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Isolamento e caracterização molecular do vírus da cinomose canina, análise de antivirais e produção de uma proteína M recombinante

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biotecnologia e Biodiversidade da Universidade de Brasília, para obtenção do título de Doutor em Biotecnologia e Biodiversidade.

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SUMÁRIO

LISTA DE ABREVIATURAS E SÍMBOLOS

µg	micrograma
µL	microlitro
°C	graus Celsius
CD	*Canine distemper*
CDV	*Canine distemper virus*
CDV M_p	CDV Matrix protein
CPE	*cytopathic effect*
DNA	ácido desoxirribonucleico
ELISA	*enzyme-linked immunosorbent assay*
h	hora
IFA	*immunofluorescence assay*
kb	kilobase
kDa	kiloDalton
LB	meio de cultura Luria-Bertani
MD	*molecular dynamic*
min	minutos
mL	mililitro
nm	nanômetros
nt	nucleotídeos
ORF	*open reading frame*
pb	pares de bases
PBS	*phosphate-buffered saline*
RdRp	RNA polimerase dependente de RNA
RNA	ácido ribonucléico
RNA	ácido ribonucleico mensageiro
RT-PCR	*reverse transcription polymerase chain reaction*
sec	século
seg	segundos
TBS	*tris-buffered saline*
TBST	*tris-buffered saline* with Tween 20
VDS	VerodogSLAM cell

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RESUMO

O *canine distemper virus* (CDV), gênero *Morbillivirus*, tem emergido e sido reconhecido como um patógeno altamente contagioso e letal de cães domésticos (*Canis familiaris*). CDV também infecta e ameaça uma grande variedade de animais selvagens, incluindo, por exemplo, as famílias *Canidea* (cães selvagens, raposa, lobo, cão-guaxinim), *Mustelidae* (furão, gambá, visom, lontra) e *Procyonidae (*guaxinim, quati). Estudos sobre o CDV são necessários por conta das diversas lacunas envolvendo o assunto, entre as quais estudos de prevalência e caracterização molecular, terapias antivirais e produção de insumos laboratoriais para o diagnóstico. Dessa forma, o presente estudo foi realizado e está constituído em seis capítulos. No primeiro capítulo foi realizada a revisão bibliográfica envolvendo a temática em questão. O segundo capítulo se refere à combinação dos dados obtidos do inquérito molecular, incluindo também os dados da literatura sobre dectecção molecular e sorológica do vírus nas diversas regiões do mundo. Embora este capítulo tenha sido publicado na forma de meta-análise, destaca-se a inserção dos nossos dados experimentais no estudo, permitindo a geração de dados mais robustos quanto à frequência de positividade viral, juntamente com a análise de diversas variáveis potencialmente envolvidas com o fator infecção viral. Ainda em relação ao segundo capítulo, frisa-se que o mesmo envolveu a coleta de amostras biológicas de cães com suspeita clínica de cinomose, procedentes do município de Jataí-Goiás. Em suma, houve 34% (48/141) de positividade para o RNA viral nas amostras. Essas amostras também serviram de base para a realização dos capítulos três e quatro. O terceiro capítulo envolveu o isolamento e sequenciamento completo do CDV. Para tanto, pela primeira vez foram desenvolvidos dezenas de primers que permitiram flanquear todo o material genético. Assim, houve sucesso na amplificação e sequenciamento completo de uma amostra (JA88/20, 15,624 nt, GenBank: MW460905). Interessantemente, a linhagem isolada se separou num ramo e formou um subgenótipo dentro do genótipo América do Sul-I/Europa. No quarto capítulo, as amostras laboratorialmente positivas para o CDV, foram testadas quanto à amplificação dos genes estruturais M (1008 nt), F (1989 nt) e H (1824 nt). Mediante a utilização de novos primers, foi obtido sucesso na amplificação de quatro amostras (4/48). A partir da caracterização molecular destes genes estruturais, foi observada a circulação na região de uma única linhagem durante o período de estudo. Surpreendentemente, os sequenciados revelaram assinaturas moleculares únicas dos isolados no local. O quinto capítulo está relacionado à dinâmica molecular das proteínas M e N, juntamente com o fornecimento de conhecimentos a respeito da geração de drogas antivirais. Os resultados obtidos de dinâmica molecular mostraram que os modelos gerados são de alta qualidade. A partir destes modelos, foi verificado que os resíduos aminoacídicos considerados chave, das proteínas M e N, estão em local acessível, representando assim excelente sítio para a ancoragem de moléculas com potenciais antivirais. Finalmente, no sexto capítulo a proteína M foi expressa em *Escherichia coli* (BL21) e purificada. Também houve sucesso no emprego da proteína recombinante como substrato no ELISA. Os dados, mostraram que houve acurácia laboratorial na distinção entre amostras IgG anti-CDV positivas e negativas. Em síntese, a proteína recombinante produzida confirma nossa hipótese referente ao seu emprego em ensaios de imunodetecção.

Palavras chave: *Canine morbillivirus*; vírus da cinomose canina; *Paramixovirus*; cães domésticos; filogenia viral; expressão proteica

ABSTRACT

Canine distemper virus (CDV), genus *Morbillivirus*, has emerged and has been recognized as a highly contagious and lethal pathogen in domestic dogs (*Canis familiaris*). CDV also infects and threatens a wide variety of wild animals, including, for example, the *Canidea* (wild dogs, fox, wolf, raccoon dog), *Mustelidae* (ferret, opossum, mink, otter) and *Procyonidae* families (raccoon, coati). The studies on the CDV are necessary due to the several gaps involving the subject, including prevalence and molecular characterization studies, antiviral therapies and production of laboratory supplies for the diagnosis. Thus, the present study was carried out and consists of six chapters. In the first chapter, a bibliographic review involving the subject in question was carried out. The second chapter refers to the combination of data obtained from the molecular survey, also including data from the literature on molecular and serological detection of the virus in different regions of the world. Although this chapter was published in the form of a meta-analysis, the inclusion of our experimental data in the study stands out, allowing the generation of more robust data regarding the frequency of viral positivity, together with the analysis of several variables potentially involved with the viral infection factor. Still in relation to the second chapter, it is emphasized that it involved the collection of biological samples from dogs with clinical suspicion of distemper, coming from the municipality of Jataí-Goiás. In sum, CDV RNA was detected in 34% (48/141) of dogs suspected to have distemper. These collected samples served as the basis for the realization of chapters three and four. The third chapter involves the complete viral isolation and DNA sequencing. For this purpose, for the first time dozens of primers were developed that allowed the flanking of all genetic material. Thus, there was success in the amplification and complete sequencing of a sample (JA88/2020, 15,624 nt, GenBank: MW460905). Interestingly, the isolated strain separated into a branch and formed a subgenotype within the South America-I/Europe genotype. In the fourth chapter, the laboratory samples positive for CDV, were tested for the amplification of M (1008 nt), F (1989 nt) and H (1824 nt) structural protein genes. Through the use of novel primers, it was successful in amplifying four samples (4/48). From the molecular characterization of structural genes, circulation in the region of a single lineage was observed during the study period. Surprisingly, the sequences reveal unique molecular signatures of the isolates at the site. The fifth chapter is related to the molecular dynamics of M and N proteins, together with the generation of knowledge regarding the rational generation of drugs against CDV. The results obtained from molecular dynamics showed that the models generated are of high quality. From these models, it was verified that the amino acid residues considered key for M and N proteins are in an accessible location, thus representing an excellent site for the anchoring of molecules with potential antivirals. Finally, in the sixth chapter, M protein was expressed in *Escherichia coli* (BL21), purified. There was also success in the use of recombinant protein as a substrate in the ELISA. The data showed that there was laboratory accuracy in distinguishing between positive and negative anti-CDV IgG samples. In summary, the recombinant protein produced confirms our hypothesis regarding its use in immunodetection assays.

Keywors: Canine morbillivirus, canine distemper virus, *Paramyxovirus*, Domestic dogs, viral phylogeny, Protein expression

OBJETIVOS

Objetivo(s) Geral

- Realizar a caracterização molecular de isolados do CDV em amostras biológicas de cães com suspeita clínica de cinomose da região de Jataí-Goiás;
- Desenvolver modelos das proteínas M e N pela simulação de dinâmica molecular e promover estudos referentes ao desenho racional de drogas antivirais;
- Produzir antígeno recombinante da proteína do CDV com finalidade de insumo laboratorial.

Objetivos Específicos

1) Promover o diagnóstico laboratorial do CDV, mediante a detecção do gene N pela "*nested*" RT-PCR, a partir de amostras biológicas coletada de cães com suspeita de cinomose, procedentes do hospital veterinário (Universidade Federal de Jataí) e do Centro de Controle de Zoonoses do município de Jataí-Goiás;

2) Promover a caracterização molecular dos genes M, F e H, a partir das amostras CDV positivas confirmadas no laboratório;

3) Isolar o CDV das amostras clínicas RT-PCR positivas para a cinomose, empregando as células VeroDogSLAM para fins de isolamento viral;

4) Desenvolver novos primers para a amplificação do vírus isolado, juntamente com sequenciamento de DNA do genoma viral;

5) Gerar modelo de alta qualidade das proteínas M e N do CDV com propósito de estudo racional de drogas antiviriais;

6) Utilizar o vetor plasmidial pET30(a), contendo o gene da proteína M do CDV para transformar *Escherichia coli*, linhagem BL21;

7) Expressar e purificar a proteína viral recombinante em *Escherichia coli*;

8) Utilizar o *Western blot* para análise de reatividade de anticorpos específicos contra a proteína;

9) Desenvolver um teste de ELISA para detecção de anticorpos da classe IgG utilizando a proteína M recombinante purificada.

CAPÍTULO 1.

INTRODUÇÃO GERAL E REVISÃO BIBLIOGRÁFICA

INTRODUÇÃO GERAL

O *canine morbillivirus*, também conhecido como *canine distemper virus* (CDV), pertence à família *Paramyxoviridae*, sendo encontrado mundialmente infectando cães domésticos e carnívoros silvestres (Martinez-Gutierrez & Ruiz-Saenz, 2016; von Messling, 2017). A cinomose canina, decorrente da infecção pelo CDV, é uma doença multissistêmica, contagiosa e com elevada taxa de mortalidade (Loots *et al*., 2017). Esta virose representa um importante problema de saúde pública animal, principalmente por conta da sua endemicidade e reemergência em várias regiões que tinham a doença outrora controlada pela vacinação (Lan *et al*., 2006; Fischer *et al*., 2016). Adicionalmente, outro fator que chama a atenção é que mesmo em populações de animais vacinados tem se observado surtos (Riley & Wilkes, 2015). Embora vários fatores possam ser atribuídos à falha vacinal, o fator diferenças antigênicas, entre as linhagens do CDV de campo e vacinal, têm chamado mais a atenção dos pesquisadores (Dong *et al*., 2015; Bhatt *et al*., 2019).

O CDV é uma partícula envelopada contendo em seu interior RNA de fita simples, não segmentado e de sentido negativo. O genoma viral é constituído por seis genes que codificam as proteínas estruturais N, P, M, F, H e L. As glicoproteínas denominadas de hemaglutinina (H) e fusão (F) estão inseridas na superfície da partícula viral, tendo papel chave na adsorção e fusão do *virion* a célula hospedeira. O espaço entre o envelope e o nucleocapsídeo é preenchido pela proteína de matriz (M), participando da arquiterura da partícula viral. A nucleoproteína (N) está envolvida na encapsidação do genoma, protegendo o material genético viral. Por fim, as proteínas L (*Large*) e P (fosfoproteína) estão envolvidas na transcrição e replicação do RNA viral (Lamb & Kolakofsky, 2013; Rendon-Marin *et al*., 2019).

O CDV é caracterizado por mutações, mudanças evolutivas com alta diversidade genética, o que eventualmente pode levar a falha vacinal. Devido à variabilidade genética, no momento são descritas 17 linhagens principais, incluindo América 1-5, Europa selvagem, Ártico, África do Sul, América do Sul-I/Europa, América do Sul 1-3, *Rockborn-like* e Ásia 1-4. Essas classificações estão relacionadas à origem geográfica onde as linhagens foram detectadas (Budaszewski *et al*., 2016; Fischer *et al*., 2016; Anis *et al*., 2018; Piewbang *et al*., 2019; Romanutti *et al*., 2020). No tocante a caracterização

molecular viral, a principal justificativa do presente estudo se baseia na necessidade de aprofundar pesquisas moleculares, do CDV circulante em regiões distantes de locais que tem concentrado estudos sobre a cinomose (Costa *et al*., 2019). Consequentemente, estudos de sequenciamento do genoma e genes completos contribuem na mensuração real da diversidade viral por permitir identificar diferenças regionais na epidemiologia molecular do CDV no território brasileiro. Ademais, constante vigilância epidemiológica molecular é importante para detectar e compreender mudanças moleculares do patógeno, devido a sua adaptabilidade as várias pressões externas, incluindo a vacinal (Lan *et al*., 2006; Dong *et al*., 2015).

A infecção viral, em animais susceptíveis, ocorre, principalmente, pela inalação de aerossóis provenientes de animais infectados (von Messling, 2017). O vírus se replica dentro de macrófagos, nos tecidos do trato respiratório superior, disseminando para as amídalas e gânglios linfáticos regionais. Posterior à infecção viral, é observado uma doença de amplo espectro clínico, variando desde casos clinicamente inaparentes até casos de encefalite aguda e fatal (von Messling, 2017). Devido à emergência e elevada taxa de letalidade da cinomose, variando de 30 a 80%, faz-se necessário à pesquisa por antivirais, os quais podem reduzir a mortalidade e auxiliar na contenção da disseminação da doença (Deem *et al*., 2000).

As proteínas M e N são alvos interessantes para antivirais devido ao seu importante papel no ciclo de replicação dos paramixovírus. A busca por fármacos associados a resíduos aminoacídicos chave da proteína N podem desestabilizar a formação do complexo ribonucleoproteico, o qual é formado por polímeros da proteína N cobrindo o RNA viral e se associando à polimerase viral (proteína L). A ribonucleoproteína participa da síntese do RNA viral e é o núcleo estrutural da partícula viral. Já em relação à proteína M, entre as suas funções se destacam o orquestramento da montagem e liberação das partículas virais da membrana plasmática da célula hospedeira (Iwasaki *et al*., 2009; Lamb & Kolakofsky, 2013). Recentemente, Gast e colaboradores (2021) descobriram dois microdomínios protéicos como alvos promissores na busca por antivirais, pois estes inibem a formação de dímeros e a montagem do vírus (Gast *et al*., 2021). Diante dos desafios, novas abordagens *in silico*, para a testagem de drogas antivirais, constituem um caminho na aceleração do tempo e na economia de recursos financeiros, a fim de obter-se moléculas antivirais. A vista disso, simulações de dinâmica molecular das proteínas M e N resultam em modelos confiáveis para serem testados com compostos ligantes oriundos

de banco de dados *on line*, permitindo análise preliminar deles antes de seguir para os testes *in vitro* e *in vivo*.

Dentre as diversas proteínas do CDV, crescente atenção vem surgindo para a proteína de matriz (M) (Iwasaki *et al*., 2009). Conforme estudo de Dietzel e colaboradores, foi observado que a proteína influencia a infectividade e modula a virulência (Dietzel *et al*., 2011). Recentemente foi lançada a hipótese do potencial uso desta proteína como alvo antiviral (Gast *et al*., 2021). Aqui, hipotetizamos que a proteína M pode ser um alvo interessante para ensaios de imunodiagnóstico. Entre as justificativas para o uso desta proteína, enfatizam-se os seguintes pontos: a proteína é considerada conservada e não glicosilada; possuindo apenas 38 kDa em tamanho; é a proteína mais abuntante da partícula viral; possui mecanismos chave no ciclo de replicação e virulência da partícula viral (Lamb & Kolakofsky, 2013). Desta forma, optamos por escolher estudar a proteína devido à quantidade reduzida de pesquisas relacionadas às suas características biológicas, principalmente quanto a sua aplicabilidade em ensaios de detecção de anticorpos anti-CDV.

1. REVISÃO BIBLIOGRÁFICA

1.1 Histórico do CDV

A cinomose canina foi primeiramente reportada no ano de 1730, com inicial confinamento as Américas (Blancou, 2004; Uhl, 2019). Convém destacar que cães da Europa foram introduzidos pela primeira vez nas Américas por Cristóvão Colombo em 1493, entretanto a chegada destes animais não foi acompanhada por epidemias nas populações de cães europeus, ou nativos, durante os anos 1500-1600 (Varner & Varner, 1983). Interessantemente, os cães importados do Velho Mundo tiveram um papel muito importante para os exploradores e conquistadores europeus; neste quesito merece atenção os relatos das raças *Greyhound* (Galgo inglês) e *Mastiff* (Mastim Inglês), as quais eram extremamente valorizadas e usadas na caça e guerra. O fato da cinomose estar presente nas Américas antes da chegada dos europeus é um assunto pouco esclarecido por conta da ausência de relatos de doença semelhante à cinomose em cães no período do contato inicial europeu (Uhl, 2019).

O primeiro relato crível da cinomose canina foi feito pelo cientista Don Antonio de Ulloa, membro da missão geodésica francesa de 1735 para medir a linha do equador, o qual observou a doença no Equador e Peru (Uhl, 2019; Ulloa, 1772). Sua descrição incluiu várias características clínicas clássicas, incluindo que a doença era comparável em gravidade à varíola em humanos, o que também foi relatado por observadores subsequentes. Ulloa também relatou que a doença iniciava com abatimento e perda de apetite antes de progredir para convulsões, vômitos de sangue, fraqueza e incapacidade da coordenação motora (Blancou, 2004).

Outras observações de Ulloa referem-se aos seguintes fatos: 1) de que os cães geralmente contraem a doença durante o primeiro ano de vida e, quando se recuperam, nunca voltam a tê-la. Esse padrão sugere que, no momento de suas observações, a doença estava bem estabelecida e endêmica (Blancou, 2004); 2) de que a doença era desconhecida na Europa, fato este apoiado por observações de outros cientistas (Fleming, 1882; Jenner, 1815); 3) Ulloa também relatou que a epidemia de cinomose de 1767 na Louisiana, era a mesma doença que ele havia descrito 20 anos antes como comum em cães na América do Sul (Blancou, 2004; Uhl, 2019).

Na Europa os primeiros relatos sobre a cinomose ocorreram na década de 1760; cerca de 20 anos após a doença ter sido descrita na América do Sul. Os surtos europeus iniciais tiveram como característica uma marcante letalidade para o novo patógeno

microbiano que entrou em contato com uma nova população; incluindo um relato de 900 cães perecendo da doença em Madri (Blancou, 2004).

Os cientistas da época, incluindo Edward Jenner, que escreveu um dos primeiros artigos científicos sobre a cinomose canina, notaram que a doença era completamente desconhecida na Europa antes de meados de 1700 (Jenner, 1815). Por consequência, muitos cientistas aceitaram as descrições da cinomose no Novo Mundo como indicativo de sua origem (Blancou, 2004; Nambulli *et al*., 2016; Uhl, 2019). Dessa forma, a eventual disseminação da epidemia é suportada pelas inferências de que a mesma foi importada para a Europa das Américas, em particular da América do Sul, uma vez que não foi descrita nas colônias norte-americanas até a década de 1760 (Fleming, 1882; Blancou, 2004; Nambulli *et al*., 2016; Uhl, 2019).

Durante o século XIX e início do XX, os cientistas e veterinários que estudaram a cinomose canina acreditavam que a doença veio de pessoas devido às suas semelhanças com o sarampo humano; de fato, um tratamento recomendado para cães aflitos era a injeção com o sangue de seus donos (Blancou, 2004; Uhl, 2019). Pelo fato dos anticorpos contra o vírus do sarampo poder proteger contra o CDV, então existe fundamentação biológica para esta terapia (Vries *et al*., 2014).

Existem relatos históricos que sugerem que os *morbillivírus* do sarampo e da peste bovina evoluíram como patógenos humanos e bovinos no Velho Mundo, enquanto o CDV patogênico canino, intimamente relacionado, surgiu no Novo Mundo e, posteriormente, se espalhou para a Europa. O momento da primeira descrição da cinomose na América do Sul sugere que a origem do CDV estava relacionada à chegada de europeus e do vírus do sarampo, mas não de cães europeus. Portanto, a inferência é de que os seres humanos, em vez dos cães, serviram como fonte inicial de infecção ao patógeno viral responsável pela cinomose (White, 1961; Uhl, 2019).

Em continuidade a disseminação do vírus, causando surto de cinomose na Europa, foi observado casos na Itália e Irlanda em 1764, e depois na Rússia em 1770. Já no início do século XIX, mas precisamente em 1809, o curso e as características clínicas da doença foram inicialmente descritas por Edward Jenner. Em 1870, desde que a doença incidiu em cães de todas as raças e idades no Reino Unido, ela foi descrita como o flagelo da raça canina (Headley *et al*., 2012).

Com o início do século XX, importantes conhecimentos foram sendo estabelecidos sobre a etiologia da temida cinomose canina. Henri Carré (1870-1938), em 1905, demonstrou ser a doença causada por um vírus, contrariando a hipótese de outro estudo

de que ela era causada por salmonelas, brucelas, pasteurelas ou a outros microrganismos bacterianos (von Messling, 2017).

No ano de 1960, Koprowski e Imagawa classificaram o vírus da cinomose no gênero dos vírus do sarampo e peste bovina (Meulen e Hall, 1978). Esta classificação persiste até o momento devido a grande similaridade entre os vírus destas espécies.

Ainda no século XX, grandes avanços vieram no campo das vacinas para a prevenção da doença. A primeira vacina foi criada em 1923 por Puntoni, a qual foi feita a partir da inativação viral por formalina do tecido cerebral de cão doente (Rikula, 2008). No entanto, a proteção com as vacinas inativadas foram limitadas.

As vacinas "vivas atenuadas" surgiram na década de 1950, decorrentes de sua produção por atenuação do CDV através de passagens em ovos de galinha embrionados. Posteriormente, diversas cepas vacinais foram desenvolvidas como as cepas *Lederle*, *Onderstepoort* e *Rockborn* (Rikula, 2008).

1.2 Propriedades do CDV

CDV, atualmente chamado *Canine morbillivirus*, pertence ao gênero *Morbillivirus* (família *Paramyxoviridae*). Possuem aspecto pleomórfico e geralmente têm *virions* esféricos com diâmetro ao redor de 150 nm, envelopados e o seu material genético é RNA fita simples, sentido negativo e não segmentado (von Messling *et al*., 2003).

A Figura 1 ilustra a partícula do CDV com destaque para a organização genômica das seis proteínas estruturais e das duas não estruturais. O genoma contém 15690 nucleotídeos e codifica oito proteínas. A estrutura do genoma inclui seis genes (N-P-M-F-H-L) organizados em forma linear, separados por regiões intergênicas não traduzidas (UTRs) que são relativamente uniformes em comprimento (Kolakofsky *et al*., 2016; Rendon-Marin *et al*., 2019). O genoma viral é flanqueado por regiões extracistrônicas que o delimitam. Desta forma, a extremidade 3' possui uma região de aproximadamente 50 nucleotídeos, denominada líder, que sinaliza para o complexo polimerase o local de início da transcrição ou replicação; enquanto a extremidade 5', denominada de trailer, constitui o final do genoma viral e possui cerca de 50 nucleotídeos. Também é importante destacar que os genes são monocistrônico. Interessantemente, o gene da fosfoproteína (P) codifica a proteína P, juntamente com as proteínas não estruturais C e V, as quais são codificadas por mudança da fase de leitura e edição do RNA, respectivamente (Lamb & Kolakofsky, 2013). A seguir, as proteínas do CDV são discutidas em separado.

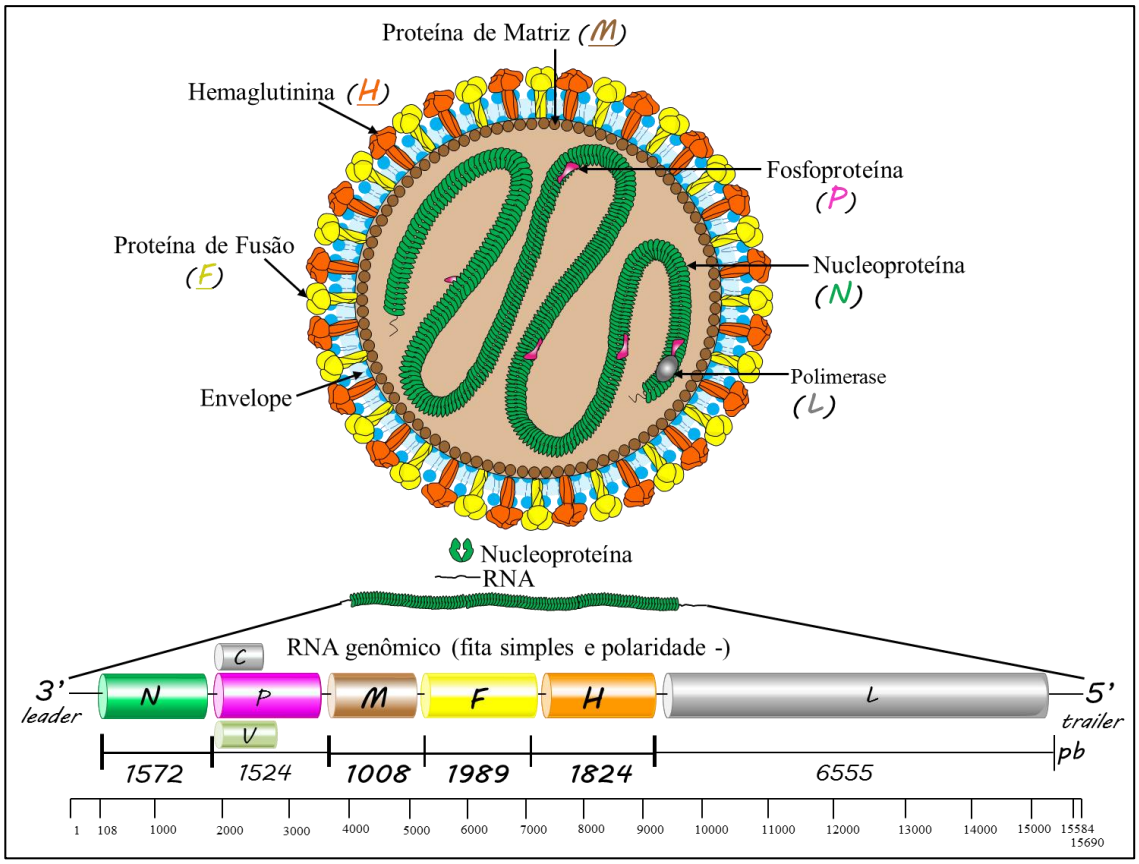

Figura 1. Representação esquematizada da partícula viral dos paramixovirus. O vírion é esférico e envelopado. O RNA é fita simples com polaridade negativa. No caso dos *Morbillivirus*, o genoma pode resultar na produção de seis proteínas estruturais e duas não estruturais. Visto que cada um dos seis genes contém uma ORF, as proteínas são produzidas individualmente. Adaptado de ViralZone (https://viralzone.expasy.org/556?outline=all_by_protein).

No envelope viral estão inseridas duas glicoproteínas integrais de membrana: a hemaglutinina (H) e a glicoproteína de fusão (F). A função primordial da glicoproteína H está relacionada à adsorção viral na célula hospedeira. Além do mais, pelo fato de ter considerável variabilidade genética, tornou-se possível a realização de estudos filogenéticos e filodinâmicos baseados na divergência genética e epidemiologia molecular, respectivamente. Portanto, a proteína H tornou-se o alvo mais adequado para investigar a variabilidade e evolução dos paramixovírus. Outro destaque da proteína H refere-se a sua atividade hemaglutinante para alguns paramixovírus; essa afinidade da hemaglutinina com os receptores celulares compostos de ácido siálico ocasiona a aglutinação de eritrócitos de mamíferos e aves, possibilitando a realização de testes diagnósticos como o da inibição da hemaglutinação (Ito & Iwasa, 1976; von Messling, 2001). Em relação à proteína F, salienta-se o seu papel fundamental na dispersão viral no hospedeiro por mediar sua fusão à célula hospedeira (Lamb & Kolakofsky, 2013).

A proteína do nucleocapsídeo (N) participa da encapsidação do RNA genômico e, baseado na expressão gênica, serve como um *template* para a transcrição e replicação pela polimerase viral. Também tem a função de agrupar os componentes virais quando interage com a proteína M (von Messling, 2001; von Messling, 2017).

A fosfoproteína (P) é de natureza altamente fosforilada e o seu gene constitui um exemplo de compactação da informação genética, tendo em vista que através de mudanças na fase de leitura, bem como na edição do RNA, pode ser gerado três proteínas distintas a partir do mesmo gene: P, C e V. No tocante a funcionalidade proteica, nota-se que a proteína P é um componente do complexo replicase para a síntese de RNA, embora não seja essencial à replicação viral pode ser um importante determinante de virulência. Ao passo que a proteína C possivelmente teria um papel na inibição seletiva da síntese de RNA, auxiliando na transição da transcrição primária para a replicação do genoma viral. Quanto à proteína V, sua função também não está claramente elucidada, mas sugere-se que esta possui influência na síntese de RNAm. Também foi observado que as proteínas V e C possuem participação na evasão da resposta inata pelo vírus (von Messling, 2017).

A proteína de matriz (M) preenche o espaço entre o nucleocapsídeo e o envelope viral, constituindo assim a proteína mais abundante no vírion. Portanto, interage com o envelope lipídico e serve como um ponto de ancoragem entre a ribonucleoproteína e as duas glicoproteínas de superfície (F e H). Essa interação é vital durante o processo de morfogênese, maturação e brotamento das novas partículas. Além disso, a proteína M está implicada no controle dos níveis de síntese de RNA (von Messling, 2017).

Por outro lado, a proteína L (large) é a menos abundante nos vírions e sua função está relacionada com a atividade catalítica da RNA-polimerase RNA-dependente (RdRp). A sequência dos 6554 nucleotídeos do gene é muito conservada entre os membros de uma mesma subfamília (von Messling, 2017).

1.3 Dados Epidemiológicos

Pelo fato de o CDV não persistir de forma infecciosa após a resolução de uma infecção, julga-se que é necessária uma fonte constante de indivíduos suscetíveis para a proliferação do CDV na população (von Messling, 2017). No entanto, considerando a ampla gama de hospedeiros do CDV, a circulação do vírus não depende apenas do tamanho da população de cães, mas do tamanho da população total combinada de todas as espécies vulneráveis a infecção na área. Além disso, os fatores envolvidos no contato

entre essas espécies serão cruciais para a presença constante do vírus (Figura 2) (Rikula, 2008).

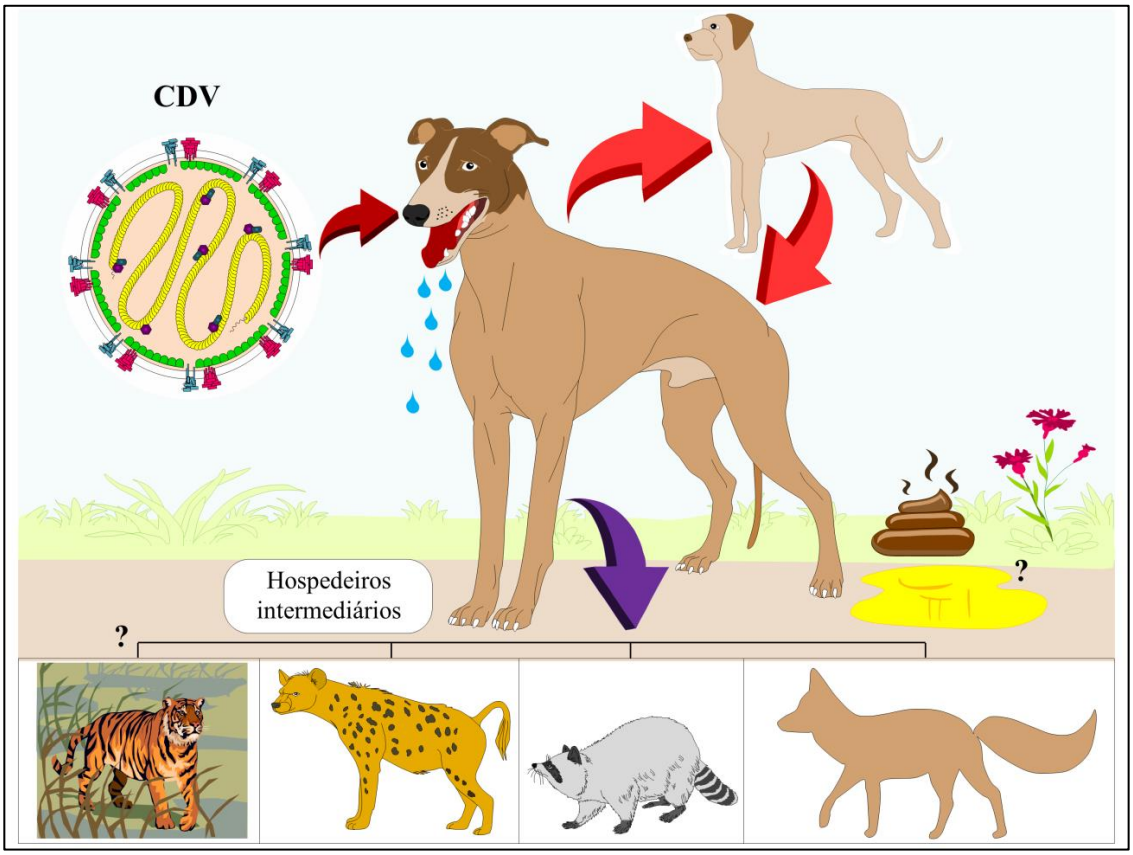

Figura 2. Ciclo de transmissão envolvendo o CDV. A transmissão ocorre por meio do contato ou fluidos orais, respiratórios e oculares aerossolizados e exsudatos contendo o patógeno. Durante a fase aguda da infecção, outras excreções e secreções (p. ex.: urina, fezes, pele) pode conter o vírus. Adicionalmente, o CDV pode ser transmitido para ampla variedade de seres vivos, incluindo os animais de vida selvagem. Os animais de vida selvagem são propensos à infecção pelo CDV, principalmente em locais que tenham alta densidade de cães não vacinados e que adentram no ambiente selvagem (Loots *et al*., 2017). Fonte: Elaborada pelo autor.

O desvio antigênico, nas cepas do CDV do tipo selvagem, pode causar crescentes surtos nas populações de cães e animais selvagens (Rendon-Marin *et al*., 2019; Viana *et al*., 2015). Dessa forma, vários genótipos do CDV foram mostrados circulando simultaneamente em uma população (Ke *et al*., 2015). Todavia, o vírus é considerado como tendo apenas um tipo antigênico (Rikula, 2008). Dessa forma, Haas *et al*. (1999) não encontraram grande diversidade em genes H e ensaios de neutralização entre isolados recentes do tipo selvagem e a cepa vacinal.

Nos países desenvolvidos, durante a década de 1950, foi produzida uma eficaz vacina anti-CDV, a qual foi amplamente utilizada na década de 60 com uma drástica redução do impacto do CDV na população canina (Harder & Osterhaus, 1997). Todavia,

pelo fato de serem vacinas de “vírus vivo” atenuado, ainda retêm alta patogenicidade quando aplicada em animais sem a devida avaliação. Neste aspecto incluem-se estudos que tem observado a ocorrência de diversos casos da doença mesmo em animais vacinados; indicando uma reação vacinal ou reduzida imunidade gerada pela vacina (Ek-Kommonen *et al*., 1997).

Referente ao espectro de hospedeiros do CDV observa-se que, desde o ano de 1990, têm aumentado os relatos de espécies de animais selvagens infectadas. Conforme revisão sistemática de Martinez-Gutierrez & Ruiz-Saenz (2016), a frequência de estudos e da positividade do CDV nas famílias *Canidae*, *Felidae*, *Mustelidae*, *Procyonidae*, *Ursidae*, *Hyaenidae* e *Phocidae* têm considerável taxa de infecção e mostram alta probabilidade destes animais serem infectados ao longo da sua vida.

Estudos sorológicos e moleculares do CDV têm sido realizados ao longo dos últimos anos em alguns países (Figura 3). Conforme é ilustrada na imagem, a frequência de positividade, da infecção viral, no geral foi alta, porém existe um aspecto de menor positividade para aquelas regiões considerada desenvolvida, pois a vacinação contra a cinomose continua cara na maioria dos países em desenvolvimento (Costa *et al*., 2019; Dorji *et al*., 2020).

No tocante a caracterização molecular do CDV, nota-se um perfil molecular baseado na sua distribuição geográfica. Dessa forma as cepas do CDV, baseado em variações do gene H, agrupam-se em, pelo menos, 11 linhagens genéticas designadas como América I (cepas vacinais clássicas), América II, Ásia-I, Ásia-II, Europa, Europa Selvagem, Ártico, África do Sul, América do Sul-I e América do Sul-II (Budaszewski, 2013).

A epidemiologia do CDV no Brasil, assim como acontece em diversas regiões do mundo, é pouco estudada e existem diversas lacunas de informações (Rosa *et al*., 2012; Castilho *et al*., 2007). Assim, os poucos relatos de pesquisas sorológicas e moleculares, concentram-se em algumas regiões dos estados Sul-Sudeste do país, com variações de positividade de 8 a 70% (Costa *et al*., 2019). Estes dados mostram a necessidade de estudos epidemiológicos com o intuito de maior conhecimento para vigilância e análise de medidas de controle de saúde pública animal.

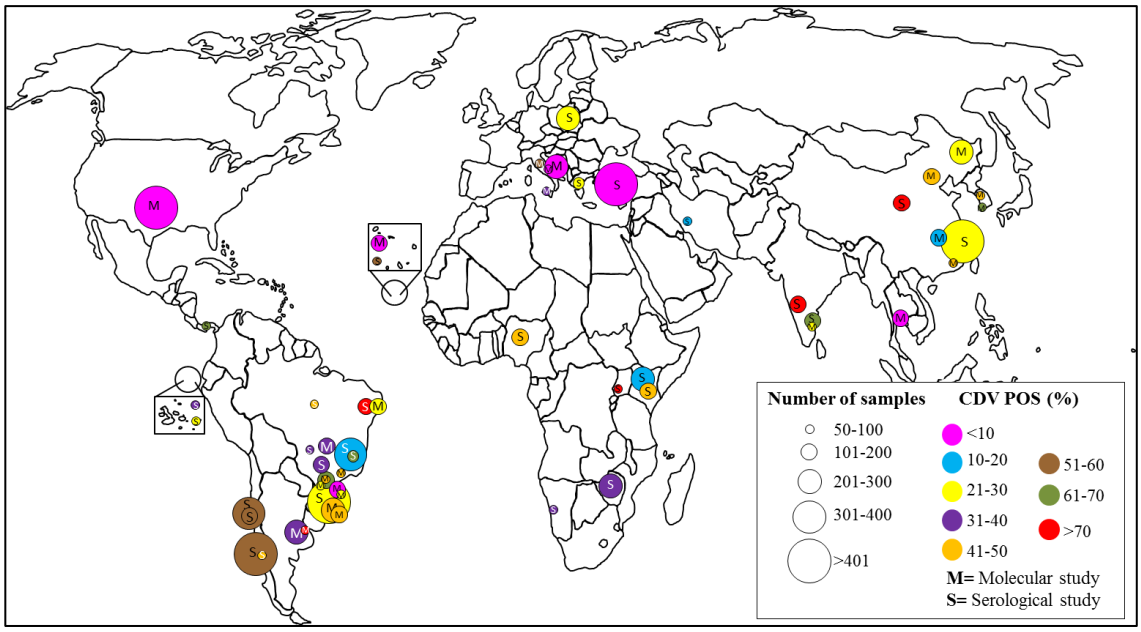

Figura 3. Distribuição geográfica de estudos analisando o nível de infecção viral. Para mais detalhes consultar: https://doi.org/10.1371/journal.pone.0217594.

1.4 Patogenia e Sinais Clínicos da Cinomose

A principal rota de entrada do CDV no hospedeiro ocorre após a infecção do trato respiratório com os aerossóis de ambientes contaminados (Loots *et al*., 2017). O vírus inicialmente se replica dentro das células mononucleares nos tecidos do trato respiratório superior e, então rapidamente, se espalha para as amígdalas e linfonodos regionais. A infecção viral acontece devido o tropismo pelas células que expressam o receptor SLAM, presente nos timócitos, linfócitos ativados, macrófagos e células dendríticas (Rendon-Marín *et al*., 2019) (Figura 4). Consequentemente, o tropismo viral para estas células explica os efeitos imunossupressores, que possivelmente refletem a destruição mediada por vírus das células imunes, bem como a modulação da resposta imune inata (von Messling, 2017).

Após a multiplicação nos gânglios linfáticos regionais, o vírus espalha-se sistematicamente através das células B e T infectadas (von Messling, 2017). A viremia associada às células primárias coincide com o primeiro pico de febre e resulta em infecção de tecidos linfóides por todo o corpo. As células mononucleares recém-infectadas levam a uma viremia secundária associada ao segundo pico febril. A infecção resultante de células epiteliais no pulmão, bexiga e pele ocorre através da interação com o receptor nectina-4 (Rendon-Marín *et al*., 2019).

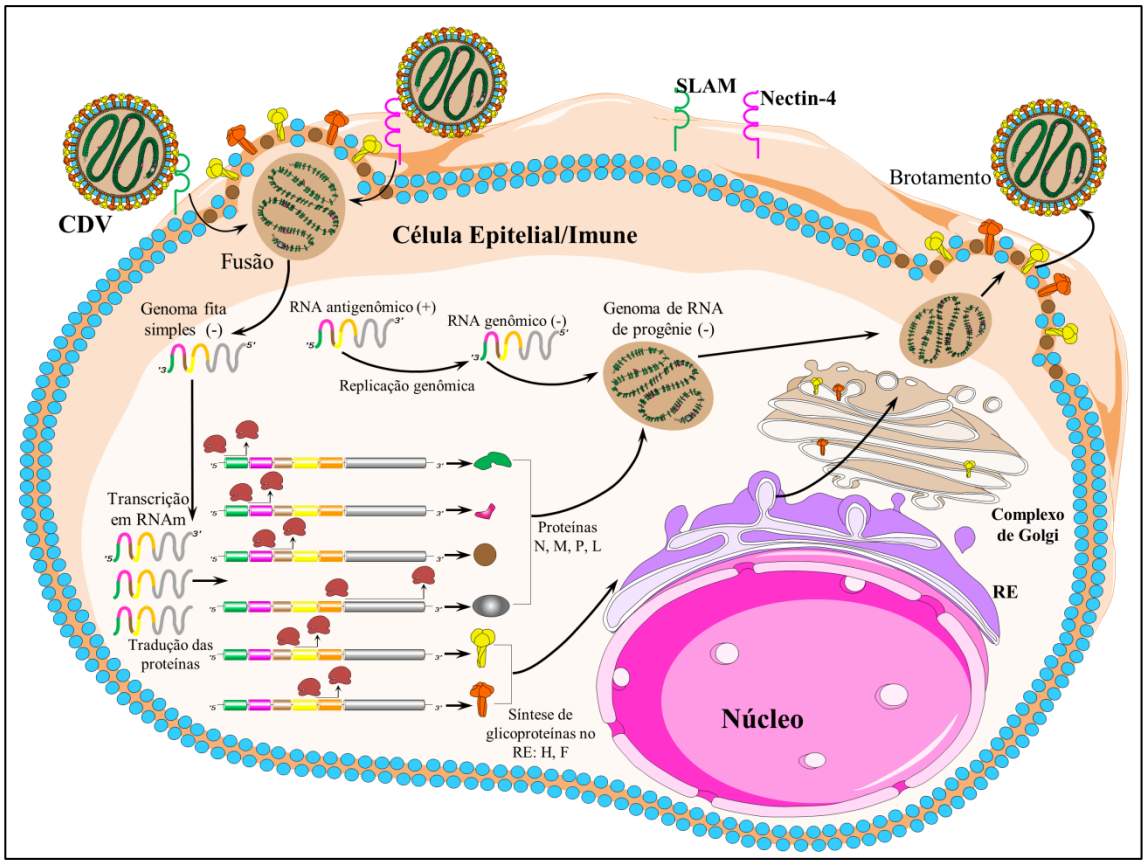

Figura 4. Ciclo de replicação do CDV. A etapa de reconhecimento das partículas virais envolve os receptores das células hospedeiras (SLAM/CD150 ou nectina-4). Após a fusão, acontece a liberação da ribonucleoproteína no citoplasma. Os processos de replicação, transcrição e brotamento da partícula viral são ilustrados. Adaptado de Rendon-Marin *et al*., 2019.

O período de incubação em média é de 14 dias, variando de 14 a 18 dias (Greene, 2006). No tocante as características clínicas, quando um cão é infectado pelo CDV, manifestações catarrais ou uma nervosa, ou a combinação de ambos e, ainda, uma manifestação nervosa crônica podem ser observadas. Na fase aguda, os vírus podem ser encontrados em todas as secreções do animal infectado (von Messling, 2017). Esta fase é seguida por vários sinais clínicos, incluindo um aparecimento de erupção cutânea, corrimento nasal e ocular grave, conjuntivite e anorexia, seguida por sinais gastrointestinais e respiratórios que são frequentemente complicados por infecções bacterianas secundárias e distúrbios neurológicos (Beineke *et al*., 2009; Rendon-Marin *et al*., 2019).

Na doença grave generalizada (Figura 5), os cães infectados primeiro desenvolvem febre após um período curto de incubação de 3-6 dias. A posteriori, uma segunda resposta febril inaugura a fase mais grave da infecção que coincide com a disseminação sistêmica do vírus e acompanha a leucopenia acentuada (von Messling, 2017).

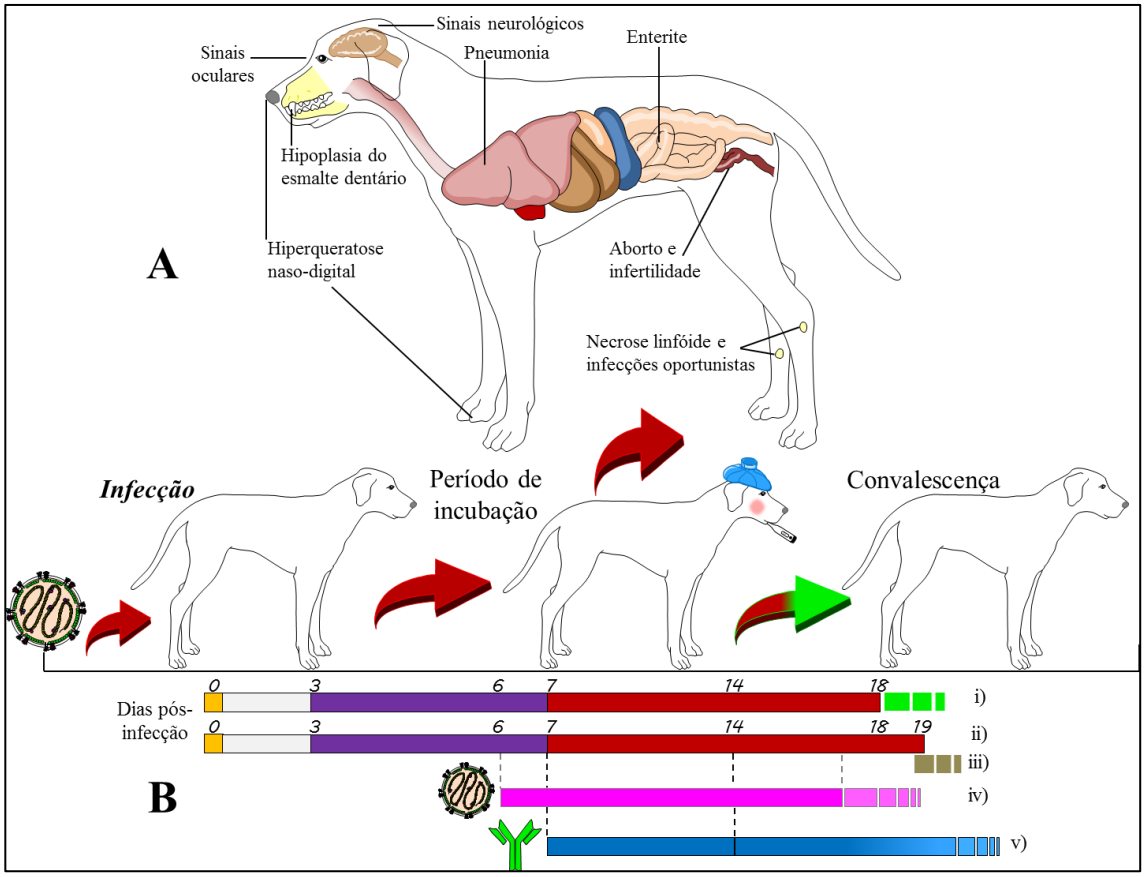

Figura 5. A imagem esquematizada (A) refere-se aos órgãos afetados nos cães com cinomose durante a fase aguda ou crônica da doença. Na ilustração B constam as seguintes informações da doença generalizada e grave: o período de incubação tende a ser mais curto (3-6 dias) e o primeiro sintoma a surgir é a febre. Todavia, uma segunda resposta febril (7-18 dias pós-infecção) inaugura a fase mais grave da infecção que coincide com a disseminação do CDV e acompanha a leucopenia profunda. Neste momento os sinais incluem o quadro clínico da Fig. 5A, com destaque para anorexia, inflamação do trato respiratório superior com secreção nasal serosa a mucopurulenta, conjuntivite e depressão. Alguns cães mostram principalmente sinais respiratórios, enquanto outros desenvolvem sinais gastrointestinais. Os sinais respiratórios estão associados à inflamação e lesão do trato respiratório superior, causando tosse produtiva, seguida por bronquite e pneumonia intersticial. As manifestações neurológicas que são indicadores de mau prognóstico surgem de 1-3 semanas.. Na porção inferior da Fig. 5B observa-se o seguinte: em (i) é ilustrado a evolução temporal de um cão que progrediu para a fase de convalescença; (ii) mostra que por volta, ou depois, da 3ª semana pós-infecção o animal pode vir a óbito por conta das complicações neurológicas, p. ex. ataxia, mioclonia, tremores, convulsões, moribundo; (iii) o animal pode evoluir para forma crônica da doença; (iv) mostra que antes do surgimento dos sinais clínicos o CDV já pode estar sendo eliminado no ambiente. Para alguns casos a eliminação do vírus pode prolongar-se até 90 dias pós-infecção; (v) a produção de anticorpos IgM anti-CDV aparece de forma precoce ao surgimento dos sinais clínicos e continua até 3 meses pós-infecção. O período da janela imunológica do IgG é pouco esclarecido, mas provavelmente é detectável poucos dias após o início da doença (von Messling, 2017). Fonte: Elaborada pelo autor.

As manifestações neurológicas da cinomose usualmente ocorrem em 1-3 semanas após o início dos sinais agudo, porém também pode aparecer após infecção subclínica (Figura 5). Nesta fase clínica, os animais podem apresentar os sinais de mioclonia,

nistagmo, ataxia, déficits na reação postural e tetraparesia ou plegia (Amude *et al*., 2007; Koutinas *et al*., 2004). No entanto quando tem aumento na produção de anticorpos neutralizantes específicos, ocorre recuperação do animal por conta desta melhora no sistema imunológico. Como resultado, embora o vírus seja removido do sangue periférico e de diferentes órgãos, o mesmo pode permanecer em alguns tecidos, incluindo órgãos linfoides, úvea, sistema nervoso central (SNC) e pata traseira. Além disso, alguns animais infectados exibem desenvolvimento retardado, propensão a doenças e uma resposta imune moderada com alguns sinais clínicos precoces imperceptíveis (Schobesberger *et al*., 2005; Rendon-Marin *et al*., 2019).

1.5 Diagnóstico do CDV

1.5.1 Métodos de Detecção Sorológico

Imunofluorescência (IFA)

IFA é uma técnica útil para demonstrar à presença de anticorpos, ou antígeno viral, em esfregaços de impressões da conjuntiva, esfregaço sanguíneo e biópsias de pele (antemortem) ou seções de tecido de pulmão, intestino, estômago, rim, cérebro e bexiga coletados na necropsia. Também é possível realizar o IFA usando células infectadas com o CDV e fixadas na lâmina para detecção de anticorpos contra o vírus. Em síntese na IFA são utilizados anticorpos marcados com corantes fluorescentes para revelar a formação de complexo vírus-anticorpo (Potgieter *et al*., 1989; Athanasiou *et al*., 2017).

O IFA tem sido avaliado quanto à acurácia diagnóstica, com resultados de sensibilidade de 79% e especificidade de 100% quando comparado ao padrão ouro (PCR) (Athanasiou *et al*., 2017). Por outro lado quando o padrão ouro foi o ensaio mais sensível de *nested* PCR, a sensibilidade do IFA foi reduzida para 50%. Apesar de que para este caso tenham se analisado somente as formas subclínica e crônica da doença (Józwik & Frymus, 2005).

Enzyme-Linked Immunosorbent Assays (ELISA)

Um dos principais ensaios sorológicos utilizados para a detecção de anticorpos, ou antígenos, contra o CDV é o ELISA. Diferentes plataformas do ELISA têm sido desenvolvidas com o propósito de contribuir no diagnóstico acurado e vigilância da infecção viral. Uma das plataformas exploradas é a produção da proteína N e sua adsorção aos micropoços da placa de ELISA, permitindo-se assim a detecção de anticorpos específicos contra o vírus (Messling *et al*., 1999; Fernandes *et al*., 2018).

Outras plataformas disponíveis se fundamentam na produção de anticorpos monoclonais contra uma proteína do CDV. Nesse sentido, Zhang e colaboradores tem gerado anticorpos monoclonais contra a proteína F, permitindo que estas possam ser utilizadas na detecção de anticorpos neutralizantes contra o vírus (Zhang *et al*., 2020).

Teste imunocromatográfico

Em questão de praticidade os testes imunocromatográficos constituem a melhor escolha na detecção de anticorpos, ou antígenos do CDV. Basicamente, a técnica consiste no fluxo lateral da amostra biológica que corre por capilaridade em uma membrana de nitrocelulose. Geralmente é utilizado ouro coloidal ligado a um anticorpo como sistema detector (An *et al*., 2008). Em sua maioria os kits comerciais para a cinomose envolvem o princípio da imunocromatografia, exemplificando os kits: 1) ImmunoComb VacciCheck® Parvovirose & Cinomose & Hepatite IgG; 2) Cinomose Ag test kit/Easy Alere; 3) SensPERT™ Cinomose.

Ensaio de soroneutralização para o CDV

A metodologia do ensaio de soroneutralização viral é considerada padrão ouro na titulação de anticorpos. Basicamente, a técnica consiste no cultivo de células suscetível a infeção viral em poço de placas, as quais são infectadas e depois é determinada a titulação viral em "*tissue culture infective dose* 50" (TCID50). Em sequência, o soro em diluições seriadas é adicionado nos poços contendo as células infectadas com titulação viral conhecida. Os resultados são expressos como a recíproca diluição do soro em que pelo menos 50% da infectivdade do vírus em 100 TCID50 foi bloqueado (Gencay *et al*., 2004). Entretanto, este método é laborioso para ser usado na rotina laboratorial por ser uma técnica demorada, exigir pessoas qualificadas e envolver custo financeiro elevado, incluindo os custos do cultivo de célula, manutenção do estoque viral, equipamentos sofisticados como estufa de CO_2 e cabine de segurança biológica.

1.5.2 Isolamento Viral

O isolamento viral é uma metodologia laboriosa para a rotina laboratorial de diagnóstico, pois é demorado, de alto custo financeiro e com reduzida possibilidade de detecção. Por outro lado, com o vírus isolado em cultivo celular existe abundância de material alvo para ser analisado por diversas metodologias, incluindo molecular, sorológica e morfológica (von Messlinf, 2017).

No isolamento do CDV as cepas selvagens não podem ser isoladas em células tradicionais como MDCK, Vero e fibroblastos de embrião, possivelmente devido a ausência do receptor celular SLAM canino (von Messling, 2017). Inicialmente o isolamento deve ser feito mediante o co-cultivo de linfócitos de animais suspeitos com linhagens que expressam a molécula CD150 (SLAM). Outras possibilidades de isolamento das cepas de campo são mediante o uso de células Vero modificadas, as quais expressam o SLAM de cães, e célula B95a, uma linhagem de células linfoides de sagui. Logo após este isolamento viral, o vírus pode ser adaptado a se desenvolver nas células tradicionais supracitadas (von Messling, 2017).

Uma das características da replicação do CDV em cultivos celulares é a formação de sincícios; esta alteração morfológica é determinada principalmente pela proteína H. Outras alterações de efeito citopático se referem ao desprendimento de células da monocamada e inclusões intracitoplasmáticas nos cultivos infectados (von Messling, 2017).

1.5.3 Detecção do RNA Viral

O pioneirismo da utilização da PCR, e suas variantes no diagnóstico do CDV, ocorreu em meados da década de 1990 (Yoshida, 1998; Shin *et al*., 1995). No entanto, apenas no período atual é que a RT-PCR está se tornando um método padrão ouro quando o assunto é detecção molecular. Todavia, a distinção entre vírus de campo e vacinal requer ensaios especializados de RT-PCR que não estão rotineiramente disponíveis. Neste contexto, Fischer *et al*. (2013) desenvolveram primers para detecção e distinção entre as cepas selvagens e vacinais do CDV, mas a dificuldade na utilização desta metodologia em laboratórios de rotinas está relacionada ao alto custo de insumos laboratoriais e equipamentos para a realização da PCR em tempo real.

Os ensaios de RT-PCR podem ser feitos a partir de uma grande variabilidade de amostras biológicas infectadas, incluindo swabs conjuntivais, células mononucleares do sangue, amostras sanguíneas, urina, fezes e qualquer amostra de tecidos que inclua epitélio (Fischer *et al*., 2013). Para tanto é possível utilizar a PCR convencional, ou a PCR quantitativa que devido ao alto custo financeiro ainda continua restrita a poucos laboratórios.

1.6 Antivirais contra a cinomose

Ainda não existe um protocolo terapêutico específico para o tratamento dos cães acometidos pela cinomose. No entanto, existe tratamento sintomático e de suporte, exemplificando: terapia antimicrobiana para casos de enfermidades bacterianas concomitantes; antipiréticos; antieméticos; expectorantes e broncodilatadores; anticonvulsivantes; corticosteroides; suplementação vitamínica e mineral (Crivellenti & Crivellenti, 2012; Portela *et al*., 2017).

A complexidade no desenvolvimento de drogas antivirais fundamenta-se nos poucos alvos disponíveis contra o vírus. Logo se torna difícil a detecção de uma droga terapêutica sem perigo citotóxico para as células do paciente, pois os vírus são agentes intracelulares que dependem da célula hospedeira para realizarem o processo de biossíntese e geração da progênie viral. Os sítios de atuação de um antiviral envolvem a inibição de eventos específicos na etapa do ciclo de replicação viral (Bule *et al*., 2019). Na Figura 6 podem ser visualizados possíveis sítios de atuação de drogas antivirais contra o CDV.

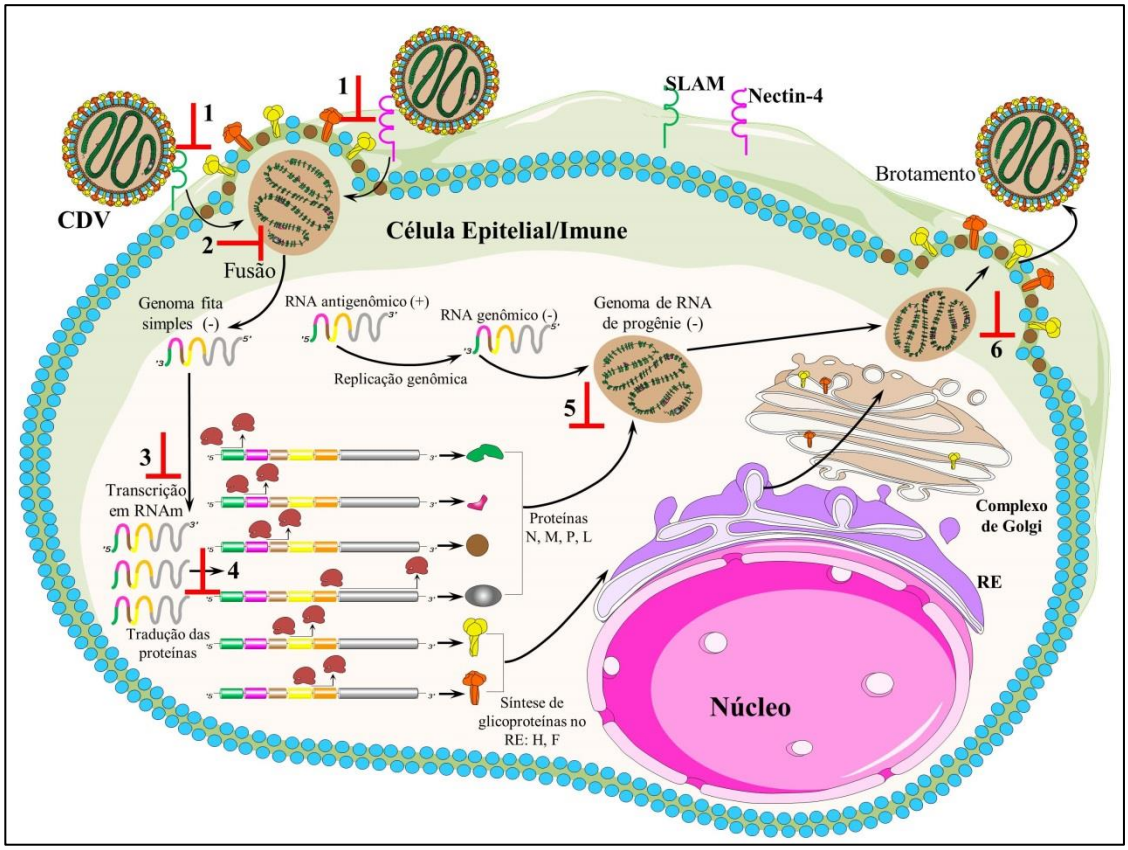

Figura 6. Etapas do ciclo de replicação viral e locais alvo para ação dos antivirais. Os potenciais alvos de antivirais podem ser: 1) interferência na adsorção do vírus; 2) inibição da fusão viral; 3) inibição da transcrição do RNA genômico em RNA mensageiro; 4) interferência na tradução e processamento das proteínas virais; 5) interferência na montagem das proteínas virais; 6) interferência na liberação da partícula viral. Fonte: Elaborada pelo autor.

A pesquisa de antiviral contra a cinomose é algo considerado recente porque estudos pioneiros, buscando desenvolver ferramentas terapêuticas antivirais específicas *in vitro*, são do início do séc. XXI (Leyssen *et al*., 2005). Neste quesito, a primeira molécula a ser testada, e ter efeito antiviral contra os paramixovírus, foi a ribavirina (1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide). A ribavirina é um análogo de nucleosídeo sintético com estrutura semelhante à guanosina, possuindo atividade de amplo espectro contra vírus de DNA e RNA (Graci & Cameron 2006; Lanave *et al*., 2006). Essa molécula está disponível no comércio, porém sua testagem em cães, naturalmente infectados pelo CDV, apresentou efeitos colaterais incluindo anemia hemolítica (Mangia *et al*., 2014). Além do mais, será preciso novos estudos com propósitos de melhor entendimento da eficácia da droga *in vivo* e dos seus efeitos colaterais.

Outra molécula sintética com efeito antiviral *in vitro* foi o 6-methylmercaptopurine riboside (6MMPr). 6MMPr inibe a síntese de purinas, e seus efeitos antivirais foram demonstrados contra flavivírus, incluindo os vírus da dengue, febre amarela e hepatite C (Carvalho *et al*., 2017). Merece destaque que no estudo de Carvalho e colaboradores, 6MMPr se apresenta como candidato promissor para aplicações clínicas contra a infecção por CDV, devido sua seletividade terapêutica ter sido superior a da ribavirina.

Compostos naturais, incluindo extratos das plantas *Plumbago zeylanica*, *Carissa edulis*, metabólitos secundários de plantas (compostos fenólicos), também foram testados como inibidores da replicação do CDV. Os resultados mostraram diminuição da síntese de RNA viral e redução na liberação da progênie viral. Todos esses compostos ainda estão em fase inicial de testagem como moléculas antivirais. Novamente, frisa-se a necessidade de mais estudos para a geração de resultados robustos de sua eficácia e segurança na rotina clínica (Galina *et al*., 2011; Bagla *et al*., 2012; Carvalho *et al*., 2013; Whu *et al*., 2017).

Metabólito oriundo de alga marinha também foi testado como antiviral *in vitro* contra o CDV (Damonte *et al*., 2004). O fucoidan, polissacarídeo sulfatado encontrado na matriz da parede celular da alga *Cladosiphon okamuranus*, conseguiu inibir a infecção viral *in vitro*. Todavia, visto que a linhagem do CDV testada era atenuada por ser cepa vacinal, será preciso analisar as atividades de eficácia e citotoxicidade do fucoidan com cepas virais de campo (Travejo-Avila *et al*., 2014). Em suma todos os dados de antivirais contra a cinomose estão em fase inicial de teste, sendo de extrema importância à continuidade de pesquisas nesta área por conta da demanda crescente de veterinários, tutores de animais e pela doença permanecer com alta incidência e letalidade em cães

(Galina *et al*., 2011; Bagla *et al*., 2012; Carvalho *et al*., 2013; Trejo-Avila *et al*., 2014; Whu *et al*., 2017)

1.7 Vacinas anti-CDV

A partir da vacinação foi obtido o controle efetivo da cinomose em diversas regiões do mundo. Diversas vacinas estão disponíveis no mercado como a Inomune®, Nobivac®, Duramune®, Recombitek®, Eurican® e Vanguard®. Em sua maioria as vacinas possuem vírus atenuado na sua formulação, incluindo a Inomune®, Nobivac®, Duramune®, Eurican® e Vanguard®. Anterior ao desenvolvimento destas vacinas, uma das vacinas pioneiras surgiu na década de 1950 e tinha em sua constituição "vírus vivo" atenuado, resultante de linhagem isolada conhecida como *Onderstepoort*. A disponibilidade desta vacina representou o maior progresso científico no combate a doença (Rikula, 2008).

As vacinas constituídas das linhagens *Onderstepoort* ou *Rockborn* são tradicionais no campo da vacinologia contra a cinomose. No entanto, a linhagem *Onderstepoort* é a mais utilizada nas formulações. Também existem vacinas que possuem em suas formulações outras cepas de "vírus vivo" atenuado, incluindo a *Synder Hill* e *Lederle* (Martella *et al*., 2008; Martella *et al*., 2011). Em relação às aplicações das vacinas, contendo estes tipos de formulações, recomenda-se aplicação de dose em filhotes após o declínio dos anticorpos maternos. Geralmente, a imunização ocorre entre seis e oito semanas de idade e com doses de reforço após intervalo de 21 a 30 dias (Biazonno *et al*., 2001).

Alguns relatos de encefalite pós-vacinal têm sido registrados, possivelmente devido aos seguintes fatores: 1) atenuação inadequada do vírus que pode ocorrer em determinados lotes de vacinas multivalentes; 2) vacinação de animais imunossuprimidos; 3) ou por conta de diferenças antigênicas entre as cepas vacinais e selvagens, com geração de anticorpos vacinais não neutralizantes o suficiente para evitar infecção pela cepa de campo (Blixenkrone-Moller *et al*., 1993; Martella *et al*., 2011). Logo, com o propósito de evitar reações adversas novas estratégias de vacinas recombinantes têm surgido. Nesse sentido, a vacina utilizando o poxvírus aviário como vetor dos genes das glicoproteínas F e H do CDV, são incapazes de se replicar na célula hospedeira, mas podem induzir resposta imune apropriada do hospedeiro sem possibilidade de efeito adverso grave (Stephensen *et al*., 1997; Buczkowski *et al*., 2014).

1.8 Cinomose no Brasil

A cinomose continua endêmica e com altas taxas de infecão em diferentes regiões do Brasil (Fischer *et al*., 2016; Costa *et al*., 2019). Por outro lado, um país de extensão continental como o Brasil, possui reduzido número de pesquisas, principalmente as do tipo de caracterização molecular das cepas virais circulantes (Budaszewski *et al*., 2014; Freitas *et al*., 2019). Soma-se a este fato, a escassez de dados referentes à caracterização molecular de genes completos, juntamente com a ausência de estudos envolvendo o isolamento viral para fins de caracterização do genoma. Para o presente vírus, é sabido que o mesmo é caracterizado por mutações, mudanças evolutivas e alta diversidade genética (Anis *et al*., 2018). Portanto, a vigilância epidemiológica molecular é importante para permitir o desenvolvimento de intervenções de saúde pública que previnam surtos e contribuam no aprimoramento de métodos de diagnóstico. À vista disso, no presente estudo dados epidemiológicos de infecção pelo CDV foram obtidos através da coleta de amostras biológicas (soro e/ou plasma, swab nasal/ocular) de cães com suspeita clinica de cinomose no município de Jataí-Goiás.

As taxas de mortalidade das infecções pelo CDV, para a maioria dos animais suscetíveis, têm variado de 30 a 80% (Deem *et al*., 2000). Adicionalmente, casos da doença têm ocorrido até mesmo em cães vacinados e em outras espécies de animais economicamente importantes (Beineke *et al*., 2015; Pope *et al*., 2016). Estas informações mostram o quanto a cinomose ainda impacta na saúde animal. Agrava-se a esta situação o fato de inexistir antiviral disponível no comércio contra a doença. Logo é preciso explorar novas abordagens que busquem agentes antivirais para combater a doença e reduzir a incidência e mortalidade dos animais acometidos. Para tanto, o presente estudo propôs gerar modelos das proteínas M e N, através de ferramentas de bioinformática. A posteriori, estes modelos serão usados para o desenho racional de drogas que possam interferir no ciclo de replicação viral. Assim resíduos aminoacídicos chave, encontrados nestas proteínas, foram avaliados quanto a sua exposição na superfície e analisado se estes resíduos teriam afinidades com outros ligantes. A partir desta análise, espera-se a triagem racional de moléculas com potencial antiviral *in silico* e posterior testagem *in vitro*.

Em relação ao diagnóstico laboratorial muitos dos testes laboratoriais, usados na detecção laboratorial do CDV, incluem a RT-PCR em tempo real e a RT-PCR convencional. Todavia, a utilização dessas técnicas demanda laboratórios bem equipados, além de demandar profissionais altamente qualificados. Já os ensaios em tiras contendo

ouro coloidal imune são caros e inadequados para a triagem de um grande número de amostras (Elia *et al*., 2006; Zhang *et al*., 2020). Em contraste, ELISA é uma técnica que apresenta algumas vantagens, incluindo a sua aplicação em triagens sorológicas envolvendo alta quantidade de amostras. Portanto, um dos propósitos deste estudo foi expressar a proteína de Matriz do CDV com o intuito de ser utilizada no ELISA, permitindo a sua aplicabilidade na detecção clínica do CDV e pesquisa de status imune em inquéritos soroepidemiológicos. Em específico, a escolha desta proteína M, baseou-se nos seguintes fatores: 1) em contraste as outras proteínas estruturais do CDV, pouco é conhecido sobre esta importante proteína, principalmente quanto as suas características de interação com anticorpos; 2) a proteína M desempenha importante papel na replicação viral, incluindo montagem e auxílio na liberação da partícula viral da célula infectada; 3) a proteína M tem baixa massa molecular (~38 kDa) e é altamente conservada entre os CDV (Sidhu et al., 1993); 4) por último a sua escolha está relacionada ao estudo de Dietzel e colaboradores, os quais observaram que a proteína modula a virulência do vírus (Dietzel *et al*., 2011). A partir disso, levantamos a hipótese de que a proteína M pode constituir um alvo a ser utilizado no diagnóstico da infecção pelo CDV.

CAPÍTULO 2

MOLECULAR AND SEROLOGICAL SURVEYS OF CANINE DISTEMPER VIRUS: A META-ANALYSIS OF CROSS-SECTIONAL STUDIES

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2. MOLECULAR AND SEROLOGICAL SURVEYS OF CANINE DISTEMPER VIRUS: A META-ANALYSIS OF CROSS-SECTIONAL STUDIES

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2.1 Abstract

Background

Canine morbillivirus (canine distemper virus, CDV) persists as a serious threat to the health of domestic dogs and wildlife. Although studies have been conducted on the frequency and risk factors associated with canine distemper virus (CDV) infection, there are no comprehensive data on the current epidemiological magnitude in the domestic dog population at regional and national levels. Therefore, we conducted a cross-sectional study and included our results in a meta-analysis to summarize and combine available data on the frequency and potential risk factors associated with CDV infection.

Methods

For the cross-sectional study, biological samples from dogs suspected to have canine distemper (CD) were collected and screened for viral RNA. Briefly, the PRISMA protocol was used for the meta-analysis, and data analyses were performed using STATA IC 13.1 software.

Results

CDV RNA was detected in 34% (48/141) of dogs suspected to have CD. Following our meta-analysis, 53 studies were selected for a total of 11,527 dogs. Overall, the pooled frequency of CDV positivity based on molecular and serological results were 33% (95% CI: 23-43) and 46% (95% CI: 36-57), respectively. The pooled subgroup analyses of clinical signs, types of biological samples, diagnostic methods and dog lifestyle had a wide range of CDV positivity (range 8-75%). Free-ranging dogs (OR: 1.44, 95% CI: 1.05-1.97), dogs >24 months old (OR: 1.83, 95% CI: 1.1-3) and unvaccinated dogs (OR: 2.92, 95% CI: 1.26-6.77) were found to be positively associated with CDV infection. In contrast, dogs <12 months old (OR: 0.36, 95% CI: 0.20-0.64) and dogs with a complete anti-CDV vaccination (OR: 0.18, 95% CI: 0.05-0.59) had a negative association.

Conclusion

Considering the high frequency of CDV positivity associated with almost all the variables analyzed in dogs, it is necessary to immediately and continuously plan mitigation strategies to reduce the CDV prevalence, especially in determined endemic localities.

2.2 Introduction

Canine morbillivirus (previously known as canine distemper virus (CDV)) is one of the major pathogens in canine populations, as it causes one of the most contagious and fatal diseases for domestic dogs (*Canis familiaris*) [1,2]. CDV is enveloped with single-stranded, negative sense and nonsegmented RNA genetic material, belonging to the genus *Morbillivirus* (family *Paramyxoviridae*) [3]. Viral transmission occurs via aerosols or by direct contact of susceptible animals with the various fresh body secretions of infected

animals [4]. Consequently, CDV infection results in canine distemper (CD), which is a severe disease with multisystemic clinical signs [5]. Despite the existence of a vaccine, several reports highlight CDV, calling attention to the increased activity, genetic diversity and reemergence of other infections in the world [6-8].

Regarding the diagnosis of CD, it is essential to use laboratory tests with better accuracy for viral detection. This is due to the broad clinical spectrum of signs of the disease, making clinical diagnosis difficult since nonspecific clinical signs may be confused with several other infectious diseases [9-11]. Therefore, laboratory tests, including serological and molecular surveys, have been carried out over the past few years to describe the epidemiological profile of CDV in some localities [12,13]. In these laboratory tests, methodological variants aim at the specific identification of genetic material, antigens and proteins (IgG and/or IgM) related to CDV. For this purpose, several biological samples, including nasal, ocular and saliva secretions and blood, feces, urine and infected tissues have been used mainly for PCR, immunochromatography (IC), seroneutralization (SN), immunofluorescence (IFA) and ELISA [14-16] analyses. However, there are still several gaps related to CDV epidemiology, including the following: 1) the frequencies of infections in domestic dogs are still poorly characterized; 2) the sample size of most studies is relatively small; 3) there is no robust analysis of the risk factors associated with CD/CDV; 4) there is no synthesis of the current epidemiological picture regarding the burden of the disease and its frequency according to various clinical signs, diagnostic methods and types of biological samples analyzed.

In view of the significant impact of CDV infections on the health of domestic dogs, which are the main reservoir hosts, and the lack of data on the epidemiological characteristics of these infections in the world, this observational study and meta-analysis aimed to determine and better understand the individual and pooled frequency patterns of detectable CDV using various molecular and serological tests.

2.3 Methods

2.3.1 Cross-sectional study

With the main purpose of laboratory diagnosis of CDV, the present study was approved by the ethics committee on the use of animals of the Universidade Federal de Goiás (Protocol Number: 054/17). Samples from domestic dogs showing clinical signs suggestive of CD were collected between 2017 and 2019. The collection sites were the

Veterinary Hospital of the UFG and the Control Center of Zoonoses of the municipality of Jataí, located in the Center-West region of Brazil.

After blood samples were collected in tubes (BD Vacutainer® PPT 13x100 mm, 5 ml) and nasal specimens were collected with flocked swabs placed into 1 ml universal transport medium (UTM (Copan, Brescia, Italy)) for the purpose of molecular diagnostic testing, the plasma and UTM were separated and used for the detection of viral RNA. Initially, the RNA was extracted using a QIAamp Viral RNA commercial kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. Subsequently, following adaptations of the protocol of Castilho et al. and Frisk et al. [17,18], reverse transcription, PCR and nested PCR were performed for the purpose of partial detection of the CDV nucleoprotein (N) gene. After the addition of the possible amplicons in the 1.5% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen; Carlsbad, USA), the amplification product was analyzed under ultraviolet light. The molecular identity of the PCR product of expected size (287 bp) was confirmed by DNA sequencing (ACTGene Análises Moleculares Ltda., RS, Brazil).

2.3.2 Systematic review and meta-analysis

The present meta-analysis followed the methods developed in the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) protocol, which refers to rules and guidelines for systematic reviews and meta-analyses (S1 File) [19]. Additionally, we recorded the study protocol in SYRCLE (Systematic Review Center for Laboratory Animal Experimentation) (www.syrcle.nl). We also deposited our laboratory protocols at protocols.io, which can be viewed at https://dx.doi.org/10.17504/protocols.io.2umgeu6.

Search strategy. After defining the research protocol, we performed a systematic search in the PubMed, SciELO and ScienceDirect databases. Articles in the English, Spanish and Portuguese languages that were screened from July to October 2018 were selected. At this stage, to refine the studies of interest, a combination of descriptors was used ("*canine distemper virus*," "*canine distemper*," "*viruses in dogs*," "*dogs*," "*domestic dogs*," "*canis familiaris*," and "*canis lupus familiaris*"). We also sought additional studies through screening the references of selected articles and highly cited reviews of the topic of interest.

The next step involved the analysis of the selected articles containing the previously mentioned descriptors. To do so, the following inclusion criteria were used: 1) original articles published in scientific journals that contained information on serological and molecular surveys for the detection of CDV in domestic dogs; 2) studies containing data related to the proportion/rate of viral infection by laboratory tests; 3) seroepidemiological surveys for the detection of anti-CDV antibodies that included data concerning groups of animals not vaccinated against CDV; 4) data secondary to CDV positivity to analyze risk factors such as gender, age, vaccine status, breed, coinfection and lifestyle (free-ranging dogs versus non-free-ranging dogs); and 5) studies that used the most conventional ante-mortem detection tests. Regarding the exclusion criteria, the following parameters were adopted: 1) absence or confusing specification of the outcome of interest regarding the CDV positivity of laboratory tests; 2) revisions, book chapters, and seroprevalence studies not involving domestic dogs; and 3) small scale studies with a sample size <50.

Data analysis. For all selected studies, the following data were extracted: first author, year of publication, place of study, baseline characteristics of the studies including mean age, sex percentage, dog lifestyle, method of diagnosis, number of dogs investigated for CDV infection, proportion of positive animals, and clinical sign of CD and vaccine status. The main outcomes of interest in the data analysis were: 1) the proportion of CDV cases (laboratory confirmed to clinically suspected CD-positive dogs); 2) the proportion of cases with recent and/or previous CDV infection that were laboratory confirmed among apparently healthy dogs; and 3) the proportions of positive cases compared to the types of biological samples, clinical signs, diagnostic methods and origin of the studies. The secondary outcomes represented the determination of previously cited risk factors compared to the CDV positivity. For the bias risk analysis, a modified Joanna Briggs Institute and Strengthening the Reporting of Observational Studies in Epidemiology checklist were used [20,21]. In addition, the quality assessment of the studies referred to a modified method composed of the participant selection methodology, laboratory tests and outcome variables (S2 File).

Statistical analyses. Data collection required for analysis of the primary and secondary outcomes were initially extracted using Microsoft Excel (S3 File). Several tables were generated containing dichotomous data (occurrence or not of an event of interest) for the relative and cumulative calculation of the frequencies of the outcomes of interest, and a

95% confidence interval (CI) was used whenever possible. For all of the meta-analysis procedures, STATA IC/64 version 13.1 software was used (Stata Corporation, College Station, TX, USA). In STATA, the metaprop, metafunnel and metaninf commands were used for data analysis and the generation of forest and funnel plots. The relative frequency was determined by the number of cases (CDV positivity) divided by the total number of animals screened, and the results were expressed as percentages. The variance of each frequency estimate (known as ES (Effect Sizes)) was calculated as pq/n, where p is the frequency, q is 1 – p, and n is the total number of animals screened [22]. 95% Confidence intervals (CI) for the average ES were calculated with the formula: 95% CI = ES ± 1.96 * SE, where SE is the standard error (SE = √(pq/n)). To ensure proportionate weight distribution to studies presenting extreme frequency (near 0 or 1), we applied the Freeman-Tukey arcsine methodology [23,24]. In addition, the dichotomous data of the selected studies were extracted and plotted in a 2x2 table to obtain individual and combined odds ratios (ORs). The I^2 test was also used to assess the existence of heterogeneity between studies (I^2=75-100%, $p<0.05$) [25]. Due to the nature of the studies, the existence of heterogeneity was expected; therefore, we chose to use the random effects model for the meta-analysis as proposed by DerSimonian-Laird [26]. We performed a sensitivity analysis to test the effect of the individual influence of each study on the overall estimate, and a subgroup analysis was also performed to reduce the existence of heterogeneity. In addition, we evaluated the existence of publication bias by visual inspection of Begg's funnel plot as well as by Egger's test calculations [27,28].

2.4 Results

2.4.1 Cross-sectional study

To diagnose, contribute to molecular surveillance and trace the epidemiological profile of CDV in the study region, 141 clinical samples were collected from dogs exhibiting signs suggestive of CD. The mean age was 39 months (range 2-204), and most dogs were females (53%). Regarding clinical signs, in addition to ocular and nasal secretions, there was a predominance of neurological complications (myoclonus, ataxia, and paralysis of the limbs) and systemic complications (apathy and prostration). Because of these complications, 76% (107/141) of dogs died; CDV RNA was detected from nasal and blood samples in 34% (48/141) of dogs. The molecular identity was obtained by sequencing the amplicons generated by nested PCR, and the sequenced amplicons were identified with 99% homology to the partial segment of the CDV N gene.

2.4.2 Systematic review and meta-analysis: characteristics of included studies

Initially, during the search for articles in the digital databases and additional records from other sources, 439 reference studies were found (S1 Table). After application of the inclusion and exclusion criteria, we refined the results, and 53 eligible studies constituted the present meta-analysis [14,16,29-79]. The flowchart of this selection step is shown in **Fig 1**.

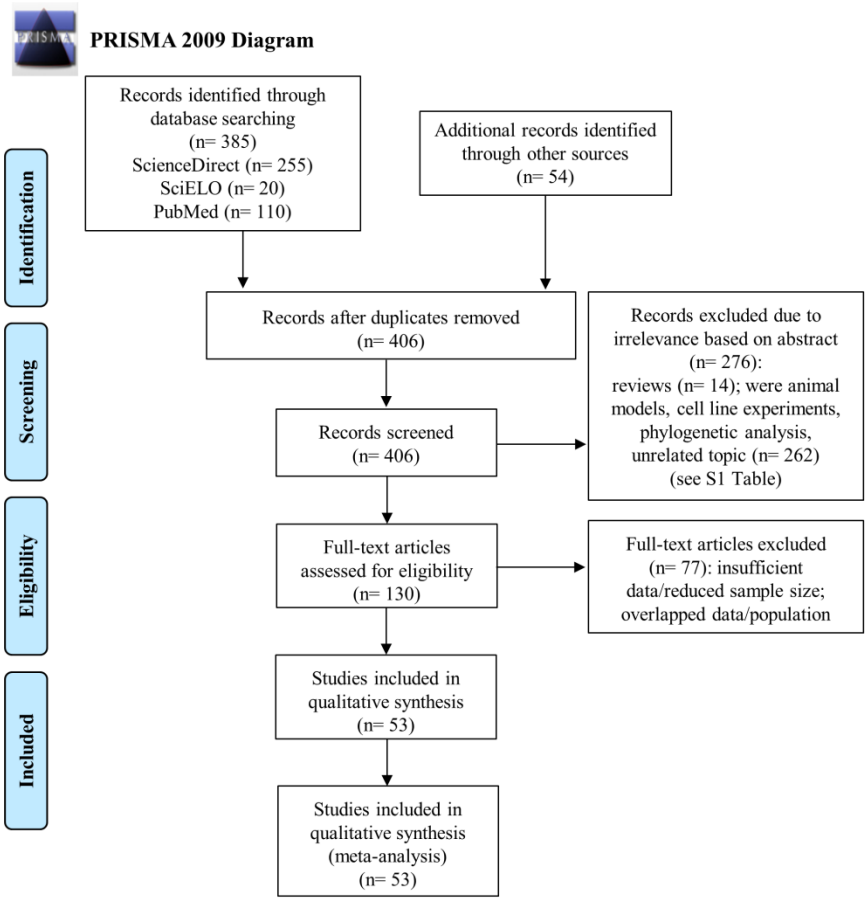

Fig 1. PRISMA 2009 flow diagram of observational studies included in the meta-analysis. https://doi.org/10.1371/journal.pone.0217594.g001

The 53 articles selected in addition to the data from our study produced a total sample of 11,527 domestic dogs included in the CDV infection analysis (S2 Table). The age range of these animals was considerably heterogeneous, ranging from 40 days to 18 years. In some studies, the mean age was 34.5 months (±41.2) [30,42-44]. Regarding the general age profile of the animals, only a few authors specified this profile in detail. In this context, there was an approximate ratio of 2.5:1:2.4 in relation to the number of dogs included in the classification of <12 months of age, 12-24 months and >24 months of age,

respectively. Thus, the majority of the animals included in the risk analysis were pups (<12 months, n=2581) and adults (>24 months, n=2497). In contrast to the cross-sectional study, there was a higher proportion of males than females (1.4:1).

Regarding the regions of origin of the selected articles, studies were conducted in 21 countries of the American (n=6), Asian (n=6), African (n=6) and European (n=3) continents. Most of the samples consisted of regions of China (n=3104), Brazil (n=2916) and Chile (n=1055). More details regarding the regional distribution of CDV infection rates are shown in **Fig 2** (S3 Table).

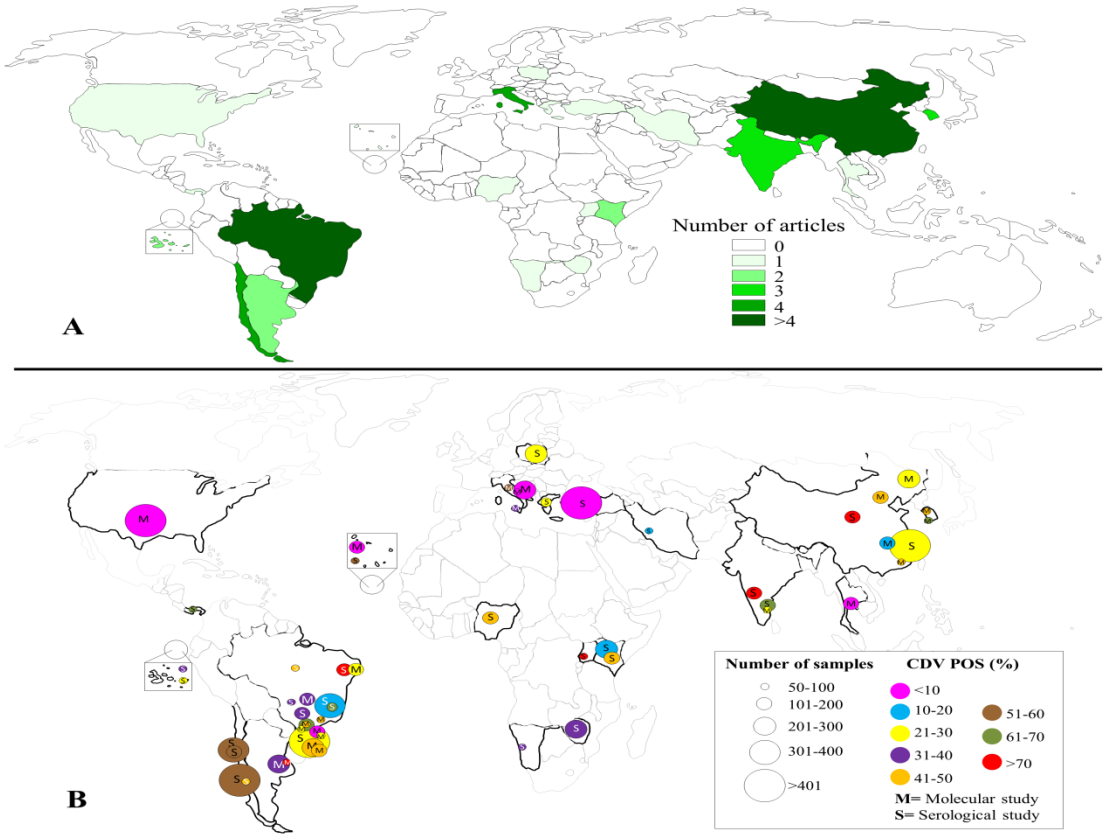

Fig 2. World map with the geographical distribution of the studies. The color intensity categories represent the number of studies included in the meta-analysis (A). The individual estimated frequency of laboratory confirmed CDV positivity in domestic dogs is shown (B). The CDV positivity is represented by the different colors and the total number of animals screened by the size of the circles. https://doi.org/10.1371/journal.pone.0217594.g002

2.4.3 A meta-analysis to estimate the pooled frequency of the CDV

As a result of laboratory confirmation by molecular surveys, the overall estimate of the combined frequency of CDV infection was 33% (95% CI: 23-43), with considerable evidence for regional epidemiological variations (**Figs 3A** and **2**). For serological surveys (antibody survey anti-CDV), the pooled frequency was 46% (95% CI: 36-57), while analysis based on antigenic results was 37% (95% CI: 25-50).

To better understand the picture of current studies in relation the frequency of CDV infection versus clinical signs of animals, **Fig 3B** was generated. Consequently, a higher viral positivity was observed when systemic clinical signs were present (75%, 95% CI: 34-100), followed by systemic and neurological (56%, 95% CI: 36-76), ocular (55%, 95% CI: 22-85), neurological (41%, 95% CI: 30-52), gastrointestinal (29%, 95% CI: 19-40) and respiratory signs (25%, 95% CI: 4-53).

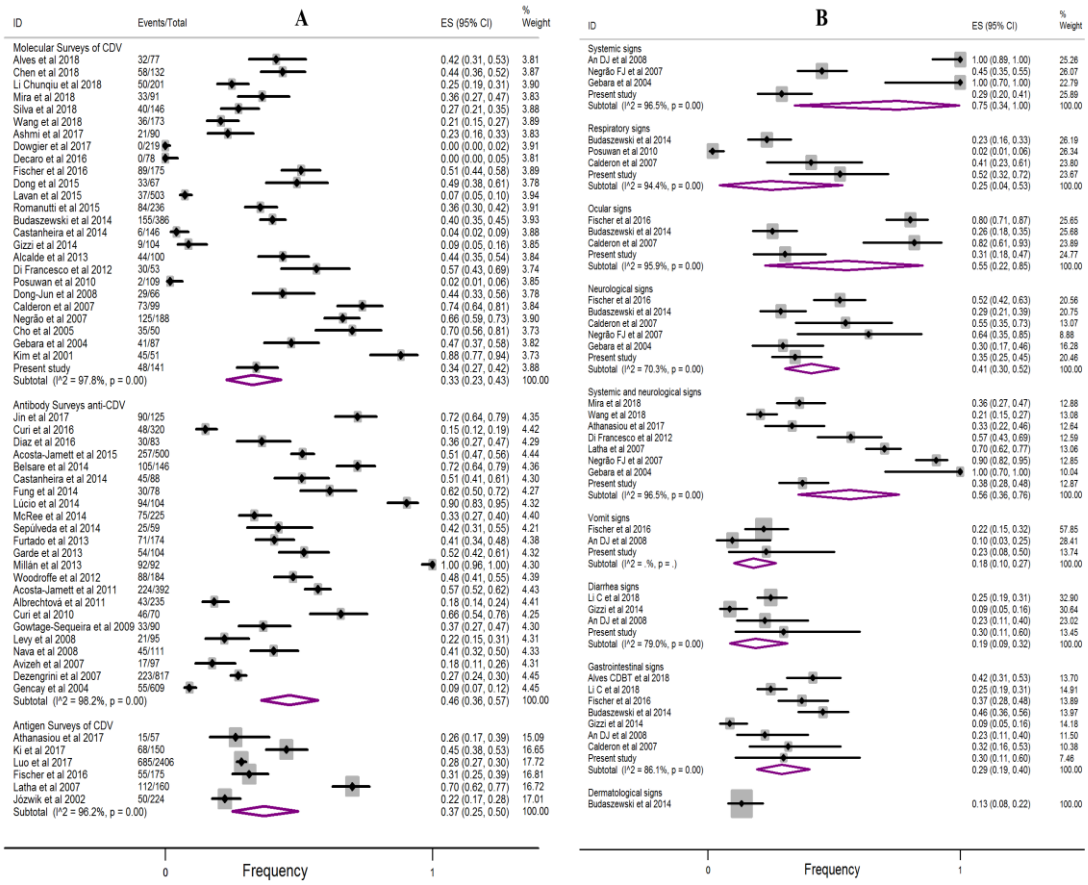

Fig 3. Forest plot of the frequency of laboratory confirmed CDV in biological samples from domestic dogs. Fig 3A shows the analysis of the subgroups of molecular, antibody and antigen surveys. Fig 3B shows the subgroup regarding the clinical signs of dogs (molecular surveys). The length of the line indicates the 95% confidence interval for each study, and the diamonds represent the pooled estimate. ID = identification of study; ES = effect size; Events = CDV POS. https://doi.org/10.1371/journal.pone.0217594.g003

Another study question was related to the determination of the levels of CDV infection according to the type of biological sample analyzed in the laboratory. As a result, greater positivity was observed for samples from ocular fluids (54%, 95% CI: 37-72), urine (51%, 95% CI: 40-62) and blood (46%, 95% CI: 36-57 (serological assays)). For the other types of biological samples, reduced positive frequency rates were found in blood lymphocytes (38%, 95% CI: 29-48), blood (37%, 95% CI: 24-50 (molecular

assays)) and nasal fluids (33%, 95% CI: 0-81) (**Fig 4A**). A lower frequency of viral infection was observed in fecal samples (18%, 95% CI: 5-35) and mucous fluid (11%, 95% CI: 4-21), which refers to the mixing of biological samples composed of nasal, ocular, oropharyngeal, oronasal and genital tract swabs.

In the forest plot (**Fig 4B**), the proportion of CDV positivity is shown, and the data are related to the diagnostic method used in conjunction with the type of biological sample. Of note, a higher proportion of positivity occurred when using the ELISA assays (62%, 95% CI: 47-77), IC (46%, 95% CI: 28-64), RT-PCR (41%, 95% CI: 33-50), nested PCR (38%, 95% CI: 28-49) and SN (38%, 95% CI: 26-50). However, a lower proportion of positive CDVs occurred when using quantitative PCR assays (8%, 95% CI: 0-23) and IFA (26%, 95% CI: 17-36).

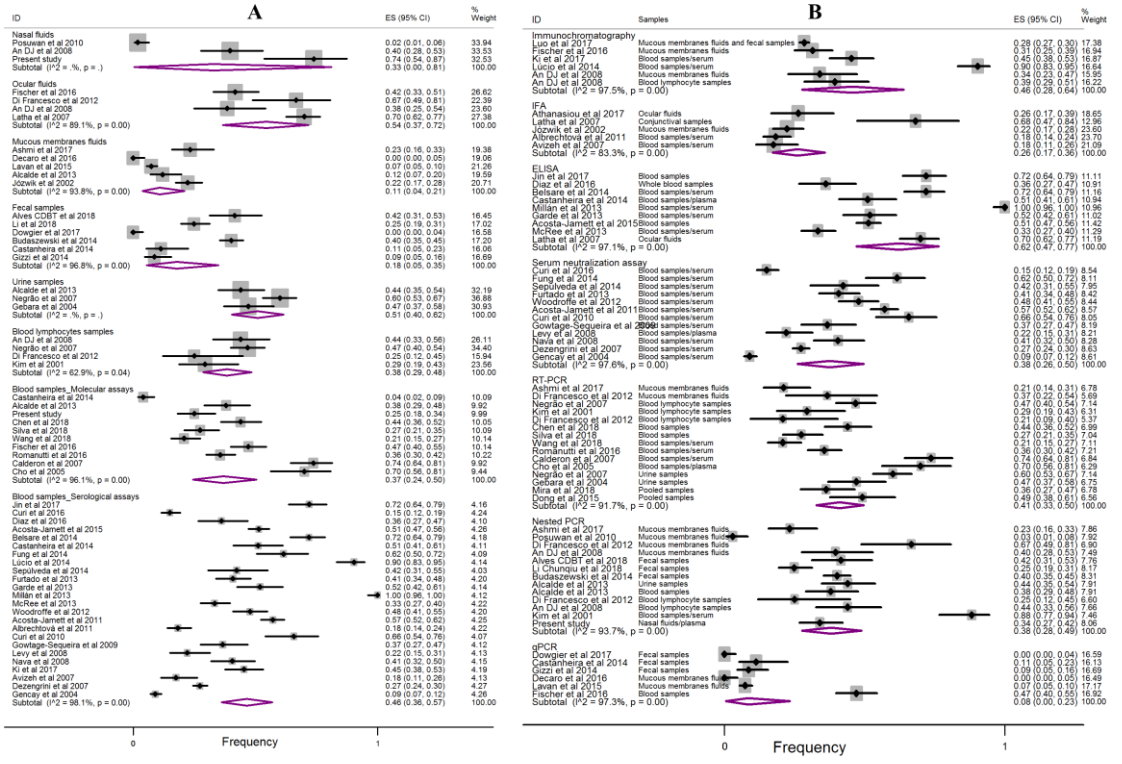

Fig 4. Fig 4A shows the forest plot of CDV positivity according to the type of biological sample surveyed. For Fig 4B, the forest plot is related to the diagnostic method. https://doi.org/10.1371/journal.pone.0217594.g004

When the main behavioral factor was analyzed, a high proportion of positivity was observed in the free-ranging dogs (**Fig 5**). Thus, the proportion of CDV positivity was higher for free-ranging dogs (55%, 95% CI: 40-70) compared to the overall estimates of 37% (antigen surveys) and 46% (antibody surveys), which represented the pooled data of non-free-ranging and free-ranging animals.

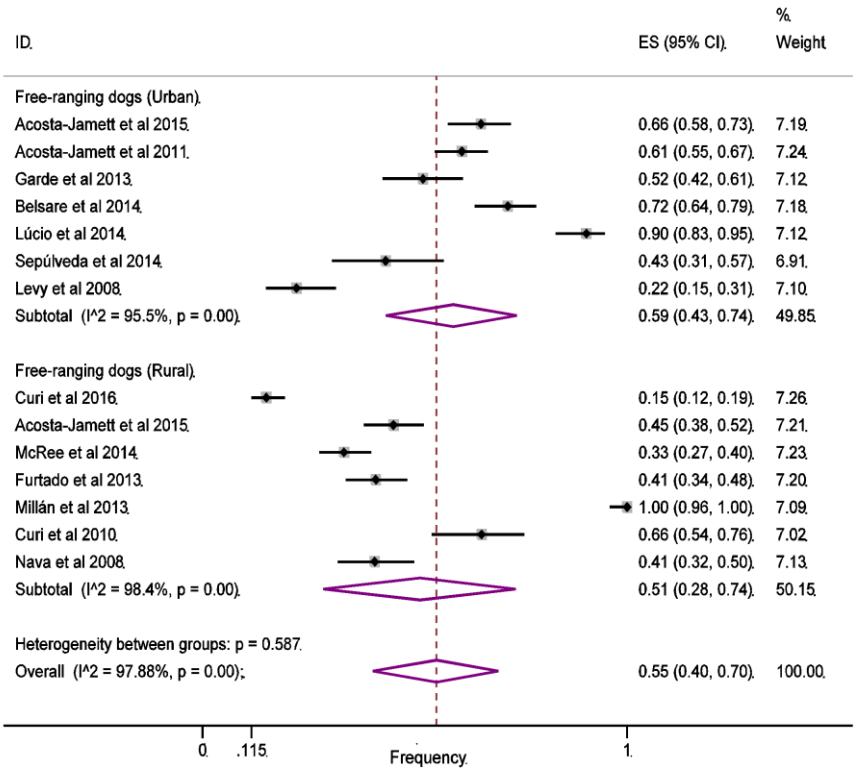

Fig 5. Forest plot showing the frequency of CDV positivity in relation to free-ranging dogs. https://doi.org/10.1371/journal.pone.0217594.g005

For the analysis of the frequency of viral coinfection, only four studies [14,27,30,67] provided the necessary data for the calculation of the proportions. The common viral pathogens involved in CDV coinfection were canine parvovirus (35%, 95% CI: 21-49), canine adenovirus (4%, 95% CI: 1-10) and canine coronavirus (24%, 95% CI 15-34) (S1 Fig).

In the analysis of the positivity rate of CDV over time, the included studies ranged from 1998 to 2018. Thus, we analyzed whether there was any trend between the positivity rates and the year of collection of the biological samples. As a consequence, the results more closely approximated a visual steady trend, as shown in S2 Fig.

2.4.4 A meta-analysis to evaluate risk factors associated with CDV positivity

For the purpose of testing potential risk factors associated with CDV positivity, the following variables were analyzed: gender; breed; age; free-ranging; vaccine status; and coinfection. In **Fig 6**, the results of the ORs for these variables are shown. In summary, a positive association with CDV positivity was observed in relation to the following

variables: free-ranging dogs (OR=1.44, 95% CI: 1.05-1.97); age of dogs >24 months (OR=1.83, 95% CI: 1.10-3.05); and unvaccinated dogs (OR=2.92, 95% CI: 1.26-6.77). In contrast, there was a negative association with vaccinated dogs (OR=0.18, 95% CI: 0.05-0.59), dogs <12 months old (OR=0.36, 95% CI: 0.20-0.64) and dogs that were coinfected with canine parvovirus (OR=0.21, 95% CI: 0.13-0.33). The other variables, such as gender, breed and incomplete vaccination status, had no association.

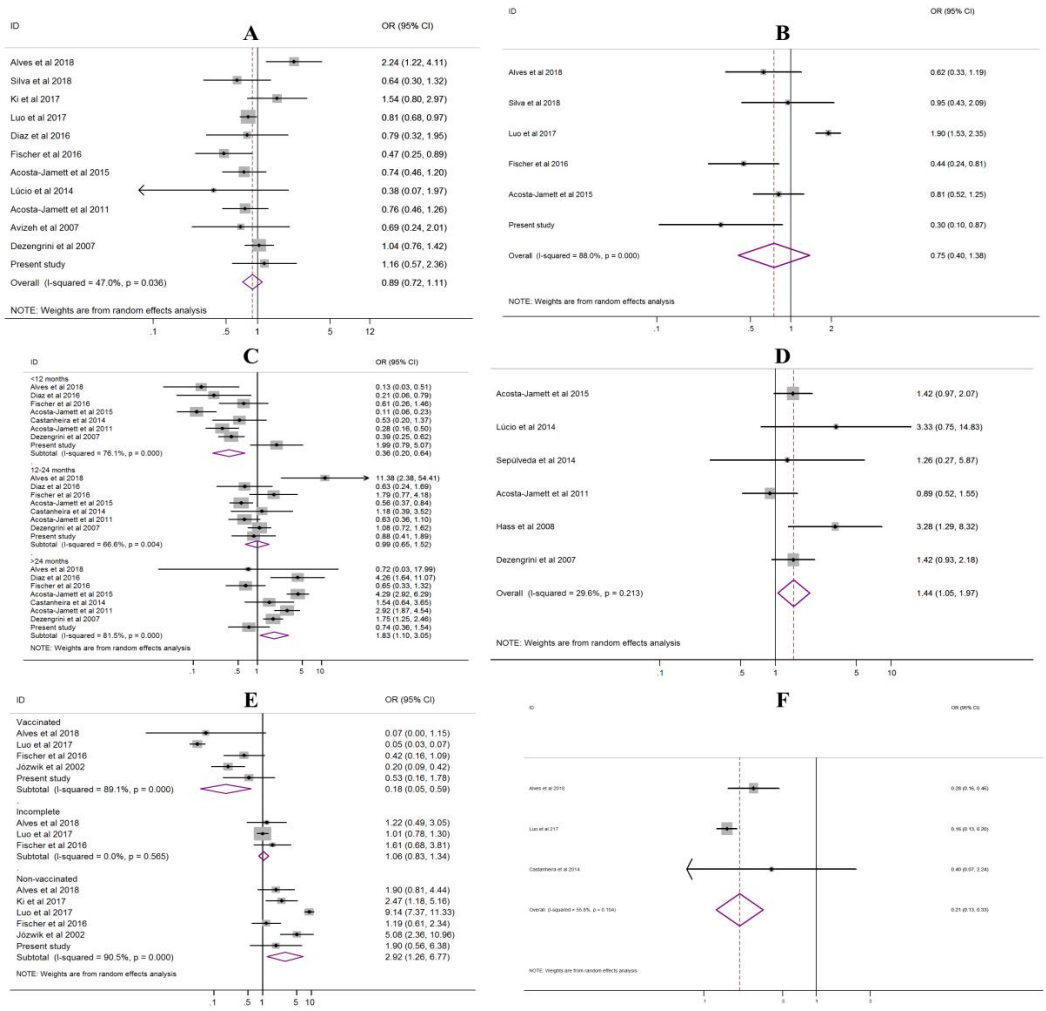

Fig 6. Forest plot of the odds ratio (OR) for CDV positivity in domestic dogs. A = gender, male versus female; B = purebred versus mixed-breed; C = age,<12 months, 12–24 months, and >24 months; D = free-ranging dogs versus non-free-ranging dogs; E = vaccinated versus incomplete and nonvaccinated; F = coinfection, CDV versus canine parvovirus. https://doi.org/10.1371/journal.pone.0217594.g006

2.4.5 Sensitivity analysis and publication bias

When performing the sensitivity analysis to assess the weight of each individual study on the combined frequency through the removal of individual studies, there was no study that significantly affected the combined frequency (S4 Table). In addition, subgroup analyses were performed. For the majority of the results, high heterogeneity

was observed (I^2>75%). Low heterogeneity was found only in the subgroup of clinical signs (vomit, diarrhea and dermatological signs; I^2=0.0). It should be emphasized that these results were expected, given that in observational epidemiological studies there is a considerable occurrence of diversity due to the study design, detection methodology and epidemiological variations.

In the analysis of publication bias, asymmetry of the funnel plot was noted for the CDV positivity frequency subgroups molecular surveys, serological surveys and free-ranging dogs (S3 Fig). However, when analyzing the asymmetry by Egger's test, significant bias was observed only for the subgroup serological surveys (P=0.02).

2.5 Discussion

In this cross-sectional study and meta-analysis, frequencies and analysis of risk factors for CDV infection in domestic dogs were investigated. Interestingly, the results showed a high frequency of viral positivity obtained from serological and molecular assays. Therefore, we found that almost a third of suspected CD-infected and almost half of apparently healthy dogs were CDV-positive (33-46%; 95% CI: 23-57) (Fig 3A). These general data show the high likelihood of dogs being exposed to CDV throughout their lives and show their prominent role in the viral transmission chain [80]. In view of this, the importance of epidemiological studies of CDV is highlighted as it is a valuable tool in monitoring viral dissemination and in the development of animal public health strategies.

Among the viruses that affect dogs, CD is the most relevant disease after rabies due to its considerable dissemination and severity potential [81]. However, it has been shown that half of the CDV infections are subclinical or so mild that they do not require veterinary care [82]. However, mild disease may develop into severe disease in dogs, and in this case, the initial clinical condition, which is often restricted to fever, respiratory, ocular signs, apathy and inappetence, may result in severe impairment of the gastrointestinal tract (vomiting and diarrhea) and central nervous system (paraparesis or tetraparesis with sensitive ataxia and myoclonus) [5,80,81,83]. Thus, neurological signs may be progressive, and the onset of sequelae tends to generate an expectation of poor prognosis, which reflects a reduced survival rate. In this context, few studies have described the outcome of CDV-positive dogs [36,42,71]. Here, we observed a fatality rate of 55% (95% CI: 47-64, I^2: 0%) for animals with predominantly neurological signs, demonstrating how dangerous CD is.

When obtaining the frequencies of CDV infection in relation to clinical signs and types of biological samples, the factors associated with greater positivity were dogs with systemic, systemic-neurological and ocular signs in conjunction with samples of ocular fluids, blood and urine. The determination of which sample to analyze depends on the method of detection and the opportunity to collect the biological material representative of the evident clinical signs [84]. Thus, in a suspected case of CD, those animals with exuberant ocular and nasal secretions tend to provide good clinical material for screening since swabs of ocular and nasal secretions specimens are easy to obtain at an early stage of CDV infection [85]. For animals with early systemic signs, including fever, prostration and inappetence, the indicated specimen choice would be blood and/or urine. Some studies have verified that urine is a good biological sample for the detection of CDV RNA [36-38], and although the authors used RT-PCR, which is less sensitive than nested PCR, results continued to show its excellent application for laboratory diagnostic purposes.

As briefly mentioned, regional epidemiological variations of CDV may be based on study design, detection methodologies and epidemiological aspects, which include differences in the populations studied. All of these factors contribute to the variations in CDV frequency; there have been individual studies with positivity rates varying from 0 to 100% in regions of Italy [55,61] and Uganda [69], respectively. However, most of the articles reported a frequency of positivity between 30 and 50% (Fig 3A). In our experimental study, the lowest observed frequency (25%) in blood samples compared to the overall estimate (37%) may have occurred due to differences in the studied populations and/or due to the period of infection (Fig 4). Several dogs had already presented neurological impairment with clinical signs present several days prior; therefore, the possibility of finding viral RNA in the plasma was reduced, even when using nested PCR.

In the detection methodology, the serological tests included ELISA, IC, IFA and SN. The molecular assays included PCR and its variants. Currently, all of these test methodologies are financially accessible for use in the laboratory, but the IFA and PCR variants require reagents and equipment of higher financial cost, and because of this, their satisfactory use in the laboratory will depend on the number of samples to be examined. In addition, regarding the use of these assays in the present meta-analysis, higher positivity rates were reported from ELISA, IC and RT-PCR assays; therefore, in the future, data regarding laboratory accuracy should be investigated. For ELISA assays,

only a few authors analyzed the sensitivity and specificity parameters, with indices varying from 93 to 100% and from 83 to 100% for sensitivity and specificity, respectively [53,86]. However, further studies are needed to better understand the diagnostic accuracy, including a higher number and varied types of biological samples. In the SN assay, known as the gold standard in antibody detection [87], the positivity rate of 38% (95% CI: 26-50) was lower to the global average, and approximately 1/2 of the seroepidemiological studies used SN. Regarding nested PCR, referred to as the gold standard in the diagnosis of CDV RNA, there was excellent sensitivity; however, the laboratory accuracy is susceptible to variation, including the sample collection during the clinical manifestations of acute CDV infection, the type of sample collected, the RNA extraction protocol and the primers used [88]. In these examples, it is not possible to estimate the probability of false-negative and/or false-positive results in our individual frequency estimates, but due to the robust global n-sample, this diagnostic bias tends to be reduced for the combined frequency estimate.

As a complementary investigation, we performed an analysis of the risk factors for CDV positivity. Consistent with the previous literature, we found no association between viral infection and the breed and gender of dogs, showing that regardless of whether dogs are male, female, purebred or mixed-breed, susceptibility to the etiological agent does not differ significantly between them [41,49,67,76,89]. In contrast, partial inconsistency with the literature was found in the negative association with pups [49,56,76]. Consequently, a higher risk of pups being affected by CD has been reported, but our results showed no association (OR= 0.59, 95% CI: 0.15-2.33). However, in the subgroup of pups without CD, there was a negative association between positivity (OR= 0.27, 95% CI: 0.16-0.46), indicating the vulnerability to CDV infection in this subgroup. In relation to adult dogs (> 24 months), the positive association observed was probably related to the fact that with the passage of time, the probability of exposure to the viral agent in the environment increases. In addition, another factor that increases the risk of infection is related to the behavioral factors of free-ranging or stray dogs. The free-ranging lifestyle of dogs likely means that they are not vaccinated and are constantly exposed to canine populations, which may justify the higher positivity of CDV in free-ranging dogs (55%) (Fig 5).

In the analyses of risk factors, it is important to highlight that it was impossible to perform ORs adjusted, such as grouping the vaccination status of the animals in relation to different ages and associating them with CDV infection by the fact of most of the selected studies were based on the inclusion of unvaccinated animals. Additionally, the

absence of vaccination status in each region evaluated may have contributed to the existence of biases in the results obtained. Also, it is important to highlight the potential existence of vaccination status biases in the dog lifestyle (free-ranging dogs) results considering that there were no data in the literature in order to adjust the OR. Thus, it is interesting to mention that the OR would be influenced by the fact that free-ranging dogs, especially non-vaccinated dogs, tend to be more exposed to CDV because of the greater possibility of contact with other non-vaccinated dogs and eventually CDV infected, especially in urban environments. However, for two studies [32,53] among those included in the analyses of risk factors, we can infer the absence of biases of the vaccine status factor in the variables of age, race, sex and behavioral style of the animals in the regions of the Santa Cruz (Galapagos) and Cape Verde, considering that they are regions with no vaccination programs.

Domestic dogs are a source of CDV transmission for wildlife. This is because dogs acting as major reservoirs can infect and cause disease in wildlife [90,91]. This issue highlights the impact of dog diseases on wildlife conservation, as they enter these habitats and have contributed to the emergence of fatal CD outbreaks. Examples include CD epidemics in dogs and other wild species that have threatened populations of African lions (*Panthera leo*) in the Serengeti ecosystem and Ethiopian wolves (*Canis simensis*) [92,93]. Additionally, the diversity of animals susceptible to CDV infection is broad as shown in a systematic review by Martinez-Gutierrez [94] who did not include domestic dogs in their analysis. Interestingly, in that analysis, the taxonomic families with the largest number of existing studies were *Canidae* followed by *Felidae* and *Mustelidae*. The median seropositivity of CDV was 35.6%, 34.1% and 41.1% for *Canidae*, *Felidae* and *Mustelidae*, respectively [94]. These data, as well as our data, show how common the circulation of CDV is in these groups of animals.

This systematic review and meta-analysis had several strengths. First, due to recent observational studies, there was a need for an updated systematic review. Second, this seemed to be the first meta-analysis on the intended subject. Third, it was possible to conduct multiple analyses of relevant factors by subgroups for the present theme. Fourth, to eliminate antibody positivity from immunization in serological surveys, care was taken to collect data restricted to animals not vaccinated against CDV. Despite the strengths of our study, which generated enough power to implement a comprehensive analysis, there is still room for future improvements that will depend on the quality of the data from future, improved studies. There were some limitations in this meta-analysis that must be

considered when interpreting the results. First, heterogeneity was observed in most of the analyses; therefore, heterogeneity in the subgroup was still relatively significant, and the results should be interpreted with caution. Second, there was a partial possibility of publication bias due to the asymmetrical funnel plot and the result of Egger's test; in other words, there was the possibility that unpublished articles were not included in our metadata. Third, the results of our analysis were mainly based on unadjusted estimates, which may have led to some bias in the results. Thus a more accurate analysis would be possible if individual crude data were available.

2.6 Conclusion

In summary, in the current meta-analysis (including our present study), the frequency rate of CDV positivity among molecular surveys was 33% (95% CI: 23-43) and among serological surveys, the rate was 46% (95% CI: 36-57), with considerable regional epidemiological variations in clinical signal parameters, biological samples, detection methods and animal lifestyle. Variables of adult (>24 months), free-ranging and unvaccinated dogs were found to be predictors of CDV infection. In contrast, complete vaccination, coinfection with parvovirus and pups (<12 months) had a negative association. Therefore, considering the high frequency of CDV positivity found across almost all variables analyzed, it is necessary to plan immediate and continuous mitigation strategies aiming to reduce infection levels, especially in certain endemic localities. In view of this, constant epidemiological surveillance, control of street dog populations, and more knowledge and access for dog owners to the complete CDV vaccine scheme is essential.

2.7 Supporting information

S1 File. PRISMA 2009 checklist.
https://doi.org/10.1371/journal.pone.0217594.s001

S2 File. Critical appraisal checklist for the quality assessment of studies.
https://doi.org/10.1371/journal.pone.0217594.s002

S3 File. Crude data extracted for the generation of the meta-analysis.
https://doi.org/10.1371/journal.pone.0217594.s003

S1 Table. List of excluded full text papers with proper justification.
https://doi.org/10.1371/journal.pone.0217594.s004

S2 Table. The characteristics of the studies included in the meta-analysis.

https://doi.org/10.1371/journal.pone.0217594.s005

S3 Table. Frequency of CDV infection regarding the regions of origin of the articles. https://doi.org/10.1371/journal.pone.0217594.s006

S4 Table. The sensitivity analysis to estimate of the frequency of CDV positivity. https://doi.org/10.1371/journal.pone.0217594.s007

S1 Fig. Frequency of coinfection between CDV with canine parvovirus (CPV), canine adenovirus (CAdV) and canine coronavirus (CCoV). https://doi.org/10.1371/journal.pone.0217594.s008

S2 Fig. CDV positivity by year of biological sample collection. The variation corresponds to the 95% CI. https://doi.org/10.1371/journal.pone.0217594.s009

S3 Fig. Funnel plot for CDV frequencies in subgroups molecular surveys (A), serological surveys (B) and free-ranging dogs (C). https://doi.org/10.1371/journal.pone.0217594.s010

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CAPÍTULO 3

FIRST COMPLETE GENOME SEQUENCE AND MOLECULAR CHARACTERIZATION OF CANINE MORBILLIVIRUS ISOLATED IN CENTRAL BRAZIL

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3. FIRST COMPLETE GENOME SEQUENCE AND MOLECULAR CHARACTERIZATION OF CANINE MORBILLIVIRUS ISOLATED IN CENTRAL BRAZIL

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3.1 Abstract

The Brazilian regions are still highly endemic areas for *Canine morbillivirus* (canine distemper virus [CDV]). However, little is known regarding the genetic variability of the strain circulating in several Brazilian regions. Here, we report the first full-length genome and molecular characterization of CDV isolated from domestic dogs in the Brazilian Center-West region. Sequence alignment and phylogenetic analyses based on deduced amino acid and nucleotide sequences showed that the isolated strain is characterized as the South America-I/Europe genotype. However, it segregates into a CDV subgenotype branch. Interestingly, both H and F proteins have a gain of a potential *N*-glycosylation sites compared to the Onderstepoort vaccine strain. Therefore, this study provides a reference to further understand the epidemic and molecular characteristics of the CDV in Brazil.

3.2 Introduction

Canine morbillivirus (also known as canine distemper virus [CDV]) is a highly contagious and deadly pathogen of dogs and wildlife[1,2]. CDV, which belongs to the genus *Morbillivirus* in the *Paramyxoviridae* family, is enveloped with single-stranded, negative sense, and non-segmented RNA genetic material[3]. The principal mode by which dogs are infected is through airborne exposure to respiratory droplets carrying infectious virus or by direct contact of susceptible animals with the various fresh body secretions of a CDV-positive dog or wild animal. CDV infection can result in canine distemper (CD), with clinical signs including the onset of cutaneous rash, serious nasal and ocular discharge, conjunctivitis, and anorexia, followed by gastrointestinal and respiratory signs[4,5]. Although CD results in multisystemic clinical signs[6], viral infection shows a high

incidence of neurological complications, and neurological signs may be progressive, generating sequelae and an expectation for a poor prognosis[7,8].

CDV is characterized by mutations, evolutionary changes with high genetic diversity, and eventual vaccine failure[9-12]. Regarding the complete viral genome, it contains 15,690 nucleotides (nt) and encodes six structural proteins, termed nucleocapsid (N), phospho (P), large (L), matrix (M), hemagglutinin (H), and fusion (F), and two accessory non-structural proteins (C and V). The glycoproteins H and F are inserted on the surface of the viral particle; they play a key role in adsorption and fusion, respectively, from the virion to the host cell. The M protein fills the space between the envelope and the ribonucleoprotein, thus contributing to viral morphology and the packaging and budding process in the host cell membrane. The N protein stands out because it encapsulates the genome and protects the genetic material. The L and P proteins are involved in viral RNA transcription and replication. Finally, the P, C, and V proteins play a role in RNA synthesis, aid in the transition from primary transcription to replication of the viral genome, and potentially influence messenger RNA (mRNA) synthesis, respectively[5,8,12].

Currently, there are at least 17 major CDV genetic lineages, including America-1 to America-5, Europe Wildlife, Arctic, South Africa, South America-1/Europe, South America-1 to South America-3, Rockborn-like, and Asia-1 to Asia-45,10. Due to its greater genetic variability, the H and F genes have been the main choices for determining CDV genetic lineages[13,14]. In addition, these classifications are related to the geographic origin where the lineages have been detected. While Brazil is considered endemic for CD, with high disease incidence rates[15], there have been limited studies conducted in the country related to virus isolation and molecular characterization of the circulating wild-type strains. To date, no studies have examined the full-length genome to characterize the Brazilian CDV field. Therefore, to elucidate the genetic basis of the protein diversity of CDV, we conducted amino acid and nucleotide sequence analysis of a recent field isolate, with a focus on the H and F genes, which are the most suitable targets to investigate the CDV variability and evolution[5,13,14].

3.3 Materials and Methods

3.3.1 Ethics statement

All animal procedures were approved by the Animal Care Committee of the Federal University of Goiás, Goiânia, Brazil (approval ID: 054/17). All experiments were

performed in accordance with relevant guidelines and regulations. The owners of all animals signed informed consent forms approved by the ethics committee. The biological samples, from dogs showing clinical signs suggestive of CD, were collected from the Veterinary Hospital of the Federal University of Jataí and the Control Center of Zoonoses of the municipality of Jataí, located in the Center-West region of Brazil.

3.3.2 Reverse transcription–polymerase chain reaction (RT-PCR)

The ocular/nasal specimens from dogs were collected with flocked swabs placed into 1 mL universal transport medium (UTM; Copan, Brescia, Italy). These samples in UTM were separated and used for the detection of viral RNA and cryopreserved for virus isolation. Initially, the RNA was extracted using a QIAamp Viral RNA commercial kit (QIAGEN, Hilden, Germany) according to the manufacturer's specifications. Briefly, the method involved synthesis of a complementary DNA (cDNA) strand with a denaturation mix consisting of 1.0 µL (10 pmol/µL) random hexamers (Promega, inc), 0.5 µL nuclease-free water (Thermo Scientific, inc), and 8.5 µL total RNA; this mix was denatured at 70°C for 5 min and immediately incubated on ice. The RT mix solution consisted of 4 µL 5X Reverse Transcriptase Buffer, 1.8 µL (50 mM) $MgCl_2$, 1.7 µL (2.5 mM) dNTPs, 1 µL (20 units) RNase inhibitor, and 1.5 µL GoScript enzyme (Promega, inc). The RT mix was added to the denaturation mix and reverse transcription was performed in a total volume of 20 µL in an Amplitherm PCR Thermal Cycler for 10 min at 25°C followed by 90 min at 42°C; the reaction was terminated by heating to 70°C for 15 min. Following protocol adaptations reported by Castilho et al.[16] and Frisk et al.[17], reverse transcription, PCR, and nested PCR were performed for the purpose of partial detection of the CDV N gene. After the addition of the possible amplicons in the 1.2% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA), the amplification product was visualized under ultraviolet light. The molecular identity of the expected PCR product (287 base pairs [bp]) was confirmed by DNA sequencing.

3.3.3 Cell culture and virus isolation

VerodogSLAM (VDS) cells were obtained from the Department of Veterinary, Laboratory of Immunobiological and Animal Virology, at the Federal University of Viçosa, Brazil. VDS cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, D7777, Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B (Vitrocell, Campinas,

Brazil). Cells were cultured at 37°C in a humidified incubator containing 5% CO_2. In addition, zeocin (R25001, Gibco) antibiotic was added (1% final concentration) for stable maintenance of canine signaling lymphocytic activation molecule (SLAM) tag expression, which is one of the cellular receptors for CDV.

An aliquot of cryopreserved PCR-positive swab samples for CDV was thawed and filtered through a 0.22-μm filter and used as an inoculum for virus isolation. Thus, CDV isolation was attempted using VDS cells. Consequently, confluent VDS cells in 6-well plates were washed twice with phosphate-buffered saline (1X) and inoculated with 400 μL sample and 200 μL DMEM. After 1–2 h incubation with gentle shaking, more DMEM supplemented with 2% FBS and 1% antibiotic was added to each well. Inoculated cells were incubated at 37°C with 5% CO_2. The cell culture plate was observed each day under an inverted microscope to determine whether a cytopathic effect (CPE) developed. If there was a CPE, the supernatant was collected and confirmed to be positive for CDV by RT-PCR. All positive cell cultures were subjected to molecular typing with whole genome sequencing. However, if no CPE was observed 7 days post-inoculation, the supernatants were inoculated on new VDS cells for a second passage. Finally, if the CPE tests and RT-PCR results were negative after three passages, the virus isolation result was considered negative.

3.3.4 Primer design and whole genome sequencing

CDV-specific primers (Supplementary Table S1) were designed based on the reference sequences for the target species, which were selected from an analysis of complete sequences accessed through the Virus Pathogen Database and Analysis Resource (ViPR)[18] and nucleotide sequences available in GenBank[19].

The primers were designed using Geneious Primer (2020.2)[20] to cover the complete genome sequence of all CDV strains. The primers were designed to have a melting temperature (Tm) between 52 and 68°C and not to form hairpin loops or primer dimers. In addition, the NCBI BLAST tool was used to confirm the specificity of the primers for CDV. Supplementary Table S1 shows the primers created and used for full-length cDNA amplification and sequencing. DNA fragments corresponding to the full-genome of the isolated virus were amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen). This enzyme was used to amplify the genome in 25 overlapping fragments (**Fig. 1**). The PCR products were then purified with the QIAquick PCR purification kit

according to the manufacturer's protocol. Purified amplicons were sequenced bidirectionally using an ABI3.500 genetic analyzer (Applied Biosystems).

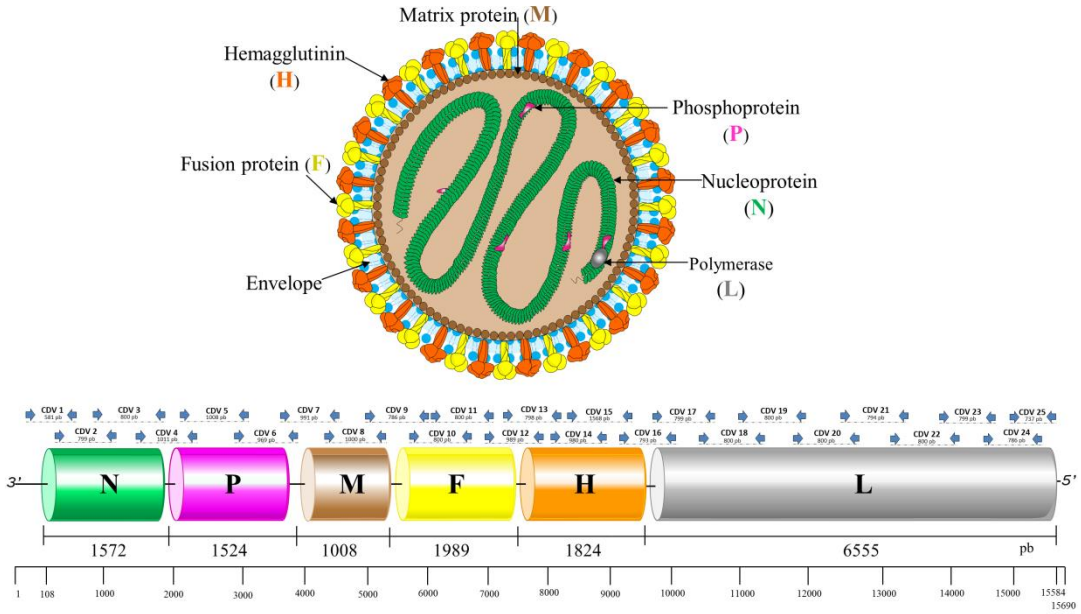

Fig 1. Schematic organization of the canine distemper virus (CDV) genome and primer design.

3.3.5 Phylogenetic analysis and molecular characterization of CDV

Phylogenetic analysis was performed using the nucleotide/amino acid sequence, as well as the sequences of 24 reference strains for which full genome sequences were available in GenBank and ViPR. Sequences were edited and aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program in the Geneious software package. A phylogenetic tree was constructed, based on the open reading frame (ORF) sequences of CDV, using the neighbor-joining method in the Geneious software package. Bootstrap analysis was carried out on 10,000 replicate data sets.

Potential *N*-glycosylation sites of H and F proteins were predicted using the NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/NetNGlyc/)[21].

Selection pressure on the F/H proteins was evaluated using four methods: Single Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), Mixed Effects Model of Evolution (MEME), and Fast Unconstrained Bayesian AppRoximation, for inferring selection (FUBAR) on the Datamonkey web server[22]. A *p* value less than 0.05 for MEME and FEL and a posterior probability higher than 0.9 for FUBAR were considered suggestive of positive selection. The Genetic Algorithm for Recombination Detection (GARD) analysis in Datamonkey was performed to detect the recombination breakpoints in the H gene alignment of the wild-type CDV isolated in this study.

3.4 Results

3.4.1 Detection of the N gene in clinical specimens

Biological samples from a total of 30 dogs with clinical suspicion of CD were collected in 2019. Preliminary identification of CDV was done using nested RT-PCR targeting the conserved region of the N gene. A total of 18 samples were positive and showed a specific band at 287 bp in an agarose gel. VDS cells were then inoculated with the samples. Gross lesions such as detachment of cells and the syncytial effect were observed. Again, the nested RT-PCR was employed on the harvested VDS cells to confirm the isolation of a virus, designated JA88/2020. **Fig. 2** presents the results with the synthesized information.

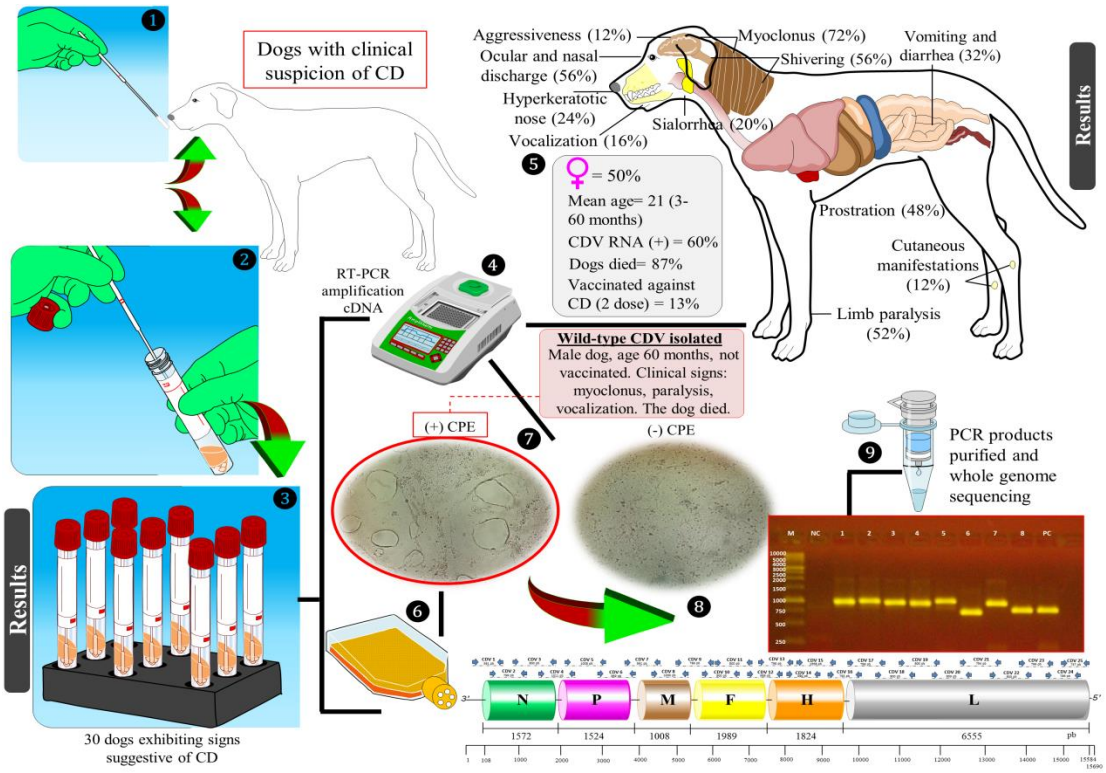

Fig 2. This schematic diagram reflects the experimental workflow. (1–3) In 2019, samples were collected from 30 domestic dogs showing clinical signs suggestive of CD. (4 and 5) CDV RNA (N gene) was detected from nasal samples in 60% of dogs. Also shown is the frequency of clinical signs for dogs that were CDV RNA positive. (6 and 7) Virus isolation was confirmed based on cell culture (one sample caused a cytopathic effect [CPE]) as well as reverse transcription–polymerase chain reaction (RT-PCR). (8 and 9) Subsequently, degenerate primers were generated to sequence the isolated CDV genome. 1.2% Agarose gel electrophoresis image (lanes contain PCR products from cDNA extracted from dog's nasal swab [lane M, pb DNA standard marker], [lane NC, negative control], [lane 1-4, CDV 4-7 primers], [lane 5-8, CDV 12/25/14/17 primers], [lane PC, positive control]).

3.4.2 Complete genome phylogenetic analysis

The JA88/2020 genome was fully sequenced (**Fig. 2**), and the complete nucleotide sequence has been deposited in GenBank under accession number MW460905. The whole genome sequencing comprises 15,624 nt with 100% consensus obtained by Sanger sequencing. The RNA contains six ORFs at 42–1,613 nt (N gene); 1,735–3,258 nt (P gene); 3,366–4,373 nt (M gene); 4,869–6,857 nt (F gene); 7,013–8,836 nt (H gene); and 8,964–15,518 nt (L gene). The 5′ untranslated region (UTR) and 3′-UTR are 106 and 41 nt long in the viral RNA, respectively. In addition, to identify the type of the CDV detected, the strain sequence was compared with those of 24 reference strains. Based on the standard criteria for classification of CDV lineages (nucleotide and amino acid sequence identity), JA88/2020 is part of the South America 1/Europe (SA1/EU) lineage: Its complete coding sequence displays 97.4% nucleotide and 98.5% amino acid similarity with the prototype strain Uy251 (**Fig. 3**). Interestingly, the genome-wide phylogenetic tree revealed that the strain isolated in this study segregates into a cluster of previously reported CDV lineages.

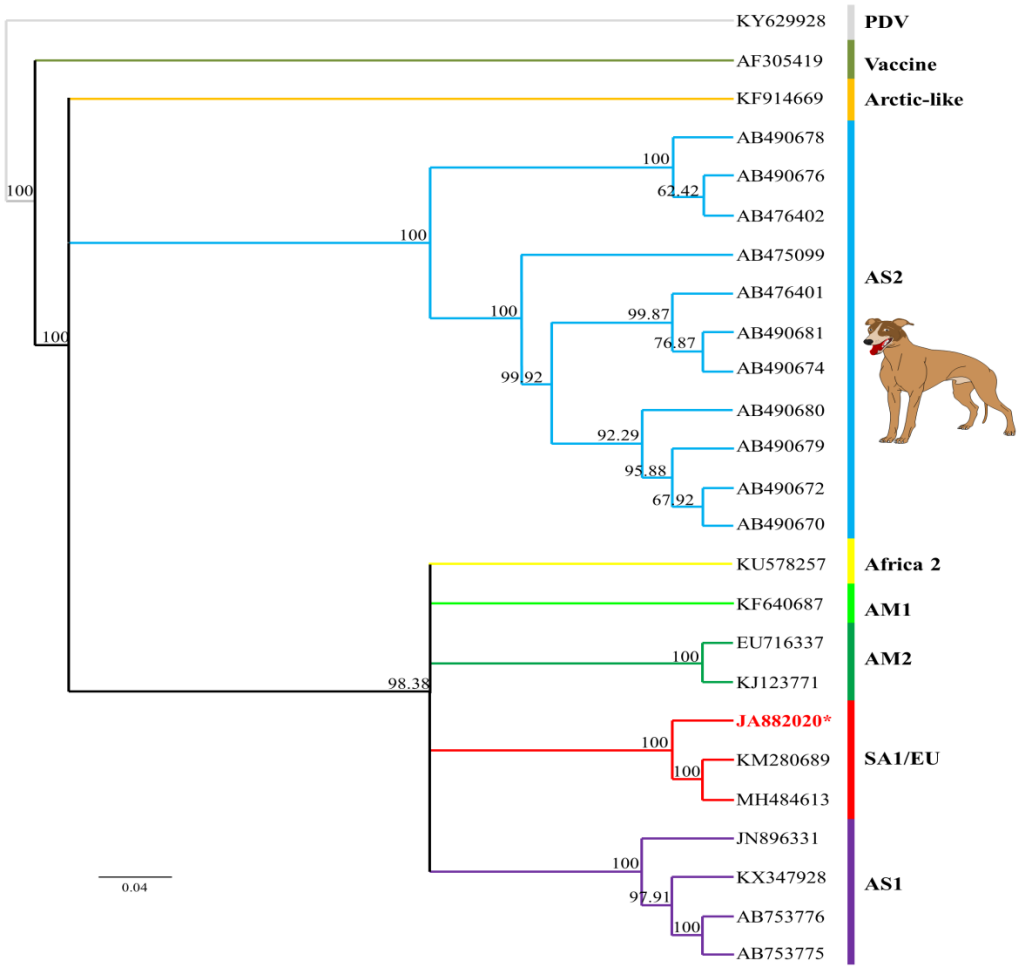

Fig 3. A phylogenetic tree based on amino acid sequences between the detected CDV and reference strains. Bootstrap values (> 50%) are shown at each node of the tree using

10,000 replicates. The scale bar below the tree represents a genetic distance of 0.04 amino acid substitutions per site. The CDV isolate identified in this study is indicated in bold and with an asterisk.

Phylogenetic trees of each CDV gene (N, P, M, F, H, and L) were also made. The topology of trees has branch positioning similar to that of the genome (**Fig. 4**). When comparing each individual gene between JA882020 and SA1/EU, the nucleotide/amino acid identities are as follows: 98%/99.2% (N gene), 98%/98.6% (P gene), 97.12%/99.1% (M gene), 96.7%/97.3% (F gene), 97%/96% (H gene), and 97.2%/99.2% (L gene)

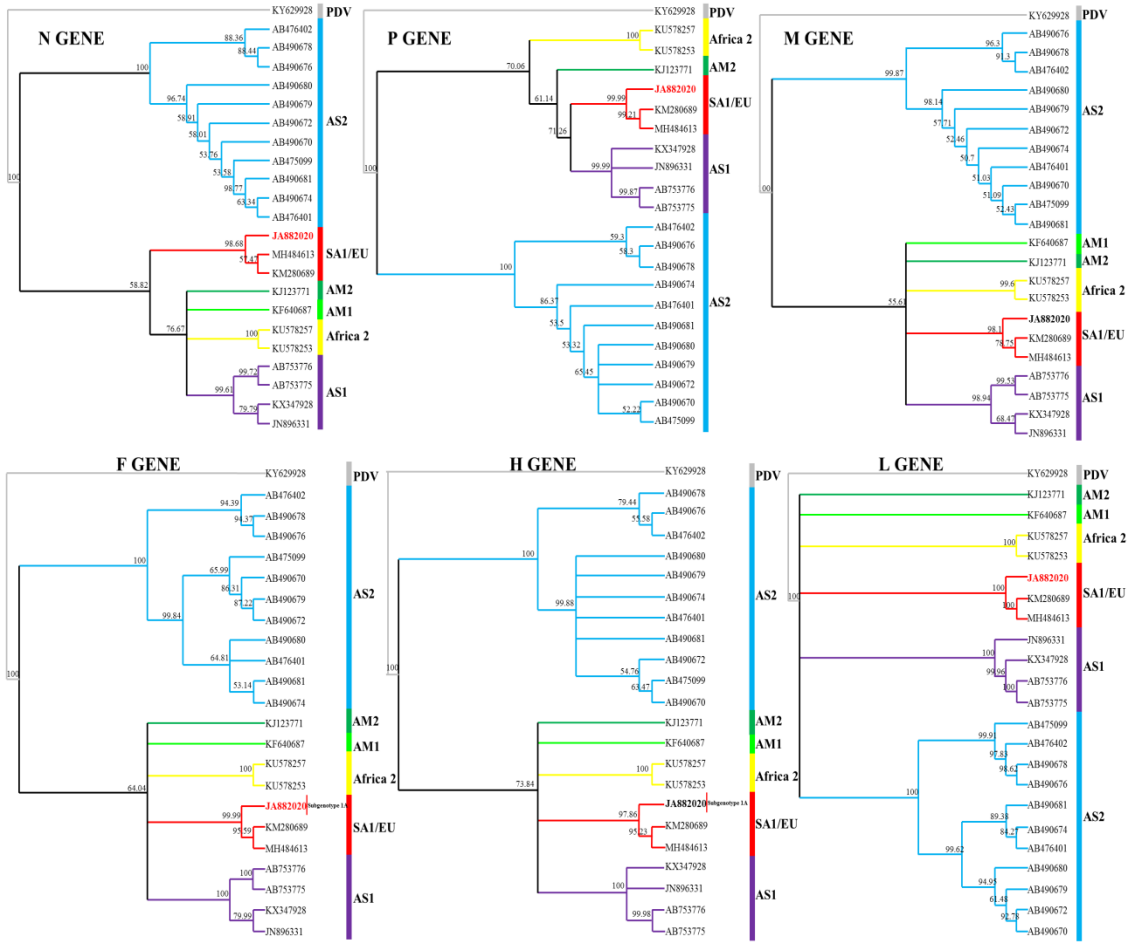

Fig 4. Phylogenetic relationships between CDV strains based on N, P, M, F, H, and L gene sequences. Bootstrap values are shown at each node of the tree. The phylogenetic tree was constructed by the neighbor-joining method using 10,000 bootstrap replicates. The CDV isolate identified in this study is indicated in bold.

3.4.3 CDV subgenotype and amino acid analysis of the H and F proteins

Following the criteria of at least 95% amino acid identity to define a genotype and 98% to define a CDV subgenotype, for H amino acid identity, we identified a subgenotype in the SA1/EU lineage (subgenotype 1A; 3.95% amino acid variation). Regarding the F amino acid identity, for the Fsp fragment gene sequences, we arbitrarily

extrapolated the classification and also found a subgenotype within the SA1/EU lineage (subgenotype 1A; 4.4% amino acid variation).

The deduced full-length H and F amino acid sequences were aligned with the Onderstepoort vaccine strain and with wild-type strains from other parts of the world (**Figs. 5** and **6**). For the H protein, there are 60 amino acid variations. In the H amino acid sequences, nine substitutions at positions 161, 172, 218, 227, 291, 332, 363, 401, and 506 are specific to the new JA88/2020 isolate. For the F protein, there are 62 amino acid variations. In the F amino acid sequences, six substitutions at positions 71, 105, 208, 386, 612, and 644 are specific to the new JA88/2020 isolate.

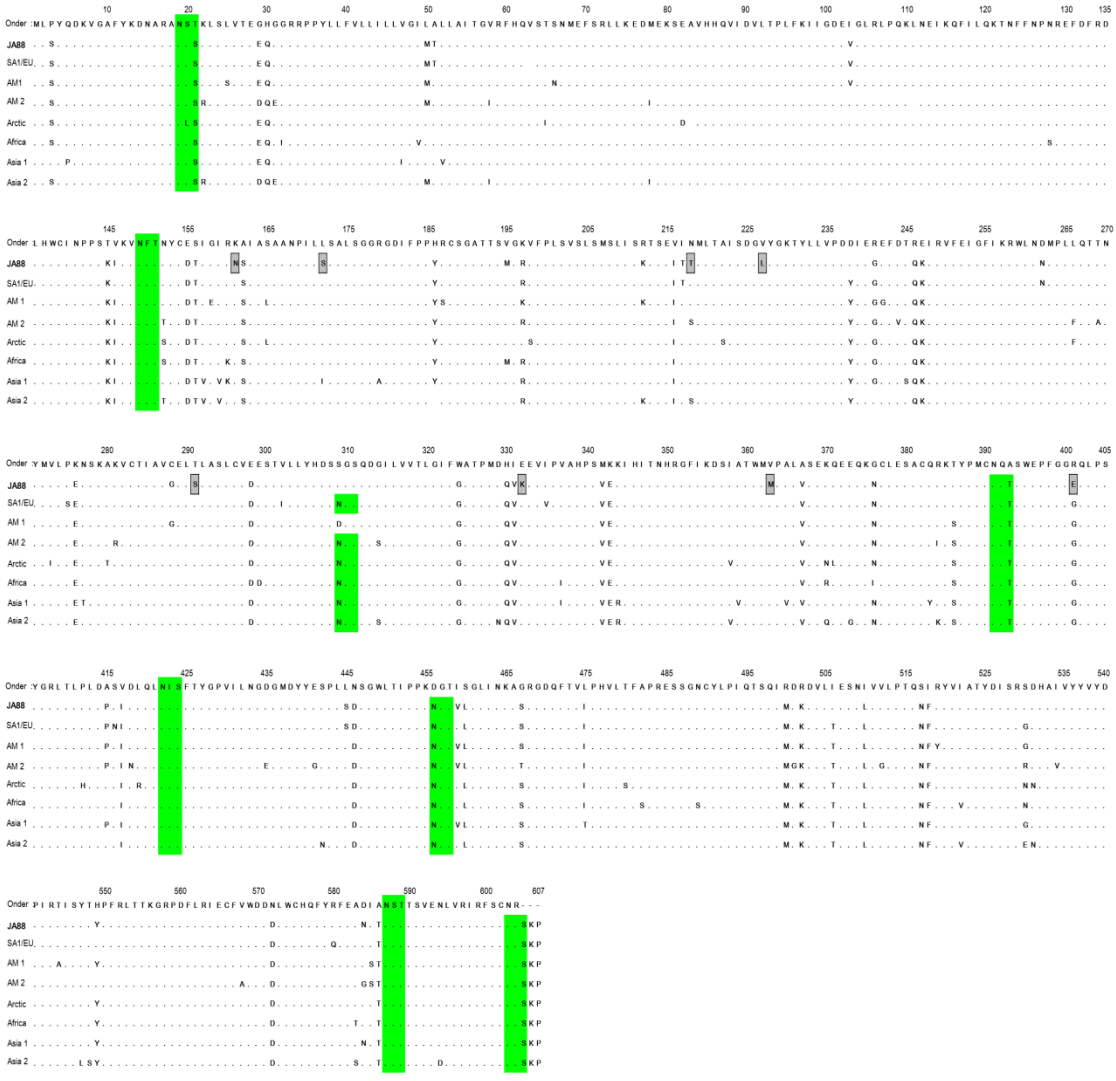

Fig 5. Amino acid sequence alignment of the CDV H protein from the Onderstepoort vaccine strain (AF305419); a wild-type strain from Brazil; and seven wild-type strains from Uruguay (SA1/EU: KM280689), the United State of America (AM1: EU716337; AM2: AY542312), Italy (Arctic: KF914669), South Africa (KY971528), China (AS1: JN896331), and Japan (AS2: AB490670).

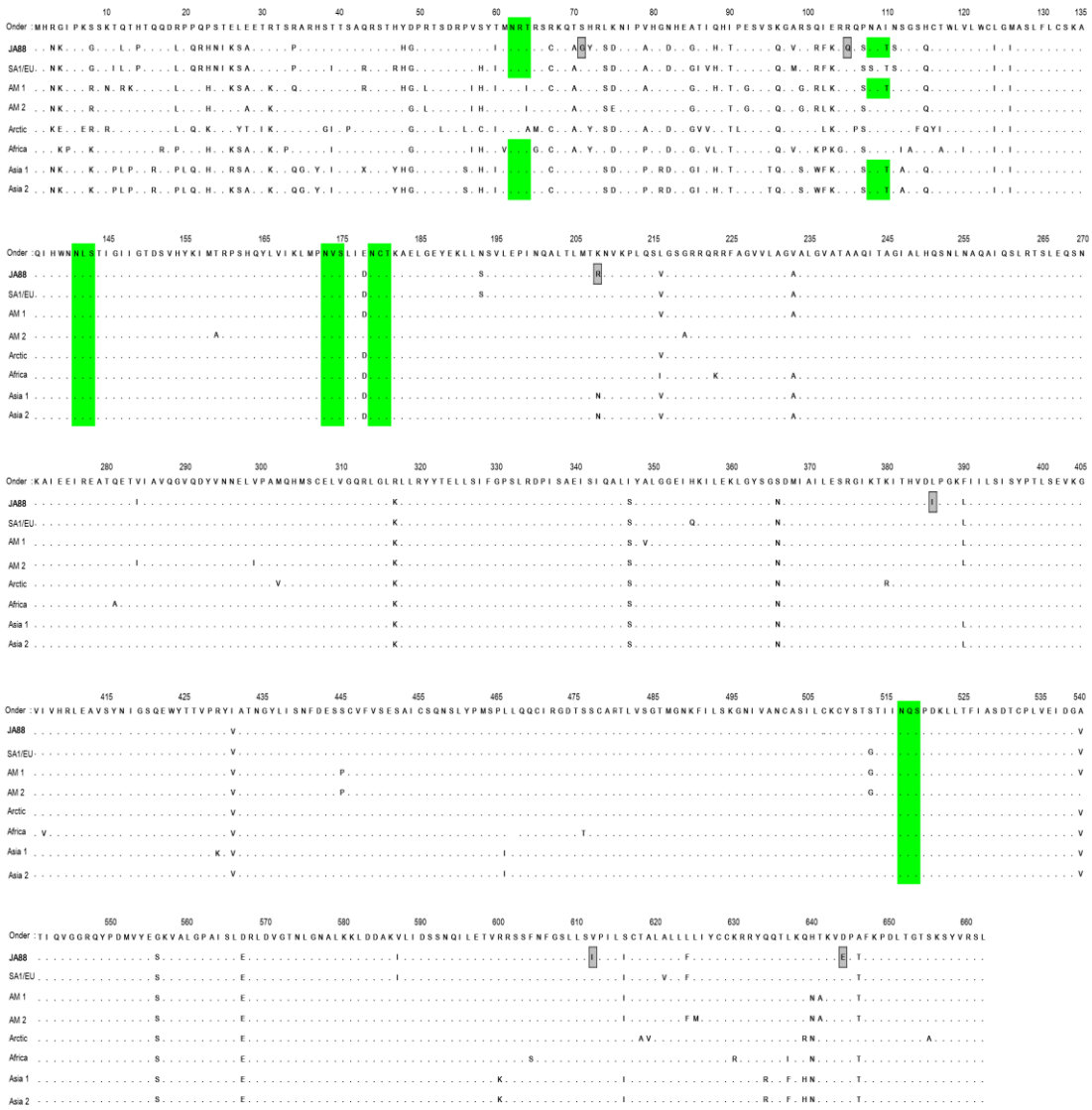

Fig 6. Amino acid sequence alignment of the CDV F protein from the Onderstepoort vaccine strain; a wild-type strain from Brazil; and seven wild-type strains from Uruguay (SA1/EU: KM280689), the United State of America (AM1: EU716337; AM2: AY542312), Italy (Arctic: KF914669), South Africa (KY971528), China (AS1: JN896331), and Japan (AS2: AB490670).

3.4.4 H and F protein *N*-glycosylation sites analysis

Seven *N*-glycosylation sites are predicted at amino acid residues in the H protein: 19, 149, 391, 422, 456, 587, and 603. Our analysis also showed that these are conserved sites for *N*-glycosylation in the H protein for the SA1/EU, America 1/2, Africa, Asia-1/2, and Arctic lineages. Surprisingly, a potential *N*-glycosylation site at position 309 is lost in JA88/2020 compared with the other lineage from SA1/EU. Regarding the F protein, there are six *N*-glycosylation sites at amino acid residues 62, 108, 141, 173, 179, and 517,

which are common sites for other lineages. In addition, an *N*-glycosylation site at amino acid residue 108 is lost in the reference lineage from SA1/EU (Uy251).

3.4.5 Selection pressure and recombination within CDV

Selection pressure analysis of the F protein from all CDV strains revealed 18 positively selected codon sites. Of the 18 positive sites, three were detected by several methods (Supplementary Table S2). Codons 71, 208, 612, and 644, potential positively selected codon sites, were detected by FEL. Codons 21, 87, and 101 were detected by FUBAR and MEME. Codons 61 and 302, potential negatively selected codon sites, were detected by FEL. One hundred codon sites predicted under negative selection were detected by FUBAR (Supplementary Table S2). Finally, recombination events for codons 549–662 were found for CDV by analysis with GARD from the Datamonkey package.

Selection pressure analysis of the H protein revealed 14 positively selected codon sites. Of the 14 positive sites, three were detected by several methods (Supplementary Table S2). Codons 172, 218, 227, 291, 309, 401, and 530, potential positively selected codon sites, were detected by FEL. Codon 530 was detected by FEL, FUBAR, and MEME. Seventy-five codon sites predicted to be under negative selection were detected by FUBAR (Supplementary Table S3).

3.5 Discussion

In this work, we amplified the complete coding and intergenic regions of the JA882020 SA1/EU strain obtained from a dog's nasal swab. The sample came from a 5-year-old male that showed clinical manifestations of myoclonus, paralysis, and vocalization; the animal ultimately died. Importantly, degenerate primer sets were generated to sequence this isolate. Furthermore, we contribute to the identification of sequence variability, and this information is also valuable for selecting appropriate primers and excluding false-negative PCR results.

The putative natural recombination events of the CDV F gene have been reported by our group, and the results in this study correlate with other previous findings[23]. Recombination events have also been reported in the H gene[10,24-26]. Consequently, the introduction of genetic mutations and recombination result in significant genetic variability of RNA viruses and may lead to the emergence of new viral lineages. Therefore, it is necessary to monitor these events to understand the genetic evolution of CDV.

One of the main benefits of monitoring mutations in infectious agents is to associate whether possible non-synonymous mutations are contributing to the prevalence of a more contagious and/or pathogenic strain. It is worth mentioning the molecular epidemiological surveillance of two glycoproteins on the CDV viral surface: H and F. To evaluate the possibility that key residues are involved in virulent CDV, Zipperle et al.[27] identified the key residues in the H protein (Y525, D526, and R529) that are involved in controlling SLAM-binding activity. SLAM and nectin-4 are CDV host cell receptors, which are expressed on activated T and B lymphocytes; epithelial, glial, and dendritic cells; and macrophages[5]. Consequently, based on the amino acid mutations of the viral isolate in this study, these key residues (H protein) have been conserved. However, it is important to search for new key residues in other proteins such as F to understand factors involved in virulent CDV.

Better molecular characterization of the CDV epidemic in Brazil is needed because CDV infection in dogs is high and deadly. Few studies have analyzed molecular epidemiology and carried out molecular analysis of full-length genes from Brazilian CDV lineages[28,29]. Therefore, some researchers have performed complete sequence analysis of the full-length F and H genes[14,30]. These reports have demonstrated the predominance of one genotype in Brazil: SA1/EU. However, two co-circulating lineages have already been detected, including the South America-II lineage. Moreover, similar results to our study have been described regarding CDV subgenotypes found in biological samples from dogs in Brazil and elsewhere[30,31]. CDV genotypes possibly differ due to geographic distribution rather than by host species. In this context, for a country as large as Brazil, concomitant circulation of different CDV genotypes is possible. Given this possibility, extensive molecular epidemiological surveys are required to determine the circulating (sub)genotypes[32].

The *N*-glycosylation sites in the F and H proteins are essential for their correct folding, transport, and cell surface expression. Hence, it is necessary to ask what is the importance of monitoring *N*-glycosylation sites in viral proteins? Among the possible answers, the following stand out: in previous studies, researchers have hypothesized that reduced *N*-glycosylation contributes to attenuate CDV pathogenesis, and that an increase in *N*-glycosylation may eventually result in vaccine failure[33,34]. Here, we observed an extra putative *N*-glycosylation site in the F protein compared with the Uruguay sequence (Uy251). Strikingly, four additional *N*-glycosylation sites in the F and H proteins were

found compared with the vaccine strain, thus showing its importance for modulating virulence.

In conclusion, this study is unique because it is the first that has isolated and identified the full CDV genome from Brazil. In sum, the isolated strain is characterized as the SA1/EU genotype, but it is segregated into a CDV subgenotype branch. Sequence analysis of more CDV field isolates from different Brazilian geographic regions is needed to investigate differences between (sub)genotypes. In addition, immunological investigation might be required to determine and monitor the biological relevance of circulating CDV (sub)genotypes and their importance for future drug and new vaccine development.

3.6 Supplementary data

Table S1. Primers used for RT-PCR amplification of the CDV genome

Primer	Nucleotide sequence (5'-3')	Position	bp[1]	Gene(s)
CDV 1F	MCYTAGDGRACAAGGTCAGG	72-91	581	N
CDV 1R	ACTGCTTTAGCGAGCARKAT	652-633		
CDV 2F	TCAACTCTGTTTGYGGTCTT	430-449	799	N
CDV 2R	CCRRCRGATCTTCTRACCAT	1228-1209		
CDV 3F	TGAAACRGCRCCGTAYATGG	1028-1047	800	N/P
CDV 3R	GACATGRTAGGCCTGYTCYTC	1827-1807		
CDV 4F	GCTGGTCCCAAGCAATCTCA	1344-1363	1011	N/P
CDV 4R	ACATCAGCTGCTCTGTCTGG	2354-2335		
CDV 5F	GCGGTGAAGAGGTTAAGGGA	2156-2175	1008	P
CDV 5R	ATCCGATTGCCGAGCTAGAC	3163-3144		
CDV 6F	ACATTAACCCAGAGCTCCGC	2975-2994	969	P/M
CDV 6R	TCGTCTGATAGTCGAGTGATGC	3943-3922		
CDV 7F	CGAACTGCAGGTGTCAAGGA	3737-3756	991	M
CDV 7R	AGGCGAAGTTCAAAACCCCA	4727-4708		
CDV 8F	CAGCGATGATCAGGGTCTTT	4415-4434	1000	F
CDV 8R	GATCTTATAATGGACACTGTCAGTC	5414-5390		
CDV 9F	GAGWCCRRDACCTCCCRRGS	5019-5038	786	F
CDV 9R	RACYCCCTGAACRGCAATGA	5804-5785		
CDV 10F	CGTTTTGCAGGAGTGGTRC	5604-5622	800	F
CDV 10R	TTGTTGCCCATMGTYCCAGAY	6403-6383		
CDV 11F	YGTCTCAGARTCAGCMATTTG	6278-6298	800	F/H
CDV 11R	GYTGGACTACYTGAGCCCTA	7077-7058		
CDV 12F	CTGGTCACACGTCTTACCCG	6939-6958	989	F/H
CDV 12R	GGCTTTGGAATTCTCCGGGA	7927-7908		
CDV 13F	TYGGCAGCAAAHCCYATCM	7571-7589	798	H
CDV 13R	MACYGGACCRTATGTAAAYGAK	8368-8347		
CDV 14F	TGACCGCTATCTCAGACGGA	7746-7765	980	H
CDV 14R	AGAAATCGTCCGGATTGGGT	8725-8706		
CDV 15F	YTGTCTRGAGTCDGCTTGTC	8206-8225	1424	H/L
CDV 15R	TGATTCACCTYTYACAAAGACAGGR	9629-9605		
CDV 16F	YATGCRAAGCTCACARTGGTTC	9494-9515	793	L
CDV 16R	AGGAGATGCATGGACAGGRA	10286-10267		
CDV 17F	MGCAGCAGARAATGTAMGGA	10124-10143	799	L
CDV 17R	ACTRCTYGGRGARGAGACGG	10922-10903		
CDV 18F	AAGAAAGACTCMCATCGBGGY	10827-10847	800	L
CDV 18R	GCTCGGGTCTCRTCCACTA	11626-11608		
CDV 19F	AGCCTTRAGACAGAGRTTRCATGA	11444-11467	800	L
CDV 19R	TYGCACCHGTGATYGTGTT	12243-12225		
CDV 20F	CCYATGCTGAAAGGAYTRTTCCAT	12111-12134	800	L
CDV 20R	CTCTTGTCYCTKAGTCGRTGAGC	12910-12888		
CDV 21F	CWACYGTGTACTCRTGGGCCT	12574-12774	794	L
CDV 21R	TCCAGARGGYCGGTGATARTG	13547-13527		
CDV 22F	CYGCDTTAATKGGYGAYGAYG	13408-13428	800	L
CDV 22R	ARCYAGRTCATCTTTHGGTGG	14207-14187		
CDV 23F	RAGRAGAGGBTCWATYAAGCAGA	14051-14073	799	L
CDV 23R	ACTTCKMAGAAAATGTGGGAGTG	14849-14827		
CDV 24F	TGGAAGARCTGTCTGCYATA	14710-14729	786	L
CDV 24R	TKGGGATTGTYGTCAGGATKA	15495-15475		
CDV 25F	CAACAGATTTTGCGAGTCGGT	14961-14981	737	L
CDV 25R	CAGACAAAGCTGGGTATGATAACT	15697-15674		

Table S2. Codon sites predicted under positive selection with p-value threshold of 0,1

Gene	FEL	MEME	FUBAR
F	71, 208, 612, 644	21, 53, 87, 98, 101, 105, 112, 311, 354, 546, 654	21, 87, 101
H	172, 218, 227, 291, 309, 401, 530	218, 349, 386, 471, 500, 530	530

Table S3. Codon sites predicted under negative selection

Gene	FEL	FUBAR
F	61, 302	9,18, 66, 78, 83, 93, 103, 109, 119, 124,129, 133, 138,140, 147, 149, 150, 162, 168, 172, 176, 180, 196,197, 209,212, 218, 222, 229, 230, 239, 251, 258, 267, 292, 296, 298, 300, 306, 308, 315, 331, 332, 335, 350, 351, 356, 361, 362, 365, 370, 378, 380, 382, 386, 389, 394, 402, 403, 410, 412, 421, 435, 440, 441, 442, 451, 464, 465, 477, 483, 485, 486, 490, 495, 499, 506, 521, 523, 529, 530, 531, 533, 536, 538, 545, 549, 554, 569, 613, 623, 625, 633642, 645, 647, 650, 651, 655, 657
H		6, 9, 40, 62, 75, 80, 81,106, 112, 129, 132, 134, 141, 144, 147, 154, 162, 177, 180, 226, 245, 253, 264, 278, 285, 290, 300, 320, 322, 331, 339, 343, 347, 351, 352, 353, 380, 393, 398, 400, 406, 410, 414, 423, 424, 427, 429, 433, 452, 453, 454, 456, 464, 474, 476, 481, 492, 494, 518, 520, 528, 533, 536, 554, 556, 558, 566, 570, 571, 572, 576, 578, 581, 589, 598, 603

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CAPÍTULO 4

MOLECULAR CHARACTERIZATION OF THE VIRAL STRUCTURAL PROTEIN GENES OF CANINE MORBILLIVIRUS

Este capítulo está em fase de preparação e será submetido para publicação de forma modificada numa revista científica. Costa VGd, Saivish MV, Krüger RH, Moreli ML: **Molecular characterization of the viral structural protein genes of canine morbillivirus**.

4. MOLECULAR CHARACTERIZATION OF THE VIRAL STRUCTURAL PROTEIN GENES OF CANINE MORBILLIVIRUS

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4.1 Abstract

To investigate the molecular epidemiology and genetic diversity of canine distemper virus (CDV), a total of 141 blood samples/nasal swab from domestic dogs were screened. CDV RNA was detected in 48 (34%) clinical specimens. We generate the PCR products for full-length F, H and M genes sequencing of four samples previously CDV RNA positive. Sequences of the structural protein genes showed that the South America-I/Europe genotype was in circulation in the Midwest region of Brazil. Interestingly, phylogenetic analysis of CDV structural proteins reveals unique molecular signatures of the local isolates. In sum, this report constitutes the first study of the molecular characterization of structural protein genes of CDV found in domestic dogs of the present region, and it contributes with valuable information regarding the CDV molecular epidemiology.

4.2 Introduction

The canine morbillivirus, formerly known as canine distemper virus (CDV), belonging to the *Paramyxoviridae* family, is found worldwide infecting domestic dogs and wild carnivores [1,2]. Viral infection can cause disease (canine distemper, CD) of complex clinical signs, which is highly contagious and have a high death rate. [3,4]. Therefore, CD represents an important animal public health problem, mainly because of its high incidence in the poorest regions and also because it is considered to be emerging in several other species of carnivores [1-4].

CDV is an enveloped particle, containing non-segmented, negative-sense single-stranded RNA virus [5,6]. Its genome consists of six genes: hemagglutinin (H) and fusion protein (F), which are two glycoproteins that are inserted on the surface of the viral particle, having a key role in the adsorption and fusion, respectively, of the virion to host cell; the space between the envelope and the nucleocapsid is filled by the matrix protein (M), which is important for viral maturation. For nucleoprotein (N), it stands out that it encapsulates the genome and, thus, has the function of protecting the genetic material; proteins L (large) and P (phosphoprotein) are involved in the transcription and replication of viral RNA [7]. Of these proteins, CDV H and F glycoproteins are interesting targets for the study of genetic diversity because they are highly variable among circulating viral

strains [8,9]. In addition, in recent times, CDV M protein has attracted attention because of its important contribution to infectivity and virulence of wild-type CDV [10].

CDV infections have been a cause of concern in many parts of the world, it has increasingly become known as a worldwide multi-host pathogen [3,11-14]. Therefore, in Brazil it is no different, but it has been observed that Brazilian regions are still highly endemic areas for viral infection [15]. However, little is known regarding the genetic variability of the strain circulating in several Brazilian regions. Here, we generate the PCR products for full-length F, H and M genes sequencing from blood samples/nasal swab of domestic dogs (*Canis familiaris*), which had their samples collected in a city located in the Central region of Brazil.

4.3 Materials and Methods

4.3.1 Ethics statement

All sample collection and processing of biological samples was performed following the protocol approved by the animal ethics committee of Goias Federal University (Protocol Number: 054/17).

4.3.2 Clinical case, RNA isolation and RT-PCR for diagnosis of CDV

Samples from domestic dogs showing clinical signs suggestive of CD (respiratory manifestations, hyperkeratosis, neurological signs, systemic troubles or combination of them) were collected between 2017 and 2019. The collection sites were the Veterinary Hospital of the UFG and the Control Center of Zoonoses of the municipality of Jataí, located in the Center-West region of Brazil.

The blood samples were collected in tubes (BD Vacutainer PPT 13x100 mm, 5 ml) and nasal specimens were collected with flocked swabs placed into 1 ml universal transport medium (UTM (Copan, Brescia, Italy)) for the purpose of molecular diagnostic testing, the plasma and UTM were separated and used for the detection of viral RNA.

We extracted viral RNA from 140 µl of blood sample, or nasal swabs, using a QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Reverse transcription was performed at 42 °C for 60 min using the random primers (Promega, inc). For detection of the CDV infection, we used the forward primer and the reverse primer for amplification of the nucleoprotein (N) [16,17] (Table 1). Amplification reactions were carried out under the following conditions: 94 °C for 5 min; followed by 35 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 90 s; and then 72

°C for 10 min. For nested PCR, the amplification reactions were carried out under the following conditions: 94 °C for 3 min; followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 60 s; and then 72 °C for 5 min. After the addition of the possible amplicons in the 1.5% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen; Carlsbad, USA), the amplification product was analyzed under ultraviolet light. The molecular identity of the PCR product of expected size (287 bp) was confirmed by DNA sequencing (ACTGene Análises Moleculares Ltda., RS, Brazil).

4.3.3 PCR for detection of CDV F, H and M genes

A protocol for the amplification of the F, H and M genes was used for the subsequent purpose of DNA sequencing and phylogenetic analysis. Only CDV N positive samples were used for this part. For this purpose, ten primer pairs were designed based on the conserved regions of CDV (**Table 1**). The primers were designed using Geneious Primer (2020.2)[20] to cover the complete genes sequence of all CDV strains.

Table 1. Oligonucleotides used in the PCRs assays for CDV M, F, and H genes characterization.

Primer	Nucleotide sequence (5'-3')	Position	bp[1]	Gene(s)
CDV 1F	ACATTAACCCAGAGCTCCGC	2975-2994	969	P/M
CDV 1R	TCGTCTGATAGTCGAGTGATGC	3943-3922		
CDV 2F	CGAACTGCAGGTGTCAAGGA	3737-3756	991	M
CDV 2R	AGGCGAAGTTCAAAACCCCA	4727-4708		
CDV 3F	CAGCGATGATCAGGGTCTTT	4415-4434	1000	F
CDV 3R	GATCTTATAATGGACACTGTCAGTC	5414-5390		
CDV 4F	GAGWCCRRDACCTCCCRRGS	5019-5038	786	F
CDV 4R	RACYCCCTGAACRGCAATGA	5804-5785		
CDV 5F	CGTTTTGCAGGAGTGGTRC	5604-5622	800	F
CDV 5R	TTGTTGCCCATMGTYCCAGAY	6403-6383		
CDV 6F	YGTCTCAGARTCAGCMATTTG	6278-6298	800	F/H
CDV 6R	GYTGGACTACYTGAGCCCTA	7077-7058		
CDV 7F	CTGGTCACACGTCTTACCCG	6939-6958	989	F/H
CDV 7R	GGCTTTGGAATTCTCCGGGA	7927-7908		
CDV 8F	TYGGCAGCAAAHCCYATCM	7571-7589	798	H
CDV 8R	MACYGGACCRTATGTAAAYGAK	8368-8347		
CDV 9F	TGACCGCTATCTCAGACGGA	7746-7765	980	H
CDV 9R	AGAAATCGTCCGGATTGGGT	8725-8706		
CDV 10F	YTGTCTRGAGTCDGCTTGTC	8206-8225	1424	H/L
CDV 10R	TGATTCACCTYTYACAAAGACAGGR	9629-9605		

The RT reaction was carried out in a volume of 20 µl. eight point five microliters of RNA and 1.5 µl of Random primer (0.75 µg) were heated for 5 min at 70 °C and chilled on ice (5 min). Ten microliters of 1.7 µl mix dNTP (2.5 mM), 2,5 µl of DTT (100 mM), 4 µl of 5×buffer, 1 µl of RNase inhibitor (20 units/µl) and 1.5 µl of RT (240 units) were

added. The mixture was incubated for 5 min at 25 °C. Again, the mixture was incubated for 90 min at 42 ° C. The RT enzyme inactivation step was for 15 min at 70 °C.

PCR was conducted in a reaction volume of 50 µl containing 10 µM forward primer, 10 µM reverse primer, 4 µ mix dNTPs (2.5 mM), 5 µl 5x PCR buffer (Invitrogen), 0.4 µl Taq DNA polymerase High Fidelity (2 U) (Invitrogem) and 8 µl of template DNA. The initial denaturation at 94 °C for 2 min was followed by 50 cycles at 94 °C for 15 s, 58 °C for 30 s and 68 °C for 75 s. Taq DNA polymerase High Fidelity was used to amplify the genome in 10 overlapping fragments. Thus, the expected positive PCR products of 969 bp (pair 1), 991 bp (pair 2), 1,000 bp (pair 3), 786 bp (pair 4), 800 bp (pair 5), 800 bp (pair 6), 989 bp (pair 7), 798 bp (pair 8), 980 bp (pair 9), and 1424 (pair 10) were visualized on either 1-1.2% agarose gels. DNA from a previously confirmed CDV- positive sample was used as a positive control.

4.3.4 Sequencing of structural genes

PCR products with the expected size were selected for sequencing. Thus, amplicons were then purified with the QIAquick PCR purification kit according to the manufacturer's protocol. Purified amplicons were sequenced bidirectionally with the same primers using an ABI3.500 genetic analyzer (Applied Biosystems).

4.3.5 Phylogenetic analysis and molecular characterization

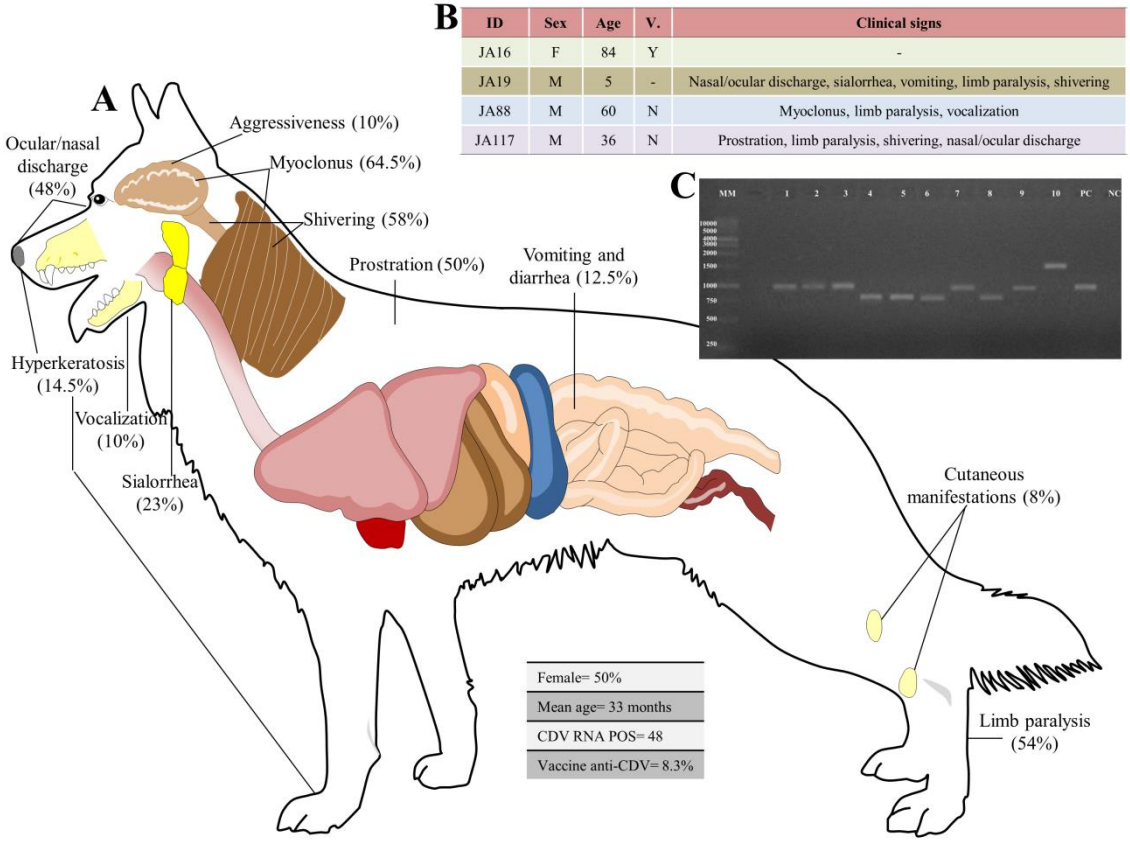
Phylogenetic analysis was performed using the nucleotide/amino acid sequence, as well as the sequences of 24 reference strains for which full genome sequences were available in GenBank and ViPR. Sequences were edited and aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program in the Geneious software package. A phylogenetic tree was constructed, based on the open reading frame (ORF) sequences of CDV, using the neighbor-joining method in the Geneious software package. Bootstrap analysis was carried out on 10,000 replicate data sets.

4.4. Results

4.4.1 Clinical features and detection of N gene

In order to determine the genetic variability of dog CDV in Jataí county, for a total of 141 dog samples collected, there was positive RT-PCR results for CDV RNA in 48 (34%, 48/141). In **Fig 1**, the clinical signs for 48 CDV positive RT-PCR samples are outlined below. However, out of this total of positive samples, only in four it was

possible to amplify the F, H and M genes. This set of samples is our main target in this study.



ID	Sex	Age	V.	Clinical signs
JA16	F	84	Y	-
JA19	M	5	-	Nasal/ocular discharge, sialorrhea, vomiting, limb paralysis, shivering
JA88	M	60	N	Myoclonus, limb paralysis, vocalization
JA117	M	36	N	Prostration, limb paralysis, shivering, nasal/ocular discharge

Fig 1. Clinical signs and general data for positive CDV samples. **(A)** Schematic image containing the frequency of clinical signs for the 48 positive samples from CD. **(B)** Data referring to the four clinical specimens that had the M, F, and H genes sequenced. **(C)** Agarose gel electrophoresis from a representative clinical specimen containing the primers used to amplify the M, F and H genes. Lane M = DNA ladder (250-10000 bp). Lane 1: PCR products of 969 bp (pair 1). Lane 2: Amplicon of 991 bp (pair 2). Lane 3: Amplicon of 1,000 bp (pair 3). Lane 4: Amplicon of 786 bp (pair 4). Lane 5: Amplicon of 800 bp (pair 5). Lane 6: Amplicon of 800 bp (pair 6). Lane 7: Amplicon of 989 bp (pair 7). Lane 8: Amplicon of 798 bp (pair 8). Lane 9: Amplicon of 980 bp (pair 9). Lane 10: Amplicon of 1424 bp (pair 10). Lane PC: positive control (amplified CDV H gene= 1000 bp). Lane NC: negative control (distilled water).

4.4.2 Sequence analysis of the structural protein genes

Phylogenetic trees of the structural protein genes (F/H/M) were plotted to analyze the genetic diversity and relationships among our four clinical specimens as a comparison with reference strains. The phylogenetic tree of the Brazilian CDV subjected to RT-PCR and nucleotide sequencing in this study is shown in **Fig 2**. Phylogenetic analysis showed that the Brazilian CDV belonged to one phylogenetic group (SA-I/EU) during 2018–2019.In addition, sequence comparison of CDV genes with DNASTAR software showed that three gene segments of the four clinical specimens shared 98.2–99.5% nucleotide identity. BLAST analysis was conducted on each sequence to identify related reference

viruses available in GenBank. The F, H, and M genes were found to be the most closely related to South America-I/Europe (SA-I/EU), with nucleotide identities ranging from 96.9 to 97.5%.

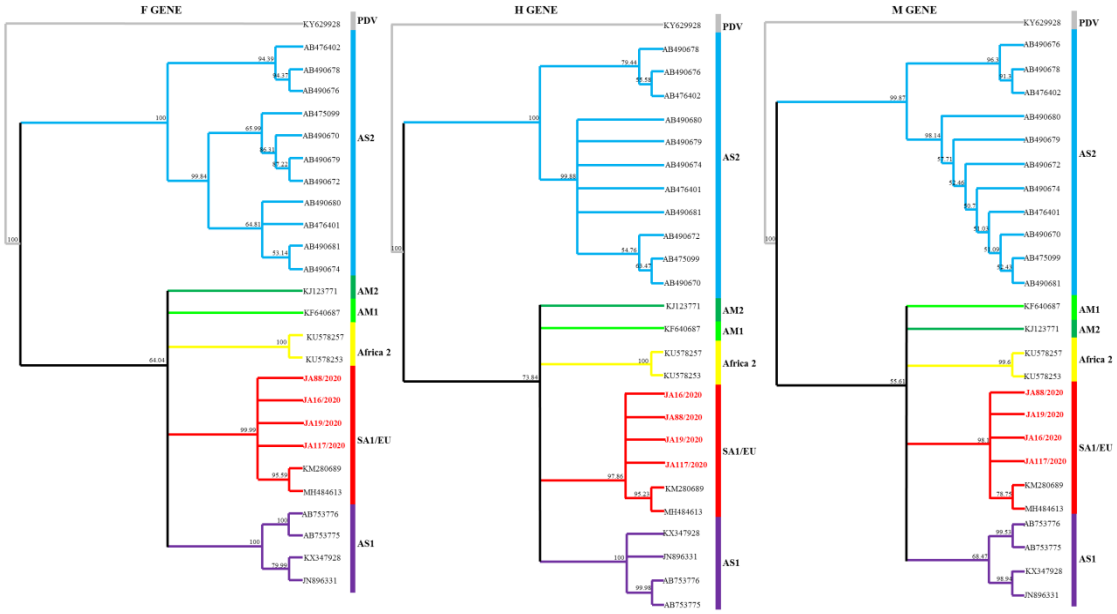


Fig 2. Phylogenetic relationships between CDV strains based on M, F, and H gene sequences. Bootstrap values are shown at each node of the tree. The phylogenetic tree was constructed by the neighbor-joining method using 10,000 bootstrap replicates. The four clinical specimens of this study were indicated in red color.

Amino acid changes for each gene segment of the four clinical specimens were compared to the closest CDV (Reference sequence [Uy251]). Regarding the JA88 sample, in F, H, and M, 14, 20, and 4 amino acid changes were found. For JA16 sample, in F, H, and M, 14, 22, and 2 amino acid changes were found. For JA19, in F, H, and M, 9, 19, and 2 amino acid changes were found. Finally, for the JA117 sample, in F, H, and M, 13, 18, and 2 amino acid changes were found (**Fig 3-4**).

Genotypes were defined as clusters of viruses with ≥95% sequence convergence. In contrast, <98% of amino acid sequence convergence can be classified as a new subgenotype of the CDV. This classification has been based mainly on the amino acid differences of F and H proteins. Interestingly, the sequencing of F and H proteins of the four clinical specimens has more than 2% amino acid difference, indicating that it is a CDV subgenotype.

Fig 3. Multiple alignment of the amino acid sequences of CDV F protein.

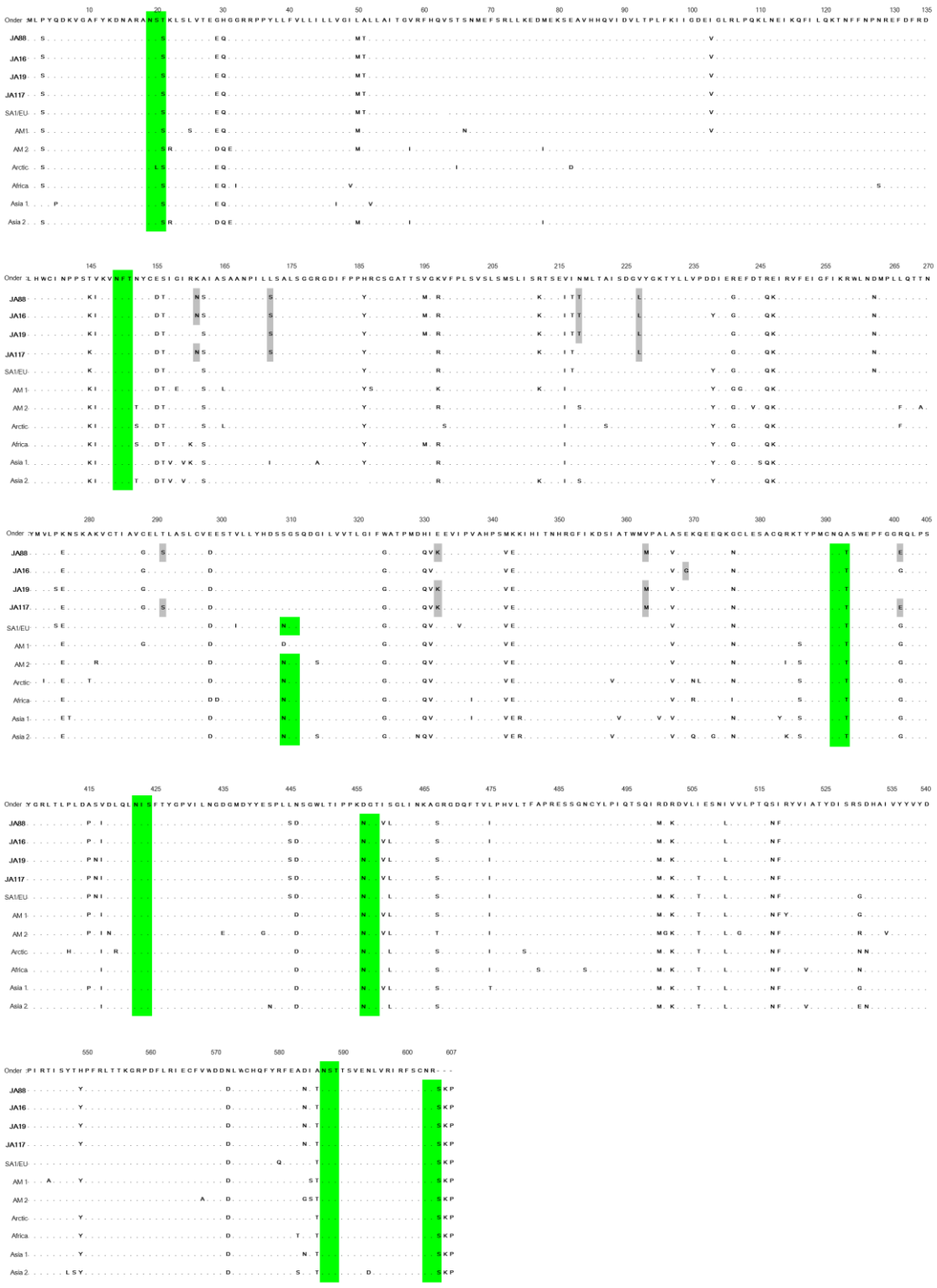


Fig 4. Multiple alignment of the amino acid sequences of CDV H protein.

N glycosylation has been shown to be important for the correct folding, transport, virulence and function of other paramyxovirus fusion and attachment glycoproteins [17-19]. Six potential N-linked glycosylation sites in F (62, 108, 141, 173, 179 and 517) and seven in H (19, 149, 391, 422, 456, 587 and 603) were detected in the strain identified in

this study. The specific polypeptide for N-linked glycosylation is defined as (Asn-X-Ser/Thr), where X can be any amino acid, except proline (P) or aspartic acid (D) [20] (**Fig 3-5**).

4.5. Discussion

Molecular and phylogenetic analyses of full-length F, H, and M genes of four dog samples were successfully recovered. Thus, phylogenetic analysis was performed based on the structural protein genes, which are critical for viral adsorption, penetration, assembly, and release [7,10]. In addition, these genes were compared with reference sequences from all over the world. Curiously, the results showed that all four strains belonged to one genotype: SA-I/EU. However, it is segregated into a CDV subgenotype branch.

Currently, there are at least 17 major CDV genetic genotypes, including America-1 to America-5, Europe Wildlife, Arctic, South Africa, South America-1/Europe, South America-1 to South America-3, Rockborn-like, and Asia-1 to Asia-4 [7,21,22]. The nomenclature of this multiple strains shows that they tend to follow a geographical pattern of distribution [23]. Due to its greater genetic variability, the H and F genes have been the main choices for determining CDV genotypes [8,9,24,25]. Recently, the initial region of the F gene has attracted the attention of molecular epidemiology studies, as this region of interest contains only 135 amino acids, but it is highly variable and may be a less difficult target to amplify than the complete H gene, which is long (1824 nt). We hypothesized that the main factor that may explain the low number of clinical specimens (8.3%, 4/48) that had their structural genes amplified, the following stand out: in this RNA region the transcription level is proportionately lower in comparison to the genes located in the 3´ terminal region of the genome [26-30]. Also due to the complexity of this region in the formation of RNA hairpin may have hindered access to primers.

The unique molecular signatures of the CDV M/F and H genes were identified through visual inspection from amino acid positions (F: S71G, R105Q, K208R, L386I, V612I, D644E, H: K161N, L172S, N218T, V227L, E332K, V362M). The analysis of polymorphisms featuring unique molecular signatures has been carried out by Fischer; with the observation of a unique molecular pattern in a viral lineage from Rio Grande do Sul. Further research will be important to ascertain whether these unique molecular signature patterns are stable and whether they are involved in the gain or not of some

characteristic of the virus, such as an increase or reduced virulence in these strains (Fischer et al., 2016).

The high frequency of mutations, due to the high rate of mistakes of the RNA-dependent RNA polymerase, as well as frequent recombination are considered the main mechanisms of CDV evolution, leading to the emergence of novel strains, subtypes and genotypes within species [21,31]. Molecular epidemiology surveillance of CDV is very important because cases of vaccine failure have occurred. One of the causes for this may be antigenic differences between the vaccine strains and the currently circulating wild-type strains [32]. In this case, large amino acid differences have been observed between Onderstepoort vaccine strain and wild strains. Exemplifying this concern, we can mention that most of the neutralizing epitopes are found on the H protein, which has been shown to be highly variable in the constitution of its amino acids according to the genotypes [22]. Therefore, drastic changes in this region in a wild-type CDV can compromise the efficient binding of neutralizing antibodies generated by the vaccine.

South America stands out for its high genetic diversity and incidence of the virus, even though studies on the characterization of the CDV have been carried out in only 40% of the countries in the region [15]. A similar scenario is observed in Brazil, as only a few molecular characterization studies have been carried out, with the predominance of the SA-I/EU genotype has been seen, however these studies have been concentrated in a small portion of the country [9,33,34]. Thus, it is possible that future large-scale epidemiological studies describe novel CDV strains, genotypes, circulating in South America, such as Duque's recent research, which has proposed the circulation of a novel intercontinental CDV genotype in Colombia, called "South America/North America-4" genotype.

4.6 Conclusion

In conclusion, in the present study, the detection and molecular analysis of structural protein genes (F/H/M) of four wild-type CDV are reported. Interestingly, phylogenetic analysis of structural protein genes showed that the four detected CDV RNA form a distinct branch within the cluster corresponding to the SA-I/EU. This work is expected to stimulate further large-scale epidemiological studies and enhance our understanding of the genetic characteristics and evolution of CDV.

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CAPÍTULO 5

MOLECULAR DYNAMICS ANALYSIS OF CANINE MORBILLIVIRUS M AND N PROTEINS AND INSIGHTS TO RATIONAL DRUG DESIGN

Este capítulo está em fase de preparação para submissão à revista científica. Costa VGd, Saivish MV, Menezes GL, Silva, RA, Krüger RH, Moreli ML: **Molecular Dynamics Analysis of Canine morbillivirus M and N proteins and insights to rational drug design**.

5. Molecular Dynamics Analysis of Canine morbillivirus M and N proteins and insights to rational drug design

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5.1 Abstract

Canine morbillivirus (canine distemper virus [CDV]) infection is still a major global challenge for carnivorous animals with huge mortality and morbidity. There are no antiviral therapy available for the treatment of CDV infection. Therefore, a study aimed at antivirals against the disease known as distemper is important to reduce its mortality and reduce the spread of CDV. For this purpose, we produced high quality models of M and N viral proteins, which play a key role in the viral replication cycle. With the M and N proteins molecular dynamics simulation, pockets containing amino acids were found to be key for interaction with potential antivirals. The results showed that the 241-IEKMGL-246 and 183-TAPDTAADSEMRR-195 regions of the M and N proteins, respectively, are promising pockets for the search of inhibitory molecules. In sum, the data generated here contribute to the search in banks of molecules and their rational screening in order to obtain antivirals against CDV.

5.2 Introduction

Canine distemper virus (CDV) infection is still a major global challenge for carnivorous animals with huge mortality and morbidity. The transmission of the pathogen occurs through direct contact, or from oral/nasal fluids, and aerosolized respiratory fluids in an appropriate environment, where the infected animal has recently been [1-4]. Since the CDV belongs to the group of *paramyxoviruses*, in domestic dogs infection by the pathogen is highly contagious and causes a disease with respiratory manifestations, hyperkeratosis, neurological signs, systemic troubles or combination of them [5-7]. The treatment consists of supportive care and antibiotics to prevent secondary bacterial infections. There are no antiviral therapy available for the treatment of CDV infection [8].

CDV belongs to the genus *Morbillivirus*, has a lipid envelope that contains non segmented single-stranded RNA genome of negative sense, which encodes for two surface glycoproteins (hemagglutinin [H] and the fusion protein [F]), the viral nucleocapsid protein (N), the phosphoprotein (P), the matrix protein (M), the and the large RNA-dependent RNA polymerase (L). V and C are non-structural accessory

proteins. M and N proteins are interesting targets for antivirals, due to their key role in the replication cycle of paramyxoviruses. The search for drugs that are associated with key amino acid residues of N protein can destabilize the formation of the ribonucleoprotein complex, which is formed by polymers of N protein covering viral RNA and associating with viral polymerase (L protein). Ribonucleoprotein participates in the synthesis of viral RNA and is the structural nucleus of the viral particle. The M protein has the function of orchestrating the assembly and release activity of the viral particles from plasma membrane of the host cell [9,10]. In this study, we introduced *in silico* strategy for insights to rational drug design. Models of M and N proteins were generated by molecular dynamics simulation, following the analysis of key amino acid residues for the search of inhibitors. With this data it will be possible to screen compounds for drugs in a database and subsequent *in vitro* testing [11].

5.3 Methodology

5.3.1 Molecular modeling and molecular dynamics simulation

CDV M and N proteins were first modeled using I-TASSER server after submission of amino acid sequence retrieved from GenBank (AIL92333; AIL92331.1) [12]. The obtained structure was then submitted to Molprobity server for model validation [13]. Specific protonation of histidine residues at pH 7.4 was predicted by H++ server [14].

GROMACS 5.1.2 software was used to execute molecular dynamics simulations and evaluate protein stability in water solution. The protein model and ion were modeled with AMBER ff99SB-ILDN force field and the water molecules with TIP3P type. This system was inserted in a cubic periodic box with a minimum distance equal to 12 Å away from any protein atom. This box was solvated with water molecules and neutralized with chloride ions. Hydrogen atoms related to solute covalent bonds were constrained by the LINCS algorithm [15], while to maintain rigid internal structure of the water molecules, SETTLE algorithm was used [16].

The system was submitted to initial energy minimizations with steepest descent algorithm with complete protein restriction and without any restriction until the maximum tolerance of 250 kJ/mol was not exceeded, or until reaching the limit of 5000 steps. A constant force of 1000 kJ/mol•nm^2 was applied for positional restraints on all heavy atoms when protein had its position restrained. In the non-bonded interactions, a

cutoff was applied at 1.0 nm, and to calculate the long-range electrostatic interactions the particle mesh Ewald method was applied.

The system was subjected to a 100 ps simulation of NVT and NPT to balance the thermodynamic variables, with the protein restricted in its positions, the temperature was adjusted to 310 K. In the step of pressure variation (NVT), the temperature adjustment was performed with Berendsen thermostat algorithm [17], with a relaxation constant of 0.1 ps, and the initial atomic velocities were generated from Maxwell-Boltzmann distribution equations [18]. In the simulation, where the volume can vary (NPT), the pressure was kept constant by the Parrinello-Rahman barostat algorithm and leap-frog algorithm was used to integrate motion equations in these previous and follow steps [19].

After these initial steps, protein structure was submitted to production run in NPT ensemble at 310 K for 200 ns, 2 fs of time step, one bar pressure and without any protein restriction.

The Root Mean Square (RMSD) profile was calculated between trajectory frame and first trajectory frame as reference. This is a manner to measure spatial variation o between two structures. In order to evaluate residue fluctuation around an average position to determine its flexibility, Root Mean Square Fluctuation (RMFS) was performed.

The g_cluster (GROMACS) program was used to determine the conformations that were most found along the trajectory. In this case, all structures with RMSD values less than the determined cut-off for any element in a cluster were added to the primary cluster. The conformations were grouped considering all backbone atoms of the protein based on the RMSD profile with cut-off of 0.25 nm. The algorithm described by Daura et al. [20] was used from the gromos option chosen as a method of determining the clusters.

To visualize protein behavior throughout the simulation, UCSF Chimera and Visual Molecular Dynamics (VMD) visualization programs were used [19,21].

5.3.2 Mapping druggable pockets in relevant M and N monomer MD Structures

The prediction of the general ability of a target protein to be inhibited by low molecular weight compounds is called druggability [22]. The determination of protein pockets with potential for druggability was carried out through the DoGSiteScorer server (https://proteinsplus.zbh.uni-hamburg.de/#dogsite).

5.4 Results

5.4.1 M and N molecular dynamic simulation

For M protein, initially model predicted by I-TASSER server had 77.8% of all residues in favored regions and 92.2% in allowed regions according to Ramachandran plot (Suppl. **Fig. S1**). After protein refinement in ModRefiner server [31] these values increase to 91.9% and 98.5% for favored and allowed regions, respectively which suggests a model quality enhancement.

For N protein, model predicted by I-TASSER server had 74.7% of all residues in favored regions and 86.9% in allowed regions according to Ramachandran plot (Suppl. **Fig. S2**). After protein refinement in ModRefiner server [31] these values increase to 86.6% and 95.7% for favored and allowed regions.

After model validation, M protein was submitted to 200 ns MD simulation. In the first 35 ns of simulation, it was observed an increasing of RMSD values along the trajectory what illustrates conformational adjustment (Suppl. **Fig. S3**). The 200 ns MD simulation analyses indicated protein stability, especially after 90 ns, when values ranged from 0.55 to 0.65 nm. When we analyze per residue stability through RMSF values, some flexible regions are observed specially in highly solvent exposed loop regions. The RMSD per residue per time shows two regions (N-terminal regions and residues 200-210) are unstable along all trajectories. On the other hand, ß-sheet and alfa-helices structures have lower RMSF values. Through the cluster analysis, settled in a 0.25 nm cut-off, eleven conformational groups were observed, and cluster 1 was the one that remained for most time in simulation. The differences between clusters occurred at loop segments flexibility and no major changes were observed among structures. Also, the Ramachandran plot for cluster 1 showed 93.4% of all residues were in favored regions and 99.4 % were in allowed regions indicating a conformational improvement through MD.

After model validation, N protein was submitted to 100 ns MD simulation (Suppl. **Fig. S3**). The N protein, which initially presented an increase in the RMSD value as a result of the conformational adjustment of the protein, reaching stability in approximately 50 ns of simulation in a range close to 0.6 nm of RMSD. In the heatmap, where the RMSD per residue is analyzed over the simulation time, it is possible to observe that these three regions remain unstable throughout the entire simulation, unlike the first region. All of them, with the exception of the 378-410 region, which is composed mainly of alpha-helix, are formed by a loop with high exposure to the solvent. In the Suppl. **Fig. S3 (4D)** graph of the representative structure of cluster # 1. There is an improvement in

the quality of the model, reflected by the number of residues in favorable regions that went from 86.7% to 89.0% and in permitted regions that went from 95.7% to 98.1%. The results indicate a high quality model of CDV N protein obtained in the MD where improvement in the spatial conformation of the residues was observed.

5.4.2 Mapping druggable pockets

In the analysis of cavities with potential for pocket druggability performed by the DoGSiteScorer server (https://proteinsplus.zbh.uni-hamburg.de/#dogsite) a site involving key residues was found in M protein: 241-IEKMGL-246 (**Fig 1**). This site was classified as the second best in the analysis, with a volume of 1332.86 Å3 and an area of 1585.89 Å2. The value of drugscore, a metric used by the server that takes into account the shape, size and hydrophobicity of the cavity, was 0.81, on a scale ranging from 0 to 1, the closer to 1, the greater chance of being a site for anchoring molecules.

In protein N cavity analysis, the second best cavity according to the DoGSiteScorer server also had key residues: 183-TAPDTAADSEMRR-195. This site has 1145.36 Å3 in volume and 1700.11 Å2 in area. As with M protein, 0.81 of drugscore was also observed, both of which are cavities of CDV proteins considered to be promising for a possible search for inhibitors.

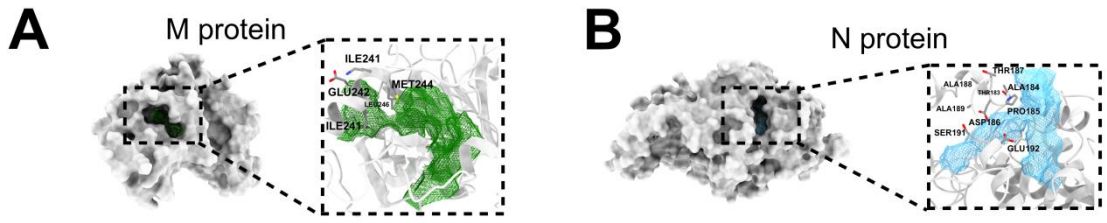


Fig 1. Pocket analyses of 200 ns (M protein) and 100 ns (N protein) monomer simulation. Representation analysis of CDV M pocket showing its surface in green mesh and related residues in sticks. (B) Representation analysis of CDV N pocket showing its surface in blue mesh and related residues in sticks.

5.5 Discussion

In the present study, high quality models of CDV M and N proteins were generated by molecular dynamics simulations. Subsequently, interesting pockets were researched and their characteristics of druggability were estimated. For M protein, the residues considered key were 85-ARPEEL-90, 133-SVFSAN-138 and 241-IEKMGL-246. Only 241-IEKMGL-246 was presented in the form of an excellent pocket, since it had a

drogscore index close to 1 (0.81). For protein N, amino acid residues considered key were 183-TAPDTAADSEMRR-195 and 345-NSMGGLNFGRS-355. Only 183-TAPDTAADSEMRR-195 was presented in the form of an excellent pocket, since it had a drogscore index close to 1 (0.81).

The amino acids considered key to M protein are regions that can interfere with the formation of dimers [23,24]. A binding molecule in this region can inhibit the formation of dimers and protomers, which would inhibit the assembly and release of the viral particle. Recently, Gast and collaborators observed that the process of oligomerization and release of the viral particle from the cell is controlled by two microdomains: 87-PEELLKEATLL-97 and 236-YCKLKIEKM-244. Interestingly, our analysis encompassed a considerable part of these amino acids. Gast and collaborators draw attention to these microdomains and highlights that they constitute novel attractive targets for drug design [25].

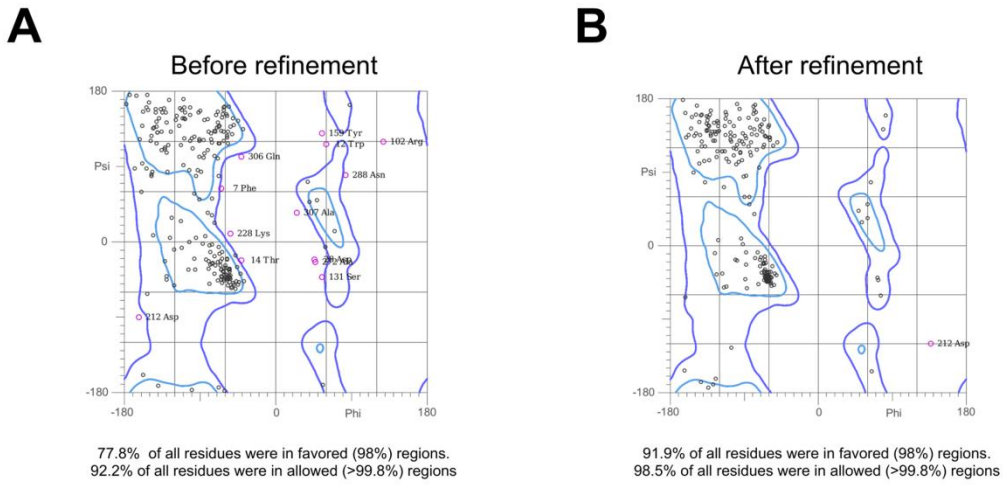
Regarding to the key amino acids of N protein, it were considered so because there are regions close to the N protein-RNA interaction [26-28]. In other words, there is a possibility that ligands in this region compromise the ribonucleoprotein complex. However, future studies will be necessary to better characterize this region of the ribonucleoprotein complex.

The computational methods have been used for the rational development of drugs [29]. In this sense, we seek to define whether key regions of the CDV proteins are prone to ligands molecules because they present regions of pockets that facilitate this interaction. Therefore, this study will continue on the path of screening molecules in the database with the potential to be an antiviral. Later on, the *in silico* data will be compared with *in vitro* antiviral tests. The search for antivirals is a long one, but it is necessary in view of the severity that the disease causes in several species of animals.

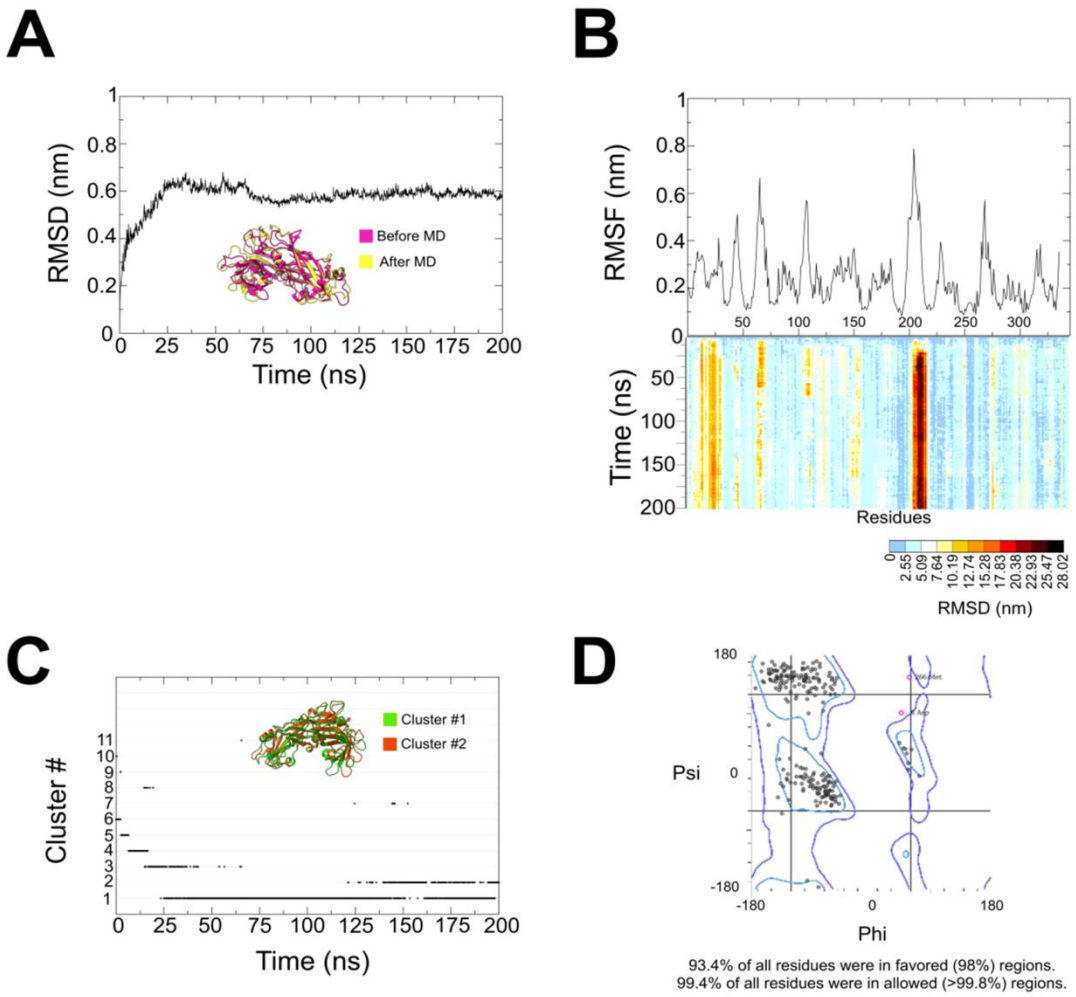
5.6 Conclusion

In sum, high quality models of CDV M and N proteins were generated by molecular dynamics simulations. By analyzing pockets containing key amino acids, it was observed that the 241-IEKMGL-246 and 183-TAPDTAADSEMRR-195 regions of the M and N proteins, respectively, are promising pockets for the search of inhibitory molecules.

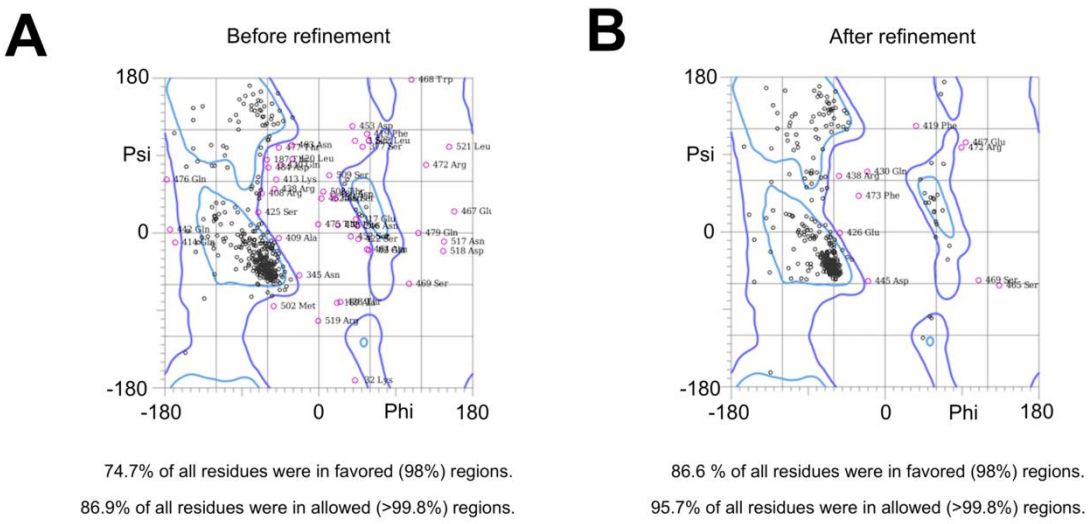
5.7 Supplementary data



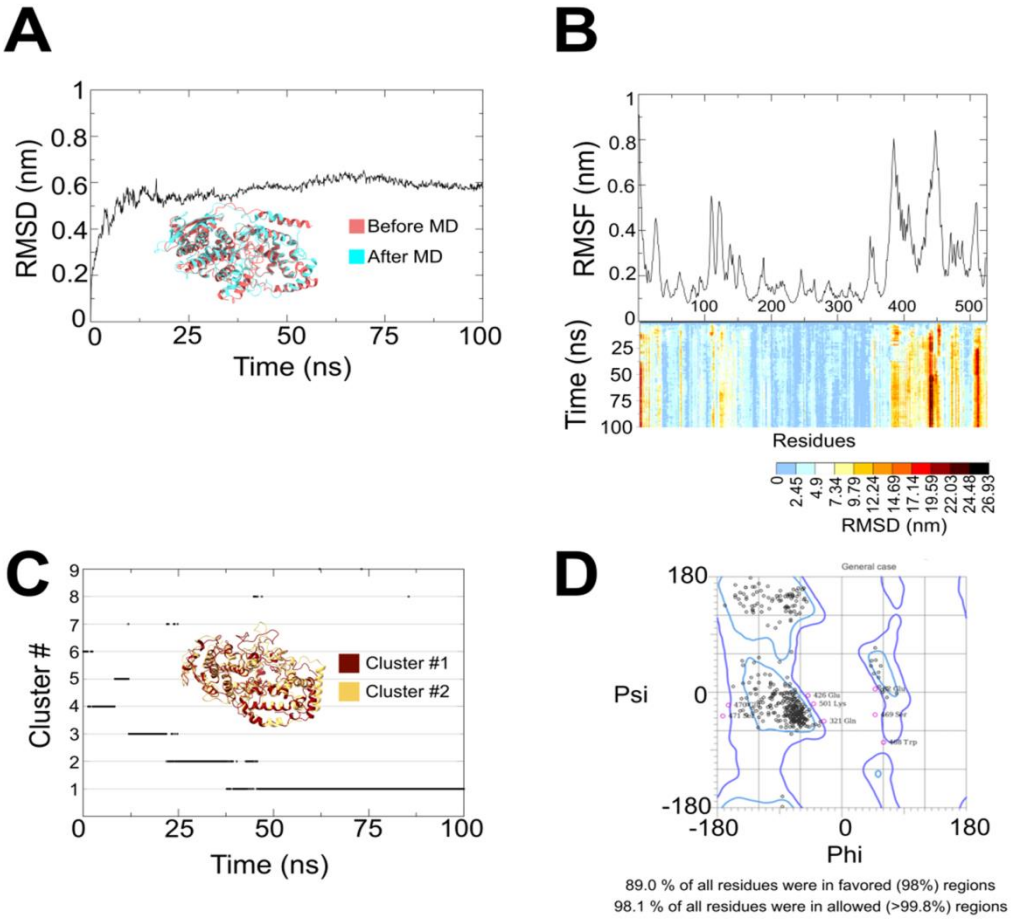
Supplementary Fig. 1 – Ramachandran plot of M protein model. (A) Ramachandran plot of I-TASSER M protein output model showing 77.8% of residues in favored (light blue line delimiter) regions and 99.2 % of residues in allowed (dark blue line delimiter) regions. (B) Ramachandran plot of C protein after refinement in ModRefiner server. An increase to 91.9% of residues in favored regions and 98.5% of residues in allowed regions were observed.



Supplementary Fig. 2 – Results of molecular dynamics simulation. (A) RMSD graphic of M protein showing structure stability after ~ 90 ns of simulation. Structures (represented in pink and yellow cartoons) before and after simulation are shown inserted in the graphic. (B) RMSF graphic showing some unstable regions. These regions are mainly high solvent exposed loop structures. Below, the heatmap of RMSD per residue per time shows two regions (N-terminal regions and residues 200 - 210) are unstable along all trajectory. (C) Results from the cluster analyses of protein trajectories obtained during the simulation. A cut-off point of 0.25 nm was selected to include the major structures during the simulations and eleven conformation groups were obtained. The graph shows the stabilization of cluster #1 after ~25 ns and cluster #2 appeared after ~137 ns. These two clusters oscillated between them at the end of simulation. Both main clusters (1 and 2) obtained during the simulation are shown in the inserted figure (green and orange cartoons). It can be noticed the main differences are in loop segments (D) Ramachandran plot after MD simulation describing an increase in residues in favored regions (93.4%) and in allowed regions (99.4 %).



Supplementary Fig. 3 – Ramachandran plot of N protein model. (A) Ramachandran plot of I-TASSER N protein output model showing 74.7% of residues in favored (light blue line delimiter) regions and 86.9 % of residues in allowed (dark blue line delimiter) regions. (B) Ramachandran plot of N protein after refinement in ModRefiner server. An increase to 86.6% of residues in favored regions and 95.7% of residues in allowed regions were observed.



Supplementary Fig. 4 – Results of molecular dynamics simulation. (A) RMSD graphic of N protein showing structure stability after ~ 50 ns of simulation. Structures (represented in pink and yellow cartoons) before and after simulation are shown inserted in the graphic. (B) RMSF graphic showing some unstable regions. These regions are mainly high solvent exposed loop structures. Below, the heatmap of RMSD per residue per time shows four regions (120-125, 378-410, 434-454 and 505-511) are unstable along all trajectory. (C) Results from the cluster analyses of protein trajectories obtained during the simulation. A cut-off point of 0.3 nm was selected to include the major structures during the simulations and nine conformation groups were obtained. The graph shows the stabilization of cluster #1 after ~25 ns and cluster #2 appeared after ~50 ns. (D) Ramachandran plot after MD simulation describing an increase in residues in favored regions 89%) and in allowed regions (98%).

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CAPÍTULO 6

EXPRESSION, PURIFICATION AND CHARACTERIZATION OF THE CANINE MORBILLIVIRUS M PROTEIN FROM *ESCHERICHIA COLI*

Este capítulo está em fase de preparação e será submetido de forma modificada numa revista científica. Costa VGd, Saivish MV, Menezes GL, Rodrigues RL, Silva, RA, Krüger RH, Moreli ML: **Expression, purification and characterization of the Canine morbillivirus M protein from *Escherichia coli***.

6. EXPRESSION, PURIFICATION AND CHARACTERIZATION OF THE CANINE MORBILLIVIRUS M PROTEIN FROM *ESCHERICHIA COLI*

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6.1 Abstract

Canine morbillivirus (canine distemper virus, CDV) is one of the highly pathogenic carnivore viruses. In the current study, the CDV M protein (CDV M_p) was expressed in *Escherichia coli* as histidine-tagged protein. The purified CDV M_p was tested for their reactivity with sera from CDV in naturally infected domestic dogs by immunoblot and ELISA tests. Panels of reference sera from dogs with and without CD were used to detect CDV-specific IgG in an indirect ELISA using the CDV M_p as coating antigen. A High degree of agreement was observed between ELISA and serodiagnostic techniques results for clearly reactive and non-reactive sera. On this basis, our data indicate that CDV M_p and antibodies to them may be an alternative and cheap source of diagnostic reagents for the development of serological assays for CDV. Additionally, here we report the molecular dynamics of this viral protein with regard to new insights into the structure and antigenic/immunogenic nature.

6.2 Introduction

Canine distemper (CD) is a highly contagious disease with high lethality rate that occurs worldwide, caused by *Canine morbillivirus* (previously named canine distemper virus [CDV]) [1-3]. Particularly, domestic dogs (*Canis familiaris*) are the animals most affected by CD, as the disease mainly affects young dogs, causing respiratory, gastrointestinal and neurological complications [1,4]. CDV is acquired via aerosols or by direct contact of susceptible animals with the various fresh body secretions of infected animals [5].

CDV belongs to the genus *Morbillivirus* in the *Paramyxoviridae* family, which is enveloped with single-stranded, negative sense, and non-segmented RNA genetic material. Regarding the viral genome, it encodes six structural proteins, termed hemagglutinin (H), fusion (F), nucleocapsid (N), phospho (P), large (L), and matrix (M), and two accessory non-structural proteins (C and V). The P, C, and V proteins play a role in RNA synthesis, aid in the transition from primary transcription to replication of the viral genome, and potentially influence messenger RNA (mRNA) synthesis, respectively. The surface glycoproteins H and F play a key role in adsorption and fusion, respectively, from the virion to the host cell. The N protein stands out because it encapsulates the genome and protects the genetic material. The L and P proteins are involved in viral RNA

transcription and replication. Finally, the M protein fills the space between the envelope and the ribonucleoprotein, thus contributing to viral morphology and the packaging and budding process in the host cell membrane [6,7].

In view of the broad clinical spectrum of CD, the use of laboratory tests that allow the correct diagnosis of the causative pathogen is extremely important for the choice of clinical management and preventive measures [8]. During an acute CDV infection there is induction of a virus-specific antibody response. Therefore, the detection of antibodies titers against CDV proteins can be obtained by using the complement fixation, precipitation, cytotoxic and indirect immunofluorescence tests, however, not suitable for routine diagnostics in veterinary laboratories because considerable time is spent on exams, in addition to high financial cost [9]. In contrast, enzyme-linked immunosorbent assay (ELISA) has proved to be an interesting detection technique for routine diagnostic [9-11].

ELISA tests are a convenient method for confirmation of clinical cases, infection surveillance, and evaluation of the immune status post-vaccination in dogs. Here we focus on expressing a protein and testing it in immunodiagnosis, thus allowing the specific detection of IgG antibodies anti-CDV. The reason for choosing the CDV M_p (protein) was based on the research by Dietzel and collaborators [12], who noted that the CDV M_p influences the infectivity of the viral particle and modulates the virulence of the wild-type CDV. In addition, the CDV M_p is abundant among the structural proteins of CDV. It is also noteworthy that the nucleotide sequence of the M gene is highly conserved among different CDV strains [6,7,13,14]. Therefore, the CDV M_p may be used as a candidate diagnostic reagent for determining CDV infection. Additionally, we have also shed light on the knowledge of the 3-D structure of CDV M_p and shown if it has antigenic/immunogenic regions exposed through molecular dynamics with bioinformatics tools.

6.3 Materials and methods

6.3.1 Expression of recombinant protein

With the nucleotide sequence defined, the respective synthetic gene (GenBank: AIL92333), containing a polyhistidine-tag (6xHis), was inserted into the vector (pET30_a) by GenOne Biotechnology (Rio de Janeiro, Brazil). Thus, the recombinant plasmid (pET30(a)_CDV M_p was transformed into competent *E. coli* BL21 (DE3) (Sigma-Aldrich, St. Louis, MO) for protein expression. For certification that the gene of

interest was into the transformed expression vectors, M gene was amplified from the cDNA clone (*E. Coli* BL21_CDV M_p) using the following set of primers developed by us: 5'-TGCGGCCGCTTAGAGAATTT-3' (forward primer); 5'-AGGGGAATTGTGAGCGGATAA-3' (reverse primer) [15,16].

Expression of the CDV M_p was performed using selected clones containing pET30(a)_CDV M_p. In order to select the optimal expression conditions, different induction times (0, 2, 4, 6 and 8 hours [h]) at 37 °C, under the induction of 1 mM IPTG, were tested. Finally, at 37 °C, 1 mM IPTG induced for 6 h was determined as an effective expression condition. Therefore, the bacteria suspension was inoculated into 200 mL Luria Bertani (LB) medium supplemented with kanamycin (60 μg/mL) and incubated at 37 °C and 200 rpm until OD_{600} reaches 0.8. IPTG was added to a final concentration of 1 mM, and the cultures were further grown for 8h at 37°C. The cell pellets were collected by centrifugation at 5000×g for 30 minutes, and the expression of the recombinant protein was evaluated by 10% glycine SDS-PAGE, and Western blot analysis.

6.3.2 Purification of CDV M_p

The harvested cell pellet containing recombinant CDV M_p (200 mL culture medium) was resuspended in ice-cold binding buffer/lysis buffer [17]. Subsequently, the cell suspension was sonicated in 10 repeating cycles of 20 seconds with a 45 s cooling intervals between cycles (Branson Sonifier 250 [Branson Ultrasonic Corp., Danbury, CT]). The suspension was centrifuged at 12000×g for 20 minutes to obtain the supernatant of the cell lysate, which is filtered by 0.45 μm (polyethersulfone membrane). The bound his-tagged recombinant protein was purified using Ni-NTA chromatography (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. All protein purification steps were performed at 4 °C. Thus, the recombinant CDV M_p was recovered from the column and the eluted fractions were used for further 10% SDS-PAGE evaluation, followed by Coomassie brilliant blue R-250 staining. The Bicinchoninic Acid (BCA) protein Assay Kit was used to determine the protein concentration. The concentration is calculated by plotting the absorbance data of the sample against a standard curve of Bovine Serum Albumin (BSA) [18].

6.3.3 Western blot

For western blot analysis, the protein samples from the 10% SDS-PAGE, were transferred to nitrocellulose membrane (0.2 μm [Bio-Rad Laboratories, Hercules, CA]),

constant current, 360 mA, 75 min (Trans-Blot transfer membrane, Bio-Rad). Regarding the transfer buffer, it contains 3g Tris-base, 14.4g Glycine and 100 mL methanol. After blotting, the proteins were visualized non-specifically by Ponceau S (Sigma-Aldrich). Shortly thereafter, the transferred nitrocellulose membrane was blocked with Tris buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.4), and 0.1% Tween-20 (TBS-T) containing 5% (w/v) nonfat dried milk for 2 h, Dog anti-CDV polyclonal antibody (1:500 dilution) was incubated for 2h at 37 °C; after 4 washing with TBS-T, Horseradish peroxidase (HRP) labeled anti-dog IgG secondary antibody (1:1000 dilution [KPL, Milford, MA, USA]) was incubated at 37 °C for 1 h, then washed 5 times with TBS-T, then added DAB (3,3'-diaminobenzidine) HRP substrate solution (Sigma-Aldrich).

6.3.4 Dog sera

The 28 CDV-positive sera were collected at the Centro de Zoonoses and Veterinary Hospital from Jataí county, Brazil. Positive sera were representative of the following animals: 1) dogs with clinical sign of CD (PCR positive) and a paired blood sample, collected weeks later, and positive anti-CDV IgG (indirect immunofluorescence assay [IFA]) . This standard technique has been described elsewhere [19]; 2) vaccinated against CD with positive IFA results. PCR results were also used in combination with ELISA and IFA results to estimate test accuracy [15,16]. In contrast, sera from six healthy donors served as negative control, which are young dogs (3-6 months) not vaccinated against CD and non-reactive IgG (IFA). In addition, five dogs affected by other viruses (canine parvovirus [CPV]) served as negative control for cross-reactivity testing.

6.3.5 ELISA

Briefly, a 96-well, flat-bottom ELISA microplate (Nunc, Chicago, USA) were coated with 0.5-5 μg of purified CDV M_p per ml in a bicarbonate/carbonate coating buffer (pH 9.6) at 4 °C overnight. Plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T) and blocked with 5% (w/v) nonfat dried milk at 37 °C for 2 h. As diluent solution, PBS with 0.1% Tween 20 and 5% nonfat dried milk was used. After blocking, the plates were washed three times with PBS-T, the diluted sera (1:100) was added to the wells (100 μl/well) and incubated at 37 °C for 1 h. The plates were washed six times and incubated with 100 μl of HRP-conjugated anti-dog IgG (1:1,000) at 37 °C for 1 h. After washing eight times with PBS-T, 50 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) was added to the wells, the plate was incubated for

15 min at 37 °C, and the reaction was stopped by adding 50 µl of 2 M HCl. Optical densities (OD) values were read at 450 nm using an absorbance microplate reader (Thermoplate). Intra-assay reproducibility was evaluated using 3 replicates (on each of 3 plates) of 34 serum samples. In the panel of samples to be screened, the cut off value was determined by the mean OD of the negative sera ± 3 standard deviations (SD). Finally, the optimal conditions were obtained by dilutions that gave the maximum difference in absorbance at 450 nm between positive and negative serum (P/N) of the samples.

6.3.6 Model building and structural analysis

6.3.6.1 Molecular modeling and molecular dynamics simulation

CDV M_p was first modeled using I-TASSER server after submission of amino acid sequence retrieved from GenBank (AIL92333) [20]. The obtained structure was then submitted to Molprobity server for model validation [21]. Specific protonation of histidine residues at pH 7.4 was predicted by H++ server [22].

GROMACS 5.1.2 software was used to execute molecular dynamics simulations and evaluate protein stability in water solution. The protein model and ion were modeled with AMBER ff99SB-ILDN force field and the water molecules with TIP3P type. This system was inserted in a cubic periodic box with a minimum distance equal to 12 Å away from any protein atom. This box was solvated with water molecules and neutralized with chloride ions. Hydrogen atoms related to solute covalent bonds were constrained by the LINCS algorithm [23], while to maintain rigid internal structure of the water molecules, SETTLE algorithm was used [24].

The system was submitted to initial energy minimizations with steepest descent algorithm with complete protein restriction and without any restriction until the maximum tolerance of 250 kJ/mol was not exceeded, or until reaching the limit of 5000 steps. A constant force of 1000 kJ/mol•nm^2 was applied for positional restraints on all heavy atoms when protein had its position restrained. In the non-bonded interactions, a cutoff was applied at 1.0 nm, and to calculate the long-range electrostatic interactions the particle mesh Ewald method was applied.

The system was subjected to a 100 ps simulation of NVT and NPT to balance the thermodynamic variables, with the protein restricted in its positions, the temperature was adjusted to 310 K. In the step of pressure variation (NVT), the temperature adjustment was performed with Berendsen thermostat algorithm [25], with a relaxation constant of 0.1 ps, and the initial atomic velocities were generated from Maxwell-Boltzmann

distribution equations [26]. In the simulation, where the volume can vary (NPT), the pressure was kept constant by the Parrinello-Rahman barostat algorithm and leap-frog algorithm was used to integrate motion equations in these previous and follow steps [27].

After these initial steps, protein structure was submitted to production run in NPT ensemble at 310 K for 200 ns, 2 fs of time step, one bar pressure and without any protein restriction.

The Root Mean Square (RMSD) profile was calculated between trajectory frame and first trajectory frame as reference. This is a manner to measure spatial variation o between two structures. In order to evaluate residue fluctuation around an average position to determine its flexibility, Root Mean Square Fluctuation (RMFS) was performed.

The g_cluster (GROMACS) program was used to determine the conformations that were most found along the trajectory. In this case, all structures with RMSD values less than the determined cut-off for any element in a cluster were added to the primary cluster. The conformations were grouped considering all backbone atoms of the protein based on the RMSD profile with cut-off of 0.25 nm. The algorithm described by Daura et al. [28] was used from the gromos option chosen as a method of determining the clusters.

To visualize protein behavior throughout the simulation, UCSF Chimera and Visual Molecular Dynamics (VMD) visualization programs were used [27,29].

6.3.6.2 Screening antigenic/immunogenic amino acids in CDV M_p model

In order to show that CDV M_p is antigenic/immunogenic and the location of these sites in the generated protein model, DNAStar software was used to predict the candidate sequences [30].

6.4. Results

6.4.1 Expression and purification of CDV M_p

In RT-PCR (reverse transcription-polymerase chain reaction) protocol **[15,16]**, the PCR product corresponding to the expected size (1110 base pairs), in the transformed clones, appeared in the 1.2% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen; Carlsbad, USA) (Supplementary data: **Fig. S1**). This is indicated that pET30(a)_CDV M_p was successfully delivered into these protein expression competent cells. In contrast, bacterial cells transformed with the vector without the gene showed no amplicon in the agarose gel.

The recombinant CDV M_p was expressed in an insoluble form and purified by affinity chromatography. Thus, BL21 cells transformed with recombinant CDV M_p expressed CDV M_p after a 6 h incubation period. As seen in **Fig. 1A**, a protein of about 38 kDa was detected in protein extracts from *E. coli* cultures transformed with CDV M DNA. Thus, the CDV M_p was purified through Ni-NTA columns and Western blots were performed using purified protein. A total of two dog sera were tested for IgG antibody against CDV M_p by Western blot assay. **Fig. 1B** illustrates the detection of anti-CDV M_p IgG with recombinant CDV M_p by Western analysis.

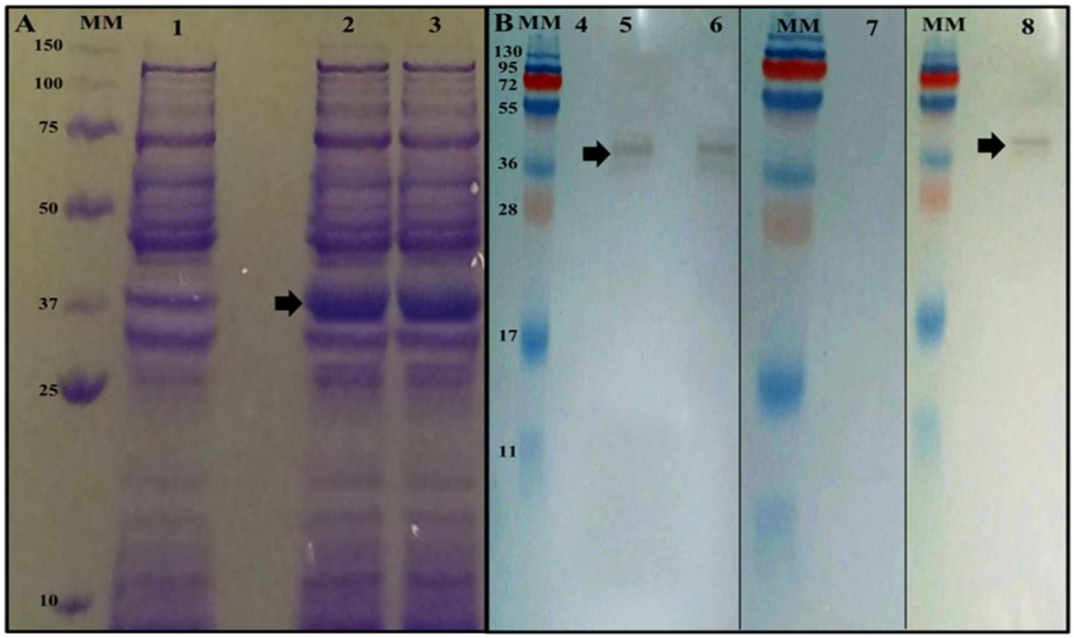

Fig 1. Expression of CDV M_p in *E. coli* and immunodection. The M_p expression was followed every hour using SDS-PAGE (**a**). Lane MM: protein molecular weight marker, Lane 1: Uninduced *E. coli*, Lanes 2, 3: Lysates of transformed bacteria at 6 and 8 h post-IPTG induction, respectively. Purification analysis of CDV M_p by Western blot analysis (**b**). The immunoblotting was performed using serum known to be anti-CDV IgG as primary antibody. The Western blot results are shown in lanes 5, 6, and 2, Lanes 4: represent purified *E. coli* lysate without the M gene in pET30, Lane 7: young dog serum known not to be infected with CDV. Arrows indicate the position of the M_p with a molecular weight of approx. 38 kDa.

6.4.2 IFA and ELISA CDV M_p

Detection of appropriate anti-species IgG was done by placing an appropriate sample, containing virus-infected cells on glass slides. At this moment, it deserves to be highlighted that VerodogSLAM cells were used for the cultivation of the virus. Soon, the IFA was used as the reference method to detect the presence of anti-CDV IgG. Thus, a total of 28 and 6 dog sera were positive and negative anti-CDV IgG, respectively (**Fig. S2**).

In the checkerboard ELISAs CDV M_p, the optimal concentration of CDV M antigen and the dilution for serum samples were set at 2 µg/ml and 1:100, respectively. This was based on the standard that the OD450 value of positive serum was highest than negative reference serum with the lowest background. Posteriorly, a total of 39 dog sera were reacted against the CDV M_p in an indirect ELISA. Reference negative sera, which were confirmed to be free of CDV antibodies by an IFA, were used to determine a cut-off and to establish a level of nonspecific background. Consequently, the cut off value was 0.155, soon the samples with mean OD more than 0.155 were considered positive, and those with mean OD less than or equal to 0.155 were considered negative (**Fig. 2**). The sensitivity of the ELISA CDV M_p was 85.7 (95% confidence interval [CI]: 68.5 to 94.3). Regarding to true negative rate, the specificity was 90.9% (95% CI: 62.2 to 98.3) while accuracy was 87% when compared to the reference IFA.

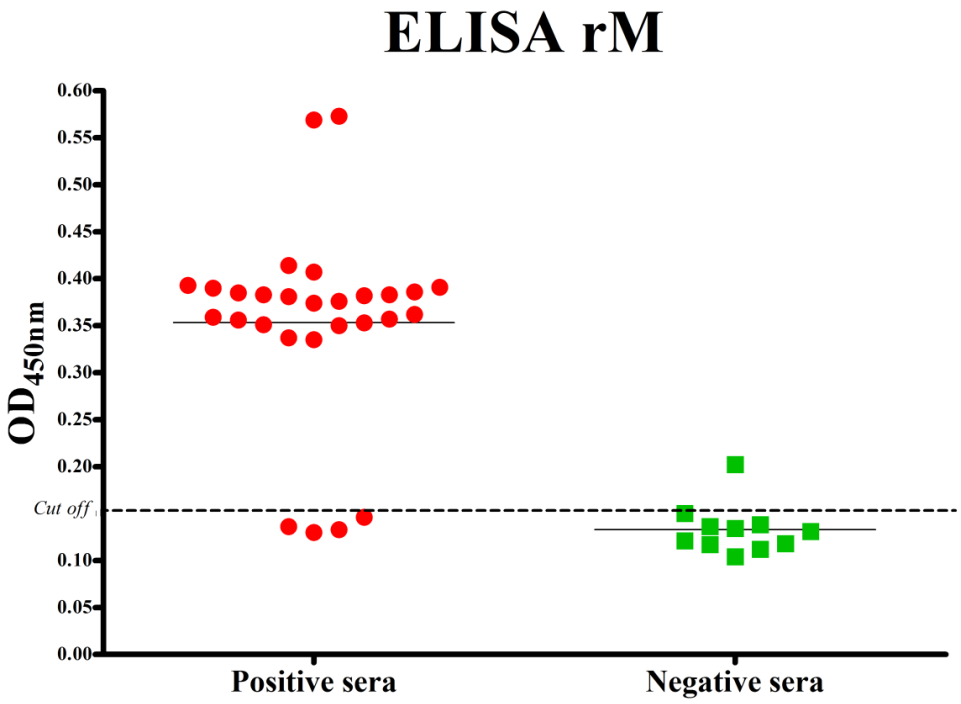

Fig 2. ELISA result of IgG anti-CDV indicating the serum antibody concentration in positive and negative sera. The cut off value was 0.155. Horizontal bars show median values.

Regarding the inter-assay (between-run) reproducibility, three replicates of each sample were run in different plates. In summary, reproducibility was considered good because the coefficient of variation (CV) was between ~5% and 18%. For more details see **Table S1**.

6.4.3 Structural modeling and antigenicity analysis of CDV M_p

Initially, model predicted by I-TASSER server had 77.8% of all residues in favored regions and 92.2% in allowed regions according to Ramachandran plot (Suppl. **Fig. S3**). After protein refinement in ModRefiner server [31] these values increased to 91.9% and 98.5% for favored and allowed regions, respectively, which suggests a model quality enhancement.

After model validation, M protein was submitted to 200 ns MD simulation. In the first 35 ns of simulation, it was observed an increasing of RMSD values along the trajectory what illustrates conformational adjustment (Suppl. **Fig. S4**). The 200 ns MD simulation analyses indicated protein stability, especially after 90 ns, when values ranged from 0.55 to 0.65 nm. When we analyze per residue stability through RMSF values, some flexible regions are observed specially in highly solvent exposed loop regions. The RMSD per residue per time shows two regions (N-terminal regions and residues 200-210) are unstable along all trajectories. On the other hand, ß-sheet and alfa-helices structures have lower RMSF values.

Through the cluster analysis, settled in a 0.25 nm cut-off, eleven conformational groups were observed, and cluster 1 was the one that remained for most time in simulation. The differences between clusters occurred at loop segments flexibility and no major changes were observed among structures. Also, the Ramachandran plot for cluster 1 showed 93.4% of all residues were in favored regions and 99.4 % were in allowed regions indicating a conformational improvement through MD.

Regarding the location in the protein model generated from potential antigenic amino acid and epitopes regions, it can be seen in **Fig. 3**, **4** and **5**. For more details on the locations of these amino acids, see **Tables S2**, **S3** and **S4**.

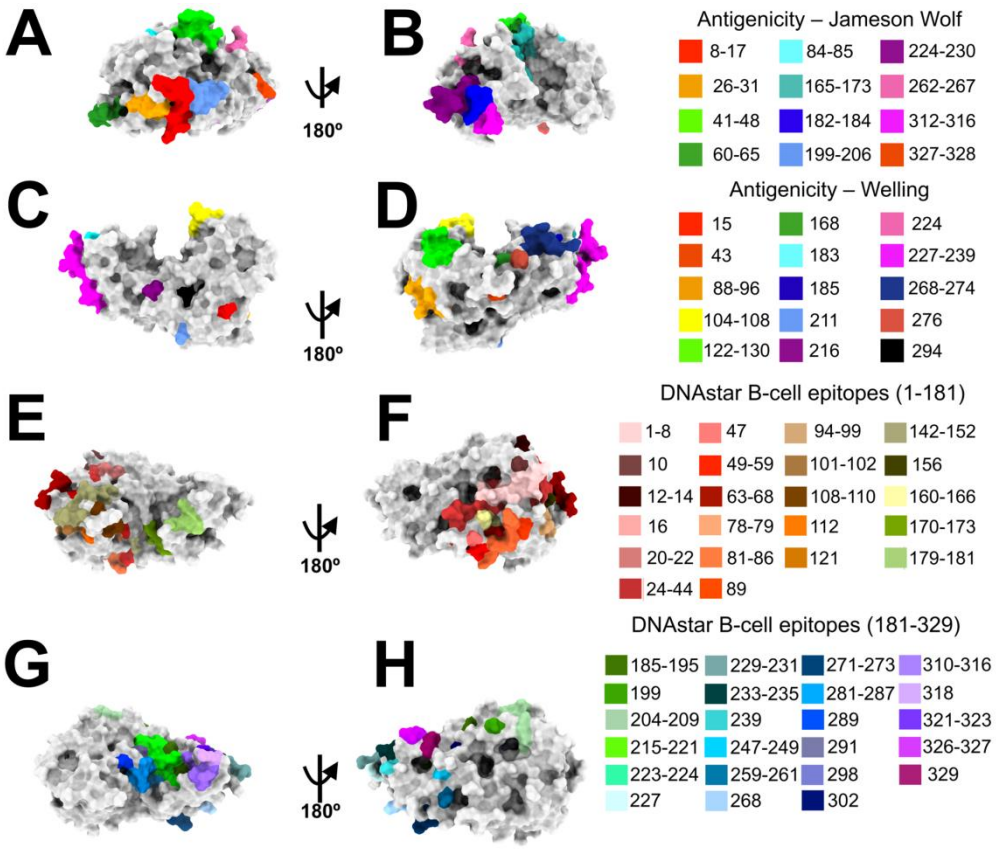

Fig 3. CDV M_p structure obtained from Molecular dynamics simulations showing the location of possible antigenic and epitopes sites.

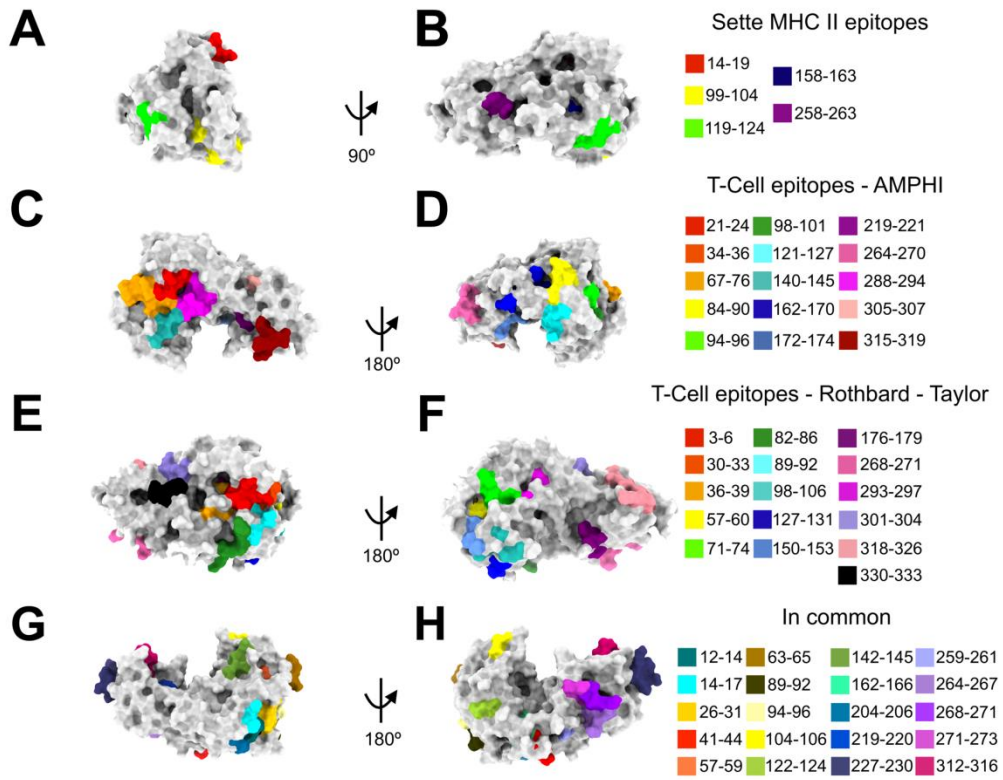

Fig 4. CDV M_p structure obtained from Molecular dynamics simulations showing the location of possible antigenic and epitopes sites. "In Common"= represents amino acids that overlapped by different types of analyzes (for more details see Tables S2-S4).

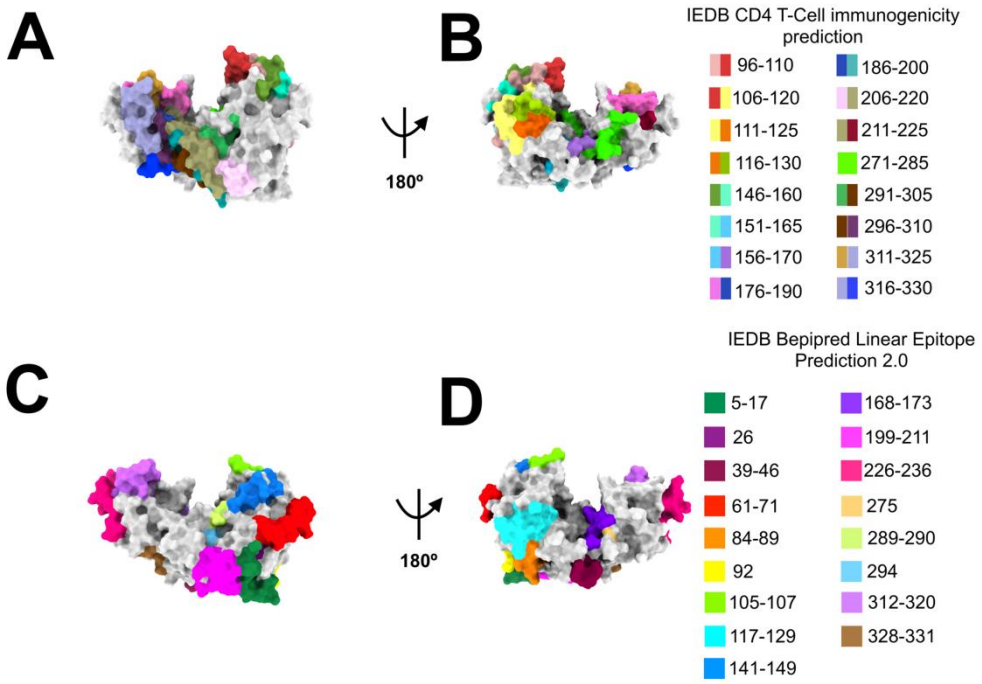

Fig 5. CDV M_p structure obtained from Molecular dynamics simulations showing the location of possible antigenic and epitopes sites.

6.5 Discussion

Initially, in our experiments the CDV M_p accumulated in insoluble fraction. To solve this problem we used a protocol adapted for the expression and purification of insoluble proteins in *E. coli* [17]. However, even then soluble recombinant CDV M_p was recovered in low yields (100 µg/1 L culture) when expressed in *E. coli*. As is known from other studies, the formation of insoluble aggregates within inclusion bodies can be difficult to purify in high yield [32,33]. An alternative to improve yields would be the insertion of the chaperone gene associated with the plasmid vector promoter. In the future, this strategy should be tested as chaperones can contribute to stabilize and reduce the interaction between protein aggregates [34].

The choice to express CDV M_p, and test its ability to interact with specific detection of IgG antibodies, was based on the study by Dietzel and colleagues, who noted: CDV M_p influences the infectivity and modulates the virulence of the viral particle. In other words, the authors observed the extreme importance of M_p in this process, since a recombinant wild-type CDV was modified with the vaccine M gene. Surprisingly, recombinant viral particles inoculated in ferrets caused only mild illness. It is also noteworthy that the authors observed the inefficient H protein incorporation on the surface of the viral particle [12]. This factor shows the involvement of CDV M_p, since this protein can affect the membrane integrity [35,36]. In summary, these data show the importance of further studies involving this viral protein to understand its molecular mechanisms.

Still in relation to the choice of studying serology anti-CDV M_p, we emphasize that it has been neglected in this regard, in view of the absence of studies. On the other hand, some researchers have analyzed the production of antibodies against other proteins of the CDV: N, P, F and H [37]. However, a more established use for serological diagnosis was concentrated on N protein [9,10]. Consequently, it is important to analyze and scale the role of these proteins in the sensitive and specific detection of anti-CDV antibodies. Furthermore, the expression and purification of CDV proteomics allows characterization of the function, structure and interactions of proteins of interest [38].

Serologic diagnosis of CDV can be achieved by serum-virus neutralization test and IFA; however these methods are not ideal for large-scale routine testing. In contrast, ELISA is a rapid, sensitive, and inexpensive test for the detection of antibodies. The IgG ELISA presented here adds a new perspective to serodiagnosis of CDV infections in dog. The high sensitivity (85%) and specificity (90%) of the IgG ELISA are supported by

results obtained in similar IgG capture assay as in Messling's study, which found sensitivity of 93% and specificity of 83%; however the assay was based on a recombinant CDV N protein [10]. Another difference is that the panel of samples tested was superior to ours. Further investigations are needed to evaluate a number of larger samples and to evaluate for how long it is possible to detect IgG antibodies in serum after vaccination, our CD.

The number of PCR positive samples with second collection was reduced. Therefore, the analysis of the accuracy of the IFA with PCR was not possible. Most of the CDV positive samples refer to dogs that received booster doses of the distemper vaccine. It is known that IFA can generate false positive results, so further analysis testing the protein with the microneutralization test will be important, as this is the gold standard in detecting anti-CDV antibodies.

The CDV M_p could be purified only denaturing protocols. Thus, future studies will be needed to assess whether protein remained immunogenic. Another need to be done will be to test its cross-reactivity with samples known to be positive for other viruses, including canine coronavirus, canine adenovirus, canine parainfluenza virus and CPV.

Finally, the model of the generated CDV M_p and the potential epitopes or antigenic determinants allow collaborate with our thesis by showing that the protein is an interesting target for studies involving diagnosis or immunogens. In this regard, it is expected that the study will encourage new research, especially those that address its direct application in the field of diagnosis and vaccines against the virus.

6.6 Conclusion

In conclusion, the ELISA rM established in the present study showed that the CDV M_p was only able to react specifically with the antisera anti-CDV not with antisera from CPV, and healthy animals. Subsequently, in a correlation experiment, the ELISA rM indicated high identity to the IFA in the total number positive and negative for antibodies to CDV. Additionally, we obtained a 3D model of the protein and exposed potential antigenic and epitopes sites.

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6.8 Supplementary data

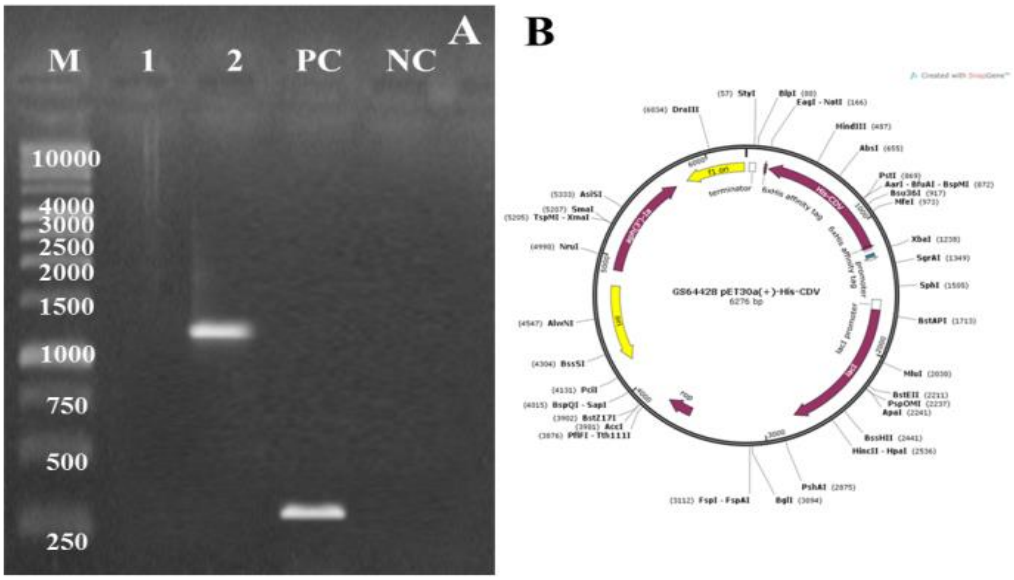

Fig. S1. Agarose gel (1%) electrophoresis of PCR products amplified. Lane M = DNA ladder (250-10000 bp). Lane 1: recombinant plasmid, without the gene M, purified from transformed *E. coli*. Lane 2: Amplicon of the M gene (1110 bp) obtained from E. coli containing the recombinant vector. Lane PC: positive control (amplified CDV N gene= 287 bp). Lane NC: negative control (distilled water). B) The synthetic gene was cloned into NotI/NdeI digested pET30a(+). The certification that the plasmid contained the gene of interest in the correct position was confirmed by DNA sequencing

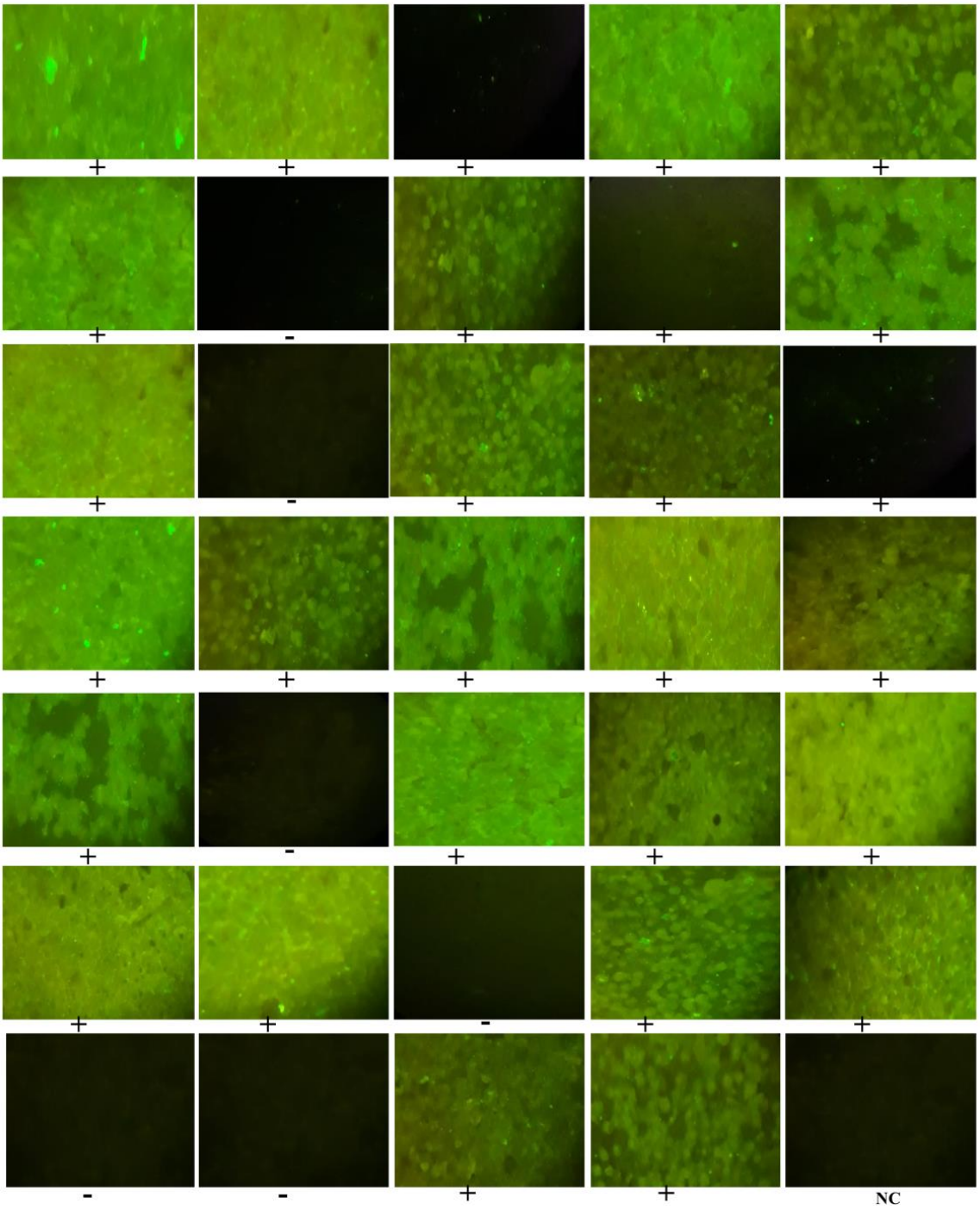

Fig. S2. IFA results using sera from dogs. (+)= Positive anti-CDV IgG; (-)= Negative anti-CDV IgG. NC= Negative control (VerodogSLAM cell not infected with wild-type CDV) (x200).

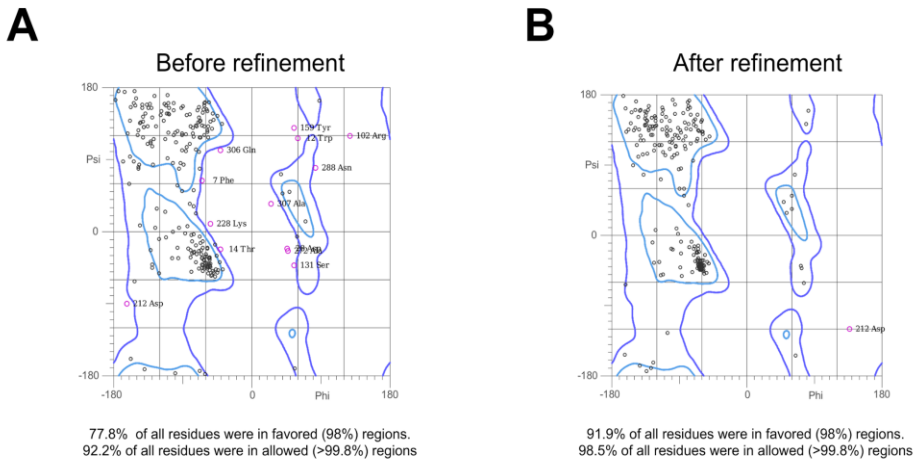

Fig S3. Ramachandran plot of C protein model. (A) Ramachandran plot of I-TASSER C protein output model showing 77.8% of residues in favored (light blue line delimiter) regions and 99.2 % of residues in allowed (dark blue line delimiter) regions. (B) Ramachandran plot of C protein after refinement in ModRefiner server. An increase to 91.9% of residues in favored regions and 98.5% of residues in allowed regions were observed.

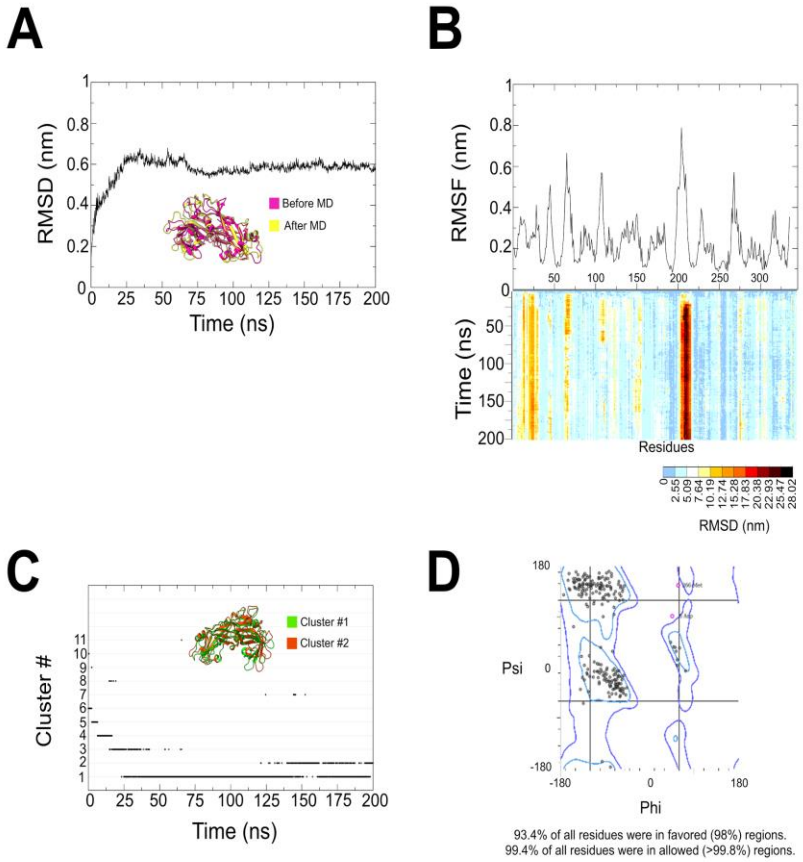

Fig S4. Results of molecular dynamics simulation. (A) RMSD graphic of C protein showing structure stability after ~ 90 ns of simulation. Structures (represented in pink and yellow cartoons) before and after simulation are shown inserted in the graphic. (B) RMSF graphic showing some unstable regions. These regions are mainly high solvent

exposed loop structures. Below, the heatmap of RMSD per residue per time shows two regions (N-terminal regions and residues 200 - 210) are unstable along all trajectory. (C) Results from the cluster analyses of protein trajectories obtained during the simulation. A cut-off point of 0.25 nm was selected to include the major structures during the simulations and eleven conformation groups were obtained. The graph shows the stabilization of cluster #1 after ~25 ns and cluster #2 appeared after ~137 ns. These two clusters oscillated between them at the end of simulation. Both main clusters (1 and 2) obtained during the simulation are shown in the inserted figure (green and orange cartoons). It can be noticed the main differences are in loop segments (D) Ramachandran plot after MD simulation describing an increase in residues in favored regions (93.4%) and in allowed regions (99.4 %).

Table S1. The coefficient of variation (CV) of positive and negative sera between runs. For inter-assay (between-run) reproducibility, three replicates of each sample were run in different plates.

Number of antisera	I (OD value_X)	II (OD value_X)	III (OD value_X)	X	SD	CV (%)
1	0,615	0,547	0,543	0,569	0,033	5.8
2	0,370	0,322	0,362	0,351	0,0211	6.01
3	0,127	0,162	0,120	0,136	0,018	13.24
4	0,607	0,531	0,582	0,573	0,031	5.41
5	0,358	0,385	0,406	0,383	0,019	4.96
6	0,44	0,382	0,419	0,414	0,024	5.8
7	0,394	0,413	0,345	0,383	0,028	7.31
8	0,402	0,384	0,335	0,374	0,028	7.49
9	0,375	0,400	0,312	0,362	0,037	10.22
10	0,437	0,338	0,400	0,391	0,041	10.49
11	0,304	0,377	0,390	0,357	0,038	10.64
12	0,338	0,389	0,426	0,385	0,036	9.35
13	0,436	0,385	0,337	0,386	0,041	10.62
14	0,410	0,315	0,444	0,390	0,054	13.85
15	0,316	0,347	0,385	0,350	0,028	8
16	0,384	0,378	0,315	0,359	0,031	8.64
17	0,392	0,320	0,348	0,353	0,029	8.22
18	0,328	0,379	0,422	0,376	0,038	10.11
19	0,370	0,403	0,448	0,407	0,032	7.86
20	0,114	0,154	0,123	0,130	0,017	13.08
21	0,404	0,415	0,325	0,381	0,040	10.5
22	0,166	0,111	0,166	0,148	0,026	17.57
23	0,327	0,351	0,392	0,356	0,027	7.58
24	0,112	0,146	0,140	0,133	0,014	10.53
25	0,346	0,297	0,369	0,337	0,03	8.9
26	0,378	0,350	0,418	0,382	0,028	7.33
27	0,394	0,419	0,368	0,393	0,020	5.09
28	0,305	0,340	0,360	0,335	0,022	6.57
29	0,207	0,217	0,182	0,202	0,014	6.93
30	0,101	0,116	0,139	0,118	0,015	12.71
31	0,111	0,141	0,111	0,121	0,014	11.57
32	0,113	0,133	0,091	0,112	0,016	14.29
33	0,100	0,116	0,0975	0,104	0,008	7.69
34	0,124	0,133	0,164	0,140	0,016	11.43

Table S2. IEDB_CD4 T cell immunogenicity prediction and IEDB_ Bepipred Linear Epitope Prediction 2.0

IEDB_CD4 T cell immunogenicity prediction			IEDB_ Bepipred Linear Epitope Prediction 2.0		
Start	**End**	**Peptide**	**Start**	**End**	**Peptide**
96	110	LLDIVVRRTAGVKEQ	5	17	YDFDESSWDTKGS
106	120	GVKEQLVFYNNTPLH	26	26	Y
111	125	LVFYNNTPLHILTPW	39	46	DPGLGDRK
116	130	NTPLHILTPWKKVLT	61	71	DNDGLGPPIGR
146	160	LIPLDIAQRFRVVYM	84	89	TARPEE
151	165	IAQRFRVVYMSITRL	92	92	K
156	170	RVVYMSITRLSDDGS	105	107	AGV
176	190	GMFEFRSRNALAFNI	117	129	TPLHILTPWKKVL
186	200	LAFNILVTIQVEGDV	141	149	CNAVNLIPL
206	220	NLSMFKDHQVTFMVH	168	173	DGSYRI
211	225	KDHQVTFMVHIGNFS	199	211	DVCSSRGNLSMFK
271	285	NAQLGFKKILCYPLM	226	236	RKKNQAYSADY
291	305	LNRFLWRLECKIVRI	275	275	G
296	310	WRLECKIVRIQAVLQ	289	290	ED
311	325	PSVPQDFRVYNDVII	292	292	N
316	330	DFRVYNDVIISDDQG	294	294	F
			312	320	SVPQDFRVY
			328	331	DQGL

Table S3. DNASTAR - predicted epitopes and antigenic regions

Antigenicity – Jameson Wolf			**Antigenicity – Welling**			**DNAstar B-cell epitopes**		
Start	**End**	**Peptide**	**Start**	**End**	**Peptide**	**Start**	**End**	**Peptides**
8	17	DESSWDTKGS	227	239	KKNQAYSADYCKL	81	81	G
26	31	YPDGRL	268	274	KALNAQL	82	86	RTTAR
41	48	GLGDRKDE	276	276	F	89	89	E
60	65	EDNDGL	294	294	F	94	95	AT
84	85	TA	**DNAstar B-cell epitopes**			96	96	L
165	173	LSDDGSYRI	**Start**	**End**	**Peptide**	97	97	L
182	184	SRN	1	6	MTEVYD	98	98	D
199	206	DVCSSRGN	7	8	FD	99	99	I
224	230	FSRKKNQ	10	10	S	101	101	V
262	267	CTGKMS	12	12	W	102	102	R
312	316	SVPQD	13	14	DT	108	108	K
327	328	DD	16	16	G	109	110	EQ
Antigenicity – Welling			20	22	PIL	112	112	V
Start	**End**	**Peptide**	24	35	TTYPDGRLVPQV	121	121	I
15	15	K	36	36	R	142	142	N
43	43	G	37	40	VIDP	143	144	AV
88	96	EELLKEATL	41	41	G	145	148	NLIP
104	108	TAGVK	42	44	LGD	149	150	LD
122	130	LTPWKKVLT	47	47	D	151	152	IA
168	168	D	49	58	CFMYIFLLGI	156	156	R
183	183	R	59	59	I	160	161	MS
185	185	A	63	65	DGL	162	163	IT
211	211	K	66	67	GP	164	165	RL
216	216	T	68	68	P	166	166	S
224	224	F	78	79	LG	170	173	SYRI

Table S3. DNASTAR - Predicted epitopes and antigenic regions (continued)

Sette MHC II epitopes			**T-cell epitopes – Rothbard – Taylor**			**DNAstar B-cell epitopes**		
Start	**End**	**Peptide**	**Start**	**End**	**Peptide**	**Start**	**End**	**Peptide**
14	19	TKGSLA	30	33	RLVP	216	218	TFM
99	104	IVVRRT	36	39	RVID	219	219	V
119	124	LHILTP	57	60	GIIE	220	221	HI
158	163	VYMSIT	71	74	RTFG	223	224	NF
258	263	LHIRCT	82	86	RTTAR	227	227	K
T-Cell epitope AMPHI			89	92	ELLK			N
Start	**End**	**Peptide**	98	106	DIVVRRTAG			QA
21	24	ILPT	127	131	KVLTS	233	235	SAD
34	36	QVR	150	153	DIAQ	239	239	L
67	76	PPIGRTFGSL	176	179	GMFE	247	249	VFA
84	90	TARPEEL	268	271	KALN	259	261	HIR
94	96	ATL	293	297	RFLWR	268	268	K
98	101	DIVV	301	304	KIVR	271	273	NAQ
121	127	ILTPWKK	318	326	RVYNDVIIS	281	281	C
140	145	VCNAVN	330	333	GLFK	282	287	YPLMEI
162	170	ITRLSDDGS	**DNAstar B-cell epitopes**			289	289	E
172	174	RIP	**Start**	**End**	**Peptide**	291	291	L
219	221	VHI	179	179	E	298	298	L
264	270	GKMSKAL	180	180	F	302	302	I
288	294	NEDLNRF	181	181	R	310	316	QPSVPQD
305	307	IQA	185	185	A	318	318	R
315	319	QDFRV	186	195	LAFNILVTIQ	321	321	N
T-cell epitope – Rothbard – Taylor			199	199	D	322	323	DV
Start	**End**	**Peptide**	204	204	R	326	327	SD
3	6	EVYD	205	206	GN	329	329	Q

Table S4. Overlapping CDV M peptide sequences

DNASTAR			
Start	**End**	**Peptide**	**Methods**
12	14	WDT	Antigenicity – Jameson Wolf x DNAstar B-cell epitopes
14	17	TKGS	Sette MHC II epitopes x Antigenicity – Jameson Wolf
26	31	YPDGRL	DNAstar B-cell epitopes x Antigenicity – Jameson Wolf
41	44	GLGD	Antigenicity – Jameson Wolf x Antigenicity – Welling x DNAstar B-cell epitopes
57	59	GII	T-cell epitopes – Rothbard – Taylor x DNAstar B-cell epitopes
63	65	DGL	Antigenicity – Jameson Wolf x DNAstar B-cell epitopes
89	92	ELLK	Antigenicity – Welling x T-cell epitopes – Rothbard – Taylor
94	96	ATL	Antigenicity – Welling x DNAstar B-cell epitopes x T-Cell epitopes AMPHI
104	106	TAG	Antigenicity – Welling x T-cell epitopes – Rothbard – Taylor
122	124	LTP	Sette MHC II epitopes x Antigenicity – Welling x T-Cell epitopes AMPHI
142	145	NAVN	T-Cell epitopes AMPHI x DNAstar B-cell epitopes
162	166	ITRLS	DNAstar B-cell epitopes x T-Cell epitopes AMPHI
204	206	RGN	DNAstar B-cell epitopes x Antigenicity – Jameson Wolf
219	221	VHI	T-Cell epitopes AMPHI x DNAstar B-cell epitopes
227	230	KKNQ	Antigenicity – Jameson Wolf x Antigenicity – Welling x DNAstar B-cell epitopes
259	261	HIR	Sette MHC II epitopes x DNAstar B-cell epitopes
264	267	GKMS	Antigenicity – Jameson Wolf x T-Cell epitopes AMPHI
268	271	KALN	Antigenicity – Welling x T-cell epitopes – Rothbard – Taylor
271	273	NAQ	DNAstar B-cell epitopes x Antigenicity – Welling
312	316	SVPQD	Antigenicity – Jameson Wolf x DNAstar B-cell epitopes

CAPÍTULO 7

CONCLUSÃO GERAL

Considerando os resultados obtidos, pode-se concluir:

- A frequência de infecção pelo CDV estimada em cães clinicamente suspeitos de cinomose em Jataí, Goiás, foi de 34% (48/141) (Intervalo de Confiança 95%: 27-42), sendo este resultado similar a outros estudos conduzidos em diferentes regiões do Brasil;

- Foi isolado um CDV de campo, o qual teve o seu genoma sequênciado (GenBank: MW460905). Conclui-se que este isolado pertence a um subgenótipo agrupado no genótipo América do Sul-I/Europa;

- Genes das proteínas estruturais (M/F/H) de quatro amostras (4/48) foram sequenciados e também classificados no genótipo América do Sul-I/Europa, demonstrando, assim, que estas amostras apresentam alta homologia aminoacídica entre si, possuindo assinaturas moleculares únicas. Conclui-se da circulação de um único subtipo de CDV em nossa Região no período de 2018-2019;

- Através das simulações de dinâmica molecular das proteínas M e N foram obtidos modelos de alta qualidade. As regiões aminoacídicas 241-IEKMGL-246 e 183-TAPDTAADSEMRR-195 foram consideradas *pockets* promissores para interação com ligantes moleculares. Com isso abre-se a possibilidade de triagem virtual de moléculas que interajam com estas regiões e sejam testadas *in vitro* e *in vivo* quanto ao seu potencial de antiviral.

- A produção da proteína M recombinante reage no teste de *immunoblot* com soro de cães que foram infectados pelo CDV. A proteína M atua adequadamente como antígeno em testes de ELISA para a detecção de anticorpos IgG contra o CDV.

PERSPECTIVAS

Durante este estudo foi produzido um modelo de panfleto (Anexo), o qual possui linguagem didática, e acessível para a população, sobre a importância de se conhecer a cinomose e proteger os cães contra a doença. Espera-se que este modelo seja divulgado e contribua para a prevenção da doença mediante a conscientização do público sobre o que é a doença e quais as medidas de prevenção da cinomose.

Outras perspectivas são apresentadas logo a seguir:
Continuar os estudos de caracterização molecular dos CDVs mais dribuídos na região a fim de detectar possíveis genótipos e subgenótipos circulantes;

Realizar o *docking* molecular das proteínas M e N virais com compostos ligantes dos *pockets* de interesse;

Realizar ensaios *in vitro* (cultivo de células) e *in vivo* (furões) testando os efeitos antivirais das moléculas promissoras obtidas nos ensaios de interação *in silico*;

Analisar a reatividade da proteína M recombinante frente a um painel maior de soros obtidos de cães clinicamente suspeitos de cinomose;

Analisar a reatividade cruzada da proteína comparando-a com um painel sorológico maior de amostras reativas para outros vírus, incluindo os coronavírus, adenovírus e parvovírus canino.

Caracterizar os anticorpos envolvidos na interação com a proteína recombinante;

Produzir outras proteínas recombinantes do CDV e caracterizar os anticorpos envolvidos.

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ANEXO

Aprovação da Comissão de Ética no Uso de Animais em Pesquisa

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE GOIÁS
PRÓ-REITORIA DE PESQUISA E INOVAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA

Goiânia, 16 de dezembro de 2019.

PARECER CONSUBSTANCIADO REFERENTE AO PEDIDO DE EMENDA DO PROTOCOLO Nº. 054/17

I. IDENTIFICAÇÃO:

1. **Título do projeto**: Estudo molecular para identificação e caracterização do vírus da cinomose em amostras de cães
2. **Pesquisador Responsável:** Marcos Lázaro Moreli/Vivaldo Gomes da Costa
3. **Unidade/Órgão do pesquisador:** UAE Ciências da Saúde - Jataí
4. **Pesquisadores Participantes:** Andréa Vitor Couto do Amaral, Marielena Vogel Saivish, Luciana Garcia Oliveria Presotto
5. **Unidade onde será realizado:** Unidade Especial de Ciências Agrárias, Hospital Veterinário, Jataí
6. **Data de apresentação do protocolo a CEUA:** 09/05/17
7. **Data da apresentação do Pedido de Emenda: 20/11/2019**
8. **Solicitações:**
   - ✓ Prorrogação de prazo, de 01/12/19 a 31/12/2020

II - Parecer da CEUA:

Informamos que a *Comissão de Ética no Uso de Animais/CEUA* da Universidade Federal de Goiás, após análise das adequações solicitadas, **APROVOU** o pedido de emenda da proposta acima referida, e a mesma foi considerada em acordo com os princípios éticos vigentes.

Reiteramos a importância deste Parecer Consubstanciado, e lembramos que o(a) pesquisador(a) responsável deverá encaminhar à CEUA-PRPI-UFG o Relatório Final baseado na conclusão do estudo e na incidência de publicações decorrentes deste, de acordo com o disposto na Lei nº. 11.794 de 08/10/2008, e Resolução Normativa nº. 01, de 09/07/2010 do Conselho Nacional de Controle de Experimentação Animal-CONCEA. O prazo para entrega do Relatório é de até 30 dias após o encerramento da pesquisa, prevista para conclusão em **31 de dezembro de 2020**.

III - Data da reunião: 16/12/2019

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE GOIÁS
PRÓ-REITORIA DE PESQUISA E INOVAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA

C E RT I F I C A D O

Certificamos que a proposta intitulada "**Estudo molecular para identificação e caracterização do vírus da cinomose em amostras de cães**", registrada com o protocolo **nº 054/17**, sob a responsabilidade de **Marcos Lázaro Moreli** e **Vivaldo Gomes da Costa** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Universidade Federal de Goiás (UFG), em reunião de **16/12/2019.**

- Finalidade: ( ) Ensino (X) Pesquisa Científica
- Vigência da autorização (início e fim): 16/12/2019 a 30/12/2020
- Espécie/linhagem/raça: *Canis lupus familiaris*
- Nº de animais autorizados: 150
- Peso/Idade: indiferente
- Sexo: machos e fêmeas
- Origem (fornecedor): Hospital Veterinário Regional Jataí

Dra. Marina Pacheco Miguel
Coordenadora da CEUA/PRPI/UFG

Tabela 1. Os dados representam as informações coletadas e o resultado da triagem molecular do CDV para as amostras biológicas de cães com suspeita de cinomose

ID	Data	I[#]	Sex	M.B.	V[*]	Sinal clínico	CDV[¶]	Desfecho
1	28/06/17	84	F	Soro	Não	Apatia, inapetência, SO	**POS**	Óbito
2	20/03/17	8	F	Soro	Sim	Febre, vômito, diarreia, SO	NEG	
3	20/03/17	60	M	Soro	Não	Inapetência, vômito	NEG	Óbito
4	22/03/17	5	F	Soro/SN	Sim	SO/SN, apatia, diarreia, vômito, tosse	**POS**	Óbito
5	20/03/17	60	M	Soro	Não	Apatia, inapetência, vômito, SO	NEG	
6	23/03/17	60	M	Soro	Sim	Apatia, inapetência, ofegância	NEG	
7	30/03/17	1.5	F	Soro	Não	Mioclonia, apatia, vômito	NEG	
8	30/03/17	2	F	Soro	Não	Inapetência, diarreia	NEG	Óbito
9	28/03/17	120	F	Plasma	Não	-	NEG	Óbito
10	10/05/17	12	F	Soro	Sim	Apatia, inapetência	NEG	Óbito
11	09/05/17	144	F	Soro	Não	-	NEG	Óbito
12	28/06/17	6	F	Soro/SN	Sim	Inapetência, vômito	**POS**	Óbito
13	31/07/17	7	F	Soro	Sim	Inapetência, febre, SO	NEG	
14	09/08/17	5	-	Plasma	Não	Vômito	NEG	Óbito
15	19/10/17	24	-	Plasma	Não	SO, SN	NEG	
16	21/09/17	84	F	Soro	Não	-	**POS**	Óbito
17	21/09/17	24	F	Soro	-	SN, paralisia dos membros (PM), prostração, diarreia	NEG	Óbito
18	21/09/17	12	F	Plasma	-	SN, Mioclonia, PM, prostração, sialorreia, conjuntivite, incoordenação	**POS**	Óbito
19	21/09/17	5	M	Plasma	-	SN, sialorreia, conjuntivite, vômito, incoordenação	**POS**	Óbito
20	21/09/17	12	M	Plasma/ SN	-	PM, prostração, Mioclonia, incoordenação	NEG	Óbito
21	26/09/17	12	F	Plasma/ SN	-	Agressividade, PM, prostração, incoordenação	NEG	Óbito
22	26/09/17	24	M	Plasma/ SN	-	PM, prostração, sialorreia, conjuntivite, incoordenação	NEG	Óbito
23	02/10/17	60	M	Plasma/ SN	-	SN, PM, prostração, conjuntivite, incoordenação	NEG	Óbito
24	20/09/17	18	F	Plasma	Não	SO	NEG	Óbito
25	03/10/17	96	M	Soro	Não	Mioclonia, ataxia, convulsões, SO, tosse	NEG	Óbito
26	05/10/17	84	F	Plasma	Não	-	NEG	Óbito
27	10/10/17	72	M	Plasma/ SN	-	PM, prostração, incoordenação	NEG	Óbito
28	09/10/17	60	F	Plasma	Sim	Vômito, diarreia	NEG	
29	10/10/17	24	F	Plasma	Não	Mioclonia, ataxia, diarreia	NEG	
30	11/10/17	36	M	Plasma/ SN	-	PM, prostração, sialorreia, conjuntivite, incoordenação	NEG	Óbito
31	11/10/17	60	F	Plasma	-	SN, mioclonia, PM, prostração, sialorreia, conjuntivite, incoordenação	**POS**	Óbito
32	18/10/17	84	F	Plasma	Não	Apatia, ataxia, convulsões	NEG	
33	18/10/17	2	F	Plasma	Não	Inapetência, vômito	NEG	
34	18/10/17	12	F	Plasma	Não	Inapetência, apatia	NEG	
35	19/10/17	36	F	Plasma	Não	Inapetência, apatia	**POS**	
36	19/10/17	60	F	Plasma	Sim	-	**POS**	Óbito
37	23/10/17	84	F	Plasma	Não	-	NEG	
38	24/10/17	5	M	Plasma	Não	Apatia	**POS**	Óbito
39	25/10/17	12	F	Plasma	-	PM, mioclonia, prostração, sialorreia, conjuntivite, incoordenação	NEG	Óbito
40	25/10/17	12	F	Plasma	-	PM, conjuntivite, incoordenação	NEG	Óbito
41	27/10/17	108	M	Plasma	Sim	SN, dermatopatias, conjuntivite	NEG	Óbito

ID	Data	I[#]	Sex	M.B.	V[*]	Sinal clínico	CDV[¶]	Desfecho
42	26/10/17	12	M	Plasma	Não	SO, vômito	NEG	
43	30/10/17	12	M	Plasma	Não	SO, vômito	NEG	
44	01/11/17	36	M	Plasma	-	Incoordenação	NEG	Óbito
45	07/11/17	108	F	Plasma	Sim	-	NEG	
46	07/11/17	2	F	Plasma	Não	Mioclonia, incoordenação	**POS**	Óbito
47	07/11/17	24	F	Plasma	Não	PM, sialorreira, incoordenação	NEG	Óbito
48	07/11/17	204	F	Plasma	-	Mioclonia, PM, sialorreira, incoordenação	**POS**	Óbito
49	22/11/17	120	F	Plasma	-	SN, PM, prostração, incoordenação	NEG	Óbito
50	23/11/17	60	M	Plasma	-	Agressividade, PM, prostração	NEG	Óbito
51	23/11/17	24	F	Plasma	-	SN, mioclonia, PM, prostração, sialorreia, incoordenação	**POS**	Óbito
52	24/11/17	24	F	Plasma	-	Dermatopatias, PM, prostração, incoordenação	NEG	Óbito
53	28/11/17	8	F	Plasma	-	PM, prostração, conjuntivite	NEG	Óbito
54	28/11/17	24	F	Plasma	-	PM, prostração	NEG	Óbito
55	01/12/17	12	F	Plasma	-	SN, mioclonia, PM, prostração, conjuntivite	**POS**	Óbito
56	05/12/17	48	F	Plasma	-	SN, PM, prostração, conjuntivite, incoordenação	NEG	Óbito
57	07/12/17	24	M	Plasma	-	CN, mioclonia, PM, prostração, conjuntivite, incoordenação	**POS**	Óbito
58	07/12/17	36	M	Plasma	-	SN, PM, sialorreia, conjuntivite, incoordenação	NEG	Óbito
59	14/12/17	6	M	Plasma/ SN	-	SN, mioclonia, PM, prostração, sialorreia, conjuntivite, incoordenação	**POS**	Óbito
60	15/12/17	72	M	Plasma	Não	PM, prostração, incoordenação	NEG	Óbito
61	24/11/17	36	F	Plasma	Não	Inapetência, mioclonia, apatia, SO	NEG	
62	04/01/18	-	M	Plasma/ SN	Não	SN, agressividade, PM, prostração, sialorreia, conjuntivite, incoordenação	NEG	Óbito
63	11/01/18	24	M	Plasma	Não	Agressividade, PM, incoordenação	NEG	Óbito
64	16/01/18	24	F	Plasma	-	SN, PM, sialorreia, conjuntivite, incoordenação	NEG	Óbito
65	19/01/18	84	M	Plasma	Não	PM, prostração, incoordenação	NEG	Óbito
66	29/01/18	60	M	Plasma	Não	Inapetência, ofegância, SO	NEG	
67	26/01/18	-	M	Plasma	Não	Inapetência, diarreia, vômito	NEG	
68	26/01/18	-	F	Plasma	Não	Mioclonia, paralisia	**POS**	Óbito
69	29/01/18	-	M	Plasma	Não	Sinal sistêmico/neurológico (não se move)/SO	NEG	
70	08/02/18	48	M	Plasma	Sim	-	NEG	
71	08/02/18	24	F	Plasma	Sim	Dermopatia/dermatopatia	NEG	
72	09/02/18	24	M	Plasma	Não	PM, mioclonia, prostração, conjuntivite, incoordenação	**POS**	Óbito
73	09/02/18	48	M	Plasma	Sim	PM, prostração, sialorreia, incoordenação	NEG	Óbito
74	19/02/18	48	F	Plasma	-	PM, prostração	NEG	Óbito
75	19/02/18	36	M	Plasma	-	PM, prostração, incoordenação	NEG	Óbito
76	19/02/18	72	M	Plasma	-	SN, mioclonia, PM, prostração, sialorreia, conjuntivite, diarréia, incoordenação	**POS**	Óbito
77	19/02/18	60	M	Plasma	Não	SO, mioclonia, vômito	NEG	
78	21/02/18	24	F	Plasma	-	Agressividade, PM, prostração	NEG	Óbito
79	21/02/18	96	M	Plasma	Não	Mioclonia, prostração	NEG	
80	26/02/18	36	M	Plasma	Não	Dermatopatia, mioclonia, SO	NEG	
81	27/02/18	72	F	Plasma	-	PM, prostração	NEG	Óbito
82	27/02/18	72	M	Plasma	-	Mioclonia, PM, prostração,	**POS**	Óbito

						incoordenação		
ID	**Data**	**I[#]**	**Sex**	**M.B.**	**V[*]**	**Sinal clínico**	**CDV[¶]**	**Desfecho**
83	27/02/18	12	M	Plasma	-	Mioclonia, PM, prostração, diarreia, incoordenação	**POS**	Óbito
84	27/02/18	12	F	Plasma	-	PM, prostração, conjuntivite, diarreia, incoordenação	NEG	Óbito
85	28/02/18	36	M	Plasma	-	PM, incoordenação	NEG	Óbito
86	02/03/18	96	M	Plasma	Não	-	**POS**	
87	05/03/18	84	F	Plasma	Não	Vômito, tosse	NEG	
88	03/04/18	36	F	Plasma	Não	Hiperqueratose, incoordenação	**POS**	Óbito
89	05/04/18	48	M	Plasma	-	PM	NEG	
90	09/05/18	8	M	Plasma	Não	SN, mioclonia, PM, prostração, sialorreia, incoordenação	**POS**	Óbito
91	09/05/18	60	M	Plasma	Não	Mioclonia, PM, incoordenação	**POS**	Óbito
92	10/05/18	24	M	Plasma	-	PM, prostração, conjuntivite, incoordenação	NEG	
93	10/05/18	60	M	Plasma	Não	Agressividade, PM, prostração	NEG	
94	29/05/18	48	F	Plasma	Não	PM	NEG	Óbito
95	14/06/18	20	M	Plasma	-	SO/SN, hiperqueratose, vocalização	**POS**	Óbito
96	01/10/18	24	F	Plasma	-	Sialorreia, incoordenação	NEG	Óbito
97	01/10/18	24	F	Plasma	-	PM, prostração, sialorreia, conjuntivite, incoordenação	NEG	Óbito
98	10/09/18	-	F	Plasma	-	Mioclonia, incoordenação	**POS**	
99	03/10/18	48	F	Plasma	Não	Prostração, PM	NEG	Óbito
100	17/10/18	18	F	Soro	Não	Agressividade, PM, prostração	NEG	Óbito
101	23/10/18	12	M	Plasma	Não	Sialorreia, incoordenação	NEG	Óbito
102	25/10/18	36	M	Plasma	-	PM, sialorreia	NEG	Óbito
103	29/10/18	48	M	Plasma	Não	Paralisia, prostração, diarreia	NEG	Óbito
104	30/10/18	12	F	Plasma	Não	SN/SO, dermatopatia	**POS**	Óbito
105		48	F	Plasma	-	Paralisia e incoordenação	NEG	Óbito
106	19/11/18	12	F	Plasma	Não	Agressividade, mioclonia, incoordenação, dermatopatias	**POS**	Óbito
107	21/11/18	96	F	Plasma	Não	Paralisia, prostração, incoordenação	NEG	Óbito
108	04/12/18	36	M	Nasal	Não	Agressividade, paralisia, prostração, incoordenação	**POS**	
109	06/12/18	6	F	Plasma	Não	Incoordenação	NEG	Óbito
110	07/12/18	48	M	Plasma	Não	PM, incoordenação	NEG	Óbito
111	07/12/18	36	M	Plasma	Não	Agressividade, incorordenação	NEG	Óbito
112	09/12/18	120	F	Plasma	-	PM, prostração	NEG	Óbito
113	13/12/18	24	F	Plasma	Não	PM, prostração	NEG	
114	18/12/18	12	F	Plasma	Não	PM, prostração	NEG	Óbito
115	19/12/18	8	F	Plasma	Não	PM, prostração, hiperqueratose	**POS**	Óbito
116	03/01/19	12	M	Plasma	Não	PM, mioclonia, incoordenação	**POS**	Óbito
117	03/01/19	36	M	Nasal	Não	Paralisia, prostração, incoordenação, SN/SO	**POS**	Óbito
118	04/01/19	3	F	Nasal	Não	Diarreia, vômito, SN/SO, hiperqueratose	**POS**	Óbito
119	08/01/19	36	M	Nasal	Não	Mioclonia, incoordenação, SN/SO	**POS**	Óbito
120	08/01/19	12	F	Nasal	Não	Prostração, diarreia, vômito, SN/SO, dermatopatia	**POS**	Óbito
121	17/01/19	36	M	Nasal	Não	Mioclonia, PM, prostração	**POS**	Óbito
122	17/01/19	3	M	Nasal	Não	Mioclonia, incoordenação, diarreia, vômito, SN/SO, sialorreia	**POS**	Óbito
123	24/01/19	60	M	Nasal	Não	Agressividade, incoordenação	NEG	Óbito
124	28/01/19	36	F	Nasal	Não	Agressividade, incoordemação	NEG	Óbito
125	30/01/19	12	F	Plasma	Não	Mioclonia, PM, prostração, SN/SO, sialorreia	**POS**	Óbito

ID	Data	I[#]	Sex	M.B.[#]	V[*]	Sinal clínico	CDV[¶]	Desfecho
126	04/02/19	24	M	Nasal	-	Paralisia/sialorreia	NEG	Óbito
127	13/02/19	36	M	Nasal	-	Mioclonia, paralisia, alteração de comportamento, hiperqueratose, dermatopatia	**POS**	Óbito
128	20/02/19	36	F	Plasma	Não	Mioclonia, paralisia, tremor, vocalização, SN/SO	**POS**	Óbito
129	20/02/19	36	F	Nasal	Sim	Mioclonia, paralisia, tremor, vocalização, hiperqueratose	**POS**	Óbito
130	21/02/19	24	M	Nasal	Não	Mioclonia, paralisia, vocalização, SN/SO, prostração	**POS**	Óbito
131	25/02/19	60	M	Nasal	Não	Mioclonia, tremor, paralisia, vocalização, SN/SO	**POS**	Óbito
132	26/02/19	5	M	Nasal	Não	Mioclonia, tremor, paralisia, alteração de comportamento, sialorreia	**POS**	Óbito
133	26/02/19	24	M	Nasal	Não	Mioclonia, tremor, paralisia, diarreia, vômito, SN/SO	**POS**	Óbito
134	27/02/19	24	M	Nasal	Não	Paralisia, prostração, alteração de comportamento	NEG	Óbito
135	28/02/19	4	M	Nasal	-	Tremor, alteração de comportamento, vocalização	NEG	Óbito
136	07/03/19	6	M	Plasma	Sim	PM, prostração, incoordenação	NEG	Óbito
137	07/03/19	12	F	Nasal	Não	PM, prostração	NEG	Óbito
138	11/03/19	24	F	Nasal	Sim	PM, prostração	NEG	Óbito
139	15/03/19	6	F	Nasal	Sim	Tremor, sialorreia, vocalização	NEG	Óbito
140	18/03/19	24	F	Nasal	Não	Mioclonia, PM, prostração, hiperqueratose	**POS**	Óbito
141	01/04/19	24	M	Nasal	Não	Agressividade, paralisia, mioclonia, incoordenação	**POS**	Óbito

#Idade; #Material biológico; *Vacina anti-CDV; ǂInício sintomas; ¶Resultado nested RT-PCR. SO= swab oral; SN= swab nasal; PM= paralisia do membro

Panfleto sobre a Cinomose Canina

Mas o que é: **CINOMOSE**?

A cinomose é uma doença extremamente grave que pode acometer vários animais carnívoros, incluindo os cães. A doença em cães é conhecida como cinomose canina, sendo mais suscetíveis a doença os cães filhotes jovens.

Como a doença é transmitida?

A doença da cinomose é ocasionado por um vírus, existindo várias formas de contágio do vírus. Dessa forma, o contágio pode ocorrer por meio da urina, saliva e/ou secreções ocular e nasal do cão que está doente.

Como posso suspeitar de cinomose canina?

O diagnóstico da doença é difícil, pois envolve diversos sinais clínicos. A vista disso é necessário um veterinário para confirmação, ou não, da doença.

ATENÇÃO: Abaixo estão os sinais clínicos que podem sugerir cinomose:

FASES E SEUS SINAIS CLÍNICOS:

1. Oftálmica
Secreção nos olhos e conjuntivite severa

2. Respiratória
Secreção nasal, tosse e pneumonia

3. Tegumentar
O cão apresenta pústulas abdominais e hiperqueratose dos coxins plantares (pele das patas ressecadas e descamadas)

4. Digestiva
Vômito e diarreias

5. Neurológica
Tremores musculares, incoordenação motora, convulsões

6. Outros
Febre, apatia, perda de peso

Existe medicamento contra a cinomose?

Não há medicamentos antivirais eficazes para combater a doença. No entanto, veterinários podem prescrever medicamentos para combater e aliviar os sintomas associados ao mal-estar como medicamentos contra a febre...

Como posso prevenir a doença no meu cão?

- Consultas periódicas no veterinário
- Vacinação contra a cinomose
- Evitar que os filhotes com calendário de vacinação dos 90 dias ainda incompleto circulem em ambientes públicos
- Animais sadios não devem ter contato com o cão doente (este deve ficar isolado durante todo o tratamento).
- Evitar passear com o cão sem guia.

Lembre-se: Manter sempre em dias a vacinação de seu animal de estimação!!!

APÊNDICE

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