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Hemagglutinin Gene Diversity of Canine Distemper Virus from Clinical Samples in Brazil

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ABSTRACT

Canine distemper virus (CDV) affects both domestic dogs and wild animals, resulting in high rates of morbidity and mortality. The viral genome encodes six different proteins. Hemagglutinin (H) is the most variable one, being the basis for molecular phylogenetic analysis, and may lead to antibody recognition mismatch and vaccine failure. The study aimed to analyze the H gene of the CDV in field samples from naturally infected dogs in Brazil.24 urine samples and eye discharge swabs were collected from naturally infected dogs in endemic areas of Brazil. Viral ribonucleic acid (RNA) of collected samples was extracted, converted to cDNA and amplified by polymerase chain reaction (PCR). PCR amplicons of H gene were purified and sequenced for phylogenetic analysis. The protein sequences of the samples were subjected to a Genbank search and aligned with the amino acids sequence of the CDV vaccine strain, "Onderstepoort", to explore their similarity profiles. Sequencing of the fragments that represent the entire H gene sequence demonstrated five distinct isolates, two of them sequenced in full-length. The phylogenetic analysis of the isolates showed that they belong to the cluster of Europe/South America1, differing substantially from the currently used vaccine strains. Deduced amino acid sequence analyzed, showed that the specific substitutions at the SLAM receptor site of the H gene, previously linked to the emergence of diseases in new hosts, and were not detected in these sequences. However, the R580Q substitution detected in the isolated strain H_CDV_A5_PE (GenBank: MK423863) is considered imminently deleterious to the efficiency of fusion and expression of the surface of the binding protein. These findings demonstrate that the genetic variability identified in field strains of the distemper virus in endemic areas in Brazil shows genetic alterations that may lead to antibody mismatch.

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The canine distemper virus (CDV) belongs to the family *Paramyxoviridae* and *Morbillivirus* genus **(Rima *et al.*, 2019)**. The CDV is the etiologic agent of canine distemper, a serious, immunosuppressive and highly contagious systemic disease that affects a wide range of terrestrial and marine carnivores and non-human primates **(Loots *et al.*, 2018)**.

The CDV is a negative single-strand RNA virus with non-segmented genome and the viral RNA encodes at least eight proteins: hemagglutinin (H), fusion protein (F), envelope-associated matrix (M), phospoprotein (P), viral polymerase (L), and nucleoprotein (N), and two secondary nonstructural proteins (C and V), encoded as extra-transcriptional

units, incorporated in the P gene **(Freitas *et al*., 2019)**. The Hemagglutinin (H) plays an important role in host-specific immunity. Its main function is to bind to receptors on the host cell and interact with the fusion protein to assist in the attachment and fusion of the viral envelope to the host cell membrane, a mechanism that allows the virus to enter the cell **(Panzera *et al*., 2015)**. The Nucleocapsid (N) protein gene makes up a more conserved region of the CDV genome **(Castilho *et al., 2007*; Headley *et al.,* 2012)**. At post-transcriptional time, the N protein has a high production and association with the nucleic acid, taking charge of protecting the genome against digestion by nucleases by serving to encapsulate the genome in a nucleocapsid resistant against RNase action **(Liu *al*., 2019)**. Therefore, some phylogenetic studies are based on N gene sequence analysis since there is less genetic variability among isolates. However, the H protein exhibits significant genetic variability in the genome, being the focus of epidemiological studies and phylogenetic analyses involving CDV **(Von Messling *et al*., 2006; Martella *et al*., 2011; Mira *et al*., 2018; Bhatt *et al*., 2019; Duque-valencia *et al.*, 2019; Kodi *et al*., 2021)**.

Although the incidence of the disease can be dramatically reduced by using vaccines, cases of vaccinated puppies, that have acquired the disease, have been reported **(Da Costa *et al*., 2021)**. Despite the various explanations mentioned, the exact cause of the vaccine failure remains unknown. As CDV vaccine strains have become widely used, their interaction with wild-type strains is an important issue requiring further investigation **(Da Costa *et al*., 2021)**. Recent studies bringing new data regarding the molecular epidemiology of this disease in Brazil, showed a predominance of a viral genotype, called Europe/South America 1, phylogenetically distant from the clade of vaccine strains, reflecting the need to intensify the control measures currently employed **(Budaszewski *et al*., 2014; Cortez *et al*., 2017; Freitas *et al*., 2019; Tao *et al*., 2020)**.

Some polymorphisms described in preliminary studies point to viral mutations of interest, which may lead to losses in the ability of fusion and surface expression of the binding protein, viral adaptation to new host species, severity of clinical disease. N-glycosylation sites are critical for the correct folding, transport and function of viral fusion and attachment glycoproteins **(Sawatsky and Von Messling, 2010)**. The alteration in the number of N-glycosylation sites may favor the reduction of the pathogenic potential of infection or even lead to failures in the vaccine response of hosts **(Iwatsuki *et al*., 1997; Sawatsky and Von Messling, 2010)**. The importance of the identification of these polymorphisms and predictions of N-glycosylation sites in newly described isolates contribute to an analysis of the viral epidemiological behavior.

However, there is still a lack of genetic data regarding many Brazilian regions, especially those with a high population of stray dogs. Therefore, this article aimed to analyze the CDV Hemagglutinin gene in naturally infected dogs' field samples from different regions of Brazil.

MATERIALS AND METHODS

1. Ethical approval and Informed consent:

The experiment was conducted in accordance with the norms set forth by the CEUA/UFRPE (Ethics Committee on Animals Use of Federal Rural University of Pernambuco, Brazil) (approval no.8919/2017).

2. Samples

Urine and eye discharge swabs collected from 24 infected dogs from different regions of Brazil from a sample bank belonging to Tecsa® Laboratórios (Belo Horizonte, MG, Brazil) were kindly provided for this experiment. The samples were processed at the Laboratory of Virology and Experimental Therapy (LaViTE) of the Aggeu Magalhães Institute (IAM-FIOCRUZ/PE), located in Recife-PE, Brazil.

3. RNA extraction and reverse transcription

RNA from clinical samples of infected animals (urine, eye discharge swabs) was extracted using QIAamp Viral RNA Mini Kit (Qiagen®, Germany), according to manufacturer's instructions and eluted to a final volume of 200µl. After extraction, RNA was reverse transcribed with specific reverse primer P2 (Table 1) **(Frisk *et al*., 1999)**. For this step, ImProm-II™ Reverse Transcription System (Promega®, USA) was used, according to manufacturer's recommendations.

4. PCR amplification

The PCR reactions were performed in two steps: the first step consisted of a screening and the second, an amplification step of the positive samples, targeting the H gene, used for subsequent sequencing. PCR Master Mix kit (Promega®, USA) was used according to manufacturer's recommendations. In the screening reaction, samples were submitted to the protocol proposed by Frisk and others **(Frisk *et al*., 1999)**, with modifications, targeting the Nucleoprotein (N) gene **(Frisk *et al*., 1999)**. PCR conditions were as follows: initial denaturation at 95ºC for 2 minutes; 40

cycles of denaturation at 95ºC for 1 minute, annealing at 59ºC for 2 minutes and extension at 72ºC for 1 minute; followed a final extension step of 72ºC for 5 minutes.

A second PCR reaction was performed on positive samples to amplify the RNA of gene H. From these samples, reverse transcription was performed with specific primer H5R, following the protocol described in section 2.3. Genome amplification from positive samples was conducted using 0.25µl of cDNA from each clinical sample, commercial PCR Master Mix, and forward and reverse primers from the complete CDV hemagglutinin sequence, based on the protocol of Negrão and collaborators, with modifications **(Negrão *et al.*, 2013)**. Proposed modifications to some nucleotides have been highlighted in red (Table 1). The cycle temperatures in this second PCR reaction were: initial denaturation at 95°C for 2 minutes; 40 cycles of denaturation at 95°C for 1 minute, annealing at 45°C for 1 minute, and extension at 72°C for 1 minute; followed by a final extension step of 72°C for 5 minutes.

The verification of possible amplicons occurred by electrophoresis in 1.2% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA), the resulting amplification product was visualized under ultraviolet light.

Table 1: Oligonucleotide primers designed from the H gene of the CDV virus for RT-PCR reaction described by Negrão and collaborators **(Negrão *et al.*, 2013)**. In red, the nucleotides modified in our study:

Primer	Gene	Sequence (5´- 3´)
NAH1[a]	H	CCAACAGACACTCAAGCA
NAH2[a]		AACCGTAACCCAATCTCAT
NAH3[b]	H	AGATTGCTGAAAGAGGATA
NAH4[b]		ACATACCTTGGCTTTGGAA
NAH5[c]	H	GTGGAGCTACTACTTCAG
NAH6[c]		TGTCAACCGCCCATAAGAT
NAH7[d]	H	CTGAGAAACAAGAAGAACAA
NAH8[d]		TCATCCCACACAAAACATT
NAH9[e]	H	GTTTATTATGTTTATGACCC
NAH10[e]		ATTCTCTCTTTGATATTACG

5. DNA sequencing

PCR products of H gene amplification were purified with PureLink™ PCR Purification Kit (Invitrogen®, USA), following the manufacturer's instructions. Purified products were quantified by spectrometry, using NanoDrop™ 2000 (Thermo Fisher Scientific®, USA). The primers used on sequencing were the same used for the amplification of H gene (Table 2).

Table 2: Oligonucleotide primers used in our study. For the screening step (conventional PCR), the N gene region was targeted, based on Frisk and collaborators **(Frisk *et al*, 1999)**. For the second PCR step (prior to gene sequencing), the H gene was targeted, based on the protocol by N Negrão and collaborators **(Negrão *et al*., 2003)**, with modifications marked in red. a: Primers PCR **(Frisk *et al*., 1999)**; b: Primers RT-PCR **(Negrão *et al*., 2013)** with modifications:

Primer	Gene	Sequence (5´- 3´)	Direction	Nucleotide position
P1[a]	N	ACAGGATTGCTGAGGACCTAT	+	769–789
P2[a]		CAAGATAACCATGTACGGTGC	-	1055–1035
H1F[b]	H	CCGACAGACATTCAAGCA	+	6833-6850
H1R[b]		AACCGTAACCCAACCTCAT	-	7380-7398
H2F[b]	H	AGATTGCTGAAAGAGGATA	+	7292-7310
H2R[b]		ACATACCTTGGCTTTGGAA	-	7909-7927
H3F[b]	H	GTGGAGCTACTACTTCAG	+	7644-7661
H3R[b]		TGTCAACCGCCCATAAGAT	-	8290-8308
H4F[b]	H	CTGAGAAACAAGAGGAACAA	+	8181-8200
H4R[b]		TCATCCCACACAAAACATT	-	8772-8790
H5F[b]	H	GTTTATTATGTTTATGACCC	+	8681-8700
H5R[b]		ATTCTCTCTTTGATATTACG	-	9168-9187

Purified samples were sequenced with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems®, USA). All fragments were analyzed by the DNA automatic sequencer ABI Prism 3100 (Applied Biosystems®, USA), available at Núcleo de Plataformas Tecnológicas of IAM/FIOCRUZ. Obtained sequences were aligned using DNASTAR®-Lasergene® SeqMan Pro™ software (© 1988-2017).

Table 3: Detail of nucleotide sequences by genogroup with GenBank accession codes, used to create the phylogenetic tree for this study:

Access code (GenBank)	Host	Place	Year	Genotype
EU143737	Vaccine strain- Onderstepoort	USA	2007	**America 1**
FJ461701	Vaccine strain- Nobivac DHPPI	South Africa	2008	**America 1**
FJ461710	Vaccine strain- Canigen DHPPI	South Africa	2006	**America 1**
AF378705	Vaccine strain- Onderstepoort	USA	2008	**America 1**
Z35493	Vaccine strain- Convac	Denmark	1994	**America 1**
JN896987	Vaccine strain- Snyder Hill	USA,Boston	2011	**America 1**
AF259552	Vaccine strain- Snyder Hill	Russia, Irkutsk	2000	**America 1**
AY649446	Raccoon	USA	2004	**America 2**
AY498692	Raccoon	USA	2003	**America 2**
JX912968	Dog	Brazil, Porto Alegre	2013	**Rockborn-like**
AY964114	Dog	USA	2005	**Rockborn-like**
AF178039	Panda	China	1999	**Rockborn-like**
GU810819	Dog	Italy	2010	**Rockborn-like**
FJ461702	Dog	South Africa	2008	**Rockborn-like**
DQ191767	Dog	Taiwan	2005	**Asia 1**
AB212964	Dog	Japan	2005	**Asia 1**
AB212963	Dog	Japan	2005	**Asia 1**
AB212730	Dog	Japan	2005	**Asia 2**
AY297454	Dog	Japan	2003	**Asia 2**
AY297453	Dog	Japan	2003	**Asia 2**
X84998	*Phoca sibirica*	United Kingdom	1995	**Artic**
Z47760	Dog	Denmark	1995	**Artic**
AF172411	Dog	China	1999	**Artic**
Z77672	Dog	Germany	1996	**Europe 1**
Z77673	Dog	Germany	1996	**Europe 1**
DQ494317	Dog	Italy	2006	**Europe 1**
DQ494318	Dog	Italy	2006	**Europe 1**
X84999	Raccoon	United Kingdom	1995	**Europe 2**
Z47759	Mink	Denmark	1995	**Europe 2**
GQ214369	Stone marten	Austria, Vienna	2009	**Europe 2**
EU098104	Dog	Brazil, Londrina	2007	**South America 1**
EU098105	Dog	Brazil,Londrina	2007	**South America 1**
JX912958	Dog	Brazil, Porto Alegre	2013	**South America 1**
JX912963	Dog	Brazil, Porto Alegre	2013	**South America 1**
KX434626	Dog	Brazil, Botucatu	2016	**South America 1**
EU098102	Dog	Brazil, Londrina	2007	**South America 1**
JX912959	Dog	Brazil, Porto Alegre	2013	**South America 1**
JN215475	Dog	Uruguay, Montevideo	2011	**South America 1**
JN215476	Dog	Uruguay, Montevideo	2011	**South America 1**
JN215473	Dog	Uruguay, Montevideo	2011	**South America 1**
JN215477	Dog	Uruguay, Montevideo	2011	**South America 1**
JN215474	Dog	Uruguay, Montevideo	2011	**South America 1**
JX912971	Dog	Brazil, Porto Alegre	2013	**South America 1**
JX912975	Dog	Brazil, Porto Alegre	2013	**South America 1**
JX912976	Dog	Brazil, Porto Alegre	2013	**South America 1**
FJ392652	Dog	Argentina	2008	**South America 1**
JX912978	Dog	Brazil, Porto Alegre	2013	**South America 1**
KT429766	Dog	Brazil, Londrina	2012	**South America 1**
KX434626	Dog	Brazil, Botucatu	2016	**South America 1**
MG827088	*Tamandua tetradactyla*	Brazil, Cuiabá	2018	**South America 1**
FJ392651	Dog	Argentina	2008	**South America 2**
KC257463	Dog	Argentina, Buenos Aires	2012	**South America 2**
KC257464	Dog	Argentina, Buenos Aires	2012	**South America 2**
KF835423	Dog	Colômbia, Medellin	2013	**South America 3**
KF835424	Dog	Colômbia, Medellin	2013	**South America 3**
KF835425	Dog	Colômbia, Medellin	2013	**South America 3**

6. Phylogenetic analysis

The obtained sequence data were assembled and edited in a total length of 1824 bp using the program SeqMan (software package DNAStar Lasergene, Madison, WI, USA) (Table 3). To verify the integrity and similarity of the sequences, the samples were submitted to a public database search - NCBI using the tool BLAST. The Neighbor-Join (NJ) and BioNJ algorithms were used in an array of distances between the estimated pairs using the Maximum Composite Likelihood (MVI) approach, based on the full-length H gene sequence. NJ trees were constructed using MEGA X software **(Kumar *et al.*, 2018)**. The best fit model for the sequence data set was the 2-parameter Kimura model **(Kimura, 1980)**. The reliability of the trees was estimated by the bootstrap analysis (BS) of 1000 sets of pseudo replicated data **(Felsenstein, 1985)**.

7. Amino acid analysis of H protein

Sequences of 607 amino acids were generated from open reading frames (ORFs) of protein H from each one of new described strains. Potential N-glycosylation sites of H protein were predicted using the NetNGlyc 1.0 Server **(Bhatt *et al.*, 2019)**.

RESULTS

Biological samples from 24 infected animals from different regions of Brazil from a sample bank belonging to Tecsa® Laboratórios (Belo Horizonte, MG, Brazil) were used in this study. The preliminary identification of CDV occurred by conventional PCR, targeting the Nucleoprotein (N) gene. Among the samples tested, only five were positive and then classified for the next step of amplification of the Hemagglutinin (H) gene. After sequencing, it was confirmed that the amplified fragments corresponded to the gene of interest. However, after the data analysis, it was concluded that only two of the five samples had the H gene sequenced full length, the sequences H_CDV_A2_MG, coming from a domestic dog from Belo Horizonte, Minas Gerais state, and H_CDV_A5_PE, coming from a domestic dog from Recife, Pernambuco state. The sequences of the isolates analyzed in this study were deposited in GenBank with the following access codes: H_CDV_A5_PE (MK423863); H_CDV_A8_PE (MK423859); H_CDV_A10_PE (MK423862); H_CDV_A16_PE (MK423861); H_CDV_A2_MG (MK423860).

All isolates showed 99% similarity (NCBI Blast tool) when compared with strains from other Brazilian regions and 98% similarity with strains from Argentina and Uruguay. In order to extend our study, 56 CDV sequences from different groups were selected in GenBank® (https://www.ncbi.nlm.nih.gov/genbank/) and aligned with the isolates described in this study. Phylogenetic analysis performed with the Neighbor-Joining method based on the nucleotide sequences of the H gene formed a phylogenetic tree, represented in Fig. 1.

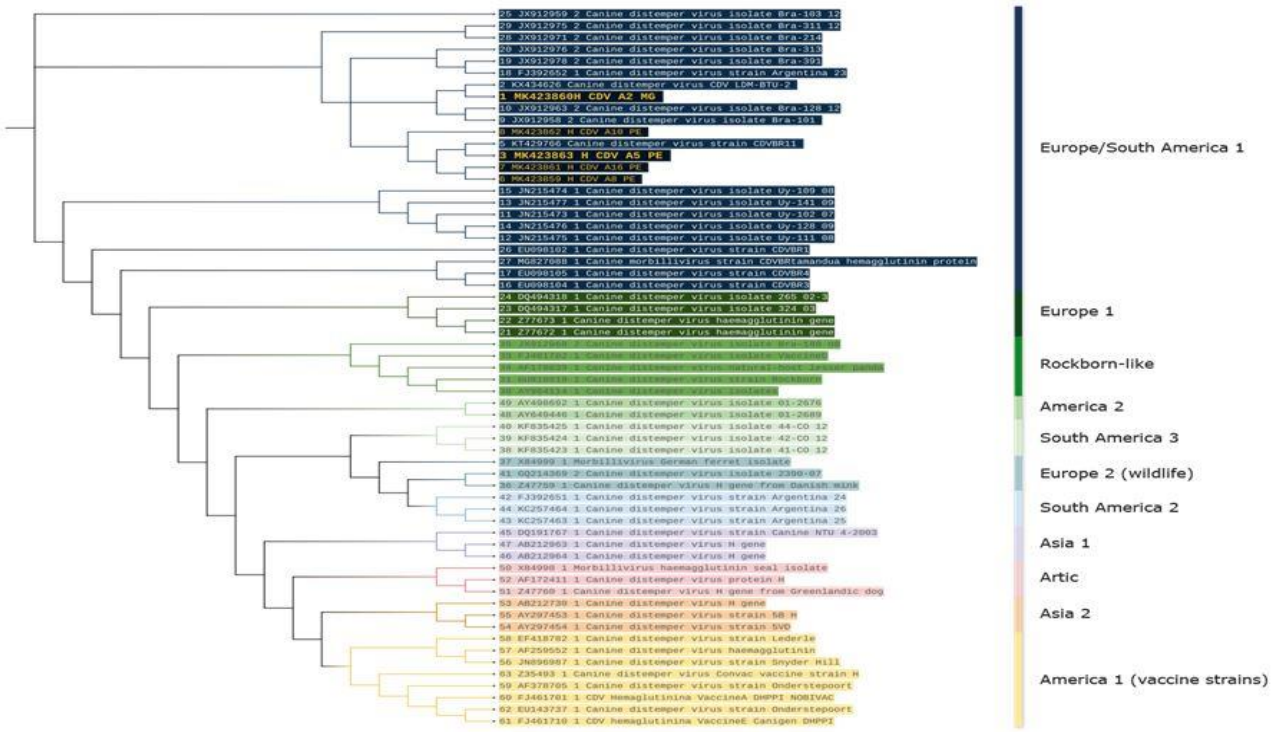

Fig.1: Phylogenetic relationship among CDV strains selected in Genbank (sequence identifications used in Tab. 3), based on the Hemagglutinin protein gene.

The CDV isolates identified in this study is indicated in yellow bold in the clade Europe/South America 1. The main CDV lineages are highlighted and differentiated by color scales in the corresponding arms, named America 1, America 2, Artic-like, Asia 1, Asia 2, Europe/South America 1, Europe 2, South America 2, South America 3 and Rockborn-like. The Neighbor joining tree was reconstructed by the 2-parameter Kimura model, using 1000 bootstrap replicates.In this tree, it was possible to observe that isolates from Pernambuco and Minas Gerais were related to strains of the Europe/South America-1 genogroup (EU/SA-1), in which are also grouped the other Brazilian isolates already described, as well as isolates from Argentina and Uruguay.

Based on the analysis of the amino acid residues presented by the open reading frames (ORFs) of the described isolates and the main polymorphisms described in literature, represented here in Table 4, it was possible to verify the presence of an important substitution at position 580 (R580Q) in the isolates from Pernambuco, as represented in Fig. (2).

Fig. 2: Analysis of amino acid sequence polymorphisms in the new isolates described in this study in comparison with vaccine strain “Onderstepoort” (access: EU143737). Sequences of 607 amino acids generated from the protein H ORFs of each of the newly described strains were aligned with the protein sequence of the vaccine strain “Onderstepoort” of CDV, in which the presence of alterations in specific regions was analyzed. The positions of interest were depicted in trees with reconstructions of the CDV Hemagglutinin gene and the signaling in the region of interest in the alignment depicting the modifications was also highlighted. (a) Branches colored according to state at position 580. (b) Branches colored according to state at position 549. (c) Branches colored according to state at positions 525, 526, and 529.

Also based on amino acid analysis, prediction of N-glycosylation sites was performed, detecting the presence of five predisposed regions (19-21/ 149-151/ 309-311/ 391-393/ 422-424/ 456-458/ 587-590/ 603-605), described in Fig.(3), all of which have been described in previous studies.

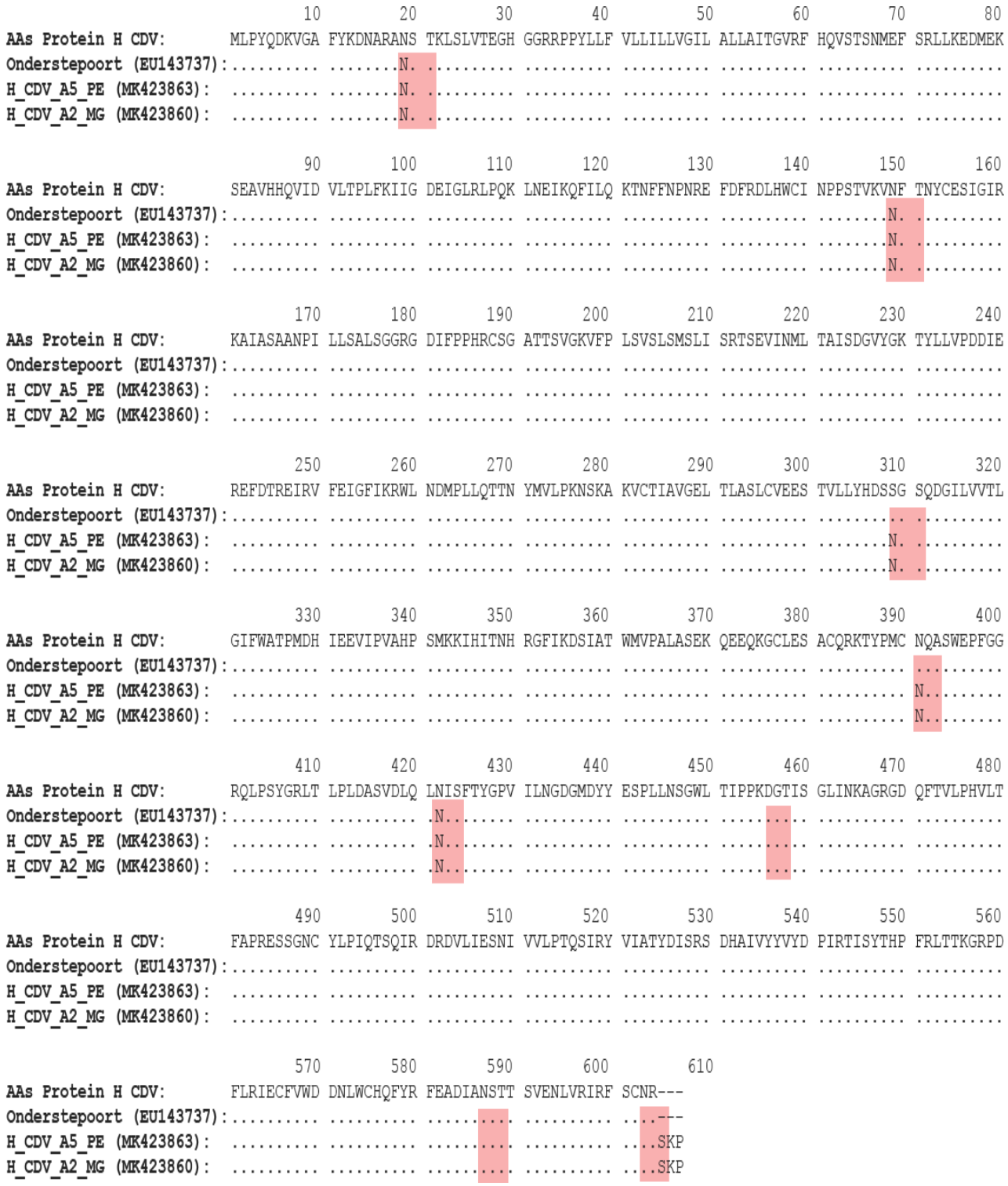

Fig. 3: Prediction of N-glycosylation sites from alignment of deduced amino acid sequences of protein H from newly described sequences and from CDV vaccine strain. Alignment of the amino acid sequence of the CDV H protein of the vaccine strain “Onderstepoort” (EU143737) and two new Brazilian isolates described in this study: H_CDV_A5_PE (MK423863) and H_CDV_A2_MG (MK423860). The pink region represents the areas associated with potential N-glycosylation sites (N-X-S/T). The dots (.) refer to identity.

DISCUSSION

Some previous phylogenetic studies are based on partial sequence analysis of the N gene, since there is less genetic variability among isolates when compared to the H gene, which is justified by the fact that the N gene is part of the most conserved region of the CDV genome **(Castilho *et al*., 2007; Headley *et al*., 2012)**. However, studies involving the inference of the H gene lead to more robust analyses **(Tan *et al*., 2011)**, since this glycoprotein is under constant evolutionary pressure, causing frequent naturally occurring mutations **(Bolt *et al*., 1997; Mochizuki *et al*., 1999; Pardo *et al*., 2005; Martella *et al*., 2006)**. In our study, we used the analysis of the N gene and the H gene, the first working as a preliminary way to select positive samples for CDV, since this gene has greater conservation and is more feasible to make it a target in PCR. The analysis based on the inference of the H gene was used to evaluate the variability of this gene in the population of naturally infected dogs in Brazil, besides allowing a parallel involving the isolates described with the vaccine strains.

Studies suggest that there are three lineages of the virus circulating among South American countries, where previously only two lineages were described **(Espinal *et al*., 2014; Sarute *et al*., 2014**). The described lineages involve the clade denominated Europe/South America1, where it could be estimated that the CDV strains were introduced into Europe through Italy following its expansion and spreading throughout the continent and South America. It is assumed that the entry of the Italian ancestor in South America was through Brazil, then spreading through Uruguay and Argentina **(Panzera *et al*., 2012; Budaszewski *et al*., 2014)**.When analyzing the phylogenetic relationships based on nucleotide alignment between the isolates described in the present study, based on a 566 bp segment of the H gene, and other CDV sequences stored in Genbank, it was observed that the strains described here grouped to the clade denominated Europe/South America-1 (EU1 / SA1), corroborating with findings of **(Rosa *et al*., 2012; Budaszewski *et al*., 2014; Cortez *et al*., 2017)**.The Brazilian CDV strains analyzed are genetically related to the strains circulating in Uruguay, Argentina and Europe, and no relationships were found with South American strains 2 and 3, also observed by **Cortez and colleagues (2017)**.

Research shows considerable antigenic divergence between field isolates and commercial vaccine strains **(Hashimoto and Mochizuki, 2001; Si *et al*., 2010)**. Among the group of animals positive for CDV infection studied by **Fischer and colleagues (2016)**, 19% of animals had undergone a vaccination regimen, reinforcing the description of other studies of CDV outbreaks in vaccinated animals (**Simon-Martinéz *et al*., 2008; Liu *et al*., 2021**), and raising questions about the vaccination coverage and induction of neutralizing antibodies offered by commercial vaccine formulations. This further suggests the need for the development of an updated CDV vaccine formulation. **(da Costa *et al*., 2021)**. Due to the use of samples from a sample bank, it was not possible to evaluate the clinical profile of the animals from which the clinical samples originated in order to correlate the presence of infection with the presence of the vaccine protocol. However, the CDV strains presented in this study presented in a group distinct from the clade composed of the vaccine strains (America 1), in agreement with preliminary studies **(Martella *et al*., 2006; Panzera *et al*., 2012; Rosa *et al*., 2012; Negrão *et al*, 2013; Cortez *et a*l., 2017; da Costa *et al*., 2021)**, suggesting evolution in a distinct manner between field strains and vaccine strains because of divergence in selection pressures in different viral populations **(Cortez *et al*., 2017)**.

The amino acid exchange at position 580 (R/Q) of the CDV H gene is considered unusual *in vivo* and studies associate this exchange as being possibly detrimental in the ability of the fusion and expression of the binding protein surface *in vitro* **(Sattler *et al*., 2014)**. In this context, one of the new Brazilian isolates presented stands out, originating from an infected dog from the city of Recife, Pernambuco, northeast region of Brazil, H_CDV_A5_PE. **Fischer and collaborators (2016)**, found similar results, in which they report the presence of the potentially detrimental amino acid substitution at position 580 (R580Q) as a hallmark of the epidemic in the EU/SA 1 genogroup. However, another Brazilian isolate, H_CDV_A2_MG, from an infected domestic dog from the city of Belo Horizonte, Minas Gerais, Southeast region of the country, did not show the mutation, again corroborating with **Fischer and collaborators (2016)**, in which they present that two groups of CDV can be identified in Brazil: a smaller and older group composed of 580R strains and a larger and more recent group of 580Q strains.

Mccarthy and collaborators (2007) described a mutation at position Y549H, related to virus host adaptation. Domestic dogs and wild canids are more commonly affected with 549Y strains, whereas 549H strains are more commonly described in non-canid hosts **(Mccarthy *et al*., 2007; Nikolin *et al*., 2012)**. In our study, we observed that position 549 of the newly described sequences contained a Tyrosine (Y). A preliminary study in Brazilian dogs, showed a frequency of 11.9% of the Y549H mutation, with a peculiar occurrence in mixed breed animals, which are often found in Brazilian territory in urban and rural areas, often with wandering behavior, which may suggest a contact with wild fauna infected by CDV and occurrence of mutation phenomena in viral molecular

epidemiology **(Fischer *et al.*, 2016)**. In the present study, it was not possible to identify the breed of the animals to which the biological samples belonged, however, it is known that they were animals under human guardianship, since the samples came from a private veterinary laboratory bank (Tecsa Laboratories®), which processes only samples sent by veterinarians who care for animals under human guardianship.

Some polymorphisms in the CDV hemagglutinin protein have been reported in association with adaptation to viral fitness and clinical disease severity. **Zipperle and colleagues (2010)**, evaluated the possibility that some specific residues are associated with CDV virulence activity. These specific residues of the H protein, described as Y525, D526 and R529, are associated with the lymphocyte activation signaling molecule (SLAM) H gene receptor binding site, present in host cells, expressed on activated T- and B-type lymphocytes, as well as epithelial, glial, dendritic and macrophage cells **(McCarthy *et al.*, 2007)**. In our study, these specific residues of the H protein were conserved, since the new sequences were isolated from animals that had field strain infection.

The changes that occur in the post-translational step are critical to the role of many proteins. N-glycosylation sitesare important for the correct folding, transport and function of fusion and attachment glycoproteins **(Sawatsky and Von Messling, 2010)**. Studies have hypothesized that a reduction in N-glycosylation sites favors reduced pathogenesis associated with CDV infection, and conversely, an increased presence of N-glycosylation may be associated with episodes of vaccine failure **(Iwatsuki *et al.*, 1997; Sawatsky and Von Messling, 2010)**. Here, we present new isolates H_CDV_A5_PE and H_CDV_A5_PE, whose showed in predictive analysis, seven probable N-glycosylation sites, (19, 149, 391, 422, 456, 587, and 603), also observed by **Haas *et al.*, (1997), Pardo *et al*. (2005) and Espinal *et al*. (2014)**. **Da Costa and colleagues (2021)**, describe that the seven predicted sites show as conserved sites for N-glycosylation in CDV protein H for specimens associated with genogroup SA1/EU, America 1/2, Africa, Asia-1/2, and Arctic, corroborating with our finding.

When we performed an analysis on the N-glycosylation sites of the Onderstepoort vaccine strain (GenBank: EU143737), we observed the presence of only four predictive sites (19, 149,422 and 587), three sites less than the isolates described here, which reflects the importance of this prediction for virulence modulation analysis of field strains, also observed by **da Costa *et al*., (2021).**

When analyzing the phylogenetic relationships based on nucleotide alignment between the strains described in the present study, based on the 1,824bp H gene, and the CDV sequences stored in Genbank, it was observed that the new strains described were grouped into the cluster called Europe/South America-1 (EU1/SA1).

CONCLUSION

The data presented in the present study expand the phylogenetic studies in South America, a special focus on the regions contemplated in this research, where records of the CDV H gene analysis were not yet described. It also strengthens the previous findings that the Brazilian strains are inserted in the cluster of Europe/South America1, besides presenting themselves in a distant cluster from the vaccine strains. This encourages further studies to elaborate an updated CDV vaccine formulation with broader coverage, perhaps reducing the incidence and consequently the endemic fashion of the disease.

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Declaration of Conflicting Interests

The authors declare that they have no conflict of interest.

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