Canine coronavirus in Australian dogs

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Objective To estimate the frequency of serum antibodies (IgG and IgM) to canine coronavirus (CCV) in the Australian dog population and evaluate the role of CCV as a causative agent of gastroenteritis.

Design A serological survey of antibodies to CCV among different dog populations.

Procedure The development and characterisation of an indirect ELISA for the detection of antibodies (IgG and IgM) to CCV was undertaken. Sera collected from both diarrhoeal and non-diarrhoeal dogs from various populations throughout Australia were tested for these antibodies to CCV.

Results Serum samples (1396) collected from 1984 to 1998 were tested for the presence of IgG antibodies to CCV. Samples were divided into two categories on the basis of the number of dogs housed together. The groups were either an open population containing dogs housed as groups of three or less, or kennel populations. Sera from 15.8% of the open population and 40.8% of kennelled dogs were positive for CCV antibodies. The prevalence of antibodies varied from zero to 76% in kennelled dogs. About 23% of 128 dogs positive for IgG antibodies to CCV were also positive for IgM antibodies to CCV, indicating recent CCV infection. Of those dogs that were presented with clinical signs of gastroenteritis such as diarrhoea and vomiting(n = 29), 85% were positive in the IgM ELISA and 85.7% in the IgG ELISA for antibodies to CCV. In comparison, for those dogs presented without any history of gastroenteritis only 15% were positive for IgM and 30% positive for IgG.

Conclusion Serological evidence indicates that infection with CCV in dogs is widespread throughout the Australian mainland. The prevalence of antibodies varies greatly among different populations, with an average of 40.8% positive in kennelled populations and 15.8% in the open population.

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Key words: Dogs, canine coronavirus, gastroenteritis, ELISA, serological survey.

CCV was first recovered from the faeces of dogs during an epidemic of diarrhoeal disease in Germany.[1] Enteritis was subsequently induced in neonatal dogs by oral inoculation of canine coronavirus (Strain 1-71) isolated from the original outbreak.[2] Enteritis accompanied by diarrhoea was characterised by atrophy and fusion of intestinal villi. The infection was not fatal and was self-limiting.[2] Subsequently CCV has been isolated from other epidemics of canine diarrhoea and has a worldwide distribution.[3-5]

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The extent to which CCV is important as a gastroenteric pathogen in the Australian dog population is presently unknown, although CCV and CVLP have been previously reported in Australia.[6-8] In these reports, electron microscopy of faecal samples was used as the detection method with a relatively small number of samples.

In this study we determine the prevalence of serum IgG and IgM antibodies to CCV from a larger number of dogs sampled from throughout Australia.

Materials and methods

Virus and cells

CCV TN-449 strain and CRFK cell line were obtained from Fort Dodge Laboratories, Iowa, USA.

Cell culture

CRFK cells were propagated in 75 cm^2 cell-culture flasks at 37°C (Corning, Australia) in growth medium containing EMEM (Trace Biosciences, Australia), 2 mM L-glutamine, 0.05% LAH and 10% FBS not heat inactivated (CSL Ltd, Parkville). Maintenance medium for maintaining cells without division consisted of EMEM, 2 mM L-glutamine, 0.05% lactalbumin hydrolysate and 5% FBS not heat inactivated.

Positive and negative serum antibodies to CCV

Positive control serum (pooled sera from dogs inoculated with CCV TN-449) and negative control serum (pooled sera from specific pathogen free dogs) were obtained from Fort Dodge Laboratories, Iowa, USA. These serum samples were further verified by serum neutralisation assay as described by Mochizuki et al.[9]

ELISA antigen preparation

Roller bottles of 1500 cm^2 surface area (Corning, Australia) with CRFK cells at 90% confluency were infected with CCV (TN-449) at a multiplicity of infection of 0.1. After incubation for 48 h at 37°C, cells were harvested by scraping with a cell scraper and resuspended in 10 mL of PBS. Cells were washed three times by centrifugation at 2000 *g* for 10 min and resuspended in 10 mL of PBS containing 0.3% sodium deoxycholate and incubated for 30 min on ice. The suspension

CCV	Canine coronavirus
CPV	Canine parvovirus
CRFK	Crandell feline kidney
CVLP	Coronavirus like particles
ELISA	Enzyme linked immunosorbent assay
EMEM	Essential Mininum Eagle Salts Medium
FBS	Foetal bovine serum
HRP	Horse radish peroxidase
LAH	Lactalbumin hydrolysate
OD	Optical density
PBS	Phosphate buffered saline

was ultracentrifuged for 30 min at 90,000 *g* (4°C) and the supernatant used as antigen for the ELISA. Control antigen was prepared in the same manner from uninfected cells.

Indirect ELISA methods

Indirect ELISAs for the detection of serum IgG or IgM antibodies to CCV were developed on the basis of previously published assay methods.[10,11]

Control and CCV TN-449 antigens were diluted separately to 2 µg per 0.1 mL in carbonate-bicarbonate buffer and incubated overnight (4°C) in 96-well flat-bottomed Immunoplates (Nunc, Denmark) with 0.1 mL per well of diluted antigen. The solution was decanted from the plates and 0.1 mL per well of 0.1% skim milk in PBS-blocking agent was added. After incubation for 1 h at 37°C, plates were washed once with PBS containing 0.05% Tween 20. Positive and negative control sera and test sera were serially diluted in PBS containing 0.05% Tween 20 with 10% foetal bovine serum (CSL Biosciences, Australia), inactivated at 57°C for 1 h and added at a volume of 50 µL per well. Dilutions were incubated for a further hour at room temperature before the plates were decanted and washed three times with PBS containing 0.05% Tween 20. Fifty µL per well of a 1:6000 dilution of either HRP-conjugated rabbit anti-dog IgG (Sigma, Australia) or HRP-conjugated goat anti-dog IgM (Nordic, The Netherlands) was added and the plates incubated for 1 h at room temperature. Following a further three washes, 100 µL per well of 0.1 mg per mL tetramethylbenzidine microwell peroxidase substrate (Kirkegarrd & Perry Laboratory, USA) was added and incubated for 10 min. One hundred µL of 0.3M phosphoric acid was added to stop the reaction and the absorbance was read at 450 nm. Each serum dilution was performed in duplicate, with the absorbance value of the control antigen deducted from the CCV TN-449 antigen absorbance value to give the final value. An absorbance value greater than 0.1 was considered a positive reaction. This value was at least two times higher than background values from negative control sera (unpublished data).

Population

Serum samples (n = 1396) were collected from dogs in two different housing groups. One group contained dogs housed in groups of no more than three animals (open population) while the other group contained dogs from commercial breeders, rescue shelters and remote settlement colonies where dogs were housed as a group of 12 or more animals (kennel populations) (Table 1).

Open population - In this group, 1107 serum samples were collected from veterinary pathology laboratories, universities and by field sampling from dogs submitted for general pathology, surgery or survey purposes. These dogs were domestic pets from both suburban and rural areas, generally kept singly or in pairs. The sample areas included all the Australian states and territories, with sera collected from 1984 to 1998.

Kennel populations - In this group, 289 serum samples were collected from three commercial breeders, a university study, three rescue kennels and four remote settlement colonies.

Serum samples

All serum samples were stored at -30°C before use. All samples were tested for IgG antibodies and 128 randomly selected IgG positive sera were tested for IgM antibodies to CCV. Fourteen IgG-negative serum samples derived from dogs at sites where episodes of gastroenteric infection had recently occurred, were also tested for IgM antibodies to CCV.

Statistical analysis was carried out using unpaired *t*-tests (Statview version 4.5; Abacus Concepts Inc, USA).

Table 1. Detection by ELISA of IgG antibodies to canine coronavirus in Australian dog populations.

Housing	No. tested	No. positive[a]	% positive
Open population			
New South Wales	276	66	23.9
Northern Territory	20	0	0
South Australia	91	7	7.7
Queensland	266	73	27.4
Western Australia	200	13	6.5
Victoria	109	6	5.5
Tasmania	1	1	100
Mixed (Australia)[b]	144	9	6.3
Total	1107	175	15.8
Kennel populations			
Breeding colony 1 (NSW)	33	25	75.8
Breeding colony 2 (NSW)	30	14	46.7
Breeding colony 3 (NSW)	24	18	75.0
University study (VIC)	44	13	29.5
Rescue shelter 1 (QLD)	16	3	18.8
Rescue shelter 2 (SA)	23	9	39.1
Rescue shelter 3 (VIC)	25	18	72.0
Remote settlement colony 1 (NT)	35	15	42.9
Remote settlement colony 2 (NT)	29	2	6.9
Remote settlement colony 3 (NT)	18	0	0
Remote settlement colony 4 (NT)	12	1	8.3
Total	289	118	40.8
Grand total	1396	293	21.0

[a]An absorbance value of greater than 0.1 (OD. 450 nm) was considered a positive reaction.
[b]State not recorded

Results

IgG antibody to CCV

Open population - Of the 1107 dogs tested, IgG antibodies to CCV were detected in 175 (15.8%), (Table 1). From this group, clinical histories were obtained for 162 dogs. Twenty-eight of these dogs had signs of gastroenteritis such as diarrhoea, vomiting and weight loss and all 28 dogs were negative for gastroenteric pathogens such as CPV as determined by independent pathology laboratories. Twenty-four of the 28 were positive for CCV antibody (IgG), with ELISA OD results equivalent to those obtained from experimentally infected dogs (unpublished data). Antibodies (IgG) to CCV were detected in 40 of the remaining 134 for which histories were obtained but which did not have diarrhoea or any other signs of gastroenteric infection at the time of presentation.

Kennel populations - Of the 289 group-housed dogs, IgG antibodies to CCV were detected in 118 (40.8%). Ten of the 11 different kennel populations tested contained CCV antibody-positive dogs.

The range of OD values determined by indirect ELISA for serum IgG antibodies to CCV varied from 0.100 (the cut-off

value for a positive result) to 0.590. For the open population group the majority (81%) of readings were in the range 0.100 to 0.199. In comparison 58.5% of values for the kennel populations were greater than an OD of 0.199.

IgM antibody to CCV

Twenty-nine of the 128 (22.7%) randomly selected, IgG antibody-positive sera were also found to be positive for IgM antibodies to CCV. Clinical histories were available for 20 of these 29 dogs and 17 (85%) had clinical signs of diarrhoea.

Among the IgG negative sera, fourteen samples were also tested for IgM antibodies to CCV. These samples were selected on the basis of a high prevalence of gastroenteric disease in the kennelled population at the time of sampling (Breeding colony 1, NSW, 8 samples) or evidence from clinical records of a recent episode of gastroenteritis in the open population dogs sampled (6 samples). Seven (50%) of these dogs were positive for anti-CCV IgM antibodies.

Discussion

The prevalence of CCV antibodies in different dog populations throughout the world has been found to range from 6 to 75%, with as high as 80% reported in kennelled populations.[11-13] We report the first serological study of canine coronavirus antibodies in the Australian dog population. The prevalence of CCV antibody was 15.8% (0 to 27.4%) in dogs housed singly or in small groups (open population) but a significantly higher ($P < 0.0001$) prevalence of 40.8% (0 to 76%) was found among kennelled dogs.

CCV has been reported previously in Australia based on electron microscopic examination of faeces; these two studies showed 7.1% of 154 dogs positive, and 2.9% of 102 dogs positive.[7,8] In the open population of 1107 dogs tested we found 15.8% positive for anti-CCV IgG antibody, which reflects past exposure and infection with CCV whereas the electron microscopic studies detected only those dogs currently infected and shedding virus in their faeces.

Dogs positive for CCV antibody were found in every Australian state and territory, including remote areas of the Northern Territory. Schnagl and Holmes[6] had also previously reported CVLP in remote areas of Western Australia and Northern Territory. One dog with antibodies to CCV was found in Tasmania, although no other sera were tested from that state. We conclude that CCV exists in dogs throughout Australia.

In the open populations, most ELISA results fell within an OD range of 0.1 to 0.199 (unpublished data). Within the kennel populations, approximately 60% of OD readings were greater than 0.199 ($P > 0.0001$). The difference in the occurrence of exposure among dogs from kennel populations and the open population has been observed previously.[11-13] The higher frequency of exposure found in kennel populations presumably represents the increased opportunity for exposure due to the different housing and social interactions.

In the three commercial breeding colonies from NSW examined in this study, severe gastroenteric disease resulting in variable fatality among young pups had been previously observed (M Lindsey personal communication). In these cases CPV was reported as the causative agent. The current study identified high titres of CCV antibody in these kennels during the same period. IgM antibodies to CCV were also detected in these populations, indicating current CCV infection at the time of the gastroenteritis epidemics. It is probable that a mixed infection of CCV and CPV was occurring and the presence of CCV may have contributed to the severity of the enteritis. Appel[14] and Brunner and Swango[15] demonstrated increased severity of clinical signs of both CCV and CPV in mixed infections, particularly in young and stressed animals. It is therefore difficult to assess the clinical implications of CCV infection alone, because mixed infections or other factors may influence the course of disease.

The 24 dogs that were found to be positive for CCV antibody and had signs of gastroenteritis, may represent examples of CCV induced disease as these dogs were also negative for many of the other common gastroenteric pathogens such as CPV (unpublished data). A highly significant correlation ($P < 0.0001$) was found between the presence of anti-CCV IgG antibodies and diarrhoea, with 24 of 28 dogs (85.7%) that presented with diarrhoea being IgG positive compared with 40 of 134 (30%) dogs that presented without diarrhoea. Approximately 23% of 128 dogs positive for IgG antibodies to CCV were also positive for IgM antibodies to CCV indicating infection within 14 days of blood samples being collected.[16] Seven dogs were identified as IgG antibody negative and IgM antibody positive to CCV. These dogs represent examples of animals exposed to CCV within 2 to 5 days before blood samples were collected and that had not had time to produce a detectable IgG antibody response. All seven of these dogs had signs of gastroenteritis. Eighty-five percent of dogs that had clinical signs of gastroenteritis attributed to CCV infection were positive for IgG and IgM antibody to CCV. In comparison, for those dogs presented without any history of gastroenteritis only 15% were anti-CCV IgM positive ($P < 0.0001$). Our study indicates that CCV is widespread in the Australian dog population and suggests that the presence of CCV antibodies is associated with gastroenteritis.

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OBITUARY

Marjorie Ann Reid

Marjorie Reid was a remarkable woman who epitomised the wealth and breadth of veterinary training, by successfully accepting challenges that ranged from practice, laboratory, fieldwork and senior veterinary administration. Marjorie, who was born in 1926, graduated from the Royal Veterinary College in 1950 and was one of a small, but elite group of women in the veterinary profession in England. She had always had a strong sense of adventure and pioneering spirit and, in 1951, she commenced practice in South Africa. Later, she moved to Kenya and joined the well known Veterinary Research Institute at Kabete near Nairobi. Marjorie enjoyed working in Kenya, where there was a constant challenge to use basic veterinary principles to provide a diagnostic service for commercial and wild animals. Her two daughters were born during this period in Kenya. During 1963 she immigrated to Australia and became the first female veterinarian at the Institute of Medical and Veterinary Science in Adelaide. It was during this period she became interested in bacteriology and began to develop her skills in this subject.

Always seeking adventure, in 1966 she joined the Department of Agriculture, Stock and Fisheries in Papua New Guinea and was based at the Kila Kila Veterinary Laboratory in Port Moresby. When she arrived the laboratory was a converted airport control tower from the Second World War. This tested her strengths in more ways than one, not only in bacteriology, but also filling in as Port Moresby's veterinarian on occasions. With her colleagues, Ifor Owen and Noel Talbot, she showed a remarkable ability to use good practical commonsense to simplify what seemed to be highly complex issues. When the new laboratory was built, she was able to demonstrate her considerable laboratory skills to all and was a good but firm teacher to many. This was a happy and productive period of her life. Marjorie was very good with animals of all species ranging from the usual to exotic species such as crocodiles. Because of her combination of ability and character, she was the 'preferred vet' in a strongly male-dominated society, which reflected her high standing in the community. Many at first underestimated her strength of character and discipline; everyone soon learnt not to loaf about when Marjorie was around.

In 1974, Marjorie was invited back to South Australia to organise the Brucellosis and Tuberculosis Eradication Scheme in cattle. Again true to form, she was the first woman to join the Department of Agriculture in South Australia. Due to her efforts and management skills she orchestrated a successful Eradication Scheme. The effort to coordinate all aspects of the Brucellosis campaign, with limited resources in a big state, was a difficult task. Marjorie was appointed Principal Veterinary Officer in 1976 and Deputy Chief Veterinary Officer for South Australia in 1986.

She retired in 1990 to live in the Adelaide Hills, devoting some of her time on a voluntary basis to the Guide Dogs for the Blind. Marjorie was the first woman in many of her appointments and she was able to achieve this due to her strong character, abundant wit and sense of humour. She enjoyed a good argument or discussion and, with her formidable memory for dates and wide reading, she was not an easy person to get the better of. Those who tried, felt the sharpness of her wit. An agitated Marjorie was a sight to behold. There are still anecdotes circulating around PNG about Marjorie, when someone accidentally locked her in the cold room.

In 1998 she moved to Sydney to be closer to her family and died on 6 June 2000, after a long illness during which she showed her characteristic strength of character. The veterinary profession is very proud of Marjorie Reid's contribution to the welfare of animals in Australia and internationally.

JW Copland