

Research Article



Construction of Envelope Domain III Based Recombinant Tetravalent Dengue Vaccine

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ABSTRACT

Dengue virus (DENV) belongs to the Flavi virus genus of the Flaviviridae family which are capable of causing self-limited dengue fever (DF) or even life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). More than 125 countries are known to be dengue endemic. The development of dengue tetravalent vaccine is the most challenging of all because infection with any of the four dengue virus serotypes confers lifelong homotypic immunity but subsequent infection with a different serotype leads to more complicate and life-threatening clinical conditions such as DHF and DSS. In the present study, we aim to fuse ED III region of all four dengue virus serotypes into a mammalian expression vector to make a single recombinant tetravalent ED III dengue vaccine construct for its subsequent use as a novel vaccine candidate because it is exposed to the surface and thus becomes the primary target for antibody-mediated neutralization. Therefore, we constructed recombinant tetravalent dengue vaccine by fusing ED III region of all four dengue virus serotype by PCR amplification using overlapping primers having 16–22 bp homologies at their 5' ends than the routine method of using restriction endonuclease digestion and ligation and cloned into mammalian expression plasmid (pVAC1-mcs) to develop seamless fusion proteins. Here we choose pVAC1-mcs mammalian expression vector for construction of recombinant tetravalent dengue vaccine as it is a DNA vaccine vector which is specially designed to stimulate humoral immune response. Cloning was confirmed by colony PCR and also by restriction endonuclease enzyme digestion. The sequence was submitted to the GenBank (Accession Number: KF855114). Protein expression was tested in 293T cells by using immuno fluorescence assay. We have successfully attempted the construction of recombinant tetravalent dengue envelope domain III as a novel vaccine candidate. In future we can use this construct to study the efficacy in mice.

Keywords: Dengue, Domain III, Envelope, HEK293T cells, Recombinant, Tetravalent vaccine.

INTRODUCTION

Dengue virus (DENV) belongs to the Flavivirus genus of the Flaviviridae family. DENV is transmitted to humans mainly by *Aedes* mosquitoes. There are four serotypes of DENV which are capable of causing self-limited dengue fever (DF) or even life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). It is present in every World Health Organization (WHO) region of the world and more than 125 countries are known to be dengue endemic.^{1,2} Annually millions of DENV infections, hundreds of thousands of hospitalizations, and tens of thousands of deaths related to dengue occurs.³ The development of any human vaccine is highly challenging, complex and an expensive process.⁴ There are numerous dengue vaccine candidates in the pre-clinical and clinical development.⁵⁻¹⁰ However, the development of dengue tetravalent vaccine is the most challenging of all because infection with any of the four dengue virus serotypes confers lifelong homotypic immunity but subsequent infection with a different serotype leads to more complicate and life-threatening clinical conditions such as DHF and DSS.⁵ Therefore, a safe and effective tetravalent dengue vaccine that can simultaneously induce a high level, long-lasting immune response against all four serotypes may help to overcome this problem. In the present study, we aim to fuse ED III region of all four dengue virus serotypes into a mammalian expression vector to make a single recombinant tetravalent ED III dengue vaccine construct

for its subsequent use as a novel vaccine candidate because envelope protein becomes the top priority for the researchers involved in vaccine development as it is exposed to the surface and thus becomes the primary target for antibody-mediated neutralization. Domain III of envelope protein (EDIII) contains an immunoglobulin-like structure and is found to be involved in host cell binding.¹¹ In addition it possesses multiple and serotype specific neutralizing epitopes making it a suitable candidate for vaccine development.¹²⁻¹⁵ Neutralizing antibodies produced against domain III may block the entry of the virus into the cell.¹¹

MATERIALS AND METHODS

Dengue virus propagation

All the procedures were conducted in accordance with guidelines under animal protocols approved by the Institutional Animal Ethics Committee (Ethics Committee Approval No. HITRT/IEC/05/2011 dated 17th January 2011).

Dengue virus serotypes 1 to 4 (Dengue-1 strain P 23086, Dengue-2 strain P 23085, Dengue-3 strain 633798 and Dengue-4 strain 611319) were procured from the National Institute of Virology (NIV), Pune, India and propagated in one to four-day old suckling Swiss Albino mice brains. Briefly, 20µL of virus suspension was inoculated intra-cerebrally and mice were observed daily



for twenty-one days for the appearance of symptoms such as paralysis or difficulty in walking, lethargy, and/or disorientation. Moribund brain tissue was then harvested, homogenized, centrifuged and the filtered supernatant was stored at -80°C until further use.

RNA extraction and cDNA preparation

Viral RNA was extracted using QIAamp[®] Viral RNA Mini kit (Qiagen, India) as per manufacturer's instructions. Eluted RNA was stored at -80°C till used for cDNA synthesis. cDNA was synthesized using BluePrint[™] 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Japan) and stored at -20°C until further use.

Construction of linearized vector

The pVAC1-mcs (InvivoGen, USA), is a DNA vaccine vector specifically designed to stimulate a humoral immune response by intramuscular injection was used for the study. This vector was digested using a mixture of *EcoRI*-HF (New England Biolabs Inc., USA) and *BamHI*-HF (New England Biolabs Inc., USA) restriction enzymes. Briefly, 1.0

$\mu\text{g}/\mu\text{L}$ of vector was linearized using these enzymes by incubating the mixture at 37°C for 2 hrs followed by heat inactivation at 65°C for 20 min. The linearized vector was electrophoresed on a 1.5% agarose gel and stained with SYBR[®] Safe (Invitrogen, India). The plasmid was then extracted and purified using QIAquick[®] Gel Extraction kit (Qiagen, India) and its concentration was determined using NanoVue[™] spectrophotometer (GE Healthcare, UK). The linearized purified vector was stored at -20°C until further use.

Oligonucleotide Design

Dengue envelope domain III (ED-III) primers

Primers used to amplify domain III region of the envelope protein gene of dengue virus serotype 1 to 4 were selected from sequences available at GenBank. The specificity of primers was checked by using NCBI BLAST program (available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences of domain III primers are given in Table 1.

Table 1: Primer sequences for dengue envelope domain III.

Primer Name	Sequence (5' to 3')	Product length	GenBank Reference
D1DIII F	TGG CAG AGA CCC AGC ATG GAA CC	299 bp	JF297577
D1DIII R	CCT TCG TGC TCC TCG GGC GG		
D2DIII F	TCA TAC TCC ATG TGC ACA GGA	306 bp	JQ993227
D2DIII R	GCC GAT AGA ACT TCC TTT CTT		
D3DIII F	ATG AGC TAT GCA ATG TGC TTG	296 bp	JN697379
D3DIII R	CCC TTC CTG TAC CAG TTG ATT TT		
D4DIII F	ATT GAG AAT TAA GGG AAT GTC A	307 bp	JQ915085
D4DIII R	CCT GAA CCA ATG GAG TGT TAA		

Dengue envelope domain III overlapping primers

Overlapping primers having 16 to 22bp homologies at their 5' ends were designed and used for fusion of envelope domain III regions of all four dengue virus serotypes by PCR. Sequences of overlapping primers used in the study are given in Table 2.

Table 2: Overlapping dengue ED III primer sequences used for cloning

Primer Name	Sequence (5' to 3')
D1DIII F-cloning	AAC TCT GCC AGG ATC CTG GCA GAG ACC CAG CAT GGA ACC
D1DIII R-cloning	TCC TGT GCA CAT GGA GTA TGA CCTT CGT GCT CCT CGG GCG G
D2DIII F-cloning	TCA TAC TCC ATG TGC ACA GGA
D2DIII R-cloning	CAA GCA CAT TGC ATA GCT CAT GCC GAT AGA ACT TCC TTT CTT
D3DIII F-cloning	ATG AGC TAT GCA ATG TGC TTG
D3DIII R-cloning	TGA CAT TCC CTT AAT TCT CAA TCC CTT CCT GTA CCA GTT GAT TTT
D4DIII F-cloning	ATT GAG AAT TAA GGG AAT GTC A
D4DIII R-cloning	CAT CAG TGG TGA ATT CCC TGA ACC AAT GGA GTG TTA A

Vector primers

PVAC1-mcs plasmid vector primers were synthesized using the sequences available on manufacturer's website (<http://www.invivogen.com/sequencing-primers>). Sequences of pVAC1-mcs primers have been mentioned in Table 3. All the above mentioned primers were commercially synthesized by M/s Eurofins Genomics India Pvt. Ltd. Bangalore, India.

Table 3: Primer sequences for pVAC1-mcs vector

Primer Name	Sequence (5' to 3')
pVAC1-mcs F	TGT ATA GGA TGC AAC TGC TG
pVAC1-mcs R	GAA ACA AAC AGT TCT GAG ACC G

Amplification of envelope domain III region

Polymerase chain reaction was performed by using ED-III primers (Table 1) for serotypes 1 - 4 ($0.8\mu\text{M}$) using Clontech Advantage HF2 PCR kit (Clontech, USA) as per the manufacturer's instructions. The cycling conditions used were initial DNA denaturing step at 94°C , 5 min followed by 35 cycles of denaturation (94°C , 30s), annealing ($48-60^{\circ}\text{C}$, 30s) and extension (68°C , 30s) and a



final extension step at 68°C for 10 min. The amplified products were electrophoresed on a 1.5% agarose gel stained with SYBR® Safe (Invitrogen, India) and visualized on a gel documentation system (BIO-RAD Molecular Imager® ChemiDoc™ XRS+ with Image Lab™ software, USA). PCR products were gel purified using the QIAquick® Gel Extraction kit (Qiagen, India). The concentrations of purified PCR products were determined by using NanoVue™ spectrophotometer (GE Healthcare, UK). Sequencing of the purified PCR products was outsourced (M/s Ocimum Biosolutions India Pvt. Ltd., Hyderabad) for confirmation of correctly amplified regions.

Tetravalent envelope domain III gene construction

Creation of amplified products having 5' matching ends using overlapping primers in PCR

PCR for dengue serotype 1 - 4 was performed using Clontech Advantage HF2 PCR kit with overlapping primers (Table 2). The master mix preparation and thermal cycling conditions were same as mentioned for amplification of envelope domain III region. PCR products were gel purified using the QIAquick® Gel Extraction kit (Qiagen, India). The concentrations of purified PCR products were determined by using NanoVue™ spectrophotometer (GE Healthcare, UK).

Ligation of envelope domain III amplified products by using overlapping primers

Purified amplified products having 5' matching ends products were then used as template in subsequent amplification reactions for ligation. In order to make a tetravalent envelope domain III gene construct, initially Dengue serotype-1 and Dengue serotype-2 were ligated by PCR using Clontech Advantage HF2 PCR kit, using 0.8 μM of each D1DIII F and D2DIII R overlapping primers. The cycling conditions were initial DNA denaturing step at 94°C, 5 min followed by 35 cycles of denaturation (94°C, 30s), annealing (65°C, 30s) and extension (68°C, 30s) and a final extension step at 68°C for 10 min. Similarly Dengue serotype-3 and Dengue serotype-4 were ligated by PCR using D3DIII F and D4DIII R overlapping primers. Ligated DNA fragments were purified by using the QIAquick® Gel Extraction kit (Qiagen, India). Both the ligated purified products were used as a template in subsequent PCR and further ligated by using D1DIII F and D4DIII R overlapping primers. Ligated DNA fragments were purified by using the QIAquick® Gel Extraction kit (Qiagen, India). The concentration of ligated purified PCR product was determined by using NanoVue™ spectrophotometer (GE Healthcare, UK). Sequencing of the purified PCR products was outsourced (M/s Saf Labs Pvt. Ltd., Mumbai India) for final confirmation. The tetravalent envelope domain III gene construct was then used for cloning reaction.

Insertion of the tetravalent construct into the vector and transformation

Cloning was carried out using Gibson Assembly® Master Mix kit (New England Biolabs Inc., USA) as per the

manufacturer's instructions. Briefly, the cloning reaction mixture (20.0 μL) contained 2X Gibson Assembly Master Mix (10.0 μL), linearized vector, ≈1200bp ligated tetravalent envelope domain III gene insert and PCR grade water. A 2:1 insert: vector molar ratio was used in the reaction. The cloning reaction was incubated for 60 min at 50°C and then placed on ice. A volume of 80.0μL TE Buffer (pH 8.0) was added to the above cloning reaction tube and mixed. Transformation was carried out using GENECOMP XL-1 Blue chemically competent cells using heat shock method, as described by the manufacturer (Genetix Biotech Asia Pvt. Ltd., India). The transformant culture was spread on Fast-media Zeo agar (InvivoGen, USA) containing Zeocin as a selection marker and incubated at 37°C overnight. Controls included competent cells transformed with circular pVAC1-mcs plasmid vector, competent cells transformed with linear pVAC1-mcs plasmid vector, and competent cells control. Zeocin resistant colonies were screened for recombinant clones by colony PCR.

Colony PCR

Colony PCR reactions were performed using 2X Fermentas PCR Master Mix, PCR grade water and 0.8 μM each of vector primers (Table 3). PCR cycling conditions included DNA denaturing step at 94°C, for 5 min followed by 35 cycles of denaturation (94°C, 30 s), annealing (50°C, 30 s) and extension (72°C, 80 s) and a final extension step at 72°C for 10 min. Colony PCR products were electrophoresed on a 1.5% agarose gel stained with SYBR® Safe (Invitrogen, India). A diagrammatic presentation of construction of recombinant tetravalent dengue vaccine shown in figure 1.

Plasmid extraction and RE digestion

Plasmid extraction was performed using Nucleo-pore® SureSpin® Plasmid Mini Kit (Genetix Biotech Asia Pvt. Ltd.) as per the manufacturer's instructions. The purified plasmid was stored at -20°C until further use. The concentration of purified plasmids was determined by using NanoVue™ spectrophotometer (GE Healthcare, UK). The presence of gene insert was confirmed by RE digestion using *EcoRI*-HF RE-mix (New England Biolabs Inc., USA) and *BamHI*-HF (New England Biolabs Inc., USA) restriction enzymes. Sequencing of the purified plasmid was outsourced (M/s Saf Labs Pvt. Ltd., Mumbai India) for final confirmation.

Cell transfection and immunofluorescence assay

To evaluate the protein expression of ED III in the vaccine construct, 293T cells were transfected with vaccine construct or with empty vector by using Xfect™ kit of Clontech Laboratories, Inc. as per the manufacturer instructions using 1μg DNA. At 48 hrs post transfection, cells analyzed by IFA as described previously with modification. Briefly, cells were washed twice with PBS, air dried and fixed with ice-cold acetone: methanol mixture (1:1) and incubated with 1:200 dilution of mouse hyperimmune sera against recombinant clone for 30 min



at 37°C. Positive transfected cells were detected with fluorescein-conjugated goat anti mouse IgG secondary antibody (Sigma Aldrich, USA) under inverted fluorescent microscope (Carl Zeiss, USA).

RESULTS AND DISCUSSION

Amplification of ED III of four dengue serotypes

The dengue ED III primers used in this study as mentioned in Table 1 and 2 successfully amplified ≈300bp fragment of each dengue virus serotype in PCR, which was confirmed by sequencing.

Construction of tetravalent ED III gene insert for cloning

In the present study we used a unique end joining capability of the overlapping primers to fuse ED III of four dengue serotypes than the routine method of using restriction endonuclease digestion and ligation. We designed overlapping primers having 16–22bp homologies at their 5' ends and fused ED III of dengue serotype 1 and 2 by PCR. Similarly ED III of dengue serotype 3 and 4 also fused by PCR to get ≈600bp fragments respectively (Fig 2A). In order to make a tetravalent ED III gene insert for cloning we finally fused above two products by PCR to get ≈1200bp fragment which was confirmed by sequencing.

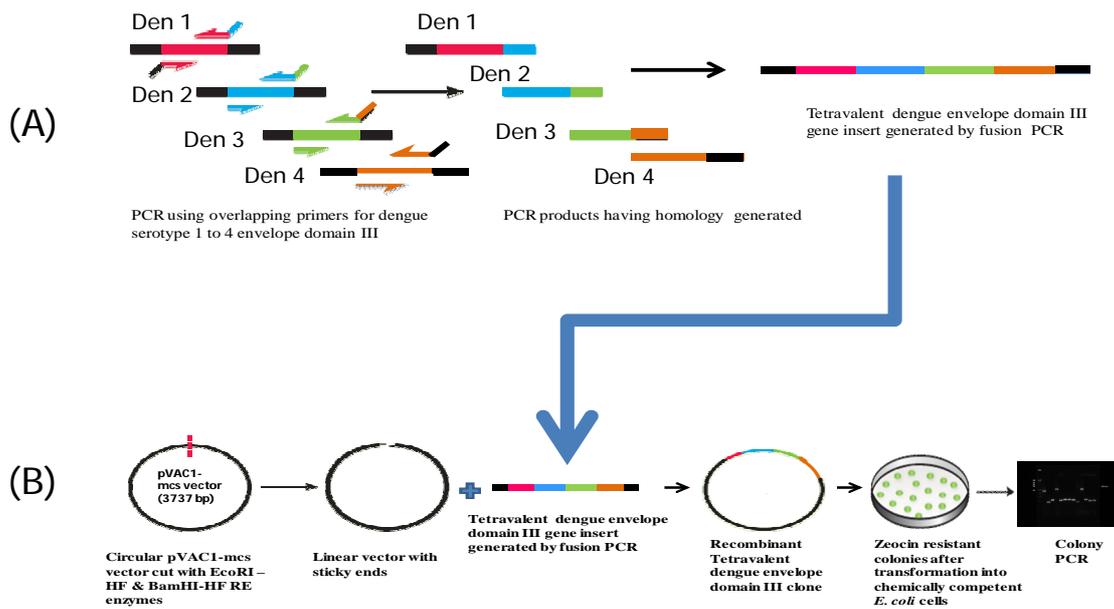


Figure 1: Diagrammatic presentation of (A) construction of tetravalent dengue ED III gene insert and (B) construction of recombinant tetravalent vaccine construct.

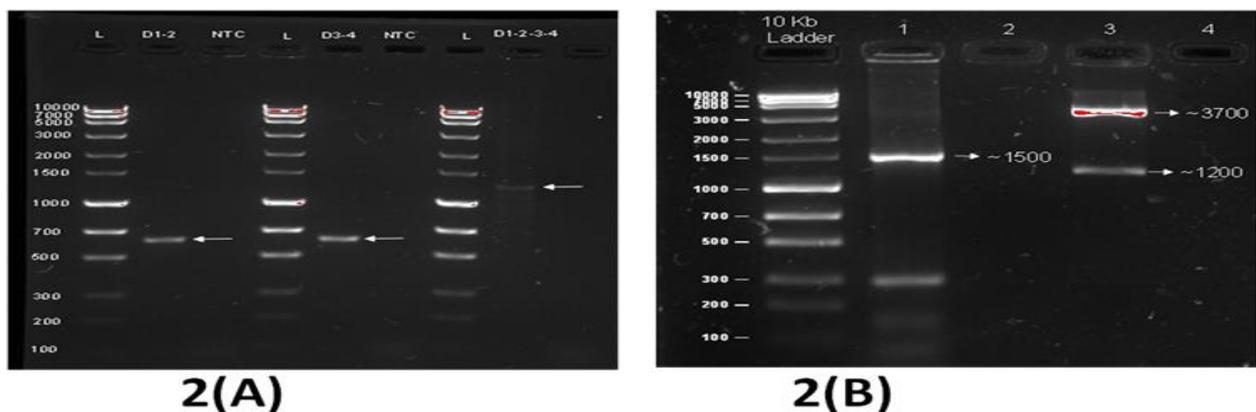


Figure 2: (A) Well marked as L: ladder, D1-2: fusion PCR product of DEN 1 and 2, D3-4: fusion PCR product of DEN 3 and 4, D1-2-3-4: fusion PCR product of DEN 1-2 and DEN 3-4, NTC: No template control and (B) Colony PCR and RE digestion (well marked as 1: colony PCR showing ≈ 1500bp band, well marked as 3: RE digestion of Colony PCR product shows vector band at ≈ 3700bp and gene insert band at ≈ 1200bp, Wells marked as 2 & 4 are empty).

Recombinant tetravalent dengue ED III DNA vaccine construction

To make a recombinant tetravalent DNA vaccine construct we choose pVAC1-mcs mammalian expression vector for cloning because it is a DNA vaccine vector

specifically designed to stimulate humoral immune response. Also it has a strong rhesus monkey elongation factor 1-α (rhEF1-α) promoter that shares 92.9% homology with its human counterpart and displays a similar activity like human EF1- α promoter. Other



features like SV40 enhancer for gene expression in a large host range and presence of IL-2 signal sequence makes it a suitable vaccine vector. It is well known that humoral immune response specifically neutralizing antibodies plays a pivotal role in the prevention of the dengue virus infection. Most of them are targeted against envelope protein particularly against ED III as it was exposed on surface and thus the primary target for neutralizing antibodies. Also it possesses type and subtype specific neutralizing epitopes that elicit only neutralizing antibodies. In dengue infection Antibody Dependent Enhancement (ADE) phenomena has been suggested for DHF and DSS because of presence of other epitopes which elicit cross reactive, non-neutralizing antibodies. These cross reactive, non-neutralizing epitopes are absent in ED III region. So we decided to take the advantage of the important characteristics of ED III to develop a recombinant tetravalent vaccine construct to avoid the risk of ADE. Dengue vaccine must be tetravalent so as to induce a balanced immune response against all four serotypes and provide long-term protection. Over the last few decades significant efforts have been made to develop an effective dengue vaccine which gives protection against all four serotypes simultaneously (tetravalent vaccine) but have gained limited success to date. Despite other vaccine types, recombinant DNA vaccines against flaviviruses provide promising approach towards understanding of the key factors that elicit an effective immune response. The ability of generating antigen-specific immune responses, stability, ease of preparation in large scale with high purity, simplicity of delivery, and safety makes the recombinant DNA vaccines an attractive approach for developing an effective tetravalent dengue vaccine¹⁶. Many researchers working on recombinant DNA vaccine approach have previously developed monovalent vaccine against dengue. Most of them targeted envelope, premembrane or both, ED III, and NS1 gene.^{13,17-27} Efforts for developing tetravalent recombinant ED III DNA vaccine against dengue have been made by few researchers.^{12,15,28,29} Different approaches have been used by these researchers to develop recombinant tetravalent vaccine constructs. Chen et al (2007) fused four ED III into a single bacterial expression vector and expressed proteins for efficacy study in mice, while J. Mota et al (2005) and Rajamanonmani et al (2009) constructed four different recombinant clones and pooled them together as a tetravalent vaccine candidate. Leng et al (2009) developed a consensus sequence of ED III from the sequences available in GenBank and used as a vaccine candidate. Etemad et al (2008) developed a chimeric Ed III antigen and expressed in *Pichia pastoris* for developing neutralizing antibodies against all four serotypes. Here we have described the different approach to construct the recombinant tetravalent vaccine candidate by using overlapping primers to fuse ED III regions of all four dengue virus serotypes by PCR than the routine method of using restriction endonuclease (RE) digestion and ligation. The fused single tetravalent gene insert was then

cloned into a mammalian expression vector to express fusion protein. The sequence was submitted to the GenBank (Accession Number: KF855114).

Colony PCR and RE digestion

The Zeocin resistant colonies were screened for recombinant clones by colony PCR by using vector primers. The vector primers amplified ≈ 1.5 kb fragment of the desired clone having gene insert (Fig 2B). The presence of gene insert was confirmed by restriction endonuclease enzyme digestion of purified plasmid. The restriction endonuclease enzymes digestion showed two bands. The vector band was about ≈ 3.7 kb and insert band was about ≈ 1.2 kb in size (Fig 2B). Orientation of recombinant plasmid was confirmed by sequencing.

Cell transfection and IFA

For the development of an effective DNA vaccine it is very essential to efficiently transcribe the genome in the expression system so as to correct and efficient synthesis of recombinant protein. Our recombinant tetravalent vaccine construct was expressed adequately in 293T transfected cells (Figure 3).

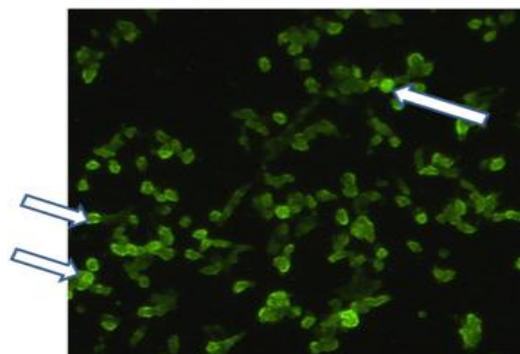


Figure 3: IFA showing apple green fluorescence of positive 293T cells transfected with recombinant clone (marked with arrow).

CONCLUSION

Dengue vaccine must be tetravalent so as to induce a balanced immune response against all four serotypes and provide long-term protection. Over the last few decades significant efforts have been made to develop an effective dengue vaccine which gives protection against all four serotypes simultaneously (tetravalent vaccine) but have gained limited success to date. Despite other vaccine types, recombinant DNA vaccines against flaviviruses provide promising approach towards understanding of the key factors that elicit an effective immune response. The ability of generating antigen-specific immune responses, stability, ease of preparation in large scale with high purity, simplicity of delivery, and safety make recombinant DNA vaccines attractive candidates for developing an effective tetravalent dengue vaccine. In the present study, we have successfully constructed recombinant tetravalent dengue envelope domain III vaccine candidate, which can be subsequently used for efficacy study in mice.

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