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Immunogenicity of a novel inactivated canine adenovirus type 2 variant vaccine for dogs

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Purpose: The immunogenicity of vaccines containing the canine adenovirus (CAdV) type 2 (CAdV-2) variant has not yet been reported. We prepared a novel inactivated CAdV-2 variant vaccine using the CAV2232-41 strain, and evaluated its safety and immunogenicity in raccoon dogs.

Materials and Methods: The growth kinetics of CAV2232-41 were determined using Madin-Darby Canine Kidney (MDCK) cells. The nucleotide sequences of CAV2232 and CAV2232-41 were determined by next-generation sequencing. To generate the CAdV-2 variant vaccine, CAV2232-41 propagated in the MDCK cells was inactivated with 0.1% formaldehyde. Two vaccines were prepared by blending inactivated CAV2232-41 with Cabopol and Rehydragel adjuvants. Safety and immunogenicity of the CAV2232C and CAV2232R vaccines were evaluated in guinea pigs. Safety and immunogenicity of the CAV2232C vaccine were also evaluated in raccoon dogs. The virus neutralizing antibody (VNA) titer against CAV2232-41 was measured in sera collected from immunized guinea pigs and raccoon dogs.

Results: CAV2232-41 showed the highest viral titer on days 4–6 post-inoculation and had a deletion in the E3 gene, which was confirmed as a CAdV-2 variant. Guinea pigs inoculated with CAV2232C showed slightly higher VNA titers than those inoculated with CAV2232R 2 weeks after booster vaccination. Raccoon dogs immunized with the CAV2232C vaccine developed high mean VNA titers, while non-vaccinated raccoon dogs were antibody-negative.

Conclusion: The CAV2232C vaccine is safe and induces a protective VNA titer in raccoon dogs.

Keywords: Adenovirus, E3 protein, Raccoon dogs, Vaccine

Introduction

Canine adenoviruses (CAdVs), belonging to the Mastadenovirus genus, are divided into CAdV type 1 (CAdV-1) and CAdV type 2 (CAdV-2) based on their biological, pathogenic, and genetic characteristics [1-3]. The host spectrum of CAdVs is quite broad and includes dogs and raccoon dogs. The clinical symptoms caused by CAdVs are different owing to differences in cell tropism [4]. CAdV-1 and CAdV-2 cause infectious hepatitis and laryngotracheitis, respectively, and their pathogenicity is different. CAdVs are classified as virulent and low pathogenic [2]. Dogs infected with CAdV-2 alone exhibit weak clinical symptoms; however, simultaneous infection with canine distemper, bacteria, and CAdV-2 leads to serious clinical symptoms including bron-

chopneumonia [5,6]. Commercial attenuated CAdV vaccines reduce CAdV infections in dogs; however, the emergence of novel CAdV-2 variants in wild raccoon dogs poses new challenges [7]. New CAdV-2 variants may exhibit different pathogenic profiles and immune escape mechanisms, necessitating reassessment of current vaccination strategies and development of novel vaccines.

In Korea, CAdV-1 infection has been reported in Eurasian rover otter and fennec fox in 2007 and 2014, respectively [8,9]. CAdV-2 has been isolated from dogs and raccoon dogs in 2018 and 2022 [7,10]. The isolation of a CAdV-2 variant from raccoon dogs indicates a potential shift in the host range and adaptability of the virus. The presence of this variant in wild raccoon dog populations complicates the epidemiological landscape and raises concerns about cross-species transmission and the possibility of new reservoirs [11]. The effectiveness of current CAdV vaccines against CAdV-2 variant remains uncertain, prompting an urgent need for comprehensive immunogenicity studies [12,13]. Therefore, the evaluation of new variant vaccines is crucial to ensure continued protection of both domestic and wild canid populations and prevent the spread of CAdVs into other species.

In this study, we investigated the safety and immunogenicity of an emerging CAdV-2 variant vaccine. By isolating a CAdV-2 variant from raccoon dogs and preparing a novel trial-inactivated CAdV-2 variant vaccine, we aimed to evaluate the immunogenic response of raccoon dogs to the vaccine.

Materials and Methods

Viruses

A CAdV-2 variant strain, CAV2232, isolated from feces of a raccoon dog was passaged 41-times in Madin-Darby Canine Kidney (MDCK) cells (ATCC CCL-43; ATCC, Manassas, VA, USA) and was named CAV2232-41. The strain was used as an antigen for preparing the test vaccine. The CAdV-1 (Utrecht; National Center for Biotechnology Information accession no., M73811) and CAdV-2 (APQA1701; Korean Veterinary Culture Collection accession no., VR200005) strains were used for the virus neutralization test using raccoon dog serum.

Viral growth kinetics and propagation

Viral growth dynamics was assessed as previously reported [10]. Briefly, MDCK cells grown in 25-cm^2 cell culture flasks were inoculated with CAV2232-41 at 200 50% Tissue Culture Infectious Dose ($TCID_{50}$)/mL and harvested daily for a period of 7 days. After repeating the freeze-thaw process thrice, harvested virus was centrifuged and subjected to viral titration in 96-well microplates. Viral titers of CAV2232-41 were determined and quantified as $TCID_{50}$/mL. The virus was propagated after determining the highest viral titer. In brief, MDCK cells grown to 90% confluency in a 175-cm^2 flask were washed 3 times with phosphate-buffered saline (pH 7.2) and inoculated with CAV2232-41. The flask was placed within a CO_2 incubator for 1 hour, and the inoculum was replaced with 30 mL fresh alpha Minimum Essential Medium (MEM). CAV2232-41-infected MDCK cells were harvested 5 days post-inoculation. The flask was subjected to three freeze-thaw cycles, and the collected virus-containing fluid was used as the vaccine antigen.

Next-generation sequencing

Viral DNA was extracted from CAV2232 and CAC2232-41 suspensions using an AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea) following the manufacturer's instructions. The genomes of two viruses were sequenced (Sanigen Inc., Anyang, Korea) using next-generation technology. Nucleotide sequence alignments were carried out using Clone Manager 6 ver. 6.0 (Sci-Ed Software, Denver, CO, USA).

Preparation of vaccines

CAV2232-41 with a viral titer of $10^{6.5}$ $TCID_{50}$/mL was inactivated by incubating with 0.1% formaldehyde at 37°C overnight. The inactivation of CAV2232-41 was verified by the absence of viral growth in MD CK cells over 5 days. Two kinds of adjuvants, including aluminum hydroxide (Rehydragel LV; Chemtrade Logistics Inc., Toronto, ON, Canada), and Cabopol (Lubrizol, Wickliffe, OH, USA), were used. The second vaccine designated CAV2232C was prepared using 2 mg/mL Cabopol.

Animal experiments

All animal protocols were approved by the Institutional Animal Care and Use Committees of Animal and Plant Quarantine Agency (approval numbers, 2023-666 and 2023-690). Two 8-week-old guinea pigs were intramuscularly inoculated with 2 mL of CAV2232R or CAV2232C for safety testing. For immunogenicity, 16 guinea pigs were divided into two groups, and each guinea pig was immunized twice with one dose each of CAV2232C and CAV2232R vaccine at 2-week intervals. Blood was collected from the immunized guinea pigs. Ten wild raccoon dogs treated at a wildlife rescue center were used to assess the safety and immunogenicity of test vaccines. For the

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safety test, two raccoon dogs were subcutaneously inoculated with two doses (4 mL) of CAV2232C and observed for 14 days. For the immunogenicity test, six raccoon dogs were inoculated with one dose (2 mL) of CAV2232C twice at 2-week intervals. Two raccoon dogs were used as untreated controls. Blood samples were collected at 2-week intervals.

Virus neutralization test

The virus neutralization test was performed as previously described [10]. In brief, a 50 μL heat-inactivated serum of raccoon dogs or guinea pigs was diluted two-fold with alpha MEM, and an equal volume of 200 $TCID_{50}$/0.1 mL CAdVs was added into each well and incubated at 37℃ for 1 hour. Then, 100 μL alpha MEM containing 20,000 MDCK cells was added to each well. The plates were placed within an incubator with 5% CO_2 for 5 days. MDCK cells in each well were observed under a microscope to confirm cytopathic effect caused by the virus. Virus neutralizing antibody (VNA) titers against each CAdV were expressed as the reciprocal of dilution factor of serum that completely inhibited viral growth.

Statistical analysis

The values are expressed as mean±standard deviation. All statistical analyses were conducted using GraphPad Prism ver. 5.0 (GraphPad Software, San Diego, CA, USA; https://www.graphpad.com). Significant differences between groups were analyzed using one-way analysis of variance followed by Dunnett's multiple comparison test.

Results

Characterization of the CAV2232 strain

CAV2232-41 had the highest viral titer of $10^{6.5}$ $TCID_{50}$/mL on days 4–6 post-inoculation (Fig. 1). The full genome sequences of CAV2232 and CAV2232-41 were compared with that of the APQA1701 strain. CAV2232-41 had a deletion in the gene encoding the early region (E3) protein (Fig. 2A). The E3 gene of CAV2232-41 had 414 nucleotides, which was 681-nucleotide shorter than that of the APQA1701 strain (Fig. 2B). CAV2232-41 with only 414 nucleotides in the E3 gene was confirmed as

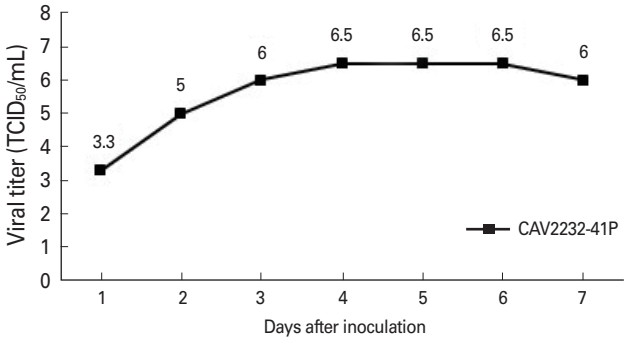

Fig. 1. Growth kinetics of the CAV2232-41 according to the day of harvest from MDCK cells. The CAV2232-41 strain showed the highest viral titer of $10^{6.5}$ $TCID_{50}$/mL on day 4 to 6 post-inoculation. $TCID_{50}$, 50% Tissue Culture Infectious Dose.

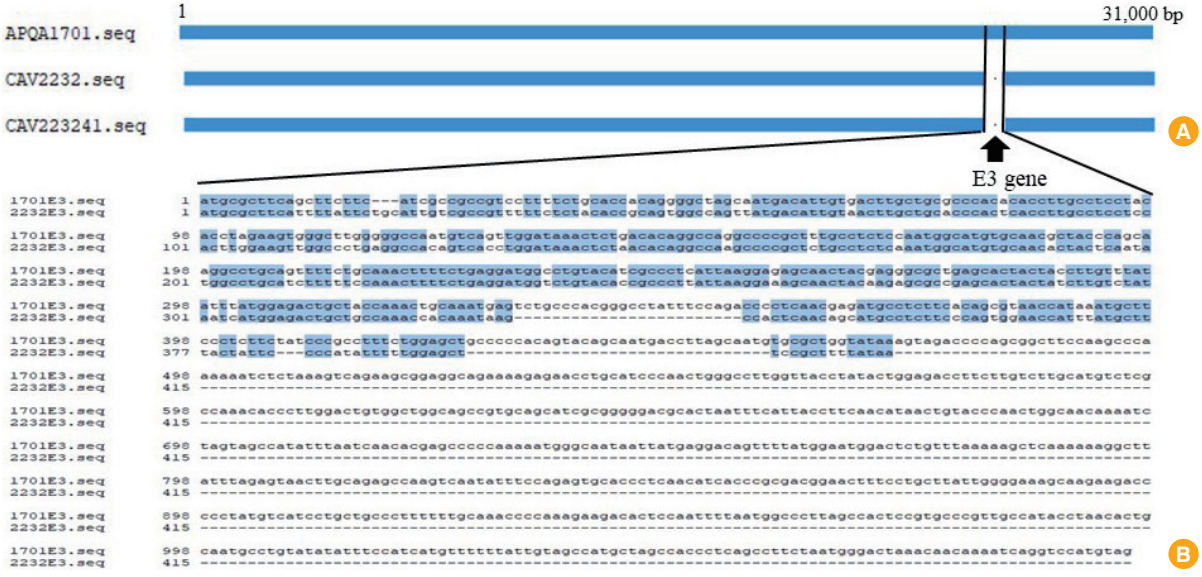

Fig. 2. Comparison of the entire nucleotide sequence among APQA1701, CAV2232 parent, and CAV2232-41 strain **(A)** and alignment of E3 genes between APQA1701 and CAV2232-41 strain **(B)**. The E3 gene of CAV2232-41 had 441 nucleotides, which was 681 shorter than that of APQA1701.

Table 1. Safety test of inactivated CAdV-2 variant vaccines in animals

Types of vaccine	Animal species	No. of animals	Dose/route	Clinical symptoms[a)]	Observation period (day)	Mean VNA titer
CAV2232R	Guinea pigs	2	2 mL/IM	Normal	7	-
CAV2232C	Guinea pigs	2	2 mL/IM	Normal	7	-
CAV2232C	Raccoon dogs	2	4 mL/SC	Normal	14	64

CAdV-2, canine adenovirus type 2; VNA, virus neutralizing antibody; IM, intramuscular; SC, subcutaneous.
[a)]Clinical symptoms, such as fever, cough, runny nose, and diarrhea, were monitored.

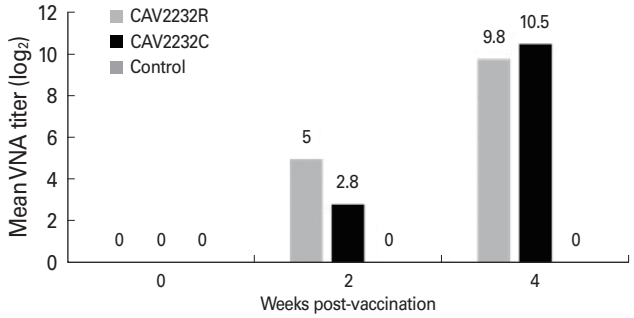

Fig. 3. Immunogenicity of CAV2232R and CAV2232C in guinea pigs. Guinea pigs immunized with CAV2232C induced slightly higher virus neutralizing antibody (VNA) titers than guinea pigs of inoculated with CAV2232R 2 weeks after the second inoculation.

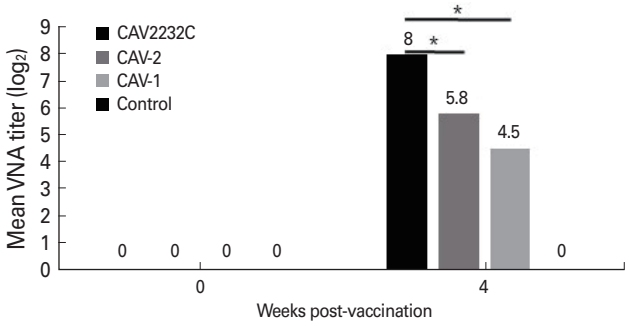

Fig. 4. Immunogenicity of CAV2232C in raccoon dogs. The raccoon dogs that received the second vaccination developed a virus neutralizing antibody (VNA) titer of 256 (2^8). After 4 weeks, the VNA titer of the immunized raccoon dogs could not be confirmed because they were released into the field in the area where they were captures.

a CAdV-2 variant.

Safety and immunogenicity of CAdV-2 variant vaccines in animals

Guinea pigs inoculated with CAV2232R or CAV2232C were clinically normal for 7 days. Raccoon dogs inoculated with two doses of CAV2232C remained clinically normal for 14 days and had a mean VNA titer of 64 (Table 1). To verify the immunogenicity of CAV2232R and CAV2232C vaccines, we immunized guinea pigs with two types of trial-inactivated CAV2232-41 vaccines. Guinea pigs inoculated with CAV2232R or CAV 2232C showed mean VNA titer of 891.4±29.8 or 1,448.2±210.5, respectively, 2 weeks after booster vaccination (Fig. 3). Based on the VNA titers of guinea pigs immunized with two types of CAV2232-41 vaccines, we selected CAV2232C as the target animal vaccine. Raccoon dogs immunized with CAV2232C had a mean VNA titer of 256±28 against the CAV2232 antigen, whereas unvaccinated raccoon dogs remained negative for VNA titers (Fig. 4). Raccoon dogs inoculated with CAV2232C had VNA titers of 55.7±25.8 and 22.6±24.5 against CAdV-2 and CAdV-1, respectively.

Discussion

Significant studies, such as isolation of CAdV-2 from dogs and raccoon dogs, have been conducted to prevent CAdV infections in Korea. Although CAdVs do not cause high mortality, they can threaten the lives of dogs when coinfected with other pathogens [13]. Interestingly, CAdV isolates have recently been reported in Korean and raccoon dogs [7,10]. We have previously reported that a new vaccine containing the Korean CAdV-2 isolate designated APQA1701-40P is safe and induces high VNA titers in dogs [14]. However, despite this progressive study, new CAdV-2 vaccines using the latest isolates from pets have not yet been industrialized, and CAdV infections have been continuously reported in canine populations [15-18]. Antibodies against CAdV-2 have also been identified in livestock such as cattle and pigs [13]. In addition, a new CAdV-2 variant, CAV2232, with a mutation in the E3 gene has been identified, which is involved in evading the host immune response, thereby suggesting the need for a new CAdV-2 variant vaccine. Therefore, we report the genetic characteristics of a CAdV-2 variant and safety and immunogenicity of a new inactivated CAdV-2 variant vaccine.

Analysis of the genetic characteristics of CAdVs will expand

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our understanding of CAdV and improve preventive measures. Two CAdVs, APQA1601 and CAdV-2/18Ra-54, isolated from dogs and raccoon dogs, respectively, had genetic characteristics similar to that of the vaccine strain, Toronto A26/61, indicating that the vaccine strain may be transmitted to dogs and raccoon dogs [7,10]. The CAV2232-41 strain exhibited unique genetic characteristics with an E3 gene of 441 bases. Interestingly, the shortened base of E3 gene of the CAV2232 strain was classified into a clade different from those of CAdV-1 and CAdV-2 in the phylogenetic tree. In addition, the CAV2232 strain showed 91.6% homology with the hexon gene of the Korea/2020/18Ra-65 strain, which was recently isolated from Korean raccoon dogs [7]. Another CAdV-2 strain, APQA1701-40P, with a deletion in the E1b-19K gene, showed only 86.9% homology with the fiber gene of the CAV2232 strain [14,19]. Genetic analysis of CAdVs can expand the scope for developing adenoviral vectors for gene therapy [20]. The identification of CAdV-2 variants in raccoon dogs is important for establishing preventive measures against transmission of this virus from raccoon dogs to companion dogs.

Many adjuvants, including aluminum hydroxide and Cabopol, have been used in veterinary vaccines [21]. Selecting the optimal adjuvant for the CAdV-2 variant is essential for improving immune response to the vaccine. We evaluated the safety and immunogenicity of the CAdV-2 variant vaccines containing adjuvants. The vaccines were safe for guinea pigs and induced high VNA titers after 2 weeks of booster vaccination. Similar results have been reported for an inactivated Cabopol-adjuvanted goose hemorrhagic polyomavirus vaccine [22]. Cabopol induces rapid differentiation of T cells and interferon producing cells [23]. Based on the immunogenicity in guinea pigs, the CAV2232C vaccine containing Cabopol adjuvant was selected as the target animal vaccine.

Immunogenicity testing of the CAV2232C vaccine in raccoon dogs provided an estimate of its efficacy. Previously, we have reported that an inactivated CAdV-2 vaccine of the APQA1701-40P strain induces protective immunity in dogs [14]. In the present study, raccoon dogs inoculated with CAV 2232C developed a high VNA titer of 256 2 weeks after the second vaccination, indicating that the CAV2232C vaccine can induce sufficient protective potential in raccoon dogs and is predicted to be protective for dogs. The protective antibody titer against CAdVs is more than the VNA titer ≥32 [24]. The mean VNA titers against CAdV-2 and CAdV-1 in serum were 55.7 and 22.6, respectively, indicating that the VNA titers for the homologous CAdV antigen were high, whereas those for the heterologous CAdV-1 strain were lower than the protective antibody level. Further studies on the pathogenicity of the CAV2232 strain in dogs are required because it is difficult to conduct pathogenicity or vaccine efficacy tests for the CAdV-2 variant in wild raccoon dogs.

In conclusion, the CAV2232C vaccine is safe and likely protective for raccoon dogs against CAdV infection.

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