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Short communication

RT-PCR and sequence analysis of the full-length fusion protein of Canine Distemper Virus from domestic dogs

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During 2007–2014, 84 out of 236 (35.6%) samples from domestic dogs submitted to our laboratory for diagnostic purposes were positive for Canine Distemper Virus (CDV), as analyzed by RT-PCR amplification of a fragment of the nucleoprotein gene. Fifty-nine of them (70.2%) were from dogs that had been vaccinated against CDV. The full-length gene encoding the Fusion (F) protein of fifteen isolates was sequenced and compared with that of those of other CDVs, including wild-type and vaccine strains. Phylogenetic analysis using the F gene full-length sequences grouped all the Argentinean CDV strains in the SA2 clade. Sequence identity with the Onderstepoort vaccine strain was 89.0–90.6%, and the highest divergence was found in the 135 amino acids corresponding to the F protein signal-peptide, Fsp (64.4–66.7% identity). In contrast, this region was highly conserved among the local strains (94.1–100% identity). One extra putative N-glycosylation site was identified in the F gene of CDV Argentinean strains with respect to the vaccine strain. The present report is the first to analyze full-length F protein sequences of CDV strains circulating in Argentina, and contributes to the knowledge of molecular epidemiology of CDV, which may help in understanding future disease outbreaks.

Canine Distemper Virus (CDV) is a member of the Morbillivirus genus within the Paramyxoviridae family. The virus possesses a single-stranded negative-sense RNA genome of approximately 15 kb, which encodes eight viral proteins: Matrix (M), Fusion (F), Hemagglutinin (H), Nucleocapsid (NP), Polymerase (L), Phosphoprotein (P), and the nonstructural proteins V and C (Summers and Appel, 1994). F and H are glycoproteins that are responsible for viral attachment, entry, and membrane fusion. These two glycoproteins are more variable than other CDV proteins, and possess the major antigenic determinants that induce protective immune responses against CDV. These features have made them suitable for the study of genetic and antigenic diversity (Bolt et al., 1997).

Although CDV vaccines have been routinely administered to dogs for many years, canine distemper remains as a major infectious disease of dogs as well as of other wild animals (Lopez-Pena et al., 1994; Harder and Osterhaus, 1997; Myers et al., 1997; Ferreyra et al., 2009). Most CDV commercial vaccines are formulated with the Onderstepoort strain, which was isolated in the 1950s (Iwatsuki et al., 2000; Lednicky et al., 2004). A recombinant vaccine based on a canary pox vector expressing the H and F genes is also available (Pardo et al., 1997).

Recent data have shown a worldwide increase in the incidence of the disease, even in vaccinated dog populations (Blixenkrone-Moller et al., 1993; Decaro et al., 2004; Lan et al., 2006; Calderon et al., 2007; Martella et al., 2008; Budaszewski et al., 2014).

Most molecular epidemiological studies on CDV have been focused on the H gene, and recently also on the F protein signal-peptide (Fsp) coding region (Iwatsuki et al., 2000; Uema et al., 2005; Martella et al., 2006; Calderon et al., 2007; Panzera et al., 2012; Sultan et al., 2009; Espinal et al., 2014; Sarute et al., 2013, 2014b). The open reading frame of the CDV F protein encodes 662 amino acids (aa), comprising three regions: the Fsp region (aa 1–135), and two subunits, F2 (aa 136–224) and F1 (aa 225–662), which are generated via post-translational proteolysis.

The presence of three lineages of CDV in South America has been reported based on the analysis of the Fsp region (Sarute et al., 2013, 2014b), but only one full-length sequence of the F gene is available (Sarute et al., 2014a). In the present study, we analyzed the genetic diversity within the full-length F gene of CDV strains currently circulating in Argentina and compared it with worldwide circulating wild-type and vaccine strains.

A total of 236 clotted blood dog samples were submitted to our laboratory for diagnostic purposes between 2007 and 2014.

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Total genomic RNA was extracted directly with Trizol Reagent (Life Technologies, Carlsbad, CA, USA), and RT-PCR amplification of a fragment corresponding to the NP gene (287 bp) was performed as previously described (Frisk et al., 1999; Calderon et al., 2007). CDV-specific RNA was detected in 84 out of the 236 (35.6%) samples analyzed. Fifty-nine out of the 84 positive samples (70.2%) were from dogs which had been vaccinated against CDV at least once, while 19 (22.6%) were from unvaccinated dogs. The vaccination status of the remaining dogs was unknown. Although the existence of unreported incomplete vaccination schedules and/or problems related to the vaccine administered cannot be ruled out, the finding of infection and disease in vaccinated dogs, which has also been seen in other regions of the world (Blixenkrone-Moller et al., 1993; Decaro et al., 2004; Uema et al., 2005; Calderon et al., 2007; Budaszewski et al., 2014), suggests the emergence of new wild-type CDV strains with antigenic properties different from those of the vaccine viruses (Bolt et al., 1997; Iwatsuki et al., 2000; Martella et al., 2008; Bae et al., 2013).

Fifteen CDV positive RNA samples (Table 1) were used to amplify the full-length F gene to allow comparative sequence analysis. Four sets of primers were designed to amplify fragments covering the full-length F gene (Fw/1Rev, 2F/3Rev, 4F/5Rev and 6F/Rev) (Table 2). RT-PCR reactions were performed using a Mastercycler gradient thermal cycler (Eppendorf) and the One-step RT-PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Reverse transcription was performed at 45 °C for 45 min. For PCR amplification, a preliminary step at 95 °C for 15 min was made, followed by 35 cycles of denaturation for 1 min at 95 °C, annealing at 50 °C (for primer pairs Fw/1Rev and 6F/Rev) or at 54 °C (for primers 2F/3rev and 4F/5Rev) for 1 min, and extension for 1 min at 72 °C. A final extension step at 72 °C for 5 min was added in all cases. The amplified DNA fragments were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced in both directions, by an automated dideoxy-mediated chain termination method (Macrogen Inc, Korea). Sequence analyses were performed using the EditSeq program of the DNAstar software package. The nucleotide sequences were submitted to GenBank (accession numbers: KT224718 to KT224732, Table 1).

The deduced full-length F amino acid sequences were aligned with the Onderstepoort vaccine strain and with wild-type strains from other parts of the world (Fig. 1). All local wild-type strains were closely related (97.3–99.8% identity). When they were compared with the Onderstepoort commercial vaccine strain, the sequence identity was 90.6–91.2% at the nucleotide level and 89.0–90.6% at the amino acid level.

The alignment of the Fsp region (the first 135 aa sequences) of local CDV strains and the Onderstepoort vaccine strain showed an identity that varied from 64.4 to 66.7%. Conversely, the sequences of subunits F1 and F2 were relatively less variable, showing 95.3–96.8% identity between wild-type and vaccine strains. This is in agreement with that reported by researchers in other parts of the world (von Messling and Cattaneo, 2002; Sultan et al., 2009; Lee et al., 2010; Bae et al., 2013).

The sequences of the Argentinean strains shared the five predicted potential N-glycosylation sites (N-X-S/T) reported for the Onderstepoort vaccine strain, but also one additional potential site between residues 3 and 5 (N-K-T) (Fig. 1). Moreover, sixteen conserved cysteine residues were identified in vaccine and wild-type strains. All fifteen Argentinean strains contained two additional cysteine residues at amino acid positions 67 and 72 (Fig. 1). Both changes (one potential N-glycosylation site and two extra cysteine residues) were present in all the Argentinean strains sequenced and were localized in the Fsp region (Fig. 1). Although the Fsp region is absent from mature virions, it has been reported that this is an important region for localization of the precursor F0 in the Golgi network and that it may indirectly affect the fusion activity

Table 1
Summary of general information of Argentinean CDV wild type strains and F gene sequence accession numbers.

Strain	Year	Age (months)	Sex	Breed	Vaccination status	Accession number
Arg24	2005	4	M	MB	V	KT224718
Arg26	2010	48	M	LR	V	KT224719
Arg27	2011	7	M	BX	V	KT224720
Arg28	2011	36	F	SH	NV	KT224721
Arg29	2012	6	F	GR	V	KT224722
Arg30	2012	3	M	GR	V	KT224723
Arg31	2012	3	F	MB	NA	KT224724
Arg32	2012	4	F	R	V	KT224725
Arg33	2012	4	M	GS	V	KT224726
Arg34	2012	2	F	LR	V	KT224727
Arg35	2012	12	M	MB	V	KT224728
Arg36	2012	NA	F	SP	V	KT224729
Arg37	2013	3	M	MB	V	KT224730
Arg38	2013	8	M	MB	V	KT224731
Arg39	2014	3	M	LR	V	KT224732

F, female; M, male; MB, mixed breed; GR: Golden Retriever; LR, Labrador Retriever; GS, German Shepherd; R, Rottweiler; BX, Boxer; SP, Shar Pei; SH, Siberian Husky; V, vaccinated; NV, non-vaccinated; NA, no information available.

Table 2
Primers designed for RT-PCR amplification of the full-length CDV F gene.

Primer	DNA sequence (5'–3')	Direction	Genomic position[a]	Product size (bp)
Fw	cgagatctagggtccaggacatagcaagc	Sense	4850–4871	594
1Rev	agttttatgaccaagtac	Antisense	5426–5444	
2F	tgggattatcgggactga	Sense	5369–5387	558
3Rev	gggccaaatattgacaac	Antisense	5909–5927	
4F	gtccctgctatgcaacat	Sense	5829–5847	483
5Rev	ggagttctggctacaaatg	Antisense	6293–6312	
6F	tgtgtattcgtctcaga	Sense	6270–6287	797
Rev	Cgaggcctaagacgtgtgaccagagtgctttag	Antisense	7042–7067	

[a] Genomic position corresponding to the F gene sequence of the Onderstepoort strain (GenBank AF305419).

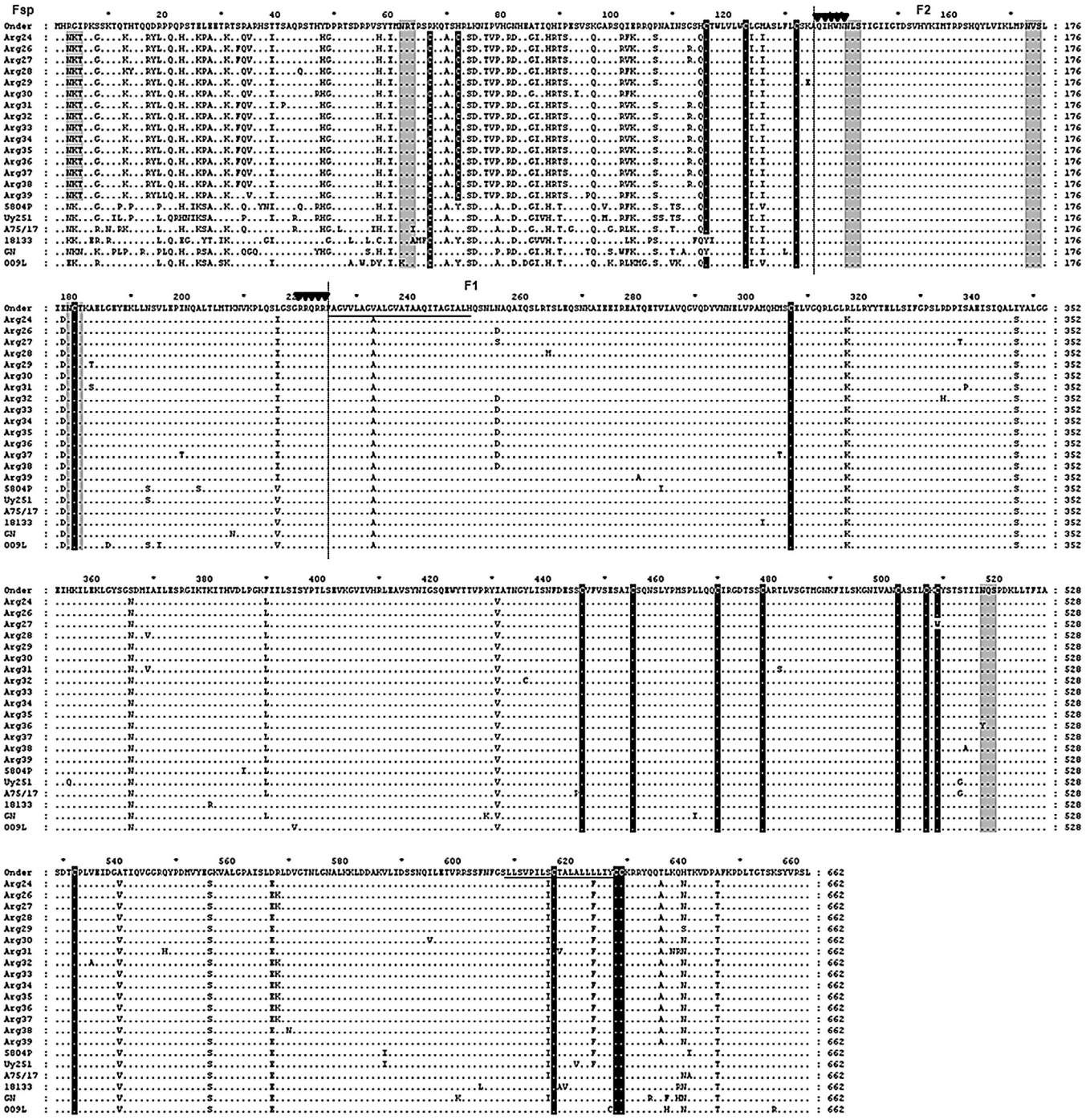

Fig. 1. Amino acid sequence alignment of the F protein of the vaccine Onderstepoort strain (Onder, top sequence), 15 wild-type CDV strains from Argentina and 6 wild-type strains from Germany (5804P), Uruguay (Uy251), Switzerland (A75-17), USA (18133), China (GN) and Japan (009L). Dots represent identical amino acids. Only differences from the Onderstepoort sequence are shown. Potential N-linked glycosylation sites (N-X-S/T) are shaded grey; cysteine residues are shaded black; cleavage sites are indicated (▼). Hydrophobic regions are underlined.

of the F protein (von Messling and Cattaneo, 2002; Plattet et al., 2007).

The presence of an additional glycosylation site and two extra cysteine residues, which participate in intra-molecular disulfide bonds, in this region, might result in alterations of the protein structure, with a consequent potential modulation in the cleavage-dependent activation of the F0 protein precursor or in its transport to the sub-cellular region where proteolytic cleavage occurs (Alkhatib et al., 1990; Harder et al., 1996). It has been hypothesized that this might influence certain viral biological properties, such as virulence (von Messling and Cattaneo, 2002; Plattet et al., 2007).

Furthermore, the two predicted cleavage sites identified in the F protein precursor (AQIHW in the C-terminus of the signal peptide region and RRQRR in the N-terminus of the F1 region) (Kovamees et al., 1991; Visser et al., 1993; Iwatsuki et al., 1998) were conserved in all the Argentinean strains (Fig. 1).

Finally, a neighbor-joining phylogenetic tree with DNA sequences of local and worldwide wild-type strains, together with vaccine strains, was constructed using Mega version 6.0 software

Table 3
CDV F gene sequences used in the phylogenetic analysis.

Strains	Origin	Accession numbers
Onderstepoort	USA	AF014953
Onderstepoort	United Kingdom	AF305419
SnyderHill	USA	JN896987
00-2601	USA	AY443350
98-2646	USA	AY542312
98-2654	USA	AY466011
98-2645	USA	AY445077
164071	USA	EU716337
01-2689	USA	AY649446
A75-17	Switzerland	AF164967
007Lm	Japan	AB474397
66L	Japan	AB475100
007Lm-1vp	Japan	AB462810
50Cbl	Japan	AB476403
50Con	Japan	AB476402
009L	Japan	AB475101
55L	Japan	AB475099
011C	Japan	AB476401
M25CR	Japan	AB475097
25259	USA	AY964114
HL	China	EF596901
21261	USA	AY964112
18133	USA	AY964108
SC01	China	EF596902
ZD01	China	EF596904
GN	China	EF596900
NM	China	EF596903
MS01	China	EF445055
GZ2	China	JN381189
GS0812-4	China	HQ850148
BS0610	China	EU934234
HeB(07)1	China	EU327874
HeB(07)2	China	KP064126
JL(07)1	China	EU327875
TW-TP1	Taiwan	EU191985
TW-HL2	Taiwan	EU191989
TW-KS15	Taiwan	EU192026
TW-KL6	Taiwan	EU191993
TW-TC4	Taiwan	EU191997
TW-KS14	Taiwan	EU192025
TW-KM5	Taiwan	EU192011
TW-KM2	Taiwan	EU192008
Th12	Thailand	AB509344
81ND	Japan	AB509341
50Sc	Japan	AB509345
83mLN	Japan	AB509343
82Con	Japan	AB509342
Ac96I	Japan	AB512286
19876	USA	AY964110
5804P	Germany	AY386316
Uy251	Uruguay	KM280689

(Tamura et al., 2013). The full-length sequences of the F gene were phylogenetic analyzed to identify the evolutionary relationship between the 15 CDV Argentinean strains and 52 international CDV strains, including the Uruguayan strain Uy251 (Sarute et al., 2014a), which is the only South American strain with F full-length sequence found in the GenBank (Table 3). Eight clades were identified by F full-length sequence analysis (Fig. 2). The 15 wild-type Argentinean strains were closely related to each other, sharing the SA2 clade (divergence of 1.1% among them), which had an evolutionary divergence of 8.8% with respect to the vaccine clade.

In conclusion, the present report is the first to analyze full-length F protein sequences of CDV strains circulating in Argentina, and is a contribution to the knowledge of molecular epidemiology of CDV, which may help in the interpretation of future disease outbreak. This study confirms and extends previous results related to the variability of CDV wild-type strains with respect to vaccine strains.

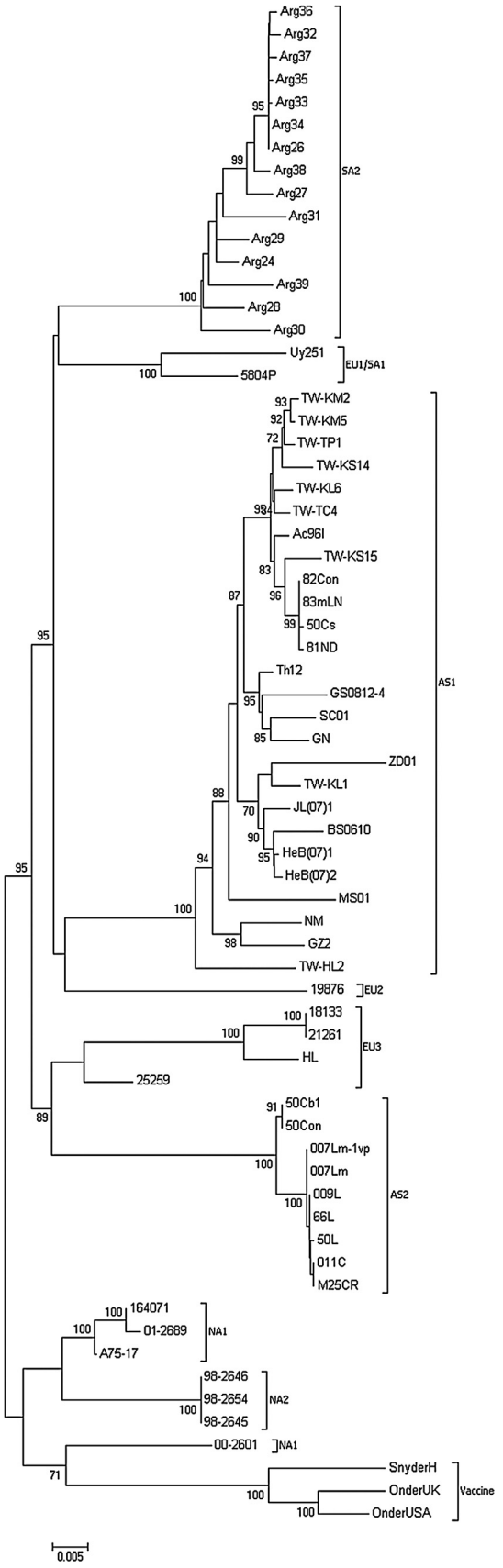

Fig. 2. Neighbor-joining phylogenetic tree based on the nucleotide sequences of full-length F genes from representative international (GenBank accession numbers detailed in Table 3) and Argentinean CDV strains (Table 1). Bootstrap support was calculated using 1000 data replicates. SA2, South America 2; EU1/SA1, Europe 1/South America 1; EU2, Europe 2; AS1, Asia 1; EU3, Europe 3; AS2, Asia 2; NA1, North America 1; NA2, North America 2.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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