Virology Journal

RESEARCH

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Prime-boost immunization with inactivated human adenovirus type 55 combined with an adjuvant enhances neutralizing antibody responses in mice

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Abstract

Background Human adenovirus type 55 (hAd55) infection can lead to acute respiratory diseases that often present with severe symptoms. Despite its persistent prevalence in military camps and communities, there are no commercially available vaccines or vaccine candidates undergoing clinical evaluation; therefore, there is an urgent need to address this. In this study, we evaluated the immunogenicity of inactivated hAd55 isolates and investigated the effects of adjuvants and various immunization intervals.

Methods and results To select a vaccine candidate, four hAd55 strains (6–9, 6–15 (AFMRI 41014), 28–48 (AFMRI 41013), and 12–164 (AFMRI 41012)) were isolated from infected patients in military camps. Sequence analysis revealed no variation in the coding regions of structural proteins, including pentons, hexons, and fibers. Immunization with inactivated hAd55 isolates elicited robust hAd55-specific binding and neutralizing antibody responses in mice, with adjuvants, particularly alum hydroxide (AH), enhancing antibody titers. Co-immunization with AH also induced hAd14-specific neutralizing antibody responses but did not induce hAd11-specific neutralizing antibody responses. Notably, booster immunization administered at a four-week interval resulted in superior immune responses compared with shorter immunization intervals.

Conclusions Prime-boost immunization with the inactivated hAd55 isolate and an AH adjuvant shows promise as a potential approach for preventing hAd55-induced respiratory disease. Further research is needed to evaluate the efficacy and safety of these vaccine candidates in preventing hAd55-associated respiratory illnesses.

Keywords Human adenovirus type 55, Acute respiratory disease, Respiratory infection, Inactivated viral vaccine, Neutralizing antibody

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Introduction

Human adenoviruses (hAds) are non-enveloped, double-stranded DNA viruses. Over 85 types, encompassing approximately 50 serotypes, have been identified and categorized into seven species (A-G) [1]. Each species exhibits distinct tissue tropism: species B, C, and E predominantly target the respiratory tract, while species F and G affect the gastrointestinal tract. These viruses cause various diseases, including gastroenteritis, diarrhea, and respiratory ailments. Children under four are primarily affected, but outbreaks also occur in hospitals, long-term care facilities, and military camps. Both young children and immunosuppressed adults are more susceptible to severe infections. Severe pneumonia can occasionally occur in immunocompetent individuals, sometimes resulting in fatalities.

Human adenovirus type 55 (hAd55) is generated via a hexon gene rearrangement between hAd11 and hAd14 [2]. This strain has emerged as a significant pathogen that causes acute respiratory diseases (ARD) in military encampments and broader communities [2–7]. Initial cases of hAd55 infection were documented in Spain in 1969, followed by subsequent reports from Singapore, China, France, Israel, the United States, and South Korea [3, 6–12]. Notably, 538 cases of infection were documented in military camps in South Korea between 2013 and 2018. Furthermore, despite the ongoing COVID-19 pandemic, an additional 60 infection cases among students were reported in China in 2020 [6, 7, 13]. Compared to other adenovirus genotypes, hAd55 induces more pronounced symptoms of pneumonia. This heightened virulence appears to be intricately linked to the specific tropism of hAd55 for airway and alveolar stem cells, coupled with its elevated infectivity attributable to the heightened affinity of its fibers for cellular receptors [11, 14–16]. Given the recent incidence of hAd55 infections and the pronounced disease severity observed within both military encampments and civilian communities, there are growing concerns regarding potential global transmission events similar to other respiratory infectious diseases such as severe acute respiratory syndrome (SARS), Middle East Respiratory Syndrome (MERS), and COVID-19 [17–24].

Similar to hAd55 in military encampments in South Korea, hAd4 and hAd7 have demonstrated significant prevalence in U.S. military training camps, with viral infections emerging as the primary causes of ARDs [25, 26]. Estimates indicate that 80% of recruits experience adenovirus infection during initial training, leading to hospitalization in approximately 20% of these cases [27]. Live oral vaccines for hAd4 and hAd7, introduced in 1971, led to a substantial decline in ARD cases [28, 29]. However, vaccine production ceased in 1996 owing to economic constraints, leading to a resurgence of adenovirus cases [30, 31]. The reintroduction of these vaccines in 2012, accompanied by year-round vaccination campaigns, resulted in an approximately 100-fold reduction in adenovirus infection rates among U.S. military recruit trainees and prevented an estimated 13,000 cases of acute febrile illness annually [32]. The fiscal analysis conducted in the study demonstrated that the vaccine results in annual savings of $50 million. This is based on an initial investment of approximately $107 million and the avoidance of illness-related expenditures, estimated at $3,838 per case. These outcomes underscore the potential viability of developing a vaccine targeting hAd55, which could significantly reduce the burden of hAd55-associated respiratory diseases.

The adenovirus capsid comprises three principal structural proteins: hexon, a penton base, and fiber, with hexon and fiber recognized as the primary antigens [33]. Investigations involving hAd3, hAd5, and hAd7 have shown that the hexon protein serves as a primary antigen-eliciting neutralizing antibody (nAb) [34–36]. Comprehensive analyses of the hAd55 hexon, including sequence alignment and three-dimensional structural elucidation through homologous modeling, have identified four distinct neutralizing epitopes [37]. Immunization studies investigating individual hexon peptides or hAd3 vectors expressing specific epitope peptides have corroborated the generation of nAbs that are not only specific to the respective peptides but also effective against hAd55 in murine models [37, 38]. These findings underscore the potential of developing hexon-specific nAbs as a viable strategy for developing hAd55 vaccine candidates. In another study investigating human sera that exhibits neutralizing activity against hAd55, the presence of nAbs targeting both hexons and fibers was confirmed [39]. In addition, removing fiber-specific antibodies (Abs) from sera significantly reduced the neutralizing efficacy against hAd55. This observation implies that nAbs against the fibers may also confer protective immunity against hAd55 infection. Consequently, strategies aimed at inducing nAbs against both hexons and fibers represent a compelling approach for ameliorating hAd55 infection. Therefore, an inactivated hAd55 vaccine targeting both hexons and fibers is a promising candidate for further vaccine development.

In the present study, we investigated the immunogenicity of inactivated hAd55 isolates in mice. In addition, we identified the optimal adjuvant and immunization interval that can enhance the humoral responses induced by the inactivated hAd55 vaccine.

Methods

Cells

Vero (European Collection of Authenticated Cell Cultures (ECACC) 88020401) and A549 (Korean Cell Line

Bank (KCLB) 10185) cells were cultured in minimum essential medium (MEM; Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FBS; Gibco) and penicillin-streptomycin (100 U/mL) (Gibco). The cell lines were sub-cultured every 3 to 4 days and maintained at 37 °C in an incubator with a 5% CO_2 atmosphere.

Vero cells were used for hAd amplification and A549 cells were used for the virus titration by plaque assay.

Viruses

hAd11 (KBPV-VR-63) was obtained from the Korea Bank of Pathogenic Viruses and hAd14 (ATCC-VR-15) was purchased from the ATCC. hAd55 was isolated from respiratory specimens from patients infected with adenovirus attending the Armed Forces Capital Hospital, with approval (AFMC-18004-IRB-18-005) from the Life Research Ethics Committee of the Armed Forces Medical Command. To isolate hAd55, 1×10^6 A549 cells were seeded in a 25T flask (SPL, Korea) one day before the experiment. Before virus inoculation, 800 µL of respiratory sample was mixed with 100 µL of nystatin (Sigma, N1638) and 100 µL of penicillin/streptomycin (Gibco BRL, 15140-122), reacted at 4 °C for 1 h, centrifuged at 2,000 rpm for 20 min at 4 °C, and the supernatant was collected. The A549 cell culture medium from the previous day was removed and washed twice using phosphate-buffered saline (PBS), and 500 µL of the pretreated sample was inoculated and allowed to adsorb for 1 h. Following this, 5 mL of inoculation medium (MEM containing 2% FBS) was added, and the mixture was incubated at 37 °C, 5% CO_2, and 90% humidity and observed for 5 days. After confirming the cytopathic effects (CPE), the supernatant was collected and centrifuged at 12,000 rpm at 4 °C, and the supernatant was collected to detect adenovirus via real-time (RT)-PCR.

hAd genotype identification

For genotyping of the isolated adenovirus, DNA was extracted from 200 µL of the isolated virus solution using the blood and tissue DNA purification kit (QIAGEN, Germany), according to the manufacturer's instructions. RT-PCR amplification and quantification were performed using a UF-150 Real-Time PCR System (Genesystem, Korea). The RT-PCR conditions for the genes were set as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 3 s, 58 °C for 4 s, and 72 °C for 4 s. The hAd55-specific primer sequences were 5′-CAGTGGATTGCAG AAGGT-3′ (forward), 5′-CAGCATAAATCGGTTTACT TTCTTC-3′ (reverse), and 5′-FAM-CCCGTAAAAGCT GAAGCTGA-BHQ1-3′ (probe).

Phylogenetic analysis

For phylogenetic analysis of hAd55, concatenated whole genomes of adenoviruses were used to calculate evolutionary distances. Multiple sequence alignments (MSA) were performed using MEGA X (version 10.0.5) [40]. The maximum composite likelihood model was used to construct neighbor-joining phylogenetic trees [41]. The phylogeny was tested using the bootstrap method, which was replicated 1,000 times.

For MSA analysis, full-length genome information from 15 hAd55 strains available on GenBank was collected, and four isolates from this study and four strains (6–9, 6–15 (AFMRI 41014), 28–48 (AFMRI 41013), and 12–164 (AFMRI 41012)) were analyzed using NCBI BLAST (version 2.2.26). The full-length genome sequence of the hAd55 strain AMFC16-0007 (KY471318.1) was used as the reference strain.

The resulting trees were visualized using FigTree software (version 1.3.1) (http://tree.bio.ed.ac.uk/software/figtree/).

Virus culture

Vero cells were prepared one day before the experiment by seeding 5×10^6 cells in T-75 flasks (Thermo Fisher Scientific, Rochester, NY, USA). The cells were infected with hAd at 1, 2.5, or 5 multiplicities of infection (MOI) in Opti-MEM (Gibco, Carlsbad, CA, USA) containing the diluted virus but without FBS. Visible CPE resulting from the generation of infectious adenoviruses were typically observed 2–4 days post-infection. After 2–4 days, the supernatant was harvested and centrifuged (Labogene, Seoul, South Korea) at 3,000 rpm for 10 min at 4 °C to remove the cell debris pellets. The clarified supernatant was then filtered using a 0.45 µM bottle-top filter (Corning, NY, USA). The viral titers were measured using a standard plaque assay.

Virus titration

To titrate hAds, 2.5×10^5 A549 cells were seeded into each well of a 12-well plate (NUNC, Roskilde, Denmark) one day before the experiment. The cultured viruses were serially diluted via a 10-fold dilution, transferred to a 12-well plate with confluent A549 cells, and the virus-cell mixture was incubated for 2 h at 37 °C. The supernatants then were removed, and the cells were covered with maintenance medium (2% FBS) containing 0.8% agarose (Lonza, Rockland, ME, USA). After 7 days of incubation, the cells were fixed with 4% paraformaldehyde (PFA, Hanlab, Chungju, South Korea). Plaques were visualized by staining with 2.3% crystal violet (Sigma-Aldrich Ltd., UK). The viral titers were expressed as plaque-forming units (PFU)/mL, calculated using the formula: PFU = [(number of plaques per well) × (dilution)]/(inoculum volume).

Virus purification

The cultured hAd11, hAd14, and hAd55 were purified using CaptoCore 700 resin (Cytiva, Middlesex County, MA, USA), and chromatographic experiments were performed using an Äkta™ Pure liquid chromatography system (GE Healthcare, Vélizy, France). The flow rates used in the chromatographic experiments were set according to the manufacturer's recommendations. Initially, the resin was equilibrated with PBS. After the cultured virus was loaded, the fractions were collected. These fractions were loaded onto Amicon® Ultracentrifugation filters (Merck Millipore, Billericia, MA, USA) and centrifuged at 4,000 rpm for 20 min at 4 °C to obtain concentrated hAd55.

hAd55 inactivation

The purified hAd55 was incubated with 0.01 mol of formaldehyde (Sigma-Aldrich Ltd., UK) at 37 °C for 24 h. After loading the inactivated virus onto Amicon® Ultracentrifugation filters (Merck Millipore), 5 mL of PBS (Gibco) was added to the samples, and the mixture was centrifuged at 4,000 rpm for 20 min at 4 °C. This purification process was repeated five times to remove formaldehyde. Subsequently, the inactivated virus was collected and stored in a deep freezer at -80 °C. To titrate the inactivated virus, an equal volume of live virus was subjected to the above-mentioned procedure without the addition of formaldehyde, and the viruses were titrated using the method described above and the viral titer was used to represent the titer of the inactivated virus.

Immunogenicity assessment in mice

All mouse experiments were conducted following protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the International Vaccine Institute according to the guidelines outlined in IACUC PN 2019-006 (approved on 5 June 2019). Six-week-old female BALB/c mice were purchased from Koatech (Pyeongtaek, South Korea). For assess the immunogenicity of 6–15 (AFMRI 41014), mice were immunized with 5×10^5, 1×10^6 or 2×10^6 pfu of inactivated hAd55 (6–15 (AFMRI 41014)) intramuscularly two times via two-week interval. Blood samples were collected from the retro-orbital plexus of anesthetized mice 2 weeks after the first and second immunizations. The mice were anesthetized for 5 minutes using 2% isoflurane gas inhalation. hAd55-specific binding Ab and nAb titers in the collected blood samples were measured using an enzyme-linked immunosorbent assay (ELISA) and the plaque reduction neutralization test (PRNT), as described below.

To evaluate the immunogenicity of hAd55 strains isolated from infected patients, mice were immunized intramuscularly with 1×10^6 PFU of inactivated hAd55 isolates (6–9, 6–15 (AFMRI 41014), 28–48 (AFMRI 41013), or 12–164 (AFMRI 41012)), with or without Imject alum adjuvant (Thermo Fisher), twice with a 2-week interval. The total injection volume was 50 µl, with PBS as the diluent. The Imject alum was diluted to one-fourth of the total volume, with the mixture containing 0.5 mg of aluminum hydroxide. Sera collection and Ab titers were assessed described in the dose-experiment.

For the adjuvant study, mice were immunized using the same regimen described above with 6–15 (AFMRI 41014) isolate combined with various adjuvants, including Imject alum (diluted to one-fourth of the total volume, containing 0.5 mg of aluminum hydroxide; Thermo Fisher), AddaVax (with equal volumes of antigen and AddaVax mixed; Invivogen, USA), dmLT (1 µg/mouse), alum hydroxide (AH; Invivogen, USA), or alum phosphate (AP; Invivogen, USA). AH was mixed with varying dilution factors ranging from 1:1 to 1:1/9 of the total sample volume. The amounts of AH included were 0.05 mg (1:1/9 dilution), 0.0625 mg (1:1/7 dilution), 0.083 mg (1:1/5 dilution), 0.125 mg (1:1/3 dilution), and 0.25 mg (1:1 dilution). Similarly, AP was mixed with dilution factors ranging from 1:1 to 1:1/9 of the total sample volume. The amounts of AP included were 0.025 mg (1:1/9 dilution), 0.03125 mg (1:1/7 dilution), 0.0417 mg (1:5 dilution), 0.0625 mg (1:3 dilution), and 0.125 mg (1:1 dilution). The injection volume totaled 50 µl, with PBS used as the diluent. The adjuvant dosages used in this experiment were selected based on the manufacturer's suggestion (AddaVax, AH and AP) or results from internal mouse experiments (Imject Alum and dmLT). Antibody titers were analyzed using the same method as described above.

To assess the effect of vaccination interval on hAd55-specific antibody induction, mice were immunized intramuscularly with 1×10^6 PFU of 6–15 (AFMRI 41014) isolate, with or without AH (1:1/7 dilution), twice at intervals of 1-, 2-, or 4 weeks. Blood samples were collected before the second immunization and 2 weeks after the second immunization and analyzed using ELISA and PRNT.

Enzyme-linked immunosorbent assay (ELISA)

The inactivated hAd55, initially at a concentration of 6×10^7 PFU/mL, was diluted in a 1:100 with PBS (Thermo Fisher, USA), and 100 µL of the dilution was added to each well of a 96-well plate (Thermo Fisher, USA). The plates were incubated overnight at 4 °C. The wells were washed three times with washing buffer (PBS with 0.05% tween-20 (Sigma, USA)), and 100 µL of blocking buffer (2% BSA (Sigma, USA) in PBS containing 0.05% Tween-20) was added to each well, followed by a 1-hour incubation at 37 °C. Mouse serum samples were diluted 1:30 in an antibody buffer (0.5% BSA in PBS containing 0.05% Tween-20). After blocking, the wells were washed

three times, and 100 µL of the prepared sera samples was added into each well, followed by 5-fold serial dilutions. The plates were incubated overnight at 4 °C. The wells were then washed three times, and 100 µL of goat anti-mouse HRP-labeled IgG (Southern Biotech, USA), diluted 1:3,000 in antibody buffer, was added to each well and incubated for 1 h at 37 °C. After washing three times, 100 µL of TMB solution (Millipore, USA) was added for color development. The reaction was terminated by adding 100 µL of 1 N HCl (Merck, USA) to each well. Absorbance was measured at 450 nm using a SpectraMax 340PC384 microplate reader (Molecular Devices, USA), and the obtained data were analyzed using SoftMax Pro 5.4.1 software (Molecular Devices) to determine the titers (log2).

Plaque reduction neutralization test (PRNT)

The presence of neutralizing antibodies against hAd11, hAd14, and hAd55 in the sera of vaccinated animals was assessed using the PRNT. A549 cells were prepared the day before the experiment by seeding 2.5×10^5 cells per well in a 12-well plate (NUNC, Roskilde, Denmark). Serum samples from immunized mice, serially diluted two-fold starting from 1:20, were mixed in a 1:1 ratio with 100 PFU of hAd11, hAd14, or hAd55 and incubated for 60 min at 37 °C. The mixtures were transferred to a 12-well plate with confluent A549 cells for 2 h at 37 °C. After incubation, the mixtures were removed, and the cells were overlaid with maintenance medium (2% FBS) containing 0.8% agarose (Lonza). After 7 days of incubation, the cells were fixed with 4% PFA (Hanlab), and plaques were visualized by staining with 2.3% crystal violet (Sigma-Aldrich Ltd., UK). The $PRNT_{50}$ was determined as the serum dilution that resulted in a 50% plaque reduction compared with the plaque formation observed in the virus-only control.

Statistical analysis

The data on graphs is presented as the means with error bars representing the standard deviation (sd). Statistical analyses were performed using the unpaired *t*-test. Statistical significance was defined as $P<0.05$.

Results

Isolation and genotypic analysis of hAd55

To select an hAd55 vaccine candidate, four hAd55 (6–9, 6–15 (AFMRI 41014), 28–48 (AFMRI 41013), and 12–164 (AFMRI 41012)) strains were isolated from males with an average age of 22 years, with no underlying diseases. The patients were presented with mild symptoms, including fever, cough, and sore throat. To analyze the genotypes of the hAd55 isolates, PCR and RT-PCR were performed using an hAd55-specific primer set. PCR analysis revealed 175 hAd55-specific base pair bands in all samples, as confirmed by electrophoresis (Fig. 1A). Subsequent RT-PCR assays using SYBR green or TaqMan probes consistently detected hAd55-specific amplification across all samples (Fig. 1B and D). Additionally, culturing the isolated samples in A549 cells resulted in typical CPE (Fig. 1C). Through genotypic and phenotypic analyses, all isolated viruses were identified as hAd55.

Phylogenetic and sequence analysis of the isolated hAd55

Phylogenetic analysis confirmed that all the hAd55 isolates belonged to the hAd55 clade (Fig. 2A). A more comprehensive analysis of the hAd55 clade showed that adenoviruses isolated from Korea and China diverged in 2016 and evolved differently (Fig. 2B). These findings suggest that the predominant hAd55 strains in Korea and China form distinct clusters within their respective regions, indicating regional and temporal evolution through point mutations. The phylogenetic tree analysis further revealed that the isolated hAd55 strains from Korea in the first half (pneumonia cases) and second half (mild respiratory symptoms) of 2016 constituted a separate cluster among strains isolated from patients with pneumonia and those with common cold symptoms. The MSA results revealed no mutations in the genes, including structural proteins such as penton, hexon, and fiber, when compared with the reference hAd55 strain AMFC16-0007 (KY471318.1) (Fig. 2C and D). However, single nucleotide polymorphisms (SNPs) were identified in the untranslated region (UTR). These findings highlight the genetic conservation of key structural genes among the hAd55 isolates as well as the variability in the non-coding regions.

Immunization with inactivated hAd55 induced both binding and neutralizing Abs in mice

Prior to assessing the immunogenicity of the hAd55 isolates, we first determined the optimal dose of inactivated hAd55 without adjuvant using the 6–15 (AFMRI 41014) strain. Mice were immunized intramuscularly with 5×10^5, 1×10^6, or 2×10^6 pfu of inactivated hAd55 (6–15 (AFMRI 41014)), administered twice at a two-week interval. Sera collected from the immunized mice were then analyzed for hAd55-specific binding and neutralizing antibody titers (Fig. 3A). Although there was no statistically significant difference in the hAd55-specific binding antibody titers induced by the three different doses, the 1×10^6 pfu dose of inactivated hAd55 (6–15 (AFMRI 41014)) elicited higher hAd55-specific neutralizing antibody titers compared to the 5×10^5 and 2×10^6 pfu doses (Fig. 3B and C). Consequently, we selected 1×10^6 pfu as the immunization dose for the subsequent experiments.

Although the four hAd55 isolates share identical structural proteins without mutations, we tested all the isolates for immunogenicity due to their differences from

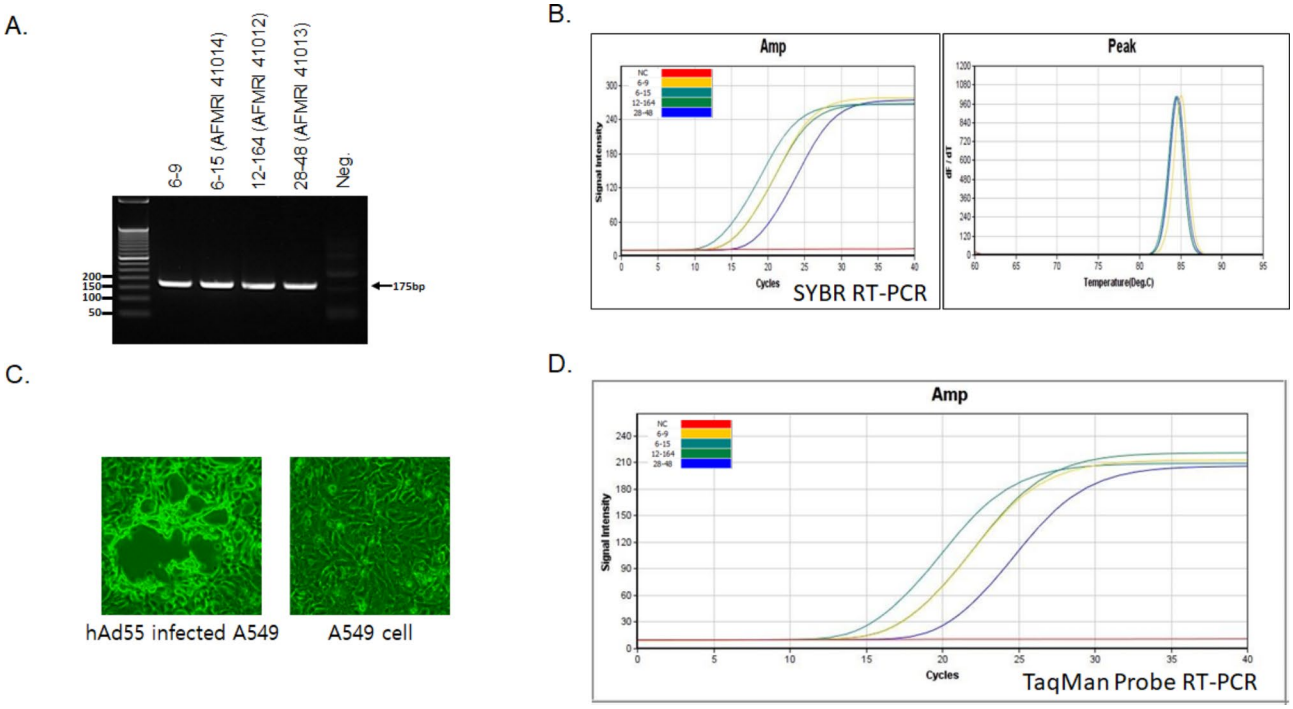


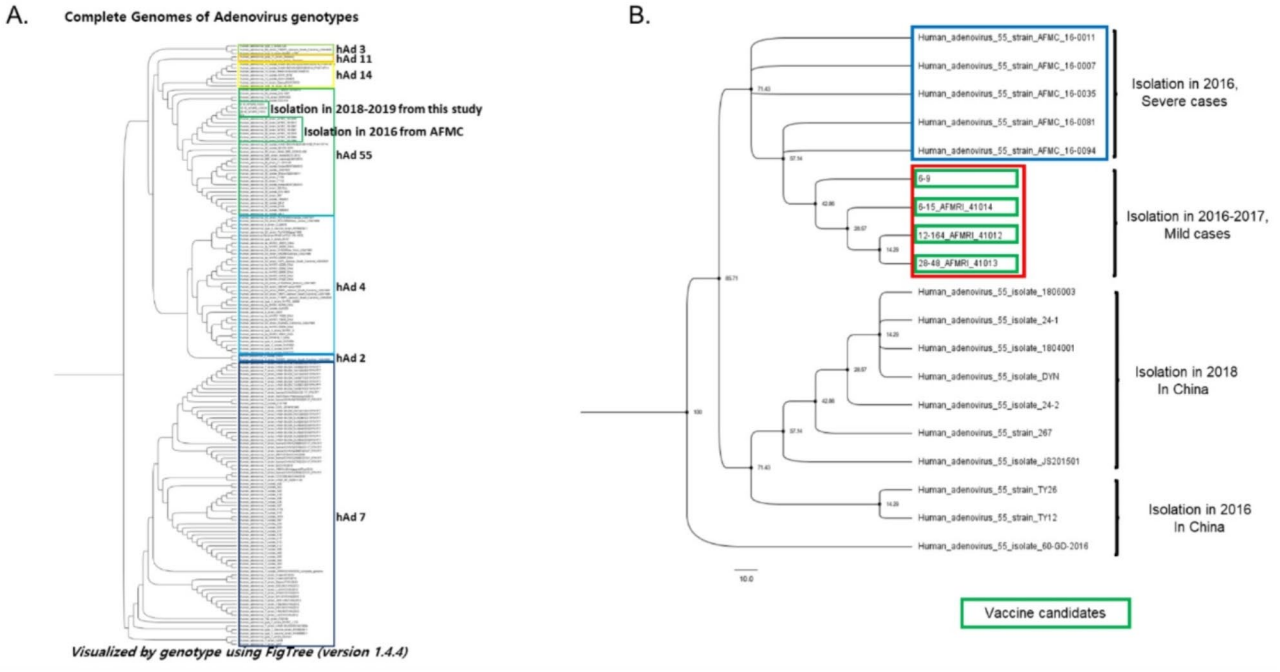
Fig. 1 Isolation and genotyping of human adenovirus type 55 (hAd55). **(A)** Conventional PCR for vaccine candidates. (**B)** Real-time PCR using SYBR. (red: negative control (NC); yellow: 6–9; dark blue: 6–15 (AFMRI 41014); sea blue: 12–164 (AFMRI 41012); blue: 28–48 (AFMRI 41013)). (**C)** The cytopathic effects (CPE) in A549 cells induced by hAd55. (**D)** Real-time PCR using a TaqMan probe for the hAd55-specific primer. (red: negative control (NC); yellow: 6–9; dark blue: 6–15 (AFMRI 41014); sea blue: 12–164 (AFMRI 41012); blue: 28–48 (AFMRI 41013)). Neg.: negative control

previously identified isolates in South Korea. Therefore, it was necessary to conduct a comprehensive characterization of the isolates, including an assessment of their immunogenicity. To evaluate the immunogenicity of hAd55 isolated from infected patients, mice were immunized with inactivated hAd55 isolates (6–9, 6–15 (AFMRI 41014), 28–48 (AFMRI 41013), or 12–164 (AFMRI 41012)), with or without Imject alum, and hAd55-specific binding Ab titers were measured using ELISA. Across all groups, co-immunization with inactivated hAd55 isolates and Imject alum induced higher hAd55-specific binding Ab titers than immunization with inactivated hAd55 isolates alone (Fig. 4A). Thirteen days after the first immunization, the group immunized with inactivated 12–164 (AFMRI 41012) combined with Imject alum exhibited elevated hAd55-specific binding Ab responses compared with the other groups. However, following booster immunization, mice injected with the inactivated 6–9 isolate and Imject alum demonstrated higher hAd55-specific binding Ab levels compared to other inactivated hAd55 isolates combined with Imject alum. Although not statistically significant, the inactivated 6–15 (AFMRI 41014) isolate induced higher hAd55-specific binding Ab levels than the 28–28 (AFMRI 41013) or 12–164 (AFMRI 41012) isolates.

Subsequently, nAb titers were evaluated using the PRNT in mice immunized with inactivated 6–9 or 6–15 (AFMRI 41014) isolates 27 days after the first immunization (Fig. 4B). Although not statistically significant, Imject alum increased the hAd55-specific nAb titers induced by immunization with inactivated 6–9 or 6–15 (AFMRI 41014) isolates. Moreover, immunization with inactivated 6–9 or 6–15 (AFMRI 41014) combined with Imject alum induced similar hAd55-specific nAb titers in mice. These findings suggest that inactivated hAd55 can elicit hAd55-specific Ab responses that are further enhanced by co-immunization with Imject alum adjuvant.

Co-immunization of inactivated hAd55 with alum hydroxide increased the Ab responses

Given that Imject alum, which contains AH, increased hAd55-specific Ab responses following immunization with an inactivated hAd55 isolate, we investigated whether other adjuvants could similarly enhance Ab responses and identified the most effective adjuvant. All adjuvants induced higher hAd55-specific binding Ab titers in mice than in mice immunized with inactivated 6–15 (AFMIR 41014) alone (Fig. 5A). Thirteen days after the first immunization, hAd55-specific binding antibody titers were similar across the groups. However, after booster immunization, AH (at ratios of 1:1/5 and 1:1/7) induced higher hAd55-specific binding Ab responses than the other adjuvant groups. Although there is no



C.

	Position *	Reference	Isolates								
		16-0007	16-0011	16-0035	16-0074	16-0081	16-0094	16-0096	6-9	6-15	12-164	28-48
SNPs	223	T	T	**A**	T	T	T	T	T	T	T	T
	285	T	T	**A**	T	T	T	T	T	T	T	T
	346	A	**G**	A	A	A	A	A	A	A	A	A
	1,477	T	T	T	T	T	**K**	T	T	T	T	T
	24,461	G	G	G	G	G	G	G	G	G	G	**A**#

: hexon assembly-associated protein region (no amino acid change)

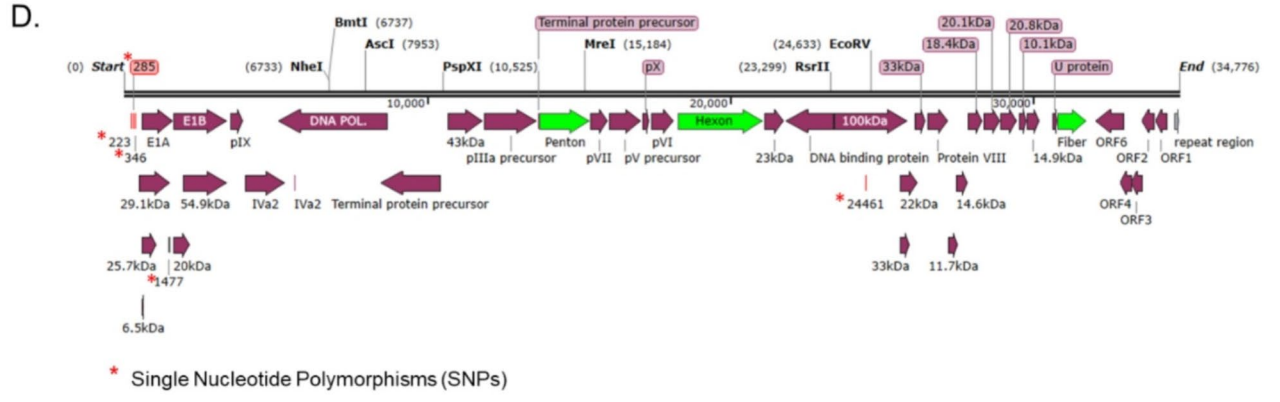


Fig. 2 Phylogenetic analysis of the hAd55 isolates. **(A)** Complete genomes of adenovirus genotypes. (**B)** Phylogenetic tree for hAd55 isolates. (**C)** Single nucleotide polymorphism (SNP) analysis of hAd55 isolates. (**D)** The specific locations of SNPs within the genomic nucleotide sequence of hAd55

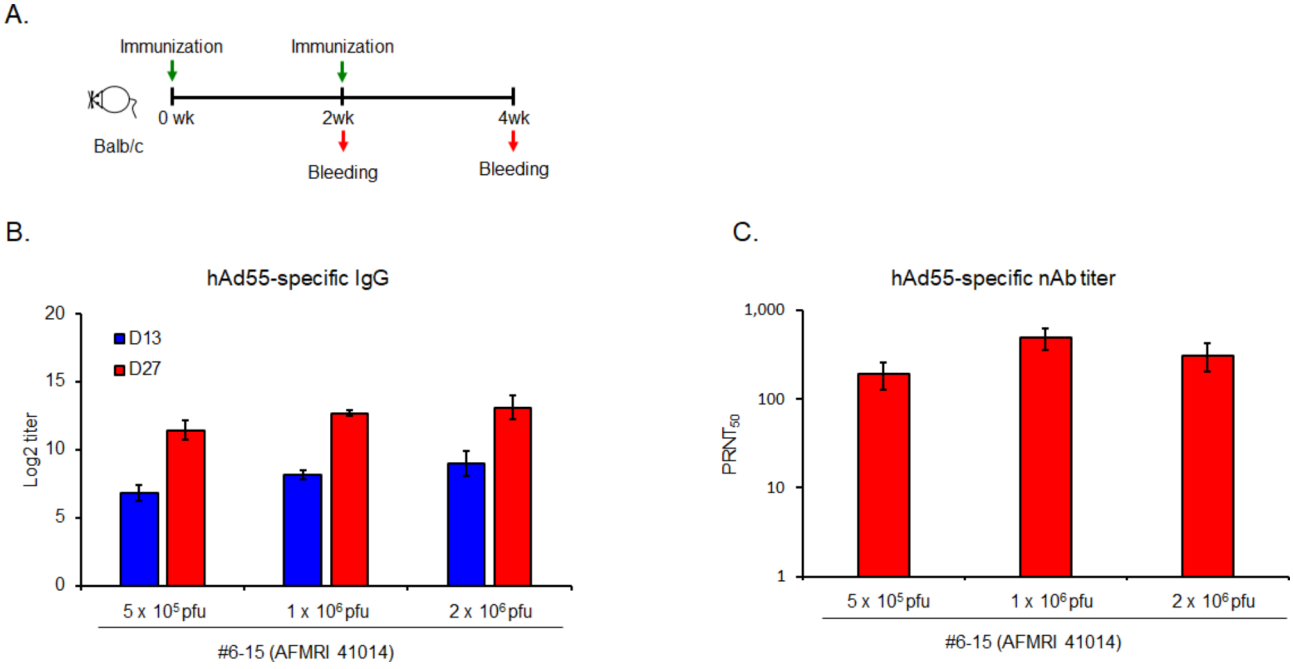


Fig. 3 Immunogenicity assessment of inactivated hAd55 (6–15 (AFMRI 41014)) at three-different doses. Mice were immunized intramuscularly with 5×10^5, 1×10^6, or 2×10^6 pfu of inactivated hAd55 (6–15 (AFMRI 41014) two times via two-week interval **(A)**. hAd55-specific binding antibody titers **(B)** and neutralizing antibody titers **(C)** were analyzed by ELISA or PRNT using sera collected two weeks after the first and second immunizations (for ELISA) or after the second immunization (for PRNT)

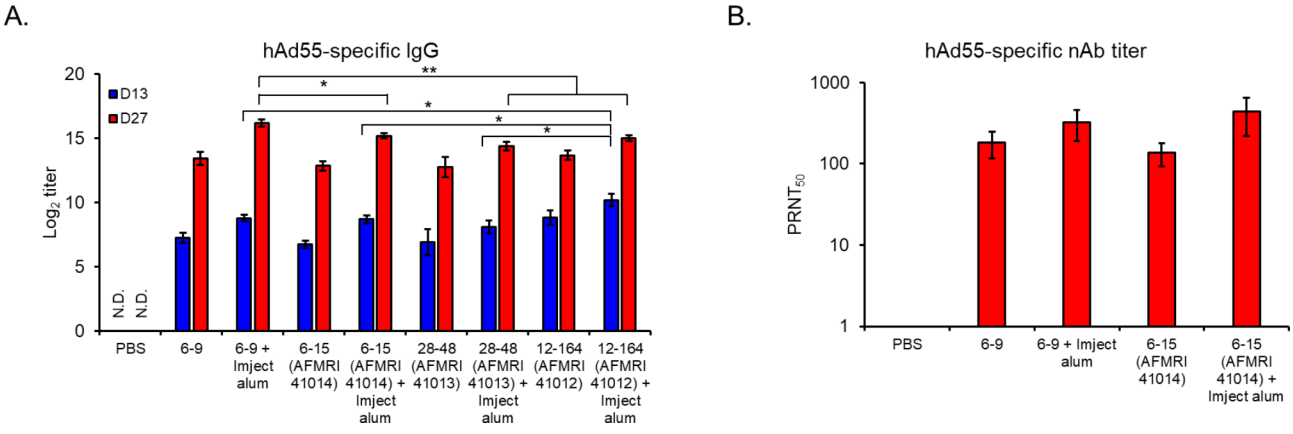


Fig. 4 Immunogenicity assessment of inactivated hAd55 isolates. Mice were immunized intramuscularly with PBS or hAd55 isolates (6–9, 6–15 (AFMRI 41014), 28–48 (AFMRI 41013), or 12–164 (AFMRI 41012)), with or without Imject alum, twice at two-week intervals. (**A**) To measure hAd55-specific binding antibody titers, sera were collected two weeks after each immunization, and an enzyme-linked immunosorbent assay (ELISA) was performed. (**B**) To assess hAd55-specific neutralizing antibody (nAb) titers, the plaque reduction neutralization test (PRNT) was performed using sera collected two weeks after the second immunization. N.D.: not detected, $^*P<0.05$, $^{**}P<0.01$

statistical difference in the induced nAb titers between 1:1/5 diluted AH and 1:1/7 diluted AH, the variation in nAb titers were higher in the 1:1/5 AH group compared to the 1:1/7 AH group (Fig. 5B). Mice immunized with 1:1/5 to 1:1/9 ratio concentrations of AH exhibited increased hAd55-specific nAb titers in a dose-dependent manner. These results suggest that co-immunization with inactivated hAd55 and AH, as opposed to other adjuvants, is an effective strategy for augmenting hAd55-specific Ab responses.

Immunization with inactivated hAd55 combined with AH induced hAd14-specific neutralizing antibodies but not hAd11-specific neutralizing antibodies

Since hAd55 is generated via hexon gene rearrangement between hAd11 and hAd14, we investigated whether immunization using inactivated hAd55 (AFMRI 41014)

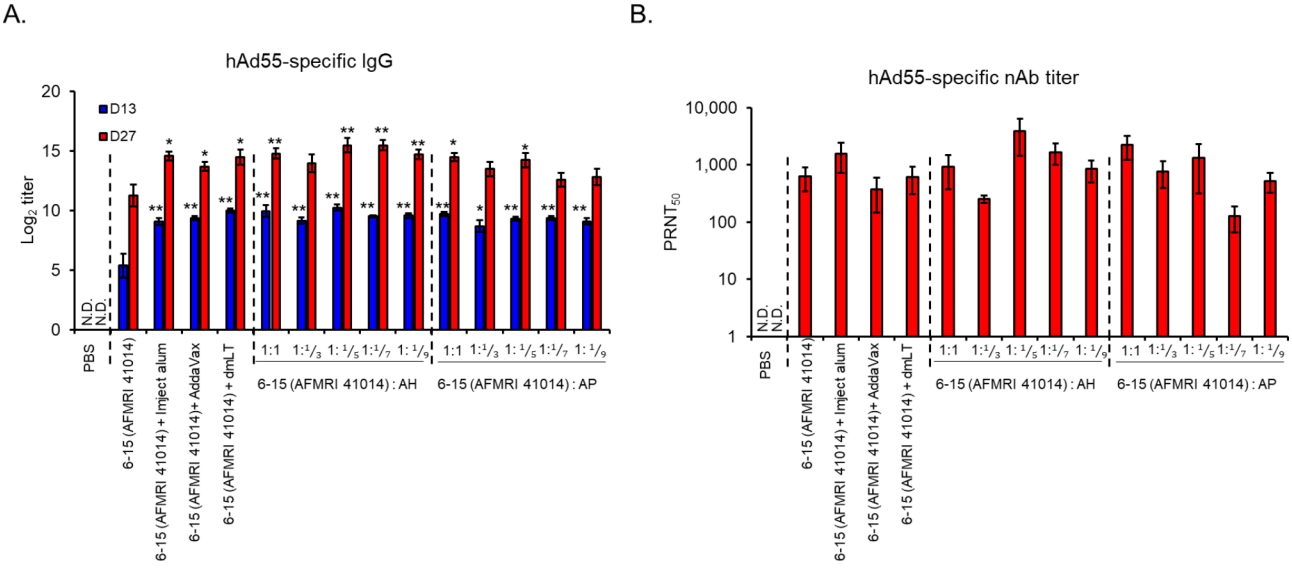
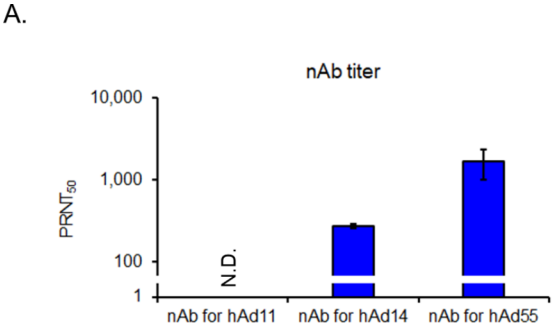


Fig. 5 Comparison of adjuvants for inactivated hAd55. Mice were immunized intramuscularly with the hAd55 isolate 6–15 (AFMRI 41014), with or without adjuvants (Imject, AddaVax, dmLT, AH, or AP), or PBS twice at a two-week interval. Various amounts of alum adjuvant were tested for AH and AP, as described in the Materials and Methods section. The adjuvant effects were assessed in terms of hAd55-specific binding antibody titers (**A**) and nAb titers (**B**). Neutralizing antibody titers were assessed using sera collected 2 weeks after the second immunization. AH: alum hydroxide; AP: alum phosphate; N.D.: not detected. *$P < 0.05$ vs. 6–15 (AFMRI 41014), **$P < 0.01$ vs. 6–15 (AFMRI 41014)



B.

	PRNT$_{50}$		
	For hAd11	For hAd14	For hAd55
6-15 (AFMRI 41014) + AH (1:1/7 ratio)	0	276	1,683

Fig. 6 Assessment of hAd11 and hAd14-specific nAb induction following immunization with inactivated hAd55 combined with adjuvants. Sera from mice immunized with 6–15 (AFMRI 41014) plus AH (1:1/7) were assessed for hAd11- and hAd14-specific nAb titers using a PRNT. Bar graph (**A**) and table (**B**) for PRNT$_{50}$ values of hAd11, hAd14, and hAd55. N.D.: not detected

combined with adjuvant AH (1:1/7 ratio) induces nAbs against hAd11 or hAd14.

Sera from mice immunized with inactivated hAd55 (AFMRI 41014) and either AH (1:1/7 ratio) exhibited no detectable neutralizing activity against hAd11 (Fig. 6A). However, notable neutralizing activity was observed against hAd14, with PRNT$_{50}$ values of 276. In comparison, the nAb titers for hAd55 were significantly higher, with PRNT$_{50}$ values of 1,683.43 for AH (1:1/7 ratio) (Fig. 6B). Relative to the nAb titers for hAd55, the hAd14-specific nAb titers induced by inactivated hAd55 combined with AH (1:1/7 ratio) was 16.40%. These results indicate that immunization with inactivated hAd55 (AFMRI 41014) combined with adjuvants can induce nAb responses against hAd14 in mice.

Two-time immunization with inactivated hAd55 at a four-week interval induced a stronger Ab response than shorter intervals

The immunization interval can significantly influence the magnitude of vaccine-induced Ab responses. Therefore, we sought to determine the optimal immunization interval to enhance hAd55-specific Ab responses. Mice were immunized twice with the inactivated 6–15 (AFMIR 41014) isolate combined with AH at intervals of one, two, or four weeks, and the sera were analyzed for hAd55-specific binding Ab and nAb titers (Fig. 7A). Mice co-immunized with the inactivated 6–15 (AFMIR 41014) combined with AH twice with a 4-week interval exhibited higher hAd55-specific binding Ab titers than those immunized at 1- or 2-week intervals (Fig. 7B). However, two-time immunization with a two-week interval resulted in higher hAd55-specific binding Ab titers

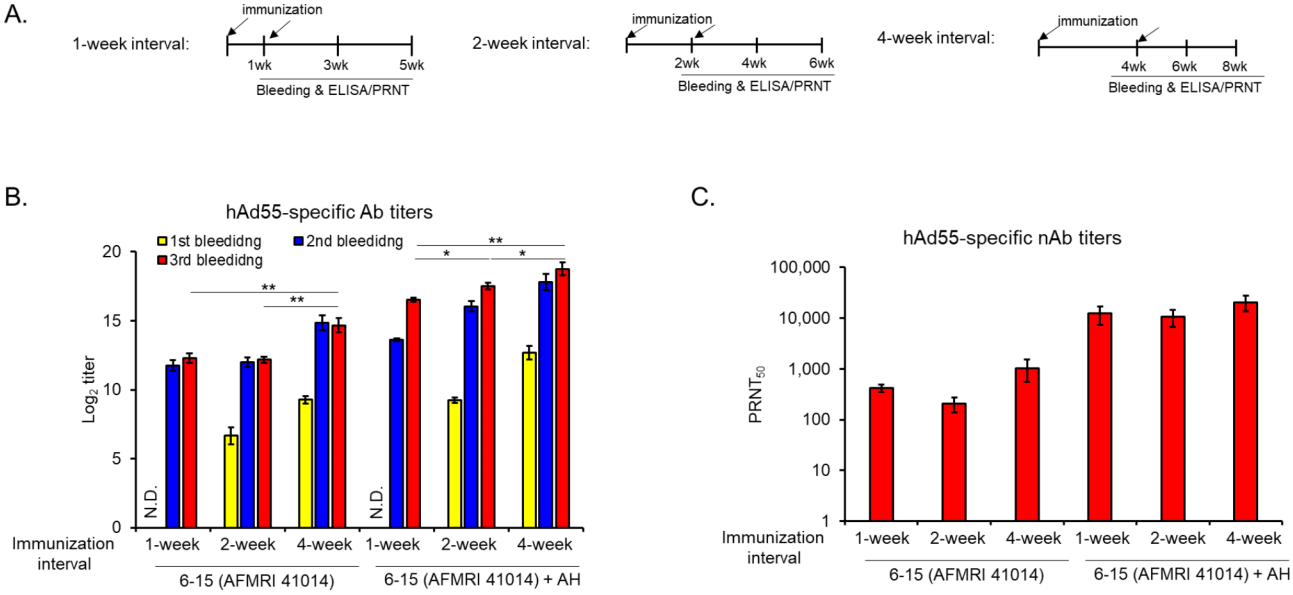


Fig. 7 Comparison of immunization intervals for inactivated hAd55. **(A)** Mice were immunized intramuscularly with the hAd55 isolate (6–15 (AFMRI 41014)), with or without AH, twice at one-, two-, or four-week intervals, and sera were collected at the indicated time points. (**B**) hAd55-specific binding antibodies were assessed by ELISA in sera collected 0, 2, and 4 weeks after the last immunization. (**C**) hAd55-specific nAb titers were assessed by PRNT in sera collected two weeks after the last immunization. AH: alum hydroxide, N.D.: not detected, $^{*}P < 0.05$, $^{**}P < 0.01$

compared to a one-week interval. While there was no statistically significant difference in neutralizing Ab titers between the groups (Fig. 7C), booster immunizations with a 4-week interval appeared to induce hAd55-specific neutralizing Abs in mice more effectively. In conclusion, our findings suggest that immunization with inactivated hAd55 (6–15 (AFMIR 41014)) combined with AH twice with a four-week interval represents an optimal immunization regimen for inducing hAd55-specific binding Abs in mice.

Discussion

In the present study, we evaluated the immunogenicity of inactivated hAd55 isolates and investigated the effects of adjuvants and various immunization intervals. Our results demonstrated that inactivated hAd55 derived from infected patients can induce hAd55-specific nAbs in mice and that Ab responses can be increased by co-immunization with AH, a human-applicable adjuvant. We also demonstrated that a two-time injection at a four-week interval was the optimal immunization regimen for inactivated hAd55 in mice.

In a previous study, a chimeric adenovirus incorporating hAd3-replated hexon hypervariable region 5 with the corresponding region from hAd7, along with the hAd55 hexon, was investigated as a potential vaccine candidate [38]. This trivalent vaccine candidate elicited hAd55-specific nAbs along with type 3- and 7-specific nAbs in mice. While hexon-specific Abs play a crucial role in preventing adenovirus infection, fiber-specific Abs also contribute significantly to the prevention of viral infection [39]. Additionally, immunization with inactivated hAd55 induced higher hAd55-specific nAb titers in mice than immunization with inactivated hAd14 containing hypervariable regions of the hAd55 hexon, the main epitope region of the hexon. Therefore, solely targeting hexons, as chimeric adenovirus vaccines do, may not be an optimal strategy. Conversely, a vaccine approach that induces antibodies targeting both hexons and fibers, such as employing the whole virus as a vaccine candidate, as demonstrated in this study, may confer superior protective efficacy and represent a promising approach.

Although live hAd4 and hAd7 vaccines are used by the U.S. military and live chimeric virus vaccine candidates were explored in a previous study, we opted to use an inactivated formulation of the hAd55 vaccine candidate because this formulation has a superior safety profile to live adenovirus. Furthermore, oral administration of live hAd4 and hAd7 can lead to gastrointestinal tract infections, resulting in viral shedding in the stool for up to 28 days after vaccination [42]. This viral shedding poses a risk of transmission to individuals in close contact with infected patients [43]. Moreover, live viral vaccines have the potential for recombination, which may generate novel adenovirus types if they co-infect with other adenoviruses within the same cells [44]. Additionally, live viral vaccines cannot generally be used in immunocompromised or pregnant individuals, limiting their applicability [45]. Despite the typically lower immunogenicity of inactivated vaccines compared to live variants, our study demonstrated that inactivated hAd55 induced high titers of hAd55-specific binding Ab and nAb responses

in mice. This suggests that the inactivated hAd55 vaccine exhibits both safety and immunogenicity, making it a viable formulation for vaccination strategies. Furthermore, this study underscores the potential safety and effectiveness of inactivated hAd55 vaccines, suggesting implications for broader vaccine utilization, particularly in populations where live viral vaccines may pose risks or limitations.

Although inactivated hAd55 could induce hAd55-specific Abs in mice, whether these induced antibodies can confer protection against hAd55 infection or mitigate disease severity could not be investigated as an animal infection model has not yet been established. In a previous study, the susceptibility of transgenic mice expressing CD46 or desmogrein-2 (DSG-2) to hAd55 infection was investigated [46]. Three- and seven-days post-infection with hAd55 carrying the secreted alkaline phosphatase (SEAP) reporter gene, SEAP activity and the hAd55 genome were detected in the sera and livers of the infected transgenic mice, respectively. However, physiological changes resembling those observed in humans, such as lung inflammation and a loss of body weight following hAd55 infection, were not documented in the study. Establishing a mouse infection model that accurately reflects human physiological responses is crucial for evaluating the protective efficacy of vaccines and their potential for vaccine-associated disease enhancement (VADE). Given that no existing animal model exhibits lung inflammation post-viral infection, we are currently generating transgenic mice expressing CD46, DSG-2, or a combination of both receptors. Upon successful establishment of an appropriate animal infection model, we will assess the vaccine candidate for its protective efficacy and potential for VAED. This forthcoming investigation will provide valuable insights into the protective capabilities and safety profile of the inactivated hAd55 vaccine candidate, paving the way for further advancements in vaccine development and disease prevention strategies.

hAd14 is also prevalent in Korea military training camps (data now shown). Given that hAd55 is derived from both hAd11 and hAd14, we aimed to determine whether immunization with inactivated hAd55 combined with AH could induce nAbs against hAd11 and hAd14. Our study found that immunization with inactivated hAd55 (6-15 (AFMRI 41014)) combined with AH in a 1:1/7 ratio induced hAd14-specific nAb responses in immunized mice but did not induce hAd11-specific nAb responses. These results suggest that immunization with inactivated hAd55 may confer protection against hAd14 infection. Future studies will assess the protective efficacy of the inactivated hAd55 vaccine candidate against hAd14 infection following the establishment of a murine infection model.

The amino acids of the hAd11 and hAd55 hexons share a higher similarity (98.42%) compared to those of hAd14 and hAd55 (91.95%) [2]. In contrast, the amino acid similarity between the hAd14 and hAd55 fibers (99.08%) was higher than that between the hAd11 and hAd55 fibers (92.33%). Additionally, the cross-neutralizing activities of hAd14 and hAd55 in human serum have been associated with fiber-specific antibodies [39]. Thus, the neutralizing activity against hAd14 appears to be associated with the induction of fiber-specific Abs following immunization with inactivated hAd55 (6-15 (AFMRI 41014)) combined with adjuvants. Although relatively low antibody responses against hAd14 were induced by this immunization, it remains uncertain whether this hAd14-specific nAb response is sufficient to protect against hAd14 infection and/or prevent respiratory diseases in mice. Further studies can evaluate this using an animal model of infection. Additionally, it is unclear whether hAd11-specific nAb responses can be induced in humans after immunization with inactivated hAd55 combined with adjuvants. Therefore, the cross-neutralizing effects of inactivated hAd55 combined with adjuvants need to be assessed through clinical studies.

Phylogenetic analysis of the four isolates revealed differences between the domestic and foreign strains (Fig. 2B). Notably, no significant variations were observed in hAd55 isolated at the Armed Forces Capital Hospital in 2016. Furthermore, structural proteins such as hexon, fiber, and penton had consistent compositions across domestic and foreign isolates (data not shown). However, SNPs were detected in the UTRs of several strains. Further investigation is needed to elucidate the impact of these UTR SNPs on viral proliferation and virulence.

Conclusion

We identified a promising vaccine candidate against hAd55. The immunogenic potential demonstrated by the vaccine candidate in mice suggests that inactivated hAd55 combined with an adjuvant shows promise as a vaccine candidate. However, further investigation into the protective efficacy and safety profiles of the vaccine candidate is needed to evaluate its potential for translation into human use.

Abbreviations

Ab	antibody
AH	alum hydroxide
AP	alum phosphate
ARD	acute respiratory diseases
CPE	cytopathic effects
hAd55	Human adenovirus type 55
MSA	multiple sequence alignment
nAb	neutralizing antibody
PRNT	plaque reduction neutralization test

Acknowledgements

Not applicable.

Author contributions
S. H. Seo wrote the article and analyzed and interpreted the data; J. A. Choi designed the animal experiments and analyzed and interpreted the data; D. I. Jeong, Y. Park, E. Yang and S. Jung performed the experiments; T. Kwon analyzed the phylogenic tree; S. H. Kwon and M. Song supervised this study.

Funding
This work was funded by the Korea Disease Control and Prevention Agency (KDCA) [project number: 2019-ER5502-00].

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All animal experiments were approved by the Institutional Animal Care and Use Committee of International Vaccine Institute.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 7 August 2024 / Accepted: 5 September 2024
Published online: 16 September 2024

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