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Design of a multi-epitope peptide vaccine candidate against chandipura virus: an immuno-informatics study

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ABSTRACT

Chandipura virus (CHPV) is an emerging pathogen responsible for acute encephalitic syndrome (AES) in pediatric population in India. Several outbreaks of CHPV have been reported from different states of India since the year 2003. At present there is no vaccine or therapeutic measures available to curtail the disease. In this study, we have identified both T-cell and B-cell epitopes of different antigenic proteins of CHPV like Nucleoprotein (N), Phosphoprotein (P) and Matrix protein (M) along with the immuno-dominant glycoprotein (G) and conducted *in silico* characterization for the same. The idea is to design a multi-epitope peptide construct using the epitopes, which were found to be non-toxic, non-allergenic and possessing high immunogenicity. The final multi-epitope construct named as: MEC-CHPV, comprised of β-defensin adjuvant at N-terminal for enhancement of immunogenicity followed by fourteen B-cell epitopes, four Helper T-cell epitopes and six Cytotoxic T-cell epitopes. The characterization of designed construct was carried out in terms of physicochemical parameters, antigenicity and allergenicity. The 3D structure prediction was performed. Molecular docking and molecular-dynamics simulation of MEC-CHPV with Toll like receptors (TLR-3 and TLR-8) showed stable interactions. *In silico* cloning of MEC-CHPV in pET30a(+) expression vector was also conducted using codon optimization. The *in silico* immune-simulation indicated a typical immune response against MEC-CHPV when used as a potential vaccine. This study provides a cost-effective and time-saving way to design a peptide vaccine candidate against CHPV using immuno-informatics approach. Development of the MEC-CHPV construct may pave the way for future laboratory experiments.

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KEYWORDS
Chandipura virus; multi-epitope construct; molecular docking; vaccine; in silico cloning; molecular dynamics

1. Introduction

Chandipura virus (CHPV), an arbovirus belongs to the genus *Vesiculovirus* of the family *Rhabdoviridae*. The virus is primarily associated with an encephalitic illness in the pediatric population, clinically characterized by high-grade fever of short duration, vomiting, altered sensorium, generalized convulsions and decerebrate posture leading to grade IV coma and death within 48 hrs of hospitalization (Rao et al., 2004). The virus was isolated for the first time from human serum sample during the year 1965 from Maharashtra, India (Bhatt & Rodrigues, 1967). In the year 2003, CHPV was found to be responsible for a large encephalitis outbreak with case fatality rate as high as 78% in children in many districts of Andhra Pradesh, India (Rao et al., 2004). Thereafter, the CHPV activity is being continuously reported from Gujarat, Maharashtra, Andhra Pradesh and Telangana states (Sudeep et al., 2016). The disease is transmitted by sand flies (Tesh & Modi, 1983). The virus has also been isolated from a hedgehog and Phlebotomine sand flies from Nigeria and Senegal from West Africa (Ba et al., 1999; Fontenille et al., 1994; Kemp, 1975). Apart from this, the presence of anti-CHPV antibodies was detected in monkeys from Sri Lanka (Peiris et al., 1993).

Chandipura virus is a negative sense, SS RNA virus with an approximate genome length of ~11 kb. Viral genome encodes five structural proteins Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M), Glycoprotein (G) and Large Protein (L). The N protein encapsidates viral RNA into a nuclease resistant forms protecting it from cellular RNAses. L and P protein together form viral RNA dependent RNA polymerase complex; where catalytic functions for RNA polymerization, capping and Poly-A polymerase resides within the L protein while, P protein acts as a transcriptional activator (Banerjee, 1987). Matrix protein lies on the inner surface of virion in between viral membrane and encapsidated genome RNA and is mainly associated with interaction with host-cell machinery. Glycoprotein G is an envelope glycoprotein which acts as a major antigenic determinant.

Although, experimental work to develop effective vaccine against CHPV was carried out earlier i.e. attempts to develop inactivated and sub-unit (Jadi et al., 2011; Venkateswarlu & Arankalle, 2010), at present no vaccine against CHPV is available in the market. Currently, in the post genomic era, computational based vaccine designing approaches are gaining importance on account of availability of dependable immuno-informatics tools (Nandy & Basak, 2016). The multi-

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epitope vaccine (MEV) consisting of highly antigenic epitopes (B-cell, HTL, and CTL epitopes) derived from the proteins, which are highly pathogenic and aid in the induction of potent humoral and cell-mediated immune responses. Similar approach has been used to develop next generation vaccine candidate against different viral agents (Adhikari et al., 2018; Azim et al., 2019; Salvador et al., 2019). In this study, we have used different immuno-informatics approaches to design an immunogenic multi-epitope peptide vaccine candidate against CHPV using its N, P, M and G proteins, considering the role of individual proteins in survival of the virus. The utilization of different structural proteins and careful selection of viral peptides may lead to development of a robust construct to elicit relevant and specific humoral as well as an efficient cell-mediated immune response against CHPV.

2. Methods

2.1. Sequence retrieval

FASTA formatted amino acid sequences of CHPV proteins were retrieved from the GenBank NCBI (National Center for Biotechnology Information). Twenty Nucleoprotein, eighteen phosphoprotein, twelve matrix protein and fourteen glycoprotein sequences were used for antigenicity analysis as well as for studying epitope conservancy.

2.2. Multiple sequence alignment

The immunogenic proteins of CHPV were aligned using MEGA 6.0 software with ClustalW alignment tool (Tamura et al., 2013). This was used to study the conserved region in different CHPV proteins.

2.3. Epitope prediction

All structural proteins of CHPV, except polymerase, were firstly subjected to evaluation of their antigenic potential using VaxiJen 2.0 server available at http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html (Doytchinova & Flower, 2007). The antigenic proteins of CHPV i.e. N, P, M and G protein were used for prediction of potential B and T-cell epitopes. Different tools available at the Immune Epitope Database (IEDB) analysis resource (http://www.iedb.org/) were used to analyze the immunogenic proteins. The reference sequence of CHPV G protein (GenBank accession number-ADO63668), P protein (GenBank accession number-ADO63666), M protein (GenBank accession number- ADO63667) and N protein (GenBank accession number-ADO63665) was used as an input in IEDB tools. The predicted epitopes were analyzed for conservancy using epitope conservancy tool available online (Bui et al., 2007).

2.3.1. B-cell epitope prediction

The regions of antigenic molecules which are recognized by B-cell receptor to further elicit immunoglobulin response are termed as B-cell epitopes. The computational prediction of B-cell epitopes is mainly characterized by their surface accessibility and their antigenic reactivity. Different epitope prediction tools for stringent selection of B-cell epitopes available at Immune Epitope Database (IEDB) at http://tools.iedb.org/bcell/ were used for this purpose. Linear B-cell epitopes were predicted by Kolaskar and Tongaonkar antigenicity method (Kolaskar & Tongaonkar, 1990) to identify the antigenic determinants on proteins based on the physicochemical properties of amino acid residues as well as by BepiPred 2.0 method, (Jespersen et al., 2017) predictions of which were based on a random forest algorithm trained on epitopes from antibody-antigen protein structures. The B-cell epitopes shared by these two prediction criteria's were further evaluated regarding allergenicity and toxicity using AllerTop (https://www.ddg-pharmfac.net/AllerTOP/) and Toxinpred (http://crdd.osdd.net/raghava/toxinpred/) online tools respectively for defining final B-cell epitopes.

2.3.2. T cell epitope prediction

2.3.2.1. Prediction of CTL (Cytotoxic T-cell) i.e. MHC class I epitopes

During year 2003, while investigating Chandipura outbreak from Andhra Pradesh, host genetics studies were carried out on CHPV infected individuals and racially matched controls. This indicated associations of HLA-A*03, HLA-A*69:01 and HLA-A*26 alleles with CHPV infection (NIV, unpublished data). These MHC class I alleles were further utilized for MHC-I binding predictions by the IEDB analysis resource (IEDB; http://www.iedb.org/) using reference protein sequence from 2003 outbreak strain 034627 using IEDB recommended method (Moutaftsi et al., 2006) with peptide length of nine amino acids. The predicted epitopes were primarily shortlisted with percentile rank of $\leq$5 for predicting peptides with high binding affinity. The peptides detected by all the three alleles under consideration were analyzed for immunogenicity using IEDB MHC class I immunogenicity prediction tool and epitope conservancy analysis for selection of most conserved immunogenic epitopes.

2.3.2.2. Prediction of HTL (Helper T-cell) i.e. MHC class II epitopes

Based on the data from Allele Frequency Net Database online repository available at (http://www.allelefrequencies.net/pops.asp), the most prevalent HLA class II alleles from the geographical areas affected by CHPV (2003 onwards) were considered for prediction of HTL epitopes. Andhra Pradesh-Telangana region located in southern India had the following dominant HLA-DRB1 alleles: HLA-DRB1-04, HLA-DRB1-07 and HLA-DRB1-15 (Dedhia et al., 2015), whereas Gujarat region had: HLA- DRB1-03 HLA-DRB1-11, HLA DRB1-13 and HLA-DRB1-15 alleles (Singh et al., 2012). MHC Class II peptides were predicted on N, P, M and G protein sequences using MHC class II epitope prediction tool (Wang et al., 2008) available at IEDB. The epitopes which were having binding potential for greater than or equal to six alleles out of eight alleles which were used for the prediction (Eight alleles

used for the prediction of epitopes were: HLA-DRB1*03:01, HLA-DRB1*04:01, HLA-DRB1*04:05, HLA-DRB1*07:01, HLA-DRB1*09:01, HLA-DRB1*11:01, HLA-DRB1*13:02 and HLA-DRB1*15:01) were identified and analyzed for their immunogenicity potential using CD4 T-cell immunogenicity prediction tool (http://tools.iedb.org/CD4episcore/), available at IEDB using IEDB recommended method, which combines the predictions from MHC binding (using 7-allele method) and immunogenicity method. The immunogenic epitopes were further screened for their ability as potential inducers of interleukin 4 (IL-4) secretion, typical of Th2 cells, this was done using IL4pred, an *In Silico* Platform for Designing and Discovering of Interleukin-4 inducing peptides developed by Dhanda et al., available at (https://webs.iiitd.edu.in/raghava/il4pred/index.php), using Hybrid method (Dhanda et al., 2013).

2.4. Design of multi-epitope construct and 3D structure prediction, structure refinement and validation

Predicted B and T-cell epitopes were used for designing of a multi-epitope construct (MEC). The arrangement of the B and T-cell epitopes after the N-terminal β-defensin adjuvant was done using different linkers as EAAAK, KK, GGGGS and AAY was done for construction of effective immunogenic molecule. The 'EAAAK' linker is a rigid linker and known to facilitate the separation of bi-functional fusion proteins (Arai et al., 2001) thus, used to link adjuvant with first B-cell epitope. Further, the B-cell epitopes were joined together using bi-lysine (KK) linker to preserve their independent immunogenic activities (Gu et al., 2017). The 'GGGGS' and 'AAY' linkers were used to merge the HTL and the CTL epitopes, respectively to enable the efficient recognition of epitopes within the MEC.

The construct was further subjected to prediction of 3D structure using RaptorX server available at (http://raptorx.uchicago.edu/StructPredV2/predict/) (Peng & Xu, 2011) followed by refinement with ModRefiner server available at https://zhanglab.ccmb.med.umich.edu/ModRefiner/ (Xu & Zhang, 2011). Validation of the predicted 3D structure of the MEC was carried out through Ramachandran plot analyses as implemented in at PROCHECK (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html) as well as RAMPAGE server available at (http://www-cryst.bioc.cam.ac.uk/rampage) (Lovell et al., 2003). The final validated construct was named as MEC-CHPV.

2.5. Allergenicity, antigenicity prediction and physicochemical characterization of designed construct

The probable non-allergenic behavior of MEC-CHPV was predicted using AllerTop v.2.0 and the antigenic nature was predicted using VaxiJen v2.0 server. The Physicochemical characterization of MEC-CHPV was carried out using ProtParam tool from Expasy's server available at https://web.expasy.org/protparam/.

2.6. Protein-protein docking and molecular dynamics simulations

To determine the binding affinity of MEC-CHPV with TLR-3 and TLR-8 immune receptor, molecular docking with PatchDock server available at https://bioinfo3d.cs.tau.ac.il/PatchDock/php.php (Schneidman-Duhovny et al., 2005) was implemented. The 3D structure of human TLR-3 (PDB ID-2A0Z) and TLR-8 (PDB ID-3W3G) receptor was retrieved from RCSB protein data bank. Ten top ranking complexes in terms of electrostatic interactions and free binding energy were further refined using FireDock server http://bioinfo3d.cs.tau.ac.il/FireDock/php.php (Mashiach et al., 2008).

To evaluate the stability of the MEC-CHPV complexed with TLR-3 and TLR-8, molecular dynamics simulations were performed using GROMACS-2020.2 molecular dynamics package (Abraham et al., 2015). Molecular dynamics analyzed the bonded as well as the non-bonded interactions between the protein molecules using the OPLS-AA/L all atom force field (Kaminski et al., 2001). System was equilibrated and charge neutralization was carried out by addition of suitable ions as per protocols established. The system pressure and temperature were equilibrated at 1 bar and 300°K for equilibration period of 100 ps respectively. In each case, the MD simulations were set for 10 ns and RMSD were determined to obtain a stable protein-protein complex (Shukla et al., 2018).

The PROtein binDIng enerGY prediction (PRODIGY) protocol online services (https://bianca.science.uu.nl/prodigy/) to estimate the binding affinity of our vaccine construct (MEC-CHPV) with TLR-3 as well as with TLR-8. The program calculates binding affinity (Protein-protein) in terms of ΔG (Xue et al., 2016). All calculations were performed using the recommended default parameters for the software.

2.7. Codon optimization and in silico cloning

To facilitate the cloning and maximal protein expression of the MEC-CHPV using bacterial vector, E. coli strain, K12 was selected. The codon optimized construct sequence was generated as cloning insert for pET-30a(+) vector under *Nde*I and *Xho*I restriction sites at N and C terminal respectively. The reverse translation and codon optimization of the construct was carried out using Java Codon adaptation Tool (JCat) server available at http://www.jcat.de/ (Grote et al., 2005). The codon optimized sequence with restriction sites was inserted into the pET-30a(+) vector using DNA construct design tool named as GenSmart available at https://www.genscript.com/gensmart-design/ to ensure the expression of the MEC-CHPV.

2.8. Immune simulation

In order to characterize the immunogenicity and immune response profile of MEC-CHPV, *in silico* immune simulation was carried out using the C-ImmSim server available at http://150.146.2.1/C-IMMSIM/index.php. It is an agent-based computational immune response simulator that utilizes position-specific score matrix (PSSM) and machine learning

methods for predicting epitope and immune interactions, respectively (Rapin et al., 2010). It describes both humoral and cellular response of a mammalian immune system against vaccine construct. The minimum recommended time interval between vaccine doses is four weeks for most of the currently used vaccines (Castiglione et al., 2012). Therefore, three injections, with a dose of 1000 vaccine proteins each, containing no LPS (random seed = 12345) were administered four weeks apart at 1, 84 and 168 time-steps (wherein, first dose is injected at time = 0 and each time-step is equivalent to 8 h in real-life). The simulation was conducted for 1050 simulation steps. Rests of the simulation parameters were kept default.

2.9. Study design

Chandipura virus proteins N, P, M and G from strain CHPV-034627 (Human, 2003 outbreak) were considered for the study. B and T-cell epitopes were predicted for all the proteins using bioinformatics tools. MHC class I epitopes were primarily selected based on their ability to get recognized by alleles (HLA-A*03, HLA-A*69:01 and HLA-A*26) susceptible for CHPV infection, whereas, MHC class II epitope prediction was carried out using most prevalent alleles (HLA-DRB1 alleles: HLA- DRB1-03, HLA-DRB1-04, HLA-DRB1-07 HLA-DRB1-11, HLA DRB1-13 and HLA-DRB1-15) in CHPV endemic region. The predicted epitopes were further analysed for allergenicity and toxicity using different *in silico* tools. The selected B and T-cell epitopes were linked together to form a multi-epitope protein (MEC) with immunological adjuvant at the N-terminal end. 3D structure of designed construct was predicted and validated. The validated multi-epitope construct was named as MEC-CHPV. Analysis of different physicochemical properties of MEC-CHPV was carried out. It was further analyzed for its binding with human immune-receptors (TLR-3 and TLR-8) using PatchDock and FireDock molecular docking protocols. Molecular dynamics simulations were performed to study the stability of the MEC-CHPV when complexed with TLR-3 and TLR-8 separately. In order to facilitate the bulk expression of MEC-CHPV, the *in silico* cloning was carried out using codon optimized sequence and *E. coli*, K-12 strain as host in pET-30a(+) vector. Finally, the immunogenicity and immune response profiling of MEC-CHPV was studied using C-ImmSim, an *in silico* immune simulation tool (Figure 1).

3. Results

3.1. Antigenicity prediction of CHPV proteins

The evaluation of different structural proteins of CHPV by VaxiJen 2.0 online tool revealed the antigenic potential of these proteins with antigenicity scores for N protein: 0.4770, P-protein: 0.5082, M protein: 0.5076 and G protein: 0.5118 with a threshold value of 0.4 for viral antigens.

3.2. Prediction of B-cell epitopes

Linear B-cell epitopes for CHPV proteins were predicted using Kolaskar and Tongaonkar method and BepiPred 2.0 method. Threshold of 1.035 for Kolaskar and Tongaonkar method and 0.5 for BepiPred 2.0 method was considered for selection. The epitopes predicted by both methods were selected for further analysis of allergenicity and toxicity. This lead to identification of four B-cell epitopes *(4-QVFCISTG-11, 13-TVSVCLPA-20, 49-LSLLRSHVYDG-59 and 374-RNVVVWLA-381)* in N Protein, two in P protein *(120- TLSVPQNLSALQLLQ-134 and 148-GCRLLQIS-155)* three in M protein *(58-PSLGIQTLKLQYKCVVNIN-76, 128-DRGVVEYH-135 and 141-RALVFHSLGPS-151)* which were 100% conserved. In case of the G protein, of the five B-cell epitopes, three namely, *143-SEFLVIMITPHHVGVD-158, 184-NSSVWIP-190 and 457-KNPVELVTG-465* were 100% conserved and the rest *221-GAIVFKS-227and 321-LSPLDLSYLAS-331* were >85% conserved among the G-protein sequences from 1965 to 2014 (Supplementary Table 1).

3.3. Prediction of CTL epitopes

The prediction of MHC class I epitopes were performed using allelic preference for HLA-A*03:01, HLA-A*26:01 and HLA-A*69:01 with stringent percentile cut-off ≤ 5. In case of overlapping peptides identified by different alleles, the contiguous sequence was considered as an epitope. Selection criterion for the most probable MHC class I epitope included: i) identification/recognition by all the three alleles (mentioned above) and ii) positive immunogenicity score and high conservancy. These criteria lead to the identification of two contiguous epitopes (*245- EVFTWVFNK-253 and 329- TTAALLFAYAIGR −341*) in the N protein, one epitope (*159- ETFTREWNILTNK-171*) in the M protein and three epitopes (*8-SVILLISFILISFITPSY-20, 86-VTTCDFRWYGPK-97 and 482- LVVVLIYGVLR-492*) in the G protein (Supplementary Table 2).

3.4. Prediction of HTL epitopes

The predicted HTL epitopes using alleles prevalent in the susceptible population from Gujarat and Andhra Pradesh/Telangana region were shortlisted on the basis of stringent percentile cut-off ≤ 5. For overlapping peptides, identified by more than six out of eight alleles taken for prediction, the contiguous sequence was considered as an HTL epitope. The selected epitopes were further screened for immunogenicity potential and conservancy. This lead to the short-listing of four contiguous epitopes:

134- WLPTLIFGLYRVSRATQVEYKKTL-157,
184- NAWANDSNFTKIVAAVDMYFHHFKKSDHAPIRFG-217,
243- IEEVFTWVFNKSVQD DLLRMMTPGQEIDQA-272 and
284-
LSTKSPYSSTKNPSFHFWGQLTAFLVKSARAKNALVPVDIAYHELTTAAL-LFAYAI-339, in the N protein. Three epitopes identified in the M protein were found to be probable allergens; no epitopes were identified for P and G protein having ability to get

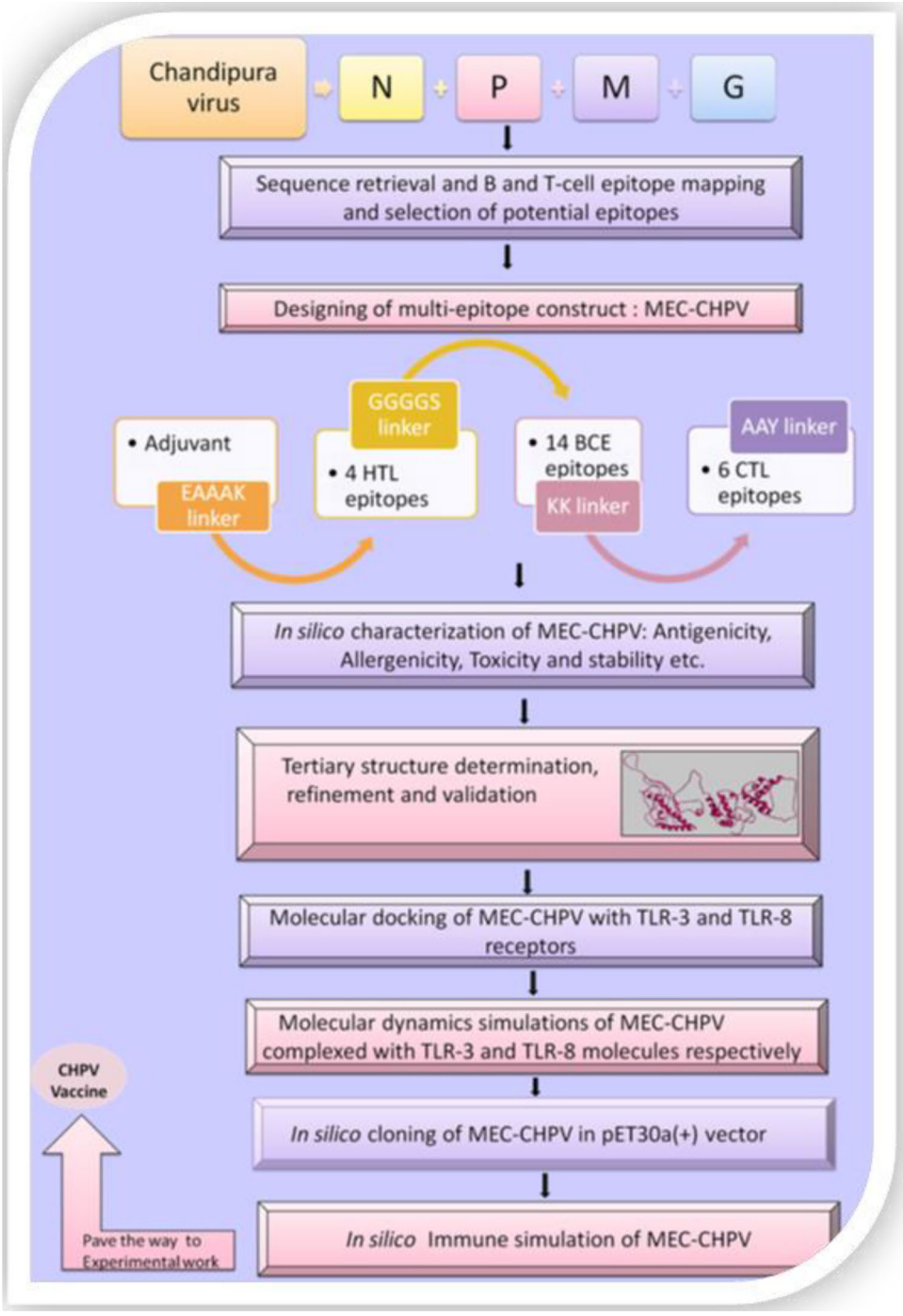

Figure 1. Schematic representation of immuno-informatics workflow used for designing the multi-epitope vaccine candidate exploring different CHPV proteins.

recognized by six or more alleles considered in the study (Supplementary Table 3).

3.5. Multi-epitope construct

The MEC-CHPV consists of 479 amino acid derived from 24 merged peptide sequences (Supplementary file: 1) comprising of 14 B-cell epitopes, 4 HTL epitopes and 6 CTL epitopes and the N-terminal β-defensin adjuvant. The N terminal β defensin adjuvant is followed by 'EAAAK' linker followed by the HTL epitopes separated by 'GGGGS' linkers followed by the B-cell epitopes separated by 'KK' linkers and then the CTL epitopes separated by 'AAY' linkers.

3.5.1. Allergenicity, antigenicity and physicochemical characterization of the multi-epitope construct

The MEC-CHPV was found to be non-allergenic, non-toxic with antigenicity score of 0.6248 (>0.54, cut-off for viral antigen) as estimated by the Vaxijen server. The physicochemical characterization reveals the molecular weight to be 53.13 kDa with the theoretical isoelectric point (PI): 9.98. This

indicates the basic nature of the protein. The estimated half-life was 30 h in mammalian reticulocytes, *in vitro*, >20 h in yeast, and >10 h *E. coli*, *in vivo*. The instability index (II) was predicted to be 36.38, (less than the cut-off 40) indicating that the construct is stable. The grand average of hydropathicity (GRAVY) was predicted as −0.033. The negative GRAVY value indicates hydrophilic nature of the protein.

3.5.2. 3D structure prediction, refinement and validation of multi-epitope construct

Tertiary structure prediction of the MEC-CHPV construct, was carried out using RaptorX server, with all residues being modeled (with a P-value $= 4.26 \times 10^{-8}$). Models with P-value $<10^{-3}$ indicate the goodness of prediction (Peng & Xu, 2011). Other model parameters like, overall uGDT (unnormalized global distance test) score was 293. The uGDT score: >50 shows good model quality. This 3D model was further refined using ModRefiner server (Figure 2). The validation of refined structure was carried out by Ramachandran plot analysis using RAMPAGE and PROCHECK servers. The RAMPAGE analyses revealed that the MEC-CHPV model is stable and valid (92.5% occupancy in the favoured region, 6.9% residues in the allowed region of Ramachandran plot. The PROCHECK results indicated that 81.4% residues lies in the favoured region, 17.2% and 0.5% in the additional and generously allowed regions of the Ramachandran plot respectively. Both analyses reveal good model quality. The minimized energy of the refined tertiary structure of MEC-CHPV was calculated using SPDBV server and was found to be −10,308 kJ/mol (Figure 3).

3.6. Molecular docking

Molecular docking of immune receptor (Human TLR-3: PDB ID-2A0Z and TLR-8: PDB ID-3W3G) was carried out with the MEC-CHPV using PatchDock server. Top twenty solutions were submitted to FireDock for refinement, the FireDock refinement resulted in ten best complexes. The top ranking docking poses generated by FireDock were further analyzed for binding affinities using the PRODIGY (PROtein binDIng enerGY prediction) web server available at https://bianca.science.uu.nl/prodigy/ (Xue et al., 2016). The results of molecular docking studies between MEC-CHPV and TLR molecules revealed that the proposed peptide construct has high affinity to both TLR-3 and TLR-8. The best docked complex with TLR-3 showed docking score of 18756 (PatchDock score) and binding affinity of $\Delta G = -33.8$ kcal/mol (PRODIGY). This complex also had 23 intermolecular H-bonds. The docked complex of MEC-CHPV with TLR-8, showed a docking score of 19066 (PatchDock) and binding affinity of $\Delta G = -11.3$ kcal/mol (PRODIGY). This complex showed 8 intermolecular H-bonds. Figure 4(A, B), displays the best complex of MEC-CHPV with TLR-3 and TLR-8 respectively.

3.7. Molecular dynamics simulations of the docked complex

For the evaluation of stability of the docked complexes of the MEC-CHPV with TLR-3 and TLR-8, molecular dynamics simulation was performed in GROMACS-2020.2. The pressure and temperature were equilibrated by applying NPT and NVT ensembles with a period of 100 ps. For MEC-CHPV complexed with TLR-3, the minimized potential energy was estimated to be -9.968×10^6 kJ/mol. The RMSD plot for the complex is shown in Figure 5. This indicated an initial deviation of 0.2 nm gradually increased with time till 8 ns and then becomes steady and stable at RMSD ∼ 0.85 nm until 10 ns. The RMSF plot (Figure 5(B)) indicated fluctuations in the MEC-CHPV covering the amino acid stretch from 220 to 240 and 460 to 479 (C-terminal). This indicated the flexibility and stability of the docked complex.

For the MEC-CHPV complexed with TLR-8, the minimized energy was found to be -8.9774×10^6 kJ/mol. The RMSD plot for the complex is shown in Figure 6(A). This indicated an initial value of 0.2 nm which gradually increased with time till 7 ns and then became steady and stable at RMSD ∼ 0.8 nm until 10 ns. The RMSF plot (Figure 6(B)) showed maximum fluctuation of the MEC-CHPV covering the amino acid stretch 220 to 240 and then at the C-terminal: 460–479. Findings indicate that the complex is stable.

3.8. Codon optimization and in silico cloning

Amino acid sequence of MEC_CHPV (479 AA) was reverse translated and codon optimized for *E. coli* (strain K12) using JCat server. A 1437 nucleotide sequence was obtained. The codon adaptation index (CAI) of optimized sequence was 0.964 with 48.57% GC content indicating possibility of good expression in bacterial strain K12. The desirable CAI should be >0.8 and GC% should lie between 30-70% to obtain a good expression level (Sharp & Li, 1987). The pET30a (+) expression vector was used to design the recombinant plasmid by inserting 1437 nucleotide sequence with *Nde*I restriction site at N terminal and *Xho*I at C terminal. The resultant recombinant vector was 6693 bp in length. Figure 7 gives a visual representation of the recombinant vector.

3.9. Immune simulation

I*n silico* immune response of the MEC-CHPV was generated by C-ImmSim immune simulator. The immune simulation results indicate a typical immune response (Figure 8). The primary response was identified by high levels of IgM. Secondary and tertiary responses were marked by increase in levels of IgG1 + IgG2, IgM and IgM + IgG antibodies with a corresponding decrease in antigen concentration (Figure 8(A)). Observations on the cytokine levels showed rise in interferon-γ and IL-2 levels after the first injection and retained peaks following repeated exposures to the antigen (Figure 8(B)). The inset in Figure 8(B) indicated the rise in IL-2 levels and the Simpson index D, which is a measure of

Figure 2. Refined tertiary structure of MEC-CHPV.

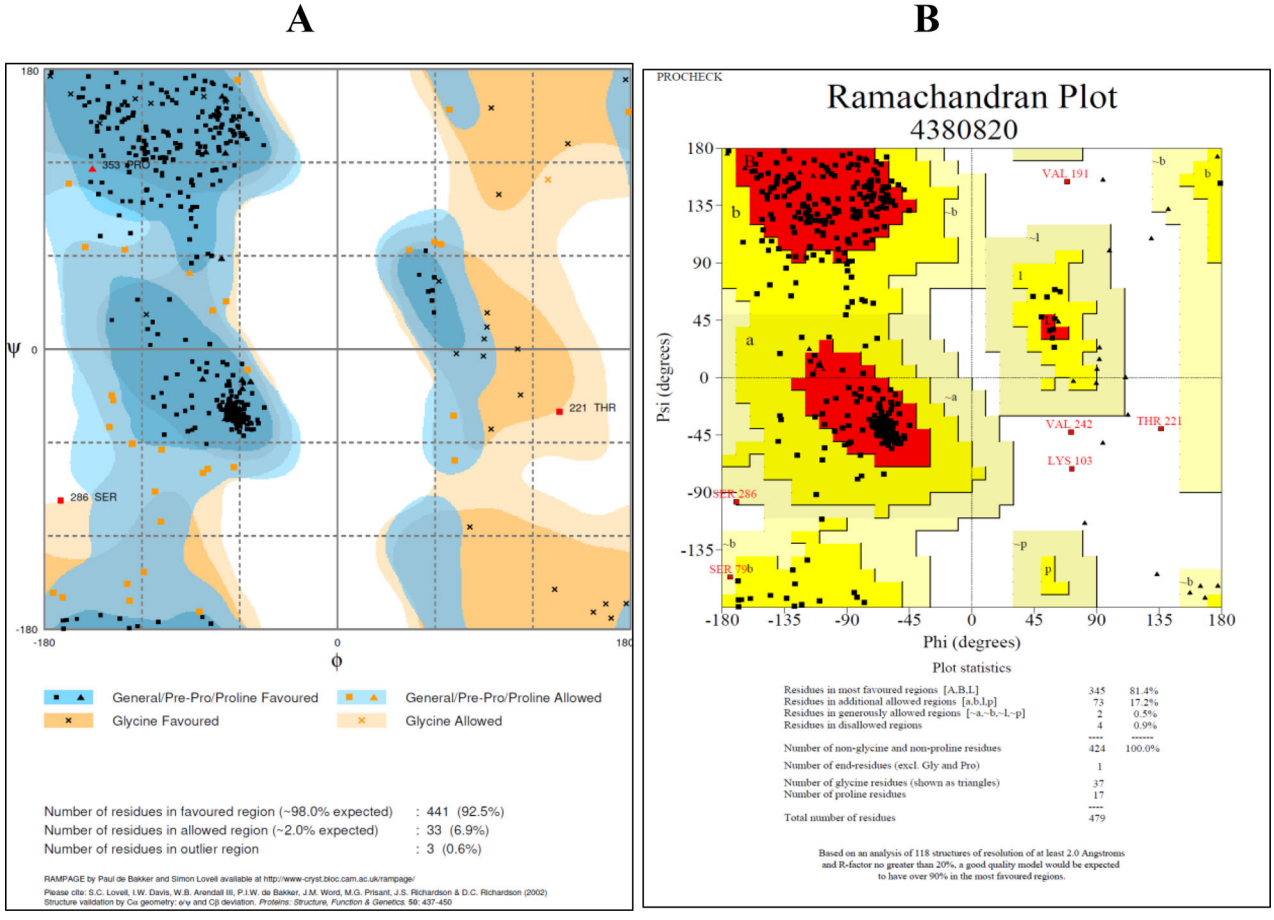

Figure 3. Ramachandran plot assessment using (A) RAMPAGE and (B) PROCHECK servers for validation of tertiary structure of MEC-CHPV.

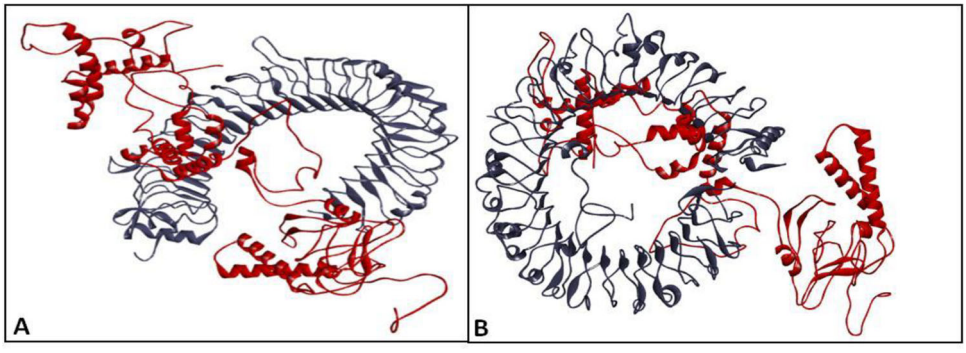

Figure 4. Molecular docking of MEC-CHPV with immune receptor. (A) Docked complex of MEC-CHPV with TLR-3 and (B) Docked complex of MEC-CHPV with TLR-8.

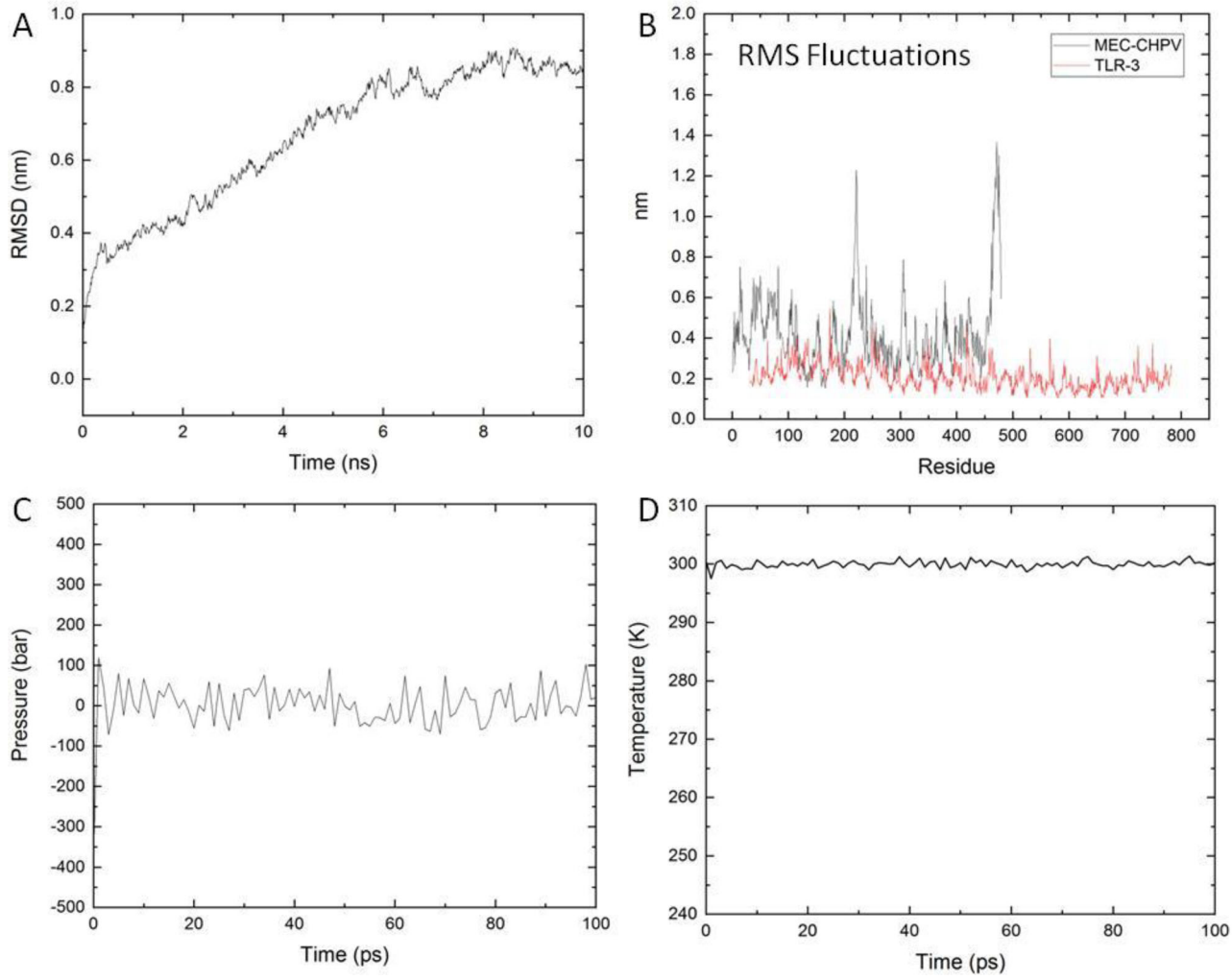

Figure 5. The results of molecular dynamics simulation for MEC-CHPV complexed with TLR-3. A) Root mean squared deviation (RMSD) plot of receptor-ligand complex. B) Root mean square fluctuation (RMSF) plot representing the fluctuations of the side chain residues for both the proteins. C) Plot of Pressure vs time, and D) Plot of temperature vs time.

diversity in the clonal specificity. A low level of D suggests a lower diversity in the immune response.

4. Discussion

Chandipura virus is responsible for AES, mainly in paediatric population of Gujarat, Telangana, Andhra Pradesh and Maharashtra states of India. Till today, no preventive or therapeutic measures are available in the market. In depth study of different viral proteins using computational approach may be helpful in devising the protective measures against it. The purpose of the present study was to conduct antigenic characterization of CHPV proteins and design a multi-epitope protein using immuno-informatics approaches.

Our earlier study on immune-dominant G protein of CHPV using neutralizing monoclonal antibody using laboratory experiments and *in silico* antigen-antibody interaction, led to the identification of conserved neutralizing epitope in the fusion domain (Pavitrakar et al., 2018). Although neutralizing antibodies are mainly targeted against the surface-expressed G protein for CHPV, the other viral proteins namely N, P and M proteins have also been found to elicit immune response as evident from the interaction of these proteins with polyclonal serum raised against CHPV in western blot analysis (Jadi et al., 2011). Role of these viral antigens in the induction of protective immunity has been assessed in rabies virus ribonucleoprotein (RNP) and it was observed that, mice/raccoons immunized with RNP alone resisted lethal peripheral challenge with homologous or heterologous rabies virus strains, thus establishing the crucial role of RNP in the induction of protective immunity (Dietzschold et al., 1987). Considering this, recently, Realegeno et al. (2018) have developed an ELISA-based method for the detection of rabies virus nucleoprotein-specific antibodies in human ante-mortem samples. Thus, the role of internal structural proteins as immunogens has already been established for rhabdoviruses related to CHPV.

Development of safe and effective vaccine against infectious disease is an important intervention for prevention of disease. Conventional vaccine development approaches mainly includes inactivated or attenuated vaccines derived by attenuating the virulence of the pathogen by physical or chemical methods. Progress in the field of immuno-informatics allows us to identify the potential immunogenic fragments of viral proteins (Skwarczynski & Toth, 2014). The

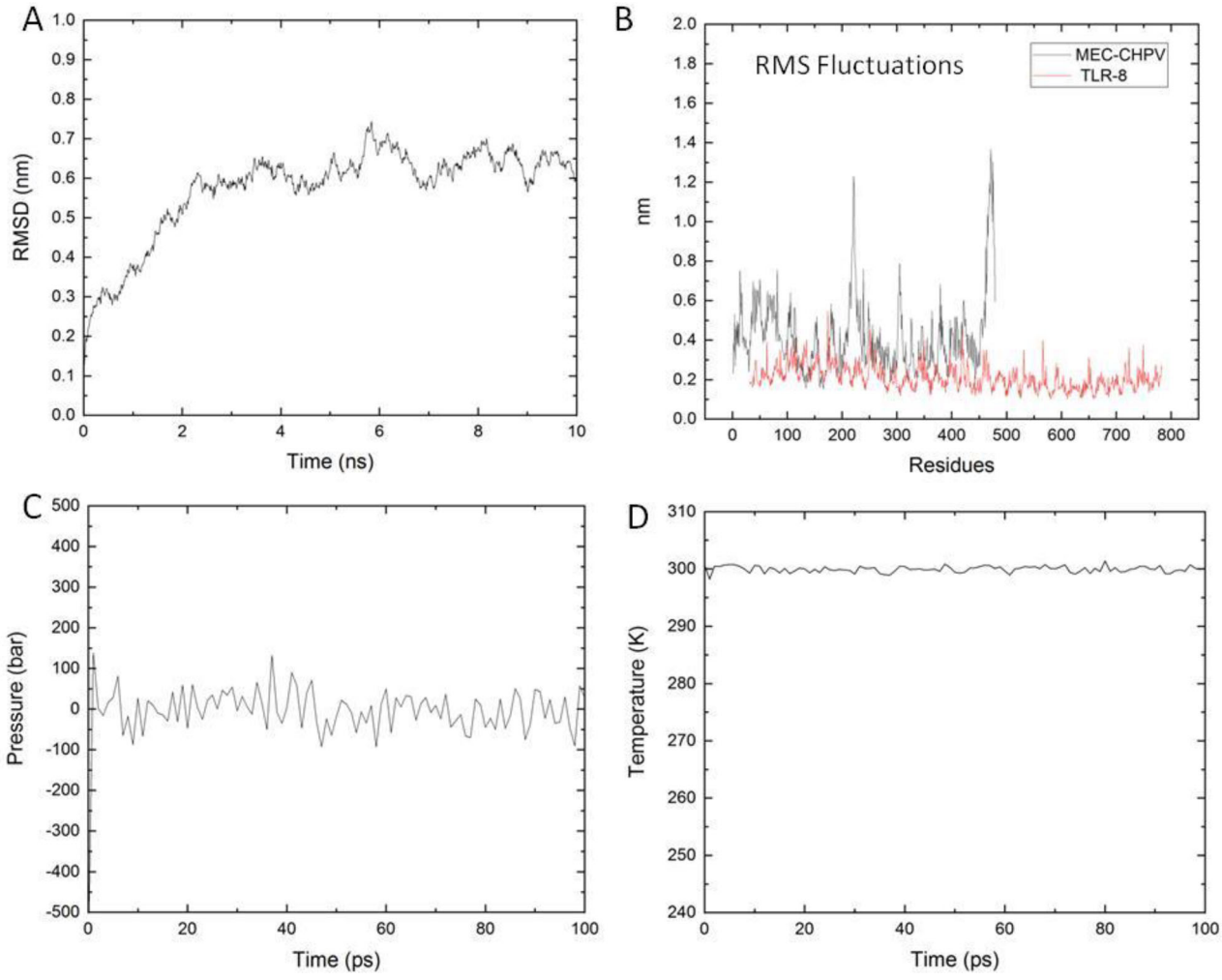

Figure 6. The results of molecular dynamics simulation for MEC-CHPV complexed with TLR-8. A) Root mean squared deviation (RMSD) plot of receptor-ligand complex. B) Root mean square fluctuation (RMSF) plot representing the fluctuations of the side chain residues for both the proteins. C) Plot of Pressure vs time, and D) Plot of temperature vs time.

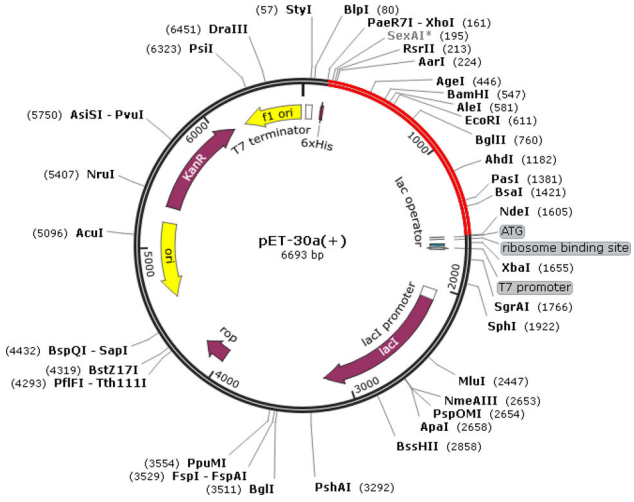

Figure 7. *In silico* cloning of MEC-CHPV in bacterial expression vector pET30a(+). The red colored portion represents sequence coding for MEC-CHPV while remaining black color part represents vector backbone.

epitope-based vaccines exhibit several advantages over conventional vaccines, including high specificity, good safety, ease of production and storage, and stability. In order to avoid the degradation of the antigenic peptides by host proteases epitope-based vaccines are generally designed with a suitable delivery system or an adjuvant. One of these immunological adjuvants is β-Defensin. These antimicrobial peptides are involved in the innate immune response of the host and are responsible for stimulating innate and adaptive immune responses by recruiting naïve T-cells and immature DCs through interactions with corresponding immune receptors (e.g. CCR6 or TLRs) (Narula et al., 2018). Using immunoinformatics methods, Ali et al. have designed a multi-epitope vaccine for dengue that included Tc (Cytotoxic T-cell) and Th (Helper T-cell) cell epitopes with β-defensin as a molecular adjuvant at the N-terminal (Ali et al., 2017). Similarly, researchers have developed an anti-chikungunya and human norovirus multi-epitope vaccine which also included B-cell and T-cell binding epitopes with β-defensin added as a built-in adjuvant (Azim et al., 2019; Narula et al., 2018).

In the current study, we designed a multi-epitope protein, MEC-CHPV, consisting of B and T-cell epitopes of N, P, M and G protein of CHPV with β-defensin as an immunological adjuvant at the N-terminal. The CHPV strain responsible for major outbreak in the year 2003 was chosen for prediction of B and T-cell epitopes. The different proteins of CHPV were assessed for their antigenic potential using VaxiJen server.

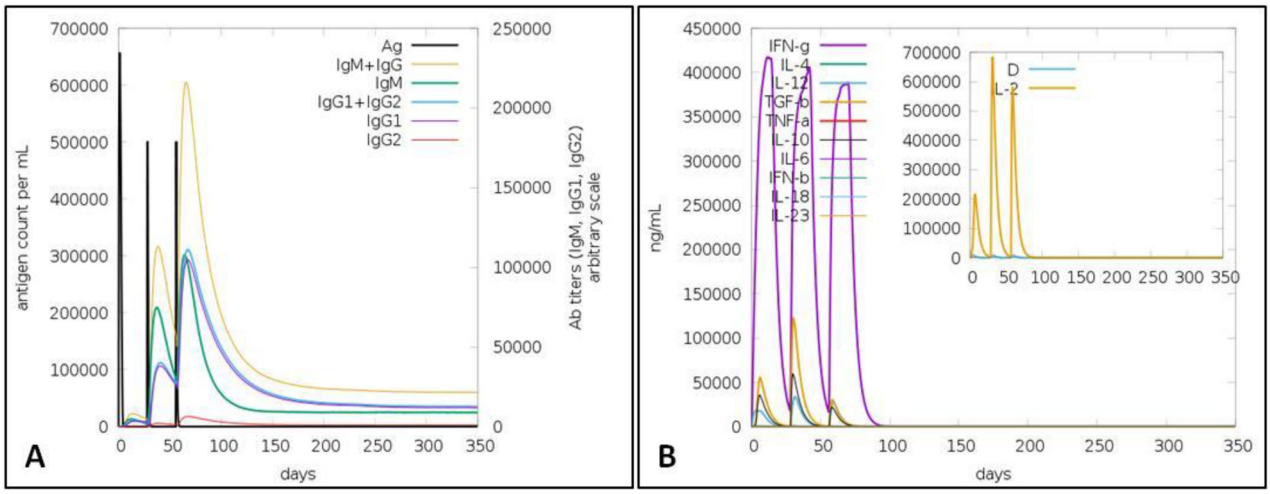

Figure 8. *In silico* immune simulation of MEC-CHPV using C-ImmSim. A) Immunoglobulin production in response to three doses of the antigen. B) Cytokine responses after three doses of the antigen. Inset is the levels of IL-2 post injections and Simpson index, D.

Since, B-cells are mainly responsible for eliciting antibodies against specific pathogen where as CTL response selectively kills the infected cells restricting further spread of infection, we considered that any peptide vaccine should ideally consist of B and T-cell epitopes. The B-cell epitopes commonly predicted by both Kolaskar Toangaonkar and BepiPred methods were used for further screening for allergenicity, toxicity and conservancy. This led to identification of 14 linear B-cell epitopes from all the four proteins.

T-cell epitopes mainly, MHC class I epitopes for N, P, M and G protein were primarily selected based on their ability to get recognized by alleles (HLA-A*03, HLA-A*69:01 and HLA-A*26) susceptible for CHPV infection (allele identification - as per experimental work done during CHPV outbreak investigations – NIV archival data) followed by immunogenicity prediction (IEDB tools). The efforts resulted in identification of six MHC class I epitopes by combining few overlapping epitopes identified by all the three alleles under consideration. Four MHC class II epitopes were considered as significant because of their ability to get recognized by six or more alleles prevalent in the endemic region as well as other scrutinizing criteria like allergenicity and toxicity. The epitopes were found to be potential IL-4 inducers as well.

MEC-CHPV was constructed by incorporating 14 B-cell epitopes and 6 MHC class II and 4 MHC class I epitopes linked by different linkers (KK: for the B-cell epitopes, GGGGS: for the MHC II and AAY: for the MHC I epitopes). The adjuvant β defensin (45 amino acids) was added to the N terminal followed by EAAAK linker peptide to ensure the efficient separation of bi-functional fusion protein.

The predicted 3D structure of MEC-CHPV was found to be valid by Ramachandran plot analysis and *in silico* analysis of physicochemical properties showed its stable nature. As per literature, the innate immune response through TLR molecules in case of RNA viruses is mainly governed through TLR-3, TLR-7 and TLR-8. The up-regulation of TLR-7 in related vesiculovirus i.e. vesicular stomatitis virus (Lund et al., 2004) is well established. Molecular docking analysis of our designed construct MEC-CHPV with human TLR-3 and TLR-8 molecules showed stable interactions. Molecular dynamics simulations revealed that both complexes were highly stable. The *in silico* cloning of codon optimized sequence in prokaryotic expression vector pET30a(+) demonstrated effective expression of vaccine construct.

Induction of a typical immune response, as indicated by in silico immune simulation using C-ImmSim tool, suggested that repeated exposure to MEC-CHPV antigen resulted in enhancement of immune responses. C-ImmSim results indicated rise in the levels of IL-2 and interferon-γ, which is in line with the report of high levels of IL-2 and interferon-γ in the recovered individuals from CHPV infection (Tripathy et al., 2005). In a nutshell, the current data suggest that use of MEC-CHPV as a vaccine candidate against CHPV infection may induce high levels of antiviral cytokines (IL-2 and interferon-γ) along with the induction of humoral immune responses.

All our findings indicate that the intended vaccine candidate MEC-CHPV may undergo laboratory analyses involving *in vitro* and *in vivo* studies for the development of potential vaccine against Chandipura virus in future.

5. Conclusion

The advancement of immuno-informatics tools in post genomic era enables us to explore these platforms in development of multi-epitope based vaccine with higher accuracy and in a cost effective manner with low risk. In this study, we have applied a series of immuno-informatics tools in a sequential manner to find a peptide vaccine candidate that may prove effective against Chandipura infection. These findings paved the way for future experimental work.

Disclosure statement

The authors declare that they have no conflict of interest.

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