Tropical Biomedicine 43(1): 5-15 (2026)
https://doi.org/10.47665/tb.43.1.002

RESEARCH ARTICLE

Sustained antibody response to a linear epitope of Nipah virus fusion protein in human survivor serum samples

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ARTICLE HISTORY

Received: 31 July 2025
Revised: 12 November 2025
Accepted: 14 November 2025
Published: 31 March 2026

ABSTRACT

Nipah virus (NiV), a pathogen with pandemic potential, lacks approved treatments or vaccines, highlighting the urgent need for research on immune-targeted antigenic determinants. A significant gap persists in NiV research, as studies on the fusion (F) protein critical for viral entry as well as the B cells epitopes, have primarily focused on computational prediction rather than experimental validation of immunogenic epitopes obtained through immunoinformatics approach. This study focused on the conserved F protein across NiV human isolates and employed an immunoinformatics approach to predict linear B-cell epitopes capable of direct immune activation. Predicted epitopes were screened for antigenicity, toxicity, and allergenicity. Molecular docking analysis was performed to evaluate its binding affinity with B-cell receptors (BCRs). To validate its immunogenicity, the LF6 peptide was synthesized and used in an indirect ELISA to test sera from cohort previously infected with NiV as well as with negative cohort. Epitope LF6 (LISNIEIGFCL) was identified as a strong candidate based on its immunogenic properties. Molecular docking showed favorable binding of LF6 with BCR. ELISA results revealed that one sera from the survival cohort showed positive response which is an IgG antibody response 2-fold higher (0.343) than the cut-off value (0.0171). This study provides the first reported evidence linking computational predictions with functional immune reactivity for a B-cell epitope of NiV. The findings suggest that LF6 has the potential to elicit specific immune responses. However, given the small sample size, further validation in larger cohorts is essential to confirm LF6's vaccine relevance.

Keywords: Paramyxoviridae; B lymphocytes; humoral immunity; ELISA; IgG antibody.

INTRODUCTION

Nipah virus (NiV) first emerged in Malaysia in 1998, causing an outbreak that resulted in 105 deaths among 265 reported cases (Chua, 2003). The infection typically presents with acute respiratory symptoms and severe neurological complications, often progressing to febrile encephalitis. Subsequent outbreaks in Bangladesh and India have been reported, with case fatality rates ranging from 33% to 100% (Chattu *et al*., 2018). Due to its high mortality rate, potential person-to-person transmission, and lack of approved treatments or vaccines, NiV is classified by the World Health Organization (WHO) as a priority pathogen under its R&D Blueprint for emerging infectious diseases (World Health Organization, 2019). NiV from the *Paramyxoviridae* family, is a single-stranded, non-segmented, negative-sense RNA virus. Its genome encodes six structural proteins (nucleoprotein, phosphoprotein, matrix, fusion, glycoprotein, and large RNA polymerase) and three non-structural proteins (C, V, W) from the P gene (Singh *et al*., 2019). The G and F surface glycoproteins in NiV lipid envelope are crucial for viral infection.

The NiV fusion protein (F), a class I fusion protein like others in paramyxoviruses, has a globular head with three domains, a C-terminal stalk, transmembrane domain, and cytoplasmic tail (Lamb & Jardetzky, 2007). Initially synthesized as F0, it is cleaved by host cathepsins into disulfide-linked F1 and F2 subunits. When G protein binds to the ephrinB2/B3 on host cells, the F protein undergoes conformational changes, releasing the hydrophobic fusion peptide into the host membrane, facilitating viral and host membrane fusion (Aguilar *et al*., 2016). The G and F proteins, exposed on the surface, are key targets for therapeutic antibodies, showing promise in prophylaxis and post-exposure treatment for henipaviral disease (Avanzato *et al*., 2019; Byrne *et al*., 2023). Stabilized prefusion F proteins have triggered strong neutralizing antibody responses in preclinical studies (Dang *et al*., 2019; Langedijk *et al*., 2024). Similarly, recombinant subunit vaccines using prefusion F or chimeric F/G

Published by Malaysian Society of Parasitology and Tropical Medicine.

proteins have demonstrated protective efficacy in animal models (Loomis *et al*., 2020).

Despite extensive NiV research, no antiviral therapies or vaccines have been approved. Recent immunoinformatics advances have enabled epitope-based vaccine design, a cost effective, rapid approach for diseases like tuberculosis, SARS-CoV, and NiV (Ashfaq *et al*., 2021; Habib, 2021). Studies using immunoinformatics identified the potency of T-cell and B-cell epitopes eliciting strong immune responses against pathogens (Kubiszeski *et al*., 2022). The aim of this study is to assess the immunogenicity of the identified potential B-cell epitope using post-infected serum from human survivors. In this study, the NiV F protein was selected for epitope prediction due to its conserved, immunogenic regions, allowing precise vaccine candidate identification with minimal resources compared to traditional screening. Selected epitopes were evaluated for epitope-based vaccine potential against NiV. A synthetic peptide corresponding to one F protein epitope was produced, and its immunoreactivity was tested with NiV survivors' sera to evaluate its immunodominant properties.

MATERIALS AND METHODS

Sequence retrieval and target protein selection

The complete amino acid sequence of the NiV F protein (accession number: NC_002728) was retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/). Conserved regions were identified via NCBI BLASTp (Basic Local Alignment Search Tool), selecting alignment pairs with the highest bit scores and low E-values. Only sequences from strains isolated from Malaysia, India and Bangladesh, with *Homo sapiens,* were included for further analysis. Multiple sequence alignment was performed using Cluster Omega. Conserved amino acid sequences from the NiV F protein were analyzed for transmembrane topology using TMHMM v0.2 server (Krogh *et al*., 2001). Surface-exposed regions of the F protein were selected for epitope prediction, as they are most accessible to the immune system.

Prediction of linear B-cell epitope

A probability-based prediction of linear B-cell epitopes on the NiV F protein was performed using multiple immunoinformatics web servers. The IEDB server (http://tools.iedb.org/bcell/) was used to evaluate sequence characteristics such as surface accessibility (Emini's method) and antigenicity (BepiPred 2.0), with a threshold score of 0.5 applied to identify candidate peptides. ABCpred was also employed, using a fixed epitope length of 16 amino acids (hexadecamers) and a selection cutoff of 0.80. Non-overlapping epitopes exceeding this threshold were retained. Furthermore, SVMTrip was utilized with a 20-residues window size to predict linear B-cell epitopes, offering further insight into potential antigenic regions. Predicted epitopes from all servers were aligned using Mega-X version 11, and only epitopes identified by at least 2 tools were shortlisted as candidates for downstream analysis.

Prediction of MHC-II binding helper T-cell epitope

For helper T-cell epitope binding prediction, The Consensus method 2.22 available on the IEDB server (https://www.iedb.org/mhcii/) was employed. This method integrates multiple algorithms, including NN-align, SMM-align, CombLib, Sturniolo and NetMHCIIpan. HLA class II alleles reported in previous studies (Majee *et al*., 2021; Mohammed *et al*., 2020; Rahman *et al*., 2022) were analyzed, focusing on -DR, -DP, -DQ supertypes prevalent in South and Southeast Asian populations. Helper T-cell epitopes were selected based on two criteria: (i) 15-mer peptides with a binding percentile rank below 20%, and (ii) epitopes overlapping with predicted B-cell epitopes. Interacting HLA-II alleles for each epitope were recorded for further evaluation. Population coverage of selected epitopes associated with specific MHC class II alleles were assessed using the IEDB Population Coverage tool (http://tools.iedb.org/population), which estimates the proportion of individuals likely to respond to the selected specific MHC-II epitopes based on HLA genotyping data from South and Southeast Asia (Bui *et al*., 2006).

Screening of predicted epitopes

The predicted epitopes were evaluated for antigenicity, allergenicity, toxicity, conservancy, and homology, followed by assessment of their potential to induce interferon and interleukin responses. Antigenicity was predicted using VaxiJen v2.0 (https://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html) with a threshold of 0.4 to identify peptides likely to be recognized by the immune system (Doytchinova & Flower, 2007). Allergenicity was assessed using AllerTOP v2.0 (https://www.ddg-pharmfac.net/AllerTOP/) (Dimitrov *et al*., 2014a) and AllergenFP (http://libdb.csu.edu.cn/rwt/PUBMED/http/MSTGPLLRNBRYE5LGMFST65UFPE/AllergenFP) to exclude allergenic peptides (Dimitrov *et al*., 2014b). Toxicity prediction was checked using ToxinPred (http://crdd.osdd.net/ragha va/toxin pred/) with a non-toxic threshold set at 0.1 (Gupta *et al*., 2013). To ensure specificity, a BLASTp homology screening was conducted against the IEDB's database, applying an E-value <1 and 100% identity threshold, confirming no homology with human proteins. Conservancy was analyzed using the IEDB Epitope Conservancy Analysis tool against NCBI protein sequences, and epitopes with ≥90% conservation were retained (Dhanda *et al*., 2013). Predicted helper T-cell epitopes were further tested for interferon and interleukin production using IFN-epitope (http://crdd.osdd.net/raghava/ifnepitope/index.php), IL4pred (https://webs.iiitd.edu.in/raghava/il4pred/predict.php) and IL-10Pred (http:// crdd.osdd.net/raghava/IL-10pred/) servers (Nagpal *et al*., 2017). Only epitopes that passed all the above screening parameters were selected as candidate epitopes for downstream analysis.

Secondary structure analysis and homology modelling of epitopes

The PSIPRED 4.0 server (http://bioinf.cs.ucl.ac.uk/psipred/) was used to predict the secondary structure of the NiV F protein, identifying α-helices, β-sheets, and coil regions. Subsequently, the PEP-FOLD 3.5 (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/) was employed to generate three-dimensional structural models of the shortlisted linear B-cell and MHC class II epitopes. Amino acid sequences of the corresponding HLA alleles were retrieved from the Immuno Polymorphism Database (IPT-IMGT/HLA) (https://www.ebi.ac.uk/ipd/imgt/hla/allele.html) and used to identify structural templates from the Protein Data Bank (PDB). The dominant allele DPA1*02:01/DPB1*01:01 (PDB ID: 7ZAK) was modelled using homology modelling platform SWISS-MODEL (https://swissmodel.expasy.org/). Model quality was evaluated using QMEAN scoring, where *z*-scores between 0 and 1 indicated a reliable model (Benkert *et al*., 2010). Additionally, Ramachandran plot analysis was performed to ensure the stereochemical quality of the predicted structures, requiring >90% of residues to be located within the most favored regions, excluding glycine and proline. Only high-quality models were considered for further molecular docking.

Molecular docking

All crystal structures generated via PEP-FOLD 3.5 were subsequently prepared for molecular docking using AutoDock Tool by removing water molecules, adding polar hydrogens, and assigning Kollman charges to the receptor molecules. Molecular docking was conducted using AutoDock Vina v1.1.2 to analyze binding interactions between predicted epitopes and their corresponding B-cell receptors (BCR) or MHC class II molecules (Eberhardt *et al*., 2021). Crystal structures used included BCR (PDB ID: 5IFH) and HLA-DPA1*02:01/DPB1*01:01 (PDB ID: 7ZAK). Grid box dimensions were set at 86, 41, 10 Å (X, Y and Z respectively), with an exhaustiveness parameter of 8. The lowest binding affinity (△G, in kcal/mol) were recorded for each epitope-ligand complex. Interactions were visualized using PyMOL v2.5.5, and hydrogen bonds between peptide residues and receptor binding sites were examined.

Blood sample collection

Blood samples was collected from individuals who recovered from NiV infection and healthy donors with no history of NiV exposure. Each participant provided 8 mL of whole blood in SST tubes (Becton, Dickinson and Company (BD), USA). Samples were centrifuged at 1500 x g for 10 min at 25°C. The isolated serum was carefully harvested from the upper portion of the serum separator tube and stored at -80°C until further use.

Peptide synthesis

The synthesis linear B-cell epitope, LF6 (LISNIEIGFCL), was done by GenScript Biotech (USA). The lyophilized peptide was reconstituted in DMSO to a final concentration of 4.9 mg/mL and stored at -80°C.

Enzyme-linked immunosorbent assay (ELISA)

The 96-well microtiter plate were coated with 50 µL/well of peptide LF6 at 2 µg/mL in coating buffer (0.1 M carbonate buffer, pH 9.6) and incubated overnight at 4°C. Plates were washed 5x in washing buffer (1x PBS, 0.05% Tween 20, pH 7.4) and blocked with 300 µL/well of blocking buffer (PBS with 5% skim milk) for 2 h at room temperature. Serum samples were diluted 1:50 in blocking buffer at 100 µL/well, followed by incubation for 2 h at 37°C. After washing, wells were incubated with goat anti-human IgG-HRP (1:40,000; Merck, Germany) for 1 h at 37°C, plates were washed five times and developed by addition of TMB Microwell Peroxidase Substrate (100µL/well; KPL SeraCare, USA) for 25 min at room temperature. The reaction was quenched by addition 100 µL/well of 2M H_2SO_4 and absorbance was measured at 450 nm using an Infinite® M200 microplate reader (Tecan Group Ltd, Switzerland). Samples were tested in triplicates and run in duplicates. The cut-off value was calculated as the mean OD_{450} of negative controls + (3x standard deviation).

Inter and Intra-Assay Variation

To assess assay reliability, 5 serum samples were randomly selected for intra- and inter-assay variability analysis. For intra-assay variation, each sample was tested in triplicate on a single plate. Inter-assay variation was evaluated by repeating the assay on two separate plates under identical conditions. The coefficient of variation (CV%) was calculated, and a CV <20% was considered acceptable (Lee & Hall, 2009; Lexmond *et al*., 2011).

Statistical analysis

All data were analyzed using IBM SPSS Statistics v30 (IBM Corp, Armonk, NY, USA). Group comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. A *p*-value < 0.05 was considered statistically significant.

Study approval

Individuals who had recovered from NiV (*n* = 5) and healthy non-infected individuals (*n* = 5) were enrolled in this study. Written informed consent was obtained from all participants prior to sample collection. The study protocol was approved by the Medical Research Ethics Committee (MREC) of University Malaya Medical Centre (MREC ref. 2023419-12373) and conducted in accordance with the Declaration of Helsinki.

RESULTS

Sequence retrieval and conserved regions identification

A total of 9 fusion (F) protein sequences from different NiV strains originating from various countries, and only those isolated from *Homo sapiens*, were retrieved from the NCBI database for multiple sequence alignment. A region of 1-546 amino acids was found to be conserved across all given F protein sequences (Figure S1).

Selection of target regions for epitope prediction (Transmembrane domain selection)

Based on TMHMM-2.0 webserver, the outer region sequence was found to be position at 131-495 amino acid sequence of F protein (Figure S2). This region was subsequently utilized for antigenic epitope prediction.

Prediction and screening of linear B-cell

The ABCpred server predicted 24 linear B-cell epitopes of the NiV F protein with cut-off scores above 0.8. In contrast, BepiPred 2.0 on the IEDB server identified 13 linear epitopes with scores exceeding 0.5. Additionally, Kolaskar and Tongoankar's antigenicity yielded 13 epitopes, while Emini's surface accessibility method identified 9 epitope candidates. Only epitopes predicted by at least two of these servers were selected for further analysis. Regions overlapped ranged between region 21 to 535. Predicted epitopes with antigenic scores above 0.4 were retained, followed by screening for non-toxicity, non-homologous, non-allergenicity and ≥90% sequence (Table 1).

Table 1. Predicted linear B-cell epitopes of NiV F protein and its antigenicity, allergenicity, toxicity, conservancy and homology

Protein	Position	No	Sequence	Antigenicity	Allergenicity	Toxicity	Conservancy (>=90%)	Homology
F	163-177	LF1	ETAEKTVYVLTALQD	0.4397	Negative	Negative	Conserved	NH*
	265-277	LF2	QIIYVDLSSYYIIVR	0.4038	Negative	Negative	Conserved	NH*
	295-307	LF3	ELLPVSFNNDNSE	0.9023	Negative	Negative	Conserved	NH*
	306-321	LF4	SEWISIVPNFILVRN	0.5788	Negative	Negative	Conserved	NH*
	309-318	LF5	ISIVPNFILV	1.0418	Negative	Negative	Conserved	NH*
	322-332	LF6	LISNIEIGFCL	1.2952	Negative	Negative	Conserved	NH*
	323-334	LF7	LISNIEIGFCLIT	1.1531	Negative	Negative	Conserved	NH*
	332-343	LF8	LITKRSVICNQD	0.9334	Negative	Negative	Conserved	NH*
	384-395	LF9	FANCISVTCQCQ	0.5609	Negative	Negative	Conserved	NH*
	437-448	LF10	NYNSEGIAIGPP	0.9214	Negative	Negative	Conserved	NH*
	443-456	LF11	IAIGPPVFTDKVDIS	0.9779	Negative	Negative	Conserved	NH*

*NH = non-homologous, indicating the sequence does not show similarity to known human proteins.

Table 2. List of potential MHC-II epitopes of NiV F protein with its antigenicity, allergenicity, toxicity, conservancy, homology and IFN-γ, IL-4, IL-10 inducer predictions

Protein	Position	No	Sequence	Antigenicity	Allergenicity	Toxicity	Conservancy (>=90%)	Homology	IFN-γ, IL-4, IL-10
F	323-337	HF1	ISNIEIGFCLITKRS	1.8639	Negative	Negative	Conserved	NH*	Inducer
	286-300	HF2	TEIQQAYIQELLPVS	0.5086	Negative	Negative	Conserved	NH*	Inducer
	473-487	HF3	YIKEAQRLLDTVNPS	0.4412	Negative	Negative	Conserved	NH*	Inducer

*NH = non-homologous.

Table 3. Summary of docking results from F protein linear B-cell epitopes with B-cell receptor (BCR) and MHC-II epitope with MHC-II allele (HLA-DPA1*02:01/DPB1*01:01)

Protein	No	Sequence	Binding affinity (kcal/mol)
Linear B-cell epitopes			
F	LF6	LISNIEIGFCL	-8.8
	LF9	FANCISVTCQCQ	-8.1
	LF10	NYNSEGIAIGPP	-8.4
MHC class II epitope			
F	HF1	ISNIEIGFCLITKRS	-14.7

Prediction of potential MHC class II epitopes of F protein

Using the IEDB Consensus tool, 2,288 MHC class II epitopes were predicted from the NiV F protein with percentile ranks below 0.20. These candidates were further screened for antigenicity, toxicity, allergenicity, conservancy, and human homology. Three epitopes (ISNIEIGFCLITKRS, TEIQQAYIQELLPVS, and YIKEAQRLLDTVNPS) met all criteria and were identified to induce IL-4, IL-10 and IFN-γ cytokines (Table 2). The top ranked helper T-cell epitopes with their associated MHC-II alleles were showed in Table S1. Population coverage analysis showed near-complete coverage in South Asia (99.99%) and 93.73% in that of Southeast Asia (Table S2). The distribution percentage of the epitope's hits population of South Asia and Southeast Asia are shown in Figure S3.

Prediction of secondary structure of NiV F protein

According to the finding of PSIPRED secondary structure (Figure S4), it depicted that most of the predicted linear B-cell epitopes consists of protein residues mapped to β-strands and random coil regions. Alignment summary of these secondary structures and the residue positions of each epitope are shown in Figure S5.

Molecular docking

Peptides LF6, LF9, and LF10 were selected as final candidates based on docking models, each demonstrated binding energies less than cutoff value -8.0 kcal/mol. Notably, peptide LF6 exhibited the strongest binding affinity with BCR at -8.8 kcal/mol (Figure 1), followed by LF10 (-8.4 kcal/mol; Figure 3) and LF9 (-8.1 kcal/mol; Figure 2). For MHC class II binding, epitope HF1 (ISNIEIGFCLITKRS) formed a stable complex with HLA-DPA1*02:01/DPB1*01:01, displaying a binding energy of -14.7 kcal/mol (Table 3; Figure 4). Structural analysis revealed that LF6 epitope formed 6 hydrogen bonds with BCR, involving heavy chain residues Asp 62, Ser 63, Arg 87, and light chain residue Gly 102. The interaction of HF1 epitope with MHC-II complex showed 7 hydrogen bonds with α-chain residue Asn 93 and β-chain residues Gln 42, Trp 88, Lys 98, Arg 104, and Asp 103.

Serum IgG response to epitope LF6 from NiV-F protein

Among the survival cohort, S5 exhibited positive response indicated by OD_{450} value 0.343 which above the cut off value (cut off value= 0.171) at a dilution of 1:50 in NiV-F ELISA. No samples from the negative cohort showed detectable response above the cut off value at the same dilution of 1:50. Statistical analyses demonstrated that sample S5 exhibited significantly higher values compared to both negative control sera and other test sera groups, with $p < 0.001$ when compared to negative controls, and $p < 0.05$ relative to the other sera (Figure 5). The assay reliability of the LF6-indirect ELISA was confirmed by intra- and inter-assay variability tests. Five randomly selected samples yielded coefficient of variation (CV) values below 15%, suggesting high assay reproducibility and technical validity (Table S3).

DISCUSSION

Nipah virus (NiV), a zoonotic paramyxovirus with mortality rates up to 70%, poses a severe public health threat, underscored by recent outbreaks in India (2024) and Bangladesh (2023) (Thiagarajan, 2024; World Health Organization, 2024). The NiV fusion (F) protein, which mediates viral entry through membrane fusion, is a prime target for epitope-based vaccine development due to its structural transition from a prefusion to a postfusion conformation. Similar to previous studies on paramyxovirus and pneumoviruses such as respiratory syncytial virus (RSV), its prefusion conformation has been shown to elicits robust neutralizing antibody responses by inhibiting membrane fusion (Steff *et al*., 2017; Avanzato *et al*., 2019; Dang *et al*., 2019). Our study is the first to experimentally validate linear B-cell epitopes of the NiV F protein, using immunoinformatics predictions and post-infection human sera, bridging a critical gap in NiV vaccine research. Unlike previous in silico studies targeting the broader NiV proteome of both F and G proteins (Sakib *et al*., 2014; Ojha *et al*., 2019; Kaushik, 2020; Majee *et al*., 2021; Banico *et al*., 2024), our focus on F protein-specific linear epitopes, and to confirm its immunoglobulin G (IgG) reactivity in NiV survivors.

Using ABCpred, SVMTrip, and BepiPred 2.0, we predicted 11 linear B-cell epitopes prioritizing those with favorable antigenicity, allergenicity, toxicity and homology. Secondary structure analysis favored surface-exposed β-turns and random coils for B-cell receptor (BCR) accessibility (Teng *et al*., 2018). LF6 (LISNIEIGFCL) emerged as the lead B-cell epitope, with high antigenicity score (1.2952), non-allergenicity, and a flexible coil structure, optimizing its recognition by BCRs. To note, LF6 overlaps with a highly antigenic MHC-II epitope, HF1 (ISNIEIGFCLITKR), surpassing a similar epitope reported by Sakib *et al*. (2014). Suggesting dual B-cell and T-cell activation, an attribute important for vaccine designs (Chukwudozie *et al*., 2021; Khan *et al*., 2023).

Molecular docking using AutoDock Vina confirmed LF6 showed strong affinity to BCR (-8.8 kcal/mol) (Figure 1). This binding affinity falls within the affinity range reported for known immunogenic peptides in SARS-CoV-2 and RSV (-7 to -11.6 kcal/mol) (Anandhan *et*

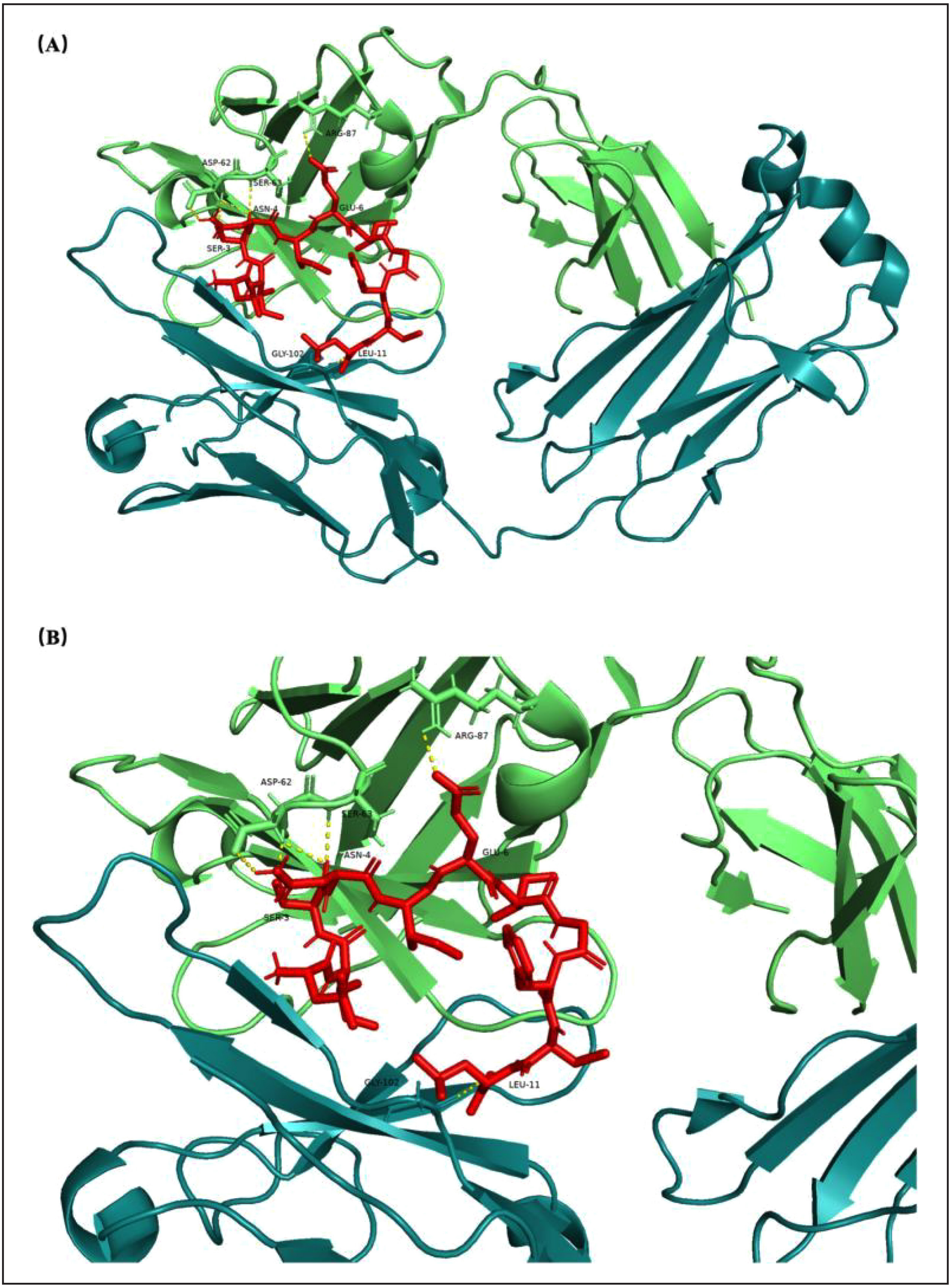

Figure 1. Interface of LF6 epitope of NiV F protein in complex with BCR. (A) A cartoon representation of surface interaction and (B) magnified interacting residues of LF6 epitope (Red) with BCR heavy chain (lime colour) and light chain (dark turquoise colour). Hydrogen bonds were represented by yellow coloured dashed lines.

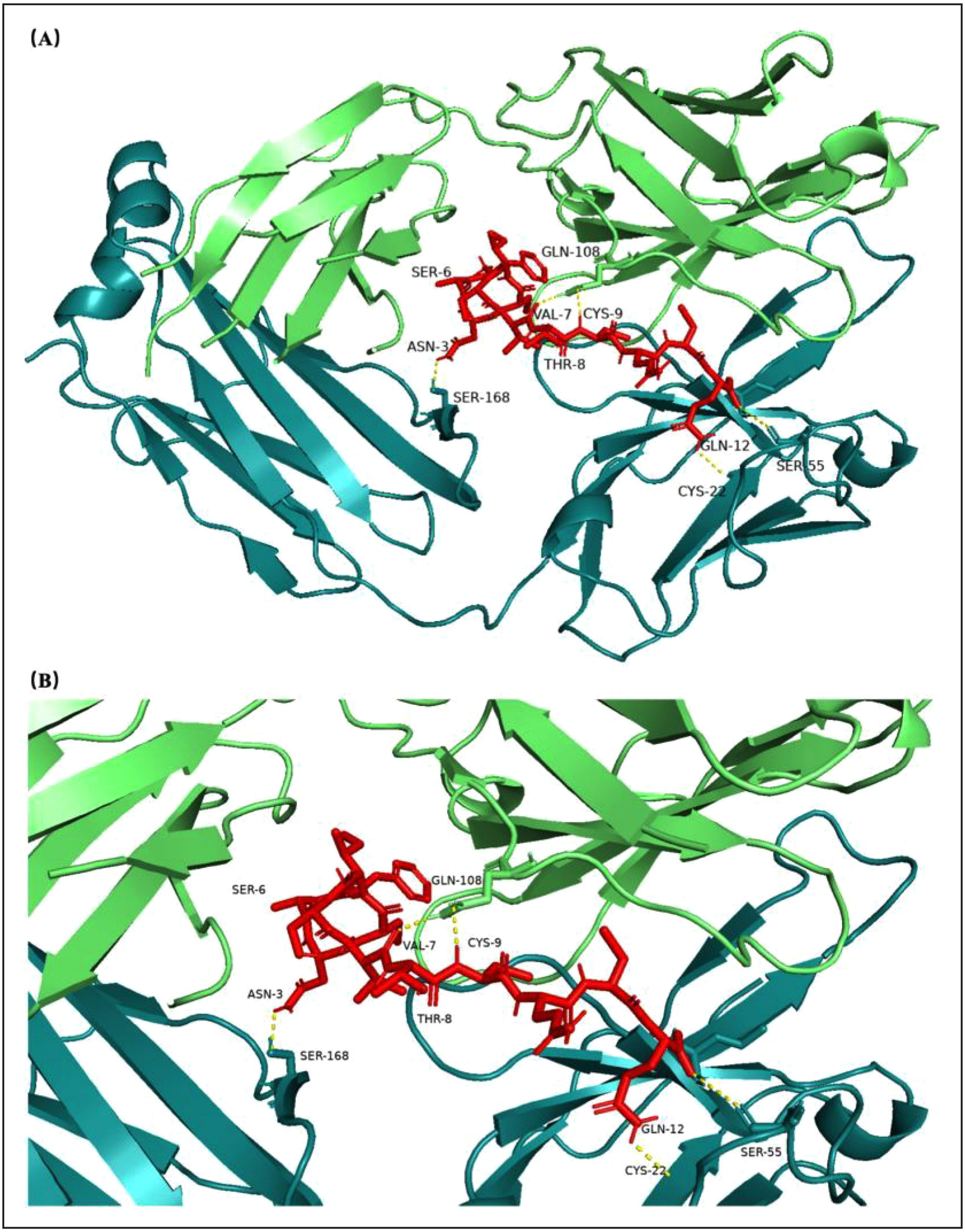

Figure 2. Interface of LF9 epitope of NiV F protein in complex with BCR. (A) A cartoon representation of surface interaction and (B) magnified interacting residues of LF9 epitope (Red) with BCR heavy chain (lime colour) and light chain (dark turquoise colour). Hydrogen bonds were represented by yellow-coloured dashed line.

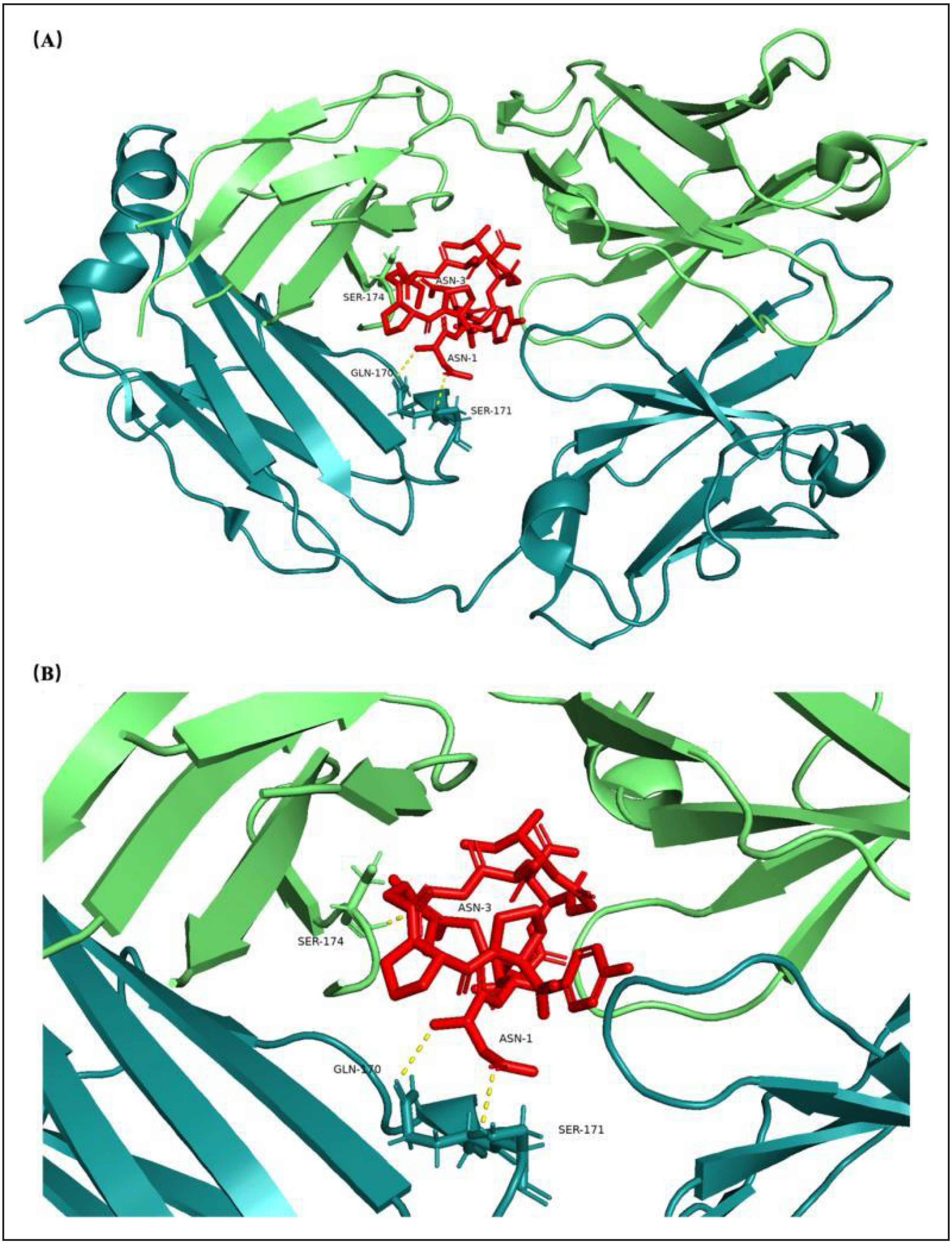

Figure 3. Interface of LF10 epitope of NiV F protein in complex with BCR. (A) Cartoon representation of surface interaction and (B) magnified interacting residues of LF10 epitope (Red) with BCR heavy chain (lime colour) and light chain (dark turquoise colour) and labelled with respective amino acid residues. Hydrogen bonds were represented by yellow coloured dashed line.

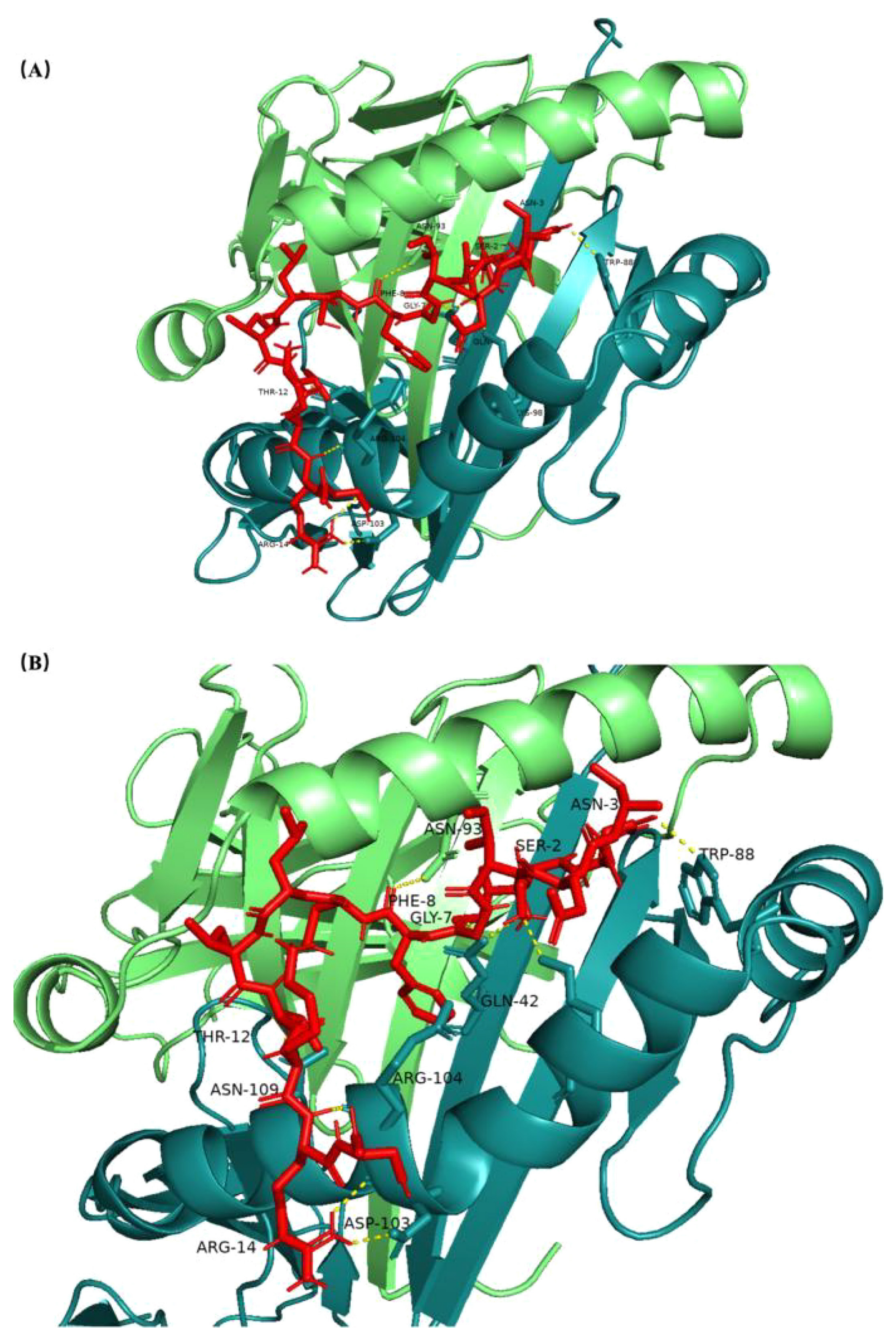

Figure 4. The interaction between MHC class-II epitope HF1 (red colour) and MHC allele α-chain (lime color) and β-chain (dark turquoise colour). The interaction was visualized with PyMol shows the interacting residues and yellow colour shows the hydrogen bond. (A) Cartoon representation of surface interaction and (B) magnified interacting residues of HF1 epitope with HLA-DPA1*02:01/DPB1*01:01 as shown as sticks and labelled with respective amino acid residues. Hydrogen bonds were represented by yellow coloured dashed line.

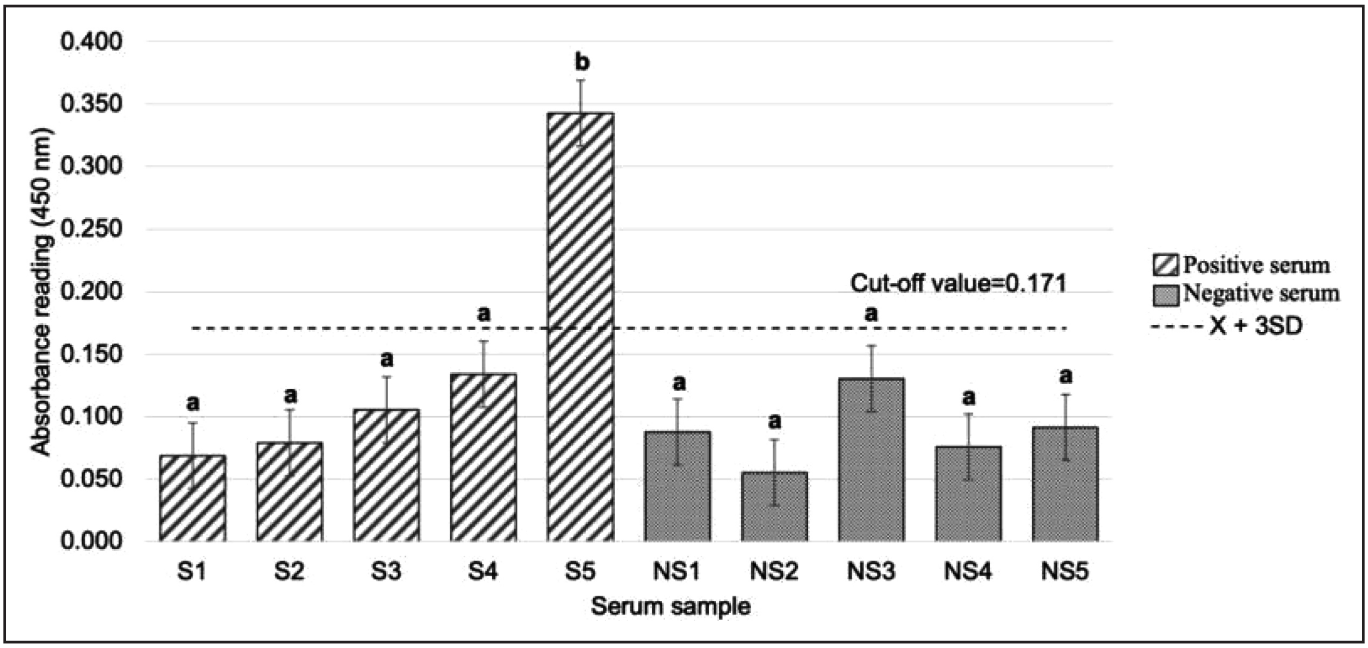

Figure 5. Antibody responses in NiV post-infected individuals and healthy negative sera against the predicted linear B-cell epitope of NiV F protein. Synthesized peptide was tested with 5 post-infected individuals and 5 serum samples from healthy individuals using indirect ELISA. The experiments were all performed in sample triplicates and plotted with the average of triplicates. The following cut-off value was established as the comparison threshold, the dotted line indicates the mean of three negative samples + (3x standard deviation). Samples above the dashed line OD_{450nm} >0.1707 indicating positivity to NiV F protein. ANOVA with Tukey's multiple comparison test was performed. Bars denoted by the same letter are not statistically significant ($p > 0.05$).

al., 2023; Chaurasiya *et al*., 2023). Simultaneously, the overlapping HF1 epitope interacted with MHC class II molecule showed a robust affinity (-14.7 kcal/mol) and observed multiple hydrogen bonding (Figure 4) thereby supporting LF6's broad immunogenic potential. LF6's random coil structure further enhances binding flexibility; a trait linked to epitope immunogenicity (Hubbard, 2001). However, it's important to note that AutoDock Vina assumes rigid receptor structures, limiting dynamic insights (Forli *et al*., 2016). To address this, necessitating molecular dynamics simulations, as applied in some recent henipavirus vaccine modeling studies, would allow improved predictions into binding stability (Masum *et al*., 2024; Shabbir *et al*., 2025). Additionally, docking against diverse human HLA alleles and survivor-derived antibodies could assess population coverage and therapeutic potential of both LF6 and HF1, a strategy gaining traction in immunological research (Muik *et al*., 2024). These findings collectively present LF6, supported by HF1, as a promising candidate for a multi-epitope vaccine, with docking-guided optimization lend compelling justification for LF6's immunogenic potential.

Sera from five NiV survivors, collected 26 years after the 1998 Malaysia outbreak, were tested for NiV-specific IgG binding to the synthetic LF6 via indirect enzyme-linked immunosorbent assay (ELISA). Optical density at 450 nm (OD_{450}) was compared against five healthy control sera to determine a cut-off value. One survivor sera exhibited a robust IgG response (OD_{450} exceeding the cutoff = 0.171), indicating possible LF6's recognition by long-lived memory B cells. This finding suggests that LF6 corresponds to a conserved epitope capable of eliciting durable immunity, consistent with reports of sustained humoral responses in paramyxovirus infections (Turner *et al*., 2021; Mariotti *et al*., 2024). Sequence alignment confirmed LF6 conservation across 95% of NiV F protein sequences (Malaysia and Bangladesh strains), supporting its potential as cross-protective subunit vaccine candidate. However, four survivors showed weaker or negligible reactivity, likely due to age-related immune decline, which reduces B-cell clonal diversity and antibody titers, as observed in long-term paramyxovirus immunity (Henry *et al*., 2019). Clinical symptoms variability and infection severity may affect humoral responses, with severe cases linked to higher antibody levels than mild or asymptomatic infection (Park *et al*., 2022; Tegeler *et al*., 2022). The small sample size (n=5), constrained by the rarity of NiV survivors and ethical restrictions, limits generalizability but reflects the challenge of studying rare pathogens. Assay sensitivity was reduced by weak hydrophobic interactions between the short LF6 peptide and the ELISA plate, a known limitation in peptide-based assay (Marin *et al*., 2005; Mosadeghi & Heydari-Zarnagh, 2018). Nevertheless, LF6's detection in one survivor underscores its potential to be developed as subunit vaccine and warranting validation in larger, diverse cohorts and using complementary methods, such as neutralization tests. These findings underscore LF6's promise and the need to investigate age-dependent immune dynamics and epitope performance across diverse populations.

CONCLUSION

This study is the first to integrate immunoinformatics and experimental approaches to provide preliminary validation of the NiV F protein epitope LF6, leveraging bioinformatics predictions with indirect ELISA to assess its immunogenic potential. Among five survivors' sera collected 26 years post-1998 NiV outbreak, one sample exhibited a robust IgG response, aligning with observations of long-lasting antibody persistence in viral infections, suggesting LF6 targets a conserved region suitable for sustained immunity. However, the limited positive response underscores the need for larger sample sizes to enhance statistical power and address variability. Technical challenges such as weak van der Waals interactions with short peptides in ELISA, suggest the optimizing peptide length or conjugation may enhance detection sensitivity. Despite these limitations, LF6 represents a promising step toward epitope-based NiV vaccines, which could offer preventive and prophylactic advantages with fewer adverse effects compared to whole-protein vaccines, a benefit emphasized in recent trials (Song *et al*., 2024). Its dual B- and T-cell immunogenicity further positions

LF6 as a versatile candidate for multi-epitope constructs, consistent with advances in vaccine design (Pastor *et al*., 2024). Future efforts should prioritize expanded serological validation, cross-reactivity assessments with henipaviruses, and in vivo studies in models like hamsters or ferrets to evaluate protective efficacy, paving the way for clinical translation.

Conflict of interest statement

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

This study was funded by Ministry of Higher Education Malaysia via Fundamental Research Grant Scheme (FRGS/1/2022/SKK06/UM/03/1), Ministry of Higher Education Malaysia for niche area research under the Higher Institution Centre of Excellence (HICoE) program (MO002-2019 and TIDREC-2023) and UM International Collaboration Grant (ST040-2023). Ms Ting Li Jie express gratitude to Ministry of Higher Education Malaysia for providing MyBrainSc scholarship.

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