PAPER

# High-cell-passage canine coronavirus vaccine providing sterilising immunity

**OBJECTIVES:** To evaluate the ability of a high-cell-passage canine coronavirus vaccine to immunise dogs against challenge with a field isolate of the virus.

**METHODS:** Three dogs that had previously tested seronegative and virus-negative for canine coronavirus were inoculated twice, at 21-day intervals, with the vaccine and kept under observation. Two seronegative and virus-negative dogs served as unvaccinated controls. For safety tests, two additional dogs were inoculated oronasally with 10 times the vaccinal dose and no reactions were observed. Faecal samples were collected daily from the vaccinated dogs after the first and second inoculations. Both vaccinated and control dogs were challenged two weeks after the second vaccination with a field canine coronavirus strain. Blood samples were collected for serological tests before vaccination and at weekly intervals after vaccinations and challenge.

**RESULTS:** Virus was not detected in faecal samples after the first or second vaccinations by virus isolation assays and PCR. Significantly, the vaccinated dogs did not have clinical signs after challenge and no virus shedding was observed. The two unvaccinated control dogs had moderate enteritis, and virus was detected in cell cultures starting from three days postchallenge (dog 1) and two days postchallenge (dog 2), and by PCR for 23 median days.

**CLINICAL SIGNIFICANCE:** This study showed the efficacy of a high-cell-passage canine coronavirus vaccine in preventing infection of dogs by virulent virus and, specifically, its ability to induce sterilising immunity.

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## INTRODUCTION

Coronaviruses, of the family *Coronaviridae*, are large, enveloped, positive-stranded RNA viruses and are responsible for some highly prevalent diseases in human beings and domestic animals.

Canine coronaviruses (CCoV) cause mild to moderate enteritis in dogs. In young pups, or when the virus occurs with concurrent pathogens, diarrhoea may be severe with dehydration, loss of appetite and even death (Appel 1987). Infected dogs generally shed CCoV in their faeces for six to nine days after infection (Keenan and others 1976), although the virus has been detected in faeces up to six months after clinical signs have ceased (Pratelli and others 2001a, 2002b, Pratelli 2006). CCoV is highly contagious, and once the virus is established in a kennel, the spread of the infection is difficult to control. Crowding, unsanitary conditions, stress during training and other environmental conditions appear to favour the development of clinical disease (Evermann and others 1980, Yasoshima and others 1983, Pratelli and others 1999b, 2001b, Decaro and others 2004a). Disinfection of kennels and equipment with 3 per cent hypochlorite solution is effective in killing CCoV, but it does not prevent dog-to-dog transmission.

The efficacy and duration of the immunity provided by CCoV vaccines are controversial (Carmichael 1997). A recent study described the low efficacy of an inactivated CCoV vaccine in reducing viral shedding in the faeces of dogs after challenge (Pratelli and others 2003b). The safety and immunogenicity of an experimental modified-live CCoV vaccine administered intramuscularly or oronasally were subsequently shown (Pratelli and others 2004). In this study, PCR showed excretion of the vaccinal virus in the faeces after oronasal inoculation. The present study reports the results of a vaccination trial of a high-cell-passage CCoV vaccine administered via the oronasal route.

## MATERIALS AND METHODS

### Animals

Seven three-month-old stray dogs, four females and three males, were included in the present study. The dogs tested negative by PCR for CCoV antigens in the faeces and CCoV antibodies in the

sera. Baseline body temperature and white blood cell (WBC) count were determined for each dog by calculating an average from the measurements taken three days before vaccination. The experimental study was performed according to the animal health and wellbeing regulations and was authorised by the Italian Ministry of Health (authorisation number: 67/2002-C). After completion of the study, all dogs were adopted by private individuals, except one that remained in the study kennel. The policy of the kennel and local ordinances requires that the dogs be humanely cared for.

## Vaccine

The modified-live CCoV vaccine strain (strain 257/98-3c) used was originally isolated from a dog with mild enteritis (Pratelli and others 2004). The virus at the 40th passage on canine cells (A-72) was subsequently passaged an additional 20 times. The new vaccine stock, CCoV-hcp, had an infectivity titre of $10^{5·75}$ tissue cultured infectious dose $(TCID)_{50}$/50 µl, and tests using standardised methods for aerobic and anaerobic bacteria, mycoplasmas, mycetes and contaminant viruses were negative. The viral suspension was sterilised by passage through a filter of 0·22 µm (Millex; Millipore Corporation). Cell cultures infected for 24 hours were tested for pestivirs using an immunofluorescence test with monoclonal antibodies. To evaluate the presence of both moulds and yeasts, the vaccine stock was cultured onto Sabouraud dextrose agar with 0·5 g/l chloramphenicol (BioLife Italiana S.r.l.), and incubated at 25°C for seven days and 30°C for 14 days.

The safety of the CCoV-hcp vaccine was evaluated in two dogs that were given oronasal inoculations of the vaccine at a dose 10 times greater than that used in the efficacy test. The two control dogs were given 7 ml by the intranasal route plus 3 ml orally. Following inoculation, the dogs were housed individually and examined daily for 28 days for signs of illness, including WBC counts and body temperatures. Individual faecal samples were collected for the virological assays that were carried out from three days before the test through to day 28. On day 28, blood was taken from all dogs for serological testing.

## Experimental design

After an acclimatisation period of 10 days, three dogs were kept isolated in separate rooms and handled by separate operators. Each dog was then vaccinated by the oronasal route with 4 ml of CCoV-hcp (1 ml by nasal route and 3 ml by oral route). Twenty-one days later, the dogs received a second oronasal dose of the vaccine as before. Two dogs were housed separately and were not vaccinated (control group).

After the first and second vaccinations, the dogs were observed to watch for any adverse local or systemic reactions, and faecal samples were collected daily for 21 days post-first vaccination (dpfv) and for 14 days post-second vaccination (dpsv).

Fourteen days after the second dose, both the vaccinated and control dogs were challenged with a CCoV field strain (strain 144/01) that had been isolated from a diarrhoeic pup (Marsilio and others 2002). The virus was propagated in primary canine embryonic kidney cell cultures for three passages and stored at −70°C. Each challenged dog received 4 ml of viral suspension (1 ml intranasally and 3 ml orally) with a titre of $10^{5·75}$ $TCID_{50}$/50 µl. On the day of challenge, and for 14 days post-challenge (dpc), both the vaccinated and the control dogs were examined for signs of illness, and virus shedding was monitored daily. Because CCoV does not usually cause clinical disease in experimentally challenged susceptible dogs, the challenge study was monitored by evaluating median days of viral shedding.

Blood samples were collected from the three vaccinated dogs before vaccination and on seven, 14 and 21 dpfv and on seven and 14 dpsv. Additional samples were collected from both vaccinated and control dogs, at seven and 14 dpc. All were tested for antibody responses to monitor seroconversion.

## Virological analysis

Faecal samples collected from the vaccinated and control dogs were tested by virus isolation and PCR assays. To attempt virus isolation, samples were homogenised (10 per cent weight/volume) in minimal essential medium, treated with antibiotics (5000 IU/ml penicillin, 2500 µg/ml streptomycin and 10 µg/ml amphotericin B) and inoculated in duplicate onto freshly trypsinised A-72 cells in 24-well plates containing glass slides. The monolayers were observed daily for any cytopathic effects, and after 72 hours the cells were fixed in cold acetone and examined by an immunofluorescence test using a coronavirus monoclonal antibody. Samples were considered to be negative if no cytopathic effects or immunofluorescence occurred after three serial passages.

PCR assays were performed as previously reported (Pratelli and others 1999a). Viral RNA was extracted from clinical specimens using an RNeasy kit (Qiagen). The target sequence was a fragment of the gene encoding for the membrane protein M of CCoV, which amplified a 409 base pair fragment. Reverse transcription was performed in a total reaction volume of 20 µl containing 1× PCR buffer (50mM KCl and 10mM Tris-HCl, pH 8·3), 5mM $MgCl_2$, 1mM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 1 U RNAse inhibitors, 2·5 U murine leukaemia virus reverse transcriptase and 2·5 U random hexamers. Synthesis of complementary DNA was carried out at 42°C for 30 minutes, followed by a denaturation step at 99°C for five minutes. The mixture was brought up to a total volume of 100 µl, containing 1× PCR buffer, 2mM $MgCl_2$, 2·5 U Amplitaq Gold DNA polymerase (Perkin Elmer Cetus) and 50 pmol of each primer. Amplification was performed under the following PCR conditions: 35 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute. Negative and positive samples, faeces from specific pathogen-free dogs and CCoV strain S/378, respectively, were used as PCR controls. The final products were detected by gel electrophoresis, ethidium bromide staining and UV light transillumination.

## Serological analysis

ELISA and virus neutralisation tests were carried out on all serum samples, as described by Pratelli and others (2002a). In duplicate 96-well microtitration plates (Falcon-Becton, Dickinson Labware), serial twofold dilutions of each serum sample, starting from 1:2, were mixed

with 100 $TCID_{50}$/50 µl of CCoV, strain S/378, with an infectivity titre of $10^{6·5}$ $TCID_{50}$/50 µl. The plates were kept at room temperature for 90 minutes and then 20,000 freshly trypsinised A-72 cells were added to each well. Plates were incubated for five days at 37°C. The virus neutralisation antibody titre was expressed as the reciprocal of the highest serum dilution that completely inhibited viral cytopathic effects. Antibody titres lower than 1:2 were considered negative.

Immunoplates (NUNC; Roskilde) were coated with 25 µg/ml CCoV type II antigen, strain 45/93 (Buonavoglia and others 1994), diluted in carbonate buffer (15mM $Na_2CO_3$, 35mM $NaHCO_3$ and 0·02 per cent $NaN_3$, pH 9·6) and incubated overnight at 4°C with shaking. The plates were washed in phosphate-buffered saline (PBS) buffer containing 0·05 per cent Tween 20 (PBS-T) and then treated with a blocking solution of 0·2 per cent gelatin in carbonate buffer for 90 minutes at 37°C and again washed with PBS-T. Each dog serum, diluted 1/50 in PBS-T, was added in duplicate and the plates were incubated for 90 minutes at 37°C. After a washing cycle, peroxidase-conjugated goat anti-dog immunoglobulin IgG (Sigma Chemicals), diluted in PBS-T, was added to each well and the plates were incubated for one hour at 37°C.

After another washing cycle, freshly prepared substrates were placed into each well. The solution consisted of 10 mg 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt ([ABTS] Sigma Chemicals) in 5 0ml 0·05M phosphate citrate buffer, pH 5·0, containing 25 µl/100 ml hydrogen peroxide. After adding the stop buffer solution (1 per cent sodium dodecyl sulphate), the optical densities (ODs) were determined at 405 nm using an automatic ELISA reader (Biorad; Hercules). 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. Samples with OD values more than 0·040 were considered to be positive.

Faecal secretory IgA antibodies were evaluated by ELISA (Decaro and others 2004b) in all dogs after vaccination and challenge, and two weeks after challenge in the control dogs. Briefly, immunoplates were coated with CCoV antigen diluted in carbonate buffer and treated with blocking solution. Faecal samples, diluted 1/25 in PBS-T, were added in duplicate, and the plates were incubated for 90 minutes at 37°C. The washing cycle was then repeated and 100 µl of goat anti-dog IgA horseradish peroxidase conjugate (Bethyl Laboratories) was added to each well. The plates were then incubated for one hour at 37°C. After a washing cycle, 10 mg of freshly prepared substrate, ABTS, was added and the OD at 405 nm determined. The cut-off value was defined as OD less than 0·060.

Virus neutralisation titres were expressed as geometric means and OD values as median values.

## RESULTS

The two dogs that received oronasal inoculations of 10 times the vaccinal dose to test the safety of the high-cell-passage vaccine did not have any local or systemic reactions and their WBC counts remained normal throughout the observation period. Faecal samples collected for 28 days post-vaccination (dpv) were consistently negative by virus isolation and PCR assays. Serological tests (virus neutralisation and ELISA) performed on 28 dpv indicated that the two control dogs had developed an immune response (Table 1).

After the first vaccination, all faecal samples collected from the three dogs used in the efficacy test were consistently negative by both virus isolation and PCR tests. The sera collected on seven and 14 dpfv also tested negative by virus neutralisation and ELISA. The sera collected on 21 dpfv had low antibody titres (1:2) in the virus neutralisation test and the ELISA showed a moderate reactivity (median OD 0·048). The median ELISA IgA OD value was 0·046 at day 0 and increased at 21 dpfv to 0·344 (Table 2).

As expected, CCoV was not detected by virus isolation or PCR in the faeces of the dogs after the second vaccination or during the entire observation period (14 dpsv). The virus neutralisation test showed a slight increase in the antibody titre (1:4) from seven to 14 dpsv. With the ELISA test, high antibody values were observed both on seven dpsv (median OD 0·255) and 14 dpsv (median OD 0·345). ELISA IgA median OD values were 0·356 on seven dpsv and 0·372 on 14 dpsv (Table 2).

After challenge with field strain 144/01, the vaccinated dogs did not develop clinical signs, and virus isolation and PCR did not detect viral shedding. Neutralising antibodies in the vaccinated dogs increased progressively to approximately 1:25 (geometric mean) on 14 dpc. The OD values from the ELISA test also increased progressively, reaching a median value of 0·411 on 14 dpc. The median ELISA IgA OD value on seven dpc was 0·402, and it increased to 0·473 on 14 dpc (Table 2).

The two control dogs developed mild diarrhoea for an average of three days after challenge, and virus was isolated in cell cultures from their faeces starting from three dpc (dog 1) and two dpc (dog 2). By PCR, virus was detected from one to 14 dpc (dog 1 and dog 2). Virus neutralisation tests showed that antibodies had increased by 14 dpc (geometric mean 22·4). The serum ELISA test also showed the highest OD values on 14 dpc (median OD 0·250). Faecal ELISA IgAs had a median OD of 0·116 on 14 dpc (Table 2).

**Table 1. Results of the safety test performed with the canine coronavirus-hcp vaccine**

| Dogs | Virus shedding days | | Antibodies (28 dpv) | |
|---|---|---|---|---|
| | VI | PCR | VN | ELISA |
| 1 | 0 | 0 | 4 | 0·152 |
| 2 | 0 | 0 | 8 | 0·164 |

dpv Days post vaccination, VI Virus isolation, VN Virus neutralisation titre

**Table 2. Serological responses of vaccinated and control dogs after vaccination and challenge**

| Days | Vaccinated dogs (n = 3) | | | Controls (n = 2) | | |
|---|---|---|---|---|---|---|
| | Serum antibodies | | Faecal IgAs | Serum antibodies | | Faecal IgAs |
| | VN* | ELISA† | ELISA† | VN* | ELISA† | ELISA† |
| 21 dpfv | 2 | 0·048 | 0·344 | nd | nd | nd |
| 14 dpsv | 4 | 0·345 | 0·372 | nd | nd | nd |
| 14 dpc | 25·11 | 0·411 | 0·473 | 22·4 | 0·250 | 0·116 |

VN Virus neutralisation titre, dpfv Days post-first vaccination, dpsv Days post-second vaccination, dpc Days post-challenge, nd Not determined
*Geometric mean
†Median value

## DISCUSSION

CCoV was discovered as a causative agent of enteritis in dogs in 1971 (Binn and others 1974). The importance of this pathogen has been underestimated probably because of the difficulty in identifying the virus using conventional virological assays (Pratelli and others 2000) and in reproducing clinical disease in dogs under experimental conditions. Situations that cause stress in dogs, such as poor nutrition or overcrowding, and concurrent infections with other pathogens have been hypothesised to contribute to the severity of CCoV disease in field cases (Evermann and others 1980, Yasoshima and others 1983, Pratelli and others 1999b, 2001b, Decaro and others 2004a).

Fulker and others (1995) produced clinical disease in dogs after CCoV inoculation, followed by treatment with the immunosuppressive drug dexamethasone. In that study, the authors showed that an inactivated CCoV vaccine could induce an immune response that protected dogs against clinical signs. Because CCoV, in the absence of immunosuppression, does not cause clinical disease in experimentally challenged dogs, monitoring the reduction of viral shedding has been used to test the efficacy of CCoV vaccines. In a recent study, it was found that the inactivated vaccine had poor efficacy in reducing faecal shedding of CCoV following infection with a field strain of the virus (Pratelli and others 2003b). Subsequently, the safety and efficacy of a modified-live CCoV vaccine (strain 257/98-3c) was evaluated in 14 dogs. The study showed that there were no adverse reactions in dogs after the inoculation of the modified-live vaccine and that viral nucleic acid was detected by PCR for six dpfv in dogs that had been inoculated oronasally (Pratelli and others 2004).

The aim of the present study was to develop a safe and immunogenic vaccine that would not result in virus shedding. For this purpose, the modified-live CCoV vaccine (Pratelli and others 2004) was further attenuated (60 passages) and tested for its ability to immunise dogs. Because CCoV is considered a poor immunogen, dogs were vaccinated twice by the oronasal route. Of interest was the significant immune response observed in the sera and in the faeces of all dogs after the second inoculation. After challenge at 14 dpsv, protection from CCoV infection was complete because no viral shedding was observed by either virus isolation or PCR tests. Protection against CCoV infections has generally been associated with the presence of specific antibodies on the mucosal surface. Therefore, mucosal IgA in the vaccinated dogs (Table 2) might be responsible for the protection observed (Ogra and others 1980, Saif 1996, Murphy 1999, Decaro and others 2004b).

Although CCoV infections do not appear to be a major cause of life-threatening enteritis in dogs, severe illness as a consequence of dual infections has been observed (Evermann and others 1980, Yasoshima and others 1983, Pratelli and others 1999b, 2001b, Decaro and others 2004a). Because multiple infections are common in high-density populations, such as in unvaccinated kennels, and that dogs may shed the virus for as long as six months after the clinical signs have ceased (Pratelli and others 2001a, 2002b), it seems that immunisation of dogs to produce a sterilising immunity would have beneficial epidemiological effects in controlling the spread of CCoV in high-risk dog populations.

Epidemiological monitoring of the evolution of CCoV is particularly important for the development of rational prophylaxis. For example, documentation of recombination events affecting CCoV may further explain the evolutionary processes leading to the emergence of new virus strains, serotypes or subtypes, as has occurred with SARS-CoV, with the new genotype of CCoV (Pratelli and others 2003a) and with the new pathogenic variant of CCoV isolated from the organs of dog with severe lesions (Buonavoglia and others 2006).

The present study has shown the efficacy of a high-cell-passage CCoV vaccine in preventing infection of dogs by virulent virus and, specifically, its ability to induce sterilising immunity.

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