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.

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 2004a). 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...
Canine coronavirus vaccine

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 passed an additional 20 times. The new vaccine stock, CCoV-hcp, had an infectivity titre of 10^7.5 virus 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 stabilised by passage through a filter of 0.22 µm (Milllex; Millipore Corporation). Cell cultures infected for 24 hours were tested for pestiviruses using an immunofluorescence test with monoclonal antibodies. The final products were detected by gel electrophoresis, ethidium bromide staining and UV light transillumination.

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 oral 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 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).

Fifteen 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 3 ml of viral suspension (1 ml intranasally and 3 ml orally) with a titre of 10^7.5 TCID50/50 µl. On the day of challenge, and for 14 days post-challenge (dpch), 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 MgCl2, 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 MgCl2, 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.
with 100 TCID$_{50}$/50 μl of CCoV, strain S/378, with an infectivity titre of 10$^{5.5}$ 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$_2$CO$_3$, 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 0.25 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 diluted 1/25 in PBS-T, were added 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 (dppv) were consistently negative by virus isolation and PCR assays. Serological tests (virus neutralisation and ELISA) performed on 28 dppv 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 dppv also tested negative by virus neutralisation and ELISA. The sera collected on 21 dppv 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 dppv 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 dppv). The virus neutralisation test showed a slight increase in the antibody titre (1:4) from seven to 14 dppv. With the ELISA test, high antibody values were observed both on seven dpps (median OD 0.255) and 14 dppv (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 dpsv 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 dppv) |
|-------------------------------------------|-----------------|-----------------|
|                | VI | PCR | VN | ELISA |
| 1              | 0  | 0   | 0  | 0.152 |
| 2              | 0  | 0   | 0  | 0.164 |

dppv Days post vaccination, VI Virus isolation, VN Virus neutralisation titre
Canine coronavirus vaccine

### 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 fecal 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 dpso, 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.

### Acknowledgements

The author is grateful to the researchers in the infectious diseases section of the Department of Animal Health and Well-being at the Veterinary Faculty of Bari, Italy, for their assistance. The author thanks Dr G. Chappuis, from Merial in France, for kindly supplying the monoclonal antibodies used in the immunofluorescence test, and Professor L. E. Carmichael for supplying the S/378 strain of CCoV.

### References

Apfel, M. J. (1987) Canine coronavirus. In: Virus Infections of Carnivores. Ed M. J. Appel. Elsevier Science Publishers, Amsterdam, The Netherlands. pp 115-120

Binn, L. N., Lazar, E. C., Kedim, K. P., Hussell, D. L., Macmillan, R. H. & Stouffer, A. J. (1974) Recovery and characterization of a coronavirus from military dogs with diarrhea. Proceedings, Annual Meeting of the United States Animal Health Association 78, 359-366

Buonavoglia, C., Decaro, N., Mantella, V., Elia, G., Capuolo, M., Decaro, C., Castaglied, M. & Tempesta, M. (2006) Canine coronavirus highly pathogenic for dogs. Emerging Infectious Diseases 12, 492-494

Buonavoglia, C., Marsilio, F., Cavalli, A. & Tricarico, P. G. (1994) L’infezione da coronavirus del cane: indagine sulla presenza del virus in Italia. Notiziario Farmaceutico Veterinario 2/94

Camposole, L. E. (1997) Vaccines for dogs. In: Veterinary Vaccinology. Eds P.-P. Pastoret, J. Blancou, P. Vannier and C. Verschueren. Elsevier, New York, NY, USA. pp 326-335

Decaro, N., Camero, M., Greco, G. Z., Zizzo, N., Tinelli, A., Capuolo, M., Pratelli, A. & Buonavoglia, C. (2004a) Canine distemper and related diseases: report of a severe outbreak in a kennel. New Microbiologica 27, 177-182

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

| Days | Vaccinated dogs (n = 3) | Controls (n = 2) |
|------|------------------------|-----------------|
|      | Serum antibodies       | Serum antibodies |
|      | VN* ELISA1             | VN* ELISA1      |
|      | Faecal IgAs            | Faecal IgAs     |
| 21 dpfv | 0.048 | nd               |
| 14 dpso | 0.345 | nd               |
| 14 dpc | 0.411 | nd               |

| Days | Vaccinated dogs (n = 3) | Controls (n = 2) |
|------|------------------------|-----------------|
|      | Serum antibodies       | Serum antibodies |
|      | VN* ELISA1             | VN* ELISA1      |
|      | Faecal IgAs            | Faecal IgAs     |
| 21 dpfv | 0.344 | nd               |
| 14 dpso | 0.473 | nd               |
| 14 dpc | 22.4  | 0.250            |

**Notes:**
- VN: Virus neutralisation titre, dpfv: Days post-first vaccination, dpso: Days post-second vaccination, dpc: Days post-challenge. nd: Not determined
- *: Geometric mean
- 1: Median value

---

**Journal of Small Animal Practice • Vol 48 • October 2007 • © 2007 British Small Animal Veterinary Association**
Pratelli, A. (2006) Genetic evolution of canine coronavirus and recent advances in prophylaxis. Veterinary Research 37, 151-200
Pratelli, A., Buonavoglia, D., Martella, V., Decaro, N., Marsilio, F., Buonavoglia, D., Tempera, M., Lazzari, A. & Buonavoglia, C. (2000a) Diagnosis of canine coronavirus infection using nested-PCR. Journal of Virological Methods 84, 91-94
Pratelli, A., Elia, G., Martella, V., Paliferi, A., Corrente, M., Torelli, A., Corrente, M., Greco, G., Buonavoglia, D., Gentile, M., Tempera, M. & Buonavoglia, C. (2002a) Prevalence of canine coronavirus (CCoV) antibodies in dogs in Bari, Italy, by an enzyme-linked immunosorbent assay. Journal of Virological Methods 102, 67-71
Pratelli, A., Elia, G., Martella, V., Torelli, A., Decaro, N., Marsilio, F., Buonavoglia, D., Tempera, M. & Buonavoglia, C. (2002b) M gene evolution of canine coronavirus in naturally infected dogs. Veterinary Record 151, 758-761
Pratelli, A., Martella, V., Decaro, N., Torelli, A., Camero, M., Corrente, M., Elia, G., Cavalli, A., Corrente, M., Greco, G., Buonavoglia, D., Gentile, M., Tempera, M. & Buonavoglia, C. (2003a) Genetic diversity of a canine coronavirus detected in pups with diarrhoea in Italy. Journal of Virological Methods 110, 9-17
Pratelli, A., Martella, V., Elia, G., Decaro, N., Alberti, A., Buonavoglia, D., Tempera, M. & Buonavoglia, C. (2003b) Variation of the sequence in the gene encoding for transmembrane protein M of canine coronavirus (CCoV). Molecular and Cellular Probes 15, 229-233
Pratelli, A., Martella, V., Elia, G., Tempera, M., Giarda, F., Capuccio, M., Carnechi, L. E. & Buonavoglia, C. (2001b) Severe enteric disease in an animal shelter associated with dual infections by canine adenovirus type 1 and canine coronavirus. Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health 48, 385-392
Pratelli, A., Tempera, M., Greco, G., Martella, V. & Buonavoglia, C. (1999a) Development of a nested PCR for the detection of canine coronavirus. Journal of Virological Methods 80, 11-15
Pratelli, A., Tempera, M., Roperto, F. P., Saggioro, P., Carchioli, L. E. & Buonavoglia, C. (1999b) Fatal coronavirus infection in puppies following canine parvovirus 2b infection. Journal of Veterinary Diagnostic Investigation 11, 550-553
Pratelli, A., Torelli, A., Decaro, N., Corrente, M., Elia, G., Roperto, S., Tempera, M. & Buonavoglia, C. (2003b) Efficacy of an inactivated canine coronavirus vaccine in pups. New Microbiologica 26, 151-155
Pratelli, A., Torelli, A., Decaro, N., Martella, V., Camero, M., Tempera, M., Martini, M., Carnechi, L. E. & Buonavoglia, C. (2004) Safety and efficacy of a modified-live canine coronavirus vaccine in dogs. Veterinary Microbiology 99, 43-49
Safi, J. L. (1996) Mucosal immunity: an overview and studies of enteric and respiratory coronavirus infections in a swine model of enteric disease. Veterinary Immunology and Immunopathology 54, 163-169
Yamgure, A., Fushiki, M., Doi, K., Kigawa, A., Tsakia, H. & Oshikawa, A. (1983) Case report on mixed infection of canine parvovirus and canine coronavirus—electron microscopy and recovery of canine coronavirus. Nippon Juigaku Zasshi 45, 217-221