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Immunogenicity and protective efficacy in dogs of an MF59™-adjuvanted vaccine against recombinant canine/porcine coronavirus

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ABSTRACT

Recently, canine coronavirus (CCoV) strains with putative recombinant origin with porcine transmissible gastroenteritis virus (TGEV) were shown to be widespread in Europe. In this study, a killed vaccine against TGEV-like CCoV strains, included in the new subtype CCoV-IIb, was developed through inactivation with betapropiolactone and emulsification with MF59™ adjuvant. Safety, immunogenicity and efficacy of the developed vaccine were evaluated in vivo. Five 10-week-old beagle pups were administered (three weeks apart) two vaccine doses, whereas two animals served as unvaccinated controls. The vaccine was shown to be safe as no local neither systemic reactions were observed after first and second dose administration. Serum antibodies against CCoV were detected in vaccinates starting from study day 14 (by enzyme-linked immunosorbent assay) or 28 (by virus neutralisation test). Subsequent challenge with virulent CCoV-IIb resulted in the development of mild gastroenteric disease in control pups, whereas vaccinates did not display clinical signs. Faecal shedding of the challenge virus occurred in both treatment groups, but vaccinated dogs were found to shed very low viral titres in comparison to controls. The developed vaccine may help control the CCoV-IIb-induced disease (and active virus circulation) in environments, such as kennels and shelters, where the pathogenic potential of this virus is greater as a consequence of predisposing factors and concurrent infections.

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1. Introduction

Canine coronavirus (CCoV) is a member of the newly established genus *Alphacoronavirus* of the family *Coronaviridae*, order *Nidovirales*. CCoV is strictly related to feline coronavirus type I (FCoV-I) and type II (FCoV-II), transmissible gastroenteritis virus of swine (TGEV) and its respiratory variant porcine respiratory coronavirus (PRCoV). Based on the similarities in their genomic organisation, all these viruses have been now included in a unique viral species *Alphacoronavirus-1* [1]. CCoV has a classical faecal–oral route of transmission and colonises the top of the villi of the enteric tract, being responsible for mild, self-limiting enteritis. Infected pups usually recover spontaneously from CCoV-induced disease [2]. However, hypervirulent CCoV strains have been reported in the last years [3] and a pantropic variant [4] has been associated to systemic, sometimes fatal disease in pups under natural [5] and experimental conditions [6–8].

Two CCoV genotypes have been identified so far, namely CCoV type I (CCoV-I) and CCoV type II (CCoV-II) [9]. These genotypes are variously distributed worldwide, with a predominance of CCoV-II in Europe [10] and Asia [11,12]. In addition, CCoVs with a recombinant origin between CCoV-II and TGEV have been identified in the faeces of dogs with diarrhoea and have been found to be widespread in dogs populations. Accordingly, CCoV-II has been further classified into two subtypes, CCoV-IIa and CCoV-IIb, including "classical" CCoVs and TGEV-like strains, respectively [13]. Subtype CCoV-IIb has been reported in several European countries [14,15], as well as in Japan [12]. Limited antigenic cross-reactivity has been observed between subtype IIa and IIb CCoVs and this has raised some concerns about the real efficacy of the CCoV vaccines available in the market (prepared with subtype IIa) against the TGEV-like strains [13,15].

The aim of the present study was to develop an inactivated vaccine adjuvanted with MF59™ against CCoV-IIb and to evaluate its safety, immunogenicity and efficacy in beagle pups.

2. Materials and methods

2.1. Cells and viruses

Canine A-72 cells used for virus cultivation were grown in Dulbecco's minimal essential medium (D-MEM) supplemented with

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10% foetal calf serum. CCoV-IIb strains 341/05 and 174/06 were isolated from the lungs of an Italian 14-week-old great dane pup and a Hungarian 10-week-old chihuahua pup, respectively [13]. In the present study, virus 341/05 was used for vaccine preparation, whereas the Hungarian strain served as challenge virus. CCoV-IIb strain 341/05 was chosen as vaccine virus since it contains a 154-nucleotide deletion in ORF7b that could be used in the future as vaccine genetic marker, whereas strain174/06 was used as challenge virus since it had a distinct geographical origin. The two strains had been found to be strictly related at the genetic level, displaying a nucleotide identity of more than 96% in the nearly full-length genome [13].

For virus isolation, the lung samples were homogenised (10%, w/v) in D-MEM containing antibiotics (penicillin 5000 IU/mL, streptomycin 2500 μg/mL, amphotericin B 10 μg/mL). Viral growth was monitored constantly by an immunofluorescence (IF) assay using a monoclonal antibody (MAb) that binds the *Alphacoronavirus-1* N protein and a goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Sigma Aldrich srl, Milan, Italy). Both viruses induced a cytopathic effect in the inoculated monolayers and tested positive by the IF assay. The cell media of the third serial passage were collected, centrifuged at $3000 \times g$ for 15 min to remove cell debris, aliquoted and stored at −70 °C until their use. Viral titres of isolates 341/05 and 174/06 were $10^{5.75}$ and $10^{5.50}$ $TCID_{50}$ mL^{-1} of viral suspension, respectively.

2.2. Vaccine preparation

Isolate 341/05 was inactivated with 1:2000 betapropiolacton (0.05%, v/v) and the inactivated suspension, containing a total protein amount of 441 μg mL^{-1} as determined by spectophometer analysis, was mixed 1:1 with MF59^{TM} adjuvant (Novartis Vaccines and Diagnostics, Siena, Italy). Vaccine stock was aliquoted in 1-mL doses and stored at +4 °C.

2.3. Sterility test

The stock vaccine was tested for sterility from aerobe and anaerobe bacteria, mycoplasmas and mycetes using standardised methods. The presence of contaminant viruses was searched for in the viral suspension prior to adding the adjuvant by means of (RT-)PCR assays for detection of canine parvovirus type 2 (CPV-2) [16], canine distemper virus (CDV) [17], canine adenoviruses (CAdVs) [18], canine herpesvirus 1 [19], rotaviruses [20], reoviruses [21], and caliciviruses [22].

2.4. Experimental study

The experimental study was performed according to the European animal health and well-being regulations and was authorised by the Italian Ministry of Health (authorization no. 81/2010-C). Seven 10-week-old beagle pups were housed at the Infectious Disease Unit of the Animal Hospital, Faculty of Veterinary Medicine of Bari. The dogs had tested negative for CCoV RNA by a real-time RT-PCR assay [23] carried out on the faeces and for CCoV antibodies by an ELISA test [24] carried out on serum samples. All dogs were housed individually in separate boxes, fed twice daily with a commercial dry dog food and provided water ad libitum. After an acclimatization period of one week, five animals (pups #1 to #5) were vaccinated by subcutaneous administration of two doses, three weeks apart (study days 0 and 21), of 1 mL of the experimental vaccine, whereas two dogs (pups #6 and #7) were maintained unvaccinated by receiving subcutaneously two doses of 1 mL of sterile saline solution (placebo). In order to assess injection site reactions after each vaccination, the first vaccine administration was on the right hand side of the interscapular space, whereas the second dose was administered on the left hand side.

On day 35 (two weeks after booster administration), animals were administered a total of 3.0 mL of challenge material (isolate 174/06) with a titre of approximately $10^{5.5}$ $TCID_{50}$ mL^{-1}. The challenge dose was established according to previous studies on CCoV vaccination [26,27]. Approximately 0.5 mL of challenge material was administered per nostril (1.0 mL total), and 2.0 mL was administered orally. Animals were observed for 21 days after challenge for specific clinical signs of CCoV infection. A single veterinarian, who was not aware of the treatment group assignments, was responsible for daily clinical observations in all dogs. At the end of the animal phase (study day 56), animals were kept in the animal facility and tested every five days for CCoV shedding from faecal samples. Animals were assigned to private owners once the laboratory results indicated the animals were not shedding CCoV for three consecutive tests.

2.5. Safety test

Vaccine safety was evaluated by the observation of local and systemic reactions after each vaccination. Qualitative assessment of injection site reaction was made by palpation of the injection site for the occurrence of pain and/or reaction, whereas systemic reactions were assessed by clinical inspection. Examinations for local and systemic reactions were performed twice on the day of vaccination (pre-vaccination and 5 h post-vaccination) and once daily for four days after each vaccine administration.

2.6. Immunogenicity test

Vaccine immunogenicity was evaluated by assessing the CCoV-antibody response after each vaccine dose administration. Vaccinated and control dogs were bled for serum collection at days 0 (day of first-dose administration), 7, 14, 21 (day of booster administration), 28 and 35 (day of challenge). Antibody titres were also evaluated after challenge (study days 42, 49 and 56). Serum samples were stored at −20 °C until analysis by using enzyme-linked immunosorbent assay (ELISA) and virus neutralisation (VN) tests, as previously described [6,7,23].

For ELISA test, microtitre plates were coated with CCoV antigen and, after treatment with blocking solution and repeated washing, 1:50 dilutions of the plasma samples were added to each well. Plates were incubated for 90 min at 37 °C, washed four times and incubated for 60 min at 37 °C with anti-dog IgG-goat peroxidase conjugate (Sigma–Aldrich srl, Milan, Italy). After another washing cycle, 10 mg of freshly prepared substrate, 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate]diammonium salt (ABTS, Sigma–Aldrich srl) in 50 mL of 0.05 M phosphate citrate buffer (pH 5.0) was added to each well and the optical density at 405 nm (OD_{405}) was determined.

For VN tests, serial two-fold dilutions of heat-inactivated sera were mixed with 100 $TCID_{50}$ of strain 341/05 (CCoV-IIb) or S378 (CCoV-IIa) in 96-well microtitre plates. After pre-incubation at room temperature for 90 min, 2×10^4 A-72 cells were added to each well. The plates were read after four days of incubation at 37 °C. VN titres were calculated using the Spearman–Karber method and expressed as the highest serum dilution able to neutralise the virus.

2.7. Efficacy test

Vaccine efficacy was evaluated by challenging the vaccinated dogs with virulent TGEV-like strain 174/06, two weeks post-second vaccination, and assessing the prevention or reduction of clinical signs and of viral shedding in comparison to unvaccinated control dogs. Clinical examinations were performed on all dogs, once daily

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