

# Enzyme-linked immunosorbent assay for the detection of canine coronavirus and its antibody in dogs

Kotaro Tuchiya\*, Taisuke Horimoto, Masayuki Azetaka, Eiji Takahashi\*\* and Shin-ichiro Konishi\*\*\*

*Department of Veterinary Microbiology, Faculty of Agriculture, University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113, Japan*

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## ABSTRACT

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Two methods of enzyme-linked immunosorbent assay (ELISA) were developed for the diagnosis of canine coronavirus (CCV) infection in dogs. One ELISA, in which CCV-infected CRFK cell lysate is used as antigen, is for the detection and titration of antibody against CCV, and the other ELISA uses the double antibody sandwich method for the detection of CCV antigen. The first ELISA procedure demonstrated antibody responses in dogs inoculated with CCV, as did the virus neutralization test; the second ELISA detected specific CCV antigen in feces and organ homogenates of inoculated dogs.

## INTRODUCTION

Canine coronavirus (CCV) has been identified as one of the causative agents of viral enteritis in members of the Canidae. The presence of CCV infection has been reported in many countries (Binn et al., 1975; Foreyt and Evermann, 1985; Schnagl and Holmes, 1978; Appel et al., 1979; Vandenberghe et al., 1980; Tingpalapong et al., 1982; Yasoshima et al., 1983). Canine parvovirus, canine rotavirus, and astroviruses may also be responsible for canine viral enteritis (Carmichael and Binn, 1981). CCV infection and other

\*Present address: Nippon Institute for Biological Science, 2221-1, Shinmachi, Ome, Tokyo 198, Japan.

\*\*To whom correspondence should be addressed.

\*\*\*Present address: Department of Animal Health I, School of Veterinary Medicine, Azabu University, 1-17-71, Fuchinobe, Sagamihara, Kanagawa 229, Japan.

viral enteric infections are highly contagious and spread rapidly through dog populations. Clinical signs of CCV infection vary from inapparent to those of rapidly fatal gastroenteritis. It is impossible to distinguish between the various causes of gastroenteritis by clinical signs. Mixed infections with two or more agents, including bacteria and parasites, occur in nature, which further complicates the diagnosis of canine enteritis (Vandenbergh et al., 1980; Yasoshima et al., 1983).

In the diagnosis of CCV infection, electron microscopy, virus isolation by cell culture and the immunofluorescent assay (IFA) have been used for the detection of CCV or its antigen (Binn et al., 1975; Keenan et al., 1976; Williams, 1980). The virus neutralization test (NT) and indirect IFA have also been used in serodiagnosis and serosurveys (Binn et al., 1975; Keenan et al., 1976; Appel et al., 1980; Helfer-Baker et al., 1980; Foreyt and Evermann, 1985). These procedures take a long time and are not suitable for handling many samples. Recently the enzyme-linked immunosorbent assay (ELISA) has been used for the detection of anti-viral antibodies and virus antigens (Voller et al., 1976; Kapikian et al., 1979; Callebaut et al., 1982; Crouch et al., 1984). In the present report two ELISA procedures for the detection of anti-CCV antibody and CCV antigen are described.

## MATERIALS AND METHODS

### *Virus and cells*

CCV strain 1-71 (Binn et al., 1975) was obtained from the American Type Culture Collection (U.S.A.). This virus was passaged in primary dog kidney cells and then in CRFK cells, derived from domestic cat kidney by Crandell et al. (1973), and was obtained from Dr. J.F. Weaver, Naval Bioscience Laboratory, University of California, U.S.A. The feline cell line fcwf-4 (Horzinek et al., 1982) was kindly supplied by Dr. N.C. Pedersen, University of California, U.S.A., and was used for virus isolation experiments.

### *Experimental infection of dogs with CCV*

One litter of seven 21-day-old puppies was inoculated with CCV strain 1-17. Three puppies were inoculated orally (dogs D1, D2 and D3;  $10^{3.6}$  TCID<sub>50</sub> of virus and three intranasally (dogs D4, D5 and D6;  $10^{2.2}$  TCID<sub>50</sub>); one puppy (dog D7) was kept as a non-inoculated control. Serum samples were serially collected from each dog and examined for CCV antibody by ELISA and NT.

Rectal, nasal, and throat swabs were also collected from the dogs. These specimens were suspended in 3 ml of Eagle's minimum essential medium containing 10% tryptose phosphate broth, 1% fetal calf serum, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and antibiotics, and the suspensions were clarified by low-speed centrifugation. The supernatants were stored at  $-20^{\circ}\text{C}$  until examined. Some dogs were killed at post-

inoculation day (PID) 5 (dogs D3 and D6) or PID 11 (dogs D2 and D5) and necropsied. Each organ was divided into two parts; one part was quenched in n-hexane at  $-70^{\circ}\text{C}$  for viral antigen detection by IFA, and the other was stored at  $-20^{\circ}\text{C}$  for virus isolation by cell culture and viral antigen detection by the ELISA developed in this study. For these assays frozen materials were thawed and 10% homogenates were prepared in the medium used for the swab specimens.

#### *Antisera against coronaviruses*

Antisera against CCV strain 1-17 were prepared in rabbits. Virus antigens purified by the method of Makino et al. (1983, 1984) were mixed with Freund's complete adjuvant, and injected into the foot pad. Three weeks later each rabbit received a similar injection, and sera were obtained 1 week after this booster inoculation. Serum from a cat experimentally infected with feline infectious peritonitis virus (FIPV) was kindly supplied by Dr. R. Goitsuka (Department of Veterinary Medicine, University of Tokyo, Japan). This serum had a neutralizing titer of 600 000 against CCV strain 1-71.

#### *ELISA procedure for the detection of antibody to CCV*

CRFK cells, which were inoculated with CCV strain 1-71 at a multiplicity of infection of 0.1, were incubated at  $37^{\circ}\text{C}$  for 24 h. The infected cells were scraped off using a rubber policeman and washed three times with phosphate buffered saline (PBS), and  $10^8$  cells were resuspended in 5.5 ml of PBS containing 0.3% sodium deoxycholate (Difco, U.S.A.). The suspension was incubated on ice for 30 min, centrifuged at  $90\,000\times g$  for 30 min, and the supernatant was used as the ELISA antigen. Mock infected cells were treated in the same manner and used as the control antigen. The protein contents of these antigens were measured by the method of Lowry et al. (1951). These antigens were stored at  $-70^{\circ}\text{C}$  until use.

For the ELISA, virus antigen and control antigen were diluted to an appropriate concentration in PBS, and 100- $\mu\text{l}$  volumes of the dilutions were dispensed into the wells of 96-well flat-bottomed Immunoplates (Nunc, Denmark). After overnight incubation at  $4^{\circ}\text{C}$ , the solutions were decanted and the sensitized wells were preincubated with 100  $\mu\text{l}$ /well of PBS containing 0.1% bovine serum albumin (Armour, U.S.A.) for 1 h at  $37^{\circ}\text{C}$ , followed by a single wash with PBS containing 0.05% Tween 20 (PT). Test and control sera were serially diluted two-fold in PT containing 10% calf serum (PTCS), and 50- $\mu\text{l}$  aliquots were dispensed into each well of virus antigen-coated and control antigen-coated plates. After incubation for 1 h at room temperature, wells were washed three times with PT and again incubated with 50- $\mu\text{l}$  volumes of peroxidase-conjugated goat anti-dog IgG (Cappel, U.S.A.) for another hour at room temperature. The wells were again washed, and 100  $\mu\text{l}$  of substrate solution was placed in each well. The solution consisted of 50 ml

0.1 M citric acid, 50 ml 0.2 M disodium hydrogen phosphate, 30 mg 2,2-azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt (ABTS, Boehringer Mannheim GmbH, West Germany), and 10  $\mu$ l 30% hydrogen peroxide. After 1 h of incubation at room temperature, optical density was measured at 405 nm with a Titertek Multiskan (Flow Laboratories, U.S.A.). The ELISA value of each sample was obtained by subtracting the absorbance of the control antigen-coated well from that of the virus antigen-coated well; an ELISA value greater than 0.1 was considered to be positive for the reaction. The ELISA antibody titer was expressed as the reciprocal of the highest positive serum dilution.

#### *Virus neutralization test*

Test serum was heated at 56°C for 30 min, then serially diluted two-fold and mixed with an equal volume of virus solution containing 200 plaque-forming units/0.2 ml of CCV strain 1-71 and was incubated at 37°C for 1 h. The mixture was assayed by the plaque-counting method (Tuchiya et al., 1987), and the neutralization titer was expressed as the reciprocal of the serum dilution that reduced the plaque count to 50% of the control value.

#### *ELISA procedure for the detection of CCV antigen*

The double antibody sandwich method was used with anti-FIPV cat serum as the capture antibody. Cat anti-FIPV serum and anti-CCV antibody-free serum were diluted at an optimal concentration in 50 mM carbonate buffer, pH 9.6, and 100  $\mu$ l of each serum was dispensed into individual wells of 96-well Immunoplates. After overnight incubation at 4°C, serum solutions were decanted and wells were preincubated with 100  $\mu$ l/well of PBS containing 0.1% bovine serum albumin for 1 h at 37°C followed by one wash with PT. Specimen solutions were diluted 1:2 in PBS containing 0.1% Tween 20, and 100- $\mu$ l aliquots were distributed into each well of the anti-FIPV, serum-coated and anti-CCV antibody-free, serum-coated wells. After overnight incubation at 4°C, wells were washed with PT three times and incubated with 50- $\mu$ l volumes of rabbit anti-CCV serum in PTCS for 1 h at room temperature. The wells were washed and again incubated with goat anti-rabbit IgG peroxidase conjugate (Miles-Yada Ltd., Israel) in PTCS for another hour at room temperature. The wells were then washed, and the amount of conjugate bound to each well was determined as described above. The ELISA value of each specimen was obtained by subtracting the absorbance of the anti-CCV antibody-free, serum-coated well from that of anti-FIPV serum-coated well. The cut-off value was calculated by the method of Smirnov (1941).

#### *Antigen absorption test*

The specificity of the antigen-detection ELISA was examined using the absorption test. Rectal swab specimens with a high ELISA value from dogs D1

and D4 were mixed with equal volumes of coronavirus antibody-negative and -positive dog serum, and the mixtures were incubated at 4°C overnight. The mixtures were then tested by ELISA as described above. The reduction of the ELISA value by treatment with anti-CCV serum and the lack of reduction with antibody-free serum indicated the specificity of the procedure.

#### *Virus isolation*

Specimens from experimental dogs were inoculated with CRFK and fcfw-4 cells, and each culture medium was blindly passaged three times. Isolated cytopathic agents were identified as CCV by the IFA technique described below.

#### *IFA*

The localization of viral antigens in the frozen sections of organs from experimental dogs was demonstrated by an indirect IFA technique using rabbit anti-CCV serum and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Miles-Yada Ltd., Israel).

## RESULTS

### *Determination of optimal antigen concentration for antibody-detection ELISA*

Antigen concentrations ranging from 4 to 0.125  $\mu\text{g}/\text{well}$  were coated onto Immunoplates and the optimal antigen concentration for the procedure was determined. As shown in Fig. 1, antigen protein concentrations from 4 to 1  $\mu\text{g}/\text{well}$  reacted similarly with CCV antibody-positive canine serum, whereas the ELISA value decreased at concentrations below 1  $\mu\text{g}/\text{well}$ . An antigen concentration of 1  $\mu\text{g}/\text{well}$  was therefore used for subsequent experiments.

### *Determination of optimal dilution of capture antibody for antigen-detection ELISA*

The optimal concentration of anti-FIPV serum used as the capture antibody was determined by checker-board titration. Serially diluted anti-FIPV

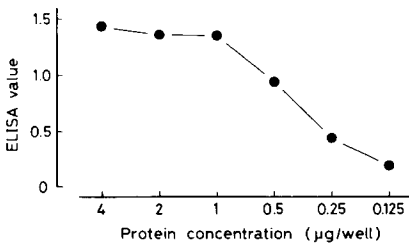


Fig. 1. Titration curve of ELISA antigen used for antibody-detection ELISA.

serum was coated onto wells, and each of the dilutions of CCV antigen that had been prepared from CCV-infected CRFK cells was reacted to the wells. The amount of antigen captured by each well was determined as described in Materials and Methods. As shown in Fig. 2, antibody diluted 1:5000 to 1:40 000 reacted similarly with respective dilutions of CCV antigen, and there was a sharp decrease in the ELISA value at dilutions below 1:80 000. Anti-FIPV serum was therefore diluted 1:20 000 to 1:40 000 for the subsequent experiments.

*Antibody responses in experimentally infected dogs measured by ELISA and NT*

ELISA and NT antibody responses of two experimental dogs are shown in Fig. 3. Dog D1, inoculated orally with CCV, produced detectable NT antibody at PID 7, and the titer reached its maximum level at PID 14. On the other hand, ELISA antibody was first detected at PID 11 and reached its maximum level at PID 22. Subsequently, ELISA titers were slightly lower than NT titers. The intranasally inoculated dog D4 also produced NT and ELISA an-

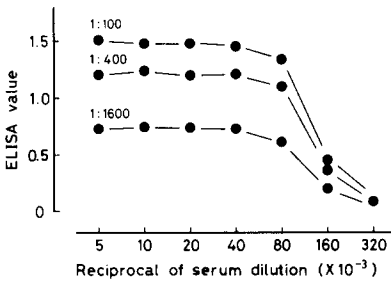


Fig. 2. Checker-board titration curves of capture antibody used for antigen-detection ELISA. CCV antigen was diluted to 1:100, 1:400, or 1:1600, and reacted to wells coated with serial dilutions of capture antibody.

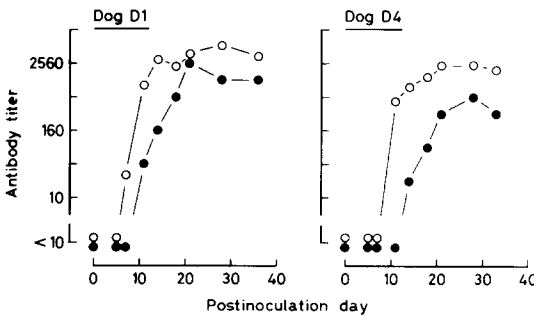


Fig. 3. Antibody responses to CCV in experimental dogs D1 and D4 assessed by ELISA (●) and NT (○).

tibody; as in dog D1, ELISA antibody was first detected later than NT antibody, and the ELISA titer was slightly lower than the NT titer. The non-inoculated control dog D7 produced neither NT or ELISA antibody.

*Detection of CCV and its antigen in experimentally infected dogs*

ELISA values of rectal swab specimens collected from dogs D1, D4 and D7 are shown in Fig. 4. The values for uninfected control dog D7 remained below 0.1 throughout the test period, and had a mean value of 0.033 and a standard deviation of 0.016 ( $n=12$ ). The cut-off value was 0.101. Fig. 4 therefore shows that CCV was excreted in the feces of dog D1 from PID 3 to PID 11, and of dog D4 from PID 5 to PID 11. The specificity of the ELISA was examined by the absorption test. After incubation of the ELISA-positive specimens with CCV antibody-positive serum, ELISA values were all reduced below 0.1; values were not reduced after incubation with CCV antibody-free serum.

CCV excretion in the feces of experimentally infected dogs is shown in Table 1. In orally infected dogs, CCV was first detected at PID 3–4, whereas intranasally infected dogs began to excrete at PID 4–6. Excretion of CCV occurred until PID 11 in all dogs examined. No CCV excretion was detected by ELISA in nasal and throat swabs in any dog. Although virus isolation was attempted using CRFK and fcwf-4 cells, both of which are known to be susceptible to CCV, no CCV was isolated, even from ELISA-positive specimens.

Some dogs were killed at PID 5 or PID 11, and their organs were subjected to virus isolation and antigen detection. As shown in Table 2, cytopathic agents were isolated only from the kidney and mesenteric lymph node of dog D3, and were identified as CCV by IFA. However, CCV antigen could not be detected in the kidney of dog D3, either by ELISA or by IFA. CCV antigens were detected in almost all parts of the small intestine of infected dogs by both ELISA and IFA. Of four mesenteric lymph nodes, two (including one from dog D3) were ELISA-positive for CCV antigens, but were IFA-negative. No CCV was detected in other organs by ELISA, IFA, or virus isolation experiments.

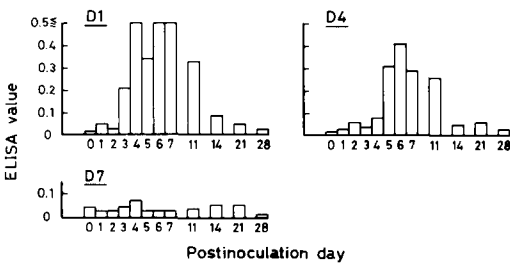


Fig. 4. ELISA value of fecal specimens from experimental dogs D1, D4, and D7 measured by antigen-detection ELISA.

TABLE 1

Detection of CCV in fecal specimens from experimentally infected dogs by ELISA

Dog no.	Route of inoculation <sup>1</sup>	Days after inoculation												
		0	1	2	3	4	5	6	7	11	14	18	22	28
D1	O	-	-	-	+	+	+	+	+	+	-	-	-	-
D2	O	-	-	-	-	+	+	+	+	+	-	-	-	-
D3	O	-	-	-	+	+	+	+	+	+	-	-	-	-
D4	IN	-	-	-	-	-	+	+	+	+	-	-	-	-
D5	IN	-	-	-	-	-	-	+	+	+	-	-	-	-
D6	IN	-	-	-	-	+	+	+	+	+	-	-	-	-
D7	Not inoculated	-	-	-	-	-	-	-	-	-	-	-	-	-

<sup>1</sup>Six dogs were inoculated with CCV orally (O) or intranasally (IN); dog D7 was not inoculated (control).

<sup>2</sup>Dogs were killed for necropsy.

TABLE 2

Detection of CCV antigens by ELISA and IFA, and virus isolation from organs of experimentally infected dogs

Organs	Dog no. (postinoculation day at necropsy)											
	D2 (11)			D3 (5)			D5 (11)			D6 (5)		
	EIA	IFA	Isol <sup>1</sup>	EIA	IFA	Isol	EIA	IFA	Isol	EIA	IFA	Isol
Tonsil	- <sup>2</sup>	-	-	-	-	-	-	-	-	ND	ND	ND
Lung	-	-	-	-	-	-	-	-	-	ND	-	ND
Liver	-	-	-	-	-	-	-	-	-	ND	-	ND
Spleen	-	-	-	-	-	-	-	-	-	ND	-	ND
Kidney	-	-	-	-	-	+	-	ND	-	ND	-	ND
Mesenteric lymph node	-	-	-	+	-	+	-	-	-	+	-	ND
Duodenum	+	+	-	+	+	-	+	+	-	-	-	ND
Jejunum	+	+	-	+	+	-	+	+	-	+	-	ND
Ileum	+	+	-	+	+	-	+	+	-	+	+	ND

<sup>1</sup>CCV or its antigens were detected by ELISA (EIA), IFA, or virus isolation experiments (Isol).

<sup>2</sup>+, CCV detected; -, CCV not detected; ND, not done.

## DISCUSSION

In the present study, an ELISA method using detergent-solubilized antigen from CCV-infected cells was developed for the detection of antibody against the virus in canine serum. Enveloped viruses are commonly inactivated by treatment with detergents, and solubilized antigens have therefore been widely



used as non-infectious antigens in ELISA. We chose sodium deoxycholate for antigen solubilization because it is reported that the presence of the detergent, whatever the concentration, does not interfere with the adsorption of antigens to the solid phase (Jeansson et al., 1982). The original solubilized antigen solution contains about 2 mg/ml of protein, so 1 ml of antigen would be sufficient for 20 antigen-coated plates. The antigen preparation and assay procedures developed in this study are simple, so that this ELISA method will be useful in large-scale epidemiological surveys of CCV infection in the field. In the experimental infection, ELISA antibody was first detected about 3 days after the appearance of NT antibody. We suspect that this was because our ELISA procedure detects only IgG subclass antibodies and does not detect antibodies of other classes – probably IgM – that may be produced during the early stage of infection and detected by NT.

An ELISA procedure for the detection of CCV antigens was also developed in this study. Anti-FIPV serum was used as the capture antibody because FIPV is antigenically related to CCV (Pedersen et al., 1978; Horzinek et al., 1982), and FIPV-infected cats produce high antibody titers. The anti-FIPV serum used in this study did not need purification of Ig or specific antibody fraction, and could be used at 1:40 000 dilution.

Although CCV was not isolated from feces in experimental infections, high ELISA values were observed in specimens from inoculated dogs for a limited period of time, but were not found in specimens from the non-inoculated dog at any time. Conclusive diagnosis requires isolation of the virus from feces, but attempts to isolate the virus have been unsuccessful in many previous reports (Cartwright and Lucas, 1972; Williams, 1980; Marshall et al., 1984) as well as in the present work. The reason for this is uncertain. As shown in Table 2, we also failed to isolate virus from necropsied samples of ELISA-positive organs; however, the results of ELISA were correlated with those of IFA for almost all specimens. The high ELISA values observed for fecal specimens were reduced after incubation of the specimen with dog serum containing anti-CCV antibody, but was not reduced by incubation with antibody-free serum. These observations indicate the specificity of our ELISA procedure for the detection of CCV antigen in feces and organ homogenates; it is important that any ELISA method used in the field detects only CCV antigens and not other antigens such as canine parvovirus, canine rotavirus, food antigens etc. Although we were unable to examine the reactivity of the two antisera (capture and detector antibodies) with antigens other than CCV, these antisera were prepared from healthy animals of species other than the dog (cat and rabbit), so it seems unlikely that both sera simultaneously possess the same antibodies against other canine pathogens and food antigens. We used polyclonal antisera in this study, but it is likely that the use of monoclonal antibody as the detector will improve specificity.

In conclusion, our method was more simple, rapid and reliable for the de-

tection of CCV than virus isolation, electron microscopic examination and the IFA technique, and was able to detect antigen in specimens as small as rectal swabs.

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