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20 **Short Communication**

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22 **Natural distemper infection in stone martens (*Martes foina*): from infection to neutralizing**  
23 **antibodies**

24

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#### 56 **Declaration of Competing Interest**

57 Declarations of interest: none.

58 **Abstract**

59 We report an outbreak of canine distemper virus (CDV) among stone martens (*Martes foina*) in  
60 Italy. After being rescued in Northern Italy between April and June 2018, six subjects were kept in  
61 a wildlife and exotic animal rescue center in Bologna province. Subjects have been monitored for  
62 15 months in captivity. Within this time-lapse, two subjects died, while among the remaining four,  
63 only one showed clinical symptoms referable to distemper. Surviving subjects have been regularly  
64 tested for CDV by means of reverse transcriptase-PCR from conjunctival and oropharyngeal swabs  
65 for eleven months. The identified viruses belonged to the Wildlife-Europe CDV genetic subgroup.  
66 Neutralizing antibodies were detected at the end of the eleven months, when all subjects tested  
67 reverse transcriptase-PCR negative. Our findings confirm the circulation of the Wildlife-Europe  
68 CDV genetic subgroup (Europe 1/South America 1 lineage) within the Italian wildlife, and improve  
69 knowledge on viral infection in stone martens.

70

71 **Keywords:** distemper; phylogenetic analysis; serology; stone marten; wildlife

72 Canine distemper is distributed worldwide and constitutes one of the most serious contagious  
73 diseases of carnivores, fluctuating in disease presentation (Greene and Vandeveld, 2012) and  
74 causing high lethality rate. Mustelids are a diverse group of carnivorous mammals known to be very  
75 susceptible to distemper (Philippa et al., 2008), displaying clinical signs similar to those in canids  
76 including anorexia, oculo-nasal mucopurulent discharge, multifocal dermal hyperkeratosis,  
77 emaciation, severe pruritus, intermittent diarrhoea, pneumonia, and rapid death (Mos et al., 2003;  
78 Williams et al., 1988). The stone marten (*Martes foina*) is widely distributed in Eurasia (IUCN,  
79 2015), where is considered as possible canine distemper virus (CDV) wildlife reservoir (Myers et  
80 al., 1997; Philippa et al., 2008). Nevertheless, no information is available on spontaneous infection  
81 within this species. We report an outbreak of canine distemper in six stone martens held in Monte  
82 Adone wildlife rescue center in Bologna province.

83 This was a retrospective study; no stone martens were sampled exclusively for the purposes of this  
84 study. Only samples taken to diagnose the aetiology of the clinical disease and to monitor viral  
85 shedding and immunity before reintroduction into the wild, were used. Subjects were rescued in  
86 Northern Italy in 2018: two (#110A, #110B) at the end of April, three at the beginning of June  
87 (#246A, #246B, #246C) and one (#298) at the end of the same month (**Appendix A:**

88 **Supplementary material**). All the stone martens were young, found in a state of apparent  
89 abandonment in anthropised areas (near human dwellings), and entered the structure in good  
90 general conditions, with the exception of #298, a male that showed mild sensory depression, which  
91 led to suspect to a car collision. Nonetheless, the subject fully recovered in five days. At the  
92 entrance of the subjects in the facility, an observation period (ranging from 2 to 30 days) was  
93 carried out in a separate isolation area before moving them to a fenced outdoor common area. In  
94 July 2018, on day 21 from rescue, subject #246A (female) showed hyperthermia and violent  
95 convulsions and died after 19 days (day 40 from rescue). One week later, subject #110A (female)  
96 was found dead, without showing any pathological sign in the previous days. On both subjects  
97 #246A and #110A, a post mortem CDV one-step real-time reverse transcription-PCR (RT-qPCR)

98 was performed on the RNA extracted from foot pads (**Appendix A: Supplementary material,**  
99 Scagliarini et al., 2007) sampled in July 2018. Histological analysis was performed only on subject  
100 #246A. In addition, qualitative two-step reverse transcription-PCR (RT-PCR) was performed to  
101 amplify the hemagglutinin (H) gene of CDV identified in animals #246A and #110A (**Appendix A:**  
102 **Supplementary material,** Demeter et al., 2007); these were subsequently sequenced, analysed by  
103 BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and aligned with 116 CDV reference strains  
104 (**Appendix A: Supplementary material**) retrieved from GenBank  
105 (<https://www.ncbi.nlm.nih.gov/genbank/>). Phylogeny was reconstructed by MEGA X version  
106 10.1.7 (Kumar et al., 2018).

107 Ten days after death of subject #110A, at day 43 from its rescue, subject #298 showed visual  
108 impairment, ocular nasal discharge, as well as lip ulcers and was quarantined. No symptoms were  
109 reported during the following period for the remaining three subjects.

110 The four surviving animals were quarantined and viral RNA shedding monitored to identify when  
111 they were no longer potentially infectious. Starting from the end of August 2018, all subjects were  
112 tested seven times within nine months for CDV RNA by one-step RT-PCR from conjunctival and  
113 oropharyngeal swabs (**Appendix A: Supplementary material,** Frisk et al., 1999). At the end of the  
114 isolation period (June 2019), presence of neutralizing antibodies was also investigated to assess  
115 whether animals were protected from reinfection before reintroduction into the wild (**Appendix A:**  
116 **Supplementary material,** Confer et al., 1975).

117 Histology on entrails from subject #246A revealed the presence of both enteritis with de-  
118 epithelialized, shortened and fused intestinal villi as well as interstitial pneumonia. Post-mortem  
119 RT-qPCR for CDV from foot pads of subjects #246A and #110A tested positive with a quantity of  
120  $17$  and  $2.8 \times 10^3$  viral RNA copies/mg of tissue, respectively. Analyses performed on complete  
121 CDV H gene nucleotide sequences (1824 bp) obtained from #246 and #110A (GenBank ID  
122 MW015089 and MW015090) showed a unique non-synonymous nucleotide mutation in position  
123 268 (corresponding to amino acid residue 90) that distinguished them with an overall nucleotide

124 identity >99,8% to the closest strain available in GenBank (ID MN044701). This strain was  
125 identified in a fox in North-Eastern Italy in 2018 (Bianco et al., 2020). Phylogeny revealed that the  
126 infection was caused by viruses belonging to the Wildlife-Europe CDV genetic subgroup, Europe  
127 1/South America 1 lineage (Bianco et al., 2020; Panzera et al., 2015) (**Figure 1**), closely related to  
128 other CDV recently identified in wildlife and dog.

129 In **Table 1** the results of RT-PCR on oropharyngeal and conjunctival swabs sampled from survived  
130 animals are reported. Stone martens #298 and #110B tested positive until the third month (October  
131 2018), while the remaining two subjects #246B and #246C tested positive until the fifth month  
132 (December 2018). Virus neutralization (VN) test revealed positive antibody titres of: 1:32 for  
133 subject #246C, 1:64 for both #110B and #298, and 1:128 for #246B.

134 Here we report a natural outbreak of canine distemper in six stone martens that showed different  
135 courses: two of them died, one showed mild signs while the remaining three displayed no  
136 symptoms. For all the animals the exact time of exposure is unknown. Nevertheless, it can be  
137 speculated that subject #246A might have been the source of infection, entering the structure during  
138 an incubation phase of the disease and developing convulsive crisis in July 2018, after 21 days, and  
139 dying 19 days later, in line with the data described in ferrets (Perpiñán et al., 2008). Furthermore, a  
140 concurrent subclinical infection of subject #298 cannot be excluded. Indeed, the mental dullness  
141 presented by the subject, initially ascribed to a car collision, might have been caused by the  
142 transient fever that occurs within the first days post CDV infection. Subsequent spread of the virus  
143 in the organism would have produced nasal and ocular discharge and lip ulcers 38 days later. As a  
144 matter of fact, asymptomatic carriers have been documented among other mustelids such as minks  
145 (Zhao et al., 2015). Also in dogs, it is estimated that at least 50% of CDV infections are subclinical  
146 (Peterson and Kutzler, 2011). The intermittent but protracted detection of the viral RNA  
147 (attributable to the sampling technique, the storing procedure between sampling and testing and the  
148 RT-PCR assay sensitivity) suggests that viral RNA excretion long lasted in all subjects. More  
149 specifically, in subjects #298 and #110B the viral excretion lasted at least three months while in the

150 remaining two subjects at least five months. This persistence recalls the pathogenesis described in  
151 dogs, where shedding of virus can continue for as long as three to four months (Sykes, 2014).  
152 Animals shed the virus in all secretions and since CDV is known for not persisting long in the  
153 environment, mildly affected and recovering infected animals may play an important role in  
154 maintaining transmission cycles in shelters (Miller and Hurley, 2009). This is particularly important  
155 within rescue centres, housing animals of unknown medical histories and endangered wildlife. In  
156 fact, CDV is considered of growing concern due to its propensity for host-switching and emergence  
157 in new species, including endangered species (Feng et al., 2016; Sulikhan et al., 2018)..  
158 Clinical manifestations and histological results confirmed that the gastrointestinal tract, the  
159 respiratory and nervous systems were the most affected in stone martens, as already documented in  
160 other mustelids (Perpiñán et al., 2008).

161 Morbilliviruses induce long-term immunity, in particular, immune response towards CDV infection  
162 is considered lifelong (Schultz et al., 2010), thus detection of seropositive animals implies  
163 protection against the virus. Sero-conversion of mink, in experimental conditions, has been  
164 described to take place at 14 days post infection (dpi) reaching the highest titres at 21 dpi (Zhao et  
165 al., 2015). Since we did not test subjects so early we cannot compare our results, nevertheless, it is  
166 assumable that, as described in dogs, clinical signs resolve and the virus is cleared from most body  
167 tissues when antibody titre increases (Greene and Vandeveld, 2012). Indeed, the neutralization test  
168 is still considered the gold standard for measuring protection against infection, and antibody titres  
169 correlate with the level of protection (Appel and Robson, 1973).

170 The identified viruses belonged to the Wildlife-Europe CDV genetic subgroup (Europe 1/South  
171 America 1 lineage). This wildlife well-adapted CDV genetic group has been detected in Europe  
172 (Monne et al., 2011). In Italy, it also caused the first spillover event, from wildlife to a non-  
173 vaccinated domestic dog (Bianco et al., 2020). Coevolution and phylogenetic clustering of CDV  
174 identified in both wildlife and domestic dogs have previously been reported (Piewbang et al., 2020),  
175 describing an intricate epidemiological dynamics characterised by multiple host infections and



176 interspecies transmission (Duque-Valencia et al., 2019). In this light, the central role of wildlife in  
177 the disease epidemiology should be exploited (Anis et al., 2020), enhancing passive surveillance of  
178 the wide range of susceptible animal species to broaden the evolutionary analysis of CDV, a  
179 pathogen that exhibits a high mutation rate, to undertake reliable wildlife conservation strategies.

180

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## 188 **Appendix A: Supplementary material**

189 Supplementary data to this article can be found online at doi...

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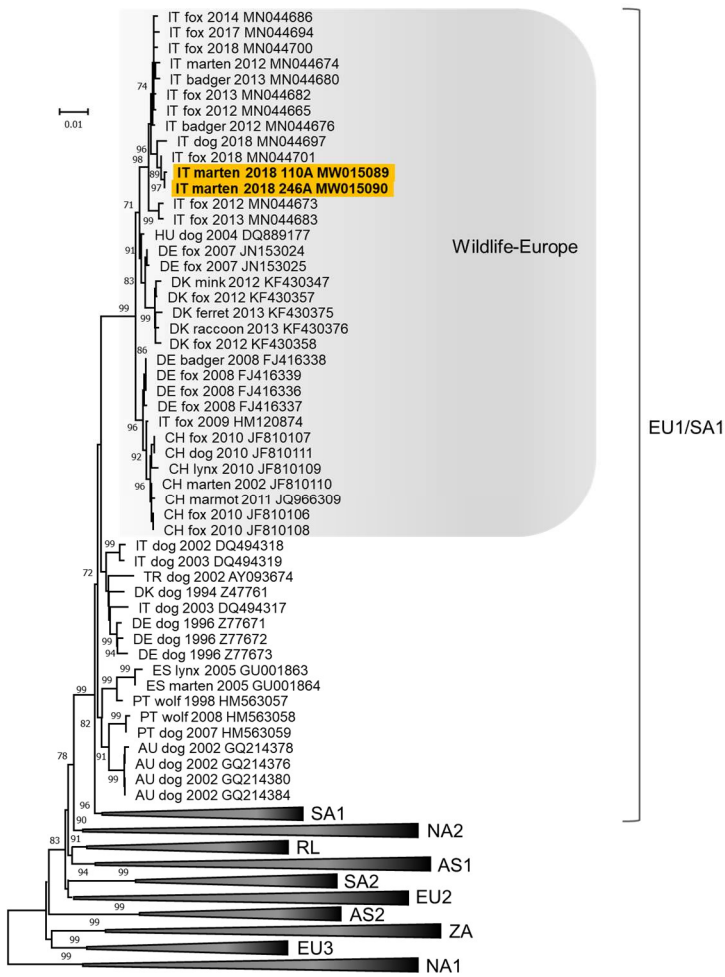
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294 **Figure captions**



295  
 296 **Figure 1.** Unrooted phylogenetic tree constructed on the canine distemper virus (CDV)  
 297 hemagglutinin (H) gene nucleotide sequences.

298 Phylogeny was carried out on the CDV H gene nucleotide sequences generated in this study (1824  
 299 nucleotides) and with sequences of 116 CDV reference strains obtained from the GenBank database  
 300 (**Appendix A: Supplementary material**) using the software MEGA X version 10.1.7 (Kumar et  
 301 al., 2018). Phylogenetic tree was constructed using Neighbor-Joining method and the best-fit model  
 302 of nucleotide substitution was determined using the Find Best DNA/Protein Model function  
 303 implemented in MEGA X. The Tamura 3-parameter (T92) model with gamma distribution resulted  
 304 optimal for the nucleotide alignment. The robustness of individual nodes on the phylogeny was  
 305 estimated using 1000 bootstrap replicates and bootstrap values >70 were indicated at the  
 306 corresponding node. The CDV strains included in the phylogenetic analysis are named with:

307 acronym of nation, host species, year of identification, lab numbers only for sequences generated in  
308 this study, and the GenBank accession number. When the year of identification was not available,  
309 the year of deposition of the nucleotide sequence in the GenBank database was indicated. Canine  
310 morbillivirus genetic lineages: America 1 (NA1), America 2 (NA2), Europe 1/South America 1  
311 (EU1/SA1), Europe 2/Europe-Wildlife (EU2), Europe 3/Arctic like (EU3), Asia 1 (AS1), Asia 2  
312 (AS2), South Africa (ZA), South America 2 (SA2) and Rockborn like (RL). The genetic lineages  
313 not containing CDV sequences obtained in this study are compressed. In grey: Wildlife Europe  
314 genetic subgroup. Highlighted: sequences generated in this study.  
315

316 **Table 1.** Results of reverse transcriptase-PCR (RT-PCR) on oropharyngeal and conjunctival swabs  
 317 sampled from survived animals

Subject and samples	RT-PCR results at different sampling times						
	August 24, 2018	September 12, 2018	October 16, 2018	December 19, 2018	February 20, 2019	May 27, 2019	April 17, 2019
<b>#110B</b>							
OFS	N	N	N	N	N	N	N
CS	P	N	P	N	N	N	N
<b>#246B</b>							
OFS	P	N	N	N	N	N	N
CS	N	N	N	P	N	N	N
<b>#246C</b>							
OFS	N	N	N	N	N	N	N
CS	P	N	N	P	N	N	N
<b>#298</b>							
OFS	P	P	P	N	N	N	N
CS	P	P	P	N	N	N	N

318

319 CS: conjunctival swab. N: negative. OFS: oropharyngeal swab. P: positive.