

A Spike protein-based subunit SARS-CoV-2 vaccine for pets: safety, immunogenicity, and protective efficacy in juvenile cats

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Abstract

Whereas multiple vaccine types have been developed to curb the spread of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) among humans, there are very few vaccines being developed for animals including pets. To combat the threat of human-to-animal, animal-to-animal and animal-to-human transmission and the generation of new virus variants, we developed a subunit SARS-CoV-2 vaccine which is based on recombinant spike protein extracellular domain expressed in insect cells then formulated with appropriate adjuvants.

Sixteen 8-12-week-old outbred female and male kittens (n=4/group) were randomly assigned into four treatment groups: Group 1, Antigen alone; Group 2, Sepivac SWE™ adjuvant; Group 3, aluminum hydroxide adjuvant; Group 4, PBS administered control animals. All animals were vaccinated twice at day 0 and 14, intramuscularly in a volume of 0.5 mL (Groups 1-3: 5 µg of Spike protein). On days 0 and 28 serum samples were collected to evaluate anti-spike IgG, inhibition of spike binding to angiotensin-converting enzyme 2 (ACE-2), neutralizing antibodies to Wuhan-01 SARS-CoV-2 D614G (wild-type) and Delta variant viruses, and whole blood for hematology studies. At day 28, all groups were challenged with SARS-CoV-2 wild-type virus 10⁶ TCID₅₀ intranasally. On day 31, tissue samples (lung, heart, and nasal turbinates) were collected for histology, viral RNA detection and virus titration.

Parameters evaluated in this study included safety, immunogenicity, and protection from infection with wild-type SARS-CoV-2 virus. After two immunizations, both vaccines induced high titers of serum anti-spike IgG, ACE-2 binding inhibitory and neutralizing antibodies against both wild-type and Delta variant virus in the juvenile cats. Both subunit vaccines provided protection of juvenile cats against virus shedding from the upper respiratory tract, and against viral replication in the lower respiratory tract and hearts. These promising data warrant ongoing evaluation of the vaccine's ability to protect cats against SARS-CoV-2 Delta variant and in particular to prevent transmission of the infection to naïve cats, before proceeding with large-scale field trials.

Introduction

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) is the pathogenic agent that causes the disease COVID-19. Although genetically closely related viruses have been isolated from *Rhinolophus* bats (horseshoe bat), the exact source of SARS-CoV-2 and route of its introduction into the human population has not been established [1]. The biological properties of coronaviruses (CoVs), including ability to cross interspecies barriers, enable its emergence in populations of various animals, including companion animals as cats (*Felis catus*) an area requiring further study [2].

There are estimated 76 million pet dogs and 96 million pet cats living in approximately 70% of the U.S. households [3]. According to Mars Petcare, Russia's pet population reaches 40.8 million cats and 22.6 million dogs, living in 59% of Russian households [4]. A relevant concern in the control of the ongoing COVID-19 pandemic is the risk domestic animals could play in the maintenance and transmission of

SARS-CoV-2. Assessing this risk implies quantifying transmission from humans-to-animals, from animals-to-animals and from animals-to-humans [5]. Large epidemics in farmed minks have confirmed this risk for that specific species [6].

The role of cats is of particular interest, because they are in close contact with humans and frequently in contact with other cats. Case reports on cats living in the same household with COVID-19 patients, suggested that the transmission may have occurred from humans to pets [7–9]. As detailed in the literature field [10–13] and experimental data [14–18] cats are susceptible to infection, can show mild clinical signs and may transmit infection to other cats. The large cats (snow leopards, tigers, and lions) are also susceptible to infection with deaths being reported in zoos. SARS-CoV-2 RNA was detected by RT-PCR in cat lung, heart, kidney, liver, spleen, and intestine with particular high levels of infectious virus being recovered from the lung and heart [19]. The estimated reproduction number R_0 in cats was calculated to be higher than 1, suggesting cats could play a role in the transmission and maintenance of SARS-CoV-2. In the same series of studies [5], it is noted that levels of virus shedding in infected household cats were as high as those observed experimentally, with reported shedding levels as high as $10^{8.5}$ RNA copies/swab sample or RT-PCR Ct values as low as 21. Considering infected cats shed high levels of virus and droplet transmission is possible, the risk for cat-to-human transmission may be significant [5]. Infected cats shed virus for prolonged periods suggesting cats may play a role in viral epidemiology by transmitting the virus onwards, generating new variants or acting as a virus reservoir [20].

While the global focus is on the human vaccine roll-out, eradication of SARS-CoV-2 infections in animals could be a vital part of pandemic recovery. According to the World Health Organization, vaccines may be useful to protect susceptible animal species and prevent generation and transmission of viral mutations [21]. To combat the threat of human-to-animal, animal-to-animal and animal-to-human transmission and the generation of new variants, we developed a subunit SARS-CoV-2 vaccine called NARUVAX-C19 (pets) based on recombinant spike protein extracellular domain expressed in insect cells that was then formulated with adjuvant.

In this study, we evaluated the safety, immunogenicity, and protective efficacy in juvenile cats of a recombinant spike protein vaccine formulated with either alum or Sepivac SWE™ adjuvant. This was based on our experience developing a human vaccine against COVID-19 (called NARUVAX-C19) [22], based on recombinant spike protein formulated with a nanoemulsion oil (SWE) adjuvant that in hamster studies provided complete protection and blocked virus transmission to naïve contact animals. This was the first study to test the safety, immunogenicity, and protective efficacy in juvenile cats of our recombinant spike protein vaccine against COVID-19.

Materials And Methods

Facility, Bioethics, and Biosafety statement

All experiments with infectious SARS-CoV-2 were performed in the biosafety level 3 (BSL-3) and animal biosafety level 3 (ABSL-3) facilities in the Central Reference Laboratory (CRL) of the M. Aikimbayev National Scientific Center for Especially Dangerous Infections (NSCEDI) of the Ministry of Health of the Republic of Kazakhstan (MoH RK), which was completed in 2017 and accredited according to ISO 35001:2019 Biorisk management for laboratories and other related organizations, by the DEKRA (a German company and member of the international accreditation associations IAF and DAkkS) in 2020. The BSL-3 and ABSL-3 facilities used were designed by the standards outlined in Biosafety in Microbiological and Biomedical Laboratories (5th edition). Features of the BSL-3 and ABSL-3 facilities include controlled access, entry/exit through a shower change room, effluent decontamination, negative air-pressure, double-door autoclaves, gas decontamination ports, HEPA-filtered supply and double-HEPA-filtered exhaust air, double-gasketed watertight and airtight seals, and airtight dampers on all ductworks. The structure of the BSL-3 and ABSL-3 facilities were pressure-decay tested regularly.

All activities inside the BSL-3 and ABSL-3 labs are monitored by trained guards via video cameras. Only authorized personnel that have received appropriate training can access the facility. Experienced personnel work in pairs in the facilities. Our staff wear powered air purifying respirators that filter the air, when they culture the virus and handle animals (juvenile cats); the researchers are disinfected before they leave the room and then shower on exiting the facility. The facility is secured by appropriate procedures approved by the NSCEDI institutional biosafety officers. All facilities, procedures, training records, safety drills, and inventory records are subject to periodic inspections and ongoing oversight by the institutional biosafety officers who consult frequently with the facility managers. The research program, procedures, occupational health plan, security, and facilities are reviewed annually by a MoH RK official.

The animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (8th edition). The protocol on Bioethics was approved by the Institutional Animal Care and Use Committee of the NSCEDI of MOH RK (Approval number 105/2021-04-19).

Cells

Vero E6 cells were obtained from American Type Culture Collection (Vero 76, clone E6, CRL-1586, ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco) and antibiotic/antimycotic (anti/anti; Gibco) solution and incubated at 37°C with 5% CO₂.

Virus

SARS-CoV-2 strain hCoV-19/Kazakhstan/KazNAU-NSCEDI-4635/2020 (GISAID accession no. EPI_ISL_1208952; belongs to the Wuhan variant with D614G and M153T mutations in the Spike protein) and was isolated from nasopharyngeal swab in a 31-year-old man with COVID-19 infection in Almaty, Kazakhstan. We also used the Delta variant (B.1.617.2) of SARS-CoV-2 isolated on August 17, 2021, from nasopharyngeal swab of a COVID-19 patient in Zhanaozen, Kazakhstan. Both strains were isolated at BSL-3 laboratory of the NSCEDI. The strains are passaged twice in Vero E6 cells and stocks frozen at -80

°C in DMEM containing 4.5 g/L D-glucose, 1 mM Sodium Pyruvate, 2% FBS, 1% non-essential amino acids (Gibco), and 25mM HEPES (Gibco). Virus stock was titrated on Vero E6 cells using Reed and Muench method [23] based on eight replicates for dilution and plaques were counted 72 h post infection to determine 50% tissue culture infectious dose (TCID₅₀) per mL.

Recombinant Spike protein vaccine design

The detailed methodology of obtaining recombinant Spike protein ECD has been described previously [24]. Briefly, the spike protein was identified from the SARS-CoV-2 genomic sequence in NCBI (access number: NC 045512) [25]. The codon-optimized insect cell expression cassette was cloned into pFASTBac1 and baculovirus was generated according to standard Bac-to-Bac procedures. The recombinant baculovirus was multiplied in Sf9 cells until the third passage and then used to infect *Trichoplusia ni* (Tni) cells to express the protein. After 72 h of infection, the cell culture supernatant was purified by centrifugation and the recombinant ECD spike protein was purified on a HisTrap Excel column using an AKTA chromatography system, concentrated by ultrafiltration and replaced with PBS, sterilized by filtration. The sequence of the spike protein was confirmed by mass spectroscopy, SDS-PAGE gel, and Western blotting. Endotoxin was detected with the PyroGene™ Endotoxin Detection System (Cat. No. 50-658U, LONZA, Walkersville, MD, USA), and residual DNA content in the final vaccine product was determined with the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher, P7589) according to manufacturers' instructions.

Preparation of vaccine formulations

SARS-CoV-2 spike protein was formulated with a squalene containing oil-in-water (O/W) nanoemulsion type adjuvant Sepivac SWE™ (SWE Adj; Seppic, France) in a ratio of 50: 50 (by volume) (NARUVAX C-19 vaccine) [22], and a wet gel suspension of aluminum hydroxide Alhydrogel® adjuvant 2% (Alum Adj; InvivoGen, USA) at a ratio of 1:1 (by volume), the following three vaccine formulations were prepared: 1) SWE Adj Spike - 5 µg; 2) Alum Adj Spike - 5 µg; 3) Antigen alone Spike - 5 µg. Phosphate-buffered saline (PBS) was used as a negative control sample. All samples were sterile and contained less than 2 EU bacterial endotoxins per dose.

Experimental design of animal studies

Sixteen 8- to 12-week-old outbred juvenile cats (females and males, each n=8), weighing approximately 500-700 grams, were obtained from a research colony maintained at the M. Aikimbayev National Scientific Center for Especially Dangerous Infections (NSCEDI; Almaty, Kazakhstan). Juvenile cats were screened negative for feline enteric coronavirus antibody prior to transfer. The negative SARS-CoV-2 status of the animals was confirmed by a commercial PCR kit (NSCEDI, Kazakhstan) after arrival of the animals to our Animal Biosafety Level 3 (ABSL-3) facility. Animals were housed in 0.65 m x 0.85 m x 1.15 m cages in the facility with 35-45% humidity at 22-23°C and with air exchange at least 16 times per hour.

The number of animals used was determined by following the “minimum-quantity-principle” in our protocol. Juvenile cats (♂ n=2 and ♀ n=2, per each group) were assigned into four treatment groups (Table 1): Group 1, antigen alone vaccinated animals (on days 0 and 14 post-first vaccination [PFV] received intramuscularly 0.5 mL of Spike - 5 µg); Groups 2, SWE Adj vaccinated animals (on days 0 and 14 PFV received intramuscularly 0.5 mL of Spike - 5 µg with Sepivac SWE™ adjuvant); Groups 3, Alum Adj vaccinated animals (on days 0 and 14 PFV received intramuscularly 0.5 mL of Spike - 5 µg with Alhydrogel® adjuvant 2%); Group 4, control group (on days 0 and 14 received intramuscularly 0.5 mL of PBS). First and second doses were administered to respective juvenile cats in the right rear and left rear legs, respectively.

Table 1

Design of the experimental SARS-CoV-2 vaccine study

Group	No. of animals	Vaccine formulation	Adjuvant	Days of vaccination	Days of challenge/euthanasia
1	4 (♂2, ♀2)	Spike - 5 µg	-	0 and 14	28/31
2	4 (♂2, ♀2)	Spike - 5 µg	Sepivac SWE™	0 and 14	28/31
3	4 (♂2, ♀2)	Spike - 5 µg	Alhydrogel® adjuvant 2%	0 and 14	28/31
4	4 (♂2, ♀2)	Control (PBS)	-	0 and 14	28/31

On day 28 PFV, animals of all groups were lightly anesthetized with 11 mg/kg by intramuscular injection ketamine hydrochloride (Dechra Veterinary Products, KS, US) and challenged with hCoV-19/Kazakhstan/KazNAU-NSCEDI-4635/2020 strain intranasally via pipette into the nares (200 µL per nare) for a total volume of 400 µL; animals were observed until fully recovered from anesthesia. Virus back-titration was performed on Vero E6 cells immediately following inoculation, confirming that kittens received 10⁶ TCID₅₀. All kittens were anesthetized on day 31 PFV (3 days post challenge) using the ketamine (11 mg/kg), humanely euthanized (pentobarbital >80mg/kg) and necropsied to collect tissue samples.

Clinical evaluations and sample collection

The cats were monitored at least once daily for clinical signs (fever, anorexia, lethargy, respiratory distress, inappetence, depression, recumbency, coughing, sneezing, diarrhea/loose stool, vomiting, vocalization, injection site reaction [for approximately 30 min immediately after each injection, and were examined daily for at least 7 days after each injection], injection site licking, stinging and transient fever) by a licensed veterinary practitioner. Body weights and temperatures (rectal) were documented daily every morning for the duration of 7 days after each vaccination and within 3 days after challenge.

Serum (in clot activator tubes) and whole blood (in EDTA tubes) samples were collected from juvenile cats via leg venipuncture on days 0, 28, and 31 PFV and stored at -20°C until used in ELISA (IgG antibodies detection), surrogate virus neutralization test (sVNT; ACE-2 cell receptor blocking), virus neutralization test (VNT; Wuhan and Delta variants neutralizing antibodies detection), and hematology (blood cell counts). Oropharyngeal swabs were collected on days 0 and 3 post challenge. The swabs were soaked in DMEM prior to obtaining the oropharyngeal samples. After collection, the swabs were placed in a tube containing 1 mL of DMEM with anti/anti solution and stored at minus 80°C . Prior to use, the swabs were vortexed for 1 minute in preparation for the viral RNA detection and virus titration assays.

Tissue collection included: lung, heart, and nasal turbinates. Necropsy tissues were halved and then placed into either 1 mL tubes and frozen at minus 80°C , or into standard tissue cassettes. Fresh frozen tissue homogenates were prepared by thawing tissue and placing 200 mg (± 50 mg) of minced tissue in a tube containing 1 ml DMEM culture medium and a chrome-steel beads (BioSpec Products). Homogenization was performed with the TissueLyser II (Qiagen) for 30 s at 30 hertz and repeated 2 times. Supernatant was retained after centrifugation for the viral RNA detection and virus titration. Tissues in standard tissue cassettes were fixed in 10% neutral-buffered formaldehyde and processed for histology.

Blood cell counts

Complete blood cell counts were performed using fresh EDTA blood samples run on an automated HTI MicroCC-20 VET Veterinary Hematology Analyser (High Technology Inc., North Attleboro, MA) according to the manufacturer's recommended protocol using the HTI MicroCC-20 VET reagent pack and recommended calibration controls. Blood cell analysis included 20 parameters: white blood cells (WBC), number of lymphocytes (LYM#), number of mid-sized cells (MID#; MID cells include less frequently occurring and rare cells correlating to monocytes, eosinophils, basophils, blasts and other precursor white cells that fall in a particular size range), number of granulocytes (GRA#), percentage of lymphocytes (LYM%), percentage of mid-sized cells (MID%), percentage of granulocytes (GRA%), red blood cells (RBC), hemoglobin (HGB), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), red blood cell distribution width-coefficient of variation (RDW-CV), red blood cell distribution width-standard deviation (RDW-SD), hematocrit (HCT), platelet (PLT), mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), and platelet-large cell ratio (P-LCR). Two control samples (one normal and one abnormal) were included in each assay run.

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

SARS-CoV-2-specific RNA was detected using a RT-qPCR assay. Briefly, tissue homogenates (lung, heart, and nasal turbinates) and oropharyngeal swabs in DMEM were mixed with an equal volume of RLT RNA stabilization/lysis buffer, and 200 µL of sample lysate was then used for extraction using the QIAmp Viral RNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, 140 µL of each sample was mixed with 560 µL of buffer AVL containing carrier RNA and incubated for 10 min at room temperature. After addition of 560 µL of 100% ethanol, the samples were passed through purification columns by centrifugation. The columns were washed sequentially with 500 µL of buffer AW1 and 500 µL of buffer AW2, and RNA was eluted using 40 µL of RNase-free water. To quantify SARS-CoV-2 RNA levels, we used the commercial real-time RT-PCR kit (NSCED1) according to the manufacturer's instructions. The following primer pairs targeting to the N gene of the SARS-CoV-2 virus were used: F 5' - GGGGAAGTTCTCCTGCTAGAAT; R 5' - CAGACATTTTGCTCTCAAGCTG. Amplification was performed as follows: 50°C for 10 min, 95°C for 2 min, then 45 cycles consisting of 95°C for 15 s, 60°C for 30 s and a default melting curve in the RotorGene® machine (QIAGEN, USA). When the Ct values (cycles) on the FAM/Green and JOE/Yellow channels were ≥ 40 , the samples were considered negative for SARS-CoV-2.

Anti-Spike binding IgG ELISA

Ninety-six well microplates (Nunc MaxiSorp, #2297421, Invitrogen, USA) were coated with pre-titrated 0.5 µg/ml recombinant Spike protein on commercial buffer (ELISA Coating Buffer, #B288159, BioLegend) overnight. Plates were blocked using ELISA Assay Diluent (#421203, BioLegend) 200 µl/well and incubated under constant shaking (300-330 rpm on a PST-60HL thermal shaker, BIOSAN) for 1 h at room temperature. The plates were washed four times with ELISA Wash Buffer (# 421601, BioLegend). Juvenile cat serum samples were titrated two-fold from dilutions 1:160 to 1:163840, 100 µl samples were added from each dilution to the wells and incubated under constant shaking (300-330 rpm) for 1.5-2 h at room temperature. After washing (4x), secondary Goat Anti-Cat IgG Fc (HRP) biotinylated antibodies (1:10000, #ab112801, Abcam, MA, USA) was added and the plates were incubated (1 h at room temperature with shaking). After additional washing (4x), plates were incubated with HRP Streptavidin (# 405210, BioLegend, 1:1000, 100 µl/well) for 30 minutes at room temperature with shaking. Finally, plates were washed (5x) and added ready-to-use TMB substrate (#N301, Thermo Fisher Scientific, 100 µl/well). The color reaction was stopped by adding a stop solution (#B308260, BioLegend, 100 µl/well), and the optical density was measured (measuring wavelength 450 nm, reference wavelength 630 nm) on a Stat Fax 2100 analyzer (Awareness Tech). The cut-off value for determining the titer was calculated based on the average optical density (OD) values of the wells containing only the buffer (blank) + three standard deviation.

Surrogate virus neutralization test

SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT) Kit (L00847; GenScript, Piscataway, USA) was used to detect antibodies inhibiting the binding of spike RBD to human ACE2 according to the manufacturer's instructions. Each sample was tested in duplicate. Briefly, samples and controls were

incubated with horseradish peroxidase-conjugated RBD (HRP-RBD) at 37 °C for 30 min. The mixtures were added to a hACE2-coated capture plate and incubated at 37 °C for 15 min. The plates were then washed, removing the HRP-RBD neutralizing antibody complexes and allowing the unbound HRP-RBD and HRP-RBD non-neutralizing antibody complexes to remain bound to hACE2. TMB solution was added and allowed to incubate at room temperature for 15 minutes, after which the reaction was stopped with a stop solution. The OD of each well was measured by spectrophotometry at 450 nm. The percentage of inhibition of the sample was calculated as $(1 - \text{Average OD of the sample} / \text{Average OD of the negative control}) \times 100\%$. A sample with an inhibition percentage $<30\%$ was considered "negative" and $\geq 30\%$ was considered "positive" for SARS-CoV-2 neutralizing antibodies. The following neutralizing antibody values were determined according to the level of inhibition: low (30-59%), medium (60-89), and high (≤ 90).

Virus neutralization test

All serum samples were assayed for SARS-CoV-2 neutralizing antibodies at BSL-3 laboratory of the CRL through virus neutralization test (VNT) described in Amanat et al., 2020 (without staining step) [26]. Briefly, serum samples were heat-inactivated at 56°C for 60 min. Then, 80 µL of three-fold serially diluted sera (for final dilutions with the virus of 1:10 to 1:7290) were pre-incubated with 80 µL of 100 TCID₅₀/mL of SARS-CoV-2 (hCoV-19/Kazakhstan/KazNAU-NSCEDI-4635/2020, the Wuhan variant with D614G and M153T mutations in the Spike protein and hCoV-19/Kazakhstan/KazNARU-NSCEDI-5526/2021, the Delta variant) in DMEM for 60 min at 37 °C with 5% CO₂. The 120 µl per well of virus-serum mixture was then cultured in duplicate on Vero E6 cells in 96-well plates (#3596, Corning™). After 60 min, all the virus-serum mixture was removed and 100 µl of each respective serum dilution added to the cells. Finally, 100 µl per well of DMEM containing 2% FBS, supplemented with anti-anti solution (Gibco) was added. The neutralization titers were determined at 5 days post-infection. The titer of a sample was recorded as the reciprocal of the highest serum dilution that provided at least 100% neutralization of the reference virus, as determined by visualization of cytopathic effect (CPE). Samples were considered seropositive when a dilution of at least 1:10 reduced the formation of CPE.

Virus Titration Assay

Confluent Vero E6 cells in 96-well plates were infected with 200 µl of 10-fold dilutions (10^{-1} to 10^{-7}) of the tissue homogenates (lung, heart, and nasal turbinates) and oropharyngeal swabs. After a 60 min incubation, the virus inoculum was removed, the cells were washed once with PBS, and then DMEM with 2% FBS and anti-anti solution was added and incubated for 5 days at 37 °C under 5% CO₂. The plates were observed daily for the presence of CPE by means of an inverted optical microscope. The end-point titers were calculated according to the Reed & Muench method [23] based on six replicates for titration. Virus titers are expressed as log₁₀ TCID₅₀/mL.

Statistical analysis

The GraphPad Prism 9.0.0 program (GraphPad Software, San Diego, CA, USA) was used for constructing graphs and statistical analysis of the experimental data. Differences in hematological parameters, antibody titers, viral load in swabs and tissues between animal groups were assessed using Tukey's multiple comparisons test or Dunnett's multiple comparisons test. The detection limit of the infectivity titer was $1.2 \log_{10}$ TCD₅₀/ml. The detection limit of IgG titers was 1:125 and that of neutralizing antibodies was 1:10. For all comparisons, $P < 0.05$ was considered a significant difference. In the figures, * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ and **** = $P < 0.0001$.

Results

Immunogenicity of spike protein-based vaccine candidates in juvenile cats

Immunogenicity in juvenile cats of our vaccine candidates were evaluated at day 14 after a booster intramuscular immunization. In the juvenile cats, SWE-adjuvanted spike protein (NARUVAX C-19) induced significantly higher anti-spike IgG titers (GMT 97,420) than immunization with spike protein alone (GMT 14,480) ($P < 0.02$) (Figure 1A) with alum-adjuvanted spike protein being intermediate (GMT 48,710). Anti-spike IgG titers induced by all spike protein formulations significantly exceeded levels in saline immunized controls (GMT 160).

All (4/4) cats that received SWE-adjuvanted or alum-adjuvanted spike protein vaccines had high levels of RBD-ACE2 blocking antibodies (Figure 1B). Immunization with spike protein alone induced RBD-ACE2 blocking antibody at medium to high levels. RBD-ACE2 blocking antibody levels in SWE-adjuvanted, Alum-adjuvanted and spike alone vaccines were significantly higher than control unvaccinated animals.

Next, sera were assessed for neutralizing activity against wild-type D614G or the Delta variant (Figure 1C &D). Consistent with the high levels of specific IgG, RBD-ACE2 blocking antibodies were highest in the SWE-adjuvanted (GMT 945) and alum-adjuvanted (GMT 450) spike immunized animals which were both higher than for animals that received spike protein alone (GMT 220). Similarly, neutralizing antibody titers against wild-type D614G of SARS-CoV-2 virus were significantly higher in SWE-adjuvanted and alum-adjuvanted vaccinated animals than in control animals (Figure 1C).

Cross-neutralizing antibody responses against the SARS-CoV-2 Delta variant were measurable in 100% of cats that received SWE-adjuvanted (GMT 315) and alum-adjuvanted (GMT 150) spike protein, whereas they were not detectable in 25% of animals that received spike alone. Generally, neutralizing antibody titers against the Delta variant were ~3-fold lower than against the wild-type virus across all vaccine groups (Figure 1D).

Safety and efficacy study of a spike protein-based vaccine candidates in juvenile cats

Safety after vaccination

Minor vocalization was observed in both control and vaccinated juvenile cats after the prime and booster dose administration. The only injection site reaction observed was mild swelling which resolved within 24 h in one SWE adjuvant immunized animal. Transient fevers (39.5-39.7°C) that resolved within 24 h post-injection was observed in all groups of vaccinated cats (spike alone = 1/4; SWE Adj = 2/4 and 1/4; Alum Adj = 2/4 and 1/4) after the first and second dose administration, respectively (Table 2).

Table 2

Distribution of immediate local reactions and transient fever within each treatment group

Vaccination	Adverse event	Control cats (n=4)	Vaccinated cats (n=4)		
			Antigen alone	SWE Adj	Alum Adj
Prime	Vocalization	1 (25)	1 (25)	2 (50)	1 (25)
	Injection site reaction	–	–	1 (25)	–
	Injection site licking	–	1 (25)	2 (50)	1 (25)
	Stinging	–	–	–	–
	Transient fever (39.5-39.7°C)	–	1 (25)	2 (50)	2 (50)
Booster	Vocalization	1 (25)	1 (25)	2 (50)	1 (25)
	Injection site reaction	–	–	–	–
	Injection site licking	–	–	–	–
	Stinging	–	–	–	–
	Transient fever (39.5-39.7°C)	–	1 (25)	1 (25)	1 (25)

Data are n (%); See Methods section for abbreviations.

Clinical signs after vaccination and challenge

Body temperature and clinical signs were recorded for the duration of 7 days after each vaccination (prime and booster) and for 3 days after challenge. No remarkable abnormal clinical signs were observed after vaccination or challenge. Body temperatures of vaccinated and challenged (1×10^6 TCID₅₀ wild-type

SARS-CoV-2 virus) juvenile cats remained normal (temperature < 39.5°C) throughout the challenge period [Figure 2A]. Body weights of all juvenile cats increased throughout the study as expected for young animals without overt clinical disease [Figure 2B].

Complete blood counts were performed on days 0, 28, and 31 days for the vaccinated and challenged animals. Overall, no significant changes in any blood cell parameters (WBC, LYM#, MID#, GRA#, LYM%, MID%, GRA%, RBC, HGB, MCHC, MCH, MCV, RDW-CV, RDW-SD, HCT, PLT, MPV, PDW, PCT, and P-LCR) were observed and counts remained within normal limits for most animals during the study. There was a significant increase in the mean corpuscular volume (MCV) in the control and spike alone vaccinated groups compared to the SWE- and Alum vaccinated groups of juvenile cats after challenge [Table S1].

Viral load in the respiratory tract and cardiovascular system

Viral load was determined in oropharyngeal swabs and suspensions of nasal turbinates, heart and lungs on day 3 after challenge by RT-qPCR (expressed in cycles/Ct) and viral titers by culture on Vero E6 cells (\log_{10} TCID₅₀/mL).

In the control group, SARS-CoV-2 RNA was positive day 3 post-challenge by PCR in 4/4 oropharyngeal swabs (100%), 3/4 lungs (75%), 2/4 hearts (50%) and 3/4 nasal turbinates (75%). Similarly, in the spike alone group, viral RNA was detected in a similar number of oropharyngeal swabs (75%), lungs (50%), heart (25%) and nasal turbinates (75%) to the control animals. By contrast, there was a complete absence of viral RNA (negative sample Ct of over 40) in heart samples in the groups that received SWE- adjuvanted- and alum- adjuvanted and in lung samples collected from SWE- adjuvanted groups (Figure 3A). However, detectable viral RNA by PCR was still seen in some oropharyngeal (25%) and nasal turbinate (75%) samples in the adjuvanted vaccine groups.

Infectious virus by CPE assay correlated with detection of viral RNA by PCR. Notably, infectious virus was not recovered from the oropharyngeal swabs, lung and heart samples in the groups that received SWE- adjuvanted and alum- adjuvanted spike vaccine. However, infectious virus was still detectable in nasal turbinates from some animals in the adjuvanted spike vaccine groups, although the virus titer was significantly lower ($P < 0.0001$; $P = 0.0035$) than in the control group (Figure 3B). These data show that the SWE- adjuvanted and Alum- adjuvanted spike vaccines were highly efficient in blocking viral replication in the lower respiratory tract and heart but were unable to fully prevent infection in the upper respiratory tract. Overall, the SWE- adjuvanted spike vaccine was associated with the lowest viral loads across all tissues although this was not significantly different to the alum- adjuvanted group.

Discussion

The COVID-19 pandemic has reminded all of us about the threat that zoonotic diseases pose to humanity. In fact, 60% of all infectious diseases affecting humans fall into this category, and 75% of the new human infections that have emerged over the past three decades are of animal origin [27]. This underscores the importance of a 'One Health' approach to disease control: the well-being of humans is

inextricably linked to the health of the animals and environment in which we are all living [28]. Vaccination is an effective method of preventing a wide range of human and animal diseases. Veterinary research into animal coronaviruses has led to several successful veterinary coronavirus vaccines and understanding of the potential challenges to develop a safe and effective veterinary vaccine against SARS-CoV-2 [29]. While the global focus remains on the human vaccine roll-out, SARS-CoV-2 protection in animals could be a vital part of pandemic recovery. While more than 190 COVID-19 vaccine candidates are reported to be in various stages of development [30], only two veterinary SARS-CoV-2 vaccines have been reported, Carnivac-Cov an inactivated vaccine commercially available in Russia [31] and an experimental recombinant spike protein vaccine produced by Zoetis that has been used in Zoo animals in the USA [32]. Carnivac-Cov vaccine has been designed for carnivores (arctic foxes, cats, rats, mink) [21]. An alphavirus replicon-based vaccine expressing the stabilized spike protein is also being developed which has been reported to induce protective immunity and prevent transmission of SARS-CoV-2 between cats [20].

Our research team consisting of the International Center for Vaccinology at the Kazakh National Agrarian Research University of the Ministry of Agriculture of the Republic of Kazakhstan (KazNARU) and NSCEDI in collaboration with Vaxine Pty Ltd, Australia, has been developing a protein subunit vaccine against COVID-19 for animals. The vaccine is prepared from Spike protein ECD with an amino acid sequence corresponding to wild-type SARS-CoV-2 (Wuhan strain) together with some stabilizing mutations that is then codon optimized and expressed in insect cells using the baculovirus transfection system. This spike protein ECD antigen has been previously been shown to be immunogenic, protective, and safe in mice and ferrets in an Australian-developed vaccine called COVAX-19/SpikoGen [30] that has successfully passed phase III clinical trials and in October 2021 received emergency use authorization from the Iranian FDA [24]. This spike protein ECD formulated with an SWE adjuvant (NARUVAX-C19 vaccine) provided complete protection of Syrian hamsters against SARS-CoV-2 infection and prevented transmission to naïve animals placed in the same cage as the challenged animals [22].

Given subunit vaccines often have low immunogenicity, adjuvants are generally needed to ensure vaccine responses that are more robust and long-lasting [33, 34]. In the current studies we combined the spike ECD protein with either Alhydrogel® 2% or the previously used SWE adjuvant. Alhydrogel is a wet gel suspension of aluminum oxyhydroxide that is commonly used an adjuvant in human vaccines [35, 36]. A recent COVID-19 vaccine study using an alum-stabilized Pickering emulsion (PAPE) showed enhancement of RBD-specific IgG1 and IgG2a and IFN- γ -secreting T cells [34].

Spike ECD formulated with either alum or SWE adjuvants demonstrated no safety issues with all juvenile cats remaining healthy post-vaccination. All formulations were well tolerated during prime and booster vaccinations, with mild short-lived local reactions that just included short-term vocalization and licking of the injection site. The only moderate injection site reaction occurred in a cat in the SWE-adjuvant group and was considered to be associated with the adjuvant, as oil emulsion adjuvants are known to cause injection site reactions. Rectal temperatures after prime and booster vaccinations were not elevated and the juvenile cats continued to gain weight throughout the study period. Hematological data showed no

significant differences in the twenty measured hematologic parameters between vaccinated and control groups and indicate the safe profile of (Antigen alone, SWE Adj, and Alum Adj) in juvenile cats. Overall, all three vaccine formulations showed an acceptable tolerability and safety profile with outcomes consistent with tolerability in cats reported for an inactivated influenza virus vaccine [37].

The data highlights the importance of adjuvants, in this case either SWE or Alum to spike protein immunogenicity [38], with highest overall responses whether measured by spike-binding IgG, RBD-ACE2 blocking antibodies, and homologous and variant neutralizing antibodies in the SWE-adjuvanted group. This translated into robust protection against challenge with wild-type (D614G) SARS-CoV-2 virus. Notably, only the animals immunized with SWE-adjuvanted or Alum-adjuvanted spike ECD had no recoverable infectious virus in day 3 oropharyngeal swabs, heart, and lung samples. This could suggest a potential for our spike ECD vaccine formulations to inhibit the release of virus by infected animals into the environment for transmission to others, in addition to protecting like other COVID-19 vaccines against SARS-CoV-2 virus invasion into the lower respiratory tract and heart.

However, during hematological examination of blood samples, we additionally found significant increase in the mean corpuscular volume (MCV) between the control animal (PBS) and Antigen alone vaccinated groups compared to the SWE- and Alum vaccinated groups of juvenile cats after challenge. This is also consistent with the data from Curukoglu et al. [39] which have demonstrated the first human to domestic cat transmission of SARS-CoV-2 with N501Y mutation (Alpha variant) in Northern Cyprus, where a slight increase in the MCV was detected, and cat did not show serious symptoms. In view of the above, this work demonstrates that the SWE-adjuvanted- and Alum-adjuvanted Spike vaccines against SARS-CoV-2 virus was able to induce high levels of virus-neutralizing antibodies in serum of vaccinated juvenile cats and that the induced response was able to prevent infection of the upper and lower respiratory tract, and the cardiovascular system. Testing for the presence of infectious SARS-CoV-2 virus in juvenile cat's heart samples in our studies was related to the finding that myocardial injury and fulminant myocarditis can occur from direct viraemic effect of COVID-19 on the myocardial cells of human [40] and increased incidence of SARS-CoV-2 infected cats that were diagnosed with compatible suspected myocarditis during their examinations [41].

Our vaccine has several distinct features compared to studies of other SARS-CoV-2 vaccines designed for cats, namely the inactivated Carvivac-CoV vaccine that also uses aluminum hydroxide adjuvant [42] and a Replicon Particle vaccine based on Venezuelan equine encephalitis virus expressing a stabilized spike antigen [20]. This latter vaccine induced neutralizing antibody responses against wild-type (strain USA-WA1/2020) and Alpha variant (B.1.1.7) in sera and provided protection against wild-type SARS-CoV-2 challenge in domestic male and female cats with none of the vaccinated animals being observed to shed detectable virus orally or nasally after challenge.

In the case of our study, we applied a shorter immunization period with an interval of 14 days between prime and booster vaccinations, compared to 21 days for the comparable vaccines, and we performed an earlier challenge (14 versus 21 days after booster vaccination). Second, we used a lower administered

volume of 0.5 mL instead of the 1.0 mL Canivac-CoV vaccine. Notably, these other vaccines, unlike ours, have not reported the ability to cross-neutralize the Delta virus variant.

Some limitations of this study need to be acknowledged. The small number of animals used per group was determined by animal welfare guidelines and the “minimum-quantity-principle”, thereby preventing us from using larger group sizes. Nevertheless, the results were highly consistent between the 4 cats in total and the 2 males and 2 females within each group, with statistical differences seen when the adjuvanted vaccine groups were compared to saline controls and/or spike ECD alone immunized animals. Furthermore, the immunogenicity and protective efficacy seen in these juvenile cats of the NARUVAX C-19 vaccine formulation with SWE adjuvant were consistent with the findings previously obtained for the same vaccine in Syrian hamsters [22]. Another important limitation is the lack of data on long-term protection, on virus transmission and on protection against challenge with the Delta virus variant, as only cross-neutralising antibody data against Delta variant was able to be collected in this study. Future planned studies will explore all these parameters and in particular the ability of our vaccines to prevent virus transmission from vaccinated to naïve cats placed in the same cage as challenged animals, which will be important given the recent appearance of highly transmissible Delta virus strains in companion animals [43].

Conclusion

In conclusion, the present research consists of a comprehensive safety and efficacy evaluation of in juvenile cats of a two-dose immunization regimen with a recombinant Spike ECD with either of two commonly used adjuvants. Our data show the benefits of the selected vaccination regimen in terms of safety, tolerability, immunogenicity, and protection against virus replication in the upper and lower respiratory tract and cardiovascular system of juvenile cats. Considering these promising data, this vaccine will now be evaluated for protection of cats against challenge with Delta variant and ability to prevent its transmission to naïve animals, as well as the durability of protection with the plan to then proceed to large-scale animal field trials.

Declarations

Conflict of interest statement

The authors declare a potential conflict of interest and state it below.

NP is affiliated with Vaxine Pty Ltd which holds the rights to COVAX-19 vaccine. None of the other authors has any financial or personal interest with any organization that could inappropriately influence or bias the research activity presented in this manuscript.

Ethics statements

The animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (8th edition). The protocol on Bioethics was approved by the Institutional Animal Care and Use Committee of the NSCEDI of Ministry of Health of the Republic of Kazakhstan (Approval number 105/2021-04-19).

Author contribution statement

Conceptualization: KaissarT

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Formal analysis: KaissarT

Funding acquisition: KaissarT, Tlektesy, ToktasynY

Investigation: KairatT, MO, NT, NM

Methodology: KaissarT, KairatT, MO

Project administration: KaissarT, KairatT

Resources: KaissarT

Software: KaissarT

Supervision: KaissarT

Validation: KairatT, MO

Visualization: KairatT, MO

Writing ± original draft: KairatT, MO

Writing ± review & editing: KaissarT, NP

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Figures

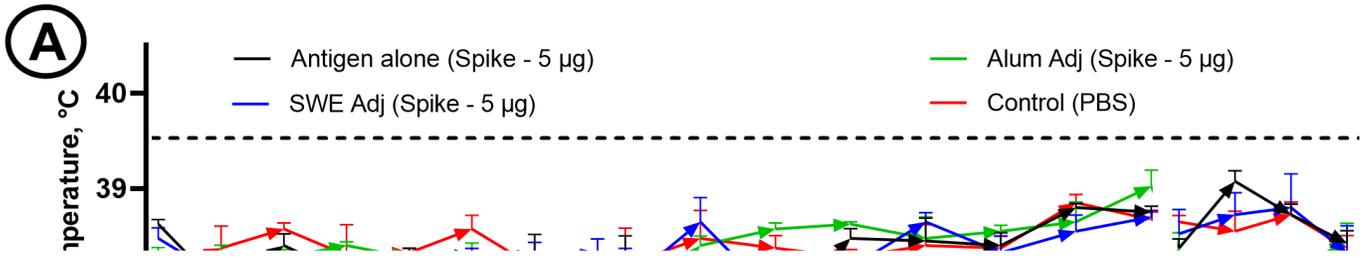


Figure 1

Spike-specific IgG (A), RBD-ACE2 blocking antibody (B) and neutralizing antibody (C, D) levels in juvenile cats 14 days after booster immunization. Viral neutralizing antibodies were assessed against wildtype D614G (C) and Delta variant (D) viruses. Differences between groups assessed using Tukey's multiple comparisons test.

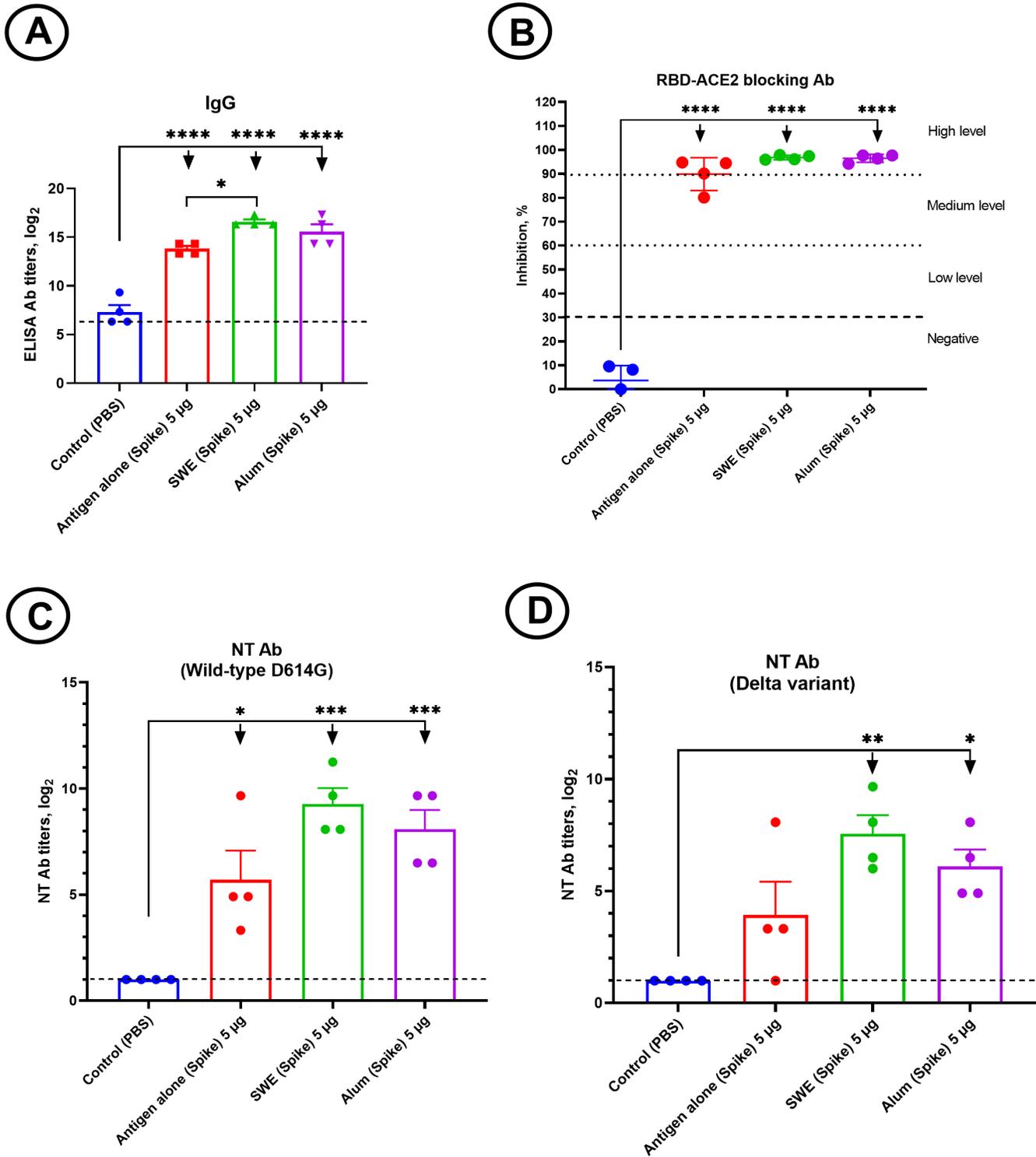


Figure 2

Rectal temperature (A) and body weight (B) in juvenile cats after vaccination and challenge. The rectal temperature ≥ 39.5 °C was considered as fever (dashed line). Data shown are mean \pm SEM of 4 juvenile cats in each group.

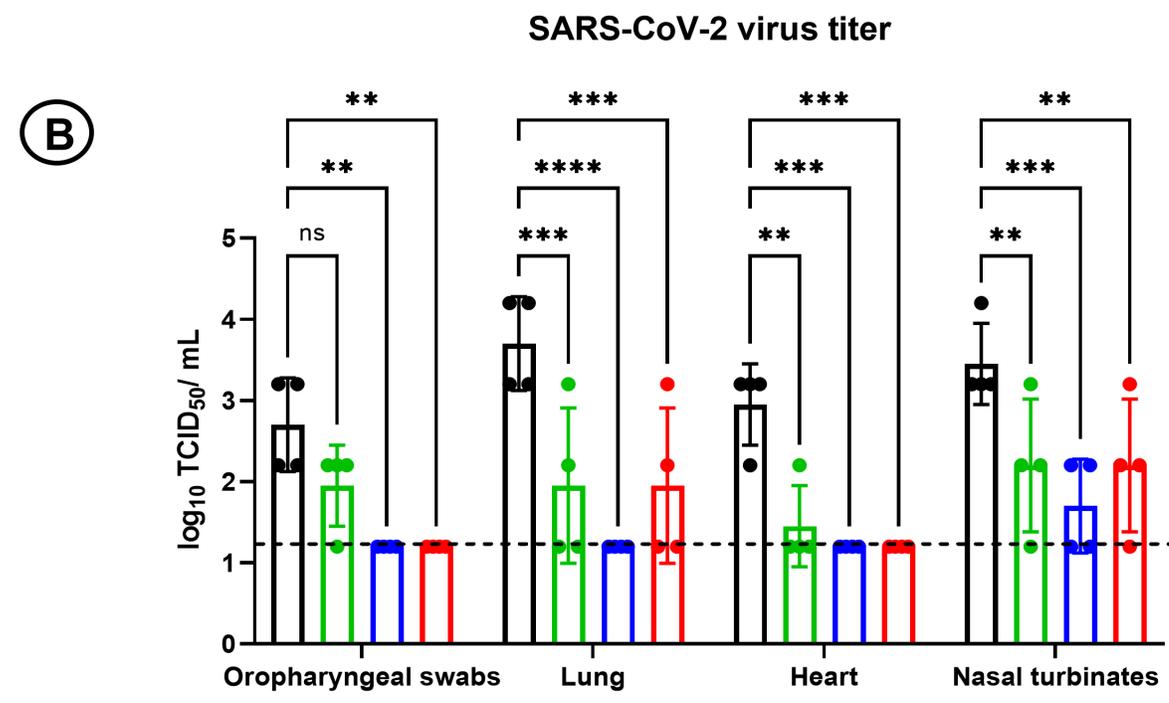
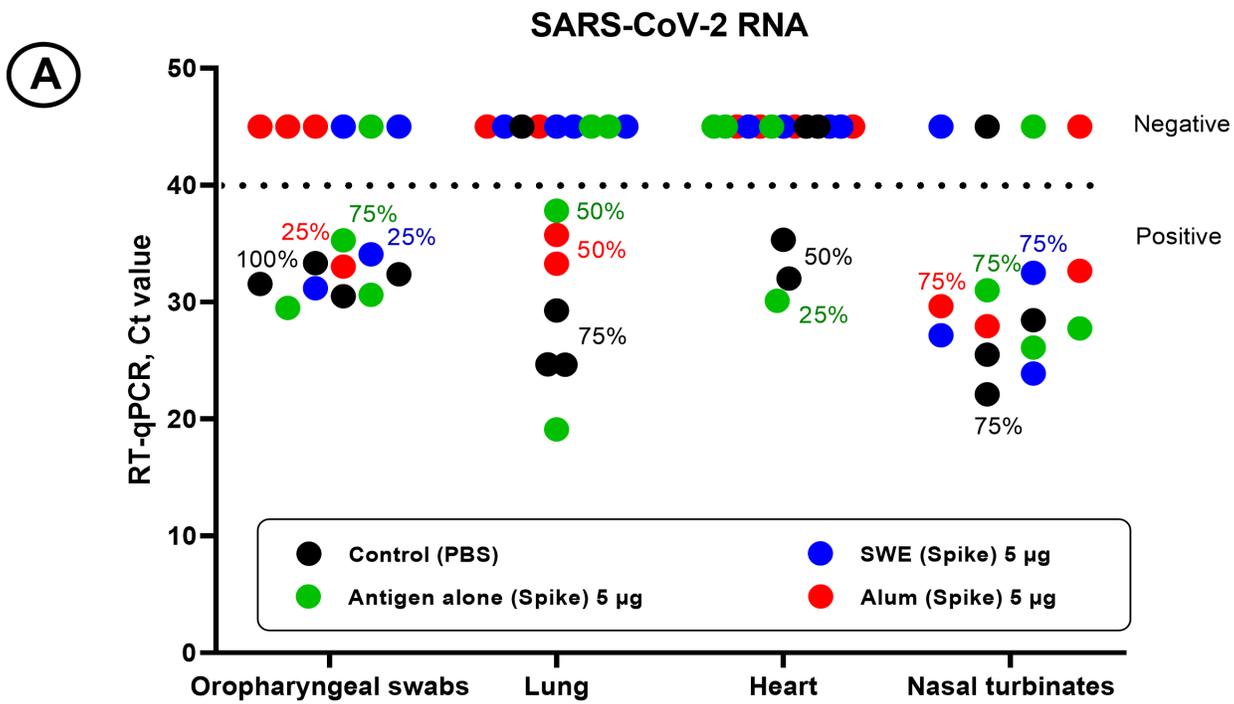


Figure 3

Viral load in the respiratory tract and cardiovascular system of juvenile cats. Animals inoculated with SARS-CoV-2 virus were euthanized on day 3 after challenge, and their organs were collected for viral RNA detection and virus titration. Shedding and presence of viral RNA (A) and viral titers (B) in oropharyngeal swabs, lung, heart, and nasal turbinates. The horizontal dashed lines in (A) and (B) indicates the upper and lower limits of detection, respectively.

Supplementary Files

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- [SupplementaryMaterial.docx](#)