DOI: 10.7589/2018-11-270

Journal of Wildlife Diseases, 56(1), 2020, pp. 239–242

Genomic Characterization of Canine Circovirus Detected in Red Foxes (*Vulpes vulpes*) from Italy using a New Real-time PCR Assay

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ABSTRACT: Data on canine circovirus circulation among red foxes (*Vulpes vulpes*) are limited. We report the detection of canine circovirus in a red fox from Italy. The virus was closely related to strains from dogs (*Canis lupus familiaris*) rather than those from foxes, suggesting a possible transmission between the two species.

Canine circovirus (CanineCV) was first described in 2012 in domestic dogs (*Canis lupus familiaris*) with no clinical signs and subsequently in dogs with necrotizing vasculitis, hemorrhagic gastroenteritis, lymphadenitis, and granulomatous diseases (Kapoor et al. 2012; Li et al. 2013; Decaro et al. 2014). Despite these reports, the real pathogenic potential of the virus in dogs has not yet been fully clarified (Anderson et al. 2017; Dowgier et al. 2017). Data on the distribution of CanineCV in wildlife are limited. Since 2015, the virus has been detected in wild carnivores from UK, Croatia, and Italy, but the circulation of CanineCV in other countries has not been confirmed (Bexton et al. 2015; Lojkic et al. 2016; Zaccaria et al. 2016; Lempp et al. 2017).

Canine circovirus identified in dogs and red foxes (*Vulpes vulpes*) share a genome (2,063 nucleotides) identity at >80%, confirming that they belong to the same viral species (Zaccaria et al. 2016; Rosario et al. 2017). Nevertheless, phylogenetic analyses showed apparent clustering within two distinct groups: dog and fox viral sequences (Zaccaria et al. 2016). Because of these genetic differences it is possible that molecular diagnostic methods, developed on the CanineCV sequences detected in dogs, may not be effective if used on foxes and vice versa. The possible viral transmission between domestic and wild canids requires the use of diagnostic assays that are able to detect circoviral strains in all carnivores (Zaccaria et al. 2016).

The aim of our study was to assess the presence of CanineCV in Italian red foxes using a molecular assay targeting a genomic region that is highly conserved among all the viral sequences available to date, and to evaluate the genomic features of the identified viruses. We tested feces and tissues from 32 red foxes collected for a previous survey (Balboni et al. 2013) and stored them at –20 C. Foxes were shot in 2011 during the regular hunting season in the province of Pisa (Tuscany, Italy) and all foxes showed good body condition score at postmortem examination. There were 13 (41%) males and 19 (59%) females; 17 (53%) were adults (>1 yr) and the remaining 15 (47%) were juveniles (<1 yr). The animals averaged 4.7 kg in weight (3.5–8.1 kg). Fecal samples were collected from all foxes; however, only 17 of 32 had livers and kidneys available for collection.

Samples were screened using a newly developed SYBR Green real-time PCR assay (qPCR) targeting a fragment of 132 nucleotides in the intergenic region between the 3′ ends of the two major open reading frames. The intergenic region was chosen as molecular target because, on the basis of an alignment of 32 nucleotide sequences retrieved from the GenBank database, it was highly conserved among all CanineCV infecting wild and domestic canids. The primers used for the qPCR are reported in Table 1 and the thermal cycling consisted of 95 C for 5 min, followed by 45 cycles of 95 C for 15 s and 60 C for 1 min. The reactions were performed using the PowerUp SYBR Green master mix

TABLE 1. Primers used for the detection, amplification, and sequencing of canine circovirus DNA in red foxes (*Vulpes vulpes*) collected in 2011 in Italy using a newly developed real-time PCR assay.

Assay	Primer	Sequence (5′–3′)	Position (nucleotides)[a]	Amplicon size (nucleotides)[a]
qPCR[b]	CaCV 909–931 qPCR-For	5′-CTGAAAGATAAAGGCCTCTCGCT-3′	909–931	132
	CaCV 1,020–1,040 qPCR-Rev	5′-AGGGGGGTGAACAGGTAAACG-3′	1,020–1,040	
PCR[c]	CaCV 1,020–1,040 For	5′-CGTTTACCTGTTCACCCCCCT-3′	1,020–1,040	1,932
	CaCV 909–931 Rev	5′-AGCGAGAGGCCTTTATCTTTCAG-3′	909–931	
	CaCV 3′–3′ For	5′-ATGGTGGGATGGCTACGATG-3′	606–625	936
	CaCV 3′–3′ Rev	5′-CAAGGAAGAGGGAATGCTACAAG-3′	1,519–1,541	

[a] Refers to canine circovirus isolate 214, GenBank accession number JQ821392.

[b] Real-time PCR.

[c] End-point PCR.

and the StepOnePlus real-time PCR system (Applied Biosystems, Waltham, Massachusetts, USA). Melting experiments were performed after the last extension step by a continuous increment from 55 C to 98 C and specific melting temperature ranged from 93.2 C to 93.6 C. The limit of detection of the assay, corresponding to five copies of the target DNA, was experimentally determined (Qurollo et al. 2017). Samples showing target DNA amount greater than or equal to the limit of detection and a specific melting peak in both replicates were considered positive.

A rolling circle amplification, using the TempliPhi 100 amplification kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), was performed on the positive sample to increase the amount of circular DNA. Subsequently, viral DNA was amplified by end-point PCR using two couples of primers (Table 1) and a proofreading DNA polymerase. The amplicons obtained were directly sequenced. The assembled nucleotide sequences were translated into amino acid sequences using BioEdit 7.0.5 (Hall 1999) and analyzed by BLAST web interface (National Center for Biotechnology Information 2017). A nucleotide alignment with reference sequences from GenBank (National Center for Biotechnology Information 2017) and the phylogenetic analyses were carried out using MEGA version 6.0.6 (Tamura et al. 2013).

Circovirus DNA was detected in 3% (1/32) of fecal samples. None of the kidneys and livers was positive. Unfortunately, tissue samples of the positive fox, an adult female weighing 5.8 kg, were not available. A 2,001-nucleotide sequence was obtained and deposited in GenBank (MH454599). It shared from 86% to 96% nucleotide identity with 22 reference strains of CanineCVs from dogs and 86% with four strains from foxes. The amino acid identity of the putative replication-associated protein was 89–97% with dog viral strains and 89–91% with fox viral strains, whereas the putative capsid protein showed an amino acid identity of 93–98% and 88–91% with the dog and fox viral strains, respectively. Phylogenetic analysis showed that the identified virus grouped with the CanineCVs from dogs, forming a group with strains detected in Thailand and separated from those detected in wolves and dogs in Italy (Zaccaria et al. 2016). CanineCV sequences from foxes retrieved from GenBank formed a separate cluster with canine strain UCD3-478 KC241983 (Fig. 1).

Our report demonstrated that red foxes in Italy may be exposed to CanineCV. In the only survey previously conducted in the same country, 24 red foxes from the Abruzzi and Molise regions tested negative using two real-time PCR assays specific for dog and fox strains, respectively (Zaccaria et al. 2016). The prevalence of circovirus infection detected by a molecular approach in the red foxes tested in our study is significantly lower than that reported in the UK, where the virus was detected in 47% (7/15) of healthy foxes and 76% (13/17) of foxes with neurologic signs

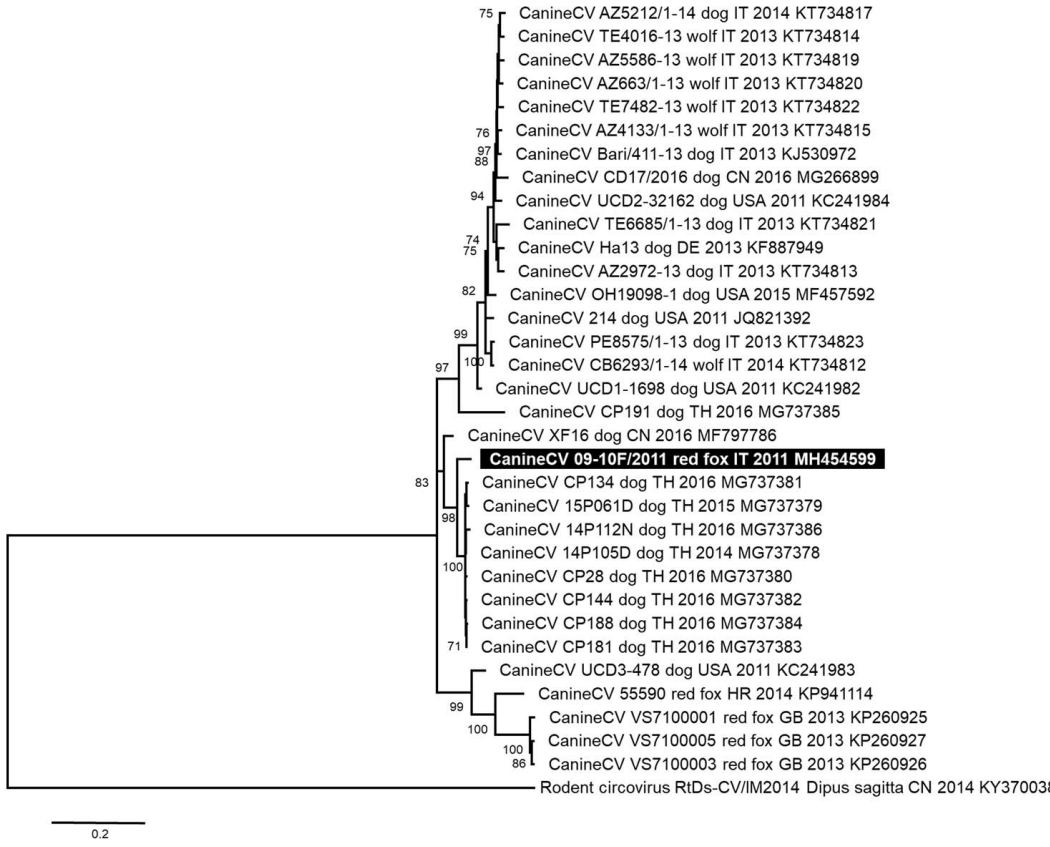

FIGURE 1. Rooted phylogenetic tree constructed by the maximum likelihood method using the general time reversible model with canine circovirus (CanineCV) nucleotide sequences. The phylogenetic analysis includes a CanineCV nucleotide sequence identified in this study in a fecal sample of an Italian red fox (*Vulpes vulpes*) and 32 CanineCV reference sequences retrieved from GenBank and a rodent circovirus reference sequence used as outgroup. A fragment of 1,902 nucleotides, including the two major viral open reading frames, were used to generate the tree. Bootstrap values greater than 70%, calculated on 1,000 replicates, are indicated on the respective branches. Identification of the sequences undergoes the following nomenclature: organism, strain, host, country, collection date, and GenBank accession number. The nucleotide sequence generated in this study is highlighted.

(Bexton et al. 2015). The different diagnostic tests used make it difficult to compare our data with other studies, but a higher virus circulation in the UK and an association between infection and the development of neurologic signs can be speculated. The sequence analyses performed in this study suggest that foxes and dogs can share strictly related circoviruses and that CanineCV could possibly be transmitted from dogs to wild canids, or vice versa. Further investigation is needed to increase the number of fox isolates and better define the genetic epidemiology of circovirus infection in canids.

The authors thank Andrew Rich, resident of the European College of Veterinary Pathologists at the University of Liverpool, UK, for revising the manuscript.

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Submitted for publication 13 November 2018.
Accepted 18 February 2019.