Occurrence of different Canine distemper virus lineages in Italian dogs

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Summary
This study describes the sequence analysis of the H gene of 7 Canine distemper virus (CDV) strains identified in dogs in Italy between years 2002-2012. The phylogenetic analysis showed that the CDV strains belonged to 2 clusters: 6 viruses were identified as Arctic-like lineage and 1 as Europe 1 lineage. These data show a considerable prevalence of Arctic-like-CDVs in the analysed dogs. The dogs and the 3 viruses more recently identified showed 4 distinctive amino acid mutations compared to all other Arctic CDVs.

Presenza di diversi lineage *di Canine distemper virus in cani in Italia*

Parole chiave
Arctic-like CDV,
Cane,
Canine distemper virus (CDV),
Europe 1 CDV,
Gene H,
Italia.

Riassunto
L'articolo riporta l'analisi della sequenza del gene H di 7 ceppi di Canine distemper virus (CDV) identificati in cani, in Italia, tra il 2002 e il 2012. L'analisi filogenetica ha mostrato l'appartenenza dei 7 ceppi a 2 gruppi genetici: 6 ceppi al *lineage* Arctic-like e un ceppo al *lineage* Europe 1. I dati raccolti hanno mostrato una considerevole prevalenza nei cani testati di CDV appartenenti al *lineage* Arctic-like, evidenziando negli ultimi 3 ceppi virali identificati la presenza di 4 mutazioni aminoacidiche distintive rispetto agli altri ceppi di Arctic-like CDV.

Currently, 9 genetic lineages of Canine distemper virus (CDV) are recognized throughout the world: America 1, America 2, Asia 1, Asia 2, Europe Wildlife, Arctic-like, South Africa, Europe 1/South America 1 and South America 2. In addition, new CDV lineages were recently proposed to include viruses circulating in Asia (Asia 3) and viral strains related to Rockborn vaccine strain (Calderon *et al.* 2007, Martella *et al.* 2011, Panzera *et al.* 2012, Zhao *et al.* 2010). The attachment/haemagglutinin (H) is the most variable CDV protein and has been used throughout the years to distinguish the different genetic lineages and to classify the CDV circulating strains. Furthermore, the 3' end of H gene codifies for important amino acid domains involved in interaction between virus and cellular receptor and it has been hypothesized that residues 530 and 549 might affect the viral tropism (McCarthy *et al.* 2007, Nikolin *et al.* 2012).

The CDV-related disease in canine population has generally been controlled by live attenuated vaccines, but outbreaks of CDV are still reported worldwide also in vaccinated animals (Martella *et al.* 2007). Moreover, sequencing of several CDV strains belonging to different genetic lineages has highlighted considerable genetic and antigenic diversities (especially in the H gene/protein). However the effects that these mutations may have on the virulence remain unclear, as well as the susceptibility of different carnivore hosts and the ability of the currently available vaccines to protect from infection (McCarthy *et al.* 2007, Nikolin *et al.* 2012, Sekulin *et al.* 2011).

Three CDV genetic lineages are currently circulating in Italy: the Europe 1 lineage (historically spread in the dogs as well as in wild animals), also named Europe 1/South America 1 because it also circulates in South America, the Europe Wildlife (prevalently diffused in wildlife and sporadically reported in

dogs) and the Arctic-like lineage (native to the Arctic ecosystem and recently detected in Italian dogs) (Martella *et al.* 2006, Martella *et al.* 2007, Monne *et al.* 2011, Panzera *et al.* 2012). Furthermore, a distinct viral subgroup insight the Europe 1 lineage (named Wildlife Europe 2006-2009) has been shown to be responsible for the recent epidemic in Alpine wildlife, both in Italy and in Central Europe (Monne *et al.* 2011, Sekulin *et al.* 2011). Until the year 2000, only the Europe 1 lineage had been circulating in the Italian dog population. Instead, after the year 2000, several cases of Arctic-like CDVs infection were reported in dogs. Between 2002 and 2006, Martella and collaborators revealed cases of Arctic-like CDVs infection in Central and Southern Italy (Martella *et al.* 2006, Martella *et al.* 2007) and, between 2000 and 2007, cases of Arctic-like CDVs infection were also reported in the North-Eastern Italy (Monne *et al.* 2011). In contrast, a survey conducted on 53 dogs with symptoms attributable to CDV infection from Central Italy only identified CDV strains belonging to the classical Europe 1 lineage (Di Francesco *et al.* 2012). It is therefore yet to be understood if the genotype Arctic-like is permanently established in the Italian canine population or if it represents occasional findings in certain geographic areas.

In order to get further information on the CDV strains circulating in Italy, 7 CDV strains from Emilia Romagna and Lazio were analysed. They were capable of causing clinical signs in infected dogs as referred by local veterinary surgeons (Table I). With this aim, viral RNA was extracted from the footpad or brain using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and molecular diagnosis of CDV was done by TaqMan based real-time polymerase chain reaction (RT-PCR) (Scagliarini *et al.* 2007). The CDV H gene was amplified using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and the set of primers C previously described by Demeter and colleagues (Demeter *et al.* 2007). The extremity 3' of H gene was directly sequenced obtaining a fragment of 582 bp in length, corresponding to the last 193 amino acid residues of the H protein. The obtained nucleotide sequences were aligned and compared with 119 reference sequences available from the GenBank database[1] using the CLUSTALW software implemented in BioEdit sequence alignment editor version 7.0.9. The phylogenetic relationships were evaluated using MEGA version 5.05, with the best-fit model of nucleotide substitution determined using the function Find Best DNA/Protein Model implemented in the program. Pairwise genetic distances were calculated by Tamura-Nei model with γ distribution that resulted optimal for all the sequence data and phylogenetic trees were constructed using the neighbor-joining method. Bootstrap values were determined by 1000 replicates to assess the confidence level of each branch pattern. The nucleotide sequences obtained have been lodged within the GenBank sequence database under accession numbers: KF184985-KF184991.

The nucleotide sequences demonstrate that the analysed CDV strains belonged to 2 clusters. The first cluster included 6 Arctic-like lineage viruses (444.2002, 456.2003, 64.2004, 99.2011, 319.2012 and 352.2012) that showed an identity of 97.7-99.8% among themselves and of 97.2-100% with other Arctic-like strains. In particular, 444.2002 showed a complete identity with 3 North Italian CDVs identified in dogs in 2000-2001 (HM443711, HM443719 and HM443720). The last identified CDV strain (741.2006) belonged to Europe 1 lineage, showing an identity of 93.2-94.8% with viruses of the first cluster and of 95.7-99.8% with Europe 1 strains. Notably, the nucleotide identity calculated by comparing the viruses of the first cluster with Artic-like reference strains decreases progressively from oldest samples to samples collected more recently. Furthermore, the identities between 741.2006 and 99.2011, 319.2012 and 352.2012 were found to be lower than those calculated with the Arctic-like reference strains.

[1] www.ncbi.nlm.nih.gov/genbank/.

Table I. *Details of 7 CDV strains identified in dogs in Italy between years 2002-2012.*

Virus	Sampling date	Breed	Sex	Age	Origin	Vaccination status	Symptoms
444	2002	Labrador retriever	M	2m	Emilia-Romagna	Yes	R + N
456	2003	German shepard	F	2m	Emilia-Romagna	No	R + N
64	2004	Cane Corso	F	1y 3m	Emilia-Romagna	Yes	GI + N + C
741	2006	Mixed-breed	Un	Un	Emilia-Romagna	Un	Un
99	2011	Mixed-breed	F	5m	Lazio	Un	GI + N
319	2012	Mixed-breed	M	2m	Lazio	Un	GI + N
352	2012	Mixed-breed	F	2m	Lazio	No	N

M = male; F = female; m = months; y = years; R = respiratory symptoms; N = neurological symptoms; GI = gastrointestinal symptoms; C = cutaneous lesions; Un = unknown.

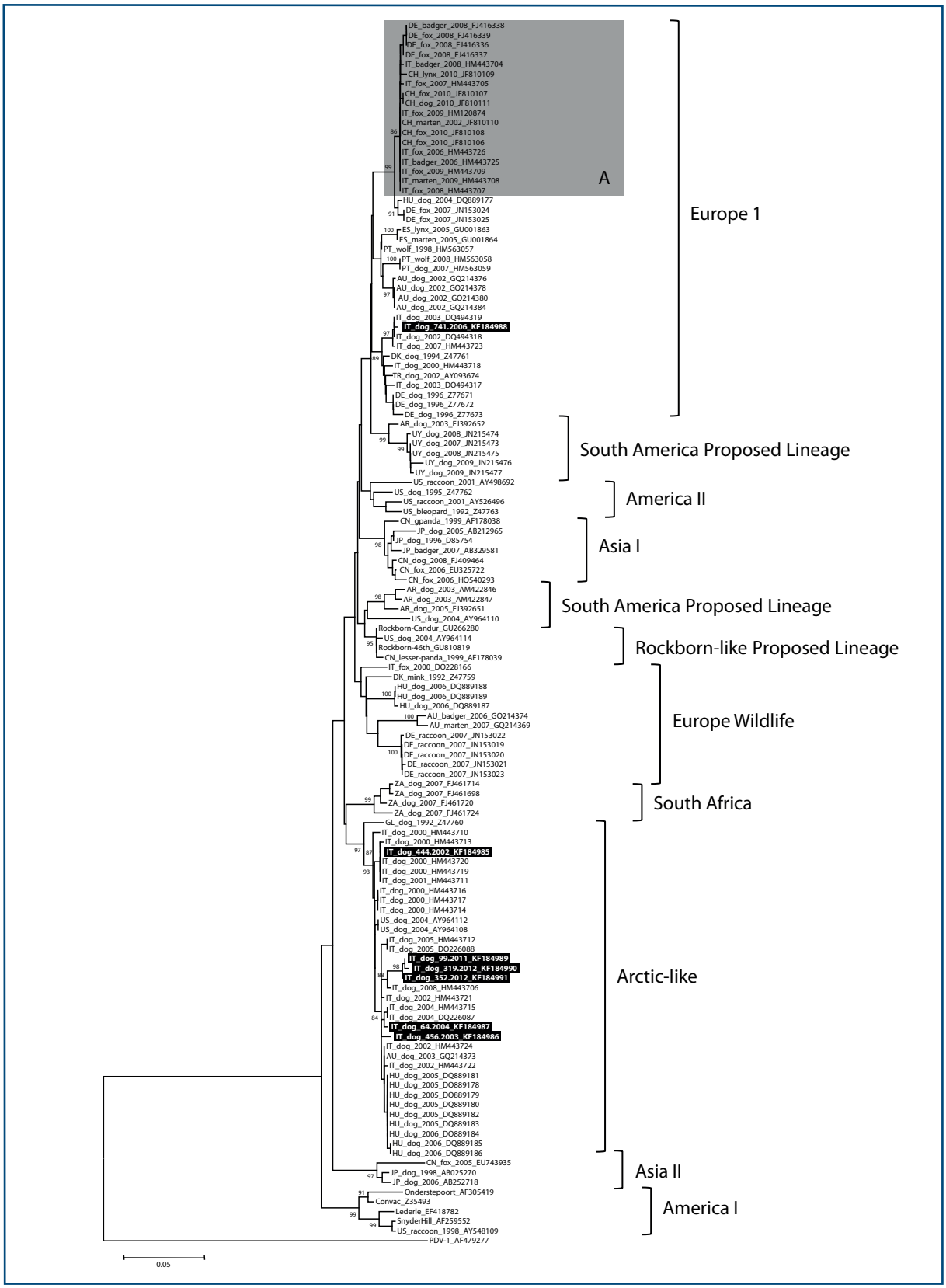

Figure 1. *Rooted phylogenetic tree constructed on nucleotide sequences of the 3' fragment of H gene of the CDV genome (582 base pairs).*
The phylogenetic tree was constructed using the neighbor-joining method with the nucleotide sequences generated in this study and with sequences of 118 CDV reference strains obtained from the GenBank database and a Phocine distemper virus as outgroup. Bootstrap values greater than 80% are indicated on the respective branches. The CDV strains included in the phylogenetic analysis are named with: acronym of nation, host species and year of identification (with lab numbers for sequences generated in this study), plus the GenBank accession number. When the year of identification was not available, the year of deposition of the nucleotide sequence in the GenBank database was indicated. Highlighted in black: sequences generated in this study. Highlighted in gray: Wildlife Europe 2006-2009 subgroup.

These findings are further supported by the phylogenetic tree, which depicts 6 viruses clustering in the Arctic-like lineage and 741.2006 in the Europe 1/South America 1 lineage (Figure 1). Furthermore, 99.2011, 319.2012 and 352.2012, formed a monophyletic clade inside the Arctic-like lineages, strictly related with a North Italian CDV identified in 2008 (HM443706).

The H protein of the 6 Arctic-like CDVs exhibited residues asparagine (N) at position 530 and tyrosine (Y) at position 549, in accordance with all Arctic-like strains sequenced until now; whereas, in accordance with all Europe 1 strains identified in domestic dogs, 741.2006 exhibited residues glycine (G) at position 530 and tyrosine (Y) at position 549 (Martella *et al.* 2007, Monne *et al.* 2011). Some differences were detected in other amino acid positions, as shown in Figure 2. In particular, 99.2011, 319.2012 and 352.2012 exhibit methionine (M) at position 445 and serine (S) at position 559 which were never reported previously in other CDVs. Furthermore, these latter Arctic-like viruses exhibit threonine (T) at position 417 and asparagine (N) at position 435 previously reported in another North Italian CDV strain HM443706.

These data show a considerable spread of Arctic-like lineage in the sampled dogs, in agreement with previous reports on CDV strains collected in Italy which had revealed the presence of Arctic-like CDVs in dogs since 2000 and confirm the dissemination of this novel genotype in Italy (Martella *et al.* 2006, Martella *et al.* 2007, Monne *et al.* 2011). The Arctic-like lineage is native to the Arctic ecosystem and was usually related to infection of wild animals; it was only occasionally associated with outbreaks in domestic dogs from country geographically distant from Italy, such as Hungary, United States of America, China and Greenland (Blixenkrone-Möller *et al.* 1992, Demeter *et al.* 2007, Pardo *et al.* 2005). Subsequently to the arrival of Arctic-like CDVs in Italy, probably due to a significant movement of viruses from the East-Central Europe to Italy in consequence of intense trade of dogs (Martella *et al.* 2006), the increasing detection of these viruses might suggest that strains belonging to Arctic-like lineage are becoming progressively endemic in Italy. Furthermore, the 3 viruses more recently identified (99.2011, 319.2012 and 352.2012) showed 4 distinctive amino acid mutations compared to all other Arctic CDVs.

Although the study shows a high prevalence of Arctic-like CDV in sampled dogs, the number of viral

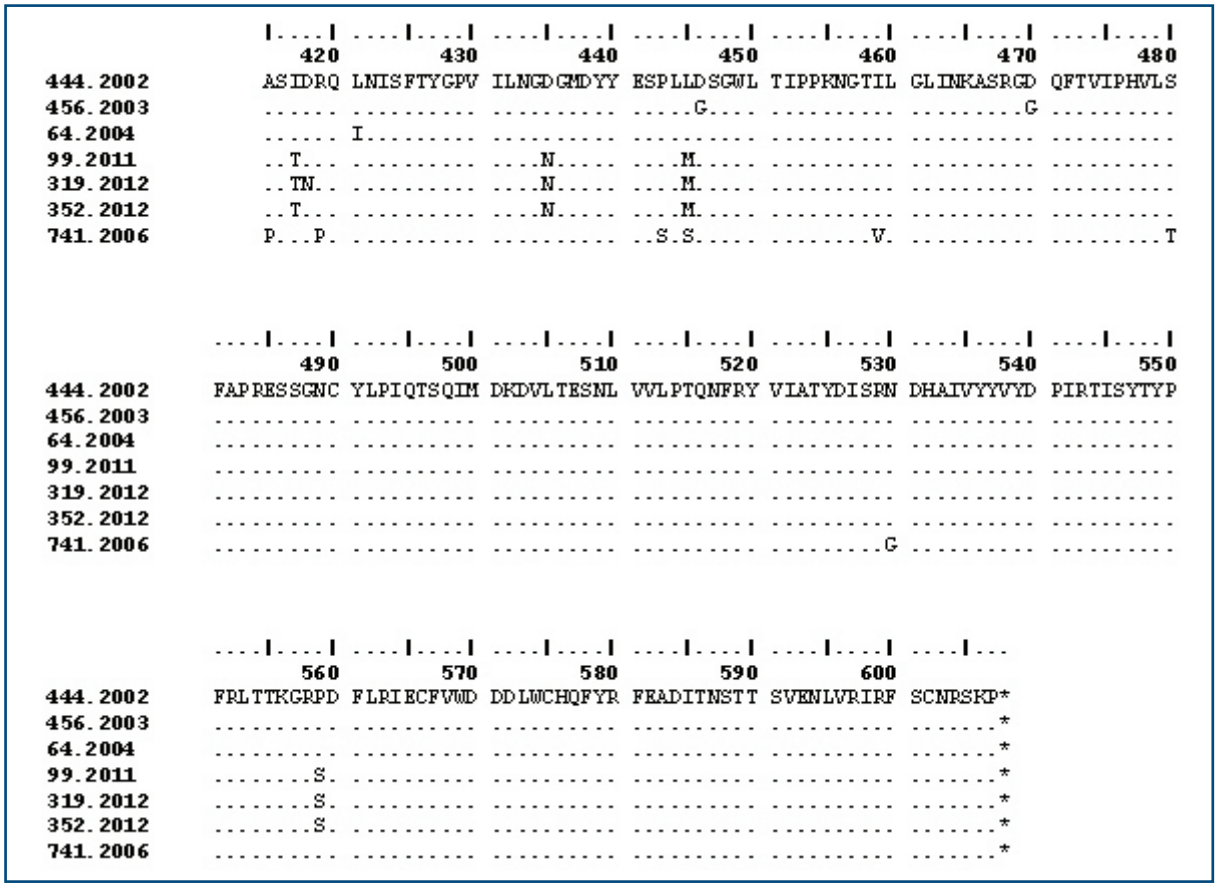

Figure 2. *Amino acids differences in the sequence tract of H gene between the 7 viral strains identified in dogs in Italy between years 2002-2012.* The ruler at the top shows the amino acid positions corresponding to the entire H protein.

strains analysed should be increased, extending the monitoring activities also to other geographical areas, to properly assess the incidence of Arctic-like CDV strains in Italian dogs. Further studies would be necessary to understand what benefits the new amino acid mutations can confer to the Arctic-like CDVs and in which way these may affect their spreading in Italy and in Europe.

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