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Circovirus in domestic and wild carnivores: An important opportunistic agent?

Guendalina Zaccaria [a], Daniela Malatesta [a], Gabriella Scipioni [a], Elisabetta Di Felice [a], Marco Campolo [b,c], Claudia Casaccia [a], Giovanni Savini [a], Daria Di Sabatino [a], Alessio Lorusso [a,*]

[a] Virology Unit, Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise (IZSAM), Teramo, Italy
[b] Veterinary Practitioner Centro Veterinario Einaudi, Bari, Italy
[c] Zoo delle Maitine, Pesco Sannita, Benevento, Italy

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ABSTRACT

Circoviruses are relatively novel pathogens with increased importance in canids. In this study, we first screened the presence of dog circovirus (DogCV) by molecular methods from a total number of 389 internal organ samples originating from 277 individuals of domestic dogs and wild animals including wolves, foxes and badgers. All the animals originated from Central-Southern Italy, specifically from Abruzzi and Molise regions, areas hosting several natural parks. DogCV was detected in 9/34 wolves (P=26.4%; IC 95%: 14.6–43.1%), 8/209 dogs (P=3.8%; IC 95%: 1.9–7.3%), 0/24 foxes (P=0%; IC 95%: 0–13.8%), 1/10 badgers (P=10%; IC 95%: 1.79–40.4%). However, all DogCV positive animals were shown to be infected at least by an additional key pathogen, including canine distemper virus (CDV) and canine parvovirus type 2. All wolves, but one, presenting DogCV in the internal tissues suffered from CDV infection. The DNA purified from 17 DogCV infected organs was used for whole genome sequencing and phylogenetic analysis.

Introduction

Circoviruses (CVs) are non-enveloped viruses with a single-strand circular DNA genome (≈ 2 kb), belonging to the family *Circoviridae*. According to the International Committee on Taxonomy of Viruses (ICTV, http://www.ictvonline.org/virustaxonomy.asp), two genera, *Circovirus* and *Gyrovirus* are officially recognized within the family *Circoviridae*. Moreover, a third genus, *Cyclovirus,* has been recently proposed to be part of this family (Biagini et al., 2011; Li et al., 2010). CVs have an ambisense genome organization with 2 major inversely arranged ORFs, ORF1 and ORF2, encoding for the rolling circle replication initiator protein gene (Rep) and a capsid protein gene (Cap), respectively (Biagini et al., 2011).

CVs have been detected in several animal species including birds, pigs and dogs (Biagini et al., 2011; Kapoor et al., 2012; Todd, 2004). There are multiple potential clinical outcomes of CV infection including respiratory and enteric disease, dermatitis, and reproductive problems. Moreover, a circovirus was identified very recently in serum samples from foxes with unexplained neurologic signs by using viral metagenomics (Bexton et al., 2015).

Dog circovirus (DogCV) has been described in cases of dogs with vasculitis and/or haemorrhagic enteritis in USA and Italy (Decaro et al., 2014; Li et al., 2013). Regardless of the prevalence of circovirus infection, the pathogenic role of DogCV in single or polymicrobial infections is undetermined as well as the prevalence of this virus in other wild carnivores.

In this study we describe the detection and molecular characterization of DogCV from the internal organs of dogs and wild carnivores including wolves and badgers collected in Abruzzi and Molise regions (Central-Southern Italy) during 2013 and 2014. The full-length genomes of 17 DogCV strains were determined and compared with extant American and European strains, including the recent CVs detected in foxes. As far as we know, this is the first large set of CV sequences available from carnivore hosts.

Results

DogCV DNA was found by rtPCR$_{DogCV}$ in 32 out of 389 samples (Table 1) belonging to 18 different individuals out of 277 (P=6.5%; IC 95%: 4.1–10%) with a diverse prevalence according to the tested

* Corresponding author. Tel.: +39 086 133 2440; Fax: +39 086 133 2251.
E-mail address: a.lorusso@izs.it (A. Lorusso).

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Table 1
Case history, tissues tested, C_T values of DogCV and FoxCV rtPCRs and GenBank accession number of DogCV genomes sequenced in this study. C_T cycle threshold values of rtPCRs; M, male; F, female; –, negative; ND, not determined; CPV-2, canine parvovirus type 2; CDV, canine distemper virus; *T. britovi*, *Trichinella britovi*.

Sample name	Species	Year	Age	Sex	Anamnesis	Matrix	$C_T$rtPCRs		GenBank accession number	Other pathogens detected
							DogCV	FoxCV		
AZ601	Wolf	2013	< 1 year	M	Trauma	Intestine	39	–	ND	CPV-2, CDV
AZ663	Wolf	2013	< 1 year	F	Euthanized, neurological symptoms	Spleen	34	35	KT734820	CDV
						Brain	39	41	ND	
						Intestine	38	41	KT734824	
						Lung	43	43	ND	
AZ982	Wolf	2013	< 1 year	M	Trauma	Intestine	40	–	ND	CPV-2, CDV, *T. britovi*
						Spleen	36	–	ND	
TE2648	Wolf	2013	< 1 year	F	Found dead	Intestine	43	–	ND	CDV, *T. britovi*
						Lung	33	45	ND	
						Brain	38	–	ND	
AZ2972	Dog	2013	< 1 year	M	Gastrointestinal and respiratory symptoms	Intestine	29	41	KT734813	CPV-2, disseminated aspergillosis
PE3302	Dog	2013	< 1 year	M	Gastrointestinal and respiratory symptoms	Intestine	42	35	ND	CPV-2
						Lymph nodes	40	33	ND	
TE4016	Wolf	2013	< 1 year	M	Found dead	Spleen	24	37	KT734814	CDV
						Lung	32	44	ND	
						Brain	32	39	ND	
AZ4133	Wolf	2013	> 1 year	F	Found dead	Spleen	26	40	KT734815	CPV-2, CDV, *T. Britovi*
						Intestine	31	47	KT734827	
AZ4438	Badger	2013	> 1 year	M	Trauma	Intestine	33	34	KT734816	CPV-2
AZ5586	Wolf	2013	> 1 year	M	Trauma	Lung	30	37	KT734819	CDV
LN5921	Dog	2013	3 months	F	Found dead	Intestine	39	–	ND	CPV-2
TE6685	Dog	2013	< 1 year	F	Gastrointestinal and neurological symptoms	Lung	32	22	KT734825	CDV
						Brain	34	27	KT734821	CDV
TE7482	Wolf	2013	< 1 year	M	Trauma	Intestine	26	43	KT734822	CPV-2, CDV
PE8575	Dog	2013	< 1 year	F	Found dead	Intestine	19	28	KT734826	CPV-2
						Lymph nodes	25	35	KT734823	
TE28415	Dog	2013	6 months		Gastrointestinal symptoms	Intestine	42	43	ND	CPV-2
TE31406/1	Dog	2013	3 months	M	Gastrointestinal symptoms	Intestine	38	–	ND	CPV-2, CDV, *Coccidia spp*
AZ5212	Dog	2014	> 1 year		Found dead	Liver	27	41	KT734817	CPV-2, *Klebsiella oxytoca*
						Lung	38	46	KT734818	
CB6293	Wolf	2014	> 1 year	M	Trauma	Intestine	32	46	KT734828	CPV-2
						Lymph nodes	22	40	KT734812	

animal species. Indeed DogCV was found in 9/34 wolves (P=26.4%; IC 95%: 14.6–43.1%), 8/209 dogs (P=3.8%; IC 95%: 1.9–7.3%), 0/24 foxes (P=0%; IC 95%: 0–13.8%), 1/10 badgers (P=10%; IC 95%: 1.79–40.4%). Significant difference has been demonstrated between the prevalence of DogCV in dogs and wild carnivores (chi-square=9.99; p-value=0.002, α=5%). Furthermore, more significant difference was observed between domestic dogs and wolves (chi-square=22.15; p-value=0.000, α=5%). 13/18 of the DogCV-positive animals were young individuals (< 1 year). DogCV C_T values ranged from 19 to 43 by rtPCR$_{DogCV}$. Spleen, lung, brain, liver, intestine and lymph nodes were the organs where DogCV has been detected. In general, lower DogCV C_T values were evidenced in the lymphatic tissues including spleen and lymph nodes and all DogCV-positive animals resulted co-infected with at least one additional key pathogen. Roughly, DogCV presence was indeed most frequently associated to canine parvovirus type 2 (CPV-2) and/or canine distemper virus (CDV), also in a polymicrobial infection (Table 1). The number of tested animals infected with CDV, CPV-2, DogCV or other viruses is listed in Table 2. Samples obtained from foxes turned negative by rtPCR$_{FoxCV}$. Likewise, also DNA extracts from samples of dogs, wolves and badgers previously demonstrated to be negative by rtPCR$_{DogCV}$ were also negative by rtPCR$_{FoxCV}$. On the other hand, nearly all samples that were demonstrated to be rtPCR$_{DogCV}$-positive were also shown to be positive by rtPCR$_{FoxCV}$ but with, in general, higher C_T values (range 22-47, Table 1). Seven samples were positive by rtPCR$_{DogCV}$ but negative by rtPCR$_{FoxCV}$ whereas four samples from two dogs (PE3302 and TE6685) had lower C_T values when tested by rtPCR$_{FoxCV}$ (Table 1). The full-genome sequences of 17 DogCV strains were obtained from 11 animals (6 wolves, 4 dogs, 1 badger) and deposited in GenBank (accession numbers KT734812-KT734828, Table 1). We sequenced DogCV genome from two different organs of the same animal in six cases (AZ663, AZ4133, TE6685, PE8575, AZ5212 and CB6293); in all cases but one, the sequences obtained from two different organs of the same animal

Table 2
Number of positive individuals for selected viral pathogens within the tested 277 animals. DogCV, dog circovirus; CPV-2, canine parvovirus type 2; CDV, canine distemper virus; SuHV1, Suid herpesvirus 1; CHV, canine herpes virus. CPV-2 DNA was detected by means of the Canine Parvovirus (CPV) kit (Genekam Biotechnology AG, Germany); CHV by means of VeTeK™ CHV Detection kit (iNtRON Biotechnology, South Korea); SuHV1 by means of ADIAVET™ PRV real time (Adiagene-Biomérieux, France); CDV by real-time RT-PCR (Frisk et al., 1999).

Viruses	Dogs	Wolves	*Foxes*	Badgers
Only DogCV	0/209	0/34	*0/24*	0/10
Only CDV	23/209	10/34	*1/24*	0
Only CPV-2	133/209	15/34	*14/24*	4/10
Mixed CDV/CPV-2	12/209	0/34	*0*	1/10
Mixed DogCV/CDV	1/209	3/34	*0*	0
Mixed DogCV/CPV-2	6/209	1/34	*0*	1/10
Mixed DogCV/CDV/CPV-2	1/209	5/34	*0*	0
SuHV1	2/209	0	*0*	0
CHV	2/209	0	*0*	0
None of the above	29/209	0/34	*9/24*	4/10

showed 100% nucleotide (nt) identity. Indeed, only strain AZ5212 showed a synonymous substitution at the protein capsid gene level between the sequences retrieved from liver and lungs. Taking into account one single DogCV sequence per animal, nt identity within the obtained sequences of this study ranged from 94.4% to 99.1% whereas nt identity with the prototype Italian DogCV strain Ba 411-13, detected in puppies with fatal hemorrhagic enteritis in 2013 (Decaro et al., 2014), ranged from 95.6% (strain AZ6685 from a dog) to 98.8% (strain AZ4133 from a wolf). The overall nt identity between our and two European strains (DogCV Ha13 strain from the blood of mongrel dog and strain FUBerlin-JRS from the spleen of an unknown source from Germany), American DogCVs and FoxCVs from the UK ranged from 82.9% to 98.4%. Specifically, DogCV sequences obtained in this study bear the highest sequence identity with the European strains (98.4%), whereas nt identity when compared to FoxCVs and DogCV UCD-3 strain detected in UK and USA, respectively, was lower (82.9–86.3%). DogCVs sequenced from wolves (considering one single sequence per animal) showed nt identity ranging from 95.9% to 98.4%. The wolves from which DogCV whole genomes were sequenced originated from different areas of the Abruzzi and Molise regions (Fig. 1). All wolves but CB6293 (from Molise region) were demonstrated to be CDV-positive and all of them originated from the CDV outbreak of 2013.

When analysed independently, the capsid and the replicase coding sequence (CDS) regions showed an overall nt identity to other DogCVs and FoxCVs of 81.3–99.3% and 81.0–98.9%, respectively. All 17 DogCV strains sequenced in this study were of 2063 nt in length, like all other DogCVs sequences publicly available including the recently reported circoviruses of foxes from UK. Viral genomes of the 17 Italian DogCV strains contained two open reading frames (ORF 1 and 2), on complementary strands in opposite orientation, encoding the putative viral replicase (303 amino acids, aa) and capsid protein (270 aa), respectively. Moreover, two intergenic noncoding regions (135 and 203 nt in length) were located between the start and stop codons, respectively, of the two major ORFs. All the analysed sequences contained in the 5'-intergenic region a characteristic stem-loop structure with a conserved 9-nt motif (TAGTATTAC) for initiation of the rolling-circle replication (Kapoor et al., 2012). A phylogenetic analysis employing the complete genome of the 17 DogCV strains and of representative CVs was performed (Fig. 2). In the neighbour-joining tree, DogCV strains grouped with porcine circoviruses (PCVs) clearly separated from circoviruses of birds. Based on the genetic relatedness of sequences available so far, two distinct clusters may be identified among carnivore circoviruses. All sequences obtained in this study grouped with all extant European DogCV strains and most of the American DogCVs, whereas the North American DogCV strain UCD3 and FoxCVs grouped apparently together into a separate cluster. Circoviruses of the same putative cluster share $>89\%$ nt identity in the genome sequence. The phylogenetic analysis was further verified using maximum likelihood and maximum parsimony analyses, which showed the same tree topology (data not shown).

Discussion

Recent studies have revealed that DogCV circulates in USA and Europe in the canine population. It has also been shown to be responsible alone for severe disease in dogs and foxes (Decaro et

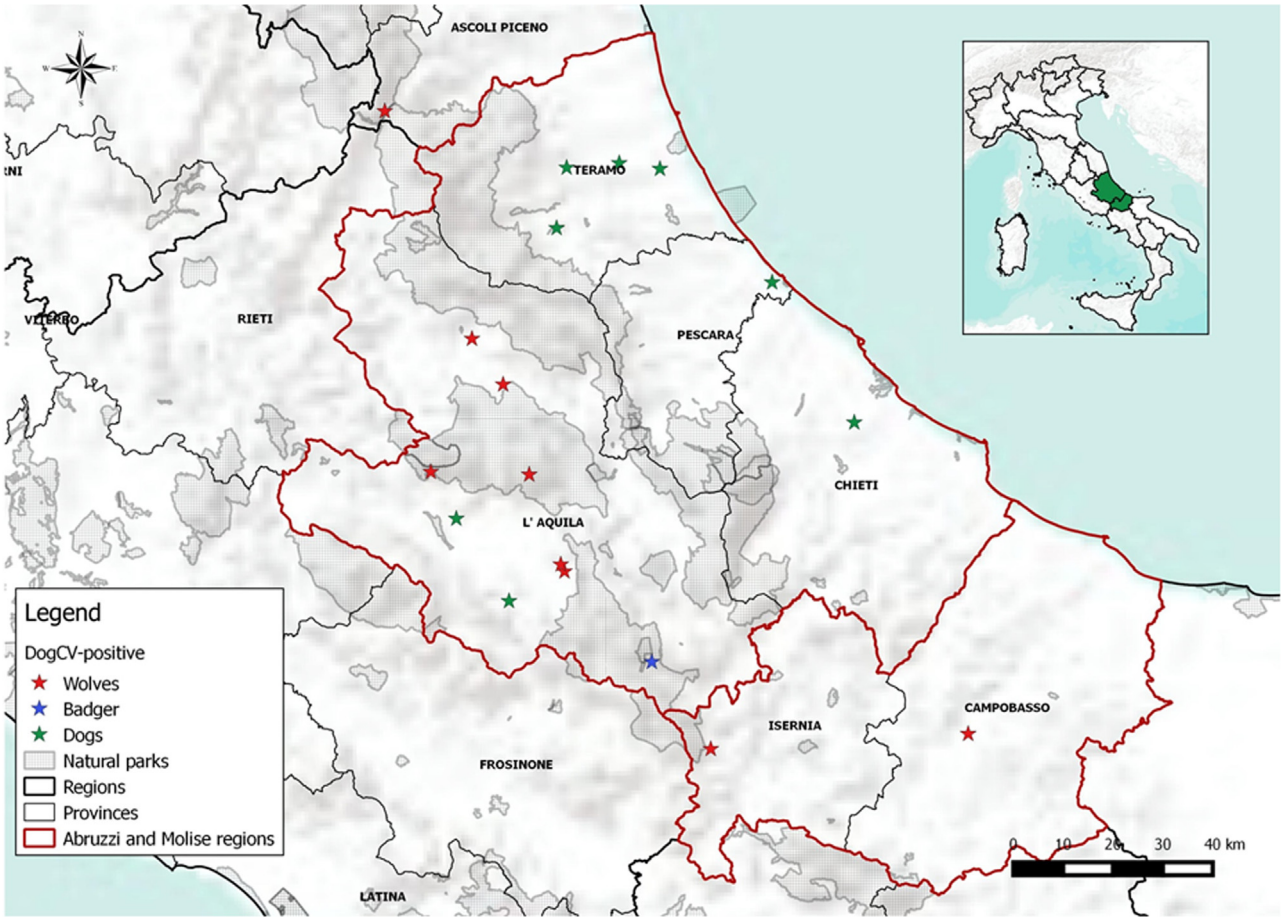

Fig. 1. Map illustrating the distribution of DogCV positive animals in Abruzzi and Molise regions. Map was generated using Quantum GIS (QGIS) software, version 1.8.0.

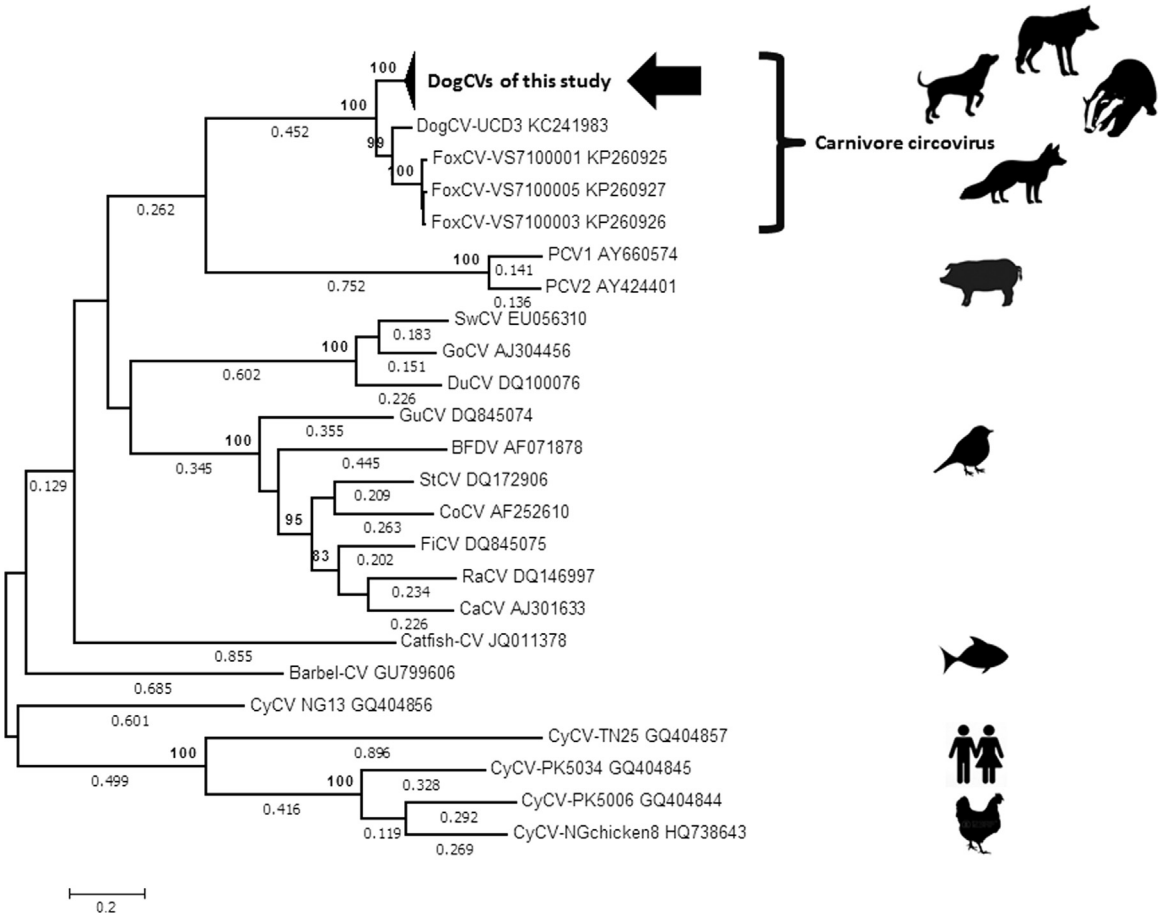

Fig. 2. Neighbour-joining tree inferred from multiple nt sequence alignment of whole genome sequences of the 17 DogCV strains detected in this study. Sequences from representative *Circoviridae* members from different geographical areas available on Genebank, were recruited. Bars indicate the estimated numbers of nt substitutions per site. Bootstrap values ≥ 70 are indicated. FoxCV-VS7100005, KP260927; FoxCV-VS7100003, KP260926; FoxCV-VS7100001, KP260925; DogCV Ba411-13, KJ530972; DogCV-Ha13, KF887949; DogCV-FUBerlin-JRS, KT283604; DogCV-214, JQ821392; DogCV-UCD1, KC241982; DogCV-UCD2, KC241984; DogCV-UCD3, KC241983; Porcine circovirus 1 (PCV1), AY660574; Porcine circovirus 2 (PCV2), AY424401; Finch circovirus (FiCV), DQ845075; Starling circovirus (StCV), DQ172906; Raven circovirus (RaCV), DQ146997; Canary circovirus (CaCV), AJ301633; Columbid circovirus (CoCV) AF252610; Gull circovirus (GuCV), DQ845074; beak and feather disease virus (BFDV), AF071878; Cygnus olor circovirus (SwCV), EU056310; Goose circovirus (GoCV), AJ304456; Duck circovirus (DuCV), DQ100076; Barbel-CV, GU799606; Catfish-CV, JQ011378; Cyclovirus (CyCV) NG13, GQ404856; CyCV TN25, GQ404857; CyCV PK5034, GQ404845; CyCV PK5006, GQ404844; and CyCV NGchicken8, HQ738643.

al., 2014; Li et al., 2013; Bexton et al., 2015). However, its pathogenic role remains yet to be determined particularly in a co-infection process. Indeed, in the present study, DogCV was not detected alone but in combination with other key pathogens including CPV-2 and CDV. DogCV was detected in the internal organs of domestic dogs and also of nine wolves and one badger, thus confirming that viral circulation is not only restricted to dogs but it is also widespread in wild animals (Bexton et al., 2015).

DogCV DNA was detected in the 3.8% (IC 95%: 1.9–7.3%) of tested dogs, which is approximately the same prevalence that has been previously reported in dog serum samples (Kapoor et al., 2012; Li et al., 2013), but lower than the prevalence reported in faecal samples of healthy (6.9%) and diarrhoeic (11.3%) dogs from the USA (Li et al., 2013). In particular conditions, DogCV may be responsible for systemic infection. For PCVs, these conditions may include host characteristics, virus properties, or co-infection processes with other pathogens (Rose et al., 2012) as it is the case of this study. By contrast, we detected 9/34 wolves infected with DogCV with an overall prevalence of 26.4% (IC 95%: 14.6–43.1%) which is significantly higher than the one observed in dogs. Although wolves were co-infected with the same pathogens of dogs including CPV-2 and CDV and that both belong to the same species *Canis lupus*, DogCV was more significantly detected in wolves. Whether this phenomenon affecting DogCV distribution depends by host, viral or environmental conditions, that are broadly different between domestic and free-ranging animals, is hard to say. It is worth to mention that wolves, according to the collection sites of the carcasses, seem to be, in general, geographically unrelated as they were collected up to 170 km apart, thus excluding a more sustained direct transmission of DogCV within wolves. All wolves, but CB6293 collected in the Molise region, suffered from CDV infection caused by the same CDV Arctic lineage strain (Di Sabatino et al., 2014; Marcacci et al., 2014) which massively circulated in these areas during 2013. However, as direct contact with the unvaccinated/infected dog population was likely responsible for the epidemic of CDV in the wildlife ecosystem in which migration of juvenile individuals may have played a pivotal role for CDV geographical expansion (Lorusso and Savini, 2014), this can also be true for circoviruses which, in turn, is more resistant into the environment.

Few factors may have biased our analysis. Indeed one could reasonably argue that the majority of DogCV positive animals (13/18) were young individuals and that DogCV prevalence may be related with the age of the susceptible carnivore population. CPV-2 and CDV were the viral agents most commonly associated with DogCV infection in our set of samples and these viruses are commonly detected in young individuals in which they cause severe disease. Although we receive daily at IZSAM samples from domestic dogs and wildlife with a broad range of age, carcasses of puppies or young individuals succumbed following systemic

disease or trauma are within the most common samples to be processed and analysed. Therefore, whereas on one hand we observe an apparent association between CPV-2 or CDV infection with DogCV in our set of samples, on the other hand we cannot exclude an important role of DogCV in adult individuals alone or in a co-infection process with other pathogens. Indeed, our analysis may be biased in favour of young individuals and conclusions cannot be drawn. Trauma is one of the most common causes of death for wildlife from the areas of analysis. Furthermore, especially when dealing with carcasses of wildlife, autolysed specimens were common. Potentially, autolysis may have biased the presence of certain viral agents in favour of those which are more resistant in the environment including CPV-2 and DogCV. However we were able to detect CDV, which is in turn less resistant, also from carcasses in advanced status of decomposition.

Neither DogCVs nor FoxCVs were detected in foxes and FoxCVs were apparently not detected as well in dogs, wolves and badgers. In order to identify FoxCV we employed a FoxCV-specific rtPCR assay (Bexton et al., 2015). $rtPCR_{FoxCV}$ was further employed to detect FoxCVs in dogs, wolves and badgers. 25/32 $rtPCR_{DogCV}$ positive samples turned to be positive by $rtPCR_{FoxCV}$. 7/32 $rtPCR_{DogCV}$ positive samples turned negative by $rtPCR_{FoxCV}$ whereas four samples from two dogs (PE3302 and TE6685) had lower C_T values when tested by $rtPCR_{FoxCV}$. In *silico* sequence analysis demonstrated 100% nt identity in the forward primer and probe sequence of $rtPCR_{FoxCV}$ with strain TE6685 and only 1/23 nt mismatch in the reverse primer sequence. On the other hand, DogCV strain TE6685 showed 1/20 and 2/22 mismatches with probe and reverse primer sequence of the specific $rtPCR_{DogCV}$, respectively. In *silico* analysis of all DogCV sequences determined in this study, with the exception of TE6685, show 2/21, 1/22 and up to 2/23 mismatches with forward primer, probe and reverse primer sequences of $rtPCR_{FoxCV}$, respectively. The presence of these mismatches may explain the lower efficiency of $rtPCR_{FoxCV}$ than $rtPCR_{DogCV}$ in detecting our DogCV-positive samples. The opposite scenario between the two rtPCRs may also be true. Indeed 5/23, 3/22, and 2/20 mismatches between FoxCV sequences and forward primer, reverse primer and probe sequences of $rtPCR_{DogCV}$, respectively, do exist. Unfortunately, we did not have the chance to test $rtPCR_{DogCV}$ with reference FoxCV strains as we did not detect any FoxCVs in the field. As for $rtPCR_{FoxCV}$ primers and probe sequences, in *silico* analysis shows 100% match with FoxCVs including the last FoxCV sequence obtained from the faeces of a fox from Croatia (KP941114). Furthermore, although strain TE6685 was clearly demonstrated to be DogCV by sequencing, we cannot exclude with absolute certainty the contemporary presence of FoxCV in that dog. Overall, future surveillance activities for carnivore circoviruses in domestic and wild animals need to take into account these genetic differences by employing both molecular tests or alternatively, by setting up a universal rtPCR assay for dog and fox circoviruses.

In the phylogenetic analysis, DogCVs and FoxCVs grouped together in a separate clade apparently arranged in two clusters. It is important to point out that CVs of the same species should share $>75\%$ and $>70\%$ nt identity in the complete genome and capsid protein encoding gene sequences, respectively (Biagini et al., 2011). Based on this criterion and considering that the lowest nt sequence identities within circoviruses detected to date are 82.9% and 81.3% in the complete genome and the capsid CDS, respectively, we propose the existence of a unique carnivore circovirus species that includes DogCVs detected in dogs, wolves, badgers and FoxCVs. Moreover, the FoxCV sequence from Croatia clusters together with FoxCVs from the UK (data not shown). Whether or not CVs circulate in carnivore hosts as a cloud of divergent sequences belonging to one of the two current clusters or more yet undetected is, based on the current data, hard to say and further studies of viral metagenomics are warranted. According to our results, nt identity between DogCVs infecting the same individual was 100% in six cases; only DogCV AZ5212 from a dog showed a single synonymous nt difference at the protein capsid gene level between the sequences obtained from liver and lungs, respectively.

The pathogenic role of carnivore circovirus in domestic and wild animals is still not clear and further studies are required in order to understand the ability of these viruses to cause disease or alternatively, complicate concurrent viral and bacterial infections, as it is the case of PCV2 (Opriessnig and Halbur, 2012). As proved for PCV2, also carnivore circovirus seems to show a tropism for lymphatic tissues (Li et al., 2013) at least in dogs, wolves and badgers as it was detected with higher titres in spleen and lymph nodes tissues thus complicating the immunosuppressive status of the host. Interestingly, in one of the tested dogs (AZ2972) invasive aspergillosis with fungal dissemination to internal organs was also diagnosed.

The presence of carnivore circovirus may help to explain the different outcomes and severity of the disease often observed in CDV/CPV-2 infected animals. However, one limitation of the study is represented by the absence of a complete and consistent sampling from the carcasses thus hampering a proper statistical analysis of our results.

Additional carnivore circovirus surveillance in wild animals is required to clarify the epidemiology of CVs and more efforts are required to identify and, more in general, to characterize co-infection processes. As far as we know, attempts to isolate DogCV have not yet been successful, thus surveillance methods are limited to viral detection by molecular assays and no seroprevalence information is available. As for the high prevalence of carnivore circovirus in Italian wolves and fox in the UK and the high genetic relatedness within strains isolated from dogs and wild animals, the possibility that these latter can act as reservoir for domestic dogs or viceversa cannot be ruled out.

Materials and Methods

A total number of 389 samples from different organs (spleen, tonsil, lymph nodes, liver, intestine, lung, kidney, brain) belonging to 277 animals including 209 dogs (*C. lupus*), 34 Apennine wolves (*C. lupus*), 24 foxes (*Vulpes vulpes*) and 10 badgers (*Meles meles*) were collected in the Abruzzi and Molise regions (Central-Southern Italy, Fig. 1) during 2013-2014 and tested for the presence of DogCV by molecular methods. These regions contain significant protected natural habitats including the National Park of Abruzzi, Lazio and Molise, the Gran Sasso and Monti della Laga and the Majella National Parks. Remarkably, these regions, during 2013, experienced a severe outbreak of canine distemper virus (CDV, Lorusso and Savini, 2014). The CDV outbreak also involved wildlife animals (Di Sabatino et al., 2014). The age of the tested animals was wide-ranging. Biological samples were mainly collected from dogs of private owners deceased after severe diseases and from carcasses of wild animals found dead within natural areas. Some of the carcasses of wild animals were in an advanced state of decomposition and were sent to the laboratories of the IZSAM, a public institution for Animal Health and Food Safety operating for the Italian Government and the Abruzzi and Molise regions, for *post-mortem* examination. Samples from dogs and wild animals were tested for the most common bacterial, viral and parasitic pathogens of carnivores according to the necropsy recommendations.

Samples were homogenised with phosphate-buffered saline buffer (PBS) with antibiotics and 100 μl of the supernatants were used for DNA extraction by using BioSprint 96 One-For-All Vet Kit

Table 3
Primers used for the amplification and sequencing of DogCV DNA.

Primer	Sequence (5′-3′)	Position (nt)	Amplicon size (nt)
DogCV1_Fw	CGTATCCGGAGACCTACGTCA	422–442[a]	2040
DogCV1_Rev	TTCGTCTCTTTCAGTTTCACAACAG	374–398[a]	
DogCV_2Fw	TTGGGATGTAGAAGTGTCTGGGG	1194–1216[a]	1439
DogCV_2Rev	TCCATGCAGTACCGGCTCTT	550–569[a]	

[a] Based on the cds numbering of the gene encoding the replicase protein.

(Qiagen) following manufacturer's instructions. Nucleic acids were stored at −80 °C until use. DNA extracts were screened using a real-time PCR (rtPCR) assay with specific primers and probe for the Rep encoding gene of DogCV, rtPCR$_{DogCV}$ (Li et al., 2013). Briefly, rtPCR was performed on a QuantStudio 7 Flex Real-Time PCR System kit (Applied Biosystem, Foster City, CA, USA) with TaqMan Universal PCR Master Mix (Applied Biosystem). The thermal cycling consisted of 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. By using the same Real-Time PCR system kit and thermal cycling profile, DNA extracts from fox samples were screened using a rtPCR assay with primers and probe targeting the Rep encoding gene of fox circovirus (FoxCV), rtPCR$_{FoxCV}$ (Bexton et al., 2015). Furthermore, all DNA extracts purified from dogs, wolves and badgers which turned positive or negative by rtPCR$_{DogCV}$ were screened by the rtPCR$_{FoxCV}$.

As circoviruses have a circular genome, the entire genome of 17 samples was obtained by inverse PCR using two primer pairs (Table 3), designed to amplify two overlapped amplicons, with PfuUltra II Fusion HS DNA Polymerase kit (Agilent Technologies). The PCR protocol consisted of an initial denaturation at 95 °C for 2 min followed by 40 cycles, each consisting of 95 °C for 30 s, 55 °C for 20 s and 72 °C for 90 s and a final elongation at 72 °C for 3 min.

In order to obtain the full-length genome of 9 of the 17 positive samples with high cycle threshold (C_T) values (range 30–42) in rtPCR$_{DogCV}$, a rolling circle amplification (RCA) protocol was previously performed to increase the circular genomic DNA, using the TempliPhi 100 amplification kit (Amersham Bioscience) (Rector et al., 2004). Subsequently, viral DNA was amplified by PCR as described above.

All PCR products were sequenced using classical dideoxy Sanger sequencing, performed on an ABI Prism 3130 Genetic Analyzer (Applied Biosystem). Sequence reads were assembled with DNAStar software package (DNAStar Inc., Madison, WI, USA). Phylogenetic analysis employing the obtained 17 DogCV whole genome sequences and extant DogCV and representative CVs were conducted using MEGA version and the evolutionary distances were computed using the maximum composite likelihood method. Statistical support was provided by bootstrapping over 1000 replicates and bootstrap values >70 are indicated at the corresponding node (Tamura et al., 2013). Other tree-building methods, including maximum parsimony and maximum likelihood, were used to confirm the topology of the neighbour-joining tree.

We also tried to isolate DogCV by direct inoculation of MDCK (Madin-Darby canine kidney) and D17 (canine osteosarcoma) cell lines with a dog liver (AZ5212) and a wolf lymph nodes (CB6293) homogenates. However, all the attempts were unsuccessful.

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