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A subunit vaccine candidate based on the Spike protein of SARS-CoV-2 prevents infectious virus shedding in cats

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ABSTRACT

Of the numerous animal species affected by the SARS-CoV-2 virus, cats are one of the most susceptible, and cat-to-cat transmission has been described. Although cat-to-human infection has not, as yet, been demonstrated, preventive measures should be taken in order to avoid both viral infection in cats and transmission among them. In this respect, the application of an effective vaccine to at-risk populations would be a useful tool for controlling the disease in this species. Here, we test a new vaccine prototype based on the Spike protein of the virus in order to prevent infection and infectious virus shedding in cats. The vaccine employed in experimentation, and which is easily produced, triggered a strong neutralizing antibody response in vaccinated animals. In contrast to that which occurred with control animals, no infectious virus was detected in the oropharyngeal or rectal swabs of vaccinated cats submitted to a SARS-CoV-2 challenge. These results are of great interest as regards future considerations related to implementing vaccination programs in pets. The value of cats as vaccination trial models is also described herein.

1. Introduction

Since the end of 2019, the entire world has been under the threat of the pandemic triggered by the severe acute syndrome coronavirus 2 (SARS-CoV-2), the causative agent of Coronavirus Disease 2019 (COVID-19) (Pérez-Campos Mayoral et al., 2020). The virus presumably originated in bats and jumped to humans using an intermediate host that has not, as yet, been identified (Lau et al., 2020; WHO, 2020; Wong et al., 2020). The zoonotic origin of the disease signifies that it is necessary to investigate the role played by susceptible animals. Of all the animals affected, cats are one of the most susceptible species (Halfmann et al., 2020; Shi et al., 2020). The close relationship between cats and humans has led to the assessment of the transmissibility and pathology of SARS-CoV-2 in cats in several studies (Halfmann et al., 2020; Chiba et al., 2021). Moreover, several cases of natural infection in cats have been reported worldwide (OIE, 2021a), which has resulted in uncertainty for veterinarians, pet owners and the scientific community. Although most of the cats naturally and experimentally infected did not show severe clinical signs, gross and histology lesions have been described in experimental assays (Chiba et al., 2021), raising the question of whether the disease is significant for this species. However, a severe case of SARS-CoV-2 infection has been described in a 4-year-old cat. This caused serious respiratory distress and culminated in humane euthanasia (Carvallo et al., 2021). Other 14 companion animals (8 dogs

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and 6 cats) that tested positive to SARS-CoV-2 have died to date worldwide (OIE, 2021a), although comorbidities may have been involved in these deaths. As a precautionary measure, it is still, therefore, necessary to avoid SARS-CoV-2 infection in domestic cats for reasons of both the animals' welfare and public health. Preventive actions, such as averting direct contact between COVID-19 positive people and cats, may serve as an effective first line of control. However, further caution might be required in animals exposed to high virus density environments and in cases in which people at risk of developing severe COVID-19 disease live with pets, as has been highlighted by the expert panel of the OIE (OIE, 2021b). In this respect, the development of an effective and safe vaccine against SARS-CoV-2 for cats could be a good approach by which to evade potential infections in this species.

Several vaccine candidates against SARS-CoV-2 have been already developed for humans, including a live virus, inactivated vaccines, a viral vector, a subunit, and nucleic acid vaccines (Dong et al., 2020). Each vaccine type has several advantages and disadvantages. Of the different types, subunit vaccines are developed on the basis of synthetic peptides or recombinant proteins. As this type of vaccine contains only specific viral antigenic fragments, several concerns related to incomplete inactivation or virulence recovery are eliminated, in contrast to inactivated or live-attenuated virus and viral vectored vaccines, respectively (Du et al., 2008; Deng et al., 2012). Subunit vaccines are, therefore, considered safe candidates for SARS-CoV-2 vaccine development, since they can target specific neutralizing epitopes, triggering an efficient host immune response, and can be used in immunocompromised patients because there are fewer side effects (Motamedi et al., 2021). A human SARS-CoV-2 subunit vaccine phase 3 study concerning efficacy and safety demonstrated 90% general efficacy and 100% protection against moderate and severe diseases (Novovax, 2021). In the case of SARS-CoV-2, the Spike (S) protein plays a key role in the entry of the virus into the cell, since it contains the receptor-binding domain (RBD) which interacts with the cellular membrane, thus allowing virus fusion (Motamedi et al., 2021). The S protein is, therefore, the major antigen to induce protective neutralizing antibodies by blocking the interaction between the viral RBD and the cell, preventing viral infection (Bukreyev et al., 2004). Since the S protein plays a role in both virus entry and viral RNA release into the cytoplasm, a vaccine against the S protein should induce antibodies with which to block viral entry (Motamedi et al., 2021). Although RBD might appear to be a good target for vaccines, a recent study that isolated monoclonal antibodies found that most of them targeted the areas outside the RBD (Seydoux et al., 2020), signifying that the whole S protein may be a better option. However, subunit vaccines are less immunogenic and require adjuvants in order to increase their immunogenicity (Park et al., 2016). Adjuvants are commonly defined as agents that are added to formulations of vaccines so as to enhance the immunogenicity of antigens (Lee and Nguyen, 2015). As adjuvants help proteins become effective vaccines by inducing strong and long-lasting protective immune responses, the selection of this agent remains critical for vaccine development.

In this paper, we show the testing of a new vaccine prototype based on the S viral protein (subunit vaccine) combined with a strong adjuvant previously tested for the development of vaccines against tuberculosis (Roy et al., 2019). According to our results, this subunit vaccine triggers a strong neutralizing antibody production and limits viral replication to the local upper respiratory tract, thus preventing infectious viral shedding. This vaccine prototype may, therefore, be an effective tool with which to control SARS-CoV-2 infection in cats and avoid viral transmission in this species. This may be especially important in the case of immunosuppressed cats living with people at risk, where they may be a potential source of infection, and in those places in which a lot of animals are crowded together, such as animal protection centers.

2. Material and methods

2.1. Experimental design

Four 5–6 month old (two male and two female) specific-pathogen-free (SPF) cats were obtained from Isoquimen (Laboratory Animal Breeder of SPF and conventional beagle dogs and cats). Temperature microchips were implanted in the cervical region of all the animals. These animals spent two weeks adapting and socializing in a large cage located in a Biosafety Level 2 (BSL2) area at the VISAVET Health Surveillance Centre (Madrid, Spain). Animals were then taken to the BSL3 area where they were given water and dry food, ad libitum, and where wet food was added two or three times a week.

In the BSL2 area, two animals (VAC1 and VAC2) were vaccinated intramuscularly with two doses of the vaccine 21 days apart. When fourteen days had passed after the second dose [five weeks after the first dose: 35 days post-vaccination (DPV)], they were taken to the BSL3 area together with two naïve non-vaccinated cats (infection control animals, CINF1 and CINF2). The four cats were then infected with 1 mL of 3.16×10^5 TCID50 of SARS CoV-2 MAD6 via the intranasal route (500 μL per nare). Animals VAC1 and CINF1 were euthanized on 16 DPI (day post-infection) and animals VAC2 and CINF2 were euthanized on 21 DPI (Fig. 1).

Before any of the procedures were carried out, the animals were sedated with dexmedetomidine 0,01 mg/kg (Dexmopet 0.5 mg, Fatro Ibérica S.L, Barcelona, Spain) and butorphanol 0,4 mg/kg (Torphadine 10 mg/mL, Fatro Ibérica S.L, Barcelona, Spain) according to the protocol described by (Nagore et al., 2013). All the animals were euthanized with 3–5 mL of sodium pentobarbital, which was applied intravenously (Dolethal, VETOQUINOL Especialidades Veterinarias, S.A, Madrid, Spain).

2.2. Isolator and suits

In the BSL3 area, the animals were located in pairs inside cages that measured 124.8 × 51.6 × 60 cm in an isolator (BioFlex® B90 Flexible Film Trolley Isolator, Livingston, UK) with HEPA filters that renovate air from time to time depending on the pressure chosen. This has negative pressure, as does the box and the BSL3 area in which the experiment took place. The more negative the pressures, the higher the air renovation rate. The suits used to enter the box and sample the animals were SubiTUS®, designed for handling BSL3 pathogens. During the experiment, the isolator was fixed at −50 Pascals (negative pressure) with 45 air renovations occurring every hour.

2.3. Animal sampling

The four cats were observed every day in order to search for clinical signs, such as fever, bodyweight loss, and depression, along with respiratory and digestive clinical signs. Special feeders with temperature microchip readers [Sure Petcare Feeders, Sure Petcare (SureFlap Ltd., Cambourne, United Kingdom)] were used. These special feeders allowed us to record the animals' body temperature each time they ate. All samples were taken while the animals were sedated (dexmedetomidine 0.01 mg/kg, butorphanol 0.4 mg/kg) (Nagore et al., 2013). After the first dose of the vaccine had been administered, blood samples were taken two or three times a week. After infection, blood, oropharyngeal and rectal swabs, along with surface sponges from the hair of the animals for environmental RNA detection, were collected every day during the experiment. Blood was obtained via venipuncture in the cephalic, jugular, or internal saphenous vein. Whole blood was collected in EDTA (Ethylenediaminetetraacetic acid) tubes, while blood for serum samples was collected in a tube without an anticoagulant and later centrifuged to obtain the serum. Swabs were collected in DeltaSwab® Virus with 3 mL of viral transport media (MTV) (Deltalab S.L., Cataluña, Spain). Dry Sponges 3 M™ (3 M, Minnesota, USA) were used to detect

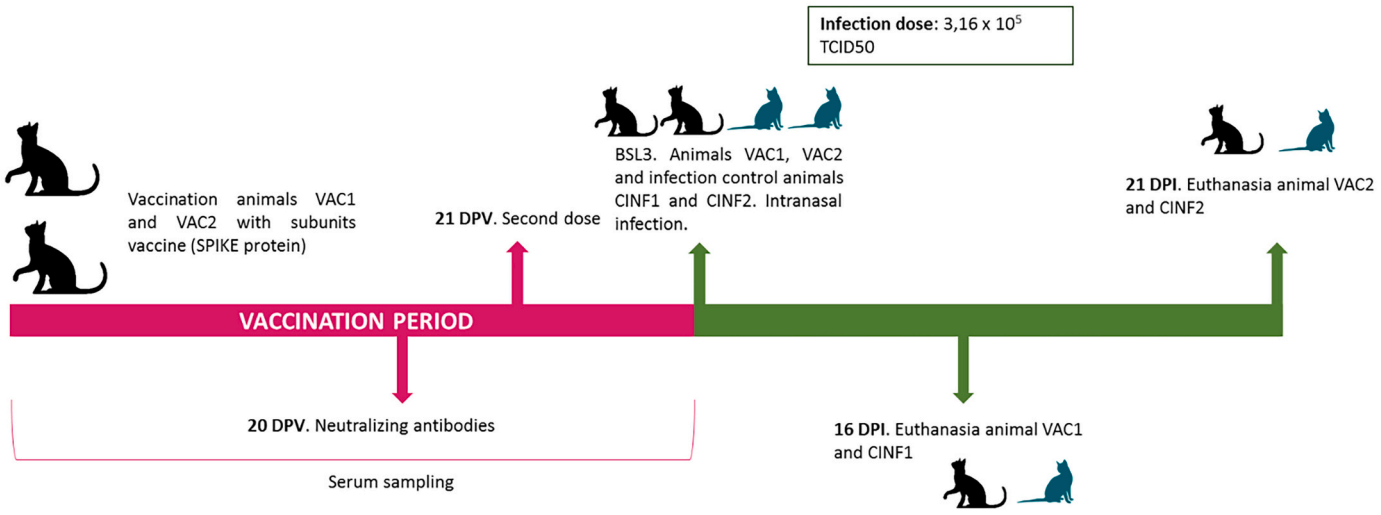

Fig. 1. Outline of the procedure carried out during the experiment on animals VAC1, VAC2, CINF1, and CINF2.

environmental RNA on the animals' skin and hair (Fernández-de-Mera et al., 2020; Barroso-Arévalo et al., 2021).

The four cats were subjected to a systematic necropsy in order to assess the pathological changes in tissue samples. Samples of the brain, nasal turbinates, thymus, tonsils (palatine, pharyngeal and lingual), salivary glands (parotid and mandibular), trachea, the lobes of each lung (right and left cranial, right and left caudals, middle and accessory), heart, spleen, liver, kidney, adrenal gland, gonads, stomach, several sections of the intestine (duodenum, jejunum, ileum, ileocecal valve, colon, and rectum), and lymph nodes (submandibular, parotid, retropharyngeal, tracheobronchial, mediastinal, gastrohepatic, mesenteric and ileocecal) were fixed in 10% buffered formalin and routinely processed for histopathological studies and were introduced into 50 mL tubes with 5 mL of PBS and homogenized to be analyzed by means of PCR. All the samples were immediately processed and analyzed in order to avoid degradation of viral RNA.

2.4. Ethics and animal welfare

Animal care and procedures were performed by following the guidelines of good experimental practices according to the Code of Practice for Housing and Care of Animals Used in Scientific Procedures, approved by the European Economic Community in 1986 (86/609/EEC amended by the directive 2003/65/EC) and Spanish laws (RD 53/2013). The protocol was also approved by the Ethics Committee of Madrid Community (reference PROEX 251.6/20) and by the Complutense University of Madrid's Ethics Committee for Animal Experiments (Project License 14/2020). The approved protocol included a detailed description of the efforts made to provide environmental enrichment and avoid the animals' unnecessary suffering, including humane endpoints and guidelines for euthanasia.

2.5. Vaccine production

The vaccine used in this study was a subunit vaccine based on the Spike protein of the SARS-CoV-2 virus. It was produced by using the full-length SARS-CoV-2 Spike (trimer form), D614 (Reference: PX-COV-P049; Proteogenix), in combination with an adjuvant consisting of 500 µL of Montanide Gel 02 PR (Reference: 36084×, Seppic SA) and 0.5 mg of inactivated porcine isolate bacille Calmette-Guerin (BCG) (Roy et al., 2019) resuspended in 500 µLof PBS (Phosphate-buffered saline) (1 mg/mL). The vaccinated animals were subjected to two doses of the vaccine. The first (dose/animal) contained 25 µg of the Spike protein solution +0.5 mL BCG/PBS + 500 µL of Montanide Gel 02 PR, while the second contained 25 µg of the Spike protein solution +500 µLof PBS + 500 µL of Montanide Gel 02 PR (without the porcine isolate BCG).

2.6. Virus and cells

The SARS-CoV-2 MAD6 isolated from a 69-year-old male patient from Madrid (Spain) was kindly provided by Dr. Luis Enjuanes from the National Biotechnology Centre (CNB) at the Higher Council for Scientific Research (CSIC). This strain belongs to the B.1 (Pango v.3.1.162021–11-04) lineage

Vero E6 cells [provided by the Carlos III Health Institute (Madrid, Spain) or ATCC®, (Manassas, Virginia)] were prepared in order to reproduce the SARS-CoV-2 stocks. Cells were incubated at 37 °C under 5% CO_2 in Gibco Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (Lonza Group Ltd., Basel, Switzerland) and supplemented with 100 IU/mL penicillin, and 100 µg/mL streptomycin and 10% fetal bovine serum (FBS) (Merck KGaA, Darmstadt, Germany). SARS-CoV-2 titers were determined by means of a tissue culture infectious dose (TCID50) assay.

2.7. RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA from oropharyngeal and rectal swabs, surface sponges and tissues was extracted using the column-based High Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. Total RNA was suspended in RNase/DNase-free water and stored at −80 °C.

SARS-CoV-2 RNA was detected using the envelope protein (*E*)-encoding gene (Sarbeco) and two targets (IP2 and IP4) of the RNA-dependent RNA polymerase gene (RdRp) in an RT-qPCR protocol established by the WHO according to the guidelines that can be found at (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance) (Corman et al., 2020). The primer sets used are detailed in Table 1. Real-time RT-PCRs were carried out by employing the SuperScript III Platinum One-Step RT-qPCR Kit (ThermoFisher, Massachusetts, USA) according to the protocol described above, using a CFX Connect™Real-Time PCR Detection System (BioRad, Berkeley, USA). A positive Ct cut-off of 40 cycles was used. A result was considered positive when the sample attained a positive result for at least two of the three targets analyzed and was confirmed by the sequencing of the positive PCR product, according to the OIE guidelines (OIE, 2020).

Table 1
Primer sequences and amplified fragment sizes in base pairs.

Primer target	Sequence 5′ – 3′	PCR fragment size
Gene RdRp/ nCoV_IP2		
nCoV_IP2 – 12669Fw	ATGAGCTTAGTCCTGTTG	
nCoV_IP2 – 12759Rv	CTCCCTTTGTTGTGTTGT	108 bp
nCoV_IP2 – 12696b Probe(+)	AGATGTCTTGTGCTGCCGGTA [5']Hex [3']BHQ – 1	
Gene RdRp/ nCoV_IP4		
nCoV_IP4 – 14059Fw	GGTAACTGGTATGATTTCG	
nCoV_IP4 – 14146Rv	CTGGTCAAGGTTAATATAGG	107 bp
nCoV_IP4–14,084 Probe(+)	TCATACAAACCACGCCAGG [5']Fam [3']BHQ – 1	
Gene E/ E_Sarbeco		
E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	
E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	125 bp
E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG [5']Fam [3']BHQ – 1	

2.8. Virus isolation

A subset of samples (oropharyngeal swabs) obtained from the animals that tested positive when employing the RT-qPCR were subjected to virus isolation in Vero E6 cells in order to evaluate the infectivity of their sheds. These cells were cultured in RPMI supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin (growth medium). The cells were then seeded in 96-well culture plates and cultured at 37 °C with 5% CO2 for 24 to 48 h, after which they were inoculated with 10 µL of the direct sample contained in VTM (oronasal or rectal swabs). Mock-inoculated cells were used as negative controls. The cultured cells were maintained at 37 °C with 5% CO_2, and CPE and cellular death were observed on a daily basis. After 6 days, the cell cultures were frozen, thawed, and subjected to three passages with inoculations of fresh Vero E6 cell cultures with the lysates, as described above. SARS-CoV-2 molecular detection was performed by employing RT-qPCR on the supernatants obtained from every passage in order to confirm the presence/absence of the virus in the cell culture and virus recovery by means of the decrease in the Ct.

2.9. Neutralizing antibody detection

A SARS-CoV-2 surrogate virus neutralization test (GenScript, Leiden, Netherlands) was used as a screening test for neutralizing antibody detection, according to the manufacturer's instructions.

All positive results were evaluated using virus neutralization test (VNT). Briefly, 25 µL of two-fold serially diluted sera were incubated with 25 µL of 100 TCID50/mL of SARS-CoV-2 in 96-well plates at 37 °C with 5% CO_2. At 1-h post-incubation, 200 µL of Vero E6 cell suspension were added to the virus-serum mixtures, and the plates were incubated at 37 °C with 5% CO_2 for 3–4 days. The neutralization titers were determined 3–4 days post-infection. The titer of a sample was recorded as the reciprocal of the highest serum dilution that provided 100% neutralization of the reference virus, as determined by visualizing the cytopathic effect (CPE).

2.10. Evaluation of the cellular immune response by studying Interleukine-6 and Interferon-gamma

Cellular immune response in both vaccinated and control cats was evaluated by employing quantitative ELISA kits for the detection of Interleukine-6 (IL-6) (MyBioSource, California, USA) and Interferon-gamma (IFN-γ) (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. For these analyses, sera from the four cats on days 1, 3, 5, 7, 9, 11, 13, and 16 post-infection were used.

2.11. Histopathological analysis

Tissue samples were fixed in 10% phosphate-buffered formalin for 24–72 h, and then immediately dehydrated in ethanol, immersed in xylol, and embedded in parafin wax by employing an automatic processor. Sections of 4 µm were stained with hematoxylin and eosin and examined microscopically with a Modular Microscopy BX43 (Olympus, Shinjuku, Tokyo, Japan). The evaluation of the lesions was blinded as regards which animal was being analyzed. The lesions scored in the lung were congestion, pleural oedema, alveolar oedema, perivascular oedema, small fibrin thrombi, interstitial and alveolar haemorrhages, perivascular cell infiltrate, interstitial cell infiltrates with alveolar septal thickening, hyperplasia of alveolar macrophages, hyperplasia of type II pneumocytes, bronchial glandular hyperplasia and mucus in bronchi. Each of these findings was described and graded on a semi-quantitative scale based on severity and the percentage of tissue involved as follows: 0, absent (0%); 1, minimal (<10%); 2, mild or focal (11–25%); 3, moderate or multifocal (26–50%); 4, marked or plurifocal (51–75%); 5, severe or diffuse (>75%).

The Fraser Lendrum technique was used on serial sections of formalin-fixed samples to confirm the presence of fibrin, following standard methods. Moreover, the avidin-biotin-peroxidase complex (ABC) method for immunohistochemistry (IHC) was performed for the detection of the Von Willebrand Factor (Factor VIII of coagulation cascade), as previously described by Risalde et al. (2013).

2.12. Statistical analyses

Data analyses were performed using SPSS 20 (IBM, Somar, NY, USA). Differences in Ct values among different periods of sampling were assessed for statistical significance using the non-parametric Mann-Whitney *U* test, since the data obtained for all variables had a skewed distribution. All statistical tests were set at a significance level of 95%; i.e., $p < 0.05$

3. Results

3.1. Clinical signs

None of the vaccinated and control cats showed any clinical signs of disease throughout the study. Their temperatures were registered every day, and underwent a slight variation over time, but on no occasion did the temperature exceed 39 °C.

3.2. Immune response in vaccinated animals (immunization period)

Both animal VAC1 and animal VAC2 were monitored by means of SVNT and VNT in order to detect neutralizing antibody production from 1 DPV to the challenge. Neutralizing antibody production started at 20 DPV in both animals, with low titers (1/64 and 1/32 for VAC1 and VAC2, respectively). Five days after the second vaccination dose (26 DPV), the neutralizing antibody titers increased significantly ($p < 0.01$), reaching titers of 1/256 in animal VAC1 and 1/128 in animal VAC2.

3.3. Viral replication and isolation, and neutralizing antibody production after the infection

No viral replication was detected in animal VAC1 throughout the experiment. There were some positive samples to PCR with low viral RNA loads on the hair and skin sponges. However, in the case of animal VAC2, viral replication was detected in oropharyngeal swabs from 1 DPI until the day of euthanasia (21 DPI). Positive results were also obtained from rectal swabs and hair sponges on 11 DPI and 5 DPI, respectively, for

animal VAC2. Viral replication in the infection control animals (CINF1 and CINF2) was evidenced from 1 DPI to the day of euthanasia in both animals, with higher viral RNA loads based on Ct values in the first days until 8–10 DPI (before antibody production) when compared with the rest days of the experiment (after antibody production) (CINF1: $p < 0.001$, U = 1.500; CINF2: $p = 0.003$, U = 3.500) (Fig. 2).

Attempts to carry out viral isolation were performed on several samples from VAC2, CINF1, and CINF2, since VAC1 did not attain any positive results to the PCR in any of the samples analyzed. Selected samples included oropharyngeal swabs taken on 2 DPI, 5 DPI, 8 DPI, 11 DPI, and 14 DPI. Virus isolation was possible in samples obtained from CINF1 (2 DPI and 5DPI) and CINF2 (2 DPI, 5 DPI, and 8 DPI), while no infectious virus was isolated from the samples obtained from VAC2.

Antibody production in the vaccinated animals remained stable throughout the experiment, with high titers (1/256) and slightly higher levels of VNT in animal VAC1 than in VAC2 (less than 1 fold). On the contrary, the control infection animals started to produce antibodies on 7 DPI, and had growing titers, which peaked on 14 DPI and then became stable (Fig. 3).

3.4. Interleukine-6 and Interferon-gamma evaluation

IL-6 concentrations from all the cats are represented in Fig. 4. Non-significant differences were observed in the concentrations of this cytokine between vaccinated and infection control cats ($p = 0.150$, U = 90). As reflected in the graph, levels in serum varied similarly in the four animals.

IFN-γ concentrations from all the cats are shown in Fig. 5. Again, no significant differences were observed between vaccinated and control infection animals ($p = 0.075$, U = 80)., although a tendency was observed: concentrations of IFN-γ in serum were slightly higher in the first 7 days post-infection compared to the last ones (8 to 16 DPI) ($p = 0.001$, U = 32). It is noteworthy that CINF1 animal showed a peak in the third day after infection, achieving the highest value on 3 DPI.

3.5. Viral replication in animal tissues

No viral replication was detected in any of the after-necropsy tissues of animal VAC1 on 16 DPI, while viral RNA was detected in some of animal VAC2's tissues (soft palate, pharyngeal tonsil, the base of the tongue, parotid salivary gland and jejunum) on 21 DPI (Fig. 4).

Viral replication in the control infected animals' tissues was higher than in those vaccinated. Animal CINF1, which was euthanized on 16 DPI, had the highest viral RNA loads on the base of the tongue, and in the parotid salivary gland and mandibular salivary gland, with relatively high values also in the soft palate, right and left cranial lobes, ileum and ileocecal valve (Fig. 5). With regard to animal CINF2, which was euthanized five days after CINF1 (21 DPI), viral replication was detected with higher levels in the tracheobronchial lymph node, the base of the tongue, pharyngeal tonsil, soft palate, and nasal turbinates (Fig. 6).

3.6. Anatomopathological study

Gross lesions were concentrated in the lungs, and the four animals had intense congestion and alveolar oedema (Fig. 7A-D). These vascular changes gave rise to an increased size of the lungs in the vaccinated animals (Fig. 7A, B). Moreover, splenic congestion and splenomegaly were detected in animals VAC2 and CINF1, probably because of the barbiturate overdose. However, these findings were more severe in the non-vaccinated animal.

Microscopic examination confirmed that the organs most affected after SARS-CoV-2 infection were the lungs. The predominant histological pattern found in all the infected animals, therefore, corresponds to the exudative and early proliferative phases of diffuse alveolar damage, especially in the center of the pulmonary lobe. Findings associated with this exudative phase of diffuse alveolar damage included alveolar and interstitial oedema, proteinaceous exudates in alveoli, congestion, dilated alveolar ducts and collapsed alveoli, perivascular oedema and light interstitial haemorrhages (Fig. 7E-H). Overall, these alterations were more constant and severe in animals CINF1 and CINF2, depending on the lesion (Fig. 8). Platelet–fibrin thrombi positive to Factor VIII in small and medium vascular vessels were found in VAC2, CINF1 and CINF2 (Fig. 7F). The features of the pulmonary lesions found were also focally associated with patterns of early proliferative phases in all the animals, and an interstitial pneumonia with the presence of inflammatory lymphomonocytic infiltrates together with deposits of fibrin were observed along the slightly thickened interalveolar septa (Fig. 7G, H). Some areas had abundant alveolar macrophages along with type II

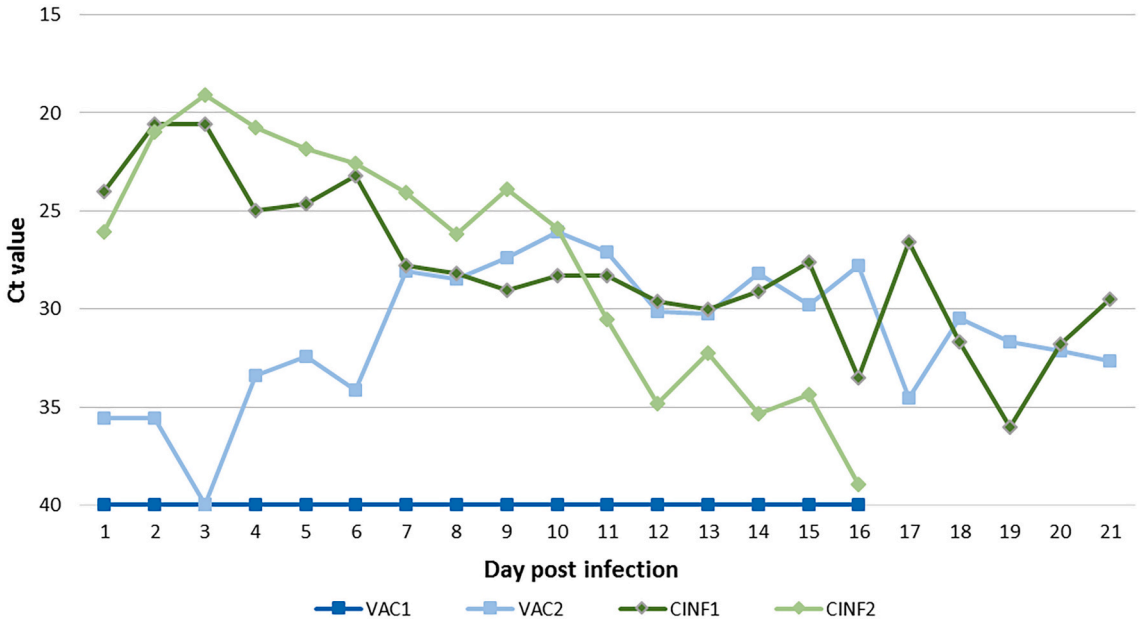

Fig. 2. Average value of Ct in oropharyngeal swabs of animals VAC1 (euthanized on 16 DPI), VAC2 (euthanized on 21 DPI), CINF1 (euthanized on 21 DPI), and CINF2 (euthanized on 16 DPI), from 1 DPI to 21 DPI. Ct of 40 corresponds to a negative result of RT-qPCR.

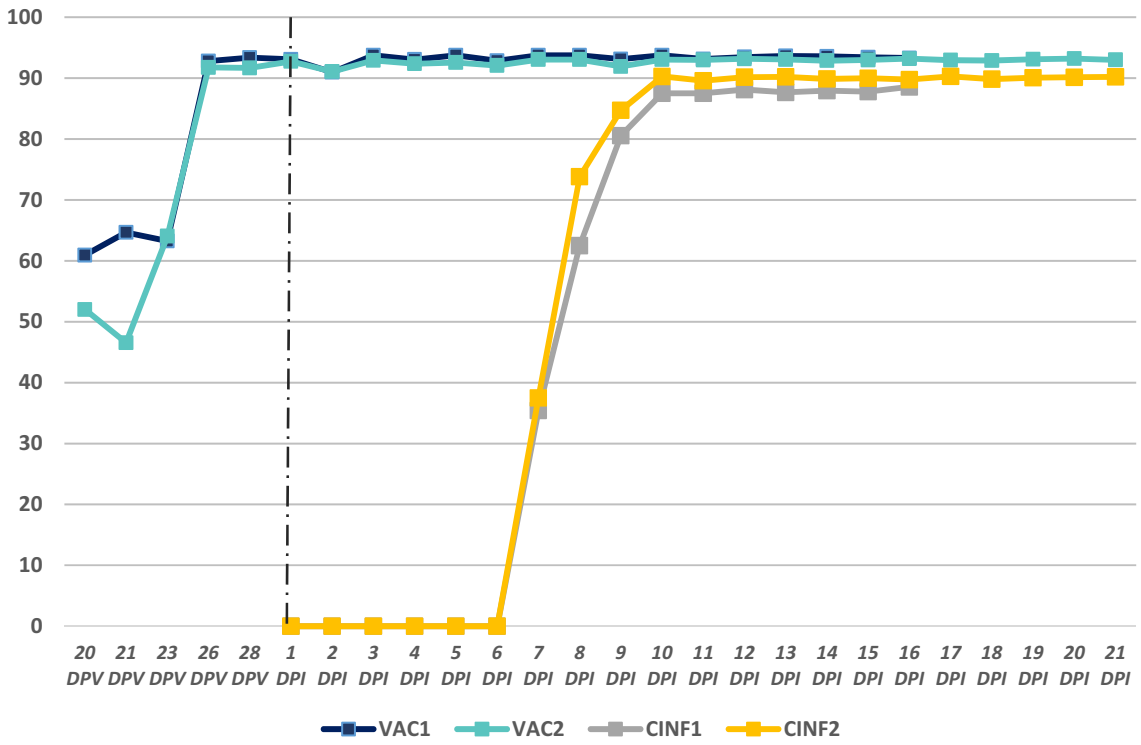

Fig. 3. Percentage of inhibition in VAC1 and VAC2 from 20 DPV (day post-vaccination; one day before the second dose of the vaccine), and CINF1 and CINF2 from 1 DPI (day post-infection). The discontinued line indicates the day of infection.

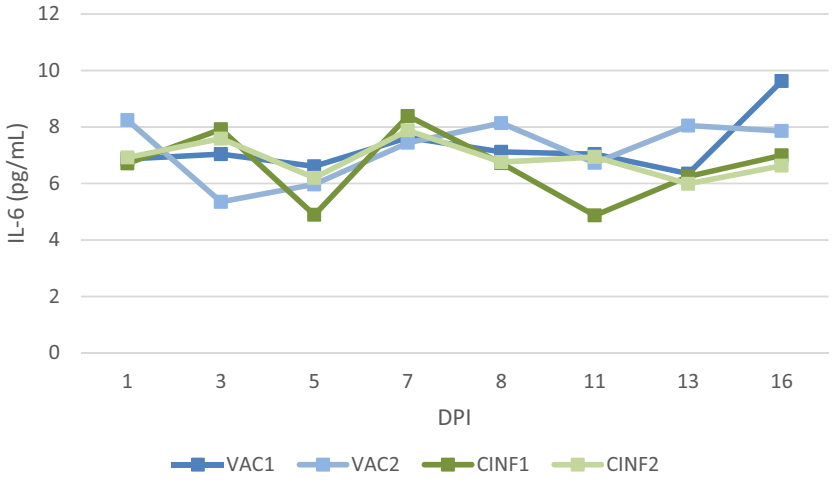

Fig. 4. Interleukin 6 (IL-6) levels in serum in vaccinated cats (VAC1 and VAC2) and control infection cats (CINF1 and CINF2) based on the sampling time (DPI: day post-infection).

pneumocytes hyperplasia (Fig. 7E), especially in animal CINF2 (Fig. 8). A histological examination of the main bronchi and bronchiolar branches revealed mild glandular hyperplasia, with lumina often containing residual dense mucoid material in animal VAC2 (Fig. 8). (See Figs. 9 and 10).

Histopathological lesions were not present in the other organs analyzed in any of the infected animals, with the exception of the microvacuolar and macrovacuolar cortical lipid degeneration observed in the adrenal glands of VAC2 and CINF2, respectively.

4. Discussion

It has been demonstrated that cats are susceptible to SARS-CoV-2 infection under natural conditions (Hamer et al., 2020; Zhang et al., 2020; Zhao et al., 2021), and this has also been shown by means of experimental infections (van den Brand et al., 2008; Bosco-Lauth et al., 2020; Shi et al., 2020; Barroso-Arévalo et al., 2022). Indeed, several cases of human-to-cat transmission have been reported worldwide throughout the pandemic (OIE, 2021a). Since cats are present in many homes, and the rate of human infection worldwide is high, the risk of human-to-cat transmission is relatively high. Moreover, cats have been proposed as being good models for mild SARS-CoV-2 infection because of the gross and histological lesions that they have in different organs as a consequence of viral replication (Chiba et al., 2021). Although several studies have proven that most cats do not have any clinical signs during infection (Chiba et al., 2021; Barroso-Arévalo et al., 2022), with the

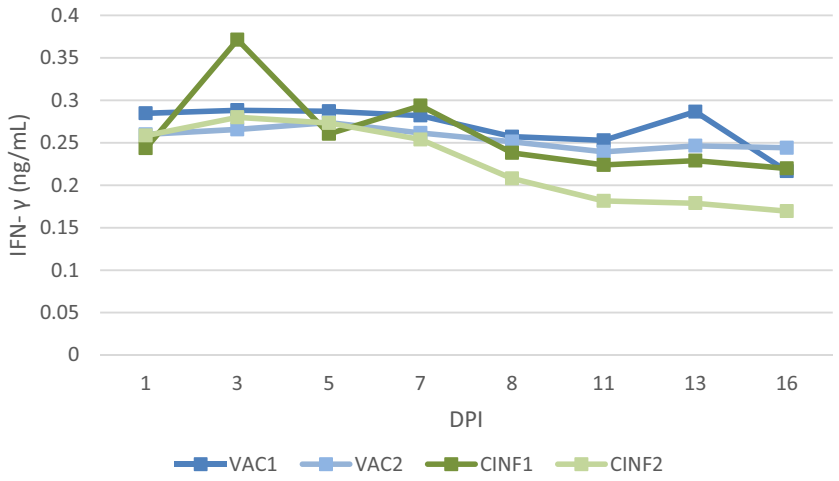

Fig. 5. Interferon-gamma (IFN- γ) levels in serum in vaccinated cats (VAC1 and VAC2) and control infection cats (CINF1 and CINF2) based on the sampling time (DPI: day post-infection).

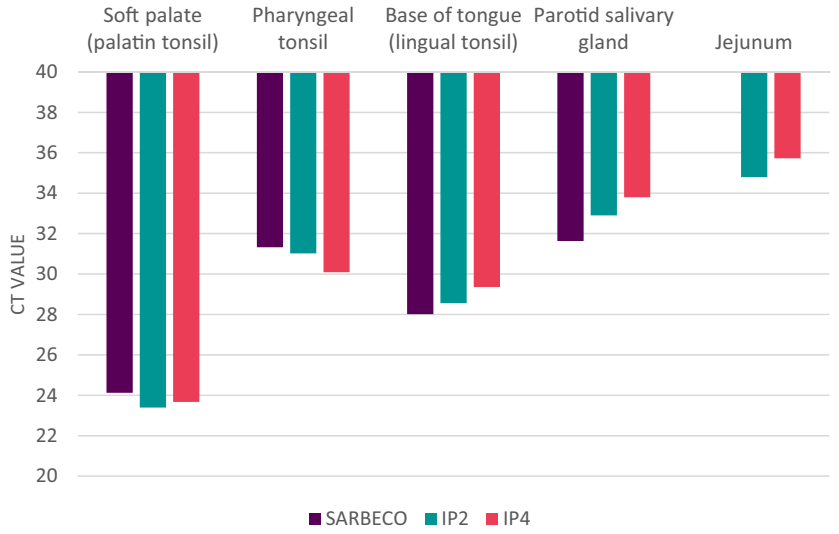

Fig. 6. Viral loads based on Ct values in tissues from animal VAC2 euthanized on 21 DPI. Only tissues positive to two or three targets (Sarbeco, IP2, and IP4) are represented.

exception of very young animals, (Shi et al., 2020), their role in the current pandemic has not yet been clarified, since they may potentially act as reservoirs for the virus. Furthermore, infected cats may act as a source of infection for susceptible people, since virus isolation from cat samples has been demonstrated previously (Hamer et al., 2020; Barroso-Arévalo et al., 2021). This scenario is particularly important in the case of immunosuppressed or older people, whose pets may be a potential zoonotic source of SARS-CoV-2. Control measures may also be necessary in the case of immunosuppressed cats, in which viral replication may be higher and infectious virus may potentially be shed. The application of control measures by which to prevent SARS-CoV-2 infection in cats is, therefore, still necessary. In this respect, vaccination may be an excellent tool with which to reduce both human-to-cat and cat-to-cat transmission, especially in the case of cats living with COVID-19-positive owners. It would also be interesting for outdoor or stray cats, since the risk of infection may be higher in the street since away a high number of animals live together in comparison with house cats..

Here, as a part of a preliminary study, we test a new vaccine candidate based on the Spike protein of the SARS-CoV-2 virus combined with the BCG adjuvant in cats, demonstrating that this subunit vaccine produces a strong neutralizing antibody response in this species. This vaccine prototype also avoided viral replication in one of the vaccinated cats and limited viral replication to the local upper respiratory tract, preventing infectious virus shedding, in the other vaccinated cat. However, the vaccine did not prevent histological lesions in the lung, which were mainly of vascular origin. These outcomes are in line with previous research in which the authors reported that the Spike protein is, on its own, capable of impairing endothelial function via the down-regulation of ACE2 (Lei et al., 2021). This study, therefore, also paves the way for cats to be used as a model in vaccination trials for vaccine development, providing new information about the significance and the histopathological findings generated by the different vaccine prototypes for SARS-CoV-2. However, the conclusions derived from this study should be treated with caution, since a low number of animals were used. This low *n* was selected taking into account the three Rs principle, which emphasizes the use of the lower number of animals possible. This work, therefore, try to lay the foundation for future studies and contribute to the knowledge in vaccines for preventing SARS-CoV-2 active infection in animals.

The vaccine triggered a powerful humoral immune response based on neutralizing antibodies in both vaccinated animals. Although antibody production started 20 days after the first dose of vaccine was

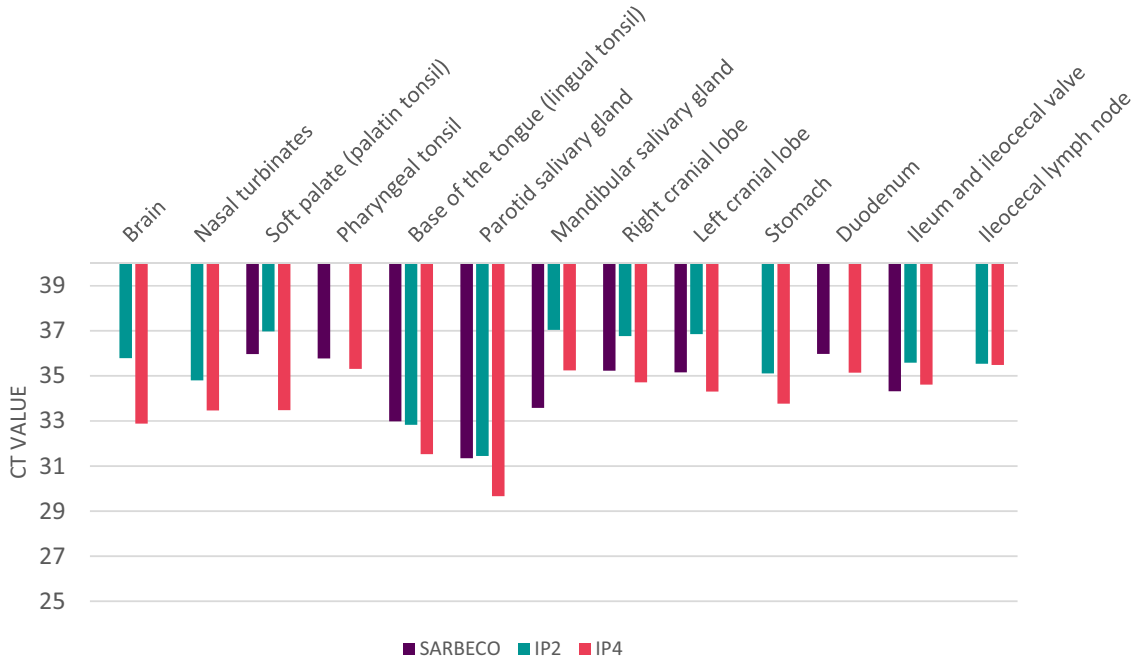

Fig. 7. Viral loads based on Ct values in tissues from animal CINF1 euthanized on 16 DPI. Only tissues positive to two or three targets (Sarbeco, IP2, and IP4) are represented.

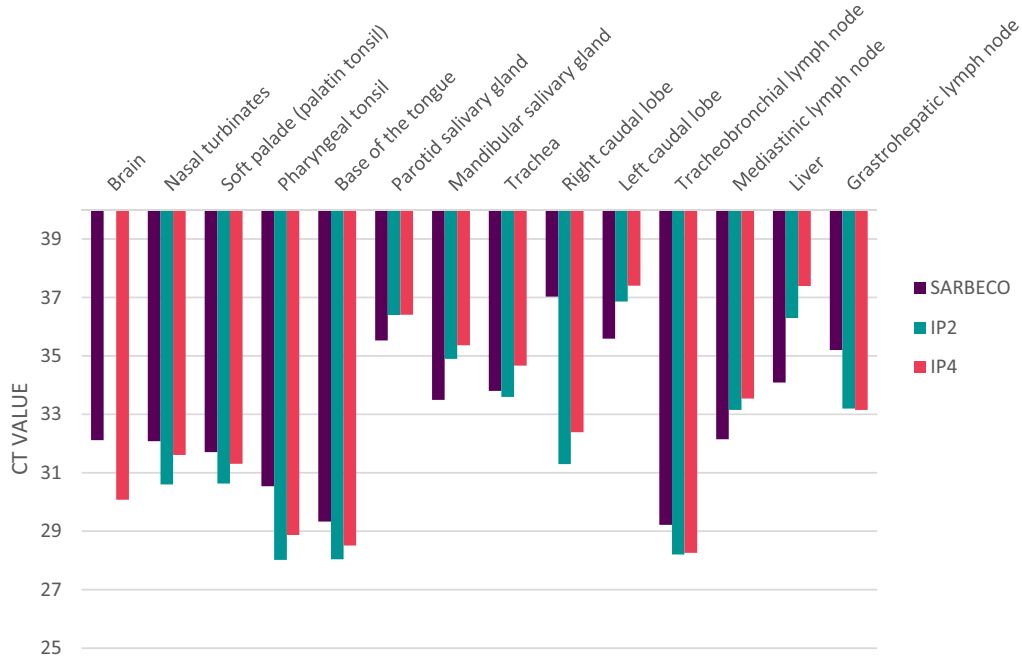

Fig. 8. Viral loads based on Ct values in tissues from animal CINF2 euthanized on 21 DPI. Only tissues positive to two or three targets (Sarbeco, IP2, and IP4) are represented.

administered, an increase in their production was observed five days after the second dose (26 DPV). These results show that the vaccine is capable of generating a strong antibody response in cats but requires around three weeks to develop it. These outcomes are in line with those observed in vaccines used for the human population (Polack et al., 2020; Baden et al., 2021). This vaccine prototype also included the BCG as an adjuvant, which is a strong immunostimulatory of innate immunity (Tanner et al., 2019). Although the BCG has usually been used for tuberculosis, several studies have described the cross-protective effects of the BCG vaccine in non-tuberculosis-related diseases (JAMA, 1932; Ohrui et al., 2005; Stensballe et al., 2005). In terms of physiology, this cross-protective effect of the BCG may be owing to "trained immunity", a concept that refers to the long-term functional reprogramming of innate immune cells. According to this definition, trained immunity is activated by exogenous or endogenous attacks and leads to an altered response toward a second challenge after the return to a non-activated state (Netea et al., 2020). In this respect, multiple studies have associated the trained immunity triggered by the BCG with better resistance to

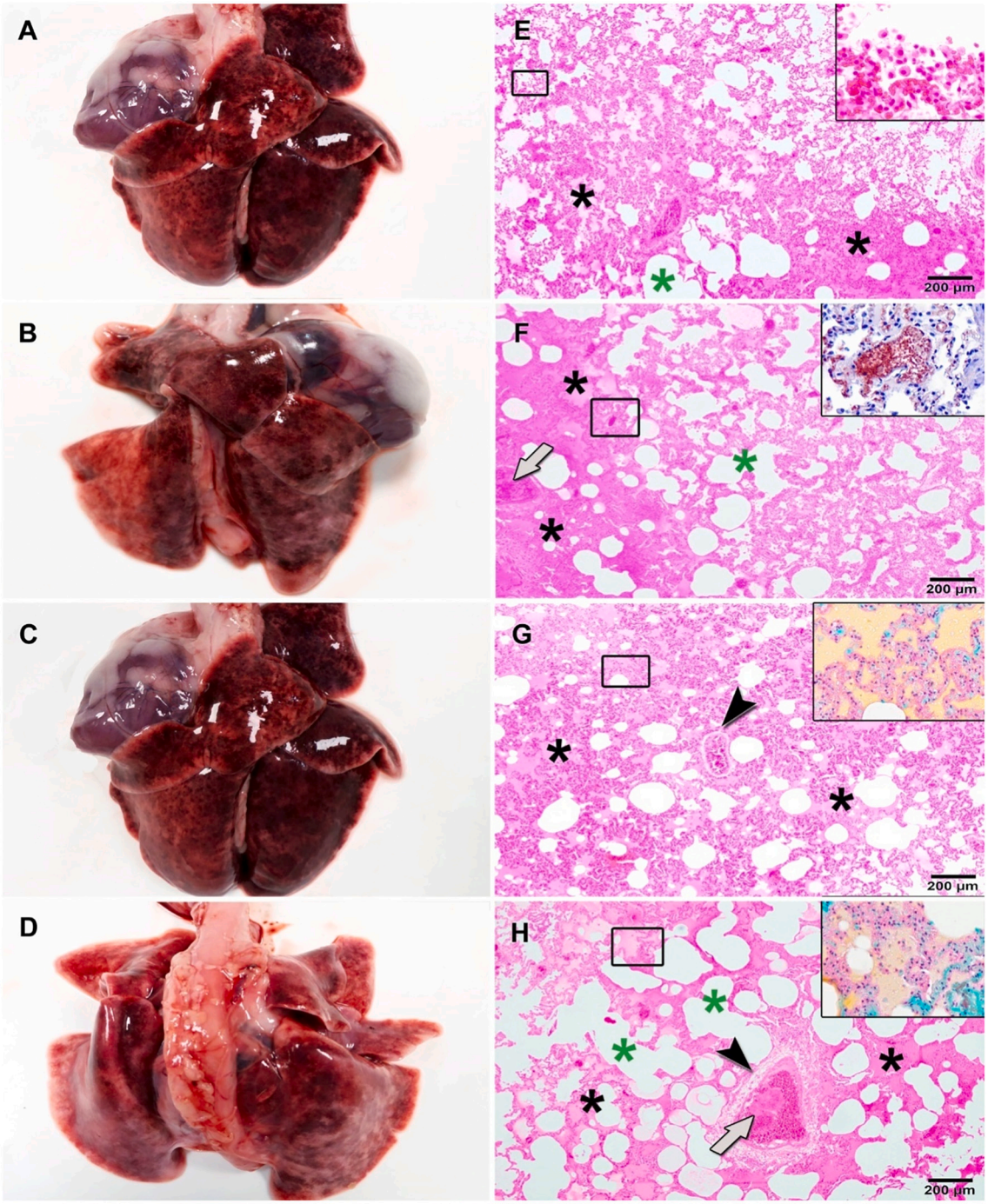

Fig. 9. Pulmonary lesions found in vaccinated and non-vaccinated cats after SARS-CoV-2 infection. The lungs of VAC2 and CINF2 had intense alveolar congestion and edema (black asterisk) (A and C, respectively), while, the presence of these lesions was moderate in VAC1 and CINF1 (B and D, respectively). E) Lung of VAC1, with mild alveolar edema (black asterisks), small areas of atelectasis (green asterisk), and focal alveolar macrophages hyperplasia (inset). F) Lung of VAC2, with moderate alveolar edema with some collapsed alveoli (black asterisks) and small atelectasis areas (green asterisk), along with the presence of microthrombi in small and medium vascular vessels (grey arrow), associated with a great number of platelets positive to the anti-Factor-VIII antibody when tested using immunohistochemistry (inset). G) Lung of CINF1, with severe alveolar edema and proteinaceous exudates (black asterisks), perivascular edema (black arrowhead) and a slight thickening of the alveolar septa owing to mononuclear cell infiltrations and deposits of fibrin positive to Fraser Lendrum technique (inset, pink). H) Lung of VAC2, with moderate alveolar edema (black asterisks) and small atelectasis areas (green asterisks), perivascular edema (black arrowhead) and thrombi (grey arrow) in medium vascular vessels, along with thickening of the alveolar septa owing to mononuclear cell infiltrations and deposits of fibrin positive to Fraser Lendrum technique (inset, pink). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

COVID-19 disease (Gursel and Gursel, 2020; Ozdemir et al., 2020). The potential of BCG in combination with vaccination against COVID-19 should, therefore, be explored in much greater depth (Gonzalez-Perez et al., 2021).

Both the vaccinated and the control animals were challenged with1 mL of 3.16×10^5 TCID50 of SARS CoV-2 via the intranasal route (500 µL per nare). While both CINF1 and CINF2 had similar patterns of viral replication with a systemic distribution, the vaccinated animals had different responses to the infection. Despite having gross and histological lesions in the lungs, VAC1 tested negative for viral RNA in all the

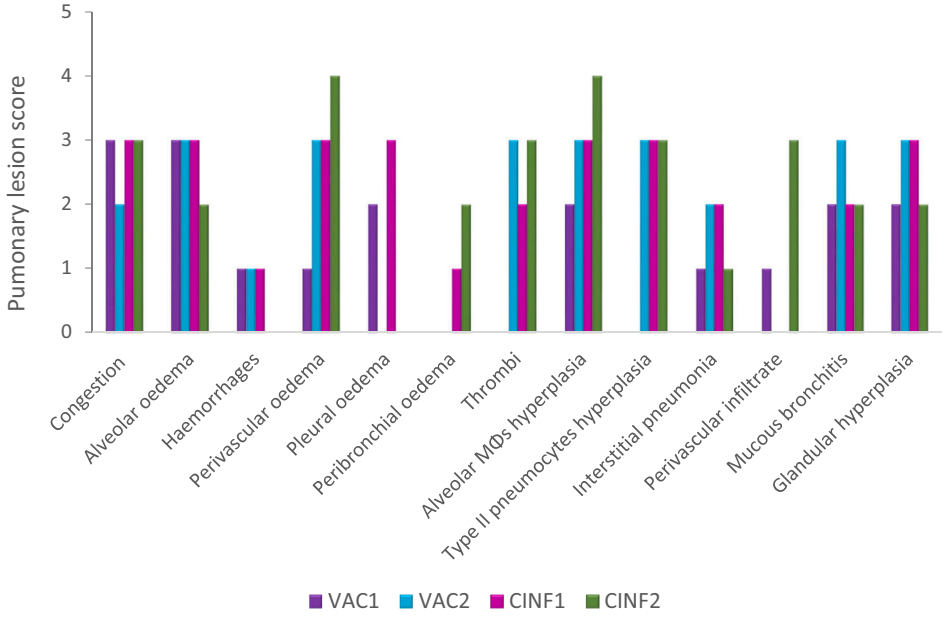

Fig. 10. Pulmonary histopathological findings and their corresponding semi-quantitative grades of evaluation in VAC1, VAC2, CINF1, and CINF2 cats infected with SARS-CoV-2.

samples taken throughout the experiment, even the day after nasal inoculation, which suggests that viral clearance was truly effective and strong in that animal. The same occurred in an experimental study in which cats were infected and reinfected with SARS-CoV-2 in order to confirm the protection provided by the first infection. No infectious virus was detected in nasal or rectal swabs, but the animals were found to have pulmonary lesions after the necropsy (Chiba et al., 2021). However, the other vaccinated animal (VAC2) attained positive results to oropharyngeal swabs tested using PCR from the moment of infection until the end of the experiment, despite high titers of neutralizing antibodies being maintained during the whole experimental period. Although viral loads based on Ct PCR results were low for this animal, the protection triggered by the vaccine was not sufficiently strong to avoid viral replication. This case may be similar to those infections that occur in vaccinated humans who test positive for PCR despite being immunized (until July 2021, 5.5% of people vaccinated in accordance with the complete guidelines in Spain became infected) (ConSalud.es, 2021). However, according to the viral isolation results, the vaccine prevented infectious virus shedding from this animal, which is a key requirement for determining vaccine efficacy (Hodgson et al., 2021). The differences between the two vaccinated cats, which were of the same age and from the same background, could be explained by individual physiological factors. Furthermore, the infection control animals had higher levels of viral RNA in the oropharyngeal swabs from 1 DPI to 8–10 DPI. According to the virus isolation results, an infectious virus was shed by the control animals during the first 8 days post-infection, showing a decrease in viral RNA loads from that day on, probably owing to antibody production. However, while the viral isolation results showed that the control animals shed infectious virus, no infectious virus was isolated from any of the samples obtained from the vaccinated animals, demonstrating that the vaccine prevented infectious viral shedding in both animals. These results are, therefore, promising, since the vaccinated animals did not shed any infectious virus, thus preventing them from acting as a source of infection and limiting possible spillover. The same occurred in the experiment carried out by (Langereis et al., 2021) on cats in which an alphavirus replicon-based vaccine expressing a stabilized Spike antigen was proven to prevent transmission from infected to naïve cats and to induce protective immunity, yet none of the vaccinated animals shed detectable virus orally or nasally.

We also studied the presence and load of viral RNA in tissues. No viral RNA was detected in any tissues from VAC1, despite the fact that lung lesions of a vascular origin were found in this animal. Furthermore, low loads of viral RNA were detected in five tissues from VAC2, all of which were in the upper respiratory tract, with the exception of the jejunum. On the contrary, a greater number of tissues from the infection control animals tested positive for the PCR, demonstrating that the vaccine is effective as regards limiting viral replication in animal tissues. In these control animals, which were euthanized at 16 and 21 DPI, respectively, viral RNA was detected in the lungs, which contrasts with previous experiments in which virus clearance was observed by 10–11 DPI (Chiba et al., 2021). Moreover, the adrenal glands of animals VAC2 and CINF2 had cortical lipid degeneration, characterized by a vacuolization within the cortical cells. This finding can be associated with an individual spontaneous appearance, age-related changes, or secondary stress caused by toxins or infections (National Toxicology Program, 2020). In this respect, the first two options are quite unlikely, as they appeared in several young animals. This pathological finding has also been described in people with severe COVID-19, and is associated with an alteration to the corticosteroids synthesis (Freire Santana et al., 2020). These hormones play a potential role in inhibiting pro-inflammatory cytokine production and controlling the host inflammatory response (Hui et al., 2015), signifying that adrenal damage may compromise the regulation of inflammation and vascular procoagulant alterations, a mechanism that could be present in those animals that have a higher presence of microthrombi. In general, gross and histological lesions were greater in the control infection cats, and the lesions that were observed in both the vaccinated animals were mainly of a vascular origin, which suggests that it may be easy to reverse them. However, more studies should be conducted in order to explore the causes of the apparition of vascular histological lesions in vaccinated animals, since the same may be occurring in the case of the human population. In this respect, and as stated above, cats would be an interesting option for vaccination trial models.

Here, we also investigated the cellular immune response by evaluating two cytokines that have been reported to be involved in the immune system response against SARS-CoV-2 infection in humans: IL-6 and IFN-γ. IL-6 seems to play a key role in COVID-19 associated respiratory failure (Gubernatorova et al., 2020) since this cytokine

concentration appears altered in severe cases of the disease. Our results, however, did not evidence any relevant difference between vaccinated and control infection cats. Therefore, IL-6 does not appear to be involved in the effective immune response triggered by the vaccine tested in this study in this species. It is important to take into account that none of the animals presented a symptomatic disease, and, therefore, the levels of cytokines were probably maintained within the normal ranges over time. The other evaluated cytokine was the IFN-γ. It is a proinflammatory cytokine which increases in patients with moderate disease and peaks in patients with severe disease (Karki et al., 2021). This could be related to the increase of this cytokine the first days after infection, since these higher levels coincided with the days in which the infection control animals had the higher viral loads in the oropharyngeal swabs. In the case of vaccinated cats, the increase in serum levels of this cytokine during the first days after infection may be related to an efficient immune response, allowing the immune system to avoid viral replication. In addition, IFN-γ production by CD4 + T cells has been reported to be triggered by several vaccines against SARS-CoV-2 (Folegatti et al., 2020; Muik et al., 2020), which suggest that this immune-modulatory cytokines play a key role in the coordination of the immune response to counter a viral intrusion (Vabret et al., 2020). Further studies should evaluate the role of the cellular immune response in vaccination trials in animals, to better understand the mechanisms underlying effective protection against the infection.

In conclusion, this study has demonstrated the efficacy of a new vaccine candidate for the cat population and has shown the value of this species as a model for SARS-CoV-2 vaccination trials. This point could be crucial regarding studying the protective effect of vaccines developed for new variants of the virus since cats could constitute a more realistic approach than in vitro experiments. Moreover, one of the main advantages of this vaccine prototype is its easy production, which may facilitate the vaccination of cat populations at high risk of SARS-CoV-2 infection.

Author agreement

All authors have seen and approved the final version of the manuscript.

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Authors' contributions

Conceptualization: SB-A, LS-M, LD, MD, JMS-V; Data treatment: SB-A, LS-M, JMS-V; Formal analysis: SB-A, LS-M, JMS-V; Funding Acquisition: JMS-V, MAR, LD, MD; Investigation: SB-A, LS-M, TG-S, MAR, IG-B; Methodology: SB-A, LS-M, MD, IG-B, MAR, JMS-V; Project Administration: JMS-V; Resources: LD, JMS-V; Software: SB-A, LS-M, TG-S; Supervision: LD, JMS-V, MAR; Validation: SB-A, LS-M, LD, JMS-V; Visualization: SB-A, LS-M; Writing – Original Draft Preparation: SB-A, LS-M, MAR, JMS-V; Writing – Review & Editing: SB-A, LS-M, LD, IG-B, TG-S, MAR, JMS-V.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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