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Domestic Dog Origin of Canine Distemper Virus in Free-ranging Wolves in Portugal as Revealed by Hemagglutinin Gene Characterization

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ABSTRACT: Serologic evidence for canine distemper virus (CDV) has been described in grey wolves but, to our knowledge, virus strains circulating in wolves have not been characterized genetically. The emergence of CDV in several non-dog hosts has been associated with amino acid substitutions at sites 530 and 549 of the hemagglutinin (H) protein. We sequenced the H gene of wild-type canine distemper virus obtained from two free-ranging Iberian wolves (*Canis lupus signatus*) and from one domestic dog (*Canis familiaris*). More differences were found between the two wolf sequences than between one of the wolves (wolf 75) and the dog. The latter two had a very high nucleotide similarity resulting in identical H gene amino acid sequences. Possible explanations include geographic and especially temporal proximity of the CDV obtained from wolf 75 and the domestic dog, taken in 2007–2008, as opposed to that from wolf 3 taken more distantly in 1998. Analysis of the deduced amino acids of the viral hemagglutinin revealed a glycine (G) and a tyrosine (Y) at amino acid positions 530 and 549, respectively, of the partial signaling lymphocytic activation molecule (SLAM)-receptor binding region which is typically found in viral strains obtained from domestic dogs. This suggests that the CDV found in these wolves resulted from transmission events from local domestic dogs rather than from wildlife species.

Key words: Canine distemper virus (CDV), domestic dog, grey wolf (*Canis lupus*), hemagglutinin (H) gene.

During the last two centuries, wolf (*Canis lupus*) populations have suffered dramatic declines in Europe, leading to genetically isolated subpopulations (Lucchini et al., 2004; Ramirez et al., 2006). Two subpopulations are recognized in Portugal (Blanco et al., 1992; Pimenta et al., 2005). The larger subpopulation, north of the Douro River valley, is connected to the main Iberian population whereas the smaller subpopulation, south of the Douro River, has been demographically isolated for decades. In the context of conservation, infectious diseases may have a considerable impact on population size (Daszak et al., 2000; Woodroffe et al., 2004; Woolhouse and Gowtage-Sequeria, 2005). Canine distemper is a disease that may cause mortality in wildlife species, as recently described in Europe (Martella et al., 2010; Sekulin et al., 2011). Serologic evidence for canine distemper has been described in wolves (Johnson et al., 1994; Sobrino et al., 2008; Santos et al., 2009) but, to our knowledge, virus strains circulating in wolves have not been characterized genetically. The canine distemper virus (CDV) is covered by an envelope containing the surface glycoprotein H (hemagglutinin) which is crucial for attachment to its host cell receptor, the signaling lymphocytic activation molecule (SLAM; Tatsuo et al., 2001; von Messling et al., 2001). The emergence of CDV in non-dog hosts has been assessed and amino acid substitutions at sites 530 and 549 of the H protein are possibly involved in the determination of host tropism (Seki et al., 2003; McCarthy et al., 2007; Sekulin et al., 2011). Here we describe the genetic characterization of the H gene of wild-type CDV obtained from two young, free-ranging Iberian wolves and a domestic dog (*Canis familiaris*; Fig. 1).

Wolf 3, a subadult male, was found dead in 1998 in northeastern Portugal (41°49′28″N, 6°41′36″W). Necropsy revealed emaciation and atelectasis and intense red hepatization of the lungs. Wolf

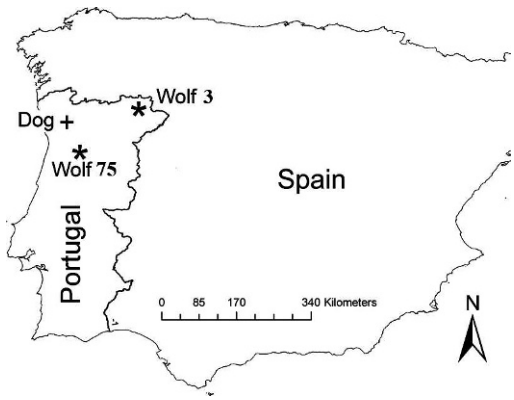

FIGURE 1. Sampling sites of two grey wolves (*Canis lupus*) and one domestic dog (*Canis familiaris*) infected with canine distemper virus. Wolf 3 was obtained in 1998 and wolf 75 in 2008; the domestic dog was obtained in 2007.

75, a male pup of the population south of the Douro River (41°0′30″N, 7°54′30″W), was found dead in 2008. Necropsy revealed emaciation, dehydration, oral hemorrhages and edema, emphysema, and red hepatization of the lungs. Lung tissue samples were collected from both animals and preserved at −30 C until further analyses. For comparison, the CDV H gene from a domestic dog obtained in 2007 from Braga District (41°23′06″N, 8°24′42″W) was also sequenced. This sample consisted of peripheral blood mononuclear cells from a dog with clinical canine distemper confirmed by diagnostic PCR (Mochizuki et al., 1999). RNA was extracted from the supernatant of tissue homogenates using the QIAamp Viral RNA Kit (Qiagen, Hilden, Germany). The extractions of the wolf samples were made separately from the canine sample. Complementary DNA was synthesized using random priming and Moloney murine leukemia virus [M-MLV] reverse transcriptase (Invitrogen, Carlsbad, California, USA). Four overlapping primer pairs were designed using the software Primer3 (Rozen and Skaletsky, 2000): Primers 1F: 5′-GGGCTCAGGTAGTCCARCAA-3′ and 1R: 5′-CCTCCGGAGAGTGCTGATAA-3′; primers 2F: 5′-GCGATACAATTGGGATCAGA-3′ and 2R: 5′-TGGGTGAGCAACAGGTATCA-3′; primers 3F: 5′-TAGGGATATTTGGGGCAACA-3′ and 3R: 5′-TCCATAATCTGGGATGTTTGAA-3′; and primers 4F: 5′-ATCCCCCATGTGTTGACATT-3′ and 4R: 5′-RGACCTCAGGGTATAGAATCTGG-3′. An annealing temperature of 57 C was used. The amplicons were directly sequenced in both directions using the amplification primers above. Sequences were aligned with other H gene sequences obtained from GenBank using the software Bioedit Version 7 (Hall, 1999). Phylogenetic analyses were carried out by the neighbor-joining method using the software Mega4 (Tamura et al., 2007). The GenBank accession numbers are HM563057–9.

The nucleotide sequences obtained from wolf 75 and the domestic dog were very similar, displaying only two synonymous nucleotide polymorphisms at positions 960 and 1065 of the H gene. In contrast, the sequence obtained from wolf 3 had 33 and 31 nucleotide polymorphisms when compared to sequences from wolf 75 and the domestic dog, respectively, resulting in 15 amino acid polymorphisms. A phylogenetic analysis was performed for the H gene sequences of representative CDV sequences from the six recognized lineages (genotypes): Asia-1, Asia-2, Europe, Arctic, America-1, and America-2 (Haas et al., 1997; McCarthy et al., 2007). The Portuguese wolf and dog sequences clustered within the genogroup "Europe" near CDV sequences obtained recently from domestic dogs in Austria (Fig. 2). Deduced amino acid sequences of the H protein had six to seven polymorphic sites between wolf 75 and the Austrian dog sequences (GenBank references GQ214384, GQ214376, GQ214380, GQ214378). Between wolf 75 and the Austrian dog sequences, 12–13 variable amino acids were found.

In a report of a recent serosurvey, we suggested that CDV infection was epidemic in free-ranging Canidae in Portugal

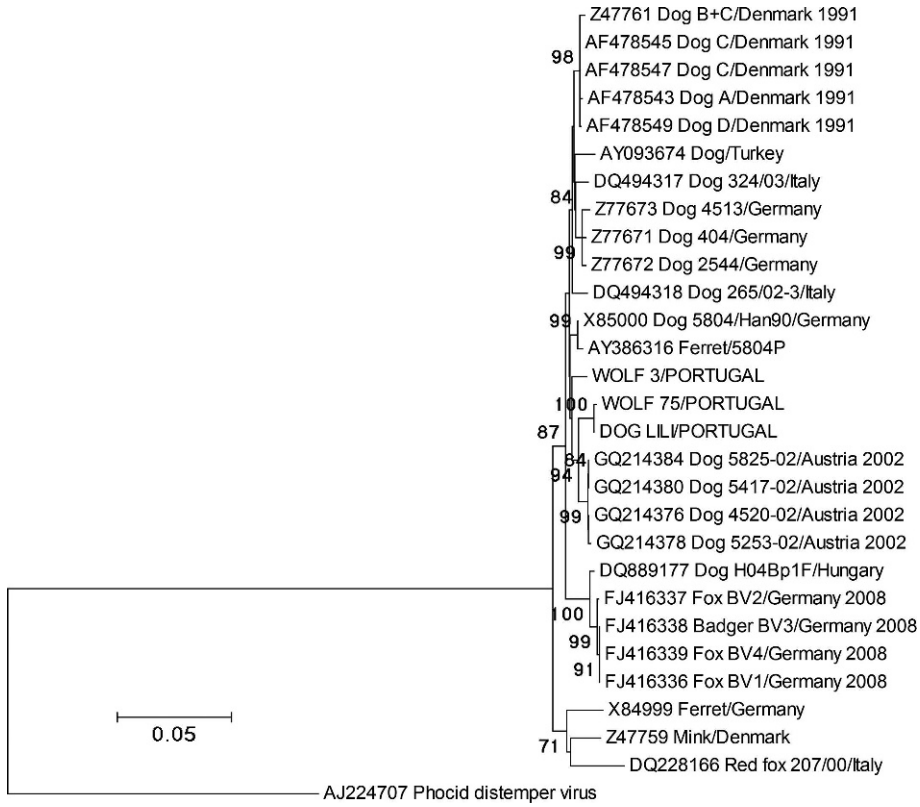

FIGURE 2. Phylogenetic tree based on the complete nucleotide sequence of the canine distemper virus H gene of strains representing the two main clusters of "Europe" (upper branch) and "European wildlife" (lower branch, containing X84999, Z47759, DQ228166). The sequences obtained in Portugal, from two grey wolves (wolf 3 and wolf 75) and a domestic dog, are shown in capital letters. Bootstrap values >70% are indicated at the nodes. A phocid distemper virus was used to root the tree.

(Santos et al., 2009). Here, we present the characterization of the H gene of CDV obtained from two free-living wolves. Possible explanations for the close genetic similarity between wolf 75 and the dog include geographic and especially temporal proximity; both were taken in 2007–2008 within a distance of 60 km. The sample of wolf 3 was taken 10 yr earlier approximately 150–200 km distant. Similar geographic and temporal clusters have been observed for canine CDV sequences obtained in Denmark in 1991, Germany in 1996, Austria in 2001, and in fox and badger sequences obtained in 2008 in Germany (Blixenkrone-Møller et al., 1993; Sekulin et al., 2011). Sequence analysis of the H gene of all three Portuguese CDV strains revealed a glycine (G) and a tyrosine (Y) at amino acid positions 530 and 549 of the partial SLAM-receptor binding region. This is typically found in viral strains obtained from dogs, suggesting that the CDV found in these wolves resulted from transmission events from local domestic dogs rather than from other wildlife. In other wildlife species, amino acid substitutions have been found at these sites, suggesting a functional role of residue 549 in host switches (von Messling et al., 2001; McCarthy et al., 2007; Sekulin et al., 2011). It is possible that mutations at these sites are not required due to the genetic proximity between dogs and wolves. To our knowledge, this is the first partial genetic characterization of CDV

circulating in wolves. In Europe, both directions of CDV transmission have been described; distemper in dogs resulting from spillover from wildlife (Martella et al., 2010) and vice versa (Meli et al., 2010). Because domestic dogs are abundant within the distributions of wolves in Portugal, and regular vaccination of dogs in rural areas is not common, we postulate that dogs may act as reservoirs of infection for wildlife. Considering the particular vulnerability of the Iberian wolf, the introduction of active control measures, such as increasing the vaccine coverage in local domestic dog populations, would be desirable and could reduce the impact of CDV.

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LITERATURE CITED

Blanco, J. C., S. Reig, and L. de la Cuesta. 1992. Distribution, status and conservation problems of the wolf *Canis lupus* in Spain. Biological Conservation 60: 73–80.

Blixenkrone-Møller, M., V. Svansson, P. Have, C. Orvell, M. Appel, I. R. Pedersen, H. H. Dietz, and P. Henriksen. 1993. Studies on manifestations of canine distemper virus infection in an urban dog population. Veterinary Microbiology 37: 163–173.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife: Threats to biodiversity and human health. Science 287: 443–449.

Haas, L., W. Martens, I. Greiser Wilke, L. Mamaev, T. Butina, D. Maack, and T. Barrett. 1997. Analysis of the haemagglutinin gene of current wild-type canine distemper virus isolates from Germany. Virus Research 48: 165–171.

Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Johnson, M. R., D. K. Boyd, and D. H. Pletscher. 1994. Serologic investigations of canine parvovirus and canine-distemper in relation to wolf (*Canis lupus*) pup mortalities. Journal of Wildlife Diseases 30: 270–273.

Lucchini, V., A. Galov, and E. Randi. 2004. Evidence of genetic distinction and long-term population decline in wolves (*Canis lupus*) in the Italian Apennines. Molecular Ecology 13: 523–536.

Martella, V., A. Bianchi, I. Bertoletti, L. Pedrotti, A. Gugiatti, A. Catella, P. Cordioli, M. S. Lucente, G. Elia, and C. Buonavoglia. 2010. Canine distemper epizootic among red foxes, Italy, 2009. Emerging Infectious Diseases 16: 2007–2009.

McCarthy, A. J., M. A. Shaw, and S. J. Goodman. 2007. Pathogen evolution and disease emergence in carnivores. Proceedings of the Royal Society B–Biological Sciences 274: 3165–3174.

Meli, M. L., P. Simmler, V. Cattori, F. Martínez, A. Vargas, F. Palomares, J. V. López-Bao, M. A. Simón, G. López, L. León-Vizcaino, R. Hofmann-Lehmann, and H. Lutz. 2010. Importance of canine distemper virus (CDV) infection in free-ranging Iberian lynxes (*Lynx pardinus*). Veterinary Microbiology 146: 132–137.

Mochizuki, M., M. Hashimoto, S. Hagiwara, Y. Yoshida, and S. Ishiguro. 1999. Genotypes of canine distemper virus determined by analysis of the hemagglutinin genes of recent isolates from dogs in Japan. Journal of Clinical Microbiology 37: 2936–2942.

Pimenta, V., I. Barroso, F. Alvares, J. Correia, G. Costa, L. Moreira, J. Nascimento, F. Fonseca, S. Roque, and E. Santos. 2005. Situaão populacional do lobo em Portugal: Resultado do censo nacional 2002/2003. *In* Relatório Técnico, ICN, ed. 2005. (Lisbon, Instituto de Conservaão da Natureza/Grupo Lobo), Lisbon, Portugal. pp. 158.

Ramirez, O., L. Altet, C. Enseñat, C. Vilà, A. Sanchez, and A. Ruiz. 2006. Genetic assessment of the Iberian wolf *Canis lupus* signatus captive breeding program. Conservation Genetics 7: 861–878.

Rozen, S., and H. J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. *In* Bioinformatics methods and protocols: Methods in molecular biology, S. Krawetz and S. Misener (eds.). Humana Press, Totowa, New Jersey. pp. 365–386.

Santos, N., C. Almendra, and L. Tavares. 2009. Serologic survey for canine distemper virus and canine parvovirus in free-ranging wild carnivores from Portugal. Journal of Wildlife Diseases 45: 221–226.

Seki, F., N. Ono, R. Yamaguchi, and Y. Yanagi. 2003. Efficient isolation of wild strains of canine distemper virus in vero cells expressing canine SLAM (CD150) and their adaptability to marmoset B95a cells. Journal of Virology 77: 9943–9950.

Sekulin, K., A. Hafner-Marx, J. Kolodziejek, D. Janik, P. Schmidt, and N. Nowotny. 2011. Emergence of canine distemper in Bavarian wildlife associated with a specific amino acid

exchange in the haemagglutinin protein. Veterinary Journal 187: 399–401.

Sobrino, R., M. C. Amal, D. F. Luco, and C. Gortazar. 2008. Prevalence of antibodies against canine distemper virus and canine parvovirus among foxes and wolves from Spain. Veterinary Microbiology 126: 251–256.

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

Tatsuo, H., N. Ono, and Y. Yanagi. 2001. Morbilliviruses use signaling lymphocyte activation molecules (CD150) as cellular receptors. Journal of Virology 75: 5842–5850.

von Messling, V., G. Zimmer, G. Herrler, L. Haas, and R. Cattaneo. 2001. The hemagglutinin of canine distemper virus determines tropism and cytopathogenicity. Journal of Virology 75: 6418–6427.

Woodroffe, R., S. Cleaveland, O. Courtenay, M. K. Laurenson, and M. Artois. 2004. Infectious disease in the management and conservation of wild canids. *In* Biology and conservation of wild canids, D. W. MacDonald and C. Sillero-Zubiri (eds.). Oxford University Press, Oxford, UK. pp. 123–142.

Woolhouse, M. E., and S. Gowtage-Sequeria. 2005. Host range and emerging and reemerging pathogens. Emerging Infectious Diseases 11: 1842–1847.

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