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Immunogenicity of a new inactivated vaccine against feline panleukopenia virus, calicivirus, and herpesvirus-1 for cats

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Abstract

Feline panleukopenia virus (FPV), feline calicivirus (FCV), and feline herpesvirus type-1 (FHV-1) are major infectious pathogens in cats. We evaluated the immunogenicity of a new vaccine containing inactivated FPV, two FCVs, and FHV-1 in animals. An FPV, two FCVs, and an FHV-1 isolate were continuously passaged 70, 50, 80, and 100 times in CRFK cells. FP70, FC50, FC80, and FH100 were propagated and used as vaccine antigens. Two inactivated feline virus vaccines, feline rehydragel-adjuvanted vaccine (FRAV) and feline cabopol-adjuvanted vaccine (FCAV) were prepared and inoculated into mice and guinea pigs. Humoral immune responses were measured using hemagglutination inhibition (HI) for FPV and virus-neutralizing antibody (VNA) for two FCVs and FHV-1 tests. Serial passages in CRFK cells resulted in increase in titers of FPV and two FCVs but not FHV-1 The FCAV induced higher mean HI and VNA titers than the FRAV in guinea pigs; therefore, the FCAV was selected. Cats inoculated with FCAV developed a mean HI titer of 259.9 against FPV, and VNA titers of 64, 256, and 3.2 against FCV17D03, FCV17D283, and FHV191071, respectively. Therefore, cats inoculated with the FCAV showed a considerable immune response after receiving a booster vaccination.

Keywords: feline panleukopenia virus; feline calicivirus; felid herpesvirus 1; inactivated vaccine; cats

Introduction

Feline panleukopenia virus (FPV), feline calicivirus (FCV), and feline herpesvirus type-1 (FHV-1) cause infectious diseases in felines worldwide. FPV is a parvovirus in the family *Parvoviridae* and is fatal and highly contagious in young kittens and exhibits strong resistance to the environment [1,2]. The clinical symptoms caused by FPV include high fever, anorexia, vomiting, dehydration, and hemorrhagic diarrhea, resulting in death of > 50% of untreated infected kittens [3]. FCV is a Vesivirus in the family *Caliciviridae*, is highly contagious, and causes oral ulcerations and conjunctivitis. FCV infection manifests in two forms in cats < 1 year of age. The clinical signs of the classic form are fever and nasal and ocular discharge. The clinical symptoms of virulent systemic disease (VSD) are pyrexia, cutaneous edema, ulcerative dermatitis, anorexia, and jaundice with sudden death [4,5]. FHV-1 is a Varicellovirus, in the subfamily *Alphaherpesvirinae* of the family *Herpesviridae*, and causes feline viral rhinotracheitis. The clinical signs of

FHV-1-infected cats are fever, nasal discharge, conjunctivitis, keratitis, and pneumonia [6]. FHV-1 infection of the trigeminal ganglion can be reactivated by high stress or immunosuppression, resulting in continuous shedding of infectious virus [7].

In South Korea, inactivated or live-attenuated FPV, FCV, and FHV-1 vaccines are used for disease prevention and management for kittens [6]. Cats are recommended to be vaccinated at 8–12 weeks of age, followed by a further two doses every 3 weeks [8–10]. Genetic variation at key sites in FPV, FCV, and FHV-1 occur over time, altering host range, cell tropism, and pathogenicity. FPV is antigenically similar to canine parvovirus (CPV). CPV are evolving genetic variation into CPV2a/b/c [11], while FPV has expanded their host range to infect monkeys as well [12]. Because the VSD form of FCV causing sudden death has been identified within the past 10 years, a dual-strain FCV vaccine has been developed that induces broader cross-neutralizing antibodies than a single-strain FCV vaccine [13]. FHV-1 is closely related to canine herpesvirus-1, and a genetic analysis of FHV-1 isolates showed low intra-species genomic variation worldwide [14].

The strains in trivalent vaccines for cats may be antigenically different from the viruses circulating in South Korea, hampering prevention of feline viral diseases. New adjuvants have been developed to enhance the immunogenicity of inactivated vaccines. In this study, we prepared new inactivated feline vaccines based on Korean FPV, FCV, and FHV-1 isolates. After selecting an optimal adjuvant, we explored the immunogenicities of the vaccines in cats.

Materials and Methods

Cells and viruses

CRFK (CCL-94; ATCC, USA) cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) heat-inactivated fetal bovine serum and antibiotic-antimycotic solution (Gibco, USA). The FPV19D01, FCV17D03, FCV17D283, and FHV191071 strains isolated from naturally infected Korean cats in 2017 and 2019 [15–17] were passaged in CRFK cells and used to assess immune responses. FPV19D01–70P (FP70, KTCT number: 18984P), FCV17D03–50P (FC50, 18985P), FCV17D283–80P (FC80, 18986P), and FHV191071–100P (FH100, 18987P) served as seed viruses for developing a new inactivated feline vaccine.

Viral titration

FPV19D01–1P(FP1), FP70, FCV17D03–1P (FC1), FC50, FCV17D283–1P (FC2), FC80, FHV191071–1P (FH1), and FH100 titers were evaluated. Briefly, CRFK cells grown in 25 cm^2 flasks were inoculated with virus at 1,000 50% tissue culture infectious dose ($TCID_{50}$/mL) and harvested as described previously [15–17]. After three continuous freeze-thaw cycles, viral titers were determined based on fluorescence and typical cytopathic effects (CPEs). Viral titers calculated using the Reed and Muhen method are expressed as $TCID_{50}$/mL.

Polymerase chain reaction, sequencing, and whole-genome sequencing

Genomic DNA from FP1 and FP70 was extracted using a DNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions. Polymerase chain reaction amplification and cloning of the VP2 genes of two FPVs were performed as described previously [15]. Viral RNAs from FC1, FC50, FC2, and FC80 were extracted using an RNA Extraction Kit (Bioneer) according to the manufacturer's instructions. Viral DNA was extracted from FH1 and FH100 using an AccuPrep DNA Extraction Kit (Bioneer) according to the manufacturer's instructions. The complete RNA and DNA genomes of FC1, FC50, FC2, FC80, FH1, and FH100 were subjected to next-generation sequencing externally (Sanigen Inc., Korea). Nucleotide sequence alignments were generated using Clone Manager version 10, Basic Edition (Sci-Ed Software, USA).

Inactivation of FPV, FC, and FHV-1

To propagate the FP70, FC50, FC80, and FH100, CRFK cells grown in 175 cm^2 culture flasks were washed three times with phosphate-buffered saline (PBS, pH 7.2) and inoculated with 1 mL the four viruses ($10^{7.0}$ $TCID_{50}$/mL). After incubation for 1 hour, extracellular virus was removed and fresh DMEM containing 5% FBS was added. The flasks were incubated for 2 or 5 days. After three consecutive freeze-thaw cycles, virus suspension was centrifuged at 3,000 × *g* for 15 min. Each virus was inactivated in formalin (0.2%) at 37°C for 48 hours. The pH of inactivated virus was adjusted to 7.0 with 1 N NaOH. To confirm inactivation, 1 mL inactivated viral suspension was dialyzed against PBS for 24 hours and inoculated onto CRFK cells. CPEs were observed for 4 days under a microscope and the cells were fixed in cold acetone and reacted with FPV-specific monoclonal antibodies (APQA, Korea). Nuclear fluorescence was observed using a fluorescence microscope. After confirming viral inactivation, the viruses were used to prepare inactivated vaccines.

Preparation of experimental vaccines

We prepared two vaccines with aluminum hydroxide gel (Rehydragel LV; Chemtrade Logistics, Canada) and Cabopol

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(Cabopol 974P NF Polymer; Lubrizol, USA) adjuvants. The vaccine containing Rehydragel LV was prepared by adding the adjuvant to a suspension of inactivated virus to a final concentration of 10% (v/v), followed by blending at room temperature for 15 min. Four viruses (FP70, FC50, FC80, and FH100) and adjuvant were blended at a ratio of 40:15:15:20:10. For the second vaccine, Cabopol (2 mg/mL) was used as the adjuvant and the inactivated virus:adjuvant ratio was 40:15:15:25:5. The vaccine was aseptically transferred to sterilized glass bottles and stored at 4°C until use. The first and second vaccines were named feline rehydragel-adjuvanted vaccine (FRAV) and feline cabopol-adjuvanted vaccine (FCAV). Two FCV inactivated vaccines containing FCC17D03 and FCV17D283 for cross-neutralization tests were prepared with Cabopol adjuvant at a ratio of 95:5.

Animal experiments

Animal experiments were performed by the Animal and Plant Quarantine Agency of Korea in accordance with protocols approved by the Experimental Animal Ethics Committee (approval numbers 2020–441, 2020–505). The species of mice, guinea pigs and cats used were institute for cancer research, Hartley and Korean domestic shorthair. The mice and guinea pigs were bred under specific pathogen free condition. Immune sera used for examination of antigenic cross-reactivity between FCV17D03 and FCV17D283 were prepared in 6-week-old female mice. Four mice per group were immunized twice (0.2 mL) intramuscularly at a 2-week interval. Blood samples were obtained from mice and pooled sera were subjected to the virus-neutralizing antibody (VNA) test.

Four-week-old female mice were divided into three groups of five mice each. Mice in groups 1 and 2 were inoculated with inactivated FRAV and FCAV, respectively; mice in group 3 were not treated (control). Mice were inoculated with 0.2 mL vaccine intramuscularly, twice, at a 2-week interval. Two weeks after the second vaccination, blood was collected from the mice.

Eight-week-old guinea pigs were divided into three groups of five animals each. Guinea pigs in groups 1 and 2 were inoculated with 1 mL inactivated FRAV and FCAV intramuscularly, three times at 0, 2, and 6 weeks post-vaccination (WPV). Blood was collected at 0, 2, 4, 6, and 8 WPV.

Four-month-old cats seronegative for the four viruses were divided into two groups. Group 1 (seven cats) was subcutaneously immunized with one dose (1 mL) of inactivated FCAV, twice, at a 4-week interval. Group 2 (two cats) did not receive the vaccine (control). Blood for serological analysis was collected at 0, 4, 6, and 12 WPV. The serum titers of antibody against FPV, two FCVs, and FHV-1 were measured using the hemagglutination inhibition (HI) and VNA tests.

Hemagglutination inhibition test

The HI test for FPV was performed in a 96-well microplate as described previously [1], with slight modifications. In brief, 50 μL serum was treated with 25% kaolin and fresh packed pig erythrocytes to prevent nonspecific reactions. Eight hemagglutination assay units of FPV19D01 in 25 μL were added to 25 μL treated serum for the HI test. After 1 hour incubation at 37°C, 50 μL 0.6% pig erythrocytes were added. After incubation at 4°C for 1 hour, the HI titer was determined under fluorescent light.

Virus-neutralizing antibody test

VNA tests for FCV17D03, FCV17D283, and FHV191071 strains were carried out in 96-well microplates using serum inactivated at 56°C for 30 minutes. In brief, 50 μL twofold serially diluted serum was blended with an equal volume of FCV17D03, FCV17D283, or FHV191071 (200 $TCID_{50}$/0.1 mL). After incubating at 37°C for 1 hour, 100 μL CRFK cell suspension (2×10^4 cells) was added to each well. The microplates were incubated for 2 to 4 days in a humidified incubator with 5% CO_2. Each well was examined under a microscope to detect virus-specific CPE. The VNA titers were expressed as the reciprocal of the highest serum dilution showing complete inhibition of CPE.

Statistical analysis

Statistical analysis was performed using Prism version 9.3.1 (GraphPad Software Inc., USA) and Excel 2010 (Microsoft Corp., USA). Among-group differences in HI and VNA data were subjected to unpaired Student t-test and two-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. *p*-values of < 0.05 were considered indicative of statistical significance.

Results

Biological and molecular characterization of FPV, FCV, and FHV-1

We selected FP70, FC50, FC80, and FH100 to develop a new inactivated FPV, FCV, and FHV-1 vaccine after passaging in CRFK cells. As shown in Fig. 1, the FPV titer increased from $10^{6.0}$ to $10^{7.8}$ $TCID_{50}$/mL after passaging in CRFK cells. The titers of the two FCV strains increased from $10^{7.0}$ and $10^{7.2}$ to $10^{8.0}$ $TCID_{50}$/mL, respectively. There were no differences between FH1 and FH100 strains in FHV-1 titers.

Amino acid alignment of VP2 between FP1 and FP70 showed

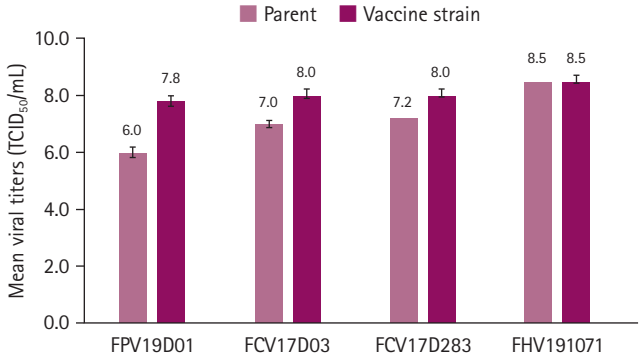

Fig. 1. Titers of feline panleukopenia virus and two feline caliciviruses increased after passage in CRFK cells. There were no differences in viral titers between FH1 and FH100. TCID, tissue culture infectious dose; FPV, feline panleukopenia virus; FCV, feline calicivirus; FHV-1, feline herpesvirus type-1.

mutations in FP70 at positions 101, 231, and 562 (Fig. 2). Next, we compared the amino acid sequence of VP1 of FC1, FC50, FC2, and FC80. Alignment of the predicted amino acid sequence of FCV VP1 between FC1 and FC50 and between FC2 and FC80 showed that FC50 and FC80 had 4 and 16 amino acid substitutions. Mutations of four amino acids in the VP1 gene of FC50 were identified at positions 150, 205, 427, and 667 (Fig. 3). Two FCV strains were tested for antigenicity using cross-neutralization assays. As shown in Table 1, antiserum to the FCV17D03 neutralized homologous virus, FCV17D03 showing high VNA titers of 111.4 ($2^{6.8}$), but did not neutralize heterologous virus, FCV17D283. The homologous-to-heterologous VNA titer ratio was zero for FCV17D03 and FCV17D283.

The complete genome sequences of FH1 and FH100 were

```
                101                                                                                  200
FPV19D01-1P     THVQIVTPWSLVDANAWGVWFNPGDWQLIVNTMSELHLVSFEQEIFNVVLKTVSESATQPPTKVYNNDLTASLMVALDSNNTMPFTPAAMRSETLGFYPW
FPV19D01-70P    I.....................................................................................................
                201                          231                                                     400
FPV19D01-1P     KPTIPTPWRYYFQWDRTLIPSHTGTSGTPTNVYHGTDPDDVQFYTIENSVPVHLLRTGDEFATGTFFFDCKPCRLTHTWQTNRALGLPPFLNSLPQSEGA
FPV19D01-70P    ............................I.....................................................................
                501                                                  562                585
FPV19D01-1P     KVAPNLTNEYDPDASANMSRIVTYSDFWWKGKLVFKAKLRASHTWNPIQQMSINVDNQFNYVPNNIGAMKIVYEKSQLAPRKLY*
FPV19D01-70P    ..........................................................L.....................*
```

Fig. 2. Alignment of the predicted amino acid sequences of the VP2 genes of FPV19D01-1P and FPV19D01-70P. Three amino acids (threonine, asparagine, and valine) were replaced with two isoleucines and leucine at positions 101, 231, and 562. FPV, feline panleukopenia virus.

```
A
                101                                                 150                                 200
FCV17D03-1P     VLMHHLIGEVAKAWDPNLPLFRLEADDGSITTPEQGTMVGGVIAEPSAQMSAAADMATGKSVDSEWEAFFSFHTSVNWSTSETQGKILFKQSLGPLLNPY
FCV17D03-50P    ..................................................V.....................................................
                201 205                                                                             300
FCV17D03-1P     LSHLAKLYVAWSGSIEVRFSISGSGVFGGKLAAIVVPPGIEPVQSTSMLQYPHVLFDARQVDPVIFSIPDLRSTLYHLMSDTDTTSLVIMVYNDLINPYA
FCV17D03-50P    ....T.................................................................................................
                401                         427                                                     500
FCV17D03-1P     AMLGIGVATDNIVPGIPDGWPDTTIPNRLTPAGHYAITNGNNNDITTRQGYESATTIINNTNFKSMYICGSLQRAWGDKKISNTGFITTASVSDNDLIPS
FCV17D03-50P    ............................D.....................................................................
                601                                                        667670
FCV17D03-1P     LPPDSFAVYRITDSNGSWFDIGIDSDGFSFVGVSAMTKLEFPLSASYMGIQLARVRLASNIRSAMTKL*
FCV17D03-50P    ...........................................................I..*

                1                                                   48                                  100
FCV17D283-1P    MCSTCANVLKFYGWDPHFQLEINPNQFLSVGFCDKPLMCCYPELLPDYGTVWDCNQSSLDIYLESILGDDEWASTFEAIDPIVPPMHWSEAGKIFQPHPG
FCV17D283-80P   ..................................................C.....................................................
                201                                                 241 245 249252             268  273     280  285          299
FCV17D283-1P    YLEHLSKLYVAWSGSVEVRFSISGSGVFGGKLAAIVVPPGMEPVRGTSVLQCPHVLFDARQVDPVIFAIPDLGNSLYHLVSDTDATSLVIMVYNDLINPY
FCV17D283-80P   ..................................I...QS..M..Y..............T....R......M....T.............1.
                401  406                       435   441                                                 497
FCV17D283-1P    GAKLGIGIATDYIVPGIPDGWPDTTIPEKLIPAGNYAITDLSANDIKTKAAYESAIGIINNTNFKGMFICGSLQRAWGDKKISNTAFITVGTIKENNIEP
FCV17D283-80P   .....V...........................D.....V.....................................................D...
                501             519                                                                 600
B
FCV17D283-1P    SNVIDQSKIAIFQDNHVNTSVQTSDITYAILGYTGIGEEAIGATRDRVARISVLPETGARGGNHPIFYKNTMKLGYVISAIDVFNSQILHTSRQLSLNNY
FCV17D283-80P   ................E.....................................................................................
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Fig. 3. Alignment of the predicted amino acid sequences of the VP1 gene of FCV17D03-1P and FCV17D03-50P (A) and of FCV17D283-1P and FCV17D283-80P (B). Four amino acids (two alanines, asparagine, and threonine) were replaced with valine, threonine, aspartic acid, and isoleucine at positions 150, 205, 427, and 667. There were 16 amino-acid substitutions in the VP1 gene of FCV17D283-80P. FCV, feline calicivirus.

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compared to detect genetic mutations after 100 passages. As shown in Fig. 4, FH100 exhibited two nucleotide substitutions in the infected cell protein 0 (ICP 0) gene. Compared to the FH1 genome, 11 nucleotides were replaced in the full genome of FH100 (data not shown).

Immunogenicity of inactivated FPV, FCV, and FHV-1 vaccine

Two inactivated FPV, FCV, and FHV-1 vaccines were prepared using aluminum hydroxide gel and Cabopol adjuvants. As shown in Fig. 5, FPV-, FCV-, and FHV-1-specific antibodies were induced by the two vaccines. The FRAV induced an HI titer of 242.5 ($10 \times 2^{4.6}$) against FPV19D01 and a VNA titer of 194.0 ($2^{7.6}$) against FCV17D03 in mice. The FCAV induced a VNA titer of 256.0 (2^{8}) and 5.3 ($2^{2.4}$) against FCV17D283 and FHV-1 in mice. The mouse humoral immune response results precluded selection of an adjuvant because the HI and VNA titers against the four viruses were not consistent.

Immunized guinea pigs developed HI or VNA titers after the second vaccination (Fig. 6). However, the HI and VNA titers tended to decrease at 2 weeks after the second vaccination. After the third vaccination, the antibody levels against the four viruses increased. Although the differences were not significant, the FCAV induced a slightly higher HI titer of 844.5 ($10 \times 2^{6.4}$) against FPV than the FRAV in guinea pigs at 8 WPV. Guinea pigs inoculated with FCAV developed VNA titers of 111.4 ($2^{6.8}$) against FCV17D03 and 13.9 ($2^{3.8}$) against FCV17D283, compared to 10.5 ($2^{3.4}$) and 1.7 ($2^{0.8}$) for FRAV-inoculated guinea pigs at 2 WPV ($p < 0.05$). Guinea pigs had the highest VNA titers of 388.0 ($2^{8.6}$) and 168.9 ($2^{7.4}$) against two FCVs at 8 WPV, which were significantly higher than the control ($p < 0.001$). The FCAV induced a VNA titer of 16.0 (2^{4}) against FHV-1, compared to 6.1 ($2^{2.6}$) for the FRAV. Based on the immune responses of guinea pigs, the FCAV was selected for cats.

Cats were inoculated with the FCAV twice, 4 weeks apart to evaluate the immune response. As shown in Fig. 7, the cats showed the highest mean HI titer of 259.9 ($10 \times 2^{4.7}$) to FPV, VNA titers of 64.0 (2^{6}) or 256.0 (2^{8}) against FCV17D03 and FCV17D283, and of 3.2 ($2^{1.7}$) against FHV-1 at 6 WPV; however, these had decreased to 226.3 ($10 \times 2^{4.5}$), 168.9 ($2^{7.4}$), 55.7 ($2^{5.8}$), and 2.8 ($2^{1.5}$) by 12 WPV. The HI and VNA titers did not increase in unvaccinated cats.

Table 1. Results of cross-neutralization between FCV17D03 and FCV17D283

Virus strain	Immune serum prepared in mice	
	FCV17D03	FCV17D283
FCV17D03	111.4 ($2^{6.8}$)	< 2
FCV17D283	< 2	157.5 ($2^{7.3}$)

FCV, feline calicivirus.

Discussion

FPV, FCV, and FHV-1 infections can be highly contagious in cats. Infections with these viruses mainly occur in the nasal or oral mucosa. Therefore, vaccination is the most effective prophylactic method and have contributed to reducing feline infections by FPV, FCV, and FHV-1. Although FPV, FCV, and FHV-

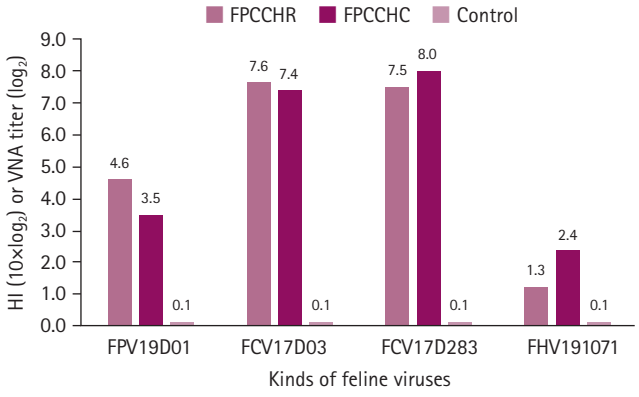

Fig. 5. Immune response to feline rehydragel-adjuvanted vaccine and feline cabopol-adjuvanted vaccine in mice. Hemagglutination inhibition (HI) titers of FPV and virus-neutralizing antibody (VNA) titers against two FCVs and FHV-1 are shown as mean values. FPV, feline panleukopenia virus; FCV, feline calicivirus; FHV-1, feline herpesvirus type-1.

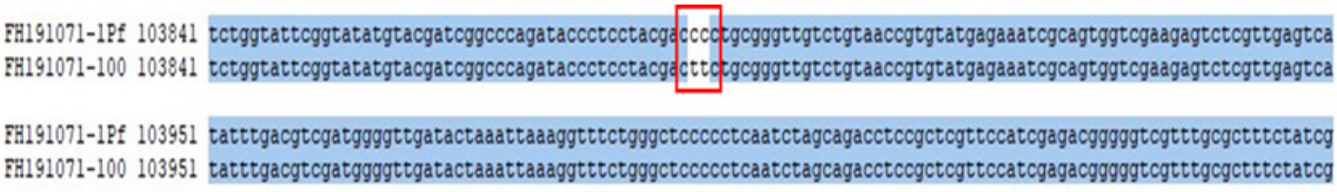

Fig. 4. Alignment of the predicted nucleotide sequences of the feline herpesvirus type-1 infected cell protein 0 (ICP 0) gene of FH1 and FH100. The site where the nucleotides are replaced is indicated by a red box.

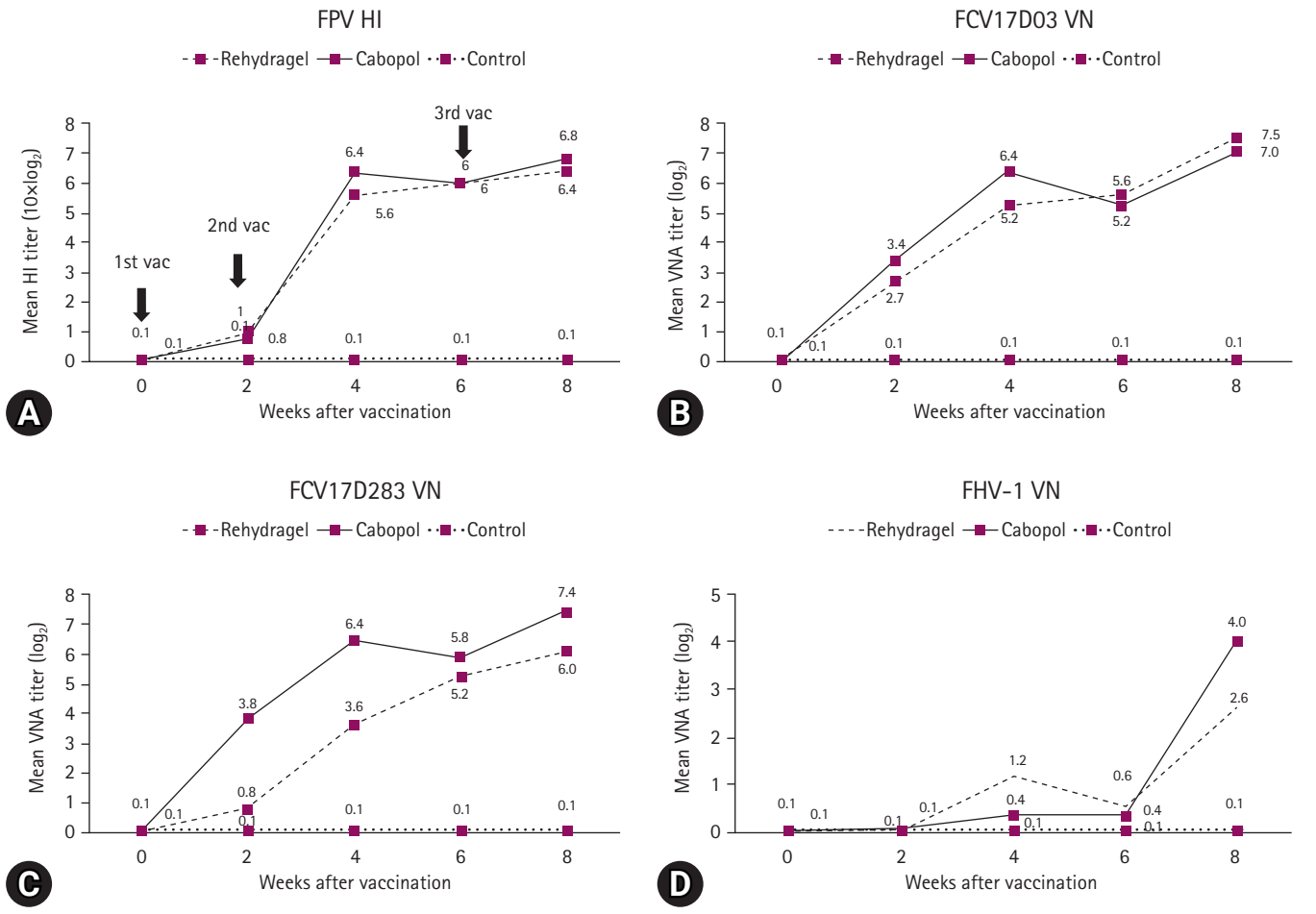

Fig. 6. Immune response in guinea pigs inoculated with two FPCCH vaccines. Vaccination of guinea pigs was carried out three times at two-week intervals. Hemagglutination inhibition (HI) titers (A) against feline panleukopenia virus (FPV) and virus-neutralizing antibody (VNA) titers (B-D) against two feline caliciviruses (FCVs) and feline herpesvirus type-1 (FHV-1). The VNA titers of feline rehydragel-adjuvanted vaccine and feline cabopol-adjuvanted vaccine against FCV17D03, and FCV17D283 differed at 2 week post-vaccination.

1 based vaccines have been used for cats in South Korea since the 2000s, FPV and FCV infections continue to be detected [18,19]. The low seropositive rates of FCV of 58.3% and FHV-1 of 44.0% in China could indicate improvements in the vaccine [20]. Genetically mutated FPV and FCV isolates have been identified in naturally infected Korean cats and continue to circulate [15–17], facilitating the development of a new vaccine against FPV, FCV, and FHV-1.

In this study, we selected representative feline viruses circulating in Korean cats as vaccine strains. Feline viruses such as FPV and FCV tend to evolve rapidly, so that variants can be found in the field [12,13]. Therefore, it is preferable to replace vaccine strains suitable for the national environment. Because the VP2 nucleotide homologies of the eight Korean FPV isolates were > 99.5%, FPV19D01 was selected as the vaccine strain based on its proliferative capacity [15]. The nucleotide homology of the VP2 gene of FCV isolates was 79.2% to 99.2% among five Korean FCV isolates; FCV17D03 and FCV17D283 showed 75.8% and 76.6% homologies with the current FCV vaccine strain, F9 [16]. A cross-neutralization test using serum from mice inoculated with two FCVs showed high VNA titers against homologous viruses. Two FCV strains were selected based on the divergence among five FCV isolates [16]. FHV191071 was selected as a vaccine strain based on its genetic characteristics [17].

The improved proliferative capacity obtained by serial passages in cells, mutations of amino acids, and deletion or insertion of genes enable differentiation of vaccine strains from wild-type FPV, FCV, and FHV-1. Artificial stresses such as ultraviolet light and continual passages can lead to mutation in viral genes [21]. FHV191071 was passaged 100 times after exposure for 30 seconds to UV light, triggering substitutions of 11 nucleotides

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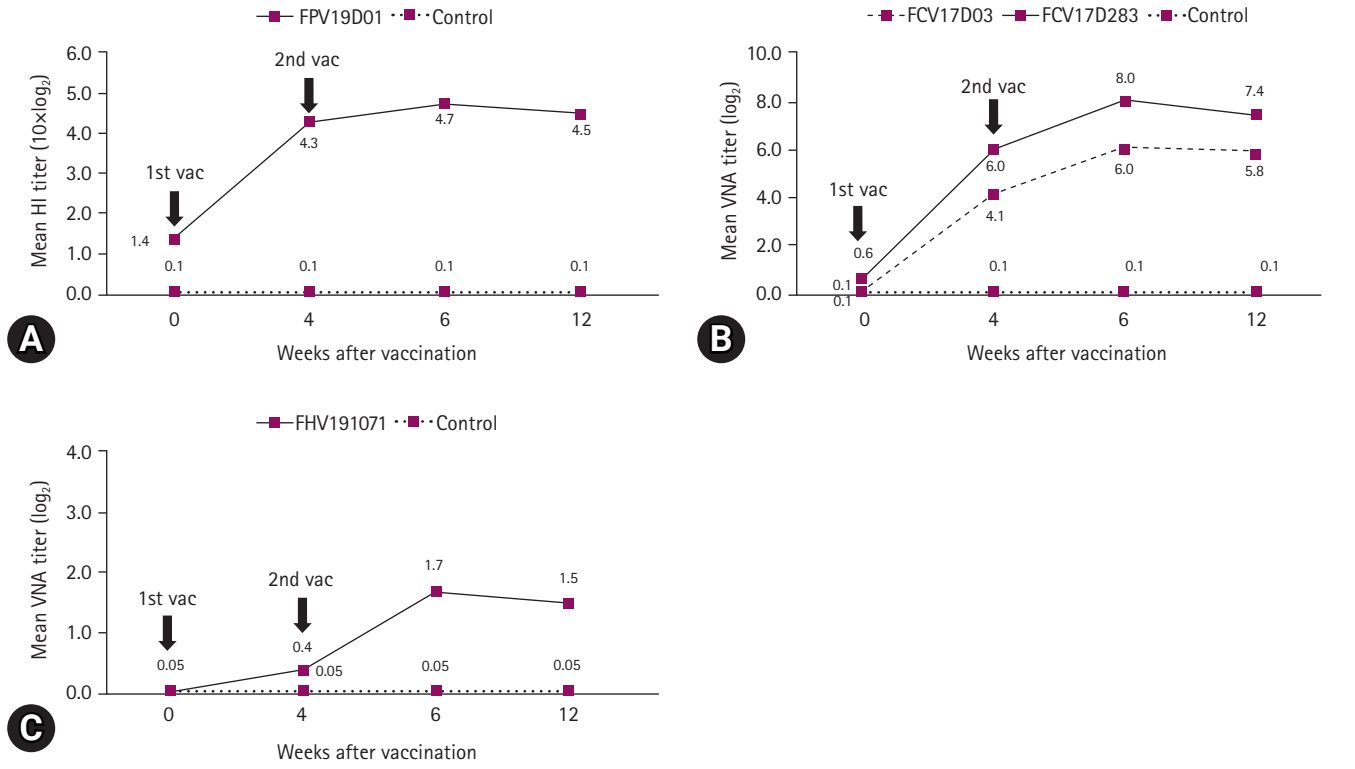

Fig. 7. Hemagglutination inhibition (HI) and virus-neutralizing antibody (VNA) titers against (A) feline panleukopenia virus (FPV), (B) two feline caliciviruses (FCVs), and (C) feline herpesvirus type-1 (FHV-1) in cats inoculated with the feline cabopol-adjuvanted vaccine.

in the entire genome of FH100. These mutations may distinguish parent or field FHV-1 strains from FH100, and thus can be considered genetic characteristics of the vaccine strain.

If an inactivated vaccine is to exhibit high immunogenicity, it is essential to select an optimal adjuvant. After evaluating the immunogenicity of two types of inactivated FRAV and FCAV in guinea pigs, we selected the FCAV for cats. The Cabopol adjuvant has been used to enhance cellular and humoral immunity in veterinary vaccines such as those against porcine reproductive respiratory syndrome virus and Newcastle disease virus [22,23]. Equine influenza vaccine containing Carbopol induced a higher level of longer-lasting antibodies than a vaccine containing aluminum phosphate in ponies [24]. Cats immunized with the FCAV vaccine developed a high mean HI titer of 259.9 ($10 \times 2^{4.7}$) at 2 weeks after booster vaccination. Because an HI titer ≥ 1:40 is protective [25], the FCAV is likely to protect against infection with wild-type FPV. The cats also developed high VNA titers of 64 and 256 against FCV17D03 and FCV17D283, respectively. These VNA titers against FCVs were similar to those in cats inoculated with a trivalent (FPV, FCV, FHV-1) inactivated vaccine developed in 2005 containing FCV-225 [26]. By contrast, vaccinated cats had a relatively low VNA titer of 3.2 against FHV-1. Many cats vaccinated with the conventional FHV-1 vaccine failed to show a more than fourfold increase in VNA titer [27]. VNA titer may be influenced by the dose and frequency of vaccination. In this study, we vaccinated cats twice to evaluate the immunogenicity of the FCAV vaccine. Therefore, tertiary vaccination for cats should be considered to increase the VNA titer against FHV-1. We did not conduct a challenge test with virulent virus in vaccinated cats; therefore, further studies are required.

In summary, we prepared an inactivated FCAV containing FP70, FC50, FC80, and FH100, featuring a 3-amino-acid mutation in the VP2 protein of FP70, 4- and 16-amino-acid substitutions in the VP1 proteins of FC50 and FC80, and 11 nucleotide substitutions in the full genome of FH100. The FCAV was more immunogenic in guinea pigs than the FRAV. The FCAV induced significant HI and VNA titers against FPV, FCV, and FHV-1 in cats.

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