Unveiling patterns of viral pathogen infection in free-ranging carnivores of northern Portugal using a complementary methodological approach

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Abstract

Pathogen surveillance in free-ranging carnivores presents challenges due to their low densitie and secretive nature. We combined molecular and serological assays to investigate infections by viral pathogens (Canine parvovirus (CPV), Canine distemper virus (CDV) and Canine coronavirus (CCoV)) in Portuguese carnivores (Canis lupus, Vulpes vulpes, Lutra lutra, Martes foina, M. martes, Meles meles, and Genetta genetta) over a period of 16 years. Additionally we explored spatio-temporal patterns of virus occurrence in Canis lupus. Our study identified CPV DNA in all carnivore species with an overall prevalence of 91.9%. CPV was detected in all sampled years and seasons in Canis lupus, supporting its enzootic nature. CDV RNA was mainly detected in the Canidae family, with viral nucleic acid recorded between 2005 and 2008 with a peak prevalence of 67% among the wolf population, followed by a sharp decline, suggesting an epizootic behaviour of the virus. Antibodies show that mustelids and viverrids were often exposed to CDV. CCoV was first recorded by molecular methods in wolf samples in 2002, remaining in the wolf populations with marked fluctuations over time. The dual serological and molecular approach provided important epidemiological data on pathogens of wild carnivores in Portugal. These programmes should also include monitoring of other potential reservoir hosts such as domestic cats and dogs.

Keywords: Canine parvovirus; canine distemper virus; coronavirus; real-time PCR; Iberian wild carnivores; serology.

1. Introduction

There is a great diversity of known pathogens among free ranging populations of carnivores, which can be transmitted to both domestic and stray animals. Thus, epidemiological surveillance and improvement of current methodologies for pathogen detection are crucial to understand the ecology, impact and dynamics of the diseases [1]. General knowledge about disease dynamics in free-ranging carnivores is sparse, mainly due lack of epidemiological information concerning pathogen distribution among wild populations [2].

Viruses are important pathogens of wild carnivores that can affect populations through increased mortality and/or decreased general health [3]. Canine parvovirus (CPV) and canine distemper virus (CDV) are known pathogens of domestic and wild carnivores worldwide [4-8]. Although CPV infection is a relatively new disease first reported in the late 1970s in domestic dogs [5], the virus has spread rapidly [7, 9]. In Europe, the presence of CPV has been documented in a variety of free-ranging carnivore populations from canids to mustelids and viverrids, through serology or molecular methods [7, 10-12]. Additionally, Duarte et al. 2013 [10] reported high exposure to parvoviruses in mesocarnivores from Portugal, but to date, no cases of mortality among wild carnivores have been reported in Iberian Peninsula [13, 14].

Canine distemper virus (CDV) is a highly contagious viral pathogen mainly transmitted by aerosols through respiratory secretions, and responsible for a lethal systemic disease in dogs and other carnivores [15]. Domestic dogs have largely been responsible for introducing CDV to previously unexposed wildlife [16, 17]. In Iberian Peninsula, the virus has already affected wild carnivores, being responsible for death or disease of common genet (*Genetta genetta*) [18], red fox (*Vulpes vulpes*) [8] and Iberian lynx (*Lynx pardinus*) [19]. Direct mortality due to canine distemper in wolves was documented in Portugal [20], and several cases have been reported in other parts of Europe (e.g. [21].

In contrast, knowledge on coronaviruses (CCoV) in free-ranging carnivores is very limited. Serological surveys have been providing evidence that some of these species may serve as hosts for this viral pathogen [11]. Nevertheless, CCoV seems to be enzootic worldwide in dogs [22, 23], which likely transmit the virus to wild carnivores [11]. In Europe, wolf population have been found positive for this virus in France and Italy, although with prevalence <9% [11]. Despite the sampling of several Iberian populations of wild carnivores, only a single coronavirus-positive has been reported from a mongoose (*Herpestes ichneumon*) [24-26].

Several carnivore species have suffered dramatic declines in Europe during the last two centuries, with human activities leading to fragmented populations and consequently genetic isolation [27, 28]. Although infectious diseases are not usually the main factor affecting the survival of these species, virulent pathogens can act as a mortality source and cause epidemics. In fact, small fragmented populations, such as those of Iberian wolves, are thought to become more vulnerable to disease outbreaks [2, 29]. Multi-host pathogens, such as CPV, CDV or CCoV, can be maintained in the system through domestic dogs or other reservoir hosts, potentially impairing the viability of smaller more vulnerable wolf populations [30].

Despite all three viruses having already been reported in the Portuguese territory in wild carnivores, either by detection of antibodies (Abs) or nucleic acids (see [8, 10, 31], an assessment of the potential threat posed is hampered by the difficulty of overlaying the different methods for analyses. The available diagnose tools used (serology or molecular) or the target material (scats or body tissues) impair, to some extent, the interpretation and comparison between datasets. Thus, with the present study we intend to investigate occurrence of infection and spatio-temporal patterns of selected viral pathogens (CDV, CCoV and CPV) circulating in free-ranging populations of carnivores from northern Portugal. The use of combined approaches that include serological (Abs) and molecular detection as means for an enhanced perception of the results and inter study comparisons is discussed. The resulting information has critical implications for understanding of disease dynamics in these species and broader conservation policies.

2. Materials and methods

2.1. Sampling and study areas

The study was conducted on 7 species of free-ranging carnivores, in a total of 62 individuals: 54 canids - Iberian wolf *Canis lupus signatus* (n=42), and red fox *Vulpes vulpes silacea* (n=12); 2 mustelids - Eurasian otter *Lutra lutra* (n=1) and Eurasian badger *Meles meles* (n=1); 3 viverrids - common genet *Genetta genetta* (n=2), stone marten *Martes foina* (n=3), and pine marten *Martes martes* (n=1). Samples were collected from animals opportunistically found dead between 1995 and 2011 in northern Portugal, including four protected areas (Peneda-Gerês National Park, Montesinho Natural Park, Douro Internacional Natural Park and Alvão Natural Park) and in Beira Interior region comprising the Serra da Estrela Natural Park (Fig. 1). The main known cause of death was accidental road kills and shootings (Table 1).

Samples were stored at -20ºC at the Institute for the Conservation of Nature and Forest (ICNF) tissue depository (BTVS/ICNB) and in the Monitoring System of Dead Wolves (SMLM/ICNF). The collection site, gender, age and the preservation status was recorded for each animal (Table S1). Sampled tissues from the Iberian wolf included liver, spleen and lymph node; liver, spleen, small intestine/rectum and lungs for the red fox and for the remaining animal species small intestine/rectum and lungs.

2.2. Molecular screening

Tissue homogenates were performed in phosphate buffered saline (PBS) solution and directly used for co-extraction of total DNA and RNA with the DNeasy tissue and blood kit (Qiagen, Germany), according to the manufactures instructions. Nucleic acids were extracted in a separate laboratory and after quantification in a Nanodrop 2000c (Thermoscientific) were kept at -80ºC until analysed. According to the organ availability, lung, liver or spleen tissue extracts were prepared as previously described [32], stored at -20ºC and later used for specific Ab detection.

We conducted Molecular screening with a TaqMan® quantitative PCR system already described [33, 34]. CPV DNA was amplified by quantitative PCR (qPCR) in a 20 µl reaction with 25 ng of template, using the TaqMan® Gene Expression 2× Master Mix (Applied Biosystems), 0.3µM of primer forward, 0.3µM of primer reverse and 0.25µM of TaqMan probe. CDV and CCoV RNA were amplified by one step reverse transcription-qPCR (rt-qPCR) using the TaqMan® RNA-to-Ct (TM) 1 step kit, 0.3µM of primer forward, 0.3µM of primer reverse and 0.25µM of TaqMan probe in a 20 µl reaction with 25 ng of template.

We performed the amplification in the Applied 7300 instrument (Applied Biosystems) and the cycling conditions comprised an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 1 minute at 60°C. When the template was RNA the amplification cycle included an initial reverse transcription step at 48°C for 15 minutes.

Reagents assemble and template addition was performed in separate areas to prevent sample contamination. As positive control for CPV, we used tenfold dilutions of CPV-2-780 916 Cornell strain (Tetradog®, Merial). Regarding CDV and CCoV each recombinant plasmid was used as positive control as already described [34]. Water was used as negative control representing 10% of the total samples tested in each run.

2.3. Serological assays

We used lung, liver or spleen tissue extracts to detect CDV and CPV Abs by Indirect Enzyme-linked immunoassay commercial kits Ingezim Parvo Canino 1.5.CPV.K.1 and

Ingezim Moquillo IgG 1.5.CDG.K.1 (Ingenaza, Spain), according to the manufacturer instructions. Tissue extracts were tenfold diluted for CDV and CPV testing. The anti-dog conjugate provided in the kits was used for detection of the primary Antigen (Ag)/Abs complex in the canid samples (wolves, red foxes); in the viverrid and mustelid samples it was replaced by the Protein A-Peroxidase from Staphylococcus aureus/horseradish (Sigma-Aldrich) [35].

2.4. Statistical analyses

For wolf and red fox we investigated the possible association between molecular findings and age (cub, juvenile, adult) and sex (male, female) of the animals with Fisher's exact test. Taking advantage of the larger sample size of wolf, we used the same test to assess the effect of season (winter/ spring vs. summer/ autumn) on the infection status by all the three viruses. To do so, we only considered the interval of years in which each virus was detected. Both statistical analyses were computed using IBM SPSS Statistics 25.0 software. A 95% confidence interval (CI) was calculated using the Wilson score interval method without correction for continuity (the Richard Lowry's VassarStats online calculators: http://vassarstats.net/prop1.html).

Lastly we tested if the samples preservation status would have an effect on Abs detection also using Fisher's exact test.

3. Results

3.1. CPV: Serology and viral nucleic acid detection

We detected CPV DNA in all the species with an overall prevalence of 91.9% (Table 2). Particularly in *C. lupus*, CPV DNA was detected in all sampled years (Table 3; Fig. 2) with prevalence of infection close to 100% across the four seasons. Thus, time of the year had no significant effect on the infection status in wolves (Fisher's Exact Test, *p*=1.000). Samples showed an average Ct of 34.6, ranging from 41.9 to 12.5 Cts (standard deviation 4.4). *C. lupus* liver samples showed a higher frequency of viral DNA positivity, but positive results were also obtained from the spleen and lymph node. *Vulpes vulpes* were also highly positive (83.3%; Table 2) with viral DNA detected in the lungs, liver, spleen and small intestine. Both mustelids tested positive for CPV with viral DNA detected in the small intestine in *L. lutra* and lungs in *M. meles* (Table 2). Among the few screened viverrids, *G. genetta* yielded the lowest prevalence with 1 out of 2 animals positive (Table 2) in the lung. Both marten species generated positives in lungs and small intestine.

Antibodies against CPV were found in 60.0% of the samples, including 33 canids where seroprevalence in wolves reached almost 74% (Table 4). Nevertheless, evidence of a positive association was found between CPV seropositivity in wolves and the sample conservation status (Fisher's Exact Test, *p*=0.001), where better preserved samples had a higher likelihood of detecting Ab. Among mustelids, the two sampled individuals of *M. foina* had been exposed to CPV.

3.2. CDV: Serology and viral nucleic acid detection

CDV RNA was detected in two families (*Canidae* and *Mustelidae*) with an overall low prevalence (8.1%) (Table 2). The organs with the higher yields were the liver and spleen in *C. lupus*, but the intestinal tract in *V. vulpes* and *G. genetta*. CDV RNA was only recorded in 2005 and 2008 (Table 3; Fig. 2). The wolf populations reached a frequency of 67% (Table 3: 2/3) in 2005, followed by a decrease in 2008 (Table 3: 20%; 1/5). We found a higher prevalence during the summer/ autumn months and progressively decreasing towards spring (Fig. 2). However, the small sample size did not allow for a statistically significant effect of seasonality (Fisher's Exact Test, *p*=0.083).

CDV Abs were detected from 1997 onwards (Table 3) in 65.0% of the samples where both canid species presented relatively high prevalences (*C. lupus* 61.9%; *V. vulpes* 72.7%) (Table 4). We found seropositive samples in all species but *G. genetta* (Table 4).

3.3. CCoV: Viral nucleic acid detection

Through molecular analyses, we obtained an overall prevalence of CCoV of 32.3%. CCoV RNA was detected in all three families. Thirteen *C. lupus* (31.0 %; Table 2) tested positive in the spleen and four *V. vulpes* (33.3%; Table 2) in the spleen and small intestine. Positive results were also found in the two *G. genetta* and the single *L. lutra*, both in the rectum (Table 2).

The virus was first detected in a single fox in 1999. However, CCoV RNA was not recorded in our wolf samples until 2002. We did not detect the virus also in 2005 and after 2010(Table 3). Although the data shows higher prevalence in the cooler months with a trending decrease in detection from autumn to summer (with no positive animals found in the warmer months; Fig. 2), the differences are not statistically significant (spring/ summer vs. autumn/ winter: Fisher's Exact Test, p=1.000).

3.4. Co-infection by multiple viruses

We recorded co-infections by multiple pathogens (two or three viruses) across the three families, representing 37.9% (22/58) of the sampled species. CPV was the most frequent virus involved in mixed infections with nucleic acid from additional viruses detected in 95.5% (21/22) of the co-infected individuals. Among these, CCoV mixed infections with CPV were most common in both wolves and red foxes (Fig. 3; Table 5). Unfortunately for most mixed infections, there was no information on the cause of death or any additional data that could help with the diagnosis.

Co-infection by the three viruses was detected in a single individual of *C. lupus* (Fig. 3; Table 5). Co-infection by CDV/CCoV was also infrequent and detected in one juvenile of *G. genetta* (Table 5).

No significant associations were detected between age (Fisher's Exact Test, $p \geq 0.339$), or sex (Fisher's Exact Test, $p \geq 0.505$), and viral nucleic acid positive samples in any of the two canid species.

4. Discussion

Diagnosis and detection of viruses and other pathogens in wildlife populations can be extremely difficult. Long-term collection of samples allowed us to show that all three taxonomic families (*Canidae*, *Viverridae* and *Mustelidae*) have been exposed to CPV, CDV and CCoV in northern Portugal. Viral detection in free-ranging animals was confirmed by molecular and serological data.

4.1. Canine parvovirus

Our study identified a high prevalence of CPV among all surveyed species. This seems to be particularly significant in the two species of canids, where wolves presented an infection rate close to 100%, although with a high variability of viral loads (ranging from 41.9 to 12.5 Cts). CPV was detected in all sampled years and across all seasons in *C. lupus*, suggesting its enzootic nature in this study area. We believe that the social habits of Iberian wolves [36] may increase the frequency of intra-specific contacts and thus promoting the pathogen transmission. The findings mimic the results obtained by Duarte et al. (2013) [10], where some southern populations of mesocarnivores in Portugal also reached 100% prevalence, using the same methodological approach. In foxes, for example, CPV DNA was detected in almost 79% of the tissue samples, not far from 83.3% obtained in our study and within the confidence interval.

Contrastingly, other European studies described a much lower prevalence, ranging from 3.5 to 15.2% in wolf scats in Italy [11, 12], or 12.1% obtained in France [11, 20], and even a total lack of viral DNA detection in tissue samples from several species (Germany [6].

CPV infection is typically established by oronasal route by contact with contaminated faeces and after initial replication in lymphoid organs CPV localizes predominantly in the intestinal tract [37]. Whenever matched tissue samples were available, mustelids and viverrids tested positive in the lung but not in the small intestine, contrasting with the canid samples, particularly from wolves, in accordance with this pattern of virus dissemination.

The pathogen's high stability in the environment and the ability to induce carrier states in susceptible hosts [38], support our findings. The high resistance of CPV to adverse environments [39], the higher sensitivity and specificity of qPCR, and the availability of target tissues for viral replication *in vivo* [10], may explain the higher detection of CPV DNA, with variability of the viral load. Nevertheless, there was a lower seroprevalence of CPV, as positive samples were approximately 30% lower compared to results obtained through molecular assays. This discrepancy may be explained by several factors including: the use of lung, liver or spleen tissue extracts as the biological matrix for Ab detection; the use of the anti-dog conjugate in the ELISA assay for canids samples, and the lower sensitivity of ELISA assays for Abs detection versus qPCR for nucleic acid detection [37, 40]. Additionally, we found a link between sample preservation status and seropositivity. Yet, the seropositivity rate obtained suggests a high rate of CPV exposure, but a low rate of CPV induced mortality [4], suggested by the perdominant low viral load detected in the samples.

4.2. Canine distemper virus

CDV was mainly detected in the Canidae family with an overall prevalence below 10%. Viral RNA was only recorded between 2005 and 2008 with a prevalence peak in the first year among the wolf population (67%) followed by a drop down, suggesting an epizootic behaviour of the virus. Similar infection pattern has been documented in several animal species worldwide [41], including in Europe [21, 42, 43], with CDV often implicated in disease outbreaks and mortality events. The highly infectious nature of CDV [44] may explain the sudden increase in prevalence.

CDV serological data shows a much higher proportion of positive samples (65%) compared to nucleic acid detection. In addition to canids, we confirmed that mustelids and viverrids have also been exposed to CDV. The observed high seroprevalence suggests a high rate of circulation among the carnivore in northern Portugal and confirms these species as being highly susceptible to CDV infections. Considering CDV epidemiology, our finding may indicate CDV spill-over into wild populations, quite possibly from domestic and stray dogs acting as disease reservoirs [16, 20, 30, 42].

4.3. Canine coronavirus

CCoV RNA was first recorded in our wolf samples in 2002, remaining with marked fluctuations over time. Both canid species present similar overall prevalence values. In comparison, the incidence was much lower in Italian and French wolf populations using faecal samples [11], which is not surprising given the low stability of viral particles in scats [45]. CCoV RNA has been detected in dog tissues across Europe [46], but despite previous efforts, only a single positive mongoose has been recorded in Portugal [10]. Thus, our results represent the first record of viral infections in wild populations of *C. lupus* and *V. vulpes* in Iberia, as well as for *L. lutra* and *G. genetta*.

Although our prevalence data are poorly informative about the impact of infection and disease in the populations, previous studies have shown that CCoV infection seems not to be frequently associated with fatal disease; instead, it often causes mild, self-limiting enteritis followed by rapid recovery [47]. Nevertheless, mortality may occur as a consequence of mixed infections with any of the two studied virus, CPV and/ or CDV [47, 48].

4.4. Viral co-infection

A proportion close to 40% of the carnivores was involved in mix infections where CPV dominated most of the combinations in all affected species. The latter is not surprising giving the high prevalence of CPV among the carnivore populations, as previously discussed. Conversely, when compared to previous studies [10, 11], we found the overall proportion of co-infections we obtained to be relatively high.

Infections by CCoV seem to be aggravated when occur simultaneously with CPV [49, 50] and/or CDV[51], which could explain seven of the unknown deaths in wolves where we detected any of the two combinations and even one individual infected by the three viruses. Yet, Mech & Goyal (2011) [52] showed that the negative impact of CPV seems to be attenuated once the virus becomes enzootic in a population, which could explain the apparent lack of outbreaks in our system, even with such a high prevalence of co-infections. However, this suggestion should be interpreted with caution due to the absence of demographic data and survival of pups for the populations here considered.

As our data does suggest that CDV is not enzootic in the northern Portugal populations of wolf, individuals may be more vulnerable to CDV infection but as well as to co-infection with CDV and CCoV. Nevertheless, only a single individual was detected with this mixed infection but cause of death was unknown.

Lastly, dual infection by CDV/CPV seems to be overall less frequent, at least in wild carnivores in European as we only detected in three canids in our study. The lack of case reports makes it hard to predict impacts and consequences of infections. However, research on other species highlighted a case of fatal canine distemper infection in African wild dogs with concurrent infection by CPV in 2/6 individuals, leading the authors to believe that co-infections may have contributed to the fatal outcome in some of the dogs [53].

4.5. Broad implications and final considerations

Free-ranging carnivores are of vital importance to the stability and integrity of most ecosystems, but some viral infectious diseases have shown the potential to negatively impact wild populations and cause declines [54, 55]. Almost half of the known infectious diseases of free-ranging carnivores are of viral origin [3]. Although the three viruses here studied have been associated with mortality in wild and domestic canids worldwide, and some viruses could, theoretically, threaten the viability of small isolated populations (e.g. CPV; [29]), their impact in wild carnivore populations is still largely unknown, particularly among the northern Portuguese populations. Despite high prevalence levels of CPV and CDV our populations, none of these pathogens seem to have been associated with declines in Iberia, or listed among the major threats to the wild carnivores [56]. This can be explained by a greater impact of fragmentation (road-kills) and illegal hunting, and/ or simple lack of robust data and regular disease surveillance. Nevertheless, our results suggest a high and common exposure to CPV and CDV, with a higher seroprevalence of Abs when compared to similar studies conducted in the Iberian Peninsula [8, 31]. Furthermore, we detected different antibody titers towards CPV and CDV, suggesting different contact timings between the animals and the viral pathogens. Immune response towards CPV and CDV infection is considered lifelong, thus detection of seropositive animals implies exposure but also protection against the virus [57].

Considering the availability of recent data regarding CDV and CPV in Portugal [10, 31; this study], it is suggested an active viral circulation among the animal population studied. Unvaccinated dogs, as well as wolf prey species, or scattered infectious faeces/urine, make the transmission of viral pathogens between populations a likely scenario [58]. The control and widespread vaccination of domestic dogs and cats could reduce the potential spill-over of pathogens. Additionally, constant evolution of virus through mutations in the genome [59, 60] present a challenge for pathogen detection and consequently for adequate wild carnivore populations' management. This means that the results obtained in this and other studies may underestimate the infectious pressure, particularly when a single diagnostic test is used. Thus, continuous surveillance of viral pathogens and longitudinal studies in stray and wild animal populations are crucial to detect new viral variants potentially escaping both the host immune system and detection methods.

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Declarations of interest

The authors declare that they have no competing interests.

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