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CIRCULATION OF CANINE VIRUSES IN FREE-RANGING ITALIAN WOLVES (CANIS LUPUS ITALICUS) FROM THREE ITALIAN REGIONS

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ABSTRACT

In this study, the duodenum, spleen, tongue, and lungs were sampled from 56 Italian wolves (*Canis lupus italicus*) who died in Emilia-Romagna, Tuscany, and Calabria between August 2017 and July 2020.

The aim of the study was to evaluate the presence and spread of DNA and RNA viruses in the wolf population examined, relating the virological results to: (i) year of sampling, (ii) region of origin, (iii) sex, (iv) age, (v) season, (vi) genetic determination of the species, (vii) nutritional conditions, (viii) causes of death, (ix) matrices examined. In addition, the presence or absence of co-infections was evaluated.

Through molecular methods of real-time PCR and RT-PCR, the presence of genomic DNA of three important DNA viruses was investigated, i.e.: Canine Parvovirus type 2 (CPV-2), Canine Adenovirus type 1 (CAdV-1), Canine Adenovirus type 2 (CAdV-2). Furthermore, the presence of genomic RNA of the important RNA viruses, Canine Enteric Coronavirus (CCoV) and Canine Distemper Virus (CDV), was also investigated.

The results of real-time PCR molecular investigations showed that the virus with the highest prevalence in the wolf population studied was CPV-2, found in 78.6% of subjects (44/56). The prevalence of CAdV was 17.9% (10/56), in particular CAdV-1 (12.5% - 7/56) and CAdV-2 (5.4% - 3/56). The results of the molecular investigations in RT-PCR of the two RNA viruses (CCoV and CDV) did not give positive results in the study population.

In this study it was observed that the majority of wolves that resulted positive were in good nutritional conditions, thus excluding a direct cause of death from CPV-2, CAdV-1, and CAdV-2 infections. Moreover, the prevalence obtained in this study suggests that, during the years here studied, the circulation of CAdV-1 and CAdV-2 in Italian wolves of the three sampled regions was sporadic, proving consistent with sporadic and short-lived introductions of the virus in these populations. However, the situation for CPV-2 is different as there was a circulation that suggests a pattern of continuous and lasting endemic exposure over time.

Keywords: Canine Adenovirus type 1-2; Canine Enteric Coronavirus; Canine Distemper Virus; Canine Parvovirus type 2; *Canis lupus italicus*; DNA virus, gray wolf, RNA virus, viral circulation

CHAPTER ONE: INTRODUCTION

1.1. BACKGROUND

1.1.1. The species: Canis lupus, Linnaeus 1758

The gray wolf (*Canis lupus*) is the second largest predator in Europe after the brown bear (*Ursus arctos*).

Due to its large distribution across a wide variety of habitats, its phenotypic variation (in terms of weight, color, and body size) is extremely high (Boitani, 2000). Moreover, additional morphological differences, such as the size and shape of the skull, allowed the identification of nine subspecies of *Canis lupus,* all within the Eurasian area (Sokolov and Rosolino, 1985).

The use of molecular tools, such as DNA sequencing (Vila *et al.*, 1997) and phylogenetics, confirmed that the gray wolf is the only ancestor of domestic dogs (*Canis familiaris* or *Canis lupus familiaris*) (Fig. 1).

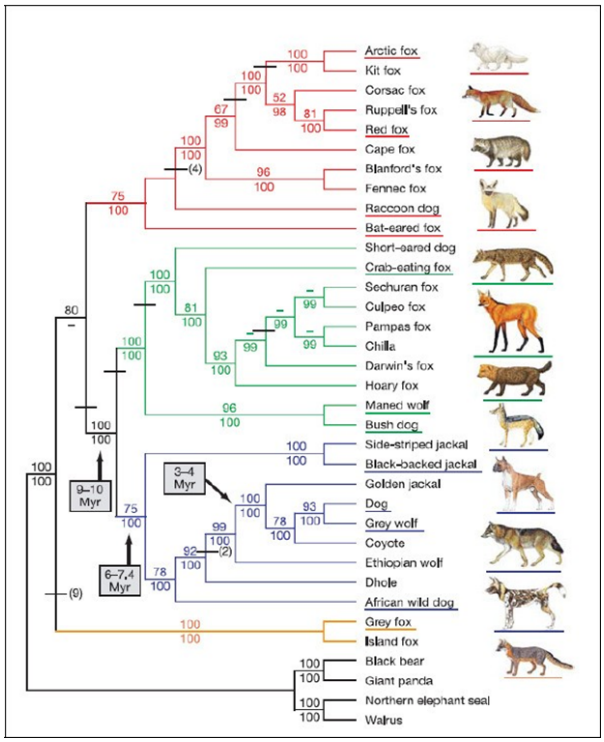


Fig. 1 - Phylogenetic relationships among *Canidae* lineages derived from nuclear sequence data.

The Italian wolf boasts peculiar phenotypic characteristics such as a black stripe on the frontal part of the anterior legs and a typical gray-brownish coat. Recent genetic investigations (Montana *et al*., 2017) classified the Italian wolf as *Canis lupus italicus*, a subspecies of the gray wolf.

1.1.2. Species distribution

The wolf's extraordinary ecological plasticity has made it the most widespread terrestrial mammal predator in the world (Mech, 1974).

At the end of the 18th century, wolves were present in all European countries, except for Ireland and Great Britain. During the 19th century, increased human activities led to a dramatic reduction of the species throughout Europe (Delibes, 1990). In fact, this was a consequence of direct – targeted eradication efforts – and indirect – habitat loss and decrease of prey availability – elements acting in unison.

To date, the largest populations of European wolves are present in Bulgaria, Romania, Poland, Russia, and the Balkans area. Meanwhile, Scandinavia, the Iberic peninsula, and France/Italy include three isolated subpopulations (Boitani, 2003) (fig. 2).

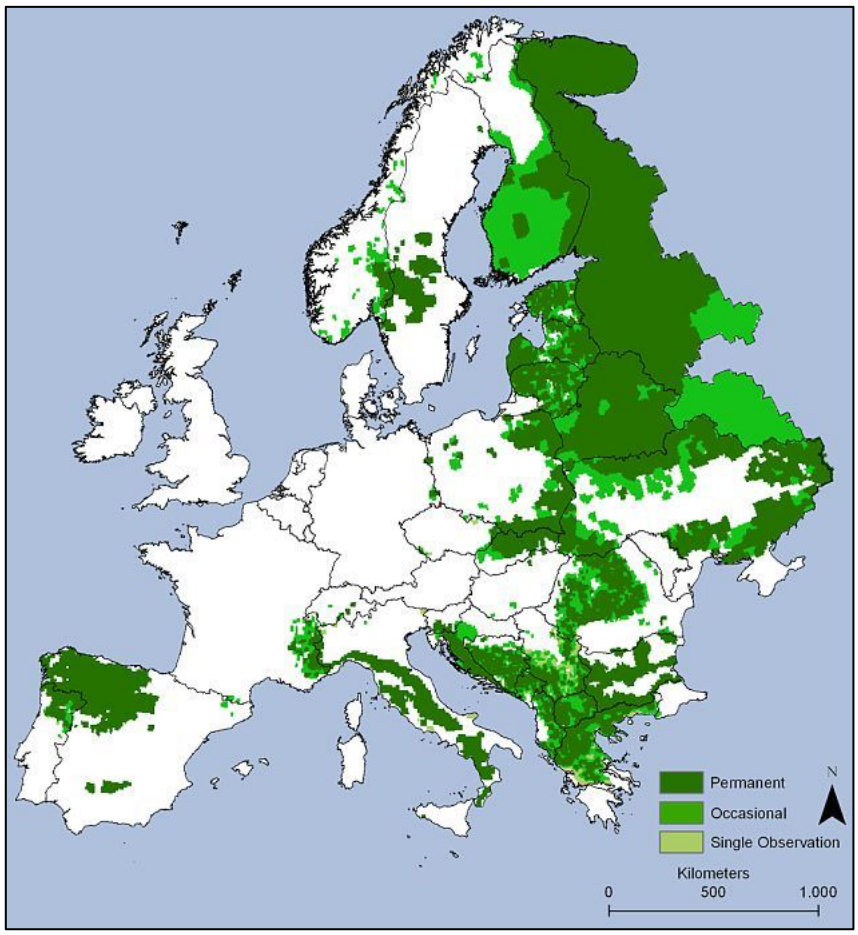


Fig. 2 - Distribution of wolves in Europe.

1.1.3. Italian population and recent re-expansion

In Italy, the wolf went through a dramatic and worrying bottleneck around the early 70s, when Zimen and Boitani (1975) estimated the presence of about just 300 wolves in the central-southern Apennines (fig. 3). In the ’80s, after they halted wolf hunting – which previously was not only legal but also paid by the State (1971) – and the species was officially protected (1976), the Italian wolf began its natural reconquest of the Apennine territory. Among all factors supporting its reconquest of the territory, the two most decisive are availability of prey (especially wild ungulates, Apollonio *et al.*, 2004) and the plasticity of the species itself in exploiting the modified landscapes (Milanesi *et al.*, 2015).

For the reasons mentioned above, in the ‘80s Italian wolves began a natural recolonization of the Apennine ridge, reaching the western Alps, Switzerland, and France (Fabbri *et al.*, 2007). To date, more than 320 packs of wolves are registered, which corresponds approximately to at least 1500 (± 300) individuals (Galaverni *et al.*, 2016), (fig. 3 and 4), but the data might probably be an underestimate.

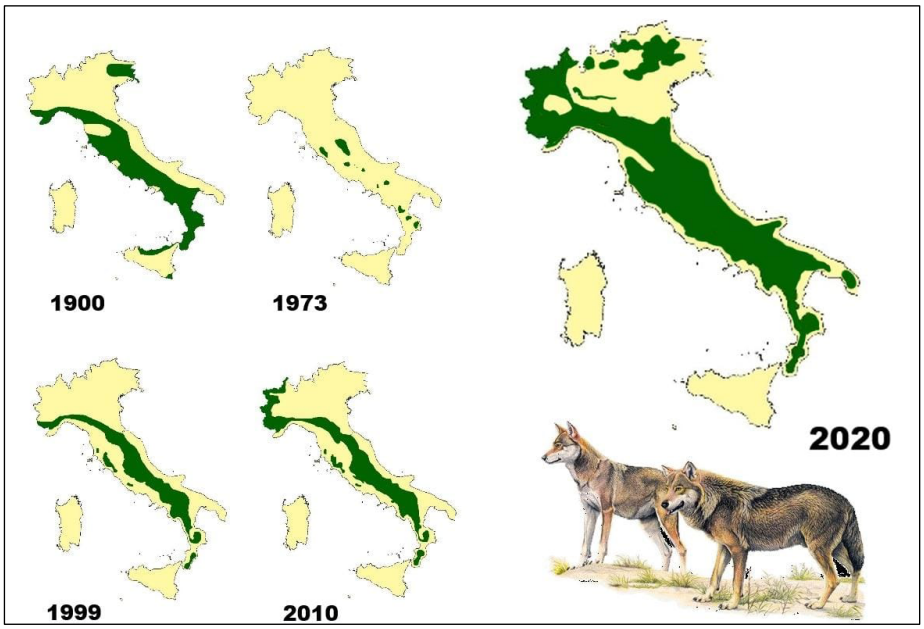


Fig. 3 - Italian wolf distribution from 1900 to 2010.

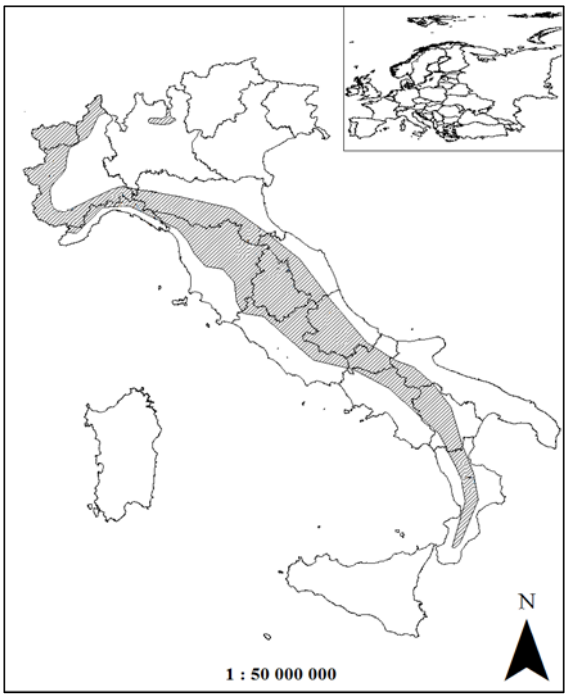


Fig. 4 - Distribution of the wolf in Italy.

1.1.4. Conservation status and recent conservation measures

Several countries have declared the aim of protecting wolves. Therefore, as a main consequence, this species is included in numerous protection regimes at both European and international levels. Importantly, as the 1996 Red List of the IUCN-World Conservation Union classifies the wolf as vulnerable, CITES (Convention on International Trade in Endangered Species of the Wild Fauna and Flora), drawn up in March 1993, listed the wolf in Appendix II (potentially endangered species), apart from Pakistan, Bhutan, Nepal, and India, where it is listed in Appendix I (species in danger of extinction) (IUCN/SSC SPECIALIST GROUP, 2021).

The wolf is included in Appendix II (strictly protected species) of the Bern Convention (Convention on the Conservation of European Wildlife and Natural Habitats), drawn up in September 1979, which adopted a detailed Recommendation on the protection of the wolf in Europe (Rec. No. 17/1989).

The EC Habitats Directive (92/43 of 21.5.1992) (European Union members only) lists the wolf in Appendix II (that requires habitat conservation) and Appendix IV (fully protected) except for the populations in Spain north of the river Duero, the populations in Greece north of the 39° longitude, and the populations in Finland in areas of reindeer management.

The European Parliament has approved (24.1.1989) a resolution (Doc. A2-0377/88, Ser. A) calling for immediate measures to support wolf conservation in all European states and adopting the IUCN Wolf Manifesto. The European Commission calls for expanding and providing financial means to support wolf conservation in Europe (Promberger and Schröder, 1993).

The EC Habitats Directive (92/43 of 21.5.1992) lists the Italian wolf in Appendix II (that requires habitat conservation) and D.P.R. 357 of 8.11.1997 of Habitats Directive in Appendix IV (fully protected) (Genovesi, 2002).

In Italy, the wolf is a strictly protected species. The Ministry of Environment and Agriculture delegated entirely to the Regions responsibility for, and in compliance with the laws, the protection of the species as well as measures for the prevention and compensation of damages caused by the wolves to livestock.

1.1.5. Threats, limiting factors and obstacles to conservation

The population of Italian wolves represents one of the few surviving populations in southern Europe after human persecutory activities. There are still many problems of coexistence with this species that has managed to naturally recolonize a large part of its lost range (Boitani and Ciucci, 1993). Limiting and threatening factors for the conservation of the *Italicus* subspecies are listed below:

- **Poaching:** generated by the conflicts between hunters and wolves, due to the competition for wild ungulates (Meriggi and Lovari, 1996), and between farmers and wolves, due to predatory activities of the latter on livestock (Duchamp *et al.*, 2004);

- **Feral dogs and wolf hybrids:** all species of the genus *Canis* have an identical number of chromosomes ($2n$=78) and they can reproduce with each other thus giving rise to hybrids (Wayne and Vilà, 2003). The domestic dog (*Canis lupus familiaris*) is the genealogically closest species to the gray wolf, which is its ancestor (Wayne and Vilà, 2006), in fact the two species separated between 11.000 and 35.000 years ago (Freedman and Wayne, 2017). The effect of hybridization can lead to phenotypic (vestigial spur in the hind limbs, the black color of the coat, depigmented nails, atypical coat, Randi, 2008; Caniglia *et al.*, 2013), physiological, and behavioral changes in wild canids (Lariviere and Crête, 1993). It should be reported that there may be hybrid individuals without atypical coats or different

phenotypic characteristics. In a recent study by Pilot *et al.*, 2018 it was possible to observe how traces of recent hybridization can also be found in wolves with the wild-type coat typical of the species.

However, the most important aspect of hybridization is the loss of genetic biodiversity typical of the *Italicus* subspecies (Galaverni *et al.*, 2017).

Hybridization between dogs and wolves seems to be more frequent in the vicinity of man-inhabited areas: where the density of wolves is low, whilst dogs are common (Nowak, 2003). In Italy, during the wolf population bottleneck, the number of feral and free-ranging dogs increased intensely, therefore raising the risk of hybridization (Galaverni *et al*., 2017).

1.2. - ETIOLOGICAL AGENTS INVESTIGATED IN THE STUDY

Various infectious diseases present in wild animals can pose a risk to humans and/or pets, but the role played by each species varies according to the disease (Wobeser, 2006).
In addition to the problems they can cause in humans and domestic animals, infectious diseases in wildlife can have implications on the mortality rate of wild animal populations, ranging from mild effects that allow natural density control to severe effects such as, at worst, the extinction of some populations (Wobeser, 2006).
In nature, wolves are subject to various diseases, however it is difficult to establish the exact prevalence since intact carcasses are found rarely. On the other hand, the situation is the opposite for wolves living in captivity. In fact, they also have a longer life than free-ranging wolves, which rarely reach old age, and therefore have a different and wider range of diseases (Mech, 2003).
Among the main viral infectious diseases of the gray wolf found on various continents, there are: Sylvatic Rabies, Canine Distemper virus, Canine Parvovirus, Canine Infectious Hepatitis, Papillomatosis, Canine Coronavirus infection (Kreeger, 2006), and sporadic infections with new viruses still little studied, as in the case of Canine Kobuviruses (CaKoVs) with one wolf resulted positive in Italy (Melegari *et al.*, 2018).
The viral agents researched in this study will be detailed in this chapter.

DNA VIRUS

1.2.1. CANINE PARVOVIRUS TYPE 2 (CPV-2)

1.2.1.1. Aetiology

At a taxonomic level, the family *Parvoviridae* includes two subfamilies. These are the *Densovirinae*, which infects arthropods, and the *Parvovirinae,* which infects vertebrate animals. Several genera are included in the latter, among which the genus *Protoparvovirus*.
The species *Carnivorous Protoparvovirus* 1 is part of this genus. It brings together the following different host variants infecting domestic and wild carnivores: *Canine Parvovirus* type 2 (CPV-2), *Feline Panleukopenia Virus* (FPV), *Mink Enteritis Virus* (MEV), and *Racoon Parvovirus* (RPV) (ICTV, 2021).
Various theories were formulated on the origin of CPV-2 that lead it back to FPV or FPV-like viruses spread in wild carnivores (Parrish, 2017).

CPV-2 appeared in the 1970s and was able to infect only dogs. Over the years it has undergone a series of genetic mutations that led to the formation of three variants: in 1980 the CPV-2a variant, in 1984 the CPV-2b variant, and in 2000 the CPV-2c variant. Firstly, they differ from CPV-2 by the amino acid present in position 426 of the VP2 protein. Secondly, they are able to replicate and spread more effectively in dogs. Moreover, they can also infect cats. To date, they have supplanted CPV-2 and are widely distributed all over the world (Greene and Decaro, 2012).

The parvovirus virion is devoid of an envelope, it measures 25 nm in diameter and has an icosahedral symmetry capsid. The capsid has 60 protein subunits, of which approximately 90% is VP2 and 10% is VP1. In addition, there is a third protein subunit, VP3, which derives from the cleavage of the terminal end of VP2. These viral proteins have a beta-sheet structure, where the beta filaments are joined by four loops that form the spikes, i.e. protrusions of the capsid (Parrish, 2017). The receptor binding sites are located on the surface of the spikes, they determine the host and tissue tropism, are the binding site with the antibodies and are responsible for the extreme resistance of the virus to the environment (Parrish, 2017).

The genome consists of a single stranded DNA molecule (ssDNA) and contains two Open Reading Frames (ORF), one of which codes for non-structural proteins useful for DNA transcription and replication, and the other codes for structural capsid proteins (Parrish, 2017).

In order to replicate, the canine parvovirus is strictly dependent on the host cells. Furthermore, as it is unable to induce the passage of cells from a resting phase to a "S" phase of replication, it targets cells that are most of the time in the "S" phase or at the beginning of the "G2" phase (Battilani, 2013). Therefore, the virus binds to specific cellular receptors, in particular transferrin, a protein that allows the entry of iron into the cell and that is present in large numbers in actively replicating cells, as they in fact require high quantities of iron (Battilani, 2013). Following binding, the parvovirus penetrates inside the cell through a process of endocytosis mediated by clathrin, subsequently the viral DNA reaches the nucleus and begins replication (Battilani, 2013).

1.2.1.2. Epidemiology

All members of the family *Canidae* are susceptible to natural infection with CPV-2. The infection has also been described among other carnivores, such as the *Felidae* and the *Mustelidae*, in mainly mild or subclinical forms (Parrish, 2017).

Parvovirus is extremely contagious and highly resistant in the external environment, where it can remain active for several months. In fact, the main route of transmission is through contact

with infected feces or material contaminated by them, such as objects, insects, or the fur of other animals (Greene and Decaro, 2012).
Canine Parvovirus is the leading cause of transmissible viral diarrhea in dogs and one of the most common veterinary infectious diseases (Sykes, 2014a).

It is reported by many authors how the infection of domestic dogs (*Canis lupus familiaris*) can have important implications in the spread of the virus in the wild population. In fact, determining factors are the presence of feral or stray owned dogs in extra-urban areas and their lack of vaccination (Bryan *et al.*, 2011; Nelson *et al.*, 2012; Miranda *et al.*, 2017). On the other hand, the encounter with pets can also be favored by wolves' approach to urban centers, as attested in a study conducted in the Tuscany region (Bassi *et al.*, 2015).
Transmission between dogs and wolves, according to Calatayud *et al.* (2019), can be related to contact with signs of presence (e.g. scats), predation, and coprophagia (Calatayud *et al.*, 2019). Furthermore, the maintenance of the infection among wolves is also because they are social animals, whose typical behaviors favor fecal-oral transmission within the population. In fact, some examples are the physical proximity among individuals, the habit of mutually sniffing the anogenital region within the same pack, and moreover the marking by urine and feces at the borders of the territory of belonging, with the subsequent inspection of such signs by other wolves (Molnar *et al.*, 2014).
Many studies were conducted to evaluate the prevalence of CPV-2 in wolves, most of which showed significant viral circulation. Below are some examples relating to the American situation and, afterwards, the European one.
Between 2000 and 2008, in the Canadian Rocky Mountains, the analysis of the serum of 99 wolves with the hemagglutination inhibition test (HI) resulted in 95% of the subjects testing positive for antibodies to CPV-2 (Nelson *et al.,* 2012). One of the authors' purposes was to assess a difference in the infection prevalence based on proximity to urban centers but given the positivity of almost all the wolves analyzed, it was not possible to draw conclusions of that sort (Nelson *et al.*, 2012).
Another study, conducted in Alaska (USA) and Yukon (Canada) between 1984 and 2000 on serum samples of 1122 animals, showed an antibody prevalence varying between 12% and 70% depending on the area, especially in adult individuals (Zarnke *et al.*, 2004).
In Yellowstone National Park (USA), a seroprevalence of 100% was found in 220 wolves and of 94% was found in 109 coyotes between 1991 and 2007. Therefore, these results were indicative of the fact that CPV-2 was endemic within the Park. Although there was not a

significant drop in the number of individuals in the population, the presence of a constant and low mortality rate induced by CPV-2 in younger animals, in weaker ones, or in those with co-infections could not be excluded (Almberg *et al.*, 2009). In contrast to the results of this study, Mech *et al.* (2008) highlighted an important correlation between the presence of antibodies to CPV-2 and the reduction in the survival rate of wolf pups in northeastern Minnesota between the years 1973 and 2004, with consequent negative implications on population growth and expansion (Mech *et al.*, 2008).

As for Europe, a study was conducted between 2004 and 2010 in Asturia (Spain) by Oleaga *et al.* (2015), which showed a seroprevalence of 61% in 84 wolves, higher in areas with a higher population density (Oleaga *et al.*, 2015).

In Portugal, however, organ samples from 227 wild carnivores, including 63 wolves, were analyzed by PCR between 1995 and 2011. CPV-2 DNA was found in only 4 animals: 3 wolves and a marten (*Martes foina*). The CPV-2 identified in the wolves were genetically similar to those previously identified in domestic dogs. Therefore, this provided evidence of the probable transmission of the infection by dogs in the wild population (Miranda *et al.*, 2017).

In Italy, a study conducted between 2006 and 2007 in the Tuscan-Emilian Apennine National Park and in the Casentinesi Forest National Park found 125 PCR negative fecal samples for canine parvovirus, while PCR investigations of two wolves found dead showed positivity for the virus DNA (Ambrogi *et al.*, 2019). Given the high prevalence values of the virus in Europe and America, the authors believe that the negativity of the stool samples was due not so much to the lack of circulation of the virus in the population, but to the absence of viral elimination in healthy subjects, as it occurs only in the early stages of infection (Ambrogi *et al.*, 2019).

Moreover, a survey was carried out on owned dogs in two National Parks, which showed that around 40% had been regularly vaccinated, about 35% had made only the first two vaccinations, and about 20% had not been vaccinated, with potential implications for the virus spread in wildlife (Ambrogi *et al.*, 2019).

Finally, a study conducted in the Abruzzo, Lazio and Molise National Park showed a 15.2% prevalence of real-time PCR on a total of 79 stool samples collected between 2006 and 2007. Four packs were detected in the area in question and all four had positive subjects. Additionally, in the same study area there was a population of feral dogs, which suggested a possible correlation with the spread of the virus among wolves (Molnar *et al.*, 2014).

1.2.1.3. Pathogenesis

In canids, the virus penetrates inside its host and the first replication occurs in the lymphoid tissues of the oropharynx, in the mesenteric lymph nodes and in the thymus. One to five days later, the viraemia occurs, following which the virus is localized in the tongue, oral mucosa, small intestine, and lymphoid tissues; other possible sites are the lungs, spleen, liver, kidneys, and myocardium. In fact, the myocardium is a typical localization when the infection occurs in the uterus or within six weeks of life, because of the active replication of the cardiac myocytes (Greene and Decaro, 2012).

The virus destroys the enterocytes of the intestinal crypts, causing maldigestion and malabsorption with consequent diarrhea. It also causes damage to the precursors of neutrophils and lymphocytes, leading to immunosuppression (Greene and Decaro, 2012).

There are often secondary bacterial infections by Gram negative bacteria and anaerobic flora that aggravate the viral damage, causing endotoxemia and disseminated intravascular coagulation (DIC), which lead to bleeding (Greene and Decaro, 2012).

Fecal elimination begins three to four days post-infection, usually before the manifestation of clinical symptoms, and continues for a few weeks (Greene and Decaro, 2012).

1.2.1.4. Clinical signs in domestic and wild canids

In canids, the most frequent clinical form is enteric, but sometimes a cardiac form or other rarer forms can occur. The enteric one is characterized by an initial profuse vomiting, followed by severe yellow-gray diarrhea, often with blood streaks, which over time leads to severe dehydration. The animal also appears anorexic, lethargic, and hyperthermic. Mortality is variable, where sepsis and DIC are the determining factors (Greene and Decaro, 2012; Parrish, 2017).

The cardiac form results from an early infection and as a result there is a serious heart damage (myocarditis), often followed by sudden death without other clinical signs. Other less frequent forms are acute diarrhea and death, acute diarrhea followed by apparent healing with death weeks or months later from congestive heart failure, or sudden congestive heart failure in apparently healthy pups between 6 weeks and 6 months of life (Greene and Decaro, 2012).

Other rarer clinical forms are the neurological one, often caused by bleeding in the central nervous system after DIC, and the cutaneous one, which manifests with an erythema multiforme (Greene and Decaro, 2012). Finally, asymptomatic and subclinical forms are frequent, particularly in wild species (Decaro and Buonavoglia, 2012).

1.2.1.5. Diagnosis

In wolves, invasive methods such as capturing animals to collect their blood can be used to obtain the samples necessary to perform laboratory investigations, as it was done, for example, in the studies by Almberg *et al.* 2009 and Nelson *et al.* 2012. Non-invasive methods can be also used, such as the removal of tissues from animals found dead or the collection of scats, an example of which is found in the study by Ambrogi *et al.* (2019). Once the samples are taken, the diagnosis can be made indirectly by searching for antibodies, for example through the ELISA, HI, and SN, or directly by searching the virus in its entirety or parts of it, such as viral antigens or genomic DNA, e.g. through PCR and real-time PCR methods (Greene and Decaro, 2012). All these methods were used in the various studies seen previously (Zarnke *et al.*, 2004; Almberg *et al.*, 2009; Nelson *et al.*, 2012; Molnar *et al.*, 2014; Oleaga *et al.*, 2015; Miranda *et al.*, 2017; Ambrogi *et al.*, 2019).

When analyzing a stool sample to directly search for the virus or parts of it, it must be considered not only the viral elimination period, but also the sensitivity and specificity of the method used. For example, ELISA highlights the viral antigen on stool samples up to 7-10 days from the start of the elimination, while PCR detects the viral DNA on stool samples up to many weeks from the beginning of the elimination (Greene and Decaro, 2012). As for the antibodies, they show up 3-4 days after the start of the infection and quickly reach high levels (Parrish, 2017) which can remain at a constant level for at least a year (Greene and Decaro, 2012).

1.2.2. CANINE ADENOVIRUS (CAdV-1 and CAdV-2)

1.2.2.1. Aetiology

The family *Adenoviridae* includes five genera: the *Mastadenovirus*, which has tropism for mammals, the *Aviadenovirus*, which infects birds, the *Atadenovirus*, which has reptiles, birds, and mammals as its host spectrum, the *Siadenovirus*, which is found in reptiles, amphibians, and birds, and the *Ichtadenovirus,* the hosts of which are fish (MacLachlan and Dubovi, 2017). Canine Adenovirus belongs to the genus *Mastadenovirus*, and there are two types: Canine Adenovirus 1 (CAdV-1), which is the causative agent of Rubarth Hepatitis or Infectious Canine Hepatitis (ICH), and Canine Adenovirus 2 (CAdV-2), which is associated with an infectious tracheobronchitis commonly called "Kennel Cough" (MacLachlan and Dubovi, 2017).

The virion has a diameter of 70-90 nm, is devoid of an envelope and presents the capsid with icosahedron symmetry, whose faces are made of 240 exons and the vertices of 12 pans. At the end of each penton there is a fiber, i.e. an extension with a terminal swelling; *Aviadenoviruses* though are an exception and have two fibers. The fibers bind to the receptors of the host cell and the pentons interact with the integrins. In this way the virus penetrates the cell and replication takes place within the nucleus (Balboni, 2013).

The genome is made of a double-stranded DNA molecule (dsDNA), the central portion of which encodes structural proteins, while the outermost portions are more variable and code for non-structural proteins (Balboni, 2013). In total there are about 40 coded proteins, of which one third are structural proteins, i.e. those that form the exons, the pentons, the fibers, and the viral core (MacLachlan and Dubovi, 2017). Among the properties of adenoviruses, there is the agglutinating capacity of erythrocytes, which is used to make diagnosis through the HI test. This property manifests when the apices of the fibers bind to the cell receptors, forming bridges between cells (MacLachlan and Dubovi, 2017).

In the external environment, adenoviruses are stable and they can last for months at both room and freezing temperatures, but they can be inactivated by high temperatures and disinfectants, such as phenol or sodium hydroxide (Greene, 2012; Sykes, 2014b).

1.2.2.2. Epidemiology

The Canine Adenovirus Type 1 has as its host domestic dogs, but also many wild animals including carnivores belonging to the family *Canidae*, such as foxes, wolves and coyotes, *Ursidae*, *Procyonidae,* and *Mustelidae* (Decaro and Buonavoglia, 2012a; MacLachlan and Dubovi, 2017). Canine Adenovirus Type 2 infections are found mainly in domestic dogs, whilst

they are poorly reported in wildlife (MacLachlan and Dubovi, 2017).

CAdV-1 is transmitted through saliva, feces, and urine, and it has been shown that viral elimination via urine in dogs can last up to nine months (Decaro and Buonavoglia, 2012a).

CAdV-2 has tropism for the respiratory system and is primarily transmitted by air (Millàn *et al.*, 2016), although it is also frequently found in scats (Balboni *et al.*, 2014).

Several studies have confirmed the worldwide spread of CAdV-1 and CAdV-2, both among wild and domestic animals. Studies have been conducted in the United States to evaluate the prevalence of Canine Adenovirus in wolf populations, e.g. Carstens *et al.'s* (2017) conducted in Minnesota (USA), Watts and Benson's (2016) conducted in Alaska (USA), and Almberg *et al.'s* (2009) conducted in Yellowstone National Park (USA). In all three studies, blood samples were taken from animals and the viral SN test was used on the serum, which allows detecting and quantifying virus-specific antibodies (Ciulli and Gallina, 2013).

The results showed a very high prevalence:

- 88% of adults and 45% of puppies out of a total of 387 samples in the study conducted in Minnesota (Carstens *et al.*, 2017);
- 90% of a total of 100 samples in the Alaska study (Watts and Benson, 2016);
- 96% of adults and 91% of puppies out of a total of 239 samples in the study conducted in Yellowstone National Park, where 110 coyotes were also sampled and a prevalence of 18% in young individuals and of 83% in adults was found (Almberg *et al.*, 2019).

These results allowed the authors to conclude that the virus is endemic (Almberg *et al.*, 2009; Watts and Benson, 2016; Carstensen *et al.*, 2017). However, it is necessary to specify that several individuals who died in the wild are not sampled, and that a positive antibody titer is indicative of infection and not of clinical disease. Furthermore, high antibody levels can persist for long periods of time (Carstensen *et al.*, 2017).

Watts and Benson (2016) speculated that the causes of the high prevalence in Alaska are mainly due to the following reasons: principally, the high transmission rate of the virus, but also the long persistence of the antibodies, or even both. Additional factors may be the harsh climate of Alaska, as the virus resists for a long time in cold environments, and the presence of other carnivores with the role of reservoir (Watts and Benson, 2016).

As for the European situation, between 2010 and 2013 in Spain a study was conducted on 54 wolves, of which 15 alive and 39 dead. A serological investigation with virus neutralization was carried out on 28 serum samples and a molecular investigation by PCR on 37 spleen and

13 scat samples. The results obtained with the two methods were similar, with a 75% prevalence of antibodies and 76% of viral DNA, in detail 70% of CAdV-1 and 6% of CAdV-2 (Millàn *et al.*, 2016). The authors hypothesized a correlation between the CAdV-1 infection being endemic and the strongly anthropized area in question, with the consequent presence of domestic dogs as a possible reservoir. In support of this hypothesis, there is the fact that country dogs are less frequently vaccinated than city dogs, and that in the past there has been evidence of an antibiotic resistance situation in Iberian wolves, an effect of environmental anthropization (Millàn *et al.*, 2016). However, an antithetical view is presented in the study conducted in Scandinavia and the Svalbard islands by Akerstedt *et al.* (2010), whose results showed a high prevalence in both wolves and foxes, which are therefore considered potential reservoirs of the infection and consequently a danger for pets, especially for unvaccinated dogs (Akerstedt *et al.*, 2010).

In this study the viral neutralization test was adopted starting from serum samples taken from 98 wolves, 275 red foxes and 60 arctic foxes, and the seroprevalence was 67.7%, 59.6% and 37.8% respectively. These are very high values, so the authors believe that the virus-induced mortality is not low. Therefore, they put forward three hypotheses in this regard, namely the poor finding of carcasses, the high resistance of the hosts, and the low virulence of the circulating virus (Akerstedt *et al.*, 2010).

In Italy there is no study that gives an estimate of the infection prevalence in the wolf population, but there are some that attest to the virus presence. One of these showed through PCR the presence of CAdV-1 DNA from a liver sample of an Italian wolf pup, then the virus was isolated and sequenced (Pizzurro *et al.*, 2017). In France, the year after the previous study, Dowgier *et al.* 2017 carried out a molecular investigation with real-time PCR on intestinal, liver, and spleen samples of a wolf that died in captivity, and they detected the presence of the DNA of a canine adenovirus genetically related to the one sequenced by Pizzurro *et al.* 2017. A genetic correspondence was also highlighted with other CAdV-1 sequenced in Italy circulating in foxes and dogs, indicating that the virus is genetically stable in the environment even in different species (Dowgier *et al.*, 2018).

In 2019 in Italy, starting from a tongue sample of an Italian wolf, Balboni *et al.* identified the DNA of CAdV-1 through real-time PCR and characterized it genetically. The viral sequence revealed a particular amino acid sequence, superimposable on that of the CAdV-1 identified in the previously mentioned French wolf (Dowgier *et al.*, 2018; Balboni *et al.*, 2019). The authors hypothesized the virus transmission between wild and domestic carnivores, because of the close correlation between the viruses circulating in the populations of wolves, foxes, and dogs

(Balboni *et al.*, 2019). It is difficult to establish which species is the reservoir of infection, however there is evidence of viral circulation in the Italian canine population which, although vaccinated, can still become infected and eliminate the virus (Balboni *et al.*, 2014). This also happens in fox populations, as attested by some studies (Akerstedt *et al.*, 2010; Tryland *et al.*, 2018; Balboni *et al.*, 2019a).

1.2.2.3. Pathogenesis

After an oronasal penetration, in canids CAdV-1 is localized in the tonsils, then takes the lymphatic pathway and through the thoracic duct reaches the bloodstream. Following viremia, it is localized in various tissues, including the liver, vascular endothelium, kidneys, and eyes, and it is then eliminated through biological secretions and excretions, such as saliva, urine, and feces (Greene, 2012).

In the liver, the extent of the damage depends on the immune response: if it is low, an acute liver necrosis develops; if it is partial, chronic hepatitis with fibrosis evolves four to five days after the infection; if it is sufficient, on the day of the infection the animal shows only mild clinical signs. The complications of hepatitis include Disseminated Intravascular Coagulation and hepatic encephalopathy (Greene, 2012).

At the renal level, the virus is first located in the glomerulus in which, following an increased antibody response, the immune complexes precipitate, then at tubular level we observe transient viruria and proteinuria. Kidney damage is limited to mild focal interstitial nephritis, which generally does not become chronic (Greene, 2012).

At the ocular level, the damage occurs only in a reduced percentage of cases, following the virus entry through the aqueous humor and its replication in the corneal endothelium, which results in anterior uveitis and corneal edema that can determine the appearance of the characteristic "blue eye" and are usually self-limited (Greene, 2012).

In canids, CAdV-2 penetrates oronasally and replicates in the mucosa of the nasal cavities, in the pharynx, in the crypts of the tonsils, and in the goblet cells of the trachea. Sometimes it can also replicate at the level of the bronchi, alveolar epithelium, and bronchial and retropharyngeal lymph nodes. Viral replication generally decreases until it ends with an increase in the host's antibody response (Ford, 2012). The most serious situations arise when the virus is located in the lower airways, causing bronchitis and interstitial pneumonia, and when the clinical situation is worsened by bacterial superinfections or viral co-infections. Mortality is rare and is found mainly in animals that are less than one month old (Ford, 2012).

1.2.2.4. Clinical signs in domestic and wild canids

The clinical symptomatology of infectious hepatitis was described mainly in captive dogs and foxes, while there is little information about other wild carnivores. In the latter, fulminant forms were reported, especially in foxes, but also infections without clinical manifestations, after the discovery of the virus in the feces of healthy animals (Decaro and Buonavoglia, 2012a).

After an incubation period of 4-6 days, the animal exhibits hyperthermia and nonspecific signs, such as decreased appetite and sensory depression. The subject also shows symptoms such as vomiting and often hemorrhagic diarrhea. Heart and respiratory rate increase. there are also cough and respiratory noises in the lower airways that are indicative of pneumonia (Decaro and Buonavoglia, 2012a; Greene, 2012).

The animal's abdomen is increased in volume, due to the accumulation of serum-hemorrhagic fluid and hepatomegaly and is also tense and painful. Moreover, the animal presents petechiae, bruising, and epistaxis, caused by hemorrhagic diathesis. The involvement of the central nervous system is manifested by hyperexcitability, nystagmus, and seizures, or with fulminant death without previous clinical signs (Decaro and Buonavoglia, 2012a; Greene, 2012).

Immunocomplex deposition has renal repercussions, i.e. glomerulonephritis and interstitial nephritis, and ocular repercussions where anterior uveitis, corneal edema, blepharospasm, photophobia, and eye drain develop. However, signs of immunocomplex deposition were rarely reported in wild animals (Decaro and Buonavoglia, 2012a; Greene, 2012).

The clinical signs of infectious tracheobronchitis in canids occur 3-10 days post-infection, typically with paroxysmal cough that sometimes can be productive. Moreover, there may also be nasal and ocular serous, mucous or mucus-purulent discharge. In severe cases where bacterial superinfections occur, animals are hyperthermic, lethargic, without appetite, and can be dyspneic in case of pneumonia (Ford, 2012).

1.2.2.5. Diagnosis

In wolves, both direct and indirect methods can be used for diagnosis. Among the indirect methods, which consist of searching for antibodies in the serum, there are the SVN test, indirect immunofluorescence, the HI, and the ELISA. All of these allow evaluating the extent of viral circulation in the population – which is not necessarily associated with clinical disease – but, except for the inhibition of hemagglutination, they do not allow the distinction between the antibodies produced against CAdV-1 and those produced against CAdV-2. Among the direct methods, there are the molecular ones, i.e. PCR or real-time PCR, which allow distinguishing the two viruses and which are used on tissue or scat samples in order to search for viral DNA

(Decaro and Buonavoglia, 2012a).

RNA VIRUS

1.2.3. CANINE DISTEMPER VIRUS (CDV)

1.2.3.1. Aetiology

Canine Distemper Virus belongs to the family *Paramyxoviridae*, subfamily *Paramyxovirinae* and genus *Morbillivirus*.

The viruses of this family are pleomorphic and have an extremely variable morphology and size. The diameter ranges from 125 to 250 nm. The virions present a lipid envelope that covers the herringbone nucleocapsid with helical symmetry (Scagliarini *et al.*, 2013). The genome consists of a single linear single-stranded RNA molecule (MacLachlan and Dubovi, 2017). The outer casing contains the fusion proteins F, and H or G attack proteins, neutralizing antigens that confer immunity against re-infections (Scagliarini *et al.*, 2013). Moreover, these are fundamental for the attack and entry into the host cells (Sykes, 2014c).

Paramyxoviridae are sensitive to heat, to ionic and non-ionic lipid detergents, to oxidizing agents, and are not very resistant in the environment. In fact, Canine Distemper Virus resists less than a day at room temperature and is deactivated by heat and drying, as well as by disinfectants (Sykes, 2014c). Replication occurs in the cytoplasm (Scagliarini *et al.*, 2013).

The distemper is a pathology of considerable importance both for domestic dogs and various world wild species. Canine Distemper Virus (CDV) is closely related to Human Measles Virus (MeV) and Rinderpest Virus (RPV). In dogs, infection involves the development of a multisystemic disease that affects the respiratory, gastrointestinal, and nervous systems (Sykes, 2014c).

1.2.3.2. Epidemiology

CDV is capable of infecting pets such as dogs and ferrets, while cats do not seem sensitive. Among wild animals, it affects many species belonging to the families *Canidae, Mustelidae, Procyonidae,* and *Felidae*. The virus eliminated from wild species can infect domestic dogs, but also the virus spread by dogs can significantly threaten wild populations. Worldwide, based on the analysis of the H gene sequence there are at least eight different CDV genotypes: Asia-1, Asia-2, America-1, America-2, Europe-1/South America-1, Europe-2, Europe-3, and South Africa (Sykes, 2014c).

Although vaccination has reduced the incidence of this pathology, CDV remains dangerous especially where there is a large number of young dogs living together and with inadequate immunity, such as inside kennels, shelters, or farms. The incidence is higher in areas where vaccination is not practiced or is carried out incorrectly (Sykes, 2014c).
Distemper is an extremely contagious disease. The main modes of transmission involve direct oronasal contact with the virus present in secretions or excretions, and the inhalation of large aerosol particles from infected dogs with clinical or sub-clinical forms (Sykes, 2014c).

Although it is a lethal disease in many carnivores, mortality from CDV in free-ranging wolves was only documented in Canada (Carbyn, 1982) and Alaska (Peterson *et al.*, 1984). In Alaska, 57 wolf sera were tested, of which 7% tested positive for exposure to CDV and they were individuals aged between 1 and 6 years. This is consistent with sporadic and short-lived introductions of the virus into populations, rather than a continuous enzootic exposure pattern (Stephenson *et al.*, 1982). Between 2000 and 2008, in two Canadian national parks and in the areas surrounding the Rocky Mountains, out of 99 wolves sampled, 94 had detectable antibodies for Canine Parvovirus, in detail 24 for CDV and 24 for both viruses (Nelson *et al.*, 2012). Compared to other studies, the prevalence of CDV was similar or slightly higher than those reported in the same populations (Zarnke *et al.*, 2004) and in others (Akerstedt *et al.*, 2010).

The large exposure of European wild carnivores to the distemper virus has been repeatedly demonstrated (Sobrino at al., 2008; Santos *et al.*, 2009; Akerstedt *et al.*, 2010).
In Europe, distemper has been known since the mid-sixteenth century and remains a current problem for domestic dogs around the world. The news related to this pathology in wildlife, however, remains few. The most important information regarding its spread in wild wolves derives from serological analyzes that demonstrate exposure to the virus, but do not say much about its epidemiology (Kreeger, 2003). The serological prevalence in Scandinavia in red foxes, arctic foxes, and wolves varies between 9.6% and 12.3%. Moreover, in Scandinavian foxes the prevalence of CDV is statistically significantly higher in adult males. CDV is not very resistant in the environment and the relatively high prevalence of antibodies in foxes and wolves found in the study presumably reflects the continuous circulation of the virus within fox and wolf populations or within other wild or domestic carnivores sensitive to the virus (Akerstedt *et al.*, 2010).

Canine Distemper Virus infects a wide range of carnivores. To assess whether wild carnivores can play a role in its epidemiology in domestic dogs, the seroprevalence of CDV was determined in Germany. The prevalence of antibodies identified in red foxes from urban areas was higher than in rural areas, indicating that foxes might have become infected as a result of contact with domestic dogs (Frölich *et al.*, 2000).

Between 1995 and 2006, in Portugal, anti-CDV antibodies were identified in wolves (*Canis lupus*), red foxes (*Vulpes vulpes*), wild cats (*Felis silvestris*), martens (*Martes foina*), and genets (*Genetta genetta*). This study suggests extensive exposure of Iberian wild carnivores to CDV (Santos *et al*., 2009).

The temporal pattern of prevalence of anti-CDV antibodies suggests that CDV is epidemic in wild canids, as reported in Spain (Sobrino *et al.*, 2008). Relatively dense populations of susceptible hosts are usually needed to support the circulation of CDV. Red foxes can reach high densities, while for wolves it is more difficult. Domestic dogs reach high densities, can maintain CDV, and act as reservoirs of infection for wild carnivores. Therefore, transmission could take place through predation or consumption of domestic dog carcasses. However, this possible role of domestic carnivores on the epidemiological cycle of CDV in wildlife has yet to be ascertained (Santos *et al.*, 2009).

A few years later in Portugal, the sequencing of the H gene of the Distemper Virus, obtained from two wild wolves and a dog, highlighted there were more differences between the sequences of the virus in the two wolves than between the sequences isolated from one of the wolves and the dog. A possible explanation of this seems to be the geographical and temporal proximity of the two latter samples' collections. The amino acid analysis of viral hemagglutinin revealed a glycine (G) and a tyrosine (Y) in positions 530 and 549 of the lymphocyte activation receptor binding region (Signaling Lymphocytic Activation Molecule - SLAM), which is typically present in viral strains obtained from dogs. This suggests that the CDV found in these wolves derived from transmissions from domestic dogs rather than from wild species (Müller *et al.*, 2011).

Recently in Abruzzo, of 30 wolf carcasses examined, 20 tested positives for Canine Distemper Virus (CDV). Furthermore, clinical signs related to this pathology were observed through camera trapping (Di Sabatino *et al.*, 2014). In 1996, Fico *et al.* reported exposure to CDV in one wolf out of four in the Abruzzo region. Its presence was confirmed even in badgers and foxes in the same area (Molnar *et al.*, 2014).

1.2.3.3. Pathogenesis

The virus initially infects the monocytes in the lymphoid tissue in the upper airways and tonsils. Subsequently, it spreads through the lymphatic system and bloodstream to the reticuloendothelial system. Viral hemagglutinin binds to the surface of the host cells at the level of the Signal Lymphocyte Activation Molecule (SLAM) (Yanagi *et al.*, 2006). SLAM is expressed in immature thymocytes, activated lymphocytes, macrophages, and dendritic cells. The virus destroys a large part of the lymphocyte population in the blood, tonsils, thymus, spleen, and lymph nodes (Beineke *et al.*, 2009).

The massive destruction of lymphocytes manifests as initial lymphopenia and a few days after the infection it causes transient fever. There is a second viremic phase associated with fever about a week after the infection, following which the infection extends to the cells of the respiratory system, gastrointestinal system, central nervous system, urinary tract, and skin, as well as white blood cells and erythrocytes. The virus is capable of infecting various cell lines and it leads to the formation of intranuclear and intracytoplasmic inclusions (Sykes, 2014c).

CDV is eliminated in all secretions and excretions from the fifth day after infection, even before the clinical signs appear. Viral elimination can continue for up to 3 or 4 months, but most frequently ends in 1 or 2 weeks (Sykes, 2014c).

Macroscopic findings include atrophy, lung congestion and consolidation, fluid intestinal contents, increased volume lymph nodes, and congestions. Pleural, pericardial and peritoneal effusion rarely can be found. In many cases, the necropsy examination shows minimal alterations.

In the histopathological examination of the brain and spinal cord, it is possible to observe neuronal necrosis, degeneration, and demyelination during chronic or subacute infections. Lymphocytic and histiocytic interstitial pneumonia and neutrophilic bronchopneumonia are found in case of secondary infection. The diagnosis of CDV infection is supported by the identification of intranuclear and intracytoplasmic eosinophilic viral bodies in different cell types, i.e. neurons, astrocytes, conjunctival epithelial cells, bladder epithelial cells, foot pad cells, and lymph node cells (Sykes, 2014c).

A widespread, severe outbreak of Canine Distemper Encephalitis was observed in wildlife in Southern Bavaria in the spring and summer of 2008 (Sekulin *et al.*, 2011). The haemagglutinin (HA) genes of six representative Canine Distemper Virus (CDV) samples originating from five red foxes and one badger during this outbreak had a Y549H amino acid substitution in the HA protein compared to sequences from two captive domesticated ferrets which succumbed to CDV in the same area 2 years earlier (Sekulin *et al.*, 2011). As this specific substitution in the

receptor-binding site has been hypothesized to contribute to the emergence of CDV and its spread to novel hosts, the outbreak in wildlife in Southern Bavaria might, directly or indirectly, be associated with a Y549H amino acid exchange (Sekulin *et al.*, 2011).

1.2.3.4. Clinical signs in domestic and wild canids

In dogs with clinical signs related to the upper airway, CDV is an important differential diagnosis. Moreover, this pathology can imitate canine parvovirus. The clinical signs vary according to the viral strain, the immune status, the age of the host, and the presence of any co-infections. In many dogs, the infection is subclinical, only in a few cases it progresses to death. The incubation period is between 3 and 6 days. In the case of respiratory involvement, dogs have fever, nasal discharge, bilateral serous conjunctivitis, and non-productive cough. Virus-induced immunodepression can lead to secondary bacterial infections where the drain becomes mucopurulent, bronchopneumonia is associated with tachypnea and productive cough, and lethargy and loss of appetite develop. The involvement of the gastrointestinal tract is manifested by vomiting, diarrhea, electrolyte disturbances, and dehydration. The subject can develop an immune response which however does not allow them to completely eliminate the virus. This is how chronic manifestations occur involving the uvea, lymphoid organs, hind limbs, and central nervous system (Sykes, 2014c).

Neurological signs occur in 30% of infected subjects, usually 1-6 weeks after the acute onset of the disease. These are progressive clinical signs, which tend to persist even after healing. "Old Dog Encephalitis" is a progressive immune-mediated demyelinating leukoencephalomyelitis induced by CDV weeks or years after the healing of the acute infection. Despite the name, it does not necessarily affect older dogs. The most common neurological manifestation includes monoclonal spasms, dulling of the sensory system, tremors, opisthotonos, ataxia, quadriparesis, paraparesis, compulsive behavior, vestibular signs, and apparent blindness (Sykes, 2014c).

In the chronic form, at the ocular level uveitis, chorioretinitis, dry keratoconjunctivitis, corneal edema, corneal ulcer, and optic neuritis are observed (De Almeida *et al.*, 2009). The teeth can be irregular with retentions or partial eruptions, due to the hypoplasia of the enamel and dentine (Bittegeko *et al.*, 1995). In the hind footpads and in the planum nasal, hyperkeratosis is detectable. Immunosuppression is caused by lymphopenia, necrosis of hematopoietic cells in the bone marrow, and malfunction of dendritic cells. The most common secondary infections are bacterial and may involve *Bordetella Bronchiseptica, Salmonella*, and *Nocardia*. Toxoplasmosis and generalized demodicosis can also be observed (Sykes, 2014c). In minks,

CDV was detected in conjunction with *Pneumocystis Carinii* (Dyer and Schamber, 1999), while in raccoons it is associated with neosporosis (Lemberger *et al.*, 2005).

1.2.3.5. Diagnosis

CDV can be isolated in the VERO cell lineage, where cytopathic effects become evident within 24 hours of inoculation. The samples used for viral isolation include eye and nasal swabs from the drain, blood and urine samples obtained by tracheal washing, and necropsy samples. The false negatives are due to a low viral load.

It is possible to use direct immunofluorescence on smears obtained from conjunctival scraping, urinary sediment, and samples of tracheobronchial washes. Again, false negatives can occur. False positives result from a recent vaccination with live attenuated vaccines. The search for antigens on serum can be done with the ELISA test. It is a rapid and inexpensive technique of which, however, sensitivity and specificity have not yet been correctly established. RT-PCR searches for viral RNA can be done on whole blood, skin biopsies, conjunctival scrapings, urine, CSF, nasal, and oropharyngeal swabs, as well as various tissues obtained through necropsy. Using samples from different anatomical areas and using PCR with other diagnostic tests can improve sensitivity. However, even with this technique we can have post-vaccination false positives, whilst false negatives can occur in case of low viral load or RNA degradation during the transport of the samples (Sykes, 2014c).

The presence of serum CDV antibodies can be determined by the SN or ELISA tests, which search for IgG and IgM and appear to be more sensitive (Von Messling *et al.*, 1999). Serological tests can be used to assess the need for vaccination and to determine which dogs are protected and therefore are at low risk of developing the disease. Serum neutralization titers of 1:16 to 1:20 are considered post-vaccination protectants. Titers greater than 1:100 indicate the passage of maternal immunity to puppies. Negative serological results do not indicate the lack of protection because of the presence of the cell-mediated immunity (Greene and Appel, 2006). The ELISA test can also be used for a semi-quantitative measurement of CDV antibodies. The diagnosis is retrospective, acute or convalescent sera are required. False positives are possible following vaccination (Sykes, 2014c).

1.2.4. CANINE ENTERIC CORONAVIRUS (CCoV)

1.2.4.1. Aetiology

Coronaviruses are divided into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus,* and *Deltacoronavirus*. *Alpha* and *Betacoronaviruses* come from bats and infect mainly mammals, whilst *Gamma* and *Deltacoronaviruses* come from birds and are capable of infecting various species of both birds and mammals (Woo *et al.,* 2012). Notable pet coronaviruses include: Feline Enteric Coronavirus (FECV), Feline Infectious Peritonitis Virus (FIPV), Canine Enteric Coronavirus (CCoV), Ferret Enteric Coronavirus (FRECV), Ferret Systemic Coronavirus (FRSCV), and Alpaca Respiratory Coronavirus. All the above-mentioned viruses are A*lphacoronaviruses*. Moreover, there are Canine Respiratory Coronavirus (CRCoV), Equine Enteric Coronavirus (ECoV), and Alpaca Enteric Coronavirus, all of which are *Betacoronaviruses* (Erles *et al.,* 2003; Decaro and Lorusso, 2020).

Coronavirus genomes encode three classes of proteins: structural, accessory, and non-structural. Major structural proteins of coronaviruses include nucleocapsid (N), spike (S), membrane (M), and envelope (E) proteins (Haake *et al.,* 2020). Protein S is the main viral binding protein and mediator of membrane fusion and viral entry. Protein N, in close association with genomic viral RNA (gRNA), forms the helical nucleocapsid, which is stabilized through the bond with the M protein. The viral genome and the helical nucleocapsid are surrounded by a host-derived lipid bilayer, in which proteins S, E and M are anchored. The transmembrane proteins E and M are involved in the assembly and budding of the virion (Haake *et al.*, 2020). Unlike Alpha Coronaviruses, a subgroup of Betacoronaviruses is structurally more complex and have additional membrane glycoproteins, called hemagglutinin-esterase proteins (HE), which are encoded by an additional gene of approximately 1.2 kb in size (Haake *et al.,* 2020). For all Coronaviruses, protein S is believed to be the primary binding protein responsible for the attachment of the coronavirus to the cell surface. However, HEs' contribution to virion attachment and their role in tropism and tissue pathogenesis are currently not well understood (Haake *et al.,* 2020). The molecular events of the Coronavirus replication cycle are complex and begin with the attachment of the virion to the host cell, achieved by binding the viral protein S to a unique target receptor on the host cell surface. As the primary binding protein and mediator of both virus host cell membrane fusion and subsequent virus entry into the cell, protein S is critical in determining host species, tissue, and cell tropism for each Coronavirus (Wickramasinghe *et al.,* 2011). Upon the receptor binding, some conformational changes in protein S expose the fusion peptide, facilitating the fusion of viral and host cell membranes and

the subsequent release of the viral nucleocapsid into the host cell cytoplasm (Haake *et al.,* 2020). Upon cytoplasmic release of the viral nucleocapsid, positive sense genomic RNA (+ gRNA) acts as a viral messenger RNA (mRNA) for direct translation of the replicase gene complex, using the host cell's ribosomal mechanism. The replicase gene complex consists of two large Open Reading Frames (ORFs) of about 20 kb in total size (Schiller *et al.*, 1998), ORF1a and ORF1b, the latter transcribed by a ribosomal frame shift.

The genetic diversity of Coronaviruses is a consequence of both polymerase error-driven point mutations and genetic recombination among different coronavirus strains and species during a co-infection within the same host cell (Haake *et al.*, 2020). Compared to other single-stranded RNA viruses, Coronavirus mutation rates are moderate to high, despite the viral exonuclease's proofreading function (Haake *et al.,* 2020). Genetic recombination is a direct result of the discontinuous transcriptional activity of the coronaviral polymerase and probably contributes to the emergence of new viruses with altered virulence, new range of host species, and new tissue tropism (Haake *et al.,* 2020).The CCoV shows in its evolutionary history a close correlation with TGEV and FCoV, so much that over time recombination phenomena have occurred, which have led to the development of different genotypes and subtypes with mixed infections with strains of both serotypes (Wang *et al.*, 2016). There are two CCoV genotypes: the type 1 genotype (similar to FCoV) and the type 2 genotype. Within the type 2 genotype, the recombinant events with TGE led to the development of two subtypes, type 2a and 2b (Decaro and Buonavoglia, 2008). The recombination among CCoV II serotype and other Coronaviruses has led to the emergence of Canine Coronavirus variants with N-terminal spike protein domains that are broadly homologous to the Transmissible Gastroenteritis Virus (TGEV) (Decaro *et al.*, 2009). Error-driven point mutations of viral polymerase, genetic recombination among different coronavirus strains and species, and incorporation of genes from other viral taxa via non-homologous recombination demonstrate the genetic plasticity of Coronaviruses and contribute to the alarming ability of Coronaviruses to perform the "jump species" (Haake *et al.,* 2020). As Feline Coronavirus serotype II, CCoV serotype II strains replicate well in tissue culture and use APN as an entry receptor (Haake *et al.*, 2020). The cellular receptor for serotype I viruses has not been determined, as these viruses are much more difficult to propagate in tissue culture systems. CCoV serotypes I and II share nearly 96% of nucleotide identity across most of their genome, while the gene encoding protein S is much more divergent, with only 56% of sequence identity (Haake *et al.,* 2020). The continuing evolution of Canine Enteric Coronaviruses with altered virulence and tropism is likely the result of changes in the genome due to random point mutations and periodic genetic recombination.

Coronaviruses, as well as RNA viruses, have a remarkable antigenic variety and different strains with numerous mutations. Even within the same infected animal, multiple strains can originate (Quasispecies theory), allowing the virus to evade the immune system and create more virulent variants with a different tropism (Domingo, 1997). The "Quasispecies theory" is suitable for RNA viruses because of their ability to mutate frequently, but also for some DNA viruses with small genomes (Domingo et al., 1998). Of particular importance in animal Coronaviruses is FCoV, where viral subpopulations were found with a divergence ranging from 0.9 to 13.8% (Battilani *et al.*, 2003).

Jary *et al.* (2020) was the first to report minority viral populations (up to 1%) during SARS-CoV-2 infection. Quasispecies differed from day to day, as well as between anatomical sites, suggesting that the new SARS-CoV-2 appears as a complex and dynamic distribution of variants (Jary *et al.*, 2020).

1.2.4.2. Epidemiology

Coronaviridae is a highly successful family of viruses that infects many different classes and orders of vertebrates, including humans, causing diseases ranging from localized respiratory or enteric infections to systemic diseases (Haake *et al.*, 2020). Coronaviruses cause significant morbidity and mortality in pet and livestock animal species, including dogs, cats, ferrets, horses, alpacas, pigs, bovids, and poultry, as well as numerous species of wild animals. In the past 20 years, we have seen three human coronaviral pandemics (SARS, MERS and, most recently, COVID-19), all of which originated from bat Coronaviruses (Fan *et al.*, 2019; Haake *et al.*, 2020). This demonstrates the zoonotic potential of coronaviruses.

Canine Enteric Coronavirus (CCoV) is a common infection in dogs with a worldwide distribution. While not universally recognized as an important canine enteric pathogen, numerous independent studies have shown that CCoV is significantly associated with diarrhea in dogs (Dowgier *et al.*, 2017). CCoV is transmitted via the fecal-oral route, with a higher prevalence in dogs housed in dense populations, like in shelters or kennels (Stavisky *et al.,* 2010).

CCoV has also been detected in wild canids, including foxes, raccoon dogs in China (Ma and Lu, 2005), and wolves in Alaska (Zarnke *et al.*, 2001) and in Europe (Haake *et al.*, 2020). The CCoV sequences found in European wolves were up to 98-99% homologous to known CCoV sequences isolated from domestic dogs (Molnar *et al.*, 2014).

In Italy, two studies were carried out concerning the circulation of the CCoV in Italian wolves.

Di Francesco *et al.*, 2019 conducted a multi-pathogen investigation to verify the health status of two Italian wolf packs in Majella National Park. Twenty fecal samples (10 per flock) were collected, and a virological screening was performed with molecular methods against the most common canine viruses (Protoparvovirus, Distemper Virus, Adenovirus, and Coronavirus). The results were positive for Canine Parvovirus type 2b and Canine Adenovirus type 2, whilst no sequences of Canine Distemper Virus and Canine Enteric Coronavirus were detected.

In a recently published study by Alfano *et al.* (2019), samples from a dead wolf were examined for DNA and RNA viruses, detecting positivity to pantropic CCoV. The analyzed samples were intestine, heart, brain, spleen, liver, and lungs, analyzed with Real-time PCR for the detection of CPV, CCoV, CAdV-1/CAdV-2, CDV, Canine Alphaherpesvirus, and Rotavirus (Decaro *et al.*, 2013). From molecular assays, the wolf was found to have a triple infection caused by CCoV, CPV-2b, and CAdV-2. The CCoV strain was detected in the gut and in non-intestinal tissues (heart, brain, spleen) and was typed as CCoV-IIa. The spike gene of the detected pCCoV strain, named CCoV/wolf/2016/IT, showed the highest degree of Nucleotide Identity (NT) (97%) with the CCoV-IIa strain CCoV/dog/HCM27/2014 from a Vietnamese dog, followed by FCoV strains NTU156/P/2007 from a Taiwanese cat with 95% of NT identity and GZ43/2003 from a Chinese raccoon dog with 93% of NT identity.

The results of Alfano *et al.* (2019) showed that CCoV circulated in the Italian wolf population, indicating that potentially fatal infections caused by pCCoV could be expected in this species. Interestingly, the wolf pCCoV strain was only remotely related to Italian CCoVs of canine origin, showing higher genetic identities to strains circulating in Asian carnivores. Therefore, it is possible that the virus was introduced in Italy through the import of dogs or other carnivores from Asia (Alfano *et al.*, 2019).

1.2.4.3. Pathogenesis

The main target of the virus is represented by epithelial cells, essentially causing enteric and respiratory damage of varying severity. Transmission occurs mainly through fecal-oral secretions and excretions (Haake *et al.,* 2020).

Similar to the pathology of other enteric Coronaviruses, CCoV infects and replicates in the apical and lateral enterocytes of the intestinal villi (which are mature enterocytes), causing cell degeneration and/or necrosis characterized by enterocyte atrophy, loss of the cell brush border, and desquamation of necrotic cells in the intestinal lumen. The degeneration and destruction of mature enterocytes at the villi ends can lead to atrophy of the villi, which results clinically in

poor digestion, malabsorption, and diarrhea (Licitra *et al.,* 2014). A more severe form of enteritis has also been reported in CCoV infected pups in the absence of co-infection. The macroscopic pathology in one case revealed moderate, diffuse hemorrhagic enteritis and in another severe ileocecal intussusception and segmental necrotic enteritis (Haake *et al.*, 2020). In the case of pantropic CCoV infections, necropsy can reveal severe coarse lesions to the lungs, liver, spleen, and kidneys (Decaro and Buonavoglia, 2008). A case report of pantropic CCoV also described lesions such as fibrin-purulent bronchopneumonia, renal cortical infarcts, severe changes in coalescing lobular liver fat, and multifocal hemorrhage in the spleen with lymphoid depletion. In this case, chronic diffuse enteritis was associated with the presence of adult roundworms in addition to CCoV (Zappulli *et al.*, 2008).

1.2.4.4. Clinical signs in domestic and wild canids

First reported in 1971 in dogs from a canine military unit in Germany (Binn *et al.*, 1974), CCoV generally causes mild, self-limiting diarrhea in dogs, especially in young puppies.

A more severe hemorrhagic disease associated with higher mortality was reported in combination with other pathogens (Decaro and Buonavoglia, 2008), including Canine Parvovirus type 2 (Decaro *et al.*, 2006) and Canine Adenovirus type I (Pratelli *et al.*, 2001). The CCoV infection has a synergistic effect with Canine Parvovirus type 2, increasing the severity of the enteric disease (Pratelli *et al.*, 1999).

In 2005, a highly virulent variant of CCoV type 2 (strain CB/05) called pantropic CCoV was reported in Italy, which caused a systemic disease followed by a fatal outcome in puppies (Buonavoglia *et al.*, 2006). Clinical signs consisted of fever (39.5-40 °C), lethargy, loss of appetite, vomiting, hemorrhagic diarrhea, severe leukopenia, and neurological signs (ataxia, convulsions) followed by death within two days from symptom onset (Decaro and Buonavoglia, 2008).

1.2.4.5. Diagnosis

CCoV type 1 is distinguishable from CCoV type 2 by means of conventional RT-PCR assays, which are capable of selectively amplifying fragments of ORF2 and ORF5 (Decaro and Buonavoglia, 2008). Recently, TaqMan-based real-time RT-PCR tests have been established for the detection and quantification of CCoV RNA in feces of dogs with diarrhea (Decaro and Buonavoglia, 2008) and for the discrimination between the two CCoV genotypes (Decaro *et al.*, 2005). Extensive molecular analysis of fecal samples collected from the Italian canine

population revealed that CCoV infection in dogs is often characterized by the simultaneous presence of both genotypes (Decaro *et al.*, 2005).

CHAPTER TWO: MATERIALS AND METHODS

2.1. AIMS

The aim of the study is to evaluate the presence and spread of DNA and RNA viruses in the Italian wolf population (*Canis lupus italicus*) herewith analyzed. We used molecular methods to investigate the presence of DNA and genomic RNA of five important viruses that infect and cause pathologies in domestic and wild canids: *Canine Parvovirus* type 2 (CPV-2), *Canine Adenovirus* type 1 and type 2 (CAdV-1 and CAdV-2), *Canine Distemper virus* (CDV), and *Canine Enteric Coronavirus* (CCoV).

2.2. MATERIALS AND METHODS

2.2.1. Study area

The study area extends over three distinct zones within three Italian regions (Tuscany, Emilia Romagna, and Calabria) (Musto, 2020). A detailed map showing the study site is presented in Fig. 5. The open-source software QGIS 3.10 was used to create all the maps.
The study area represents a large part of the Italian territory where the species is present in a stable manner and with reproductive packs (Galaverni *et al.*, 2016). Land use is quite heterogeneous, as the study area includes parts characterized by an intensive agricultural use and by higher population density and, on the other hand, protected areas with lower levels of human presence. In detail, it includes areas in the Tuscan-Emilian-Romagnolo Apennines, in the provinces of Bologna, Ravenna, (Fig. 6) and, on the Tuscan side, the provinces of Livorno, Pisa, Pistoia, Prato, and Florence (Fig. 7).
For what concerns Calabria, the wolves came from the provinces of Cosenza, Catanzaro, Reggio Calabria, and Vibo Valentia (Fig. 8). As for the Tuscan-Emilian-Romagnolo Apennines, also in this study area the subjects came both from wooded and mountain areas and from medium or heavily populated areas. The southern Apennines is one of the areas where signs of the species' presence have always been found, even when there was no specific search for them. As for the wolves of the central-northern Apennines and the Alpine area, also in the southern Apennines the species underwent a sharp decline in the twentieth century (Zimen and Boitani, 1975). Although with small numbers, in Calabria the species has managed to resist the pressure of man, restarting with a positive trend since the end of the twentieth century (Mirabelli, 1985) to present.

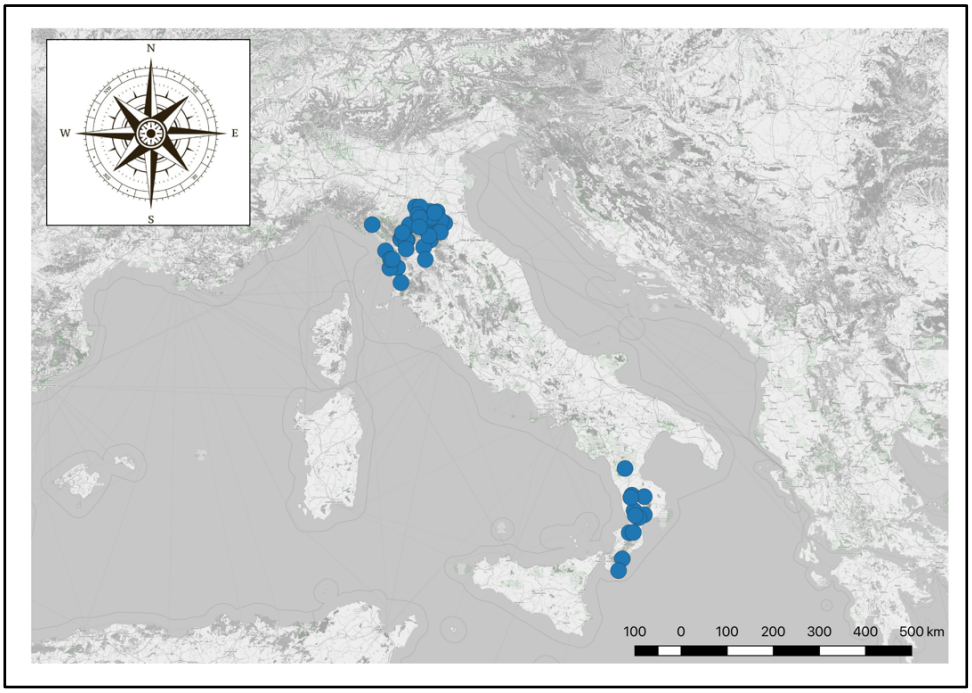


Fig. 5 – The map shows all the points of discovery of the wolf carcasses object of this study.

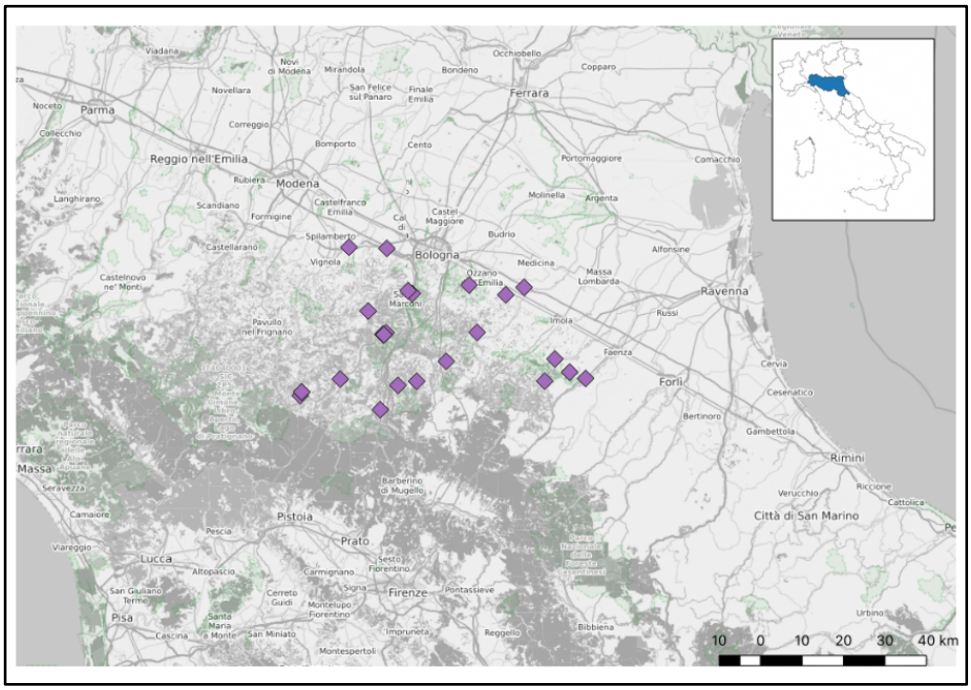


Fig. 6 – The map shows the discovery points of wolf carcasses in the study area within the Emilia-Romagna region.

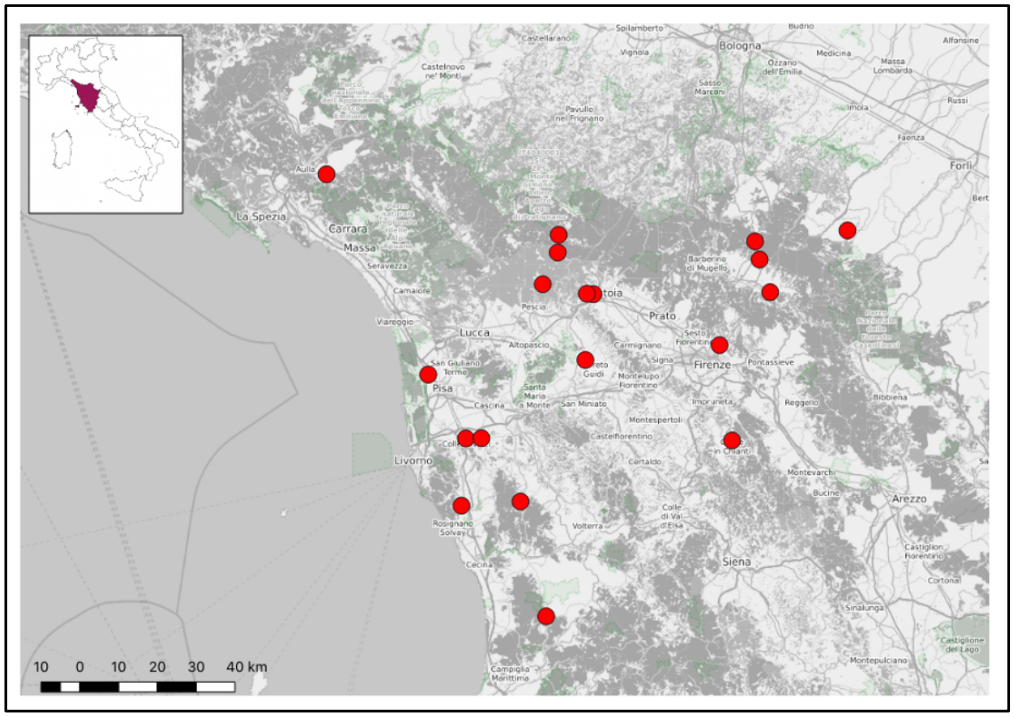


Fig. 7 – The map shows the discovery points of wolf carcasses in the study area within the Tuscany region.

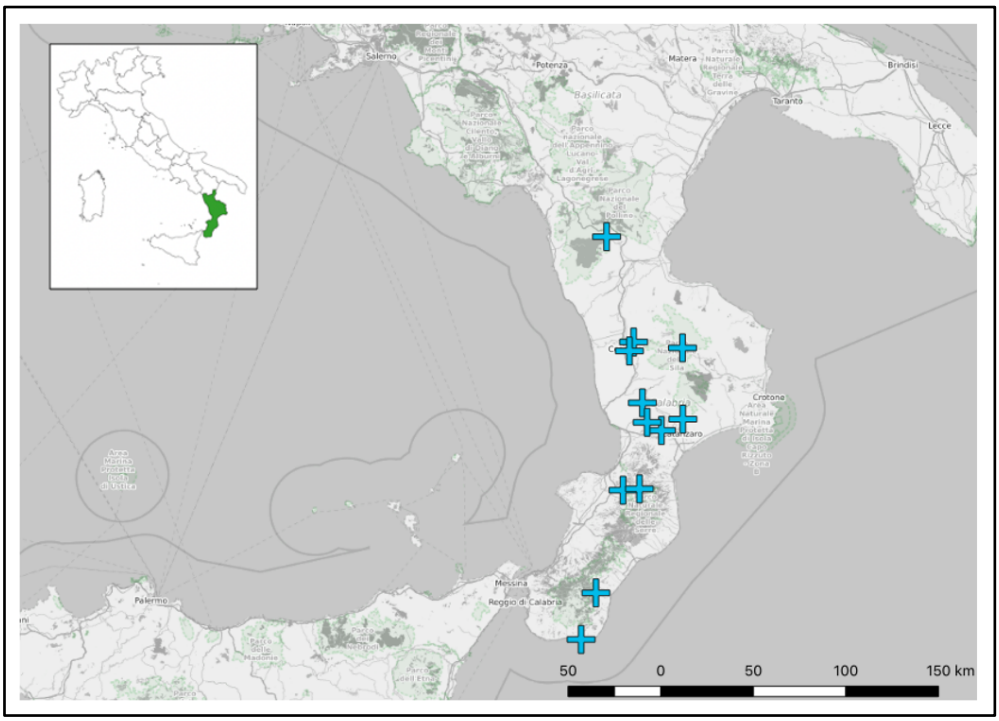


Fig. 8 - The map shows the discovery points of wolf carcasses in the study area within the Calabria region.

2.2.2. Study population and sampling methods

The study included wolves of all ages and both sexes found dead in the Emilia-Romagna, Tuscany, and Calabria regions in the period between August 2017 and April 2020 (Musto, 2020). Pure individuals (Qw>0.995), individuals with a minimal percentage of introgression of dog genes (Qw<0.995 and >0.955), and hybrid subjects (Qw<0,955) were included (Caniglia *et al.*, 2020). To evaluate the genetic profile of the wolves, tongue samples were sent to the Laboratory of the Area for Conservation Genetics (BIO-CGE) of the ISPRA (Italian Institute for Environmental Protection and Research). The DNA was extracted and amplified by PCR, then a specific mitochondrial DNA control region, diagnostic for the Italian wolf population, was sequenced and genotyped for differentiating among wolves, dogs, and hybrids of the first, second or third generation (Anderson *et al.*, 2009; Caniglia *et al.*, 2014; Randi *et al.*, 2014).

Additionally, tissue samples were taken from each wolf, including the tongue, spleen, small intestine, and lung, used for this study. The organic matrices were stored in the freezer at -20 °C until the time of the laboratory investigations.

In addition to the virological investigation object of this study, all the subjects were examined according to the National Surveillance Plan for the search of zoonotic parasitic diseases, such as *Trichinella spp*. and *Leishmania infantum*. In fact, the matrices sampled were the diaphragm and the tibial muscle for the search of *Trichinella spp*. (Kapel *et al*., 2005) and the spleen and popliteal lymph nodes for the search of *Leishmania infantum* (Gomes *et al.,* 2007). Furthermore, for all individuals the stomach and liver were analyzed to detect toxic substances, i.e. zinc phosphide, strychnine, organophosphate pesticides, metaldehyde, and anticoagulants (Berny, 2007). The search of *Sarcoptes scabiei* was not performed routinely, but only in the presence of suspicious skin lesions suggesting sarcoptic mange. In the case of mangy subjects, staging was performed according to the methodology described in Pence & Ueckermann (2002).

2.2.3. Necropsy examination

Between 2017 and 2020, necropsy examinations were carried out on 56 wolf carcasses by the Wildlife and Exotic Service of the University of Bologna and by the Experimental Zooprophylactic Institutes of Lombardy and Emilia-Romagna (section of Bologna) and of the Mezzogiorno (section of Catanzaro, Cosenza, Reggio Calabria, and Vibo Valentia) (Musto, 2020).

The carcasses analyzed in this study were collected according to the National Action Plan

for the conservation of wolves (Genovesi, 2002). The protocol requires that each wolf carcass is checked and collected by the public body in charge (depending on the regional/provincial laws, it could be a duty of the Forestry Police, Provincial Police, Local Health Authority, and/or Park Authorities). Subsequently, the carcasses are delivered to the authorized centers in order to proceed with the necropsy (Experimental Zooprophylactic Institute, University Institutes, and Park Authorities) and genetic analyses (Genovesi, 2002).

At the arrival of each carcass, a first form containing the following information was filled: subject identification data with the attribution of an ID code, place of discovery (reported as coordinates), sex, weight (in kg), and nutritional status (obtained through observation and palpation of the main points where the fat is deposited, i.e. the flank fossa, ribs, lumbar vertebrae, and pelvic bones). The animal age was estimated based on dental development, body size, and weight (Morner *et al*., 2005). In fact, all individuals examined were aged using one of the following categories: class 1: ≤ 12 months; class 2: 1–2 years; class 3: > 2 years. Additionally, only the ages of the subjects under one year and so attributed to class 1 were expressed in months. In fact, the evaluation of the months was made in relation to the reproductive cycle of Italian wolves which sees the pups' birth in May (Boitani, 1981). The biometric information and phenotypic characteristics were also recorded; in particular, the presence of spurs, stripes, white nails, and the coat coloring were noted. The necroscopic examination was finalized by taking a photographic documentation of the whole carcass and of all the fundamental steps that characterized the necropsy investigation.

After that, an external examination of the carcass was made by placing it in lateral decubitus: such position allows the direct evaluation of the subject's general nutritional and health status. Afterwards, in case of traumatic-contusive lesions we would decide if collateral radiographic investigations were needed to identify bullets, other foreign bodies, or fractures in the skeletal system. It is possible to highlight any previous injuries and to assess the state of nutrition, of the skin, of the apparent mucous membranes, and of the explorable lymph nodes.

After performing the general physical examination, the flaying is followed by putting the animal in a dorsal decubitus position. The complete necropsy examination involves the opening of the abdominal cavity first, followed by the opening of the thoracic cavity. Before proceeding with organ sampling for virological analysis, each organ was inspected and assessed individually. Information relative to cases of poisoning were also transferred

to the Mayor and the veterinary services (OM 13 June 2016, Art. 4).

2.3. LABORATORY METHODS

2.3.1. CPV-2, CAdV-1, and CAdV-2 DNA search using SYBR Green Real-time PCR (qPCR)

2.3.1.1. DNA extraction from tissue samples

The extraction of DNA from the samples of the spleen, small intestine, and tongue was performed using the commercial kit NucleoSpin Tissue (Macherey-Nagel) and was divided into eight phases (Fig. 9):

1. Sample preparation: 25 mg of tissue was cut into small pieces which were inserted inside an Eppendorf;
2. Pre-lysis of the samples: after adding 180 µl of Buffer T1 and 25 µl of proteinase K solution to the samples, they were shaken and completely covered with lysis solution, afterwards they were incubated for 1-3 hours at 56°C;
3. Lysis of the samples: the samples were shaken, then 200 µl of Buffer B3 were added and they were again shaken vigorously; subsequently, they were incubated at 70°C for 10 minutes, at the end of which they were shaken briefly;
4. Regulation of DNA binding conditions: 210 µl of 96-100% ethanol was added to the samples, which were then shaken vigorously;
5. Link between the DNA and the silica filter: the samples were inserted individually inside a NucleoSpin filter column in a Collection Tube and were centrifuged for 1 minute at 11000 g; the filtrate was then eliminated and the column with the DNA linked to the filter was reinserted inside the Collection Tube;
6. Washing of the silica membrane: two washes were carried out, 500 µl of Buffer BW was added in the first and the column was centrifuged for 1 minute at 11000 g; the waste was then eliminated and the column was inserted again in the Collection Tube; in the second washing, 600 µl of Buffer B5 was added to the column, which was then centrifuged for 1 minute at 11000 g; after that, the waste was eliminated and the column was then inserted in a new Collection Tube;
7. Drying of the silica membrane: the column was centrifuged for 1 minute at 11000 g to eliminate the residual Ethanol;

8. Elution of ultra-pure DNA: the NucleoSpin column was inserted into a 1.5 ml Eppendorf and 100 µl of Buffer BE previously heated to 70 °C was added; it was incubated at room temperature for 1 minute and then centrifuged for 1 minute at 11000 g; the Eppendorf collected the extracted DNA which was subsequently stored at -20°C until use.

For the search of CPV-2 DNA, DNA extracts from the tongue and small intestine samples were tested.

For the search of CAdV-1 and CAdV-2 DNA, DNA extracts from the tongue and spleen samples were tested.

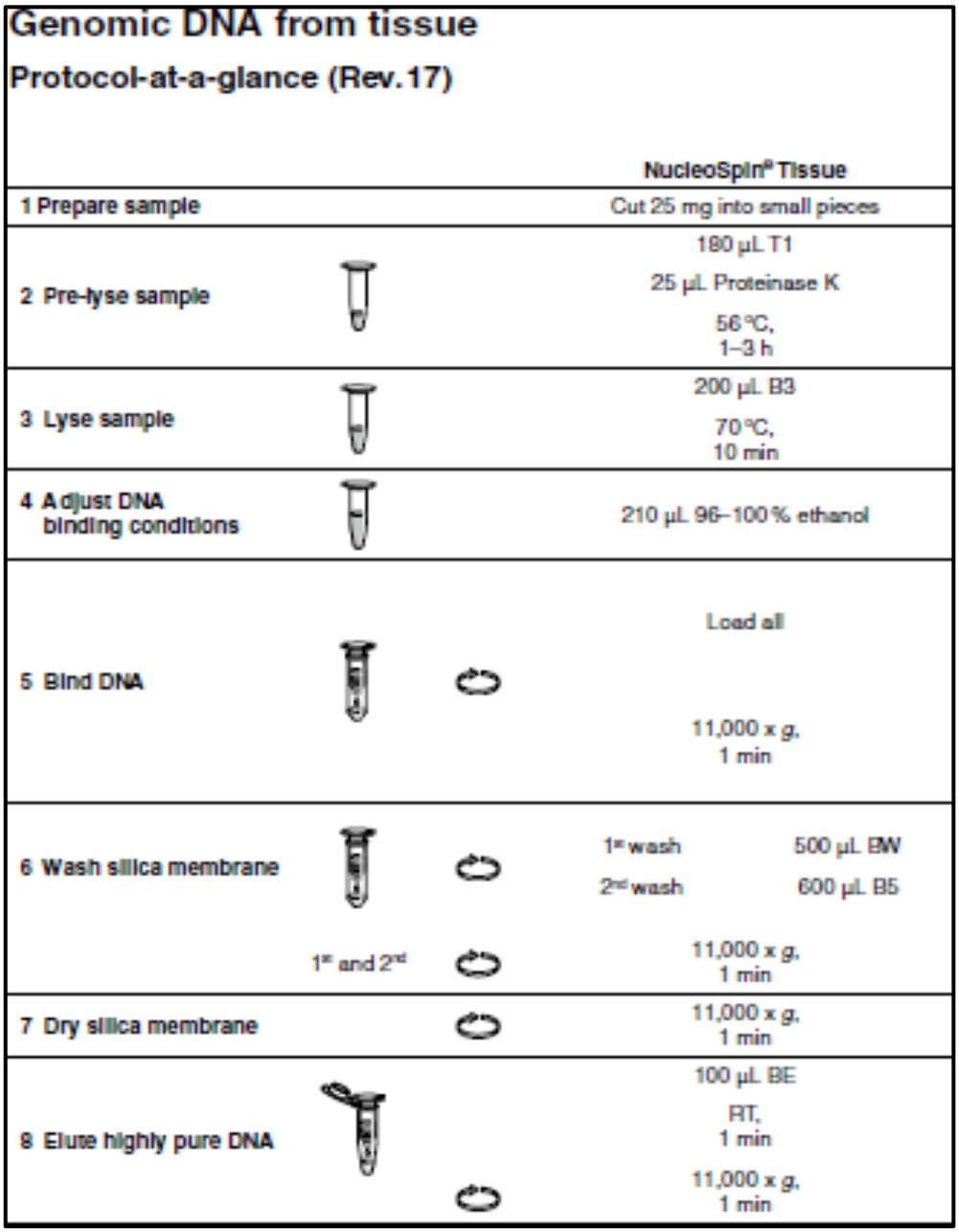


Fig. 9 - DNA extraction process from tissues.

2.3.1.2. Method used and reaction mix

Two distinct molecular methods of SYBR Green Real-time PCR were used for the viral DNA search: one for the CPV-2 DNA search and one for the CAdV-1-2 DNA search. They could detect and quantify the nucleic acids of the three viruses and, in the specific case of CAdV, also distinguish type 1 from type 2 in a single reaction. The methods adopted were validated in the INFLAB (Infectious Diseases Diagnostic Unit - CLINLAB – UNIBO) laboratories for all three viruses (Balboni *et al.,* 2015; Balboni *et al.,* 2018). The reactions were performed using the commercial kit PowerUp SYBR Green Master Mix Kit Thermo (Fisher Scientific, Life Technologies) and the platform StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Life Technologies).

The reaction mix for the three reactions is shown in Table 1:

Reaction Mix	CPV-2	CAdV-1 CAdV-2
Master Mix	10 µl	10 µl
Primer qPCR-For (10 pmol/µl)	1,2 µl	1,2 µl
Primer qPCR-Rev (10 pmol/µl)	1,2 µl	1,2 µl
Ultrapure water	5,6 µl	5,6 µl
Final volume	18 µl (+2 µl of template)	18 µl (+2 µl of template)

Tab. 1 - Reaction mixes used for Real-time PCR methods. The volumes indicated refer to a single sample.

The primers used were:

- for Canine Parvovirus (Balboni *et al.*, 2018):
  - CPV-qPCR-For - AGC TAC TAT TAT GAG ACC AGC TGA G;
  - CPV-qPCR-Rev - CCT GCT GCA ATA GGT GTT TTA A;
- for Canine Adenovirus (Balboni *et al.*, 2015):
  - CAdV-qPCR-For3 - CTG ASA CTG CWA TRM CTA TAT AYA TTT CCA;
  - CAdV-qPCR-Rev2 - GAC ATA GAR ACT CAG GAC CCA GA.

2.3.1.3. Reaction cycle

The reaction cycle was divided into three phases and was the same for all viruses: an initial denaturation phase at 95 °C for 5 minutes was followed by 45 reaction cycles, each of which was composed of:

- ♣ denaturation phase at 95 °C for 15 seconds;
- ♣ annealing phase and extension to 60 °C for 1 minute.

Finally, it was produced an amplicon of 99 bp in length for CPV-2 and one of 166 bp in length for CAdV. In fact, the nucleotide composition of the latter varied between type 1 and type 2.

2.3.1.4. Data processing

Since SYBR Green was used, it was necessary to analyze the melting curve to verify the specificity of the products obtained. To detect the melting temperature (Tm), i.e. the temperature in which 50% of the DNA is denatured, at the end of the amplification cycle. the temperature was increased in continuous increments of 1.8% and the fluorescence emitted was evaluated from the amplification product. Table 2 shows the temperature increases adopted and the Tm of the amplification targets obtained for the viruses searched.

	CPV-2	CAdV-1 and CAdV-2
Increase T	From 55 °C to 95 °C	From 55 °C to 95 °C
Tm	81 °C	CAdV-1 73 °C CAdV-2 80 °C

Tab. 2 - Temperature increases adopted and melting temperature of the amplification targets.

To quantify the target DNA detected, evaluate the efficiency of the reaction, and establish the Limit Of Detection (LOD) of each three used methods, a calibration curve was developed for each virus, within which at least six dilutions were assessed in duplicate base 10 scalars of a recombinant plasmid (pCR4 plasmid, TOPO TA Cloning Kit, Life Technologies, USA) containing the target DNA fragment and unknown samples. We started from a concentration equal to 107 copies of target DNA/µl of template up to a concentration equal to 1 copy of target DNA/µl of template.

The LOD was established at 1 copy of target DNA/µl of template for the amplification methods of CAdV-1, CAdV-2, and CPV-2.

The samples were considered positive when both repetitions for each virus were higher than the LOD and a specific melting curve was found.

During the procedure, all the necessary measures were taken to prevent any contamination. Furthermore, in order to exclude any false positive, a blank consisting of ultrapure water for molecular biology was added to the reaction.

Some samples were also tested by the Experimental Zooprophylactic Institute of Lombardy and Emilia-Romagna for the detection of CAdV-1, CadV-2, and CPV-2 DNA on the same biological matrices described in paragraph 2.3.1.1., using two different molecular assays.

The presence of CAdV DNA was investigated through a PCR assay based on the use of the primers pair HA1 and HA2 described by Hu *et al.* (2001) and of the GoTaq®Hot Start Colorless Master Mix (PROMEGA, Madison, USA) according to the manufacturer's instructions. The assay was able to detect and discriminate CAdV-1 and CAdV-2 DNA (Hu *et al.*, 2001).

The presence of CPV-2 DNA was investigated by the QuantiFast Pathogen Real-Time PCR assay + IC (Qiagen, Hilden, Germany) according to the manufacturer's instructions, using primers and probes described by Decaro *et al.* (2005a).

All the samples tested with these molecular tests gave the same diagnostic results as the samples tested with the two molecular methods of SYBR Green Real-time PCR (qPCR) described by Balboni *et al.,* 2015 and Balboni *et al.,* 2018.

2.3.2. CDV RNA search using the One-Step Reverse Transcription PCR (One-Step RT-PCR)

2.3.2.1. RNA extraction from tissue samples

Starting from lung samples (subjected to homogenization in PBS buffer in the ratio of 10% weight/volume), the RNA was extracted from 230 µL of homogenate using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

2.3.2.2. Method used and reaction mix

The RNA was eluted in 40 µL of elution buffer. The technique used was the traditional One-Step RT-PCR according to the manufacturer's instructions of the OneStep RT-PCR kit (Qiagen, Hilden, Germany) with the primers described in Frisk *et al.*, 1999. A negative reaction control (Water DNase/RNase free) and a positive reaction control (RNA extracted from the vaccine "Canigen CEPPI/L") were added to each search.

2.3.2.3. Reaction cycle and electrophoresis

The reaction was carried out in a final volume of 25 µL/sample, using the OneStep RT-PCR (Qiagen, Hilden, Germany) kit according to the manufacturer's instructions, with 20 µL of mix, primers at the final concentration of 0.6 µM, and an addition of 5 µL of RNA/sample. The reverse transcription and amplification cycle (Tab. 3) were performed with the Veriti Thermal Cyclers by Applied Biosystems.

Phase	**Temperature**	**Time**	**N° cycles**
Reverse transcription	50 °C	30 min	1
Activation *Hot Start Taq* DNA Polymerase	95 °C	15 min	1
Denaturation	94 °C	30 sec	40
Annealing	55 °C	30 sec	
Extension	72 °C	30 sec	
Final extension	72 °C	10 min	1

Tab. 3 - Cycle of reverse transcription and amplification on lung samples subjected to CDV detection.

Eight microliters of PCR products were electrophoresed in a 1.8% agarose gel prepared in 0.5 X TBE (Sigma-Aldrich ®, Saint Louis, USA) running buffer, containing SYBR Green (1 X), in the presence of a 100 bp DNA ladder (Invitrogen TM, Carlsbad, USA) as molecular marker. The gel was visualized on a UV transilluminator.

A PCR product of 287 bp was obtained in the positive samples as well as in the positive control, according to Frisk *et al*. (1999).

2.3.3. CCoV RNA search using the One-Step Reverse Transcription PCR (One-Step RT-PCR)

2.3.3.1. RNA extraction from the tissue samples

Starting from a portion of the duodenum and from the intestinal contents obtained from it (subjected to homogenization in PBS buffer in the ratio of 10% weight/volume), the RNA was extracted from 230 µL of homogenate using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

2.3.3.2. Method used and reaction mix

The RNA was eluted in 40 µL of elution buffer. The technique used was the traditional One-Step RT-PCR according to the manufacturer's instructions of the OneStep RT-PCR kit (Qiagen, Hilden, Germany) with the primers described in Simons *et al*., 2005. A negative reaction control (Water DNase/RNase free) and a positive reaction control (RNA extracted from a Positive Sample confirmed by Sanger Sequencing) were added to each search.

2.3.3.3. Reaction cycle

The reaction was carried out in a final volume of 25 µL/sample, using the OneStep RT-PCR (Qiagen, Hilden, Germany) kit according to the manufacturer's instructions, with 20 µL of mix, primers at the final concentration of 0.4 µM, and an addition of 5 µL of RNA/sample. The reverse transcription and amplification cycle (Tab. 3) were performed with the Veriti Thermal Cyclers by Applied Biosystems.

Phase	Temperature	Time	N° cycles
Reverse transcription	50 °C	30 min	1
Activation *Hot Start Taq* DNA polymerase	95 °C	15 min	1
Denaturation	95 °C	30 sec	40
Annealing	62 °C	40 sec	
Extension	72 °C	40 sec	
Final extension	72 °C	5 min	1

Tab. 4 - Cycle of reverse transcription and amplification on duodenum and fecal samples subjected to CCoV detection.

Eight microliters of PCR products were electrophoresed in a 1.8% agarose gel prepared in 0.5 X TBE (Sigma-Aldrich ®, Saint Louis, USA) running buffer, containing SYBR Green (1 X), in the presence of a 100 bp DNA ladder (Invitrogen TM, Carlsbad, USA) as molecular marker. The gel was visualized on a UV transilluminator.

A PCR product of 295 bp was obtained in the positive samples as well as in the positive control, according to Simons *et al*., 2005.

2.4. STATISTICAL ANALYSIS

Statistical analyses were implemented using the open-source software RStudio Team 2020. Descriptive statistics were done with the R Base package and *ggplot2* (Wickham, 2011).

CHAPTER THREE: RESULTS

3 - RESULTS

3.1. Study population

In the period between August 2017 and July 2020, 56 Italian wolves (*Canis lupus italicus*) found dead in Emilia-Romagna, Tuscany, and Calabria were included in the study (Musto, 2020). Tab. 4 shows the signaling data of the wolves included.

	No. of subjects	**Percentage of the study population**
Year of death		
2017	6	10,7%
2018	17	30,3%
2019	15	26,9%
2020	18	32,1%
Region of origin		
Emilia-Romagna	25	44,6%
Tuscany	19	34%
Calabria	12	21,4%
Sex		
Males	32	57,1%
Females	24	42,9%
Age		
CLASS_1	19	33,9%
CLASS_2	18	32,1%
CLASS_3	19	34%
Total individuals	**56**	**100%**

Tab. 4 - Signaling data of the sampled population.

25 (45%) subjects came from Emilia-Romagna, 19 (34%) from Tuscany, and 12 (21%) from Calabria (Fig. 10a). The provinces of origin of the carcasses were: Bologna (21/56), Vibo Valentia (1/56), Reggio Calabria (2/56), Ravenna (4/56), Pistoia (5/56), Pisa (4/56), Massa Carrara (1/56), Livorno (2/56), Florence (7/56), Cosenza (5/56), and Catanzaro (4/56) (Fig. 10b).

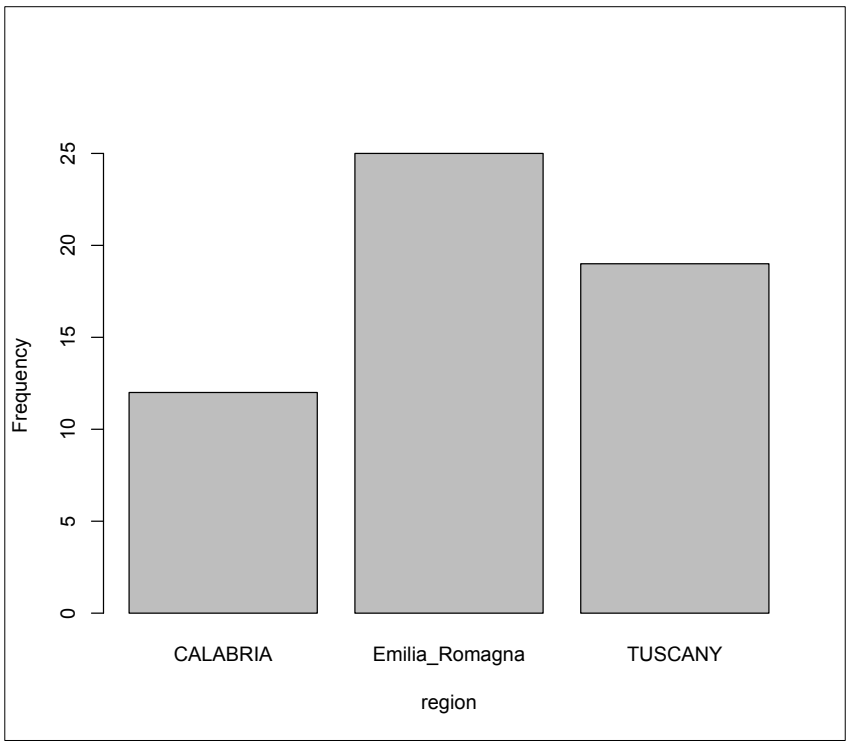


Fig. 10$_a$ – Bar plot with the sampled population's distribution by region of origin.

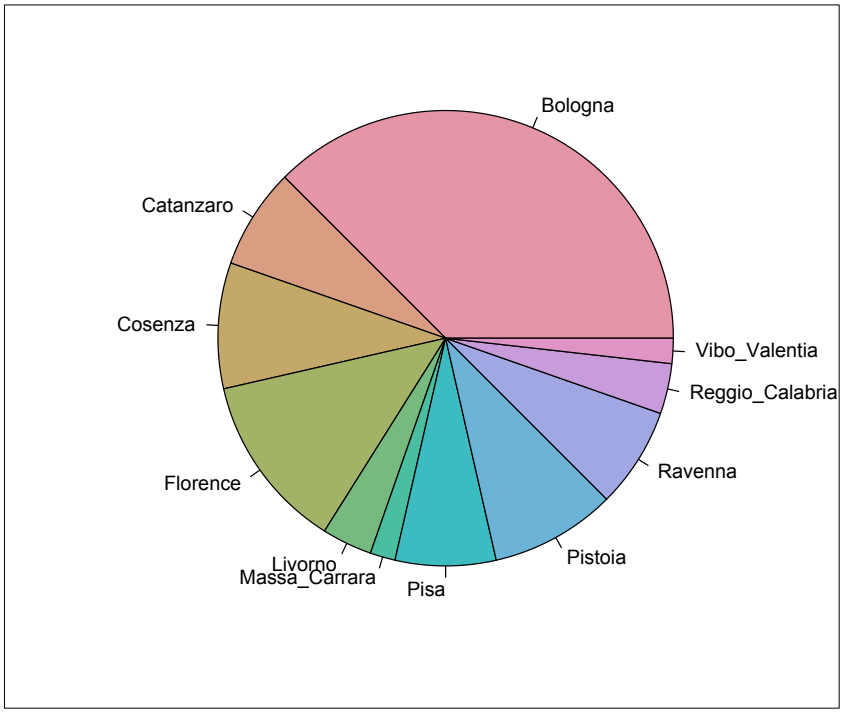


Fig. 10$_b$ – Pie chart with the sampled population's distribution by province of origin.

6 (11%) wolves died in 2017, 17 (30%) in 2018, 15 (27%) in 2019, and 18 (32%) died in 2020 (Fig. 11).

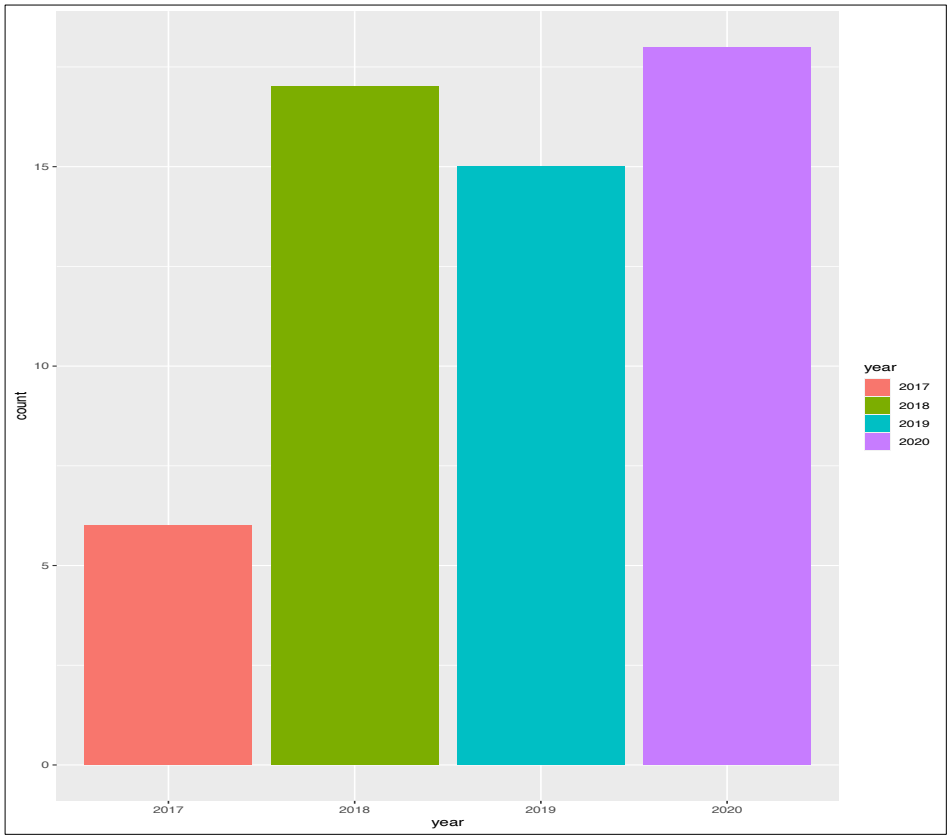


Fig. 11 – Bar plot of the sampled population by year of death and discovery. X = year of sampling, Y = n. of deceased subjects.

The population consisted of 32 (57%) male subjects and 24 (43%) female subjects (Fig.12).

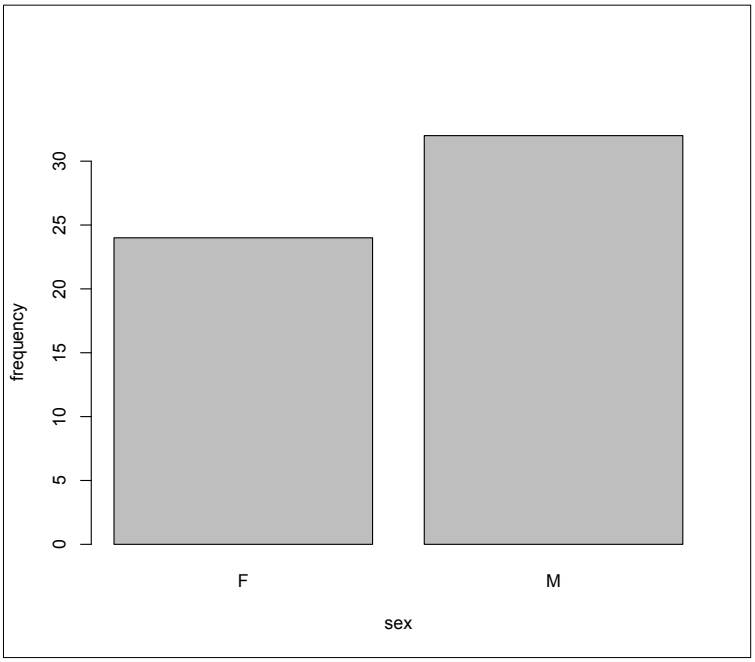


Fig. 12 – Bar chart with the population's distribution sampled by sex. F = females, M = males.

After the necropsy examination, the age of the subjects was estimated. A homogeneous distribution between the various age groups can be observed. In detail, there were 19 (34%) class 1 subjects (<1 year), 18 (32%) class 2 subjects (> 1 year <2 years), and 19 (34%) class 3 subjects (> 2 years) (Fig. 13).

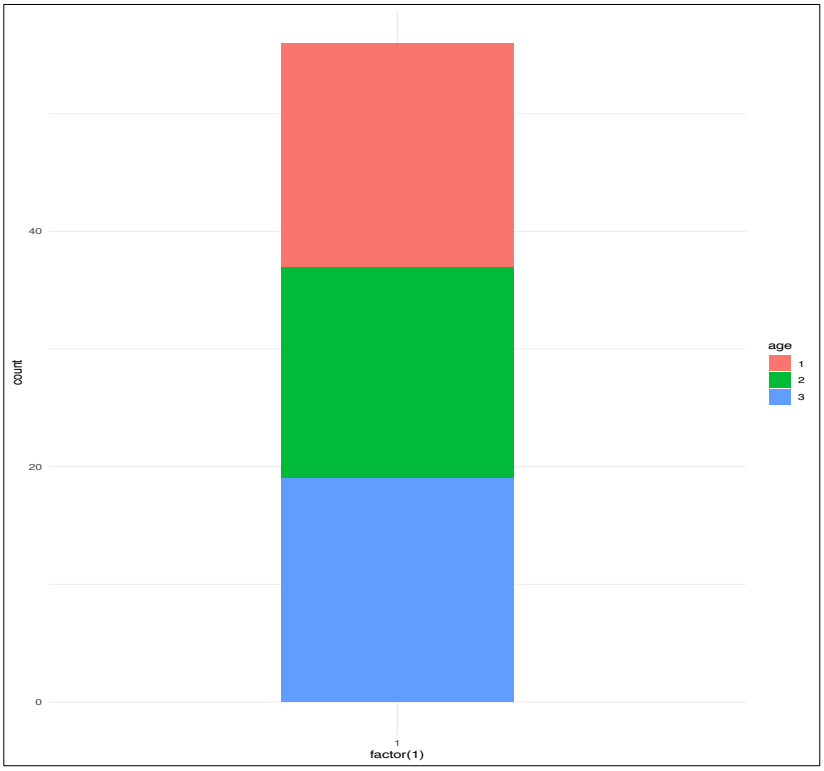


Fig. 13 – Overlapping bar plot of the sampled population divided by age group: class 1, class 2, and class 3.

The study population consisted of 56 Italian wolves, of which 73.21% (41/56) genetically identified as wolves (WOLF – Qw > 0.995), 19.64% (11/56) with a minimal percentage of introgression of dog genes (WOLF INTROGRESSED - Qw < 0.995 and Qw > 0.955), 5.36% (3/56) considered wolf *x* dog hybrids (HYBRID - Qw < 0.955), and 1.79% (1/56) not genetically determinable to any species (ND), see Fig.14a.

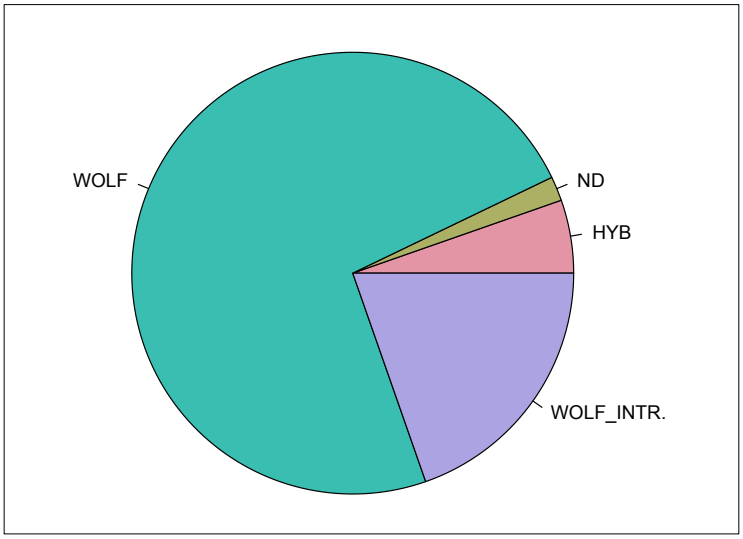


Fig. 14a – Pie chart with representation of the different genetic diagnoses of the sampled population. Legend; ND – not determinable, HYB – hybrid, WOLF_INTR. – wolf introgressed.

In addition to the attribution of species, an observation of the subjects' phenotypic characters was carried out, Fig. 14b. 76.8% (43/56) had a wild-type coat, typical of the Italian wolf. For what concerns 12.5% (7/56) of the subjects, it was not possible to perform a phenotypic evaluation due to advanced stage sarcoptic mange. 3.6% (2/56) had an atypical phenotype or at least one character not corresponding to the pattern of the species. The remaining 7.1% (4/56) had a wild-type black coat (Fig. 14b).

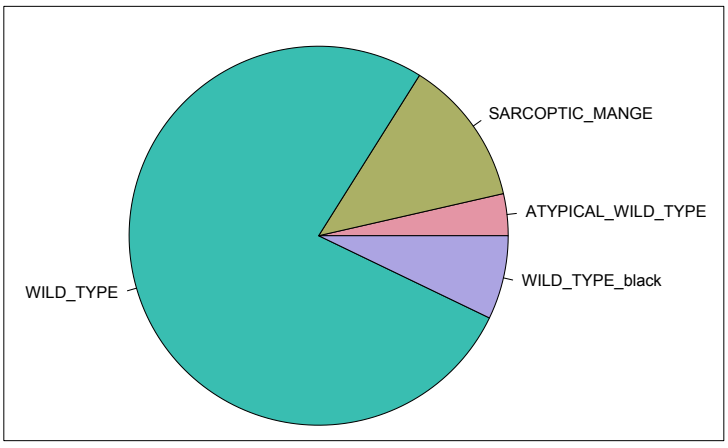


Fig. 14b – Pie chart with the representation of the different phenotypes observed in the sampled population.

The intestine, lungs, spleen, and tongue of each wolf were sampled, on which molecular methods were adopted to investigate the presence of genomic DNA and RNA of five viruses: the tongue and intestine (duodenum) to search for the DNA of Canine Parvovirus type 2 (CPV-2), the tongue and spleen to search for DNA from Canine Adenovirus type 1 and type 2 (CAdV-

1 and CAdV-2), the lung to search for Canine Distemper virus (CDV) RNA, and the intestine (duodenum) to search Canine Enteric Coronavirus (CCoV) RNA.

3.2. DNA detection of Canine Parvovirus type 2 (CPV-2)

44 of the 56 sampled wolves tested positive for CPV-2 DNA with a prevalence of 78.57% (Tab. 5; Fig. 15). The median amount of target DNA detected was 77 DNA copies/µL of extract (range 1.4 - 2.6x104).

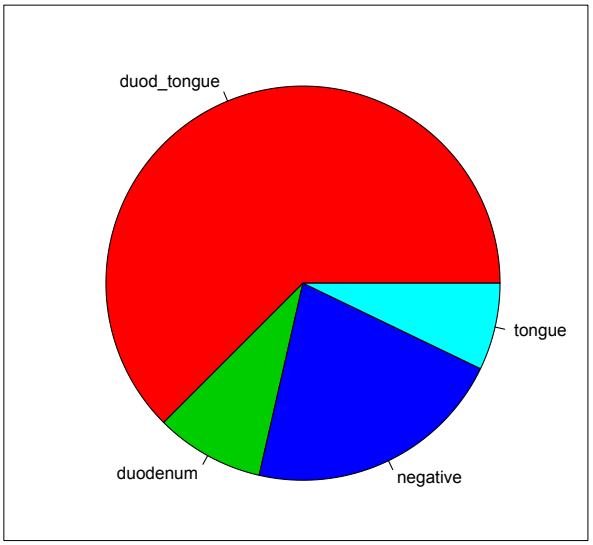


Fig. 15 – Pie chart with the prevalence of CPV-2 DNA found in the molecular investigation with Real-time PCR. Out of the total of 56 samples, 12/56 (21.5%) subjects were negative, 4/56 (7.1%) subjects were positive only in the tongue matrix, 5/56 (8.9%) subjects were positive only in the intestine matrix, and 35/56 (62,5%) subjects were positive in both matrices.

CPV-2 DNA was detected with the highest prevalence in 2018 with 34.09% (15/44) of positive subjects, followed by 2020 with 31.82% (14/44) of positive subjects, then 2019 with 22.73% (10/44), and finally 2017 with 11.36% (5/44) (Fig. 16, Tab. 5).

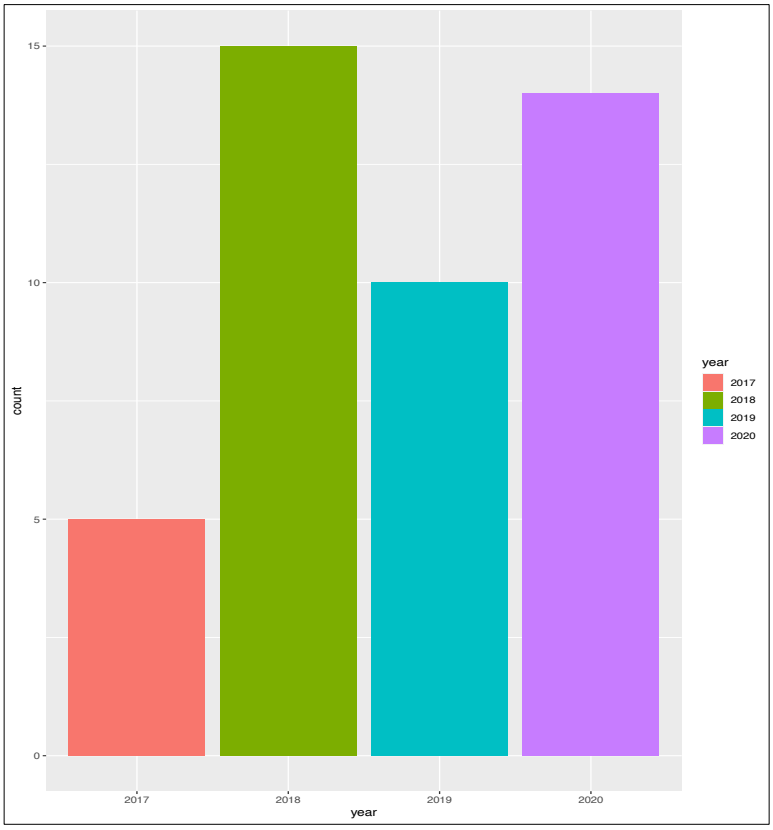


Fig. 16 – Bar plot of the annual distribution of CPV-2 positivity.

In Emilia-Romagna, CPV-2 DNA was found with a prevalence of 45.45% (20/44), in Tuscany of 29.55% (13/44), and in Calabria of 25% (11/44) (Fig. 17, Tab. 5).

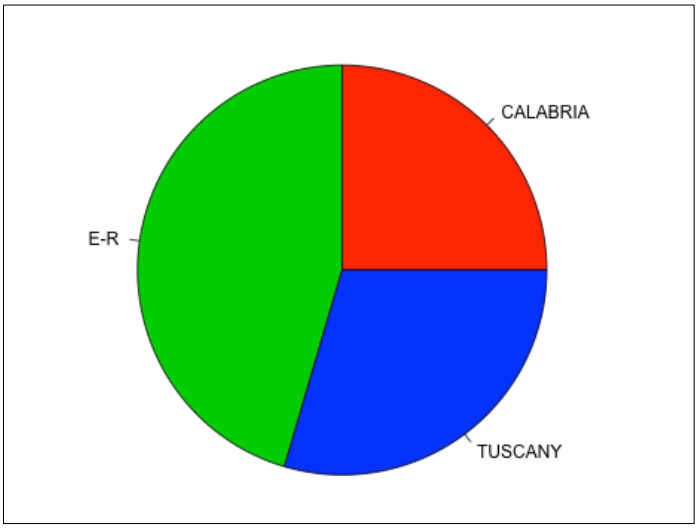


Fig. 17 – Pie chart with CPV-2 prevalence divided by sampling regions. Legend: E-R: Emilia Romagna region

Finally, the prevalence was high in all age groups, with a greater involvement of class 2 with 38.64% (17/44), followed by class 1 with 31.82% (14/44), and finally class 3 with 29.55% (13/44) (Fig. 18, Tab. 5).

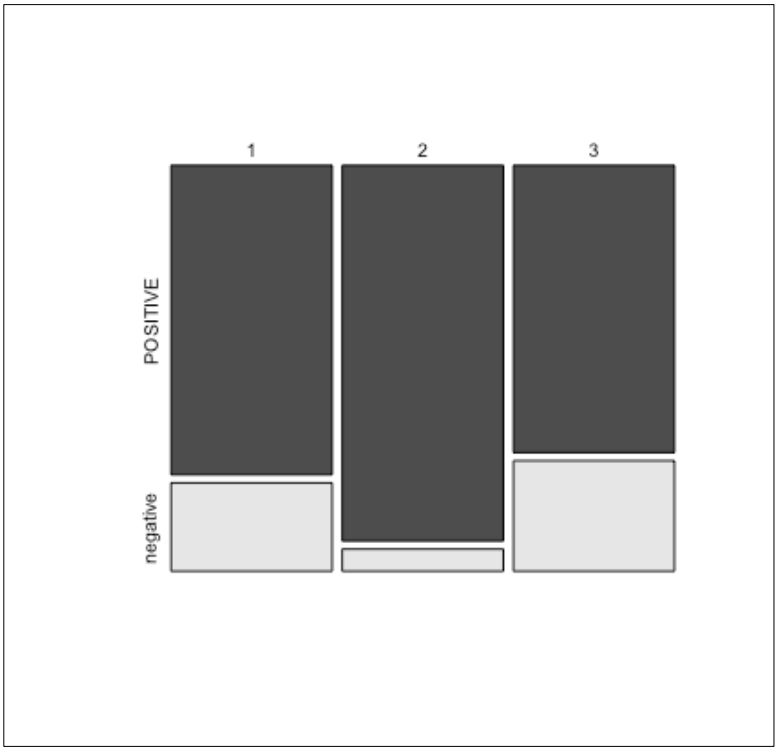


Fig. 18 – Mosaic plot with CPV-2 prevalence divided by the three age groups. X = CLASS_1, CLASS_2, CLASS_3; Y = negative, positive.

	CPV-2		CAdV-1		CAdV-2		CoCV-CDV		Tn
	N_p	P	Np	P	Np	P	Np	P	
Year of death									
2017	5	83,3 %	1	16,7 %	0	0%	0	0%	6
2018	15	88,2 %	1	5,9 %	2	11,8 %	0	0%	17
2019	10	66,7 %	1	6,7 %	1	6,7%	0	0%	15
2020	14	77,8 %	4	22,2 %	0	0%	0	0%	18
Region of origin									
Emilia Romagna	20	80%	2	8%	1	4%	0	0%	25
Tuscany	13	68,4 %	3	15,8 %	0	0%	0	0%	19
Calabria	11	91,7 %	2	16,7 %	2	16,7 %	0	0%	12
Sex									
Males	26	81,2 %	4	12,5 %	3	9,4%	0	0%	32
Females	18	75%	3	12,5 %	0	0%	0	0%	24
Age									
CLASS_1	14	73,7 %	2	10,5 %	1	5,3%	0	0%	19
CLASS_2	17	94,4 %	3	16,7 %	1	5,5%	0	0%	18
CLASS_3	13	68,4 %	2	10,5 %	1	5,3%	0	0%	19

Tab. 5 - Results of molecular investigations according to the year of death, region of origin, sex, and age. Legend: Np = number of positive individuals, P = prevalence, Tn = total number.

Tab. 5 and Fig. 19, 20, 21, and 22 show the results on the sampled matrices, depending on the sampling year.

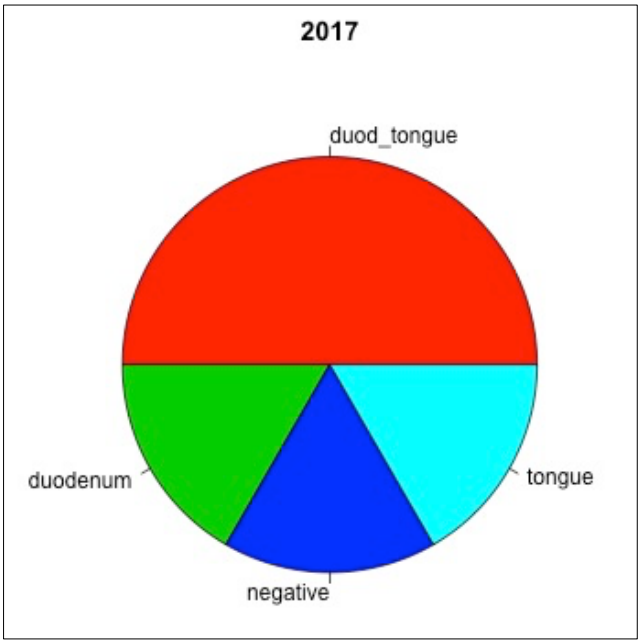


Fig. 19 – Pie chart with CPV-2 prevalence in the year 2017. On a total of 6 samples, 16.6% (1/6) were negative, 16.6% (1/6) were positive only in the tongue, 16.6% (1/6) were positive only in the duodenum, and 50% (3/6) were positive in both matrices.

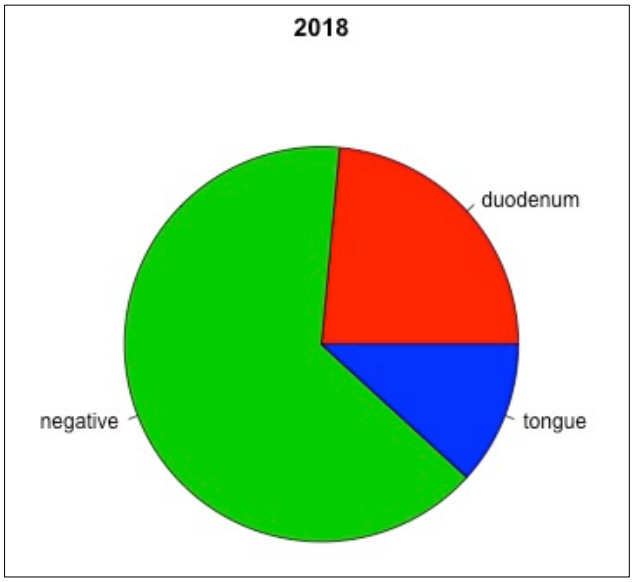


Fig. 20 – Pie chart with CPV-2 prevalence in the year 2018. Out of a total of 17 samples, 64.7% (11/17) were negative, 11.8% (2/17) were positive only in the tongue, 23.53% (4/17) were positive only in the duodenum.

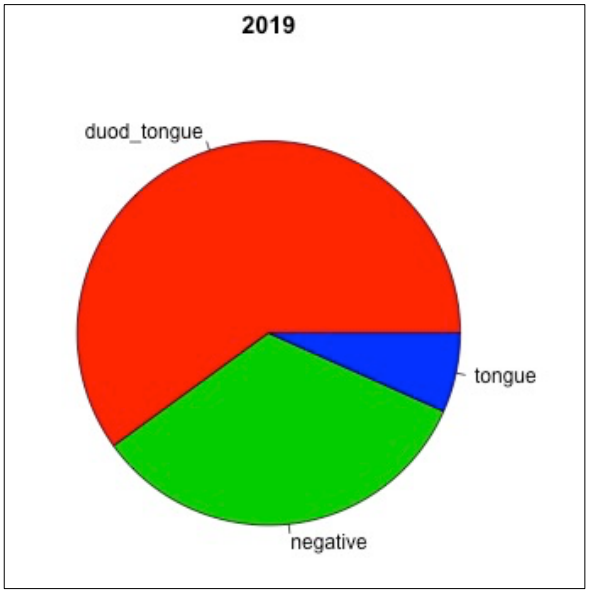


Fig. 21 – Pie chart with CPV-2 prevalence in the year 2019. Out of a total of 15 samples, 33.3% (5/15) were negative, 6.7% (1/15) were positive only in the tongue, and 60% (9/15) were positive in both matrices.

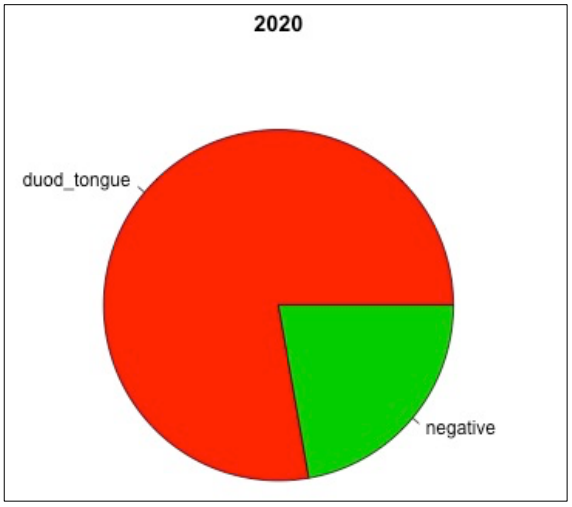


Fig. 22 – Pie chart with CPV-2 prevalence in the year 2020. Out of a total of 18 samples, 22.2% (4/18) were negative, 77.8% (14/18) were positive in both matrices.

Tab. 5 and Fig. 23, 24, and 25 show the results of the sampled matrices, according to the region of origin.

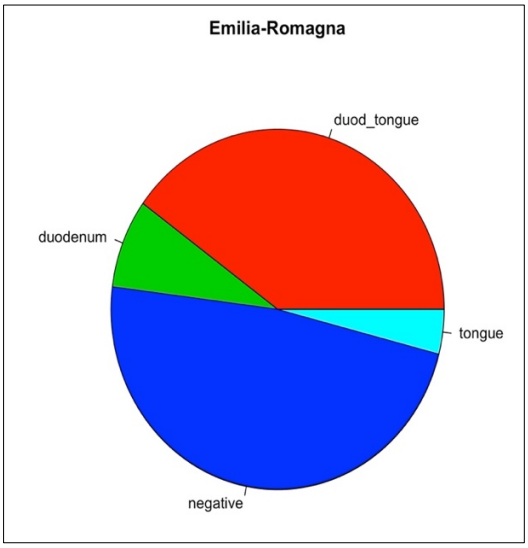


Fig. 23 – Pie chart with CPV-2 prevalence in Emilia-Romagna. On a total of 25 samples, 48% (12/25) were negative, 4% (1/25) were positive only in the tongue, 8% (2/25) were positive only in the duodenum, and 40% (10/25) were positive in both matrices.

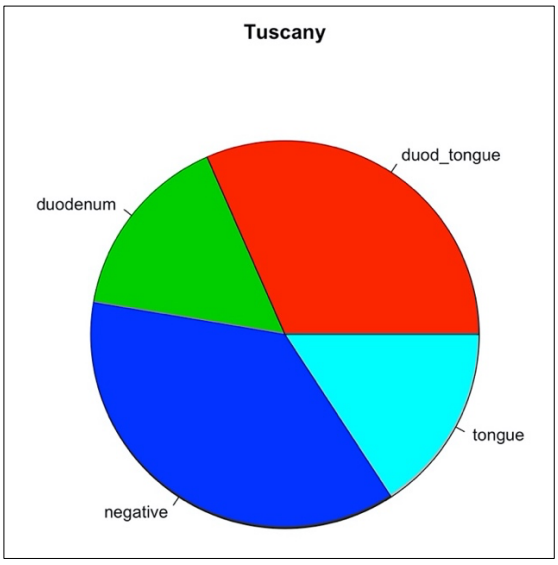


Fig. 24 – Pie chart with CPV-2 prevalence in Tuscany. Out of a total of 19 samples, 36.8% (7/19) were negative, 15.8% (3/19) were positive only in the tongue, 15.8% (3/19) were positive only in the duodenum, and 31.6% (6/19) were positive in both matrices.

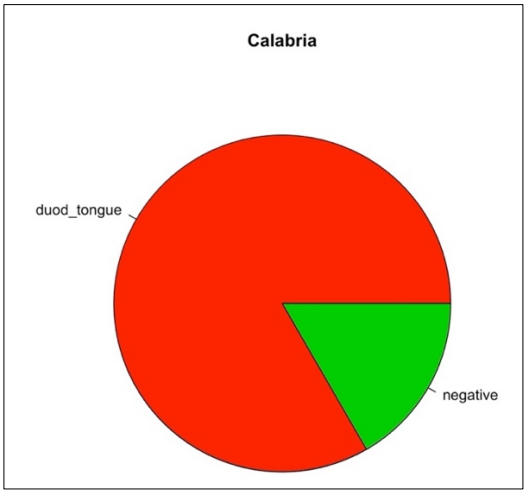


Fig. 25 – Pie chart with CPV-2 prevalence in Calabria. On the total of 12 samples, 16.7% (2/12) were negative and 83.3% (10/12) were positive in both matrices.

Tab. 5 and Fig. 26, and 27 show the results in the sampled matrices according to sex.

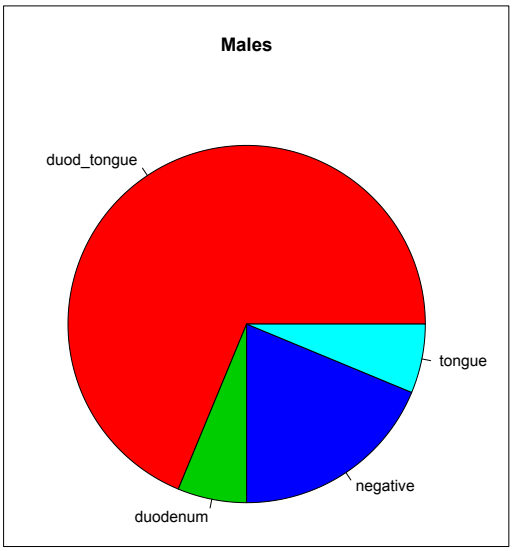


Fig. 26 – Pie chart with CPV-2 prevalence in males. Out of a total of 32 samples, 18.75% (6/32) were negative, 6.25% (2/32) were positive only in the tongue, 6.25% (2/32) were positive only in the duodenum, and 68.75% (22/32) were positive in both matrices.

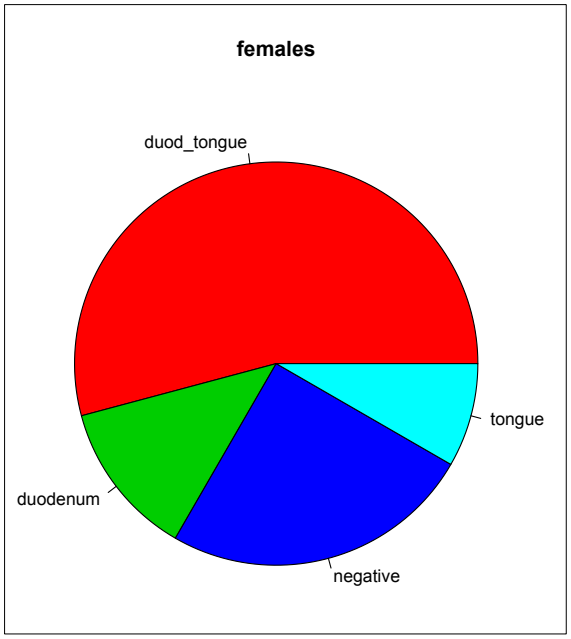


Fig. 27 – Pie chart with CPV-2 prevalence in female individuals. Out of a total of 24 samples, 25% (6/24) were negative, 8.33% (2/24) were positive only in the tongue, 12.5% (3/24) were positive only in the duodenum, and 54.17% (13/24) were positive in both matrices.

Tab. 5 and Fig. 28, 29, and 30 show the results in the sampled matrices according to the age group.

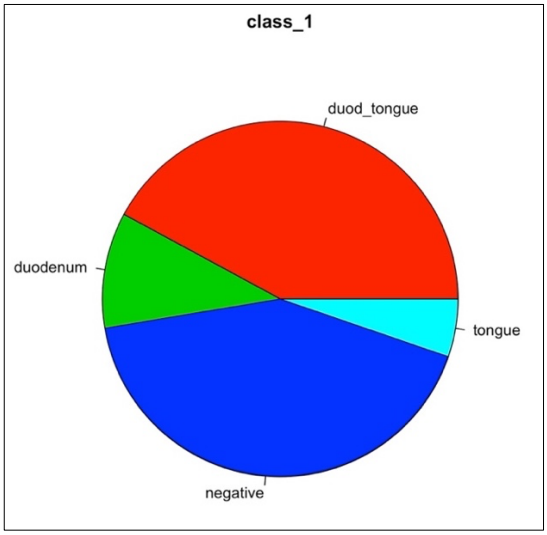


Fig. 28 – Pie chart with CPV-2 prevalence in class 1 subjects. Out of a total of 19 samples, 42.1% (8/19) were negative, 5.3% (1/19) were positive only in the tongue, 10.5% (2/19) were positive only in the duodenum, and 42, 1% (8/19) were positive in both matrices.

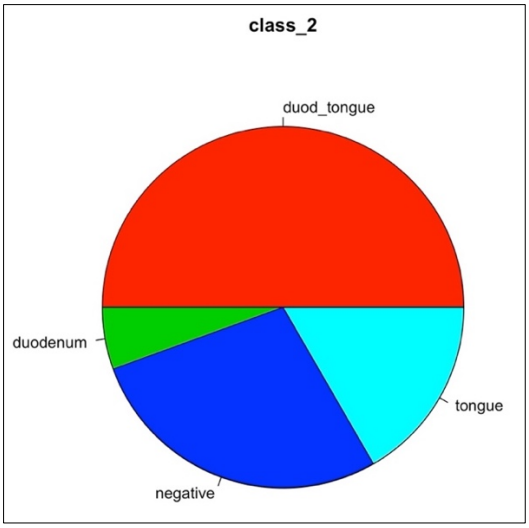


Fig. 29 – Pie chart with CPV-2 prevalence in class 2 subjects. Out of a total of 18 samples, 27.8% (5/18) were negative, 16.7% (3/18) were positive only in the tongue, 5.6% (1/18) were positive only in the duodenum, and 50% (9/18) were positive in both matrices.

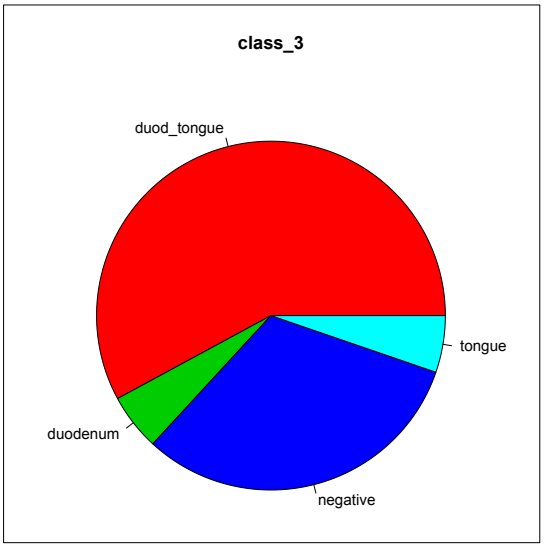


Fig. 30 – Pie chart with CPV-2 prevalence in class 3 subjects. Out of a total of 19 samples, 31.6% (6/19) were negative, 5.3% (1/19) were positive only in the tongue, 5.3% (1/19) were positive only in the duodenum, and 57.9% (11/19) were positive in both matrices.

3.3. DNA detection of Canine Adenovirus type 1 and type 2 (CAdV-1 and CAdV-2)

Ten of the 56 sampled wolves were positive for CAdV DNA with a prevalence of 17.9% (Tab. 5, Fig. 31-32). Of the positive 10 wolves, 7 were CAdV-1 DNA positive and 3 were CAdV-2 positive. 3 wolves tested positive only in the spleen matrix (all positive for CAdV-1), 3 only in the tongue matrix (all positive for CAdV-2), and 4 in both matrices (all positive for CAdV-1). The median amount of target DNA detected was 3.3x104 copies of DNA/µL of extract (range 50.4 –6.6x104).

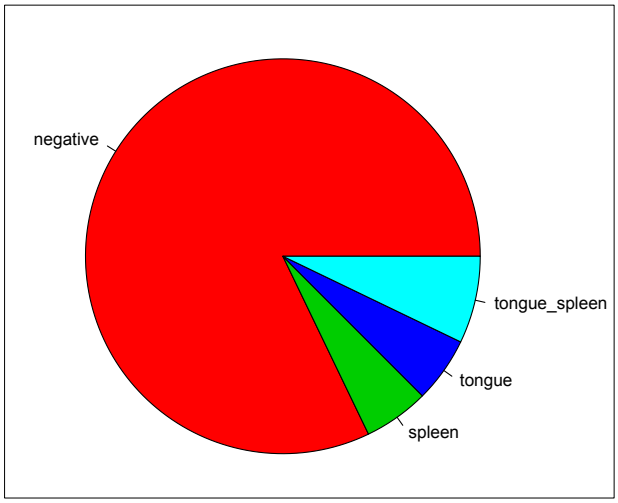


Fig. 31 – Pie chart with the prevalence of CAdV viral DNA found in the molecular investigation of Real-time PCR in the tongue and spleen samples.

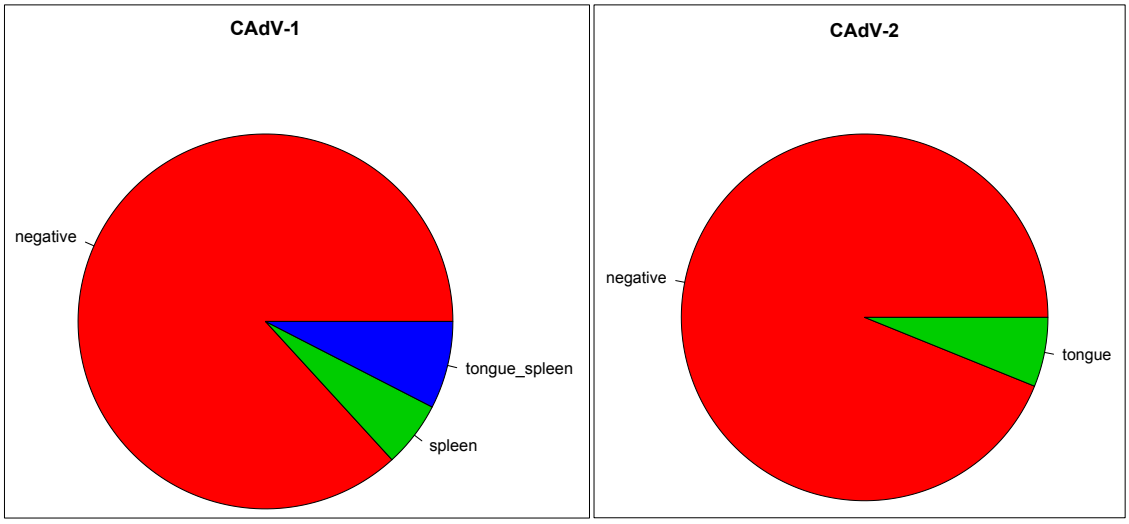


Fig. 32 – On the left, we can observe the prevalence of CAdV-1 viral DNA, with 86.8% (46/53) of negative subjects, 5.7% (3/53) of positive subjects only in the spleen matrix, and 7, 5% (4/53) of subjects positive in both matrices used. On the right, we can observe the prevalence of CAdV-2 viral DNA, with 93.9% of negative subjects and 6.1% of positive subjects only in the tongue matrix.

Finally, the prevalence was higher in class 2 represented by sub-adults with 57.1% (4/7), in class 1 with 28.6% (2/7), and 14.3% in class 3 (1/7), Fig. 33; Tab. 5.

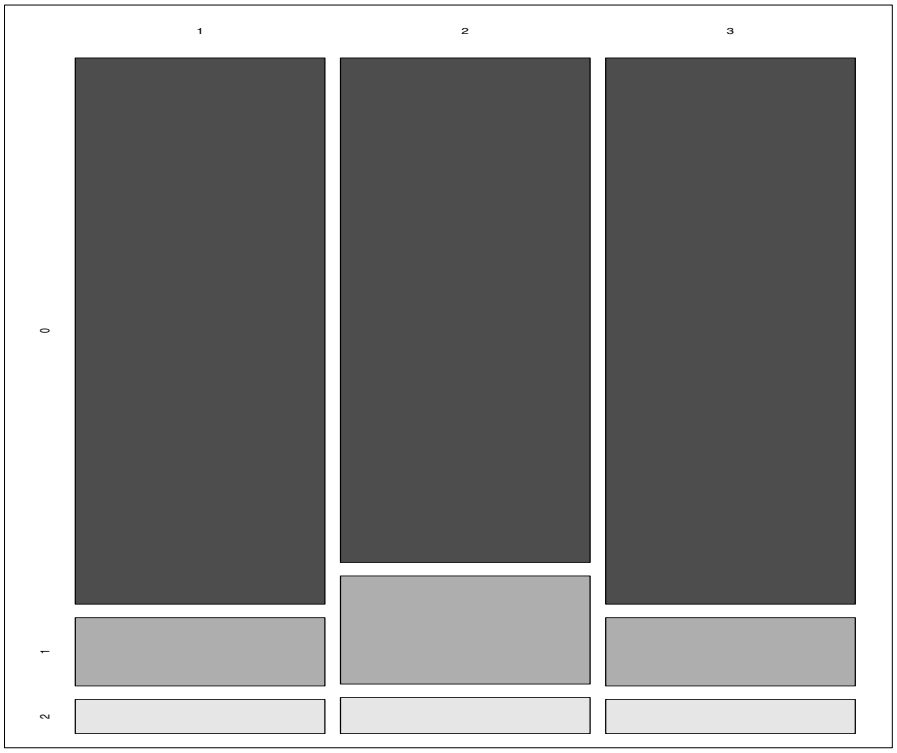


Fig. 33 – Mosaic plot of the prevalence of CAdV-1 and 2 divided by the three age groups.
X = CLASS_1, CLASS_2, CLASS_3
Y = 0: negative, 1: positive CAdV-1, 2: positive CAdV-2

CAdV DNA was detected with the highest prevalence in 2020 with 40% (4/10) of positive subjects, followed by 2018 with 30% (3/10) of positive subjects, then 2019 with 20% (2/10), and finally 2017 with 10% (1/10) of positive subjects (Fig. 34, Tab. 5).

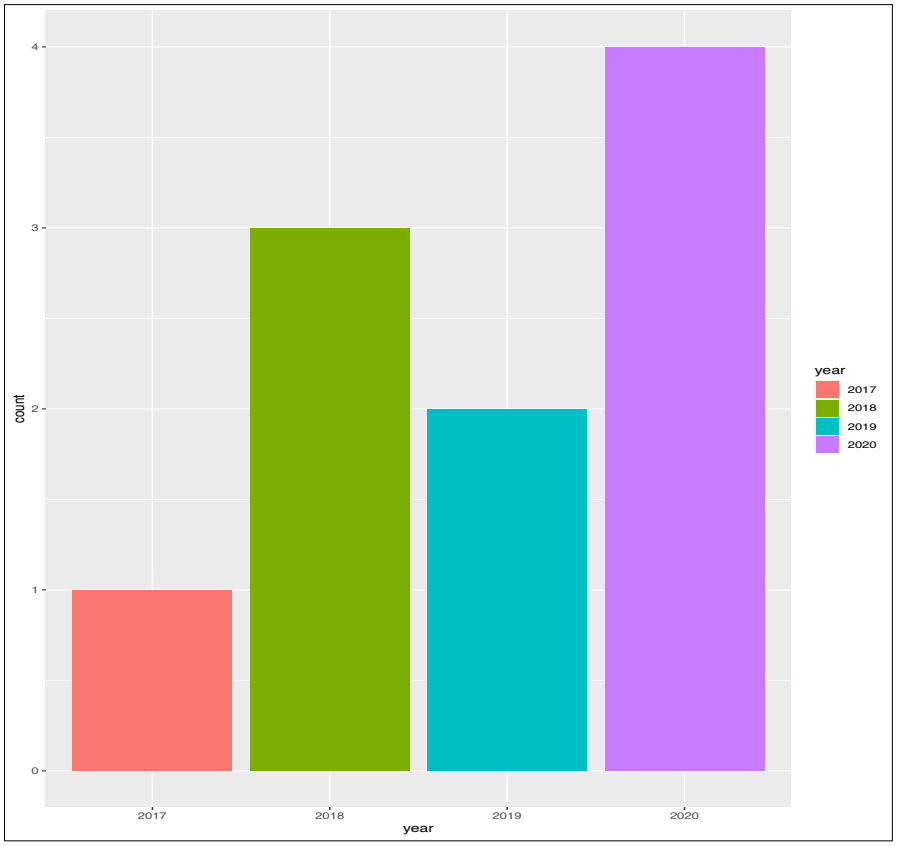


Fig. 34 – Bar plot of the annual distribution of CAdV positivity.

CAdV-1 DNA was detected in all 4 years: in 2017 in 14.3% (1/7) of subjects, in 2018 in 14.3% (1/7) of subjects, in 2019 in 14.3% (1/7) of the subjects, and finally in 2020 in 57.1% (4/7) of the subjects. CAdV-2 DNA was detected in 2 years: in 2018 in 66.7% (2/3) of subjects and in 2019 in 33.3% (1/3) of subjects. Tab. 5 and Fig. 35, 36, 37, and 38 show the results divided by subtype 1 and 2, according to the sampling year and the positive matrices.

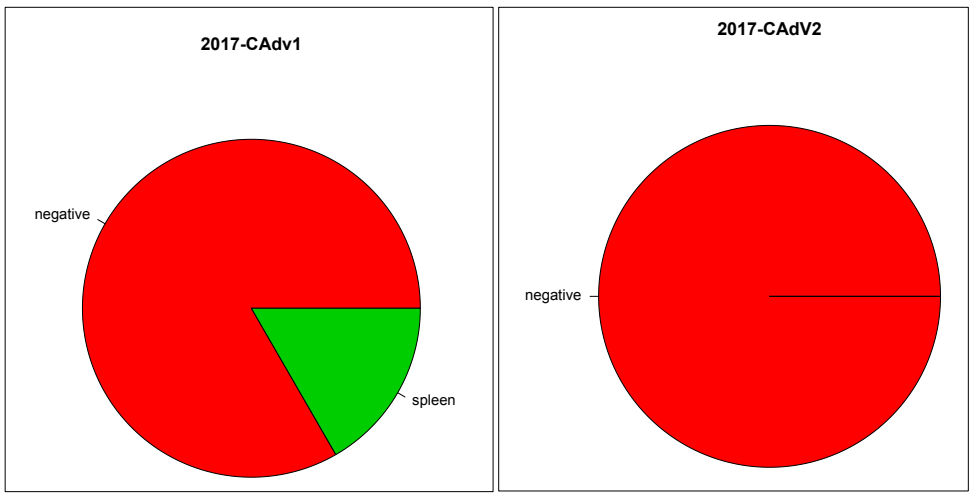


Fig. 35 – On the left, we can see the prevalence of CAdV-1 in 2017 with 83.3% (5/6) of negativity and 16.7% (1/6) of positivity in the spleen matrix. On the right, we can see the total negativity of CAdV-2 infections in the year 2017.

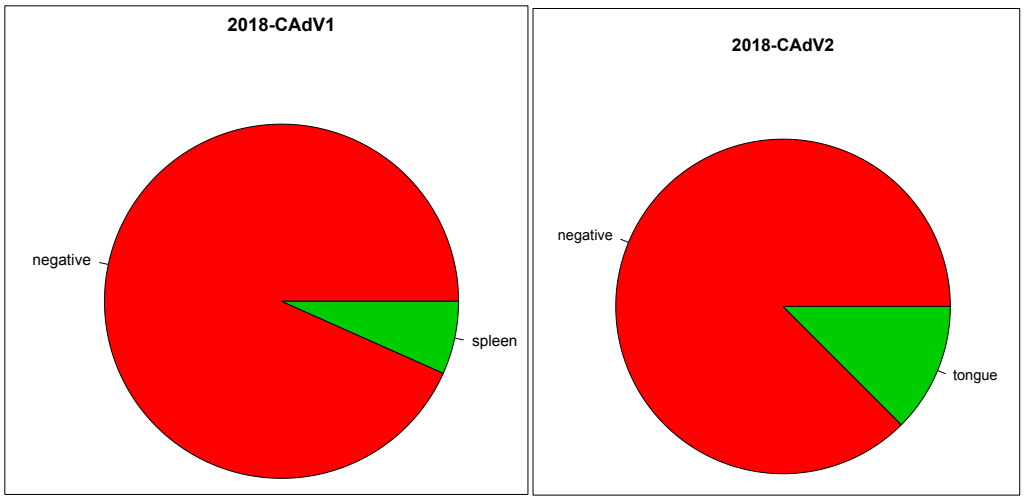


Fig. 36 - On the left, we can see CAdV-1 prevalence in 2018 with 93.3% (14/15) of negativity and 6.7% (1/15) of positivity in the spleen matrix. On the right, we can see CAdV-2 prevalence in 2018 with 87.5% (14/16) of negativity and 12.5% (2/16) of positivity in the tongue matrix.

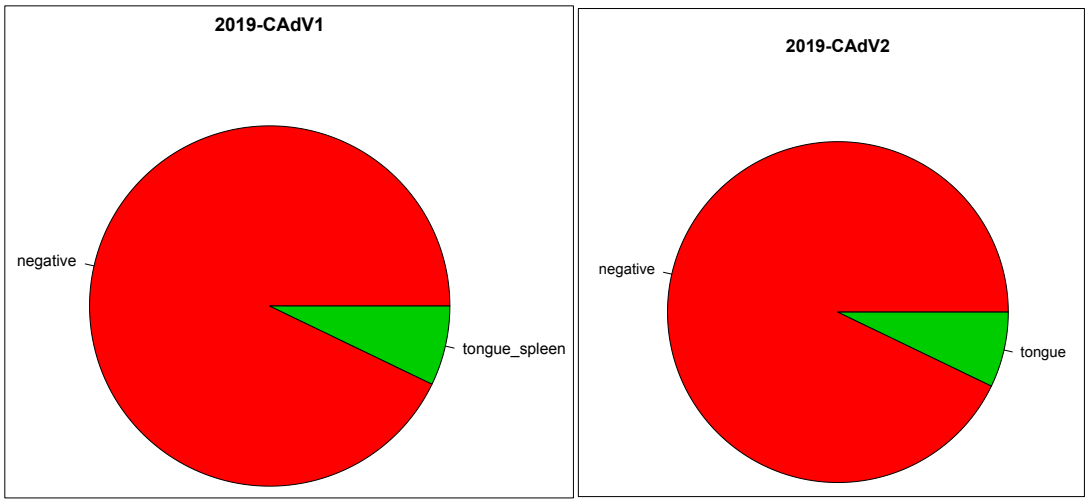


Fig. 37 - On the left, we can see CAdV-1 prevalence in 2019 with 92.9% (13/14) of negativity and 7.1% (1/14) of positivity in the spleen and tongue matrices. On the right, we can see CAdV-2 prevalence in 2019 with 92.9% (13/14) of negativity and 7.1% (1/14) of positivity in the tongue matrix.

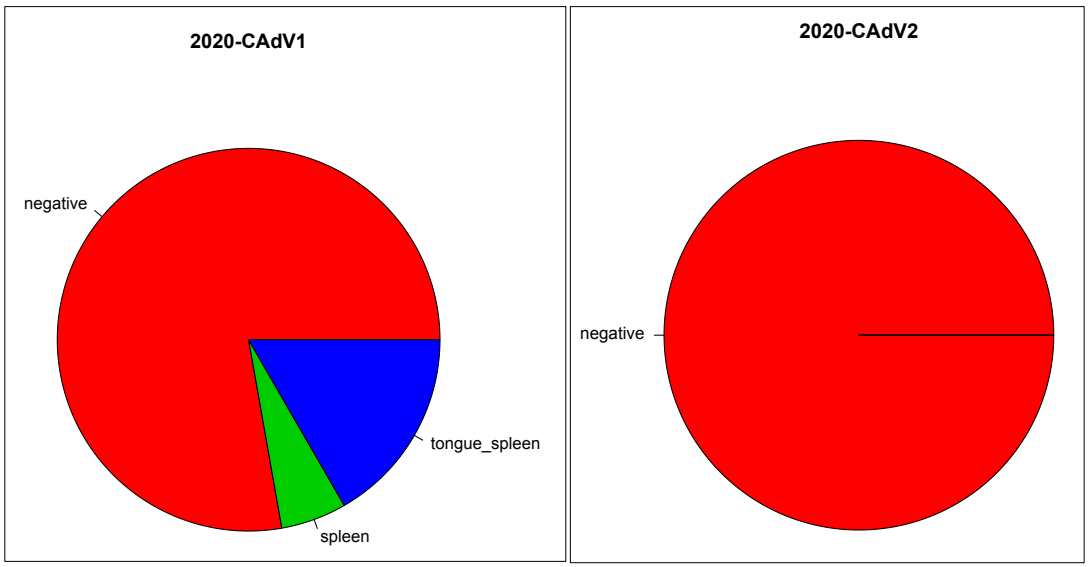


Fig. 38 - On the left, we can see CAdV-1 prevalence in the year 2020 with 77.8% (14/18) of negativity, 5.6% (1/18) of positivity in the spleen matrix, and 16.7% (3/18) of positivity in both the spleen and tongue matrices. On the right, we can see the total negativity of CAdV-2 infections in the year 2020.

In Emilia-Romagna, CAdV DNA was found with a prevalence of 40% (4/10), in Tuscany of 30% (3/10), and in Calabria of 30% (3/10) (Fig. 39, Tab. 5).

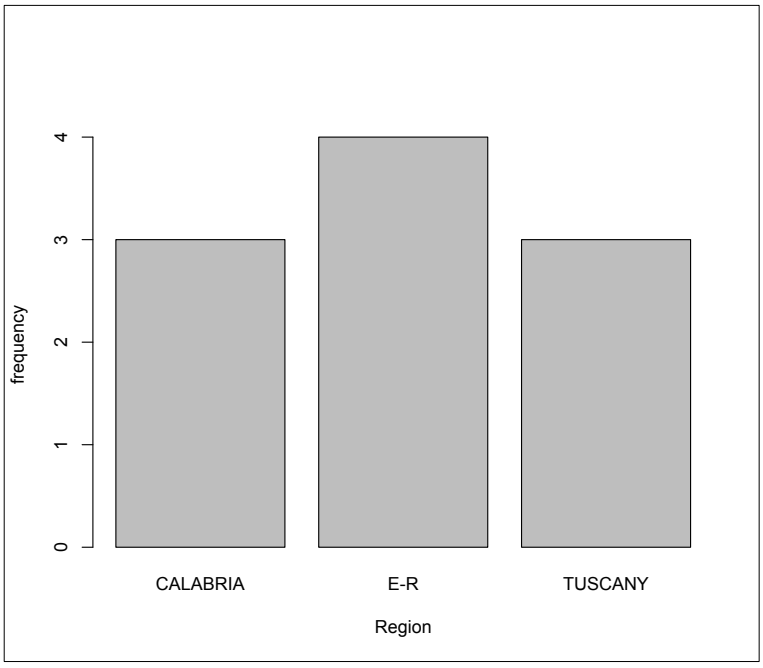


Fig. 39 – Bar plot with CAdV prevalence divided by sampling regions.

CAdV was identified in 16% (4/25) of wolves sampled in Emilia-Romagna, 15.8% (3/19) of those sampled in Tuscany, and 25% (3/12) of those sampled in Calabria. Tab. 5 and Fig. 40,

41, and 42 show the results according to the region of origin and the matrices sampled, divided between CAdV-1 and CAdV-2.

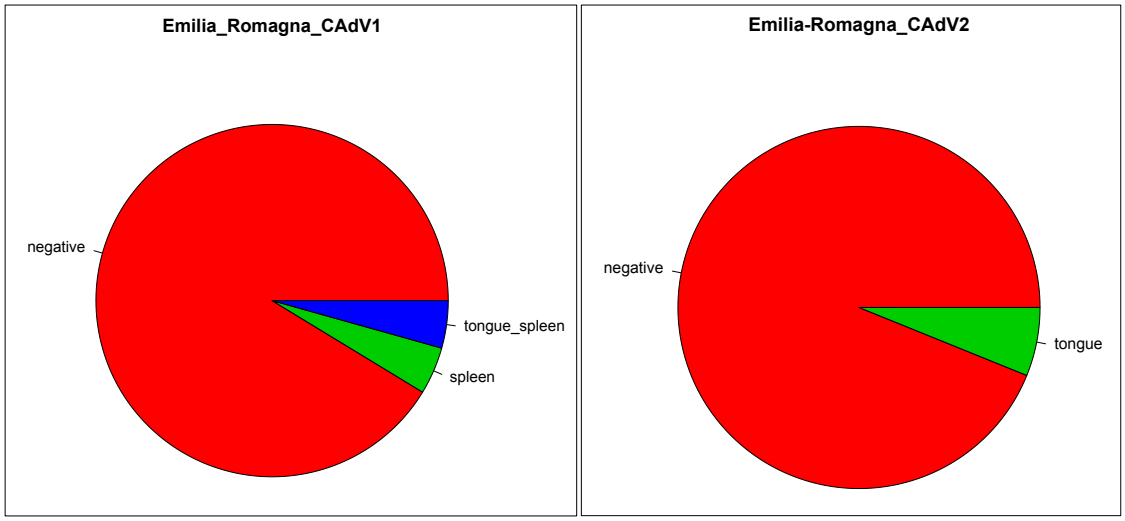


Fig. 40 – Pie chart with the prevalence of CAdV-1 on the left and of CAdV-2 on the right in Emilia-Romagna in spleen and tongue samples. On the left, we can observe the prevalence of CAdV-1DNA with 91.3% (21/23) of negative subjects, 4.3% (1/23) of subjects positive only in the spleen matrix, and 4.3% (1/23) of subjects positive in both matrices. On the right, we can observe the prevalence of CAdV-2 DNA, with 91.3% (21/23) of negative subjects and 8.7% (2/23) of subjects positive only in the tongue matrix.

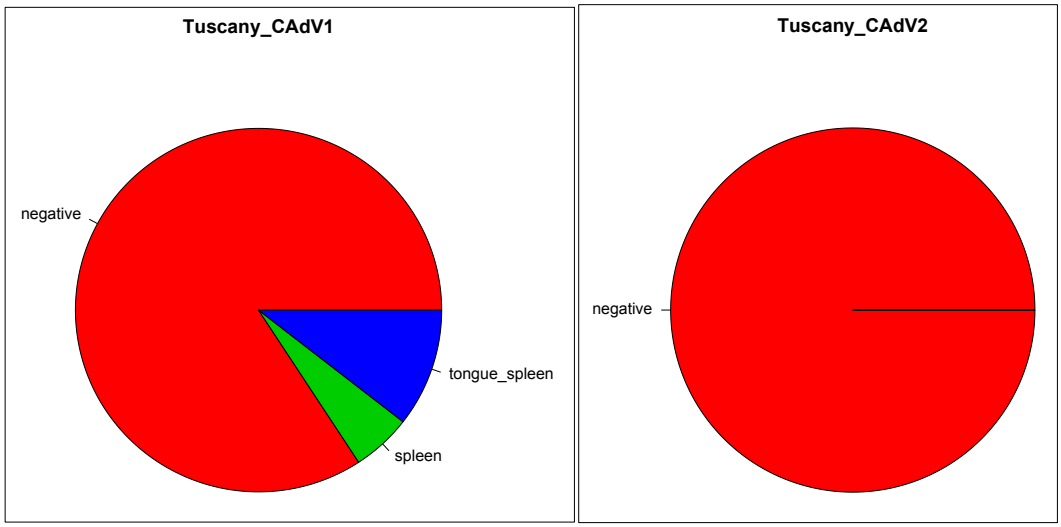


Fig. 41 – Pie chart with the prevalence of CAdV-1 on the left and of CAdV-2 on the right in Tuscany in spleen and tongue samples. On the left, we can observe the prevalence of CAdV-1 DNA with 84.2% (16/19) of negative subjects, 5.3% (1/19) of subjects positive only in the spleen matrix, and 10.5 % (2/19) of subjects positive in both matrices. On the right, we can see the absence of positive subjects for CAdV-2 in Tuscany.

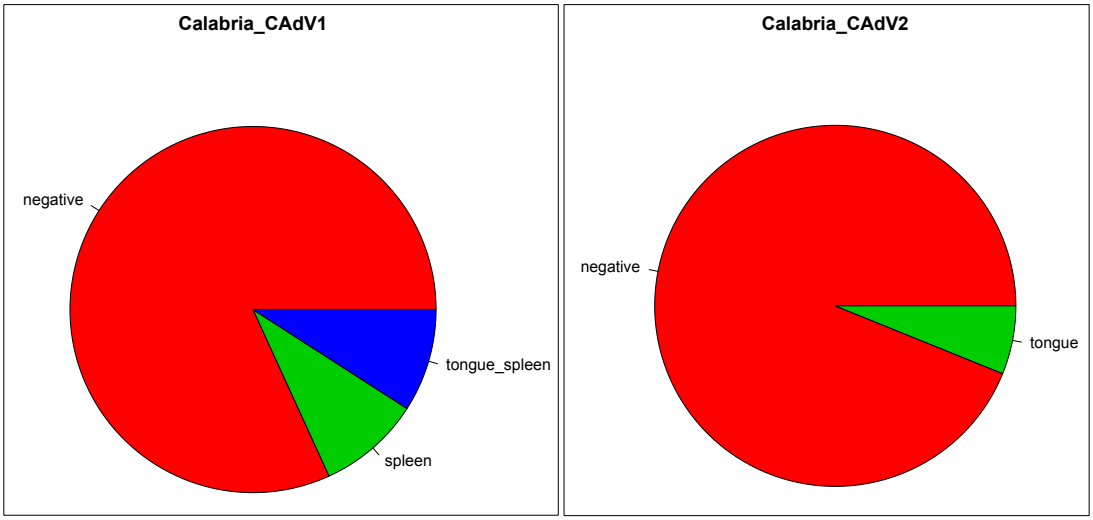


Fig. 42 – Pie chart with the prevalence of CAdV-1 on the left and of CAdV-2 on the right in Calabria in spleen and tongue samples. On the left, we can observe the prevalence of CAdV-1 DNA with 81.8% (9/11) of negative subjects, 9.1% (1/11) of subjects positive only in the spleen matrix, and 9.1% % (1/11) of subjects positive in both matrices. On the right, we can observe the prevalence of CAdV-2 DNA with 90% (9/10) of negative subjects and 10% (1/10) of subjects positive only in the tongue matrix.

Tab. 5 and Fig. 43 and 44 show the results of CAdV-1 and CAdV-2 prevalence, in function of sex and the matrices sampled.

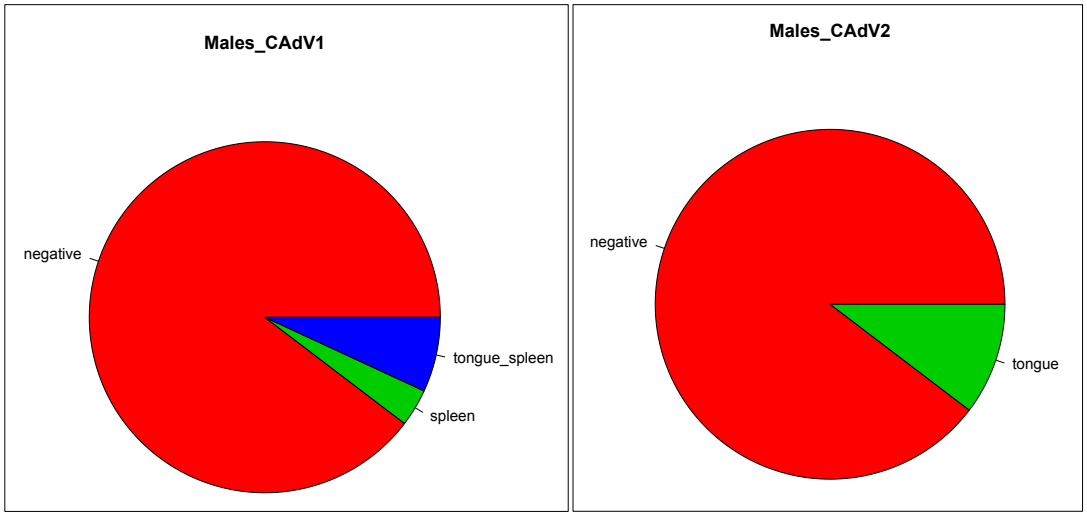


Fig. 43 – Pie chart with the prevalence of CAdV-1 on the left and of CAdV-2 on the right in male individuals in spleen and tongue samples. On the left, we can observe the prevalence of CAdV-1 DNA with 89.7% (26/29) of negative subjects, 3.4% (1/29) of subjects positive in only the spleen matrix, and 6.9 % (2/29) of subjects positive in both matrices. On the right, we can observe the prevalence of CAdV-2 DNA with 89.7% (26/29) of negative subjects and 10.3% (3/29) of subjects positive only in the tongue matrix.

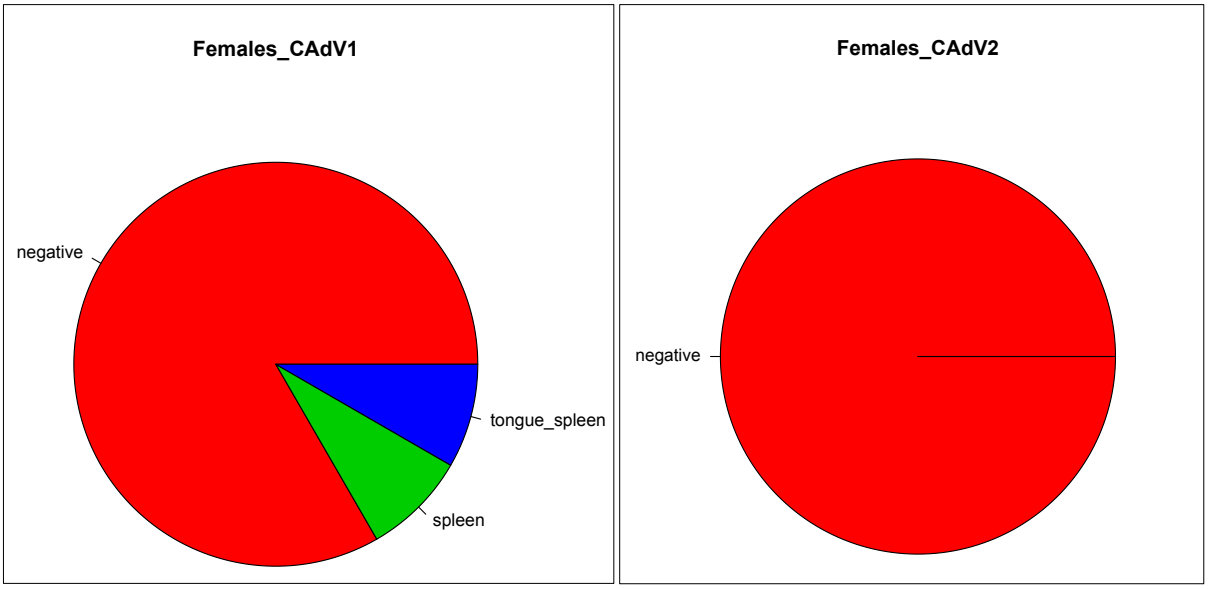


Fig. 44 – Pie chart showing the prevalence of CAdV-1 on the left and of CAdV-2 on the right in female individuals in spleen and tongue samples. On the left, we can observe the prevalence of CAdV-1 DNA with 83.3% (20/24) of negative subjects, 8.3% (2/24) of subjects positive only in the spleen matrix, and 8, 3% (2/24) of subjects positive in both matrices. On the right, we can observe the absence of CAdV-2 positive female individuals.

Tab. 5 and Fig. 45, 46, and 47 show the results of CAdV-1 and CAdV-2 prevalence, according to the age groups and the matrices sampled.

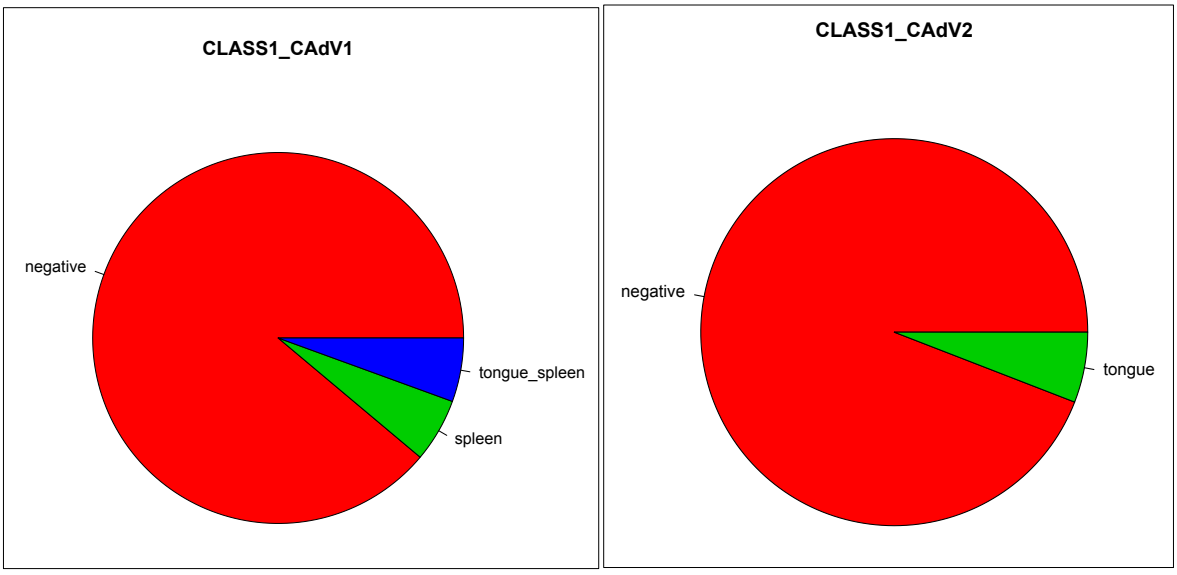


Fig. 45 – Pie chart with the prevalence of CAdV-1 on the left and of CAdV-2 on the right in class 1 subjects (<1 year) in spleen and tongue samples. On the left, we can observe the prevalence of CAdV-1 DNA with 88.9% (16/18) of negative subjects, 5.6% (1/18) of subjects positive only in the spleen matrix, and 5.6% (1/18) of subjects positive in both matrices. On the right, we can observe the prevalence of CAdV-2 DNA with 94.1% (16/17) of negative subjects and 5.9% (1/17) of subjects positive only in the tongue matrix.

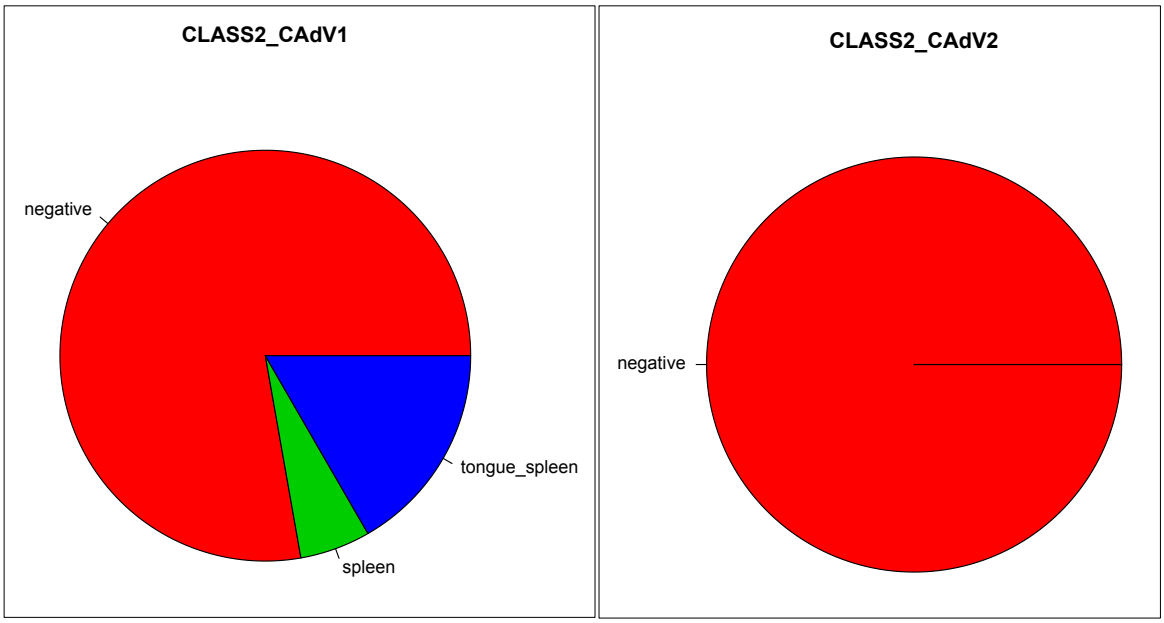


Fig. 46 – Pie chart with the prevalence of CAdV-1 on the left and of CAdV-2 on the right in class 2 subjects (> 1 year <2 years) in spleen and tongue samples. On the left, we can see the prevalence of CAdV-1 DNA with 77.8% (14/18) of negative subjects, 5.6% (1/18) of subjects positive only in the spleen matrix, and 16.6% (3/18) positive in both matrices. On the right, we can observe the absence of CAdV-2 positivity among class 2 subjects.

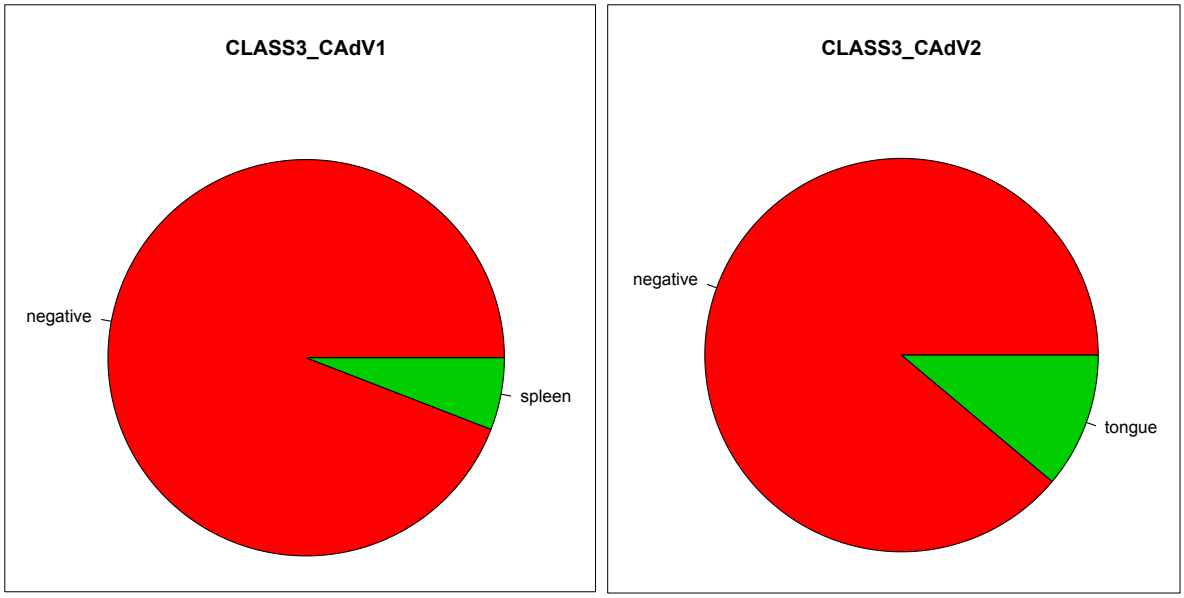


Fig. 47 – Pie chart with the prevalence of CAdV-1 on the left and of CAdV-2 on the right in class 3 subjects (> 2 years) in spleen and tongue samples. On the left, we can observe the prevalence of CAdV-1 DNA with 94.1% (16/17) of negative subjects and 5.9% (1/17) of subjects positive only in the spleen matrix. On the right, we can observe the prevalence of CAdV-2 DNA with 88.9% (16/18) of negative subjects and 11.1% (2/18) of subjects positive only in the tongue matrix.

3.4. Presence of single and multiple infections

Of the 56 wolves sampled, 8 (14.29%) wolves tested negative for all the etiological agents investigated. For the other 48 positives, 87.5% (42/48) of the wolves tested had single viral infections, while the remaining 12.5% (6/48) were co-infected with more than one of the viruses investigated (Fig. 48).

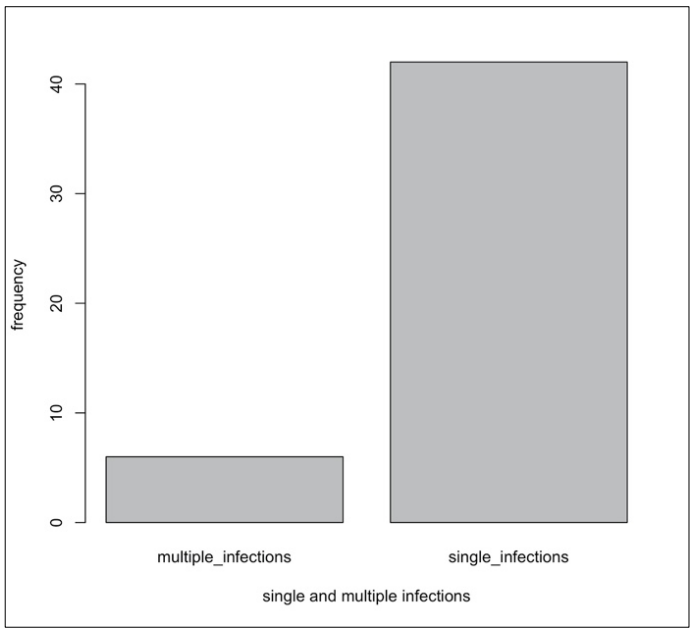


Fig. 48 – Bar plot with the distribution of the sampled population according to the type of viral infection.

90.48% (38/42) of single infections were caused by CPV-2, while 9.52% (4/42) by CAdV 1-2. In particular, each CAdV type had a frequency of 4.76% (2/42), (Fig. 49).

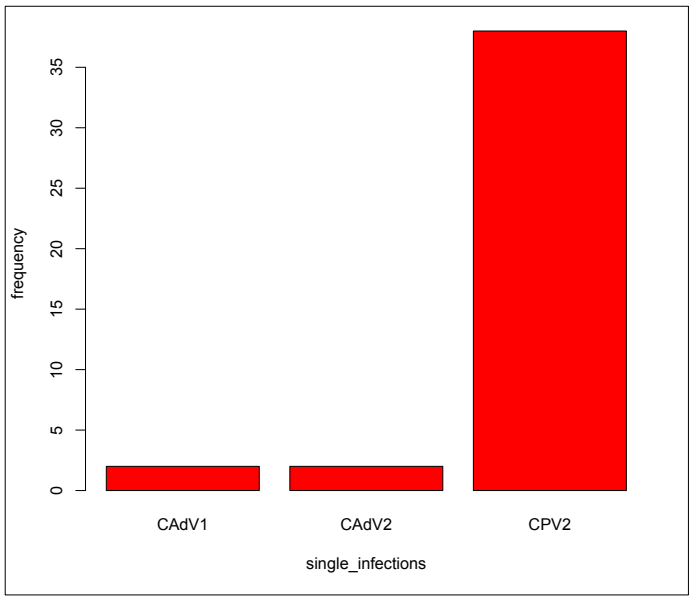


Fig. 49 – Bar plot with the graphic representation of the single viral infections found in the sampled population.

In multiple infections, Canine Parvovirus was always present, but with different associations: CAdV-1 was present in 83.33% (5/6) of cases and CAdV-2 in 16.67% (1/6), (Fig. 50).

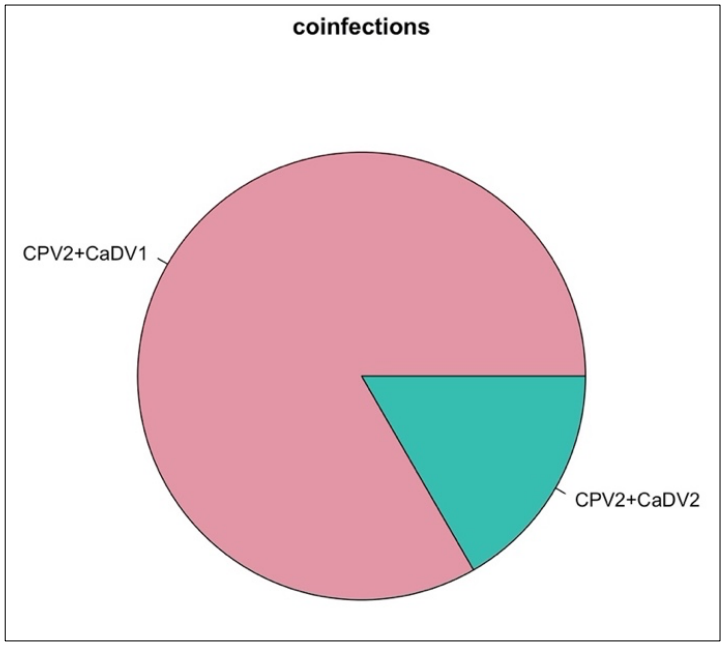


Fig. 50 – Pie chart with the representation of multiple viral infections found in the sampled population.

3.5. RNA detection of Canine Distempervirus (CDV) and Canine Enteric Coronavirus (CoCV)

None of the 56 wolves sampled in this study tested positive for viral RNA attributable to Canine Distemper virus – CDV – and Canine Enteric Coronavirus – CoCV. For these reasons, it was not possible to carry out plots, correlations, and statistical analyzes.

Tab. 5 is reported once more below, including the summary of all the results broken down by sampling year, regions, sex, and age classes. It shows the negativity of all subjects to these two etiological agents.

	CPV-2		CAdV-1		CAdV-2		CoCV-CDV		Tn
	N_p	P	Np	P	Np	P	Np	P	
Year of death									
2017	5	83,3 %	1	16,7 %	0	0%	0	0%	6
2018	15	88,2 %	1	5,9 %	2	11,8 %	0	0%	17
2019	10	66,7 %	1	6,7 %	1	6,7%	0	0%	15
2020	14	77,8 %	4	22,2 %	0	0%	0	0%	18
Region of origin									
Emilia Romagna	20	80%	2	8%	1	4%	0	0%	25
Tuscany	13	68,4 %	3	15,8 %	0	0%	0	0%	19
Calabria	11	91,7 %	2	16,7 %	2	16,7 %	0	0%	12
Sex									
Males	26	81,2 %	4	12,5 %	3	9,4%	0	0%	32
Females	18	75%	3	12,5 %	0	0%	0	0%	24
Age									
CLASS_1	14	73,7 %	2	10,5 %	1	5,3%	0	0%	19
CLASS_2	17	94,4 %	3	16,7 %	1	5,5%	0	0%	18
CLASS_3	13	68,4 %	2	10,5 %	1	5,3%	0	0%	19

Tab. 5 – Results of molecular investigations according to the year of death, region of origin, sex, and age. Legend: Np = number of positive individuals, P = prevalence, Tn = total number.

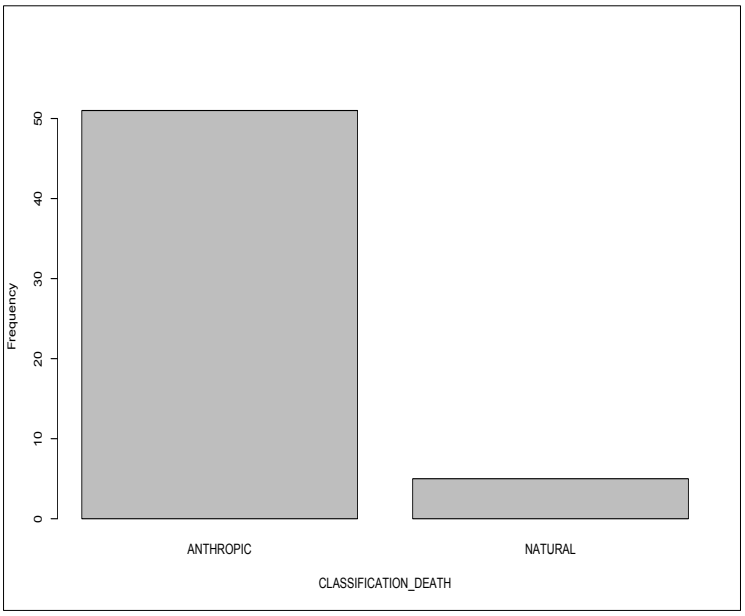
3.6. Necropsy results

The causes of mortality were grouped into two main categories: anthropic and natural, defined briefly below.

Anthropic: it included vehicle collisions (caused by cars and trains) and illegal killings (i.e. poisoning and shooting);

Natural: it included health-related causes of mortality (i.e. presence of disease and/or starvation) and intraspecific competition.

The causes of mortality in the population studied were mainly caused by anthropogenic activities with a percentage of 91,1% (51/56), while natural causes constituted the 8,9% (5/56) (Fig. 51). More information relating to each category is detailed below.



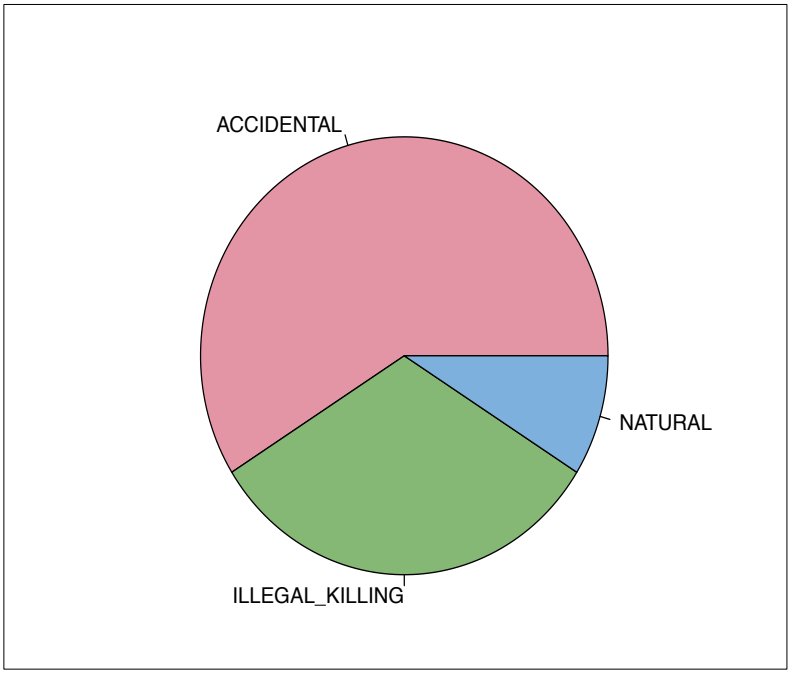


Fig. 51 - Above, there is a bar plot with the two main causes of mortality, divided into "anthropic" (91,1%) and "natural" (8,9%), and the relative frequencies. Below, a pie chart with the classification of mortality dividing the anthropic kind into "accidental" (n = 33/51 - 64,7%) and "illegal killing" (n = 18/51 - 35,3%).

3.6.1. Anthropic causes of wolf mortality

The most frequent cause of anthropogenic death was linked to traumatic injuries associated with vehicle collisions. Specifically, of the total, 55,4% (n = 31) was caused by cars, while 3,6% (n = 2) from trains.

The second most common category was also anthropogenic, as represented by illegal killing events 32,1% (n = 18). Particularly, 19,6% of the total died from poisoning (n = 11), followed by 12,5% (n = 7) killed by gunshots.

3.6.2. Natural causes of wolf mortality

Compared to anthropogenic causes of death, natural causes were reported less often. In fact, only 8,9% (n = 5) of the 56 subjects examined was classified as natural mortality. Specifically, mortality from severe cachexia and widespread sarcoptic mange was found in 2 subjects (n = 2 – 3,6%), whilst mortality caused by intraspecific conflicts was present in the other 3 subjects (n = 3 – 5,4%).

3.6.3. General physical status

At the general inspection that characterizes the early stages of the necropsy, there was a clear prevalence of individuals in good and excellent physical condition, with body and muscle development appropriate to the subspecies, estimated age, and sex.

The subjects with good/excellent health conditions – denominated “good status” – represented 71.4% (n = 40) of the total of 56 subjects examined, while the subjects in poor physical condition, emaciated, cachectic, and with sarcoptic mange – denominated “bad status” – accounted for 28.6% (n = 16) of the total collected (Fig. 52).

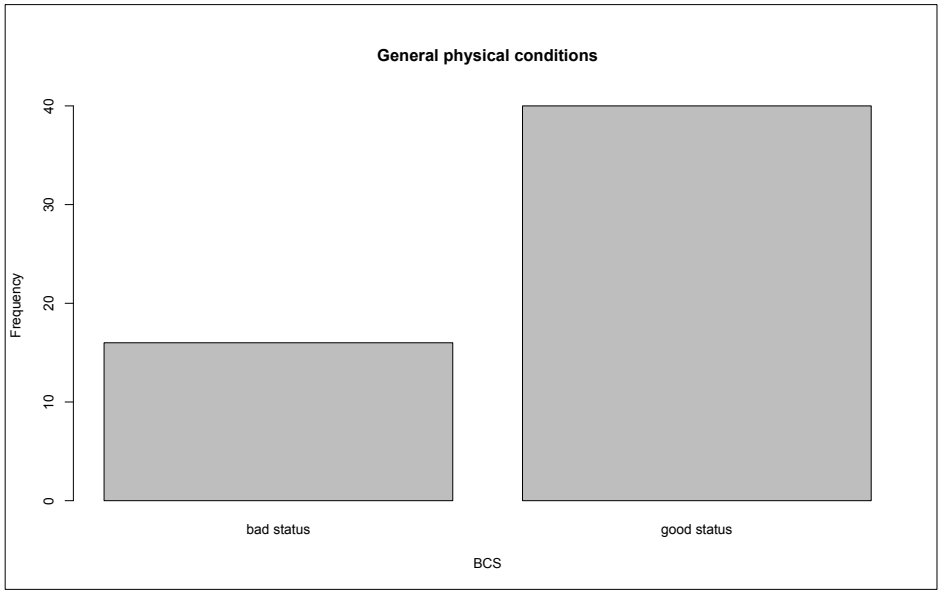


Fig. 52 – Bar plot showing the two main physical conditions of the sampled population.

Within the "bad status" group there were subdivisions (Fig. 53). One subject (1/56, 1.8%) presented poor condition, was severely cachectic, and had a fractured mandible. Seven subjects (7/56, 12.5%) presented poor condition, were cachectic, and had sarcoptic mange spread over the whole body. Eight subjects (8/56, 14.3%) presented poor nutritional conditions without associated contributing causes.

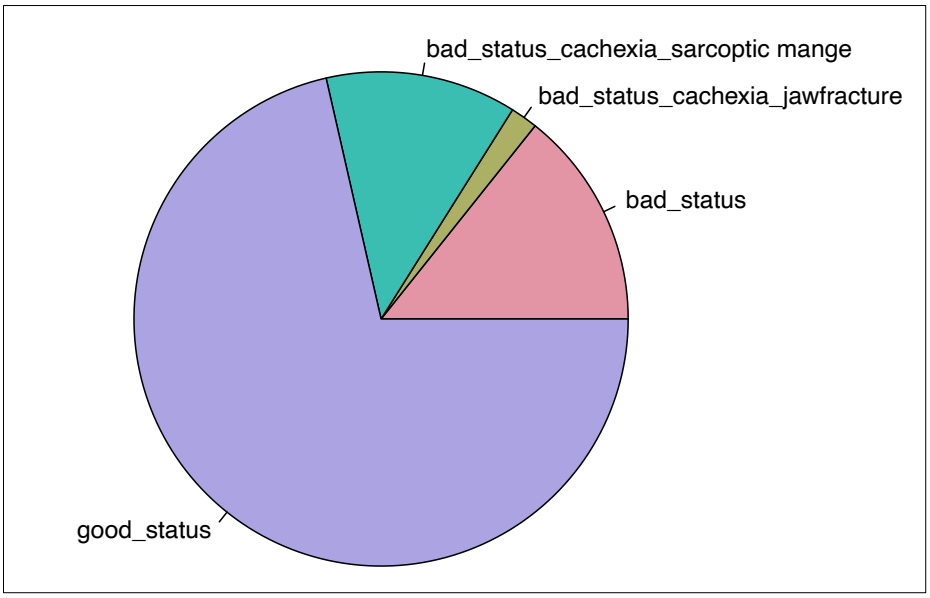


Fig. 53 – Pie chart with the "good status" group and the detailed subdivision of the "bad status" group.

Positivity to CPV-2 and CAdV 1 and 2 was higher in wolves who had a good state of nutrition. This data indirectly demonstrates that the wolves studied did not die because of the etiological agents and they were most likely asymptomatic positive subjects.

Causes of death of the sampled population

The main cause of death was car accident (31/56, 55.4%), followed by poisoning by anticoagulants (11/56, 19.6%), killing by firearm (7/56, 12.5%), intraspecific aggression by another wolf or dog (3/56, 5.4%), and finally cachexia and train collision in equal measure (2/56, 3.6 %) (Fig. 54).

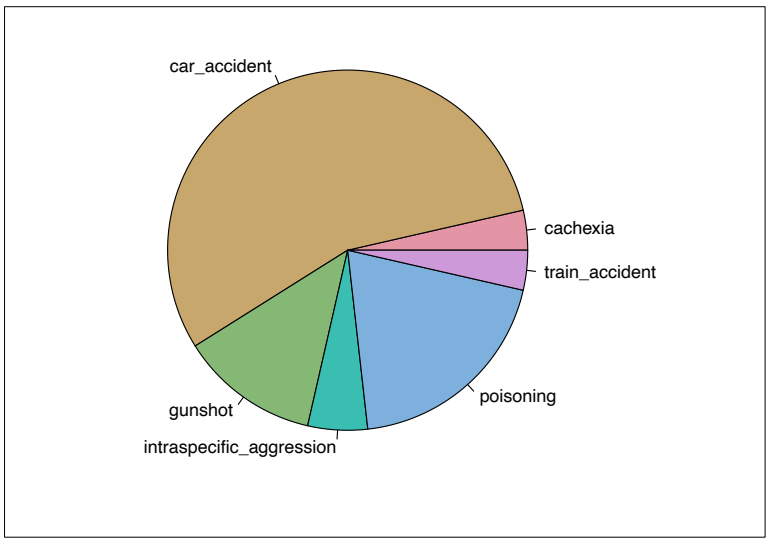


Fig. 54 – Pie chart with all the causes of death observed in the study population.

Of the 33 wolves killed by cars or trains, as many as 27 (81.8%) were in good physical condition. Of the 11 poisoned, 6 (54.5%) were in good physical condition, while 5 (45.5%) were in poor physical condition. Of the 7 wolves killed by gunfire, 4 (57.1%) were in good physical condition, while 3 (42.9%) were defied.

All three subjects killed in an attack by wolves or dogs were in good physical condition. Between the two wolves who died of cachexia, there was a wolf with a fractured jaw that prevented him from drinking and eating, with a slow and progressive starvation until death. The other subject was severely undernourished, probably because of the advanced sarcoptic mange covering all its body.

From these results it can be inferred that the causes of death were not related to CPV-2 and CAdV 1-2 positivity. In fact, both among the 44 wolves positive for CPV-2 and the 10 positive for CAdV1-2, only 2 died from severe cachexia, in one case attributable to the fracture of the jaw and in the other to the sarcoptic mange generalized on the whole body, a condition that prevented the subject from being an effective predator and that caused high expenditure of energy reserves.

CHAPTER FOUR: DISCUSSION

4 - DISCUSSION

The aim of the study was to evaluate the presence and spread in the sampled Italian wolf population of three DNA viruses – i.e. Canine Parvovirus type 2 (CPV-2), Canine Adenovirus type 1 (CAdV-1), and Canine Adenovirus type 2 (CAdV-2) – and two RNA viruses – Canine Enteric Coronavirus (CCoV) and Canine Distemper virus (CDV). These viruses are of considerable importance as they can infect and cause disease in both domestic and wild canids. To reach this goal, we tested the duodenum, spleen, tongue, and lungs of the sampled population represented by 56 wolves who died in Emilia-Romagna, Tuscany, and Calabria between 2017 and 2020 (Musto, 2020). The presence of viral DNA and RNA in tissue samples was investigated by molecular methods of real-time PCR and RT-PCR. The results obtained were then related to: (i) year of sampling, (ii) region of origin, (iii) sex, (iv) age, (v) season, (vi) genetic identification of the species, (vii) nutritional conditions, (viii) cause of death and (ix) matrices examined. In addition, the presence or absence of co-infections was also evaluated.

The results of molecular real-time PCR investigations showed that the virus with the highest prevalence in the wolf population studied was Canine Parvovirus – CPV2, found in 78.6% of the subjects (44/56). The prevalence of Canine Adenovirus – CAdV was 17.9% (10/56), particularly Adenovirus type 1 (12.5%, 7/56) and Adenovirus type 2 (5.4%, 3/56).

In Italy, three studies were conducted that assessed CPV-2 spread in Italian wolves and they showed a lower prevalence than the one found in this study (Martinello *et al*., 1997; Molnar *et al*., 2014; Ambrogi *et al*., 2019). In the oldest study, dating back to 1997, conducted by Martinello *et al*. (1997) 115 wolf fecal samples from various parks in Emilia-Romagna and Tuscany were analyzed.

In the study by Martinello *et al.* (1997) the samples were analyzed using four methods: the ELISA test and the hemagglutination test to detect CPV-2 antigens, viral isolation and electron microscopy (TEM) to detect CPV-2 itself. The only positivity found came from 4 wolves of a park in Emilia-Romagna. Specifically, one sample tested positive with all three methods, two samples tested positive with the hemagglutination test and electron microscopy but not on the ELISA test, and finally one sample resulted positive on the ELISA test and electron microscopy but not with the hemagglutination test (Martinello *et al*., 1997).

The different prevalence of CPV-2 infections found in the study by Martinello *et al*. (1997) and this study may have various explanations, including the different sampling years, matrices used, and laboratory methods adopted. In fact, wolves reached their lowest population density peak in the 1970s, since then the recovery has been gradual (Bocedi and Bracchi, 2004), so, consequently, the study conducted in the late 1990s by Martinello *et al*. (1997) considered a wolf population with completely different characteristics from the present one.

Additionally, Martinello *et al.* (1997) used as matrices not organs but animal feces. Viral elimination via excreta persists only for a few weeks after infection (Greene and Decaro, 2012), therefore it can be assumed that some individuals who tested negative were in fact infected but did not eliminate the virus. Furthermore, the methods used by Martinello *et al.* (1997) were not of the molecular type and had different sensitivity and specificity values, e.g. the ELISA test shows the viral antigen in scats up to 7-10 days from the start of the elimination, while PCR detects the viral DNA in the scats up to several weeks from the start of the elimination (Greene and Decaro, 2012).

Another study was conducted by Molnar *et al*. (2014) in the Abruzzo, Lazio and Molise National Park, where 79 fecal samples were collected between 2006 and 2007 and were analyzed with real-time PCR to detect Canine Parvovirus (CPV-2) and other viruses. Twelve samples tested positive to CPV-2 with a prevalence of 15.2% (Molnar *et al*., 2014), therefore lower than what found in this study. It should be considered that the geographic area of the sampling of Molnar *et al*. (2014) does not include Emilia-Romagna, Tuscany, and Calabria. Moreover, also in this case fecal samples were analyzed in years prior to 2017-2020, hence having the same implications reported above.

In the study by Molnar *et al*. (2014) Canine Enteric Coronavirus (CCoV) and Canine Distemper virus (CDV) were also detected. CPV-2 and CCoV co-infections were found in 2 out of 79 samples, with a prevalence of 2.5%. In this thesis, the percentage of co-infections detected was 10.7% (6/56), therefore higher than in their study. However, co-infections in our study were CPV-2 and CAdV type 1 and 2.

The third study was conducted by Ambrogi *et al*. (2019) in two parks in Tuscany where 125 wolf fecal samples were collected between 2006 and 2007. These tested negative for Canine Parvovirus with PCR. Furthermore, molecular investigations with PCR or real-time PCR were also conducted on organic matrices of 9 wolves found dead. The results highlighted the negativity of the tissue samples for CDV, CCOV, and CAdV type 1 and type 2, however two wolves aged between 6 and 18 months tested positive for Canine Parvovirus with PCR (Ambrogi *et al*., 2019). In this case, the prevalence of CPV-2 infection found using molecular

methods on tissue samples was higher than the one highlighted on fecal samples, albeit lower than what highlighted in the study subject of this thesis.

The reasons may include the smaller number of individuals sampled (9 compared to 56) and the lower population density of wolves, since the samplings were performed more than ten years before the ones of this thesis. Furthermore, it must be considered that the epidemiological situation of CPV-2 in the wild population could have changed over the years and therefore the prevalence of infection could in fact have increased in more recent years.

In the study by Ambrogi *et al*. (2019) the wolves tested positive were young subjects, corresponding to the age groups of pups and sub-adults. In this study, the prevalence of these categories was high, respectively 31.82% (14/44) for class 1 and 38.64% (17/44) for class 2. In adults there was found a prevalence of 29.55% (13/44).

In Europe, different prevalence values of CPV-2 infection were reported depending on the geographical area considered. Firstly, in Spain two studies showed a similar prevalence, particularly of 61% in the serum of 84 wolves with the ELISA test (Oleaga *et al*., 2015) and 67.6% in organic matrices of 37 wolves using real-time PCR methods (Calatayud *et al*., 2019). Additionally, in Portugal a study reported a prevalence of 4.7% in organic matrices of 63 wolves using PCR methods (Miranda *et al*., 2017). Finally, in a study conducted in Bulgaria on 57 fecal and/or intestinal samples from wild carnivores, only one wolf was positive for CPV-2 with real-time PCR (Filipov *et al*., 2016).

These four studies were conducted over overlapping time periods, i.e. 2004-2010 (Oleaga *et al*., 2015), 1994-2012 (Calatayud *et al*., 2019), 1995-2011 (Miranda *et al*., 2017), and 2004-2014 (Filipov *et al*., 2016). The presence of Canine Parvovirus was therefore highlighted in various European countries, albeit with different prevalence. It can vary depending on various factors, such as the number of subjects sampled or contact with other infected and eliminator canids. In particular, these can be other wolves (therefore a determining factor for virus transmission can be the population density), but also dogs. In this last case, determining factors may be the proximity of wolves to inhabited centers and the presence in the wolves' territories of stray or owned dogs without the owners' control.

The studies by Filipov *et al*. (2016) and Calatayud *et al*. (2019) did not provide data on the signaling of the study population, while Miranda *et al*. (2017) and Oleaga *et al*. (2015) described the signaling of the wolves tested. Only the signaling data from the study by Oleaga *et al.* (2015) are reported below for they refer to a population with a significantly higher number of positive individuals (52/84 compared to 3/63).

Firstly, positive wolves were both males and females in almost equal quantities, with 63%

(27/43) positive males and 61% (25/41) positive females. Instead, in the study of this thesis there was a slight difference between sexes, with 81.2% (26/32) of males and 75% (18/24) of females positive.
Secondly, regarding the age groups, Oleaga *et al*. (2015) detected 25% (5/20) of positive pups, 58% (14/24) of positive sub-adults, and 82% (33/40) of positive adults. In contrast, in the study object of this thesis, a different distribution of positivity by age groups was highlighted, with a homogeneous prevalence in each category.

In Italy there are some studies that attest to the presence of Canine Adenovirus type 1 and type 2 in the Italian wolf population. In one of these, conducted by Pizzurro *et al*. (2017), the DNA of CAdV-1 was detected by PCR in a liver sample of a 4-month-old male wolf, which died in 2015 in Molise (Pizzurro *et al*., 2017). In another study, CAdV-1 DNA was highlighted with real-time PCR in a tongue sample of a 12-24-month-old male wolf, which died in Tuscany in 2014 (Balboni *et al*., 2019).
In both cases the positive animals were male, a result in line with this study, in which the only CAdV-2 positive individuals were 3 males and 57% of the CAdV-1 positive individuals were also males (4/7). As for the age groups, in the research by Pizzurro *et al*. (2017), an individual under the age of one year was positive for CAdV-1, while in the study by Balboni *et al*. (2019), the CAdV-1 positive wolf was a sub-adult. On the other hand, in the study object of this thesis it can be stated that Canine Adenovirus type 1 and type 2 are homogeneously distributed in the three age groups.
The sampling region of the research by Pizzurro *et al*. (2017), Molise, is different from those considered in this study (Emilia-Romagna, Tuscany, Calabria), whilst the sampling region of the study by Balboni *et al*. (2019), Tuscany, is among those here considered. In the wolf populations covered by this study, no significant difference was found in the distribution of the two types of Adenovirus, in fact CAdV-2 DNA was found both in Calabria and in Emilia-Romagna, whilst CAdV-1 DNA was detected both in northern Italy (Emilia-Romagna), in central Italy (Tuscany), and in southern Italy (Calabria).
There is a study that highlights the absence of DNA of CAdV-1 and of CAdV-2 in a population of 9 wolves sampled in Tuscany between 2005 and 2007, in which molecular PCR investigations on organic matrices were carried out (Ambrogi *et al*., 2019). Instead, the study object of this thesis and the study conducted by Balboni *et al*. (2019) detected the presence of the virus in the wolf population considered in Tuscany. Therefore, it is possible that the absence of the viral genome's detection in the study by Ambrogi *et al*. (2019) is due to the sampling

years (2005-2007), in which the wild population could have been numerically lower, or to a viral circulation low enough to make scarce the possibility of identifying positive subjects through laboratory methods.

Di Francesco *et al.* (2019) analyzed by molecular methods twenty wolf fecal samples from central Italy (Abruzzo), detecting 3 samples positive for CAdV-2 (15%). Our study is not comparable to the results of Di Francesco *et al.* (2019) since different matrices were analyzed, moreover the positivity or negativity of the fecal samples is strictly related to the elimination time of the virus.

In our case, the situation was opposite to what was found in other European countries, in particular for what concerns CAdV-1. In fact, in wolves sampled in Spain between 2010 and 2013, a prevalence of 70% of CAdV-1 DNA and 7% of CAdV-2 DNA, evidenced by PCR in 37 spleen samples and 13 stool samples, is reported in the literature (Millàn *et al*., 2016).

Furthermore, a similar result was obtained from a study conducted in Scandinavia and in the Svalbard Islands, which showed a seroprevalence of 67.7% in serum samples from 98 wolves investigated with the viral neutralization test (Akerstedt *et al*., 2010). In the study by Akerstedt *et al*. (2010), a statistically significant difference in CAdV-1 antibody positivity was reported between juveniles (less than one year of age) and adults (older than one year of age), which had a higher percentage of antibody positivity.

The results of the molecular investigations of RT-PCR highlighted the negativity both for Canine Distemper virus (CDV) and for Canine Enteric Coronavirus (CCoV), for this reason they will be treated together in the discussion.

There are still few studies on these two etiological agents in Italian wolves. A previously mentioned study by Molnar *et al*. (2014) searched for Canine Enteric Coronavirus (CCoV) and Canine Distemper virus (CDV) in 79 biological wolf matrices, showing only 2 samples positive for CCoV with a prevalence of 2.5 %.

In the other Italian study, conducted by Ambrogi *et al*. (2019), molecular investigations were led in organic matrices of 9 wolves found dead and on 125 fecal samples. The results highlighted the negativity for CDV and CCoV in tissue samples (Ambrogi *et al*., 2019), as well as the study by Di Francesco *et al*. (2019) confirmed the total negativity in the search for CDV and CCoV in biological matrices of wolves.

Compared to other Italian studies, the prevalence of CDV was high in an Italian region, Abruzzo, where exposure to CDV in one in four wolves had already been ascertained in 1996 (Fico *et al*., 1996). This data was also confirmed in the work of Di Sabatino *et al*. (2014) where

out of 30 wolf carcasses examined, 20 tested positive for Canine Distemper Virus (CDV).
In the recently published study by Alfano *et al*. (2019), they examined specimens from a dead wolf, detecting positivity for pantropic CCoV (pCCoV). From molecular assays, the wolf was found to have triple infection caused by CCoV, CPV-2b, and CAdV-2. Despite it being only one individual, the results of Alfano *et al*. (2019) showed that CCoV circulated in the Italian wolf population, thus indicating that potentially fatal infections caused by pantropic CCoV could be expected in this species.
It is not possible to draw the European panorama of the viral circulation of CCoV in gray wolves and compare it to our results since no literature was found on the subject.
Low circulation was observed for Distemper virus in Portugal between 1995 and 2006, where anti-CDV antibodies were identified in wolves (11.1%) and red foxes (9.1%). Although low, this study suggests an exposure in wild Iberian carnivores to CDV (Santos *et al*., 2009). In Spain, the prevalence is low to moderate with anti-CDV antibodies in 18.7% of wolves and in 17.1% of red foxes (Sobrino *et al*., 2008).

In this study, we analyzed the causes of death of the sampled wolves, relating them to the virological results. The cause of death is to be considered as any injury, disease, or disorder that triggers the physiological imbalance that directly leads to the death of the individual (Brooks Brownlie and Munro, 2016).
The main cause of wolf mortality examined in this study was related to humans, as well as in other studies conducted in Europe (Huber *et al*., 2002; Morner *et al*., 2005; Lovari *et al*., 2007; Liberg *et al*., 2012) and USA (Fuller, 1989; Murray *et al*., 2010; Treves *et al*., 2017). In our study, for what concerns human-related mortality, the main one was accidental collision with vehicles, followed by illegal killings by poisoning, and finally by firearms.
As said, the first cause of illegal killing was poisoning. In fact, in Italy poisoning as a cause of death of wildlife is becoming an increasingly important issue showed by the increased number of toxicological tests, as a result of the application of the ministerial decree of 18 December 2008. In a recent study conducted in Emilia-Romagna over a period of 10 years, more than 35% of the 603 analyzed animals, including wolves, was found to have been poisoned (Rubini *et al*., 2019). Therefore, these data demonstrate the importance of the toxicological investigation on all dead animals, even when the cause of death appears to be of another nature.
The presence of poached subjects suggests that, although the subspecies is subjected to rigorous protection, there is still a strong conflict between man and this large carnivore. In fact, in our study area, natural mortality was low (8.9%). These data are in line with some studies made in

Europe (Huber *et al*., 2002; Lovari *et al*., 2007) and in Minnesota (Fuller, 1989) and with data on anthropogenic mortality. Indeed, multivariate models revealed that the increasing of wolf population density boosted a higher anthropogenic risk while reducing the risk of natural mortality (Murray *et al*., 2010).

In our study, sarcoptic mange was present in 7 subjects (12.5%) and, apart from one wolf, it was not a cause of death. On the other hand, it was always a contributing cause of death since it negatively affected the fitness of the infested subjects. For what concerns the subjects positive for the viruses detected, in this study it was possible to observe that the majority were in good nutritional conditions. As a consequence, this data leads us to conclude that the positivity for the three etiological agents CPV-2, CAdV-1, and CAdV-2 was not a direct cause of death. Similarly, these data are in line with other European studies showing that infectious diseases in wolves, except for sarcoptic mange, do not appear to be a factor negatively impacting these populations (Morner *et al*., 2005).

4.1. STUDY LIMITS

A limitation of this study is represented by the fact that the sampling applied was of opportunistic type, i.e. based on the 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 the case of poaching mortality where there is a strong interest in hiding carcasses (Liberg *et al.*, 2012) or in some cases of natural mortality in the woodland environment. In fact, the type of environment and the altitude play a decisive role in the discovery of the corpses, as possibly inaccessible areas such as mountain ones can significantly reduce the possibility of findings and therefore of sampling.

In a study of viral circulation in the wild, these difficulties are a limitation that can affect the final interpretation of the results. The statistical and methodological bias may be due to the fact that more wolves of an age group or of a sex class might be found, as well as the sampling year, the season, and other important variables may influence the findings. Consequently, this non-homogeneity of the sampling would mean that the results obtained are not representative of the Apennine population.

In conclusion, for the above reasons in this study all the results were related to the population sampled, in that period, and in those study areas, avoiding speculative conclusions and inferences about the Apennine population.

Although researchers dealing with endangered and threatened species should strive to get the most out of any data source, proper methodology and recognition of potential sources of bias should be common practice to get the right foundation in wildlife studies (Anderson, 2001).
Additionally, with this study we also want to underline the importance and usefulness of finding carcasses in nature to obtain biological matrices. In fact, it must be recognized that for studies on elusive species such as large carnivores, the finding of corpses is often the only opportunity of close contact with them that allows for structuring of transversal research, albeit maintaining a precautionary interpretative approach (Lovari *et al.*, 2007a).

Researchers working with endangered, low-density, and elusive wildlife such as large carnivores are constantly challenged to obtain robust and reliable nationwide datasets (Ciucci *et al.*, 2007), and Italian wolves make no exception. Indeed, it should be remembered that the Italian wolf is included in Appendix II - Annex A of CITES and requires specific authorizations for capture, including for research purposes. For this reason, serological sampling (systematic and organized) on live wolves is extremely difficult and complex, also because of the social structure and the elusive nature of wolves which does not lend themselves well to capture actions and invasive direct approaches.

Finally, another limitation of the study concerns the type of samples analyzed, i.e. organic matrices of free-ranging wolves found dead in the wild. This implies that not necessarily every carcass was found shortly after the death of the animal. Consequently, there is the possibility that the genetic material of present viruses degraded and were not detected by the molecular method used, thus giving an underestimated viral presence in the considered population.

5 - CONCLUSIONS

In the Italian wolf population examined, the study highlighted the circulation of CPV-2 with a high prevalence (78.6%) and of CAdV-1 and CAdV-2 with a lower prevalence (12.5% for CAdV-1, 5.4% for CAdV-2). There is no evidence of circulation of CDV and of CCoV in the population studied.
For the subjects positive for the three DNA viruses detected in this study, it was possible to observe that the majority of them were in good nutritional conditions and the most frequent cause of death was by accident and due to trauma from collision. In light of the results, it can be stated that CPV-2, CAdV-1, and CAdV-2 infections in the wolves sampled in this study are

predominantly asymptomatic and do not constitute a direct cause of death. The results are in line with other European studies showing that infectious diseases in wolves, except for sarcoptic mange, do not appear to be a factor with negative impact on this species.

Although in our population the positivity for DNA virological agents were not the direct cause of death, it is good to remember how exposure to these DNA and RNA viruses can still have important implications not only for the wolf population, but also for other canids, both wild and domestic. The transmission of these viruses can take place in both directions, i.e. from domestic animals to wild ones and vice versa. In fact, to date it is not entirely clear which species acts as a reservoir for the others and further research would be appropriate.

However, some assumptions, supported by studies reported in this paper, were made. The most indicative studies to evaluate whether wild carnivores may play a role in the epidemiology of virological agents with a wild-domestic interface observed greater viral circulation in urban wildlife – mainly red foxes since they are more present than gray wolves – compared to rural or wooded one. Consequently, this could demonstrate that the infection reservoirs for wild carnivores are domestic dogs.

Confirming the domestic reservoir theory there is also the fact that relatively dense populations of susceptible hosts are usually needed to support the circulation of a pathogen, a situation present only for dogs (consisting in millions of individuals on national soil) and certainly not for Italian wolves. However, it is important to report that Italian wolves in Italy have a positive growth trend and are in expansion, with increasingly frequent episodes of presence in peri-urban contexts. Therefore, this suggests that, along with the population, the prevalence of these viruses will also increase, and they will begin to circulate with a higher frequency in the environment.

The prevalence obtained in this study suggests that, during the years here studied, circulation of CAdV-1 and CAdV-2 in Italian wolves of the three sampled regions was sporadic, proving consistent with sporadic and short-lived introductions of the virus in these populations. A different situation occurred for CPV-2, where the observed circulation suggests a pattern of continuous and lasting endemic exposure over time.

6 - REFERENCES TO IMAGES

Fig. 1. Photo taken from: Lindblad-Toh *et al.*, 2005

Fig. 2. Photo taken from: https://mapsontheweb.zoom-maps.com/image/76734596815

Fig. 3. Photo by the Reddit user u/risingmagpie based on the following links:
http://www.provincia.bz.it/agricoltura-foreste/fauna-caccia-pesca/fauna/lupo-alto-adige/declino-riaffermazione-lupo.asp
https://www.researchgate.net/publication/282321869_One_no_one_or_one_hundred_thousand_how_many_wolves_are_there_currently_in_Italy
https://www.iononhopauradellupo.it/
https://www.lifewolfalps.eu/
https://www.inaturalist.org/taxa/42048-Canis-lupus

Fig. 4. Photo taken from: Meriggi *et al.*, 2011

Fig. 5 – 6 – 7 – 8. Maps obtained with the open-source program QGIS

Fig. 9. Photo taken from the Kit NucleoSpin Tissue (Macherey Nagel, Düren, Germany)

From Fig. 10 to Fig. 54. Plots obtained with the open-source program RStudio 2020

7 – ABBREVIATIONS

APN: aminopeptidase N

°C: Celsius degrees

CAdV-1: Canine Adenovirus type 1

CAdV-2: Canine Adenovirus type 2

CCoV: Canine Coronavirus

CDV: Canine Distemper Virus

CITES: Convention on International Trade of Endangered Species

CPV: Canine Parvovirus

CPV-2: Canine Parvovirus type 2

CRCoV: Canine respiratory coronavirus

CSF: Cerebrospinal fluid

DIC: Disseminated Intravasal Coagulation

DNA: Deoxyribo Nucleic Acid

dsDNA: double-stranded Deoxyribo Nucleic Acid

ELISA: Enzyme-Linked ImmunoSorbent Assay

FCoV: Feline Coronavirus

g: grams

HE: Hemagglutinin-esterase

HI: Hemagglutination Inhibition

ICH: Infectious Canine Hepatitis

ISH: In Situ Hybridization

LOD: Limit Of Detection

MeV: Human Measles Virus

MEV: Mink Enteritis Virus

ORF: Open Reading Frames

PCR: Polymerase Chain Reaction

RNA: Ribo Nucleic Acid

RPV: Raccoon Parvovirus

RT: Reverse Transcription

RT-PCR: Reverse Transcription-PCR

SARS: Severe Acute Respiratory Syndrome

SARS-CoV2: Severe Acute Respiratory Syndrome Coronavirus

SLAM: Signaling lymphocytic activation molecule

SN: Serum-Neutralization

SVN: Serum Virus Neutralization

TEM: Transmission electron micrograph

TGEV: Transmissible Gastroenteritis Virus

Tm: melting temperature

8 - REFERENCES

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