Research Article

Investigation of The Prevalence of Respiratoric Coronavirus in Dogs in Kirikkale and Surrounding Provinces

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Abstract

Diseases in the lungs and other respiratory organs of dogs are usually cured with appropriate treatment. If left untreated, serious problems such as hypoxemia and sepsis occur. Canine respiratory coronavirus (CRCoV) was first identified in the United Kingdom in 2003 in a group of shelter dogs with respiratory disease. It is now detected in dogs worldwide. The disease can be found in dogs with and without clinical respiratory signs. This study aims to investigate to what extent the respiratory coronavirus is effective in the formation of respiratory complaints and to determine its prevalence in dogs. Within the scope of the thesis, 100 dogs with respiratory problems were included in the study. These were dogs brought to Kırıkkale University Veterinary Faculty Hospital, municipal shelters, and private veterinary clinics in Kırıkkale and its surroundings. Nasal and pharyngeal swab samples were analyzed using the RT-PCR technique to detect the presence of CRCoV. Analyzes showed that the incidence of CRCoV in dogs in Kırıkkale and its surroundings was 2% (2/100). This is the first study conducted in Turkey to detect the presence of canine respiratory coronavirus (CRCoV). Therefore, it will form a basis for future studies and provide an insight to the researchers.

Keywords: Dogs, espiratory coronavirus, respiratory system.

Introduction

Respiratory system diseases in dogs rank high in prognostic terms due to their potential to cause death. Therefore, early diagnosis of respiratory system diseases is important. Some respiratory system diseases can be easily diagnosed with simpler diagnostic methods, however, in others, advanced examinations are necessary. Upper respiratory system diseases usually occur in the nasal cavities and frontal sinuses, nasopharynx and laryngopharynx, larynx and extra thoracic trachea. Lower respiratory system diseases occur in the organs in the thorax.

Respiratory disease in dogs is generally observed in places where dogs live or are kept in groups, such as shelters, daycare facilities and animal hospitals. Infectious respiratory disease in dogs has a multifactorial etiology. The most accurate definition is canine infectious respiratory disease complex (CIRDC), formerly known as kennel cough, shelter disease. Many bacteria and viruses may cause CIRDC.

Canine respiratory coronavirus (CRCoV) is a member of the Coronaviridae family and was identified in 2003. It is classified by 4 separate genera depending on their characteristics. These are alpha, beta, delta and gamma. This virus is a betacoronavirus and it is closely related to human coronavirus HCoV-OC43 and bovine coronavirus (BCoV). CRCoV showed 96% amino acid identity with BCoV in its variable spike protein. The presence of CRCoV in dogs was first detected in detailed studies conducted on dogs with CIRDC. It was also frequently identified in samples taken from the trachea of dogs with mild clinical signs (Erles K., 2003). The causative agent cannot be determined by looking at clinical symptoms because respiratory disease has a multifactorial etiology. There are many effective methods and techniques for early diagnosis of the disease. Many serological methods are difficult and costly to use in practice. The use of PCR and RT-PCR methods in rapid serological identification provides benefits in terms of both convenience and cost (Lai MYY, 2005).

Materials and Methods

Material of Animal

This study was conducted with the approval of Kırıkkale University Animal Experiments Local Ethics Committee (Date 29.09.2022, Meeting No: 2022/05, Decision No: 24). 100 dogs with respiratory problems of different ages, breeds and genders in Kırıkkale and its surroundings were used in the study. The dogs were examined at Kırıkkale University Veterinary Faculty Research and Application Hospital. Then, samples were collected to determine the presence of Canine Respiratory Coronavirus, which is involved in the etiology of the Infectious Respiratory Diseases Complex of Dogs.

Clinical Examination

The dogs were brought to Kırıkkale University Veterinary Faculty Research and Application Hospital with respiratory system complaints such as cough, runny nose, sneezing and wheezy breathing. The dogs were clinically examined and recorded in terms of body temperature, pulse, respiratory rate, nasal discharge, tear discharge, and lung sounds.

Blood Count

2 ml of blood was taken into each tube with EDTA (Ethylene Diamine Tetra Acetic Acid) from Vena Cephalica Accecorius for hematological controls. Erythrocyte (RBC), Leukocyte (WBC), Hematocrit (HCT), Average Erythrocyte Volume (MCV), Hemoglobin (Hb), Average Corpuscular Hemoglobin (MCH), Average Corpuscular Hemoglobin Concentration (MCHC) values were measured on the fully automatic blood counting device (Mindray BC-5000 Vet) at Kırıkkale University Veterinary Faculty Research and Application Hospital.

Radiographic Examination

Radiographic examinations of the dogs were performed using the Digital X-ray Device (International Medical Device IMD Basic 100-30) at Kırıkkale University Veterinary Faculty Research and Application Hospital. Direct thorax radiographs were taken in ventrodorsal and laterolateral positions by dosing 10-40 mAs, 40-80 kV, 0.3 seconds depending on the size of the dogs.

Molecular Tests

Collecting Samples

Nasal and pharyngeal swabs (n = 100) were collected from dogs. A total of 100 samples, including nasal swabs and pharyngeal swabs, were simultaneously collected from 100 dogs. The collected samples were transferred to Molecular Transport an Lysis Reagent (NucleoGene Biotechnology Company, Istanbul, Turkey) tubes. The tubes were vortexed and brought to the laboratory. They were stored at 4°C until RNA isolation process.

Nucleic Acid Extraction

Nucleic acid extraction was performed using the NucleoGene N32 nucleic acid automated extraction system (NucleoGene Biotechnology Company, Istanbul, Turkey) in compliance with the manufacturer's recommendation. 200 µl was taken from the samples, which were brought to the laboratory and stored in Molecular Transport an Lysis Reagent (NucleoGene Biotechnology Company, Istanbul, Turkey) tubes at 4°C. They were transferred to Well 1 of NucleoGene Viral NA Extraction Kit Plates. RNA isolation of 32 samples was completed in approximately 30 minutes by selecting the relevant program from the device. The RNAs of the samples were taken from Well 6 as 50 µl and transferred to new nuclease-free 1.5 ml tubes and they were named after RNA isolation was completed. The process continued until RNA isolation of all samples was completed. When each isolation process was completed, the RNAs were put into – 80 C and stored until the Real Time PCR process.

Real Time PCR Process

Real-time PCR is used to measure the absolute amount of a target sequence or to compare relative amounts of a target sequence among samples. This technique is monitored in real time by amplification of a specific fluorescent signal that binds to the target sequence.

NucleoGene Canine Respiratory Coronavirus One Step RT-qPCR Detection Kit (Research use only) (NucleoGene Biotechnology Company, Istanbul, Turkey) was used for the diagnosis of CRCoV infection. The kit contains primary probes that detect the virus with the CoV M gene. Synthetic positive and negative controls were used in each Real Time PCR process. Tests were performed in duplicate. Reading was made by selecting the FAM channel on the device. Curves were observed in the positive control and positive samples. No curves were observed in the negative control and other samples. All Real Time PCR processes were carried out using the AGS4800 Real Time PCR device. Preparation of Real Time PCR reagents and operating conditions in the Real Time PCR device are specified in the tables below.

Statistical Evaluation

The values in the study were digitalized in a computer environment and descriptive statistical information (average, standard deviation, etc.) was obtained. The data were shown as percentages as well as descriptive statistical information since they were not compared with any group.

Results

Clinical Examination Findings

The dogs were brought to Kırıkkale University Veterinary Faculty Research and Application Hospital with respiratory system complaints such as cough, runny nose, sneezing and wheezy breathing. The body temperatures of 16% (n=16) of the dogs were not within the normal range during clinical examinations. 11 of these dogs had high body temperatures (hyperthermia) and 5 had low body temperatures (hypothermia). The body temperatures of the remaining 84 animals were found to be normal. The respiratory rates of the dogs were examined. The respiratory rates of 36 dogs were not within the normal range. 34 of these dogs had hyperventilation and 2 had hypoventilation. The heart rate of 33 dogs was outside the normal range. 32 of these dogs had tachycardia and 1 had bradycardia. Other clinical examination findings were nasal discharge in 53 dogs, eye discharge in 13 dogs, cough in 16 dogs, wheezy breathing in 12 dogs and sneezing in 1 dog. The data obtained as a result of the clinical examination of the dogs are presented in table 3 and figure 1. It was determined that nasal discharge was a common clinical finding in positive animals, and no other clinical findings were observed.

Hematological Examination Findings

Asepsis-antisepsis was performed in the application environment. 2 ml of blood was taken into each tube with EDTA (Ethylene Diamine Tetra Acetic Acid)

from Vena Cephalica Accecorius for hematological controls. Blood samples were measured on the fully automatic blood counting device (Mindray BC-5000 Vet) at Kırıkkale University Veterinary Faculty Research and Application Hospital. Complete blood analyzes of the dogs were measured. Measurements showed minimal changes in leukocyte counts. The most significant finding was in leukocyte counts although it is not specific. Leukocytosis was observed in some of the cases. The presence of leukopenia was also detected in some others. Hematological data are shown in Table 4.

Radiographic examinations of the dogs were performed using the Digital X-ray Device (International Medical Device IMD Basic 100-30) at Kırıkkale University Veterinary Faculty Research and Application Hospital. Direct thorax radiographs were taken in ventrodorsal and laterolateral positions by dosing 10-40 mAs, 40-80 kV, 0.3 seconds depending on the size of the dogs. The radiological examinations were performed. Non-specific findings for CRCoV infection were observed, such as prominence in the trachea and branching in the lungs (prominence of the bronchi).

RT-PCR Findings

Real-time PCR is used to measure the absolute amount of a target sequence or to compare relative amounts of a target sequence among samples. This technique is monitored in real time by amplification of a specific fluorescent signal that binds to the target sequence. The Ct values observed in the graph are the values that indicate the amount of the target gene in the cell. Low Ct values indicate high amounts of targeted nucleic acid. High Ct values indicate lower (or even very small) amounts of the target nucleic acid. Ct values below 29 cycles typically indicate abundant nucleic acid and Ct values above 38 cycles indicate minimum amounts. Nasal and pharyngeal swab samples taken from 100 dogs in the study were analyzed for the M gene by RT-PCR for the diagnosis of CRCoV infection. 98 nasal swab samples were negative while 2 of them were positive for the M gene.

RT-PCR Results and Assessment of Individual Data (gender, age, etc.)

In this study, the CRCoV infection was investigated in samples collected from dogs of different breeds and ages in Kırıkkale and its surroundings. 84% (n=100) of the dogs were mixed breed and the remaining 16% (n=100) consisted of various races. The samples that gave positive results in RT-PCR examinations and detected to have CRCoV were from mixed breed dogs.

The presence of CRCoV infection was identified in dogs aged 1 year and above rather than in younger dogs unlike CCoV according to studies in the literature. The average age of the samples in the project study in Turkey was 2.36 (n=100). The average age of positive samples in the RT-PCR study was 1.50 (n=2). Studies with more samples are required to clearly determine the age predisposition. There is no information in the literature studies about the gender predisposition of CRCoV infection. 49% (n=100) of the samples were from the female dogs and the remaining 51% (n=100) were from the male dogs in this study.

Table 1. RT-PCR Component Table

Components	Volume
NucleoGene Canine Respiratory Coronavirus Reaction Mix	5 µl
NucleoGene Canine Respiratory Coronavirus Oligo Mix	10 µl
Sample, Negative or Positive Control	5 µl
Total Final Volume 20 µl	

Table 2. RT-PCR Operating Conditions

Steps	Cycle Numbers	Temperature	Duration
Application of UNG	1	25 °C	1 minute
Reverse Transcriptase	1	50 °C	10 minutes
Pre-denaturation	1	95 °C	10 minutes
Amplification	45	95 °C 60 °C1	15 seconds 30 seconds
1 Data was collected by taking readings from the FAM channel.			

Table 3. Classification of clinical examination data of the samples used in the study

Clinical Findings		Number of Animals (n)
Body Temperature	Hyperthermia	11
	Normothermia	84
(38,3 – 39,2 oC)	Hypothermia	5
Respiratory Rate	Hyperventilation	34
	Normal	64
(18 -24 respiration/ min)	Hypoventilation	2
Heart Rate	Tachycardia	32
	Normal	67
(70-120 heartbeat/min)	Bradycardia	1
Nasal Discharge		53
Eye Discharge		13
Cough		16
Wheezy Breathing		12
Sneezing		1

Table 4. Hematological data of samples

	n	x ± Sx min-max
RBC (106/mm3)	100	7,46 ± 0,36 5,82 - 8,90
WBC (10^3/µL)	100	9,66 ± 0,42 5,12 - 19,26
HCT (%)	100	45.12 ± 1,92 34,30 - 53,50
Hb (g/dL)	100	14,42 ± 0,68 11,44 - 17,50
MCV (fL)	100	64,86 ± 0,84 60,10 - 68,90
MCH (pg)	100	20,60 ± 0,32 20,10 - 21,80
MCHC (g/dL)	100	33,24 ± 0,26 31,70 - 34,10

Figure 1. Percentage distribution of examination

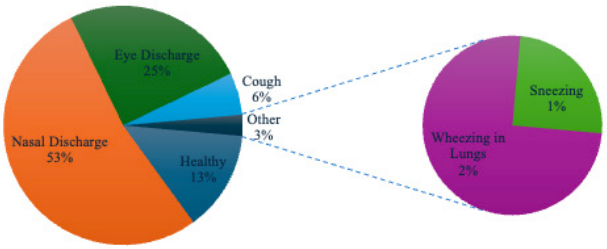


Figure 2. 22-24. Real-Time PCR graph of canine coronavirus detected in cycles (ct: 23.26)

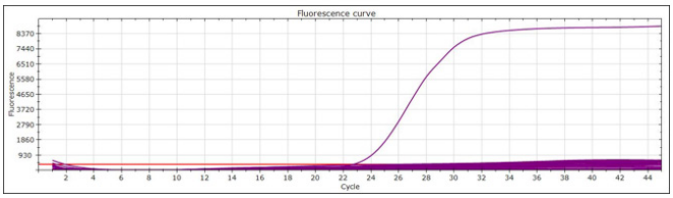
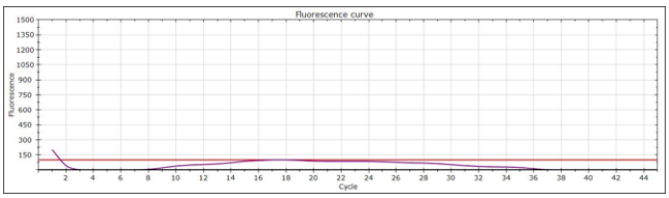


Figure 2. Example of a negative result with no peak (Case 33)



Discussion

Dogs became friendly animals as a result of domestication dating back approximately 14,000 years ago. People have benefited from both hunting and tracking abilities of dogs. The first documents of dog domestication in Anatolia were found in the neolithic murals in Çatalhöyük and they date back to 9000 BCE. The phenomenon of pet farming has become increasingly important since then. There is a dog population of over 500 million in the world. Therefore, issues such as the coexistence of dogs and humans and animal health come forward. The direct effects of contagious viral infections on animal health and the fact that they will also affect human health due to their zoonotic potential has made the research of these types of viruses popular. CRCoV infection is the subject of this study. It has infectious potential and is one of the viral diseases that affect the respiratory system. Nasal and pharyngeal swab samples were taken from 100 dogs and they were examined virologically. The presence of CRCoV nucleic acid was identified in 2 dogs (2%) via RT-PCR. These results constitute the first data on CRCoV infection in Turkey.

There are different reports about the importance and incidence of CRCoV infection in dogs in the world in terms of population and multiple living conditions (Arsevska, 2018). Many researchers believe that transmission of CRCoV infection is quite easy in

multiple living conditions and in conditions where more than one animal shares the same habitat, it may be possible to encounter CRCoV infection more frequently than in animals living alone because the virus spreads with viremia lasting up to 2 weeks and it has a high probability of spreading by oral transmission (Erles K., Toomey, 2003). CRCoV is a new canine coronavirus that is different from CCoV. CRCoV is frequently detected in dogs with clinical respiratory signs and it may cause CIRD complex (Erles K., and Brownlie J., 2005).

Many bacteria and viruses can be involved in CIRD. Bordetella bronchiseptica (Bb), canine adenovirus type 2 (CAV-2), canine distemper virus (CDV), canine herpesvirus (CHV) and canine parainfluenza virus (CPiV) are traditionally the main agents. Canine influenza virus (CIV), canine respiratory coronavirus (CRCoV), canine pneumovirus (CnPnV), Mycoplasma cynos and Streptococcus equi subspecies zooepidemicus (S. zooepidemicus) are new pathogens that have recently emerged in the development of CIRDC. Canine bocavirus and canine hepacivirus have been weakly associated with respiratory disease in isolated dogs (Priestnall, 2014).

Canine respiratory coronavirus (CRCoV) is a member of the Coronaviridae family and was identified in 2003. It is classified by 4 separate genera depending on their characteristics. These are alpha, beta, delta and gamma. This virus is a betacoronavirus and it is closely related to human coronavirus HCoV-OC43 and bovine coronavirus (BCoV). CRCoV showed 96% amino acid identity with BCoV in its variable spike protein. The presence of CRCoV in dogs was first detected in detailed studies conducted on dogs with CIRDC (Erles K., 2003). It was also frequently identified in samples taken from the trachea of dogs with mild clinical signs. The causative agent cannot be determined by looking at clinical symptoms because respiratory disease has a multifactorial etiology. There are many effective methods and techniques for early diagnosis of the disease. Many serological methods are difficult and costly to use in practice. The use of PCR and RT-PCR methods in rapid serological identification provides benefits in terms of both convenience and cost (Lai MYY, 2005). Control of CRCoV includes vaccination and improvement of kennel conditions, taking into account factors such as sanitation, population density, ventilation and quarantine procedures (Kerstin Erles and Joe Brownlie, 2008).

Unlike CECoV (Canine Enteric Coronavirus), CRCoV (Canine Respiratory Coronavirus) has been detected more frequently in dogs aged 1 year and above. Serum samples were collected from dogs in North American states in a prevalence study conducted by Simon L. in 2006. CRCoV infection has been investigated. It was a comprehensive study and it allowed the age sensitivity of the virus to be examined. Seropositive dogs were mostly 7-8 years old 68% (n=1000) (Simon L., 2006). 100 dogs in different age groups, over 1 year old, were tested with the RT-PCR technique to detect the CRCoV infection in Turkey. The positive samples in the study were between the ages of 2-4. The dogs under 1 year old will be less likely to be CRCoV positive compared to older dogs (>1) according to the study.

Canine respiratory coronavirus (CRCoV) was discovered in an extensive study conducted by Erles to identify the source of persistent respiratory complaints despite vaccinations in dog shelters in the United Kingdom. Erles collected blood/serum samples from a dog shelter in London. The number of CRCoV seropositive samples in the shelter was 30% (n = 123) as a result of Elisa antigen tests (Erles K., 2003). The main cause of respiratory complaints in dog shelters was considered to be Bordetella Bronchiseptica until recently. Advanced techniques such as Elisa and RT-PCR were used to resolve complaints that persist despite vaccinations in shelters. Blood/serum samples, nasal-pharyngeal swabs and lung samples taken from dogs were examined. Multiple pathogens have been identified to cause respiratory complaints (A. Mitchell, 2013). Erles and Brownlie collected blood serum samples from dog shelters in London and Warwickshire in another study conducted in 2005. They examined blood serums. They found a CRCoV antibody seropositivity rate of 22.2% (n=54) in London and 54.2% (n=59) in Warwickshire (Erles K., Brownlie J., 2005). Apart from the United Kingdom, Ellis conducted a study in Canada in 2005 and examined 126 dogs with respiratory complaints. 2 dogs were CRCoV positive (Erles K., Brownlie J., 2005). CRCoV was investigated in a different region for the first time in 2005. The positive results found in Canada showed that the new type of coronavirus was not only limited to the United Kingdom.

In 2006, Simon L. conducted a study to determine the prevalence of canine respiratory coronavirus in North America. Serum samples of 1000 dogs were collected from various states. They were examined using the Elisa technique. CRCoV seropositivity prevalence was 54.7% (n=1000) (Simon L., Priestnall, 2006). CRCoV prevalence studies conducted in different regions indicate that canine respiratory coronavirus has a high morbidity (spread) rate. Canine respiratory coronavirus (CRCoV) infection was detected at a prevalence rate of 2% (n=100) in Kırıkkale, Turkey and its surroundings after countries such as the United Kingdom, Canada and North America.

Although the rate is seen as low as 2% in the study, it is actually quite important. Because this ratio indicates the presence of antigen, that is, the agent itself. However, the reason why the rate is high in other studies is that they are studies based on the presence of antibodies. If this study was based on the detection of the presence of antibodies in blood serum, the rate would be high, but it was thought that it would not be able to make an opinion about cross-reactions or would positively increase the rate in animals that survived the disease.

The CRCoV pathogen alone causes non-specific respiratory symptoms. However, when they form a mixed infection with other pathogens, the symptoms are more severe and the risk of pneumonia increases (T. LeRoith, 2012). 2 CRCoV positive dogs had no symptoms other than mild nasal discharge in this study. Information was given about the clinical findings only at the time of sampling in these animals because at what stage of disease the animals were not known, and also only 2 animals had CRCoV.

CRCoV has a viremia period of two weeks in the living body ; for this reason, no obvious markers are seen on lung radiography. Studies have proven that evaluating CRCoV infection with CIRCD is clinically a more accurate approach than addressing CRCoV infection alone. Animals with respiratory problems had also similar radiographic findings in this study.

It is not possible to comment on the determination of racial predisposition of the CRCoV virus due to the narrow scope of the study on racial sensitivity and the lack of genomic analysis. The CRCoV pathogen has a high spread rate when evaluated in terms of morbidity. There should be more sample groups and genetically advanced techniques should be used for racial predisposition studies.

Conclusion

There is a dog population of over 500 million in the world. World Health Organization data demonstrates that there are 3.5 million stray dogs in Turkey. The coexistence of dogs and humans has brought animal health issues to the fore from the earliest times to the present day.

The main cause of respiratory complaints in dog shelters was considered to be Bordetella Bronchiseptica until recently. Advanced techniques such as Elisa and RT-PCR were used to resolve complaints that persist despite vaccinations in shelters. Blood/serum samples, nasal-pharyngeal swabs and lung samples taken from dogs were examined. Multiple pathogens have been identified to cause respiratory complaints (A. Mitchell, 2013).

New studies show that there are many viruses that have not yet been discovered. Canine respiratory coronavirus is different from the canine enteric coronavirus, which has been known to cause enteric symptoms in puppies for years, in terms of the group it is included in and the system it affects. This is the first study conducted in Turkey to detect the presence of canine respiratory coronavirus (CRCoV). CRCoV prevalence studies conducted in different regions indicate that canine respiratory coronavirus has a high morbidity (spread) rate. Canine respiratory coronavirus (CRCoV) infection was detected at a prevalence rate of 2% (n=100) in Kırıkkale, Turkey and its surroundings after countries such as the United Kingdom, Canada and North America.

The study shows that there is a need for a more profound examination of CIRDC infection, which cannot be prevented with vaccines in shelters in Turkey, and that a more comprehensive vaccine is essential by monitoring current strains in vaccine studies.

It is necessary to collect more samples to determine the age, breed and gender predisposition of CRCoV infection. Advanced techniques should be used to obtain the relevant data. CRCoV infection has been proven to exist within the borders of Turkey with this study.

The study will constitute an example for future studies since it is the first study conducted to determine canine respiratory coronavirus (CRCoV) in Turkey. It is crucial to increase the number of samples and use advanced diagnostic techniques in future studies. More studies should be carried out to investigate the prevalence of CRCoV nationwide.

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