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► To cite this version:

Isabella Monne, Alice Fusaro, Viviana Valastro, Carlo Citterio, Manuela Dalla Pozza, et al.. A distinct CDV genotype causing a major epidemic in Alpine wildlife. *Veterinary Microbiology*, 2011, 150 (1-2), pp.63. 10.1016/j.vetmic.2011.01.009 . hal-00687334

HAL Id: hal-00687334

<https://hal.science/hal-00687334>

Submitted on 13 Apr 2012

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Accepted Manuscript

Title: A distinct CDV genotype causing a major epidemic in Alpine wildlife

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PII: S0378-1135(11)00013-7
DOI: doi:10.1016/j.vetmic.2011.01.009
Reference: VETMIC 5147

To appear in: *VETMIC*

Received date: 29-6-2010
Revised date: 28-12-2010
Accepted date: 10-1-2011

Please cite this article as: Monne, I., Fusaro, A., Valastro, V., Citterio, C., Pozza, M.D., Obber, F., Trevisiol, K., Cova, M., De Benedictis, P., Bregoli, M., Capua, I., Cattoli, G., A distinct CDV genotype causing a major epidemic in Alpine wildlife, *Veterinary Microbiology* (2010), doi:10.1016/j.vetmic.2011.01.009

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **A distinct CDV genotype causing a major epidemic in Alpine wildlife**

2

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16

1 **ABSTRACT**

2 Canine distemper virus (CDV) infection represents an important conservation threat to
3 many carnivore species and has contributed to the population decline of several wild
4 terrestrial and aquatic mammalian species. Since 2006, the Alpine region of North-
5 Eastern (NE) Italy has been experiencing a severe and widespread outbreak of CDV
6 affecting the wild carnivore population. In this study we performed an extensive
7 phylogenetic and molecular evolutionary analysis of CDV identified during the recent
8 wildlife epidemic in the Alpine region. Our analysis yielded data on the evolutionary
9 dynamics of the Alpine wildlife CDV epidemic and revealed the emergence and spread
10 of a single genetic cluster of CDV. The wide distribution of the novel cluster combined
11 with the identification of a specific amino acid mutation, which is believed to increase
12 the ability of the virus to replicate in a wider host range, raises concerns over the
13 possible implications of the spread of this virus on the conservation of endangered
14 wildlife species.

15
16 **Keywords:** Canine distemper virus; wildlife; wild carnivores; evolutionary dynamics;
17 Haemagglutinin gene (H)

18
19

1

2 **Introduction**

3 Canine Distemper virus (CDV) belongs to genus Morbillivirus, family Paramyxoviridae
4 and is the causative agent of a severe infectious disease affecting a broad range of wild
5 and domestic carnivores. This virus possesses a non-segmented single stranded negative
6 RNA genome encoding six proteins. Among them, the haemagglutinin (H) protein is a
7 key component of the virus, being responsible for the first virus-host cell interaction and
8 initial virus entry (Appel, 1987; Martella et al., 2008). The H protein binds to the
9 signalling lymphocyte activation molecule (SLAM) on the membrane of the host target
10 cells resulting in cellular infection. Mapping of the distribution of mutations at sites
11 under positive selection pressure has shown that key amino acid substitutions associated
12 with host specificity fall into the SLAM-binding region of the H protein (McCarthy et
13 al., 2007).

14 The H gene and its encoded protein are subject to a higher genetic and antigenic
15 variation respectively than other viral genes. For this reason it has been the primary
16 focus of previous molecular epidemiological studies of CDV. Seven clusters have been
17 identified based on the genetic relationship between complete H gene sequences of
18 distinct CDV strains, namely America-1, America-2, Arctic- Like, Asia-1, Asia-2,
19 Europe-1 and Wildlife (Martella et al, 2006; McCarthy et al., 2007).

20 CDV infection represents an important conservation threat to many carnivore species
21 and has contributed to the population decline of several wild terrestrial and aquatic
22 mammalian species (Murray et al., 1999; Kennedy et al, 2000). The mountainous region
23 located in South Central Europe (the Alps) is home to several large endangered
24 carnivore species such as the Eurasian lynx (*Lynx lynx*), the wolf (*Canis lupus*) and the
25 brown bear (*Ursus arctos*), all susceptible to CDV infection (Daoust et al., 2009;
26 Marsilio et al., 1997; Martella et al., 2008). Since 2006, the Alpine region of North-

1 Eastern Italy has been experiencing CDV outbreaks affecting the wild carnivore
2 population. Several foxes, badgers and a stone marten, which were found dead or
3 showed neurological and respiratory signs, tested positive for CDV infection. The early
4 CDV cases were identified only in the North-Eastern part of the Italian Alps, but since
5 2007 infections have spread southwest reaching the Pre-Alps and urbanized areas of the
6 region, thus posing a risk to other wild carnivores and susceptible pets (Figure 1). To
7 date it is unclear how CDV reached these regions, propagated and evolved and whether
8 multiple viral strains of the virus have been involved in this epidemic.

9 This study describes the dynamics of the recent CDV wildlife epidemic through an
10 extensive molecular and phylogenetic analysis of the Italian CDVs identified between
11 2006 and 2009.

12

13 **Materials and methods**

14

15 *Samples*

16 The 96 CDV positive samples analyzed in this study were collected between October
17 2006 and March 2009 from three regions namely Veneto, Friuli Venezia Giulia and
18 Trentino Alto Adige located in the north-eastern part of Italy (figure 1). All the samples
19 were collected from wild animals, in particular foxes (n=66) (*Vulpes vulpes*), badgers
20 (n=29) (*Meles meles*) and one stone marten (*Martes foina*). 75 out of 96 animals testing
21 positive for CDV were found dead. The remaining animals displayed one or more of the
22 following clinical signs: myoclonus, ataxia, ocular-nasal discharge and dyspnea. In
23 addition, 23 CDVs detected in domestic dogs from the same regions between 2000 and
24 2007 were included in this study. Information regarding the wildlife CDVs viruses
25 analyzed here (collection date and location) are recorded in table S1 in the supplemental
26 material.

1

2 *Nucleotide sequencing*

3 Viral RNA was extracted from organ samples (lungs and brains) using the Nucleospin
4 RNA II Kit (Machery-Nagel, Duren, Germany). One step RT-PCR amplification
5 (Qiagen, Hilden, Germany) was performed using specific primers (primer sequences
6 available on request). Sequences were generated using the Big Dye Terminator v3.1
7 cycle sequencing kit (Applied Biosystem, Foster City, CA - USA). The products of the
8 sequencing reactions were cleaned-up using PERFORMA DTR Ultra 96-Well kit (Edge
9 BioSystems, Gaithersburg, MD - USA) and sequenced in a 16-capillary ABI PRISM
10 3130xl Genetic Analyzer (Applied Biosystem, Foster City, CA - USA). Sequences of
11 the H gene (from nucleotide 120 to 1824) were aligned and compared with 118
12 sequences representative of viruses belonging to the phylogenetic clades identified so
13 far and available on GenBank (Europa-1, Wildlife, Arctic-like, Asia-1, Asia-2, Asia-3,
14 America-1 and America-2).

15 Representative CDV nucleotide sequences obtained in this study are available from
16 GenBank under accession numbers from HM443704 to HM443726.

17

18 *Phylogenetic analysis*

19 A phylogenetic tree was inferred for the H gene using the maximum likelihood (ML)
20 method available in PAUP* (Swofford, 2003). The best-fit model of nucleotide
21 substitution was identified by MODELTEST (Posada et al., 1998) as the general time
22 reversible GTR + Γ_4 + I model, with the frequency of each substitution type, the gamma
23 distribution of among site rate variation with four rate categories (Γ_4) and the proportion
24 on invariant site (I) estimated from the empirical data (parameter values available from
25 the authors on request). Because of the very large size of the dataset, the nearest-
26 neighbor-interchanges branch swapping method was employed. To assess the robustness

1 of individual nodes on the phylogenetic tree, we performed a bootstrap resampling
2 analysis (1000 replications) using the neighbor-joining method, incorporating the ML
3 substitution model.

4

5 *Substitution rates and selection pressure*

6 Rates of nucleotide substitution per site, per year and the time of the most recent
7 common ancestor (tMRCA) of the samples analyzed in this study were estimated using
8 Bayesian Markov Chain Monte Carlo (MCMC) approach implemented in the BEAST
9 package version 1.4.8 (Drummond & Rambaut, 2007). Uncertainty in the data is
10 reflected in the 95% highest probability density (HPD) values. Chain lengths were run
11 for sufficient time to achieve coverage as assessed using the Tracer v1.4 program
12 (Rambaut & Drummond, 2007). Two molecular clock models, namely strict (constant)
13 and uncorrelated lognormal relaxed clock, were compared analyzing values of the
14 coefficient of variation (CoV) in Tracer (Rambaut & Drummond, 2007), such that CoV
15 values >0 are evidence of non-clock-like evolutionary behaviour. Because of the
16 typically complex population dynamics we used the Bayesian Skyline plot as a
17 coalescent prior. The GTR + Γ_4 model of nucleotide substitution was used.

18 To determine the nature of selection pressures acting on H gene of CDV, we computed
19 the mean ratio of nonsynonymous (d_N) to synonymous (d_S) substitutions per site (d_N/d_S)
20 using the Single Likelihood Ancestor Counting and the Fixed Effects Likelihood
21 methods available through DATAMONKEY web interface (Pond et al., 2005).

22 Fixed amino acid changes along major branches of the phylogeny were identified using
23 the parsimony algorithm available in the MacClade program (Maddison & Maddison,
24 2000).

25

26

1 **Results**

2 *Phylogenetic analysis*

3 The H gene of 96 CDVs detected in the North-Eastern (NE) part of Italy from wild
4 species, mainly foxes and badgers, between 2006 and 2009 was analyzed. The
5 phylogenetic tree revealed that all the isolates clustered together and with 4 viruses
6 detected in Bavarian wildlife (South Germany) in 2008 (similarity ranged from 99.7%
7 to 100%) within the clade designated as Europe 1 (figure 2). This clade contains viruses
8 detected in the domestic canine population from various European countries, such as
9 Denmark, Germany, Turkey, United Kingdom, Hungary and also Italy. Italian and
10 German wildlife CDVs (Sekulin et al., 2010) were the only viruses belonging to this
11 clade collected from wild animals. Within this clade, all the H gene sequences of CDVs
12 obtained from Italian and Bavarian wildlife formed a separate monophyletic group
13 (figure 2) supported by high bootstrap value (100). The highest genetic relationship
14 (from 99.1% to 99.5%) for the strains collected in wild species was found with the H
15 gene sequence of a CDV strain identified in an Hungarian puppy in 2004 (Demeter et al,
16 2007). To facilitate epidemic description, the group formed by the Italian and Bavarian
17 wild CDVs and the Hungarian strain will be termed in the present study as “Wildlife
18 Europe 2006-2009” (WE/06-09).

19 Five CDV viruses, identified between 2000-2007 from Italian domestic dogs (two CDV
20 sequences were obtained in the present study), grouped within the Europe 1 clade but
21 clustered in a separate branch of the phylogenetic tree showing a percentage of
22 similarity lower than 98% with the H gene of CDV strains identified in wild carnivores.

23 The remaining sequences of 21 strains identified from the domestic dog population of
24 the North-Eastern Italian area fell in the Arctic lineage, previously reported in 2
25 distemper episodes in Southern Italy (Martella et al., 2006). This lineage was originally
26 detected in the susceptible population of the Arctic ecosystem and it was recently

1 identified in Eastern Europe (Demeter et al., 2007).

2

3 *Evolutionary dynamics of Europe 1.1 CDV*

4 The rate of evolutionary change, recorded as nucleotide substitution per site per year
5 (subs/site/year), was estimated using a bayesian coalescent method for the Italian
6 viruses belonging to WE/06-09 cluster. The lower 95% HPD of CoV values of the
7 relaxed molecular clock were approximately 0; therefore, a strict molecular clock model
8 was used to estimate evolutionary dynamics. The mean substitution rate of 10.53×10^{-4}
9 subs/site/year (95% HPD $5.2-16 \times 10^{-4}$ subs/site/year) was within the range typically
10 observed for CDV (Pomeroy et al., 2008). To further explore the evolutionary origin of
11 this group we estimated the time of the most recent common ancestor (tMRCA). The
12 mean tMRCA was May 2006 (95% HPD June 2005 – September 2006), approximately
13 five months before the identification of the first virus (6th October 2006) belonging to
14 this cluster, suggesting a recent introduction of this strain into Northern Italy.

15

16 *Selection pressure and molecular characterization*

17 Using the codon-based maximum likelihood methods SLAC (Single Likelihood
18 Ancestor Counting) and FEL (Fixed Effects Likelihood) we computed the ratio of
19 nonsynonymous (d_N) to synonymous (d_S) substitutions (d_N/d_S) at every codon in the
20 alignment of the H gene of 214 sequences of CDV representative of the seven clusters
21 (sequences available in Genbank) and in the alignment of WE/06-09 (101 sequences).
22 In both cases there was no evidence of positive selection. However, the d_N/d_S was
23 higher in WE/06-09 cluster (0.44) than in all the 214 CDV sequences (0.26).

24 Analysis of the complete alignment using the FEL method allowed to identify nine
25 residues under influence of positive selection at a significance value of $p < 0.1$ and five
26 at the more stringent significance value of $p < 0.05$. Three positive selected sites at a

1 significance value of $p < 0.1$ and one at a significance value of $p < 0.05$ were confirmed
2 under the SLAC method (table 1). Interestingly, viruses of the WE/06-09 cluster
3 possessed four amino acid signatures (substitutions L266F, I315V, S/T590I, R597H,
4 Y549H) and three of them were at sites identified under the influence of positive
5 selection (positions 266, 590 and 549). In particular, residue 549 is located in propeller
6 beta-sheet 5 of the SLAM-binding domain and has been demonstrated to be involved in
7 modulation of the fusion activity in CHOdogSLAM cells (von Messling et al., 2005).
8 Analysis of 567 amino acids (40-607) of the Italian isolates sequenced in this study
9 showed 7 potential N-linked glycosylation sites (N-X-S/T) at positions 149-151, 309-
10 311, 391-393, 422-424, 456-459, 587-589 and 603-605. Only one sequence possesses 6
11 potential glycosylation sites (the site at position 603 is missing).
12 Several amino acid substitutions (range: 48-51 substitutions) were observed between
13 Italian viruses isolated from non-dog species and vaccine strains (Onderstepoort,
14 Lederle, Convac, Snyder Hill) in the H protein portion analysed in this study.

15

16 **Discussion**

17

18 Before 2006, all CDV isolates from wild animals in Europe fell within the Wildlife and
19 Arctic like clades (Martella et al., 2006; Mamaev et al., 1995). The only reported Italian
20 virus which was isolated from a fox (Martella et al., 2006) belonged to the Wildlife
21 clade. Our study describes, for the first time, the emergence and spread of a novel
22 genetic cluster of CDV in wildlife populations of the Alps. This novel genetic group,
23 herein denoted as WE/06-09 cluster, consists almost exclusively of viruses identified
24 from wild carnivores. A single virus from a domestic species, detected in an Hungarian
25 dog in 2004 (Demeter et al, 2007), falls within this monophyletic group although it is
26 distributed in a well distinct branch of the tree. Phylogenetic analysis of available

1 sequences in Genbank revealed that the circulation of the WE/06-09 cluster is not
2 confined to Italian wildlife species. Four CDV sequences obtained from Bavarian wild
3 carnivores (three foxes and one badger) in 2008 (Sekulin et al., 2010) clustered with the
4 Italian CDV, suggesting a wide geographic spread of this new CDV variant in European
5 wildlife. These findings raise concerns over the potential impact of infection on
6 conservation programs for endangered carnivores in Europe. Furthermore, the
7 identification of substitution Y549H in the H protein of all CDV strains analyzed
8 underlines the urgent need to provide more insight into the pathogenic properties of this
9 novel strain. A sequence-based study published by McCarthy et al. (2007) showed that
10 the Y549H substitution occurs predominantly in CDV isolates obtained from non-dog
11 host species. It was also reported in a highly virulent strain for ferrets, obtained by
12 repeated passages in these animals (von Messling et al., 2003). Interestingly, this
13 mutation was not observed in the Hungarian canine virus and in the sequences of CDV
14 identified in Italian dogs belonging to Europe 1 clade, suggesting that it appeared only
15 after the introduction of the strain into the wild population.

16 Large scale sequence analysis of CDV obtained during the Italian outbreak revealed that
17 the wildlife species of the North Eastern region of the Italian Alps have been affected by
18 an epidemic wave caused by viruses belonging to a single genetic clade. According to
19 our evolutionary analysis, the common ancestor of the CDV strains, involved in the
20 Italian outbreak, dates back to May 2006 (95% HPD June 2005 – September 2006)
21 approximately five months before the first confirmed case of CDV infection (6th
22 October 2006) in Alpine carnivores was reported. Molecular dating suggests that the
23 ancestor of the WE/06-09 cluster was surprisingly recent and that within a few months
24 this virus had managed to spread over a wide geographical area in Northern Italy. Ten
25 months following its initial detection, the spread of the WE/06-09 cluster had already
26 involved 4 provinces and 21 municipalities of three distinct Italian regions (figure 1).

1 Evaluation of the phylogenetic relationships demonstrated that the CDV described in
2 this study possesses a high genetic similarity (99.1% - 99.5%) with a virus isolated from
3 a dog in Hungary in 2004, indicating that the novel cluster circulated in Eastern or
4 Central Europe at that time. Also of note is that viruses isolated from dogs in Italy
5 between 2000 and 2008 and belonging to the Arctic clade were in the same cluster as
6 the viruses from Hungary. This supports the hypothesis of a significant movement of
7 viruses between Italy and East-Central Europe and intense legal and illegal trade of
8 dogs involving several Eastern European countries may explain the diffusion of the
9 Arctic-like strain in Italy (Martella et al., 2006).

10 Our data does not shed light on how the novel virus was introduced into the wild
11 population of carnivores (e.g. by human-related activities, contacts with domestic
12 carnivores or movement of wild animals). However, data obtained in this study suggest
13 that their subsequent spread was primarily linked to the transboundary movements of
14 wildlife species.

15 A variety of factors may have influenced the emergence and the spread of this large
16 epidemic event, such as demographic dynamics or population immunity. While no data
17 were available on the immune status of the susceptible population in the area before the
18 epidemic, some factors with relation to population dynamics can be considered. The last
19 few decades have seen a dramatic increase in Alpine wild ungulate populations,
20 especially in those areas where human activities linked to the forest have been
21 abandoned (Rizzoli, et al 2009). The increase in ungulate populations has represented an
22 important factor for the re-colonization by large carnivores in Europe (Linnell, et al,
23 2007), and it is likely to have favored the abundance of medium sized carnivores such
24 as foxes. This medium sized opportunistic carnivore can adapt its home range size and
25 contours to resource availability, thus dynamically influencing the total abundance of a
26 population insisting over an area (Lucherini et al., 1996). As frequently occurs in highly

1 contagious and virulent viruses such as CDV (Morner et al., 2002), the increase in host
2 concentration may have enhanced the efficacy of virus transmission.

3 In Italy, the WE/06-09 cluster has been detected circulating among foxes, badgers and a
4 stone marten, without any apparent spill over to the domestic canine population. In fact,
5 CDVs circulating in dogs obtained from the same geographic area and period grouped
6 in well separate branches of the phylogenetic tree. This may be due to the lack of
7 contact between the two populations or to the strong adaptation of this strain to the wild
8 hosts.

9 Available data concerning CDV outbreaks in the European wild carnivore populations
10 are limited to a few reports from Germany and Italy (Frolich et al., 2000; Martella et al.,
11 2002; Sekulin et al., 2010). Furthermore, the available information reports phylogenetic
12 analyses of a small number of samples and so there are little or no data concerning the
13 evolution of this virus in Europe. This appears to be the first comprehensive molecular
14 study on a large collection of CDV from wild carnivores in the European continent,
15 providing important basic information on the evolutionary and epidemiological
16 dynamics of an infection which threatens endangered species and pets. This work also
17 prompts a call for improved CDV surveillance in European wildlife populations to
18 obtain a reliable picture of the spread of the novel CDV genotype in this continent.

19

20

21 **Acknowledgments**

22 This work was financially supported by the Italian Ministry of Health through the RC
23 IZS VE 07/08 and RC IZS VE 06/09 projects.

24 We are grateful to Luigi Bertolotti (University of Studies of Torino, Faculty of
25 Veterinary Medicine, Grugliasco, TO, Italy) and Francesca Cagnacci (Research and
26 Innovation Centre, Environment and Natural Resources Area, Edmund Mach

1 Foundation, TN, Italy) for their technical collaboration. Dr William Dundon, Dr
2 Samantha Kasloff and Francesca Ellero (IZSVE) are gratefully acknowledged for the
3 revision of the manuscript.

4

5

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Figure 1. Geographical distribution of the 96 CDV strains from wildlife species analysed in the present study.

Figure 2. Maximum-likelihood (ML) phylogenetic tree for the Haemagglutinin gene of selected CDV strains. Sequences of CDV viruses analyzed in this study are highlighted in yellow. The seven main CDV clusters are identified by distinct colours. The numbers at each branch point represent bootstrap values (black). GenBank accession numbers of the CDV sequences downloaded from the public database are included.

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Table 1. Summary of the selection pressures acting in the H gene of CDV analyzed

	SLAC positively selected sites (codon position)		FEL positively selected sites (codon position)	
	p < 0.1	p < 0.05	p < 0.1	p < 0.05
CDV H gene			266	
			314	
			401	266
	401		412	401
	412	549	417	412
	549		460	549
			549	583
			583	
		590		



