

## Seroprevalence of canine respiratory coronavirus in breeding kennels in Croatia

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STEVANOVIĆ, V., M. MAURIĆ MALJKOVIĆ, K. GRACIN, I. BENVIN, V. STAREŠINA, S. KOVAČ, A. ŠKRINJARIĆ, S. HADINA, I. ZEČEVIĆ, K. MARTINKOVIĆ, J. HABUŠ, Z. ŠTRITOF, M. PERHARIĆ, M. CVETNIĆ AND LJ. BARBIĆ: Seroprevalence of canine respiratory coronavirus in breeding kennels in Croatia. *Vet. arhiv* 93, 239-246, 2023.

### ABSTRACT

Canine respiratory coronavirus is a relatively new addition to the list of pathogens causing canine infectious disease complex. The virus is highly contagious, with a high prevalence in the dog population worldwide, especially in shelters. This study aimed to establish the presence and risk factors associated with infection in privately owned dogs and breeding colonies.

This study was the first to demonstrate the presence of canine respiratory coronavirus in Croatia. Out of the 257 serum samples, 35.03% of dogs from breeding kennels and 43% of pet dogs tested enzyme-linked immunoassay positive, but the difference was not statistically significant. Sex was not an important risk factor, but the seropositivity rate increased with age. Mixing of dogs during hunting, training and dog shows was not associated with a higher seroprevalence in the breeding colonies. Daily cleaning and disinfection showed little effect on the infection spread.

The study was done on a limited sample. However, it still provides evidence that the epizootiology of this disease is complex. There is no available vaccine for canine respiratory coronavirus, and further studies on environmental and risk factors will give the valuable data needed to prevent this disease.

**Keywords:** canine respiratory coronavirus; seroprevalence; breeding colonies; ELISA; Croatia

### Introduction

Canine infectious respiratory disease (CIRD), also known as “kennel cough” or infectious tracheobronchitis, is one of the most prevalent infectious diseases of dogs worldwide. It is a

highly contagious, upper respiratory infection, characterised by the acute onset of paroxysmal cough, nasal and ocular discharge. Signs can last for days or weeks, and are usually mild to

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moderate in intensity (BUONAVOGLIA and MARTELLA, 2007). In more severe cases, bronchopneumonia can lead to death in puppies and immunocompromised animals (APPEL and BINN, 1987; RADHAKRISHNAN et al., 2007; DEAR, 2014).

As the name “kennel cough” suggests, outbreaks of CIRDC are most common in dogs housed in overcrowded environments, such as shelters, boarding kennels, breeding kennels and pet shops (ERLES et al., 2004; FORD, 2012). On the other hand, CIRDC is also often diagnosed in household dogs. Due to the high infectivity of the causal pathogens, any mixing of dogs, such as during socialisation, dog shows or veterinary clinic visits, is associated with the risk of infection (MOCHIZUKI et al., 2008; SINGLETON et al., 2019).

Many viral and bacterial pathogens may be involved in the pathogenesis of CIRDC as single or multiple infections. Several viral pathogens are traditionally considered the causative agents of CIRDC: canine parainfluenza virus (CPIV) (APPEL and PERCY, 1970), canine adenovirus type 2 (CAV-2) (DITCHFIELD et al., 1962), canine distemper virus (CDV) (DAY et al., 2020) and canid alphaherpesvirus type 1 (CaHV-1) (KARPAS et al. 1968; RONSSE et al., 2002). In recent years several new viruses, including the canine respiratory coronavirus (CRCoV) (ERLES et al., 2003), canine pneumovirus (CnPnV) (RENSHAW et al., 2010), and canine influenza H3N8 (CIV) (CRAWFORD et al., 2005) have been implicated in the development and persistence of CIRDC.

One of the most recent additions to the list of causative agents of CIRDC is canine respiratory coronavirus (CRCoV). The virus was first isolated from dogs in a shelter in the United Kingdom (ERLES et al., 2003). It is a member of the *Betacoronavirus* genus in the *Coronaviridae* family. Canine respiratory coronavirus is closely related to human OC43, severe acute respiratory syndrome coronavirus (SARS), Middle East respiratory syndrome coronavirus (MERS), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and bovine coronavirus (BCoV). Since its

first description, CRCoV specific antibodies have been recorded in dogs in many countries worldwide (PRIESTNALL et al., 2006; PRIESTNALL et al., 2007; MITCHELL et al., 2017; MORE et al., 2020). This study aimed to investigate the seroprevalence of CRCoV in breeding colonies in Croatia, and any possible association between the infection and host and management risk factors.

## Materials and methods

*Dog population and serum sampling.* Serum samples from a total of 257 dogs were analysed in this study. Among these, 157 dogs came from 25 breeding kennels in Croatia, and samples were collected during a serosurvey of canid herpesvirus 1 (CaHV-1) infections (GRACIN, 2020). At the time of sampling, data were collected including age, sex and breed. As potential risk factors, the size of the kennels, cleaning protocols, participation of dogs in hunting or dog shows and previous outbreaks of CIRDC were recorded. Training outside the kennels for assistance and military dogs was also considered a risk factor.

Additionally, 100 samples were randomly selected from the remaining sera from household dogs presented at the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine, University of Zagreb. For those animals, data regarding age, sex and breed were collected.

All serum samples were kept stored at -80 °C until testing was done.

*Serological testing.* Most of the methods described for CRCoV serological testing use the high degree of similarity between CRCoV and BCoV spike proteins and the resulting serological cross-reactivity (PRIESTNALL et al., 2006). In our study, a commercial enzyme-linked immunoassay (ELISA) kit (BIO K 392 Monoscreen AbELISA Bovine coronavirus/Competition, Bio-X diagnostics, Rochefort Belgium) was used, as previously described in MORE et al., 2020. It is a competitive ELISA, with wells coated with BCoV antigens. Samples were tested according to the manufacturer's instructions using the positive and negative controls provided in the kit. In brief, 100 µl of dog serum samples diluted 1:200 were added

to each well of the antigen-coated plate. The same volumes of the positive and negative reference sera were added to two wells. After adding 100  $\mu$ l of enzyme-conjugated BCoV antibodies to all wells, the plate was incubated for one hour at room temperature. In the next step, the plate was washed three times with the washing solution provided in the kit. After 10 minutes at room temperature, the reaction was terminated by adding 50  $\mu$ l of the manufacturer's stop solution. Optical density (OD) was recorded at 450 nm, and the results were presented as a percentage of inhibition. In other words, the sample OD was subtracted from the OD of the positive control, and divided by the OD of the positive control. According to the manufacturer's recommendations, samples with an inhibition above 20 were considered positive.

Ten known SARS-CoV-2 neutralisation test positive dog serum samples were tested to assess CRCoV ELISA specificity.

*Statistical analysis.* Descriptive statistics are presented as numbers and percentages. All statistical analyses were performed using R 4.0.5 (R Foundation for Statistical Computing, Vienna). Seroprevalence rates and exact 95% confidence intervals (95%CI) were calculated using epiR. The data obtained were analysed using the two-tailed  $\chi^2$  test or Fisher's exact test, and p values below 0.05 were considered statistically significant. The odds ratio (OR) of the bivariate risk factors and their 95%CI were calculated using epitools. Logistic regression analysis (glm) was used for calculating the OR of the multivariate risk factors and, if complete separation in the categories occurred, the Firth correction was applied using logistf.

## Results

The seroprevalence of CRCoV in breeding kennels was 35.03% (95% CI: 27.6–43.04) and in pet dogs it was higher at 43% (33.14–53.29). The observed difference was not statistically significant (OR= 1.4, 95% CI: 0.84-2.34,  $\chi^2$  p=0.36). Out of ten SARS-CoV-2 antibody-positive samples, five tested negative, and cross-reactivity was ruled out.

Both sexes were equally represented among the pet animals, with 49% male and 51% female dogs.

As expected in breeding kennels, female dogs were in the majority with 71.34% compared to 28.66% male dogs. Regardless, sex was not found to be an important risk factor for CRCoV seropositivity either in pet animals (OR=1.4, 95% CI: 0.63–3.11,  $\chi^2$  p=0.4) or breeding dogs (OR=0.9, 95% CI: 0.43–1.87,  $\chi^2$  p=0.72).

The youngest household dog was three months old, and the oldest 13 years of age. In breeding dogs, the youngest animal in this study was four months of age and the most senior, 12 years. As shown in Figure 1, among the pet dogs, the lowest percentage of positive animals was in the group younger than one year (10%). Seropositivity then steadily increased until four years of age and decreased after that. Another peak of seropositivity was found in the group of dogs between seven and eight years old (66.67%). In the breeding kennels, young dogs were less seropositive, and seroprevalence increased with age up to 100% in dogs over ten years. The statistical significance of the observed differences in seroprevalence rates between age groups is shown in Table 2.

The influence of several additional risk factors was analysed in breeding dogs. For this study, we defined small kennels as those with fewer than ten adult breeding dogs at the time of sampling, regardless of the number of puppies and young dogs for sale. In the same manner, large kennels were those with ten and more adult dogs. The size of the kennel had no significant effect on the number of seropositive animals (OR 2.18, CI 95%: 0.87–5.45, Fisher's exact test p=0.1). Any history of participation in dog shows, hunting or training, did not increase the likelihood of positive CRCoV ELISA results (Table 1.). Daily disinfection did not influence the seropositivity rate (OR=1.12 CI 95%: 0.46–2.72,  $\chi^2$  p=0.8).

Abnormal respiratory signs were detected in only seven privately owned dogs, and no meaningful statistical analysis could be done. Still, in kennels in which the owners had noticed outbreaks of CIRDC, the number of CRCoV ELISA positive dogs was not higher (OR 1.11, 95% CI: 0.31–3.89, Fisher's exact test p=1).

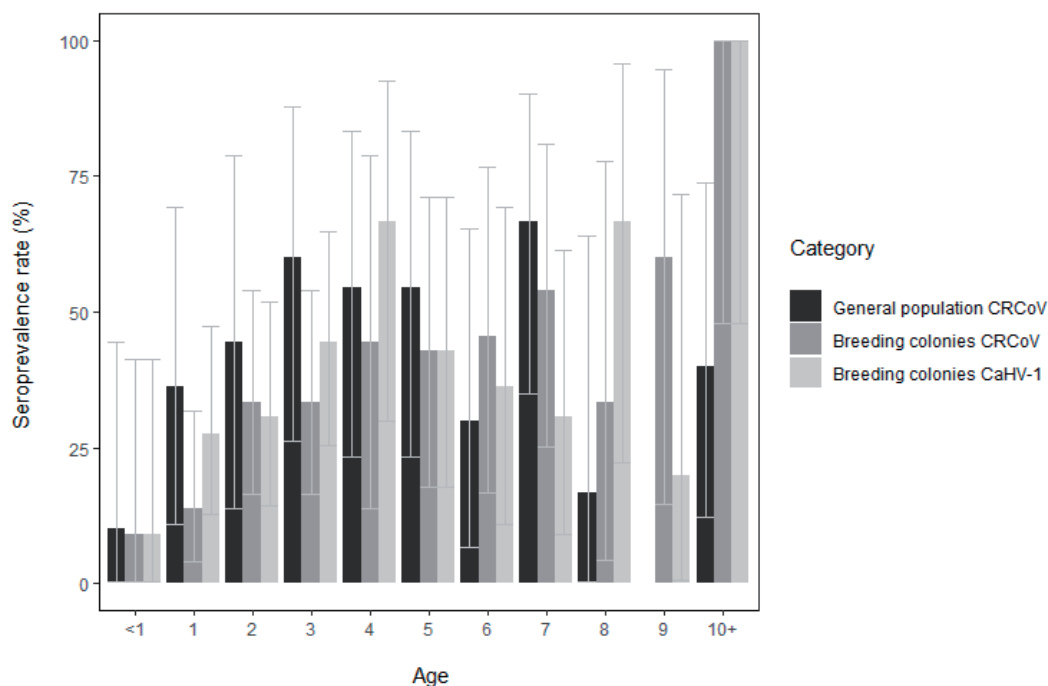


Figure 1. The age distribution of canine respiratory coronavirus seroprevalence in pet dogs (general population) and breeding colonies.

The seroprevalence of canine respiratory coronavirus in breeding colonies is shown along with the seroprevalence of canid herpesvirus 1.  
 CRCoV – canine respiratory coronavirus, CaHV-1 – canid herpesvirus 1

Table 1. Canine respiratory coronavirus – analysis of the host and environmental factors in breeding colonies.

Risk factor	N	OR	95% CI OR	P
Sex	157	0.9	0.43 – 1.87	0.78
Kennel size	152	2.18	0.87 – 5.45	0.1
Working dogs	156	0,99	0.43 – 2.25	0.98
Daily disinfection	104	1.12	0.46 – 2.72	0.8
Hunting	157	0.77	0.28 – 2.13	0.8
Dog shows	143	1.79	0.84 – 3.82	0.13
CIRD	101	1.11	0.31 – 3.89	1

N-number of tested animals, OR - odds ratio, CI - confidence interval, p - Chi-square p-value, CIRD – canine infectious respiratory disease

Table 2. The seroprevalence rate of canine respiratory coronavirus in different age groups. The significance of differences compared to the reference age group.

Age (years)	Privately owned dogs			Breeding colonies		
	Positive (%)	OR (95% CI)	P	Positive (%)	OR (95% CI)	P
<2	23.81	Ref.	-	12.5	Ref.	-
2-3	52.63	3.56 (0.96 – 14.69)	0.007	33.33	3.5 (1.24 – 11.53)	0.02
4-5	54.55	3.84 (1.08 – 15.3)	0.04	43.48	5.38 (1.61 – 20.23)	0.008
6-7	50	3.2 (0.9 – 12.68)	0.08	50	7 (2.14 – 26.06)	0.002
>8	31.25	1.45 (0.33 – 6.45)	0.61	62.5	11.67 (3.11 – 50.77)	<0.001

OR - odds ratio, CI - confidence interval, p - Chi-square p-value, Ref. – reference age group

## Discussion

This study was the first to confirm the presence of CRCoV infections in Croatia. The results presented here suggest that the infection was widespread in privately owned dogs and dogs from breeding colonies. The seroprevalence described was similar to the data reported in the United Kingdom (36%), Ireland (30.3%), the United States, Canada (59.1%) and the most recent studies in European countries (54%) and New Zealand (53%) (PRIESTNALL et al., 2006; MITCHELL et al., 2017).

To the best of our knowledge, there are no available data regarding the epizootiology of CRCoV in breeding colonies. In shelters, where many dogs are kept in confined areas, CRCoV is easily spread, and most dogs seroconvert soon after admission. The high turnover of animals and stress associated with overcrowding is a reason behind the high CRCoV infection rate (ERLES et al., 2004; ERLES and BROWNLIE, 2005). Still, the population in breeding colonies is more stable than in shelters, and the dogs have adjusted to the living conditions and handling. Accordingly, there was no significant difference in seroprevalence rate between the general population and breeding kennels. Surprisingly, dog show visits, training schools and hunting were not associated with any increased seroprevalence in kennels. It was expected that mixing animals from different sources would put animals at risk of infection and spread CRCoV in breeding colonies through animal to

animal contact. It seems that CRCoV infections are endemic and circulate in kennels, independent of new encounters. Dogs in kennels are expected to have a larger number of social contacts, but the size of the kennel did not influence the seropositivity rate.

There may be another transmission route for CRCoV. The high seroprevalence of CRCoV in dogs makes it reasonable to assume that the owners or people, in general, could be passive carriers of the virus, since its zoonotic potential has been ruled out (KRUEGER et al., 2013). Dogs and humans can be infected with BCoV, but at the moment, there is no serological test to distinguish infection with CRCoV and BCoV (ZHANG et al., 1994; KANESHIMA et al., 2007). While most dogs in this study were from an urban area, and contact with infected cattle was less likely, food as a source of BCoV infection is possible. The initial study design did not include information about feeding dogs with raw beef, and due to the retrospective nature of this study, data could not be obtained later.

Finally, little is known about the epizootiology of CRCoV infections. General prophylactic measures, such as quarantine, cleaning and disinfection, would benefit the most from this information. Coronaviruses are readily inactivated in the environment (PRATELLI, 2008), and in this study, daily cleaning and disinfection did not lower the CRCoV seroprevalence rate. Due to its short



survival in the environment, dogs that excrete the virus over a prolonged period of time, as is the case with CCoV, could be responsible for maintaining CCoV in kennels. Different management practices, such as quarantine, confinement, regular veterinary check-ups, could influence the spread of the CCoV in a kennel. Surprisingly, seroprevalence did not differ on the kennel level, which was in striking contrast to CaHV-1 seroprevalence. As mentioned before, for all dogs from breeding colonies in this study, their CaHV-1 serological status was known. Like CCoV, CaHV-1 is a primary respiratory pathogen in adult dogs (RONSSSE et al., 2005). Another similarity between these two pathogens is their high seroprevalence in Croatian dogs (GRACIN, 2020), and unlike some other respiratory viruses, vaccination is not a common practice. In CaHV-1 epizootiology, the influence of kennels on seroprevalence could be explained by different breeding practices, since this virus is sexually transmitted (POSTE and KING, 1971).

The influence of age on CCoV antibody status was analysed. In the general population, CCoV seroprevalence in dogs under one year of age was the lowest, and it was highest at ages four and five. This age-dependent fluctuation of the seropositivity rate has been described before, and it is assumed that with age the likelihood of exposure to the virus increases (PRIESTNALL et al., 2006; PRIESTNALL et al., 2007; MORE et al., 2020). On the other hand, old dogs have a less efficient immune response and more often test negative (PRIESTNALL et al., 2006; PRIESTNALL et al., 2007). In this study, only the general population seropositivity rate declined in dogs over six years of age. In the breeding colonies, the proportion of dogs that tested CCoV ELISA positive steadily increased with age in the same fashion as CaHV-1. It is highly possible that external factors, such as social behaviour, influenced these differences in age distribution between the general population and breeding kennels.

## Conclusions

Even though this study was conducted on a limited number of samples, it provides strong evidence that CCoV is widespread in Croatia, in

both the general population and breeding colonies. The first evidence of CCoV infection dates back to 1996 (ELLIS et al., 2005), so this is a new pathogen in the dog population. Information about its biology and epizootiology is mostly lacking. Still, its confirmed pathogenicity *in vivo* and *in vitro* (MITCHEL et al., 2013) and its involvement in CIRDC highlight the importance of further research. Today we are witnessing the spread of SARS-CoV-2, another respiratory betacoronavirus, in the human population. Control of SARS-CoV-2 was based on containment strategies before a vaccine was available. At this point, the same is true for CCoV infections in the dog population. New data about epizootiology will enable better control of virus spread if no vaccination is available.

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DOI: 10.1002/jmv.1890440207

Received: 27 May 2021

Accepted: 31. July 2021

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**STEVANOVIĆ, V., M. MAURIĆ MALJKOVIĆ, K. GRACIN, I. BENVIN, V. STAREŠINA, S. KOVAČ, A. ŠKRINJARIĆ, S. HAĐINA, I. ZEČEVIĆ, K. MARTINKOVIĆ, J. HABUŠ, Z. ŠTRITOF, M. PERHARIĆ, M. CVETNIĆ AND LJ. BARBIĆ: Seroprevalencija respiratornog koronavirusa pasa u Hrvatskoj. *Vet. arhiv* 93, 239-246, 2023.**

#### **SAŽETAK**

Pseći respiratorni koronavirus nedavno je dodan na listu patogena koji uzrokuju zarazni kašalj legla. Zbog svoje izrazite kontagioznosti proširio se diljem svijeta, a osobito je čest u pasa u skloništima za nezbrinute životinje. Osnovni je cilj ovog istraživanja bio ispitati proširenost infekcije u pasa u privatnom vlasništvu i uzgajivačnicama te ustanoviti čimbenike rizika koji pogoduju infekciji. Ovo je bilo prvo istraživanje koje je dokazalo prisutnost psećeg respiratornog koronavirusa u Hrvatskoj. Od 257 pretraženih uzoraka seruma 35,03 % uzoraka pasa privatnih vlasnika i 43 % uzoraka pasa iz uzgajivačnica dalo je pozitivan rezultat imunoenzimnog testa. Važno je napomenuti da razlika u seroprevalenciji između ovih dviju populacije nije bila statistički znakovita. Spol također nije bio znakovit čimbenik rizika, ali je seroprevalencija rasla s porastom dobi životinja. Miješanje životinja iz različitih uzgoja, prilikom lova, dresure ili izložbi pasa, nije dovelo do porasta seroprevalencije u uzgajivačnicama. Ni provođenje dnevne dezinfekcije nije znakovito utjecalo na seroprevalenciju. Ovo je istraživanje provedeno na ograničenom broju uzoraka, ali ipak dokazuje složenost epizootiologije infekcije psećim respiratornim koronavirusom. Kako cjepivo još uvijek nije dostupno, dodatna istraživanja čimbenika okoliša i domaćina, važnih za epizootiologiju ove bolesti, dat će važne podatke nužne za provođenje mjera prevencije.

**Ključne riječi:** pseći respiratorni koronavirus; seroprevalencija; uzgajivačnice; ELISA; Hrvatska

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