MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol.16, No.2, December 2022, p 31-36

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.16.2.31-36

Design of Adenovirus 5 Vector with Adenovirus 26 Hexon Hypervariable Region Sequence using *In Silico* Approach

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Adenovirus type 5 (Ad5) is one of the vaccine vectors, including the COVID-19 vaccine. Pre-existing immunity to Ad5 may suppress the immunogenicity and efficacy of adenovirus vectored vaccine. The neutralizing antibodies are directed specifically toward seven hypervariable regions (HVR) of hexon proteins located on the outer surface of the capsid. This study aims to design an Ad5 vector that may circumvent anti-Ad5 immunity by designing a chimera Ad5 vector with the sequence of Ad26 HVR (Ad5HVR26) using *in silico* approach. Substitution of the Ad5 HVR DNA sequence may affect the alternative splicing process of adenovirus mRNA, which then influence the protein product. The splice site prediction of Ad5HVR26 chimera vector was found at HVR5, 6, and 7. The codon change in the splice site was performed to decrease the possibility of incorrect splicing, while retaining the original amino acid sequence. The HVR substitution in chimera vector Ad5HVR26 may also affect the interaction of hexon in the capsid. The HVR2 and HVR4 hexon proteins individually interact with other hexon proteins and IX protein. Thus, two designs of the Ad5HVR26 chimera vector were created in this research. The first design was the Ad5 chimera vector with complete substitution of HVR hexon by Ad26 sequence, with codon modification on the splice site. The second design was Ad5HVR26 chimera vector without the HVR2 and HVR4 substitution to maintain the hexon protein interaction with the capsid proteins. Production of the designed vectors are needed to prove the reduction of vector neutralization by pre-existing immunity.

Key words: adenovirus type 5, chimera vector, hexon protein, hypervariable region (HVR), neutralizing antibodies, protein interaction, splice site

Adenovirus tipe 5 (Ad5) merupakan salah satu vektor vaksin yang digunakan, termasuk untuk vaksin COVID-19. Keberadaan *pre-existing immunity* terhadap Ad5 dapat menurunkan imunogenisitas dan efikasi terhadap vektor adenovirus. Antibodi netralisasi yang terbentuk diketahui mentarget secara spesifik terhadap tujuh daerah hipervariabel (*hypervariable regions*, HVR) protein hekson pada permukaan kapsid adenovirus. Penelitian ini bertujuan untuk merancang vektor Ad5 yang mampu menghindar dari imunitas anti-Ad5 melalui pendekatan vektor Ad5 khimera dengan HVR Ad26 (Ad5HVR26) dengan pendekatan *in silico*. Substitusi urutan DNA HVR Ad5 diprediksi dapat mempengaruhi proses splising alternatif dari mRNA adenovirus, yang akan mempengaruhi protein yang dihasilkan. Hasil prediksi situs splising vektor *chimera* Ad5HVR26 menunjukkan keberadaan situs baru pada HVR5, 6, dan 7. Penggantian kodon pada situs splising baru kemudian dilakukan untuk menurunkan kemungkinan terjadinya proses splising yang salah, dengan tetap mempertahankan asam amino yang dihasilkan. Substitusi HVR pada vektor chimera Ad5HVR26 juga diprediksi dapat mempengaruhi interaksi hekson pada kapsid adenovirus. HVR2 dan HVR4 pada protein hekson masing-masing berinteraksi dengan protein hekson yang lain dan protein IX. Oleh sebab itu, dua rancangan vektor khimera Ad5HVR26 dibuat pada penelitian ini. Desain pertama adalah vektor chimera Ad5 dengan substitusi lengkap dengan HVR Ad26, dengan modifikasi kodon pada situs splising. Desain kedua adalah vektor chimera Ad5HVR26 tanpa substitusi HVR2 dan HVR4 untuk mempertahankan interaksi hekson pada kapsid. Produksi vektor khimera ini perlu dilakukan untuk membuktikan penurunan *pre-existing immunity* yang mengakibatkan netralisasi vektor adenovirus.

Kata kunci: Adenovirus tipe 5, antibodi netralisasi, daerah hipervariabel (*hypervariable regions*, HVR), interaksi protein, protein hekson, situs splising, vektor chimera

Vaccination is one of the strategies to control the spread of the COVID-19 pandemic. Adenovirus type 5 (Ad5) is the most common viral vector used in gene therapy and vaccine, but it has high pre-existing immunity that may suppress the immunogenicity and efficacy of the adenovirus vectored vaccine (Sumida *et al*. 2005). Several strategies can be performed to avoid this high pre-existing immunity (Padilla *et al.* 2016, Kreppel and Hagedorn 2021). Neutralization by

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neutralizing antibodies can be avoided by removing the specific epitope present on Ad5. Hypervariable regions (HVR) is located on the outer surface of the hexon protein and is a specific epitope against different adenovirus serotypes (Mizuta *et al*. 2009, Gallardo *et al.* 2021).

Adenoviruses are a family of viruses that lack an envelope with a linear double-stranded DNA genome that has a vertebrate host (Greber 2020). The main component of the adenovirus capsid consists of hexon, penton base, and fiber proteins, while the minor components consist of IIIa, VI, VIII, and IX proteins. Adenovirus capsid with icosahedron symmetry has 20 sides composed of 12 hexon protein trimers and 12 corners, each of which consists of a penton pentamer bound to one or more fiber proteins (Flatt and Butcher, 2019). Antibodies to Ad5 consist of antibodies to fiber, penton, and hexon. However, the most dominant antibody response that can suppress Ad5 vectored-vaccine efficacy is the antibody to hexon (Bradley *et al*. 2012). Hexon is the target of neutralizing antibodies because it is much more abundant than other proteins present in Adenovirus capsid.

This study aims to design an Ad5 vector that may circumvent anti-Ad 5 immunity by designing a chimera Ad5 vector with the sequence of hypervariable region of Ad26 hexon. Adenovirus 26 was chosen because it has low seroprevalence and does not induce tumors in rodents (Pacesa 2016, Yi *et al.* 2022). In addition, vaccines with a combination of Ad5 and Ad26 vectors are expected to have high efficacy as in the Sputnik V vaccine (Logunov *et al*. 2021). The Ad5HVR26 vector could not be produced in previous research reported by Bradley *et al.,* 2012. In this study, *in silico* analysis was carried out to estimate the cause of Ad5HVR26 vector cannot be made.

Ad5-specific epitope recognized by neutralizing antibody was determined based on a literature study. The Ad5HVR26 chimera vector was constructed by replacing the HVR hexon Ad5 amino acid sequence with the Ad26 amino acid sequence. Changes that occur in the DNA sequence and the amino acid sequence of the hexon protein can affect the viability of the chimera vector. In this study, splice site analysis and analysis of hexon proteins interaction was performed to ensure that the modification would not affect the production of the chimera vector. Modifications were made to the DNA sequence of the Ad5HVR26 chimera vector to obtain a chimera vector design that can be produced based on *in silico* analyses.

MATERIALS AND METHODS

Substitution of Ad5 Hexon HVR with Ad26. Genome and amino acid sequences of the Ad5 (NCBI Reference Sequence: AC_000008.1) and Ad26 (GenBank: EF153474.1) were obtained from National Center for Biotechnology Information (NCBI) online databases (https://ncbi.nlm.nih.gov/). The amino acid sequences of the Ad5 and Ad26 hexon were aligned using Clustal Omega Multiple Sequence Alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/) to determine the HVR substitution site. The conserved area and the HVR of the Ad5 hexon protein refer to the study of Roberts (2006). To obtain the gene encoding the hexon chimera protein (Ad5HVR26), substitution of HVR was carried out at the nucleotide. Hexon gene in Ad5, Ad26, and Ad5HVR26 was translated in silico (https://web.expasy.org/translate/) and aligned to prove that the HVR replacement was appropriate.

Splice Site Prediction. Detection of the Ad5 and Ad5HVR26 gene splice sites was carried out using the Alternative Splice Site Predictor (ASSP) website (http://wangcomputing.com/assp/). The Ad5HVR26 splice site was compared to Ad5 to determine different splice sites found in Ad5HVR26. Additional acceptor splice sites in Ad5HVR26 were removed by nucleotide base substitution until they reached lowest score. Replacement of nucleotide bases is carried out while paying attention to the codon sequence so that there is no change in amino acids.

Determination of Hexon Protein Interaction. Literature study was conducted to determine the location of the interaction between hexon Ad5 protein and other capsid proteins, namely penton base, IIIa, VI, VIII, and IX protein. Protein interactions involving HVR were visualized based on the three-dimensional structure of the capsid (PDB ID: 6CGV) using PyMol.

Hexon Protein Modelling and Superimposition. Modeling of the Ad5HVR26 hexon structure was carried out with the Swiss-Model web server (https://swissmodel.expasy.org/) using the hexon amino acid sequence of Ad5 and Ad5HVR26 as target proteins. The protein used as template is Ad5 hexon monomer with highest sequence identity. The study of hexon chimera structure was carried out by visualizing the three-dimensional structure and superimposition of hexon chimera proteins using PyMol.

Table 1 Splice Site(s) in Ad5HVR26

Position (bp)		Putative Splice Site	Sequence
HVR5	13629	Alt. isoform/cryptic donor	CCTCCAGCAGgtggtagtgg
	13630	Alt. isoform/cryptic acceptor	cctccagcagGTGGTAGTGG
HVR6	13732	Alt. isoform/cryptic acceptor	ggaacttcagATAACAGTTC
HVR7	14091	Alt. isoform/cryptic donor	ACTTATCAAGgtgtaaagat
	14092	Alt. isoform/cryptic acceptor	acttatcaagGTGTAAAGAT
	14109	Alt. isoform/cryptic donor	ATTACAAATGgtaatgatgg
	14118	Alt. isoform/cryptic donor	GGTAATGATGgtgctgaaga
	14130	Alt. isoform/cryptic donor	GCTGAAGAAAgtgagtggga

Table 2 Codon substitution of Splice Acceptor Site

Position	Before Codon Substitution		After Codon Substitution	
	Sequence	Score*	Sequence	Score*
13630	CCT CCA GCA GGT GGT	3.8	CCT CCA GCG GGT GGT	0
13732	GGA ACT TCA GAT AAC	3.3	GGA ACT TCC GAT AAC	0
14092	TAT CAA GGT GTA AAG	4.2	TAT CAA GGC GTA AAG	3.3

*the score reflects splice site strength

RESULTS

Substitution of Ad5 Hexon HVR with Ad26. The gene encoding for hexon Ad5HVR26 was obtained by substitution of Ad5 hexon HVR with Ad26. The replacement of HVR sequence was carried out at the nucleotide level to obtain the DNA encoding for Ad5HVR26 hexon protein. Alignment results showed that the HVR hexon of Ad5HVR26 was different from Ad5 but the same as Ad26 HVR sequence (Fig 1).

Splice Site Prediction. Replacement of the DNA sequence of HVR Ad5 to Ad26 could cause alternative splicing errors during mRNA processing. To predict the presence of additional splice sites on Ad5HVR26, prediction of splice sites was performed using ASSP software. Predictions were made on the L3 gene, which was the gene encoding for the hexon protein. The software predicted the presence of a new splice site in L3 Ad5HVR26 gene that was not present in the L3 Ad5 gene. Additional splice sites in the L3 Ad5HVR26 gene were found, i.e. five new donor splice sites and three new acceptor splice sites (Table 1). The substitution of codons at the splice site was performed by changing the codons around the splice site to reduce prediction score (Table 2). The higher the score, the predicted splice sites sequence was closer to the consensus sequence. After codon substitution, the splice site score was lower and might reduce the possibility of splicing.

Determination of Hexon Protein Interaction. Replacement of amino acid sequence of HVR Ad5 to Ad26 could cause changes in the three-dimensional structure of the hexon protein, thus interfering the interaction of hexon protein with other proteins. Hexon protein was known to interact with each other to form a capsid structure. One of the interactions occured at amino acids 186-193, which was the HVR2 domain, and at amino acids 250-258, which was the HVR4 domain. The carboxy terminal of protein IIIa was known to interact with hexon at amino acids 250 – 258 (HVR4) (Fabry *et al*. 2005, San Martin 2012). Protein IX was also known to interact with hexon proteins at amino acids 252 – 256 (HVR4) (Liu *et al*. 2010). HVR2 and HVR4 was known to interact with other proteins to make a stable capsid during adenovirus capsid assembly. Thus, the substitution of HVR2 and HVR4 of hexon Ad5 with HVR from Ad26 might impact the formation of the capsid.

Hexon Protein Modelling and Superimposition. The effect of HVR replacement on protein interaction was predicted by comparing the hexon protein Ad5 with Ad5HVR26 structure. The structural models of the Ad5 and Ad5HVR26 hexon monomers were made using the Swiss Model with the Ad5 hexon template (PDB ID: 1P30). The HVR4 of modelled hexon from Ad5HVR26 showed slight change as compared to the Ad5 hexon monomer (Fig 2). The superimposition of the modelled structure to the template showed that HVR2 of Ad5HVR26 was longer than Ad5, while length of HVR4 was the same (Fig 3). The structural similarity of HVR2 and HVR4 between Ad5 and

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Ad26_Hexon          YNSLAPKGAPNPSQWETKEKQGT-----------TGGVQQEKDVTKTFGVAATGGINIT     168
Ad5_Hexon           YNALAPKGAPNPCEWDEAATALEINLEEEDDDNEDEVDEQAEQQKTHVFGQAPYSGINIT     180
Ad5HVR26_Hexon      YNALAPKGAPNPCEWETKEKQG-----------TTGGVQQEKDVTHVFGQAPYSGINIT     168
                    **:*********.:*:   .                . * *:: *:.** *  .*****

Ad26_Hexon          NQGLLLGTDET-AENGKKDIYADKTFQPEPQVGEENWQENEA-FYGGRALKKDTKMKPCY     226
Ad5_Hexon           KEGIQIGVE------GQTPKYADKTFQPEPQIGESQWYETEINHAAGRVLKKTTPMKPCY     234
Ad5HVR26_Hexon      KEGIQIGGTDETAENGKKDIYADKTFQPEPQIGESQWQENEA-FYGGRVLKKTTPMKPCY     227
                    ::*: :*        *:.  ***********:**.:* *.*  . .**.*** * *****

Ad26_Hexon          GSFARPTNEKGGQAKFKPVNEGEQPKDLDIDFAYFDVPG-GSPPAGGSGEEYKADIILYT     285
Ad5_Hexon           GSYAKPTNENGGQGILVKQQNGKLESQVEMQF--FSTTEATAG----NGDNLTPKVVLYS     288
Ad5HVR26_Hexon      GSYAKPTNENGGQAKFKPVNEGEQESQVEMQF--FDVPGGSPPAGGSGEEYKAPKVVLYS     285
                    **:*:****:***. :   ::*:  .::::::*  *..          . :    .::**:

Ad26_Hexon          ENVNLETPDTHVVYKPGTSDNSSEINLVQQSMPNRPNYIGFRDNFVGLMYYNSTGNMGVL     345
Ad5_Hexon           EDVDIETPDTHISYMPTIKEGNSRELMGQQSMPNRPNYIAFRDNFIGLMYYNSTGNMGVL     348
Ad5HVR26_Hexon      EDVDIETPDTHISYMPGTS-DNSRELMGQQSMPNRPNYIAFRDNFIGLMYYNSTGNMGVL     344
                    *:*::******: * *  . ..*.  : ***********.*****:**************

Ad26_Hexon          AGQASQLNAVVDLQDRNTELSYQLLLDSLGDRTRYFSMWNSAVDSYDPDVRIIENHGVED     405
Ad5_Hexon           AGQASQLNAVVDLQDRNTELSYQLLLDSIGDRTRYFSMWNQAVDSYDPDVRIIENHGTED     408
Ad5HVR26_Hexon      AGQASQLNAVVDLQDRNTELSYQLLLDSIGDRTRYFSMWNQAVDSYDPDVRIIENHGTED     404
                    ****************************:***********.***************.**

Ad26_Hexon          ELPNYCFPLNGTGTNSTYQGVKITNGNDGAEESEWEKDDA-ISRQNQICKGNVYAMEINL     464
Ad5_Hexon           ELPNYCFPLGGVINTETLTKVKPKTG----QENGWEKDATEFSDKNEIRVGNNFAMEINL     464
Ad5HVR26_Hexon      ELPNYCFPLNGTGTNSTYQGVKITNGNDGAEESEWEKDD-AISRQNQIRVGNNFAMEINL     463
                    *********.*. ...*   ** ..*    :*. ****   :* :*:*  ** :******
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Fig 1 Alignment of hexon amino acid sequence of the Ad5, Ad26, and Ad5HVR26. HVR 1-7 are highlighted in order: red, green, pink, light blue, yellow, blue, and purple.

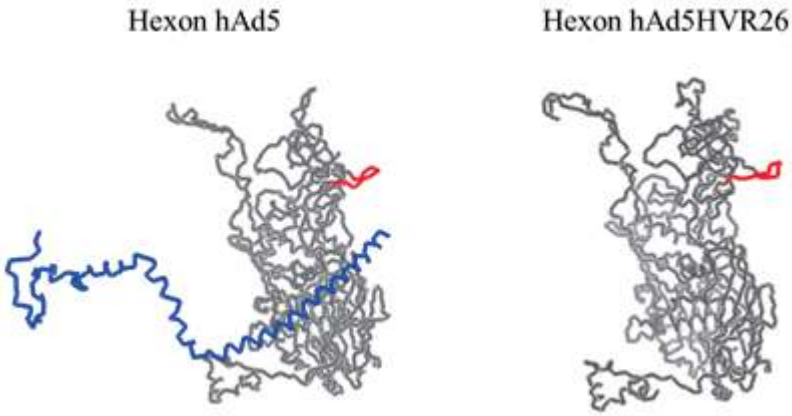

Fig 2 Modelling of hexon hAd5HVR26 monomer. The 3D structure modelling was performed using hexon Ad5 structure (PDB ID: 6CGV). Hexon protein shown in grey, HVR4 in red, and IX protein in blue.

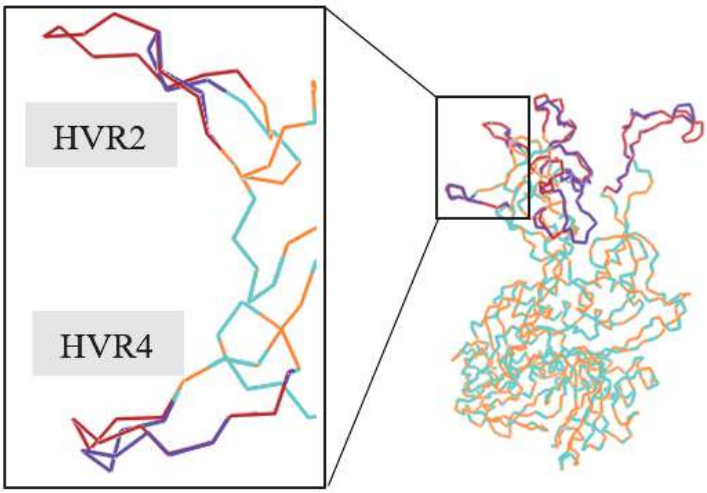

Fig 3 Superimposition of Ad5 and Ad5HVR26 hexon monomer showing the structural difference between HVR Ad5 and Ad5HVR26. Each hexon Ad5 and Ad5HVR26 monomer were shown in blue and orange, respectively. HVR hexon Ad5 and Ad5HVR26 were shown in purple and brown, respectively.

Ad5HVR26 could provide similar protein interactions in the capsid formation process.

Chimera Vector Design. Based on the splice site prediction and amino acid interaction analyses, two designs of the Ad5HVR26 chimera vector were created in this study. The first design was the Ad5 chimera vector sequence with complete substitution of 7 HVR hexon by Ad26 sequence, with codon modification on the splice site. The second design was Ad5HVR26 chimera vector without the HVR2 and HVR4 substitution in the sequence to maintain the hexon protein interaction with the capsid proteins.

DISCUSSION

The Ad5 chimera vector is formed by replacing all or part of the amino acids in the hexon with amino acids from another serotype of adenovirus. Changes in the DNA sequence in the hexon HVR can cause the appearance of a sequence of nucleotide known as splice sites. An alternative splicing error is thought to be one of the reasons why the Ad5HVR26 chimera vector could not be obtained in previous studies (Bradley *et al*. 2012). Alternative splicing errors in L3 gene can lead to gene expression errors that lie downstream L3. Gene downstream L3 are L4 and L5. The L4 gene encodes 100K, 33K, 22K and VIII protein which plays a role in capsid structure stability and transcriptional regulation. The L5 gene encodes fiber protein for vector internalization into the cell.

Introns in pre-mRNA have two distinct nucleotides at either end, GT and AG each in 5' and 3' of intron sequence. The splice site analysis was carried out with the principle of identifying the similarity of the nucleotide base sequence with the consensus sequence of the splice site. The higher the score, the sequence of splice sites is closer to the consensus order (Thanaraj and Stamm 2003). In adenovirus mature mRNA formation, the donor splice site always occurs at position 3717. Additional acceptor splice sites at positions 13630, 13732, or 14092 can lead to the formation of mature mRNA that expresses incorrect capsid-building proteins. This can lead to failure of the virion structure assembly and cause the Ad5HVR26 vector to not be generated. Therefore, codon substitutions were carried out at position 13630 (GCA→GCG), 13732 (TCA→TCC), and 14092 (GGT→GGC) to avoid alternative splicing error (Table 2). By removing additional acceptor splice sites, the chimera vector design was predicted to be produced without problem in capsid protein production.

The structure of the adenovirus capsid consists of three major proteins (hexon, penton base, and fiber) and four minor proteins (IIIa, VI, VIII, IX) which interact to form an icosahedral capsid (Reddy and Barry 2021). The capsid-forming proteins that interact with hexon are penton base proteins, IIIa, VI, VIII, and IX. Proteins IIIa and IX are located on the exterior of the capsid, while proteins VI and VIII are located on the interior of the capsid (Reddy and Nemerow 2014). Stable protein interactions can form protein complexes that are physically and functionally stable. Interactions between proteins occur at certain amino acid residues. Thus, changes in amino acids in hexon proteins, especially in HVR2 and HVR4 (Fig 1), can affect the formation of protein complexes that form the capsid. If the amino acid changes in the HVR region of the hexon affect the interaction of the hexon protein with other proteins, the adenovirus capsid structure cannot be formed.

In conclusion, two hexon sequences were designed to produce the Ad5HVR26 chimera vector. Production of the designed vectors is needed to prove the reduction of vector neutralization by pre-existing immunity. If both chimera vector designs can be produced, then a vector neutralization test could be carried out to determine which vector is better. Vaccines with the first design Ad5HVR26 vector is predicted to be more immunogenic and have higher efficacy than vaccines with second design Ad5HVR26 vector without the HVR2 and HVR4 substitution.

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