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THE CANINE ADENOVIRUS TYPE 2 (CAdV-2) IN ITALIAN WOLVES (*Canis lupus italicus*): A PRELIMINARY STUDY

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ABSTRACT

The canine adenovirus type 2 (CAdV-2) is associated with the infectious tracheobronchitis commonly called "kennel cough", cosmopolitan in dogs but little explored in gray wolves. Our goals were (i) to evaluate the presence and circulation of CAdV-2 in free-ranging Italian wolves (*Canis lupus italicus*), through the analysis of spleens and tongues collected from 56 carcasses sampled in three Italian regions between August 2017 and July 2020, and (ii) to support the validity of a matrix such as the tongue, which was never used before. Samples were screened for the presence of CAdV-2 DNA using both PCR and real-time PCR assay. Positive results were related to sampling year, location, sex, age, genetic determination of species, and matrices tested.

Three male wolves (5.4%) tested positive in tongue samples, demonstrating that the tongue is an excellent matrix for the detection of CAdV-2. To the best of our knowledge, no studies were performed to evaluate the usability of tongue samples to detect CAdV-2 DNA in grey wolves or other wild animals.

The number of wolves tested positive suggests that, during the studied years, the circulation of CAdV-2 in Italian wolves showed a low frequency, consistent with irregular introductions of the virus by dogs or other wild carnivores in these populations. This preliminary study provides new data on the ecology of CAdV-2 in Italian wolves, although future studies are needed to fully understand its real circulation at a national scale, its pathogenetic role in gray wolves, and its risk of transmission to other wild carnivores.

Keywords: Canine adenovirus type 2; *Canis lupus italicus*; Italian wolf; wildlife

The canine mastadenovirus A belongs to the genus *Mastadenovirus* (family Adenoviridae) and includes two canine types: canine adenovirus type 1 (CAdV-1), which in dogs is the causative agent of Rubarth disease or infectious canine hepatitis (ICH), while in wild carnivores causes neurological forms (encephalitis), and canine adenovirus type 2 (CAdV-2), which is associated in dogs with the infectious tracheobronchitis commonly called "kennel cough" (MacLachlan and Dubovi, 2017). CAdV-2 has tropism for the respiratory system and has mainly a respiratory transmission (Ford, 2012), though it is also frequently found in canid scats (Balboni et al., 2014). Kennel cough is an acute, highly contagious disease in dogs (Decaro et al., 2012). Mortality is rare and occurs mainly in animals that are less than one month old (Ford, 2012). The clinical signs of infectious tracheobronchitis in canids occur 3-10 days post-infection, typically with a paroxysmal sometimes productive cough (Ford, 2012).

A study by Cartensen et al. (2017) witnessed the presence of CAdV-2 in grey wolves in Minnesota (USA) with very high seroprevalence: 88% in adults and 45% in pups. This result allowed the authors to assume that the virus is endemic in Minnesota (Carstensen et al., 2017). However, it is necessary to specify that several individuals who died in the wild were not sampled, and that a positive antibody titer is indicative of infection but not of clinical disease (Carstensen et al., 2017). Additional factors that can increase the seroprevalence of the virus in the environment may be the harsh climate, as the adenovirus

resists for a long time in cold environments, and the presence of other carnivores with role of reservoir (Watts and Benson, 2016).

In Spain, a study on 37 free-ranging wolves detected 5.4% of CAdV-2 DNA by PCR on the spleen (Milàn et al., 2016) and a French study demonstrated the circulation of CAdV-2 in captive wolves (Dowgier et al., 2018).

In Italy, two study estimated the prevalence of CAdV-2 infection by molecular analyzes on non-invasively collected fecal samples of small population of wolves in central Italy (Di Francesco et al., 2019) and of another one in Northern Italy (Melegari et al., 2018). Additionally, the analysis of a dead wolf found in southern Italy tested positive for three viruses: canine pantropic coronavirus (CCoV), canine parvovirus type 2b (CPV-2b), and CAdV-2 (Alfano et al., 2019).

In light of the limited and partial data available, the aims of this preliminary study were i) to demonstrate the presence of canine adenovirus type 2 (CAdV-2) genomic DNA, ii) estimate its circulation in Italian wolves, and iii) evaluate the usability of tongue samples to detect CAdV-2 DNA.

The study area extends over three Italian regions (Tuscany, Emilia-Romagna, and Calabria), a detailed map of which is presented in Figure 1. The map was created with the open-source software QGIS 3.10 and edited with the free software *Inkscape*.

Between 2017 and 2020, necropsy examinations were carried out on 56 wolf carcasses. The animals died mainly from road collisions and secondarily from illegal killings with poison or gunshot. Five subjects died as a result of intraspecific aggression. For these we recorded subject identification data with the attribution of an ID code, place of discovery (reported as GPS coordinates), sex, weight (in kg), and nutritional status. The animal age was estimated based on dental development, body size, and weight (Morner et al., 2005). All individuals examined were aged using one of the following categories: class 1: ≤ 12 months; class 2: 1–2 years; class 3: > 2 years.

Subsequently, the nutritional status, previous lesions, appearance of the mucous membranes, and explorable lymph nodes were evaluated. A portion of lingual muscular tissue was taken and stored in 95% ethanol to genetically determine the species of the examined canids and to detect a possible presence of genetic hybridization signatures (Randi et al., 2014; Caniglia et al., 2020).

After that, the skinning and the opening of the abdominal cavity were executed, followed by the opening of the thoracic cavity. At the end of the necropsy investigations, the organs (spleen and tongue) were sampled for the detection of CAdV-2. The spleen is believed to be a good matrix in detecting CAdV DNA and is an organ frequently used for virological molecular investigations in gray wolves (Millan et al., 2016). Instead, the choice to use the tongue for molecular investigations arose from the fact that this matrix is used for the DNA detection of CPV-2 (McKnight et al., 2007) and CAdV-1 (Balboni et al., 2019). In this study we wanted to prove its usefulness for the detection also of CAdV-2 DNA.
The presence of CAdV-2 DNA was investigated by using two different molecular assays: (i) a PCR based on the use of the primers pair HA1 and HA2 described by Hu RL et al. (2001) and of the GoTaq®Hot Start Colorless Master Mix (PROMEGA, Madison, USA) according to the manufacturer's instructions; and (ii) a SYBR Green real-time PCR assay described by Balboni et al. (2015) using the PowerUp SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions.

Three of the 56 sampled wolves were positive for CAdV-2 DNA with a prevalence of 5.4% (Table 1). CAdV-2 DNA was detected in 2 years: in 2018 in 2 out of 3 subjects and in 2019 in one subject. All three positive subjects were male and equally represented in the three age classes (Table 1). In the Emilia-Romagna region, CAdV-2 DNA was found in one subject, while the other two came from the Calabria region. Regarding the species determination, there was a strong prevalence of individuals classified as "WOLF", representing the majority (2/3) of the positives, while the third subject was a wolf with dog introgression (WOLF_INTR). All 3 positives were found using the same kind of matrix, the tongue (Table 1 and Figure 2). The statistical analyses for the calculation of the prevalence and confidence intervals were implemented using the R computing environment (RStudio, 2020).

Only a few studies on the circulation of canine adenovirus type 2 (CAdV-2) in gray wolves have been published so far. However, some studies demonstrate its presence in free-ranging wolves. In Spain, Millán et al. (2016) analyzed the spleens of 37 Iberian wolves by PCR, detecting CAdV-2 DNA in 5.4% of the subjects. This study is perfectly comparable to ours. In fact, the detection rate of CAdV-2 DNA is identical in both papers (5.4%) and both analyzed organs with molecular methods. In our study, the tongues were positive

while the spleens were negative, while in the work of Millán et al. (2016) the spleens were positive but the tongues were not analyzed.

The transmission of CAdV-2 can take place in both directions, i.e. from domestic animals to wild ones, and vice versa. To date, it is not entirely clear which species acts as a reservoir for the others. However, some studies hypothesize the infection reservoirs are domestic dogs, with which wolves have contact due to strong environmental anthropization (Millán et al., 2016). They also have contact with hunting and livestock guarding dogs (Landry et al., 2020).

The number of wolves tested positive in this study suggests that, during the study period, the circulation of CAdV-2 in the Italian wolves of the three sampled regions showed a low frequency, proving consistent with irregular entry of the virus in these populations.

The data of this study were obtained from opportunistic sampling, i.e. based on random findings of dead wolves in the study areas. The limiting aspect of collecting carcasses is given by the fact that not all dying wolves are found, especially in case of poaching mortality (Liberg et al., 2012), or in some cases of deaths in a difficult to reach environment. In the studying of viral circulation in the wild, these difficulties are a limitation that can generate statistical and methodological bias that prevent inferring the data on the national population. However, it must be recognized that for studies on elusive species such as large carnivores, the finding of carcasses is often the only opportunity of close contact with them that allows structuring a transversal research, albeit maintaining a precautionary approach in interpreting the data (Lovari et al., 2007). Through the sampling of this study, we found that the tongue can be a matrix for the detection of CAdV-2, and not just CPV-2 (McKnight et al., 2007) and CAdV-1 (Balboni et al., 2019). As far as we know, no studies have never been performed to verify the validity of the tongue as a target organ of CAdV-2 in grey wolves. To verify this, more in-depth studies would be necessary, ideally using the IHC technique, capable of detecting the virus presence in the epithelium or muscle. For now, we can assume that the detection of CAdV-2 DNA is most likely due to its presence in the respiratory secretions or saliva, and that these are present on the tongue's surface.

This study regards the CAdV-2 circulation in three Italian regions. It is intended to be a preliminary study providing a small-scale idea of the CAdV-2 presence in Italian wolves. Further studies will be necessary to overcome the critical issues highlighted above.

ETHICAL STATEMENT

No ethical approvals were required as these were investigations on animals that died from other causes. No animals were killed for the purposes of this study.

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FIGURE 1 – The map shows all the points of discovery of the wolf carcasses object of this study. The red "plus" symbols represent the 3 positive wolves for CAdV-2. At the bottom right, the map shows the Italian regions covered by this study in gray.

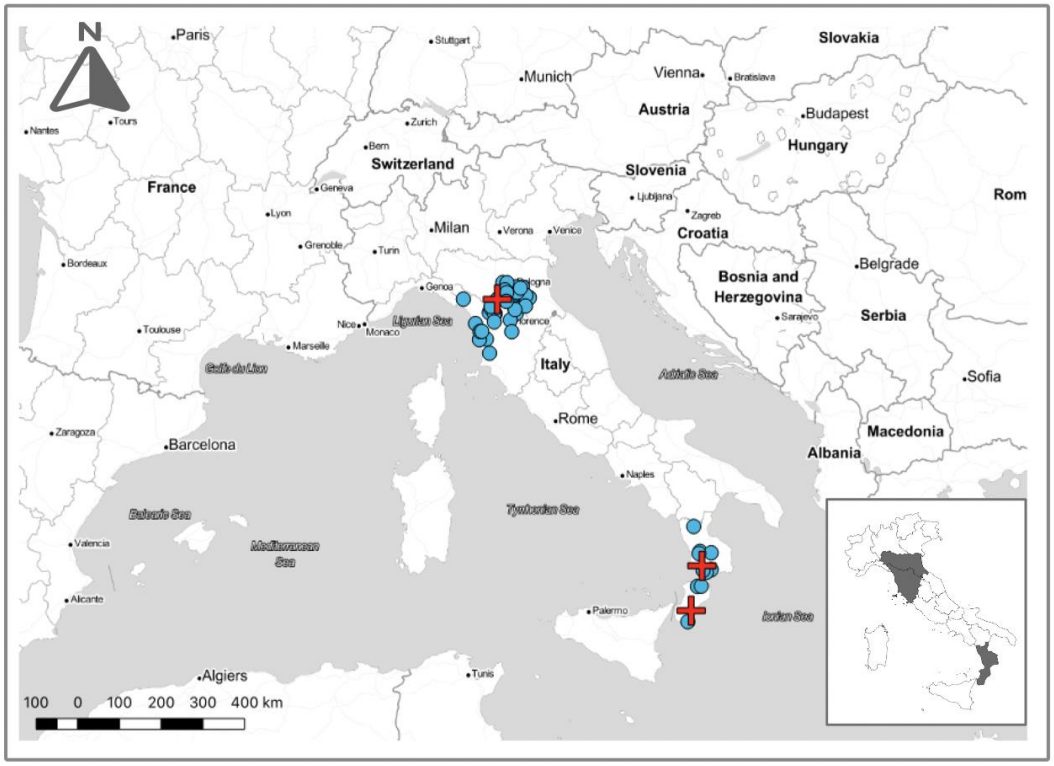

FIGURE 2 – CAdV PCR according to Hu et al. (2001). The image shows the amplification band of 1028bp obtained from one of the three positive wolves (S1) starting from two DNA extracts of the same tongue.
CE: DNA Extract Control; C-: PCR Negative Control; CAdV-1 Positive Control of 505bp; CAdV-2 Positive Control of 1028bp.

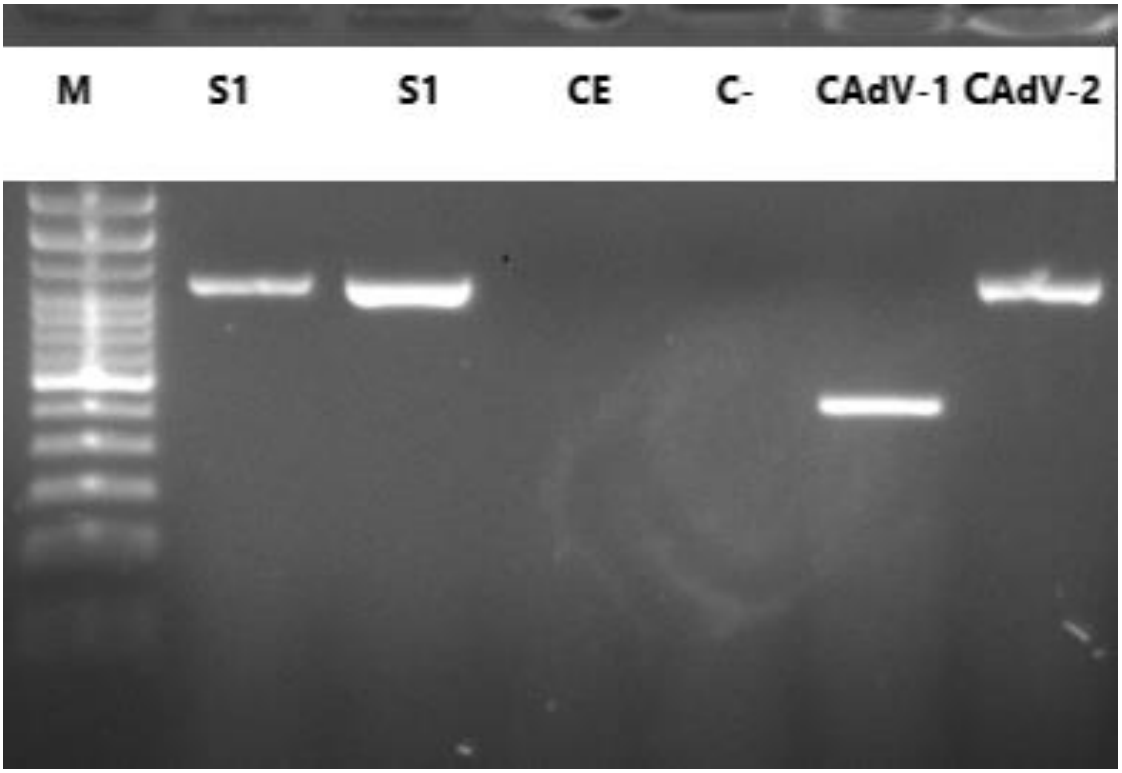

TABLE 1 - Signaling data of the population sampled with results of molecular investigations according to the year of death, location, sex, age, species identification, matrix sampled and coinfections.

Abbreviations: *n*: number; ND: not determinable; Prev: prevalence; 9% CI: lower limit of the confidence interval; 95% CI: upper limit of the confidence interval.

	No. subjects (*n*=56)	**CAdV-2 Positive (*n*=3)**	**Prev**	**9% CI**	**95% CI**
Year of death					
2017	6	0	0.00%	0,00%	45,93%
2018	17	2	11.76%	1,46%	36,44%
2019	15	1	6.67%	0,17%	31,95%
2020	18	0	0.00%	0,00%	18,53%
Region of origin					
Emilia-Romagna	25	1	4.00%	0.10%	20.35%
Tuscany	19	0	0.00%	0.00%	17.65%
Calabria	12	2	16.67%	2.09%	48.41%
Sex					
Male	32	3	9.38%	1.98%	25.02%
Female	24	0	0.00%	0.00%	14.25%
Age					
Class_1	19	1	5.26%	0.13%	26.03%
Class_2	18	1	5.26%	0.14%	27.29%
Class_3	19	1	5.26%	0.13%	26.03%
Species identification					
WOLF	42	2	4.76%	0.58%	16.16%
WOLF_INTR	10	1	10.00%	0.25%	44.50%
HYBRID	3	0	0.00%	0.00%	70.76%
ND	1	0	0.00%	0.00%	97.50%
Matrix sampled					
Spleen	56	0	0.00%	0.00%	6.38%
Tongue	56	3	5.36%	1.12%	14.87%
Tot. individuals	**56**				