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In silico construction of polyvalent multi-epitope respiratory syncytial virus subunit vaccine: focusing on prevalent HLA allele types in Korea

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ABSTRACT

Respiratory syncytial virus (RSV) is a leading cause of lower respiratory infections in infants and the elderly. The burden of RSV-related hospitalizations and deaths is significantly increasing. Recently, RSV vaccines targeting F protein have been approved, but their long-term efficacy is yet to be evaluated. This study aimed to design a polyvalent multi-epitope RSV subunit vaccine tailored to Korean HLA types using in silico methods. Nine prevalent HLA allele types in Korea were used to predict vaccine epitopes. Combinations of selected epitopes were created, and stable combinations were selected and conjugated with human β-defensin and PADRE. Fifty-five multi-epitope combinations were selected for vaccine construction, and 33 vaccine constructs induced strong immune responses in silico. The final vaccine candidate is a 228-amino acid construct weighing 23,957 Da and effectively induces humoral and cellular immune responses with high population coverage, suggesting potential for further development.

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Respiratory syncytial virus; multiepitope; in silico; recombinant subunit vaccine

Introduction

Respiratory syncytial virus (RSV), which was first isolated in 1955 from a chimpanzee with coryza at the Walter Reed Army Institute of Research, USA, is a genus Orthopneumovirus in the Pneumoviridae family, and has a single-stranded negative-sense RNA of approximately 15.2 kb, enveloped by a membrane derived from the host cell [1]. The RSV genome encodes 11 viral proteins from 10 genes: non-structural proteins NS1 and NS2, nucleoprotein (N), phosphoprotein (P), matrix protein (M), small hydrophobic protein (SH), glycoprotein (G), fusion protein (F), transcription factors M2-1 and M2-2, and large polymerase (L). Among these, G, F, and SH are envelope proteins expressed on the viral surface, while others are internal proteins. N protein binds tightly to the genome and antigenome, forming a helical nucleocapsid involved in RNA synthesis. P protein acts as a polymerase cofactor, mediating interactions within the nucleocapsid-polymerase complex. M protein supports the viral envelope and is crucial for virion morphology and RNA transcription. SH protein forms an ion channel on the viral surface and inhibits apoptosis in infected cells. G protein is a large glycoprotein involved in host cell attachment, while F protein mediates viral entry by forming a spring-loaded trimeric pre-fusion form that transitions to a post-fusion form, remaining in the infected cell membrane to form syncytia. M2-1 and M2-2 proteins are involved in transcription and genome replication regulation. L protein functions as an RNA-dependent RNA polymerase. NS1 and NS2 are known to interfere with type I interferon (IFN) induction and signalling.

RSV is categorized into two major genotypes, type A (RSV-A) and type B (RSV-B), based on the diversity of the G gene, with 13 and at least 37 genotypes, respectively. Both genotypes circulate globally and often alternate dominance annually [2]. RSV spreads rapidly through respiratory droplets, direct contact with infected individuals, or aerosols, often causing mild upper respiratory tract infections in healthy adults but leading to severe lower respiratory tract infections (LRTI) requiring hospitalization in young infants and the elderly. Globally, acute LRTI (ALRTI) are significant causes of hospitalization and death in children, and RSV is the leading cause of ALRTI in children under two years old. Almost all children are infected with RSV by age of 2 [3]. A meta-analysis in 2019 estimated the global economic burden of RSV-related ALRTI management at €4.82 billion, with 33 million cases of RSV-related ALRTI, 3.6 million hospitalizations, and 101,400 deaths among children under five [4,5].

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The antiviral ribavirin is prescribed in severe cases, but no RSV-specific therapeutics has been developed yet. The humanized monoclonal antibody (mAb) palivizumab has been used in high risk young infants to prevent RSV infection during RSV season, and recently the next-generation mAb, nirsevimab, with improved neutralization and prolonged half-life has been approved. Early RSV vaccine development faced setbacks with the failure of the formalin-inactivated RSV vaccine (FI-RSV) trials. However, recent advancements have led to the approval of three RSV vaccines [6]. Two recombinant subunit vaccines, AREXVEY and ABRYSVO, and the mRNA vaccine mRESVIA are now in clinical use, and sould offer important insights for future RSV research and prevention strategies. While all these three vaccines focus on the RSV F protein due to its conserved nature and ability to induce neutralizing antibodies, concerns about breakthrough infections due to F protein mutations remain.

In silico vaccine design technology offer a rapid and safe approach to evaluating vaccine candidates' stability, safety, and efficacy, potentially increasing the success rates of preclinical and clinical trials. In addition, in silico designed tumour and bacterial vaccines were proven to be effective in preclinical animal studies [7–9]. Human leukocyte antigen (HLA) allele diversity varies significantly across populations, determining susceptibility to vaccine antigens. This study aimed to design a polyvalent multi-epitope RSV subunit vaccine for HLA allele types prevalent in Korea, assessing its immunoreactivity in major races in the world.

Material and methods

Target protein antigen sequence retrieval and physicochemical property analysis

The target proteins for vaccine composition were selected from the 11 structural proteins of RSV including the surface proteins F and G, as well as the structural proteins N and P, which are highly expressed in infected cells [10]. Amino acid sequences for the target proteins from RSV-A strain A2 and RSV-B strain B1 were retrieved from the UniProt database (Tables S1-3). Antigenicity and physicochemical properties of the proteins were evaluated using VaxiJen v2.0 and ProtParam tools [11].

T-cell epitope prediction

T-cell epitope prediction was performed for nine prevalent HLA alleles in Korea (∗24:02, A∗33:03, A∗02:01, B∗15:01, B∗51:01, C∗01:02, C∗03:04, DRB1∗04:05:01, DRB1∗09:01:02) [12]. MHC class I epitopes were predicted using IEDB's MHC I-Binding Predictions v2.24 and NetCTLpan-1.1, with a 9-mer peptide length. MHC class II epitopes were predicted using IEDB's MHC II-Binding Predictions and NetMHCIIpan-4.3, with a 15-mer peptide lengths [13, 14].

B-cell epitope prediction

B-cell epitopes were predicted using IEDB's B cell Epitope Prediction Tools and DTU's BepiPred-3.0, with a threshold of 0.1512, to identify the top 20% of predicted sequences [15].

Epitope selection for vaccine design

The predicted epitopes were evaluated for antigenicity, allergenicity, and toxicity using VaxiJen v2.0, AllerTOP v2.0, and ToxinPred. Conservancy of the epitopes was confirmed using IEDB's Epitope Conservancy Analysis. Non-homology to the human proteome was confirmed using Protein BLAST [16–18]. Epitopes with high antigenicity, non-toxicity, non-allergenicity, and high sequence conservation were selected for vaccine design. MHC class II epitopes were also evaluated for IFN-g (IFNepitop) and IL-4 (IL4pred) inducing ability [19,20].

Vaccine design and library construction

Selected epitopes were combined with linkers (AAY for MHC class I, GPGPG for MHC class II, and KK for B-cell epitopes) as shown in Figure S1 [21,22]. By combination of epitopes, a library of 768 multi-epitope combinations was created, and evaluated for stability, allergenicity, and toxicity. Multi-epitope combinations with an aliphatic index less than 30 were selected for vaccine construction, and anti-viral human β-defensin 3 (hβD3) and pan-HLA-DP epitope (PADRE) were added to the N-terminal of selected multi-epitope combinations to enhance immune responses(Figure S2) [23].

In silico immune simulation and structure prediction

In silico immune simulation were conducted using C-ImmSimm server to predict immune responses [24]. Vaccine sequence, which strongly induces both humoral and cellular immune responses, was finally selected as polyvalent multi-epitope RSV subunit vaccine. Secondary structure of the vaccine candidate was predicted using PRISPRED, GOR IV, SOPMA, and SIMPA96 [25,26]. Tertiary structure of the vaccine candidate was modelled using I-TASSER and refined with GalaxyRefine module, followed by Ramachandran plot analysis for validation [26,27]. Population coverage for the epitopes of the vaccine candidate was performed using IEDB's Population Coverage tool to assess responsiveness across various regions, including Korea [28].

Molecular docking of the vaccine protein with human TLR4

The predicted vaccine structure was docked against the human Toll-like receptor 4 (TLR4) using the HDOCK server (http://hdock.phys.hust.edu.cn/) [29]. Finally, the

interaction between the polyvalent multi-epitope RSV subunit vaccine and the human TLR was visualized by PDBsum server (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html) [30].

Molecular dynamic simulation (MD)

iMODS server (https://chaconlab.org/multiscale-simulations/imod) [31] was used to analyze the collective motions of polyvalent multi-epitope RSV subunit vaccine. A normal mode analysis (NMA) in internal coordinates is conducted by the server to determine the stability of the vaccine protein. This server structured the dynamics of the protein complex and provided various results data, such as deformability, eigenvalues, B-factors, variance maps, co-variances, elastic networks in the atoms, and residue indexes in terms of magnitude and direction.

In silico molecular cloning and codon adaptation

A reverse translation of the vaccine protein sequence into a DNA sequence was performed with the Java Codon Adaptation Tool (JCAT) (http:// www. prodo ric. de/ JCat) because cloning uses DNA rather than proteins [32]. The sequences of the restriction enzymes NcoI (5-CCATGG-3) and XhoI (5-CTCGAG-3) were added at the 5′ and the 3′ ends of the DNA, respectively. A restriction cloning module from SnapGene (https://www.snapgene.com/) was used to clone the DNA sequence located between the restriction sites of NcoI and XhoI in the pET-28a(+) vector.

Results

Prediction and selection of B-cell and T-cell epitopes

MHC class I epitopes of RSV-A and RSV-B were predicted from P, N, F, and G proteins using MHC I-Binding Predictions v2.24 and NetCTLpan-1.1. MHC class II epitopes were predicted using MHC II-Binding Predictions and NetMHCIIpan-4.3. The top 5% of predicted sequences were extracted (Tables S4-S11). For all target proteins except the N protein, 1–5 binding sequences were predicted for all analyzed alleles. However, no binding sequences for the C*03:04 allele were predicted in the N protein for both type A and type B. After removing overlapping sequences between type A and type B, the predicted MHC class I binding sequences from P, N, F, and G proteins were 18, 15, 25, and 19, respectively. For MHC class II, the predicted binding sequences from P, N, F, and G proteins were 5, 3, 6, and 7, respectively (Tables S12-S15). For B-cell epitopes, predicted sequences with prediction values above the threshold and within the top 5% of predicted values were selected (Tables S4-S11). After removing overlapping sequences between type A and type B, the predicted B-cell epitope sequences from P, N, F, and G proteins were 9, 6, 3, and 7, respectively (Tables S12-S15).

For the predicted B-cell and T-cell epitope sequences, antigenicity (VaxiJen v2.0), allergenicity (AllerTOP v2.0), and toxicity (ToxinPred) analyses were performed. Among the predicted sequences, those with high antigenicity and without allergenicity and toxicity were selected. For P protein, 6 MHC class I, 4 MHC class II, and 4 B-cell epitopes were selected out of 18, 5, and 9 predicted sequences, respectively (Table S12). For N protein, 10 MHC class I, 2 MHC class II, and 3 B-cell epitopes were selected out of 15, 3, and 6 predicted sequences, respectively (Table S13). For F protein, 15 MHC class I, 6 MHC class II, and 3 B-cell epitopes were selected out of 25, 6, and 3 predicted sequences, respectively (Table S14). For G protein, 10 MHC class I, 4 MHC class II, and 3 B-cell epitopes were selected out of 19, 7, and 7 predicted sequences, respectively (Table S15).

Epitope conservancy analysis was performed using the Epitope Conservancy Analysis tool. For P protein, 3 out of 6 MHC class I, 2 out of 4 MHC class II, and 1 out of 4 B-cell epitope sequences showed conservation (Table S16). For N protein, 8 out of 10 MHC class I, 1 out of 2 MHC class II, and 2 out of 3 B-cell epitope sequences showed conservation (Table S17). For F protein, 7 out of 15 MHC class I, 2 out of 6 MHC class II, and 1 out of 3 B-cell epitope sequences showed conservation (Table S18). For G protein, only 1 out of 10 MHC class I epitope sequences showed conservation (Table S19). Homology analysis with human proteins confirmed that all selected epitope sequences were non-homologous to human proteins, minimizing potential cross-reactivity and adverse effects (Tables S12-S15).

Further, the cytokine-inducing potential of MHC class II epitopes was evaluated for IFN-γ and IL-4. All four MHC class II epitopes of the P protein were predicted to induce one or both cytokines. For the N protein, one of two epitopes was predicted to induce IFN-γ and IL-4. For the F protein, four out of six sequences had cytokine-inducing potential. For the G protein, three out of four epitopes were predicted to induce at least one cytokine, but all had sequence conservation below 50%, making them unsuitable as vaccine candidates (Tables S16-S19).

After comprehensive evaluation of protein stability, antigenicity, safety, sequence conservation, and cytokine-inducing potential, 25 epitope sequences (16 MHC class I, 5 MHC class II, and 4 B-cell) were selected as final vaccine design candidates for multi-epitope RSV subunit vaccine (Table 1).

Vaccine design library construction and selection

The tertiary structures of the 25 selected epitope sequences were analyzed using PEP-FOLD4, confirming stable tertiary structure formation for all sequences

Table 1. Selected epitope sequences of RSV P, N, F, G proteins.

P protein	MHC class-I (CTL)	MHC class-II (HTL)	B cell
P1	QTNDNITAR	KDSIISVNSIDIEVT	DNNEEESSYSYEEINDQTN
P2	RIDEKLSEI	ESSYSYEEINDQTND	
P3	MTNDRLEAM		
N protein	**MHC class-I (CTL)**	**MHC class-II (HTL)**	**B cell**
N1	VLDLTAEEL	ELEAIKHQLNPKDND	IAQSSTRGGSR
N2	NIMLGHASV		EYRGTPRNQDLYD
N3	TQFPHFSSV		
N4	EMGEVAPEY		
N5	NPKASLLSL		
N6	HPHFIDVFV		
N7	FPHFSSVVL		
N8	IAALVITKL		
F protein	**MHC class-I (CTL)**	**MHC class-II (HTL)**	**B cell**
F1	NICLTRTDR	PSDEFDASISQVNEK	TPVTLSKDQLSGINNI
F2	SVGNTLYYV	RQQSYSIMSIIKEEV	
F3	VQIVRQQSY		
F4	YTSVITIEL		
G protein	**MHC class-I (CTL)**	**MHC class-II (HTL)**	**B cell**
G1	LAMIISTSL		

(Figure S3). As shown in Figure A1, 96 MHC class I epitope combinations were created by linking one sequence from P, N, F, and G proteins each with an AAY linker (P3 x N8 x F4 x G1). Four MHC class II combinations were created by linking one sequence from P, N, and F proteins each with a GPGPG linker (P2 x N1 x F2). Two B-cell epitope combinations were created by linking one sequence each from P, N, and F proteins with a KK linker (P1 x N2 X F1). A total of 768 multi-epitope combinations were then created by linking MHC class I, MHC class II, B-cell epitopes in that order. All 768 multi-epitope combinations were analyzed for allergenicity and toxicity using AllerTOP v2.0 and ToxinPred, confirming no allergenicity or toxicity. Physicochemical properties were analyzed using ProtPram, revealing an average isoelectric point (pI) of 5.01. The expected half-life was over 4.4 h in reticulocytes, over 20 h in yeast, and over 10 h in *E. coli*. The instability index averaged 37.5, the aliphatic index averaged 71.39, and the grand average of hydrophobicity (GRAVY) averaged −0.7965. Fifty-five sequences with an instability index below 30 were selected as vaccine candidates (Tables S20 & S21).

Selected 55 multi-epitope combination sequences were combined into three formats: MHC class I-MHC class II-B cell (Figure S2A), B cell-MHC class I-MHC class II (Figure S2B), and B cell-MHC class II-MHC class I (Figure S2C). To stabilize the protein structure and enhance the immune responses, hβD3 and PADRE sequences were linked to the N-terminus of these multi-epitope combinations, resulting in a library of 165 polyvalent multi-epitope RSV subunit vaccines.

Physicochemical properties of these 165 vaccine sequences showed an average isoelectric point (pI) of 7.76, an expected half-life of over 30 h in mammalian reticulocytes, over 20 h in yeast, and over 10 h in *E. coli*. The instability index averaged 30.49, the aliphatic index averaged 70.53, and the GRAVY averaged −0.6529. Thirty-three sequences with a pI above 8, an instability index below 30, and an aliphatic index above 70 were selected for evaluating in silico immunoreactivity (Tables S22 & S23).

In silico immune simulation results of the 33 selected polyvalent multi-epitope RSV subunit vaccines indicated that all 33 sequences could induce antigen-specific antibody responses and cellular immune responses (CTL proliferation, TH cell proliferation, B cell proliferation, etc.) after vaccination (Figures S4-1 ∼ S4-33). Repeated antigen exposure resulted in amplified immune responses, indicating the induction of a typical secondary immune responses, i.e. memory immune responses, suggesting their potential as vaccine antigens. In general, vaccines induced strong cellular immune responses (high CTL proliferation) tended to have lower humoral immune responses (antibody generation and B cell proliferation), and vice versa. Among the 8 vaccines with excellent antibody production (Figure S4-15, S4-17, S4-19, S4-21, S4-23, S4-25, S4-28, and S4-30), two vaccines (Figure S4-19 & S4-28) induced nearly maximum levels of cytotoxic T cell proliferation, indicating that these two vaccines have both excellent humoral and cellular immune responses. Among them, one (Figure 2 and Figure S4-19), which showed superior IgG production, active B cell proliferation, and the highest binding affinity for HLA-A and HLA-B alleles, was selected as the final polyvalent multi-epitope RSV subunit vaccine candidate.

Characteristics analysis of vaccine

The antigenic composition and sequence structure of the finally selected polyvalent multi-epitope RSV subunit vaccine are shown in Figure 1. The vaccine is composed of 228 amino acid with a molecular weight of 24,957 Da, an antigenicity of 0.6157, an instability index of 29.21, an aliphatic index of 72.41, an average hydrophobicity of −0.608, and a half-life of over 30 h in eukaryotic cells, indicating it is relatively stable and hydrophilic (Table 2). It is non-toxic, non-allergenic, and non-homologous to human proteins.

In silico immune simulation predicted that all 10 epitopes used in the vaccine bind to DRB1∗04:05 and have binding affinity to at least one of the analyzed alleles, suggesting that the vaccine can activate both T-cells and B-cells. The immune simulation results showed strong induction of antibody production, with amplified immune responses upon repeated antigen administration. Effective induction of cellular immunity, such as B-cell proliferation, plasma B cell activation, helper T cell proliferation, and cytotoxic T cell proliferation was also observed, indicating that the vaccine effectively induce both humoral and cellular immune responses (Figure 2).

Secondary structure analysis of the vaccine using PRISPRED, GOR IV, SOPMA, and SIMPA96 tools revealed

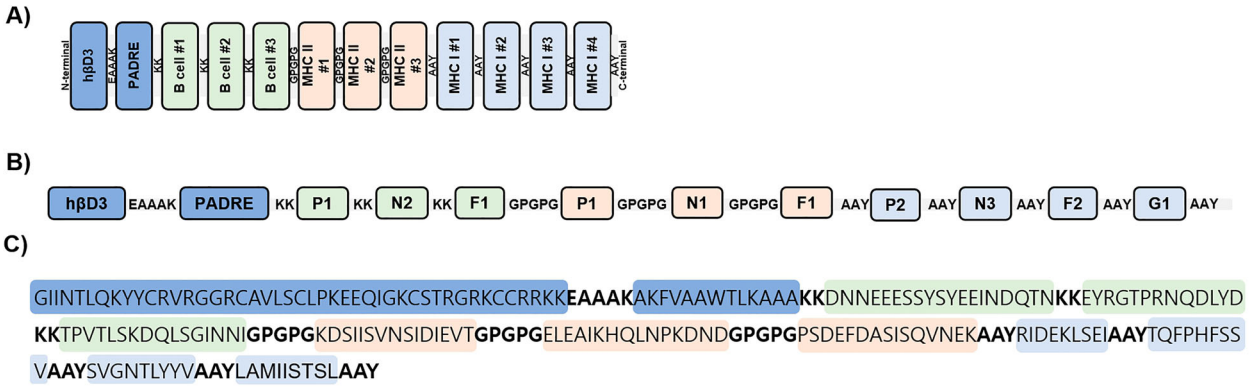


Figure 1. Schematic diagram of polyvalent multi-epitope RSV subunit vaccine construction. (A) the scheme of epitope combination of the vaccine, (B) the epitope composition of the vaccine, (C) the amino acid sequence of the vaccine

Table 2. Physicochemical characteristics of polyvalent multi-epitope RSV subunit vaccine.

Number of amino acids	Molecular weight	pI	Negative charged	Positive charged
228	24,957	8.42	28	31
Ext.coefficients	**Half-life**	**Instability index**	**Aliphatic index**	**GRAVY**
25,245	30	29.21	72.41	−0.608

that the vaccine protein had an average coil of 50.1% and the lowest β-sheet ratio of 12.9% (Figure 3, Table S24). The tertiary structure prediction using the I-TASSER online server showed that the most probable model had a confidence score (C-score) of −3.24, indicating a low reliability of the predicted structure. Therefore, the tertiary structure of the vaccine protein was refined using the GalaxyWEB server's GalaxyRefine module, and the refined 3D structure was validated using the Ramachandran plot through the BIOVIA Discovery Studio Visualizer program. The Ramachandran plot analysis showed that 92.1% of amino acids were in the allowed regions. This result has been confirmed by using PROCHECK program (Ref Laskowski RA), which also showed that 97.5% of amino acids were in the allowed regions, indicating high confidence in the final 3D structure (Figure 4).

Molecular Docking between TLR4 and the vaccine candidate was analyzed using the HDOCK server, and results of analysis revealed 434 non-bonded contacts, 6 hydrogen bonds, and 5 salt bridges between TLR4 and the vaccine candidates (Figure 5). The stability of the complex was analyzed using iMODS server (Figure 6). The 3D interaction of the complex was shown in

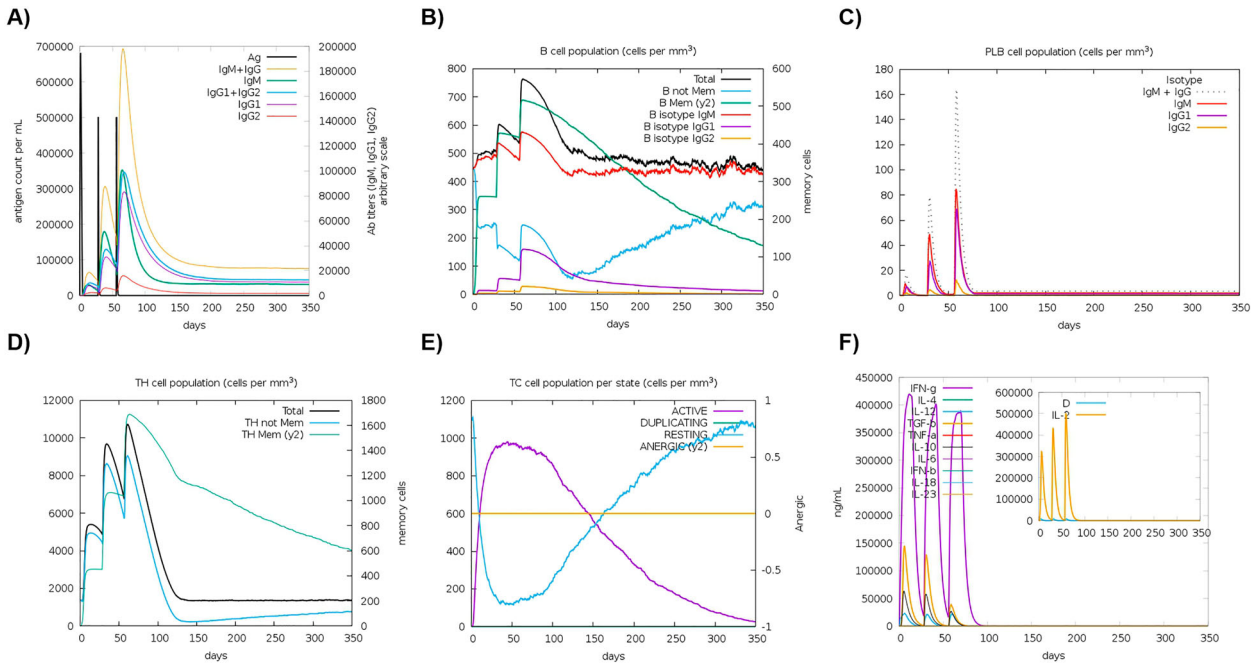


Figure 2. Predicted immunoreactivity of in silico immune stimulation with polyvalent multi-epitope RSV subunit vaccine. (A) Antigen count and antibody titres after immunization, (B) the prediction of B cell population, (C) the prediction of plasma B cell population, (D) the prediction of helper T cell (TH) population, (E) the prediction of cytotoxic T cell (TC) polulation, (F) the levels of cytokines, interleukins and interferons.

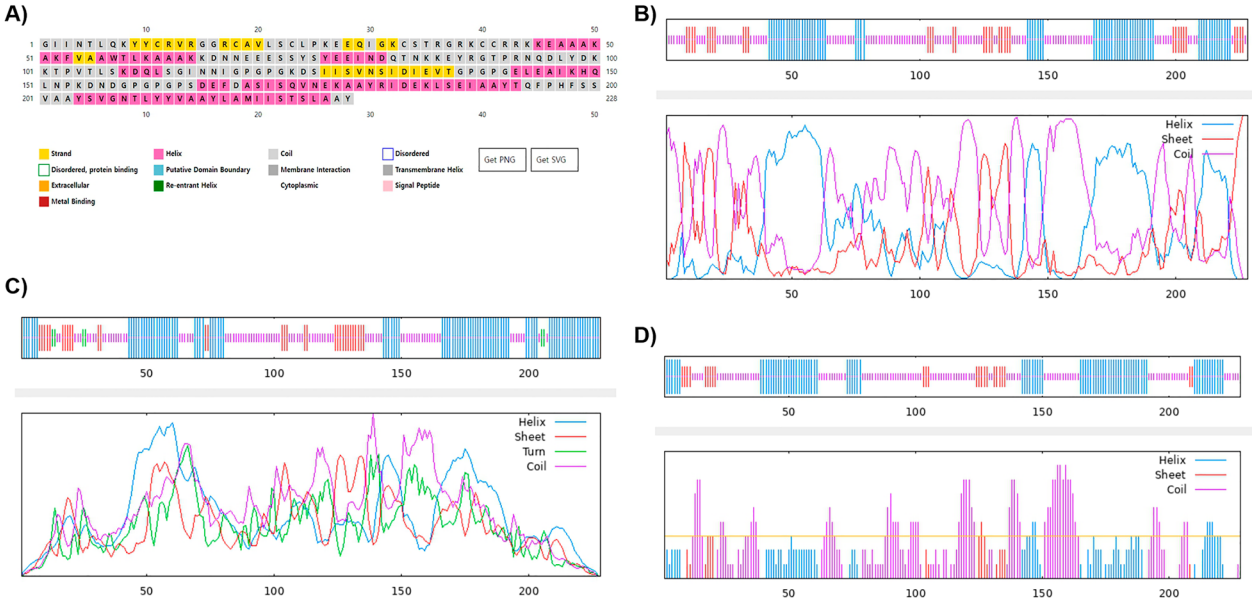


Figure 3. Results of the secondary structure analysis of polyvalent multi-epitope RSV subunit vaccine. (A) the prediction results from PRISPRED, (B) the prediction results from GOR IV, (C) the prediction result from SOPMA, (D), the prediction result from SIMPA96.

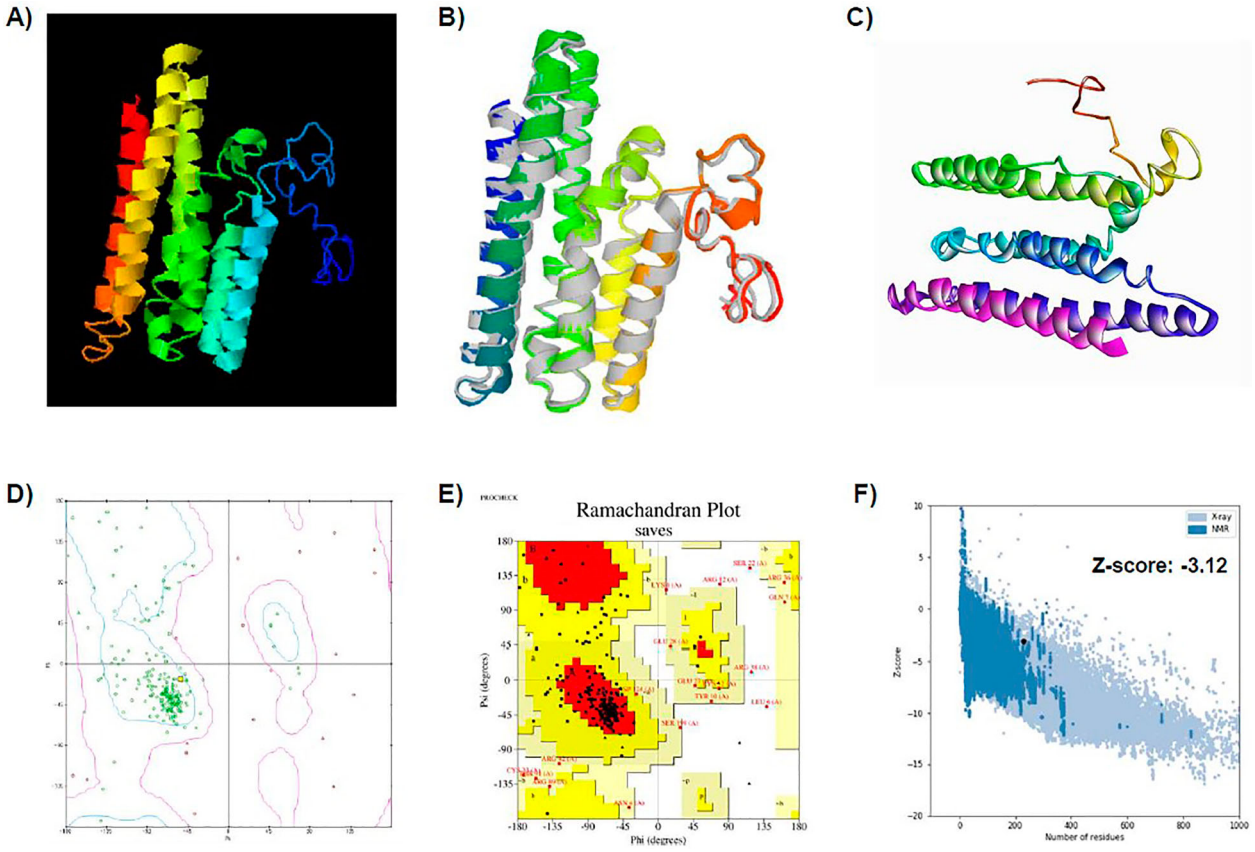
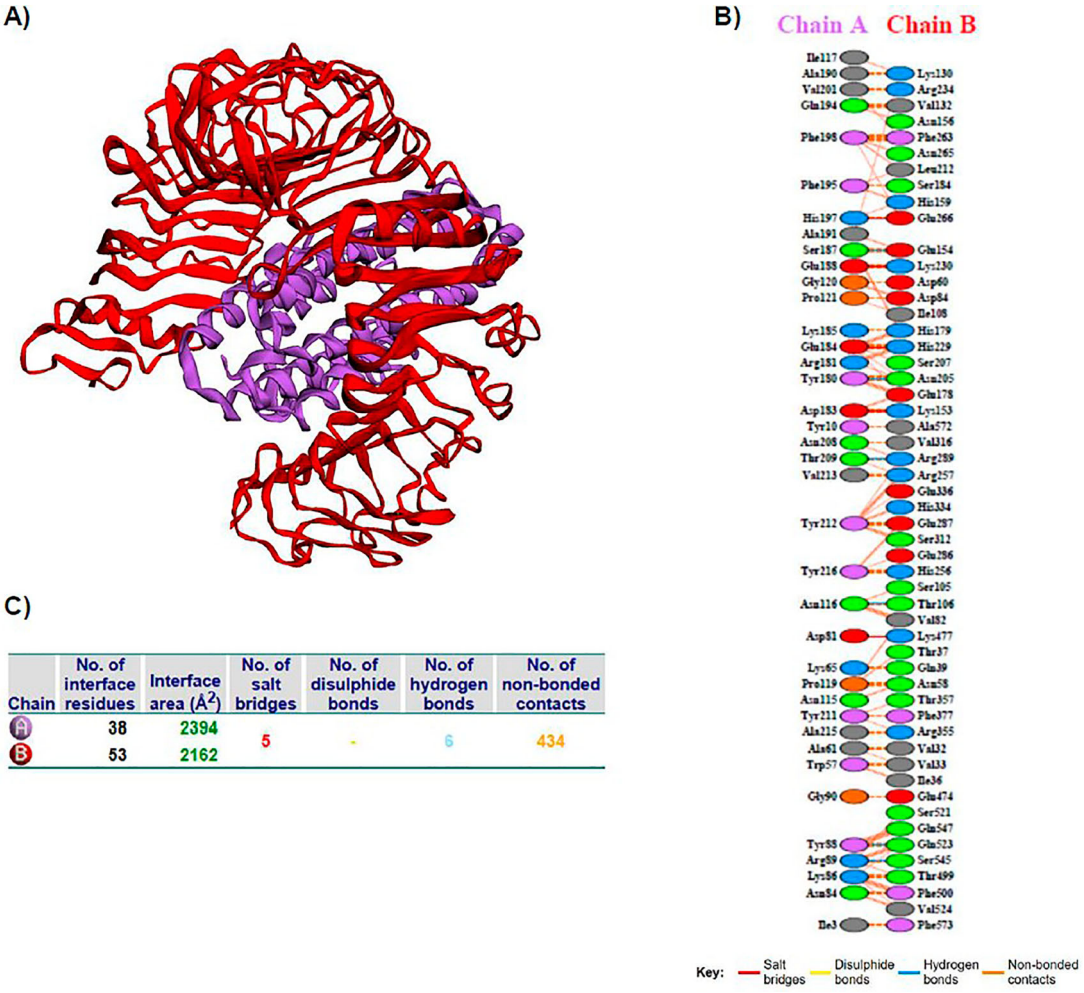


Figure 4. Prediction results of 3D structure of polyvalent multi-epitope RSV subunit vaccine. (A) protein 3D model constructed from I-TASSER, (B) Refined 3D model by GalaxyRefine, GalaxyWEB, (C) 3D model from BIOVIA Disocovery Studio Visualizer, (D & E) Ramachandran plot analysis by BIOVIA Discovery Studio Visualizer (D) and by PROCHECK (E), and (F) ProSA-server analysis.

Figure 6A, and the arrow indicates the direction of motion of amino acid residues. The complex shows low deformability (< 1), and the B factor was less deviated by the NMA (Figure 6B and C). The eigenvalue (5.36×10^{-5}) shows the motion stiffness of the complex, and an inverse connection was observed between the eigenvalue and the variance of each normal mode (Figure 6D and E). The covariance matrix and an elastic network model show a stiffer mode of the residues (Figure 6F and G). These results indicate that the vaccine candidate-TLR4 complex has reasonable stability and stiffness.



C)

Chain	No. of interface residues	Interface area (Å^2)	No. of salt bridges	No. of disulphide bonds	No. of hydrogen bonds	No. of non-bonded contacts
A	38	2394	5	-	6	434
B	53	2162				

Figure 5. Molecular docking interaction between the vaccine candidate and human TLR4. (A) human TLR4 (red colour) and the vaccine candidate (vaccine sequence #19, purple colour) docked complex. (B) Interacting residues between the vaccine candidate (chain A) and TLR4 (chain B). (C) Result of interface statistics.

The length of the codon optimized cDNA sequence of the vaccine candidate was 684 nucleotides, and the obtained Codon Adaptation Index (CAI) and the GC% content was 1.0 and 52.3%, respectively. Therefore, codon optimization was found to be acceptable, and ease of expression is guaranteed inside the E. coli. The final optimized sequence with restriction sites of NcoI (N-terminal) and XhoI (C-terminal) was closed into pET028a(+) vector, and a final length of the plasmid was 5923 bp (Figure 7).

Finally, HLA allele reactivity analysis of the 10 vaccine epitopes for representative races revealed the highest combined MHC class I+II reactivity in South Koreans (98.3%), with an average of 95.1% for the world population, and the lowest in the West Indies (90.1%) (Figure 8, Figure S5).

Discussion

During the COVID-19 pandemic, the implementation of stringent physical barriers resulted in an unprecedented global reduction in the incidence of most respiratory infections, including RSV. However, as the pandemic subsided, the occurrence of previously suppressed natural infections has resurged in the post-COVID-19 era. In some cases, the incidence of certain infections has not only increased beyond pre-pandemic levels but has also shown changes in outbreak patterns. This phenomenon is attributed to the lack of exposure to various infectious pathogens during the COVID-19 period, leading to inadequate development of protective immunity [33].

In South Korea, RSV incidence, which had decreased during the COVID-19 period, is also rising. A meta-analysis of studies conducted between 2000 and 2017 in Korea showed that the RSV positivity rate among respiratory infection samples from all age groups was 18.05%. In children with respiratory diseases, the diagnostic positivity rate exceeded 21.5%, making RSV the most frequent infectious agent [34]. Similar to other countries, RSV in South Korea poses significant risks, particularly to infants under two years of age, premature infants, or those with underlying health conditions, often leading to severe lower respiratory tract infections requiring medical intervention. However, South Korea also exhibits unique features in RSV epidemiology. At

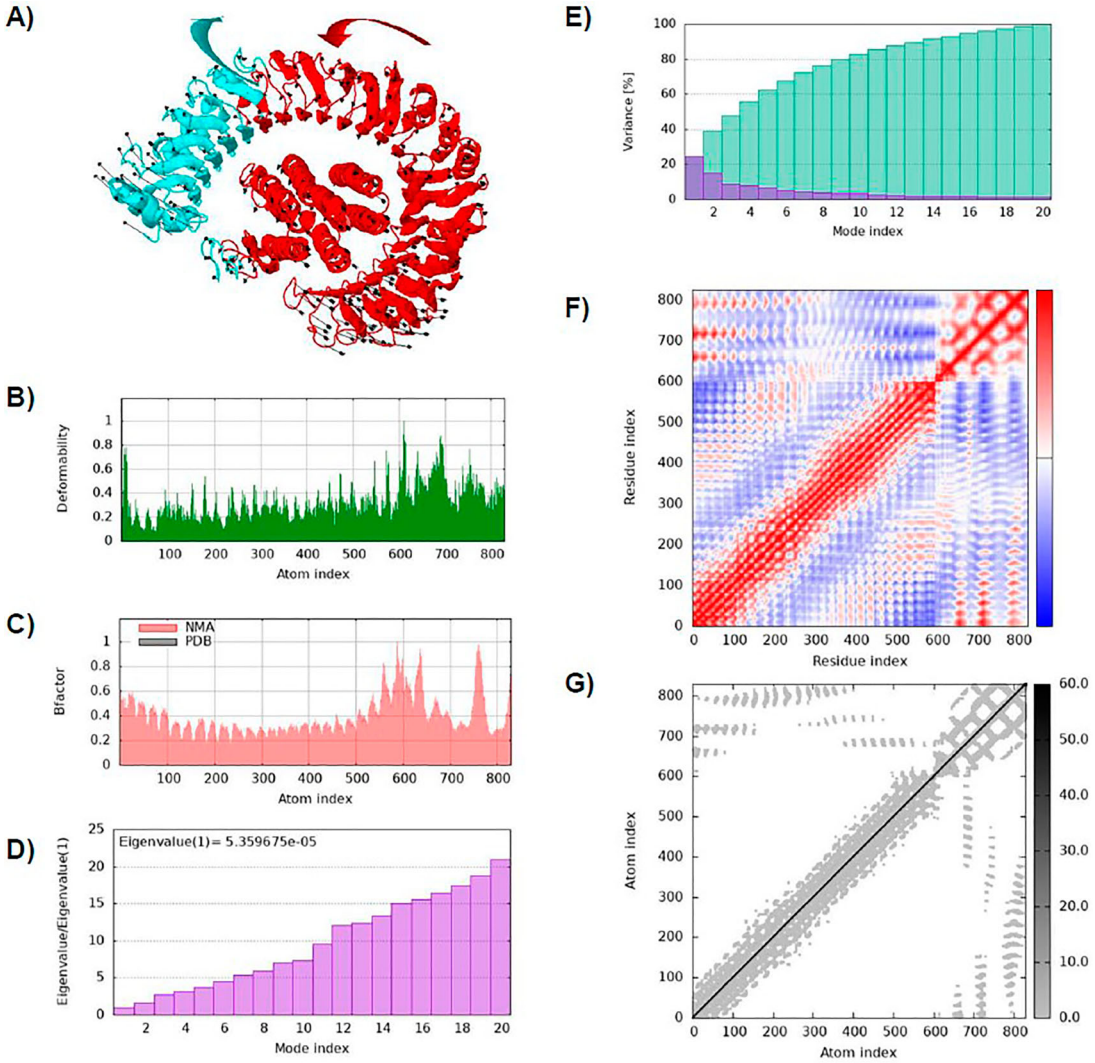


Figure 6. Molecular dynamic simulation of the vaccine and TLR4 complex. (A) the direction of the motion was shown by the red and cyan colour. (B) The stability of the vaccine was analyzed by the low main chain deformability. (C) The B factor/mobility. (D) The Eigenvalue demonstrated the protein's normal mode and the stiffness of the motion. (E) The normal mode variance, and (F) covariance index. (G) The elastic network model showed a stiffer mode of the residues.

postpartum care centres, where mothers and newborns reside for periods ranging from 1-2 weeks to over a month, RSV outbreaks have been occurs even among healthy, full-term infants with no underlying health conditions [34]. Additionally, due to the rapid transition towards an aging population and very low birth rates in South Korea, the proportion of elderly patients with lower respiratory tract diseases caused by RSV is significantly increased [35].

The introduction of Nisevimab, which demonstrates a prophylactic effect during the RSV season with a single use, and the clinical application of three F protein-targeted vaccines (AREXVEY, ABRYSVO, and mRESVIA) have garnered significant attention regarding the future of RSV management. There is also interest in whether these three vaccines, which primarily targeting relatively variable antigenic sites of the F protein as a single antigen, will be effective in preventing breakthrough infections [36]. Currently, numerous RSV vaccines employing various platforms are in different stages of development. Advances in medical technology have resulted in substantial variations in immune levels among populations, necessitating the availability of diverse vaccine platforms to overcome individual immune diversities.

The polyvalent multi-epitope RSV subunit vaccine developed in this study comprises three B-cell, three MHC class II, and four MHC class I epitopes derived from the RSV surface G and F proteins and the viral P and N proteins. To enhance the vaccine's antiviral effect and immunogenicity, hβD3 and PADRE were conjugated as adjuvants (Figure 1). This recombinant protein, consisting of 228 amino acids with a molecular weight of approximately 25 kDa, demonstrates relatively high antigenicity and safety. Additionally, all epitopes are all conserved in both RSV-A and RSV-B, aiming to eliminate the possibility of breakthrough infections. The vaccine formed a stable tertiary structure (Figure 4), and induced effective both humoral and cellular immune responses (Figure 2). In addition, the vaccine forms

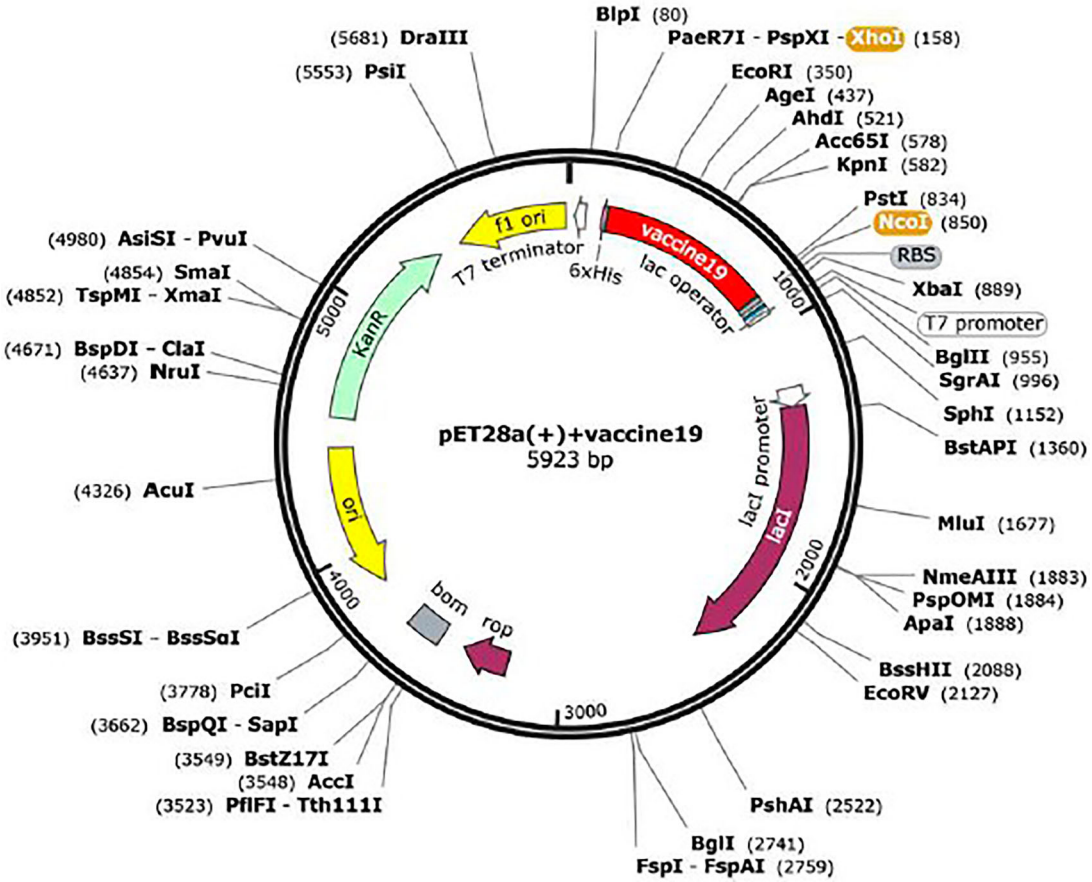


Figure 7. In silico cloning of the vaccine. In silico restriction cloning of the recombinant polyvalent multi-epitope RSV subunit vaccine sequence (vaccine #19 construct, the red segment) into the pET-28a(+) vector using NcoI and XhoI restriction sites using SnapGene software. The final product (vector and optimized codon sequence) consists of 5923 bp.

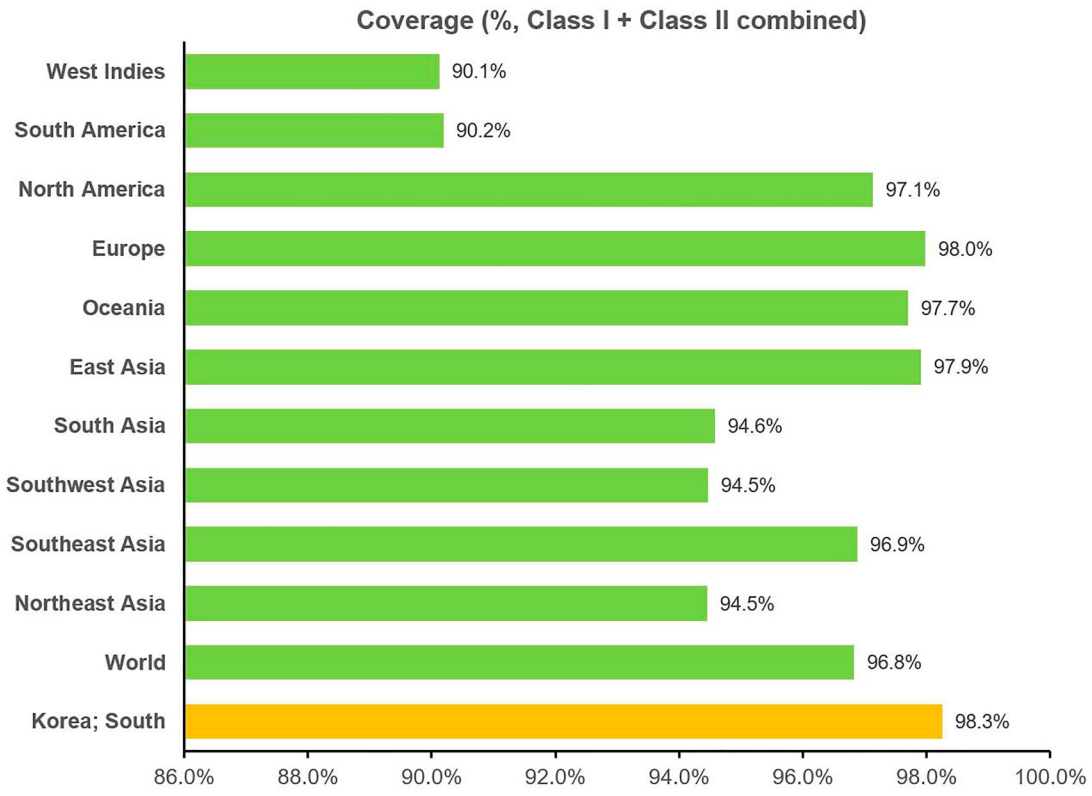


Figure 8. Results of population coverage against 10 epitopes of polyvalent multi-epitope RSV subunit vaccine.

stable complex with TLR4 (Figures 5 and 6). Population coverage for epitopes of the vaccine was average 95.1%, reaching 96.8% globally, and exceeding 90% in all analyzed regions, including the lowest in the West Indies at 90.1% (Figure 8, Figure S5).

Post-COVID-19, vaccine design studies utilizing in silico vaccine design technology are actively progressing for various infectious diseases, including RSV, Varicella zoster virus, Human Herpesvirus 6A, Variola virus, Epstein–Barr Virus, and Mucormycetes [7, 22, 37–43]. Vaccine design studies by Tahir ul Qamar M et al. focused on T cell epitopes (MHC class I and II) of RSV surface proteins [37]. These vaccines were predicted to generate effective antibody responses; however, they exhibited relatively low predictions for CTL proliferation and IFN-γ induction post-third immunization, indicating weaker cellular immune responses. Recently, Moin AT et al. demonstrated that a recombinant vaccine designed with 32 epitopes (13 MHC class I, 9 MHC class II, and 10 B-cell epitopes) from RSV P, N, F, and G proteins, conjugated with βD3 and PADRE, effectively induced both antibody production and cellular immune responses [38]. Although population coverage analysis for Koreans was not conducted, the combined coverage for Chinese, genetically closest to Koreans, was estimated at 80.12%, suggesting similar levels for Koreans. Nguyen TL & Kim H showed that a recombinant vaccine, designed with 14 epitopes (6 MHC class I, 5 MHC class II, and 3 B-cell epitopes) from RSV N, M, F, P, and L proteins, conjugated with flagellin, RS09, and a pan-HLA-DP binding epitope, strongly induced antigen-specific antibody immune responses [39]. While cellular immune response predictions were not provided, in silico simulation results from our 33-vaccine library (Figure S4-1 ~ S4-33) and study by Tahir ul Qamar M et al. [37] suggest that vaccines strongly inducing antibody production may have relatively weaker cellular immune responses, warranting further evaluation. The average population coverage for this vaccine was 35.71%, and 52.07% globally.

With three RSV vaccines already approved and undergoing clinical application, and various RSV vaccines in different stages of preclinical and clinical research, there is heightened interest in whether these vaccines will propel RSV research to the next level. However, time will be needed to confirm their clinical effects. Rapid global population movement, advancements in medicine, declining birth rates, and increasing longevity are diversifying population immunity levels, emphasizing the importance of diverse vaccine platforms. This study predicts that 10 RSV epitopes can effectively induce both humoral and cellular immune responses. However, as with all in silico design and validation studies, the effectiveness can only be confirmed through in vivo immunogenicity and protective efficacy validation via preclinical animal models and clinical trials. Future research will aim to produce the polyvalent multi-epitope RSV subunit vaccine designed in this study using various platforms, such as recombinant protein, mRNA, plasmid DNA, and viral vectors, and to evaluate in vivo immunoreactivity through preclinical animal models.

Author contributions

H.M.H. designed study, performed experiments, and wrote a draft. B.Y. analyzed data, provide critical comments and revised manuscript. MKK performed experiments, and revised manuscript. S.P. validated data, provided critical comments, and reviewed manuscript. J.H.L. conceptualized and supervised study, and wrote the manuscript. All authors have reviewed the manuscript and approved its submission.

Disclosure statement

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