

## Canine coronavirus infection in the dog following oronasal inoculation

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**The pathogenesis of canine coronavirus (CCV) infection in 10-week-old puppies was studied up to 14 days after oronasal inoculation. Mild diarrhoea was seen from three to 11 days after inoculation, approximately coincident with faecal virus shedding. Virus was initially isolated from the tonsils on day 3, and then from both small and large intestinal tissues up to 14 days after inoculation. Virus was also isolated from liver and lung. Histological changes were not seen in any tissues, but CCV antigen was detected, using a peroxidase antiperoxidase staining technique, mainly in epithelium overlying gut-associated lymphoid tissue. Virus neutralising antibody was first detected on day 10. Specific anti-CCV IgM was first detected in plasma three days after inoculation and IgG on days 4 to 7. Small amounts of anti-CCV IgG, IgM and IgA were detected in duodenal secretion, but none in bile.**

ALTHOUGH canine coronavirus (CCV) has been recognised since 1971 (Binn et al 1974) in association with moderate to severe disease in dogs (Binn et al 1974, Vandenberghe et al 1980, Williams 1980, Carmichael and Binn 1981), it is generally considered to induce only mild enteritis (Appel 1987). Clinical signs are considered to be difficult to reproduce experimentally (Appel 1987), although little work has been reported on this. Moderate signs of disease have been induced experimentally in neonatal pups but in-contact bitches showed no clinical signs (Binn et al 1974, Keenan et al 1976). In naturally occurring infections, pups of six to 12 weeks old appear to be more susceptible to disease (Cartwright and Lucas 1972, Vandenberghe et al 1980, Carmichael and Binn 1981). This may be related to the decline in maternal antibody.

There are few reports on the pathogenesis of CCV infection in dogs. In neonatal dogs the virus appears to replicate primarily in enterocytes on the villus tips of the small intestine (Binn et al 1974, Keenan et al 1976, Takeuchi et al 1976). Following natural infection, virus has been reported in the epithelium of the small and large intestine (Vandenberghe et al 1980).

Systemic spread of CCV has not been well studied although there are some, often anecdotal, reports of virus having been demonstrated in various tissues including mesenteric lymph nodes, liver, spleen, meninges and lung (Keenan et al 1976, Binn et al 1979, Greene 1984, Acree et al 1985). CCV has also been implicated in a pancreatitis/meningitis syndrome following live CCV vaccination (Martin 1985, Wilson et al 1986) and CCV has also been shown to cause systemic infection in the cat (McArdle et al 1991). Other coronaviruses, including the closely related feline infectious peritonitis virus and transmissible gastroenteritis virus of pigs, have also been found to affect various organ systems in addition to the gut (Hooper and Haelterman 1969, Mebus et al 1973, Underdahl et al 1974, Pedersen et al 1984, Weiss and Scott 1981, Thomas et al 1982, Childs et al 1983).

As the published work on the pathogenesis of CCV infection is mainly based on experimental infection of neonatal pups, the present work was undertaken in older, 10-week-old pups, in order to reproduce more closely the naturally occurring disease. A recent UK isolate was used in these studies. Since mucosal immunity is considered important in other closely related coronavirus infections, such as transmissible gastroenteritis virus (Bohl et al 1972), clinical and virological findings were related to local and systemic antibody responses.

### Materials and methods

#### *Experimental animals*

Six six-week-old pups, whelped and reared in isolation with their dam, were maintained in isolation under strict barrier conditions until they were used at 10 weeks old. They remained seronegative to CCV during this time and no virus was isolated from their faeces.

#### *Virus*

CCV-C54, isolated from a dog presented with enteritis to the Small Animal Hospital at the University of Liverpool, was used in this experiment at

the sixth passage with a titre of  $10^{6.3}$  TCID<sub>50</sub> ml<sup>-1</sup>. The original isolation was made in feline embryo A (FEA) cells (Jarrett et al 1973) but the isolate was passaged in A-72 cells (Binn et al 1980). A-72 cells, used in attempts at virus isolation in this study, were grown in Wellcome minimum essential medium (Gibco), with antibiotics and 10 per cent fetal calf serum for growth and 4 per cent fetal calf serum for maintenance.

### Experimental design

Five dogs were inoculated oronasally with 1 ml of CCV-C54. A control dog was given A-72 tissue culture fluid oronasally 14 days before the start of the experiment and killed on day 0: restricted animal accommodation precluded synchronous inoculation but environmental and dietary factors remained constant throughout the experiment.

All dogs were examined daily for evidence of disease and rectal temperatures were recorded. Daily rectal and oropharyngeal swabs were taken into 2 ml of A-72 medium for attempted virus isolation. Heparinised blood samples, taken on all days except 1, 2, 8 and 9 were separated into red blood cells, plasma and buffy coat. Virus isolation was attempted from all three samples and plasma was also stored at  $-20^{\circ}\text{C}$  for serology. EDTA samples were used for haematological examination.

Individual pups were killed by overdose with barbiturate on days 0, 3, 5, 7, 10 and 14 after inoculation and complete post mortem examinations carried out. Representative tissues (Table 1) were taken into either Bouin's fixative or unbuffered 10 per cent formalin and all were processed for histology and immunocytochemical staining. Unfixed tissues (Table 1) were also collected for virus isolation.

TABLE 1: Distribution of virus in tissues of six 10-week-old dogs inoculated oronasally with  $10^{6.3}$  TCID<sub>50</sub> CCV-C54

Tissue	Dog number (days post inoculation dog killed)					
	1 (0)	2 (3)	3 (5)	4 (7)	5 (10)	6 (14)
Duodenum	-	-C	-	-C	-	+C
Jejunum	-	-	-	+C	-	+C
Ileum	-	-	+	+C	+C	+C
Caecum	-	-	-	+	+	+
Colon	-	-	-	+	+C	+
Mes LN	-	-	-	+	+	-
Pop LN	-	-	-	-	+	-
Liver	-	-	-	+	+	-
Lung	-	-	-	+	-	-
Tonsil	-	+	-	+C	-	-

+ Virus isolated, - No virus isolated, C CCV Immunoreactive cells detected (PAP technique), Mes LN Mesenteric lymph node, Pop LN Popliteal lymph node

Stomach, spleen, kidney, pancreas, peritoneum and meninges were all negative for virus isolation and CCV-immunoreactivity

Plasma (obtained from heparinised samples collected immediately before euthanasia), bile (collected by aspiration from the gall bladder) and duodenal secretions (obtained by gently milking a section of duodenum) were tested for neutralising antibody and class specific anti-CCV immunoglobulin content.

### Serology

Antisera, bile and duodenal secretions were heat inactivated at  $56^{\circ}\text{C}$  for 30 minutes before use. Virus neutralisation tests were performed in a microtitre system using a constant virus, varying serum technique with duplicate serial twofold dilutions of each sample and 100 TCID<sub>50</sub> of challenge virus per well (Gaskell et al 1982).

### Virology

The supernatant (0.1 ml) from rectal swabs was adsorbed for 45 minutes at  $37^{\circ}\text{C}$  on to duplicate freshly confluent monolayers of A-72 cells, grown in 16 mm plastic wells (Costar). Following washing and refeeding of monolayers with maintenance medium, cultures were incubated for up to five days in 5 per cent carbon dioxide at  $37^{\circ}\text{C}$ , and examined daily for evidence of 'wet' cytopathic effect. All samples were passaged up to three times and positive samples were harvested by freeze-thawing three times and stored at  $-70^{\circ}\text{C}$ . With every assay a negative control was included by adsorbing ordinary (ie, swab free) A-72 growth medium on to the cell monolayer.

Tissues collected at post mortem examination for virus isolation were washed three times in A-72 growth medium, before grinding with small amounts of sand in Griffith's tubes on the day of sampling. Following centrifugation (700 g for 10 minutes) to pellet out residual sand particles, duplicate A-72 cell monolayers were infected with the supernatants. Samples were inoculated as above for swabs except that following adsorption the monolayers were washed twice with sterile phosphate buffered saline (PBS) A to minimise tissue induced cytotoxicity. As with faecal samples and swabs a negative (A-72 medium) control was always run concurrently. Homogenised tissues were subsequently stored at  $-70^{\circ}\text{C}$ .

Supernatants from virus positive tissues were subsequently titrated to estimate the amount of virus present. Tenfold dilutions of each sample were made in the A-72 growth medium, and 0.05 ml of each were mixed with an equal volume of growth medium in wells of a 96-well microtitre plate (Flow Laboratories). To each well was added 0.05 ml of freshly trypsinised A-72 cells in growth medium and plates were incubated at  $37^{\circ}\text{C}$  in 5 per cent carbon dioxide. Four replicates of each dilution were performed.

Results were read at 96 hours after infection and infectivity titres, calculated using the method of Reed and Muench (1938), were expressed as the 50 per cent tissue culture infective dose (TCID<sub>50</sub>) g<sup>-1</sup> of tissue.

### Bacteriology

Faecal samples obtained two and three weeks before inoculation and daily after inoculation were assessed for the presence of *Campylobacter* species, as described by Woldehiwet et al (1990).

### Histological and immunocytochemical investigation

Bouin's or formalin-fixed, paraffin-embedded tissue sections were taken on to chrome alum gelatin coated slides. Histological examination was carried out on haematoxylin and eosin stained sections. Viral antigens were demonstrated by a modification of the peroxidase anti-peroxidase (PAP) techniques described by Shu et al (1988), where overnight incubation with the primary, rabbit anti-CCV antiserum (kindly supplied by Intervet Laboratories) was carried out at 4°C. Goat antirabbit and rabbit PAP (Sigma) were used at recommended dilutions to detect the primary antiserum.

### Determination of class specific antibody development

An enzyme-linked immunosorbent assay (ELISA) was developed to detect class specific immunoglobulins to CCV. Briefly, stock antigens, CCV (grown in A-72 cells) and A-72 tissue culture fluid, were each clarified by centrifugation at 1300 g and concentrated by precipitation with 10 per cent w/v polyethylene glycol at 4°C for two hours. The precipitates, pelleted by centrifugation at 1300 g for 30 minutes and

resuspended in cold tris sodium chloride EDTA (TNE) buffer, were layered over 3 ml of 30 per cent (w/w) sucrose in TNE buffer. After centrifugation at 100,000 g for one hour, the resulting pellets were resuspended in TNE buffer to their original volumes. These antigens were used to coat activated ELISA plates (Flow Laboratories) overnight at 4°C, followed by immediate overcoating with polyvinylpyrrolidone (1 per cent) and storage at 4°C for up to one month. The plates (CCV and tissue culture fluid coated) were incubated with sera (1/100), bile or duodenal secretions (1/20) diluted in phosphate buffered saline (PBS)/Tween (0.05 per cent) for one hour at 37°C. Class specific immunoglobulins in the samples were subsequently detected with mouse monoclonal antibodies to dog IgG (1/1000) and IgA (1/200) and human IgM (1/200), followed by alkaline phosphatase conjugated rabbit anti-mouse immunoglobulin (Sigma). Each well was thoroughly washed three times with PBS/Tween between each stage. Paranitrophenyl phosphate (Sigma) was then used as the substrate in a tris/glycine buffer (pH 10.4) and the absorbance values were read at 410 nm after 30 minutes. Absorbance values for each sample were calculated by subtracting the value obtained for tissue culture fluid coated plates from that of CCV coated plates.

## Results

### Clinical signs

Moderate amounts of unformed, soft faeces were first observed in CCV-infected dogs on day 3 after inoculation and continued through to day 11 (Table 2). The dogs all remained bright throughout this period. Pyrexia was not observed at any time and

TABLE 2: Development of clinical signs and virus shedding patterns following oronasal challenge of five 10-week-old dogs with 10<sup>6.3</sup> TCID<sub>50</sub> CCV-C54

Dog number*	Day killed	Observation	Days after inoculation															
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
2	3	CS	-	-	-	-												
		VI	-	-	-	-												
3	5	CS	-	-	-	-	+	+										
		VI	-	-	-	-	-	-										
4	7	CS	-	-	-	+	-	+	+	+								
		VI	-	-	-	-	-	-	+	+								
5	10	CS	-	-	-	-	+	-	+	+	+	+	+					
		VI	-	-	-	-	-	-	-	-	+	+	-	-				
6	14	CS	-	-	-	-	-	-	+	+	+	+	-	+	-	-	-	-
		VI	-	-	-	-	-	+	-	+	+	+	-	-	+	+	-	-

+ Positive for diarrhoea (no other signs seen) or CCV isolation, CS Clinical signs, VI Virus isolation from rectal swabs  
No virus isolated from oropharyngeal swabs. No viraemia detected

\* Dog 1, uninfected cell culture control inoculated day - 14 and killed on day 0. No clinical signs or virus shedding detected

haematological parameters remained within normal limits. The control dog remained clinically normal throughout the period of observation.

#### *Virus isolation*

Virus was isolated from rectal swabs from days 4 to 12 after inoculation, but not from oropharyngeal swabs (Table 2). Tissue viral isolations are shown in Table 1. Virus was first isolated from tonsillar tissue on day 3 and then later from intestinal tissues, between days 5 and 14. Virus was isolated from other systemic tissues including liver, lung, tonsil and lymph nodes on days 7 and 10. Virus was not isolated from red blood cells, buffy coat or plasma. No virus was detected on subsequent titration of any swab or tissue except for mesenteric lymph node on day 7 (titre of  $10^{3.8}$  TCID<sub>50</sub> g<sup>-1</sup>).

#### *Bacteriology*

*Campylobacter jejuni* was repeatedly isolated from

many dogs before and following CCV inoculation. No clinical symptoms were associated with the presence of this organism before CCV inoculation.

#### *Immunocytochemical and histological findings*

CCV antigen was demonstrated by PAP staining in small intestinal tissues on days 3, 7, 10 and 14, colon on day 10 and tonsil on day 7. In intestinal tissues, antigen positive cells were mainly seen in the epithelium overlying gut-associated lymphoid tissue (Fig 1a,b); a few CCV-positive cells (one to 10 per tissue section) were seen on the upper half of villi. In the tonsil, one focus of CCV-immunoreactive cells was detected in epithelium. No histopathological changes were demonstrated in any tissues.

#### *Serology*

Serum virus neutralising (VN) antibodies were first detected on day 10 in the two remaining dogs (mean VN titre of 1/8) and increased gradually up to day 14

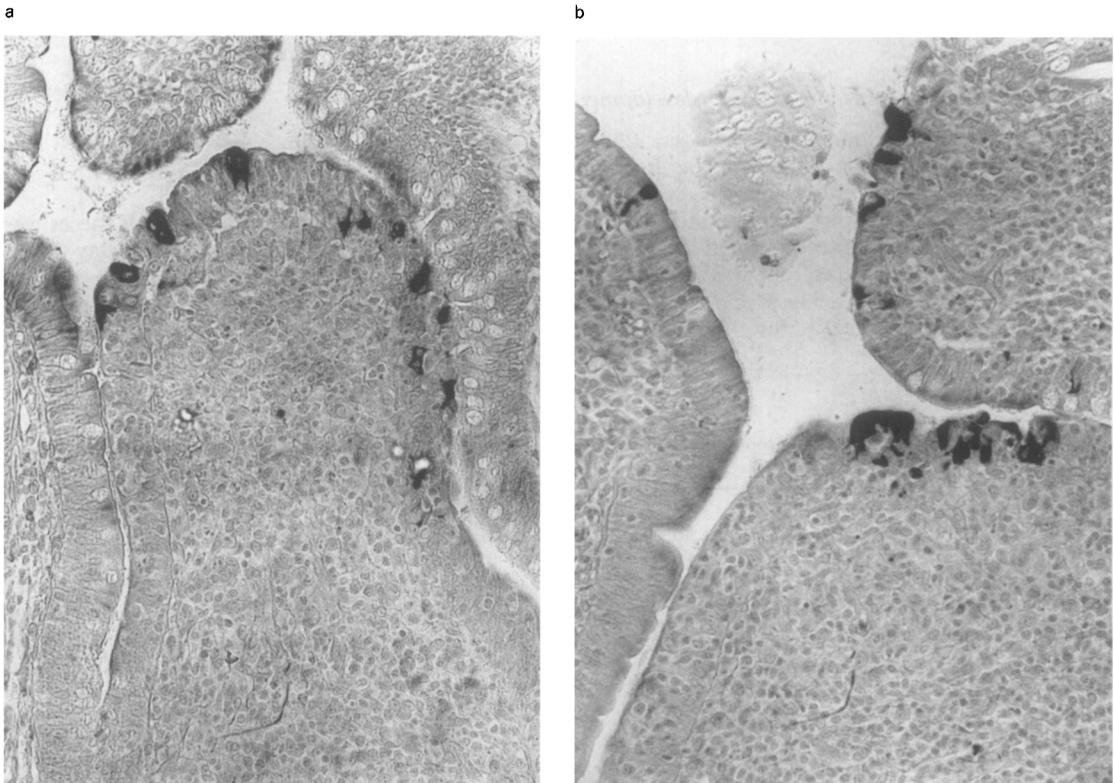


FIG 1: Demonstration of CCV-immunoreactive cells in small intestinal tissues obtained from CCV-C54 infected dogs. CCV positive cells indicated by black reaction product. (a) (left) Ileum of dog 4, killed on day 7. CCV-immunoreactivity in dome epithelial cells overlying mucosal lymphoid tissue.  $\times 195$ . (b) (right) Ileum of dog 5, killed on day 10. CCV-immunoreactivity in dome epithelial cells overlying mucosal lymphoid tissue.  $\times 195$

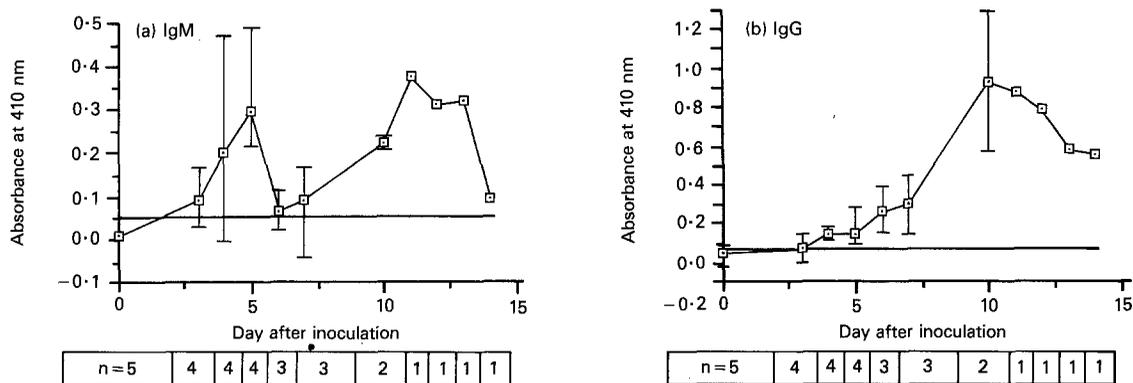


FIG 2: Development of anti-CCV IgG and IgM in plasma of CCV-C54 infected dogs. — Baseline value of control dog. n Number of dogs sampled on each occasion. ⊥ range of absorbance values on each day

(titre of 1/28). No virus neutralising antibodies were detected in duodenal juice or bile.

*Anti-CCV class specific immunoglobulin results*

**Plasma immunoglobulin response.** Specific anti-CCV-C54 IgM was detected in plasma from day 3 onwards, peaking initially at day 5 and then a second peak occurring in the one remaining dog on day 11 (Fig 2a). Specific anti-CCV-C54 IgG was detected in plasma at low levels on days 4 to 7, peaking by day 10 before declining in the one remaining dog to moderate levels over the following four days (Fig 2b). No specific anti-CCV-C54 IgA was detected in any plasma sample at any time.

**Duodenal juice immunoglobulin response.** In general, all three immunoglobulin classes to CCV-C54 were detected in duodenal juice (Fig 3). Specific anti-CCV-C54 IgM levels peaked on day 3 after infection and declined gradually over the following 11 days (Fig 3). No IgM was demonstrable on day 14. Anti-CCV-C54 IgG was first detected on day 7 and increased gradually over the following week, remaining elevated at day 14 (Fig 3). Specific anti-CCV-C54 IgA increased gradually up to day 14 approximately paralleling the IgG response (Fig 3).

**Bile immunoglobulin response.** No class specific anti-CCV-C54 immunoglobulins were detected in bile at any stage.

**Discussion**

Experimental CCV infection of 10-week-old pups produced mild diarrhoea from days 3 to 11 post inoculation, although no histological changes were seen. More severe enteritis has been produced experimentally in infected neonatal pups (Binn et al 1974,

Keenan et al 1976), but only subclinical infection has been shown in in-contact adult bitches (Keenan et al 1976) and in dogs of unspecified age (Acree et al 1985). In naturally occurring cases however, CCV infection sometimes appears to induce more serious disease both in 10-week-old pups and in older animals (Cartwright and Lucas 1972, Binn et al 1974, Vandenberghe et al 1980), although increasing age generally seems to be associated with a decrease in the severity of disease (Appel 1987). Such variation in pathogenicity may also be accounted for by other host differences, such as breed, sex or immune status, though there is no evidence for these in CCV infections.

Virus factors may also affect the outcome of infection. The titre of challenge virus may be of importance, although the viral dose used in this study was comparable to that given by Keenan et al (1976). Strain variation may also account for variations in pathogenesis. Although the C54 strain was isolated from a dog with severe haemorrhagic enteritis, some

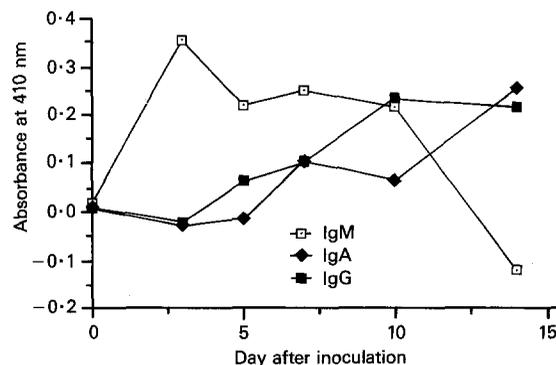


FIG 3: Development of anti-CCV IgM, IgG and IgA in the duodenal secretions of CCV.C54 infected dogs. (One dog killed on each of days 0, 3, 5, 7, 10 and 14 after inoculation)

degree of attenuation may have occurred in cell culture.

Other factors which may influence the severity of disease include stress or concurrent infection with other pathogens. In this study *Campylobacter jejuni* was isolated from all the experimental dogs from three weeks before and during the study. Although *C. jejuni* has been associated with diarrhoea in experimental dogs (Macartney et al 1988) many dogs reared in breeding colonies may be asymptomatic excretors (Newton et al 1988). No specific signs of disease were associated with the presence of campylobacter before CCV challenge in this study and no signs were seen in the uninfected control dog. Concurrent CCV/canine parvovirus infections have also been reported to induce severe disease (Tingpalapong et al 1982, Appel 1988).

The finding of faecal shedding of CCV between four and 12 days after inoculation broadly confirms a previous report (Keenan et al 1976). Similar faecal shedding patterns in the acute phase of the disease have been demonstrated for other experimental coronavirus infections, including feline enteric coronavirus (Pedersen et al 1984), bovine coronavirus and transmissible gastroenteritis virus (Morin et al 1974, Reynolds et al 1985). Persistent or recurrent virus shedding in clinically recovered animals has also been recorded in coronavirus infection in some species (Lee et al 1954, Collins et al 1987, Jones and Ambali 1987). Since decreasing numbers of dogs were examined only up to 14 days after inoculation in the present study, the long term shedding patterns of CCV were not investigated.

In contrast to previous reports of CCV and also transmissible gastroenteritis virus infections, which showed infected cells on the tips of villi (Keenan et al 1976, Takeuchi et al 1976, Shepherd et al 1979), this study demonstrates CCV infection predominantly to involve cells in dome epithelium in Peyer's patches rather than in the villus. It is possible that the infected dome epithelial cells are the membranous or M-cells described for a number of species including the dog (Egberts et al 1985). M-cells are thought to be involved in the uptake and transport of virus or other antigens from the gut lumen to the underlying lymphoid tissue (LeFevre et al 1979, Wolf et al 1981, Egberts et al 1985).

The pathogenesis of CCV appears to be more complicated than previously demonstrated. The present authors have isolated CCV from tissues other than those associated with the intestinal tract, such as lung, tonsil, liver and popliteal lymph node. The significance of this finding in the pathogenesis of the disease, in the absence of histological changes, is not clear. The mode by which other tissues were infected may be explained by viraemic spread, although this was not confirmed by virus isolation from blood.

Alternatively lung may have been infected by direct infection through inhalation of the initial inoculum and liver and mesenteric lymph node by local spread from the gastrointestinal tract. Some other coronaviruses have been shown to affect tissues outside the gastrointestinal tract (Underdahl et al 1974, Weiss and Scott 1981, Saif et al 1986).

Infection of other tissues outside the gastrointestinal tract did not appear to cause illness or tissue damage. This may be attributable to the low levels of virus present or may simply reflect efficient clearance of virus by the dogs' immune system. However, as a different CCV isolate has been shown to result in a generalised infection and cause feline infectious peritonitis-like lesions in cats (McArdle et al 1991), and CCV has been implicated in a pancreatitis-meningitis syndrome (Martin 1985, Wilson et al 1986), further studies are needed to determine what role, if any, CCV may play in systemic disease.

Although mild diarrhoea was observed, no histological changes were demonstrated. This may reflect the limited extent of viral replication in the gut mucosa demonstrated by low virus titre and PAP staining. The pathophysiology of diarrhoea is a complex subject and has been well described (Argenzio and Whipp 1980). It may be possible for CCV to infect enterocytes and induce diarrhoea, without causing marked histological damage, by altering the brush border digestive enzyme system. However, as only a few enterocytes were shown to be infected in this study the actual cause of the diarrhoea cannot be explained. The use of canine small intestinal organ cultures may allow further investigation into the effect of CCV infection on mucosal biochemistry.

The appearance of specific anti-CCV-C54 IgM in plasma, at days 3-5 after inoculation was not associated with VN antibody at this time. VN antibodies were demonstrated in these dogs by 10 days after infection, coincident with both the peak plasma IgG-anti-CCV response and the second IgM-anti-CCV peak. Previous reports showed seroconversion by 21 days, but dogs were not tested earlier than this (Binn et al 1974, Keenan et al 1976). The appearance of VN antibodies coincided approximately with the cessation of virus excretion and the disappearance of clinical signs and may therefore have contributed to recovery. However, such antibodies may not have provided any immunity to reinfection. In previous work, the authors have shown that dogs with moderate to high levels of serum CCV neutralising antibodies arising from vaccination are not protected against oral challenge (Tennant 1989).

All three classes of anti-CCV-immunoglobulins were detected in duodenal secretions and rising levels of anti-CCV-IgG and anti-CCV-IgA apparently coincided with the cessation of virus shedding. However,

their significance in recovery or in providing immunity against CCV reinfection in the dog is not clear. Secretory IgA has been associated with protection against transmissible gastroenteritis virus infection in the suckling piglet (Bohl et al 1972, Bohl and Saif 1975), and it may be that the development of local gut immunity, and possibly also local or systemic cell-mediated immunity, is more important in controlling the development of infection than are systemic antibodies.

Thus, in this study, experimental CCV infection led to the development of mild enteric infection and diarrhoea in young dogs. Under field conditions the disease may sometimes be more severe, possibly through the interactions of environmental factors and, or, other infectious agents and such interactions should be looked at further. Since CCV was isolated from organs other than the intestinal tract and has also been shown to induce feline infectious peritonitis-like lesions in cats (McArdle et al 1990), further work is required to investigate the possible role of CCV in canine systemic infections.

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