

Exploration of New Sites in Adenovirus Hexon for Foreign Peptides Insertion

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Abstract: Adenoviral vectors are now being explored as vaccine carriers to prevent infectious diseases in humans and animals. There are two strategies aimed at the expression of a vaccine antigen by adenoviral vectors. The first includes an insertion of the foreign gene expression cassette into the E1 region. The second strategy is antigen incorporation into the viral capsid proteins. To extend this methodology, we have searched for new sites at the human adenovirus serotype 5 major capsid protein hexon for a vaccine antigen insertion. To this end, we utilized sites in the hexon hypervariable region (HVR) 7, 8 and 9 to display a 15-mer peptide containing the main neutralizing epitope of porcine reproductive and respiratory syndrome virus. However, we could not rescue the viruses with the insertions of the peptide into HVR 8 and 9, consistent with the viruses being unable to tolerate insertions at these sites. In contrast, the virus with the insertion of the peptide in HVR 7 was viable - growing well in cell culture and the inserted peptide was exposed on the virion surface.

Keywords: Adenovirus, capsid-modification, hexon, vaccine.

BACKGROUND

Human adenovirus type 5 (hAd5) vector has been shown to be an excellent vaccine delivery system in humans and animals [1, 2]. The replication-defective (E1-deleted) and replication-competent (E3-deleted) hAd5 have both been used as vaccine delivery vectors. For biosafety concerns, a replication-defective hAd5 is considered to be more suitable for vaccination. Replication-defective vectors may also have deletion in the E3 region to increase the amount of foreign DNA that can be inserted. Conventional strategy for the expression of a vaccine antigen by adenoviral vector includes insertion of the foreign gene expression cassette into the E1 region. This antigen is expressed as transgene after the infection of permissive cells with recombinant adenovirus.

Apparent drawbacks of the conventional transgene expression of antigen include an inability of the hAd5-based vectors to produce a potent humoral response against certain antigens and, in some cases, the inability of the vector to express a foreign gene [3]. To overcome such hurdles, the “antigen capsid-incorporation” strategy was developed [4]. Incorporating an immunogenic peptide into the hAd5 capsid offers several potential advantages. Importantly, the processing of capsid-incorporated antigen *via* the exogenous pathway could result in a strong humoral response, similar to that generated by native hAd5 capsid proteins, and this strategy may also be useful in boosting antigen-specific antibody immune responses.

Adenoviral capsid consists of the hexon, penton base and fiber [5]. Antigenic epitopes could be incorporated into these proteins as well as into the minor capsid protein IX [6]. Importantly, hexon is the most abundant of the capsid proteins, accounting for more than 83% of the capsid protein [7]. Furthermore, hexon is shown to be a vaccine adjuvant [8].

Early analysis of the hexon protein sequences revealed that, in addition to the conserved regions, there were 7 discrete hypervariable regions (HVRs) [9]. In a subsequent study, they are reclassified as regions 1 to 9 [7]. These HVRs do not appear to be involved in the binding of any other viral proteins, and the loops at the top of the HVRs are the most amenable to modifications. It was also shown that short heterologous peptides can be incorporated within the HVRs of hexon without affecting the viability of the virus [10, 11]. Incorporating antigens into hexon is applicable towards vaccination for several pathogens including the poliovirus, pseudomonas, *B. anthracis*, influenza, malaria and HIV [12-17]. In selected studies, protective immune responses in a mouse model are documented [12, 16].

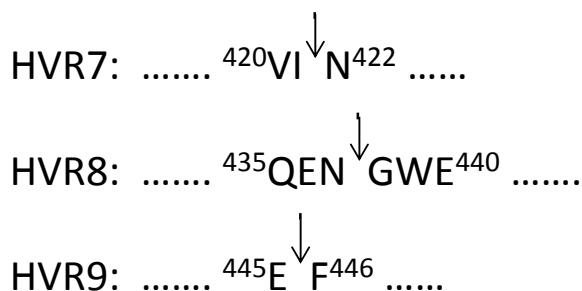
In hAd5, HVR 1 through 6, and 8 have been used for the incorporation of foreign peptides [10, 18]. In this study, we explored different sites in HVRs 7, 8 and 9 of hAd5 hexon for the insertion of a peptide corresponding to the porcine reproductive and respiratory syndrome virus (PRRSV) main neutralizing epitope B [19] of the GP5 protein. Our results provide potentially applicable information for adenovirus-vectored vaccine development involving such hexon modifications, particularly in HVR 7.

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RESULTS AND DISCUSSION

Construction of Hexon-Modified Viruses

In order to access new sites in hAd5 hexon, tailored for the incorporation of foreign peptide sequences, we genetically inserted a 15 amino acid (aa) residue peptide into HVR7, HVR8 or HVR9 region of the hAd5 hexon. The peptide consisted of embedding a 9 aa residue sequence containing the PRRSV major neutralizing epitope flanked by the LGS spacer sequences (Fig. 1).



Peptide: LGSSHLQLIYNLLGS

Fig. (1). Sites in the HVRs that were modified with a peptide containing the PRRSV major neutralizing epitope B. The arrows mark the position where the peptide was inserted. The numbers show the positions of the amino acid residues in hAd5 hexon. Peptide sequence corresponding to the PRRSV epitope is underlined.

Insertion of the DNA encoding peptide into the hAd5 hexon gene was achieved by PCR followed by bacterium-based homologous recombination. The linearized DNA of the plasmid containing the right portion of the hAd5 genome with modified hexon and the 1878 bp deletion in the E3 region was co-transfected into human embryo kidney (HEK) 293 cells together with a plasmid containing the left portion of the hAd5 genome with an E1-deletion. Homologous recombination in HEK 293 cells led to generation of the recombinant virus, termed Ad5HVR7epB. Multiple attempts to rescue the other two recombinant viruses (Ad5HVR8epB and Ad5HVR9epB) were not successful. This inability to rescue recombinant viruses with the foreign peptide insertion into HVR8 and HVR9 of hexon suggested that the insertion interferes with the formation of viral particles.

Interestingly, in a previous study [10], hexon-modified virus with incorporation of the His₆ peptide into HVR8 was reported to be viable. Our result with HVR8 is contradictory, evidently due to the differences in the peptides sequences and their lengths. Further, while the two studies are made with changes within HVR8, the specific locations of the incorporations differ as well. Notably our study uses a foreign sequence inserted between Asn437 and Gly438 (Fig. 1), while Wu *et al.* [10] placed His₆ between Pro431 and Trp439 substituting for 7 aa residues of hexon.

To corroborate the insertion of the peptide encoding DNA into the hexon gene of hAd5, the viral genome fragment was amplified using PCR from the viral DNA extracted from Ad5HVR7epB-infected cells, and the PCR product was digested with *AvrII* since the peptide encoding

DNA contained the single *AvrII* site. Predictably, a 1053 bp PCR fragment was amplified from Ad5HVR7epB DNA (Fig. 2, lane 1), and its site specific cleavage by *AvrII* provided two fragments: 650 bp and 403 bp (Fig. 2, lane 2). The results of this analysis suggested that recombinant Ad5HVR7epB contained the epitope B encoding sequence in the HVR7 of hexon.

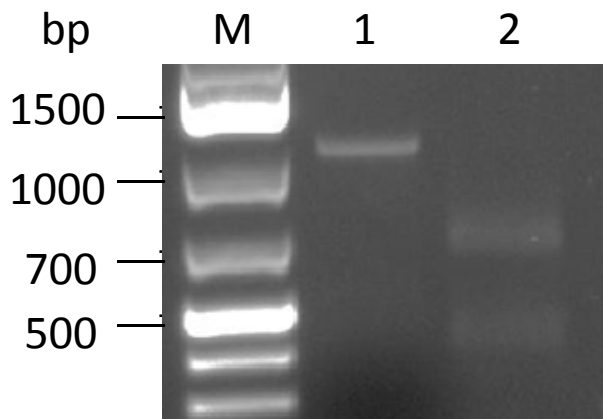


Fig. (2). PCR and restriction enzyme analysis of recombinant Ad5HVR7epB genome. Viral DNA extracted from Ad5HVR7epB infected HEK 293 cells was subjected to PCR (lane 1) and the PCR product was digested with *AvrII* (lane 2). The GeneRuler 1kb Plus DNA ladder (M) from Fermentas was used for sizing the DNA fragments. The sizes of some ladder bands are indicated.

Growth of the Hexon-Modified Virus in Cell Culture

The growth kinetics of the recombinant virus Ad5HVR7epB was analyzed in HEK 293 cells. Cultures of the cells were infected with Ad5HVR7epB or Ad5-empty (E1-deleted and partially E3-deleted hAd5 with unmodified hexon), and the cells were harvested at 12, 24, 36, 48, 72 and 96 hr post infection intervals. Virus in each sample was released by freeze-thawing and titered on monolayers of HEK 293 cells. Fig. (3) shows the insertion of the peptide in HVR7, resulting in similar virus yields to unmodified virus with maximal titers ranging from 2×10^{11} to 10^{12} TCID₅₀ per ml in a culture of 2×10^6 cells.

We also determined the viral particle/infectious particle (VP/IP) ratio for CsCl gradient purified preparations of the control virus Ad5-empty, as well as the hexon-modified virus Ad5HVR7epB. A 2.7 fold increase was observed in the VP/IP ratio of Ad5HVR7epB preparations as compared to the unmodified control Ad5-empty (Table 2). A similar observation is reported for hexon-modified hAd5 with peptide incorporation into HVR2 or 5 [11]. A typical VP/IP ratio of unmodified adenoviruses ranges from 10 to 44 [20].

Presentation of the Foreign Peptide on Virion Surface

We next examined whether PRRSV epitope was presented on the surface of the virion, using an ELISA binding assay. Different amounts of CsCl gradient purified viruses (Ad5HVR7epB and Ad5-empty) were immobilized on the wells of 96-well plates and incubated with peptide antisera. The binding of antibodies to the virions was detected with alkaline phosphatase (AP)-conjugated secondary antibody, followed by AP color reaction

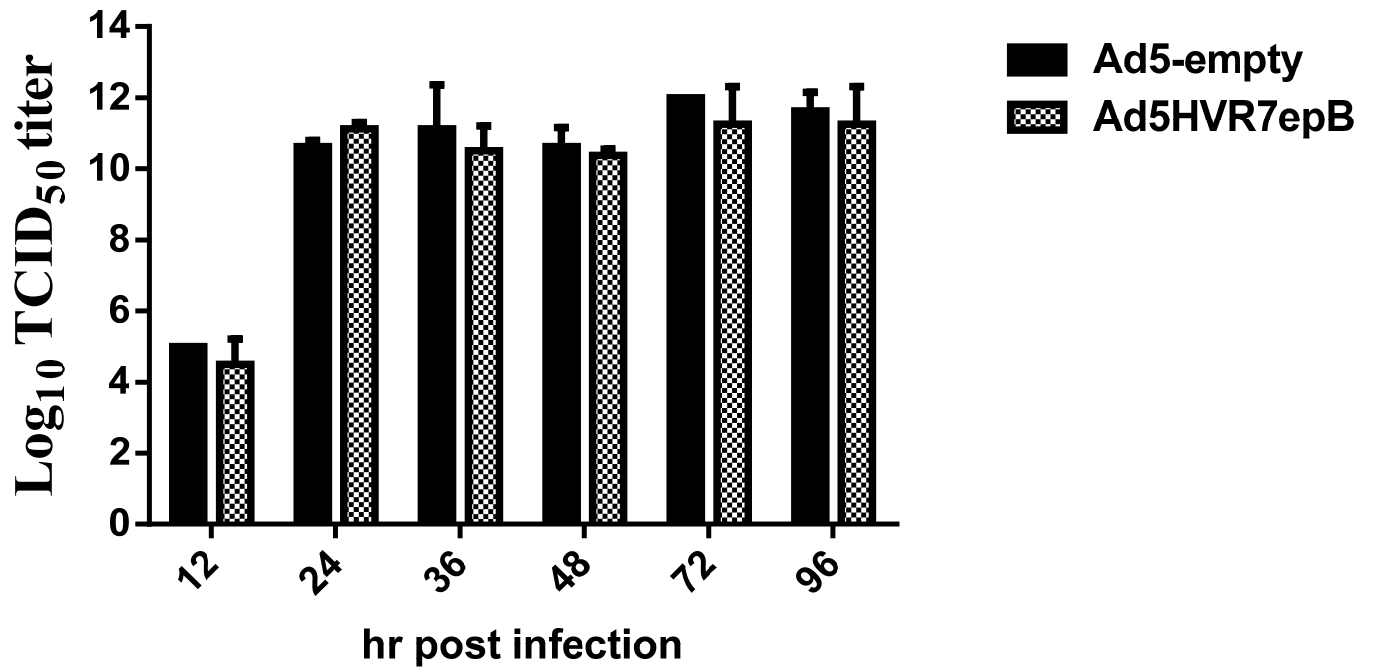


Fig. (3). Titers of hexon-modified virus Ad5HVR7epB and non-modified virus Ad5-empty. Monolayers of HEK 293 cells cultured in 6-well plates were infected with the viruses. At different times post-infection, the cells and culture media were harvested, freeze-thawed and the viruses were titrated on HEK 293 cells. Values are expressed as the mean and standard deviation of two independent experiments.

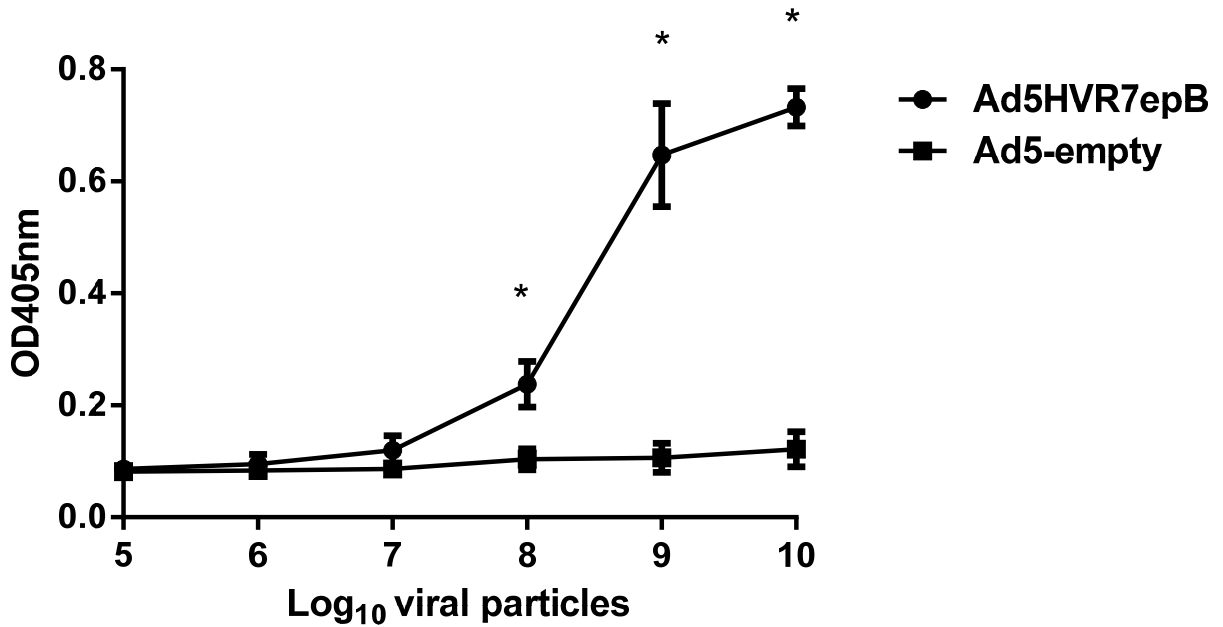


Fig. (4). The PRRSV epitope incorporated in HVR7 of hexon is accessible in the context of an intact virion. In the assay, varying amounts of purified viruses were immobilized in the wells of ELISA plate and incubated with anti-peptide antibody. The binding was detected with an AP-conjugated secondary antibody. Values are expressed as the mean and standard deviation. * indicates a P value of < 0.05.

development. Evidently, the anti-peptide antibodies bind well to AdHVR7epB virions and not to Ad5-empty (Fig. 4). This suggests that the PRRSV epitope inserted into HVR7 of hexon is exposed on the virion surface.

While a previous study showed that epitopes were exposed on the virion surface when incorporated in HVR1,

HVR2 and HVR5, and not exposed in HVR3 and HVR6, as well as poorly exposed when a peptide was placed in HVR8 [10]. In the current study, we find a new site in HVR7 for incorporation of foreign peptide into hAd5 hexon, and determine that the peptide was also exposed on the virion surface making it readily accessible for antibody binding and be potentially useful for vaccination.

CONCLUSION

Herein, we report a novel adenovirus vector (Ad5HVR7epB) with the insertion of the PRRSV main neutralizing epitope B in 3 different HVR regions of the major capsid protein hexon. While the insertion of the peptide into HVRs 8 and 9 were not tolerated by the adenovirus, the 9-mer epitope placed within HVR7 is determined to be exposed on the virion surface.

METHODS

Construction of the Plasmids pUC-Ad5hex-HVR7epB, pUC-Ad5hex-HVR8epB and pUC-Ad5hex-HVR9epB

Plasmid pUC-Ad5-hex was used as a template for PCR and as a cloning vector. This plasmid contained a 5.2 kb DNA fragment of hAd5 genome encoding a portion of the hexon gene. To insert the DNA sequence encoding the main neutralizing epitope B of PRRSV in HVR7 and obtain the DNA fragment for cloning, four consequent PCRs were performed using Phusion high-fidelity DNA Polymerase (NEB). First, a 1 kb fragment was amplified using primers BL7F1 and Nhe-R, and pUC-Ad5-hex as a template. Second, a 1 kb fragment was amplified using primers BLF2 and Nhe-R, and the product of PCR 1 as a template. Third, a 690 bp fragment was amplified using primers BL7R and Nde-F, and pUC-Ad5-hex as a template. Fourth, a 1.7 kb fragment was amplified using primers Nde-F and Nhe-R, and the mixture of the products of PCR 2 and 3 from above as a template. The final 1.7 kb PCR product was digested with *NdeI* and *NheI* and cloned into *NdeI* and *NheI* sites of pUC-Ad5-hex, creating pUC-Ad5hex-HVR7epB. Similar strategy was used to construct pUC-Ad5hex-HVR8epB and pUC-Ad5hex-HVR9epB. Specific sequences of the primers are presented in Table 1.

Construction of the Plasmids pH5R-hexHVR7epB, pH5R-hexHVR8epB and pH5R-hexHVR9epB

The plasmid pH5R-hexHVR7epB was generated by homologous DNA recombination in *E. coli* BJ5183 [21] between a 2.6 kb DNA fragment excised by *KpnI* and *NdeI* from pUC-Ad5hex-HVR7epB and *BamHI*-linearized pH5R [22]. Similar strategy was used to construct pH5R-hexHVR8epB and pH5R-hexHVR9epB. Fig. (1) shows the sites of the insertion of the peptide in the hAd5 hexon protein sequence.

Rescue of Recombinant Adenoviruses

Human embryonic kidney (HEK) 293 cells were transfected with *PacI*-digested DNAs of the plasmids pH5R-hexHVR7epB, pH5R-hexHVR8epB or pH5R-hexHVR9epB and *PacI*-digested pH5-L [22] using Lipofectamine-2000 (Invitrogen). Homologous recombination in the transfected cells led to generation of the recombinant adenovirus, and the virus was detected by developing cytopathic effect (CPE) in the cell monolayer. If CPE was not detected by the 14th day after transfection, the cells and the cell culture media were collected and freeze-thawed. This material was used to infect a fresh monolayer of HEK 293 cells.

The virus was amplified by passaging in HEK 293 cells and purified by CsCl gradient centrifugation [23]. The concentration of virus particles (VP) in purified virus preparations was determined by measuring the optical density at 260 nm as previously described [24], whereas numbers of infectious particles (IP) were determined by the tissue culture infective dose 50 (TCID₅₀) method [25].

Table 1. Synthetic oligonucleotides used for PCR to insert the sequence corresponding to the PRRSV epitope B into HVRs of Ad5 hexon.

Name	Sequence ^a
BL7F1	TTTACAACCTCCTAGGCAGCAATACAGAGACTTTACCAA
BL7R	TGTAGATGGGAAGAACCTAAAATCACACCTCCAGTGGAA
BL8F1	TTTACAACCTCCTAGGCAGCGGATGGGAAAAAGATGCTAC
BL8R	TGTAGATGGGAAGAACCTAAATTTTCCTGACCTGTTTTAG
BL9F1	TTTACAACCTCCTAGGCAGCTTTTCAGATAAAAATGAA
BL9R	TGTAGATGGGAAGAACCTAATTCTGTAGCATCTTTTT
BLF2	TTAGGTTCTTCCCCTACAGCTGATTTACAACCTCCTAGGCAGC
Nde-F	CGGCATCAGAGCAGATTGTA
Nhe-R	ATCGGTCCTTGTAGCTCTC

^aLetters representing the sequences encoding the PRRSV epitope and flanking LGS amino acid residues are in bold.

Table 2. Numbers of viral and infectious particles in purified preparations of adenoviruses.

Viruses	Viral Particle (VP), vp/ml	Infectious Particles (IP), TCID ₅₀ /ml	VP/IP
Ad5-empty	1.2 X 10 ¹²	4.5 X 10 ¹⁰	27
Ad5HVR7epB	1.8 X 10 ¹²	2.5 X 10 ¹⁰	72

Analysis of Virus Growth

HEK 293 cells grown in wells of 6-well plates were infected with Ad5-empty or Ad5HVR7epB viruses at a multiplicity of infection (m.o.i.) of 5. Infected cells were harvested at the indicated time intervals post-infection. Cells were lysed in the growth medium by freezing-thawing three times. The titres of infectious viral progeny were determined by TCID₅₀ method in monolayers of HEK 293 cells.

Isolation of Adenoviral DNA from Infected Cell Lysates and PCR

HEK 293 cells grown in T75 flasks were infected with the virus Ad5HVR7epB. After 48 hours post infection, when full CPE was observed, the cells and cell culture media were harvested followed by 3 cycles of freezing and thawing. Cell debris was removed by centrifugation at 1,500 rpm for 5 min, and the supernatant (275 µl) was used for viral DNA isolation. Briefly, the supernatant was incubated with 10 µl of DNase I (10 mg/ml) at 37°C for 30 min, followed by addition of 6 µl of 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 7.5 µl of 20 % sodium dodecyl sulfate (SDS), 1.5 µl of Proteinase K (20 mg/ml) and incubation at 50°C for 1 h. Total DNA was further extracted using a GeneClean Spin Kit (MP Biomedicals) according to the manufacturer's instructions. The isolated DNA was subjected to PCR using Fermentas (2X) PCR Master Mix and primers (5'-ATCATGCAGCTGGGAGAGTC-3' and 5'-CATTGCCAGCAACATTGAG-3') that were designed to anneal in the sites flanking the hexon HVRs. Amplification product was digested by *AvrII* and size-separated by electrophoresis in 1 % agarose gel.

Production of Peptide-Specific Polyclonal Antibodies

The peptide, containing amino acid residues of the PRRSV neutralizing epitope B (SHLQLIYNL), was synthesized by GenScript Inc. The peptide was conjugated to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) as carrier molecules. Two New Zealand White rabbits were immunized with the conjugated peptide (500 µg/rabbit) emulsified with Freund's Complete Adjuvant (FCA) followed by two injections of conjugated peptide (300 µg/rabbit) in Freund's incomplete adjuvant (FIA) at four weeks apart. Serum was collected ten days after the third injection and tested for ability to bind to the peptide by ELISA.

ELISA

The enzyme-linked immunosorbent assay (ELISA) binding assay was used to test if the peptide incorporated into the hexon protein is accessible to anti-peptide antibody at the virion level. Different amounts of purified virions ranging from 10³ to 10¹⁰ viral particles were immobilized on the wells of a 96-well plate (IMMULON, Thermo Scientific) by overnight incubation in 100 µl/well of 100 mM carbonate buffer (pH 9.5) at 4 °C. After washing with PBS, containing 0.05% Tween 20, the immobilized virus was incubated with 1:500 diluted anti-peptide rabbit serum for 4 h at 25 °C, followed by AP-conjugated goat anti-rabbit antibody (Cedarlane) incubation. Color reaction was performed with

p-nitrophenyl phosphate (Sigma-Aldrich), and optical density at 405 nm was measured with a microplate reader.

ABBREVIATIONS

aa	= Amino acid
bp	= Base pairs
CPE	= Cytopathic effect
ELISA	= Enzyme-linked immunosorbent assay
hAd5	= Human adenovirus serotype 5
HVRs	= Hypervariable regions
HEK	= Human embryo kidney
IP	= Infectious particles
m.o.i.	= Multiplicity of infection
PRRSV	= Porcine reproductive and respiratory syndrome virus
TCID	= Tissue culture infective dose
VP	= Viral particles.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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