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Cocktail Antigen Presenting Peptide Vaccine Development for Nipah Virus: An Immunoinformatic Approach to Indian and Malaysian Strain

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Abstract

Nipah virus (NiV) is an emerging zoonotic pathogen of global concern, causing serious life-threatening illness in humans. The first outbreak was reported from Malaysia in 1998. The virus is classified under the family *Paramyxoviridae.* Geographically, the deadly virus is known to be distributed in Southeast Asia. Its recent emergence in Kerala, India, indicates its public health emergency and necessitates the urgent proactive measures for the development of a safe and effective vaccine for the prevention and control of the disease. As a single-stranded RNA virus, there is no effective antiviral or vaccine available to fight this virus. Attempt on the development of vaccines had been hampered due to the highly infectious nature of the virus and the requirement of specialised bio-containment facility to handle the pathogen. Considering the potential advantages of reverse vaccinology approaches, the present study attempted to predict vaccine candidates targeting NiV virus genes encoding non-structural and structural proteins (specifically glycoprotein G, glycoprotein F, and W protein) circulating in Southeast Asia. The results of the analysis have suggested two potential vaccine candidates based on various parameters such as antigenicity, immunogenicity, non-toxicity and non-allergenicity. In view of the global interest on urgent requirement of an effective vaccine, the present study predicted candidate vaccine antigens using bioinformatic tools and their promising usefulness as candidate peptides along with ongoing vaccine discovery efforts has been discussed.

Keywords: Viral Zoonoses, Immunoinformatic, Structural and Non-structural Proteins, Emerging Infection, Reverse Vaccinology

INTRODUCTION

The emergence and re-emergence of infectious diseases are very serious public health threats worldwide,[1] and approximately 75% of these diseases are zoonotic and transmitted between humans and animals. Nipah virus is a zoonotic pathogen in humans that causes serious illnesses, such as encephalitis/respiratory diseases with fatal outcomes. It is a close relative of the Hendra virus and is classified under the family *Paramyxoviridae,* within the order: Mononegavirales and the subfamily Paramyxovirinae. It is transmitted to animals/humans by certain species of fruit bats (flying foxes), primarily *Pteropus* spp.[2] and is now distributed in many of Southeast Asian countries, namely Cambodia, East Timor, Indonesia, Malaysia, Papua New Guinea, Vietnam, Thailand, Bangladesh, and India.[3] In a recent episode of Nipah virus outbreak in India and Bangladesh, the virus was found to be transmitted between *Pteropus* bats and humans.[3,4] Due to its highly infectious nature, ease of dissemination and increased rates of mortality and morbidity from the viral outbreak, NiV has been categorized as a BSL 4 pathogen.[5]

This virus has helical symmetry and is an RNA virus with an envelope, and a negative sense, non-segmented, single-stranded genome. The genetic material of the Nipah virus contains a succession of six genes, namely nucleocapsid protein (N), matrix protein (M), phosphoprotein (P), attachment glycoprotein (G), fusion glycoprotein (F), and long polymerase (L). The Nucleoprotein, Phosphoprotein, and long polymerase proteins attach to the viral nucleic acid forming the viral ribonucleoprotein (vRNP) (Figure 1). The attachment (G) and fusion (F) glycoproteins are the causes of virion adhesion to the susceptible host and subsequent entry into the cell.[6] Gene P encodes four proteins, a phosphoprotein (P), and three non-structural proteins, namely, protein V, protein W, and protein C[7] (Table 1).

Glycoproteins on the viral surface, such as the G and F proteins present on the NiV outer membrane, are regarded as prime immune system targets due to their ability to interact with host cell receptors.[8,9] In earlier studies, Rebecca *et al.* reported that the *Paramyxoviridae* virus family possesses two glycoproteins anchored in the membrane, that serve as targets for neutralizing antibodies.[10] Pratik *et al.* reported that because of

the synchronized actions of fusion and attachment glycoproteins, it is quite natural for the Nipah virus to infiltrate the target cell following the binding process.[11] According to Brent *et al.*, the soluble recombinant G glycoprotein subunit has the potential to offer protection not only against identified strains of Henipaviruses such as NiV M and NiV B but also against HeV in four animal species. It was further confirmed that the NiV G glycoprotein is a strong immunogen for the construction of pre-F/G (HN/H) Chimera.[10,11] As stated by Yoneda *et al.*, the W-protein plays a vital role in inhibiting the synthesis of α/β interferon (IFN-α/β), thus eluding the innate immune response.[12] The structural proteins F and G as well as the non-structural protein W are included in the scope of previous investigations aimed at identifying the immunodominant protein of NiV and these findings have been confirmed.[13] Hence, we focused on the NiV structural and non-structural proteins F, G and W from the isolates from India and Malaysia, which have antigenic determinants and can confer an effective immune system defense against infection.[14]

According to the Centers for Disease Control and Prevention (CDC), the current treatment options are limited to supportive care, and approved vaccines or drugs for human use are still unavailable (http://www.cdc.gov/vhf/nipah/prevention/index.html). More conclusive evidence on the effectiveness of antiviral drugs for treating NiV infections in humans is needed due to the limited number of *ex vivo* and *in vivo* studies conducted thus far. Animal model studies have evaluated a number of antiviral drugs for the control of NiV infection such as ribavirin, remdesivir and favipiravir[15] (Table 2).

NiV has been listed by the WHO as one of the top 10 emerging viruses for which urgent research and development are needed (World Health Organization. WHO R&D Nipah Baseline Situation Analysis; WHO: Geneva, Switzerland, 2018) (https://www.who.int/emergencies/disease-outbreak-news/item/2023-DON490).

Table 1. Proteins of NiV and their functions

Type of Protein	Name of Protein	Function
Structural proteins	G Glycoprotein	Promotes virion entry by clathrin mediated endocytosis
	F Glycoprotein	Merge the viral and host cell membrane
	P Phosphoprotein	Helps for replication and transcription of virus
	N Nucleoprotein	Protects viral genome from nucleases
	M Matrix protein	Facilitates RNA progression
	L Protein	Catalyzes transcription of viral mRNA
Non-structural proteins	C Protein	Counteracts with cellular IFN
	V Protein	Inhibits host immune response
	W Protein	Blocks IFN alpha & beta

Attachment glycoprotein [G]

Fusion glycoprotein [F]

Lipid bilayer

Matrix protein [M]

Nucleoprotein [N]
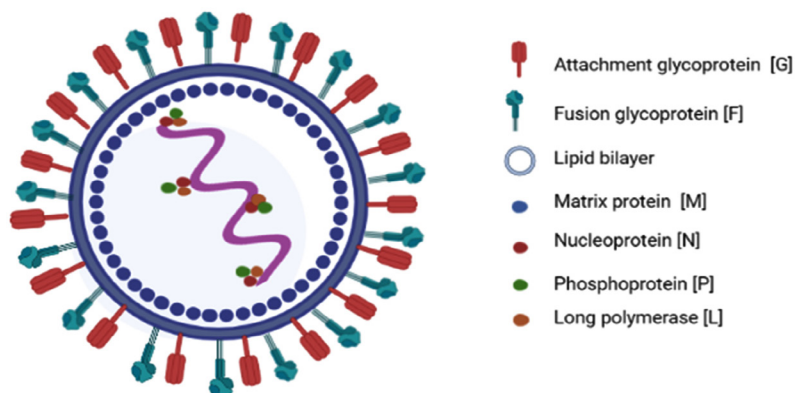
Phosphoprotein [P]

Long polymerase [L]

Figure 1. Structural representation of Nipah virus

Traditional vaccine development is quite difficult due to the extremely lethal nature of NiV, and the virus needs to be cultured *in vitro*. Several investigations are in progress for the development of vaccines against NiV infection, and majority of these investigations are based on a single protein /epitope of the protein (Table 3).

To overcome the limitations of traditional vaccine design and development, modern computational approaches have emerged, including, reverse vaccinology, systems biology, next-generation sequencing technology, and the design of epitope-based vaccines.[35,36] Singh *et al*. reported that Immunoinformatics, also known as computational immunology, combines computational capabilities with the vast amount of genetic and proteomic data collected from pathogens. This integration aims to comprehend their immune responses, and the acquired information is subsequently employed in the development of vaccines.[37] A study conducted by Martinelli revealed that using *in silico* tools for designing approaches to treat specific pathogens allows researchers to adapt procedures according to their objectives at a minimal cost.[38] Employing computational tools prior to conducting laboratory experiments is beneficial since it is a cost-effective and time-efficient approach. The term computational vaccinology refers to the utilization of computational and mathematical methods to condense extensive immunological data into a more concise and manageable format.[39] Fadaka *et al*. reported that immunoinformatics approaches are gaining increasing acceptance as the primary strategy for developing potent vaccines against various microorganisms, especially viruses.[40] According to Shawan *et al*., the application of computational vaccinology, bolstered by strategies such as vaccinomics and immunoinformatics, has positioned the world at an advantageous stage. This perspective allows for the economical and time-efficient screening and detection of antigens of interest, facilitating the development of vaccine candidates to combat emerging pathogenic invasions.[41] Targeting the right protein or gene that can provide the necessary outcomes or immune response is essential for improving the development of vaccines and medicinal agents.[42] Hence, in this study, we utilised two structural proteins (G and F) and one non-structural protein (W) for the designing of two vaccine constructs (Malaysian and Indian) using the Reverse vaccinology approach.

Table 2. Status of currently available antiviral drugs for NiV infection

Antiviral drug	Species	Remarks	Ref.
Ribavirin	Human (Malaysia outbreak)	36% decrease in death rate	15
	Syrian hamster	Not any advantageous impact	15
Chloroquine	Syrian hamster	Not any advantageous impact	15
	Ferret		15
Neutralizing antibodies	Syrian hamster	Pretreatment resulted in 100% survival; post treatment provided only partial protection	16
m102.4 antibody	Ferret	100% existence was observed once treatment was administered one day post inoculation	17
Poly(I)-poly(C12U)	Syrian hamster	An 80% existence was observed once treatment was administered two hours post inoculation and continued for 9 more days	18
VIKI-PEG 4-chol	Syrian hamster	A 40% existence was observed when treatment was administered 2 days after inoculation	19
Remdesivir	African green monkey	Prevents viremia not viral replication	20
Acyclovir	Human (Singapore outbreak)	Acting as an analogue to deoxyguanosine triphosphate	21
Favipiravir	Hamster	Highest antiviral activity	22
Rintatolimid (Ampligen)	Hamster	Blocks viral replication	23

Table 3. Currently available NiV vaccines (Under development)

Vaccine	Species	Remarks	Ref.
Vaccinia virus vector	Syrian hamster	One vaccination provided protection against the fatal challenge	24
Canarypox virus vector	Pig	Two vaccinations provided clinical disease protection	25
VSV G	Ferret	One vaccination provided protection against heterologous fatal challenge	26
	Syrian hamster	One vaccination provided protection against fatal challenge	27
	African green monkey	Induced stable and robust humoral responses with moderate cellular response	28
Adeno associated virus vector	Syrian hamster	One vaccination provided protection against fatal challenge	29
Measles virus		Two vaccinations provided protection against fatal challenge	30
	African green monkey	After two vaccinations, there is protection from clinical disease	
sG NiV	Cat	After three vaccinations, protection against clinical disease is achieved	31
sG HeV		All vaccinated animals were protected from disease	32
	Ferret	Two vaccinations provided protection against fatal challenge for up to 1 year	33
	African green monkey	Vaccination provided protection against fatal challenge	34

MATERIALS AND METHODS

Protein sequences retrieval

The sequences of the two structural proteins attachment glycoprotein G (ID: CBM41034.1, QBQ56723.1), fusion glycoprotein F (ID: CBM41033.1, QBQ56722.1), and one non-structural protein W protein (ID: QHR78997.1, QBQ56719.1) of Indian and Malaysian isolates of NiV were obtained from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein) in the FASTA format.

Protein Variability Testing (PVS)

To analyse and characterize the differences/variations in the amino acid sequences /structures of proteins (the Indian and Malaysian NiV proteins), protein variability testing was performed using the Protein Variability Server (http://imed.med.ucm.es/PVS/). This server employs various variability metrics to calculate the variability of sequences within a multiple sequence alignment.

Prediction of CTL, HTL and linear B-Cell epitopes

The IEDB (https://www.iedb.org/) Immune Epitope Database, was utilized for the prediction of cytotoxic T lymphocytes (CTL) and helper T-cell epitopes from peptides/proteins. The Net MHC pan EL4.1 and IEDB recommended the 2.22 method for the prediction process.[43] The IEDB selection process incorporates a consensus approach, which incorporates NN-align, SMM-align, CombLib, and Sturniolo, if there is an appropriate predictor for the molecule.[44] The prediction of linear B-cell epitopes was carried out by employing the ABC Pred software tool, which is accessible at http://crdd.osdd.net/raghava/abcpred/. Notably, a B-cell epitope refers to a short peptide capable of cross-reacting with an antibody, that binds to a conformational epitope.

Toxicity, allergenicity, antigenicity and immunogenicity prediction of selected epitopes

To ensure safety, all epitopes were subjected to toxicity screening with the ToxinPred2 server (https://webs.iiitd.edu.in/raghava/toxinpred2/), and only non-toxic epitopes were considered for subsequent analysis. The server

utilizes a wide range of techniques and information sources for prediction including machine learning techniques, BLAST, and MERCI. To predict the allergenicity of the epitopes, we utilized the AllerTOP v.2.0 server (https://www.ddg-pharmfac.net/AllerTOP/method.html), which is the initial server for alignment-free *in silico* prediction of allergens depending on the physical and chemical properties of protein sequences.

Table 4. NiV protein details

| No.	Name of protein	Country from which isolated	NCBI Accession number	No. of amino acids	Mol. wt (kD)	Amino acid composition (%)	
						Aliphatic	Aromatic
1	Attachment glycoprotein G	India	QBQ56723.1	602	67179.06	39.2	9.1
2	Fusion glycoprotein F		QBQ56722.1	546	60280.90	43	7.2
3	W Protein		QBQ56719.1	450	48917.63	37.7	4.9
4	Attachment glycoprotein G	Malaysia	CBM41034.1	602	67056.91	40.2	8.9
5	Fusion glycoprotein F		CBM41033.1	546	60254.87	42.6	7.0
6	W Protein		QHR78997.1	450	49521.72	36.8	4.7

(a)

(b)

(c)
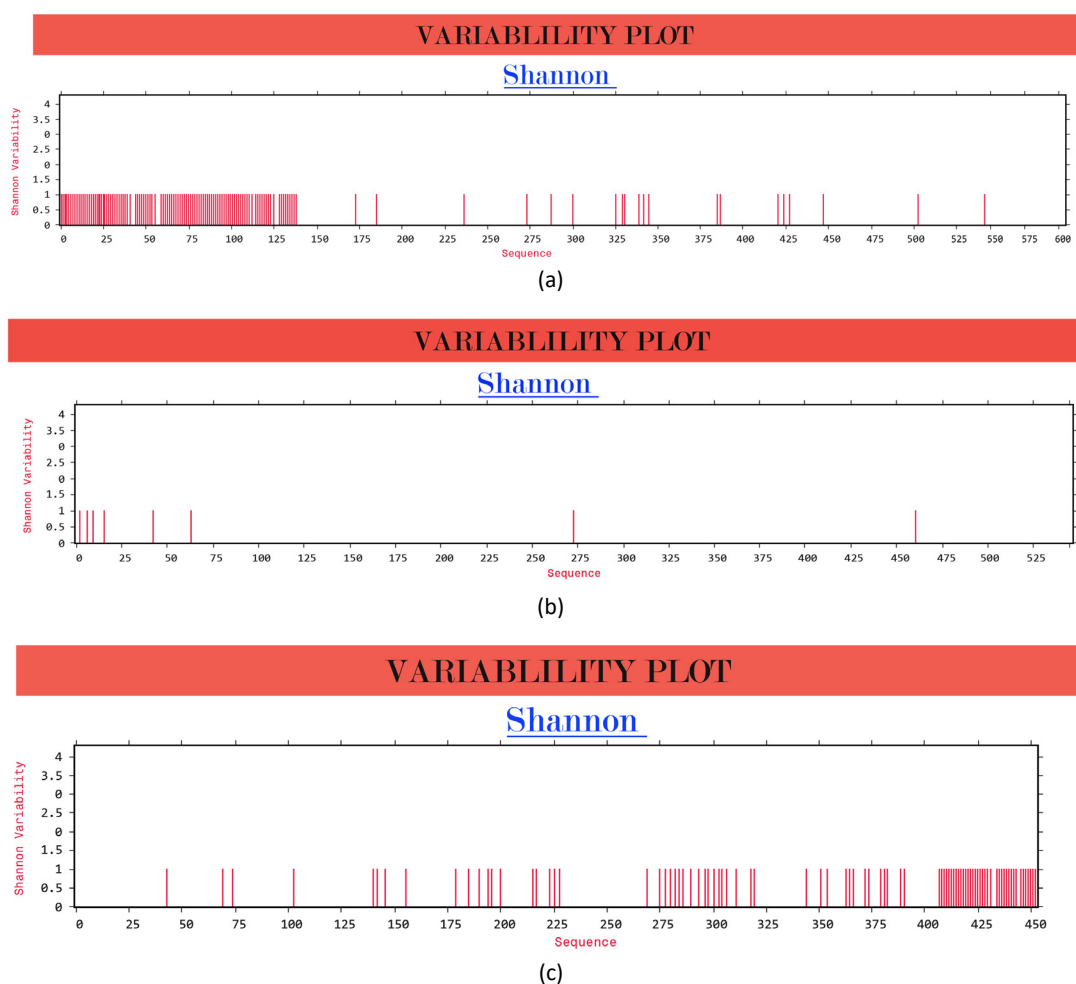
Figure 2. Protein variability analysis results. (a) G glycoprotein, (b) F glycoprotein, (c) W protein

Table 5. TCL Epitopes of G Glycoproteins from the Indian isolates

Protein & Origin	Epitopes	Toxicity	Allergenicity	Antigenicity score (Th value: 0.4*)	Immunogenicity
G Glycoprotein India	AFLIDRINW YFPAVGFLV	Non-toxin	Probable Non-allergen	0.9509 0.8143	0.3091 0.2008

*Threshold value

The VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) server was employed for the prediction of antigenicity of epitopes, and it is the first alignment-free method in which protein sequences are subjected to an autocross-covariance (ACC) transformation at a threshold of 0.4. To evaluate the immunogenicity of the predicted epitopes,
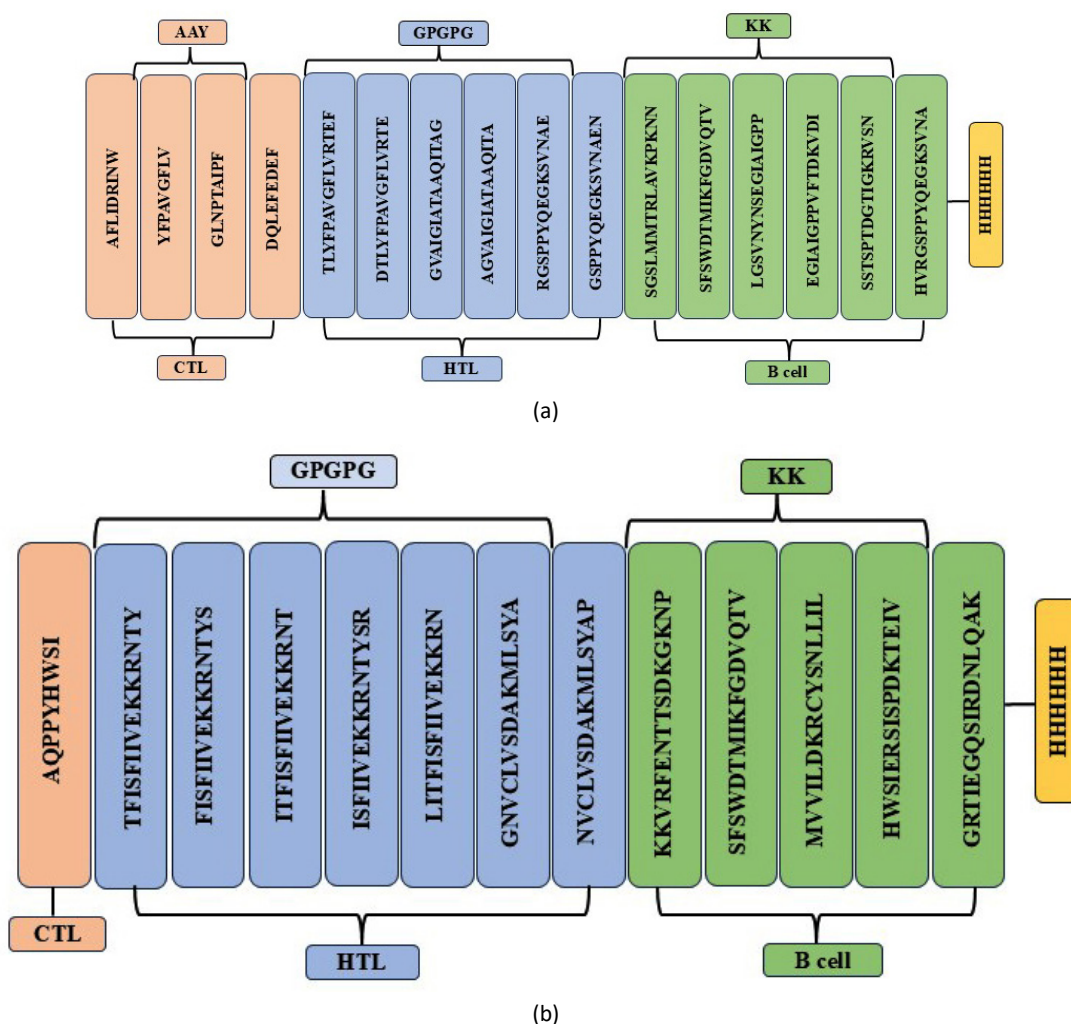
Figure 3. (a) Multiepitope vaccine construct- Indian Isolate, (b) Multiepitope vaccine construct- Malaysian Isolate

the IEDB Immunogenicity (Class I) server (https://tools.iedb.org/immunogenicity/) was utilized to predict the immunogenicity of the peptide MHC (pMHC) complex. This tool utilizes the arrangement of amino acids within the peptide and their properties.

Designing the multiepitope vaccine candidates-PNiV Vac-M and PNiV Vac-I

Antigenicity, immunogenicity, non-toxicity, and non-allergenicity play a vital role in new vaccine construction with epitopes. Two vaccine candidates were designed, PNiV Vac-M (Malaysia) and PNiV Vac-I (India). The CTL, HTL, and B-cell epitopes that passed the screening were chosen. HTL and linear B cell epitopes were linked by a GPGPG linker and the CTL epitopes were linked by an AAY linker.

Prediction of the quality and physiochemical properties of PNiV Vac-M and PNiV Vac-I

The quality attributes (toxicity, allergenicity and antigenicity) of PNiV Vac-M & PNiV Vac-I were assessed, Toxinpred 2.0 was

Table 6. HTL epitopes of G Glycoproteins from the Indian isolates

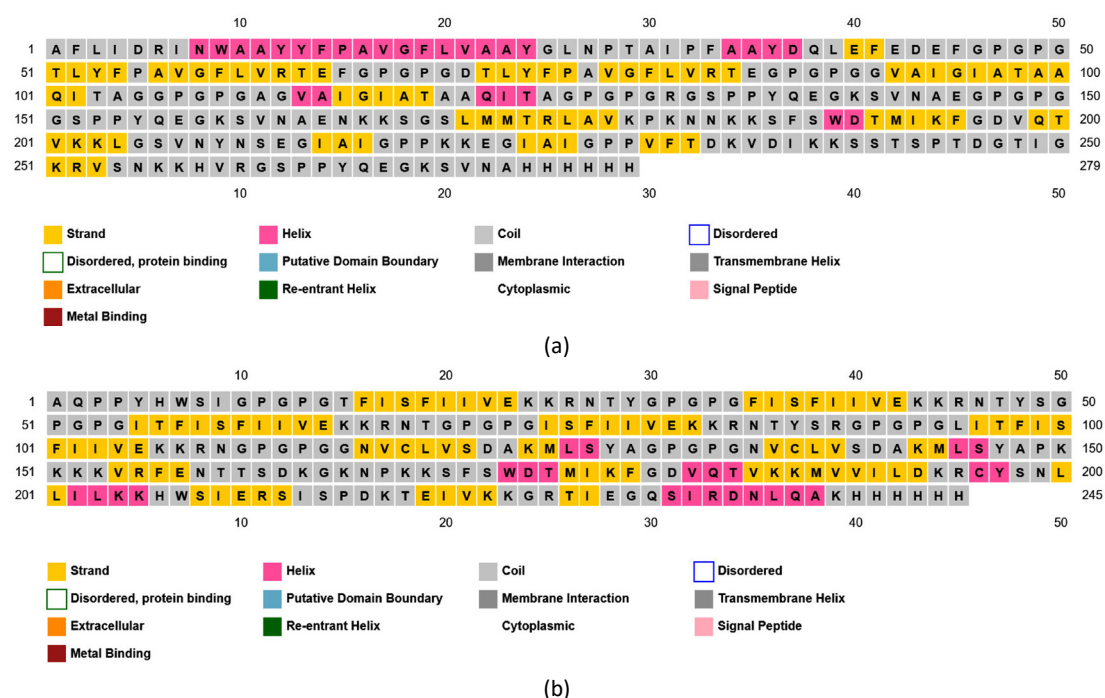| Protein & Origin	Epitopes	Toxicity	Allergenicity	Antigenicity score (Th value: 0.4)	Immunogenicity
G Glycoprotein India	TLYFPAVGFLVRTEF	Non-toxin	Probable Non-allergen	1.0010	91.0893
	DTLYFPAVGFLVRTE			0.8064	92.2596
	GVAIGIATAAQITAG			1.0404	96.1663
	AGVAIGIATAAQITA			0.9497	93.4635
	RGSPPYQEGKSVNAE			1.0176	98.5261
	GSPPYQEGKSVNAEN			0.9395	96.8956

Figure 4. 2D structure of PNiV Vac-I (a) & PNiV Vac-M (b)

used for toxicity prediction, and allergenicity was estimated via the Allertop 2.0 and VaxiJen 2.0 software tools to assess the antigenicity scores of the vaccine candidates. The Protparam tool (https://web.expasy.org/protparam/) available on the ExPASy server was used to predict physiochemical properties (molecular weight, amino acid number and composition, isoelectric point, instability and aliphatic index, extinction coefficient, net charge and hydropathicity value-GRAVY).

Two-dimensional & three-dimensional structure prediction

We used the PSIPRED (https://bioinf.cs.ucl.ac.uk/psipred/) tool to generate the secondary structure of PNiV Vac-I & PNiV Vac-M. PSIPRED is a PSI-BLAST based secondary structure prediction tool, that uses artificial neural network machine learning methods in its algorithm (https://en.wikipedia.org/wiki/PSIPRED). 3D

models of the final vaccine construct were generated by employing the Alphafold Colab (https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb).

Refinement, energy minimization & validation

For refinement of the 3D model of PNiV, the Vac-I and PNiV Vac-M Galaxy refine web server was used (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE). The Galaxy Refine server operates on a refinement methodology that underwent successful validation in refinement experiments conducted during CASP10. Top of Form To minimize the energy of PNiV Vac-I and PNiV Vac-M Forms, a Chiron server was used (https://dokhlab.med.psu.edu/chiron/login.php). Chiron swiftly minimizes the energy of protein molecules by employing discrete molecular dynamics, utilizing an all-atom representation for every residue within the protein. The PNiV Vac-I

(a)   (b)
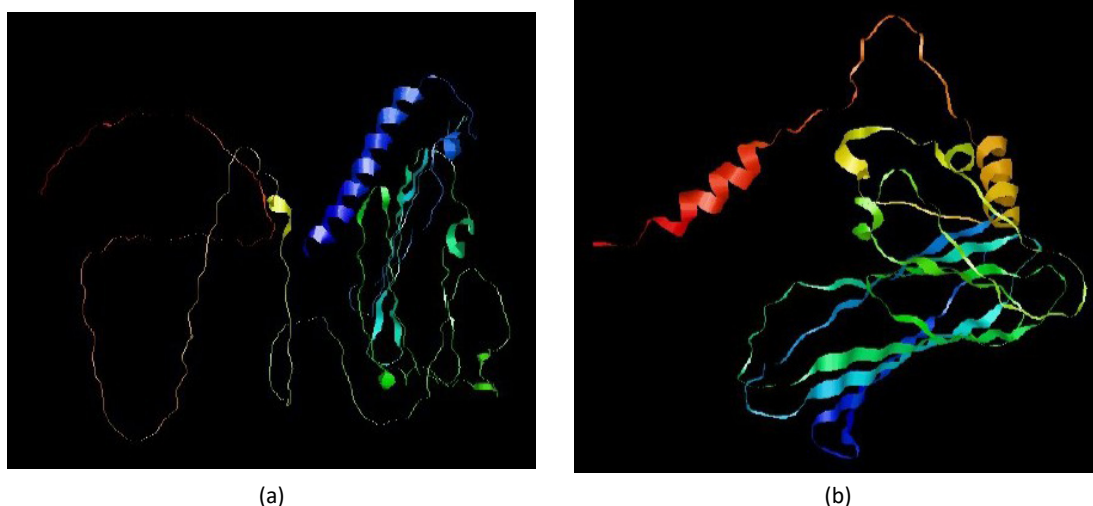
Figure 5. 3D structure of PNiV Vac-M (a) & PNiV Vac-I (b)

Table 7. B-Cell epitopes of G Glycoproteins from Indian and Malaysian isolates

Protein & Origin	Epitopes	Toxicity	Allergenicity	Antigenicity score (Th value: 0.4)
G Glycoprotein India	SFSWDTMIKFGDVQTV	Non-toxin	Probable	1.0016
	SGSLMMTRLAVKPKNN		Non-allergen	1.1763
G Glycoprotein Malaysia	SFSWDTMIKFGDVQTV			1.0016
	KKVRFENTTSDKGKNP			0.8723

& PNiV Vac-M were validated using RAMPAGE (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/).

Molecular docking of PNiV Vac-M & PNiV Vac-I with toll-like receptor 4

An effective method for assessing the cross-molecular interactions between proteins
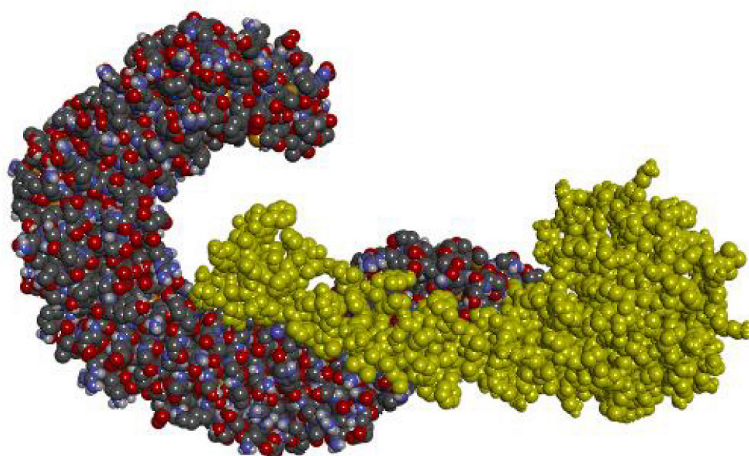
Figure 6. (a) 3D interaction diagram for PNiV Vac-I with TLR4, (b) 3D interaction diagram for PNiV Vac-M with TLR4

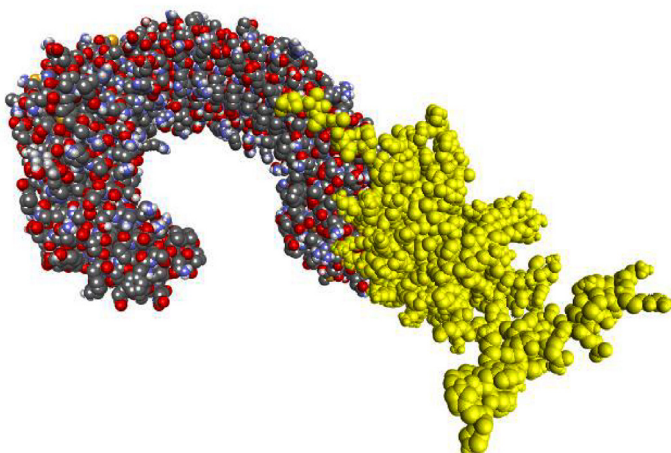and proteins is *in silico* molecular docking. The Schrodinger module version 11.8 was used to perform the protein-protein docking of the TLR4 with PNiV Vac-M and PNiV Vac-I. The protein structure was generated using the protein preparation wizard within the Maestro 11.8 version of the Schrodinger suite. The protein was pre-processed by introducing hydrogen bonds to stabilize the atomic positions and by eliminating extraneous chains, excluding water molecules, and removing heteroatom ion compounds. Energy minimization was carried out using the force field of OPLS_3e (Optimized Potentials for Liquid Simulations, version 3e). Protein-protein docking was performed using the Schrodinger Bio luminate package with default parameters according to the manufacturer's protocol.

MD simulation of the PNiV Vac-M & PNiV Vac-I -TLR4 complex

Molecular dynamics was used to simulate the docked complexes, and protein complex MD simulations were conducted using the Desmond program (Desmond 2018-4). Using the steepest descent method, the prepared systems underwent energy minimization across 2000 interactions. To equilibrate the system, Desmond's default parameters were used. Then, using the NPT (normal pressure and temperature) ensemble with a time step of 2 fs, the 100 ns MD simulations were run with equilibrated systems at 300 K and constant pressure of 1 atm.

RESULTS

Protein selection and protein sequence retrieval

Among the NiV structural proteins F (Fusion protein), G (Attachment Glycoprotein), M (Matrix protein), L (RNA Polymerase), P (Phosphoprotein) and M (Matrix protein) and the non-structural proteins C, V and W, highly antigenic and immunogenic proteins i.e., glycoprotein F (fusion), glycoprotein G (attachment) and the W protein were selected and further subjected
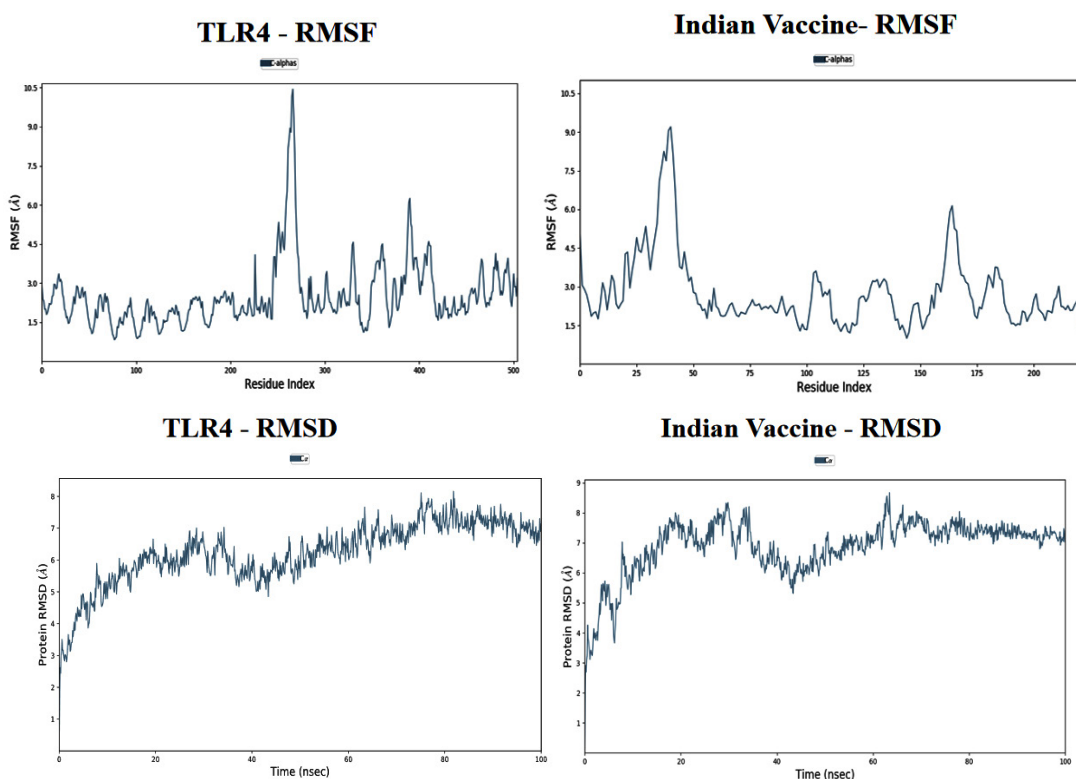
(a)

(b)

Figure 7. Results of Molecular Dynamics simulation of vaccine candidates. Root mean square deviation (RMSD) plots reflec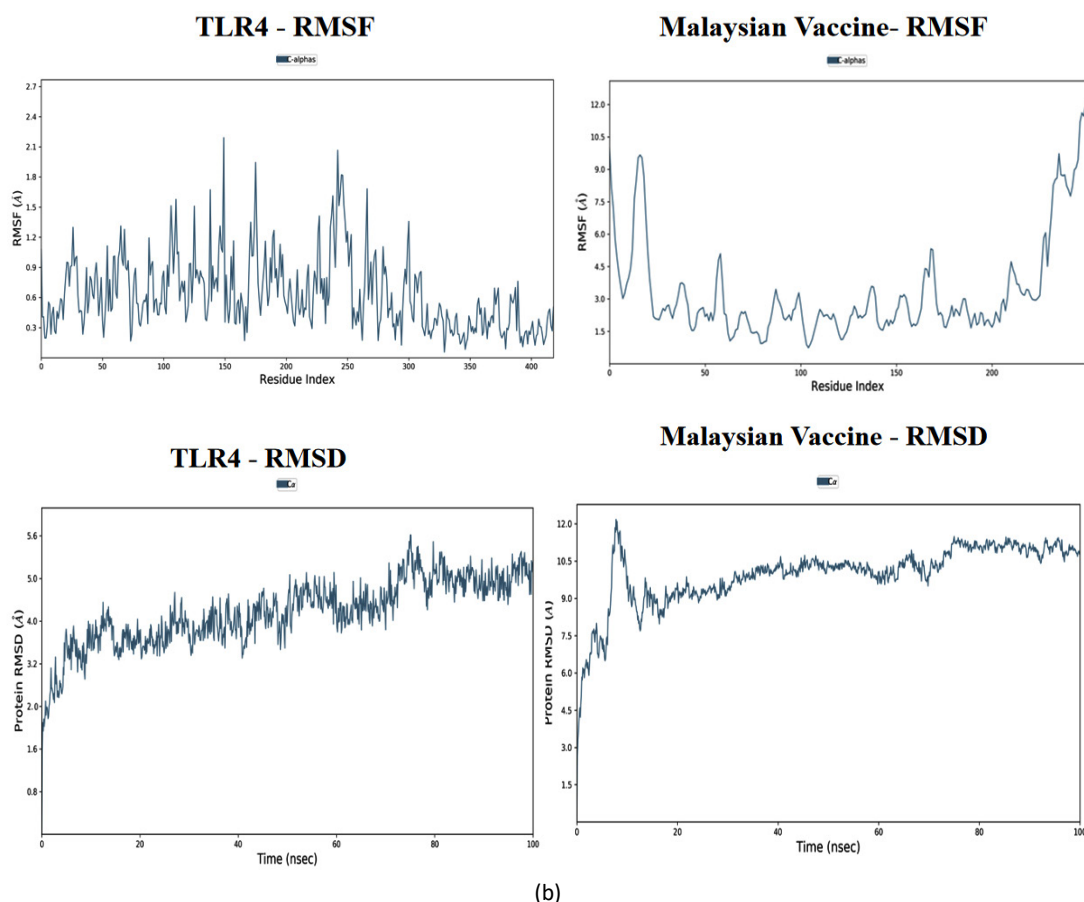ts the stability between the vaccine and TLR 4 and Root mean square fluctuation (RMSF) reflects the flexibility and fluctuation of the amino acids residues in the docked complexes. a) PNiV Vac-I b) PNiV Vac-M

to epitope prediction. The F glycoprotein, G glycoprotein and W protein sequences of Indian and Malaysian isolates/strains of NiV were obtained from the NCBI database in the FASTA format and stored along with their accession numbers for further studies (Table 4).

Evaluation of protein variability

The protein variability between structural and non-structural proteins of Indian and Malaysian isolates/strains was analysed using the protein variability server, which is based on Shannon entropy analysis.[45] The Shannon entropy (H) for every position is as follows

$$H = -\sum_{i=1}^{M} P_i \log_2 P_i$$

Here, P_i signifies the proportion of residues of amino acid type i, while M represents the total number of amino acid types. The range of H spans from 0 indicating the presence of only one residue at that position, to 4.322 where each of the 20 residues is equally represented at that position. In general, positions with H >2.0 are regarded as variable, while those with H <2 are deemed conserved. Positions are deemed highly conserved when H <1.0.

The Shannon entropy of the G, F and W proteins of structural and non-structural proteins from Indian and Malaysian isolates/strains were found to be 1. These proteins remain highly conserved without any significant variations (Figure 2).

Table 8. HTL epitopes of F Glycoproteins from the Indian & Malaysian isolates

Protein & Origin	Epitopes	Toxicity	Allergenicity	Antigenicity score (Th value: 0.4)	Immunogenicity
F Glycoprotein India	SEWISIVPNFILVRN	Non-toxin	Probable Non-allergen	0.5788	65.948
F Glycoprotein Malaysia	ITFISFIIVEKKRNT			1.7597	82.1445
	LITFISFIIVEKKRN			1.5214	76.5204
	TFISFIIVEKKRNTY			1.4822	81.2113
	FISFIIVEKKRNTYS			1.4463	77.8806
	ISFIIVEKKRNTYSR			1.2290	76.7376

Table 9. BCL epitopes of F Glycoproteins from the Indian and Malaysian isolates

Protein & Origin	Epitopes	Toxicity	Allergenicity	Antigenicity score (Th value: 0.4)
F Glycoprotein India	LGSVNYNSEGIAIGPP	Non -toxin	Probable Non-allergen	1.1587
	EGIAIGPPVFTDKVDI			0.7407
F Glycoprotein Malaysia	MVVILDKRCYSNLLIL			0.7160

Table 10. TCL epitopes of W Proteins from the Indian & Malaysian isolates

Protein & Origin	Epitopes	Toxicity	Allergenicity	Antigenicity score (Th value: 0.4)	Immunogenicity
W Protein India	GLNPTAIPF	Non-toxin	Probable Non-allergen	1.8141	0.16721
	DQLEFEDEF			1.033	0.38262
W Protein Malaysia	AQPPYHWSI			0.9115	0.10237

Prediction of CTL, HTL and linear B-cell epitopes

To predict T-cell epitopes, which are peptides identified by T-cell receptors and presented on antigen presenting cells (APCs) through class I or II molecules, the prediction tool for T-cell epitopes of the Immune Epitope Database and Analysis Resource (IEDB) was utilized. Ten epitopes were selected for each protein according to their percentile rank and good binding was indicated by a low percentile rank. These selected epitopes were further tested against MHC I supertype alleles, specifically the human leucocyte antigens of India and Malaysia HLA-A*24:02 and HLA-A*24:07, respectively.

Prediction of the HTL epitopes was carried out using the IEDB server. The default mode peptide length of 15 residues was adjusted, and the epitopes were then screened against DRB1*15 and DRB1*12:02 MHC II alleles found in India and Malaysia, respectively. The NetMHCII pan method was employed for prediction, and epitopes were chosen depending on their percentile rank, with low ranks indicating good binding properties. The humoral immune system relies heavily on the crucial participation of B lymphocytes which produce targeted antibodies against pathogens. Ten linear B-cell epitopes for each protein were predicted using the ABC web server. The epitope that received the highest score was chosen for vaccine construction (Table 5-12).

Table 11. HTL epitopes of W Proteins from the Malaysian isolates

Protein & Origin	Epitopes	Toxicity	Allergenicity	Antigenicity score (Th value: 0.4)	Immunogenicity
W Protein Malaysia	NVCLVSDAKMLSYAP	Non-toxin	Probable Non-allergen	0.8356	91.4996
	GNVCLVSDAKMLSYA			0.7717	91.4531

Table 12. BCL epitopes of W Proteins from the Indian & Malaysian isolates

Protein & Origin	Epitopes	Toxicity	Allergenicity	Antigenicity score (Th value: 0.4)
W Protein India	SSTSPTDGTIGKRVSN	Non-toxin	Probable Non-allergen	1.1938
	HVRGSPPYQEGKSVNA			0.9715
W Protein Malaysia	HWSIERSISPDKTEIV			0.6304
	GRTIEGQSIRDNLQAK			0.4629

Assessment of the toxicity, allergenicity, antigenicity, and immunogenicity of the selected epitopes

The chosen epitopes were assessed for their quality traits, i.e. toxicity, allergenicity, antigenicity, and immunogenicity. Epitopes exhibiting high antigenicity and immunogenicity, along with nontoxic and nonallergic properties, were preferred for further investigation. Sixteen and 13 epitopes were selected from the 60 epitopes of the Indian and Malaysian isolates, respectively.

Construction of the multiepitopic vaccine candidate- PNiV Vac-M & PNiV Vac-I

The development of multiepitopic vaccine candidate involves the fusion of various categories of epitopes. Two vaccine candidates were designed; one representing the Malaysian isolate, PNiV Vac-M and the other representing the Indian isolate PNiV Vac-I. PNiV Vac-M was constructed using 1 CTL, 7 HTL and 5 B-cell epitopes, and PNiV Vac-I was constructed using 4 CTL, 6 HTL and 6 B-cell epitopes. The selected immunogenic epitopes were further linked together using a KK linker (B cell), an AAY linker (HTL), and a GPGPG linker (CTL). T-helper cells targeted epitopes using GPGPG linkers, while AAY linkers were employed to associate B-cell linear epitopes. Additionally, for easy downstream processing, a 6xHis tag made of polyhistidine was appended at the C-terminus (Figure 3).

a) PNiV Vac-I
b) PNiV Vac-M

Assessment of the quality and physiochemical properties of PNiV Vac-M & PNiV Vac-I

The quality attributes (toxicity, allergenicity and antigenicity) and physiochemical properties (molecular weight, isoelectric point, amino acid number and composition, instability index, aliphatic index, extinction coefficient, net charge and hydropathicity value) of PNiV Vac-M and PNiV Vac-I were assessed. Both candidates were found to be nontoxic, nonallergenic and antigenic. The molecular weights of the vaccine candidates were 28982.80 and 27201.24 D for PNiV Vac-I and PNiV Vac-M, respectively. The amino acid residues of PNiV Vac-I and PNiV Vac-M were 279 and 245, respectively. PNiV Vac-I has a hydropathicity index of -0.292, and PNiV Vac-M has a value of -0.426. The aliphatic indices of PNiV Vac I and PNiV Vac-M were 67.53 and 77.51, respectively. PNiV Vac-I has an instability index of 26.41 and PNiV Vac-M has an instability index of 35.24. The isoelectric point of PNiV Vac-I was determined to be 9.30 and that of PNiV Vac-M was

Table 13. Physiochemical properties of the PNiV Vac-M & PNiV Vac-I vaccine candidates

Vaccine construct	Amino acid	Molecular weight (D) composition	Aliphatic index	Theoretical pI	Instability index	GRAVY	Extinction coefficient	Toxigenicity	Allergenicity	Antigenicity
PNiV Vac-I	279	28982.80	67.53	9.30	26.41	-0.292	25900	Non-toxin	Probable Non-allergen	0.7997
PNiV Vac-M	245	27201.24	77.51	10.08	35.24	-0.426	27055	Non-toxin	Probable Non-allergen	0.8002

Table 14. Molecular Docking

No.	Construct vaccine	Piper Score	Piper Energy
1.	Indian construct	-316.054	-587.658
2.	Malaysian construct	-451.548	-634.281

10.08. The extinction coefficients were 25900 and 27055 for PNiV Vac-I and PNiV Vac-M, respectively (Table 13).

Prediction of the 2D and 3D structures of PNiV Vac-M and PNiV Vac-I: refinement, energy minimization & validation

Prediction of the 2D structure was performed using PSIPRED which revealed that PNiV Vac-M had 6.0% helices, 31.6% strands, 4.8% beta strands and 57.6% random coils, whereas PNiV Vac-I had 9.6% helices, 31.1% strands, 8.9% beta strands and 50.1% random coils (Figure 4). 3D structure prediction was conducted on the designed vaccine candidates using and Alphafold software tool (Figure 5). The resulting 3D structures were refined, subjected to energy minimization and further validated via a Ramachandran plot. The resulting data indicate that the structures exhibit high-quality resolution, and atomic clashes between the residues upon steric confirmation.

Molecular docking interaction between PNiV Vac-M & PNiV Vac-I and TLR 4

The protein database provided the crystal structure of the human TLR 4 (PDB ID: 4g8a) complex. The refined PNiV Vac-I & PNiV Vac-M underwent molecular docking with the monomeric form of human TLR4 immune receptors. The crystallographic structure of the human TLR 4 complex consists of 10 chains labelled A through H (A-H), where the A and B chains represent the human TLR 4 receptor, the C and D chains correspond to lymphocyte antigen 96, and the E-H chains are associated with various forms of glucopyranose.

The protein complexes were further subjected to protein and protein docking as per the Schrodinger Bio luminate protocol with their respective target proteins. For the TLR4- PNiV Vac-I complex a Piper score of -316.054 kcal/mol and a Piper energy of -587.658 kcal/mol were

Table 15. Interaction of the Indian and Malaysian vaccine constructs with TLR4

	Indian vaccine construct vs TLR 4				Malaysian vaccine construct vs TLR 4		
No.	Protein	Protein	Distance	No.	Protein	Protein	Distance
1	A: ASN339	B: GLN38	2.6064	1	A: GLN562	B: SER54	1.9647
2	A: LYS560	B: TYR74	1.8231	2	A: GLN565	B: PRO36	2.372
3	A: GLN578	B: LEU52	2.6818	3	A: HIS566	B: GLY35	2.231
4	A: THR584	B: THR72	2.332	4	A: SER589	B: ARG94	1.7474
5	A: HIS587	B: THR72	2.2306	5	A: GLN592	B: ASN73	2.038
6	A: ASN531	B: ARG6	1.7476	6	A: ASP596	B: ARG72	1.6266
7	A: GLU608	B: TYR53	1.5927	7	A: THR626	B: ARG89	2.649
8	A: GLU586	B: THR72	1.634	8	A: GLN588	B: ARG94	1.7333
9	A: THR626	B: ARG252	2.1978	9	A: CYS585	B: ARG94	2.0301

obtained with 9 hydrogen bond interactions. For TLR4-PNiV, the Vac-M complex had a Piper score of -451.548 and a Piper energy of -634.281 with 9 hydrogen bond interactions (Table 14 and 15). The results revealed that the interactions were thermodynamically stable and graphical interaction analysis was carried out through LigPlot+ and PDBsum.

Molecular dynamics simulation

Molecular dynamic simulations of the protein-protein complexes were performed for 100 ns of simulation time, and subsequent analyses were performed on the basis of the simulation trajectory. The root means square deviation (RMSD) acts as a metric for the average alteration in the structural displacement of atoms within a specific frame compared to the reference frame. The root mean square fluctuation (RMSF) is valuable for characterizing the localized variations along the protein chain.

Based on the simulation's trajectory, the RMSD of the TLR4 protein backbone initially increased gradually until reaching 4.0 Å and then stabilized at 7.0 Å until 100 ns, and the RMSF of the complex stabilized at 3.0 Å until 100 ns. The RMSD of the PNiV Vac-I backbone increased gradually until reaching 5.0 Å and then stabilized at 7.0 to 8.0 Å 100 ns, and the RMSF of the complex stabilized at 4.5 Å until 100 ns. Both of the RMSD values were stable at 7.0 Å during the simulation period. In TLR4-PNiV Vac-M, the RMSD of the TLR4 protein backbone initially increased gradually until reaching 2.0 Å and then stabilized at 5.0 Å until 100 ns, and the RMSF of the complex stabilized at 1.5 Å until 100 ns. The RMSD of the PNiV Vac-M

backbone increased gradually until reaching 6.0 Å and then stabilized at 10.5 Å 100 ns, and the RMSF of the complex stabilized at 6.0 Å until the 100 ns. Both of the RDDs show a stable constant value of 4.5 Å during the simulation period (Figure 6 and 7).

DISCUSSION

Currently, there is no approved drug/ vaccine available for the prevention and control of the highly infectious NiV infection (Table 2 and 3). Conventional vaccine development approaches need highly specialised containment facility (BSL-3 & BSL-4) for handling live or infectious NiV. At this juncture, reverse vaccinology approaches are found very promising in design and development of effective vaccine candidate based on their quality attributes such as toxicity, allergenicity, antigenicity, immunogenicity. In this study, amino acid sequences of the structural and non-structural proteins of Indian and Malaysian isolates of NiV were retrieved from the NCBI database and stored in the FASTA format for further analysis (Table 4).

Protein variability testing is a process of analysing and characterizing the differences/ variations in the amino acid sequences or structures of proteins. This type of testing is often used to determine the extent of variation between numerous protein samples or between proteins from different organisms to identify potential variations that may affect protein functions and behaviour. The present research utilized a protein variability server (http://imed.med. ucm.es/PVS/) to analyse the variability of proteins from Indian and Malaysian sources. The results of the analysis

have shown that these proteins remained highly conserved over a period of 2.5 decades, without any significant variations. Hence, the utilization of these proteins for developing a cocktail-based multiepitope vaccine would expect to be highly effective and could subsequently progress for appropriate regulatory studies, both *in vitro* and *in vivo*.

In this study, a total of ten T-cell epitopes and linear B cells for each protein, i.e. F, G & W, based on the percentile rank were predicted, and were found are stimulating antibody-mediated immunity as well as cellular immunity. The quality attributes of each epitope were analysed using commercially available immunoinformatic tools such as VaxiJen, Allertop, and Toxinpred. Based on potential factors of vaccine candidates such as toxicity prediction, allergenicity prediction, antigenicity score, and immunogenicity prediction, the epitopes were assessed (Table 5-12). Furthermore, the epitopes were subjected to linking and constructing CAPPV candidates (Figure 3). The 2D and 3D structures of these constructed vaccines were predicted using PSIPRED and Alphafold, respectively (Figures 4 and 5).

Table 12 shows the physiochemical characteristics of those constructed vaccines- PNiV Vac-I and PNiV Vac-M, such as molecular weight, amino acid number and composition, hydropathicity index (GRAVY), aliphatic index, instability index, extinction coefficient, theoretical PI, and quality attributes such as toxicity, allergenicity and antigenicity.

The molecular weight of the peptides impacts the immune response in different ways. The size of the protein can influence its ability to be processed and presented to immune cells, and the size and shape of the protein influence its ability to interact with immune cells and signalling molecules. The molecular weight of proteins can also have an impact on stability and solubility, and the molecular weights of proteins have a great impact on immune function through a variety of cellular mechanisms.[46] The molecular weights of our vaccine constructs were 28982.80 D and 27201.24 D for PNiV Vac-I and PNiV Vac-M, respectively.

As it has been shown that the amino acid number and composition are influential factors of immune function and the risk of infectious and inflammatory diseases.[47] The present study determined that the amino acid residues of PNiV Vac-I and PNiV Vac-M were determined to be 279 and 245, respectively.

Further, it has been shown that PNiV Vac-I has hydropathicity index of -0.292 and that PNiV Vac-M has a hydropathicity index of -0.426, as the hydropathicity index (GRAVY) influences various aspects of immune functions, including antigen presentation, antibody recognition, and immune cell trafficking. Proteins that possess a GRAVY score above 0 exhibit hydrophobic characteristics, while those with a GRAVY score below 0 display hydrophilic properties. Here, both vaccine constructs were found maintaining their hydrophilic nature.

Considering the significance of aliphatic index in the thermal stability of proteins, our study, determined the aliphatic index of PNiV Vac-I and PNiV Vac-M to be 67.53 and 77.51, respectively, which indicates their thermal stability.

The instability index is a protein measurement used to ascertain its stability. Any protein with an instability index less than 40 was considered stable and those with an instability index greater than 40 were considered unstable.[48] From this study, it was found that the instability indices of PNiV Vac-I and PNiV Vac-M were 26.41 and 35.24, respectively, which indicates that both are stable.

The extinction coefficient is a measure of the amount of light absorbed by a sample at a particular wavelength and depends on the composition and structure of the sample.[49] Our study revealed that the extinction coefficients of PNiV Vac-I and PNiV Vac-M were 25900 and 27055, respectively.

The pI of a protein indicates the pH at which the protein has an overall neutral charge in a particular solution, and it can influence the interplay between the immune system and the vaccine construct in different ways. The pI has an impact on solubility, stability, antigenicity and the ability to elicit an immune response, bioavailability and distribution throughout the body.[50] According to this research, the pI of PNiV Vac-I was determined to be 9.30 and that of PNiV Vac-M was 10.08.

Analysis of the physiochemical parameters revealed that both vaccine constructs satisfied all the necessary requirements for a compound to be considered effective as a vaccine.

Molecular docking studies of PNiV Vac-M & PNiV Vac-I were conducted with TLR 4 to confirm the binding potential, and the results revealed that the interactions were thermodynamically stable. Further dynamic simulations were performed to assess the physical movements of atoms or molecules. The RMSD confirmed that PNiV Vac-I is more stable than PNiV Vac-M.

Similar efforts on the development of NiV vaccine candidates have been documented in the literature. Several investigations have focused on the design, development and production of NiV vaccines.[51] According to the studies of Benjamin *et al*., a subunit vaccine comprising a soluble glycoprotein (Sg) from Hendra virus (HeV), a related henipavirus that causes diseases in humans and equines, the HeV Sg vaccine is presently employed as an animal vaccine to protect horses against Hendra Virus (Equivac HeV, Zoetis) in Australia and is considered as a vaccine candidate for humans against Nipah virus that is still not approved by regulators.

Various approaches utilize viral vectors such as canarypox which codes for NiV G or NiV F,[25] vaccinia viruses, which code for NiV G or NiV F,[24] recombinant AAV, which codes Niv G,[29] recombinant rhabdoviruses (VSV and rabies), which express NiV G or NiV F,[52] and recombinant measles virus vectors, which express NiV G.[30] These approaches have been demonstrated to protect Pigs, Syrian hamsters, Ferrets, and/or AGMs from NiV infection (Table 3).

Recently, an attempt on developing DNA vaccine (DNA-G) and a recombinant chimpanzee adenovirus vaccine (AdC68-G), expressing NiV-G and induce long-term immunity against NiV infection was undertaken.[53]

All of these earlier studies were found focusing on a single protein/epitope of the protein only, despite the single protein-based vaccines may suffer from limited effectiveness, and susceptibility to antigenic variation and have some drawbacks that can compromise their ability to provide broad and durable protection. In our study, we focused on a cocktail approach involving highly antigenic and immunogenic NiV proteins through an immunoinformatic method, which revealed a more comprehensive and resilient immune response and reduced the time, manpower and cost associated with the design and development of newer vaccine candidates.

The vaccine candidate, which was designed using immunoinformatic methods, underwent comprehensive characterization, suggesting its strong immunogenicity, lack of toxicity and allergenicity, and stability under various suitable environmental conditions. The availability of computational tools promisingly facilitates hassle-free designing of an epitope-based peptide vaccine, that is potentially antigenic and highly immunogenic in nature.

CONCLUSION

Nipah virus infection is an emerging serious public health problem worldwide. Considering the high mortality rates and the nonavailability of a safe and effective vaccine against NiV, swift actions should be prioritized for its design and development exploiting modern innovative tools. The present study has demonstrated the application of immunoinformatic tools and successfully predicted certain promising immunogenic cocktail antigen-presenting peptides (CAPP) as promising vaccine candidates, which could be further tested for its immunogenic potential using appropriate *in vivo* methods.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

NKP and SJ conceptualized the study. SJ performed Research Co-ordination and supervision. NKP, SJ and SBP applied methodology. NKP and SJ performed computational work. NKP, SJ and RP performed data analysis. NKP wrote the manuscript. SKC, AAP, MM, KD, PB, SS, SJ and SBP reviewed the manuscript. RP, SJ, SKC, AAP, MM, KD, PB, SS and SBP edited the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not Applicable.

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