHC of the skin is a sensitive and valuable technique to confirm CDV infection in dogs (Koutinas *et al*, 2004). As reported by others, antigen expression was common in this study, demonstrated in 10 of the 12 included dogs (Gröne *et al*, 2003a; Koutinas *et al*, 2004). Labeling was similar among nasodigital and non-nasodigital areas, overall predominating in the sweat glands, epidermis, and blood vessels. Epidermis and sweat glands are the most common sites of CDV antigen expression, with the latter occasionally having a more intense labelling (Haines *et al*, 1999; Gröne *et al*, 2003a; Koutinas *et al*, 2004). Virus shedding from all body excretions, beginning at day 9 post infection, is mentioned by the literature, and based on the immunohistochemical findings of this and other studies, sweat and sebaceous gland secretions are probable sources of viral shedding to the environment (Haines *et al*, 1999; Koutinas *et al*, 2004; Greene and Vandevelde, 2012). The persistence of the CDV genome in the absence of viral protein synthesis has been demonstrated in the skin and brain of dogs with distemper (Nesseler *et al*, 1997; Gröne *et al*, 2003a). This feature is probably part of the mechanism of viral persistence in dog tissues, and could possibly explain why CDV antigen was not detected in the skin of case 6, which had a 30-day clinical evolution, and did not present inclusion bodies. It was not possible to determine why the other two skins from cases 5 and 9 were also negative.

CDV probably reaches the CNS and skin by blood, around 8-9 days post infection, which would explain the frequency of viral antigen expression in blood vessel endothelium and pericytes in this and other studies (Gröne *et al*, 2003a; Gröne *et al*, 2004b; Koutinas *et al*, 2004; Greene and Vandevelde, 2012). After reaching the skin, the virus infects keratinocytes and induces their proliferation, leading to an increase in mitotic figures and proliferating markers (Gröne *et al*, 2003b; Gröne *et al*, 2004a; Engelhardt *et al*, 2005). Why clinical hyperkeratosis in dogs appears to be more common in the nasodigital skin remains undetermined. This contrasts with non-

human primates with measles, which develop similar cutaneous changes in haired skin areas, often sparing the palms and soles (Hall *et* al, 1971; Choi *et al*, 1999). This predilection of nasodigital epithelium in dogs could indicate some kind of viral tropism for keratinocytes from these areas, maybe related to a better environment for CDV replication, facilitation of viral evasion from the immune system and/or viral persistence (Gröne *et al*, 2003a). Other possibility is that hyperkeratosis occurs in a similar frequency in haired skins, but not severely enough to cause macroscopic lesions, as it does in footpads/nasal planum. This would probably lead these lesions to be underdiagnosed by pathologists. The virus seams to easily infect haired skin, and even in the absence of gross lesions, hyperkeratosis has been detected on histological exam (Appel, 1969; Haines *et al,* 1999). Grossly normal skin areas have not been analyzed in our study, which makes it difficult to confirm or refute these hypotheses.

Other conditions associated with cutaneous hyperkeratosis in dogs, some of them also restricted to footpads and/or nasal planum, should be mentioned as possible clinical and macroscopic differential diagnoses, namely: *Malassezia* infection, zinc-responsive dermatosis, idiopathic nasodigital hyperkeratosis, familial footpad hyperkeratosis, and nasal parakeratosis of the Labrador Retriever (Gross *et al,* 2005; Mauldin and Peters-Kennedy, 2016). A complete clinical history, associated with macroscopic distribution of these lesions and absence of infectious agents in the histologic slides, in addition to CDV-associated histologic features in these skins and in other organs, allowed us to discard the possibility of these lesions being associated with other skin diseases, and should aid pathologists in this differentiation.

Funding

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). E.D. Flores has a fellowship from CNPq and W. V. Cardozo Areco has a scholarship from CAPES.

Conflict of Interest Statement

The authors declared no potential conflicts of interest with respect to the research, authorship or publication of this article.

References

Appel MJG. Pathogenesis of canine distemper. *Am J Vet Res*. 1969;**30**:1167–1182.

Blixenkrone-Molled M, Svansson V, Have P, et al. Studies on manifestations of canine distemper virus infection in an urban dog population. *Vet Microbiol*. 1993;37:163-173.

Caswell JL, Williams KJ. Respiratory System. In: Maxie MG, ed. *Jubb, Kennedy and Palmer's Pathology of Domestic Animals*. **Vol 2**. Elsevier; 2016:574–576.

Choi YK, Simon A, Kim DY, et al. Fatal Measles virus infection in Japanese Maquaces (*Macaca fuscata*). *Vet Pathol*. 1999;**36**:594-600.

Engelhardt P, Wyder M, Zurbriggen A, Gröne A. Canine Distemper Virus Associated Proliferation of Canine Footpad Keratinocytes in Vitro. *Vet Microbiol*. 2005;**107**:1-12.

Greene CE, Vandevelde M. Canine distemper. In: Greene CE, ed. *Infectious diseases of the dog and cat*. Elsevier; 2012:26-43.

Gröne A, Doherr MG, Zurbriggen A. Canine Distemper Virus Infection of Canine Footpad Epidermis. *Vet Dematol.* 2004;**151**:159-167b

Gröne A, Doherr MG, Zurbriggen A. Up-regulation of cytokeratin expression in canine distemper virus-infected canine footpad epidermis. *Vet Dermatol*. 2004;**15**:168-174a

Gröne A, Engelhardt P and Zurbriggen A. Canine Distemper Virus Infection: Proliferation of Canine Footpad Keratinocytes. *Vet Pathol*. 2003;**40**:574-578b

Gröne A, Groeters S, Koutinas A, Saridomichelakis M, Baumgärtner W. Non-cytocidal infection of keratinocytes by canine distemper virus in the so-called hard pad disease of canine distemper. *Vet Microbiol*. 2003; **96**:157-163a

Gross TL, Ihrke PJ, Walder EJ, Affolter VK. *Skin Diseases of the Dog and Cat. Clinical and Histopathologic Diagnosis*. Blackwell Science; 2005:142-190.

Haines DM, Martin KM, Chelack BJ, Sargent RA, Outerbridge CA, Clark EG. Immunohistochemical detection of canine distemper virus in haired skin, nasal mucosa, and footpad epithelium: a method for antemortem diagnosis of infection. *J Vet Diagn Invest*. 1999;**11**:396-399.

Hall WC, Kovatch RM, Herman PH, Fox JG. Pathology of Measles in Rhesus Monkeys. *Vet Pathol*. 1971;**8**:307-319.

Kelleher SA. Skin Diseases of Ferrets. *Vet Clin North Am Exot Anim Pract*. 2001;**4**:565-572.

Koutinas AF, Baumgärtner W, Tontis D, Polizopoulou Z, Saridomichelakis N, Lekkas S. Histopathology and Immunohistochemistry of Canine Distemper Virus-induced Footpad Hyperkeratosis (Hard Pad Diseases) in Dogs with Natural Canine Distemper.*Vet Pathol*. 2004;**41**:2-9.

Lipscomb TP, Mense MG, Habecker PL, Taubenberger JK, Schoelkopf R. Morbilliviral dermatitis in seals. *Vet Pathol.* 2001;**38**:724-726.

Maeda H, Ozaki K, Takagi Y, Sawashima K, Narama I. Distemper Skin Lesions in a Dog. *J Am Vet Med Assoc*. 1994;**41**:247-250.

Magdaleno-Tapial JMD, Valenzuela-Oñate CMD, Giacaman-von W, et al. Follicle and Sebaceous Gland Multinucleated Cells in Measles. The American Journal of *Dermatopathology (Basel)*. 2019;**41**:289-292.

Martella V, Elia G, Buonavoglia C. Canine Distemper Virus. *Vet Clin North Am Small Anim Pract*. 2008;**38**:787-797.

Mauldin EA, Peters-Kennedy J. Integumentary System. In.: Maxie MG, ed. *Jubb, Kennedy and Palmer's Pathology of Domestic Animals*. **Vol 1**. Elsevier; 2016:523,569.

Nesseler A, Baumgartner W, Zurbriggen A, Orvell C. Restricted virus protein translation in canine distemper virus inclusion body polioencephalitis. *Vet Microbiol*. 1997;**69**:23-28.

Perpiñán D, Ramis A, Tomás A, Carpintero E, Bargalló F. Outbreak of canine distemper in domestic ferrets (*Mustela putorius furo*). *Vet Rec.* 2008;**163**:246-250.

Figure legends

Fig. 1. Footpad, case 2. The metacarpal and digital footpads are diffusely thickened, with multiple cracks.

Fig. 2. Nasal planum, case 9. The nasal planum is diffusely thickened, with an irregular surface.

Fig. 3. Ventral abdomen, case 11. The skin is covered with multiple yellowish to brownish crusts.

Fig. 4. Peri labial and perinasal skin from the snout, case 3. The skin is thickened and covered with multiple yellowish to brownish crusts.

Fig. 5. Scrotum, case 9. Small, multifocal to coalescent areas of thickening and crusting are observed.

Fig. 6. Vulva, case 8. The external surface is thickened and covered with multiple yellowish crusts.

Fig. 7. Ortokeratotic hyperkeratosis, nasal planum, case 9. Severe epidermal hyperplasia and ortokeratotic hyperkeratosis. Hematoxylin and eosin (HE).

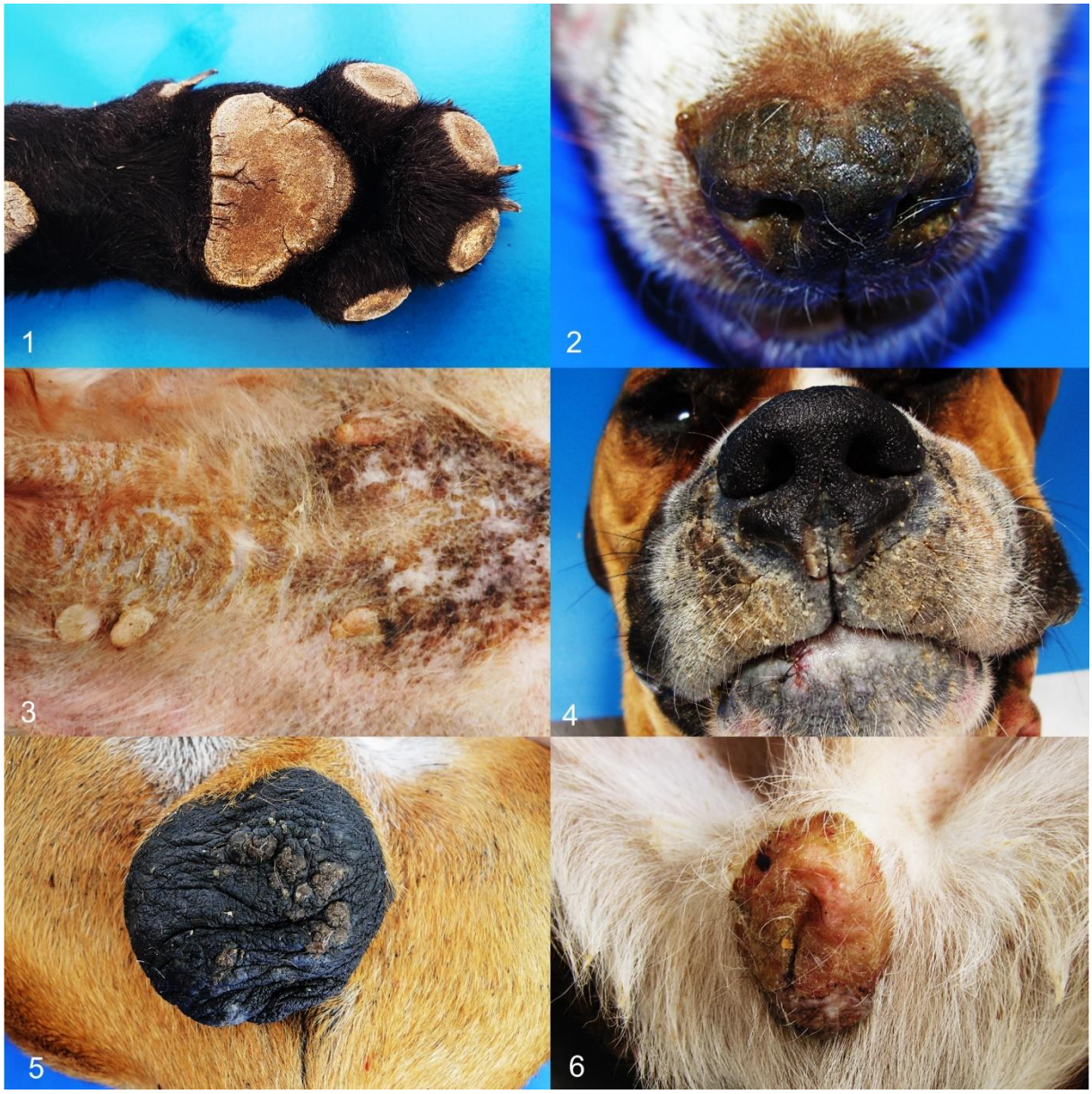
Fig. 8. Ortokeratotic hyperkeratosis and ballooning degeneration, skin from the periocular area, case 1. Keratinocytes from the superficial epidermal layers are diffusely vacuolated, with pale basophilic cytoplasm (ballooning degeneration). There is also ortokeratotic hyperkeratosis. HE.

Fig. 9. Hydropic degeneration and inclusion bodies, skin from the nasal planum, case 8. Multiple keratinocytes from the stratum spinosum are swollen, with pale eosinophilic cytoplasm (ballooning degeneration) and contain multiple cytoplasmic eosinophilic inclusion bodies (arrows). HE.

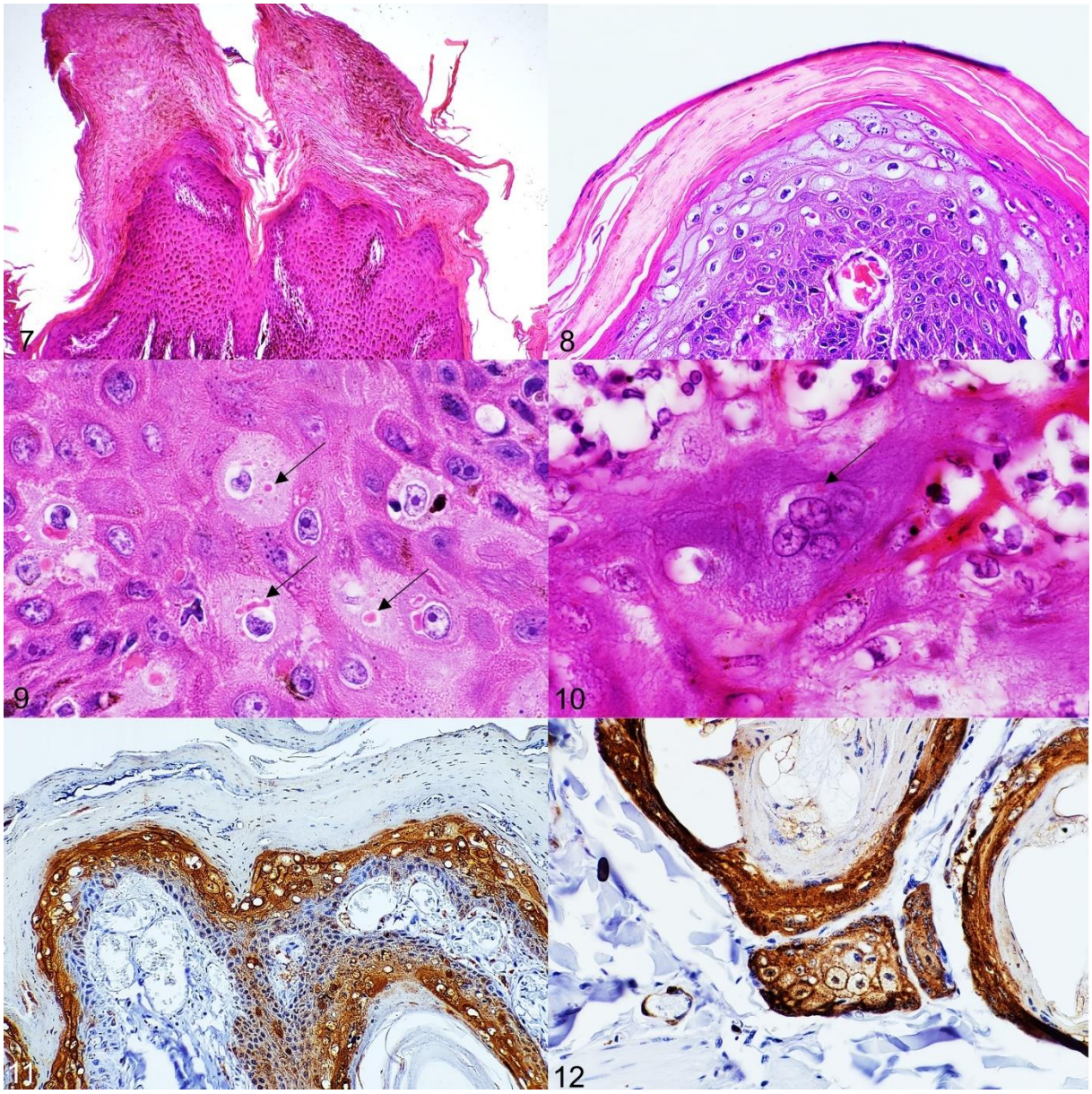
Fig. 10. Syncytial cell with multiple inclusion bodies, skin from the peri labial region, case 3. An epidermal multinucleated keratinocyte (syncytial cell) containing cytoplasmic inclusion bodies (arrow) is in the center, below a pustule. HE.

Fig. 11. Skin from the periocular area, dog, case 3. The vacuolated stratum spinosum and granulosum and follicular epithelium are strongly and diffusely labeled. Some dermal cells are also positive.

Fig. 12. Skin from the ventral abdomen, case 8. The follicular epithelium and sebaceous glands show a strong and diffuse labeling. An adjacent blood vessel is also positive.



Figures 1-6



Figures 7-12

Table 1.
Canine distemper virus-induced hyperkeratosis in 12 dogs: macroscopic distribution, presence of other histologic lesions and viral antigen expression

Nº	*Age*[a]	*Clinical evolution*	*Areas with cutaneous hyperkeratosis*	*Respiratory lesions*	*CNS lesions*	*Viral inclusions*	*Antigen detection in one or more skin lesions*	*Antigen detection in other organs*
1	132	15 days	Footpad, periocular	PNM	Absent	Brain, lung, skin, stomach	Positive	Lung, liver[b], stomach
2	72	15 days	Footpad[c]	Absent	Encephalitis	Brain	Positive	Stomach
3	24	9 days	Footpad, periocular and haired skin from the snout	PNM	Absent	Liver, skin, stomach, urothelium	Positive	Lung
4	4	n.i.	Footpad	PNM	Encephalitis	Brain, skin, stomach, urothelium	Positive	CNS, lung
5	60	10 days	Footpad, nasal planum	PNM	Absent	Brain, skin, urothelium, prostate tumor	Negative	Lung, stomach, prostate tumor
6	5	30 days	Footpad, haired skin from the snout	PNM	Encephalitis	Brain	Negative	Liver**
7	8	20 days	Footpad	Absent	Encephalitis	Skin	Positive	CNS, liver**
8	n.i.	n.i.	Nasal planum, ventral abdomen, vulva	PNM	Encephalitis	Brain, lung, mammary tumor, esophagus, skin, stomach, urothelium	Positive	CNS, lung
9	72	n.i.	Footpad, nasal planum, scrotum	Absent	Encephalitis	Brain, skin, stomach, urothelium	Positive	CNS, lung
10	n.i.	7 days	Footpad	PNM	Encephalitis	Skin	Positive	CNS, lung
11	n.i	n.i.	Footpad, ventral abdomen	Absent	Encephalitis	Skin	Positive	CNS, lung
12	48	40 days	Footpad	PNM	Encephalitis	Brain, urinary bladder	Positive	CNS, lung, pancreas

CNS, central nervous system; PNM, pneumonia; n.i., not informed; [a]Months; [b]Kupffer cells; [c]Only thoracic limbs.

Table 2.
Canine distemper virus-induced hyperkeratosis in 12 dogs: gross distribution and skin lesions submitted to histologic re-evaluation and immunohistochemistry

	Footpad	*Nasal planum*	*Other skin areas*
Dogs affected	11	3	6
Skin foci affected	11	3	8
Lesions submitted to histologic re-evaluation and IHC	9	2	6
Positivity to CDV antigen	7	1	6

CDV, canine distemper virus.

Table 3.
Histologic features in 17 skin lesions with canine distemper virus-induced hyperkeratosis

Histopathology	*Footpad*	*Nasal planum*	*Other skin regions*
Orthokeratotic hyperkeratosis	9/9	2/2	6/6
Parakeratotic hyperkeratosis	1/9	0/0	3/6
Epidermal hyperplasia	3/9	2/2	4/6
Keratinocyte hydropic degeneration	3/9	1/2	2/6
Pigmentary incontinence	0/9	2/2	2/6
Viral inclusions	6/9	2/2	6/6
Epidermal pustules	0/9	0/2	2/6
Epidermal necrosis	1/9	0/2	1/6
Sweat gland necrosis	2/9	0/2	1/6
Epithelial syncytial cells	2/9	0/2	1/6

Table 4.
Canine distemper virus antigen expression and labelling intensity in hyperkeratotic skins of 12 dogs with natural canine distemper

Dog n°	*Skin region*	*Epidermis*	*Hair follicles*	*Sweat glands*	*Sebaceous glands*	*Blood vessels*[a]	*Dermal mesenchymal cells*	*Nerves*	*Dermal leukocytes*
1	Periocular	3	3	3	1	3	0	0	1
2	Footpad	0	n/a	2	n/a	0	0	0	0
3	Footpad	3	n/a	2	n/a	2	3	0	3
	Periocular	3	3	3	3	2	2	0	0
	Snout[b]	3	3	3	3	2	2	0	1
4	Footpad	3	n/a	2	n/a	1	1	0	0
5	Footpad	0	n/a	0	0	0	0	0	0
6	Footpad	0	n/a	0	n/a	0	0	0	0
7	Footpad	1	n/a	2	n/a	1	0	0	0
8	Nasal planum	3	n/a	0	n/a	0	0	0	0
	Ventral abdomen	2	3	2	3	2	1	0	1
9	Footpad	1	n/a	1	n/a	0	0	0	0
	Nasal planum	0	n/a	0	n/a	0	0	0	0
	Scrotum	0	0	1	0	0	0	0	0
10	Footpad	3	n/a	1	n/a	0	0	0	0
11	Ventral abdomen	1	1	1	1	1	3	0	3
12	Footpad	0	n/a	3	n/a	0	0	0	0
	Total	11	5	13	4	8	6	0	5

n/a, not applicable. 3: strong labelling 2: median labelling; 1: weak labelling; 0: negative; [a]Endothelial cells and/or pericytes; [b]Perilabial.

5 DISCUSSÃO

A realização deste trabalho permitiu descrever e caracterizar lesões até então pouco exploradas na cinomose: leucomielite desmielinzante e hiperceratose em regiões de pele hirsuta. Lesões na medula espinhal são consideradas comuns em cães com leucoencefalomielite desmielinizante, mas ainda muito pouco investigadas e caracterizadas na literatura. Por outro lado, as lesões de pele hirsuta, além de pouco exploradas, são consideradas raras. Os resultados permitiram a elaboração de dois artigos científicos.

No artigo 1, constatou-se que as lesões foram multifocais, muitas vezes afetando mais de uma região medular no mesmo cão. As mesmas predominaram na região lombossacra e afetaram mais frequentemente os funículos dorsais e laterais. Interessantemente, nem todos os cães tinham sinais clínicos associados à lesão lesionada. Apesar da sabida importância e frequência das lesões medulares na cinomose, dados referentes aos locais afetados na medula são escassos e ainda pouco explorados (HIGGINS; CHILD; VANDEVELDE, 1989; KOUTINAS et al., 2002; RAINE, 1976; SCHONING; LAYTON, 1992; VANDEVELDE; KRISTENSEN, 1977). A evolução dessas lesões pode ser determinada por meio de um esquema de classificação morfológica com base nos achados histopatológicos utilizado para casos de leucoencefalite induzida experimentalmente em cães (LEMPP et al., 2014, p. 4; VANDEVELDE et al., 1981, 1982). Esse esquema de classificação de lesões de acordo com sua evolução já é bem estabelecido e utilizado em estudos anteriores (GRÖNE; ALLDINGER; BAUMGÄRTNER, 2000; SEEHUSEN et al., 2007; SEEHUSEN; BAUMGÄRTNER, 2010). Apesar disso, é difícil determinar com que frequência cada uma dessas situações ocorre em casos espontâneos de LEMC. Várias lesões de diferentes evoluções temporais podem ser observadas simultaneamente no SNC de cães afetados (BEINEKE et al., 2009), como foi visto em vários animais do nosso estudo.

No artigo 2, foram investigadas a distribuição morfológica, aspectos histológicos e detecção de antígeno viral na pele de 12 cães com hiperceratose associada à cinomose. Interessantemente, seis desses cães apresentavam lesões de hiperceratose em locais atípicos, principalmente pele com pelos, mas também vulva. A hiperqueratose induzida pelo CDV é classicamente observada nos coxins e plano nasal, sendo raramente relatada em peles com pelos (MAEDA et al., 1994; CASWELL; WILLIAMS, 2016). Os achados histológicos e a distribuição de antígeno viral foram muito semelhantes entre as peles glabras e hirsutas nesse trabalho, sugerindo que elas compartilhem da mesma patogênese. Ressalta-se a importância de

caracterizar as lesões de hiperceratose em locais atípicos, visto que o seu reconhecimento pelo clínico pode facilitar o diagnóstico, permitindo uma instituição precoce do tratamento.

6 CONCLUSÃO

O presente estudo possibilitou caracterizar lesões consideradas pouco exploradas na cinomose. A realização do estudo relatado no artigo 1 permitiu concluir que as lesões medulares são frequentemente multifocais e aleatórias, e que as mesmas nem sempre se correlacionam com os sinais clínicos apresentados pelo paciente. Desta forma, enfatiza-se a importância da coleta de medula espinhal em todos os cães com cinomose, mesmo na ausência de sinais clínicos neurológicos medulares. Por meio do artigo 2, concluiu-se que a hiperceratose associada ao CDV pode afetar pele hirsuta, e que essa apresentação não parece ser tão incomum em nossa região. As alterações histológicas foram semelhantes em todas as peles analisadas (hirsutas e glabras), o que permitiu concluir que as mesmas provavelmente compartilham a mesma patogênese. O reconhecimento da hiperceratose associada ao CDV pelo clínico – mesmo de lesões não consideradas clássicas, como as estudadas neste trabalho – é importante, pois contribui para um diagnóstico precoce, aumentando as chances de sucesso na terapia do paciente. Espera-se que os achados desses dois manuscritos possam contribuir para futuros estudos de patogênese da cinomose canina.

REFERÊNCIAS

ALLDINGER, S. et al. 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**, v. 92, n. 3, p. 273–280, 1 ago. 1996a.

ALLDINGER, S. et al. 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**, v. 92, n. 3, p. 273–280, 1 ago. 1996b.

APPEL, M. J. G.; GILLESPIE, J. H.; SIEGERT, R. **Canine Distemper Virus: Marburg Virus**. Vienna: Springer Vienna, 1972.

APPEL, M. J. G. (ED.). **Virus infections of carnivores**. Amsterdam ; New York : New York, NY: Elsevier Science ; Distributors for the U.S. and Canada, Elsevier Science Pub. Co, 1987.

APPEL, M.; SUMMERS, B. Pathogenicity of morbilliviruses for terrestrial carnivores. **Veterinary Microbiology**, v. 44, n. 2–4, p. 187–191, maio 1995.

AXTHELM, M. K.; KRAKOWKA, S. Canine distemper virus: the early blood-brain barrier lesion. **Acta Neuropathologica**, v. 75, n. 1, p. 27–33, 1987.

AXTHELM, M. K.; KRAKOWKA, S. Experimental Old Dog Encephalitis (ODE) in a Gnotobiotic Dog. **Veterinary Pathology**, v. 35, n. 6, p. 527–534, nov. 1998.

BAUMGÄRTNER, W.; ÖRVELL, C.; REINACHER, M. Naturally occurring canine distemper virus encephalitis: distribution and expression of viral polypeptides in nervous tissues. **Acta Neuropathologica**, v. 78, n. 5, p. 504–512, 1 set. 1989.

BEINEKE, A. et al. Increase of pro-inflammatory cytokine expression in non-demyelinating early cerebral lesions in nervous canine distemper. **Viral Immunology**, v. 21, n. 4, p. 401–410, dez. 2008.

BLANCOU, J. Dog distemper: imported into Europe from South America? **Historia Medicinae Veterinariae**, v. 29, n. 2, p. 35–41, 2004.

BLIXENKRONE-MÖLLER, M. et al. Antigenic relationships between field isolates of morbilliviruses from different carnivores. **Archives of Virology**, v. 123, n. 3–4, p. 279–294, set. 1992.

BOTTERON, C. et al. Canine distemper virus-immune complexes induce bystander degeneration of oligodendrocytes. **Acta Neuropathologica**, v. 83, n. 4, p. 402–407, 1992.

COLEMAN, M. Axon degeneration mechanisms: commonality amid diversity. **Nature Reviews Neuroscience**, v. 6, n. 11, p. 889–898, nov. 2005.

DECARO, N. et al. Canine distemper and related diseases: report of a severe outbreak in a kennel. **The New Microbiologica**, v. 27, n. 2, p. 177–181, abr. 2004.

DEEM, S. L. et al. CANINE DISTEMPER IN TERRESTRIAL CARNIVORES: A REVIEW. **Journal of Zoo and Wildlife Medicine**, v. 31, n. 4, p. 441–451, dez. 2000.

DUCATELLE, R.; COUSSEMENT, W.; HOORENS, J. Demonstration of canine distemper viral antigen in paraffin sections, using an unlabeled antibody-enzyme method. **American Journal of Veterinary Research**, v. 41, n. 11, p. 1860–1862, nov. 1980.

EK-KOMMONEN, C. et al. Outbreak of canine distemper in vaccinated dogs in Finland. **Veterinary Record**, v. 141, n. 15, p. 380–383, 11 out. 1997.

ELIA, G. et al. Detection of canine distemper virus in dogs by real-time RT-PCR. **Journal of Virological Methods**, v. 136, n. 1–2, p. 171–176, set. 2006.

FIGHERA, R. A. et al. Causas de morte e razões para eutanásia de cães da Mesorregião do Centro Ocidental Rio-Grandense (1965-2004). **Pesquisa Veterinária Brasileira**, v. 28, n. 4, p. 223–230, abr. 2008.

FRISK, A. L. et al. 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**, v. 37, n. 11, p. 3634–3643, 1999.

GEBARA, C. M. S. et al. Lesões histológicas 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**, v. 56, n. 2, p. 168–174, abr. 2004a.

GEBARA, C. M. S. et al. 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**, v. 56, n. 4, p. 480–487, ago. 2004b.

GEMMA, T. et al. Serological analysis of canine distemper virus using an immunocapture ELISA. **The Journal of Veterinary Medical Science**, v. 58, n. 8, p. 791–794, ago. 1996.

HARTLEY, W. J. A Post-Vaccinal Inclusion Body Encephalitis in Dogs. **Veterinary Pathology**, v. 11, n. 4, p. 301–312, jul. 1974.

HEADLEY, S. A. et al. Diagnostic Exercise: Tyzzer's Disease, Distemper, and Coccidiosis in a Pup. **Veterinary Pathology**, v. 46, n. 1, p. 151–154, jan. 2009a.

HEADLEY, S. A. et al. Molecular Detection of Canine Distemper Virus and the Immunohistochemical Characterization of the Neurologic Lesions in Naturally Occurring Old Dog Encephalitis. **Journal of Veterinary Diagnostic Investigation**, v. 21, n. 5, p. 588–597, set. 2009b.

KRAKOWKA, S.; AXTHELM, M. K.; GORHAM, J. R. Effects of induced thrombocytopenia on viral invasion of the central nervous system in canine distemper virus infection. **Journal of Comparative Pathology**, v. 97, n. 4, p. 441–450, jul. 1987.

KRAKOWKA, S.; COCKERELL, G.; KOESTNER, A. Effects of canine distemper virus infection on lymphoid function in vitro and in vivo. **Infection and Immunity**, v. 11, n. 5, p. 1069–1078, maio 1975.

KRAKOWKA, S.; KOESTNER, A. Comparison of canine distemper virus strains in gnotobiotic dogs: effects on lymphoid tissues. **American Journal of Veterinary Research**, v. 38, n. 12, p. 1919–1922, dez. 1977.

LIANG, C. T. et al. A Non-biotin Polymerized Horseradish-peroxidase Method for the Immunohistochemical Diagnosis of Canine Distemper. **Journal of Comparative Pathology**, v. 136, n. 1, p. 57–64, jan. 2007.

LUDLOW, M. et al. Using the ferret model to study morbillivirus entry, spread, transmission and cross-species infection. **Current Opinion in Virology**, v. 4, p. 15–23, fev. 2014.

MASUDA, M. et al. Characterization of monoclonal antibodies directed against the canine distemper virus nucleocapsid protein. **Comparative Immunology, Microbiology and Infectious Diseases**, v. 29, n. 2–3, p. 157–165, mar. 2006.

MIELE, J. A.; KRAKOWKA, S. Antibody responses to virion polypeptides in gnotobiotic dogs infected with canine distemper virus. **Infection and Immunity**, v. 41, n. 2, p. 869–871, ago. 1983.

MILLER, W. H. et al. **Muller & Kirk's small animal dermatology**. [s.l: s.n.].

MORI, T. et al. The biological characterization of field isolates of canine distemper virus from Japan. **Journal of General Virology**, v. 75, n. 9, p. 2403–2408, 1 set. 1994.

NEGRÃO, F. J.; ALFIERI, A. A.; ALFIERI, A. F. Avaliação da urina e de leucócitos como amostras biológicas para a detecção ante mortem do vírus da cinomose canina por RT-PCR em cães naturalmente infectados. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 59, n. 1, p. 253–257, fev. 2007.

NELSON, R. W.; COUTO, C. G. (EDS.). **Small animal internal medicine**. Fifth edition ed. St. Louis, MO: Elsevier/Mosby, 2014.

NESSELER, A. et al. Abundant expression of viral nucleoprotein mRNA and restricted translation of the corresponding viral protein in inclusion body polioencephalitis of canine distemper. **Journal of Comparative Pathology**, v. 116, n. 3, p. 291–301, abr. 1997.

POVLISHOCK, J. T. Traumatically induced axonal injury: pathogenesis and pathobiological implications. **Brain Pathology (Zurich, Switzerland)**, v. 2, n. 1, p. 1–12, jan. 1992.

POVLISHOCK, J. T.; ERB, D. E.; ASTRUC, J. Axonal response to traumatic brain injury: reactive axonal change, deafferentation, and neuroplasticity. **Journal of Neurotrauma**, v. 9 Suppl 1, p. S189-200, mar. 1992.

RIVALS, J.-P. et al. Adaptation of canine distemper virus to canine footpad keratinocytes modifies polymerase activity and fusogenicity through amino acid substitutions in the P/V/C and H proteins. **Virology**, v. 359, n. 1, p. 6–18, 1 mar. 2007.

RUDD, P. A.; CATTANEO, R.; VON MESSLING, V. Canine Distemper Virus Uses both the Anterograde and the Hematogenous Pathway for Neuroinvasion. **Journal of Virology**, v. 80, n. 19, p. 9361–9370, 1 out. 2006.

SCHONING, P.; LAYTON, C. E. Canine Distemper with Spinal Cord Lesions. **Journal of Veterinary Medicine, Series B**, v. 39, n. 1–10, p. 571–574, 12 jan. 1992.

SEEHUSEN, F. et al. Vimentin-positive astrocytes in canine distemper: a target for canine distemper virus especially in chronic demyelinating lesions? **Acta Neuropathologica**, v. 114, n. 6, p. 597–608, 15 nov. 2007.

SHIN, Y.-S. et al. Detection of Canine Distemper Virus Nucleocapsid Protein Gene in Canine Peripheral Blood Mononuclear Cells by RT-PCR. **The Journal of Veterinary Medical Science**, v. 57, n. 3, p. 439–445, 1995.

SILVA, M. C. et al. Aspectos clinicopatológicos de 620 casos neurológicos de cinomose em cães: Clinicopathological features in 620 neurological cases of canine distemper. **Pesquisa Veterinária Brasileira**, v. 27, n. 5, p. 215–220, maio 2007.

STEIN, V. M. et al. Microglial cell activation in demyelinating canine distemper lesions. **Journal of Neuroimmunology**, v. 153, n. 1–2, p. 122–131, ago. 2004.

SUMMERS, B. A.; GREISEN, H. A.; APPEL, M. J. G. Early events in canine distemper demyelinating encephalomyelitis. **Acta Neuropathologica**, v. 46, n. 1–2, p. 1–10, 1979.

SUMMERS, B. A.; GREISEN, H. A.; APPEL, M. J. G. Canine distemper encephalomyelitis: Variation with virus strain. **Journal of Comparative Pathology**, v. 94, n. 1, p. 65–75, jan. 1984.

SUMMERS, B.; CUMMINGS, J.; LAHUNTA, A. Inflammatory disease of the central nervosus system. In: **Veterinary Neuropathology**. St Louis: Mosby, 1995. p. 95–188.

TIPOLD, A. et al. Early T cell response in the central nervous system in canine distemper virus infection. **Acta Neuropathologica**, v. 97, n. 1, p. 45–56, jan. 1999a.

TIPOLD, A. et al. Partial protection and intrathecal invasion of CD8(+) T cells in acute canine distemper virus infection. **Veterinary Microbiology**, v. 83, n. 3, p. 189–203, 26 nov. 2001a.

TIPOLD, A. et al. Partial protection and intrathecal invasion of CD8(+) T cells in acute canine distemper virus infection. **Veterinary Microbiology**, v. 83, n. 3, p. 189–203, 26 nov. 2001b.

ULRICH, R. et al. Transcriptional changes in canine distemper virus-induced demyelinating leukoencephalitis favor a biphasic mode of demyelination. **PloS One**, v. 9, n. 4, p. e95917, 2014.

VANDEVELDE, M. et al. Immunological and pathological findings in demyelinating encephalitis associated with canine distemper virus infection. **Acta Neuropathologica**, v. 56, n. 1, p. 1–8, 1 mar. 1982.

WÜNSCHMANN, A. et al. Identification of CD4+ and CD8+ T cell subsets and B cells in the brain of dogs with spontaneous acute, subacute-, and chronic-demyelinating distemper

encephalitis. **Veterinary Immunology and Immunopathology**, v. 67, n. 2, p. 101–116, 1 fev. 1999.

ZURBRIGGEN, A.; YAMAWAKI, M.; VANDEVELDE, M. Restricted canine distemper virus infection of oligodendrocytes. **Laboratory Investigation; a Journal of Technical Methods and Pathology**, v. 68, n. 3, p. 277–284, mar. 1993.