Acta Scientific Veterinary Sciences (ISSN: 2582-3183)

Volume 6 Issue 3 March 2024

Research Article

Comprehensive Epitope Prediction, Molecular Docking, and *In Silico* Vaccine Construction Targeting Monkeypox Virus

Mehnaj Khatoon, Madumitha B, Swati Rani, Yamini Sri S, Uma Bharathi I, Raaga R, Sagar N, Kuralayanapalya Puttahonnappa Suresh* and Sharanagouda S Patil

Spatial Epidemiology Lab, ICAR- National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru, Karnataka, India

***Corresponding Author:** Kuralayanapalya Puttahonnappa Suresh, Spatial Epidemiology Lab, ICAR- National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru, Karnataka, India.

DOI: 10.31080/ASVS.2024.06.0845

Received: January 31, 2024

Published: February 27, 2024

Abstract

A zoonotic Orthopoxvirus called Monkeypox Virus (MPXV) causes smallpox-like infections that affect people. Worldwide, the World Health Organization, abbreviated as WHO has been recorded to be growing in quantity of monkeypox (MPX) cases since May 2022. Currently, there are lots of problems with utilizing the smallpox vaccination to prevent monkeypox, and the treatment of infections with the disease is not clinically established. Considering this necessity and the rising number of cases of treatment resistance, creating a more potent and improved vaccination against the monkeypox virus is extremely desirable. In the present study, reverse vaccinology and several other bioinformatics and immunoinformatics tools were utilized to design multi-epitopes-based vaccine against MPXV by exploring four probable cell proteins like E8L, A28, COP-A44L and COP-B7R. The potential epitopes T-cell and B-cell were predicted from the proteins and connected with the Support of adjuvants and linkers. The predicted epitopes' physiochemical properties were evaluated, and only probable antigenic, non-allergic, and non-toxic epitopes were utilized in the multi-epitope vaccine design. The 3D structure of the designed vaccine is predicted, refined and validated for molecular docking with human immune receptor TLR4 demonstrated increased binding interaction. The designed vaccine construct was reverse transcribed and modified for E. coli strain K12 preceding inclusion inside pET28a (+) vector for its heterologous cloning and expression. Immunological responses were found to be enhanced by the IMMSIM server. In conclusion, multi-epitope vaccine candidates were created and their effectiveness has been verified. The strategy developed in this study could hold considerable importance effects on the early detection and therapy of infectious diseases brought on by the monkeypox virus.

Keywords: Immuninoformatics; Monkeypox Virus; Multi-Epitope Vaccine; Molecular Docking; In-sillico Clonning; Immune Simulation

Abbreviations

MPXV: Monkeypoxvirus; WHO: World Health Organization; E8L: IMV Surface Protein; A28: Carbonic Antiydrase; COP-A44L: Hydroxysteroid Dehydeogenase; CDC: Centers for Disease Control and Prevention; MEV: Multi-Epitope Vaccine; NCBI: National Center for Biotechnology Information; BLAST: Basic Local Alignment Search Tool; CTL: Cytotoxic T Lymphocyte; HTL: Helper T Lymphocyte; MHC: Major Histocompatibility Complex

Introduction

Monkeypox is a zoonotic infection caused by the monkeypox virus (MPXV), which is an enveloped virus with a brick-shaped structure. This virus belongs to the Poxviridae family, which consists of ancient viruses with linear double-stranded DNA genomes [1]. The first isolate of MPXV was discovered in a Denmark research facility in 1958, When monkeys transported from Singapore became ill, Following then, a several of outbreaks the death rate fluctuating between 1% to 10% have been reported in countries in Central

and West Africa as well as the United States of America [2]. MPXV has a linear double-stranded DNA genome, is brick-shaped, and is quite big (200–250 nanometers) with a lipoprotein envelope [3]. MPXVs divided into two clades that is Central African Congo Basin clade than the West African clade. More reports of Human-to-human transmission in the Central African Congo Basin clade [4]. In May 2022, the (WHO) World Health Organization reported cases of monkeypox virus (MPXV). Instances of MPX have been detected in various countries across different regions. Human-to-human transmission of MPXV is facilitated through close contact with lesions, bodily fluids, respiratory droplets, and contaminated objects, including bedding. There is a possible factor that may pose a risk in connection with consumption of undercooked meat and items originating from animals with infections [5]. The initial symptoms of monkeypox (MPX) encompass fatigue, headache, fever, myalgia, and lymphadenopathy, with the latter being a distinguishing factor from smallpox. Within 1-2 days, mucosal lesions emerge in the mouth, succeeded by centrifugally concentrated skin lesions on the face, hands, and feet. The rash has the potential to extend to other body parts, with the number of lesions ranging from a few to thousands. The incubation period for MPX typically spans 6-13 days, though it can extend up to 21 days [6]. In most cases, MPX resolves on its own, but in certain cases-children, pregnant women, and those with immunosuppressive disorders-it can become quite severe. A significant portion of cases, as reported by the World Health Organisation (WHO) in 2022 and Prevention and Control of Diseases (CDC, involve men who identify as homosexual [7] A considerable danger of infection exists, nevertheless, for all individuals enters intimate confrontation with an infected people [8].

In 1970, humans in the Democratic Republic of Congo were discovered to be infected by a DNA virus identified as the monkeypox virus [9]. The first isolate of MPXV was identified in 1958 when monkeys shipped from Singapore fell sick in a research facility in Denmark [10]. On September 1, 1970, a 9-month-old kid from the Republic of the Democratic Congo was discovered to possess the first monkeypox viral infection. Outside of Africa, Additionally, the monkeypox virus has linked to isolated outbreaks and human cases [11] Furthermore, there have been reports of human MPX instances and rare clusters. The midwestern United States reported the first MPX outbreak involving humans outside of Africa in 2003 resulting in more than 50 cases [12]. On October 4th, 2018, a Nigerian traveling to Israel reported Monkeypox [13]. In May 2021, Monkeypox was reported to be present in three members of a Nigerian family traveling to the United Kingdom. Only a single case existed. recorded. (from Nigeria to Texas) in July 2021[10].In November 2021, There was one incident recorded from from Nigeria to Maryland [14]. Here report on the identification and genomic characterization of the first two MPXV cases that arrived in India in July 2022 from the United Arab Emirates (UAE) [15]. There have been monkeypox reports in countries that are non-endemic since early May 2022, and the disease is still being reported in many nations that are endemic as well. (https://www.who.int/emergencies/situations/)monkeypox-oubreak-2022; (https://www.cdc.gov/poxvirus/monkeypox/response/2022/world-map.html). There have been speculations suggesting that the epidemic may be attributed to a virus originating in West Africa, which causes milder symptoms and is commonly transmitted from person to person. Investigations are underway to identify the sources of the disease, the patterns of viral transmission, and the epidemiology of the disease [16].

The monkeypox virus does not currently have a specific treatment approved by the FDA. There are numerous antiviral drugs available to treat smallpox and other conditions that may be helpful to patients with monkeypox. (https://www.niaid.nih.gov/diseases-conditions/monkeypox-treatmen). For the prevention of monkeypox, the CDC (Centers for Disease Control) and Prevention recommended the use of two vaccines: JYNNEOS, a replication-deficient vaccinia virus vaccine [17] and ACAM2000, a cell culture-based live vaccinia smallpox vaccine [18]. The efficacy of such vaccines against a widespread monkeypox virus outbreak is unknown, either. The CDC highlighted the absence of unified evidence on the clinical efficacy or effectiveness of JYNNEOS or ACAM2000 for monkeypox disease in its September 2022 update. The CDC suggested that individuals who have received these vaccinations continue to avoid close, skin-to-skin interaction with individuals afflicted by monkeypox due to the gaps in current information on the efficiency of these vaccines in the ongoing outbreak. On the other hand, several medications, such as tecovirimat, cidofovir, brincidofovir, or vaccinia immunoglobulin, may be used, while the level of success remains uncertain balance the risks and benefits for treating monkeypox infection.

An investigation of monkeypox infection disease in sound rhesus macaques indicates that defensive immune responses against monkeypox could be considered [19] The Dryvax vaccination ensures that macaques are protected against monkeypox [14]. Numerous reports have indicated that antibodies provide significant protection against monkeypox using the non-attenuated smallpox vaccination currently used [20]. These several negative effects, however, affected both people who received the immunization and individuals who had touch with them.

The proteins A28, E8L, COP-A44L, and COP-B7R were selected as They are necessary for the membrane fusion, recognizing host immunological responses, and receptor binding [21]. A28 is an envelope protein that helps in cellular entry and it stabilizes the immunogenic form that imitates viruses with an exposed portion of the entry-fusion complex. E8L a cell surface binding protein binds to the cell surface chondroitin sulfate, to provide viral particles attaching to the target cell [22]. COP-B7R is a protein that resides in the ER. It interacts with and maintains a constantly released or cell surface-produced protein necessary for the immunological response in the endoplasmic reticulum (ER). COP- A44L regulates the immune response by inhibiting the immune system, increasing steroid production, and diminishing virulence[12]. It is vitally necessary to develop therapeutic approaches to combat new monkeypox strains. Hence, it is essential to develop an efficient and effective vaccination against the monkeypox virus. The ability of the vaccine to stimulate an immunological reaction that happens faster than the virus itself is the fundamental aspect of all immunizations [23]. In both the pathogenesis of diseases and the defense against viral infections, the immune system is essential.

In vaccine development, immunodiagnostic development, and antibody production, immunoinformatics is an important part. It incorporates several algorithms that help predict highly potential epitopes for B-cells and T-cells that are essential to the creation of peptide vaccines. Multi-epitope-based vaccination is an emerging strategy for the prevention of pathogenic diseases [24]. The virus can be induced either by a cellular or a humoral reaction of the immune system when these substances contain the essential parts of the virus and reduce undesirable elements that could be harmful outcomes [25]. It is possible to develop highly effective vaccine candidates for clinical trials using multi-epitope vaccines if they provide the capacity for fight viral infections [23]. Particularly when compared to traditional vaccinations, the synthesis of vaccines using peptide-based is exceptionally safe and economical.

This study aims to design a multiepitope vaccine (MEV) against the Monkeypox virus using immunoinformatics tools. COP-A44L, A28, E8L, and COP-B7R proteins were selected and analyzed to forecast B- and T-cell epitopes, followed by the construction of MEVs. Immunological and physicochemical suitability of the vaccine protein, stability, flexibility, and binding affinity to TLR4 immunoreceptors were all evaluated thoroughly. Finally, the vaccine protein's translation efficiency was improved through in silico cloning with optimized codons.

Materials and Methods

Data collection

A sequence of four immunogenic proteins i.e, E8L, A28, COP-B7R, and COP-A44L from the monkeypox virus was retrieved from the NCBI (National Center for Biotechnology Information) (https://www.ncbi.nlm.nih.gov/). BLASTp tool was utalized to collect the homology sequences of the protein having the simalirity >98%. Later, ClustalW tool (https://www.ebi.ac.uk/Tools/services/web_clustalo/toolform.ebi) was used to align the complete sequences of monkeypox virus immunogenic proteins to create multiple sequence alignments.

Prediction and assessment of B-cell epitope

Surface-accessible groupings of amino acids known as B-cell epitopes are recognised by secretory antibodies. B-cell epitopes were identified by ABC pred server (http://crdd.osdd.net/raghava/abcpred/) with a default threshold of 0.51 for all four proteins. The ABCpred server uses an artificial neural network to predict conserved B cell epitope areas inside an antigen sequence. This server will assist in finding epitope regions that are useful in selecting potential synthetic vaccine candidates [26].

Prediction and assessment of t-cell epitopes

Prediction of CTL Epitope

The initial stage of initiating an immune response to viral infections is the presentation of antigen by MHC-I to the Cytotoxic T Lymphocyte (CTL). The CTL epitopes are restricted to 12 MHC class I major histocompatibility complex supertypes (HLA-A01:01, HLA-A02:01, HLA-A03:01, HLA-A24:02, HLA-A26:01, HLA B07:02,HLA-B08:01,HLA-B27:05, HLA-B39:01, HLA-B40:01, HLA-B58:01 and HLA-B15:01) [27]. were predicted using the NetMHCpan -4.1 server (https://services.healthtech.dtu.dk/services/NetMHCpan-4.1/) [28]. Utilizing artificial neural networks, the service predicts a peptide's affinity to bind to any MHC-I molecules of a given sequence based on its greatest prediction score and a threshold of % rank <0.5 for epitope prediction.

Prediction of HTL Epitope

By identifying MHC-II peptides formed from exterior environment-derived extraneous proteins, has a significant part in triggering humoral and cellular immune responses. The helper T lymphocyte (HTL) epitopes were therefore predicted using the Immune Epitope Database (IEDB) Analysis Resource (www.iedb.org/) MHC class II binding prediction algorithm with default values as this server is considered to be essential for the development of immunotherapeutic vaccines. The reference set for HLA class II was the default 7-allele (HLA-DRB1*03:01, HLA-DRB1*07:01, HLA-DRB1*15:01, HLA-DRB3*01:01, HLA-DRB3*02:02, HLA-DRB4*01:01, HLA-DRB5*01:01) set. In order to enhance effective innate and adaptive immunity, the HTL epitopes also support the IFN-γ response. IFN-γ is believed to have responses that are naturally secure by preventing the spread of viruses [29]. It also activates CTL and HTL against the virus, which stimulates a diverse im-

mune system. Thus, by using the IFN-epitope server (http://crdd.osdd.net/raghava/ifnepitope/), all HTL epitopes were exposed to IFN-γ producing qualities. Using motif consistent with the appropriate score and SVM hybrid algorithms, positive epitopes for IFN-γ inducers were selected for further analysis. As a result, there should be a stable, highly antigenic, non-allergic, non-toxic, and well-soluble candidate epitope.

Multi-epitope vaccines designing

To produce a multi-epitope vaccine structure, the most promising B-cell, HTL, and CTL epitopes were associated with suitable linkers. The adjuvant of choice was 50S ribosomal protein L7/L12 to boost the vaccine immunogenicity and connected to the vaccine's N-terminal sequence, adjacent to the B-cell epitopes by EAAAK linker, followed by KK linkers [30]. Classes I and II of MHC epitopes were linked together using AAY and GPGPG linkers, respectively.

Vaccine safety prediction: Physiochemical parameter analysis

The goal of vaccination is to stimulate the host's immune system. Therefore, a stable, highly antigenic, non-allergic, non-toxic, and well-soluble candidate vaccination should be present. The physicochemical characteristics were assessed via the ProtParam tool (https://web.expasy.org/protparam/). The various output parameters consist of the content of amino acids (aa), theoretical isoelectric point (pI), molecular weight, instability index (<40), estimated half-life in vitro and in vivo, stability profile, aliphatic index, as well as grand average of hydropathy [31]. The VaxiJen v2.0 (https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) tool was utilised to predict antigenicity because it showed the highest precision (70–89%). The virus model available at the server, at a threshold of 0.4, was utilized for higher accuracy. AllerTOP v2.0 (https://www.ddg-pharmfac.net/AllerTOP/) tool was used to screen for allergenicity which offers accuracy of 87.9% and 88.7%, respectively, at a set threshold of 0.4. Using the ToxinPred server (http://crdd.osdd.net/raghava/toxinpred/) and SOLpro (http://scratch.proteomics.ics.uci.edu), respectively, the toxicity and solubility tendency discrimination in *E. coli* following overexpression were calculated [32].

Prediction of tertiary structure, refinement, and vaccine construct of validation

The transform-restrained Rosetta tool (https://yanglab.nankai.edu.cn/trRosetta/) (trRosetta) was used to determine the vaccine's linear amino acid sequence's three-dimensional structure. The trRosetta is a web-based de novo technique that uses Rosetta direct energy minimization with deep learning to offer accurate protein structure prediction [33]. Additional refining was carried out utilising the GalaxyRefine webserver (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) The server enhances protein models by refining them according to their underlying protein structure. It produces diverse refined models that exhibit variations in GDT-HA, Poor Rotamers, RMSD, MolProbity, and Clash Score, and Rama Favoured [34]. To validate the overall model quality of the refined vaccine model, ProSA web server (https://prosa.services.came.sbg.ac.at/prosa.php) is used. Based on the refinement of the model, ProSA predicts a Z-score, which reflects model quality. A negative Z-score indicates that there are no errors in the model structure. ProSA checks the local model quality and the residue scores as well. The MolProbity web server (http://molprobity.biochem.duke.edu/) was employed to confirm the structures. It gives a Ramachandran plot of the protein structure, which can be used to assess the overall quality of the modelled structure [35].

Molecular docking of designed vaccine candidates with TLR4

TLR plays a part inside the natural defence mechanism when a viral infection occurs. Human immune toll-receptor TLR4 in particular has been shown to recognise the viral envelope protein [36] To develop vaccination candidates, 3D structure of TLR4 (PDB ID: O00206) was chosen as the target protein. The prediction of binding energy between TLR-4 and the vaccine construct was conducted using the ClusPro 2.0 server (https://cluspro.bu.edu/home.php) as it is a widely used protein-protein docking tool [37]. The experiment involved uploading PDB files of receptors and ligands to the server and submitting them with default parameters. The low binding energies of the complexes indicated the highest binding affinity between the vaccine construct and receptor.

In silico cloning optimization of MPXV multi-epitope vaccine candidates

The Java Codon Adaptation Tool (JCat) (http://www.jcat.de/) was used to calculate the GC content and codon adaptation index (CAI) value of the vaccine construct in *E. coli* (strain K12), as well as for reverse translation and codon optimization. Although GC content should be between 30 and 70%, CAI helps to offer codon information, and a score of > 0.8 is considered as desirable. Furthermore, SnapGene tool was used to carry out restriction enzyme cloning, confirming the vaccine's expression construct. *E. coli* plasmid pET-28a (+) vectors were utilized to clone the final vaccine constructions' optimized gene sequences [38].

Immune simulation

Utilising the C-ImmSim server (http://kraken.iac.rm.cnr.it/C-IMMSIM/), immune response induction verified the vaccine's efficacy [39]. To detect immunological epitopes and immune interactions, this server employs a position-specific scoring matrix (PSSM). Throughout the trial, all of the default settings were used.

Results

Protein sequence retrieval

The wild-type protein sequence of the monkeypox virus was retrieved from the(NCBI) National Center for Biotechnology Information server using GenBank ID: AF380138.1 (https://www.ncbi.nlm.nih.gov/nuccore/AF380138.1) of the strain Monkeypox virus Zaire-96-1-16. Four proteins E8L (Accession ID: NP_536532.1), A28(Accession ID: QNI40000.1), COP-B7R (Accession ID: YP_010377164.1), COP-A44L (Accession ID: NP_536582.1) were selected having better immunogenic potential with the prediction score of 0.5311, 0.6212, 0.4395, 0.4098 by vaxijen respectively. In the NCBI BLASTp server, homologous strains for all four proteins of the monkeypox virus were identified. Protein sets of E8L (11 sequences), COP-A44L (6 sequences), COP-B7R (7 sequences), and A28 (4 sequences) for every protein produced after BLASTp search with NCBI BLAST tools. To obtain areas that are conserved , The technique of multiple sequence alignment was employed ClustalW tool. There was a total number of 6, 5, 2, and 2 conserved epitopes among E8L, A28, COP-B7R, and COP-A44L, respectively provided in Table 1.

Sl. No	Protein	Conserved epitopes
1.	E8L	MPQQLSPINIETKKAISD TLKTLDIHYNESKPTTIQNTG LVRINFKGGYISGGFLPNE QKIVNQLDSIRSANMSAPFDSVFYLDNLLP-STLDYFTYLGTTINHSADA CFSYYQK YIEGNKTFAIIAIVFVFILTILFLMSYSREKQN
2.	A28	MNSLSIFFIVVATAAVCLLFIQSYSIYENYGNIKEF-NATHAAFEYSKSIGGTPALDRRVQ DVDISDVKQKWRCVVYPGNGF SASIFGFQAEVGPNN SIRKENTMRQCIDFTFSDV INIDIYNPCIAPNINNTECQFLKSVL
3.	COP-B7R	MYKKTFLFVIGAVASYSNNEYTPFNK HTLKIGFTYHG
4.	COP-A44L	MAVYAVTGGAGFLGRYIVKLLISADDVQEIRVIDI-VEDPQPIT KVKVINYIQCDINDFD KVR

Table 1: Conserved sequence of monekypox virus selected gene (E8L, A28, COP-B7R, COP-A44L) obtained from ClustalW tool.

Linear B-cell epitope prediction

Based on the higher set threshold of 0.51 and the chosen protein consensus sequences, a total of 17 possible epitopes were examined using the ABCpred service. Following the epitope validation with immunogenicity features, three promising B-cell epitopes from A28 and one each from E8L, COP-B7R, and COP-A44L were predicted (Table 2).

S. No	MPXV-proteins	B-cell epitopes	ABCpred Score
1.	E8L	KTFAIIAIVFVFILTI	0.79
2.	A28	SLSIFFIVVATAAVCL VDISDVKQKWRCVVYP RKENTMRQCIDFTFSD	0.65 0.71 0.72
3.	COP-A44L	KVKVINYIQCDINDFD	0.71
4.	COP-B7R	FVIGAVASYSNNEYTP	0.85

Table 2: B-cell predicted epitopes using the conserved regions from the ABCpred server of E8L, A28, COP-B7R and COP-A44L of MPXV proteins.

Prediction and assessment of T-cell epitopes

Prediction of CTL epitope

In order to select epitopes in this study, the following criteria were established: their conservation among proteins should be 100%, their binding affinity should be the highest possible, They shouldn't cross over the human proteins and have significant immunogenicity. According to all these specifications, some promising epitopes were recognized in this study. The CTL epitopes of the protein's conserved sequences under study were identified through the NetMHCpan4.1 service. The server supports CTL epitope prediction on 12 HLA- alleles, including-A01:01, HLA-A02:01, HLA-A03:01, HLA-A24:02, HLA-A26:01, HLA-B07:02, HLA-B08:01, HLA-B27:05, HLA-B39:01, HLA-B40:01, HLA-B58:01, HLA-B15:01. Based on their low percentile rank and high prediction score compared to the HLA alleles, the epitopes were predicted. Ten potential epitopes were examined, of which five are distinct, strong-bound (thresholds 0.500) from A28 protein, one from E8L, one from COP-B7R, and three from COP-B7R protein (Table 3). To develop a vaccination that works, the epitopes with the best binding ratings for each targeted allele were selected.

Prediction of CTL epitope

Using the IEDB server, to predict HTL epitopes the default 7-allele HLA class II reference set. Each protein's final epitope measured 15 mers in length, and it was choosen for additional analysis based on percent rank and higher binding scores. Once cytokine production was further examined, all the selected HTL epitopes showed signs of IFN-γ stimulation, making them perfect applicants for creating vaccines. The predicted epitopes from each protein, Epitope prediction using the IFN-γ software yielded positive results from E8L and A28 proteins, while no predictions were obtained from the COP-B7R and COP-A44L proteins. Additionally, for vaccine construction, positive epitopes were collectively identified along with their interacting alleles and scores, are displayed in table 4.

Protein	Selected epitopes	Interacting Alleles	%Rank score
E8L	TFAIIAIVF	HLA-A*24:02	0.809
A28	ATHAAFEYSK	HLA-A*03:01	0.361
	KEFNATHAAF	HLA-A*24:02	0.979
		HLA-B*40:01	0.214
		HLA-B*40:01	0.466
	ATAAVCLLF	HLA-B*58:01	0.328
	VDISDVKQKW	HLA-B*08:01	0.569
		HLA-B*58:01	0.889
		HLA-B*58:01	1.439
	SIFGFQAEV	HLA-A*02:01	0.09
COP-A44L	VTGGAGFLGRY	HLA-A*01:01	0.494
		HLA-A*24:02	
		HLA-A*26:01	
COP-B7R	VASYSNNEY	HLA-A*01:01	0.454
	FVIGAVASY	HLA-A*26:01	0.044
		HLA-A*26:01	0.019
		HLA-A*26:01	0.054
		HLA-B*15:01	0.03
		HLA-B*15:01	0.735
		HLA-B*15:01	0.087
	HTLKIGFTY	HLA-A*01:01	0.482
		HLA-A*26:01	0.347
		HLA-B*58:01	0.253
		HLA-B*15:01	2.001
		HLA-B*15:01	0.46

Table 3: Highly conserved, Antigenic, non-allergenic, and non-toxic MHC class I predicted epitopes in MPXV.

MPXV Proteins	Epitope	Interacting allele	% Rank score	IFN-γ
E8L	DYFTYLGTTINHSAD	HLA-DRB3*02:02	0.0662	Positive
		HLA-DRB1*15:01	0.1759	
		HLA-DRB1*07:01	0.187	
		HLA-DRB3*02:02	0.0538	
		HLA-DRB1*15:01	0.1292	
A28	HAAFEYSKSIGGTPA	HLA-DRB1*07:01	0.9208	Positive
		HLA-DRB5*01:01	0.5616	
	DVKQKWRCVVYPGNG	HLA-DRB3*02:02	0.0167	Positive
		HLA-DRB1*03:01	0.0228	
	ISDVKQKWRCVVYPG	HLA-DRB1*03:01	0.0051	Positive
	SASIFGFQAEVGPNN	HLA-DRB1*15:01	0.7789	Positive

Table 4: Highly conserved, Antigenic, non-allergenic, and non-toxic MHC class II predicted epitopes in MPXV.

Multi-epitope vaccines designing

While creating a multi-epitope vaccine, we ultimately opted for CTL, HTL, and B cell linear epitopes, which were interconnected using EAAAK, KK, AAY, and GPGPG linkers, respectively. Typically, an adjuvant is necessary to boost the immune-stimulating potential of the selected epitopes. In this case, the adjuvant (50S ribosomal protein L7/L12) was tentatively incorporated via the EAAAK connector, nevertheless the B and HTL epitopes were joined together using GPGPG linkers, respectively (Figure 1). The final vaccine formulations contained 446 amino acid residues.

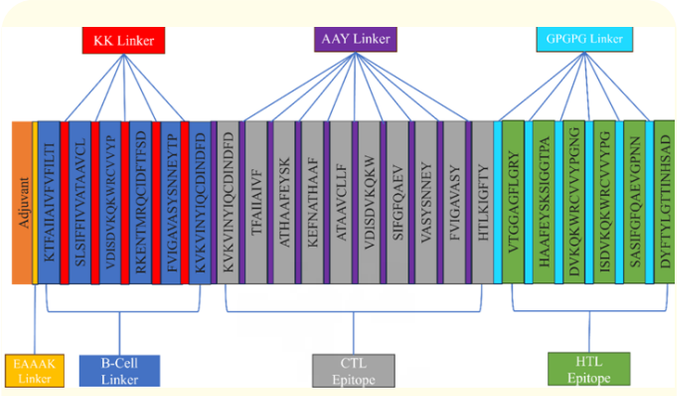

Figure 1: The final multi-epitope vaccination peptide is shown schematically. The 466-amino acid long peptide sequence containing adjuvant (orange) at the N terminal was linked with the multi-epitope sequence through an EAAAK linker (yellow). B cell epitopes are linked with KK linkers (red) and CTL epitopes are linked using AAY linkers (purple) while the HTL epitopes are linked with GPGPG linkers (sky blue).

The constructed vaccines were also investigated for their physicochemical properties, allergenicity, and the antigenicity which are displayed in table 5.

Physicochemical characterization

The developed vaccine has been shown to be nontoxic, nonallergenic, and naturally antigenic to the host. With the VaxiJen server's default threshold of 0.4%, the antigenic score was 0.707. The vaccine that was created has aweight of molecular level approximately 49 kDa, which indicates that it is highly antigenic and simple to purify. The produced vaccine can be produced on a large scale and is easily purifiable due to its low molecular weight of less than 110 kDa. The peptide's basic nature is indicated by its pI value of 7.95. Assuming that all cysteine residues are reduced, the extinction coefficient at 0.1% absorption was 59250. The protein has a half-life of 30 hours in mammalian reticulocytes (in vitro), over 20 hours in yeast (in vivo), and more than 10 hours in Escherichia coli (in vivo). These results suggest that the protein can be exposed for

Sl.No	Physicochemical property	MPXV-Vaccine
1.	Molecular weight	49586.87
2.	GRAVY score	0.146
3.	Theoretical PI	7.95
4.	Aliphatic Index	84.94
5.	Instability index	14.93
6.	Protein half-lives SOLpro	0.96

Table 5: Physicochemical properties of vaccine construct.

prolonged periods and potentially stimulate the host's immune system. Furthermore, the calculated instability index of 14.93 indicates the stability of the vaccination protein. The grand average of hydropathicity (GRAVY) of -0.146 and aliphatic index of 84.94 both indicated high thermostability and hydrophilicity properties. The SOLpro website states that protein was soluble at overexpression, with a probability of 0.96. Overall, the outcomes indicated that this construct could be a potential vaccine candidate as shown in Table 5. However, additional experimental study is necessary to confirm the veracity of these results.

Prediction of tertiary structure, refinement and validation of vaccine construct

The resulting 3D structure of the vaccine antigenic peptide was constructed using the transform-restrained rosetta tool. The modelled protein's consistency was improved by using the Galaxy Refine web server. The great quality of the anticipated structure was accomplished by energy minimization and loop refining. Five model structures were developed when the initial "crude" vaccine model was improved with the use of the Galaxy Refine web service. The refined models underwent validation through Ramachandran plots. In terms of structural quality across all constructed entities, Model 3 stood out as the most significant from the Vaccine considering various factors. i.e., GDT-HA (0.9614), RMSD (0.392), and Mol Probity (2.510). The clash value was 3.2, the low rotamers value was 0.9, and Rama favored value was 98.1. ProSA webserver to analyze the protein structure and validate the model. The model's total quality was evaluated using a Z-score value of -1.32.

Molecular docking

The TLR4 immune cell receptor is essential for producing effective immune responses (Figure 3). were generated Crystal structure using Discovery study visualizer (PDB ID: O00206). vaccine construct's interaction with the ligand-binding domain of the immunological receptor TLR4 (Figure 4) was predicted through molecular docking. This involved employing the Cluspro2.0 online protein–protein docking server to facilitate the analysis which is specifically designed for protein-protein docking. Parallel inspections of multiple models can be carried out simultaneously through

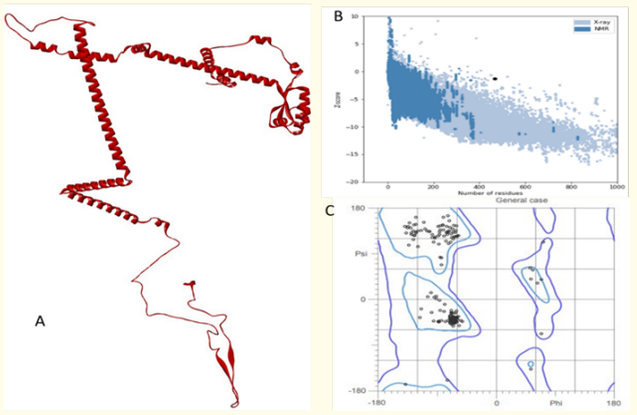

Figure 2: Demonstration of the 3D model's structural refinement, quality evaluation, and validation of the vaccine build. (A) The tertiary structure of the improved construct shows a helix, strand, and random coil (B) The ProsA Z-score (C) A Ramachandran plot of the improved model.

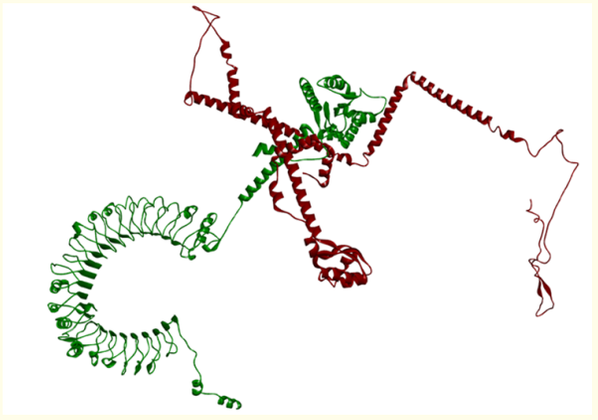

Figure 3: Crystal structure of TLR4 receptor generated using Discovery study visualizer.

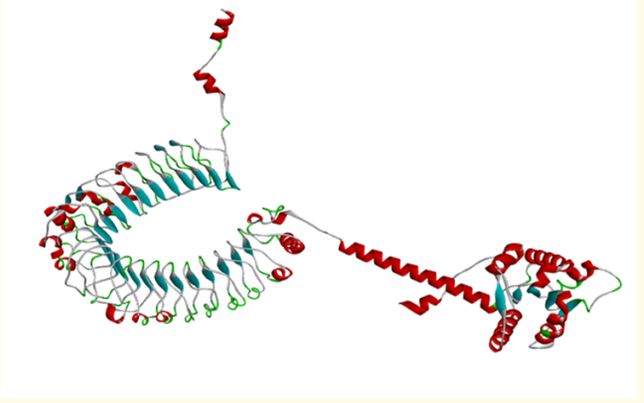

Figure 4: The docked complex of vaccine model and the TLR-4 immune receptor.

docking. The models exhibiting the lowest binding energy score was chosen, indicating strong binding affinity. Model 1 emerged as the optimal docked complex, being distinguished by the lowest energy criterion value of -1418.8. Based on these results, this MEV stands out as the most promising vaccination candidate as seen in (Figure 4).

Codon optimization and *In-silico* cloning

The sequences of the designed vaccine constructs were subjected to codon optimization using the JCat web server. The CAI value for the constructs were predicted to be ~0.97, the average GC content of the adapted sequences was ~48.99% indicating an acceptable range for higher expression of the designed vaccine in the *E. coli* host. SnapGene software was used to construct a recombinant plasmid sequence by introducing the adapted codon sequence of the final vaccine construct XhoI and XbaI, two limited endonucleases, are located at both ends of the vaccine construction into the plasmid vector pET28a (+), thereby ensuring cloning and expression in the *E.coli* system (Figure 5).

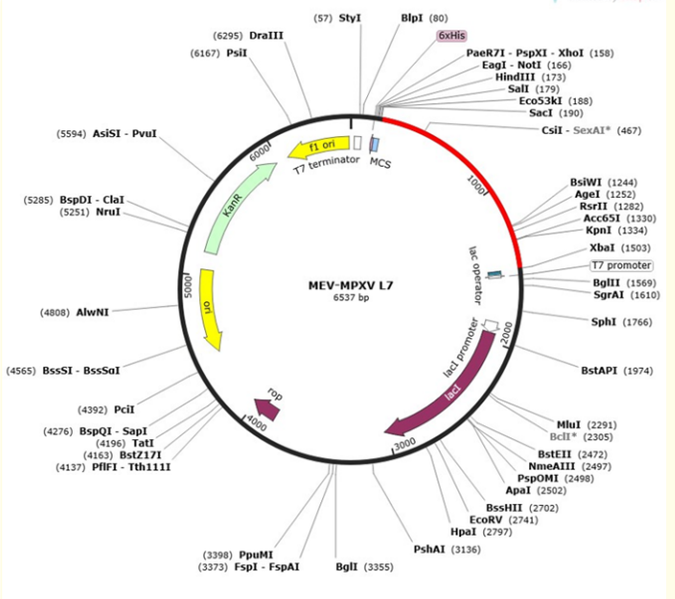

Figure 5: MPXV-V1 vaccine using pET28a (+) vector.

Immune simulation

The C-ImmSim server was utilized in order to conduct the immunological simulation. This shows a similar immunological response to a genuine immune response. The first reaction was characterized by an increase in IgM+IgG levels, which was followed by rises in IgM and IgG1+IgG2 levels, respectively. (Figure 6a) Significant numbers of B-cells were present in both the secondary and tertiary stages of the immunological response (Figure 6b) Furthermore, the results showed that Memory cells develop after additional exposure. Additionally, there was a rise in the quantity of helper (TH) cells (as seen in Figure 6c) as well as cytokines (Figure 6d).

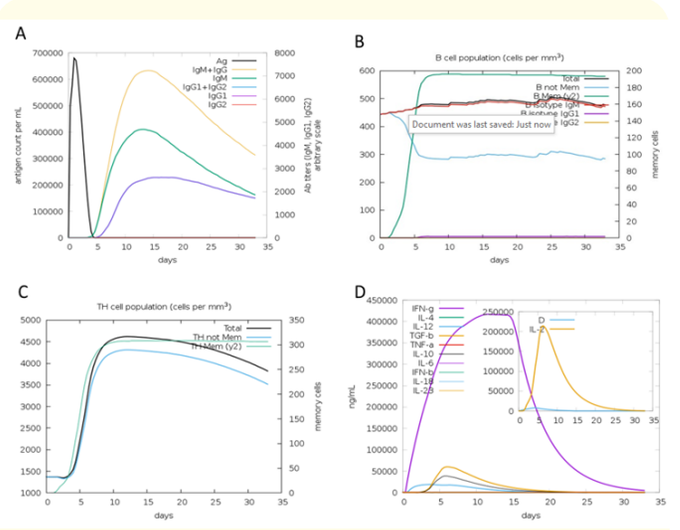

Figure 6: Immune simulation of multi-epitope vaccine: (a) immunoglobulin production on subsequent injection of antigens (shown by black lines); coloured lines indicative of immune cells class; (b) shows changes in B-cell population and memory formation; (c) production of helper T-cells; (d) elevated rates of cytokines and interleukins for effective immune response.

Discussion

The Orthopoxvirus genus and Poxviridae family comprise the pathogen that causes monkeypox. It was first noted in May 2022 that there was a current global outbreak in places where monkeypox is not endemic. The initial genome sequence of the MPXV linked to the ongoing outbreak was announced by Portugal on May 19 and verified that it is a member of the West African clade [40]. As reported cases of human monkeypox increase globally, prevention of the epidemic monkeypox virus is challenging. The MPXV vaccines that are approved provide only moderate prevention, particularly for children and those with underlying health issues [9]. Therefore, therapeutic methods are necessary to treat monkeypox virus infections that are on the upsurge. Reverse vaccinology developments and the availability of genetic and proteomic data have aided in vaccine development. Additionally, the use of modern bioinformatics tools is preferable than to use conventional methods [41]. The production of effective vaccinations with high potency, logistical viability, and better safety is made possible by epitope-based vaccines, a revolutionary therapeutic strategy. Multi-epitope vaccines have the ability to produce targeted immunogenic reactions based on conserved epitopes in entire antigenic sequences, avoiding responses against unfavourable epitopes that could trig-

ger immunopathogenic or immune-modulating reactions against the host [42]. Currently, there is no known treatment for monkeypox, consequently the only preventive measure is vaccination. The goal of this research was to develop multi-epitope MPXV vaccine design using immunoinformatics technique that might trigger immunogenic reactions in infected people. For the purpose of locating T-cell and B-cell epitopes, four membrane proteins of monkeypox virus were retrieved based on properties such antigenic behaviour, non-allergenic and non-toxic nature, and virulence capabilities, to recognize B-cell and T-cell epitopes. Utilizing this technique, vaccine developers can assess vaccine candidates suitable for experimental analysis [43].

Adaptive immunity is enhanced by T-cell epitopes (MHC-I and MHC-II). Viruses and contaminated cells are extracted from the host by MHC-I epitopes, which generate durable immunity, whereas MHC-II epitopes are in charge of triggering both cellular and humoral immune responses [44]. These epitopes stimulate CD4+ helper T cells, which leads to CD8+ T cell memory and activation of B-cell. As a result, the multi-epitope vaccine construct included the anticipated B- and T-cell epitopes of the vaccine. Using a range of linkers AAY, KK, EAAAK and adjuvant peptide sequences derived from 50S ribosomal protein L7/L12 (UniProt ID: P9WHE3). For the designed multi-epitope constructs, Vaxijen v2.0 determined high antigenicity values. All constructed vaccine designs were non-toxic and non-allergen. Its potential as a candidate for a vaccine is increased by these immunological qualities. Its immunological characteristics make it more likely to be a viable vaccine candidate. Physicochemical characteristics from the predicted vaccine constructs were also analyzed. The predicted vaccine construct's Physicochemical characteristics have been examined employing ExPASy Protparam and SOLpro, and the findings showed that it has a high level of stability and solubility. In order to comprehend interactions between antigens and receptor molecules, structural information is essential for vaccine development [30].

Examining the interaction between antigens and receptor molecules is essential for advancing vaccine development by gaining insights into the virus's structure. The trRosetta server was applied to forecast the vaccine's three-dimensional structures construct, which were further improved by the Galaxy Refine server [36]. The resulting enhanced three-dimensional structural analysis affirmed the stability of the intended configuration. Additionally, employing the Mol Probity server revealed that the refined vaccine construct exhibited the majority of residues in the favorable region of the Ramachandran plot [45]. ProSA webserver prediction was used to validate the high-quality predicted structures of the multi-epitope vaccine constructs. The molecular weights of the vaccine constructs were within the intended range (<20 kDa). These structures are highly soluble and extremely stable when expressed, according to their physicochemical characteristics. This is one of the process's initial stages [46]. To achieve this, it is essential to express the recombinant protein in a suitable host. *E. coli*-based expression systems are well-suited for the production of recombinant proteins [47]. To achieve a heightened expression of our recombinant vaccine protein in *E. coli* K12, we conducted codon optimization beforehand. The codon adaptability index of 0.97 and a GC content of 48.99 percent indicated the potential for substantial protein expression in bacteria. The designed vaccine constructs will be highly stable upon expression, according to the projected instability scores, which increases the potential of the vaccine. A critical step in validating a designed vaccine [46]. which must be expressed in an appropriate expression system, is indeed the confirmation of immunoreactivity based on serological analysis. The development of recombinant peptides is supposed to be better achieved using the *E. coli* expression system.

To determine whether designed vaccines bind to TLR4 immune cell receptors, a molecular docking analysis was conducted. In innate immunity, TLR receptors play a significant role in activating immune cells to generate adaptive immunity responses. There is evidence that TLR4 recognizes viral peptide structures and induces the release of inflammatory cytokines due to its recognition of viral peptide structures [48]. The vaccine constructs demonstrated strong binding affinities with the receptor protein's active site by employing molecular docking analysis. The level of immunogenicity of the vaccine influences its ability to generate stable immune responses. Docking poses, atom interactions, and binding free energies were used to select the best, most stable, and most effective vaccine candidate [49]. The vaccine complex exhibited strong molecular interactions with immune receptors, thereby maintaining its molecular stability within the cell. Therefore, the vaccine construct developed able to produce high gene expression in this study and strong immunological responses. Findings from the immunological simulation were consistent with reactions commonly observed in the immune system. Following repeated exposure to the antigen, there was a notable increase in the overall number of immune responses. Memorization of B-cell generation was visible. There was also the production of helper T-cells and memory T-cells. Significantly higher levels of IL-2 were observed following the first injection [50]. Using immunoinformatics techniques in silico will be helpful in the development of future laboratory assays, thereby saving time and money. The following stage is to conduct in vitro immunological assays to validate the developed vaccine, assess the immunogenicity of the multi-epitope vaccine construct, and develop a challenge-protection preclinical trial to confirm the results of this study.

Conclusion

The emergence of the monkeypox virus poses a significant and concerning threat. Utilizing immune informatics strategies, a multiepitope vaccine has been developed, recognizing the advantages of a peptide-based approach. To enhance the vaccine's efficacy, both T-cell and B-cell epitopes derived from the MPXV protein has been added to the vaccine construct. Positive responses to our vaccination are anticipated, including humoral and cell-mediated immune responses. The human TLR4 receptor and vaccine protein were discovered to have a stable and sustained binding potential and interaction. Immune responses that were effective were observed during the immunological. Its potential to effectively combat the monkeypox virus, however, will need to be determined by additional in vitro and in vivo research. The engineered protein sequence of the vaccine is ready for synthesis to facilitate expression studies. Upon successful validation of the expression studies, the vaccine construct, once isolated and purified, can be employed for preclinical and clinical investigations.

Disclosure Statement

The authors report there are no competing interests to declare.

- **Consent for publication:** Not applicable.
- **Competing interests:** The author (s) declare that they have no Competing interests.
- **Acknowledgement:** We would like to thank DBT-One Health project and DBT-ADMaC project. Special thank to Spatial Epidemiology lab, ICAR- National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI) for assistance in carrying out this research.
- **Authors Contribution:** Investigation, formal analysis, data curation, visualization, writing original draft preparation, writing and review, and editing: M.K ,M.B; Conceptualization, methodology, validation, visualization, writing-review, editing and supervision: S.R, Y.S.S, R.R, S.N, S.S. P; Resource, visualization, Conceptualization, and methodology U.B.I Supervision, resource and reviewing: K.P.S.
- **Informed Consent Statement:** N/A.
- **Ethical Statement**: The authors have no ethical statement applicable to this work.
- **Conflict of Interest:** The authors declare that they have no conflict of interest in this study.

Bibliography

1. KN Durski., *et al.* "Emergence of monkeypox in West Africa and Central Africa, 1970-2017/Emergence de l'orthopoxvirose simienne en Afrique de l'Ouest et en Afrique centrale, 1970-2017". *The Weekly Epidemiological Record* 93 (2018): 125.
2. OJ Peter., *et al.* "Transmission dynamics of Monkeypox virus: a mathematical modelling approach". *Modeling Earth Systems and Environment* 8.3 (2022): 3423-3434.
3. A Moodley., *et al.* "Reverse vaccinology approach to design a multi-epitope vaccine construct based on the Mycobacterium tuberculosis biomarker PE_PGRS17". *Immunologic Research* 70.4 (2022): 501-517.
4. N Sklenovská and M Van Ranst. "Emergence of Monkeypox as the Most Important Orthopoxvirus Infection in Humans". *Frontiers in Public Health* (2018): 1-12.
5. R Grant., *et al.* "Modelling human-to-human transmission of monkeypox". *Bull. World Health Organ* 98.9 (2020): 638-640.
6. ME Wilson., *et al.* "Human monkeypox". *Clinical Infectious Diseases* 58.2 (2014): 260-267.
7. M Naveed., *et al.* "Design of a novel multiple epitope-based vaccine: An immunoinformatics approach to combat SARS-CoV-2 strains". *Journal of Infection and Public Health* 14.7 (2021): 938-946.
8. SY Ren., *et al.* "Monkeypox outbreak: Why is it a public health emergency of international concern? What can we do to control it?". *World Journal of Clinical Cases* 10.30 (2022): 10873-10881.
9. ID Ladnyj., *et al.* "A human infection caused by monkeypox virus in Basankusu Territory, Democratic Republic of the Congo". *Bulletin of the World Health Organization* 46.5 (1972): 593-597.
10. AK Rao., *et al.* "Monkeypox in a Traveler Returning from Nigeria-Dallas, Texas, July 2021". *MMWR. Morbidity and Mortality Weekly Report* 71.14 (2022): 509-516.
11. T Kaever., *et al.* "Potent Neutralization of Vaccinia Virus by Divergent Murine Antibodies Targeting a Common Site of Vulnerability in L1 Protein". *Journal of Virology* 88.19 (2014): 11339-11355.
12. P Jeerapat. "Monkeypox: From Past to Present Outbreak". *International Journal of Current Science and Research* 05.07 (2022): 2454-2461.
13. E Hammarlund., *et al.* "Multiple diagnostic techniques identify previously vaccinated individuals with protective immunity against monkeypox". *Nature Medicine* 11.9 (2005): 1005-1011.
14. PL Earl., *et al.* "Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox". *Nature* 428.6979 (2004): 182-185.

15. PD Yadav., *et al.* "First two cases of Monkeypox virus infection in travellers returned from UAE to India, July 2022". *Journal of Infection* 85.5 (2022): e145-e148.

16. MUG Kraemer., *et al.* "Tracking the 2022 monkeypox outbreak with epidemiological data in real-time". *The Lancet Infectious Diseases* 22.7 (2022): 941-942.

17. DM Pastula and KL Tyler. "An Overview of Monkeypox Virus and Its Neuroinvasive Potential". *Annals of Neurology* 92.4 (2022): 527-531.

18. J Myers and M Maresh. "Clinical Management". in *Maternal Obesity and Pregnancy*, Berlin, Heidelberg: Springer Berlin Heidelberg (2012): 43-62.

19. JW Hooper., *et al.* "Smallpox DNA Vaccine Protects Nonhuman Primates against Lethal Monkeypox". *Journal of Virology* 78.9 (2004): 4433-4443.

20. Y Edghill-Smith., *et al.* "Modeling a Safer Smallpox Vaccination Regimen, for Human Immunodeficiency Virus Type 1-Infected Patients, in Immunocompromised Macaques". *The Journal of Infectious Diseases* 188.8 (2003): 1181-1191.

21. SF Hoque., *et al.* "Scrutinizing surface glycoproteins and poxin-schlafen protein to design a heterologous recombinant vaccine against monkeypox virus". *bioRxiv* (2020).

22. Y Wang., *et al.* "Immunogenic proteins and potential delivery platforms for mpox virus vaccine development: A rapid review". *International Journal of Biological Macromolecules* 245 (2023): 125515.

23. Z Yang., *et al.* "An *In silico* deep learning approach to multi-epitope vaccine design: a SARS-CoV-2 case study". *Scientific Reports* 11.1 (2021): 1-21.

24. S Aiman., *et al.* "Core genome mediated potential vaccine targets prioritization against Clostridium difficile via reverse vaccinology - an immuno-informatics approach". *Journal of Biological Research* 29 (2022).

25. P Zhou., *et al.* "Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin". *Nature* 556.7700 (2018): 255-259.

26. S Saha and GPS Raghava. "Prediction of continuous B-cell epitopes in an antigen using recurrent neural network". *Proteins: Structure, Function, and Genetics* 65.1 (2006): 40-48.

27. N Akhtar., *et al.* "Immunoinformatics-Aided Design of a Peptide Based Multiepitope Vaccine Targeting Glycoproteins and Membrane Proteins against Monkeypox Virus". *Viruses* 14.11 (2022): 2374.

28. B Reynisson., *et al.* "NetMHCpan-4.1 and NetMHCIIpan-4.0: Improved predictions of MHC antigen presentation by concurrent motif deconvolution and integration of MS MHC eluted ligand data". *Nucleic Acids Research* 48.W1 (2019): W449-W454.

29. S Fuse., *et al.* "Immune Responses Against Persistent Viral Infections: Possible Avenues for Immunotherapeutic Interventions". *Critical Reviews in Immunology* 28.2 (2008): 159-183.

30. K Bhattacharya., *et al.* "Multi-Epitope Vaccine Design against Monkeypox Virus via Reverse Vaccinology Method Exploiting Immunoinformatic and Bioinformatic Approaches". *Vaccines* 10.12 (2022).

31. MR Wilkins., *et al.* "The ExPASy Server 531 53 531 Protein Identification and Analysis Tools in the ExPASy Server". *Methods in Molecular Biology* 112 (1999): 531-552.

32. S Gupta., *et al.* "*In silico* Approach for Predicting Toxicity of Peptides and Proteins". *PLoS One* 8.9 (2013).

33. Z Du., *et al.* "The trRosetta server for fast and accurate protein structure prediction". *Nature Research* (2021): 5634-5651.

34. Ç Yılmaz Çolak. "Computational Design of a Multi-epitope Vaccine Against Clostridium chauvoei: An Immunoinformatics Approach". *International Journal of Peptide Research and Therapeutics* 27.4 (2021): 2639-2649.

35. ACB Antonelli., *et al.* "*In silico* construction of a multiepitope Zika virus vaccine using immunoinformatics tools". *Scientific Reports* 12.1 (2022): 1-20.

36. S Aiman., *et al.* "Multi-epitope chimeric vaccine design against emerging Monkeypox virus via reverse vaccinology techniques- a bioinformatics and immunoinformatics approach". *Frontiers in Immunology* (2022): 1-17.

37. D Kozakov., *et al.* "The ClusPro web server for protein-protein docking". *Nature Protocols* 12.2 (2017): 255-278.

38. A Sarker., *et al.* "Evaluation of scFv protein recovery from E. coli by in vitro refolding and mild solubilization process". *Microbial Cell Factories* 18.1 (2019): 1-12.

39. V Lennerz., *et al.* "Immunologic response to the survivin-derived multi-epitope vaccine EMD640744 in patients with advanced solid tumors". *Cancer Immunology, Immunotherapy* 63.4 (2014): 381-394.

40. C Veintimilla., *et al.* "The relevance of multiple clinical specimens in the diagnosis of monkeypox virus, Spain, June 2022". *Eurosurveillance* 27.33 (2021): 1-5.

41. Y Jiao., *et al.* "PABPC4 Broadly Inhibits Coronavirus Replication by Degrading Nucleocapsid Protein through Selective Autophagy". *Microbiology Spectrum* 9.2 (2021).

42. WY Zhou., *et al.* "Therapeutic efficacy of a multi-epitope vaccine against Helicobacter pylori infection in BALB/c mice model". *Vaccine* 27.36 (2019): 5013-5019.

43. K Naz., *et al.* "PanRV: Pangenome-reverse vaccinology approach for identifications of potential vaccine candidates in microbial pangenome". *BMC Bioinformatics* 20.1 (2019): 1-10.

44. Sunita N Singhvi., *et al.* "Computational approaches in epitope design using DNA binding proteins as vaccine candidate in Mycobacterium tuberculosis". *Infection, Genetics and Evolution* 83 (2020): 104357.

45. N Khatoon., *et al.* "Exploring Leishmania secretory proteins to design B and T cell multi-epitope subunit vaccine using immunoinformatics approach". *Scientific Reports* 7.1 (2017): 1-12.

46. A Gori., *et al.* "Peptides for immunological purposes: Design, strategies and applications". *Amino Acids* 45.2 (2013): 257-268.

47. R Chen. "Bacterial expression systems for recombinant protein production: *E. coli* and beyond". *Biotechnology Advances* 30.5 (2012): 1102-1107.

48. T Compton., *et al.* "Human Cytomegalovirus Activates Inflammatory Cytokine Responses via CD14 and Toll-Like Receptor 2". *Journal of Virology* 77.8 (2003): 4588-4596.

49. V Chauhan., *et al.* "Designing a multi-epitope based vaccine to combat Kaposi Sarcoma utilizing immunoinformatics approach". *Scientific Reports* 9.1 (2019): 1-15.

50. S Arumugam and P Varamballi. "*In-silico* design of envelope based multi-epitope vaccine candidate against Kyasanur forest disease virus". *Scientific Reports* 11.1 (2021): 1-15.