

ORIGINAL ARTICLE

No Serologic Evidence for Zoonotic Canine Respiratory Coronavirus Infections among Immunocompetent Adults

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Impacts

- An emerging infectious disease first seen in persons with intense canine exposures could indicate that a canine pathogen has gained the ability to spread across species.
- While seroepidemiological studies have their limitations, they are often employed as a valid first step in examining the potential for zoonotic spread of animal pathogens.
- This study supports the premise that immunocompetent adults are not at risk for canine respiratory coronavirus (CRCoV) infections; however, infrequent cross-species transmission of CRCoV cannot be ruled out.

Keywords:

Coronavirus; zoonoses; occupational exposure; communicable diseases, emerging; seroepidemiological studies

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Summary

Zoonotic diseases continue to emerge and threaten both human and animal health. Overcrowded shelters and breeding kennels create the perfect environment for amplified infectious disease transmission among dogs and present a critical opportunity for zoonotic pathogens to emerge and infect people who work in close contact with dogs. Coronaviruses' widespread prevalence, extensive host range, various disease manifestations and increased frequency of recombination events all underline their potential for interspecies transmission (*Methods Mol. Biol.* 2008, 454, 43). The objectives of this study were to determine whether people with occupational contact with dogs were more likely to have antibodies against canine respiratory coronavirus (CRCoV) compared to persons with no dog exposure. A seroepidemiological cohort study was completed, for which 302 canine-exposed and 99 non-canine-exposed study subjects enrolled in the study by providing a serum sample and completing a self-administered questionnaire. A competitive enzyme-linked immunosorbent assay (ELISA) was developed to detect human antibodies against CRCoV while controlling for cross-reacting antibodies against the human coronavirus OC43. All study subjects were negative for antibodies against CRCoV by this competitive ELISA. This study supports the premise that humans are not at risk for CRCoV infections; however, infrequent cross-species transmission of CRCoV cannot be ruled out.

Introduction

It is important to monitor the human–animal nexus for emerging zoonotic pathogens that gain the ability to cross the species barrier. This is especially true among high-risk occupational settings where animal workers have close and prolonged contact with many animals. Recently, agricultural professionals in routine contact with production

animals have been shown to have an increased risk of zoonotic infections and often experience symptomatic illnesses (Meng et al., 2002; Olsen et al., 2002; Koopmans et al., 2004; Myers et al., 2006; Gray and Baker, 2007; Gray et al., 2007a,b, 2008; Kayali et al., 2010). Dogs are a popular companion animal, but the canine industry is a less-studied occupational setting. Overcrowded shelters and breeding kennels create the perfect environment for

amplified infectious disease transmission among dogs and a critical opportunity for zoonotic pathogens to emerge and infect people who work in close contact with dogs.

First identified in 2003 in the United Kingdom, canine respiratory coronavirus (CRCoV) is a newly emerged host variant of the enteric canine coronavirus (CCoV). Similar to how other coronaviruses undergo genetic evolution, CRCoV evolved through accumulations of point mutations, insertions and deletions within the coronavirus genome (Decaro and Buonavoglia, 2008). Following its discovery, evidence of CRCoV infection or seropositivity has been documented in dogs with various clinical histories in Japan, Italy, New Zealand, Korea, Canada and the United States. CRCoV plays a role in the canine infectious respiratory disease complex (CIRD or 'kennel cough') and is now considered enzootic among dog populations (Priestnall et al., 2006; Erles and Brownlie, 2008). Human infections with CRCoV have never been reported or studied. To investigate evidence of CRCoV infections in humans, a seroepidemiological cohort study was conducted in the United States. We sought to test the hypothesis that dog workers would have a higher prevalence of antibodies against CRCoV compared to non-dog-exposed controls.

Materials and Methods

Participant recruitment and enrolment

This study was approved by the University of Iowa and the University of Florida's institutional review boards. The target population included breeders, kennel employees, veterinary personnel, animal shelter workers, greyhound racetrack employees and dog show handlers whose work or hobby involved exposure to multiple dogs. A non-exposed, non-matched control group consisted of individuals who had neither been exposed to multiple dogs as part of their work or hobby *nor* had pet dogs in their household in the last 5 years. All participants had to be at least 18 years of age and self-report no current immunocompromising conditions.

Recruitments were based on a convenience sample of the target population primarily from Iowa and Florida. Breeders, shelters and veterinary clinics were identified through state databases of licensed breeders and practicing veterinarians, as well as through internet searches. Organizations and staff were invited to participate in the study via a mailed letter with a telephone call follow-up. Enrolments typically occurred at the participants' place of employment. Recruitments also occurred at large public venues including dog shows, agility trials and trade shows. Non-exposed controls were faculty, staff and students from the University of Iowa and the University of Florida. After informed consent was obtained, participants

completed a self-administered questionnaire and permitted collection of a blood specimen via venipuncture at a single encounter. The questionnaire collected demographic data, specific dog exposure data and behavioural data including personal hygiene practices when caring for dogs. Dog-years of exposure for a specific occupation/hobby was calculated by multiplying the average number of dogs with which the subject came in close contact on a given day for the occupation/hobby by the total years worked in the occupation/hobby.

Whole blood specimens were transported on ice to the laboratory within a few hours of collection. Blood tubes were centrifuged at 3000 g for 15 min at room temperature to separate serum. All collected serum was aliquoted and frozen at -80°C .

Laboratory methods

Culturing of CRCoV and HCoV for use as a capture antigen and antagonist in a competitive ELISA

The human colorectal adenocarcinoma cell line (HCT-8) (ATCC catalog #CCL-224) was propagated in modified Roswell Park Memorial Institute (RPMI) media [RPMI 1640 (Gibco®; Invitrogen, Carlsbad, CA, USA), 10 mm HEPES buffer (Fisher Scientific, Pittsburgh, PA, USA), 5% foetal bovine serum (FBS), 10% glucose, 100 mm sodium pyruvate, 100 mg/ml streptomycin (Fisher Scientific) and 100 000 IU penicillin (Fisher Scientific)] as previously described (Erles et al., 2007). A CRCoV polymerase chain reaction (PCR)-positive canine respiratory swab provided by Dr. Edward Dubovi at Cornell University was diluted 1 : 4 in RPMI infection media (FBS dropped to 2%) and used to inoculate a suspension of freshly trypsinized HCT-8 cells. The suspension was then incubated for 1 h at 35°C with 5% CO_2 on a rocking platform. The infected suspension was then seeded onto a 150-cm² cell culture flask (Corning, Corning, NY, USA), 15 ml of infection media was added and the cells were allowed to adhere. A mock-infected flask of HCT-8 cells was included as a negative control. After 5 days, the cells had reached 80–90% confluency and were harvested as previously described (Priestnall et al., 2006).

Propagation of CRCoV was also attempted on two canine respiratory tract cell lines; however, viral titre never surpassed the threshold of that provided by culturing on HCT-8 cells. In attempts to obtain higher titres of CRCoV by serial passage on HCT-8 cells, it was observed that after 5–7 viral passages, the titre would dramatically decrease; therefore, for development of the competitive ELISA, virus at passage 1 on HCT-8 cells was used.

For use as the antagonist for a competitive ELISA, human coronavirus (HCoV) OC43 was also propagated in HCT-8 cells. Briefly, cells were seeded in 150-cm² cell

culture flasks (Corning), and upon reaching 90% confluency, monolayers were washed three times with plain RPMI 1640 media (Gibco®; Invitrogen) and inoculated with 1 ml of HCoV OC43 passage 7 (ATTC # VR-1558) at 9×10^7 TCID₅₀/ml diluted 1 : 4 in RPMI infection media. An additional 15 ml of RPMI infection media was then added, and cells were incubated at 37°C with 5% CO₂ until 70–90% cytopathic effect (CPE) was observed microscopically (24 h). This virus stock was saved as passage 8 and later blind passaged as described above (without re-calculating its TCID₅₀/ml) to ensure fresh viral culture supernatant was used during the competitive ELISA. A mock-infected flask was included as a negative control.

Real-time RT-PCR for the detection of CRCoV in cell culture

Real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed with proprietary primers and probe obtained from the University of Wisconsin to detect the presence of CRCoV in the cell culture. RNA was extracted from the HCT-8 cell culture supernatant with the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA), and from infected HCT-8 cells with the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. One step qRT-PCR was run using Superscript III Reverse Transcriptase with Platinum Taq (Invitrogen) at the following conditions: 42°C for 15 min; 95°C for 2 min; 40 cycles of 95°C for 15 s and 53°C for 30 s. Cycle threshold (C_T) values were examined to determine the number of cycles required for the fluorescent signal to cross a threshold (background) level.

As per a previous report (Priestnall et al., 2006), the protein concentration of the CRCoV, HCoV OC43 and negative control cell lysates was determined with the Pierce Coomassie (Bradford, UK) colorimetric protein assay kit (Thermo Fisher Scientific/Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Optical densities of the samples were determined using the Powerwave 340 automated microplate spectrophotometer (Biotek, Winooski, VT, USA). Referencing a standard curve with bovine serum albumin, protein concentrations (reported as $\mu\text{g/ml}$) were extrapolated.

Development of a competitive ELISA for CRCoV

To detect CRCoV antibodies in human sera while also controlling for cross-reacting antibodies against HCoV OC43, an ELISA designed to detect CRCoV antibodies in canine serum (Erles et al., 2003; Priestnall et al., 2006) was adapted. Sera from CRCoV-positive and CRCoV-negative dogs were used as positive and negative assay controls. CRCoV-infected and uninfected control cell culture

lysates were diluted to approximately 20 $\mu\text{g/ml}$ protein in carbonate-bicarbonate buffer, pH 9.6. The diluted antigen suspensions were added to alternating duplicate columns of clear 96-well flat bottom, high-binding Immulon® 2HB polystyrene microtiter plates (Thermo Scientific, Rochester, NY, USA). Plates were sealed to prevent evaporation or contamination and incubated overnight at 4°C for optimal protein binding. Next, the plates were washed three times with phosphate-buffered saline (PBS) and blocked by the addition of 300 μl of a solution of 5% non-fat milk (Nestle Carnation, Wilkes-Barre, PA, USA) in PBS for 1 h at room temperature. Plates were washed once with PBS. Undiluted human sera were mixed 1 : 1 with HCoV OC43 culture supernatant and incubated at 37°C for 1 h. Then, 50 μl of the mixture diluted 1 : 50 in dilution buffer [5% non-fat milk and 0.05% Tween® 20 (Fisher Scientific) in PBS] resulting in a final 1 : 100 dilution of the sera was added to the plates in duplicate to wells coated with CRCoV-infected and uninfected cell culture lysates. ELISA plates were incubated at 37°C for 1 h and then washed three times in wash buffer (0.05% Tween® 20 in PBS). Detection of human IgG bound to the plates was accomplished by the addition of 50 μl of goat anti-human IgG conjugated to horseradish peroxidase (HRP) (KPL) diluted 1 : 6000 in dilution buffer was added to wells where blocked human sera had been added. To detect dog IgG in the positive and negative control wells, 50 μl of rabbit anti-dog IgG conjugate conjugated to HRP (Sigma-Aldrich, St. Louis, MO, USA) diluted 1 : 5000 was added to the control wells. Following a 1-h incubation at room temperature, plates were washed three times in wash buffer. Detection of the HRP-conjugated antibodies bound to the plates was accomplished by the addition of 100 μl of tetramethylbenzidine peroxidase substrate 2 (TMB) (KPL). After a 10-min incubation in the dark at room temperature, the TMB reaction was stopped by the addition of 100 μl 1 N sulphuric acid (Fisher Scientific). Within 30 min of stopping the reaction, absorbance was read at 450 nm wavelength using the Powerwave 340 automated microplate spectrophotometer (Biotek). Values from the duplicate wells of CRCoV-coated and uninfected cell control wells were averaged for each serum sample. A serum sample was considered positive for antibodies against CRCoV when the average absorbance of the CRCoV well exceeded three standard deviations above the average absorbance of the control wells.

Student's *t*-test was used to compare continuous variables, and Wald chi-square test was used to compare categorical variables. Logistic regression was used to compare optical density (OD) levels between the exposure groups and ascertain odds ratios and associated confidence intervals. Analysis was performed using SAS v9.2 (SAS Institute, Cary, NC, USA).

Table 1. Occupations/hobbies and associated levels of dog exposure, as cited by subjects

Occupation ^a	N	Median dog-years of exposure (IQR) ^{b,c}
Breeder	101	60 (25–250)
Veterinary staff	90	79 (24–200)
Kennel staff	72	60 (30–300)
Veterinarian	63	140 (80–264)
Shelter staff	47	54 (16–160)
Trainer	38	50 (12–160)
Kennel owner	30	225 (117–520)
Groomer	23	50 (14–210)
Racetrack staff	16	540 (200–1560)
Dog show handler	12	60 (26–286)
Owner/Hobbyist	7	50 (18–90)
Researcher	2	19 (5–32)
Pet store staff	1	180 (180–180)

^aSubjects allowed to cite multiple occupations.

^bCalculated as the reported number of years multiplied by the average number of dogs per day.

^cInterquartile range.

Results

Between 2007 and 2010, a total of 302 canine-exposed subjects and 99 non-canine-exposed controls granted informed consent, completed the enrolment questionnaire and submitted a serum sample. Demographically, the gender distribution was identical between exposure groups, but the controls tended to be younger than the exposed group (means of 33 and 43 years old, respectively). Overall, the participants were more likely to be women (68%), and 79% resided in Iowa or Florida where the majority of enrolments took place. Table 1 illustrates the work/hobbies involving close contact (approximately 3 ft) with dogs, as reported by participants (respondents were allowed to indicate more than one occupation/hobby). A single occupation/hobby involved a median of 80 dog-years of exposure. Breeders tended to a median of three breeding females in their kennels.

There was no serological evidence of previous exposure to CRCoV among the study population, based upon results of the competitive ELISA. The frequency of mean OD levels indicated no apparent outliers. In addition, there was no significant difference in the mean OD levels [(average of the test wells) – (average of the negative control wells + 3 standard deviations)] between the two groups. The mean OD was –0.03 for both canine-exposed subjects and non-exposed controls, with no significant difference between the groups, examining both continuous OD data and OD levels categorized into quartiles (Table 2). The canine-positive control serum had a mean OD of 0.013, which was >3 standard deviations above its

Table 2. Serologic results for human antibodies against canine respiratory coronavirus based on optical density (OD) readings between exposed and non-exposed study groups

Variable	Exposed (n = 302)	Controls (n = 99)	P-value	OR (95% CI)
Mean OD (SD)	–0.03 (0.11)	–0.03 (0.12)	0.97	1.02 (0.7–1.5)
OD Quartiles				
First	75 (24.8)	24 (24.2)	0.80	1.1 (0.7–1.6)
Second	76 (25.2)	25 (25.3)		
Third	76 (25.2)	25 (25.3)		
Fourth	75 (24.8)	25 (25.3)		

negative control well. The canine-negative control serum had a mean OD of –0.066.

Discussion

Variations in CoV host range specificity and pathogenesis are attributed to the spike glycoprotein (Gallagher and Buchmeier, 2001). After entry into the body, CoVs attach to specific cellular receptors via the spike protein (Weiss and Navas-Martin, 2005). CRCoV is transmitted through inhalation of infected aerosolized droplets; however, its pathogenesis in dogs is still unknown. CRCoV likely elicits only a subclinical or asymptomatic disease in dogs, but damage to the respiratory epithelium during viral replication may lead to clinical secondary infections by other respiratory pathogens (Buonavoglia and Martella, 2007). CRCoV may also function as a primary pathogen for infection (Priestnall et al., 2006).

If CRCoV's spike protein was to gain affinity for the human respiratory epithelial cell receptor that HCoV OC43 employs (Weiss and Navas-Martin, 2005), CRCoV could potentially replicate in human cells and cause human infections. Based on the current published literature, this is the first study to examine the possibility of zoonotic infections with CRCoV among humans; however, results show no evidence of previous exposure to CRCoV among immunocompetent adults, as no antibodies against CRCoV were detected. There was no difference in ELISA OD between dog workers and unexposed controls.

Cross-reactivity was a substantial obstacle to overcome when designing a serological assay. A competitive ELISA was developed to control for cross-reacting antibodies and detect specific antibodies against CRCoV. The Group 2a HCoV OC43 was chosen as the CRCoV ELISA competitor owing to the high amino acid identities between various viral proteins (up to 98% homologous) (Erles et al., 2007; Lorusso et al., 2009). Strain OC43 is the HCoV most closely related to CRCoV by phylogenetic analyses (Kaneshima et al., 2006) and therefore considered most likely to cross-react with CRCoV antibodies.

This study had a number of limitations. A key limiting factor was the inherently imperfect nature of serological assays. By design, antibodies are not rigidly specific. Infection with one virus or bacterium can render a person immune to attack by a closely related pathogen, thus reducing the incidence of infections. Although fortuitous in nature, this can present a difficult obstacle in serological diagnoses. Because completely controlling for cross-reacting antibodies is often unachievable, epidemiological studies frequently employ comparison groups and statistical adjustments to control for this limitation. In the case of this study, both of these approaches did not overcome the lack of antibody specificity seen for CRCoV.

The negative results of this study suggest several possible scenarios: (i) no one in the study population has been previously exposed to CRCoV, which could be extrapolated further to suggest that CRCoV has not developed zoonotic capabilities; (ii) the assay's sensitivity was insufficient in detecting true positives; or (iii) IgG-only secondary antibodies did not detect existing IgM antibodies indicative of recent exposures. It is possible that low levels of antibodies against CRCoV were present in human sera but HCoV OC43 cross-reacted with CRCoV antibodies and prevented them from being detected.

Another significant limitation of examining novel zoonotic transmission of an infectious disease is the lack of proper positive and negative assay controls. There exist no human sera from a known human case of CRCoV. Furthermore, cross-reactivity with antibodies against HCoVs that cause up to 30% of common colds (Keyaerts et al., 2009) as well as with distantly related enteric HCoVs makes finding truly non-exposed negative control sera difficult as well. Without these human serum controls, this study employed canine serum as a basis for assay success. While appropriate and essential for this study of a potentially zoonotic disease, this parallel was not ideal as canine serum is intrinsically different from human serum, and the secondary antibodies (anti-dog IgG and anti-human IgG) require individual optimization.

In addition, the lack of prevalence data for CRCoV among dogs in the study areas weakened the strength of this study. Without prevalence data of CRCoV in the canine population, it is difficult to distinguish whether negative results indicate the pathogen is not zoonotic or whether people were not being exposed to CRCoV in the first place.

While seroepidemiological studies have their limitations, they are often employed as a valid first step in examining the potential for zoonotic spread of animal pathogens. In spite of the aforementioned study limitations, this study's novelty and public health significance made its implementation worthwhile.

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