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Serological and molecular evidence of canine enteric coronavirus in southern Italy

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Canine enteric coronavirus (CECoV) is one of the most common viruses in dogs, causing gastrointestinal disorders and, in severe cases, death. Despite being a main pathogen, research on its distribution is limited, particularly in some geographical locations, such as Campania region (southern Italy). This study investigated the serological and molecular prevalence of this virus, as well as the risk factors associated with higher exposures. A total of 258 blood and 154 fecal samples from 71 districts were collected, along with anamnestic information such as sex, breed, size, location, age, origin, lifestyle, and attitude. The serological and molecular prevalence were determined using a commercial ELISA and a previously established real-time PCR method. A total of 139/258 dogs tested positive in serological analysis, nevertheless only 5.8% tested positive in real-time PCR. Univariate and multivariate analyses revealed that hunting dogs and dogs with an outdoor lifestyle had higher seroprevalences. Furthermore, dogs in some locations had seroprevalences of up to 85%, including the bulk of PCR-positive animals. The findings of this study emphasized the widespread prevalence of CECoV in the studied area, as well as the presence of current outbreaks across several districts.

Keywords Canine enteric coronavirus, CECoV, Risk factors, Coronavirus

Coronaviruses (CoVs) are enveloped and single-stranded RNA viruses infecting a wide range of hosts, including mammals and birds. CoVs have a large and complex positive-sense RNA genome whose dimension reaches up to 32 kb[1]. These viruses belong to the order *Nidovirales*, family *Coronaviridae*, and are recently classified by the International Committee on Taxonomy of Viruses classification (ICTV) in four genera: Alphacoronavirus (including several CoVs of veterinary interest), Betacoronavirus (including some impactful human CoVs), Gammacoronavirus (which primarily affect birds), and Deltacoronavirus. CoV's disease is generally associated with respiratory and/or enteric disease[2,3]. Dogs, and canids in general, including wolves, foxes, and raccoons, are nowadays susceptible to three different coronaviruses described in literature: canine enteric coronavirus (CECoV), canine respiratory coronavirus (CRCoV), and pantropic (pCCoV)[4–6]. CECoV is an alphacoronavirus with a history of recombination events with feline enteric coronavirus (FECoV) and transmissible gastroenteritis virus of pigs (TGEV), responsible for mild or asymptomatic gastroenteritis and usually associated with other pathogens[4,7]. The disease can result in more severe forms in puppies, causing even hemorrhagic enteritis and vomiting[8]. Types I and II are nowadays known, with differences based on genomics (although they share up to 96% of the genome, type I contains an additional open reading frame) and particularly in the highly different spike protein[9]. CECoV-II is considered the "original" strain, isolated by a military dog in 1971 in Germany, and the most prevalent[6]. A further classification in two subtypes, CECoV-IIa and CECoV-IIb (also known as the TGEV-like strain), complicates the taxonomy of CECoVs. The two strains are distinguishable based on the amino acid sequence of the N-terminal domain (NTD) of the spike protein. On the other hand, CECoV-I was first identified in 2003 and is considered, with feline enteric coronavirus (FeCoV), a potential ancestor of the carnivore CoVs[6].

CRCoV, firstly isolated in 2003 in the UK before to spread throughout the world, is a Betacoronavirus genetically close to bovine coronavirus (BCoV) and is one of the etiological agents associated with canine infectious respiratory disease complex (CIRDC)[4,10]. CECoV and CRCoV are different not only in origin and tropism but also based on high dissimilarity in nucleotide and amino acid sequences[3].

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A hypervirulent and highly fatal CECoV-IIa strain, referred to as "pantropic" CCoV (pCCoV), was first isolated in Italy in 2005 and spread in several tissues, causing severe gastroenteritis and disseminated lesions in the vital organs[6,11,12].

These complex subdivisions are the expression of some peculiar CoV features as the genome structure, the low fidelity of the RNA polymerase, the misleading proofreading activity, and the selection pressure during adaptation of the virus to the new host, that cause a high frequency of recombination and mutation events during viral replication[2,13].

Among canine coronaviruses, CECoV is the most common and is transmitted via the fecal-oral route (both direct and indirect), which recognizes the infected canids as the source of infection[8]. Secondary routes of dissemination are saliva and fomites[6]. Infection may occur with a single strain; however, mixed infections with both types (CECoV-I and II) appear to be common[14]. Direct measures are mainly used against this infection, since even if an inactivated vaccine exists, it is not recommended by international guidelines. The World Small Animal Veterinary Association (WSAVA) guidelines, in fact, discourage the use of vaccines against canine enteric coronavirus since there is little evidence that these vaccines are protective or that they protect against pantropic variants[15]. Coronaviruses have large zoonotic potential, and the ability to cross species boundaries lies not only in mutation rate but also in the propensity for these viruses to recombine, with important breakpoints identified around the spike gene in other mammals[2]. Extensive homologous and heterologous recombination events have been documented in both human and animal coronaviruses, leading to the generation of various genotypes and strains. Two recent novel coronaviruses (CCoV-HuPn-2018 and HuC CoV_Z19Haiti) have been described in 2022, respectively, in Malaysia and Haiti, in patients with pneumoniae, suggesting cross-species transmission potential from dogs and highlighting the risk of CECoV spillover to humans[16,17]. Recombination phenomena can also involve other animal viruses as, for example, occurred in Cyprus in 2023, with the FCoV-23 variant born from the recombination of the feline infectious peritonitis virus (FIPV) and the pCCOV[18]. For these reasons, these viruses have an impact not only on animal health but also a potential impact on human health, especially considering the more and more strict relationship between pets and their owners.

Although the infection is considered a worldwide spread, only a few studies have reported the presence of the virus in the Italian canine population, and the apparent prevalence remains unknown. These investigations examined molecular prevalences, highlighting the pathogen's active circulation in southern Italian regions such as Apulia and Sicily[12,14,37]. The aim of this work was to evaluate, through a serological and molecular approach, the prevalence of CECV in the canine population of the Campania region, evaluating risk factors associated with higher risks of exposure/shedding.

Results

A high seroprevalence (53.9%) was obtained by testing 258 dogs in the Campania region (Table 1). A total of 139/258 animals presented specific antibodies against CECoV. A total of 45 out of 71 districts reported seropositive dogs. Among the animals that tested seropositive, 57 had a titer of 1:50, 36 of 1:100 and 46 of 1:200. Higher prevalences were found in the provinces of Avellino and Salerno, where more animals with high titers were also found (1:200). Hunting attitude and outdoor lifestyle were also found to be factors related to higher risks of exposure (Table 1). Sex, age, breed, origin, and size were not associated with higher seroprevalences. Location, attitude, and lifestyle were all significant risk variables in both the univariate and multivariate analyses (Table 2). Dogs from the provinces of Naples, Caserta, and Benevento were 5 to 10 times less likely to be exposed to CECoV than those from Avellino and Salerno. Hunting animals and those who live outside were two and three times more likely to be seropositive, respectively.

At the molecular level, the prevalences obtained were much lower than those found in ELISA. In particular, a total of 9 stool samples out of 154 (5.8%) presented CECoV RNA (Table 3). In certain instances, the molecular analysis corroborated the findings of the risk analysis conducted using the ELISA data. The cycle threshold value (Ct) of the positive samples ranged from 16.87 to 39.03. Among these 9 animals, only 2 were seronegative when tested by ELISA, another 5 had a titer of 1:50 and 2 of 1:200. The PCR-positive animals came from two provinces, Avellino ($n=3$) and Salerno ($n=4$). Furthermore, 8 out of 9 animals were hunting dogs or strays, and only one was a pet dog, and 7/9 lived outdoors (Table 3).

Discussion

Despite growing interest in coronaviruses of veterinary concern, CECoV remains one of the most poorly documented viruses among common canine pathogens. In fact, there are few serological studies conducted on a large scale at a global level. One of these was carried out in Australia between 1984 and 1998, obtaining seroprevalences of 15.8% in pet dogs and of 40.8% in kennel dogs[19]. Other studies have been carried out mainly on wildlife, revealing antibodies for CECoV in several species such as bear (4% in Slovakia), maned wolf (45.4% in Brazil), and raccoon (7%)[20–22].

A recent study carried out in the southern Italy and using a specific ELISA has identified a seroprevalence of 22.7% in wild canids (wolves and foxes) admitted to a wildlife rescue and rehabilitation center[23].

Numerous studies, however, have evaluated the exposure of dogs to CRCoV (ranging from 23 to 26%) in several countries[24–26]. Other studies have, instead, established the seroprevalence of the virus in canine populations using heterologous antigens (mainly from BCoV and TGEV, with which CRCoV shares part of the genome)[27]. Using this approach, in southern Italy (Apulia region), a seroprevalence of 32.06% was found[28].

Generally, the spread of CECoV seems higher than that of CRCoV, as demonstrated by the different seroprevalences. A study also evaluated the spread of the two infections by differentiating the specific antibodies and found prevalences of 23.3% and 86.1% for CRCoV and CECoV, respectively[29]. This difference became even

Factor	CCV					
	n	Positive	%	95%CI	χ^2	*p*
Total	258	139	53.9			
Province						
Avellino	42	36	85.7			
Benevento	37	15	40.5			
Salerno	49	35	71.4		35.9	**< 0.001**
Caserta	64	29	45.3			
Napoli	66	24	36.7			
Sex						
Male	143	79	55.2			
					0.2	0.62
Female	115	60	52.2			
Age						
Young	52	31	59.6			
Adult	126	66	52.4		0.86	0.65
Old	80	42	52.5			
Breed						
Mix	150	81	54			
					0.002	0.96
Specific breed	108	58	53.7			
Origin						
Stray	164	88	53.7			
					0.009	0.92
Housed	94	51	54.3			
Size						
Small	75	43	57.3			
Medium	135	74	54.8		1.35	0.51
Giant	47	22	46.8			
Attitude						
Hunting	52	38	73.8			
					9.66	**0.02**
Non-hunting	206	101	49			
Life style						
Indor	52	15	28.8			
					16.4	**< 0.001**
Outdoor	206	124	60.2			

Table 1. Risk factor analysis of variables potentially associated with CECoV seropositivity.

Factor	Coefficient (β)	Standard error	OR	OR CI%	*p*-value
Intercept	1.5	0.67	4.5	1.2–16.9	0.03
Province (Benevento)	-1.5	0.36	0.22	0.11–0.45	< 0.001
Province (Caserta)	-2.3	0.54	0.09	0.03–0.27	< 0.001
Province (Napoli)	-1.95	0.53	0.14	0.05–0.4	< 0.001
Lifestyle (outdoor)	1.25	0.4	3.5	1.58–7.64	0.002
Attitude (no hunting)	-0.8	0.4	0.45	0.21–0.98	0.044

Table 2. Multivariate risk factor analysis of variables potentially associated with CECoV seropositivity.

more substantial when shelter dogs were considered, with seroprevalences of 4% and 97% for CRCoV and CECoV[29].

The data collected determined a higher seroprevalence in animals of stray origin, hunting dogs and those living outdoors. All these conditions, in fact, increase the possibility of direct or indirect exposure to CECoV, considering its resistance when contained in biological fluids with suboptimal conditions, as well as during cold seasons[3]. In fact, some studies have described outbreaks in animal shelters highlighting how animal density can affect the circulation of this virus in particular epidemiological units, such as kennels. In fact, kennels are ideal

Sample ID	Province	District	Age (years)	Sex	Breed	Attitude	Size	Lifestyle	ELISA outcome (titer)
AV27	Avellino	Avella	4	Female	Mixed	Stray	Medium	Outdoor	Positive (1:200)
AV4	Avellino	Pago del Vallo di Lauro	6	Male	Pure	Hunting	Medium	Outdoor	Positive (1:50)
SA13	Salerno	Salerno	4	Male	Pure	Hunting	Medium	Outdoor	Positive (1:200)
AV3	Avellino	Lauro	1	Male	Mixed	Stray	Small	Outdoor	Positive (1:50)
SA32	Salerno	Pontecagnano Faiano	14	Male	Mixed	Stray	Small	Outdoor	Negative
SA36	Salerno	Pontecagnano Faiano	5	Female	Mixed	Stray	Giant	Outdoor	Positive (1:50)
NA12	Napoli	Frattaminore	4	Male	Pure	Pet	Medium	Indoor	Positive (1:50)
SA41	Salerno	Salerno	14	Female	Mixed	Stray	Medium	Indoor	Negative
CE34	Caserta	Caserta	5	Female	Mixed	Stray	Small	Outdoor	Positive (1:50)

Table 3. Information from real-time PCR-positive dogs.

places for pathogen exchange because they are a dynamic population in which some subjects are fixed (those who are not adopted), new entries are regularly made up of stray animals with no clinical history and may be reservoirs of pathogens, and large numbers of animals coexist in limited spaces[30].

At the molecular level, the prevalence obtained was lower (5.8%), also considering the different meaning of positivity to ELISA (exposure, even previous) and to PCR (shedding). Higher prevalences have been described in China (ranging from 17.5% to 21%) and the Netherlands (14%)[31–33]. Using similar approaches, studies conducted in the UK and in India found prevalence similar to that described in the present study, 2.8% and 5.9% respectively[34,35]. These percentages increased when animals with diarrhea were assessed (up to 30%)[36]. A molecular survey performed in Sardinia (Italy) in symptomatic dogs found a 18% of animals positive for CECoV-IIa and 10.3% for CECoV-I[36]. Another recent Italian study found CECoV RNA in 39 (13.7%) dogs in Sicily, mainly with other viral pathogens (87.2% of positive results) as CPV-2, CaAV-1, norovirus[37]. The main circulating strain was CECoV-I (51.3%) followed by CECoV-IIa (20.5%) and coinfection of this latter strain (23.1%)[37]. This differentiation, as well as sequencing activities, was not carried out in our work, indicating a limitation in the CECoV tracking. This information could prove useful in the future for tracking emerging, more virulent strains, as has occurred in the past, as well as characterizing less pathogenic ones. A recent study performed in India found a prevalence of 1.19% and 0% in symptomatic and aymptomatic dogs, respectively[38]. Another piece of evidence of low prevalence has been described in Cape Verde with only 2 out of 186 positive healthy animals[39].

These results vary widely based on the type of material used, the technique employed, and the animals tested. The same features impact the meta-analysis results and global prevalence reported in the literature. In China, a 30% prevalence was established in 2024 after analyzing 21,034 samples from 27 investigations[40]. This was consistent with the 33% obtained in 2022, whereas the worldwide average was 21.2% (that includes 43 research)[41,42]. The current investigations also found greater incidence rates in younger dogs, multi-dog households, apparently healthy dogs and season[40,41]. The finding of the virus in clinically healthy animals, as emphasized by the current study conducted in southern Italy, is not surprising and illustrates the virus's capacity to spread, as viral shedding has been reported to occur up to 6 months after infection[6].

The current study evaluated the serological and molecular prevalence of CECoV in a limited area (Campania region, southern Italy), but has highlighted key risk variables that contribute to the viral transmission. More than half of the dogs (53.9%) tested positive for specific antibodies, despite only a small number also shedding (5.8%). These elements suggest that CECoV is actively circulating among the dog population of southern Italy. Additional efforts are required to provide helpful epidemiological surveillance for this important canine pathogen, as well as to detect mutations and forecast potential changes in its epidemiological cycle.

Methods

Sampling and study area

This study was conducted in Campania, the most populated region in southern Italy (40°49′34″N 14°15′23″E). The sample size was determined using Thurshfield's formula, considering a theoretically "infinite" population, an expected prevalence of 20%, a confidence interval (CI) at 95%, and a desired absolute precision of 5%. Whole blood samples were randomly collected from clinically healthy 258 dogs belonging to 71 districts[43]. The sampling was performed in three different dog categories (stray dogs, pet dogs, and hunting dogs). Samples were transported to the laboratory, maintaining the cold chain conditions, before being centrifuged and stored at -20 °C until being analyzed. Fresh fecal samples were collected from 154 dogs due to the difficulties of collecting all (for example, empty rectal ampulla)[44,45]. A form was compiled by the veterinarian to collect anamnestic information on each sampled animal, including sex (male or female), breed (mix or specific breed), size (small, medium or giant), location (Avellino, Benevento, Caserta, Naples, Salerno), age [young (≤ 2 years), adult (> 2 and ≤ 6 years), or old (> 6 years)], origin (stray or housed), lifestyle (outdoor or indoor), and attitude (hunting dog or not)[46]. The animal study protocol was approved by the Institutional Ethics Committee of the Department of Veterinary Medicine and Animal production (Centro Servizi Veterinari), University of Naples, Federico II (PG/2022/0093420, 21st July 2022) and the sampling was performed in accordance with ARRIVE guidelines (https://arriveguidelines.org). Consent from the dog owner was obtained for the collection of samples by signing a specific document. All methods were performed in accordance with the relevant guidelines and regulations.

Serological assay

Specific CECoV antibodies were detected using Canine Coronavirus Ab ELISA (Agrolabo Spa, Italy), that was an indirect ELISA with CECoV antigen coated on the wells of the plate. Specific antibodies against CECoV were bound to the adsorbed antigen, forming the antigen-antibody complex that was detected by a specific anti-canine IgG monoclonal antibody (labeled with horseradish peroxidase).

Briefly, the reagents and samples were brought at room temperature before use. The serum samples and controls were prepared in four two-fold dilutions (1:50, 1:100, 1:200, 1:400) in an appropriate buffer. A total of 100 µL of each sample was dispensed to each well of the plates. The plates were sealed and incubated for 10 min. After three washing cycles, 100 µL of conjugated antibody was inoculated in each well and incubated for a further 10 min. After five washing steps, 100 µL of substrate followed by 100 µL of stop solution were added. A spectrophotometer at a wavelength of 450 nm was used for reading. The test validation was ensured by the optical density (OD) of the negative control being lower than 0.4 and the positivity of the positive control at dilution 1:400 too. The antibody titer was represented by the highest dilution of the samples that had an OD value higher than 0.4. The manufacturer reported sensitivity of 99% and specificity of 100%.

Molecular analysis

After collection, each specimen was immediately processed into 10% (w/v) suspensions in 1X phosphate buffered saline (PBS, pH 7.2–7.4) and centrifuged at 1500 g for 10 min. Viral RNA was isolated from the supernatants using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Each RNA sample was extracted and stored at -80 °C until processing. Before being processed by PCR, each sample was reverse transcribed using a commercial kit, iScript™ Reverse Transcription Supermix (BioRad). A previously described real-time PCR protocol was used to amplify a 99 bp fragment of ORF5[47]. Primers used were as follows: forward (TTGATCGTTTTTATAACGGTTCTACAA), reverse (AATGGGCCATAATAGCCACATAAT), probe (FAM-ACCTCAATTTAGCTGGTTCGTGTATGGCATT-TAMRA). This TaqMan probe-based protocol consisted of an initial denaturation at 95 °C for 10 min and 45 cycles consisting of denaturation at 95 °C for 15 s and primer annealing-extension at 60 °C for 1 min. RNA obtained from a reference strain (S/378) and reverse transcribed using the same procedures described above served as a control in the PCR protocols. The amplification was performed using iTaq Universal Probes Supermix (BioRad) in a volume of 20 µL[47]. A real-time apparatus, the CFX96™ Real-Time PCR Detection System (BioRad), was used for reading the results.

Statistical analysis

Risk factor analysis was performed using the information collected through the questionnaire as the independent variable and the ELISA test result as the dependent variable (positive or negative). The chi-square test was applied, considering a $p < 0.05$ as significant. Variables correlated with higher seroprevalence were further tested in a multivariate analysis (logistic regression) using the stepwise elimination method. The model was assessed for multicollinearity using the Variance Inflation Factor (VIF) and allowed the calculation of odds ratios (OR) and related confidence intervals (CIs). Fit models were compared using the Akaike Information Criterion (AIC) and selected as those that best fit the data. Statistical analysis was not evaluated for molecular prevalence due to the low number of PCR-positive animals.

Data availability

All data generated or analysed during this study are included in this published article.

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Author contributions

Gianmarco Ferrara was involved in the work design, writing and acquisition, analysis, and interpretation of data. Raffaele Lerro was involved in the analysis and interpretation of data. Ugo Pagnini and Hyun-Jin Shin were involved in writing and analysis and interpretation of data.

Declarations

Competing interests

The authors declare no competing interests.

Ethics declarations

The animal study protocol was approved by the Institutional Ethics Committee of Department of Veterinary Medicine and Animal production (Centro Servizi Veterinari), University of Naples, Federico II (PG/2022/0093420 20 July 2022) in accordance with international guidelines (ARRIVE).

Additional information

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