Virus Research 264 (2019) 8–15

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Molecular analysis of the full-length F gene of Brazilian strains of canine distemper virus shows lineage co-circulation and variability between field and vaccine strains

Luana Almeida Freitas[a], Raquel Arruda Leme[a,b], Viviane Saporiti[a,b], Amauri Alcindo Alfieri[a,b,*], Alice Fernandes Alfieri[a,b]

[a] Laboratory of Animal Virology, Department of Veterinary Preventive Medicine, Universidade Estadual de Londrina. Rodovia Celso Garcia Cid - Campus Universitário, CEP 86057-970 - Londrina, PO Box 10011, Paraná, Brazil

[b] Multi-User Animal Health Laboratory, Molecular Biology Unit, Department of Veterinary Preventive Medicine, Universidade Estadual de Londrina. Rodovia Celso Garcia Cid - Campus Universitário, CEP 86057-970 - Londrina, PO Box 10011, Paraná, Brazil

ARTICLE INFO

Keywords:
Paramyxovirus
CDV
Fusion protein
Fsp-coding region
Rockborn-like lineage
Molecular epidemiology

ABSTRACT

Canine distemper is a highly contagious systemic viral disease, with worldwide distribution that affects a wide variety of terrestrial carnivores. This study characterized full-length fusion (F) genes from 15 Brazilian wild-type canine distemper virus (CDV) strains collected between 2003–2004 ($n = 6$) and 2013–2016 ($n = 9$). Using deduced amino acid (aa) sequence analysis, 14 strains were classified into Europe 1/South America 1 (EU1/SA1) lineage, with a temporal clustering into past (2003–2004) and contemporary (2013–2016) strains. One strain clustered to Rockborn-like lineage, showing high similarity (98.5%) with the Rockborn vaccine strain. In analyzed strains, the fusion protein signal-peptide (Fsp) coding region was highly variable at the aa level (67.4%–96.2%). The Brazilian strains were more Fsp-divergent from the North America 1 (NA1) strains (24.5%–36.3%) than from the Rockborn (11.2%–14.9%) vaccine strain. Seventeen cysteine residues in the full-length F gene and four non-conserved glycosylation sites in the Fsp region were detected. The results reveal that past and contemporary CDV strains are currently co-circulating. This first analysis of full-length F genes from Brazilian wild-type CDV strains contributes to knowledge of molecular epidemiology of CDV viral infection and evolution.

1. Introduction

Canine distemper virus (CDV) is a highly contagious pathogen for domestic dogs and many wild carnivore species (Fischer et al., 2016). CDV is the etiological agent of canine distemper (CD), a severe multisystemic and globally distributed disease (Panzera et al., 2015). Clinical manifestations of CDV infections vary widely and depend on factors such as individual immune status, vaccination history, and virulence of the CDV field strain. The main clinical symptoms include dermatological, respiratory, gastrointestinal, neurological, and immunological disorders such as immunosuppression (Martella et al., 2008).

CDV is representative of *Canine morbillivirus* species, genus *Morbillivirus* within the family *Paramyxoviridae*. The virus is enveloped and exhibits a non-segmented single-stranded negative-sense RNA genome of approximately 15.7 kb (Lamb and Parks, 2013). The CDV genome encodes eight viral proteins, two of which are nonstructural (C and V) and six are structural (hemagglutinin (H), fusion (F), matrix (M), phosphoprotein (P), large polymerase (L), and nucleocapsid (N) (Martella et al., 2008).

The H and F glycoproteins are located on the virus envelope and are more variable than the other CDV proteins (Bae et al., 2013; Martella et al., 2006; Sultan et al., 2009). The H protein helps viral host cell attachment, while the F protein mediates fusion between the virus and infected cells (Sawatsky and von Messling, 2010). Both glycoproteins are the antigenic determinants that induce protective immune responses against CDV (Hirama et al., 2003; Wild and Buckland, 1997). Genetic variation in H and F envelope genes is regarded as a possible cause of the increasing number of CDV infections in dogs (Lan et al., 2006; Lee et al., 2010; Martella et al., 2006).

Sequence analysis of the H gene has been widely studied and employed to characterize CDV field strains worldwide (Espinal et al., 2014; Ke et al., 2015; Negrão et al., 2013; Riley and Wilkes, 2015). In

* Corresponding author.
E-mail address: alfieri@uel.br (A.A. Alfieri).

https://doi.org/10.1016/j.virusres.2019.02.009
Received 19 December 2018; Received in revised form 16 January 2019; Accepted 18 February 2019
Available online 20 February 2019
0168-1702/

Table 1
Oligonucleotide primers used in the One-Step RT-PCR assay for the amplification of the canine distemper virus full-length F gene.

Primer	Sequence (5' – 3')	Genomic position*	Fragment length (bp)	Reference
CDV-1 Fw	CAGGACATAGCAAGCCA	4856-4872	588	This study
1Rev	AGTTTTATGACCAAGTAC	5426–5444		Romanutti et al., 2016
2F	TGGGATTATCGGGACTGA	5369–5387	558	Romanutti et al., 2016
3Rev	GGGCCAAATATTGACAAC	5909–5927		
4F	GTCCCTGCTATGCAACAT	5829–5847	483	Romanutti et al., 2016
5Rev	GGAGTTCTGGCTACAAATG	6293–6312		
6F	TGTGTATTCGTCTCAGA	6270–6287	797	Romanutti et al., 2016
CDV-7Rev	CTGAGCCCTAAGTTTTCT	7050-7067		This study

* Genomic position corresponding to the F gene sequence of the Onderstepoort strain (GenBank accession number AF305419).

addition, the F gene, especially the fusion protein signal-peptide (Fsp) coding region, has been the focus of recent studies due to its high variability. These studies have contributed to our knowledge of CDV molecular epidemiology and characterization of CDV field strains (Lee et al., 2010; Romanutti et al., 2016; Sarute et al., 2013, 2014b).

The F gene is synthesized initially as an inactive precursor, named F0, which is then cleaved by furin (von Messling and Cattaneo, 2002). Such cleavage is activated by a cellular serine protease that acts in the Golgi complex and plays a determining role in CDV pathogenicity. The CDV F gene open reading frame encodes 662 amino acids (aa) comprising the regions Fsp (aa 1–135), F2 (aa 136–224), and F1 (aa 225–662), which are produced via post-translational proteolysis (Romanutti et al., 2016). Cleavage releases the newly formed F1 N-terminus, thus forming the biologically active protein consisting of F1 and F2 subunits linked via a disulfide bond (Scheid and Choppin, 1977; von Messling and Cattaneo, 2002). The F1 subunit contains two hydrophobic regions that are important for membrane fusion, the fusion peptide (FP) domain on its N-terminus and the trans-membrane (TM) domain on its C-terminus (Lamb and Parks, 2013).

In Brazil, CD is one of the most important viral diseases affecting domestic dogs and is the principal cause of mortality in some urban canine populations (Headley et al., 2012). Increased disease incidence rates have been demonstrated worldwide, including in Brazil, and even in vaccinated dog populations (Budaszewski et al., 2014; Headley and Graça, 2000; Martella et al., 2008; Panzera et al., 2012). Most of the CDV-based molecular epidemiological studies conducted in Brazil analyzed the N and H genes (Amude et al., 2007b; Budaszewski et al., 2014; Castilho et al., 2007; Negrão et al., 2013; Rosa et al., 2012). To date, no studies have examined the full-length F gene to characterize Brazilian CDV field, although such studies have been conducted in other Asian and American countries (Lee et al., 2010; Romanutti et al., 2016; Sarute et al., 2014a; Sultan et al., 2009). In this context, the aim of this study was to molecularly characterize Brazilian CDV wild-type strains based on full-length F gene analysis.

2. Materials and methods

2.1. Sample collection

The samples included in this study were from a collection of CDV-positive canine biological samples collected between 2003–2004 (Amude et al., 2007a) and 2013–2016. These included whole blood, urine, and pooled cerebrum, cerebellum, and brainstem tissue fragments. The central nervous system pooled tissues were obtained from autopsy of the animals soon after death. All samples were stored at −80 °C.

We selected samples that were positive for a 287-bp fragment corresponding to the CDV N gene, as determined by conventional reverse transcription-polymerase chain reaction (RT-PCR) (Frisk et al., 1999; Saito et al., 2006). Twenty-one samples collected between 2003–2004 (past samples; n = 7) and 2013–2016 (contemporary samples; n = 14) from the domestic dogs of Northern Paraná, Brazil were included in the study.

2.2. Nucleic acid extraction

Nucleic acids were extracted from 250 µL of whole blood and tissue suspension samples using TRIzol® LS Reagent (Invitrogen™ Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and from 300 µL of urine samples using the silica/guanidinium isothiocyanate method (Boom et al., 1990). The nucleic acids were eluted in 30 µL of ultrapure RNase-free diethylpyrocarbonate (DEPC)-treated sterile water (Invitrogen™ Life Technologies, Carlsbad, CA, USA). During nucleic acid extraction and the subsequent procedures, an attenuated CDV vaccine strain and aliquots of ultrapure sterile water were used as positive and negative controls, respectively.

2.3. CDV F gene amplification

Four primer sets were used to amplify the full-length (2426 bp) CDV F gene (Table 1). The amplification reactions were performed in a final volume of 50 µL for each targeted fragment using a One-Step RT-PCR kit (Invitrogen™ Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The reaction solutions contained 5 µL of extracted nucleic acid, 25 µL of 2x reaction mix (buffer containing 0.4 mM of each dNTP and 3.2 mM $MgSO_4$), 20 pmol of each primer (forward and reverse), and 2 µL of SuperScript® III RT/Platinum® *Taq* mix. Reverse transcription reactions were performed at 55 °C for 30 min, and PCR amplifications were performed at 94 °C for 2 min for denaturation, followed by 40 cycles at 94 °C/15 s, 50 °C/30 s, and 68 °C/1 min, prior to a final extension at 68 °C for 5 min using the thermocycler ProFlex™ PCR System (Applied Biosystems™, Foster City, CA, USA). The nucleic acid extractions, the RT-PCR reactions to amplify the four target fragments, and the sequencing reactions were performed in triplicate.

2.4. CDV F gene sequencing and comparative analyses

The amplicons were purified using the PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen™ Life Technologies, Carlsbad, CA, USA), quantified by a Qubit® Fluorometer (Invitrogen™ Life Technologies, Eugene, OR, USA), and sequenced in both directions with forward and reverse primers using an ABI 3500 Genetic Analyzer Sequencer with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, Foster City, CA, USA). Sequence quality analyses and consensus sequences were assembled using Phred/Phrap/CAP3 software (http://asparagin.cenargen.embrapa.br/phph/). Similarity searches were performed with sequences deposited in GenBank using Basic Local Alignment Search Tool (BLAST) software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence identity matrix was constructed using BioEdit software version 7.1.11 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Only full-length CDV F gene sequences were selected for analysis (Table 2), with the exception of a partial F gene aa sequence from a Rockborn vaccine strain

Table 2
GenBank accession numbers and origin of the full-length CDV F gene sequences used in phylogenetic analysis.

Origin	Country	Strain	Accession numbers
Vaccine	UK	Onderstepoort	AF305419
	USA	Onderstepoort	AF378705
	USA	Snyder Hill	JN896987
America	USA	18133	AY964108
	USA	21261	AY964112
	USA	25,259	AY964114
	USA	171391-513	KJ123771
	USA	01-2689	AY649446
	USA	164071	EU716337
	Argentina	Benito	KT224731
	Argentina	Pampa	KT224732
	Uruguay	Uy251	KM280689
Europe	Germany	5804P	AY386316
	Italy	CDV2784/2013	KF914669
	Switzerland	A75-17	AF112188
Asia	China	GZ2	JN381189
	China	GN	EF596900
	China	HL	EF596901
	China	SC01	EF596902
	Indian	Ludhiana	LC011102
	Japan	55L	AB475099
	Japan	009L	AB475101
	Japan	50Cbl	AB476403
	Taiwan	TW-TP1	EU191985
	Taiwan	TW-KS15	EU192026
	Thailand	270Lu	AB509346
Africa	Tanzania	Lion94SNP	JN812977

(GenBank accession number AF026244). Phylogenetic trees based on aa were obtained using the neighbor joining statistical method based on the maximum composite likelihood model (MEGA v. 6), which provided statistical support via bootstrapping with 1000 replicates. The GenBank accession numbers of the nucleotides (nt) sequences described in this study are KY057345 to KY057359.

3. Results

3.1. Phylogenetic analysis of full-length F gene

Full-length CDV F genes were successfully amplified from 15 of 21 samples, including urine (n = 6), whole blood (n = 5), and central nervous system tissues (n = 4). From samples spanning the collection period, complete F genes were characterized in 6/7 past samples and 9/14 contemporary samples. Table 3 summarizes the sampling data according to the collection year and the age, gender, vaccination, and health status of 15 dogs.

Deduced full-length F gene aa sequence analysis of CDV showed that most of the Brazilian strains clustered into the Europe 1/South America 1 (EU1/SA1) clade and separeted of the other representative strains South America 2 (SA2), Europe 2 (EU2), Asia 1 (AS1), Asia 2 (AS2), North America 1 (NA1), North America 2 (NA2), Rockborn-like, and Artic-like (Fig. 1A). A comparison of the architecture of phylogenetic trees constructed based on the deduced full-length and partial (Fsp region) F gene aa sequences revealed similar clade patterns and bootstrap values (Fig. 1B).

The Brazilian CDV strains were not grouped according to the vaccination status of the animals included in this study. Fourteen wild-type Brazilian strains were closely related to each other, sharing the EU1/SA1 clade. As expected, two clusters within this clade composed of CDV strains from samples collected between 2003–2004 and 2013–2016 were noted. However, two exceptions were observed, the BRA/UEL-BLU/13 CDV strain from 2013, which was clustered with strains collected between 2003 and 2004, and the BRA/UEL-30/04 CDV strain, which was more similar to the European 5804 P isolate than to any other Brazilian field strains.

A single CDV strain, BRA/UEL-FRD/15, was clustered into the Rockborn-like clade together with the 25,259 strain, a canine CDV strain isolated in the USA (Pardo et al., 2005). To confirm this unexpected result, a new aa alignment and phylogenetic tree were constructed based on the Fsp region; these analyses included an aa sequence from the Rockborn vaccine strain (Liermann et al., 1998). The results confirmed the close phylogenetic relationship between BRA/UEL-FRD/15 and the other Rockborn strains (data not shown).

Fsp aa sequence analysis of the Brazilian wild-type strains showed similarities ranging from 86.6% to 100%. Higher divergence (13.4%) was observed between the BRA/UEL-30/04 – BRA/UEL-SIM/13 and BRA/UEL-FRD/15 – BRA/UEL-BLU/13 strains. A comparison of the deduced aa sequences in this study with CDV strains from other countries revealed similarities ranging from 67.4% to 96.2%, with the greatest divergence (32.6%) observed for the Italian CDV2784/2013 strain. Most of the aa sequences were highly similar (89.6% to 92.5%) to the Uruguayan Uy251 strain. Exceptions included the BRA/UEL-30/04 and BRA/UEL-FRD/15 CDV strains, which presented similarities of 91.1% and 96.2% with the European 5804 P and American 25,259 strains, respectively. Alignment of the Fsp regions of the samples herein with the CDV vaccine strains included in the NA1 clade showed an even higher divergence, with values ranging from 24.5% to 36.3% (Fig. S1 in the supplementary material).

When the Fsp aa sequences of the Brazilian strains were compared with those of the Rockborn vaccine strain, lower divergence values were obtained, ranging from 11.2% to 14.9%. An exception was the BRA/UEL-FRD/15 strain, which presented the lowest divergence value (1.5%) from the Rockborn vaccine strain.

Unlike the high variation observed in the Fsp region, the aa sequences of the F2 and F1 regions were more homogeneous. The similarities among Brazilian strains for these two regions were ≥ 96.1%. When aligned with wild-type strains from other countries, the deduced aa sequence similarities ranged from 94.3% to 100% for F2 and from 94.9% to 99.5% for F1. Similarities of the Brazilian strains with the CDV vaccine strains were 93.2% to 98.8% for F2 and 94.5% to 97.4% for F1.

3.2. Cysteine residues and N-linked glycosylation sites

Seventeen cysteine residues were identified in the full-length F genes of the Brazilian CDV strains analyzed in this study. Of these cysteine residues 16 were located at the same positions of those in the reference vaccine strains. All the Brazilian wild-type strains presented one additional cysteine residue at aa position 67 within the Fsp region (Fig. 2).

Four glycosylation sites (N-X-S/T) were found in the Fsp region of the Brazilian sequences; however, none were conserved. The glycosylation sites at positions 62–64 and 76–78 were common to at least one of the vaccine strains analyzed. Two additional potential sites were identified in this region, one between residues 88–90 (N-H-T) that was specific to the BRA/UEL-MEL/16 strain and another between residues 108–110 (N-A-T) in the BRA/UEL-30/04 and BRA/UEL-FRD/15 strains (Fig. 2). In the other regions, four conserved glycosylation sites were identified, three at positions 141–143, 173–175, and 179–181 within the F2 region (Fig. S2 in the supplementary material) and one at position 517–519 within the F1 region (Fig. S3 in the supplementary material).

3.3. Cleavage sites and hydrophobic region analysis

The two conserved cleavage sites in the Fsp C-terminus (A↓QIHW) and the F2 C-terminus (RRQRR) regions were present in all the Brazilian wild-type CDV strains (Fig. S2 in the supplementary material).

The FP domain located in the F1 subunit N-terminus was highly conserved in all the CDV strains analyzed. However, the TM domain in the F1 C-terminus showed aa differences when compared to other CDV

Table 3
Sampling data according to the year of collection and animal age, sex, and health status.

Identification	Sample[a]	Year of collection	Animal data			
			Age (months)	Gender[b,c]	Vaccination status[d,e,f]	Main clinical signs
BRA/UEL-05/03	Whole Blood	2003	36	F	NV	Weakness of hind limbs, spastic tetraparesis/plegia, ataxia, and nystagmus.
BRA/UEL-13/03	Whole Blood	2003	132	F	NV	Personality change, compulsive walking, ataxia, head pressing, postural reactions deficits, and tetraparesis.
BRA/UEL-15/03	CNS	2003	62	M	V	Weakness of the hind limbs, ataxia, nystagmus, spastic tetraparesis/plegia, and myoclonus.
BRA/UEL-18/03	Whole Blood	2003	21	M	NA	Ataxia, spastic tetraparesis, nystagmu, and postural reactions deficits.
BRA/UEL-16/04	Whole Blood	2004	7	F	V	Compulsive walking, seizure, nystagmus, menace deficit, spastic tetraparesis, and postural reactions deficits.
BRA/UEL-30/04	CNS	2004	55	M	V	Ataxia, head tilt to the right, spastic tetraparesis/plegia, and nystagmus.
BRA/UEL-BLU/13	Urine	2013	96	M	NV	Weakness of hind limbs, ocular discharge, lateral recumbency, and pneumonia.
BRA/UEL-SIM/13	Urine	2013	Adult	M	NA	Head Tilt, spastic tetraparesis, hip dysplasia and pain in cervicothoracic region.
BRA/UEL-MEG/14	CNS	2014	2	F	NV	Ocular discharge, lateral recumbency, excessive vocalization, myoclonus, and dry fur.
BRA/UEL-LLA/14	Urine	2014	21	F	NA	Stupor, ataxia, spastic tetraparesis, decreased nasal mucosal sensitivity, myoclonus, and cervical pain.
BRA/UEL-FRD/15	CNS	2015	2	M	NA	Excessive vocalization, dry eyes, and bloody stools.
BRA/UEL-ZNH/16	Urine	2016	15	M	NV	Weakness of hind limbs, myoclonus, trismu, and enophthalmos.
BRA/UEL-LLK/16	Whole Blood	2016	Adult	F	NV	Ocular discharge, incoordination, myoclonus and weakness of hind limbs.
BRA/UEL-TCO/16	Urine	2016	Adult	M	NV	Pneumonia, lateral recumbency, and otitis.
BRA/UEL-MEL/16	Urine	2016	60	F	V	Spastic tetraparesis/plegia, weakness of hind limbs and pain in cervicothoracic region.

[a] CNS: Central Nervous System.
[b] M: male.
[c] F: female.
[d] V: vaccinated.
[e] NV: non-vaccinated.
[f] NA: not available.

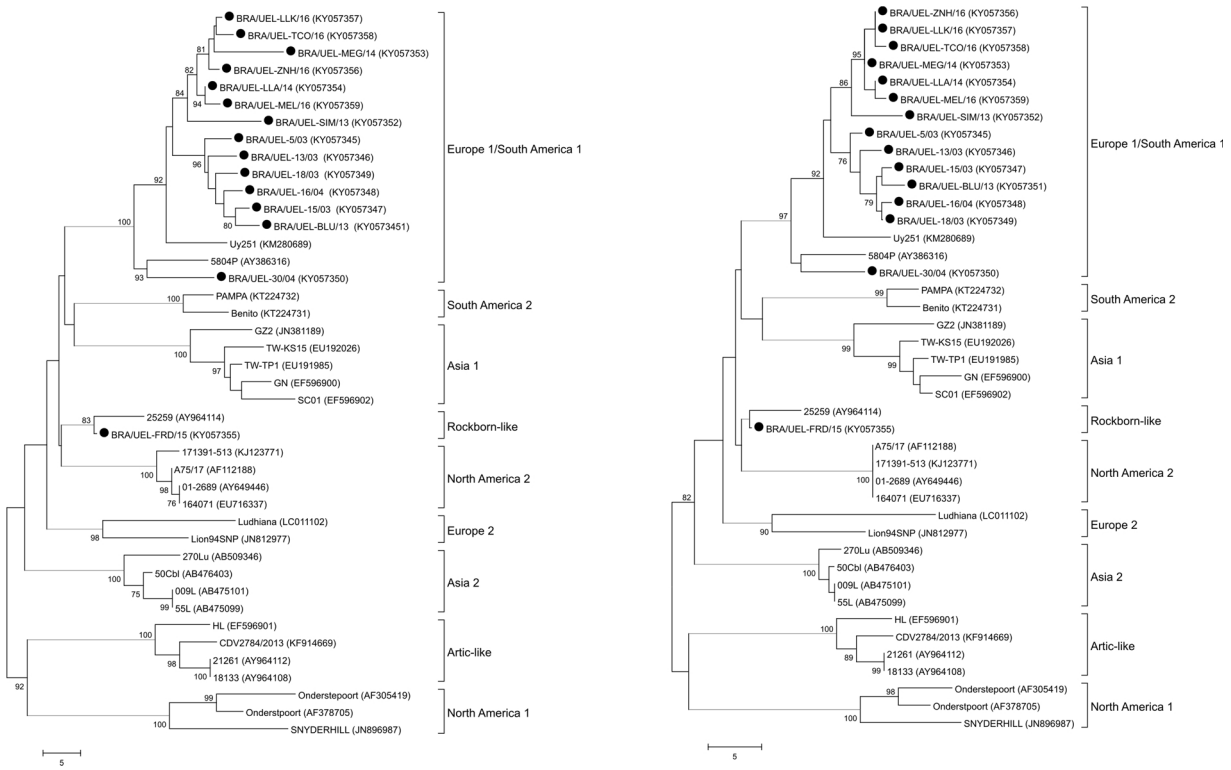

Fig. 1. Phylogenetic analysis of the deduced amino acid sequences of the full-length F genes (panel A) and fusion protein signal-peptide (Fsp) coding region (panel B) of Brazilian CDV field strains. Neighbor joining phylogenetic tree construction using the maximum composite likelihood model was employed. Bootstrap values were determined in 1000 replications; only bootstrap values ≥75% are shown. The GenBank accession numbers of representative sequences and of the CDV Brazilian strains (black circles) analyzed herein are indicated in parentheses.

sequences, including the vaccine strains. A common change at position 616 (S→I) was found in all the Brazilian isolates studied. Only the BRA/UEL-30/04 and BRA/UEL-SIM/13 strains presented a specific change at position 618 (T→I). Another common change was found at position 621 (A→V), except in the BRA/UEL-30/04 and BRA/UEL-FRD/15 strains, which exhibited the same aa as the vaccine strains. The aa at position 624 was changed from L→F for all the Brazilian isolates, except in the BRA/UEL-FRD/15 strain (Fig. S3 in the supplementary material).

4. Discussion

The available molecular data on Brazilian CDV strains are based on the N and H genes; this study represents the first full-length F gene sequence analysis of Brazilian wild-type CDV strains. The phylogenetic analysis revealed the circulation of two CDV lineages in Brazil, the EU1/SA1 and Rockborn-like strains, which agrees with the previous H gene-based studies of Brazilian wild-type strains (Budaszewski et al., 2014; Negrão et al., 2013; Rosa et al., 2012). The predominance of the EU1/SA1 lineage strain was identified herein, including high similarities of the Brazilian CDV aa sequences with the Uruguayan Uy251 strain and their clustering in a separate Argentinean strain clade, classified as SA2. The BRA/UEL-30/04 strain was closely related to the European 5804 P strain. Although there was no geographical grouping of this strain with other Brazilian strains, a temporal pattern was observed. The European 5804 P strain was obtained in 2003 after the virulent 5804 strain was serially passaged in domestic ferrets, and the wild-type 5804 strain was isolated in 1989 after a distemper outbreak in a dog shelter in northern Germany (von Messling et al., 2003). Considering that the strains analyzed in this study were collected through 2016 and that the virus was introduced to Brazil (the onset of SA1 lineage) around 1989 (Fischer et al., 2016), the EU1/SA1 lineage has presumably been circulating in Brazilian territories for approximately 25 years. Additionally, the Brazilian full-length F gene sequences within the EU1/SA1 clade formed two groups according to

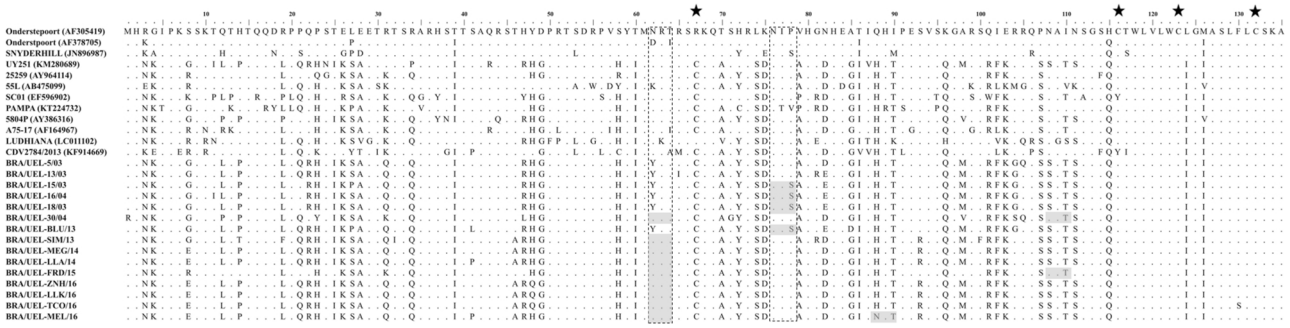

Fig. 2. Alignment of the deduced amino acid sequences of the fusion protein signal-peptide (Fsp) coding region within the F gene of the CDV strains. Only amino acids different from those of the Onderstepoort vaccine sequence (AF305419) are shown. Potential N-linked glycosylation sites (N-X-S/T) are shaded gray and open rectangles indicate glycosylation sites common to at least one vaccine strain analyzed. Stars indicate cysteine residues.

their year of collection, revealing a temporal division of the Brazilian strains into past (2003–2004) and contemporary (2013–2016) CDV strains. However, the clustering of strains obtained in 2003–2004 with that of a 2013 Brazilian strain (BRA/UEL-BLU/13) suggests the likely current co-circulation of past and contemporary CDV strains.

The high genetic identity among the strains of this study with the European and American strains suggests that CDVs in these geographical regions have a common ancestral origin. A study suggested that current CDV strains emerged in the United States in the 1880s and that their ancestor diversified through time into two ancestral clades, one that spread worldwide and originated eight current lineages and another that produced the NA1 lineage, which comprises most vaccine strains (Panzera et al., 2015). The Brazilian territorial extension together with increasing incidences of global animal displacement also support the possibility of concomitant circulation of different CDV lineages in Brazil (Negrão et al., 2013).

The Brazilian CDV strains were not grouped according to the vaccination status of the animals included in this survey. However, all the wild-type CDV strains herein were distant from the NA1 lineage, in which most vaccine strains are included. This result agrees with those of other studies that analyzed the H and F CDV gene sequences (Martella et al., 2006; Negrão et al., 2013; Romanutti et al., 2016; Rosa et al., 2012; Sarute et al., 2014b). Phylogenetic analysis of the full-length F gene was compared with that of its most variable region (Fsp); both phylogenetic trees showed similar classifications by grouping the analyzed CDV strains into the same clades. Our results revealed a 24.5–36.3% divergence in the Fsp region among the Brazilian CDV field isolates and the Onderstepoort and Snyder Hill vaccine strains. The results of the comparison of Fsp aa sequences of the Brazilian strains with that of the Rockborn vaccine isolate revealed lower divergence (11.2% to 14.9%), suggesting that the Brazilian strains are more closely related to the Rockborn vaccine isolate than to the NA1 vaccine strains. These results confirm previous reports that indicated that Fsp-coding region sequence analysis is suitable for evolutionary studies because it allows for the straightforward identification of CDV lineages (Sarute et al., 2013, 2014b; Sultan et al., 2009). In contrast to the high aa divergence in the Fsp region, lower variations were verified in the F1 and F2 regions. The latter region exhibited the most conserved aa sequences of the three F gene regions, which agrees with previous studies (Lee et al., 2010; Sultan et al., 2009; von Messling and Cattaneo, 2002).

The Rockborn vaccine strain was isolated in the 1950s from primary canine kidney cells (Rockborn, 1958); however, this vaccine strain is not located within the NA1 clade. This CDV strain was withdrawn from several markets in the mid-1990s due to suspected cases of post-vaccinal encephalitis and the resulting belief that this strain was less attenuated and less safe than other CDV strains (Budaszewski et al., 2014; Martella et al., 2011). However, Martella et al. (2011) suggested that Rockborn-like strains are still available in commercial vaccines. Fsp phylogenetic analysis revealed that the Brazilian BRA/UEL-FRD/15 strain was grouped with the 25,259 and Rockborn vaccine isolates. Classification of the 25,259 isolate as a Rockborn-like strain was performed in previous studies based on the H gene (Espinal et al., 2014; Ke et al., 2015; Martella et al., 2011). The phylogenetic result and high similarity values between the BRA/UEL-FRD/15 strain with the Rockborn vaccine isolate and the 25,259 strain (98.5% and 96.2%, respectively) in the present study confirm this classification and include the BRA/UEL-FRD/15 as a Rockborn-like strain.

The BRA/UEL-FRD/15 strain was obtained from a two-month-old pup, and limited information regarding its clinical history was available. This animal was purchased 15 days prior to the presentation of clinical symptoms, including excessive vocalization, dry eyes, and bloody stools, and no vaccination history was available. Based on these data, we formulated three hypotheses. The first was that the pup was not vaccinated and that the reported clinical manifestations were likely due to infection with a wild-type Rockborn-like CDV strain that was circulating in Brazil. The second hypothesis was that the unvaccinated pup had natural contact with an attenuated Rockborn CDV strain of vaccine origin that converted into the observed clinical manifestations. Finally, the third hypothesis was that the pup was vaccinated and presented subsequent post-vaccine encephalitis due to reversal of the Rockborn CDV strain virulence, as previously described (Hartley, 1974; Martella et al., 2011).

The two F gene cleavage sites (A↓QIHW and RRQRR) were conserved in all the CDV Brazilian strains. Cleavage in the Fsp region is necessary for F gene activation and cell surface expression. The Fsp region is cleaved at an early stage of F protein processing and is not present in the mature F protein (von Messling and Cattaneo, 2002); however, previous reports have demonstrated that some mutations within the Fsp peptide can lead to changes in the fusogenicity of the resulting protein (Plattet et al., 2007; von Messling and Cattaneo, 2002).

In this study, four non-conserved N-glycosylation sites within the Fsp region of the Brazilian strains were found; two extra N-glycosylation sites were observed in three of the 15 Brazilian CDV strains. Furthermore, an additional cysteine residue (at position 67) was identified in the Brazilian CDV sequences. N-linked glycosylation of viral envelope proteins plays critical roles in viral life cycle and virulence mechanisms, such as binding to cell surface receptors and protecting against neutralization via antibody (Kim et al., 2008; Moeller-Ehrlich et al., 2007). The various F protein glycosylation sites suggest that F proteins have different biological and/or morphological features; however, more studies are needed to evaluate these purposes. Cysteine amino acids are important factors in the formation of the intra molecular disulfide bonds and the steric protein structure (Romanutti et al., 2016; Sultan et al., 2009). This additional residue was also reported by authors who analyzed strains from other countries, indicating changes in protein structure (Romanutti et al., 2016; Sultan et al., 2009).

The membrane-anchored F1 subunit of paramyxoviruses contains two hydrophobic domains, the FP and TM segments. The FP domain was conserved in all the CDV strains of this study; the TM domain of the Brazilian strains showed aa changes when compared with the vaccine strains. Hydrophobic FP domains are conserved regions of paramyxovirus fusion-mediating glycoproteins that contain conserved glycine residues. These residues appear to play primary roles in regulating activation of the metastable native form of the F gene and fusion (Russell et al., 2001, 2004). The TM domain anchors the protein to the cell membrane; modifications in this domain can result in modulation of viral fusion protein activity, including impairment of fusion pore openings, enlargement, or fusion promotion efficiency alterations (Bissonnette et al., 2009; Muhlebach et al., 2008). Further studies based on the full-length F gene are needed to better elucidate the variable relationship among TM domains in the CDV fusion process. Together, the findings of this study suggest possible structural alterations of the F gene, resulting in the potential modulation of antigenicity, or even virulence, of these CDV strains.

The present study is the first to analyze the full-length F genes of wild-type CDV strains infecting domestic dogs in Brazil. The results presented herein indicate co-circulation of two CDV lineages in the country and suggest that the EU1/SA1 strain has been circulating in Brazil for 25 years. Co-circulation of past and contemporary CDV strains in Brazil was also demonstrated, as the analyzed CDV Brazilian strains were collected approximately 10 years apart. The CDV wild-type strains described in this study presented F gene variability when compared to the vaccine strains. However, comparisons of the Fsp regions of the Brazilian strains with those of the vaccine isolates revealed that the strains herein are more similar to the Rockborn vaccine strain than to the NA1 vaccine isolates. Thus, we conclude that the F gene is useful for investigating the molecular diversity of CDV, thus contributing to understanding the epidemiology of the virus. Changes to the fusion protein likely lead to pathogenicity and/or immunogenicity alterations; however, additional studies based on analyses of the full-length CDV F gene are required to elucidate the biological roles of this protein.

Conflict of interest

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

Acknowledgments

We thank the following Brazilian Institutes for financial support: the National Council of Scientific and Technological Development (CNPq), the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES), Financing of Studies and Projects (FINEP), and the Araucaria Foundation (FAP/PR). Alfieri, A.A., Alfieri, A.F., and Leme, R.A. are recipients of CNPq fellowships.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.02.009.

References

Amude, A.M., Alfieri, A.A., Alfieri, A.F., 2007a. Clinicopathological findings in dogs with distemper encephalomyelitis presented without characteristic signs of the disease. Res. Vet. Sci. 82, 416–422. https://doi.org/10.1016/j.rvsc.2006.08.008.

Amude, A.M., Carvalho, Gd.A., Alfieri, A.A., Alfieri, A.F., 2007b. Virus isolation and molecular characterization of canine distemper virus by RT-PCR from a mature dog with multifocal encephalomyelit. Braz. J. Microbiol. 38, 354–356. https://doi.org/10.1590/S1517-83822007000200031.

Bae, C.W., Lee, J.B., Park, S.Y., Song, C.S., Lee, N.H., Seo, K.H., Kang, Y.S., Park, C.K., Choi, I.S., 2013. Deduced sequences of the membrane fusion and attachment proteins of canine distemper viruses isolated from dogs and wild animals in Korea. Virus Genes 47, 56–65. https://doi.org/10.1007/s11262-013-0916-0.

Bissonnette, M.L., Donald, J.E., DeGrado, W.F., Jardetzky, T.S., Lamb, R.A., 2009. Functional analysis of the transmembrane domain in paramyxovirus F protein-mediated membrane fusion. J. Mol. Biol. 386, 14–36. https://doi.org/10.1016/j.jmb.2008.12.029.

Boom, R., Sol, C.J., Salimans, M.M., Jansen, C.L., Wertheim-van Dillen, P.M., van der Noordaa, J., 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28, 495–503 0095-1137/90/030495-09$02.00/0.

Budaszewski, R.F., Pinto, L.D., Weber, M.N., Caldart, E.T., Alves, C.D., Martella, V., Ikuta, N., Lunge, V.R., Canal, C.W., 2014. Genotyping of canine distemper virus strains circulating in Brazil from 2008 to 2012. Virus Res. 180, 76–83. https://doi.org/10.1016/j.virusres.2013.12.024.

Castilho, J.G., Brandão, P.E., Carnieli Jr, P., Oliveira, R.N., Macedo, C.I., Peixoto, Z.M.P., Carrieri, M.L., Kotait, I., 2007. Molecular analysis of the N gene of canine distemper virus in dogs in Brazil. Arq. Bras. Med. Vet. Zootec. 59, 654–659. https://doi.org/10.1590/S0102-09352007000300016.

Espinal, M.A., Diaz, F.J., Ruiz-Saenz, J., 2014. Phylogenetic evidence of a new canine distemper virus lineage among domestic dogs in Colombia, South America. Vet. Microbiol. 172, 168–176. https://doi.org/10.1016/j.vetmic.2014.05.019.

Fischer, C.D., Graf, T., Ikuta, N., Lehmann, F.K., Passos, D.T., Makiejczuk, A., Silveira Jr, M.A., Fonseca, A.S., Canal, C.W., Lunge, V.R., 2016. Phylogenetic analysis of canine distemper virus in South America clade 1 reveals unique molecular signatures of the local epidemic. Infect. Genet. Evol. 41, 135–141. https://doi.org/10.1016/j.meegid.2016.03.029.

Frisk, A.L., Konig, M., Moritz, A., Baumgartner, W., 1999. Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. J. Clin. Microbiol. 37, 3634–3643. https://doi.org/0095-1137/99/$04.0010.

Hartley, W.J., 1974. A post-vaccinal inclusion body encephalitis in dogs. Vet. Pathol. 11, 301–312. https://doi.org/10.1177/030098587401100403.

Headley, S.A., Graça, D.L., 2000. Canine distemper: epidemiological findings of 250 cases. Braz. J. Vet. Res. Anim. Sci. 37https://doi.org/10.1590/S1413-95962000000200009. 00-00.

Headley, S.A., Amude, A.M., Alfieri, A.F., Bracarense, A.P.F.R.L., Alfieri, A.A., 2012. Epidemiological features and the neuropathological manifestations of canine distemper virus induced infections in Brazil: a review. Semina Cienc. Agrar. 33, 1945–1978. https://doi.org/10.5433/1679-0359.2012v33n5p1945.

Hirama, K., Togashi, K., Wakasa, C., Yoneda, M., Nishi, T., Endo, Y., Miura, R., Tsukiyama-Kohara, K., Kai, C., 2003. Cytotoxic T-lymphocyte activity specific for hemagglutinin (H) protein of canine distemper virus in dogs. J. Vet. Med. Sci. 65, 109–112. https://doi.org/10.1292/jvms.65.109.

Ke, G.M., Ho, C.H., Chiang, M.J., Sanno-Duanda, B., Chung, C.S., Lin, M.Y., Shi, Y.Y., Yang, M.H., Tyan, Y.C., Liao, P.C., Chu, P.Y., 2015. Phylodynamic analysis of the canine distemper virus hemagglutinin gene. BMC Vet. Res. 11, 164. https://doi.org/10.1186/s12917-015-0491-9.

Kim, J.M., Yun, S.I., Song, B.H., Hahn, Y.S., Lee, C.H., Oh, H.W., Lee, Y.M., 2008. A single N-linked glycosylation site in the Japanese encephalitis virus prM protein is critical for cell type-specific prM protein biogenesis, virus particle release, and pathogenicity in mice. J. Virol. 82, 7846–7862. https://doi.org/10.1128/jvi.00789-08.

Lamb, R.A., Parks, G.D., 2013. Paramyxoviridae: the viruses and their replication. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 6th ed. Lippincott Williams & Wilkins, Philadelphia, pp. 957–995.

Lan, N.T., Yamaguchi, R., Inomata, A., Furuya, Y., Uchida, K., Sugano, S., Tateyama, S., 2006. Comparative analyses of canine distemper viral isolates from clinical cases of canine distemper in vaccinated dogs. Vet. Microbiol. 115, 32–42. https://doi.org/10.1016/j.vetmic.2006.01.010.

Lee, M.S., Tsai, K.J., Chen, L.H., Chen, C.Y., Liu, Y.P., Chang, C.C., Lee, S.H., Hsu, W.L., 2010. The identification of frequent variations in the fusion protein of canine distemper virus. Vet. J. 183, 184–190. https://doi.org/10.1016/j.tvjl.2008.10.001.

Liermann, H., Harder, T.C., Lochelt, M., Von Messling, V., Baumgartner, W., Moennig, V., Haas, L., 1998. Genetic analysis of the central untranslated genome region and the proximal coding part of the F gene of wild-type and vaccine canine distemper morbilliviruses. Virus Genes 17, 259–270. https://doi.org/10.1023/a:1008069805011.

Martella, V., Cirone, F., Elia, G., Lorusso, E., Decaro, N., Campolo, M., Desario, C., Lucente, M.S., Bellacicco, A.L., Blixenkrone-Moller, M., Carmichael, L.E., Buonavoglia, C., 2006. Heterogeneity within the hemagglutinin genes of canine distemper virus (CDV) strains detected in Italy. Vet. Microbiol. 116, 301–309. https://doi.org/10.1016/j.vetmic.2006.04.019.

Martella, V., Elia, G., Buonavoglia, C., 2008. Canine distemper virus. Vet. Clin. North Am. Small Anim. Pract. 38, 787–797. https://doi.org/10.1016/j.cvsm.2008.02.007. vii-viii.

Martella, V., Blixenkrone-Moller, M., Elia, G., Lucente, M.S., Cirone, F., Decaro, N., Nielsen, L., Banyai, K., Carmichael, L.E., Buonavoglia, C., 2011. Lights and shades on an historical vaccine canine distemper virus, the Rockborn strain. Vaccine 29, 1222–1227. https://doi.org/10.1016/j.vaccine.2010.12.001.

Moeller-Ehrlich, K., Ludlow, M., Beschorner, R., Meyermann, R., Rima, B.K., Duprex, W.P., Niewiesk, S., Schneider-Schaulies, J., 2007. Two functionally linked amino acids in the stem 2 region of measles virus haemagglutinin determine infectivity and virulence in the rodent central nervous system. J. Gen. Virol. 88, 3112–3120. https://doi.org/10.1099/vir.0.83235-0.

Muhlebach, M.D., Leonard, V.H., Cattaneo, R., 2008. The measles virus fusion protein transmembrane region modulates availability of an active glycoprotein complex and fusion efficiency. J. Virol. 82, 11437–11445. https://doi.org/10.1128/jvi.00779-08.

Negrão, F.J., Gardinali, N.R., Headley, S.A., Alfieri, A.A., Fernandez, M.A., Alfieri, A.F., 2013. Phylogenetic analyses of the hemagglutinin gene of wild-type strains of canine distemper virus in southern Brazil. Genet. Mol. Res. 12, 2549–2555. https://doi.org/10.4238/2013.March.11.2.

Panzera, Y., Calderon, M.G., Sarute, N., Guasco, S., Cardeillac, A., Bonilla, B., Hernandez, M., Francia, L., Bedo, G., La Torre, J., Perez, R., 2012. Evidence of two co-circulating genetic lineages of canine distemper virus in South America. Virus Res. 163, 401–404. https://doi.org/10.1016/j.virusres.2011.10.008.

Panzera, Y., Sarute, N., Iraola, G., Hernandez, M., Perez, R., 2015. Molecular phylogeography of canine distemper virus: geographic origin and global spreading. Mol. Phylogenet. Evol. 92, 147–154. https://doi.org/10.1016/j.ympev.2015.06.015.

Pardo, I.D., Johnson, G.C., Kleiboeker, S.B., 2005. Phylogenetic characterization of canine distemper viruses detected in naturally infected dogs in North America. J. Clin. Microbiol. 43, 5009–5017. https://doi.org/10.1128/jcm.43.10.5009-5017.2005.

Plattet, P., Cherpillod, P., Wiener, D., Zipperle, L., Vandevelde, M., Wittek, R., Zurbriggen, A., 2007. Signal peptide and helical bundle domains of virulent canine distemper virus fusion protein restrict fusogenicity. J. Virol. 81, 11413–11425. https://doi.org/10.1128/jvi.01287-07.

Riley, M.C., Wilkes, R.P., 2015. Sequencing of emerging canine distemper virus strain reveals new distinct genetic lineage in the United States associated with disease in wildlife and domestic canine populations. Virol. J. 12, 219. https://doi.org/10.1186/s12985-015-0445-7.

Rockborn, G., 1958. Canine distemper virus in tissue culture. Arch. Fã¼r Die Gesamte Virusforsch. 8, 485–492. https://doi.org/10.1007/bf01243067.

Romanutti, C., Gallo Calderon, M., Keller, L., Mattion, N., La Torre, J., 2016. RT-PCR and sequence analysis of the full-length fusion protein of Canine Distemper Virus from domestic dogs. J. Virol. Methods 228, 79–83. https://doi.org/10.1016/j.jviromet.2015.11.011.

Rosa, G.N., Domingues, H.G., Santos, M.M.A.Bd., Felippe, P.A.N., Spilki, F.R., Arns, C.W., 2012. Molecular detection and phylogenetic analysis of the gene H from canine distemper virus isolates circulating at the municipality of Campinas, São Paulo. Pesqui. Vet. Bras. 32, 72–77. https://doi.org/10.1590/S0100-736X2012000100012.

Russell, C.J., Jardetzky, T.S., Lamb, R.A., 2001. Membrane fusion machines of paramyxoviruses: capture of intermediates of fusion. EMBO J. 20, 4024–4034. https://doi.org/10.1093/emboj/20.15.4024.

Russell, C.J., Jardetzky, T.S., Lamb, R.A., 2004. Conserved glycine residues in the fusion peptide of the paramyxovirus fusion protein regulate activation of the native state. J. Virol. 78, 13727–13742. https://doi.org/10.1128/jvi.78.24.13727-13742.2004.

Saito, T.B., Alfieri, A.A., Wosiacki, S.R., Negrão, F.J., Morais, H.S., Alfieri, A.F., 2006. Detection of canine distemper virus by reverse transcriptase-polymerase chain reaction in the urine of dogs with clinical signs of distemper encephalitis. Res. Vet. Sci. 80, 116–119. https://doi.org/10.1016/j.rvsc.2005.03.002.

Sarute, N., Calderon, M.G., Perez, R., La Torre, J., Hernandez, M., Francia, L., Panzera, Y., 2013. The fusion protein signal-peptide-coding region of canine distemper virus: a useful tool for phylogenetic reconstruction and lineage identification. PLoS One 8, e63595. https://doi.org/10.1371/journal.pone.0063595.

Sarute, N., Delgado, M.V., Carrau, L., Benech, A., Francia, L., Pérez, R., Panzera, Y., 2014a. First genome sequence of a canine distemper virus strain from South America. Genome Announc. 2, e01009–e01014. https://doi.org/10.1128/genomeA.01009-14.

Sarute, N., Perez, R., Aldaz, J., Alfieri, A.A., Alfieri, A.F., Name, D., Llanes, J., Hernandez, M., Francia, L., Panzera, Y., 2014b. Molecular typing of canine distemper virus strains

reveals the presence of a new genetic variant in South America. Virus Genes 48, 474–478. https://doi.org/10.1007/s11262-014-1054-z.

Sawatsky, B., von Messling, V., 2010. Canine distemper viruses expressing a hemagglutinin without N-glycans lose virulence but retain immunosuppression. J. Virol. 84, 2753–2761. https://doi.org/10.1128/jvi.01813-09.

Scheid, A., Choppin, P.W., 1977. Two disulfide-linked polypeptide chains constitute the active F protein of paramyxoviruses. Virology 80, 54–66. https://doi.org/10.1016/0042-6822(77)90380-4.

Sultan, S., Charoenvisal, N., Lan, N.T., Yamaguchi, R., Maeda, K., Kai, K., 2009. The Asia 2 specific signal peptide region and other domains in fusion protein genes characterized Asia 1 and Asia 2 canine distemper viruses. Virol. J. 6, 157. https://doi.org/10.1186/1743-422x-6-157.

von Messling, V., Cattaneo, R., 2002. Amino-terminal precursor sequence modulates canine distemper virus fusion protein function. J. Virol. 76, 4172–4180. https://doi.org/10.1128/JVI.76.9.4172-4180.2002.

von Messling, V., Springfeld, C., Devaux, P., Cattaneo, R., 2003. A ferret model of canine distemper virus virulence and immunosuppression. J. Virol. 77, 12579–12591. https://doi.org/10.1128/JVI.77.23.12579-12591.2003.

Wild, T.F., Buckland, R., 1997. Inhibition of measles virus infection and fusion with peptides corresponding to the leucine zipper region of the fusion protein. J. Gen. Virol. 78 (Pt 1), 107–111. https://doi.org/10.1099/0022-1317-78-1-107.