Non-Invasive Surveillance of the Wildlife Virome in Central Italy: Insights from the Foreste Casentinesi National Park

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Article

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Simple Summary

Wild animals can host many viruses, some of which may also affect humans and domestic animals. Monitoring these viruses is challenging because capturing wild animals is stressful and often impractical. In this study, we collected feces left in the environment by several mammal species living in the Foreste Casentinesi National Park in the Tuscany side Italy. Using laboratory methods able to detect both known and unknown viruses, we found that more than one-third of the samples contained viral genetic material. Astroviruses were the most common and were detected in deer, foxes, wolves, porcupines, and small mustelids. Foxes carried the highest variety of viruses, such as parvovirus, coronavirus, and adenovirus. Because predators and scavengers may also ingest viruses from their prey, fecal samples provide information not only on individual animals but also on the broader community. Our results show that non-invasive sampling can give important insights into wildlife health, help identify new viruses early, and support biodiversity protection and public health.

Abstract

Wildlife can act as both a reservoir and a sentinel for emerging pathogens, but surveillance is often constrained by difficulties in obtaining samples without disturbing animals. This study explored the viral diversity of wild mammals inhabiting the Foreste Casentinesi National Park (Central Italy) using non-invasive fecal sampling. From 2021 to 2022, 99 fecal samples from several species were collected and analyzed by PCR and metagenomic next-generation sequencing. Of 26 pools examined, 10 (38.5%) tested positive for at least one viral target. Astroviruses were the most frequently detected, found in deer, foxes, wolves, small mustelids, and porcupines. Foxes carried the widest range of viruses, including astrovirus, parvovirus, bocavirus, kobuvirus, adenovirus, and coronavirus. Several sequences showed low similarity to known strains, suggesting divergent or novel viral lineages. Metagenomic analysis also identified members of *Circoviridae*, *Anelloviridae*, and *Picobirnaviridae*. These results provide new insights into the virome of European wildlife, including the first reports of some viruses in certain species. Overall, our study demonstrates that non-invasive surveillance is a valuable tool for monitoring ecosystem health and supports a One Health approach to early detection of viral threats.

Keywords: wildlife virome; non-invasive surveillance; next-generation sequencing

1. Introduction

Wildlife in Europe comprises more than 1,300 protected animal species, yet recent assessments indicate that 63% are unable to maintain a viable long-term presence in their natural habitats, while

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81% of Europe's natural habitats remain in unfavorable conservation status due to the loss of ecological, structural, and functional characteristics required to support native species. In this context, biodiversity conservation represents one of the most pressing global challenges, and the control of infectious diseases that may influence these dynamics plays a pivotal role [1]. Although the health impact of many pathogens identified in wildlife is still poorly understood, their ecological consequences, including infection spread and amplification in free-ranging species, must be considered. Unlike domestic animals, wildlife moves freely across landscapes and interacts with multiple species and environmental components, thereby increasing the risk of pathogen transmission, cross-species spillover (including predator–prey interactions), and opportunities for viral mutation and recombination. These processes are further amplified by anthropogenic drivers such as land-use change, habitat fragmentation, unintentional species translocations, and transformations in agricultural and livestock systems, which create conditions for bidirectional pathogen flow between wild and domestic hosts [2]. Wildlife species can therefore act both as reservoirs of emerging pathogens and as valuable sentinels for their early identification [1,3]. Monitoring wild animals offers crucial insights into ecosystem health and environmental changes, including pollution, climate change, antimicrobial resistance, and emerging infectious diseases [4]. Consequently, wildlife health surveillance has become a growing priority at national and international levels, with efforts to establish harmonized and integrated systems across Europe [5]. While its objectives are comparable to those in domestic animals, surveillance in wildlife is hindered by ecological and behavioral traits that complicate sampling. Available approaches—including live capture, carcass collection, or opportunistic sampling from hunting and culling—often restrict species coverage and may introduce bias by over- or underestimating pathogen prevalence when relying on symptomatic or dead individuals [5,6]. Non-invasive environmental sampling reduces animal impact but is prone to degradation and contamination, limiting diagnostic accuracy. Traditional molecular tools such as PCR can detect known pathogens but often fail with genetically divergent or previously undescribed agents. Next-generation sequencing (NGS) has expanded diagnostic capacity, enabling the discovery of novel viruses and host–pathogen associations [7]. Metagenomic approaches have revealed diverse viral agents, including *theilovirus*, *phlebovirus, amdovirus, kobuvirus, and picobirnavirus* in wild carnivores in Spain [8], supported zoonotic surveillance in bats in China [9], and detected *Porcine Parvovirus 4* (PPV4) and novel *Torque Teno Sus Virus* (TTSuV) variants in wild suids [10]. In red foxes, viral sequences related to *parvoviruses, astroviruses*, and *hepeviruses* showed low homology with known strains, suggesting novel viral species [11]. Similarly, astrovirus studies in Hungarian wild boars identified domestic pigs as viral reservoir [12]. Based on this framework, our study aims to develop a model for wildlife health monitoring capable of detecting viral infectious agents circulating among populations of wild animals sharing the same ecosystem, using non-invasive sampling methods with minimal impact on both the environment and animal welfare. Enhancing wildlife health surveillance through such approaches may significantly improve early warning capacities for future health threats [4].

2. Materials and Methods

2.1. Foreste Casentinesi National Park (PNFC)

The Foreste Casentinesi National Park (PNFC) covers an area of 368 km² across two regions, Emilia-Romagna and Tuscany, with elevations ranging from 400 to 1658 meters above sea level. Forests account for over 80% of the Park's total surface area. The extensive woodland coverage, the presence of diverse habitats and vegetation types, along with low human population density, make the Park an ideal environment for the presence and spread of wildlife, which is notable for its richness and diversity of species (https://www.parcoforestecasentinesi.it/). For the purposes of this study, only the portion of the Park located within the Province of Arezzo (138 km²) was considered (Figure 1).

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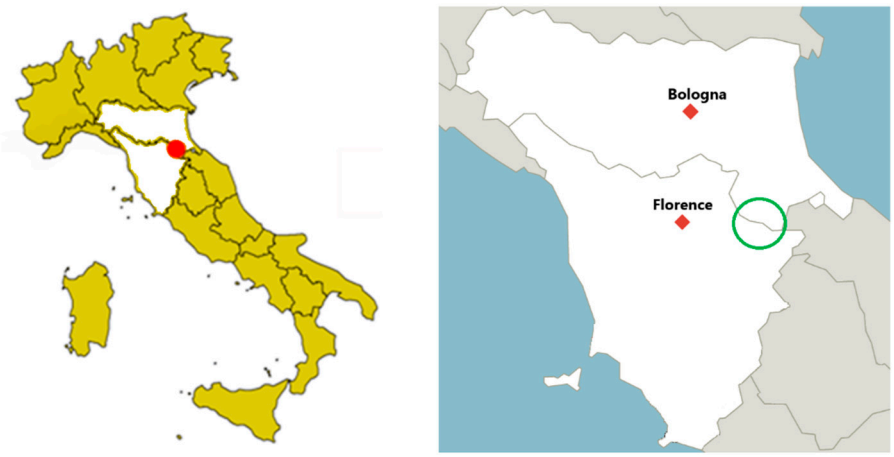

Figure 1. The PNFC sampling area, located in Tuscany and Emilia-Romagna, is shown with a green circle.

2.2. Sampling

In our study fecal sample have been chooses due to their suitability for application in large and complex ecosystems, where direct contact with wild animals is often difficult or unfeasible. Fecal samples can be collected non-invasively from a wide range of species, without the need for animal capture or handling. Moreover, fecal samples allow the detection not only of pathogens carried by individual animals, but also, in case of predator/prey interaction, of those circulating within the broader community inhabiting the same environment. Sampling activities were carried out following an agreement between the Department of Veterinary Sciences of the University of Pisa and the Foreste Casentinesi, Monte Falterona and Campigna National Park Authority (26/02/2021). The study area, corresponding to the portion of the Park located within the Province of Arezzo, was divided into 11 cells measuring 5 × 5 km. Each transect was surveyed simultaneously by 2–3 operators, who followed and examined both the main trail/path and the surrounding area, considering a buffer zone of approximately 10 meters on each side. Given the aims of the study, only fresh fecal samples from wild mammals were collected. The sampling sessions were conducted between March 2021 and June 2022. Fecal samples were attributed to species based on morphological characteristics (appearance, size), deposition pattern, and content. Freshness was assessed based on consistency, color, moisture, mucus, and odor [89,90]. All samples were collected using sterile gloves (EN455), aliquoted into Eppendorf tubes, and stored at –80 °C within six hours of collection.

2.3. Sample Processing and PCR

In this study, samples were primarily analyzed in pools for conventional PCR screening and subsequent NGS analysis. Pools consisting of 3 to 4 fecal samples were created by grouping individuals belonging to the same species from the same or neighboring transects. Nucleic acid extraction was performed using the AllPrep PowerFecal DNA/RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including optional on-column DNase and RNase digestion. After lysis and clarification, an additional filtration step was performed using 0.45 µm filters (Euroclone, Milan, Italy). Extracted nucleic acids were quantified using a Nanodrop spectrophotometer (Thermofisher scientific, Waltam, MA, USA), aliquoted, and stored at –80 °C until further analysis.

2.3.1. PCR Screening

Conventional PRC screening has been performed on each pooled sample targeting the major fecal-shed viruses. Based on an extensive literature review, species-specific viral target panels were developed, while for less-studied species such as the porcupine (*Hystrix cristata)*, badger (*Meles meles*), and small mustelids, broader panels were designed to include viruses from phylogenetically related taxa as well (supplementary data).

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When possible—particularly for species with limited or no virological data—broad-range PCR protocols were also employed [2,13–21]. This strategy maximized the information obtained from each sample and provided an indirect means of investigating elusive species that are difficult or impossible to sample directly. Primer sequences used in this study, along reference details are provided in Table 1.

Table 1. : 5′-3′ primer sequences used for PCR analysis.

	5′-3′PRIMER FORWARD	5′-3′ PRIMER REVERSE	REFERENCE
Adenovirus spp*	1° round: TNMGNGGNGGNMGNTGYTAYCC 2° round: GTDGCRAANSHNCCRTABARNGMRT	1° round: GTDGCRAANSHNCCRT ABARNGMRTT 2° round: CCANCCBCDRTTRTGN ARNGTRA	[22]
CAdV 1,2	CGCGCTGAACATTACTACCTTGTC	CCTAGAGCACTTCGTG TCCGCTT	[23]
CPV	ACAAGATAAAAGACGTGGTGTAACTC AA	CAACCTCAGCTGGTCT CATAATAGT	[24]
FPV/CPV	ACAAGATAAAAGACGTGGTGTAACTC AA	CAACCTCAGCTGGTCT CATAATAGT	[25]
Bocavirus spp*	GCCAGCACNGGNAARACMAA	CATNAGNCAYTCYTCC CACCA	[26]
CBov 1	1° round: CARTGGTAYGCTCCMATYTTTAA 2° round: TGGTAYGCTCCMATYTTTAAYGG	1° round: TGGCTCCCGTCACAAA AKATRTG 2° round: GCTCCCGTCACAAAAK ATRTGAAC	[27]
CBoV 2	AGGTCGGCCACTGGCTGT	CAGCTTAACGGCATTC ACTA	[26]
CBoV 3	1° round: CAGATTTGGGGGTCCTGCAT 2° round: ATGCCGTCACCAATCCACAT	1° round: GCACTGTCTGCGCTGA AAAA 2° round: AGCTTGTGGTGGACAG TAGC	[28]
LBoV	AGACCAGATGCTCCACATGG	TGCCTGCCACGGATTG TACC	[29]
Canine CV	CTGAAAGATAAAGGCCTCTCGCT	AGGGGGGTGAACAGG TAAACG	[30]
TTV1	CGGGTTCAGGAGGCTCAAT	GCCATTCGGAACTGCA CTTACT	[31]
TTV2	TCATGACAGGGTTCACCGGA	CGTCTGCGCACTTACT TATATACTCTA	[31]
Kobuvirus spp*	TGGAYTACAAGTGTTTTGATGC	ATGTTGTTRATGATGG TGTTGA	[32]
Astrovirus spp*	1° round a: GARTTYGATTGGRCKCGKTAYGA 1° round b: GARTTYGATTGGRCKAGGTAYGA 2° round a: CGKTAYGATGGKACKATHCC	GGYTTKACCCACATNC CRAA	[33]

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	2° round b: AGGTAYGATGGKACKATHCC		
Coronavirus spp.*	1° round: GGKTGGGAYTAYCCKAARTG *--2° round: GGTTGGGACTATCCTAAGTGTGA	1° round: TGYTGTSWRCARAAYT CRTG 2° round: CCATCATCAGATAGAA TCATCAT	[34]
BVDV	ATGCCCWTAGTAGGACTAGCA	TCAACTCCATGTGCCA TGTAC	[35]
Bopivirus spp*	CTGRGCAAGTTCACCAACAA	GTCCATGACAGGGTGA ATCA	[36]
CDV	ACTTCCGCGATCTCCACTGG	GCTCCACTGCATCTGT ATGG	[29]

For the detection of viral DNA targets, conventional PCR was performed using the Wonder Taq Hot Start Kit (Euroclone, Milan, Italy), while RNA viruses were analyzed via RT-PCR using the OneStep RT-PCR Kit (Qiagen, Hilden, Germany). For the second round of nested or seminested protocols, Wonder Taq Hot Start was again employed. All reactions were set up following the manufacturers' protocols.

PCR products were subjected to agarose gel electrophoresis to verify the presence of the amplicons of expected size. Samples showing bands of the correct size were cut from gel and purified using the EuroSAP PCR Enzymatic Clean-up Kit (Euroclone, Milan, Italy) prior to sequencing.

Sanger sequencing was performed by BMR Genomics (Padua, Italy). Resulting sequences were analyzed using the online BLAST tool to confirm target identity. High-quality sequences were further processed using BioEdit and submitted to GenBank.

2.4. Metagenomics Analysis

Although PCR using consensus primers is a useful tool for characterizing viral populations, enabling the detection of viruses even distantly related to known ones, this approach remains limited by the need for primer design and the selection of targets by the operator. To overcome these constraints and reduce operator bias, we employed a next-generation sequencing (NGS) approach, which does not rely on prior knowledge of viral sequences. Metagenomic analysis is a well-established methodology with broad detection capabilities and applicability to various biological matrices, making it a valuable tool for exploring samples potentially containing unexpected or novel viruses [37].

Four pooled fecal samples from red foxes (*Vulpes vulpes*) were subjected to metagenomic analysis to characterize the fecal virome. To enable unbiased amplification of viral nucleic acids, both RNA and DNA extracted from each pool underwent Sequence-Independent *Single-Primer Amplification* (SISPA) [38,39].

Following SISPA and quantification using a Qubit fluorometer and the dsDNA Assay Kit (Thermo Fisher Scientific, Waltam, MA, USA), RNA- and DNA-derived products were combined in a 1:1 ratio and submitted to IGA Technology Services Srl (Udine, Italy) for shotgun metagenomic sequencing. Libraries were prepared and sequenced on the Illumina NovaSeq 6000 platform (Illumina, Madison, USA), generating approximately 30 million 150 bp paired-end reads per sample

2.5. Bioinformatics

A preliminary taxonomic classification of the sequencing reads was performed by IGA Technology Services using Kraken, a high-speed and accurate program for taxonomic assignment of DNA sequences.

Subsequently, a more detailed bioinformatic analysis was conducted using Geneious Prime® 2022.2.2 (www.geneious.com), as indicated in Pacini et al. [40] supported by computing resources

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provided by the University of Pisa Data Center. Analyses were carried out on a 64-bit Windows-based virtual machine equipped with dual Intel Xeon Gold 5120 CPUs (2.20 GHz) and 128 GB RAM.

3. Results

3.1. Sample Collection and Pool Preparation

During sampling session, a total of 99 fecal samples were collected along 21 transects. Fifteen samples were excluded during processing due to advanced degradation or uncertain species attribution.

Samples were grouped into 26 pools: 2 pools of roe deer, 4 of fallow deer, 3 of red deer, 4 of red foxes, 4 of wolves, 4 of badgers, 4 of small mustelids, and 1 of porcupines.

3.2. PCR Results

PCR performed on the nucleic acids extracted from fecal samples pool returned with the following results: out of the 26 fecal pools tested, 10 pools (38.5%) were positive for at least one viral target. Viral RNA or DNA were detected in multiple species, including red deer (2/3 pools), red foxes (3/4 pools), wolves (2/4 pools), small mustelids (2/4 pools), and porcupine (1/1 pool). In contrast, all pools from roe deer, fallow deer, and badgers tested negative for the entire panel of viral targets.

Among the viruses investigated, *Astrovirus spp* was the most frequently detected, found in red deer (2/3 pools), red foxes (3/4 pools), wolves (1/4 pools), small mustelids (1/4 pools), and porcupine (1/1 pool). Several additional viruses were identified in red foxes, including bocavirus, *canine parvovirus* (CPV), *kobuvirus, canine adenovirus type* 1 (CAdV-1), and *coronavirus*, each detected in one pool. In wolves, kobuvirus was also found in one pool, while adenovirus was the only additional virus detected in small mustelids (2/4 pools).

No pools tested positive for *canine distemper virus* (CDV), bovine viral diarrhea virus (BVDV), *bovine papillomavirus* (BopV), *canine circovirus*, or *torque teno viruses* (TTV1 and TTV2).

A detailed analysis of the positive pools revealed specific associations between viral targets and host species. Astrovirus was the most frequently detected virus, observed in multiple species. Several other viral agents were identified in red foxes, wolves, and small mustelids.

3.2.1. GenBank Submission and BLAST Analysis

All confirmed positive PCR sequences were submitted to *GenBank*. Table 2 lists accession numbers alongside BLAST results, including the closest match, nucleotide identity percentage, and E-value.

Table 2.

Virus	Host	Genbank	Reference sequence	Per. Ident	E value
CPV	Red Fox	CPV/fox/169/IT OQ079554	*Canine parvovirus 2c* MF177262.1	84,83%	6e-31
CCoV	Red Fox	CCoV/fox/208/IT OQ079555	*Canine coronavirus* ON834692.1	100.00%	0.0
AdV	Small mustelids	RAdV/mustelidae/878/IT OQ079556	*Rodent adenovirus* KY369960.1	75.91%	1e-49
	Small mustelids	RAdV/mustelidae/888/IT OQ079557	*Rodent adenovirus* KY369960.1	74.74%	1e-42
AstV	Red Fox	Mastv/fox/46/IT OQ079558	*Bovine astrovirus* MW373720.1	90.82%	7e-155

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	Porcupine	Mastv/porcupine/484/IT OQ079559	*Mamastrovirus 3* MH399894.1	91,27%	2e-156
	Cervo	Mastv/reddeer/707/IT OQ079560	*Murine astrovirus* JQ408746.1	88.49%	9e-128
	Small mustelids	Mastv/mustelidae/883/IT OQ079561	*Murine astrovirus* JQ408746.1	87.95%	3e-81
	Red Deer	Mastv/redeer/885/IT OQ079562	*Murine astrovirus* JQ408746.1	88.56%	7e-129
	Wolf	CAsV/wolf/481/IT OQ079564	*Canine astrovirus* OQ198039	88.80%	3e-133
BoV	Red Fox	BoV/fox/171/IT OQ079563	*Bocaparvovirus carnivoran5* PP592987	88.55%	3-e83
KoV	Wolf	Kov/wolf/525/IT OQ079565	*Porcine kobuvirus* MH184668.1	93.30%	8e-88
	Red Fox	Kov/fox/88/IT OQ079566	*Fox kobuvirus* KF781172.1	92.49%	5e-63

3.3. Metagenomic Analysis

Metagenomic analysis was performed on 4 red fox pools. Pools were selected based on PCR results, with red foxes chosen due to their high positivity rate and limited virological data in the literature. IGA Technology Services performed a preliminary taxonomic classification using Kraken 2 [40].

In Table 3 are presented the total reads, the percentage of classified vs. unclassified reads, and the taxonomic breakdown. Among classified reads, the vast majority (76–98%) belonged to Bacteria (taxid: 2), While viral reads (Viruses, taxid: 10239) represented 0.4–2%.

Table 3. Results of the taxonomic classification using Kraken 2.

Pool Number	N° of sequences	Classified sequences * (%)	Unclassified sequences * (%)	Bacteria sequences * (%)	Viral sequences * (%)
1	1.86×10^7	21	79	76	2
2	1.79×10^7	29	72	92	1
3	2.17×10^7	26	74	89	0.7
4	2.18×10^7	59	41	98	0.4

The percentage of sequences assigned to Bacteria and Viruses is calculated over the number of classified sequences (*).

3.3.1. In-depth Bioinformatic Analysis

Further analyses were conducted using Geneious Prime®, aiming to refine viral identification from the metagenomic datasets. Sequencing reads were compared against two custom viral databases constructed through the NCBI Virus portal: one comprising all known viral sequences isolated from mammals (TaxID: 40674, excluding Homo sapiens, as of October 21, 2022), and another including viruses known to infect birds (TaxID: 8782). The mammalian virus database contained 470,053 sequences and 1,033 complete reference genomes, while the avian virus database included 297,656 sequences and 212 complete reference genomes.

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Reads were aligned to the reference genomes within these databases to generate consensus sequences, each representing a potential viral fragment identified in the sample. For each consensus sequence, the following parameters were recorded: total consensus length, percentage of reference genome coverage, number of identical sites, pairwise identity, and BLAST E-value.

To ensure reliability and reduce background noise, only consensus sequences meeting strict filtering criteria were retained for further interpretation. Specifically, retained sequences were required to be longer than 150 base pairs, to present more than 75% identical sites, a pairwise identity above 80%, and a BLAST E-value less than or equal to 10^{-100}. [40]. These thresholds were set to minimize false positives and to focus the analysis on highly confident viral identifications. Table 4 presents these results in detail.

Table 4. post data analysis of NGS results.

Pool number	Genbank	Ref- Seq	n° Reads	n° nucleotide	Pairwise Identity	e Value
1	PX314117	*Porcine picobirnavirus* MW978536.1	213	467	100%	0.0
	PX314118	*Rodent circovirus* KY370041.1	7	480	89.97%	1e-145
	PX314119	*Anelloviridae sp.* MF346362.1	2	427	99,77%	0.0
2	PX314120	*Fox adeno-associated virus* KC878874	28	1288	100%	0.0
	PX314121 PX314122 PX314123	*Red squirrel adenovirus -1* NC_035207	27	2129	69.66% 88.58% 100	1e-24 1e-177 0
	PX314124	*Pigeon adenovirus* NC024474	17	319	100%	2e-104
	PX314125 PX314126 PX314127	*Torqueteno felis virus* MT538010	25	779	92.21% 98.06% 70.71%	4e-51 7e-67 2e-30
3	PX314128 PX314129	*Porcine circo-like virus* JF713717	31	1759	90.90% 98.15%	0.0 0.0

4. Discussion

Tuscany hosts a high density of wild mammals and harbors the largest population of wild ungulates in peninsular Italy, estimated at approximately 400,000 individuals, with steadily increasing numbers. The region accounts for about 40% of the national roe deer population, 45% of fallow deer, and 30% of wild boar. The PNFC constitutes a well-preserved ecosystem with high biodiversity and ecological continuity making it particularly suitable for wildlife virological surveillance.

Fecal samples are considered among the most practical and informative matrices for virological studies due to their suitability for non-invasive sampling across a wide range of host species and their capacity to reveal both host-specific enteric viruses and environmentally acquired pathogens [41,42]. They enable the collection of data from animals that cannot be captured or directly handled and are widely used in studies on both domestic and wild animals. Numerous investigations have analyzed viruses from feces or rectal swabs collected from wild animals that were either live-captured, found dead, or culled through management programs [9,11,43–51], using either targeted molecular assays [48,49,51–53] or broader metagenomic approaches for virome characterization [43,45,46,50,54–56].

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Recently, there has been increasing interest in non-invasive environmental sampling, in response to growing awareness of the physiological stress induced by animal capture [29,57,58]. These studies include both targeted viral detection [57,58] and ecosystem-level virome characterization from feces collected in the environment [29,59]. However, only a limited number of studies have applied a multi-species sampling strategy to explore the virome at the ecosystem level.

In this study, a systematic sampling design was adopted based on predefined transects evenly distributed across the study area. The park's biodiversity, ecological continuity, varied habitats, and low levels of anthropogenic disturbance made it particularly suited for this approach. The main limitations concerned the quality and freshness of fecal samples, which were affected by environmental conditions such as temperature, precipitation, snow cover, and leaf litter. Deposition sites also influenced sample integrity, especially regarding potential environmental contamination. However, such contamination was not considered a limiting factor in this context, as the study aimed to characterize the ecosystem virome rather than assess the health status of individual animals.

Samples were processed as pooled specimens to optimize laboratory workflow and because the study area, due to its ecological continuity and limited spatial scale, could be considered a single epidemiological unit. Given the environmental origin of the samples, it cannot be excluded that some originated from the same individual or from members of the same social group in gregarious species.

The positive PCR results support the validity of the viral target selection performed during the study design phase, as most of the selected viruses were detected in at least one sample.

Furthermore, PCR screening revealed that foxes harbored the highest number of detected pathogens among the sampled species. Due to methodological constraints a limited number of samples were selected for metagenomic investigation. Fox sample pools were prioritized, as this species is one of the most widespread wild mammals, with opportunistic feeding habits that include scavenging and consumption of human waste. In recent years, fox populations have increased notably, particularly in urban areas [13,55,60]. Owing to these ecological traits, the red fox has been proposed in several studies as a sentinel species for monitoring ecosystem health, including pollution, climate change, antimicrobial resistance, and infectious diseases [61–66].

The absence of CDV was not unexpected. CDV has been reported in Italy in various carnivores, including canids, foxes, wolves, and mustelids, predominantly in animals found dead with suspected neurological symptoms [67–73]. Conversely, environmental monitoring based on fecal samples has consistently returned negative results [74,75].

Canine circovirus has only recently been identified in Italian foxes, with two independent studies reporting prevalence rates between 2% and 5%, lower than those documented in other European countries [76,77]. In a separate study on wild carnivores in Italy, the virus was not detected in foxes but was identified in nine wolves and one badger [78].

Regarding BVDV, its presence has been documented in red deer, fallow deer, and Apennine chamois in the central Apennines, but not in roe deer [79]. Although molecular evidence from the Alpine region is lacking, serological surveys suggest the potential circulation of the virus among wild ruminant populations in these areas as well [80–82].

In our study *Astrovirus* was the most frequently detected across almost all tested species, including deer, foxes, wolves, small mustelids, and porcupines. This result aligns with its known broad host. Astroviruses have previously been identified in foxes in the Netherlands and Australia through metagenomic approaches [55,65]. In the Netherlands, two novel species—Fox Astrovirus F4 and F5—were described, while Australian sequences showed high similarity to Feline Astrovirus. In our study, sequences obtained from fox pools in the PNFC (OQ079558) showed high homology with *Bovine Astrovirus* (Accession Number MW373720), suggesting the possibility of alimentary origin rather than active infection, further supporting the circulation of the virus in the study area.

Research on astroviruses in cervids is still limited. However, the virus has been identified in roe deer in Denmark (2010), in apparently healthy individuals hunted in Slovenia (2013), and in diarrheic red deer in the UK [83–85]. Additionally, in the U.S., *Astrovirus* was detected in five white-tailed deer (*Odocoileus virginianus*) with respiratory signs [86]. Two strains identified in roe deer have been

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classified as a new species—*Capreolus capreolus astrovirus* (CcAstV strains 1 and 2)—phylogenetically related to strains found in cattle, pigs, yak, porcupines, and dromedaries [84]. The sequences obtained from our deer samples (OQ079560 and OQ079562) showed relatively low nucleotide identity with both, CcAstV and *White-tailed Deer Astrovirus* (74% and 75%, respectively), but high similarity to *Murine Astrovirus* (88,49%), indicating the need for further phylogenetic analyses using more specific primers to refine the identification and contribute to the growing knowledge of astrovirus diversity in wild ruminants.

To date, there are no published reports of astrovirus infection in wolves, European porcupines (*Hystrix cristata*), or the small mustelid species sampled in this study, including weasel (*Mustela nivalis*), beech marten (*Martes foina*), pine marten (*Martes martes*), and polecat (*Mustela putorius*), making our findings the first documented detections in these species.

In a previous study Sarchese et al. reported *Porcine Astrovirus* in a wolf fecal sample, but the authors attributed this to dietary origin [41]. In contrast, the sequence identified in this study in wolf sample (OQ079564) showed homology with *Canine Astrovirus* suggesting a likely host-specific infection.

Although *Porcupine Astrovirus* has not previously been identified in *Hystrix cristata*, it has been reported in the Himalayan porcupine (*Hystrix branchyura*) in China. Phylogenetic analysis by Hu et al. demonstrated high similarity to *Porcine Astrovirus 2* [87]. Our sequence (OQ079559) showed strong homology with both Porcupine Astrovirus (nucleotide identity 86.97%; E-value 2e-113) and *Porcine Astrovirus Mamastrovirus 3* (nucleotide identity 91.27 %; E-value 2e-156), supporting the presence of closely related astroviruses in European porcupines.

Within mustelids, astroviruses have been detected in the European mink (*Mustela lutreola*), a species not present in the wild in Italy, as well as in domestic ferrets (*Mustela putorius furo*) [88,89]. *Mink Astrovirus* causes various clinical syndromes, including Shaking Mink Syndrome (SMS), pre-weaning diarrhea, and Wet Mink Syndrome (WMS) [89–91]. In ferrets, *Murine Astrovirus* has been identified. Similarly, sequence from the small mustelid pool (OQ079561) showed high similarity to *Murine Astrovirus* (88.4% nucleotide identity; E-value 8e-77) and to *Rodent Astrovirus* at percentage of identity 89.16% with E-value 5e-24. Since rodents are part of the mustelid diet, alimentary origin is plausible. However, given the detection of similar sequences in farmed ferrets fed poultry-based diets, further studies are needed to clarify transmission routes and evaluate the potential for true infection in wild mustelids [88].

Kobuviruses were also detected in foxes and wolves. Belonging to the *Picornaviridae* family, the *Kobuvirus* genus includes three species: *Aichivirus A* (Aichi virus), *Aichivirus B* (bovine kobuvirus), and *Aichivirus C* (porcine kobuvirus). In recent years, *kobuviruses* have been identified in an increasing number of host species worldwide, including domestic and wild carnivores, ruminants, suids, mustelids, rodents, and bats, indicating a continuously expanding host range [45,58,88,92,93,93–95].

In Italy, *kobuviruses* have been reported in dogs, foxes, wolves, roe deer, goats, pigs, cats, and cattle [92,93,96–99]. In our study, sequences obtained in foxes (OQ079566) by positive PCR analysis showed high nucleotide identity with *Canine Kobuvirus* (CaKoV), particularly with those previously identified in Italian foxes (KF781172; nucleotide identity 92.49%; E-value 2e-61). CaKoV is often found in co-infection with other viruses, likely acting as a secondary pathogen following primary infections by immunosuppressive agents such as *canine distemper virus* (CDV) or *canine parvovirus* [98,100,101]. Di Martino et al. reported CaKoV and *Canine Coronavirus* (CCoV) co-infection in both a fox and several dogs [92,102].

CaKoV detection in wolves has already been documented in Italy by Melegari et al. (2018), with a reported prevalence of 4.9% [93]. However, in our case, the wolf sequence identified (OQ079565) showed greater similarity to *Porcine Kobuvirus* (OQ129479.), suggesting a possible dietary origin. This finding is particularly relevant given that no wild suid samples were included in this study, as none were available at the time of sampling. Nevertheless, the detection in a wolf may indirectly reflect the circulation of *kobuvirus* in the local wild boar population.

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By metagenomic analyses and PCR assays using *Adenovirus spp* generic primers, sequences related to *Squirrel adenovirus* (PX314121; PX314122; PX314123) and rodent Mastadenovirus (MAdV) (OQ079556; OQ079557), were detected.

In recent years, SqAdV has gained increasing relevance due to the expansion of its geographical range across several European regions and its impact on the health of the Eurasian red squirrel (*Sciurus vulgaris*), with significant implications for conservation and reintroduction programs targeting this protected species [103–108]. Similar to what has been documented for Squirrel poxvirus, SqAdV was introduced into Europe by the invasive Eastern grey squirrel (*Sciurus carolinensis*), which carries the virus asymptomatically. The pathogen subsequently spread among local populations, both free-living and captive, causing frequent fatal infections [104]. SqAdV infection was first reported in the United Kingdom in 1997 and has since been documented in several countries, including Germany, Portugal, South Korea, and Italy (Piedmont and Lombardy, 2014) [103,105–108]. The detection of SqAdV-1 by shotgun assay in fox faeces (PX314121; PX314122; PX314123), likely following predation events, represents a potential warning sign for the red squirrel population inhabiting the study area. Such detection may highlight a health issue that is difficult to identify by other means, as the infection does not typically produce easily recognisable clinical signs and, in natural environments, carcasses of deceased animals are rapidly removed by predators or scavengers. Metagenomic analysis performed on fox pool 2 gained a sequence (PX314124) related to *Aviadenovirus* (E-value: 3e-107). In this case as well, the finding is likely attributable to fox predatory activity, further supporting the role of this skilled predator as a sentinel species within the ecosystem.

In this research positivity for *Adenovirus* was also confirmed by PCR in two samples from small mustelids (OQ079556, OQ079557). In England, previous studies identified novel adenoviruses from liver samples of martens and otters: Marten adenovirus 1 and 2 (MAdV-1, MAdV-2) and Lutrine adenovirus (LAdV-1) [109]. While MAdV-2 and LAdV-1 are genetically similar to bat Mastadenoviruses (Vespertilionid adenovirus type 1 and Indian flying fox adenovirus type 5) and may have evolved through co-speciation events with their hosts, MAdV-1 is closely related to *Aviadenoviruses* and has been hypothesised to result from an historic prey-to-predator species jump, given the bird-rich diet of martens. In the present study, the Adenovirus sequences detected in mustelids (OQ079556: OQ079557) showed the highest similarity to rodent Mastadenoviruses, specifically Rodent adenovirus (KY369960, nucleotide identity 74.7–75.9%; E-value 1e-42–1e-49) and Rattus norvegicus adenovirus KU258174 (69.7–70.4% identity; E-value 1e-17–4e-17). It is plausible that these viruses have an alimentary origin, a hypothesis supported by the detection of a rodent-specific astrovirus in one of the two positive pools. Nevertheless, the possibility that these adenoviruses can infect small mustelids cannot be ruled out, as suggested by the precedent of MAdV-1 and the relatively low nucleotide identity observed, leaving open the hypothesis of a prey-to-predator host-switching event.

Canine coronavirus (CCoV), Canine Parvovirus (CPV) and Canine bocavirus (CBoV) were detected by PCR in foxes from the study area. These viruses are widespread among companion animals in Italy and are primarily associated with gastrointestinal disease, though extra-intestinal manifestations are also reported, affecting domestic carnivores and, less frequently, wildlife.

Canine coronavirus, has been reported in several wild canids, including coyotes, golden jackals — a species recently expanding its range into northern Italy — wolves, and foxes, although its pathogenic role in these species remains unclear [17,110–112]. In addition, CCoV has been detected in other carnivores such as the common genet, mongoose, and Eurasian otter [111,113]. In foxes, both genotype 1 and genotype 2 have been reported in China, and CCoV has also been detected in Portugal and Italy [111,114,115]. In wolves, alongside the classical form, the highly pathogenic pantropic variant (pCCoV) has been identified in Italy; this hyper-virulent strain is associated with leukopenia and high mortality rates [110,112]. In the present study, the fox CCoV sequence (OQ079555) showed the highest identity with a genotype 2 strain previously obtained from a in Italy and from a wolf (ON834692: 100% nucleotide identity; E-value = 0.0).

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The same fox pool (number 2) also tested positive for CPV. In Europe, CPV is widely documented in numerous wild carnivore species, particularly canids and mustelids, but also ursids, procyonids, and viverrids [111,113,116–119]. A recent survey by Ndiana et al. (2021) [25] on the presence of *Carnivore protoparvoviruses* (CPV and FPV) in Italian wildlife reported an overall prevalence of 11.4%. CPV was found at high prevalence in wolves (53.4%) and badgers (60%), but appeared absent in foxes, where only FPV was detected (2.8% prevalence) and absent in wolves. These results align with literature indicating frequent detection of FPV-like viruses in foxes, leading some authors to hypothesise that foxes may have acted as an intermediate host species in the adaptation of FPV to dogs and its evolution into CPV. In contrast, CPV prevalence in foxes is usually reported as low, suggesting greater resistance compared to other wild carnivores such as wolves [113,116,117,120,121]. Our findings contrast with these patterns, as the only Carnivore protoparvovirus-1 positivity was recorded in a fox pool, while all wolf and mustelid samples — pooled or individual — tested negative. Moreover, the sequence obtained was more closely related to CPV than FPV. This is not the first detection of CPV in foxes in Italy, as it was previously reported by Zaccaria et al. [78]. The sequence in our study (OQ079554) showed the highest similarity to CPV-2c strains. Expanding sequence analysis to larger and more informative genomic regions would be valuable for more reliable phylogenetic inferences, particularly considering the evolutionary dynamics of CPV. Indeed, CPV appears to mutate more rapidly than FPV and most DNA viruses, as supported by phylogenetic analyses from Allison et al., which revealed substantial genetic diversity among parvoviruses in wildlife [18,122–124].

In recent years, a novel parvovirus, designated fox parvovirus, has been identified in foxes. Classified as Carnivore protoparvovirus-4, it is genetically distant from both CPV and FPV, as well as from Blue fox parvovirus and Gray fox Amdovirus [55,125].

Regarding other species analysed in this study, the complete absence of CPV detection in wolves was unexpected, given previous reports from Italy, including in the same study population[57,75,118,126]. For instance, Martinello et al. detected CPV in 4/49 wolf faecal samples collected in the PNFC, while all samples from three other areas — the Orecchiella Nature Park, the Alto Appennino Reggiano Regional Park, and the Alta Val Parma Reserve — tested negative [57].

Within the *Parvoviridae* family, a sequence corresponding to Fox adeno-associated virus, a novel adeno-associated virus (AAV) was identified in a fox fecal sample by NGS methodology (PX314120) [55]. AAVs belong to the genus *Dependoparvovirus*, so named because they are unable to replicate autonomously and require a helper virus, typically an adenovirus [127]. In our study, Fox AAV was detected in a pool in which two adenoviruses (*Squirrel adenovirus* and an *Aviadenovirus*) were also present; it would therefore be of interest to determine whether these viruses occurred in the same individual and whether Fox AAV was actively replicating. Several AAV serotypes have been isolated from birds and a wide range of mammalian species, including humans, but they have not been associated with clinical disease [127]. Although extensively studied for their potential as vectors in gene therapy and vaccine development, information on their circulation in domestic and wild animal populations remains limited.

Bocaparvovirus is a member of the *Parvovirinae* subfamily and has been reported in humans as well as in a wide range of domestic and wild species [29,42,56,128–131]. In both humans and animals, bocaviruses are mainly associated with gastrointestinal and respiratory disorders, although neurological manifestations have also been described, for instance in piglets infected with Porcine bocaparvovirus and in dogs infected with Canine bocaparvovirus 2 [130,132]. In wildlife, several novel bocaparvoviruses have been identified in recent years, including Pine marten bocavirus [56], Lupine bocavirus [29,55,133], and Fox bocavirus [55], all of which show genetic relationships with Porcine bocaparvovirus. In our study, the sequence obtained (OQ079563) displayed significant homology with both Feline bocaparvovirus 3 and Lupine bocavirus, suggesting the circulation of divergent strains in wild carnivores and warranting further investigations for accurate classification.

Picobirnavirus (PBV) is the only genus within the *Picobirnaviridae* family and has been identified in humans as well as in a wide range of animals, including mammals, reptiles, and birds, both

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domestic and wild [134]. The clinical relevance of these viruses remains controversial. In humans, PBVs have been detected in patients with gastroenteritis, either as sole agents or in coinfection with other enteric pathogens. In animals, they have been reported in fecal samples from both symptomatic and asymptomatic hosts, making it difficult to confirm or exclude a direct etiological role in enteric diseases, typically characterized by profuse diarrhea [134–136]. Currently, PBVs are mostly regarded as "opportunistic diarrhoeagenic pathogens," excreted under conditions of immunosuppression or stress, such as in captive or farmed animals [134,135]. In red foxes, PBV sequences have already been described. In 2013, Bodewes et al., during a metagenomic analysis of the fecal virome of foxes, identified numerous sequences related to Picobirnavirus, including some clustering with the already known *Microtus picobirnavirus* and others assigned to a novel species, designated *Fox picobirnavirus* [55]. In our study, the sequence obtained (PX314117) showed high homology with a *Porcine picobirnavirus* strain detected in pig fecal samples. *Porcine picobirnavirus* was among the first PBVs identified in livestock and has since been reported in pig farms across different geographic regions, as well as in fecal samples from wild boars [134,136–138]. Considering that the main transmission route is fecal–oral and that wild boars are widespread within the PNFC, it is plausible that the presence of PBV in fox feces reflects ingestion of water or food contaminated with fecal material from infected wild boars.

Sequences belonging to the family *Circoviridae* (PX314128; PX314129) were also identified, most likely as a result of predation or contamination of food material. These sequences were assigned to Rodent circovirus and to a Porcine circovirus-like. The latter belongs to a heterogeneous group of viruses classified among the unclassified *Circoviridae* but genetically related to *Porcine circovirus* (PCV). Some members of this group have been isolated from pigs affected by postweaning multisystemic wasting syndrome (PMWS), a condition typically associated with PCV2 [139–141]. Since sequences related to this virus were detected in a pool of fox fecal samples, it is plausible that their origin lies in wild suids rather than domestic pigs, through fecal shedding and subsequent environmental contamination. This hypothesis is supported by the fact that, although *Porcine circovirus-like* has not yet been described in wild boars, these animals are known to be susceptible to PCV2, PCV3, and PCV4 [142–145]. In Europe, PCV2 seroprevalence in wild boars ranges from 23% to 65%, while in Italy it is estimated at around 39% [142,146,147]. Furthermore, as in domestic pigs, PCV2 has been demonstrated to cause PMWS in wild boars and to be vertically transmitted from sows to fetuses, potentially leading to reproductive disorders [142,143].

The sequence attributed to *Rodent circovirus* (PX314118) showed high similarity with sequences reported by Wu et al. in a study characterizing the fecal virome of rodents and other small mammals [148]. To date, however, reports of circoviruses in rodents remain scarce.

Finally, sequences belonging to the family *Anelloviridae* were also identified. Members of this family are widely distributed in humans and animals, although their impact on host health is still unclear. The sequence obtained in our study (PX314119) showed homology with Torque teno viruses (TTV) described in rodents, with an unclassified anelloviridae and with *Rodent Torque teno virus 1*. Unexpectedly, a TTV felis was also detected in feces attributed to foxes (PX314125; PX314126; PX34127). This virus has so far been reported only in felids, both domestic and wild, including the ocelot (*Leopardus pardalis*), puma (*Puma concolor*), bobcat (*Lynx rufus*), Canada lynx (*Lynx canadensis*), and caracal (*Caracal caracal*) [149,150]. Considering that the wildcat (*Felis silvestris*) is present in the PNFC area from which the fecal pool originated, this finding may reflect either contamination or misattribution of feces. Indeed, although the assignment of samples was carried out carefully and following established criteria, it cannot be ruled out that the pool included feces from *Felis silvestris* rather than from foxes, given the morphological and compositional similarities between the feces of these two species.

Our results highlight some interesting differences compared with recent investigations carried out on wildlife admitted to the Veterinary Teaching Hospital of the University of Pisa, following recovery from the surrounding province [40,151]. In the Pisa area, a lower number of viral taxa were detected, but with higher prevalence rates, whereas in the PNFC we identified a broader viral

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diversity, although at lower prevalence. These contrasting patterns can be plausibly explained by the ecological features of the two study areas. The province of Pisa is characterized by lower biodiversity and highly fragmented habitats, where wild animals live at higher population densities; this scenario may favor the circulation and high prevalence of specific pathogens. In contrast, the PNFC harbors greater biodiversity and large, continuous habitats, which likely support the coexistence of multiple viral species but limit their widespread circulation [40,151].

Another point deserving attention is the discrepancy observed between conventional PCR assays and metagenomic sequencing performed on the same samples. Several viral targets identified by PCR were not subsequently detected through metagenomics. This discrepancy largely reflects the intrinsic properties of the two methods. PCR remains highly reliable for specific targets, offering high sensitivity and specificity, particularly when nested protocols are applied and amplification products can be re-amplified to obtain sequences suitable for downstream analyses. Conversely, metagenomic workflows rely on random amplification strategies such as SISPA, which, although valuable in contexts where viral loads are expected to be low, inevitably introduce biases. SISPA may distort genome coverage by over-representing some regions while leaving others underrepresented, thereby limiting the recovery of complete genomes. It also tends to favor the amplification of more abundant viral genomes at the expense of those less represented, and contributes to an increase in unclassified sequences [152].

Despite these limitations, metagenomics proved to be a powerful complementary tool, enabling the detection of unexpected or previously unreported viruses that would not have been included in targeted PCR screening. The combined use of conventional and next-generation molecular approaches thus broadened the spectrum of detectable viruses, allowing the identification of both known pathogens—sometimes underrepresented in the population—and novel or unexpected viruses.

Interestingly, most viral sequences were recovered from carnivorous or scavenging species, reflecting not only their own pathogens but also those of their prey or other sympatric species. This emphasizes the ecological value of the chosen sample type, which provided insight into both the pathogens affecting the sampled species and those circulating in the broader ecosystem.

Overall, our findings document the circulation of pathogens typically associated with domestic species within wildlife populations, supporting the role of wild animals as potential reservoirs, shedders, or propagators. Moreover, we identified viral agents not previously reported in the host species or in Italy, underscoring the limited knowledge of the wildlife virome and its dynamic nature. These viruses may circulate silently, cross species barriers, and occasionally expand their host range. The detection of viruses considered non-pathogenic, or for which the impact on host health remains unknown, is also noteworthy. Microorganisms, including viruses, are integral components of ecosystems and often establish balanced interactions with their hosts. However, this equilibrium is fragile and increasingly threatened by global changes such as climate change, pollution, deforestation, and habitat degradation, all of which may disrupt host–pathogen dynamics and favor the emergence of disease outbreaks—even from microorganisms traditionally regarded as harmless.

In this perspective, our study confirms the importance of continued research on infectious agents in wildlife within a One Health framework, as a fundamental step toward safeguarding both animal and human health.

5. Conclusions

This study demonstrated the effectiveness of a non-invasive approach based on environmental fecal sampling for virological surveillance of wildlife in a high-biodiversity ecosystem such as the PNFC. The integration of conventional molecular techniques and metagenomic analyses enabled the detection of a wide range of viruses, including known pathogens, emerging viruses, and agents not previously reported in certain host species or in Italy. The findings confirm the central role of carnivorous and scavenging species, such as the red fox, as ecological sentinels capable of reflecting viral circulation at the community level. The presence of viruses typically associated with domestic

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species suggests potential interactions between wildlife and anthropogenic environments, with implications for both animal and public health. Moreover, the detection of viruses considered non-pathogenic or of unknown impact highlights the need to deepen our understanding of host–virus dynamics in natural settings. From a One Health perspective, ongoing monitoring of the wildlife virome represents a crucial tool for anticipating and mitigating the risks associated with the emergence of new infectious diseases.

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Abbreviations

Canine parvovirus (CPV)
Canine adenovirus type 1 (CAdV-1)
Canine distemper virus (CDV)
Bovine viral diarrhea virus (BVDV)
Bovine papillomavirus (BopV)
Canine circovirus (CCoV)
Torque teno viruses (TTV1 and TTV2)
Adenovirus (AdV)
Rodent Adenovirus (RAdV)
Mastadenovirus (MAdV)
Postweaning multisystemic wasting syndrome (PMWS)
Rodent Mastadenovirus (RMAdV)
Squirrel adenovirus (SqAdv)
Canine astrovirus (CAsV)
Bokaviru (BoV)
Kobuvirus (KoV)
Canine Kobuvirus (CaKoV)
Capreolus capreolus astrovirus (CcAstV)
Wet Mink Syndrome (WMS)
Lutrine adenovirus (LAdV-1)
Picobirnavirus (PBV)
Porcine circovirus (PCV)
Parco Nazionale Foreste Casentinesi (PNFC)

Appendix A

[2–14]	AdV	PV	BoV	CV	TTV	V	AstV	CoV	PeV	BoPV	MoV	References
Roe deer	*AdV spp.* *	-	*BoV spp.* *	-	-	*KoV spp.* *	*AstV spp.* *	*CoV spp.* *	*BVDV*	*BopV spp.* *	-	

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Fallow deer	*AdV spp.**	-	*BoV spp.**	-	-	*KoV spp.**	*AstV spp.**	*CoV spp.**	*BVDV*	*BopV spp.**	-	[48,83,84,153–161]
Red deer	*AdV spp.**	-	*BoV spp.**	-	-	*KoV spp.**	*AstV spp.**	*CoV spp.**	*BVDV*	*BopV spp.**	-	
Red fox	*CAdV 1,2* *AdV spp.**	*CPV* *FPV*	*CBoV 1,2,3* *BoV spp.**	*CCV*	-	*KoV spp.**	*AstV spp.**	*CoV spp.**	-	-	*CDV*	[54,55,67,87,114,162–171]
Wolf	*CAdV 1,2* *AdV spp.**	*CPV* *FPV*	*LBoV* *CBoV 1,2,3* *BoV spp.**	*CCV*	-	*KoV spp.**	*AstV spp.**	*CoV spp.**	-	-	*CDV*	[57,153,162–164,166,170–173]
Badger	*CAdV 1,2* *AdV spp.**	*CPV* *FPV*	*BoV spp.**	-	*TTV 1,2*	*KoV spp.**	*AstV spp.**	*CoV spp.**	-	-	*CDV*	[54,162,166,167,174–183]
Small mustelids	*CAdV 1,2* *AdV spp.**	*CPV* *FPV*	*BoV spp.**	-	*TTV 1,2*	*KoV spp.**	*AstV spp.**	*CoV spp.**	-	-	*CDV*	
Porcupine	*AdV spp.**	-	*BoV spp.**	-	-	*KoV spp.**	*AstV spp**	*CoV spp.**	-	-	-	[45,87,177,184–187]

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