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Proteomic analysis of SARS-CoV-2 to design B and T cells Multi-epitopes Subunit Vaccine

BS Zoology (2017-2021)

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Affiliated with University of Swat

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BY

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A thesis submitted in partial fulfilment of the requirements for the degree of BS Zoology

DEPARTMENT OF ZOOLOGY

GPG JAHANZEB COLLEGE SAIDU SHARIF SWAT

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Research Completion Certificate

It is certified that the research work contained in this research project titled **Proteomic analysis of SARS-CoV-2 to design B and T cells Multi-epitopes Subunit Vaccine .”** carried out and completed by **Abdullah Syed Allouddin** and **Hussain Farooq** under my supervision during their **BS** studies in the subject of zoology.

Date:

_____

Mr. Waqar Ahmad
(Research Supervisor)

Submitted through

_____

Prof. Akhtar Munir

DECLERATION

I hereby declare that this thesis entitled “Proteomic analysis of SARS-CoV-2 to design B and T cells Multi-epitopes Subunit Vaccine.” is a genuine work of Mr. Abdullah, Mr. Syed Allouddin and Mr. Hussain Farooq of the academic requirements for the award of BS (Hons) in Zoology.

The work has not been submitted to any other organization/institution for the award of any degree/diploma. This work is carried by us under the guidance and supervision of our supervisor.

Abdullah

Signature _____

Syed Allouddin

Signature _____

Hussain Farooq

Signature _____

DEDICATION

Above all we would like to dedicate our research work to the Almighty Allah. Thank you for the guidance, strength, power of mind, protection and skills and for giving us a healthy life. All of these we offer to you.

We whole heartedly dedicate our research work to our beloved parents, whom we love beyond measures, who are our source of inspiration, they gave us strength when we thought of giving up, who always provide their moral, spiritual, emotional and financial support to us. We thank them for being our greatest support, without their support and care we are unable to do anything in any field of our life.

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Abdullah

Syed Allouddin

Hussain Farooq

APPROVAL SHEET

A thesis entitles “Molecular determination of resistant gene to rifampicin in *M. tuberculosis* via genotypic analysis” be accepted as partial fulfilling this part of the requirements for the BS degree in Zoology.

Approved by

Lecturer. Waqar Ahmad **Signature: _____**
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Akhtar Munir **Signature_____**
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Abstract

In December 2019, an outbreak of pneumonia of unknown cause surfaced in Wuhan, China's Hubei province, with an epidemiologic link to the Huanan Seafood Market. It was discovered that a newly Identified coronavirus, later named as SARS CoV-2 is responsible for the disease. As of 21 July 2020, World Health Organization (WHO) confirmed a total of 14,881,534 infections and 613,994 deaths.

Many vaccine candidates are under development and clinical trials for corona virus disease. In this study, immunoinformatics strategies were applied to four specific proteins of the newly sequenced strain of the virus. Two non-structural proteins (Orf1ab and Orf3a) and two structural proteins (M and S proteins) were selected and subjected to B and T cells epitopes prediction and a vaccine was constructed. The constructed was checked for stability, effectiveness and safety using *in silico* tools.

CTL and HTL epitopes were selected based on their high binding affinity towards MHC-I and MHC-II and a vaccine was constructed. The vaccine was found to be antigenic (0.44), non-allergenic (-0.83), stable (instability index 32) and hydrophilic (GRAVY -0.027). The vaccine was modelled for docking to evaluate its molecular level interaction with human TLR-3 and 4. Immune simulation ensured the vaccine can efficiently induce the host immune response. The vaccine sequence was reverse translated into for cloning into pET28a(+) vector for cloning into *E.coli*. The CAI value of 9.61 given by Jcat ensured the maximal expression.

The current designed vaccine is confirmed by in silico tools to be capable of inducing appropriate immune response and requires experimental validation to verify the results.

Keywords: SARS-CoV-2, COVID-19, Immunoinformatics, epitopes, vaccine, Cytotoxic T Lymphocyte, Helper T Lymphocyte

Introduction

Coronaviruses that infects mammals along with humans belongs to the Coronaviridae circle of relatives of order Nidovirales. It has a unmarried stranded fine feel RNA genome enclosed by using a non-segmented envelope (Van doorn *et al.,* 2020). Coronaviruses are the largest amongst RNA viruses belonging to Coronaviridae, Roniviridae and Arteriviridae households. Coronaviridae are unsegmented, three′ polyadenylated and five′ capped fantastic sense unmarried-stranded RNA viruses motive various breathing diseases in humans (Ksiazek *et al.,* 2003;Kuiken *et al.,* 2003). CoVs are categorized into 4 lessons: alpha, beta, delta, and gamma. Amongst them, beta and alpha CoVs have been said for infecting human beings (Drosten *et al.,* 2003).

The Coronaviruses infections are usually milder in humans, however in case of famous beta-coronaviruses, Severe Acute Respiratory Syndrome Coronavirus (SARS) and Middle East respiration Syndrome Coronavirus (MERS), the infections are intense (Ksiazek *et al.,* 2003; Kuiken *et al.,* 2003; Drosten *et al.,* 2003; De Groot *et al.,* 2013). These viruses have together precipitated more than 10,000 infections with loss of life charges of 37% and 10% respectively within the beyond a long time (WHO, 2002-2003). A whole world of Coronaviruses is predicted to be found yet, that can be a great health issue.

A latest outbreak of acute respiratory ailment comparable to viral pneumonia infection from its clinical manifestations has been diagnosed in Wuhan town of Hubei province, China given that December 2019 (WHO, China 2020). The SARS-CoV-2 is chargeable for the outbreak that has inflamed 14,881,534 people and claimed lives of 613,994 humans as of 21 July 2020 as of now (News, 2020).Exported cases had been confirmed in other provinces of China, and 209 different countries round the arena (WHO, 2020; News, 2020). The dying rate is two-3% (News, 2020). Considerably high for an infectious disorder however lower than loss of life charge of SARS-CoV and MERS-CoV. The virus may be easily transmitted from person to person immediately through air and speak to or in a roundabout way by way of infected surfaces. WHO has declared the unconventional coronavirus outbreak a worldwide emergency that has the ability to be a worldwide epidemic (Drosten *et al.,* 2003).

The new virus targeted as WH-Human 1 coronavirus (WHCV) (and has additionally been called '2019-nCoV') has a 2.9kb genome having seventy nine.Five% similarity with SARS-CoV like coronavirus formerly isolated from Bats in china. The genome includes 16 ORF-coded non-structural proteins and 4 S, E, M and N coded structural proteins (Wu *et al.,* 2020). The Chinese researchers have named the virus as 2019-nCoV (Zhu *et al.,* 2019). The new coronavirus was later named Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) by means of the International Committee on Virus Taxonomy (Zu *et al.,* 2020). The identical day on 11 February 2020, WHO named the Pneumonia like disorder as Coronavirus sickness-19 (COVID-19) wherein the 19 represents the year of outbreak? According to statistics up to 21 july 2020, the very best quantity of high-quality instances were stated in USA accompanied by way of Brazil and India.(Waris *et al.,* 2020).

There isn't any verified powerful antiviral remedy for COVID-19. The combination of lopinavir and ritonavir in a historical control examine has been associated with large healing outcome amongst SARS-CoV sufferers and may be an option for treating COVID-19 infections.(Huang *et al.,* 2020 ; News. China 2020). Two HIV capsules are idea to target the protein that replicates the viral genome considered as a likely treatment are beneath laboratory check (News, China 2020).

The improvement of an powerful vaccine is needed urgently to prevent COVID-19. The immune gadget produces antibodies against the unique portions of the pathogen which can be antigenic and identified by B and T cells of the immune system (Janeway, 2001). After infection, T cells play a important role in killing cells infected by way of the pathogen (Lodoen, 2006). Immune system responses can be induced against pathogens through vaccination and immunization (Khan *et al.,* 2019). Computationally designed vaccines are price-effective and thermodynamically strong as compared to their conventional counterparts. In this medical examine a vaccine has been designed using Computational Biology method. The in silico take a look at showed the vaccine protection, effectiveness, and thermodynamically stability.

Aims and Objectives

- To evaluate the proteomic profile of SARS COV 2
- Docking of candidate proteins for vaccine designing against SARS-CoV-2

Review literature

Tania et al. (2020) expressed that broad endeavors all throughout the planet are being made to foster a reasonable antibody against COVID-19 (Coronavirus Disease-19) brought about by SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2). A viable immunization ought to have the option to actuate high titers of killing antibodies to keep the infection from joining to the host cell receptors. In any case, to inspire the defensive degrees of antibodies, an immunization may require various dosages or help from other immunostimulatory atoms. Further, the immunization ought to have the option to actuate defensive degrees of antibodies quickly with minimal measure of antigen utilized. This abatements the expense of an immunization and makes it reasonable. As the pandemic has hit most nations across the globe, there will be a staggering interest for the immunization in a fast time. Joining an appropriate adjuvant in a SARS-CoV-2 antibody may address these necessities. This survey paper will examine the trial consequences of the adjuvanted antibody concentrates with comparative Covids (CoVs) which may be valuable to choose a suitable adjuvant for an immunization against quickly arising SARS-CoV-2. We likewise talk about the flow progress in the improvement of adjuvanted immunizations against the infection.

Chiranjib et al. (2021) expressed that as of now, immunoinformatics is assuming a huge part in epitope recognizable proof and immunization planning for different basic infections. Utilizing immunoinformatics, a few researchers are attempting to recognize and describe T cell and B cell epitopes just as plan peptide-based immunization against SARS-CoV-2. In this audit article, we have attempted to talk about the significance in versatile insusceptibility and its importance for planning the SARS-CoV-2 antibody. In addition, we have endeavored to outline a few huge central issues for using immunoinformatics for immunization planning, like the models for determination and distinguishing proof of epitopes, T cell epitope, and B cell epitope expectation

and distinctive arising instruments/data sets for immunoinformatics. In the ebb and flow situation, a couple immunoinformatics reads have been performed for different irresistible microbes and related sicknesses. Subsequently, we have likewise summed up and incorporated these current immunoinformatics concentrates in this audit article. At last, we have examined about the likely T cell and B cell epitopes and their ID and portrayal for immunization planning against SARS-CoV-2.

Abiodun et al. (2021) expressed that toward the start of the year 2020, the world was hit with a worldwide pandemic infection alluded to as SARS-CoV-2 (COVID-19) which has left a huge number of individuals dead. To control this infection, immunization configuration becomes basic. In this investigation, potential epitopes-based antibody applicants were investigated. 600 (6 0) genomes of SARS-CoV-2 were recovered from the viPR information base to produce CD8+ T-cell, CD4+ T-cell and direct B-cell epitopes which were evaluated for antigenicity, immunogenicity and non-allergenicity. The aftereffects of this examination give 19 promising up-and-comer CD8+ T-cell epitopes that firmly cross-over with 8 promising B-cells epitopes. Another 19 CD4+ T-cell epitopes were additionally distinguished that can actuate IFN-γ and IL-4 cytokines. The most moderated MHC-I and MHC-II for both CD8+ and CD4+ T-cell epitopes are HLA-A*02:06 and HLA-DRB1*01:01 separately. These epitopes additionally bound to Toll-like receptor 3 (TLR3). The populace inclusion of the monitored Major Histocompatibility Complex Human Leukocyte Antigen (HLA) for both CD8+ T-cell and CD4+ T-cell went from 65.6% to 100%. The point by point examination of the potential epitope-based antibody and their planning to the total COVID-19 genome uncovers that they are transcendently found in the area of the surface (S) and layer (M) glycoproteins recommending the likely contribution of these underlying proteins in the immunogenic reaction and antigenicity of the infection. Since most of the potential epitopes are situated on M protein, the plan of multi-epitope antibody with the primary protein is exceptionally encouraging however the entire M protein could likewise fill in as a suitable epitope for the advancement of a constricted immunization. Our discoveries give a gauge to the exploratory plan of an appropriate immunization against SARS-CoV-2.

Abiodun et al. (2021) introduced in their examination that worldwide wellbeing emergency brought about by extreme intense respiratory condition Covid 2 (SARS-CoV-2), the causal specialist of COVID-19, adversely affects human wellbeing and on friendly and financial exercises around the world. Analysts all throughout the planet need to plan and foster effective therapeutics just as antibodies against the clever COVID-19 illness. In the current investigation, we led far reaching PC helped examination on the spike glycoprotein of SARS-CoV-2 to plan a protected and powerful multiepitope antibody. In silico epitope prioritization shortlisted six HLA I epitopes and six B-cell-determined HLA II epitopes. These high-positioned epitopes were totally associated with one another by means of adaptable GPGPG linkers, and at the N-end side, the grouping of Cholera Toxin β subunit was connected through an EAAAK linker. Primary displaying of the immunization was performed, and sub-atomic docking examination unequivocally recommended a positive relationship of a multiepitope antibody with Toll-like Receptor 3. The primary examinations of the immunization TLR3 complex uncovered the arrangement of fifteen interchain hydrogen securities, subsequently approving its uprightness and soundness. Also, it was tracked down that this collaboration was thermodynamically achievable. All in all, our information upholds the suggestion that a multiepitope antibody will give defensive invulnerability against COVID-19. Be that as it may, further in vivo and in vitro tries are expected to approve the immunogenicity and wellbeing of the applicant immunization.

Tahir et al. (2020) expressed that Coronavirus illness 2019 (COVID-19) pandemic brought about by serious intense respiratory Covid 2 (SARS-COV-2) is a critical danger to worldwide wellbeing security. Till date, no totally powerful medication or antibody is accessible to fix COVID-19. In this way, a viable antibody against SARS-COV-2 is significantly required. This investigation was led to plan a powerful multiepitope based antibody (MEV) against SARS-COV-2. Seven profoundly antigenic proteins of SARS-COV-2 were chosen as targets and various epitopes (B-cell and T-cell) were anticipated. Profoundly antigenic and covering epitopes were shortlisted. Chosen epitopes demonstrated huge collaborations with the HLA-restricting alleles and 99.93% inclusion of the total populace. Consequently, 505 amino acids long MEV was planned by associating 16 MHC class I and eleven MHC class II epitopes with appropriate linkers and adjuvant. MEV develop was non-allergenic, antigenic, steady and adaptable. Besides,

atomic docking followed by sub-atomic elements (MD) recreation examinations, exhibited a steady and solid restricting proclivity of MEV with human pathogenic cost like receptors (TLR), TLR3 and TLR8. At last, MEV codons were enhanced for its in silico cloning into Escherichia coli K-12 framework, to guarantee its expanded articulation. Planned MEV in present investigation could be an expected possibility for additional antibody creation measure against COVID-19. Notwithstanding, to guarantee its security and immunogenic profile, the proposed MEV should be tentatively approved.

Methodology

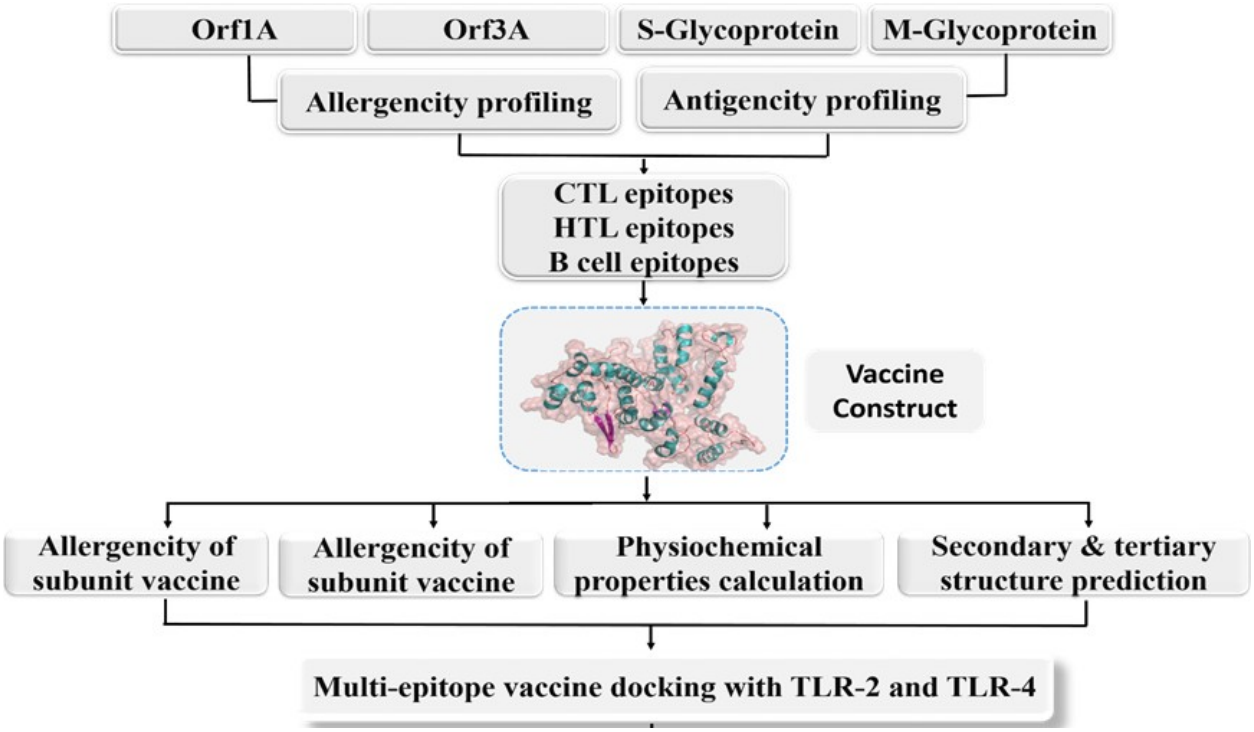

Figure 1: The methodological workflow of this scientific study.

Retrieval of proteins

The amino acid sequence of 4 proteins, orf1ab polyprotein (GenBank accession identity: QHD43415), orf3a polyprotein (GenBank accession identity: QHD43417), Surface glycoprotein (GenBank accession identity: QHD43416) and membrane glycoprotein (GenBank accession id: QHD43419) of lately recognized and sequenced novel coronavirus Wuhan-Hu-1strain or 2019-nCoV was retrieved from NCBI(Benson et al., 2002;Lu et al., 2009). Previous research had discovered orf1a have pathogenic features other than viral replication(Graham et al., 2008).

Prediction of MHC-I epitopes

NetCTL1.2 (http://www.Cbs.Dtu.Dk/offerings/NetCTL/), an internet server became applied to predict epitopes corresponding to MHC-I (CTL) at zero.Seventy five thresholds(Larsen et al., 2007). The binding affinity of the epitope for MHC-I is immediately associated with the COMB score computed by the server. Netctl1.2 prediction is based totally on epitope binding to MHC-I, proteasomal C-terminus cleavage rating, and shipping performance of Transporter Associated with Antigen Processing (TAP). Artificial neural community computes the MHC-I binding and Proteasomal C-terminus cleavage scores but TAP score is calculated through weight matrix.

Prediction of MHC-II epitopes

An online net server Immune Epitope Database (IEDB) (http://www.Iedb.Org/) (Pandey et al., 2018;Nielsen et al., 2009).Predicted MHC-II binding epitopes for a reference set of seven human HLAs particularly HLA-DRB1*03:01, HLA-DRB3*01:01, HLA-DRB1*15:0, HLA-DRB1*07:01, HLA-DRB4*01:01, HLA-DRB3*02:02 and HLA-DRB5*01:01. The server assigns IC50 values to the expected epitopes, which might be inversely related to the binding affinity closer to the MHC-II. IC50 scores of <50 nM represents excessive binding affinity. The IC50 fee <500 nM corresponds to intermediate binding affinity; however, <5000 nM is related low binding affinity of epitopes towards MHC-II. The binding affinity of the epitopes anticipated toward the MHC-II is inversely associated with the percentile rank.

Prediction of B-mobile epitopes

Receptors gift on the B-lymphocytes surface apprehend and binds B cell epitopes. ABCpred server (http://www.Imtech.Res.In/raghava/abcpred/) was employed for Linear B-cell epitopes prediction. The accuracy of the ABCpred server is 75% (zero.75 specificities and zero.49 sensitivity)(Saha et al., 2006). Moreover, discontinuous B-cellular epitopes were predicted for the 3-D shape by a web server Discotope2.0 (http://www.Cbs.Dtu.Dk/offerings/DiscoTope/) (Kringelum et al., 2012). Discotope2.0 conformational B cellular epitopes prediction is primarily based on amino acids composition ratio among residues of epitope and non-epitope. The server specificity is zero.75 while the sensitivity is zero.47, at –3.7 (default threshold).

Interferon-γ epitope prediction

IFN-epitope (http://crdd.Osdd.Internet/raghava/ifnepitope/) server become hired to are expecting interferon-gamma inducing MHC-II (HTL) epitopes(Dhanda et al., 2013). The server employs motif and SVM hybrid algorithms for the prediction of interferon inducing belongings of the epitopes. The server assigned the SVM score for every input epitope.

Vaccine creation

MHC magnificence I and II epitopes have been cautiously evaluated and selected for the construction of vaccine primarily based on their excessive binding affinity and non-allergenic nature. MHC-I epitopes have been fused by means of AAY, whereas MHC-II epitopes were joined collectively by using GPGPG linkers. The linkers have twin characteristic, separation of epitopes in order to keep away from neo-epitopes (junctional epitopes) formation and enhance presentation of epitopes. The decided on linkers are useful for differentiating the epitopes and improving their presentation(Sadi et al., 2017;Livingstone et al., 2002;Dorosti et al., 2019;Bergmann et al., 1996). Furthermore, mammalian beta defensin turned into selected as an adjuvant so that you can beautify the immunogenic assets of the vaccine assemble. The adjuvant was fused to the N-terminus of the vaccine peptide by another linker, the EAAAK(Arai et al., 2001).

Prediction of Allergenicity

Allergenicity prediction with better precision become performed by way of on-line server, the Algpred (http://www.Imtech.Res.In/raghava/algpred/)(Saha et al., 2006). The server is based on a couple of algorithms technique to compute allergenic belongings. The accuracy of the server is sort of 85% for the hybrid algorithm at threshold -0.Four. Six special strategies are used by the server for antigenicity calculation.

Prediction of Antigenicity

VaxiJen server (http://www.Ddg-pharmfac.Net/vaxijen/VaxiJen/VaxiJen.Html/)(Doytchinovaet al.,2007). Became applied to expect antigenicity of the vaccine sequence. The antigenic score predicted by VaxiJen server is thoroughly based totally at the query amino acid series physio-chemical properties as an alternative of using series alignment algorithms. VaxiJen accuracy for antigenicity is quite plenty better, 70-89%.

Physiochemical Properties

Protparam server (http://internet.Expasy.Org/protparam/)(Gasteiger et al., 2005).Became hired to calculate several physiochemical properties of the vaccine assemble, the composition of amino acids, instability index, theoretical PI, half of-life in vitro and in vivo, aliphatic index, and Grand Average of Hydropathy (GRAVY).

Secondary and Tertiary shape prediction

PSIPREDV3.Three (http://bioinf.Cs.Ucl.Ac.Uk/psipred/)(McCguffin et al., 2000). And SOPMA (https://npsa-prabi.Ibcp.Fr/NPSA/npsa_sopma.Html) servers have been utilized to are expecting the secondary structure of the vaccine collection with high accuracy. Robetta server (http://robetta.Bakerlab.Org)(Kim et al., 2004). Become used to generate a tertiary shape for given vaccine series. To are expecting the 3-d model, examine the question protein sequence into intended domains. If template shape is identified for the given amino acid series utilising PSI-BLAST, BLAST, FFAS03, or 3D-Jury, Robetta makes use of a comparative modelling method for structure era. If there's no template determined, then de novo Rosetta fragment insertion technique is employed.

Validating Tertiary Structure of Vaccine

Verification of the tertiary structure is a very vital step in tertiary shape assessment. In this take a look at, three validation servers were used to validate the protein 3-D shape. One is ProSA-web server (https://prosa.Offerings.Came.Sbg.Ac.At/prosa.Personal home page) that estimates Z-score for the given three-D shape(Wiederstein et al., 2007). The tertiary structure has inaccuracies if the calculated Z-score for the three-D structure does now not come inside the custom range for herbal proteins which is normally four-6. Prosa-web server utilizes three-D molecular viewer for highlighting and facilitating the recognition of difficult element and illustrate it in a nice plot rating. In order to compute the non-bonded interactions present inside the three-D structure, an online server ERRAT (http://offerings.Mbi.Ucla.Edu/ERRAT/) server turned into employed(Colovos et al., 1993). Another server called PROCHECK turned into used for examination of the Ramachandran plot (https://servicesn.Mbi.Ucla.Edu/PROCHECK/).

Docking the Vaccine’s Tertiary Structure With Human TLR-3 & TLR-four

Cluspro (https://cluspro.Bu.Edu/login.Hypertext Preprocessor) server turned into hired for you to locate vaccine interaction with human Toll-like receptor 3 and four(Kozakov et al., 2013;Kozakov et al., 2017). It is a extensively used docking server. The server, relying on the character of protein, and makes use of six electricity features. Ten docking outcomes are anticipated, with each docking parameter, based on centers of enormously populated clusters with lowest energy. This protocol elaborates the usage of numerous alternatives including building auxiliary restraints documents, selecting the unique energy parameters and reading the effects. Cluspro generates records in about four hours. In addition, PDBsum become used to achieve a graphical view of the interacting residues of the vaccine complexes with TLR-three and TLR-four.

Results

Retrieval of the Proteomes for B and T-cell epitopes prediction

orf1ab polyprotein (GenBank accession id: QHD43415), orf3a polyprotein (GenBank accession id: QHD43417), Surface glycoprotein (GenBank accession id: QHD43416) and membrane glycoprotein (GenBank accession id: QHD43419) were selected for vaccine designing. The amino acid sequences were subjected to B and T cells epitopes prediction for designing of a multi-epitope subunit vaccine.

Vaccine Protein Prioritization

The VaxiJen server calculated the antigenic scores for orf1ab, orf3a, S protein and M protein as 0.46, 0.49, 0.46 and 0.51 respectively (Table 1). These scores were above 0.4 which is the minimum threshold for antigenicity.

Table 1. Candidate proteins for vaccine designing

S.NO	Protein name	Genebank id	Antigenicity	Location
1	orf1ab polyprotein	QHD43415.1	0.46	Non-structural protein
2	ORF3a protein	QHD43417.1	0.49	Non-structural protein
3	surface glycoprotein	QHD43416.1	0.46	Structural protein
4	membrane glycoprotein	QHD43419.1	0.51	Structural protein

MHC-I epitopes prediction

NETCTL1.2 server predicted 272, 13, 37 and 10 MHC-I binding epitopes (9-mer) for orf1ab, orf3a, S and M correspondingly. Total 10 MHC-I epitopes were selected for vaccine designing, 2 epitopes from both orf3a and S while 3 from each orf1ab and M as shown in table 2.

Table 2. Selected CTL epitopes for vaccine construction

GenBank ID	Residue NO	Peptide sequence	MHC Binding	C-terminal cleavage affinity	TAP	Prediction score	Epitope
QHD43415.1	4842	ISDYDYYRY	0.70	0.95	2.96	3.28	Yes
	4198	KSDGTGTIY	0.68	0.97	2.75	3.21	Yes
	2889	FSAVGNICY	0.65	0.86	2.93	3.03	Yes
QHD43417.1	220	STDTGVEHV	0.43	0.54	0.21	1.94	Yes
	176	TSPISEHDY	0.33	0.95	2.93	1.69	Yes
QHD43416.1	258	WTAGAAAYY	0.67	0.73	2.86	3.11	Yes
	604	TSNQVAVLY	0.65	0.94	2.99	3.07	Yes
QHD43419.1	171	ATSRTLSYY	0.54	0.93	3.09	2.61	Yes
	196	YSRYRIGNY	0.32	0.93	3.14	1.66	Yes
	170	VATSRTLSY	0.27	0.96	3.01	1.46	Yes

MHC-II epitopes prediction

IEDB MHC-II server predicted MHC-II binding epitopes against a reference set of 7 human HLAs; HLA-DRB1*03:01, HLA-DRB3*01:01, HLA-DRB1*07:01, HLA-DRB1*15:01, HLA-DRB3*02:02, HLA-DRB4*01:01 and HLA-DRB5*01:01 for the selected candidate proteins. Ten MHC-II epitopes were selected on the basis of lowest possible percentile rank and non-allergenic nature, it was ensured the selected epitopes are not overlapping. The epitopes selected were in order as two from orf3a and S each while three from orf1ab and M provided in table 3.

Table 3. Selected HTL epitopes for vaccine designing.

GenBank ID	S.NO	Allele	Start	End	Peptide sequence	Percentile rank
QHD43415.1	72	HLA-DRB1*15:01	50	64	NMLRIMASLVLARKH	0.01
	24	HLA-DRB5*01:01	33	47	LGRYMSALNHTKKWK	0.17

QHD43417.1	2	HLA-DRB5*01:01	41	55	LVYFLQSINFVRIIM	0.9
	1	HLA-DRB4*01:01	30	44	RATATIPIQASLPFG	3.7
	1	HLA-DRB1*15:01	1	15	MDLFMRIFTIGTVTL	4
QHD43419.1	2	HLA-DRB1*07:01	31	45	RLFARTRSMWSFNPE	4.1
	2	HLA-DRB5*01:01	18	32	VGLMWLSYFIASFRL	4.2
	1	HLA-DRB1*15:01	18	32	EQWNLVIGFLFLTWI	6.1
QHD43416.1	4	HLA-DRB5*01:01	23	37	INITRFQTLLALHRS	0.32
	4	HLA-DRB3*01:01	1	15	NLVRDLPQGFSALEP	2.30

Vaccine construction

For vaccine construction, ten CTL and ten HTL epitopes were chosen based on high affinity towards the MHC-I and MHC-II respectively. High COMB score represents high binding affinity of CTL epitopes towards the MHC-I whereas lower percentile rank represents high binding affinity of HTL epitopes to MHC-II, respectively. The selected CTL and HTL epitopes were fused together by AAY and GPGPG linkers, respectively. The mammalian beta defensin was added at the N-terminus of the vaccine construct as an adjuvant to boost immune response using the EAAK linker. The arrangement of adjuvant, selected CTL and HTL epitopes in the final vaccine sequence that consists of 366 amino acids are depicted in figure 2.

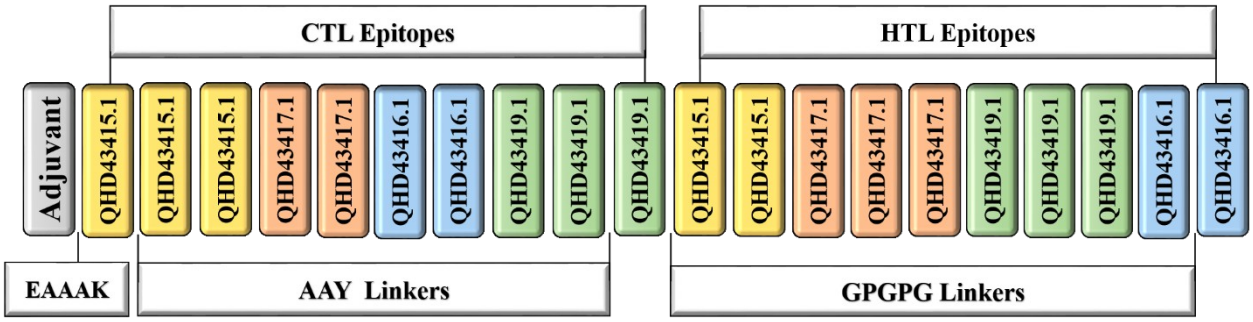

Figure 2. Figure depicting epitopes and adjuvant arrangement in vaccine sequence**.**

B-cell epitopes prediction

ABCpred server predicted a total of 14 linear B-cell epitopes (20-mer) having 0.8 or above score, the epitopes are shown in table 4 and figure 3. Discotope2.0 server identified 27 B-cell epitope residues (Table 5).

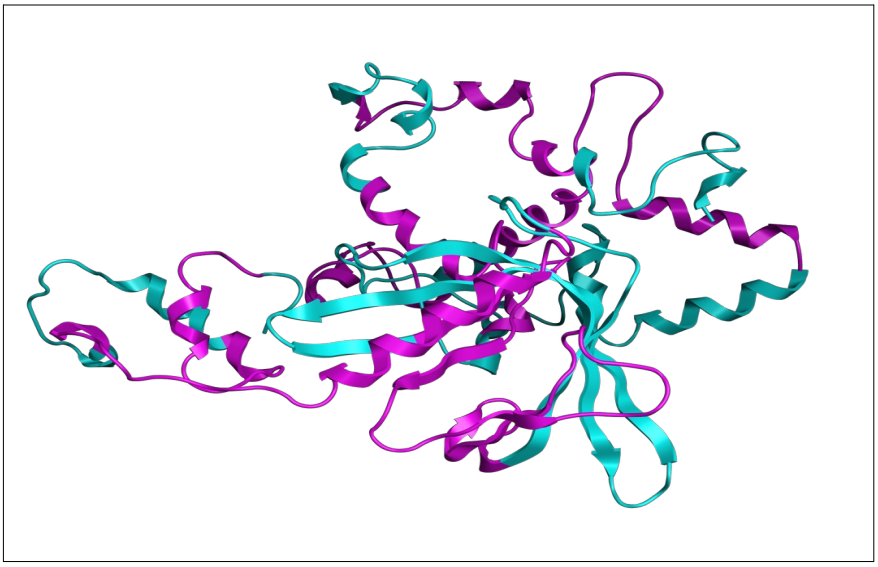

Figure 3. Light magenta colour shows Linear B-cell epitopes in vaccine 3D structure.

Table 4. Linear B cell epitopes predicted by ABCpred server

Rank	Sequence	Start position	Score
1	ALHRSGPGPGNLVRDLPQGF	342	0.89
2	GMDLFMRIFTIGTVTLGPGP	251	0.88
3	VTLGPGPGRLFARTRSMWSF	264	0.87
4	GPGVGLMWLSYFIASFRLGP	289	0.86
5	PGINITRFQTLLALHRSGPG	330	0.85
5	IIMGPGPGRATATI\IQASL	224	0.85
6	CYAAYSTDTGVEHVAAYTSP	81	0.83
6	YYRYAAYKSDGTGTIYAAYF	55	0.83
6	IASFRLGPGPGEQWNLVIGF	301	0.83
6	CAVLSCLPKEEQIGKCSTRG	18	0.83
7	PIQASLPFGGPGPGMDLFMR	238	0.82
8	SFNPEGPGPGVGLMWLSYFI	282	0.81
8	DYAAYWTAGAAAYYAAYTSN	105	0.81

9	YTSNQVAVLYAAYKTSVDCT	121	0.80

Table 5. Conformational B cell epitopes residues predicted by Discotope2.0

S.NO	Residues	Contact number	Discotope score
1	PRO	3	-3.123
2	LEU, ASN, HIS, THR, LYS, LYS. TRP	9, 14, 3, 9, 0, 4, 15	-2.496, -1.777, 0.166, 0.101, 0.940, 0.056, -3.598
3	GLY, PRO, GLY	0, 14, 12	-0.679, -1.719, -3.056
4	ARG, SER	10, 2	-3.230, -2.393
5	GLU, GLY, PRO, GLY, PRO, GLY	16, 0, 8, 2, 13, 6	-3.397, -0.584, -1.390, 0.267, -2.823, -2.835
6	PRO, GLY, GLU, GLN	3, 9, 15. 2	-0.160, -2.872, -2.041, -1.166

IFN-γ inducing epitopes prediction

IFN-epitope server predicted the MHC-II binding epitopes that can induce interferon-gamma. Three out of 10 MHC-II epitopes were predicted to be capable of inducing IFN-γ inducing ability (Table 6).

Table 6. ifn gamma inducing epitopes predicted by ifnepitope server.

GenBank ID	S.NO	Peptide sequence	Method	Results	Score
QHD43415.1	**1**	**NMLRIMASLVLARKH**	**SVM**	**POSITIVE**	**0.13**
	2	**LGRYMSALNHTKKWK**	**SVM**	**POSITIVE**	**0.41**
QHD43417.1	3	LVYFLQSINFVRIIM	SVM	NEGATIVE	-0.16
	4	**RATATIPIQASLPFG**	**SVM**	**POSITIVE**	**0.289**
	5	MDLFMRIFTIGTVTL	MERCI	NEGATIVE	1

QHD43419.1	6	RLFARTRSMWSFNPE	SVM	NEGATIVE	-0.75
	7	VGLMWLSYFIASFRL	SVM	NEGATIVE	-0.17
	8	EQWNLVIGFLFLTWI	MERCI	NEGATIVE	6
QHD43416.1	9	INITRFQTLLALHRS	SVM	NEGATIVE	0.00
	10	NLVRDLPQGFSALEP	SVM	NEGATIVE	-1.11

Prediction of Allergenicity and Antigenicity

VaxiJen server calculated 0.44 antigenic score for the vaccine sequence at 0.4 threshold indicating the potential of the vaccine to trigger immune response of the host. Algpred calculated -0.83 at -0.4 threshold indicating the non-allergenic nature of vaccine and ensured its safety.

Prediction of physicochemical properties

Protparam web server computed the physio-chemical properties of the vaccine as shown in table 7. The theoretical PI of 9.61 indicates the vaccine is basic in nature, the instability index and molecular weight were calculated to be 32 and 39.5KDa, respectively. The instability index less than 40 indicates the stable nature of the vaccine construct. Furthermore, the half-life (in vivo) of vaccine construct in E.coli and Yeast is >10 and >20 hours, respectively, and 30 hours in mammalian reticulocytes (in vitro). GRAVY and Aliphatic index were calculated as -0.027 and 75.8, respectively. The Aliphatic index ensures the thermostability of the vaccine construct.

Table 7. Physiochemical properties of final vaccine construct

S.NO	Property	Score	Prediction
1	Allergenicity	-0.83 (threshold -0.4)	Non-allergenic
2	Antigenicity	0.44 (threshold 0.4)	Antigenic
3	Molecular weight	39.5KDa	-
4	Theoretical PI	9.6	Basic
5	Half life	>10 hrs in E.coli	-
6	Instability index	32 (stable)	Stable
7	Aliphatic index	75.8	-
8	GRAVY	-0.027	Hydrophilic

Secondary structure Prediction

The secondary structure for the vaccine sequence was predicted by SOPMA and PSIPRED. The secondary structure shows 29.51% extended strands, 22.13% alpha helix, 4.92% beta turns, and 43.44% random coils in the structure as shown in figure 4

Figure 4: Figure depicting secondary structure predicted by PSIPREDV3.3. SOPMA predicted 29.51% extended strands, 22.13% alpha helix, 4.92% beta turns, and 43.44% random coils.

Prediction and Validation of Tertiary structure

Robetta server was utilized to predict the 3D structure for the vaccine sequence. The server generated five models for the query sequence. Validation tools were used to select the nearest to native structure for further analysis. ERRAT server predicted the total quality score of 92.45 for the vaccine 3D structure. Prosa-web calculated Z-score -5.08 which is lying within the standard score for natural proteins of the same size. Ramachandran analysis by PROCHECK server calculated that 84% of the residues are present in the most favoured, 15.4% present in additional allowed, 0.3% in generously allowed, and 0.3% present in the disallowed regions.

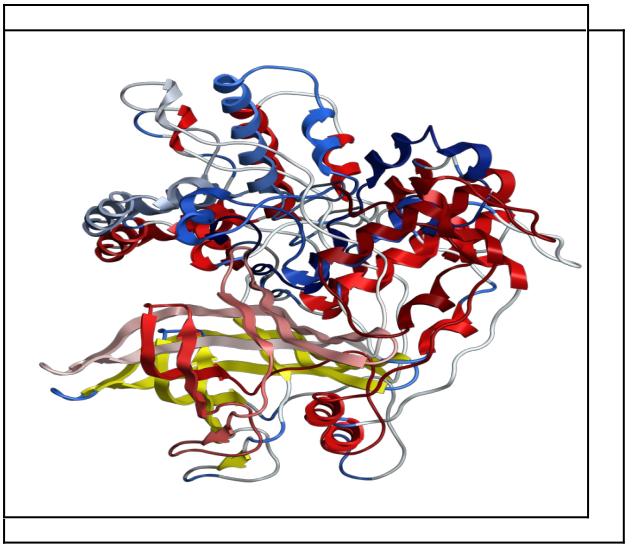

Figure 5. Final structure of vaccine (A) Depicting Helix in Red, beta-sheet in Yellow, and loop in White and Blue (B) Vaccine 3D image

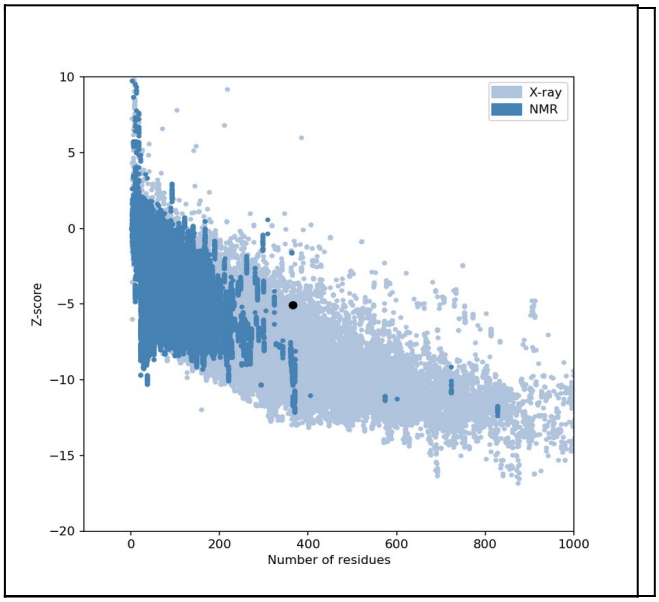

Figure 6: Validation of Final vaccine 3D model. (A)Validation by Prosa-web of 3D structure (Z-score: −5.08) (B) Ramachandran investigation showing the residues: 84% in most favoured, 15.4% in additionally allowed, 0.3% in generously allowed and 0.3% in disallowed regions.

Docking the Vaccine's Tertiary Structure with Human TLR-3 & TLR-4

Cluspro docking server was employed to evaluate the bonding of vaccine 3D model with two human TLRs namely TLR 3 and TLR-4. The server provided 10 complexes ranked on cluster size. Pymol software was used for visual evaluation and inspection of the docking complexes, finally, vaccine-TLR3 docking complex eight, and vccine-TLR4 docking complex six were selected for further analysis (Figure 7). The server assigned weighted scores -1312.4 (center) and -1412.3 (lowest energy) for selected vaccine-TLR3 complex. Similarly, it also assigned scores of -999.1 (center) and -1219.5 (lowest energy) for selected vaccine-TLR4 complex. PDBsum provided the graphical representation of residual interaction between the vaccine-TLR-3 and TLR-4, and graphical images of hydrogen bonds for both the complexes were produced.

Thirty six hydrogen bond interactions between vaccine-TLR-3 docked complex were identified displayed in supplementary table 1. The graphical image of the interaction is depicted in figure 7. Whereas, a total of thirty hydrogen bond interactions were identified in vaccine-TLR-4 docked complex as displayed in supplementary table 2. The graphical image of the interaction is depicted in figure 8.

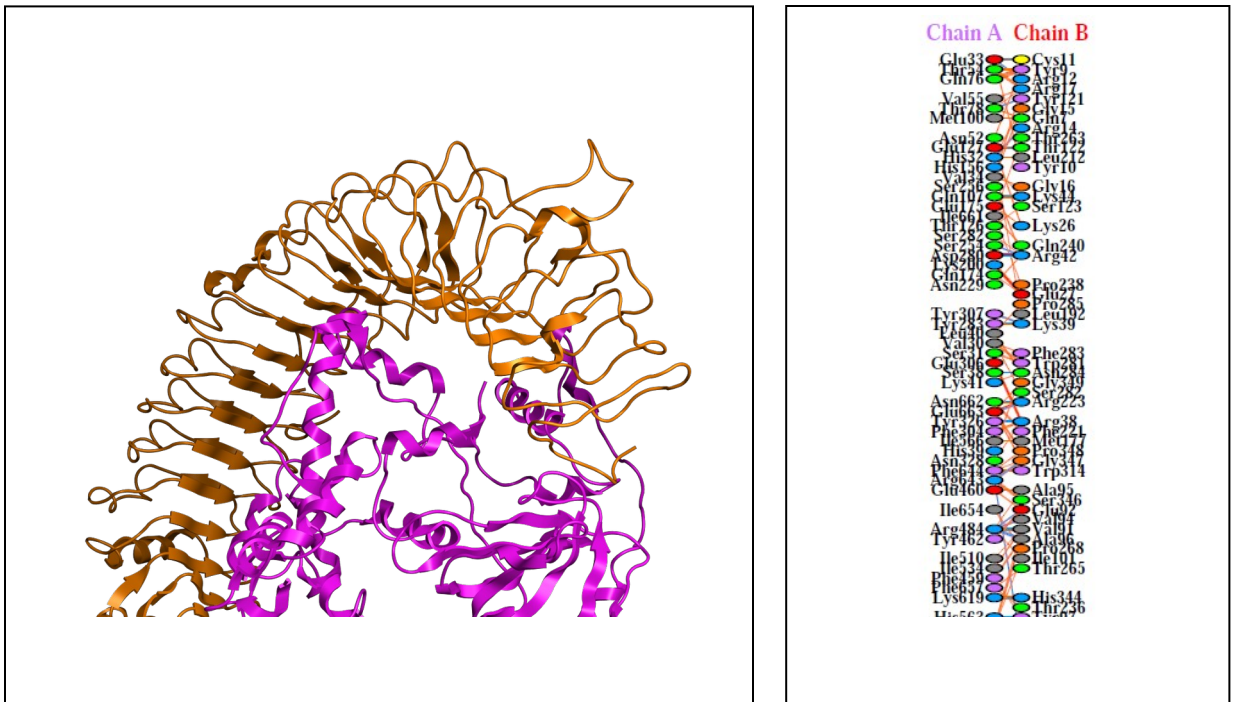

Figure 7: vaccine-TLR-3 complex. (A) The TLR-3 (receptor) is shown in Orange, while the Magenta color shows the multi-epitope subunit vaccine. (B) PDBsum file showing blue colored lines H-Bonds (Chain B: Vaccine)

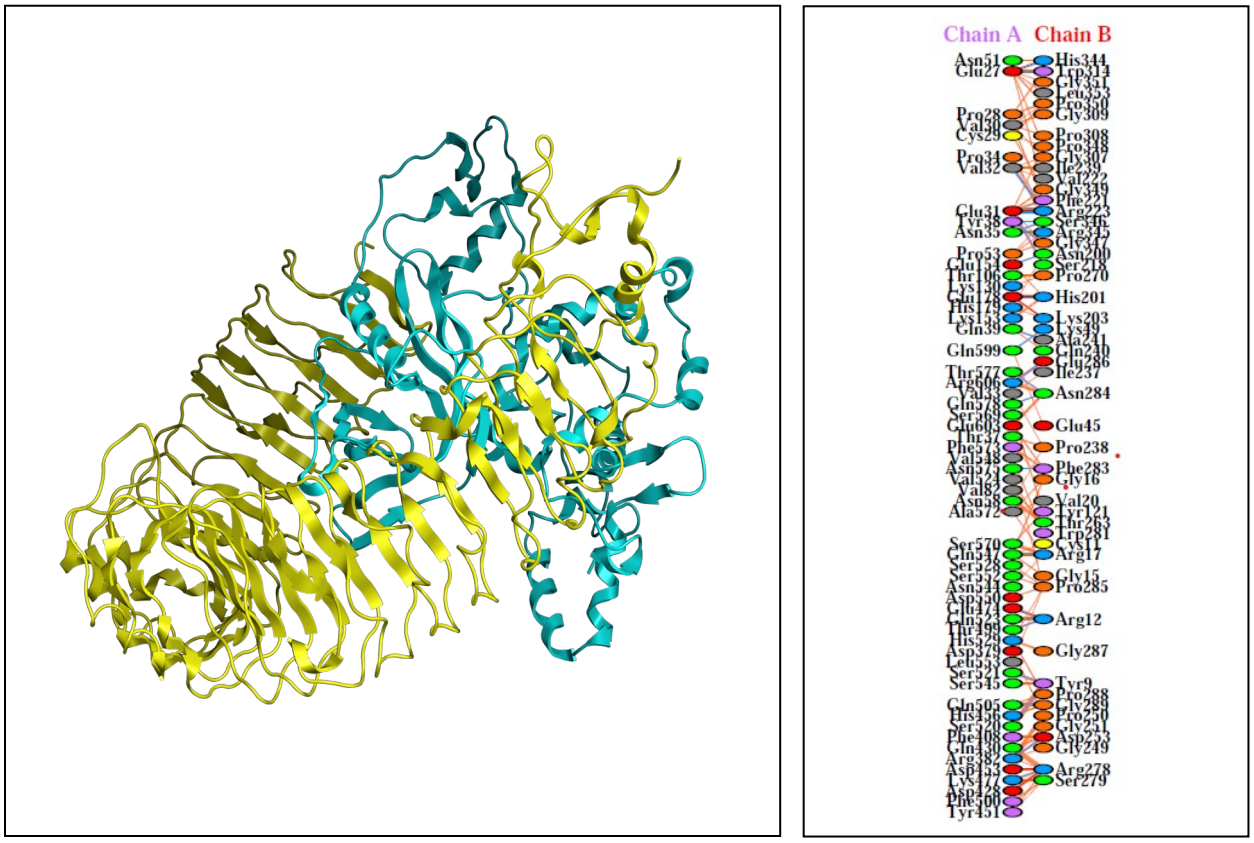

Figure 8: TLR-4 (PDB ID: j368)-vaccine-TLR-4 complex. (A) The TLR-4 (receptor) is shown in Yellow, while the Cyan color shows the multi-epitope subunit vaccine. (B) Pdbsum file showing blue colored lines H-Bonds (Chain B: Vaccine)

Discussion

Immunization is one of the most reliable and efficient methods of controlling infectious diseases. It improves the quality of public health more efficiently, quickly and cost-effectively. Traditional methods of making vaccines are effective against multiple pathogens and are used worldwide (Serruto *et al*., 2006). However, the vaccine developed by the classical approach faces several challenges, (Khan *et al.*, 2019). compared to the subunit vaccines that relies on specific immunogenic parts of pathogens unlike whole pathogen used in conventional vaccine either weakened or killed(Saha *et al*., 2006). Currently more than 100 vaccines candidates are in development but none of them is approved till now. The multi-epitope subunit vaccines have several advantages compared to traditional vaccines(Li *et al*., 2014). The vaccines produced by *in-silico* strategy enables the body's immune system to concentrate exclusively on desirable antigenic epitopes, thereby preventing non-protective reaction, autoimmune response from the recipient, and immune system evasion by the pathogen(Li *et al*., 2014).

In this scientific study the candidate proteins were chosen based on their role in facilitating the virus entry into the host cells and packaging of the viral particles. It was found that the protein molecules are inducers of host's immune response. This scientific study examined the structural and non-structural proteins of SARS-CoV-2 for vaccine designing, the prediction of its antigenic properties by VaxiJen ensured the candidate proteins ability to activate immune response. At the first stage of the immuno-informatics pipeline, the selected candidate proteins were subjected to MHC-I and MHC-II binding epitopes prediction. T-cells of the immune system easily detect MHC-I and MHC-II epitopes. MHC binding portion of the proteins are recognized by T cell receptors (TCRs) on the MHC molecules. MHC-I molecules exist on the surfaces of all nucleated cells of the body. MHC-I represents peptides to the cytotoxic T lymphocytes through the cytosolic pathway, these peptides are epitopes, either the antigenic parts of pathogens or endogenous peptides. MHC class II represents peptides to the helper T lymphocyte through the endocytic pathways, the peptides being antigenic parts of the pathogen surface. A vaccine was carefully constructed using suitable epitopes derived from the candidate proteins. The vaccine was subjected to a BLASTp algorithm to ensure no similarity with the human proteome in order to avoid triggering autoimmune response. The result shows the constructed vaccine did not show any resemblance to host cells proteins. This illustrates the argument that the selected polyproteins are perfect antigenic targets for research into the

development of a subunit vaccine against Covid-19 infection and subjected to further immuno-informatics tools for designing an effective vaccine.

A decent proportion of B and T cell epitopes are available in our final multi-epitope vaccine. online servers have verified the efficacy and safety of the vaccine (khan *et al*., 2019). The vaccine has a molecular weight of 39.5KDa, which is in the ideal range between a 30 and 60kDa for a vaccine protein. The vaccine has a 9.61 theoretical Pi that shows basic nature of the vaccine. Aliphatic index value representing the aliphatic side chains of the vaccine that are linked to thermal stability of a vaccine. Instability index score less than 40 ensures the vaccine is stable. The vaccine instability index was recorded to be 32 (Stable). Secondary and tertiary structure a protein defines protein's normal function, dynamics, interaction with other proteins and ligands. The secondary and tertiary structures for the final vaccine were generated by PSIPRED V3.3, SOPMA and Robetta, respectively. Procheck server was used to evaluate the vaccine's Ramachandran plot which revealed that most of the residues are in the most favorable region, showing that the tertiary structure generated is reliable. Cluspro server was used to determine the interaction between the vaccine and human toll like receptors i.e. TLR-3 & TLR-4. Jcat server was used to optimize the codons in order to boost the expression of vaccine protein in the E.coli system. The protein expression is directly linked with the CAI value and the GC content of the reverse translated optimized sequence. Both values show high expression of the vaccine(Khan *et al*., 2019). Model animals are usually used for the testing of newly developed vaccines before the approval for human trial but generally the vaccines are more efficient in model organisms than in human system. It is due to the complex nature of human's immune system. As a result, a safe, stable and relatively highly immunogenic vaccine against SARS COV-2 infection was developed in this research study using reliable immuno-informatics tools.

Conclusion

In this scientific study, by the use of immune-informatics approaches, we have constructed a multi-epitope subunit vaccine. This scientific work begins with the retrieval of four SARS CoV-2 Virus proteins (orf1ab, orf3a, membrane protein and surface protein) followed by prediction of immunogenic B-cell and T-cell epitopes for immune stimulation. Predicted epitopes were linked together using appropriate linkers and adjuvant to increase the immunogenicity of the effective epitopes. Allergenicity, antigenicity and physiochemical properties were analyzed to guarantee that the vaccine is reliable and safe for use. Molecular docking was performed to evaluate vaccine interactions with TLR-3 and TLR-4 human receptors. The vaccine construct was subjected to immune simulations which provide the information that the vaccine can successfully trigger cellular and humoral immunity. The last stage of this analysis was the optimization of codons for *E. coli* and reverse translation. The vaccine designed in this scientific study requires experimental validation in order to ensure its potential and safety. This study will help to control the current pandemic of Covid-19.

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