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Multi-epitope based vaccine against yellow fever virus applying immunoinformatics approaches

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ABSTRACT

Yellow fever disease is considered a re-emerging major health issue which has caused recent outbreaks with a high number of deaths. Tropical countries, mainly African and South American, are the most affected by Yellow fever outbreaks. Despite the availability of an attenuated vaccine, its use is limited for some groups such as pregnant and nursing women, immunocompromised and immunosuppressed patients, elderly people >65 years, infants <6 months and patients with biological disorders like thymus disorders. In order to achieve new preventive measures, we applied immunoinformatics approaches to develop a multi-epitope-based subunit vaccine for Yellow fever virus. Different epitopes, related to humoral and cell-mediated immunity, were predicted for complete polyproteins of two Yellow fever strains (Asibi and 17D vaccine). Those epitopes common for both strains were mapped into a set of 137 sequences of Yellow fever virus, including 77 sequences from a recent outbreak at the state of Minas Gerais, southeast Brazil. Therefore, the present work uses robust bioinformatics approaches for the identification of a multi-epitope vaccine against the Yellow fever virus. Our results indicate that the identified multi-epitope vaccine might stimulate humoral and cellular immune responses and could be a potential vaccine candidate against Yellow fever virus infection. Hence, it should be subjected to further experimental validations.

Abbreviations: AEs: adverse events; C: Capsid; E: Envelope; NHPs: Non-human primates; NS: Non-structural; PAHO: Pan American Health Organization; prM: Premembrane; SAEs: serious adverse events; YFV: Yellow fever virus

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Introduction

Yellow fever virus (YFV) is a member of the *Flaviviridae* family, which contains small enveloped viruses with RNA genomes from 9 kb to 13 kb (Simmonds et al., 2017). A single open reading frame encodes a unique polyprotein later cleaved by virus proteases (Lin, Amberg, Chambers, & Rice, 1993) and processed by the host cell (Ruiz-Linares, Cahour, Després, Girard, & Bouloy, 1989). Three structural proteins: capsid (C), premembrane (prM) and envelope (E) proteins, and seven non-structural proteins (NS): NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Rice, Lenes, Shin, Sheets, & Strauss, 1985) are produced for diverse roles into replication (Dalgarno, Trent, Strauss, & Rice, 1986) and evasion of host immune system (S. Chen, Wu, Wang, & Cheng, 2017).

Seven different Yellow fever virus (YFV) genotypes have been described: five African genotypes (West African type I

and II, East and Central Africa, East Africa and Angola) (Mutebi, Wang, Li, Bryant, & Barrett, 2001) and two South American genotypes (Types I and II) (E. Wang et al., 1996). YFV can infect humans and non-human primates (NHPs). The transmission can occur in three different cycles (sylvatic, intermediate and urban), varying according to the region of occurrence (Africa or South America) and vector mosquito species (Staples, Monath, Gershman, & Barrett, 2018). After infection in humans and an incubation period of 2–9 days (Johansson, Arana-Vizcarrondo, Biggerstaff, & Staples, 2010), YFV leads to a broad presentation spectrum, ranging from abortive and subclinical infection to nonspecific flu-like illness and even potentially lethal hemorrhagic disease (Staples et al., 2018).

Recent outbreaks in Brazil (Giovanetti et al., 2019) (Faria et al., 2018) and Angola (Wilder-Smith & Monath, 2017) have raised concerns over the increasing risk of re-emergence of

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yellow fever cases in the Americas and Africa. Exclusively in Africa, 19,000–180,000 deaths are estimated annually (Garske et al., 2014). In South and Central Americas in 2018, the Pan American Health Organization (PAHO) has confirmed new cases of yellow fever disease in six countries: Peru, Colombia, French Guiana, Bolivia, Ecuador and Brazil (Paho, 2018). According to the Ministry of Health of Brazil, 1,376 cases of yellow fever and 483 deaths were confirmed between July 2017 and June 2018, with 778 cases still under investigation (Ministério da Saúde divulga balanço de um ano da febre amarela, n.d.), exceeding reported cases in American countries in the last 50 years (Paho, 2018).

Forty-seven countries in African, South and Central American regions are endemic to yellow fever virus (Shearer et al., 2017). Globally, it has been estimated that 393 million to 472 million people live in regions with a risk of infection and require vaccination (Shearer et al., 2018).

The live attenuated vaccine against YFV was developed in the 1930s from a 17D attenuated lineage (Smith, Penna, & Paoliello, 1938; Theiler & Smith, 1937) produced from the Asibi strain. This strain was isolated from a patient with the same name and it belongs to the West African genotype (Lindern et al., 2006). Currently, there is no effective antiviral therapy (Monath & Vasconcelos, 2015) against yellow fever disease. Main strategies adopted to control it are vector control (Benelli, 2015) and the use of 17D vaccine in endemic areas, during outbreaks and to travellers across affected regions (WHO, 2014). Although it is considered a safe vaccine, adverse events (AEs) and serious adverse events (SAEs) against 17D vaccine have been reported (Biscayart et al., 2014), (Breugelmans et al., 2013), including yellow fever vaccine-associated viscerotropic disease (YEL-AVD) (Pulendran et al., 2008), which has a 60% case fatality rate (Monath, 2007) and yellow fever vaccine-associated neurological disease (YEL-AND) (Jennings et al., 1994) with a lower fatality rate (about 6%) (Monath, 2007).

The Live Attenuated Yellow Fever 17D vaccine is not recommended, or it is advised in a case-by-case analysis (World Health Organization, 2013), to several groups or conditions: elderly patients >60 years old (Lawrence, Burgess, & Kass, 2004), (Khromava et al., 2005), infants younger than 6 months (World Health Organization, 2013), patients undergoing immunomodulatory or immunosuppressive therapy (Porudominsky & Gotuzzo, 2018), with thymus disorder or whose thymus has been removed (Marfin, Eidex, Kozarsky, & Cetron, 2005), pregnant women (Tsai, Paul, Lynberg, & Letson, 1993), (Nishioka, Nunes-Araújo, Pires, Silva, & Costa, 1998), breastfeeding women (Traiber, Amaral, Ritter, & Winge, 2011), (Frieden et al., 2010) and people with egg associated hypersensitivity (Rutkowski, Ewan, & Nasser, 2013).

Therefore, the scientific community is still trying to develop an inactivated (Monath et al., 2010) or DNA (Maciel et al., 2015) vaccine against yellow fever virus. Immunoinformatics is a well-established approach and has been applied to the vaccine study of different pathogenic microorganisms, such as *Streptococcus pneumoniae* (Dorosti et al., 2019) and Alkhurma hemorrhagic fever virus (Ul-Rahman & Shabbir, 2019) besides other arboviruses as Dengue virus (Ali et al., 2017; Murphy,

Reche, & Flower, 2019; Sabetian, Nezafat, Dorosti, Zarei, & Ghasemi, 2019) and Chikungunya virus (Narula, Pandey, Khatoon, Mishra, & Prajapati, 2018). The prediction of epitopes and their interaction with the host's immune system (Backert & Kohlbacher, 2015) can provide insights on new strategies to minimize adverse events. In this study, in order to achieve a wider coverage into the human population and to minimize adverse events, we used recently sequencing data of 77 YFV genomes obtained in a recent outbreak in the state of Minas Gerais, southeast Brazil, and additional genomes from NCBI, totalizing 137 genomes for the analysis. As a result, we developed a multi-epitope based vaccine against yellow fever virus applying immunoinformatics approaches.

Materials and methods

Retrieval and selection of data

To investigate the antigenic epitopes of different proteins of Yellow Fever Virus (YFV) for an efficient vaccine, the complete polyproteins of two different YFV strains, Asibi (Q6DV88) and 17D (P03314), were retrieved in FASTA format from Uniprot (Universal Protein Resource) database (The UniProt Consortium, 2015), containing 3411 amino acids each. Asibi is the prototype Yellow fever virus sequence, isolated from a patient named Asibi in Gana, 1927 (Hahn, Dalrymple, Strauss, & Rice, 1987). The vaccine strain is derived from the Asibi isolate (Hahn et al., 1987). After a series of cellular passages for attenuation, the vaccine strain contains a set of epitopes responsible for efficient immune response, while wild strains may present a wider number of epitopes with diverse occurrences in viral populations.

Sequences of Toll-like receptor 2 (TLR-2) (PDB:2z7x) and a β-defensin agonist (Funderburg et al., 2007) - a peptide adjuvant - were also retrieved in PDB format and FASTA format, respectively. After the prediction of epitopes, they were mapped into a dataset of 137 (Supplementary material – Table 1) complete or almost complete YFV genome sequences from different locations. Among these 137 sequences, 77 sequences are from a recent outbreak in Minas Gerais state, Brazil (Faria, Kraemer, Hill, Goes de Jesus, et al., 2018) and the others were retrieved from the NCBI database. These three sets of sequences were used to construct a common multi-epitope vaccine, which should be effective against the recent endemic strains of YFV.

Epitope prediction and mapping within the individual proteins of yfv genomes

Epitope prediction of Cytotoxic T Lymphocytes (CTL)

NetCTL 1.2 server (Larsen et al., 2007) was used to predict cytotoxic T lymphocyte (CTL) epitopes for the Asibi and 17D vaccine strains. To predict CTL epitopes, NetCTL 1.2 considers three stages of antigens processing and presentation: proteasomal C-terminal cleavage, TAP transport efficiency and MHC class-I binding affinity. These three approaches are combined into a single score (Larsen et al., 2007). The affinity binding was tested to 12 MHC class-I supertypes and the CTL

Table 1. Number of CTL and HTL epitopes mapped into YFV proteins.

Protein	Number of CTL epitopes	Number of HTL epitopes
ENV	8	1
CAP	6	6
prM/Peptide pr	4	0
prM/Small envelope protein M	4	0
NS1	11	4
NS2A	11	4
NS2B	1	0
NS3	18	0
NS4A	3	0
NS4B	3	6
NS5	34	1
Total	103	22

epitopes with a score higher than 0.75 (Ali et al., 2017) were selected, representing 0.80 and 0.97 of sensitivity and specificity, respectively. Afterwards, the epitopes that were predicted in both strains were selected for the next step in the analysis.

Epitope prediction of Helper T Lymphocytes (HTL)

Polyproteins of Asibi and 17D vaccine strains were submitted to the IEDB server (<http://tools.iedb.org/mhcii/>) to predict helper T Lymphocytes (HTL) epitopes (Vita et al., 2019), (Rana & Akhter, 2016). 15-mer length epitopes were predicted for a reference set (Greenbaum et al., 2011), (Weiskopf et al., 2013) of Human MHC class II alleles. For each strain, epitopes presenting IC₅₀ value <50 nM, and percentile ranks (<2.3%) (Ali et al., 2017) were considered to have higher affinity binding to MHCII molecules. The common epitopes of both strains were selected for further analysis.

B-cells epitope prediction

Linear epitopes were predicted for the polyproteins of each strain (Asibi and 17D vaccine) using the BCPREDS server: B-cell Epitope Prediction Server (El-Manzalawy, Dobbs, & Honavar, 2008). BCPREDS applies Support Vector Machine (SVM) classifier trained to utilize subsequence kernel method. On a homology-reduced data set from the Bcipep database with 701 linear B-cell epitopes and 701 non-epitopes extracted from SwissProt, BCPred had a predictive performance of (AUC=0.758) defeating 11 previous SVM-based classifiers developed and AAP (AUC=0.7) (El-Manzalawy et al., 2008). The threshold BCPred score applied for selection was >0.8 (Ali et al., 2017). The shared epitopes among both strains were selected for further analysis.

Overlapping of cellular and humoral epitopes

Overlapping of humoral and cellular epitopes has been strategically performed to stimulate humoral and cellular immune responses (Ali et al., 2017; Shey et al., 2019). For this analysis, an in-house Python script was used. A sliding window approach was applied to verify the overlapping between the B epitopes common to both strains, compared to the groups of common CTL and HTL epitopes. The window size ranged from 2 to 9 amino acids in the case of CTL epitopes and from 2 to 15 amino acids overlapping for HTL epitopes.

Mapping of epitopes into YFV proteins

To eliminate epitopes in cleavage regions between proteins, predicted epitopes were mapped into yellow fever virus proteins using the ScanProsite tool (<https://prosite.expasy.org/scanprosite/>). Considering epitopes as motifs, ScanProsite searched each epitope against every protein file of reference strain used.

Mapping of epitopes into circulating strains of Yellow Fever Virus

The use of reference strains may not represent current epitopes and it is essential to verify whether the CTL and HTL predicted epitopes, common to both strains and overlapped with B epitopes are circulating into the viral population. To tackle this problem, we mapped the epitopes into a dataset of 137 sequences of complete or almost complete genomes of YFV retrieved from NCBI. The sequences were from different geographical locations, including 77 genomes from a recent outbreak in Minas Gerais state, Brazil (Nuno Rodrigues Faria, Kraemer, Hill, Goes de Jesus, et al., 2018). The alignment was performed using ClustalW (Larkin et al., 2007) and the analysed epitopes were visualized using Bioedit (Hall, 2013). Besides missing information regarding sequencing, epitopes with a 100% match into the recent Brazilian strains were selected as our final epitopes. Prediction and selection of epitopes are shown in (Figure 1).

Construction of multi-epitope vaccine sequence

In order to conjugate and efficiently separate the epitopes to facilitate the process of antigen-presenting (X. Chen, Zaro, & Shen, 2013), peptide linkers were positioned between each epitope. HTL and CTL overlapping or adjacent epitopes were merged into a single peptide to reduce the size of the construction (Lohia & Baranwal, 2014). CTL and HTL epitopes were linked with AAY (Chauhan, Rungta, Goyal, & Singh, 2019) and KK (Cole et al., 2013) linkers respectively. Those were experimentally tested in antigen presentation assays (Chauhan et al., 2019). A β-defensin adjuvant sequence with the help of linker EAAAK (Shey et al., 2019) was added at the N-terminal of the multi-epitope vaccine.

Evaluation of immune response

IFN-γ inducing epitope prediction

IFN-γ inducing epitopes were predicted using the IFNepitope server (<http://crdd.osdd.net/raghava/ifnepitope/scan.php>) by

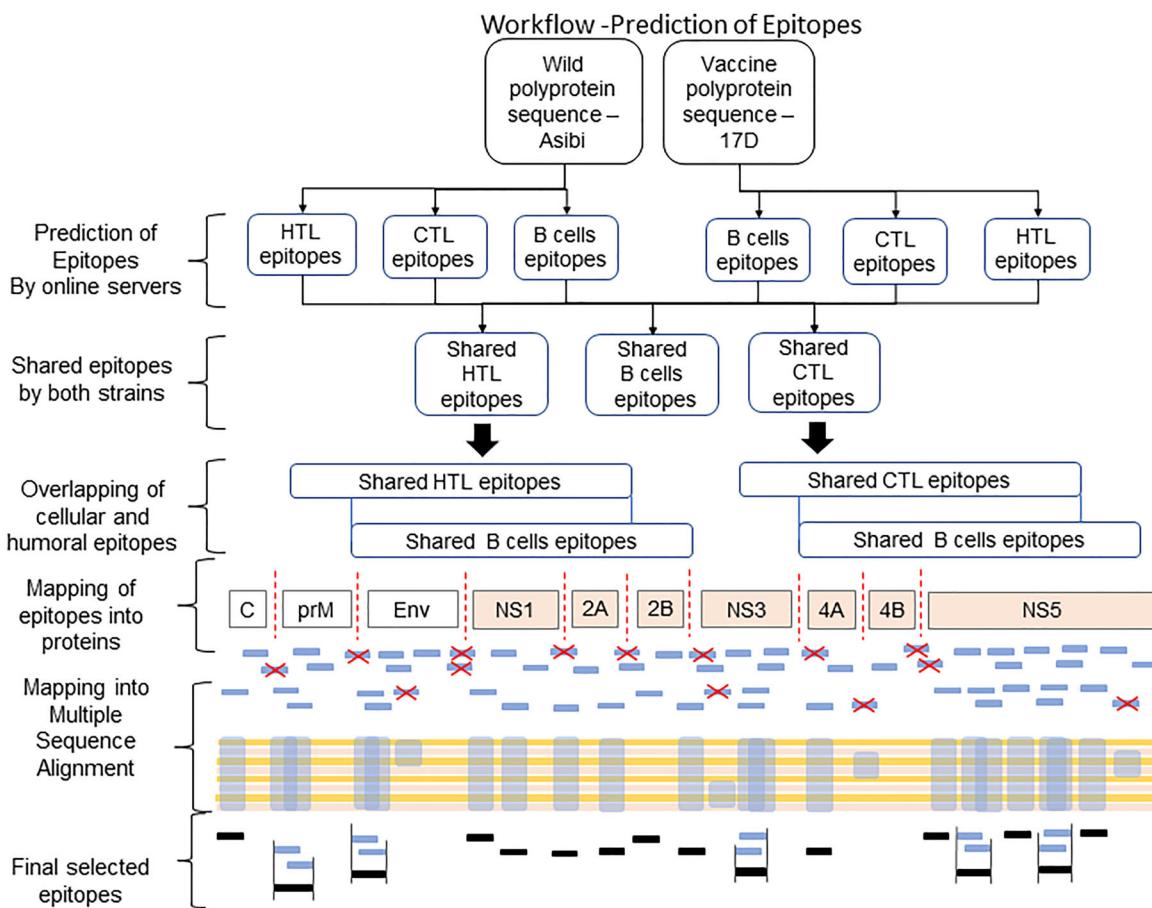


Figure 1. Complete workflow used for the selection of final epitopes.

Motif and support vector machine (SVM) hybrid approach (Dhanda, Vir, & Raghava, 2013). Through overlapped sequences on a dataset of IFN- γ inducing and non-inducing MHC class-II binder, IFNepitope identifies the epitopes capable of activating T-helper cells. IFN- γ epitope prediction score >1.0 was set as a threshold value for epitope identification.

Antigenicity and allergenicity prediction

To evaluate the multi-epitope vaccine's epitopes ability to stimulate the immune system, the VaxiJen server (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) was used. The VaxiJen prediction method does not rely on alignment or homology. Instead, it predicts protective peptides based on auto cross-covariance (ACC) transformation of protein sequences using uniform vectors of the main physicochemical properties of amino acids (Doytchinova & Flower, 2007). The antigenicity was also verified with the ANTIGENpro online server (<http://www.scratch.proteomics.ics.uci.edu/>). ANTIGENpro basically predicts the likelihood of a protein or peptide to be a protective antigen (Magnan et al., 2010). Independently of pathogen identification or alignment, ANTIGENpro uses primary sequence and ensemble methods with SVM.

Furthermore, to investigate the allergic or non-allergic characteristic, the multi-epitope sequence was submitted to AlgPred (<http://www.imtech.res.in/raghava/algpred/>) online server (Saha & Raghava, 2006). AlgPred predicts allergens based on five methods: prediction of IgE epitopes, a BLAST

search against 2890 ARPs (allergen-representative peptides), similarity analysis of known protein sequences, search MEME/MAST for allergen motifs and the use of an SVM module for the prediction of allergenicity.

Host homology analysis

Homologous protein to host may lead to autoimmunity. In order to select only novel and nonself epitopes, the multi-epitope vaccine was submitted to NCBI BLASTp (Altschul, Gish, Miller, Myers, & Lipman, 1990) to verify homology against annotated Human Proteome (Accession Number 9606).

Prediction of physicochemical and biological properties

Diverse physicochemical properties of the multi-epitope vaccine protein were predicted with ProtParam online server (<http://web.expasy.org/protparam/>). ProtParam (Gasteiger et al., 2009) returns amino acid and atomic composition, molecular weight, theoretical pI, aliphatic index, extinction coefficient, grand average of hydropathicity (GRAVY), estimated half-life for three model organisms (*Escherichia coli*, yeast and mammal cells) and the instability index. Calculated by the work of Guruprasad (Guruprasad, Reddy, & Pandit, 1990), instability index attributes weights to dipeptides according to their frequency into stable or unstable proteins. After mapping the dipeptides into our multi-epitope vaccine,

**Table 2.** Selected CTL and HTL epitopes overlapping with B-cell epitopes.

Number	Protein	CTL epitope	B-Cell epitope
1	Env	WAQDLTPW	IVDRQWAQDLTPWQSGSGG
2	Env	KTALTGAMRVTK	KTALTGAMRVTKDTNDNNLY
3	Env	NTRNMTMSMSMILV	IWVGINTRNMMSMSMILVG
4	Capsid	RPGPSRGVQ	IKQKTKQIGNRPGPSRGVQG
5	Capsid	TGKKITAHL	FNILTGGKITAHLKRLWKML
6	prM	TGNCTTNIL	GTGNCTTNILEAKYWCPDSM
7	prM	RQEKMWTGRMGERQLQK	TRQEKMWTGRMGERQLQKIE
8	NS1	SIRIDGLQYGWKTWKG	PFSRIRDGLQYGWKTWGKNL
9	NS1	CEWPLTHTI	YKECEWPLTHTIGTSVEESE
10	NS1	GPVSSHNI	PRSIGGPVSSHNHIPGYKVQ
11	NS1	PWMQVPLEV	NGPWMQVPLEVKREACPGTS
12	NS2A	EMNNNGDAM	VAVGLHFHEMNNGGDAMYMA
13	NS2A	IRPGLLIGFGLRW	IRPGLLIGFGLRTLWSPRER
14	NS3	SQRGVGVQAQGGVFHTMW	GASQRGVGVQAQGGVFHTMW
15	NS3	VAYGGSWKL	VAYGGSWKLEGRWDGEEEVQ
16	NS3	QTIDIPSEPWNNTGHDWI	DVQTDIPSEPWNNTGHDWILA
17	NS3	KTNDRKWCFC	AGLKTNDRKWCFCGPEEHEI
18	NS3	CRAPGGAKKPL	KCRAPGGAKKPLRPRWCDER
19	NS4B	PLIEGNTSLLWNGPMAV	LGPLIEGNTSLLWNGPMAVS
20	NS5	SVETDKGPLDK	SVETDKGPLDKEAIEERVER
21	NS5	DNPYRTWHCGSYV	DNPYRTWHCGSYVTKTSGS
22	NS5	PPAGTRKIMKVNN	TRAKDPPAGRTRKIMKVVN
23	NS5	QWKTAANEAVQDPKF	LEEQEIQWKTAANEAVQDPKF
24	NS5	KLSEFGKAKGSRAIW	KREKKLSEFGKAKGSRAIWY
25	NS5	DTAGWDTRI	AAMDGGFYADDTAGWDTRI
26	NS5	RPAPGGKAYMDVISRR	RPAPGGKAYMDVISRRDQRG
27	NS5	WVPQGRTTW	SAVPTSWVPQGRTTWSIHGK
28	NS5	MTTEDMLEVWNRVWI	WMTTEDMLEVWNRVWITNNP
Number	Protein	HTL epitope	B-cell epitope
29	Env	GINTRNMMSMSMIL	IWVGINTRNMMSMSMILVG
30	Capsid	FLFNILTGGKITAHLKRLWKMLD	FNILTGGKITAHLKRLWKML
31	NS1	KECEWPLTHTIGTSVEESE	YKECEWPLTHTIGTSVEESE
32	NS2A	MMIAIMEVVLRKRQGPQKM	MEVVLRKRQGPQMLVGGVV
33	NS4B	IEGNTSLLWNGPMAVSMTG	LGPLIEGNTSLLWNGPMAVS
34	NS4B	VGVMYNLWKMKTGRR	YNLWKMKTGRRGSAKTLG

the weight of dipeptides into CTL epitopes was calculated based on the number of occurrences. Epitopes conferring high instability (epitopes with total weight >100) were excluded or had the overlapping region from adjacent epitopes reduced. *Supplementary material – Table 2* was considered for the calculation of dipeptides' total weight. This stability parameter was considered in the construction of the final multi-epitope vaccine. Furthermore, the multi-epitope vaccine protein was submitted to InterProScan (Finn et al., 2017) for the biological processes, molecular functions and cellular components analysis.

Secondary structure prediction

The secondary structure is determinant in protein folding (Kwok, Mant, & Hodges, 2002). Secondary structure prediction of the multi-epitope vaccine protein was performed with the PSIPRED server (<http://bioinf.cs.ucl.ac.uk/index.php?id=779>). PSIPRED (Ward, McGuffin, Buxton, & Jones, 2003) uses two feed-forward neural networks, in which the second network refines the first one's prediction with the help of PSI-BLAST (Position-Specific Iterated-BLAST).

Tertiary structure prediction

The amino acid sequence of the final multi-epitope vaccine was submitted to RaptorX web-based server (<http://raptord.uchicago.edu/StructurePrediction/predict/>) for the prediction of tertiary

structure. RaptorX is a template-based protein modelling server that implements three strategies to the prediction of tertiary protein structure: single and multiple template threading and alignment quality prediction (S. Wang, Li, Liu, & Xu, 2016). The model quality is assessed by GDT (global distance test) for absolute global quality and p-value score for relative global quality of the predicted structure (S. Wang, Zhou, et al., 2016).

Tertiary structure refinement and validation

The refinement of the tertiary protein structure of the final multi-epitope vaccine sequence was required to enhance the global and local structural quality. The refinement of template-based protein model of the multi-epitope vaccine was performed by GalaxyRefine server (Heo, Park, & Seok, 2013). After input of the initial structure, aggressive and mild relaxation methods were applied to refine the whole prediction of tertiary protein structure. Galaxy Refine reconstructs side chains of amino acids and accomplishes side-chain repacking with consecutive relaxation by molecular dynamics simulation at the overall structure (Heo et al., 2013). In order to validate the refined tertiary structure, RAMPAGE web server (Lovell et al., 2003) (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>) was used to generate a Ramachandran plot. Based on van der Waal radius of the side chains, it permits the visualization of predicted energetically disallowed and allowed dihedral angles phi (ϕ) and psi (ψ) of each amino acid in a protein. RAMPAGE server also returns the percentage of residues in allowed and disallowed regions.

Discontinuous B-cell epitope prediction

B-cell discontinuous epitopes are estimated as approximately >90% of B-cells epitopes and may play a crucial role in the immune response. Prediction of B-cell discontinuous epitopes based on the validated tertiary protein structure of the final multi-epitope was performed by ElliPro server (<http://tools.iedb.org/ellipro/>) (Ponomarenko et al., 2008).

Protein-protein docking of epitope vaccine with TLR-2 immune receptor

TLR-2 receptor has been described as one of the Toll-like receptors responsible for the immune response against the 17D vaccine and stimulates a mixed Th2 and Th1 cell profile (Pulendran, 2009). The TLR-2 receptor structure was retrieved from the RCSB PDB database (PDB ID: 2Z7X) and edited (i.e. removal of the ligands PCJ, MAN, BMA, NDG and NAG, TLR-1 molecule, and water molecules) using Chimera (<https://www.cgl.ucsf.edu/chimera/>).

Stable folding of a protein core is generally conserved in conformational changes and it has minimally frustrated links between amino acids. Conversely, regions with high local frustration are frequently participating in allosteric or binding, which is indicative of biological activity (Jenik et al., 2012). Data from previous studies were considered to determine frustrated regions (Hajighahramani et al., 2017), (Jin et al., 2007), (Nezafat et al., 2016). Additionally, Chimera was used to visualizing TLR-2 hydrophobic regions.

The interaction of the antigenic molecule with an immune receptor molecule is required for an efficient immune response. Swarmdock server (<http://bmm.crick.ac.uk/~SwarmDock/index.html>) (Torchala, Moal, Chaleil, Fernandez-Recio, & Bates, 2013) was utilized to perform molecular docking and to assess the interaction between immune receptor (TLR-2) (PDB ID: 2z7x) and the multi-epitope vaccine. SwarmDock algorithm is a flexible modeller of protein-protein complexes that pre-process and minimizes inputted structures and performs docking through a hybrid particle swarm optimization/local search (Torchala et al., 2013). Subsequently, the algorithm minimizes, re-ranks and clusters docked poses. Democratic clustered structures were obtained as PDB format and evaluated based on energy. Also, using Chimera, each model was visualized and assessed for the number of inter-model hydrogen bonds in frustrated regions of TLR-2.

Molecular dynamics simulation of the receptor-ligand complex

Molecular dynamics (MD) is applied to understand the structure of biological macromolecules and the physical basis of their interaction and function (Karplus & McCammon, 2002), (Ode, Nakashima, Kitamura, Sugiura, & Sato, 2012). Gromacs v5.0 (Van Der Spoel et al., 2005) was used to study the structural properties and interaction between ligand (predicted vaccine protein structure) and receptor (TLR-2) at the microscopic level.

Full system MD simulation was run with the GROMOS96 43A1 force field. Neutralization of the system was achieved by

the inclusion of NaCl ions. To guarantee that the geometry of the system is adequate and there are no steric clashes using the steepest descent algorithm approach, energy minimization was performed prior to simulation. To achieve system equilibration, two steps using the leapfrog algorithm were performed: NVT and NPT ensemble. During equilibration steps, the temperature was increased up to 300K and pressure up to 1 bar. After the system equilibration, 10 ns molecular dynamics simulations were accomplished in order to analyse the trajectory

In silico cloning

In order to investigate the cloning and expression efficiency of the multi-epitope vaccine in a proper expression vector, *in silico* cloning analysis was performed. The reverse translation and codon optimization were done using Jcat (<http://www.jcat.de/>). After providing a cDNA sequence, Jcat (Grote et al., 2005) online server performs codon optimization to a chosen species and provide codon adaptation index (CAI), related to codon usage biases. The CAI score should be higher than 0.8. GC content and rare codons were analysed by Rare Codon Analysis Tool (<https://www.genscript.com/tools/rare-codon-analysis>). GC content of a sequence should range between 30 and 70%. Soon after, we carried out *in silico* unidirectional cloning using a plasmid Pet-28a(+) through SnapGene software. The BamHI and SalI restriction enzymes were present at plasmid and added to the cDNA fragment.

Results

Epitope prediction of Cytotoxic T Lymphocytes (CTL)

NetCTL 1.2 server predicted promiscuous cytotoxic T lymphocyte (CTL) epitopes for human alleles covering 12 supertypes for both strains. In total, we found 1226 epitopes for Asibi and 1211 epitopes for 17D vaccine strains. Diverse epitopes were predicted to more than one MHC I allele and they were considered as one in further analyses. The two strains shared 782 epitopes (86%), with 57 (6%) epitopes exclusive to 17D and 71 (8%) exclusive to Asibi. The shared 782 epitopes were used for further analysis.

Epitope prediction of Helper T Lymphocytes (HTL)

Helper T Lymphocytes (HTL) human epitopes of 15-mer length were predicted using the IEDB server. Considering percentile rank and IC₅₀ value, a set of 512 epitopes were selected in total for both Asibi and 17D strains. Epitopes predicted to more than one MHC II allele were considered as a single occurrence. The amount of 323 epitopes (85%) were common to both strains, with 27 (7%) epitopes exclusive to 17D and 31 (8%) epitopes exclusive to Asibi. The shared 323 epitopes were selected for further analysis.

B-cells epitope prediction

Linear B epitopes were predicted by BCPREDS to both strains. A set of 70 epitopes were predicted to Asibi and 69

to 17D strain. 51 (75%) epitopes were common to both strains and selected for further analysis.

Overlapping of cellular and humoral inducing epitopes

Applying a sliding window approach, we verified the overlapping of shared cellular epitopes in comparison with humoral epitopes. Those with overlapping of 9 amino acids to CTL and from 11 to 15 amino acids to HTL were selected as able to activate both fractions of the host immune system.

Mapping of epitopes into YFV proteins

Using the ScanPosite tool, epitopes present in cleavage sites were excluded and 103 CTL epitopes and 22 HTL epitopes were mapped into yellow fever non-structural and structural proteins (Table 1).

Mapping of epitopes into circulating strains of Yellow Fever Virus

In order to have a broad range multi-epitope vaccine, the 103 CTL epitopes and 22 HTL epitopes identified were mapped into an alignment containing 137 sequences of yellow fever virus, including 77 sequences from a recent outbreak in Minas Gerais State, Brazil. Checking the coverage of each epitope into the alignment, 84 CTL epitopes and 19 HTL epitopes were selected as epitopes common to both strains (Asibi and 17D), overlapping with B-cell epitopes, not present in cleavage sites and present in samples from a recent outbreak. To reduce the number of epitopes, overlapping epitopes present into the same protein were merged (Supplementary material – Table 3). The final epitopes listed below (Table 2) do not consider excluded unstable epitopes (Supplementary material – Table 4).

Construction of multi-epitope vaccine sequence

The final multi-epitope construct can be divided into 3 blocks:

- In the N terminal of the construct, a β -defensin adjuvant (GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK) with EAAAK linker (1–45);
- 28 CTL epitopes from non-structural and structural proteins from YFV connected by AAY linkers;
- 7 HTL epitopes from non-structural and structural proteins with the help of KK linkers.

A representation of the final vaccine construct is shown in (Figure 2).

IFN- γ inducing epitope prediction

A total of 575 IFN- γ inducing epitopes of 15-mer were predicted for the multi-epitope construct by the IFNepitope server. Merci and SVM method predicted epitopes with both

negative and positive prediction scores. Seventy-four IFN- γ inducing epitopes with prediction score >1 were selected (Supplementary Material – Table 5).

Antigenicity and allergenicity prediction

The multi-epitope vaccine antigenicity probability was predicted by ANTIGENpro and by Vaxijen 2.0. A high probability (0.900152) was predicted by ANTIGENpro, indicating the protein as a probable antigen. Although the score by Vaxijen was 0.4327, the considered threshold for the viral model was 0.4, which still shows good antigenic properties. Both results indicated that our vaccine has antigenic characteristics.

The similarity of our vaccine to known molecules with allergic and non-allergic behaviour was predicted by the AlgPred server. Prediction by SVM method based on amino acid composition and dipeptide composition had scores of -0.82142 and -0.60189, with -0.4 and -0.2 thresholds, respectively. All five methods returned negative results for allergenicity prediction as shown in (Table 3).

Homology against host

To verify if the multi-epitope vaccine has homologous proteins with the host, BLASTp was performed against the *Homo sapiens* proteome. After Blast analysis, no CTL or HTL epitopes were found as homologous to human proteins. All epitopes were kept for further analysis.

Prediction of physicochemical and biological properties

ProtParam server predicts various physicochemical properties based on the amino acid sequence. The total numbers of positively and negatively charged residues were 87 and 36, respectively. The analysis shows a molecular weight of 66 kDa and Isoelectric point (pI) 9.91, which indicates the basic behaviour of the protein. The Instability index (II) was calculated as 35, which infers the stable behaviour of our multi-epitope-based vaccine. The excluded or reduced epitopes due to their collaboration to stability are shown in (Supplementary material – Table 4). The estimated half-life of the vaccine was 30 h to mammalian reticulocytes (*in vitro*), >20 h in yeast and >10 h in *Escherichia coli*, both *in vivo*. The aliphatic index was estimated at 67.36. The higher the aliphatic index, the more stable is the protein in a broad range of temperatures. Protein hydrophilicity was calculated through GRAVY (Grand average of hydropathicity). The negative score of -0.369 represents a hydrophilic molecule able to interact with water molecules present in biological environments. The multi-epitope protein sequence resulting from the selected epitopes, linkers and adjuvant has been submitted to InterProScan. The biological processes, molecular functions and cellular components are listed below and all of them are related to flavivirus virus or eukaryotic cells (Supplementary material – Table 8).

Secondary structure prediction

Based on the amino acid sequence, Psipred online server predicted the secondary structure of our multi-epitope

Table 3. Allergenicity prediction.

Method	Result/Score	Threshold	Prediction
Prediction by mapping of IgE epitope	No IgE epitopes found	NA*	Non-allergen
MAST RESULT	No Hits found	NA*	Non-allergen
Prediction by SVM method based on amino acid composition	-0.82142838	-0.4	Non-allergen
Prediction based on SVM method based on dipeptide composition	-0.60189158	-0.2	Non-allergen
BLAST Results of ARPS	No Hits found	NA*	Non-allergen
Prediction by Hybrid Approach	Non-allergen	NA*	Non-allergen

*NA = not applicable.

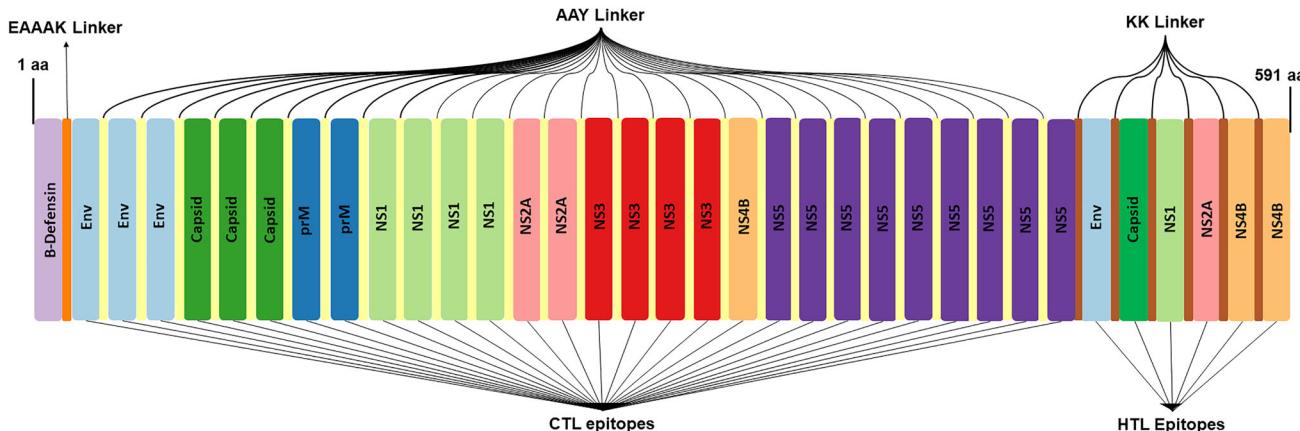


Figure 2. Schematic representation of the multi-epitope vaccine construct containing 591 amino acids. The multi-epitope vaccine sequence is composed of adjuvant sequence (light purple rectangle) at N-terminal end and linked with a multi-epitope sequence with the help of EAAAK linker (orange rectangle). CTL epitopes were connected with AYY linkers (light yellow rectangles) while HTL epitopes were merged with the help of KK linkers (brown rectangles).

vaccine. From the 591 amino acids analysed, 93 (15.73%) amino acids were involved in α -helix formation, 273 (46.19%) amino acids formed β -strands and 225 (38.07%) amino acids were involved in coil formation (Figure 3).

Tertiary structure prediction

RaptorX server predicted the tertiary structure of the multi-epitope vaccine. All the residues were modelled and 1% of regions (9 amino acids) were predicted as disordered. The absolute model quality is measured by overall uGDT (GDT), which was predicted as 148. For proteins bigger than 100 amino acids, uGDT values higher than 50 is a good indicator of the absolute model quality. Another parameter of the predicted structure is the p-value, which evaluates the relative quality of the model. Our model presented p-value of 3.09×10^{-3} , which means the vaccine is the best of a set of randomly-generated models (Figure 4A and B).

Tertiary structure refinement and validation

The predicted tertiary structure from RaptorX was processed by the GalaxyRefine server to improve the model quality. The GalaxyRefine generates 5 refined models. In order to select the best model, all five refined models were analysed through the Ramachandran plots. In the comparison (Supplementary Material – Table 6) to select the best model, GalaxyRefine refinement parameters were considered. As a result, Model 1 had 93% of residues in favoured regions and presented RMSD of 0.482, 0.9 as poor rotamers, GDT-HA of 0.9361, clash score of 20.8 and MolProbity of 2.258. Model 1 was selected as the best 3D structure and RAMPAGE online server was used to plot the Ramachandran plot. The Ramachandran plot obtained

from the original model from RaptorX has 91.3%, 6.5% and 2.2% of residues in favoured, allowed and outlier regions, respectively (Figure 4C). On the other hand, after relaxation methods from the GalaxyRefine server, 93.5% of residues were in favoured regions, 5.6% under allowed regions and only 0.8% in disallowed regions (Figure 4D).

Discontinuous B-cell epitope prediction

Conformational B-cell epitopes were identified in the final construction using the online web server ElliPro. Conformational B-cell epitopes were predicted from the final refined model of the protein vaccine. Eight conformational B-Cell epitope residues were identified at (0.5) threshold and are shown at (Supplementary material – Table 7).

Molecular docking of vaccine with TLR-2 immune receptor

TLR-2 and vaccine docking was performed by the SwarmDock server (Torchala et al., 2013). The 10 best-ranked models were chosen for analysis. After comparing all 10 complexes, the best-docked conformation was considered based on minimum energy values (-46.04) and the highest number of hydrogen bonds in frustrated regions of TLR-2 (18/24). The best-docked model of TLR-2 and vaccine complex is shown in (Figure 5). Furthermore, this vaccine-receptor complex was used for molecular dynamics.

Molecular dynamics simulation of the receptor-ligand complex

The stability of the interaction at the microscopic level of docked complex (TLR-2) and the multi-epitope-based vaccine

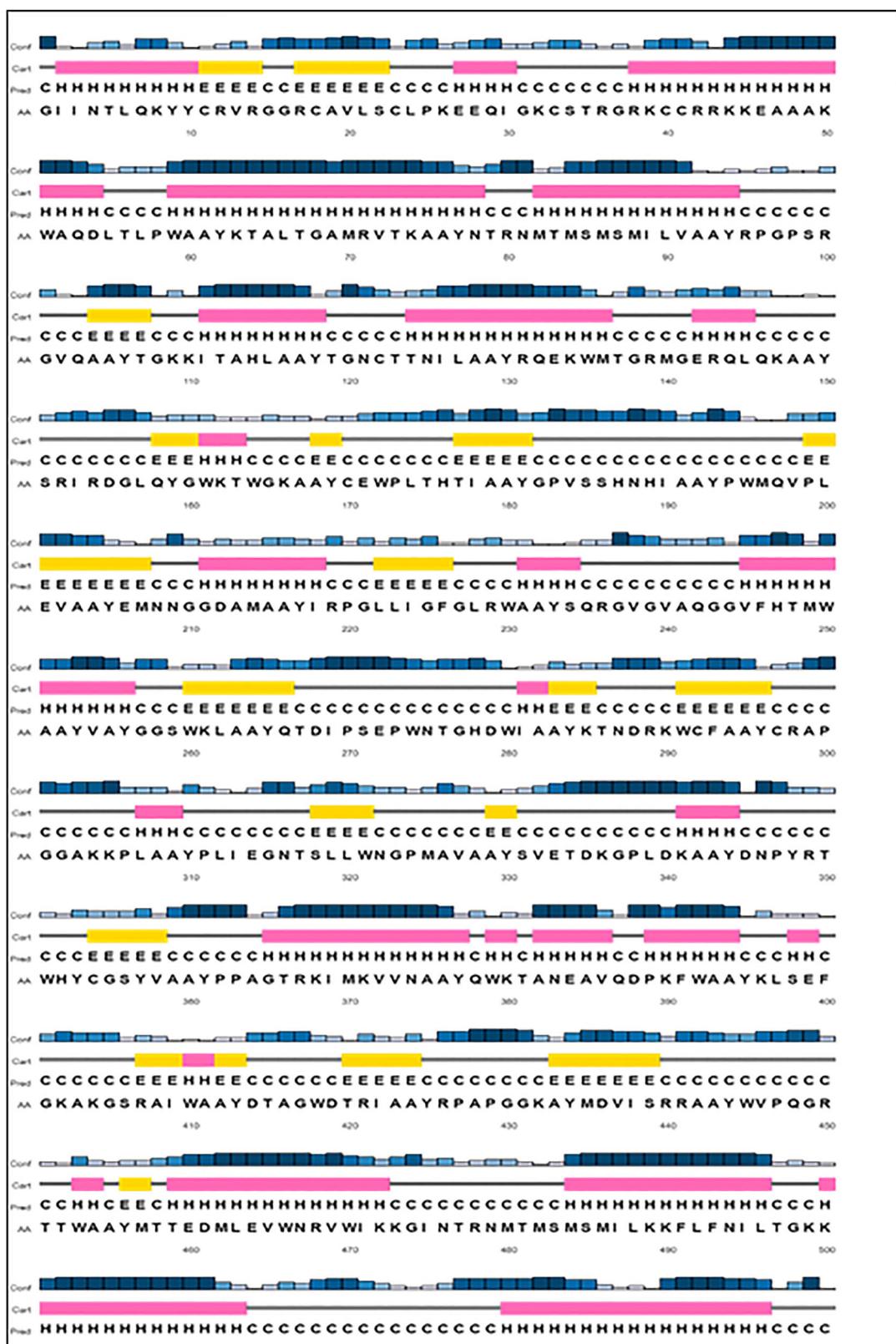


Figure 3. Schematic representation of secondary structure prediction of the multi-epitope vaccine. It shows fractions of α -helix (15.73%), β -strands (46.19%) and coils (38.07%) secondary structures.

was assessed by molecular dynamics using Gromacs 5.0 software. After energy minimization, equilibration of the system by temperature and pressure, the results were analysed using RMSD and RMSF.

The pressure plot indicated a fluctuation in pressure around 0.5 bar (Figure 6A) with 100 ps of the time interval. The temperature plot shows that the system maintained 300 K (Figure 6B) at the same time interval.

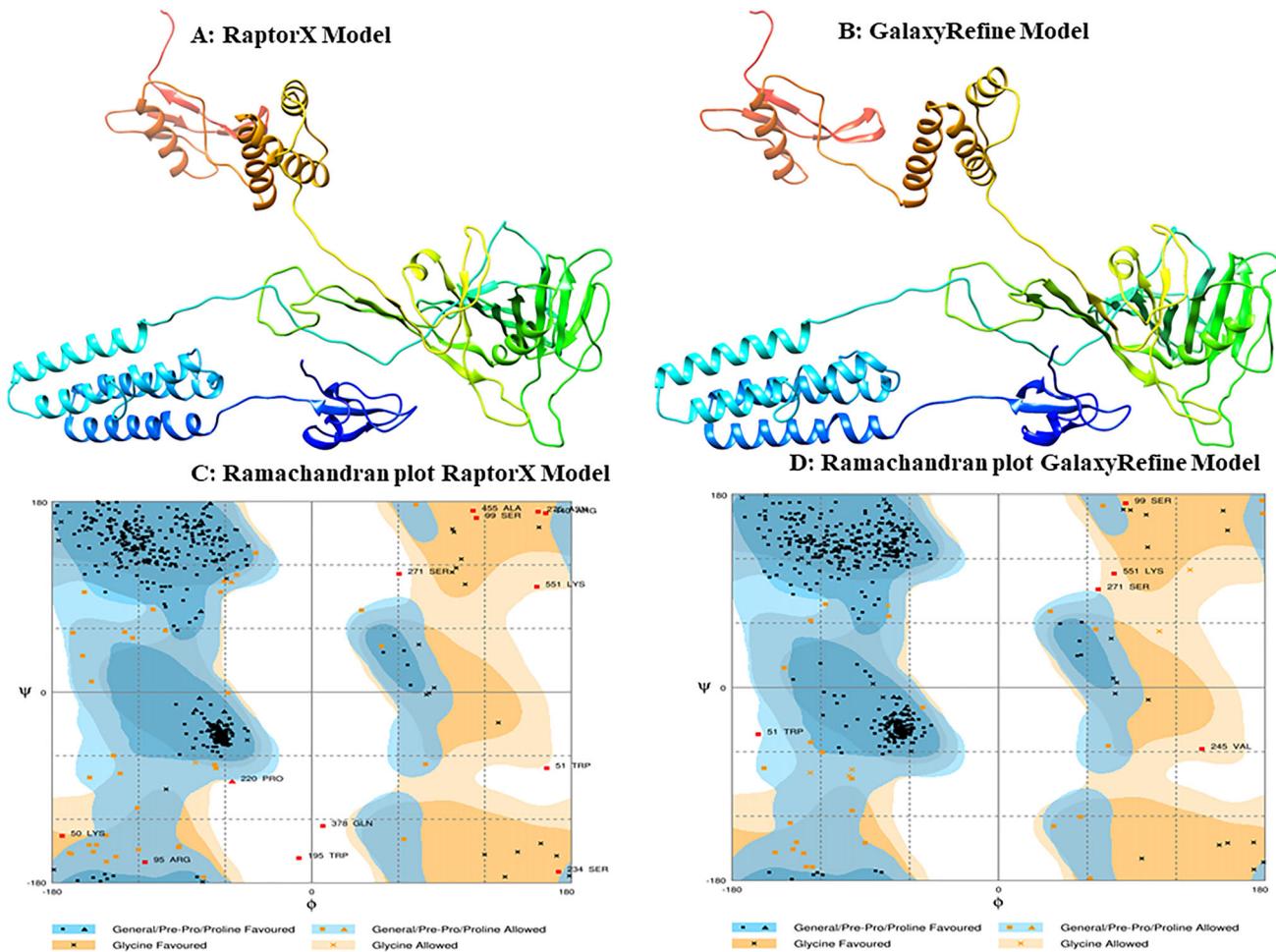


Figure 4. Multi-epitope vaccine modelling and refinement. (A) Represents the tertiary structure of the multi-epitope vaccine generated by the RaptorX server. (B) Represents the refined tertiary structure of the multi-epitope vaccine obtained from GalaxyRefine server. Validation of multi-epitope vaccine tertiary structure by Ramachandran plot: (C) RaptorX model: 91.3% of residues lie under favoured regions, 6.6% of residues fall under allowed regions and only 2.2% in outlier regions. (D) GalaxyRefine model: 93% residues were found in favoured region; 5.6% residues were found in allowed region and 0.8% residues were in outlier region.

The ligand-receptor interaction was further analysed by the root mean square deviation (RMSD) of ligand (multi-epitope vaccine) and receptor (TLR-2), which reflects the structural stability of the complex. A plot of RMSD against time points out a fluctuation ranging from 0.20 nm to 1.5 nm after 10 ns of time interval (Figure 6C). This mild fluctuation demonstrates the stability of the receptor-ligand complex at the time interval tested.

To evaluate the fluctuation of amino acid side chains, the Root Mean Square Fluctuation (RMSF) was observed (Figure 6D). A high fluctuation at the plot indicates highly flexible regions in the receptor-ligand complex while mild fluctuations show uninterrupted interaction between receptor and ligand molecules. The plot reveals mild fluctuations of RMSF values around 0.5 nm and higher peaks with RMSF value of 1.25 nm indicating highly flexible regions in the complex.

In silico cloning

Jcat server generated an optimized cDNA sequence which CAI index, calculated as 0.92, lying into the allowed range (0.8–1.0) (Figure 7A). A higher value of CAI indicates a higher chance of increased gene expression. The GC content was

calculated as 56.32% (Figure 7B) which remains in the optimal range (30–70%). *In silico* cloning was performed using SnapGene software, in order to evaluate cloning and expression of multi-epitope vaccine within the expression vector. The vector with the vaccine insert can be seen in (Figure 8).

Discussion and conclusions

Through the identification of immunogenic (B-cell, CD8⁺ and CD4⁺ T-cells) epitopes (Zhang, 2018) and applying a subtractive epitope approach, our analysis resulted in a final set of epitopes able to incite humoral and cellular responses, non-homologous to host proteins and present in circulating strains from a recent outbreak in an endemic region of Minas Gerais, Brazil.

Recently, yellow fever disease is being considered a re-emerging public threat. Outbreaks in endemic and non-endemic areas (Paules & Fauci, 2017), mainly in South America (Faria, Kraemer, Hill, Goes de Jesus, et al., 2018) and Africa (Shearer et al., 2018), but also in Asian countries, such as China (L. Wang, Zhou, et al., 2016), increased the concern about YFV control. Vaccination is highly recommended by WHO in endemic areas, regions affected by outbreaks or

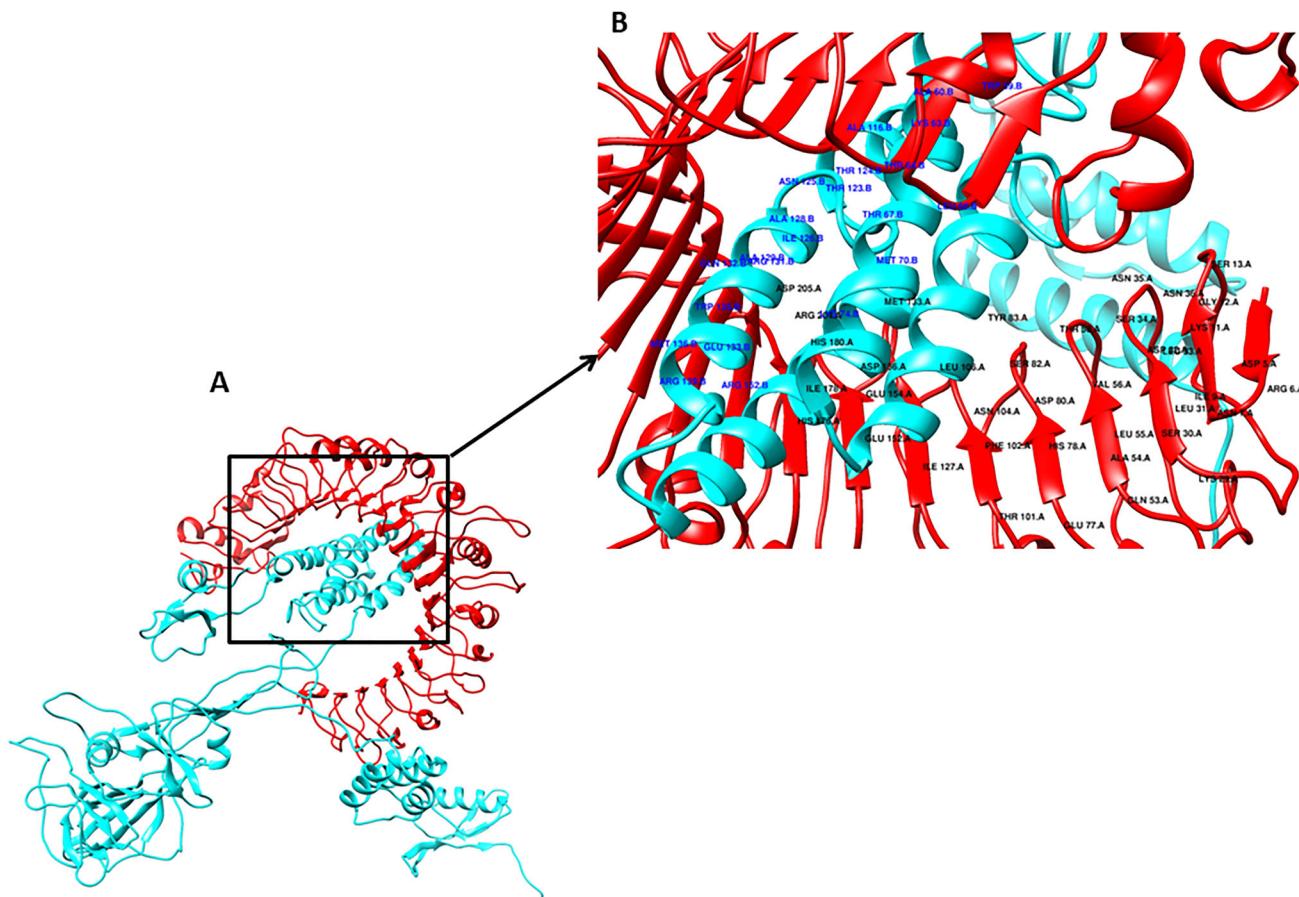


Figure 5. Ligand-receptor docked complex using the Swarmdock server. (A) multi-epitope vaccine (ligand) in cyan colour while TLR-2 (receptor) in red colour, (B) Interacting residues of multi-epitope vaccine (labelled in black) and residues of TLR-2 (labelled in blue).

epidemics, and to travellers across those regions (World Health Organization, 2013). The current YFV vaccines were established on a wild-type YFV isolated in 1927, in Ghana (the Asibi strain). After numerous mutations in its viral non-structural and structural proteins, Asibi strain was turned into 17D vaccine strain. Although considered an efficient vaccine (Lang et al., 1999), attenuated strain 17D is still a live virus with described adverse cases reported in the literature (World Health Organization, 2013), (Lawrence et al., 2004), (Khromava et al., 2005), (Porudominsky & Gotuzzo, 2018), (Marfin et al., 2005), (Tsai et al., 1993), (Nishioka et al., 1998), (Traiber et al., 2011), (Frieden et al., 2010), (Rutkowski et al., 2013).

In the current study, we proposed a multi-epitope vaccine against Yellow fever virus based on two different reference strains - Asibi and 17D vaccine strains. Despite the fact that most human vaccines were developed by classical strategies (Minor, 2015), it still is a costly, time-consuming approach. Reverse vaccinology, proposed by Rappouli (Rappouli, 2001), assumes that information contained in the genome or proteome can be used to select and prioritize antigenic proteins, making it possible to stimulate a protective and lasting immune response. In association with pan-genome analysis (Naz et al., 2019), multi-epitope-based vaccines have been proposed to a variety of pathogens, including parasites (Shey et al., 2019), (Pritam, Singh, Swaroop, Singh, & Singh, 2019), (Pandey, Kumar Bhatt, & Prajapati, 2018), bacteria (Hajighahramani et al., 2017) such as *Neisseria gonorrhoeae* (Barh, Misra, Kumar, & Azevedo, 2010), *Helicobacter pylori* (Khan et al.,

2019), *Klebsiella pneumoniae* (Dar et al., 2019) and mycobacteria (Rana, Thakur, Kumar, & Akhter, 2018) and viruses such as Kaposi's sarcoma-associated herpesvirus (Chauhan et al., 2019), Chikungunya virus (Narula et al., 2018) and Dengue virus (Ali et al., 2017). Interestingly, Malaria RTS,S vaccine (MosquirixTM), which is a set of contiguous epitopes from *Plasmodium falciparum* circumsporozoite protein (CSP), was the first candidate vaccine against malaria to reach phase-III clinical trials (RTS,S Clinical Trials Partnership, 2015).

In the current study, selected CTL and HTL epitopes were spaced by three different peptide linkers (AAY, KK and EAAAK) which have been reported to work as cleavage sites to different sets of immune cells (X. Chen, Zaro, & Shen, 2013), (Velders et al., 2001), (Li et al., 2015) and present stereochemical properties required for protein stability (X. Chen et al., 2013). Short AAY (Ala-Ala-Tyr) linker enhances antigen presentation as they are described as binding sites for TAP transporter proteins and other proteins (Nezafat, Ghasemi, Javadi, Khoshnoud, & Omidinia, 2014; Sabetian et al., 2019). The chance of neoepitope formation increases the larger the size of the multi-epitope sequence. Di-lysine (KK) linkers are non-immunogenic (Livingston et al., 2002) and have been described as enhancers of T cell antigen presentation, as they are cathepsin B cleavage sites (Takahashi, Cease, & Berzofsky, 1989). KK linkers have been used as suitable linker in multi-epitope vaccine investigations (Gu et al., 2017; Yano et al., 2005). The rigid linker EAAAK has been

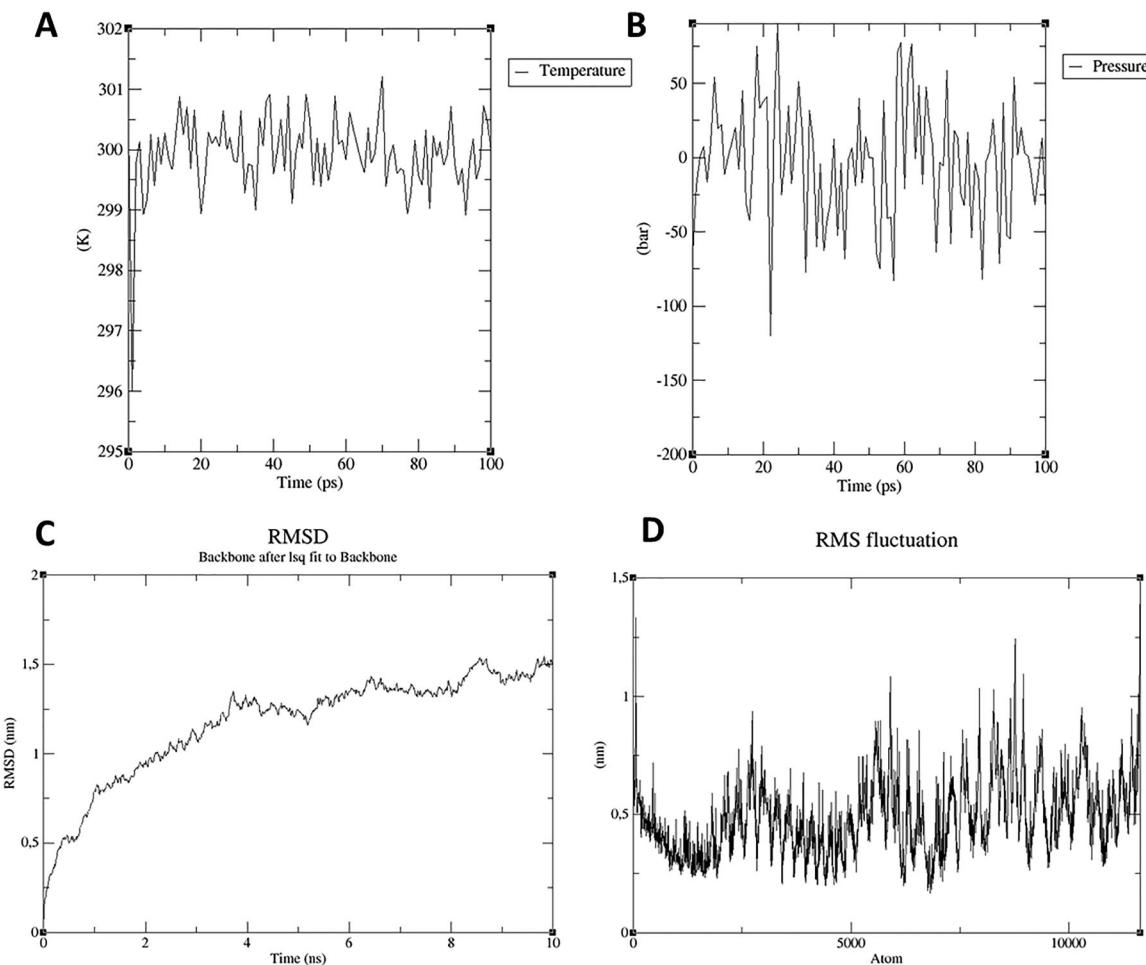


Figure 6. Molecular dynamics simulation of the ligand-receptor complex (vaccine and human TLR-2). (A) The ligand-receptor complex pressure plot shows the fluctuation of pressure during the equilibration phase of 100 ps with an average pressure value of 0.5 bar. (B) The temperature plot of the ligand-receptor complex indicates that the temperature of the system reaches 300 K and remains nearly constant around 300 K throughout the equilibration phase (100 ps). (C) RMSD - Root Mean Square Deviation of the ligand-receptor complex demonstrates no significant deviation, reflecting a stable microscopic interaction between the two molecules and (D) RMSF-RMS fluctuation plot of the ligand-receptor complex reflects the flexibility of side chain of the docked protein complex.

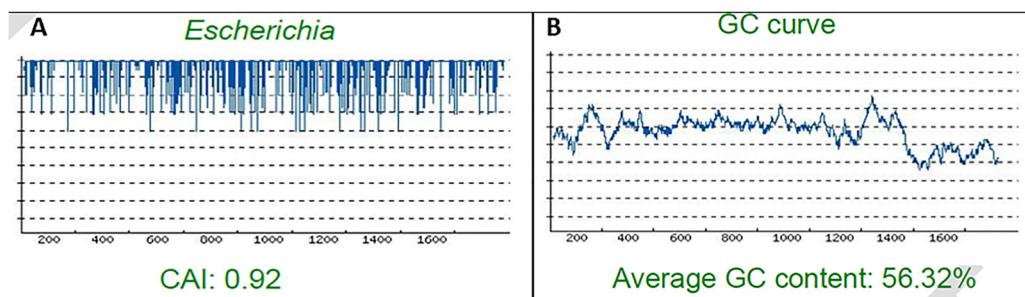
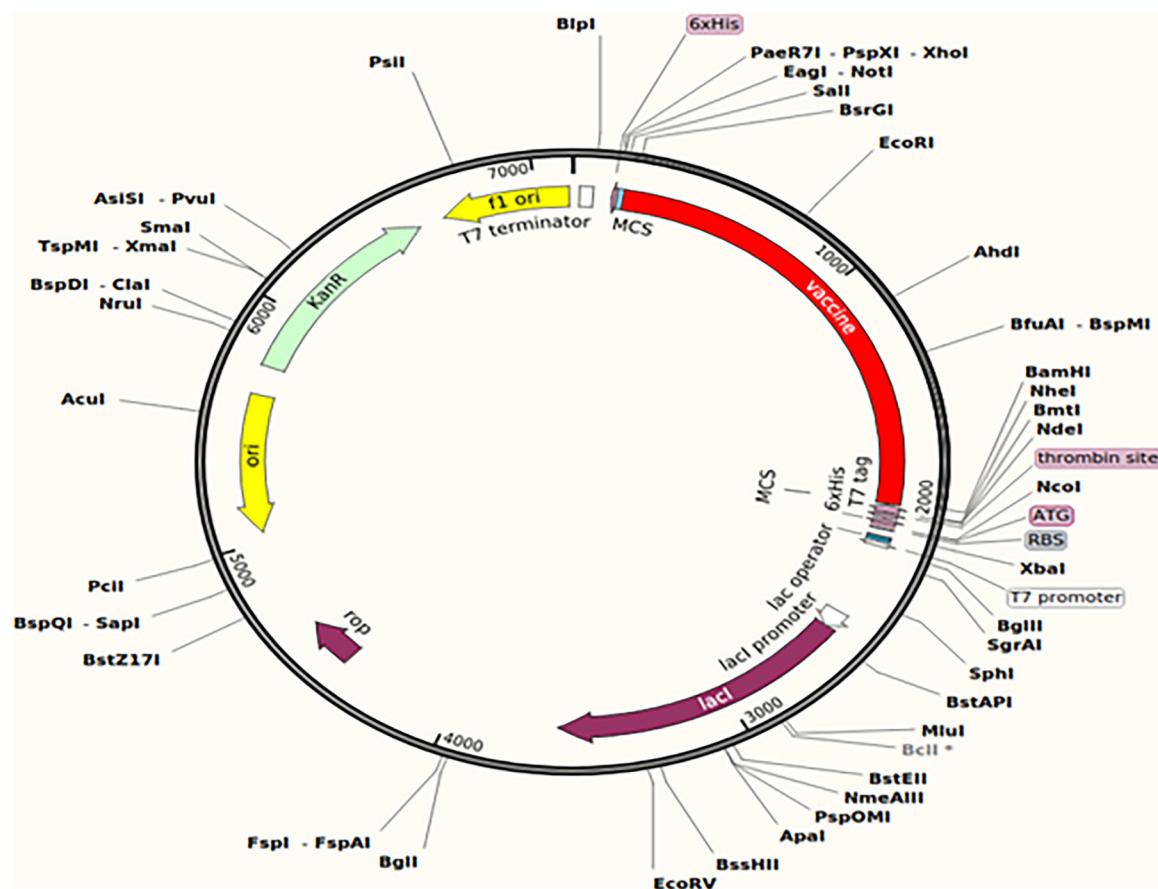


Figure 7. Distribution of codon usage frequency along the length of the multi-epitope cDNA sequence in the *E. coli* and (B) GC content percentage of cDNA originated from multi-epitope vaccine amino acid sequence.

described as capable of effectively separate and decreasing interaction between vaccine domains (here, adjuvant and YFV epitopes) as well as increasing thermal stability of the chimeric protein (H. Chen, Chen, et al., 2017; Saadi, Karkhah, & Nouri, 2017), (H. Chen, Chen, et al., 2017). Finally, adjuvants are substances able of enhancing a specific immune response and assisting the multi-epitope-based vaccine in triggering a high and lasting immune response. β -defensin, a peptide adjuvant was added at the N-terminal region to confer high antigenicity and intensify immune response (Mohan, Sharma, Bhat, & Rao, 2013; Narula et al., 2018).

In immune response process against YFV, it has been described that MHC class II epitopes, which are recognized by CD4+ T-cells, have a central role in protection against YFV (de Melo et al., 2013). As the response to CD4+ T-cells can direct Th1, Th2, and T helper 17 (Th17) cells based on their profile of cytokine secretion, activation of CD4+ T-cells can provide the mixed response described against 17 D vaccine strain (Pulendran, 2009). MHC class I epitopes have been also reported as performing an important role in the immune response against intracellular pathogens, such as viruses (de Melo et al., 2013). Similarly, neutralizing antibodies (mainly IgG type) play a crucial role in the



immune response against YFV, being one of the measures applied to confer long-lasting immune response against YFV. Therefore, the approach of overlapping CTL and HTL epitopes with B-cell epitopes ensure the presence of the different immune responses against the multi-epitope vaccine as described to the live attenuated vaccine 17D.

Although the multi-epitope vaccine proposed was intended as a proper vaccine, it could also function as a vaccine booster in the case of new mutations acquired by YFV. Evaluation of memory T-cell responses and neutralizing antibodies in primary vaccines from 45 days to 13 years after vaccination indicated a decrease in memory responses after ten years of YFV vaccination. The classical memory B-cells, the median PRNT titers and effector memory CD4⁺ and CD8⁺ T-cells were reduced at PVyear10-11 (Campi-Azevedo et al., 2016). The memory responses might be reinforced with boosters, which may include this multi-epitope vaccine as an adjuvant immunomodulating peptide, providing a specific and directed immune response against YFV.

In YFV recognition by the host immune system, TLR-2 is described along with another three Toll-Like receptors (7, 8 and 9) as critical in 17D vaccine and human cell interactions (Pulendran, 2009). The interface between TLR-2 and the multi-epitope vaccine was assessed by protein-protein flexible docking by SwarmDock. The best model (45c) presented 24 hydrogen bonds, where 18 were in highly frustrated regions of the TLR-2 receptor, corroborating protein-protein interaction occurred in previously described binding sites of

TLR-2. TLR-2 can induce both Th1 to Th2 cells and indirectly provides either antibody production or cytotoxic cellular response (Pulendran, 2009). Molecular simulation also revealed a stable structure of the multi-epitope vaccine. Our vaccine was considered a probable antigenic by two servers Vaxigen and ANTIGENpro. As allergenicity is also a characteristic component for a safe vaccine, our multi-epitope vaccine does not contain allergen characteristics according to the AlgPred server. We used robust bioinformatics approaches in this study for the identification of multi-epitope vaccine against Yellow fever virus. We hypothesize that the identified multi-epitope vaccine might be considered for prophylaxis of Yellow fever virus and hence, should be subjected to further experimental validations.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Author on contributor

SFOT, ST conceived, designed the protocol; MS, SFOT, AKJ, RK and AS collected and analysed initial data; SFOT, ST, and MG wrote the paper;

ST, MG, LCJA coordinated and led the entire project: JX revised for English correction, ST, LCJA, SCS, and MG Cross-checked all data and its analysis: All authors read and approved the manuscript.

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