

Fecal Immunoglobulin A Antibodies in Dogs Infected or Vaccinated with Canine Coronavirus

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Fecal secretory immunoglobulin A (IgA) antibodies in dogs infected or vaccinated with canine coronavirus (CCV) were evaluated by an enzyme-linked immunosorbent assay. The study was carried out with 32 fecal samples collected just before inoculation and at 28 days postinoculation. Five groups were studied: naturally infected dogs, experimentally infected dogs, dogs inoculated with a modified live (ML) CCV vaccine by the intramuscular route, dogs inoculated with an ML CCV vaccine by the oronasal route, and dogs given an inactivated CCV vaccine. Both the naturally and the experimentally infected dogs developed high levels of fecal IgAs. Interestingly, dogs inoculated with the ML CCV vaccine by the oronasal route developed levels of fecal IgA that were higher than those observed in the dogs inoculated with the same CCV vaccine by the intramuscular route or those observed in dogs inoculated with the inactivated vaccine. A relationship between the level of fecal IgAs to CCV and the degree of protection against CCV infection was observed.

Canine coronavirus (CCV) is an enteric pathogen of dogs that is responsible for mild to severe diarrhea in pups. Uncomplicated CCV infections are considered to be of minor clinical importance, but dual infections, e.g., CCV-canine parvovirus type 2 (10) and CCV-canine adenovirus type 1 (12) infections, have been observed in severely ill pups.

CCV shedding in the feces of naturally infected pups was previously demonstrated over a period of 37 days (12); recently, long-term viral shedding for up to 180 days was observed (13). Those observations led us to consider that the immunization of dogs against CCV would have favorable epidemiological effects.

Few data on the duration of immunity in infected pups are available. Although serum antibody titers in pups infected or vaccinated by the intramuscular route are generally higher than those in orally exposed pups, immunity does not occur after subsequent oral infection by CCV (1). It is known that the mucosal surface of the gastrointestinal tract is replete with organized follicles and antigen-reactive lymphoid tissues, including B cells, T lymphocytes, plasma cells, and other cellular elements involved in the induction and maintenance of the immune response. This gut-associated lymphoid tissue is considered the principal inductive site for mucosal immune responses (20). In external secretions, secretory immunoglobulin A (IgA) is involved in defending external surfaces by inhibiting the adherence of several pathogens to the surfaces of mucosal cells, thereby preventing the entry of these pathogens. Secretory IgAs contribute to immune exclusion by complexing with the antigen and limiting its luminal uptake. Consequently, protection against mucosal infections has generally been correlated with the presence of specific antibodies in the mucosal

surface (7, 8). Several studies have suggested the importance of antibodies on the mucosal surface of the small intestine as a major determinant of resistance to rotavirus illness (6). Therefore, it appears that local immunity (IgA in the gut mucosa) is essential for protection against CCV infection (1).

The aim of this study was to evaluate by an enzyme-linked immunosorbent assay (ELISA) the levels of fecal IgAs to CCV in dogs following infection or vaccination and to statistically compare the results obtained in order to define the most effective mode of vaccination.

MATERIALS AND METHODS

Fecal samples. In previous studies, 32 fecal samples were collected from 16 dogs after CCV infection or vaccination. Fecal samples were collected just before experimental infection or vaccination (time zero [T_0]) and 28 days postinoculation (T_{28}). Fecal samples from the naturally infected dogs were collected at the beginning of the enteric illness (T_0) and 28 days later (T_{28}). All samples were stored at -20°C until they were examined and grouped for this study. Group A included four fecal samples collected from two naturally infected dogs whose infections were confirmed by PCR assays for CCV (11). Group B included four samples collected from two dogs experimentally infected with CCV (17). Group C included eight samples collected from four dogs vaccinated by the intramuscular route with an experimental modified live (ML) CCV vaccine (17). Group D included eight samples collected from four dogs vaccinated with an experimental ML CCV vaccine by the oronasal route (17). Group E included eight samples collected from four dogs after vaccination by the intramuscular route with an inactivated commercial CCV vaccine (16).

ELISA for fecal IgAs. CCV-specific IgAs in the fecal samples were determined by an ELISA. The antigen for the ELISA was prepared as previously described (14). Briefly, the supernatants of Crandell feline kidney cell cultures infected with CCV strain 45/93 were harvested 96 h postinfection, clarified at $3,000 \times g$ for 20 min, and centrifuged at $140,000 \times g$ for 1 h at 4°C . Microtiter plates were coated with $100 \mu\text{l}$ of CCV antigen diluted in carbonate buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 [pH 9.6]) per well and then incubated overnight at 4°C with slow shaking. The plates were washed four times in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), treated with blocking solution (0.2% gelatin in carbonate buffer) for 90 min at 37°C , and washed four times with PBS-T. Dilutions (1/25) of each fecal sample in PBS-T were added in duplicate, and the plates were incubated for 90 min at 37°C . The washing cycle was then repeated, $100 \mu\text{l}$ of goat anti-dog IgA-horseradish peroxidase conjugates (Bethyl Laboratories Inc., Montgomery, Tex.), diluted in PBS-T, was added to each well, and the plates were incubated for 1 h at 37°C .

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TABLE 1. Fecal and serum IgA antibodies to CCV in groups of dogs following infection or vaccination^a

Group ^b	No. of dogs	No. of fecal samples	IgA level by ELISA		Serum antibody level by			
					VN test		ELISA	
			T ₀	T ₂₈	T ₀	T ₂₈	T ₀	T ₂₈
A	2	4	0.040	0.458	<2	16	0.026	0.218
B	2	4	0.041	0.116	<2	16, 32	0.057	0.250
C	4	8	0.054	0.067	<2	4	0.037	0.143
D	4	8	0.023	0.386	<2	8	0.023	0.210
E	4	8	0.027	0.041	<2	2	0.035	0.186

^a Values given for ELISA results are median OD values, and values given for the VN test results are titers.

^b Group A, naturally infected dogs; group B, experimentally infected dogs; group C, dogs vaccinated by the intramuscular route with an ML CCV vaccine; group D, dogs vaccinated by the oronasal route with an ML CCV vaccine; group E, dogs vaccinated with an inactivated vaccine.

After a washing cycle, 10 mg of freshly prepared substrate, 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]diammonium salt (ABTS; Sigma Chemical Co., St. Louis, Mo.) in 50 ml of 0.05 M phosphate citrate buffer (pH 5.0), was placed in each well, and the optical density at 405 nm (OD₄₀₅) was determined. The adjusted OD values for each sample were obtained by subtracting the OD of the mock antigen-coated well from that of the corresponding virus antigen-coated well.

The cutoff value (OD < 0.060) was defined as the mean OD plus 3 standard deviations calculated with the negative fecal samples collected from 10 dogs separately housed and previously examined for CCV antigen in their feces and antibodies in their serum.

Serum antibody determinations. For serological tests, blood samples were collected from 16 dogs on the same days as the fecal samples (T₀ and T₂₈). Virus neutralization (VN) tests and ELISAs were carried out as previously described (14), with a goat anti-dog IgG (Sigma Chemical Co.) being used for the latter test.

Statistical analysis. Statistical analysis was carried out to compare the antibody levels of the five experimental groups at T₂₈. Fecal secretory IgA antibody levels (measured by fecal optical density [fOD]), as well as serum antibody levels, determined by both VN (serum VN [sVN]) and ELISA (serum optical density [sOD]), were first submitted to a normality test by the UNIVARIATE procedure (19). Due to the deviation from normality detected for the original variables fOD and sVN, a logarithmic transformation (natural logarithm, ln_fOD and ln_sVN) was applied in order to submit data to analysis of variance. Since the sOD distribution did not significantly differ from normality, no logarithm transformation was carried out for this variable.

A one-way analysis of variance was performed by the general linear model procedure (19) fitting the linear model $y_{ij} = m + G_i + e_{ij}$, where y_{ij} is the j th observation of the dependent variable (time by time the variable analyzed was: ln_fOD, ln_sVN, sOD), m is the overall mean, G_i is the fixed effect of the i th group (five levels, corresponding to experimental groups A, B, C, D, and E), and e_{ij} is the residual error.

From the model solutions, the least-square means (LSM) were calculated for the dependent variables. The statistical significance of the differences between experimental groups was evaluated by a t test, with the Bonferroni correction being applied for multiple comparisons.

RESULTS

Serum antibody evaluations. The results of tests for antibodies to CCV in serum samples and in fecal samples are reported in Table 1.

Sera from the two dogs naturally infected with CCV (group A) at T₀ tested negative by both the VN test (with titers of <1:2) and the ELISA (median OD value, 0.026). High levels of neutralizing antibodies (with titers of 1:16) and high OD values by the ELISA (median OD value, 0.218) were found at T₂₈. In both dogs, ELISA-detected IgA OD values were higher at T₂₈ than at T₀ (median OD values, 0.458 and 0.040, respectively).

TABLE 2. LSM and standard errors of the analyzed variables^a

Group ^b	ln_fOD			ln_sVN			sOD	
	OM	LSM	SE	OM	LSM	SE	LSM	SE
A	0.458	-0.783 a	0.204	16	2.772 a	0.104	0.218 ad	0.012
B	0.116	-2.161 b	0.204	24	3.119 a	0.104	0.249 cd	0.012
C	0.069	-2.671 bcd	0.144	4	1.387 b	0.074	0.141 b	0.009
D	0.437	-0.921 a	0.144	8	2.079 c	0.074	0.209 ade	0.009
E	0.043	-3.163 d	0.144	2	0.693 d	0.074	0.184 ae	0.009

^a For the transformed variables, the original variable means (OM) are also reported. Means followed by different letters differ significantly ($P < 0.05$). Comparisons were performed with the Bonferroni correction. ln_fOD, natural logarithm of fecal secretory IgA antibodies (OD); ln_sVN, natural logarithm of serum antibodies determined by VN (titer); sOD, serum antibodies determined by ELISA (OD).

^b For a description of the groups, see Table 1, footnote b.

The sera from dogs experimentally infected with CCV (group B) were negative by both the VN test (with titers of <1:2) and the ELISA (median OD value, 0.057) at T₀, and they had high antibody titers at T₂₈ by the VN test (with titers of 1:16 and 1:32, respectively) and the ELISA (median OD value, 0.250). ELISA-detected IgA antibodies had median OD values of 0.041 at T₀ and remained at relatively low levels (median OD value, 0.116) at T₂₈.

The four dogs inoculated by the intramuscular route with the ML CCV vaccine (group C) tested negative at T₀ by both the VN test (with titers of <1:2) and the ELISA (median OD value, 0.037). However, at T₂₈, low VN titers (1:4) and median OD values of 0.143 were detected. No differences were found between T₀ and T₂₈ IgA values (median OD values, 0.054 and 0.067, respectively).

The four dogs inoculated with the ML CCV vaccine by the oronasal route (group D) tested negative at T₀ by both the VN test (with titers of <1:2) and the ELISA (median OD value, 0.023). At T₂₈, serum antibody levels for all dogs increased according to both the VN test (with titers of 1:8) and the ELISA (median OD value, 0.210). ELISA-detected IgA median OD values were 0.023 (T₀) and 0.386 (T₂₈).

Serum samples from the four dogs inoculated with the inactivated CCV vaccine (group E) tested negative at T₀ by both the VN test (with titers of <1:2) and the ELISA (median OD value, 0.035), even though serological responses could be detected by both the VN test (with titers of 1:2) and the ELISA (median OD value, 0.186) at T₂₈. The IgA levels in fecal samples at T₂₈ were similar to those observed at T₀ (median OD values, 0.041 and 0.027, respectively).

Statistical evaluation of the vaccine effects. Highly significant results were obtained from the analysis of variance of the considered variables. The probability values for the fitted linear model ranged from 0.0002 (for sOD, $R^2 = 0.85$) to 0.0001 (for ln_fOD, $R^2 = 0.94$; for ln_sVN, $R^2 = 0.98$). The LSM of the three dependent variables for the five experimental groups are shown in Table 2. The logarithmic transformation used for fecal secretory IgA antibodies and, by VN, for serum antibodies allows more powerful and robust comparisons between LSM, which are shown in Table 2.

The highest ln_fOD values were observed for groups A (-0.783) and D (-0.921), and these values did not differ statistically. Fecal IgA values for group D, i.e., dogs vaccinated by

the oronasal route, were comparable to those observed for the naturally infected dogs (group A).

An intermediate value was found for group B (-2.161), and this value differed statistically from those for groups A, D, and E. The lowest \ln_{FOD} values were observed for groups C and E. Values for both experimental group A and experimental group D significantly differed from those for groups C and E.

Significant differences were found between all pairs of \ln_{sVN} LSM except for those for experimental groups A (2.772) and B (3.119). Among the three vaccine groups, the highest \ln_{sVN} value, corresponding to the best antibody response, was again found for group D (2.079).

The same trend, but with less-significant differences, was observed for the sOD variable. In this case, the original means are not reported in Table 2, since they corresponded to the LSM obtained by one-way analysis of variance that was directly fitted to the serum antibody levels. Significant differences were detected between the C group, which showed the lowest antibody values, and the A, B, and D groups. Another significant difference was observed between responses for groups B (0.249) and E (0.184). The higher value observed for the D group with respect to the other two vaccine groups is also confirmed in this case. The statistical difference between group D and group C LSM was particularly significant.

DISCUSSION

CCV is considered a common cause of mild to severe gastroenteritis in pups, in which extensive viral replication occurs primarily in the epithelium of the small intestine. However, little is known about the immune mechanisms involved in protection. Antibodies produced by the gut-associated lymphoid tissue are the most important effectors of an immune response against enteric pathogens such as coronaviruses. Studies on the role of serum antibodies as a predictor of susceptibility to coronavirus infection and illness have yielded ambiguous results (1, 21).

The role of coproantibodies during rotavirus infection or after vaccination has been investigated by several laboratories with conflicting results (2, 3, 4, 5). In a recent study, it was demonstrated that fecal or systemic IgAs are not essential for protection from rotavirus infection, suggesting that in the absence of IgA, IgG also may play a significant role in protection from mucosal pathogens (9).

The results of our study show that fecal IgAs are involved in immune protection from CCV infection. It is well known that ML vaccines administered by the oronasal route induce the development of serological and, mainly, mucosal immune responses which confer protection against reinfection. In the present study, dogs inoculated by the oronasal route with the ML vaccine (group D) developed IgA levels in the gut that were higher than levels observed in the feces of dogs inoculated with the same vaccine by the intramuscular route (group C) or dogs inoculated with an inactivated CCV vaccine (group E).

In previous studies (16, 17), the dogs included in group D of the present study were fully protected after challenge with a CCV field strain, since the challenge virus was not detected by either virus isolation or PCR assays. It therefore seems plausible that the protection observed in these dogs may be related

to levels of fecal IgA antibodies to CCV. In contrast, the dogs inoculated intramuscularly with an ML CCV vaccine (group C) and, particularly, the dogs inoculated with the inactivated CCV vaccine (group E), developed low levels of fecal IgAs, providing partial (group C) or insignificant (group E) protective mucosal immunity. It is interesting that the fecal IgA levels of the naturally infected dogs (group A) were higher than those of the experimentally infected dogs (group B).

This result may be the consequence of a recently described new genotype of CCV, tentatively named CCV type I. This viral type has very frequently been detected in the feces of naturally infected pups (15). In such instances, the dogs were found to be simultaneously infected with both genotypes, CCVs types I and II (A. Pratelli, N. Decaro, A. Tinelli, V. Martella, G. Elia, M. Tempesta, F. Cirone, and C. Buonavoglia, submitted for publication). Since the experimental infection was carried out only with a CCV type II strain, the low IgA levels in the feces of dogs of group B may, indeed, be the consequence of a single infection. Dogs in the field are frequently found to be infected with both of the CCV genotypes.

As observed for other intestinal infections (2, 3, 4, 18), the results of our study show a direct relationship between the levels of intestinal IgA antibodies to CCV and the degree of protection against infection in dogs, as observed in a previous experiment (17). Assuming that fecal IgAs play an important protective role against CCV infection, it seems important to evaluate vaccines in regard to both the vaccine type (ML or inactivated) and the inoculation route.

The evaluation of fecal IgA antibody levels, rather than serum antibody levels, appears to be an efficient and meaningful way to evaluate the protection of dogs against CCV infection.

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