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Research paper

Highly conserved epitopes of DENV structural and non-structural proteins: Candidates for universal vaccine targets

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ABSTRACT

Dengue is a severe emerging arthropod borne viral disease occurring globally. Around two fifths of the world's population, or up to 3.9 billion people, are at a risk of dengue infection. Infection induces a life-long protective immunity to the homologous serotype but confers only partial and transient protection against subsequent infection caused by other serotypes. Thus, there is a need for a vaccine which is capable of providing a life- long protection against all the serotypes of dengue virus.

In our study, comparative genomics of Dengue virus (DENV) was conducted to explore potential candidates for novel vaccine targets. From our analysis we successfully found 100% conserved epitopes in Envelope protein (**RCPTQGE**); NS3 (**SAAQRRGR**, **PGTSGSPI**); NS4A (**QRTPQDNQL**); NS4B (**LQAKATREAQKRA**) and NS5 proteins (**QRGSGQV**) in all DENV serotypes. Some serotype specific conserved motifs were also found in NS1, NS5, Capsid, PrM and Envelope proteins. Using comparative genomics and immunoinformatics approach, we could find conserved epitopes which can be explored as peptide vaccine candidates to combat dengue worldwide. Serotype specific epitopes can also be exploited for rapid diagnostics. All ten proteins are explored to find the conserved epitopes in DENV serotypes, thus making it the most extensively studied viral genome so far.

1. Introduction

Dengue virus or as commonly called DENV is a single stranded RNA virus that infects approximately 390 million people each year (Achee et al., 2015), putting more than two-fifth of the world's population under the threat of this efficacious virus. The dengue fever has, thus become one of the most widespread disease (Sukhralia et al., 2018). The virus belongs to the family *Flaviviridae* and genus *Flavivirus* (Westaway et al., 1985; Back and Lundkvist, 2013). DENV is an arbovirus, having two known mosquito vectors *Aedes aegypti* (Gratz, 1999) and *Aedes albopictus* (Lambrechts et al., 2010). The positive stranded RNA genome of dengue virus is of 10.7 Kb size and composed of three structural proteins (Envelope, Capsid, Membrane) and seven non-structural proteins (NS1, NS2A, NS2B,NS3, NS4A, NS4B, NS5) (Imrie et al., 2010; Guzman et al., 2010; Sukhralia et al., 2018). There are atleast four serotypes and they show 65% similarity in the genome structure (Azhar et al., 2015; Ramanathan et al., 2016).

The dengue infection is caused by one of the four serotypes of DENV that are spread by *Aedes* mosquito (Kalayanarooj, 2011). During primary infection, the body develops immune responses in the form of antibodies against the particular serotype attacked (Schmid et al., 2016). But the main complexity of DENV arises during the secondary infection with another serotype, leading to serious version of dengue infection like Dengue Haemorrhagic fever (DHF) and Dengue Shock Syndrome (DSS) (Dar and Ghosh, 2015; Matheus et al., 2005; Schmid et al., 2016). This is caused due to the antibodies produced during primary attack which complicate the secondary DENV infection by a phenomenon known as Antibody Dependent Enhancement (ADE) (Durbin and Whitehead, 2011; Flipse et al., 2016). During ADE, there is a cross reaction between the antibodies of the primary infection and virus of secondary infection such that there is an increased infection in macrophages and monocytes (Whitehorn and Simmons, 2011; Durbin et al., 2010). These challenges bring the importance of an archetypal dengue vaccine which can provide life time immunity against all the serotypes (Thomas, 2011; Russell and Halstead, 2016).

Currently, the vaccine candidates that are under various stages of

Abbreviation list: DENV, dengue virus; NS, non-structural; ADE, Antibody Dependent Enhancement; RdRp, RNA-dependent RNA polymerase; NCBI, National Centre for Biotechnology Information

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clinical trial are the live attenuated viruses (Thomas et al., 2012), chimeric vaccine (Guy et al., 2015), recombinant vaccine with adjuvants (Hertz et al., 2017), reverse vaccinology (Baliga et al., 2018), purified and inactivated virions (Fernandez et al., 2015), subunit proteins and plasmid DNA (Thisyakorn and Thisyakorn, 2014). Among these, live attenuated DENGVAXIA or CYD-TDV, a tetravalent chimeric dengue vaccine (Scott, 2016), developed by Sanofi Pasteur in December 2015, is the first licensed vaccine in some Asian and Latin American countries (Pitisuttithum and Bouckenooghe, 2016; Flaschw et al., 2016). These clinical manifestations caused by the vaccine are ascribed to inefficiency of the vaccine in producing competent T- cells that protect against DENV disease (Kim et al., 2010). Moreover, the vaccine does not encode any non- structural proteins which are required by the virus to evade immune response of the host (Halstead, 2017; Morrison et al., 2012). All these studies imply that a vaccine that is tetravalent and simultaneously prevents antibody- dependent enhancement (ADE) needs to be designed urgently. These concerns led to the need for a relatively new technique of vaccine development i.e. Epitope or synthetic peptide based vaccines. As DENV has both structural and non-structural proteins for its viral activity (Oliveira et al., 2014), conserved epitopes may prove to be useful in designing synthetic peptide based vaccine. This can be easily initiated in today's time, as there is no dearth of information about genome sequences in the databases (Hasan et al., 2013; Sharmin and Islam, 2014).

Our study provides answers to all above mentioned concerns as we could successfully find conserved epitopes in structural as well as non-structural proteins. These conserved epitopes can be used for further analysis and exploited to develop the vaccine against the deadly dengue virus as well as in rapid diagnostics.

2. Materials and methods

2.1. Retrieval of sequences and alignment

A sum total of 23,622 partial sequences of structural and non-structural proteins of dengue virus were retrieved from NCBI (www.ncbi.nih.gov) (Table 1). After retrieving, the sequences were aligned using multiple sequence alignment program CLUSTAL_X (Thompson et al., 1997). These aligned sequence files were then used for further analysis.

2.2. Search for conserved sequences

Sequences were used for detecting the species-specific signature sequences or **motifs**. Motifs were obtained for each serotype of DENV individually using an online tool multiple em for motif elicitation or MEME *Suite* (Bailey et al., 2009). Motifs common for all serotypes were also obtained using the same method. In order to get a maximum number of motifs, the default setting was adjusted from 3 motifs to 10 motifs.

Table 1
Number of sequences for each protein of different serotypes of DENV analyzed for this study.

Proteins	DENV-1	DENV-2	DENV-3	DENV-4
Envelope (E)	500	500	500	500
Membrane (Prm)	1000	1000	1000	250
Capsid (C)	1000	1000	857	285
NS1	1000	1000	797	201
NS2A	100	100	250	100
NS2B	1000	100	500	100
NS3	1000	500	500	100
NS4A	500	800	1000	1000
NS4B	1000	500	500	100
NS5	1000	500	882	100

2.3. Linear B-cell epitope prediction

The motifs were then analyzed for the presence of B cell epitopes. The linear B cell epitopes were found using BCPRED and BEPIPRED tools of immune epitope database with default settings. Bepipred used Hidden Markov model for the prediction of B cell epitopes (Larsen et al., 2006; Potocnakova et al., 2016).

2.4. IEDB analysis

The immunogenicity of each epitope was checked using Kolaskar and Tangaonkar antigenicity method (Kolaskar and Tongaonkar, 1990) with a default threshold value 0.9. Hydrophilicity of the antigenic epitopes, required to check the accessibility were found using Parker Hydrophilicity method (Parker et al., 1986) at a threshold value of 3.448 (Sharmin and Islam, 2014). Epitopes were checked for their surface accessibility using Emini surface accessibility method (Emini et al., 1985) with a threshold value of 1.00. Flexibility and Beta turns were checked using Karplus and Schulz Flexibility (Karplus and Schulz, 1985) and Chou and Fasman Beta-turn methods (Chou and Fasman, 1978) respectively, with a threshold of 1.00 for both (Sharmin and Islam, 2014). Conservancy of epitopes was checked using Epitope Conservancy Analysis tool (Bui et al., 2007).

2.5. 3-D structure prediction

Models of the 3D structures of DENV proteins were downloaded from RCSB PDB server (https://www.rcsb.org/) for mapping the epitopes. Then the location of predicted epitopes in the 3-D model was found using CHIMERA visualization tool (Pettersen et al., 2004). For DENV proteins where no 3D strcture was available, I-TASSER server was used to predict the 3-D structures (Zhang, 2008; Roy et al., 2010). The predicted models were saved in pdb format files, which were later used to generate Ramachandran plot using PDBsum –PROCHECK software (Laskowski et al., 2001). This was done to verify the models generated by I-TASSER.

3. Results

In the present study, comparative genomics was performed on DENV sequences to find novel vaccine targets. For this purpose, all the ten proteins (3 structural and 7 non-structural) of DENV-1, DENV-2, DENV-3, DENV-4 were analyzed individually by retrieving their partial protein sequences and motif analysis was performed by using MEME SUITE. These conserved motif sequences were then used for B-cell epitope analysis (Table 2). For the matter of space and clarity, we are reporting the figures for one of the conserved epitopes in the main text, while the others are presented in the supplementary materials (Figs. 1–3, Supplementary data).

In total 13 epitopes were found which were further checked for antigenicity, hydrophilicity and surface accessibility. The B-cell epitopes on native proteins are generally composed of hydrophillic amino acids on the protein surface that are topographically accessible to membrane-bound or free antibody. These epitopes tend to be located in flexible regions of an immunogen and display site mobility of epitopes which maximizes complementarity with the antibody's binding site, permitting an antibody to bind with an epitope that it might bind ineffectively if it were rigid. Surface accessibility and hydrophilicity of these predicted epitopes was therefore determined (Table 3). The flexibility of these epitopes and the presence of beta turn in their structure were also evaluated. Predicted epitopes of Envelope, NS1, NS3, NS4A, NS4B and NS5 proteins were found out to be antigenic as their score was found out to be higher than the threshold value whereas, few antigens were found to be non-antigenic (Table 3). All the predicted epitopes were found to be flexible in nature (Table 3).

Table 2
Epitopes found within the conserved motif region.

PROTEIN	NO.OF EPITOPES	PREDICTED EPITOPES IN CONSERVED MOTIFS
E	2	HGTIATI**TPQAPTS**E **TRCPTQGE**PYLKEEQDQQYICRRDVVDRGWGNGCGLFGKGGVVTCAKFSC
PrM	1	DIDCWCNLTSTWVTYGTCNQAG**EHRRDKRS**VALAPHVGMGLDTRTQTWM
Capsid	1	GQGPMKLVMAFIAFLRFLA**IPPTAGIL**ARWGSFKKNGAIKVLRGFKKEIS
NS1	2	WPKSHTLWSNGVLESEMIPKSYAGPISQHNYRP**GYHTQTA**GPWHLGKLE RPQPMELKYSWKTWGKAKIVTAEVQNSTFLIDG**PNTPECPNA**SRAWNSWE
NS3	2	GKNPKNFQTMPGTFQTTTGEIGAIALDFK**PGTSGSPI**INR EGKVVGLYGN PRRCLKPVILKGPERVILAGPMPVTVA**SAAQRRGR**IGRNQNKEGD
NS4A	1	CVMASSVLLWMASVEPHWIAASIILEFFLMVLLIPEPDR**QRTPQDNQL**AY
NS4B	1	LVAHYAIIGPG**LQAKATREAQKRA**AAGIM
NS5	3	**WHYDQDHPYKT**WAYHGSYETKQTGSASSM MYFHRRDLRLAANAICSAVP**SHWVPTSRTT**WSIHAKHEWMT YQNKVVRVQRPAKNGTVMDVISSRD**QRGSGQV**GTYGLNTFTNMEAQLIRQ

Epitopes (marked in blue) found within the conserved motif region.

3.1. Conserved epitope in RNA-dependent RNA polymerase (RdRp) of DENV

Among all the proteins of dengue virus, RNA-dependent RNA polymerase (RdRp) is encoded by non-structural gene NS5 and consists of 20 important enzymatic activities needed for viral propagation. Studies have been conducted on RdRp for designing drug against dengue virus as it is known to be the largest and highly conserved protein (Noble et al., 2010; Noble et al., 2013; El Sahili and Lescar, 2017; Lim et al., 2015). Conserved motif 'YQNKVVRVQRPAKNGTVMDVISSRDQRGSGQVGTYGLNTFTNMEAQLIRQ' was found in NS5 (Fig. 1), which when inspected for presence of potential epitope resulted in 'QRGSGQV' (Table 2). Linear B-cell epitope prediction, Antigenicity prediction, Surface accessibility prediction, Hydrophilicity prediction and flexibility prediction of this region (Fig. 2A–E) showed 'QRGSGQV' as the most desirable epitope. The epitope was further checked for conservancy using IEDB Conservancy analysis and was found to be 100% conserved in all serotypes of DENV (Table 3), thus making it one of the most efficient epitope for vaccine development based on in silico analysis. PDB protein structure of dengue virus NS5 protein (4V0Q) (Zhao et al., 2015) was used to map this epitope and the epitope was found to be located in beta turn region of the RNA-directed RNA polymerase of DENV structure (Fig. 3), thus making it accessible for interaction.

3.2. Highly conserved epitope 'RCPTQGE' on structural protein - Envelope

As stated above, there are three structural and 7 non-structural proteins in DENV polyprotein. Envelope protein is highly variable and is notably displayed on the surface of DENV (Modis et al., 2005), thus making it the crucial candidate for vaccine development (Keasey et al., 2018). In our study, we could find two epitopes in Envelope protein (Table 2), but among them, epitope 'RCPTQGE' was found not only antigenic and surface accessible, but was also 100% conserved in all serotypes of DENV (Table 3, Supplementary Figs. S1–S3). On mapping this epitope on 3D structure of Envelope protein CryoEM structure of Dengue virus envelope protein heterotetramer (pdb id 3J2P), it was found to be present in beta turn region and therefore, is accessible for interaction, making it yet another highly suitable candidate for vaccine target. As the structural proteins are highly variable, therefore it was difficult to find any conserved epitope for capsid and PrM proteins.

3.3. Other highly conserved epitopes of DENV serotypes

To our surprise, four more predicted epitopes (other than the conserved epitopes of NS5 and envelope proteins) were found to be 100% conserved i.e., NS3 (**SAAQRRGR**, **PGTSGSPI**); NS4A (**QRTPQDNQL**); NS4B (**LQAKATREAQKRA**) and in all DENV serotypes (Table 3).

The three dimensional visualization of predicted epitopes of NS4A and NS4B protein was performed using I-TASSER server (Supplementary Figs. S12, S15) as the pdb structure was not available for these proteins. The predicted structure of protein sequences was validated by plotting Ramachandran graph using PROCheck software (data not shown). Remaining epitopes were found to be serotype-specific (Table 3) and hence 3D structure prediction and validation was not performed for these epitopes.

Fig. 1. Motif 'YQNKVVRVQRPAKNGTVMDVISSRDQRGSGQVGTYGLNTFTNMEAQLIRQ' containing the epitope 'QRGSGQV' as 100% conserved region in all sequences of protein NS5.

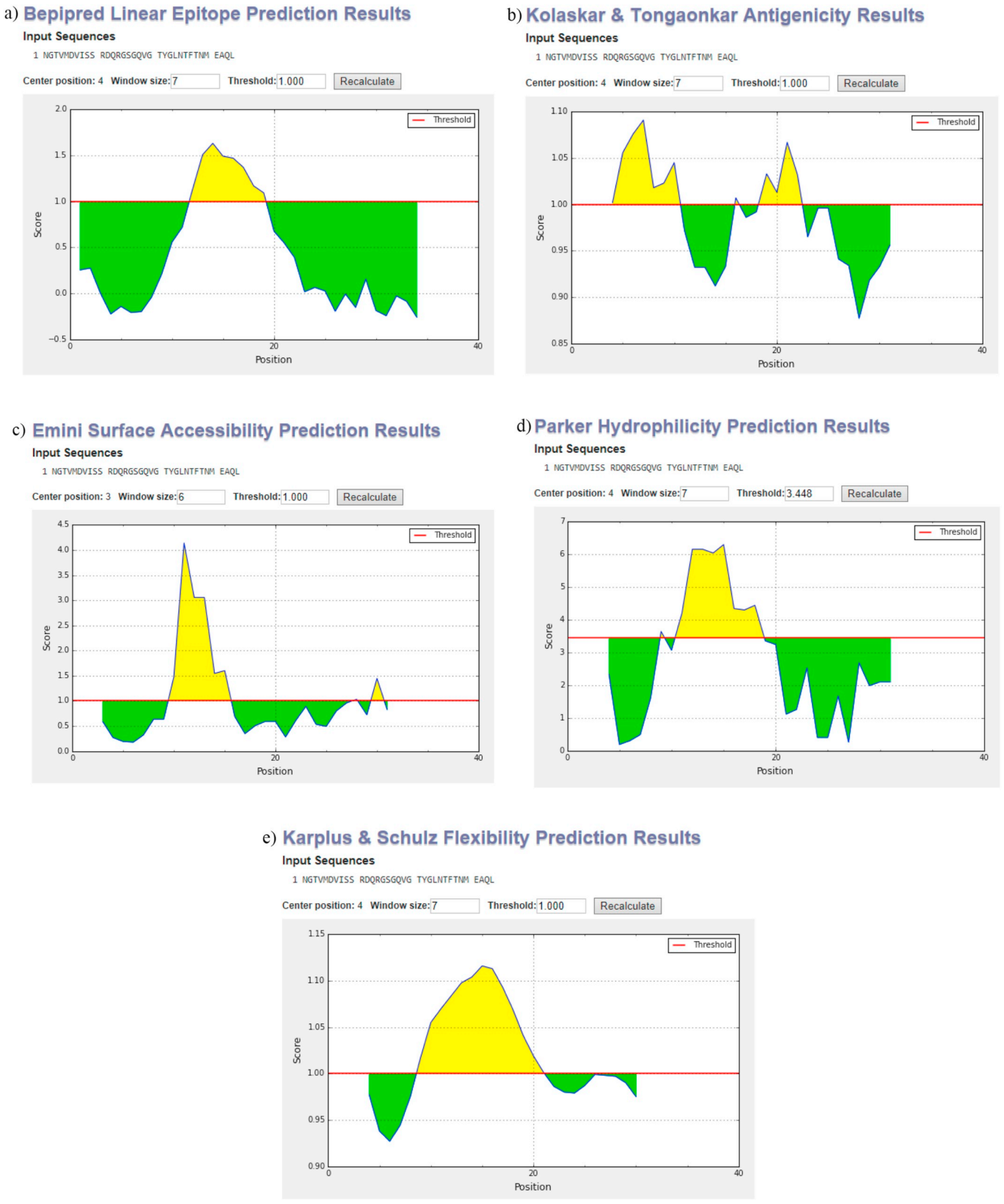

Fig. 2. (A) Linear B-cell epitope prediction, (B) Antigenicity prediction, (C) Surface accessibility prediction, (D) Hydrophilicity prediction, (E) flexibility prediction graphs of epitope 'QRGSGQV' of NS5. The graphs were plotted between the score for predicted epitope and the position of epitope in protein sequence. The red line represents the threshold value used for epitope prediction. The yellow region indicates the possible region of B-cell epitope in the protein sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

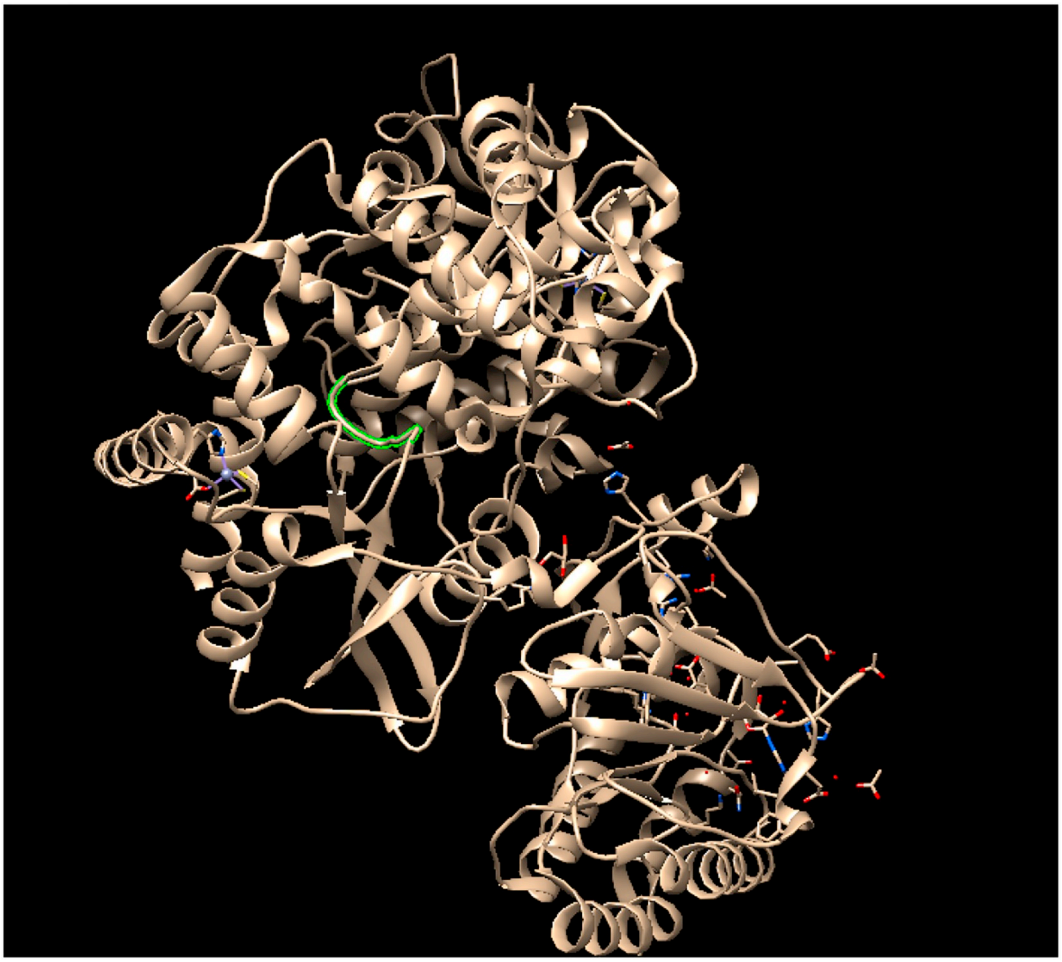

Fig. 3. Three-dimensional visualization of predicted epitope 'QRGSGQV' of NS5 protein. PDB protein structure of dengue virus NS5 protein (4V0Q) was used to map 100% conserved epitope 'QRGSGQV' using the Chimera Visualization tool and the epitope was found to be present on beta turn and surface accessible.

3.4. Serotype-specific conserved epitopes of DENV

For developing a successful vaccine candidate, epitope common in all the serotypes is a perquisite. But for developing serotype-specific drugs and in diagnostics of serotype specific strains, serotype-specific conserved epitopes may prove to be useful. Hence, epitopes like 'TPQAPTS' of Envelope protein (specific for DENV-1 serotype); Capsid protein epitopes 'EHRREKRS' for DENV-2 and 'ERRREKRS' for DENV-4; NS1 epitope 'GYH/F/ATQT/IA' and 'PN/D/ETP/S/AECPN/SA/E/T'; NS5 epitope 'WHYDQDHPYKT' in DENV-2 and 'SHWV/FPTSRTT' epitope may be exploited for developing diagnostic kits for early detection. Among the above reported serotype specific epitopes, both the epitopes of NS1 are potential candidates for distinguishing one serotype from other.

4. Discussion

Dengue is one of the most rapidly spreading mosquito-borne viral disease (WHO Dengue factsheet, 2016) and has emerged as one of the biggest threats to public health (Gubler, 2012; Thisyakorn and Thisyakorn, 2014; Artpradit et al., 2013). There are four serotypes of DENV which show 65% similarity at genomic level and share same epidemics. Yet the difference in their interactions with antibodies is enough to make the development of a common vaccine, a mammoth task (Lalla et al., 2014). The urgent need of dengue vaccine development is presented by the alarming rise in the dengue endemics (Deen, 2016; Wichmann et al., 2017). Although various vaccines are undergoing clinical trials worldwide but presently, there is no specific dengue therapeutics or vaccine available which provides overall protection against this viral infection. Recently, the only licensed DENV vaccine Dengvaxia, has been reported as a failure as it lacks DENV non-structural protein antigens, and is found to be incompetent to raise antibodies against NS1 (Halstead, 2017).

In the present study, Comparative Genomics and immunoinformatics was used as a tool to explore the potential candidates of dengue virus to find novel drug and vaccine targets. The advantage of development of an epitope based vaccine over other vaccines is its ability to induce specific immune response without undesirable effects. Also, both time and expenditure needed to screen a large number of epitopes can be saved using such computational approaches (Davidson and Doranz, 2014). Similar studies have been conducted on Human *Coronavirus* (Sharmin and Islam, 2014), *Saint Louis encephalitis virus* (Hasan et al., 2013), Rotaviruses (Morozova et al., 2018), H1N1 influenza A virus strains (Baratelli et al., 2017) and Zika virus (Dos Santos Franco et al., 2017), however, the analysis has been conducted for only some selective proteins. Therefore our study is the first one to deal with all the proteins of any viral genome for designing an efficient vaccine.

To achieve our objective, conserved epitopes have been obtained from both structural and non-structural proteins. We could successfully found six highly conserved epitopes, one from structural protein and other five from non-structural proteins. Among structural proteins, only Envelope protein showed 100% conserved epitope (**RCPTQGE**), indicating this as a good target for vaccine designing. Other structural proteins like PrM and Capsid did not show any conservancy among the

Table 3
B-cell epitope analysis.

Protein	Peptide sequence	Antigenicity prediction score	Hydrophillicity prediction score	Surface accessibility prediction score	Flexibility prediction score	Beta turn prediction score	Inference	Conservancy
THRSHOLD SCORE→		0.9	3.448	1.000	1.000	1.000		
E	TPQAPTS	1.005 Antigenic	4.171 Hydrophillic	2.599 Surface accessible	1.061	1.147	Epitope	Conserved only in D1
	RCPTQGE	1.00 Antigenic	4.629 Hydrophillic	1.0 Surface accessible	1.085	1.129	Epitope	100% Conserved
Capsid	IPPTAGIL	1.066 Antigenic	−1.0 Non-hydrophillic	0.408 Surface accessible	1.018	0.969	Non-epitope	IPPTAGVL in D3
PrM	EHRRDKRS	0.923 Antigenic	5.588 Hydrophillic	7.594 Surface accessible	1.035	1.055	Epitope	EHRREKRS in D2 ERRREKRS in D4
NS1	GYHTQTA	1.005 Antigenic	3.486 Hydrophillic	1.824 Surface accessible	1.038	1.03	Epitope	GYHTQIA in D2 GYFTQTA in D1 GYATQTA in D4
	PNTPECPNA	1.02 Antigenic	4.657 Hydrophillic	1.033 Surface accessible	1.053	1.293	Epitope	PNTPECPSA in D3 PDTSECPNE in D4 PETAECPNT in D2
NS3	SAAQRRGR	0.968 Antigenic	4.400 Hydrophillic	2.371 Surface accessible	1.093	1.156	Epitope	100% conserved
	PGTSGSPI	0.973 Antigenic	5.343 Hydrophillic	1.467 Surface accessible	1.130	1.426	Epitope	100% conserved
NS4A	QRTPQDNQL	0.992 Antigenic	5.643 Hydrophillic	3.674 Surface accessible	1.069	1.080	Epitope	100% conserved
NS4B	LQAKATREAQKRA	1.037 Antigenic	5.029 Hydrophillic	4.181 Surface accessible	1.044	1.126	Epitope	100% conserved
NS5	WHYDQDHPYKT	1.034 Antigenic	4.857 Hydrophillic	3.436 Surface accessible	1.026	1.236	Epitope	100% conserved in D2 only
	SHWVPTSRTT	1.008 Antigenic	3.529 Hydrophillic	2.635 Surface accessible	1.081	1.04	Epitope	SHWFPTSRTT in D4
	QRGSGQV	1.007 Antigenic	4.343 Hydrophillic	1.395 Surface accessible	1.113	1.137	Epitope	100% conserved

predicted epitopes. This was expected as the structural proteins are known to be highly variable even within serotypes (Modis et al., 2005). The predicted epitopes of Envelope protein (**RCPTQGE**); NS3 (**SAAQRRGR**, **PGTSGSPI**); NS4A (**QRTPQDNQL**); NS4B (**LQAKATREAQKRA**) and NS5 proteins (**QRGSGQV**) were found out to be antigenic, hydrophillic, surface accessible, flexible and consists of beta turn. Thus, these epitopes fulfill all the pre-requisite conditions of a desired epitope. Moreover, the functions of these proteins can be used as an asset for controlling the effect of the virus on the host. For instance, NS1 is an important cofactor for the formation of replication complex, and has also been exploited for the development of vaccine (Mackenzie et al., 1996; Hertz et al., 2017) whereas NS4B regulates the helicase activity of NS3 and NS4A-NS4B complex is required for genome replication (Miller et al., 2006; Umareddy et al., 2006). NS5 is the most conserved protein of DENV and its C-terminal containing RdRp (RNA dependent RNA polymerase) domain is crucial for genomic replication (Hannemann et al., 2013: Ashour et al., 2009). Therefore, we can say that each of these proteins are essential for replication of the virus inside the host and therefore, they can be used as common vaccine target for all the four serotypes of Dengue virus. Serotype specific conserved epitopes can also be exploited for detection of Dengue at early stages. During the Phase IIb and III trials, DENGVAXIA's specific response against DENV2 serotype largely became negligible. So, a vaccine developed using these epitopes could overcome this problem as these predicted epitopes are found to be conserved in all four serotypes of DENV.

5. Conclusion

Dengue Virus (DENV) has emerged as a potential threat to human health worldwide. The therapeutics against DENV are either not available in major parts of the world or if available in some endemic countries, are found to be inefficient. In this study we used computational approaches to find novel vaccine targets. It focuses to explore B-cell epitopes for all serotypes for each protein of dengue virus. We found six highly conserved epitopes in all serotypes of DENV [Envelope protein (**RCPTQGE**); NS3 (**SAAQRRGR, PGTSGSPI**); NS4A (**QRTPQDNQL**); NS4B (**LQAKATREAQKRA**) and NS5 proteins (**QRGSGQV**)]. Thus, our results suggest any of these proteins can be targeted to stimulate a specific immune response against all serotypes of DENV. These predicted epitopes would be the candidate target for the universal multi-subunit vaccine.

Nevertheless, further studies are needed to confirm the utility of these epitopes.

MV and RL conceptualized the work; MV, SB, KK, NM, SS,SG curated data; formal analysis was done by MV, PSD; MV, PSD were involved in fund acquisition and project administration; MV supervised the work; MV. SB, KK were involved in writing original draft; Review and editing was done by MV, PSD, RL.

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Ethical approval

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' information

This work is performed by the undergraduate students at Sri Venkateswara College, University of Delhi.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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