Antibody Testing Against Canine Coronavirus by Immunoperoxidase Plaque Staining

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ABSTRACT

The application of the immunoperoxidase (IP) plaque staining procedure (IP test) to the diagnosis of canine coronavirus (CCV) infection was investigated. The IP test did not react with sera from either 15 specific pathogen-free (SPF) dogs or 7 SPF dogs immunized with a multivalent vaccine, including canine parvovirus type 2, canine distemper virus, canine adenovirus type 2, and canine parainfluenza virus. To compare the IP test with the neutralizing test (NT), sera from 240 healthy dogs and from 3 experimentally CCV-infected dogs were examined. All 60 sera positive for NT antibody were positive for IP antibody, and all 180 sera negative for NT antibody were negative for IP antibody in the healthy dogs. The IP titres showed similar changes with time after CCV inoculation to those of the NT titres in the experimentally infected dogs. These findings indicate that the IP test specifically detected anti-CCV antibodies. When the IP test and NT were compared in dogs with diarrhoeic signs. 2.1% of 48 sera and 20.3% of 74 sera, which were all negative for NT antibody, were positive for IP antibody in the dogs of under one year of age and at least one year of age, respectively. The difference between the IP and NT titres ($\log_{10}$ [reciprocal of IP titre] – $\log_{10}$ [reciprocal of NT titre]) for the diarrhoeic dogs of under one year of age (2.350 ± 0.931) was significantly larger than that for the healthy dogs (0.982 ± 0.447) ($p < 0.0001$), the NT titre being negative or very low, despite a high IP titre in many diarrhoeic dogs. Hence, the IP test is more able to detect anti-CCV antibodies, especially in dogs showing clinical signs. The IP-positivity rate was significantly higher in the diarrhoeic dogs of under one year of age (48.7%) than in the healthy dogs (25.0%) ($\chi^2 = 19.844$, $p < 0.0001$), suggesting that CCV may contribute to diarrhoea in many juvenile dogs.

Keywords: antibody, canine coronavirus, diagnosis, diarrhoea, dog, immunoperoxidase plaque staining, neutralizing test, serology

Abbreviations: BSA-PBST, bovine serum albumin in phosphate-buffered saline (0.01 mol/L, pH 7.0) containing Tween 20; CCV, canine coronavirus; CRFK, Crandell feline kidney; ELISA, enzyme-linked immunosorbent assay; IP, immunoperoxidase; IP test, antibody test by immunoperoxidase plaque staining procedure; N, nucleocapsid protein; PFU, plaque-forming unit; S, spike protein; SPF, specific pathogen-free; NT, neutralizing test; PBS, phosphate-buffer saline (0.1 mol/L, pH 7.0); PBST, phosphate-buffered saline (0.1 mol/L, pH 7.0) containing Tween 20; $TCID_{50}$, median tissue culture infective dose

INTRODUCTION

Canine coronavirus (CCV) was initially isolated in Germany in 1971 by Binn and colleagues (1974), then later confirmed in Australia (Schnagl and Holmes, 1978) and the United States (Appel *et al.*, 1979). In Japan, Yasoshima and colleagues (1983) isolated CCV from a canine parvovirus (CPV)-infected dog in 1982. Together with CPV, CCV is now recognized as an important cause of diarrhoea in dogs. CCV infection is highly prevalent, but the mortality is generally low and the prognosis is good. However, mixed infections with other viruses and bacteria are frequent, and the resultant signs may be serious, especially with concurrent CPV infection (Appel *et al.*, 1979).

Electron-microscopic visualization of the virus in faeces and virus isolation from faeces using cultured cells are utilized as diagnostic techniques for CCV infection. However, the former technique requires an expensive instrument, while the latter takes days to complete and the viral activity may easily be reduced by mistreatment of the collected faeces (Tennant *et al.*, 1994). Moreoever, no virus is excreted in the faeces of some CCV-infected dogs (Kokubu *et al.*, 1998). Therefore, antigen detection in faeces is not a reliable diagnostic method. In contrast, detecting antibody against CCV is a reliable diagnostic procedure, especially using paired sera, since the antibodies remain in the blood for a prolonged period after infection.

The neutralizing test (NT) (Mochizuki *et al.*, 1987; Appel, 1988) and the enzyme-linked immunosorbent assay (ELISA) (Rimmelzwaan *et al.*, 1991; Tuchiya *et al.*, 1991; Palmer-Densmore *et al.*, 1998) have been reported as antibody tests against CCV. NT is superior in specificity, but it is not appropriate for rapid diagnosis, since it requires several days to obtain a result. In contrast, ELISA is useful for rapid diagnosis, but it may be difficult to obtain accurate measurements when non-specific reactions develop.

A highly specific enzyme immunoassay, the immunoperoxidase (IP) plaque staining procedure (IP test), which detects antibody by a specific colour reaction in plaques formed by virus-infected cultured cells in a tissue culture plate, has been applied to the diagnosis of African swine fever virus (Pan *et al.*, 1982), pseudorabies virus (Sato *et al.*, 1988) and feline infectious peritonitis virus (Kai *et al.*, 1992). In this test, non-specific reactions can easily be distinguished from the specific colour reaction in the plaques, even when non-specific colour reactions develop on uninfected cells in the background. Moreover, the procedure for this test is simple as its principle is similar to that of ELISA.

In this study, an IP test was investigated to establish a highly specific diagnostic method for CCV infection that is rapid and simple, and its value was examined in field cases and in dogs experimentally infected with CCV.

MATERIALS AND METHODS

Serum samples

To investigate the specificity of the IP test, sera were examined from 15 unimmunized specific pathogen-free (SPF) beagles of 2 months of age, and from 7 SPF beagles of 4

months age that had been immunized with a multivalent vaccine, including canine parvovirus type 2, canine distemper virus, canine adenovirus type 2 and canine parainfluenza virus (Solvey Duphar, Weeps, Holland) and had seroconverted to all these viruses. To compare the IP test with NT, 3 dogs of 3 months of age, which were negative for both IP and NT antibody against CCV, were orally inoculated with 2×10^7 $TCID_{50}$ of a strain of CCV that had been isolated in Sagamihara, Kanagawa, Japan (Hara *et al.*, 1993), and were bled at inoculation and 5, 10, 15, 20, 25 days after inoculation. Furthermore, sera were examined from 240 clinically healthy dogs, of one year or more of age, and from 187 dogs with diarrhoeic signs, including 115 dogs of less than one year of age, and 72 dogs of at least one year of age, in the general pet population. They had not received CCV vaccines.

IP test

The IP test was performed by a modification of the method reported by Kai and colleagues (1992). To prepare the antigen plate, 1.2×10^4 Crandell feline kidney (CRFK) cells (American Type Culture Collection, (ATCC), Rockville, MD, USA) were added to each well in a 96-well tissue culture plate, and grown overnight at 37°C. The cells were inoculated with 30 PFU per well of 1-71 strain of CCV (ATCC) (Binn *et al.*, 1974) and allowed to adsorb the virus for one hour at 37°C. Following adsorption, the virus suspension was removed and 0.1 ml per well of a minimum essential medium (Nissui Seiyaku, Tokyo, Japan) containing 2% fetal bovine serum and 1% methylcellulose (Nacalai Tesque, Kyoto, Japan) was overlaid onto the cells. After 40 h of incubation at 37°C, the plate was fixed with methanol for 4 h at 4°C, then washed with distilled water, dried and stored at –20°C until use. To titrate the antibodies, the stored plate was rinsed with 0.01 mol/L phosphate-buffered saline (pH 7.0) containing 0.1% Tween 20 (PBST). Then, 0.05 ml per well of the serum, which was diluted 1:40 and then serially diluted 2-fold in PBST containing 1% bovine serum albumin (BSA-PBST) was added to the plate in duplicate. After an hour of incubation at 37°C, the plate was washed three times with PBST, and 0.05 ml per well of peroxidase-conjugated rabbit anti-dog IgG (H+L) (Jackson Immuno Research Laboratories, West Grove, PA, USA), which was diluted 1:1500 with BSA-PBST, was added to the plate. After a further hour of incubation at 37°C, the plate was again washed three times with PBST and 0.1 ml per well of the substrate solution (0.0025% dianisidine, 0.1% H_2O_2 and 10 mmol/L Tris-HCl (pH 7.4)) (Towbin *et al.*, 1979) was added to the plate. After 30 min of incubation at 37°C, the plate was washed with distilled water. The titre was expressed as the highest dilution of serum with brown-coloured plaques.

Neutralizing test

The NT was performed as described by Appel (1988). Quadruplicate 0.05 ml volumes of serum were serially diluted 3-fold in a 96-well tissue culture plate and mixed with 0.05 ml of 1-71 strain of CCV, which had been diluted to 200 $TCID_{50}$/0.1 ml, and the plate was incubated for 90 min at 37°C. Following incubation, 0.05 ml of the CRFK

cells, which were suspended at $4.0 \times 10^5/\text{ml}$, were added to all the serum–virus mixtures, and the plate was incubated for 4 days at 37°C. Following this incubation, the plate was examined microscopically and the titre was calculated by the method reported by Reed and Muench (1938).

Statistical analysis

To compare the IP and NT titres, analysis by the method reported by Bland and Altman (1986) was adopted. They reported that, when the standard deviation (SD) is calculated for the difference in results obtained by two methods being compared, 95% of the samples tested are within a range of $\pm 2\text{SD}$. Following their method, 2SD of the difference between the IP and NT titres ($\log_{10}$ [reciprocal of IP titre] $- \log_{10}$ [reciprocal of NT titre]) was calculated as an index of the agreement between the IP test and NT.

RESULTS

Observation of IP plate

As shown in Figure 1, brown-coloured plaques were developed by using serum from a dog with an anti-CCV NT titre of 1:81, the highest serum dilution showing the colour development being 1:1280, which was regarded as the IP titre against CCV.

Antibody titres in SPF dogs

The sera from 15 unimmunized SPF dogs and from 7 SPF dogs inoculated with the canine multivalent vaccine did not react with CCV antigens by the IP test (IP titre $< 1:40$).
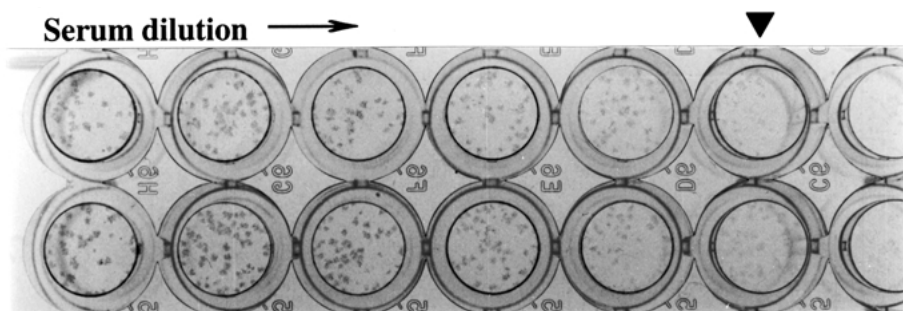

Figure 1. Colour development of plaques by immunoperoxidase plaque staining with an anti-canine coronavirus antibody-positive serum. The serum was diluted 1:40, then 2-fold serially in duplicate. The triangle (▼) indicates the end point dilution (1:1280)

Changes in antibody titres in CCV-infected dogs

Both IP and NT antibodies were detected 5 days after inoculation in two dogs (nos. 1 and 2) and by 10 days after inoculation in all three dogs, after which both titres followed similar changes with time (Figure 2). Dog no. 3 could not be bled at 25 days after inoculation.
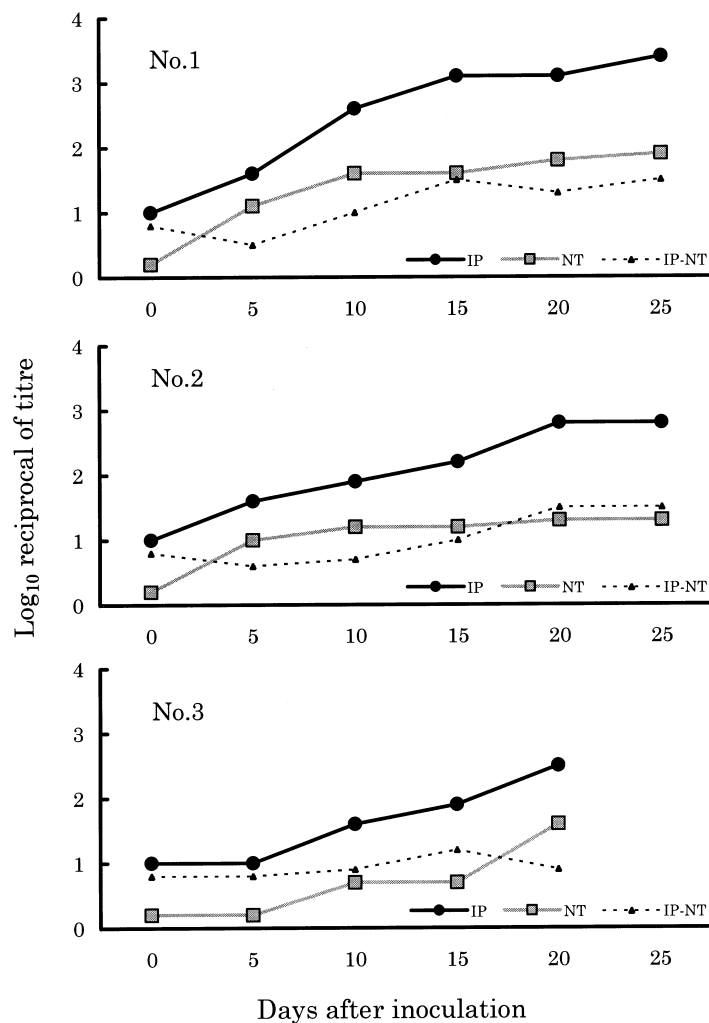

Figure 2. Changes in immunoperoxidase plaque staining (IP) and neutralizing (NT) antibody titres against canine coronavirus (CCV) in 3 dogs experimentally infected with CCV. Circles and squares indicate IP and NT titres, respectively. Triangles indicate the difference between IP and NT titres ($\log_{10}$ [reciprocal of IP titre] – $\log_{10}$ [reciprocal of NT titre])

Comparison of IP test and NT in the general pet population

When an IP titre of 1:40 or more was regarded as IP-positive and an NT titre of 1:3 or more as NT-positive, all 180 sera negative for NT antibody were also negative for IP antibody, and all 60 sera positive for NT antibody were also positive for IP antibody in the healthy dogs. In contrast, while all sera from diarrhoeic dogs positive for NT antibody were also positive for IP antibody, 15 (20.3%) of 74 sera and 1 (2.1%) of 48 sera negative for NT antibody were positive for IP antibody in the diarrhoeic dogs of under one year of age and of at least one year of age, respectively.

The rates of IP antibody positivity were 25.0% (60/240), 48.7% (56/115) and 34.7% (25/72) in the healthy dogs, diarrhoeic dogs of under one year of age and diarrhoeic dogs of at least one year of age, respectively. There was a significant difference between the healthy dogs and the diarrhoeic dogs of less than one year of age ($\chi^2 = 19.844$, $p < 0.0001$), while the difference between the healthy dogs and the diarrhoeic dogs of at least one year of age ($\chi^2 = 2.641$, $p = 0.104$) was not significant.

Scatter diagrams of the IP and NT titres in the healthy and diarrhoeic dogs are shown in Figure 3. The means and SDs of the differences between the IP and NT titres were 0.982 ± 0.447 (2SD = 0.894), 2.350 ± 0.931 (2SD = 1.862) and 1.404 ± 0.896 (2SD = 1.792) in the healthy dogs, the diarrhoeic dogs of less than one year of age and the diarrhoeic dogs of at least one year of age, respectively (Figure 4). Upon analysis by Student's *t*-test, there was a significant difference between the healthy dogs and the diarrhoeic dogs of under one year of age ($p < 0.0001$), while there was no significant difference between the healthy dogs and the diarrhoeic dogs of at least one year of age ($p = 0.033$).

DISCUSSION

There were no reactions in the IP test with antibodies against canine viruses excluding CCV using sera from vaccinated SPF dogs, and all healthy NT-negative dogs were also IP-negative. Over time after CCV infection, the IP titres showed similar changes to the NT titres. These findings indicate that the IP test can specifically detect anti-CCV antibodies. In contrast, in diarrhoeic dogs of less than one year of age, and the diarrhoeic dogs of at least one year of age, 20.3% and 2.1%, respectively, of NT-negative dogs were IP-positive. The consistencies between the IP and NT titres in the diarrhoeic dogs (under one year of age, 2SD = 1.862; at least one year of age, 2SD = 1.792) were lower than in the healthy dogs (2SD = 0.894). The difference between IP and NT titres in the diarrhoeic dogs of less than one year of age was significantly large compared to that in the healthy dogs, the NT titre being negative or very low, despite a high IP titre in many diarhoeic dogs. According to Palmer-Densmore and colleagues (1998), anti-nucleocapsid (N) protein antibodies appear in the blood circulation earlier than anti-spike (S) protein antibodies after infection with CCV. S protein carries neutralizing epitopes, while N protein is the most abundant protein in CCV (Garwes and Reynolds, 1981). As the IP test detects both the anti-S protein and the anti-N protein antibodies, it can detect the infection in dogs producing few or no antibodies
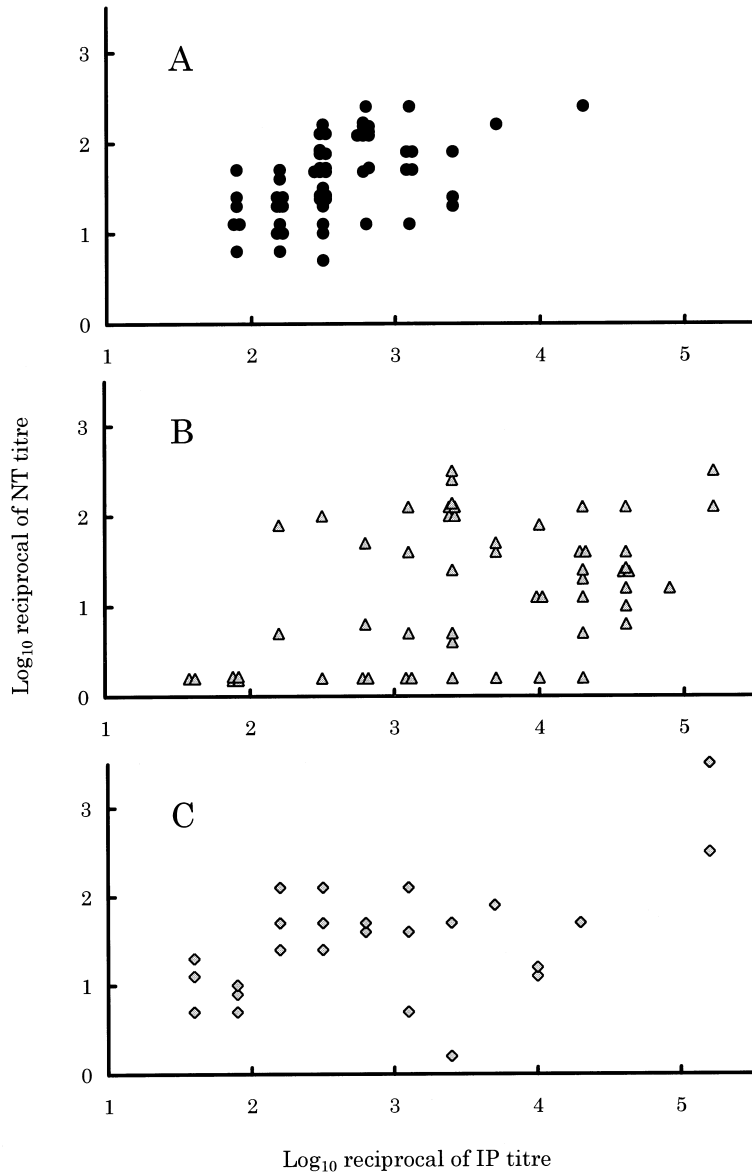
Figure 3. Scatter plot of immunoperoxidase plaque staining (IP) and neutralizing (NT) antibody titres against canine coronavirus in dogs showing positive IP antibody. $x = \log_{10}$ [reciprocal of IP titre], $y = \log_{10}$ [reciprocal of NT titre]. (A) healthy dogs ($n = 60$); (B) dogs of less than one year of age with diarrhoeic signs ($n = 56$); (C) dogs of at least one year of age with diarrhoeic signs ($n = 25$)
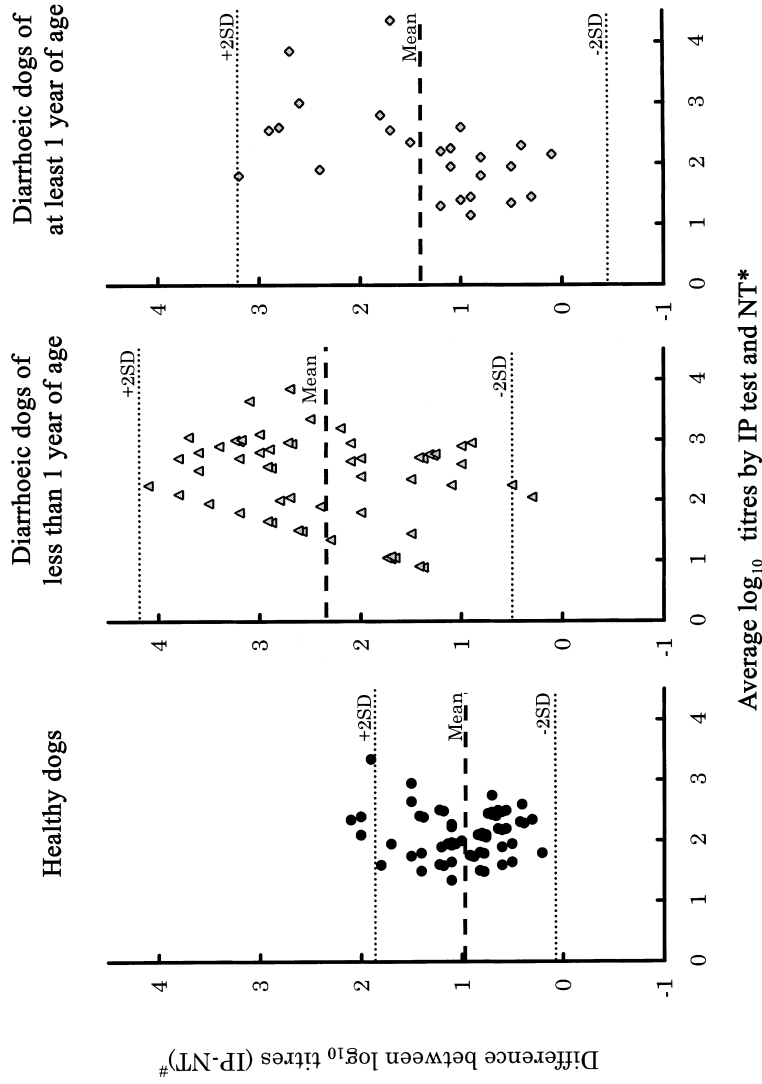
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Figure 4. Distribution of difference between immunoperoxidase plaque staining (IP) and neutralizing (NT) antibody titres against canine coronavirus in dogs showing positive IP antibody. Circles, triangles and squares indicate the differences in healthy dogs ($n = 60$), diarrhoeic dogs of less than one year of age ($n = 56$) and diarrhoeic dogs of at least one year of age ($n = 25$), respectively. *Average log_{10} titres by IP and NT = (log_{10} [reciprocal of IP titre] + log_{10} [reciprocal of NT titre])/2. #Difference between log_{10} titres (IP-NT) = log_{10} [reciprocal of IP titre] – log_{10} [reciprocal of NT] titre

detectable by the NT. Hence, the IP test should be useful for diagnosis of CCV infection, especially in dogs showing clinical signs.

The fact that the IP positivity rate for the diarrhoeic dogs of less than one year of age was significantly higher than that for the healthy dogs may indicate that CCV contributes to diarrhoea in many juvenile dogs in the general pet population.

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