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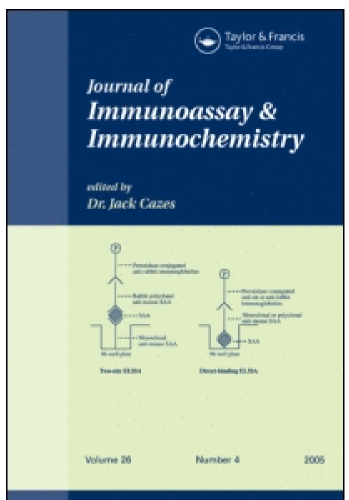

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DEVELOPMENT AND EVALUATION OF AN ELISA TO MEASURE ANTIBODY RESPONSES TO BOTH THE NUCLEOCAPSID AND SPIKE PROTEINS OF CANINE CORONAVIRUS

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ABSTRACT

A rapid and reproducible enzyme linked immunosorbent assay (ELISA) was developed for detection of canine coronavirus (CCV) specific antibodies directed to both the nucleocapsid (NC) and the spike (S) proteins. The coating antigen, a methanol-treated, S-protein enriched preparation, was produced by subjecting infected cells to Triton X-114 detergent followed by phase separation. The sensitivity of this assay was determined by following the course of infection in dogs experimentally infected with CCV. The specificity of the antibody response was determined by Western blot analysis and supported the increased magnitude of the ELISA response and the presence of serum neutralizing (SN) antibody. Due to the sensitivity and specificity of the IgG response detected by this assay it can be used to determine both virus exposure and vaccine efficacy.

KEY WORDS: CCV; Enzyme immunoassay; Coating antigen preparation; Triton X-114; Methanol

INTRODUCTION

Canine coronavirus (CCV) has been identified as one of the causative agents of viral enteritis in dogs around the world. Clinical symptoms range from inapparent [1] to rapidly fatal [2, 3] gastroenteritis. Seroconversion to CCV has

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been used to determine virus exposure and evaluate vaccine efficacy. A variety of assays, including indirect fluorescent (IFA) [4, 5, 6], enzyme linked immunosorbent (ELISA) [7, 8, 9] and serum neutralization (SN) [10, 11] have been developed in order to measure CCV-specific antibody responses. In many cases, the results of these assays are compared even though they measure different type of antibodies due to the varied composition of the antigens used for detection.

The antibodies measured by an SN assay differ from those detected by the IFA and ELISA in that they are biologically active (i.e. neutralizing). Several laboratories have further determined that the cell substrate can play a major role in the sensitivity of this assay [12]. Detection of antibody by the IFA uses CCV-infected cell monolayers, which are usually fixed with acetone, as the antigen substrate. Most of the variability in this assay arises from the fixation method and the stage at which virus replication is arrested, both of which can determine the conformation, type and amount of individual proteins available for antibody detection. Traditional ELISA assays for CCV are variable in their sensitivity and specificity due to the nature of the antigen preparation used to coat 96-well plates; ranging from purified virus [9, 13] to supernatants from infected cells disrupted by either deoxycholate detergent (DOC) [8] or sonication [7]. In general, these three antibody assay systems also differ from each other from the standpoint of time and reagents necessary to perform them, with the SN being

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the most labor intensive followed by the IFA. For this reason ELISA formats are highly desirable since they lend themselves to validation and automation.

Two desirable features for a CCV-specific ELISA is that it be sensitive enough to detect low antibody levels and that the level of antibody detected reflect the magnitude of the SN antibody response. In other words, it is important that the ELISA accurately reflect the time course of the IgG response resulting from infection or vaccination. It is fairly well established in the literature that the major neutralizing antigen of CCV is the spike or S-protein and that nucleocapsid or NC-protein, besides being immunogenic, is one of the more abundant proteins in the virus [14, 15]. In one report, an ELISA, using whole virus as the coating antigen, was able to detect CCV-specific IgG in blood samples from 10 week old puppies 4-7 days after oronasal administration of CCV [13]. However, the IgG titers did not reflect the increasing SN titers observed between 10-14 days after infection. An ELISA described by Tuchiya et al.[8], using DOC disrupted infected cell supernatants as the coating antigen, was even less sensitive in that CCV-specific IgG was first detected three days after the detection of SN antibodies in 21 day old puppies experimentally infected with CCV.

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Based on these reports we hypothesized that one reason for the lack of sensitivity and correlation with the SN response was due to the composition of the ELISA coating antigen, with the S-protein being present in a lower molar quantity than the NC protein as has been reported in structural analyses of

coronaviruses [16]. In order to test our hypothesis we developed and evaluated the sensitivity of an ELISA method using a methanol treated, S-protein enriched preparation as the coating antigen. This assay should theoretically measure all the NC and S-specific IgG antibody, therefore including the majority of virus neutralizing as well as non-neutralizing antibody, both of which are elicited as a result of vaccination or viral infection. The specificity and sensitivity of this assay was confirmed by the corresponding appearance of S and NC-protein bands in Western blots and SN antibody levels induced in dogs after experimental infection with CCV.

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MATERIALS AND METHODS

EXPERIMENTAL INFECTION OF DOGS

Ten, 13-15 week old specific pathogen-free (SPF) dogs (Liberty Labs, New Jersey, USA) were experimentally infected with a total of 1.2 x 10^5 plaque forming units of CCV strain CV-6 administered via the intranasal and oral route [13]. The virus was obtained from the USDA's National Veterinary Services Laboratory (NVSL) in the USA. Blood samples were taken at days 0, 6, 14 and 21 post infection.

ANTIGEN PREPARATION

Canine coronavirus strain C1-71 (ATCC, VR-809) was cultured in A-72 cells (ATCC, 1542-CRL) as previously described [12] using Optimem medium

(Gibco, Grand Island, NY, USA). Infected cells were harvested by mechanical agitation prior to the appearance of a cytopathic effect (CPE), pelleted at a low speed and resuspended to one-tenth the original volume in Optimem medium containing 2% Triton X-114 (Sigma Chemical Company, St. Louis, MO, USA). The concentrated mixture was then placed at 4°C for 3 h with gentle agitation resulting in an opaque solution which was further incubated at 37°C for 30 to 45 min. Finally, the mixture was centrifuged at 1,100 x g for 25 min at 30°C resulting in two distinct phases. The aqueous phase, located at the top, was analyzed and determined to be S-enriched.

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ENZYME IMMUNOASSAY FOR DETERMINATION OF IGG TITERS

An indirect enzyme immunoassay was used to quantitate the amount of CCV specific IgG induced in dogs. Briefly, each well of an Immulon I™, 96-well microtiter plate (Dynatech Laboratories, Inc) was coated with 1 µg total protein, as determined by BCA protein assay (Pierce), of the antigen preparation in 100 µl of phosphate buffered saline (PBS) and incubated at 37°C overnight in order to dry the antigen onto the well. The antigen was then fixed with 100 µl of methanol per well for 5 min at room temperature and then the plate was washed with distilled water (dH_2O) to remove the methanol. Non-specific sites were blocked by incubating each well for 2 h with 200 µl of 10% horse serum diluted in PBS. After the blocker was removed, 100 µl of each dog serum specimen, serially diluted in the blocking solution, was applied to specified wells and

incubated for 1 h at 37° C. Excess antibody was removed by washing the plates with PBS containing 0.05% Tween 20 (PBST) and each well was further incubated at 37°C for 1 h with peroxidase labeled goat anti-dog IgG (γ) (Kirkgaard and Perry Laboratories (KPL), Gaithersburg, MD, USA) After washing with PBST to remove excess conjugated antibody, each well was incubated at 20°C for 30-45 min with peroxidase substrate (ABTS, KPL). Reactivity was measured by determining the optical density (OD) at 405 and 490 nm using an automated microtiter plate reader (Molecular Devices, Menlo Park, CA). Each plate was standardized by adjusting the OD values relative to a positive control sera having an OD value of 1.25 at a dilution of 1:320.

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SERUM NEUTRALIZATION ASSAY

Serum neutralization (SN) titers were determined by diluting heat-inactivated serum samples two-fold in duplicate across 96-well tissue culture plates (Cell culture cluster dish, Costar, Cambridge, MA, USA). A standard amount of CCV C1-71 virus, between 50 and 300 $TCID_{50}$, was added in equal volumes to the diluted sera and the mixture was allowed to incubate for 90 to 120 min. After this period of time, 6.2 $x10^5$ A-72 cells were added to each well and the plates incubated in a humidified incubator at 37°C. The serum neutralization titer was determined as the reciprocal of the final serum dilution where at least one out of two wells demonstrated cytopathic effect four days following the initiation of the assay.

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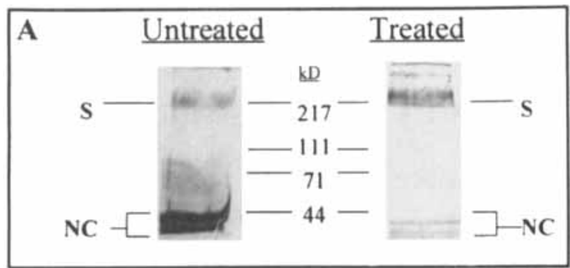
WESTERN BLOT ANALYSIS FOR ANTIGEN EVALUATION AND DETERMINATION OF IGG SPECIFICITY

Proteins from Triton X-114 treated C1-71-infected cells and untreated C1-71-infected cells were solubilized by heating at 95°C for 10 min in a buffer containing 5% (w/v) SDS, 3% (w/v) glycerol, 0.002% (w/v) bromophenol blue and 50 mM Tris (pH 6.8) and then fractionated by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [17]. After electrophoresis, the proteins were transferred onto Immobilon-P™ by electroblotting at 15 volts for 45 min at room temperature in a BioRad Trans-Blot cell (BioRad Laboratories, Richmond, CA) [18]. Non-specific sites were blocked with 1% poly(vinyl alcohol) for 5 min. The blot was incubated for 1 h at 20°C with a 1:1000 dilution of cat ascities fluid containing antibodies specific for feline infectious peritonitis virus (FIP). The specificity of FIP antibody for CCV was previously documented by Tuchiya et al [8]. After washing three times in PBST, the blot was incubated with alkaline-phosphatase conjugated anti-cat IgG (H + L) (KPL) 1 h at 20°C and developed with phosphatase substrate (BCIP/NBT, KPI). Quantitation of the reactive bands was achieved by densitometry using the whole band program of Open Windows® software (Bio-Image, Ann Arbor, MI, USA).

To illustrate the specificity of the antibody response in dogs, Immobilon™ strips containing fractionated proteins from Triton X-114 treated C1-71 infected cells were incubated with different dog sera diluted 1:20 in PBST as described above. Reactive protein bands were detected after incubation of the strips with



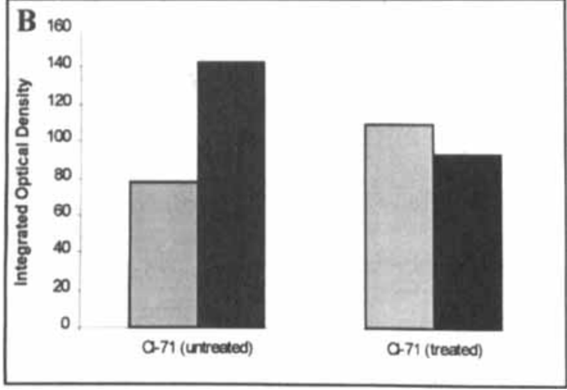


FIGURE 1. Analysis of CCV antigen preparations. Western blot reactivity of FIP-specific antiserum with the spike (S) and nucleocapsid (NC) proteins of Triton X-114 untreated and treated cells infected with strain C1-71 (A) and integrated optical density of the reactive bands corresponding to the S (▒) and NC (■) proteins (B).

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alkaline-phosphatase conjugated anti-dog IgG (H + L) (KPI) using a 1:3000 dilution followed by the substrate.

RESULTS

EVALUATION OF ANTIGEN USING WESTERN BLOT ANALYSIS

Analysis of the treated and untreated Cl-71 strain antigen preparations by Western blot is illustrated in figure 1, panel A, with the results of the

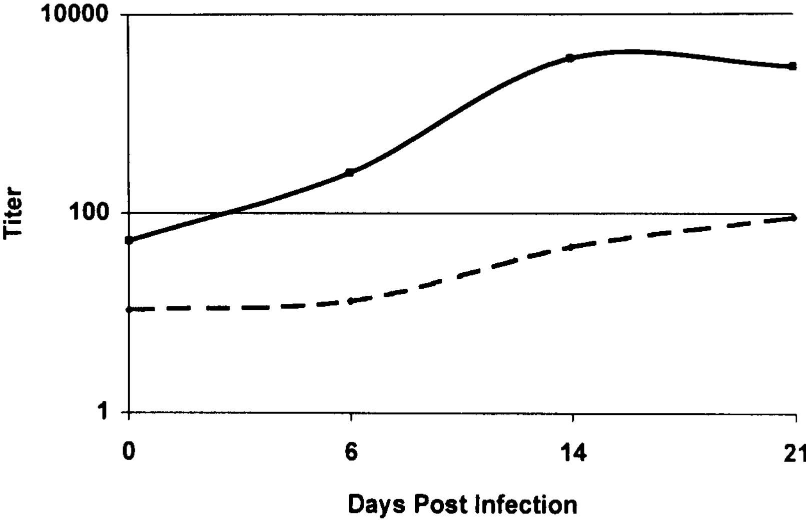

FIGURE 2. Time course of antibody response in dogs after experimental infection with CCV. ELISA (——) and SN (-------) titers represent a group mean from ten dogs.

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corresponding densitometry scan illustrated below in panel B. The calculated ratio of the integrated optical densities of the S:NC proteins is 0.55 for the untreated Cl-71 preparation and 1.1 for the treated Cl-71.

DETERMINATION OF ELISA SENSITIVITY

The sensitivity and utility of the CCV IgG-ELISA assay is demonstrated in figure 2, where the antibody response is monitored in dogs for 21 days after experimental infection with CCV. A comparison between the slopes of the lines depicting the ELISA and SN titers indicates that ELISA antibody is detected earlier after infection than SN antibody. Between days 5 and 15 post-infection, the trends of the two responses is similar. After 15 days the ELISA response

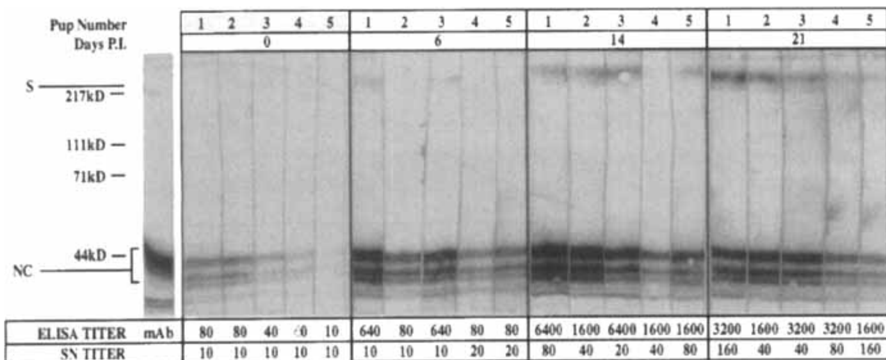

FIGURE 3. Comparison of antibody response between five dogs at 0, 6, 14, and 21 days post infection (P.I.) with CCV. Western blot reactivity of individual sera with the spike (S) and nucleocapsid (NC) proteins is illustrated with the corresponding ELISA and SN titers given below. Molecular weight markers are indicated in kD.

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reaches a plateau and then slightly decreases while the SN response continues to increase until at least day 21.

DETERMINATION OF ELISA SPECIFICITY

The Western blot in figure 3 illustrates the specificity of the response induced in each of five, selected dogs. A comparison is also made with the ELISA and SN titers at the bottom of the figure. It was interesting to find that all the dogs had a NC-specific response at day 0 and that this could be predicted by a detectable ELISA titer. In fact, the ELISA titers at day 0 ranged from 10-80 and correlated with the intensity of the NC-specific response. As expected, the SN titers for the same animals at this time point did not correlate with either the ELISA or the intensity of the Western blot response. At day 6 post-infection,

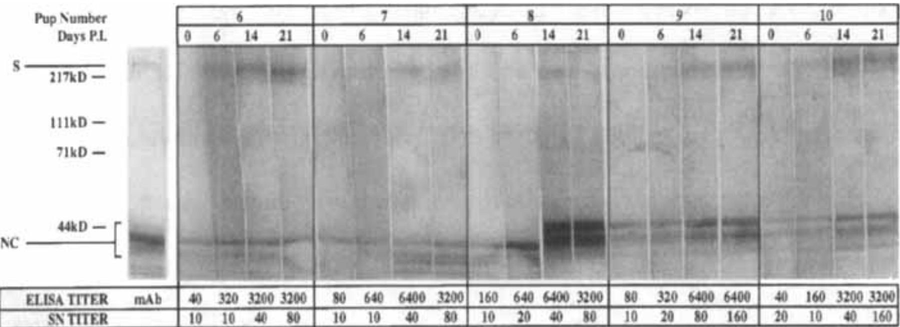

FIGURE 4. Time course of antibody response in five dogs at 0, 6, 14, and 21 days post infection (P.I.) with CCV. Western blot reactivity of individual sera with the spike (S) and nucleocapsid (NC) proteins is illustrated with the corresponding ELISA and SN titers given below. Molecular weight markers are indicated in kD.

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two pups (#1 and #3) had an S-specific response which could be predicted by the ELISA titer of 640 as compared to the other dogs which had titers of 80 and demonstrated only a NC-specific response. The SN titers did not reflect this S-specific response likely due to the decreased sensitivity of this assay as compared to the Western blot and ELISA. At days 14 and 21 post infection, all dogs were exhibiting increased S and NC-specific responses. This correlated with the increase in ELISA and SN titers, although as stated above, the ELISA response appeared to plateau and, in fact, slightly decreased at the last time point. This trend was supported by the decreased intensity of the NC-specific response illustrated in the Western blot.

Figure 4 provides a comparison between the specificity of response for the remaining five individual dogs at various times after experimental infection with

the ELISA and SN responses. Each dog illustrates an NC-specific response prior to experimental infection which can be predicated by a detectable ELISA titer. An SN titer of greater than 10 could only be detected in one of these dogs (#10). Following infection (day 6) an early NC-specific response was noted with a concomitant increase in the ELISA titer. Again, an SN titer greater than 10 was only noted in one animal (#9) at this time point. By day 14, in addition to a NC-specific response, a S-specific response was also noted. At this time point the ELISA titers increase at least 10-fold over that noted at day 6, paralleling the Western blot results. The SN titers also increased 4-fold at this time point over the day 6 titers. At day 21, all animals demonstrated an S-specific response similar or reduced in intensity to that exhibited at day 14. The ELISA titers appeared to closely parallel this response. For example, in animals # 6, 9 and 10 the S-specific intensity appeared roughly equal to that at day 14 and the ELISA titers were identical to those at day 14. In animals #7 and 8 the S-specific intensity appeared diminished and was reflected by the reduced ELISA titer. By comparison the SN titers did not parallel the Western blot results as closely as the ELISA titers.

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DISCUSSION

Studies of coronaviruses closely related to CCV, notably transmissable gastroenteritis virus (TGEV) and FIPV, indicate that two of the major structural components and immunogens of the virus are the spike (S) and nucleocapsid

(NC) protein [15, 19]. The S-protein has also been identified as the major protective antigen [20, 21, 22]. Biophysical analysis indicates that the S-protein is highly glycosylated (Mr ≅ 200 kD) and is amphipathic by nature; the majority of the molecule is located on the surface of the virion with a coiled-coil structure at the carboxy-terminus anchoring it into the membrane. The nucleocapsid protein, or ribonucleoprotein (Mr ≅ 43-60 kD) is a highly basic protein, and in the virus, it is closely associated with the nucleotides of RNA by ionic interaction. Based on studies with infectious bronchitis virus (IBV), the NC protein is considerably more abundant in the virus than the S-protein with a respective molar ratio of 4:1 [16].

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In order to accurately measure the total CCV-specific IgG antibody response to both the S and NC protein, it was important to first equalize the moles of each protein in the ELISA coating antigen preparation. Taking into consideration the biochemical nature of the CCV proteins [23], an enrichment process was developed using Triton X-114. This nonionic detergent was selected based on its biophysical properties [24], one of which is its low cloud point enabling the efficient phase separation of numerous integral membrane and hydrophilic proteins [25, 26]. Even though the basic mechanism of action for Triton X-114 on cell membranes has yet to be completely elucidated it is known to have the ability to displace and replace much of the normal lipid environment surrounding the hydrophobic domains of integral membrane proteins, while having very little

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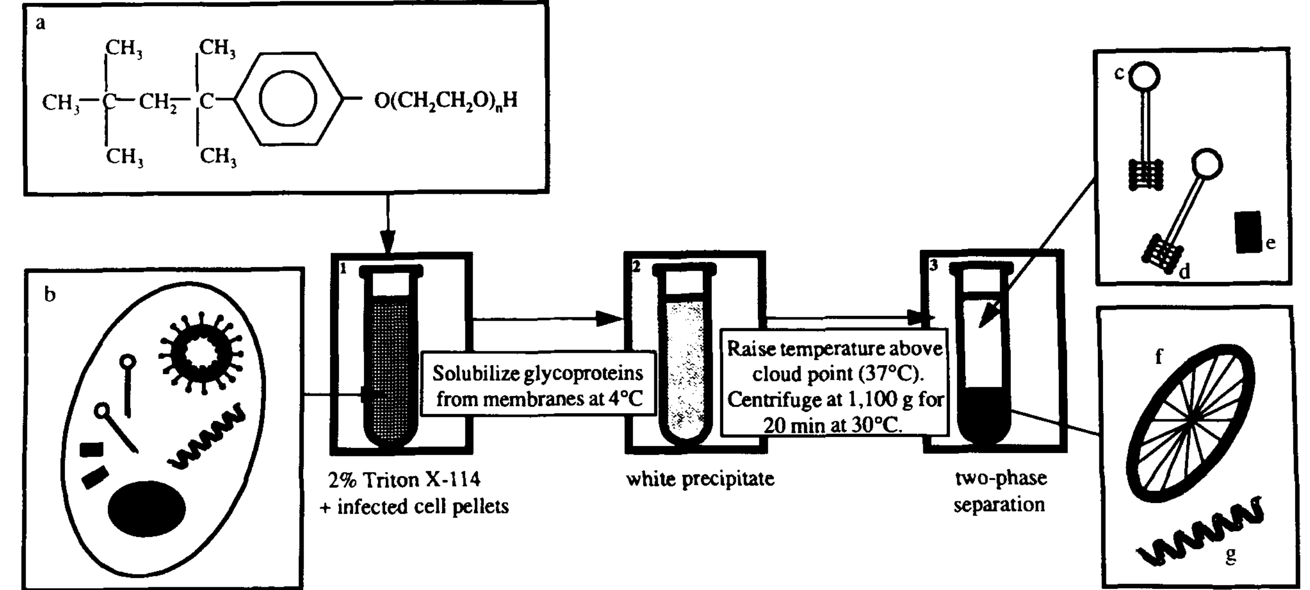

FIGURE 5. Schematic representation of phase partitioning process to produce S-protein enriched preparation. 2% Triton X-114 (a) is combined with CCV infected cell pellets containing various CCV proteins (b). After the indicated processing, the aqueous phase predominantly contains the S-protein (c) along with a small amount of detergent (d) attached to its hydrophobic carboxyterminal end and newly synthesized NC-protein (e). Mixed micellular superaggregates of detergent and remaining integral membrane proteins (f) and nucleocapsid attached to viral RNA (g) remain in the detergent phase.

binding effect on hydrophilic proteins [27]. Based on these characteristics, a schematic diagram of its mechanism of action on membranes containing CCV proteins is depicted in figure 5. Briefly, phase separation can be achieved by first solubilizing the CCV-specific glycoproteins from cellular or viral membranes with the Triton X-114 at 4°C. The solution at this temperature becomes opalescent, containing a white precipitate comprised of approximately 10% of the total membrane protein and elevated levels of cholesterol and phospholipid [23]. By increasing the temperature, mixed micellar superaggregates of detergent and various integral membrane proteins form, where they precipitate and settle into the more dense detergent phase at 37°C, well above cloud point. Hydrophilic proteins are traditionally thought to be recovered in the aqueous phase, whereas amphipathic integral membrane proteins are commonly found in the detergent phase after phase partitioning. Hooper and Bashir [28] even demonstrated that a single membrane-spanning domain or hydrophobic group is sufficient to pull a protein into the detergent phase. In our case, however, the spike glycoprotein of CCV partitions into the aqueous phase even though this integral membrane protein is amphipathic in nature. As described for other amphipathic proteins [29, 30, 31], this anomalous partitioning may be due to the highly glycosylated nature of the S-protein which prevents its hydrophobic membrane domain from being intercalated into the hydrophobic interior of the detergent micelle without disrupting the micellar structure. Also, the noncovalent association of integral membrane proteins may also predispose them to separate into the aqueous phase.

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Due to its hydrophilic nature, the NC protein was expected to be present in the aqueous phase, although the anomalous partitioning of the hydrophilic NC-protein into the detergent phase has been previously described and attributed to its association with the viral genome [24]. Based on this, it is likely that the NC present in our coating antigen preparation represents newly synthesized protein. This is consistent with the use of CCV infected cells rather than purified virus as the starting material.

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As described above, the quantity of S-protein in relation to the NC-protein in the coating preparation can be increased by Triton X-114 treatment of infected cells followed by phase separation. However, we hypothesize that both the quality and quantity of the proteins adhering to the polystyrene plate is increased by methanol treatment of this preparation. Similar to the documented increase in ordered structure of many peptide and protein antigens in halogenated alcohols [32], methanol treatment may act to preserve the secondary structure of the S-protein, in particular. This structural feature should allow for CCV-specific neutralizing antibodies to recognize and bind to conformational and cryptic epitopes. Support for this hypothesis is the relationship between ELISA and SN antibody levels in dogs after experimental infection with CCV. In addition, this treatment likely allows for better interaction between the hydrophobic portions of the protein and the polystyrene solid support, thereby retaining more of the antigen in the well. Evidence for this is the increased reactivity of CCV-specific

antiserum with methanol-fixed whole virus compared to that diluted in carbonate buffer as the coating antigen in an ELISA format (data not shown).

The utility of this ELISA method was determined by monitoring the immune response in dogs over time after experimental infection with a CCV strain which produced mild disease characterized by diarrhea, anorexia and virus shed (data not shown). It was interesting to note that all the SPF animals involved in this study had CCV-specific antibody prior to infection, as measured by our ELISA where titers ranged from 10 to 80. Western blot analysis of sera from this time point indicated that the response was directed to the NC protein. Examination of the intensity of this reaction was reflected in the magnitude of the ELISA titer. As expected, the SN assay was not able to quantify this response and, in fact, animals would have been considered seronegative by this criterion. Examination of the clinical parameters listed above would also not have indicated the presence of an infection since virus was not detected in the feces until 2 days after infection. Unfortunately, intestinal tissue was not evaluated for the presence of CCV antigen prior to experimental infection. The presence of only a NC response in the puppies prior to experimental infection is unlikely to represent the presence of maternal antibody since puppies were derived from CCV negative dams and antibody levels did not decline over time in control puppies not inoculated with CCV (data not shown). Since the NC protein is more conserved among the coronaviruses than the S protein, the NC specific response prior to

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inoculation may be due to an infection or exposure to a heterologous coronavirus [19], even though attempts were made to keep animals in isolation. In addition, the rapid elevation of IgG antibody and its maintenance until 21 days is indicative of a secondary rather than the primary response described by Tennant et al [13]. IgM was also not measured in this particular study. Nevertheless, the presence of CCV-specific antibody prior to vaccination could influence the subsequent immune response resulting in an inappropriate interpretation of vaccine efficacy. The ability of this ELISA to predict an S-specific response in the absence of detectable SN antibody demonstrates the sensitivity of this assay and illustrates the inconsistency of the SN assay when measuring low levels of antibody. Once the ELISA IgG titers increased to between 1600-6400 an SN response could be detected, however, the level of intensity of bands corresponding to the S-protein more closely reflected the ELISA titer.

It is evident from these results that the ELISA method described in this manuscript measures IgG antibody specific to both the CCV NC and S-proteins. Furthermore, the close association of the ELISA and SN titers enables the potential use of this assay to predict a protective humoral immune response in dogs. The application of detergent aided enrichment in order to produce the ELISA coating antigen as well as the methanol treatment of this preparation can be applied to other viral systems providing that the biophysical properties of the major immunogens are known.

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