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Prevalence of canine coronavirus antibodies by an enzyme-linked immunosorbent assay in dogs in the south of Italy

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Abstract

An enzyme-linked immunosorbent assay (Elisa), using as antigen canine coronavirus-infected CrFK cell supernatant, was developed to detect antibodies against canine coronavirus (CCoV). Out of a total of 109 dog serum samples, 80 which were positive by routine virus neutralisation test were also Elisa positive. Seventeen samples which were negative by the virus neutralisation test, were positive by Elisa and by the confirmatory Western blotting test. The Elisa was substantially more sensitive than the virus neutralisation test in detecting antibodies to CCoV and may be used as an alternative technique to virus neutralisation.

Keywords: Dog; Coronavirus; Antibodies; Elisa

1. Introduction

Coronaviruses are large viruses that cause respiratory, enteric and generalised disease in humans and domestic animals. Canine coronavirus (CCoV) belongs to one of the major antigenic groups of coronaviruses (Siddell et al., 1983; Spaan et al., 1988) and is related serologically and genetically to transmissible gastroenteritis virus of pigs, porcine epidemic diarrhoea virus, feline coronaviruses (FCoVs) and human coronavirus 229E (HcoV-229E) (Sanchez et al., 1990; Horsburgh et al., 1992; Wesseling et al., 1994). The viruses have enveloped virions containing a non-segmented, positive plus-stranded RNA genome that is 27–32 kb in length (Siddell, 1995) and is packaged by the nucleoprotein N (43 kDa) into a helical nucleocapsid. The ribonucleoprotein is surrounded by a lipid envelope which contains three viral glycoproteins: the spike S glycoprotein (160–

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200 kDa) forming the long club-shaped surface projections on the virion surface, the integral M glycoprotein (29 kDa) and the small membrane (E) protein (9 kDa).

CCoV was first isolated from faecal specimens of American military dogs with diarrhoeal disease (Binn et al., 1974). CCoV infects dogs of any breed or age, causing depression, anorexia, vomiting and diarrhoea in young animals. The dogs generally recover spontaneously 7–10 days after infection, but the diarrhoea may persist for more than 2 weeks. Death may occur 1–3 days after the onset of disease, especially in young pups (Carmichael and Binn, 1981).

Definitive identification of CCoV-induced disease may be achieved by detection of CCoV shed in faeces at electron microscopy or by virus isolation in cell culture. The common presence of coronavirus-like particles in faeces makes the diagnosis of CCoV by electron microscopy arduous and requires confirmation by other diagnostic methods (Athanssious et al., 1994). On the other hand, many investigators have experienced difficulties in cultivating coronaviruses in vitro (De Groot and Horzinek, 1995; Tennant et al., 1994; Pratelli et al., 1999, 2000; Wesley, 1999).

Recently, nested PCR assay (n-PCR) for the detection of CCoV with primers to the transmembrane protein M gene, has been described (Pratelli et al., 1999).

Assessment of antibodies by the virus neutralisation assay (Mochizuki et al., 1987), or by indirect enzyme-linked immunosorbent assay (ELISA) (Rimmelzwaan et al., 1991; Tuchiya et al., 1991) provides an indication of the exposure of an animal to CCoV. Detection of immunoglobulin M (IgM) and IgG against CCoV by indirect ELISA (Tennant et al., 1991; Naylor et al., 2001) determines current or previous exposure of an animal to CCoV.

The aim of this study was to improve the detection of CCoV-specific antibodies in canine sera, by using an Elisa that was compared to the virus neutralisation test and Western blotting assay.

2. Materials and methods

2.1. Virus and cells

Crandell feline kidney (CrFK) cells were grown in Dulbecco modified Eagle's medium supplemented with 10% foetal bovine serum.

A cell culture adapted CCoV strain 45/93, isolated from a dog with enteritis (Buonavoglia et al., 1994), was used throughout this study.

2.2. Canine serum samples

A total of 109 serum samples, collected from dogs of the Small Animal Clinic, Faculty of Veterinary Medicine, Bari, Italy, were employed.

2.3. Antigen preparation

The supernatants of CrFK cell cultures infected with CCoV strain 45/93, or mock infected cultures, were harvested 96 h postinfection and clarified at $3000 \times g$ for 20 min at 4 °C. Subsequently, the supernatants were centrifuged for 1 h at $140000 \times g$ at 4 °C.

The pellets were resuspended in phosphate-buffered saline (PBS, pH 7.2) at 1/80 the initial volume and used as positive and negative antigens for Elisa and Western blotting tests.

2.4. Virus neutralisation test

Serial twofold dilutions starting from 1/2 of each sample were mixed with 100 $TCID_{50}$ of CCoV 45/93 strain in 96-well microtitre plates. The plates were kept at room temperature for 90 min and then 20000 CrFK cells were added to each well. The plates were read after 4 days of incubation at 37 °C when the cytopathic effect was complete in the virus control cultures. The titre was expressed as the highest serum dilution neutralizing the virus.

2.5. Elisa

Microtitre plates (Costar) were coated with 100 μl per well of antigen diluted in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, [pH

9.6]) and incubated overnight at 4 °C with shaking. The plates were washed four times in PBS containing 0.05% Tween 20 (PBS-T), then treated with blocking solution (0.2% gelatin in carbonate buffer) for 90 min at 37 °C and washed four times with PBS-T. Dilutions of 1/50 in PBS-T of each canine serum were added in duplicate and the plates were incubated for 90 min at 37 °C.

The washing cycle described above was then repeated and 100 μl of peroxidase-conjugated caprine IgG, specific for canine IgG (Sigma Chemicals, St. Louis, MO), diluted in PBS-T were added to each well, and the plates were incubated for 1 h at 37 °C. After another washing cycle, 100 μl of freshly prepared substrate were placed in each well. The solution consisted of 10 mg 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt (ABTS, Sigma) in 50 ml 0.05 M phosphate citrate buffer, pH 5.0, containing 25 μl/100 ml hydrogen peroxide and the optical densities at 405 nm (OD 405) were determined.

The adjusted OD values of each sample were obtained by subtracting the absorbance of the mock antigen-coated well from that of the corresponding virus antigen-coated well.

2.6. Western blotting

The antigen preparations diluted 1:1 in Laemmli sample buffer were heated at 95 °C × 4 min, subjected to electrophoresis in sodium dodecyl sulphate (SDS)-polyacrylamide minigel (5–20%) and transferred onto nitrocellulose membrane (Immobilon P, pore size 0.45 μm) with a BIORAD Transblot Cell apparatus at 70 V for 2 h.

Non-specific binding sites were blocked overnight at 4 °C with 5% non-fat dry milk (Blotting Grade Blocker, Biorad) in Tris Buffered Saline (TBS; Tris 25 mM, NaCl 200 mM, pH 7.4) containing 0.05% Tween 20 (TBS-TM).

All the subsequent steps were conducted with shaking at room temperature.

After washing three times with TBS Tween 20 (TBS-T), the membrane was probed with canine serum samples diluted 1:100 in TBS-TM for 2 h. The membrane was then washed three times with TBS-T (5 min per wash) and incubated for 2 h with peroxidase labeled caprine IgG specific for canine IgG (Sigma Chemicals, St. Louis, MO).

After being washed extensively in TBS-T, DAB (3,3′-diaminobenzidine tetrahydrochloride [Sigma] in TBS [pH 7.8], 0.08% hydrogen peroxide) was used in the chromogenic reaction.

3. Results

A total of 29 of the 109 samples examined were negative by the virus neutralisation test and were examined subsequently by Western blotting. Ten of these sera were found concomitantly to be free of CCoV specific antibodies and used to adjust the Elisa cut-off value (three Standard Deviations higher than the arithmetic mean of the absorbance of concordantly negative samples). Samples with value exceeding than 0.040 were considered to be positive.

As shown in Fig. 1A, 80 of the 109 serum samples proved to be positive at the virus neutralisation test.

Nineteen samples, which were found to be free of CCoV neutralising antibodies, gave a positive signal by the Elisa. In 17 of these, CCoV specific antibodies were also found by the Western blotting test. These sera, therefore, were recorded as positive concomitantly by Elisa (Fig. 1B).

Two discrepant sera remained which gave a positive result exclusively by Elisa.

With the Western blotting test, most serum samples showed reactivity to the N and M proteins of CCoV and only a few samples also reacted against the S protein (data not shown).

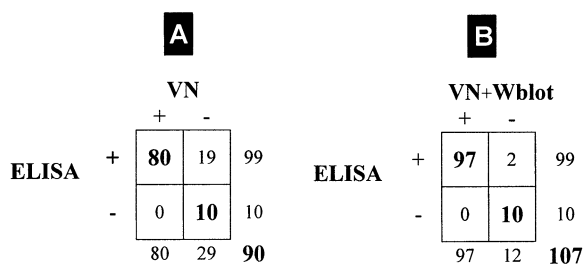

Fig. 1. Evaluation of antibodies to CCoV in dog serum samples, using Elisa compared to virus neutralisation alone (A) and in combination with Western blotting analysis (B). Values in the boxes are numbers of samples.

Considering virus neutralisation as a 'gold standard' test, Elisa had a sensitivity of 100% and a specificity of 34.5%, with an overall agreement of 82.6%. However, when the virus neutralisation test combined with the confirmatory Western blotting test, were used as the 'gold standard', the Elisa showed an improved specificity (83.3%), while the sensitivity remained unchanged (100%), with an overall agreement of 98.2%.

4. Discussion

In this study an Elisa was developed to detect CCoV-specific antibodies. To determine whether the Elisa could be used for this purpose, sensitivity and specificity were evaluated, considering the virus neutralisation test and the virus neutralisation combined with Western blotting tests as 'gold standards'.

Out of 109 sera, 19 false-positives and no false-negative samples were detected initially with the Elisa, using virus neutralisation as the standard test. In 17/19 of the false-positive samples, however, CCoV antibodies were also detected by Western blotting and these samples were thus considered to be positive.

Three of the samples, which were negative only by the virus neutralisation test, yielded consistently high OD values by Elisa; this apparently strange result is not easy to explain, but since Western blotting showed both specific and non-specific reactivity for the same samples (cellular antigens?), this may account for the high Elisa OD values.

Recently, CCoV infection has attracted scientific interest especially concerning the pathogenesis of infection in dog (Bandai et al., 1999; Naylor et al., 2001), viral genome variability (Pratelli et al., 2001) and the development of rapid and sensitive diagnostic tests (Pratelli et al., 1999; Bandai et al., 1999; Naylor et al., 2001). Routine measurements of CCoV-specific antibodies are still based on the virus neutralisation test, which is costly as well as time-consuming (at least 4 days) and requires specialised laboratories. We carried out a preliminary comparison of the virus neutralisation and the Elisa and the findings clearly revealed a discrepancy between the results of the two tests, especially in evaluating CCoV seronegativity: 17 out of the 29 samples that were negative in virus neutralisation and positive at Elisa were confirmed to be positive by the Western blotting.

Antibody determination by different methods does not necessarily give parallel results. Whereas, Elisa and Western blotting are able to detect antibodies to all major viral proteins, the virus neutralisation test only measures the neutralising antibodies and, as a result, may lack sensitivity.

The lower sensitivity of the virus neutralisation test for detecting the antibodies induced by CCoV, may provide misleading information on the epidemiological features of the infection. Above all, the virus neutralisation test may impede evaluation of the pre-existing immunological status of the dogs used in pathogenesis studies or in immunogenicity/potency trials on the vaccines employed for immunisation of dogs against CCoV infection. Because of its advantages, such as rapidity and greater sensitivity, the Elisa described may be considered more attractive than the virus neutralisation test and prove a useful tool for the serological diagnosis of CCoV infection.

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