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Virtual Prediction of King Cobra (*Ophiophagus hannah*) Neurotoxin Epitopes for Vaccine-Based Antitoxin Development

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Abstract

The King Cobra (*Ophiophagus hannah*) possesses a highly neurotoxic venom where long-chain neurotoxins (LNTX) are the primary lethal components. Current antivenoms, such as OHMAV and SABU, have constrained efficacy and regional availability, highlighting the need for innovative solutions. This study aimed to computationally predict conserved and immunogenic epitopes from LNTX proteins for vaccine development. Using IEDB tools and ProtParam, potential epitopes were screened for immunogenicity, conservancy, physicochemical properties, and their MHC-II (HLA-DRB1*07:01) binding affinity was analyzed. Three lead epitopes were identified, namely 26-WCDGFCSSRGKRIDL-40, 27-CDGFCSSRGKRIDLG-41, and 25-TWCDGFCSSRGKRID-39. These candidates demonstrated low allergenicity, favorable physicochemical properties, and broad population coverage. Structural analysis confirmed that the epitopes, connected by a linker, docked stably with the MHC-II molecule. This interaction remained stable throughout a 20 ns molecular dynamics simulation. These results provide a robust foundation for developing a novel, epitope-targeted vaccine against king cobra venom, offering a promising alternative to traditional antivenoms.

Keywords: LNTX, Neurotoxin*, Ophiophagus hannah*, Southeast Asia, Vaccine-based antivenom

1. Introduction

One of the biggest and most deadly venomous snake species in the world is the King Cobra (*Ophiophagus hannah*) (Le *et al*., 2021; Simangunsong *et al*., 2024). This species belongs to the Elapidae family, with a total body length of up to 5 metres (Gowri *et al*., 2021). This species is distributed widely throughout South and Southeast Asia, including Indonesia, China, Bangladesh, India, Nepal, Bhutan, Pakistan, and the Indochina and Indo-Malaya areas. In Indonesia, this species can be found in Sumatra, Java, Borneo, Bali, and Sulawesi (Amat and Escoriza, 2022; Charlton, 2018; Das *et al*., 2024; C. H. Tan *et al*., 2021). King Cobra predominantly inhabited primary tropical forest and shrubland habitats and could also be found in cultivated fields and suburban environments (Marshall *et al*., 2018). Its presence in rural areas poses a significant threat, especially to those who work as farmers, who are most at risk of envenomation (Adiwinata and Nelwan, 2015; Dafa and Suyanto, 2021; Simangunsong *et al*., 2024). Snakebites from King Cobra and other venomous species cause many cases of snakebite envenomation in tropical regions due to treatment delays and a lack of medical resources (Adiwinata and Nelwan, 2015; Tan *et al*., 2021). Despite this, envenomation snakebite cases are still not a concern for the government and public health community. In 2017, snakebite envenomation was reinstated according to the WHO as a neglected tropical disease, and it continues to be an open problem (Tan *et al*., 2021).

The venom of *O. hannah* is composed of several protein families, including three-finger toxins (3FTxs) specifically long neurotoxin (LNTX) and short neurotoxin (SNTX) dominating its proteome at 33,5% and 11,9%, respectively. Other protein families that can be found on *O. hannah venom* are LAAO, Snake Venom Metalloproteinase-Disintegrin (SVMP), and cysteine-rich secretory proteins (CRISPs) (Rajendiran *et al*., 2024; C. H. Tan *et al*., 2021). These neurotoxins are the primary contribution to the venom's high lethality. The long-chain neurotoxins (LNTXs) act by binding to and blocking the nicotinic acetylcholine receptors (nAChRs) at the neuromuscular junction, leading to paralysis and death. Therefore, LNTXs are an important contributor to neurotoxic effects as they produce a stronger and reversible blockage of nAChRs compared to the short-chain neurotoxins (SNTXs) (Huynh *et al*., 2022; Wong *et al*., 2021). Based on this, the effectiveness of antivenom therapy is determined by the ability of antibodies to neutralize the envenoming toxin at any rate (Danpaiboon *et al*., 2014).

The most recent monospecific antivenom specifically made against King Cobra envenomation is OHMAV, which is produced using King Cobra Venom (KCV) of Thai origin and is used in Thailand and Malaysia. On the

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other hand, OHMAV is not provided in China and Indonesia. Seeing that geographically widespread species like King Cobras may have probably speciated, the efficacy of OHMAV might not be as high against populations elsewhere, as in China or Indonesia, or even Malaysia, because of the change in venom composition and, consequently, the immune response (Tan *et al*., 2020). In Indonesia, there is a commercially available antivenom, namely SABU; also, little scientific study has been conducted regarding the efficacy of this antivenom. SABU has been depicted as unproductive or even almost ineffective in neutralising the king cobra venom (*O. hannah*) since it lacks antibodies to the main neurotoxins of long-chain (Tan *et al*., 2016).

The specificity and scalability of traditional antivenoms for King Cobra (*O. hannah*) envenomation are limited. Recent discoveries were able to foresee the presence of stable T-cell epitopes by related elapid venoms using immunoinformatics, which also demonstrates that immunoinformatics is promising as an alternative (Chan *et al*., 2023; Grahadi *et al*., 2022). Thus, this research paper aims to determine conserved and immunogenic T-cell epitopes that are based on the predominant long-chain neurotoxins (LNTXs) of *O. hannah* venom, the goal of which is to create a vaccine-based antivenom using computational methods. The methodology that is used in this study consists of epitope prediction of high MHC-II binding affinity, evaluation of immunogenicity, population coverage, conservancy, allergenicity potential, and determination of stability and molecular interactions using dynamics simulations and structural modelling. The final goal of this report is to come up with a platform that will provide an enhanced defense against the venom of King Cobras in a more efficient, secure, and widely applicable manner.

2. Materials and methods

2.1. Prediction of LNTX protein epitopes

The amino acid sequences of Long Neurotoxin (LNTX) were obtained using accession codes A01657 from the NCBI and UniProt protein databases (He *et al*., 2004). Only sequences originating from Southeast Asia were used in this study. Epitopes for LNTX were predicted using the IEDB MHC II binding prediction tool (http://tools.iedb.org/mhcii) (Grahadi *et al*., 2022; He *et al*., 2004; Wang *et al*., 2008; Wang *et al*., 2010). Epitope prediction was carried out using the IEDB-provided HLA reference allele collection, which represents the polymorphism of human MHC class II molecules, such as HLA-DRB1*07:01 (Fathollahi *et al*., 2021; Grahadi *et al*., 2022). Epitopes exhibiting a percentile rank below 5 meet the required criteria and will be utilised for further analysis (Grahadi *et al*., 2022). BepiPred 2.0 Linear Epitope Prediction was utilised to predict B-cell epitopes, utilising the IEDB B-cell analysis tool, which may be found at http://tools.iedb.org/bcell/ (Dhanda *et al*., 2018; Grahadi *et al*., 2022).

2.2. Prediction of epitope of epitopes conservancy and immunogenicity

The IEDB (Immune Epitope Database) tools were used for the prediction of epitopes' immunogenicity (http://tools.iedb.org/CD4episcore/) and calculation of epitopes' conservancy (http://tools.iedb.org/conservancy/). Epitopes were considered to be immunogenic if their immunogenicity score exceeded 70% (Dhanda *et al*., 2018). The epitopes with IEDB scores exceeding 70% were considered to be conserved (Grahadi *et al*., 2022). The epitopes that meet both conservation and high immunogenicity parameters are then subjected to further analysis (Dhanda *et al*., 2018; Kurniawan *et al*., 2020).

2.3. Allergenic properties of epitope and population coverage

Algpred (https://webs.iiitd.edu.in/raghava/algpred/submission.html) was used to predict the allergenic characteristics of the epitope by mapping the IgE epitopes and PID, MEME/MAST motif, and Blast search on the allergen representative peptides (ARPs) menu. These predictions are carried out to ensure safety when administered to humans. IEDB was used to calculate population coverage (http://tools.iedb.org/population/) (Grahadi *et al*., 2022). Predictions of population coverage were limited to areas in Southeast Asia that are the natural habitat of *O. hannah.*

2.4. Estimation of physicochemical characteristics

The ProtParam program on the ExPASy server (https://web.expasy.org/protparam/) was used to examine the physicochemical characteristics of the chosen epitopes (Duvaud *et al*., 2021). Molecular weight, isoelectric point (pI), instability index, aliphatic index, predicted half-life, and grand average of hydropathicity (GRAVY) were among the physicochemical parameters that were calculated.

2.5. Epitope structure modeling

The three-dimensional structure of the selected epitope was modeled using PEP-FOLD3 (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3). PEP-FOLD3 is a *de novo* peptide structure prediction tool. The simulation provided a structural model crucial for the subsequent analysis (Lamiable *et al*., 2016).

2.6. Molecular docking

The selected epitope's three-dimensional structure, obtained from the PEP-FOLD3 model, was converted into PDB format and then optimized through energy minimization using PyRx v0.8 (Lamiable *et al*., 2016; Mao *et al*., 2023). MHC class II molecules and selected epitopes were docked using HDock (Ruaro-Moreno *et al*., 2023). Discovery Studio was used to depict the interactions between certain epitopes and MHC class II molecules (PDB ID 3C5J) (Naveed *et al*., 2022).

2.7. Molecular dynamics simulation

YASARA software was used to run the simulation for 20 nanoseconds. The simulation environment conditions were adjusted to physiological conditions, namely at pH 7.4 and NaCl concentration of 0.9%. The temperature was kept at 310°K (equivalent to human body temperature), while the water density was set at 0.997 g/LTo evaluate the stability of the epitope backbone, the root-mean-square deviation (RMSD) is examined. The epitope is considered stable if the resulting RMSD value does not exceed 3 Å, showing that during the simulation, the epitope structure relatively stays the same (Naveed *et al*., 2022; Ruaro-Moreno *et al*., 2023).

3. Results

3.1. Prediction of LNTX protein epitopes

Based on percentile rank values less than 5, 13 epitopes from the LNTX protein for human MHC class II (HLA-DRB1*07:01) were projected to satisfy the selection criteria (Sup. Table 1). Percentile rank values are used to predict MHC class II epitopes and evaluate how strongly a peptide binds to MHC molecules. Lower percentile values indicate higher binding affinity (Rezaldi *et al*., 2021). One of the HLA Class II alleles that was extensively dispersed in Southeast Asia is HLA-DRB1*07:01, which was used in this investigation (Gustiananda *et al*., 2021). In addition, HLA-DRB1*07:01 was used in this study because the prediction results of HLA-DRB1*15:02 and HLA-DRB1*12:02 that were previously used did not show good results. All epitopes had amino acid sequence lengths varying from 15 to 18 residues, with the core peptide sequence consisting mostly of the sequence "FCSSRGRKRI". Then these 13 epitopes can be further analyzed (Table 1). Four possible epitope candidates have been identified from the B-cell epitope prediction. Only the second epitope out of the four exhibits sequence similarity to MHC class II epitopes (Table 2).

Table 1. Selected MHC class II epitopes from LNTX protein.

Allele	Core Sequence	Peptide Sequence	Percentile Rank
HLA-DRB1*07:01	FCSSRGKRI	27-CDGFCSSRGKRIDLG-41	3
		25-TWCDGFCSSRGKRID-39	3.7
		26-WCDGFCSSRGKRIDL-40	3.7

Table 2. Selected B cell epitopes from LNTX protein.

Start	End	Peptide	Length
6	19	TPDATSQTCPDGQD	14
27	39	CDGFCSSRGKRID	13
48	54	CPKVKPGVD	9
61	69	DNCNPFPTW	9

bold letters indicate epitopes that have sequence similarity to MHC class II epitopes

3.2. Prediction of epitope conservation and immunogenicity

The analysis of epitope conservation and immunogenicity was performed using the IEDB. The results indicated that 12 out of 13 predicted epitopes were conserved (Sup. Table 2), exhibiting a conservancy value of 83.33%. Most of the predicted LNTX epitopes were used in further analysis, except for the thirteenth epitope KTWCDGFCSSRGKRID, which was not categorized as conserved with a value of 66.67%. The identification of conserved epitopes with a minimal number of mutations is of importance when designing an appropriate vaccine to target a wide range of variants of *O. hannah* toxin (Bagherzadeh *et al*., 2022). The predicted LNTX epitopes were found to be immunogenic, as indicated by an immunogenicity value that was higher than 98% (Table 3). Activating CD4+ T cells requires recognizing the immunogenic LNTX epitope on MHC class II. Once activated, these helper T cells interact with B cells. This interaction initiates an immune response that results in the production of antibodies (Xu *et al*., 2022).

Table 3. Conservancy and immunogenicity of LNTX *O. hannah* MHC II predicted epitopes.

Epitope Sequences	Percent of protein sequence matches at identity <= 100% (%)	Immunogenicity Score
27-CDGFCSSRGKRIDLG-41	83.33 (5/6)	98.57
25-TWCDGFCSSRGKRID-39	83.33 (5/6)	98.74
26-WCDGFCSSRGKRIDL-40	83.33 (5/6)	98.76

3.3. Allergenic properties of epitope and population coverage

AlgPred was used for this study to predict the allergenic characteristics in order to make sure that the anticipated LNTX epitopes would not cause a reactogenic reaction in humans (Ahmad *et al*., 2021). Three primary computational methods were used to predict the allergenic potential of LNTX epitopes and IgE epitope mapping in AlgPred, namely homologue search using BLAST against the representative allergen peptide database, Support Vector Machine (SVM) based classification analyzing single amino acid and dipeptide composition, and allergenic motif search using the MEME/MAST tool (Hayes *et al*., 2015). Considering no IgE epitopes were detected, the data demonstrated that the predicted epitopes do not have the potential to be allergenic. MAST and BLAST results also showed that the predicted epitopes were categorized as non-allergens (Table 4). This non-allergenic criterion was required to produce immunoreactive peptides (Khamjan *et al*., 2023).

Table 4. The Allergenic Properties of LNTX *O. hannah* epitopes.

Amino Acid Sequences	Mapping IgE epitope	MAST Results	BLAST Result
27-CDGFCSSRGKRIDLG-41	Does not contain experimentally proven IgE epitope	Non-Allergen	Non-Allergen
25-TWCDGFCSSRGKRID-39			
26-WCDGFCSSRGKRIDL-40			

To assess the compatibility of predicted LNTX epitopes with HLA alleles that are common in Southeast Asia, which is *O. hannah*'s natural environment (Indonesia, Malaysia, the Philippines, Singapore, Thailand, and Vietnam), population coverage was estimated using IEDB methods. The prediction of population coverage was important in epitope-based vaccine design to ensure that immune recognition is maximized in target populations, as the heterogeneity of HLA allele diversity across diverse geographical regions (Khamjan *et al*., 2023). The results showed that the predicted epitopes had limited coverage, reaching only 11.26% of the population in eight Southeast Asian countries. Among these countries, Indonesia had the highest population coverage of 20.49%, while other countries ranged from 1.22% to 15.91% (Table 5). This suggested that the highly variable HLA alleles of most Southeast Asian populations were unable to detect the predicted LNTX epitopes due to their narrow range. One of the HLA Class II alleles that was extensively dispersed throughout Southeast Asia and was prevalent in Indonesia with a frequency of 13.7% is HLA 07:01, which was used in this study (Gustiananda *et al*., 2021), but other countries may have different more dominant HLA alleles so to overcome the low population coverage, future designs could integrate several Southeast Asia-specific HLA frequency data.

Table 5. The population coverage of LNTX *O. hannah epitopes.*

Countries	Population Coverage (%)
Indonesia	20.49
Malaysia	6.42
Singapore	15.91
Thailand	14.21
Philippines	1.22
Vietnam	9.28

3.4. Estimation of physicochemical characteristics

The ExPASy ProtParam tool revealed significant variability in the properties of peptide epitopes due to differences in sequence composition, length, and terminal residues. Molecular weight (161.38–202.93 Da) and isoelectric point (pI 7.74–8.96) depended on amino acid composition, with most epitopes carrying a slight negative charge at physiological pH, enhancing solubility and immunological interactions (Devi *et al*., 2021; Khamjan *et al*., 2023). The instability index (>40) indicated that all epitopes were susceptible to degradation (Raza *et al*., 2021).

Half-life varies according to the N-terminal residue: Thr/Gly epitopes persist longer (~7.2 h in mammalian cells), while Lys/Asp epitopes degrade rapidly (~1.1–1.3 h) (Khamjan *et al*., 2023; Raza *et al*., 2021). High aliphatic index (26–52) and negative GRAVY scores (–0.473 to –0.773) (Table 6) confirm these epitopes are hydrophilic (Kaynarov *et al*., 2024). Stable hydrophilic epitopes (e.g., 25-TWCDGFCSRSRGKRIDLG-39) are promising vaccine candidates, while unstable epitopes may require structural optimization (Devi *et al*., 2021).

Table 6. Physicochemical characteristics of epitopes.

Physicochemical Characteristic	26-WCDGFCSSRGKRIDL-40	27-CDGFCSSRGKRIDLG-41	25-TWCDGFCSSRGKRID-39
Moleculer Weight	1742.99	1613.83	1730.93
Theoritical pI	8.06	8.06	7.74
Estimated half-life in mammalian cells (Hours)	2.8	1.2	7.2
Estimated half-life in yeast	3 Min	> 20 Hours	> 20 Hours
Estimated half-life in *E. coli*	2 Min	> 10 Hours	> 10 Hours
Instability index	58.27	58.27	48.25
Stability	Unstable	Unstable	Unstable
Aliphatic index	52	52	26
Grand average of hydropathicity (GRAVY)	-0.473	-0,44	-0.773

3.5. Epitope Structure Modelling

PEP-FOLD3 was used to model the selected epitope after the assessment of its physicochemical characteristics, immunogenicity, allergenicity, population coverage, and conservancy. Based on the amino acid sequences of peptides, PEP-FOLD3 is a proven method for predicting their three-dimensional structures. Due to its favorable energy profile and higher structural stability, Model 1 (Sup. Figure A1) was selected for further analysis among the models that were generated. Molecular docking studies were then conducted using this model, which was provided in Protein Data Bank (PDB) format. PEP-FOLD3's prediction process consists of three primary phases. A Monte Carlo approach is used to develop and refine various models after the support vector machine first predicts the structural alphabet of peptide fragments from the input sequence. Afterwards, the top five conformations are chosen (Sánchez-Hernández *et al*., 2021). Typically, the best model for epitope modeling is the top-ranked conformation among these clusters, chosen based on criteria such as the lowest coarse-grained energy (sOPEP score) or the highest predicted TM-score. This ensures the selection of the most stable and native-like peptide structure, which is then utilized for downstream analyses like molecular docking (Lamiable *et al*., 2016).

3.6. Validation of epitope interaction with MHC class II through molecular docking

Molecular docking performed between HLA DRB1*07:01 and epitopes that had been previously modeled was done by a specific docking method. Specific docking was performed on active sites of HLA DRB1*07:01 which are GLU88, THR90, LEU92, ILE106, PHE108, ASP110, LYS111, PHE148, THR145, LEU147, ILE148, HIS149, ASN150. Molecular docking results showed differences in binding scores across all epitopes, ranging from -134 Kcal/mol to -186 Kcal/mol (Sup. Table 3). The three epitopes that have the most negative docking score are 26-26-WCDGFCSSRGKRIDL-40-40 (-186.77

Kcal/mol), 27-CDGFCSSRGKRIDLG-41 (-160.15 Kcal/mol), and 25-TWCDGFCSSRGKRID-39 (-156.10 Kcal/mol) (Figure 1). A more negative binding score indicates that the model has a higher probability of binding (Dey *et al*., 2023; Wibowo *et al*., 2021). The epitope 26-26-WCDGFCSSRGKRIDL-40-40 interacts with various types of bonds, with the most dominant interactions being hydrogen bonds and electrostatic bonds that bind to the binding site residues of HLA. The epitope 27-CDGFCSSRGKRIDLG-41 uses hydrophobic interactions as the primary mechanism for interacting with HLA. Meanwhile, the epitope 25-TWCDGFCSSRGKRID-39 interacts through hydrogen bonds, hydrophobic interactions, and electrostatic bonds (Table 7). An increased negative docking score indicates a more likely binding model, suggesting that the three epitopes will be examined further.

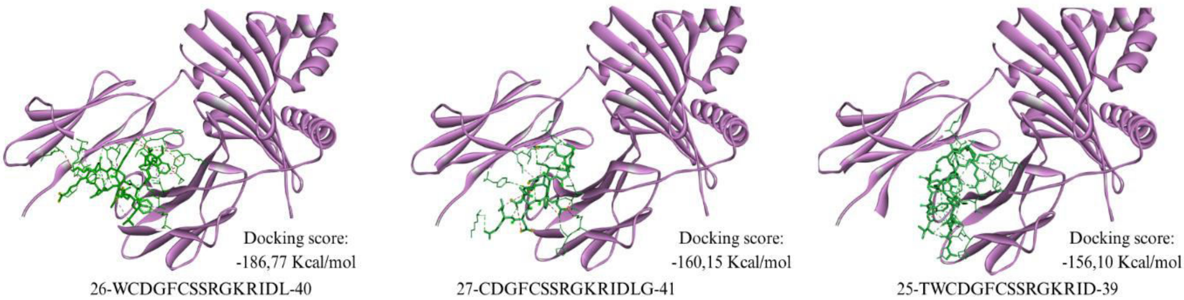

Figure 1. Top three interactions between epitopes and HLA class II.

Table 7. Top three interactions between epitopes and HLA class II

MHC II	Epitope	Docking Score (Kcal/mol)	Interaction	Amino Acids
HLA DRB1*07:01	26-WCDGFCSSRGKRIDL-40	-186,77	Electrostatic	A:LYS111, B:GLU162
			Hydrogen Bond	A:LYS111, A: THR90, A: LYS176, B: TYR102, B:HIS149, B:ALA104, B:THR145, B:LEU158
			Hydrophobic Bond	A:LEU92, B: ALA104, B:VAL143, B:LEU158, B:VAL116, A:VAL91, A:LYS176
			Other	A:PHE108
	27-CDGFCSSRGKRIDLG-41	-160,15	Hydrogen Bond	B:TYR102, B:THR145, B:LEU147, B:THR145, A:HIS177, B:LYS105, B:ILE148, B:THR145, A:THR90
			Hydrophobic Bond	B:LEU158, B:ILE148
	25-TWCDGFCSSRGKRID-39	-156,1	Electrostatic	A:GLU88, A:ASP110, A:PHE108, A:TRP178
			Hydrogen bond	A:THR90, A:LYS111, A:GLU179, A:THR93, A:VAL91, A:TRP178, A:GLU88, A:PHE108, A:HIS177
			Hydrophobic Bond	B:ILE148, A:PHE108

Bold letters indicate the same amino acid residue as the active site

3.7. Evaluation of the stability of epitope-MHC class II complexes with molecular dynamics simulations

The three epitopes with the highest docking scores, which are 26-WCDGFCSSRGKRIDL-40, 27-CDGFCSSRGKRIDLG-41, and 25-TWCDGFCSSRGKRID-39, were used for molecular dynamics simulations with the HLA DRB1*07:01 complex to determine their stability. The analysis was conducted by monitoring RMSD (Root Mean Square Deviation) fluctuations over 20 ns. The analysis results showed that all three epitopes had an average RMSD value below 3 Å. Although they had average values below 3 Å, the three epitopes exhibited different fluctuation patterns. The 26-WCDGFCSSRGKRIDL-40 epitope demonstrated the best stability with an average RMSD of 2.344 Å, with one peak reaching 3.122 Å at 8.125 ns. 27-CDGFCSSRGKRIDLG-41 exhibits similar stability with an average of 2.395 Å, but there is fluctuation at 5.6 ns, where the RMSD value reaches 3.177 Å. Meanwhile, the 25-TWCDGFCSSRGKRID-39 epitope is the least stable among the two epitopes. The average RMSD of the epitope is 2.479 Å and exhibits instability at 6.35 ns and 4–7 ns, where the maximum RMSD value reaches 3.311 Å (Figure 2). The epitope is considered stable because the resulting RMSD value does not exceed 3 Å. This indicates the epitope structure is relatively unchanged through the simulation (Dey *et al*., 2023; Naveed *et al*., 2022).

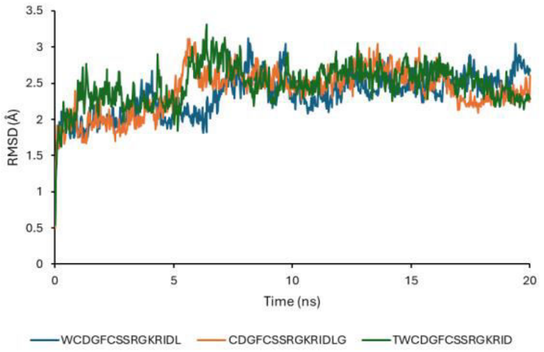

Figure 2. Simulation of the molecular dynamics of the interaction between HLA DRB1* 07:01 and the epitopes.

4. Discussion

This study predicted the potential epitopes of LNTX as the dominant component in *O. hannah* toxin. This prediction represents the initial step in the development of vaccine-based antivenoms, which are expected to prevent the increase in mortality from *O. hannah* bites with greater specificity and safety. The selection of epitopes that exhibited overlap between MHC II epitopes and B cell epitopes was a key consideration in the design of peptide-based vaccines, as it promoted effective cooperation between B cells and CD4+ T helper cells. This paper determines essential requirements of induction of high-affinity antibody responses and development of humoral immune memory (Li *et al*., 2014). The first model of the predicted epitopes included 13 MHC class II epitopes and 4 linear B cell epitopes. Among them, 12 MHC II epitopes were present at locations that overlapped with the predicted B cell epitopes, implying their ability to induce T-cell-mediated, along with antibody-mediated immunity, and therefore their suitability to be used as a candidate in the development of peptide-based vaccines. However, the epitopes that were further developed to undergo further analysis were restricted to three, since the epitopes were the only ones that satisfied all of the necessary conditions, which are conservancy, immunogenicity, allergenicity, and population coverage. The rest of the epitopes were omitted owing to non-adherence to one or more of these parameters.

The chosen epitopes were also preserved in various sequences of LNTX toxin in the proteomes of different species of *O. hannah*. Recovery of epitopes where there is a minimum change in sequence is critical in achieving universal coverage of toxin variants (Bagherzadeh *et al*., 2022). The high immunogenicity shown by the selected epitopes indicates their ability to activate CD4+ T cells and thereby initiate downstream immune responses, including B cell activation and antibody production (Xu *et al*., 2022). The safety of the selected epitopes to human use was also assessed by means of allergenicity. This assessment used several approaches, such as allergenic motif scanning, SVM-based classification, and homology searches (Ahmad *et al*., 2021), The findings showed no signs of an allergenic nature or a possibility of IgE-binding activity of the chosen epitopes, which implies they should be regarded as non-allergenic and can be safely used in the creation of a vaccine (Hayes *et al*., 2015).

The three chosen epitopes were identified as the most promising candidates because they possess very good immunogenicity, but the key weakness of the present work is that the coverage of the population is limited, and this is a critical limitation in vaccine development. Combined, these epitopes represent just 11.26 of the combined populations of eight Southeast Asian countries, a fact owing to much HLA heterogeneity in the area. The highest coverage rate was in Indonesia (20.49 3), but it was still fairly low, and other countries had even lower levels (between 1.22 3 and 15.91 3). The limitation is due to the fact that the study only focuses on one, region-specific HLA allele, HLA-DRB107-01, with a prevalence of 13.7 per cent in Indonesia (Gustiananda *et al*., 2021). Other alleles, including HLA-DRB112:02 (36.8) and HLA-DRB115:02 (24.1), which are more frequent (Hayes *et al*., 2015; Rezaldi *et al*., 2021), were excluded because of their low predicted immunogenicity. In turn, the dependence on a single allele with immunogenicity highlights one of the major tradeoffs and indicates that a single-allele approach cannot be used to protect a wide region. The future vaccine designs should thus be multi-allele. Incorporating a panel of the most frequent and immunogenic HLA Class II alleles from across Southeast Asia is the essential next step to engineer a vaccine with robust, pan-regional efficacy.

The physicochemical characteristics of the epitope were predicted to determine the stability of the epitope. Based on the results, it is known that the three selected epitopes fall into the unstable category, as shown by the stability index, where the three epitopes have a value above 40. The stability index value that exceeds 40 indicates that the epitope is unstable and easily degraded (Raza *et al*., 2021). Epitopes with longer half-lives remain in circulation for extended periods, thereby providing a greater opportunity to be recognized by immune cells. This prolonged exposure enhances the likelihood of effective antigen presentation and subsequent immune activation (Mathur *et al*., 2016). This study found that epitope 25-TWCDGFCSSRGKRID-39 showed the highest estimated half-time value among the three selected epitopes, either in mammalian cells (7.2 h), yeast (>20 h), or *E. coli* (>10 h). As indicated by the relatively high range of aliphatic index (26-52), the selected epitopes were shown to possess thermostability over a broad temperature range. The selected epitopes were hydrophilic or globular (negative GRAVY), therefore, suggesting better solubility in aqueous environments (Panda and Chandra, 2012). These acceptable stability criteria showed the potential suitability of the selected epitopes as a stable candidate for a peptide-based vaccine, while unstable epitopes may require structural optimization (Devi *et al*., 2021).

To overcome this problem, linkers can be added to the epitope to increase their stability. The increase in protein stability after the addition of linkers is due to the spatial structure of the linkers, which provides sufficient space for the protein to fold properly, thus it can function independently (Guo *et al*., 2021). EAAAK linker is a rigid alpha helix peptide responsible for maintaining the functional properties of the epitope (Fadilah *et al*., 2023). In this study, the EAAAK linker was added to the N-terminal and C-terminal of the epitope (Figure 3). The addition of the linker to the epitope successfully increased the stability of the epitope and maintained its physicochemical characteristics (Table 8).

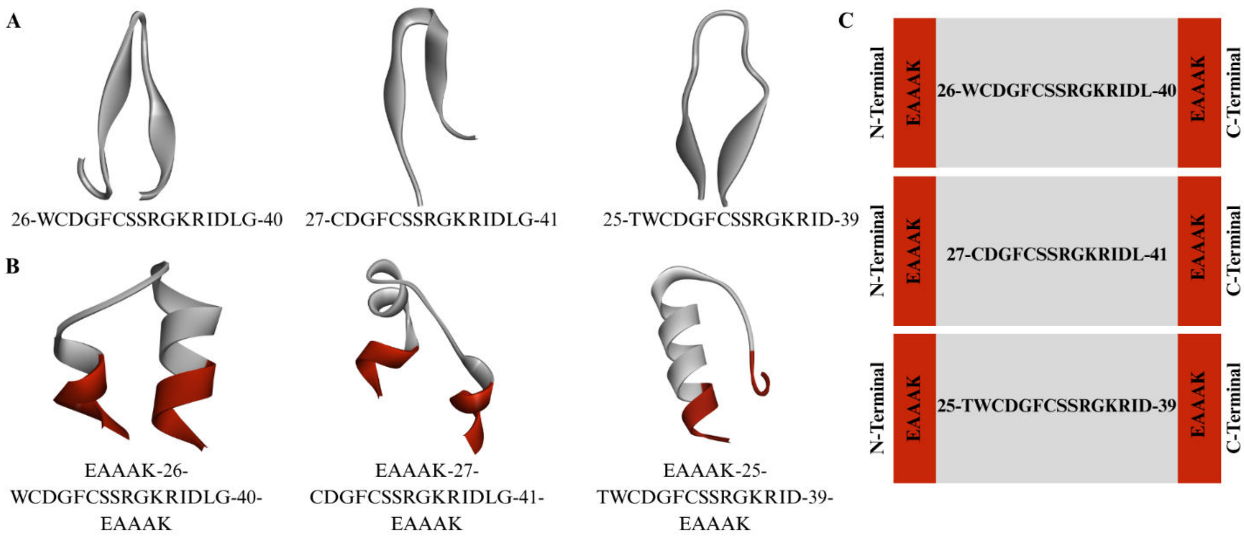

Figure 3. Design of linker conjugated epitopes. A) Initial epitope structure modeled by PEP FOLD3, B) Epitope with linker, C) Epitope sequence with linker. Red color indicated the EAAAK linker and grey color indicated the epitope sequence.

Table 8. Physicochemical characteristics of linker conjugated epitopes.

Physicochemical Characteristic	EAAAKWCDGFCSSRGKRIDL EAAAK	EAAAKCDGFCSSRGKRIDLG EAAAK	EAAAKTWCDGFCSSRGKRIDE AAAK
Molecular Weight	2684.04	2554.88	2671.99
Theoretical pI	8.12	8.12	8.12
Estimated half-life in mammalian cells (Hours)	1	1	1
Estimated half-life in yeast (Min)	30	30	30
Estimated half-life in *E. coli*	10 min	> 10 Hours	> 10 Hours
Instability index	38.96	35.94	32.95
Stability	Stable	Stable	Stable
Aliphatic index	55.20	55.20	39.60
Grand average of hydropathicity (GRAVY)	-0.444	-0.44	-0.624

Molecular docking was performed to predict how ligands and targets will align to form a stable compound (Batra *et al*., 2022). In this study, molecular docking was used to determine the ability of three epitope candidates, namely 26-WCDGFCSSRGKRIDL-40, 27-CDGFCSSRGKRIDLG-41, and 25-TWCDGFCSSRGKRID-39, to bind to the binding groove of HLA DRB1*07:01, which is human MHC-II. All three epitopes are capable of binding to HLA DRB1*07:01 in the binding groove through several kinds of bonding, including hydrophobic interactions, electrostatic bonds, and hydrogen bonds (Table 7). Among the three epitopes, the epitope 26-WCDGFCSSRGKRIDL-40 has the highest docking score.

The ability of epitopes to bind to MHC class II is linked to the role of antigens in generating antibodies (Hanyu *et al*., 2019). With varying affinities, the three identified epitopes can bind to HLA DRB1*07:01. The potential of epitopes to bind to MHC class II increases for epitopes with higher negative docking scores (H. Li *et al*., 2022). On the surface of antigen-presenting cells (APCs), CD4+ T cells will recognize epitopes that bind to the MHC II complex. This recognition aids in the differentiation of B cells into plasma cells, which in turn aids in the production of antigen-specific memory B cells and antibodies against the specific antigen by initiating an immune cascade. Because of their capacity to bind to MHC II binding sites, these three epitopes may therefore be able to initiate the production of antibodies (Moten *et al*., 2023).

The interplay between the epitopes and HLA molecules can only be elucidated by an evaluation of both structural stability and conformational flexibility, which can be measured quantitatively using the simulation of molecular dynamics (Salo-Ahen *et al*., 2020). Simulation findings show that the average root-mean-square movement (RMSD) of the three epitopes is below 3 ⁻ A in the mean, which shows a general structural integrity, although there are occasional exceedances of the limit. The RMSD profile in an epitope thus shows stability, which is a measure of its energy to form strong and sufficient interactions and have high functional efficacy. According to Hassan *et al.* (2024), an epitope's bonding ability and potential efficacy increase with its RMSD value stability. The most stable epitope is 26-WCDGFCSSRGKRIDL-40. Consequently, it possesses the most promise as a possible epitope for vaccine development.

5. Conclusions

This study provides a novel *in silico* framework for a vaccine-based antivenom by identifying three conserved, immunogenic, and stable epitopes from the LNTX of *O. hannah* venom, specifically designed to interact with the prevalent HLA-DRB1*07:01 allele. These results offer a targeted alternative to conventional antisera. Future directions must include *in vitro* and *in vivo* immunological assays to confirm neutralization efficacy, alongside the incorporation of a broader panel of HLA alleles to overcome population coverage limitations and achieve a truly pan-regional vaccine for Southeast Asia.

Data Availability

All data supporting the findings of this study are available in Figshare with the identifier DOI: 10.6084/m9.figshare.30749081 (https://doi.org/10.6084/m9.figshare.30749081). The dataset can be accessed publicly and includes all processed data and supplementary materials used in the analysis.

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