Fall 2020

A review on the development of multi-epitope vaccine candidates for SARS-CoV-2

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Abstract

SARS-CoV-2, first emerged in the Hubei province of China in December 2019. The efficient transmission of the virus from person-to-person has contributed to the global spread of infection, better known as the COVID-19 pandemic. Patients with the highly infectious disease display flu-like symptoms such as cold and fever. The COVID-19 pandemic, caused by SARS-CoV-2, has become a public health emergency with over 200 countries affected. Genome sequence analysis has discovered that the virus is similar to that of SARS-CoV and MERS-CoV. Despite this, however, attempts to control SARS-CoV-2 with antiviral agents used to treat prior SARS-CoV and MERS-CoV infections have been found to be ineffective. As the rate of infection and deaths from COVID-19 increases, the pressure to find a vaccine solution builds. Efforts to develop antiviral agents against SARS-CoV-2 continue and the potential of multi-epitope vaccines will be further explored in this review. Multiple epitope targets of the spike (S) protein of SARS-CoV-2 are analyzed for their immunogenicity, stability, safety, and potential as vaccine candidates. The vaccine constructs discussed give promise of becoming vaccine candidates but require further *in vitro* and *in vivo* experimentation.

Background

Coronaviruses are classified under the Orthocoronaviridae subfamily (order: Nidovirales, subordination: Cornidovirineae, family: *Coronaviridae*)[1] and are grouped into four genera: α-/β-/γ-/δ-CoV. Due to α- and β-CoV infecting mammals, the β-CoV is of particular interest during the 2020 coronavirus pandemic. A novel β-CoV, now known as SARS-CoV-2, was identified from pneumonia cases in Wuhan, China in December 2019. The virus was named SARS-CoV-2 by the International Committee for Taxonomy of Viruses but is more commonly known by the name COVID-19 provided by the WHO.[2] SARS-Cov-2 is a more pathogenic form compared to that of the SARS-CoV of 2002 and the MERS-CoV of 2013. SARS-CoV and MERS-CoV are both coronaviruses that also infected human populations through zoonotic transmission and spread by close human contact. The SARS-CoV-2 genome shares around 82% sequence identity with SARS- and MERS-CoV and over 92% sequence identify regarding essential enzymes and

structural proteins.[3] Such a high level of matching has uncovered common pathogenicity amongst the three coronaviruses.

Coronaviruses are a type of enveloped virus containing a positive single-stranded RNA genome. In SARS coronaviruses, four glycoproteins are present including the spike (S), membrane (M), envelope (E) and nucleocapsid (N) proteins. These glycoproteins assist in virus particle assembly, replication and release. The S protein is highly important due to its role in binding to the host cell-surface receptor and allowing the virus to enter the host cell (Figure 1). The S protein consists of the S1 and S2 subunits where the S1 unit contains the receptor binding domain (RBD) required for attaching to host cell receptors and the S2 unit has other domains needed for fusion and intracellular trafficking inside the cell.[4] Specifically, the S protein promotes cell entry by binding to angiotensin-converting enzyme 2 (ACE2) as well as to host proteases, like transmembrane serine protease 2 (TMPRSS2), that prime the S protein.[8] TMPRSS2 activates the S protein and cleaves the ACE2 receptor to initiate binding of the virus to the host cell. Virion particles enter the host cell by endocytosis and its genome attaches to the host's ribosomes. When this occurs, translation of 2 co-terminal and large polyproteins are processed further by proteolysis mediated by proteases 3CLpro and PLpro. These mediators slice large polyproteins into smaller components for folding and packaging new virions, contributing to the spread of the viral infection in the host.[3]

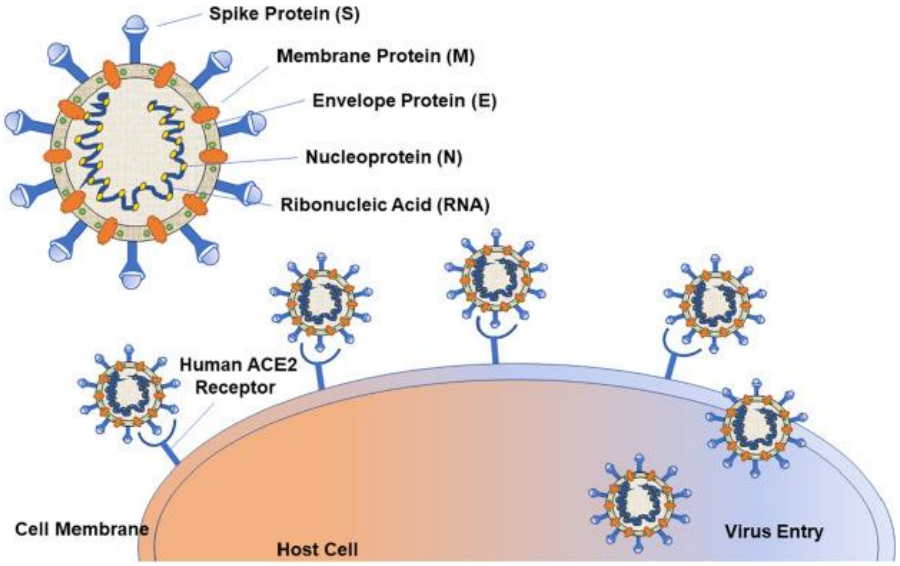

Fig. 1 Schematic representation of the SARS-CoV-2 structure and its mode of host entry.[3]

While the viral infection spreads through the host, the host cell entry of SARS-CoV-2 occurs mainly in the upper respiratory tract of the alveolar epithelial type II cells where its pathogenic effects are also observed. Studies have further clarified that the ACE2 and TMPRSS2 receptors are particularly expressed in alveolar epithelial type II cells.[16] As the viral infection continues to spread, the host immune system recognizes the virus and/or its epitopes and stimulates an innate or adaptive immune response. Pathogen recognition receptors (PRRs) present on the immune cells, toll-like receptors (TLR) 3, 7 and 8, identify the virus and initiate the production of interferon (IFN). The infection and replication of SARS-CoV-2 dampens the response of the produced IFN by dodging innate immune cells. As this occurs, the presence of monocytes, neutrophils, and adaptive immune cells initiates increased pro-inflammatory cytokine production. Within the helper T cells, Th1 and Th17 cells with viral epitopes may usher cytokine storms that result in pulmonary edema or pneumonia. Cytotoxic T cells are recruited to the site of infection to kill any infected cells in the lungs. B cells, like T cells, recognize viral proteins and activate antibody production in response.[9]

How does SARS-CoV-2 spread among humans and what are the clinical symptoms?

SARS-CoV-2 is a novel virus associated with acute respiratory disease. The infection, COVID-19, spreads via droplets, respiratory secretions, contact surfaces, and direct human contact through the respiratory tract.[3,5] The virus has a suggested incubation period of 1-14 days although the majority of cases are around 3-7 days. Patients with COVID-19 have symptoms including fever, malaise, cough, and flu-like symptoms.[6,15] Severe problems are commonly seen in elderly patients as well as those with comorbidities. Those in critical condition have displayed acute respiratory distress syndrome (ARDS), respiratory failure, multiple organ failure, and death.[7]

Possible multi-epitope vaccine use against SARS-CoV-2

SARS-CoV-2, like SARS-CoV and MERS-CoV, causes severe respiratory illness and possible death for comorbid patients. The potential adaptive mutations in the SARS-CoV-2 genome allow the virus to be highly pathogenic, making it difficult to develop drug therapeutics and vaccines.[3] Despite the difficulty, multiple pharmaceutical research companies are currently developing vaccine candidates to tackle the prevention of COVID-19 infections. There are hopes in vaccine

development as phylogenetic analysis of different SARS-CoV-2 strains' glycoproteins indicate close relation to one another, meaning that a vaccine targeting one strain would be effective against all other strains (Figure 2).

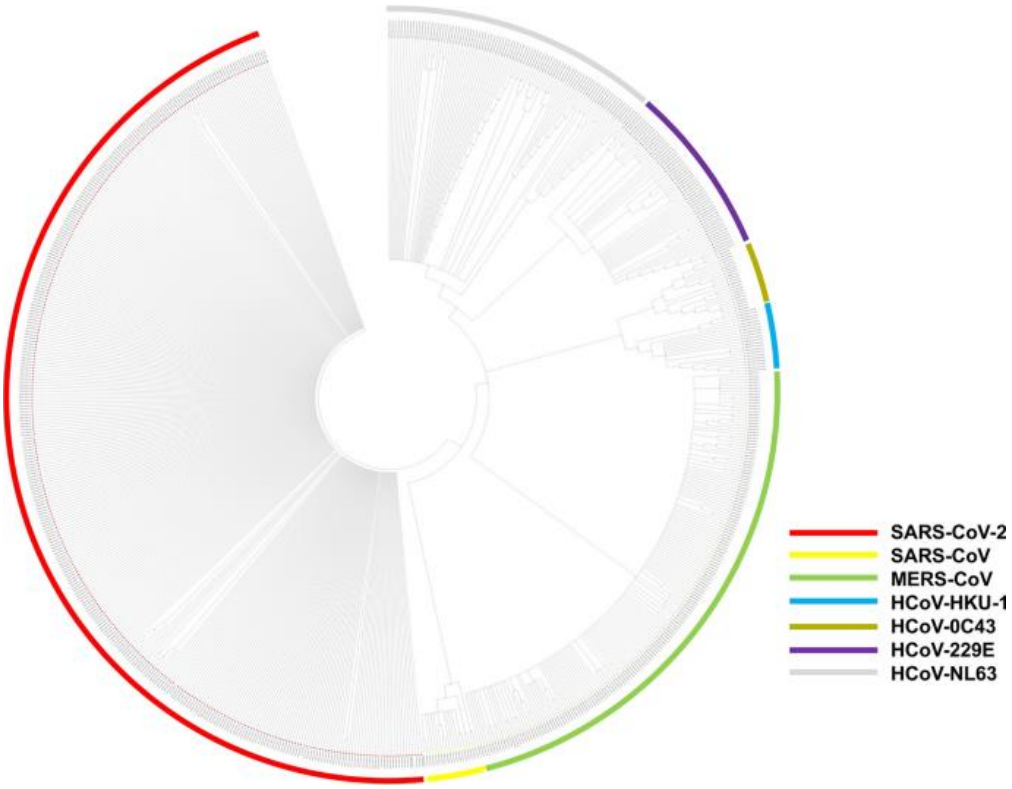

Fig. 2 Phylogenetic analysis of spike glycoprotein in HCoV-NL63, HCoV-229E, HCoV-OC43, HKU-1, MERS-CoV, SARS-CoV and SARS-CoV-2.[8]

One approach to the outbreak of SARS-CoV-2 is the development of multi-epitope vaccine constructs. These vaccines are made up of a series of or overlapping peptides that are an ideal solution to the prevention and treatment of viral infections and tumors.[18] Multi-epitope subunit vaccines are a novel strategy in the prevention and treatment of infectious diseases and are developed via immunoinformatics methods and computational analyses. This innovative approach allows researchers to identify immunogenic and highly conserved epitopes from bacterial and viral antigens. CD4+ and CD8+ epitopes can be used both separately and in combination to develop a broad range of vaccine candidates. The construct of the vaccines provide the ability to combat a variety of pathogens as well as elicit cellular and humoral responses in human hosts. Administration of these vaccines allows the mock epitopes to be

presented to the major histocompatibility complex (MHC) where their corresponding T-cell receptors proliferate and generate appropriate immune responses.[16]

Benefits and drawbacks of a multi-epitope vaccine design

Despite being a new approach to vaccine development, multi-epitope vaccines promise potential by being cost-effective, highly safe, decreasing time to design, water soluble, using natural adjuvants, stable under simple storage conditions, and providing satisfactory preclinical evaluations.[13,22] In addition, they do not require microbial culturing and surpass many wet lab experiments.[16] They are a safer option to pursue due to the absence of an entire pathogen, making the vaccine highly specific and stable. Whole pathogen immunizations include this drawback, with concerns of causing autoimmune or strong allergic reactions.[22] Regarding the design concepts, a multi-epitope vaccine construct contains unique aspects compared to classical vaccines containing a whole pathogen or single-epitope vaccines. In particular, they consist of cytotoxic T-cells, T helper cells, and B cell epitopes, all of which can induce robust cellular and humeral immune responses concurrently. With the addition of an adjuvant, the vaccine may have increased immunogenicity and long-lasting immune responses. Also, the inclusion of multiple epitopes from different virus antigens widens the range of targeted viruses. Finally, pathological immune responses or adverse effects may be reduced through the administration of a multi-epitope vaccine as components triggering this cascades are unutilized.[18] With such features, the use of multi-epitope vaccines as a therapeutic agent against viral infections, especially SARS-CoV-2, may be a future exploit.

Although promising in vaccine development, several drawbacks of a multi-epitope vaccine approach include the selection of appropriate candidate antigenic epitopes as well as the development of an effective delivery system, usually by use of an adjuvant to enhance the immune response. Additionally, peptides are poor immunogens and require the use of adjuvants or a delivery system to aid in their ability to induce an immune response. They are prone to enzymatic degradation and are often not recognized equally by the whole outbred population.[22] In the race to develop a vaccine for SARS-CoV-2, peptide-based vaccines have been outperformed by mRNA vaccine candidates. These mRNA vaccines may also be a novel approach in vaccinology but their performance has proven potent immunity that is able to induce

strong $CD8^+$ T cell responses as well as strong $CD4^+$ T cell responses unlike that in protein immunizations.[23]

Adjuvants

Following epitope selection, adjuvants are commonly used in vaccines to strengthen the stimulation of an immune response while also protecting the antigen from degradation and transporting the respective antigen to the desired tissue. Adjuvants may replace the natural danger signal triggered by infection as the lack of a whole pathogen in the vaccine construct hinders strong native danger signals.[22] Of the proposed epitope targets explored below, the use of beta-defensins as an adjuvant was a well favored method. Prior research has shown that vaccines containing defensin adjuvants activate the primary innate antiviral immune response and mediate other immunomodulatory activities against some viruses, including coronaviruses.[19] Cholera Toxin B was also explored as an adjuvant with earlier studies citing its ability to increase systemic and mucosal immune responses with protein conjugates.[21]

Review of proposed epitope targets in SARS-CoV-2

Multi-epitopic subunit vaccines have become of interest due to their role in the viral structural proteins evoking a strong immune response. Studies have indicated that humoral and cell-mediated immune responses play a role in protection from the S and N proteins of coronaviruses.[12] With multi-epitopic subunit vaccines emerging as a new strategy in vaccine development, studies on vaccine constructs for SARS-CoV-2 have emerged. These vaccine constructs are designed using immunoinformatics methods and computational analysis where epitope predictions are performed by servers and then further selected based on their antigenicity, toxicity, allergenicity, and cross-reactivity with human proteomes.[19] the predicted epitopes are attached by proper linkers. The vaccine construct is then physiochemically, immunologically, and structurally evaluated by bioinformatics tools to confirm validity and potency of the construct. The following provides a review of potential epitope targets in vaccine candidates.

In a study performed at the India Institute of Technology Mandi, three structural proteins (S, N and E) capable of inducing a humoral and cell-mediated immune response were selected for a

multi-epitopic vaccine candidate. IEDB, NetCTL, and IFNepitope servers were used to predict B lymphocyte, helper T cell, cytotoxic T cell, and IFN-γ epitopes. A multi-epitopic vaccine was constructed using 48 selected high-scoring CTLs, 4 high-affinity HTLs, and 4 B-cell epitopes. These epitopes were linked together using the CTL linker (AAY), HTL linker (GPGPG), and B epitope linker (KK). To improve the immunogenicity of the vaccine, β-defensin-TLR-3 agonist was added as an adjuvant through an EAAAK linker at the N-terminal of the vaccine construct. The vaccine candidate and human toll-like receptor-3 (TLR-3) binding stability was observed by docking and molecular dynamic simulations. Residues at the N-terminal and middle region of the vaccine construct were shown to form stable interactions with the TLR-3. The vaccine construct was validated on the Ramachandran plot indicating that 88% of residues of the vaccine model lied within the favored and allowed regions. A comparison with the RAMPAGE serve had similar results with about 90.7% residues in the favored and allowed regions. The vaccine was found to be antigenic with a score of 0.566 through VaxiJen v2.0 and 0.845 through ANTIGENPro. With the vaccine candidate being antigenic, non-allergen, nontoxic, highly stable, immunogenic in nature and capable of INF- γ production, researchers encourage continued experimentation to validate the multi-subunit vaccine by *in vitro* and *in vivo* studies.[14]

Another study with collaboration between the University of Lahore, National University of Sciences and Technology and the Khawaja Muhammad Safdar Medical College, developed three multi-epitopic peptide vaccine candidates through the use of the immunoinformatics approach by predicting B-cell and T-cell epitopes (Fig 3)[16]. The S1 and S2 domains of spike proteins were analyzed while two of the three vaccine constructs were prioritized with T-cell and B-cell epitopes. The prioritized epitopes were then modeled using linkers and adjuvants. Further observation was done via 3D models demonstrating their physiochemical properties and potential interactions with ACE2, TLR2, TLR4, and HLA Superfamily alleles. In vaccine 1, four epitopes from the S1 domain were used. Three of the four epitopes were in the N-terminal domain of the S1 protein and the fourth was in the receptor binding domain (319-541). The particular locations of these four epitopes made a great potential target for antiviral therapeutics and vaccines due to the roles of the S1 subunit and RBD in viral entry of the host cell. Vaccine 2 contrasted that of vaccine 1 by containing both weak and strong epitopes. ^{506}QPYRVVVLSFELLHA^{520} epitope was implemented due to its presence in the RBD while two

weak epitopes,^{731}MTKTSVDCTMYICGD^{745} and ^{733}KTSVDCTMY^{741}, from the S2 domain were added to observe for binding affinity. Docking with TLR2, TLR4, and ACE2 indicated that the two S2 weak epitopes bound effectivity. In the binding from ^{31}MTKTSVDCTMYICGD^{745}, Thr^{192}, Val^{197}, Lys^{186}, Thr^{187}, and Ser^{186} bound to TLR4, Lys^{186} had an affinity for the ACE2 receptor. ^{33}KTSVDCTMY^{741} was found to overlap the other S2 epitope 31MTKTSVDCTMYICGD745. The third vaccine construct was a modified version of vaccine 2 including an additional B-cell epitope ^{369}YNSASFSTFKCYGVSPTKLNDLCFT^{393} and the adjuvant beta defensin. In previous studies, β-defensin had been found to be a potent adjuvant when conjugated with MERS-CoV antigens.[20] Adding this epitope and adjuvant ensured both a cellular and humoral immune response as observed by previous studies. Vaccine 3 was found to have considerable interactions with TLRs and HLA superfamily alleles as well as interactions between Cys^{93} and Phe^{94} from the B cell epitope and between Arg^{8} and Glu^{96} from the BCR. All proposed vaccines took into account the role of the spike proteins and the likelihood of the vaccines being presented by MHC-I and MHC-II. The vaccine constructs were validated ERRAT analysis and Ramachandran plots. The ERRAT scores for vaccines 1-3 were as follows: 74.1379, 67.5676, and 74.2574, respectively. The Ramachandran plot analyses found that 97.1% residues were in the favored region for vaccine 1, 98.1% residues in the favored region for vaccine 2 and 86.5% for vaccine 3. Of the three vaccine constructs, the Ramachandran plot values indicated vaccine 1 and 2 to be higher quality in their structure. Binding affinity validated by a HADDOCK web server noted that vaccine 1 had the highest interactive ability compared to vaccine 2 and 3. In addition, the antigenic potential of the three vaccines was tested via VaxiJen v2.0. All three vaccines were found to be antigenic with scores of 0.883591, 0.946425, and 0.8853570, respectively. From these results, the suggested vaccine candidates display potential as antiviral agents. Researchers encourage further study of these potential epitope candidates by *in vitro* and *in vivo* experiments as they may block the viral interaction of ACE2 and spike glycoproteins. Further experimentation could also explain the effects of the vaccine's interactions within the host cell and the vaccine's role in the host immune response.

Vaccine combination	Epitope	Representation	MHC class/B cell	Location within spike protein	Best binding allele	Percentile rank
Vaccine 1	^{89}GVYFASTEK^{97}	E1S1	I	S1 domain	HLA-A*03:01	0.2
	^{50}STQDLFLPF^{58}	E2S1	I	S1 domain	HLA-B*15:01	0.3
	^{191} EFVFKNIDGYFKIYS^{205}	E3S1	II	S1 domain	HLA-DRB5*01:01	0.17
	^{506}QPYRVVVLSFELLHA^{520}	E4 S1	II	S1 domain	HLA-DRB4*01:01	2.9
Vaccine 2	^{89}GVYFASTEK^{97}	E1 S1	I	S1 domain	HLA-A*03:01	0.2
	^{733}KTSVDCTMY^{741}	E1 S2	I	S2 domain	HLA-A*01:01	0.63
	^{506}QPYRVVVLSFELLHA^{520}	E4 S1	II	S1 domain	HLA-DRB4*01:01	2.9
	^{731}MTKTSVDCTMYICGD^{745}	E2 S2	II	S2 domain	HLA-DRB3*01:01	6.3
Vaccine 3	^{89}GVYFASTEK^{97}	E1S1	I	S1 domain	HLA-A*03:01	0.2
	^{733}KTSVDCTMY^{741}	E1 S2	I	S2 domain	HLA-A*01:01	0.63
	^{506}QPYRVVVLSFELLHA^{520}	E4 S1	II	S1 domain	HLA-DRB4*01:01	2.9
	^{731}MTKTSVDCTMYICGD^{745}	E2 S2	II	S2 domain	HLA-DRB3*01:01	6.3
	^{369}YNSASFSTFKCYGVSPTKLNDLCFT^{393}	E5 S1	B Cell	S1 domain	N/A	N/A

Fig. 3 Finalized epitopes for vaccine constructs.[16]

An additional study based at Garden City University focused on a potential multi-epitope vaccine candidate targeting the spike glycoprotein due to its role in triggering the stimulation of the cytotoxic T lymphocytes (CTL), helper T lymphocytes (HTL) and interferon- γ (IFN- γ). A linear vaccine was constructed with 7 CTL, 8 HTL, and 3 IFN- γ epitopes linked by GPGPG linkers. GPGPG linkers were chosen due to their ability to enhance solubility and allow the adjoining domains to be approachable. Cholera Toxin B (CTB) adjuvant was attached to the N-terminal by an EAAAK linker to boost a long-lasting immune response. This particular adjuvant was chosen due to prior research proving its ability to be a potential viral adjuvant enhancing both mucosal and systemic immunity to respiratory syncytial virus by nasal vaccination.[21] The structural quality of the vaccine construct was tested and validated by Ramachandran plot, Z-score and ERRAT analysis. The Ramachandran plot indicated that 96.4% of the residues lied in the favored region, therefore verifying that the quality of the vaccine construct was reliable. The Z-score of -8.1 indicated that the linear vaccine was within range of score of other comparable size proteins. The ERRAT analysis scored the vaccine construct as 74.2947, again indicate that the modeled structure was valid. All validating methods can be referenced in Figure 4.[8] Binding affinity was validated via molecular docking to TLR 2, TLR4, MHC I receptor, and MHC II receptor. All binding affinities were found to be effective with low HADDOCK scores. The vaccine construct was found to be antigenic with a score of 0.5107 via VaxiJen v2.0. The proposed vaccine was assessed via an *in silico* immune stimulation and findings found that the vaccine was able to produce specific immune responses necessary to remove the antigen on

secondary exposure. Computational analysis from this study indicated that the vaccine was structurally stable, antigenic and immunogenic, making it a possible vaccine candidate against SARS-CoV-2.

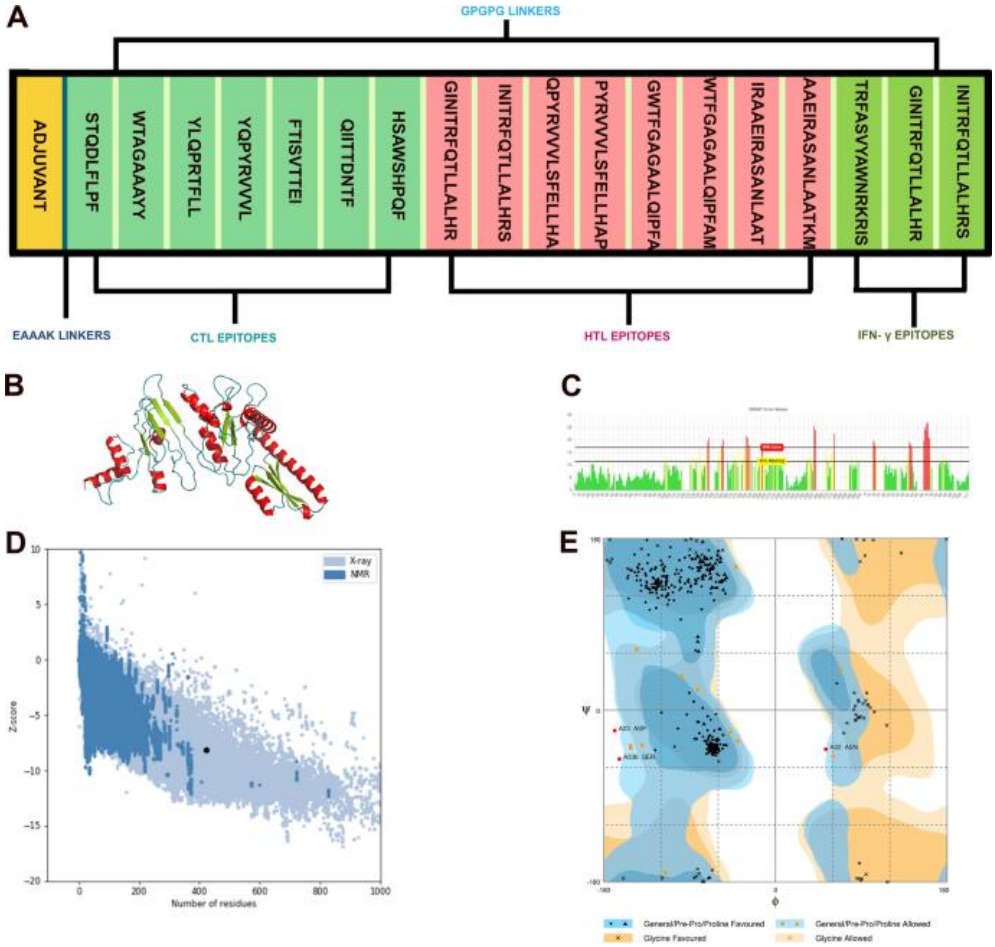

Fig 4. (**A**) Linear vaccine construct with CTL, HTL and IFN-γ depicted in sea green, pink and green boxes, respectively. EAAAK linker (deep blue) was used for linking the adjuvant and GPGPG linkers (pale green) were used for linking the epitopes. (**B**) 3D model of the final vaccine construct. Red, Limon and Blue represent the helical, sheet and loop region, respectively. (**C**) Validation of the vaccine structure by ERRAT with a score of 74.2947. (**D**) Validation of the structure with a Z-score of − 8.1 using ProSA. (**E**) Ramachandran plot analysing using RAMPAGE 96.4%, 2.9% and 0.7% in the favoured, allowed and outlier region, respectively.[8]

Lastly, another study collaborating between Guangxi University and Government College University Faisalabad (GCUF) focused on the spike glycoprotein of SARS-CoV-2. B-cell and T-cell epitopes were predicted using ABCpred and IEDB consensus method, respectively. Antigenicity and allergenicity of those selected epitopes were then checked by VaxiJen v2.0 and Allergen FPv1.0. Further analysis of the epitopes narrowed the vaccine construct to 3 CTL epitopes (S1 and M2, 6 HTL epitopes (E3 and M3) and 4 B-cell epitopes (S3 and M1). β-defensin was again used as an adjuvant and bound to the vaccine construct's N-terminal by an

EAAAK linker. The use of β-defensin was decided due to its relatively small size of 45 amino acids as well as its ability to act as an immunomodulator and antimicrobial agent. Subsequently, AAY, GPGPG and KK linkers were added to join the CTL, HTL and B cell epitopes, respectively. These linkers enhance stability, folding and expression of the vaccine construct. The vaccine construct is diagramed in Figure 5 below. Testing of the construct's structure found that it was non-allergenic, highly antigenic (0.6737), and non-toxic. The validity and quality of the construct's structure was validated by a Ramachandran plot indicating 89.4% of the residues fell in the favored region, a z-score of -4.8 and an ERRAT score 0f 82.4561. Binding affinity via molecular docking was revealed to have stable interactions between the vaccine construct and TLR3. All analyses point to a multi-epitopic subunit vaccine structure that is of good quality and has the ability to activate an effective immune response.[17] Further study through *in vitro* and *in vivo* experiments could observe the effects of the vaccine construct on SARS-CoV-2.

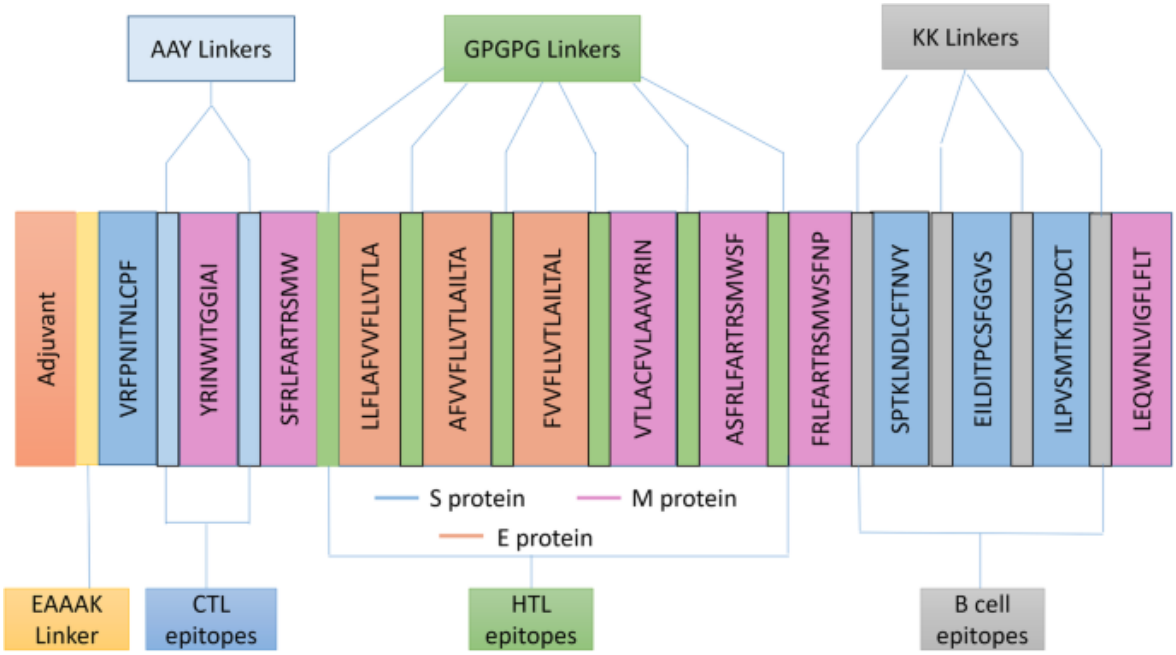

Fig. 5 Schematic diagram of MESV construct.[17]

Conclusion

While there is no current vaccine that has been approved by the FDA for COVID-19 infections, many vaccine candidates are in clinical trials. There is continued support in exploring multi-epitope subunit vaccines as these can be capable of producing an immune response. Through

immunoinformatics and computational approaches, researchers are able to select antigenic epitope targets for vaccine constructs. In addition, outside of being cost-effective, safe, time-saving, stable, using natural adjuvants and having satisfactory preclinical evaluations, multi-epitopic vaccine constructs are uncovering various subunit targets of SARS-CoV-2. Analyses of these targets have given promising potential as vaccine candidates for SARS-CoV-2. With an emerging presence in vaccine candidate studies, multi-epitopic vaccines require additional experimentation to develop the rapport of other antiviral agents such as DNA recombinant and mRNA vaccines. At this time, studies encourage *in vitro* and *in vivo* experimentation for further observation of vaccine constructs with hopes of progressing into vaccine development and clinical trials. While mRNA vaccines are at the forefront of recent SARS-CoV-2 vaccine development, multi-epitopic vaccines still promise potential in future immunization studies. Though these vaccines may not be further considered with regards to SARS-CoV-2, studies indicate that further experimentation may lead to a legitimate approach in the search for immunity in other infectious diseases.

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