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Original Article

Neutralizing Antibody Response and Efficacy of Novel Recombinant Tetravalent Dengue DNA Vaccine Comprising Envelope Domain III in Mice

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What's Known

- Previous efforts used monovalent recombinant clones pooled together as tetravalent vaccine, synthetic consensus envelope domain III (EDIII) sequence from 4 serotypes separated by proteolytic cleavage sites and expressed as a single open-reading frame (ORF) by recombinant plasmid clone, and recombinant subunit vaccine approach for expressing recombinant chimeric proteins using various expression systems like *Pichia pastoris* yeast and *E. coli*.

What's New

- Our study added to the existing body of literature a new way of developing recombinant tetravalent vaccine construct and showed its effectiveness in inducing neutralizing antibody response and protection in mice.
- We ligated EDIII from 4 different dengue virus (DENV) serotypes by PCR to make a single gene insert without using any linkers and cloned it in a mammalian expression plasmid vector to express seamless fusion protein.

Abstract

Background: Dengue is a global arboviral threat to humans; causing 390 million infections per year. The availability of safe and effective tetravalent dengue vaccine is a global requirement to prevent epidemics, morbidity, and mortality associated with it.
Methods: Five experimental groups (6 mice per group) each of 5-week-old BALB/c mice were immunized with vaccine and placebo (empty plasmid) (100 µg, i.m.) on days 0, 14 and 28. Among these, four groups (one group per serotype) of each were subsequently challenged 3 weeks after the last boost with dengue virus (DENV) serotypes 1-4 (100 LD_{50}, 20 µl intracerebrally) to determine vaccine efficacy. The fifth group of each was used as a control. The PBS immunized group was used as mock control. Serum samples were collected before and after subsequent immunizations. EDIII fusion protein expression was determined by Western blot. Total protein concentration was measured by Bradford assay. Neutralizing antibodies were assessed by $TCID_{50}$-CPE inhibition assay. Statistical analysis was performed using Stata/IC 10.1 software for Windows. One-way repeated measures ANOVA and Mann-Whitney test were used for neutralizing antibody analysis and vaccine efficacy, respectively.
Results: The recombinant EDIII fusion protein was expressed adequately in transfected 293T cells. Total protein concentration was almost 3 times more than the control. Vaccine candidate induced neutralizing antibodies against all four DENV serotypes with a notable increase after subsequent boosters. Vaccine efficacy was 83.3% (DENV-1, -3, -4) and 50% (DENV-2).
Conclusion: Our results suggest that vaccine is immunogenic and protective; however, further studies are required to improve the immunogenicity particularly against DENV-2.

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Keywords ● Dengue ● Envelope domain III (EDIII) ● Antibodies ● Neutralizing ● Vaccines

Introduction

Dengue is the global arboviral disease caused by four serologically related but antigenically distinct dengue virus (DENV) serotypes known as DENV-1, DENV-2, DENV-3, and DENV-4. DENVs are transmitted to humans by mosquitoes of the *Aedes* genus,

predominantly *Aedes ageypti* and *Aedes albopictus.*[1] Humans are the principal reservoir for DENV infections and the only host to develop clinical disease following natural infections.[2]

DENV causes a self-limiting acute febrile illness known as dengue fever (DF) to the more severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[3] The latest study reports 390 million dengue infections per year, of which 96 million infections become apparently severe.[4] Dengue infections cause major social as well as economic impact globally during large epidemics.[5-8]

Despite having an urgent global need, there is still no effective vaccine available for DENV in the market.[9] However, the recent Phase 3 clinical trial results of Sanofi Pasteur's dengue vaccine candidate have shown promising results.[10,11]

The immunity in dengue is primarily mediated by the neutralizing antibodies. The role of neutralizing antibodies has been well documented in protection.[12-17] Most of the neutralizing antibodies are directed against envelope antigen, primarily envelope domain III (EDIII).

Previous efforts for developing recombinant tetravalent EDIII vaccines used different approaches. These include construction of four different recombinant clones and pooling them together as a tetravalent DNA vaccine candidate,[18] a recombinant plasmid clone expressing single ORF comprising of synthetic consensus EDIII sequences from four serotypes separated by proteolytic cleavage site,[19] a recombinant subunit approach expressing chimeric EDIII using *Pichia pastoris* yeast,[20] and lapidated consensus EDIII in *E. coli,*[21] expression of chimeric EDIII with P64k protein from *N. meningitides* in *E. coli,*[16] and connecting two tandem EDIII sequences (DENV type1-2 and type 3-4) by Gly-Ser linker and expression in *E. coli.*[22]

We have developed a novel recombinant tetravalent DNA vaccine comprising EDIII of all four DENV serotypes ligated by PCR using overlapping primers without any linkers and cloned in a single mammalian expression plasmid vector (pVAC1-mcs, InvivoGen, USA) so as to develop a seamless EDIII fusion protein.[23] An *in silico* approach for this novel vaccine construct (the role and characteristics of the vaccine construct using its sequence and structure-based features) using various bioinformatics tools has also been studied.[24] In the present study, we report the induction of neutralizing antibody response by vaccine candidate and its efficacy in BALB/c mice.

Materials and Methods

Cell Line Procurement and Maintenance

The C6/36 mosquito larvae whole cell line was procured from the National Centre for Cell Science, Pune, India and maintained as per the instructions given. The growth medium used for C6/36 cell line consisted of 1×minimal essential medium (MEM) containing Earle's BSS (GIBCO #11430-030), 2 mM L-glutamine (GIBCO #25030-081), 0.35 g/L Na bicarbonate (GIBCO #25080-094), 0.1 mM non-essential amino acids (GIBCO #11140-050), 1.0 mM Na pyruvate (GIBCO #11360-070), 100 units penicillin, 0.1 mg/ml streptomycin; (HiMedia Laboratories #A018-5X100ML), and 10% fetal bovine serum (GIBCO #10270-106). The cells were scraped with cell scraper (Corning #CLS3010-100EA) and transferred to new tissue culture flasks (Nunc #136196). These culture flasks were incubated at room temperature (RT, 24-28 ºC) and the medium was changed twice a week. The cell culture passage was continued for subsequent assays.

DENV Procurement and Maintenance

All four DENV serotypes (Dengue-1 strain P23086, Dengue-2 strain P23085, Dengue-3 strain 633798, and Dengue-4 strain 611319) were procured from the National Institute of Virology, Pune, India. The lyophilized virus was re-suspended in 1 ml of 1×MEM (GIBCO #11430-030) and aliquoted 100 µl/vial and stored at -80 ºC until further use. DENV serotypes were propagated in C6/36 cell line to develop a cell culture adapted stock. In order to develop mouse brain adapted stock, 1 to 4 days old suckling Swiss Albino mice (6 mice per group) were inoculated intracerebrally with 20 µl of respective DENV suspension and monitored for 21 days for the appearance of signs such as paralysis or difficulty in walking. Moribund brain tissues were harvested, weighed, and homogenized in MEM medium using homogenizer. The homogenized material was centrifuged at 10,000 rpm for 30 minutes at 4 ºC, and filtered through 0.2 micron syringe filter. The filtered supernatant was stored in aliquots (100 µl/vial) at -80 ºC until further use. The complete procedure was carried out in a biosafety cabinet (BSL-2 facility) with proper aseptic precautions. The animal procedures were conducted in accordance with guidelines under animal use protocols approved by the Institutional Animal Ethics Committee (number: HITRT/IAEC/05/2011, dated 17th January 2011).

Development of a Novel Recombinant Tetravalent DENV DNA Vaccine Construct

In order to develop a novel recombinant tetravalent DENV DNA vaccine construct, we amplified EDIII region of all four serotypes using overlapping primers and ligated them by PCR to make a single tetravalent gene insert. This gene insert was cloned into a mammalian expression vector (pVAC1-mcs, InvivoGen #pvac1-mcs) between BamHI and EcoRI sites. Recombinant clones were screened by colony PCR using vector primers and confirmed by restriction enzymes (RE) digestion (BamHI, NEB #R3136T and EcoRI, NEB #R5101s) and sequencing.[23]

Preparation of Recombinant Tetravalent DNA Vaccine for Immunization

The transformed colonies, having desired recombinant clone were sub-cultured in 5.0 ml Fast-Media®Zeo TB broth (InvivoGen #fas-zn-l) and incubated at 37 ºC overnight in a shaker incubator (Sanyo, CA, USA) at 300 rpm. The next day, the starter culture was diluted 1/1000 into fresh sterile Fast-Media®Zeo TB broth (InvivoGen #fas-zn-l) and incubated at 37 ºC overnight in a shaker incubator (Sanyo, CA, USA) at 300 rpm. The recombinant clones were purified using Qiagen EndoFree® Plasmid Maxi kit (Qiagen #12362) as per the manufacturer's instructions and were used to immunize BALB/c mice.

Immunization of BALB/c Mice

Five groups of 5-week-old male BALB/c mice (6 mice per group per serotype and the fifth group for vaccine control) were inoculated intramuscularly with 100 µg of recombinant tetravalent clone in the posterior limb muscle. Similarly, five placebo mice groups (6 mice per group per serotype and the fifth group for placebo control) were inoculated intramuscularly with 100 µg of empty plasmid vector (circular plasmid without having the gene of interest). Mock control mice group (6 mice) was inoculated intramuscularly with PBS. Mice were injected on day 0 and boosted on days 14 and 28. Blood samples were collected before inoculation, before subsequent boosters (on days 13 and 27), and 2 weeks after the last booster (day 42) by retro-orbital sinus under mild anaesthesia (Isoflurane, Troikaa Pharmaceuticals Ltd., #I21145). The sera were obtained by centrifugation of coagulated blood samples (≈0.5 ml per mice) and stored at -80 ºC until use.

Determination of EDIII Fusion Protein Expression

EDIII fusion protein expression was determined by Western blot using transfection assay and pooled vaccinated mice serum. Cell lysate of 293T cells transfected with recombinant tetravalent vaccine construct was used as a sample and the empty vector plasmid (circular plasmid lacking tetravalent EDIII gene insert) transfected 293T cell lysate was used as a control. The sample and control were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto polyvinylidene difluoride (PVDF, Pall #S80306) membrane, and detected using pooled vaccinated mice serum, horseradish peroxidase (HRP)-labelled goat anti-mouse IgG antibody (Abcam #ab97265), and luminol peroxide detection reagent (Amersham ECL-Prime, GE #RPN2232) by gel documentation system (BIO-RAD Molecular Imager® ChemiDoc™ XRS+with Image Lab™ software, CA, USA).

Determination of Total Protein Concentration by Bradford Assay

The recombinant plasmid transfected 293T cells were freeze-thawed to lyse and release target protein. The lysed cells were centrifuged at 10,000 rpm for 20 minutes and the supernatant was aliquoted and stored at -80 ºC until used. Empty plasmid transfected 293T cells were used as control. Total protein concentration was determined by Bradford assay using bovine serum albumin (BSA, Sigma #P0834) as standard protein at concentration range of 5-50 µg/µl. The test and control samples (supernatant) were used as neat and absorbance was measured at 595 nm using the BioTek SynergyHT Multimode plate reader (BioTek, India).

Tissue Culture Infectious Dose 50-CPE ($TCID_{50}$)-CPE Titre Determinations

Tissue culture infectious dose 50 ($TCID_{50}$)-CPE titres were determined using C6/36 cell line and serial dilutions of DENV-1 to 4 viruses in a 96-well tissue culture plate as described by Li et al.[25] with minor modifications. The assay was performed in duplicate. In brief, one day prior to the assay, cells were seeded in a 96-well tissue culture plate in 0.2 ml of complete growth medium at a density of 2.5×10^5 cells/well and incubated overnight at 24-28 ºC so that the cells achieved 80–90% confluence at the time of virus addition. Ten-fold serial dilutions of DENV-1 to 4 were prepared in C6/36 cells maintenance medium (10^{-1} to 10^{-10}). Hundred microlitres of neat virus suspension was added to the first well, while 100 µl of each dilution was added to the next 10 subsequent wells and the last well was used as cell control (cells without virus). The plate was incubated at RT for 2 hours to adsorb the virus.

The virus suspension was aspirated off gently and the monolayers were overlaid with 200 µl per well of C6/36 cells maintenance medium. The plate was incubated at RT for 6 days and observed daily for cytopathic effect (CPE) using an inverted microscope (Carl Zeiss, USA). Infected fields were counted and 50% tissue culture infectious dose ($TCID_{50}$) of each virus was calculated as described by Reed and Muench.

Neutralizing Antibody Titre Determination

Neutralizing antibody titre (50% end-point titre of serum) was determined by $TCID_{50}$-CPE inhibition assay using C6/36 cell line, dilution of virus showing 100 $TCID_{50}$ titre and serial dilutions of pooled vaccinated mice serum in a 96-well tissue culture plate as described by Jaiswal et al.[26] with minor modifications. Pooled placebo immunized mice serum and mock-immunized (PBS) mice serum were used as control. The assay was performed in triplicate. In brief, one day prior to the assay, cells were seeded in a 96-well tissue culture plate in 0.2 ml of complete growth medium at a density of 2.5×10^5 cells/well and incubated overnight at 24-28 ºC so that the cells achieved 80–90% confluence at the time of assay. In a sterile 96-well plate, two-fold serial dilutions (1:2 to 1:256) of heat inactivated (56 ºC for 30 minutes) pooled vaccinated mice serum samples, collected at different time points (i.e. after 1st dose (day 13), after 2nd (day 27) and 3rd dose (day 42)), were prepared in C6/36 cells maintenance medium. Placebo immunized mice serum and mock-immunized (PBS) control mice serum samples were also heat inactivated (56 ºC for 30 minutes) and used as neat. Fifty microlitres of each serum dilution were mixed with 50 µl of 100$TCID_{50}$DENV-1 to 4 virus dilutions and incubated at 37 ºC for 1 hour to neutralize the virus. The virus-serum mixture was added to C6/36 cells seeded 96-well plate. The plate was incubated at RT for 2 hours to adsorb the virus. The virus-serum mixture was aspirated off gently and the monolayers were overlaid with 200 µl per well of C6/36 cells maintenance medium. The plate was incubated at RT for 6 days and observed daily for CPE using an inverted microscope (Carl Zeiss, USA). Infected fields were counted and the antiserum dilution corresponding to the 50% infectivity end-point of virus was determined by cumulative averaging using the method of Reed and Muench. The reciprocal of this dilution was expressed as the neutralizing titre.

Efficacy Study in BALB/c Mice

Three weeks after the last booster, four groups (6 mice per group) of each vaccinated and placebo controlled BALB/c mice were challenged by mouse brain adapted DENV-1 to 4 (one group per serotype). Mice were inoculated intra-cerebrally with 20 µl of 100LD_{50} of each DENV-1 to 4 (previously determined) under mild anaesthesia (isoflurane) using tuberculin syringe and $26_{1/2}$ G needle. Mice were observed daily for 21 days for the appearance of signs such as paralysis or difficulty in walking and finally death. The total number of deaths was recorded.

Statistical Analysis

Statistical analysis was performed using Stata/IC 10.1 software for Windows. For neutralizing antibody analysis one-way repeated measures ANOVA was used to compare the means between three time points; after 1st dose (day 13), after 2nd dose (day 27), and after 3rd dose (day 42). The efficacy of vaccine construct was analysed using Mann-Whitney test by comparing vaccine+challenge with placebo+challenge groups according to the following hypothesis.

Hypothesis Testing

Let µp be the mean survival time for the placebo group and let µv be the mean survival time for the vaccine group. Hence, diff=µv–µp.

Ho: Mean survival time do not differ between the two groups; diff=0 versus Ha: Mean survival time (in days) for vaccine is greater than that for placebo; diff>0.

Results

The result of Western blot, figure 1, shows the expected single EDIII fusion protein band of around 50 kDa in size, which was absent in empty plasmid vector transfected 293T cell lysate.

The total protein concentration was determined by Bradford assay. The Bradford assay showed that the protein has expressed approximately 3 times more (15.98 µg/µl) in 293T cells transfected with recombinant clone than the control (5.30 µg/µl). Table 1 shows $TCID_{50}$-CPE titre determined by Reed and Muench method.

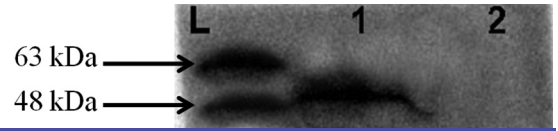

Figure 1: Western blot showing; L: Ladder (10 kDa to 250 kDa), 1: Sample (293T cells transfected with recombinant clone), 2: Control (293T cells transfected with empty plasmid).

Figure 2 shows the day-wise comparison of neutralizing antibody response in vaccinated mice.

Efficacy study showed that 5 out of 6 vaccinated mice (83.3%) were protected after challenge with DENV-1, DENV-3, and DENV-4 serotypes, while 50% (3 out of 6 mice) protection was seen in vaccinated mice challenged with DENV-2. In the placebo group, 16.6% (1 out of 6 mice) survival was observed after challenge with DENV-1 to 4. Survival estimates (in days) of vaccinated and placebo immunized mice are shown in table 2 and figure 3.

Figure 3 shows Kaplan-Meier survival estimates (in days) of vaccinated and placebo immunized mice challenged with all four DENV serotypes.

Discussion

The protection offered by neutralizing antibodies is well defined in babies of dengue-immune mothers during the early months of life[27-30] and in passively transferred studies in animals.[31-33] Most of the neutralizing epitopes have been mapped to the envelope antigen, particularly EDIII.[34-36] Thus, EDIII becomes the major target for neutralizing antibodies. Recombinant DNA vaccine approach helps in understanding the key factors that elicit an effective immune response against DENV.

In the present study, we have reported the induction of neutralizing antibody response and efficacy of our novel recombinant tetravalent dengue DNA vaccine comprising EDIII in a mouse model.

It is indispensable for recombinant DNA vaccine to correctly and efficiently synthesize protein of interest in its native conformation so as to mount an effective immune response. EDIII fusion protein expression was checked in 293T cells transfected with recombinant clone and empty plasmid and detected by Western blot (figure 1) and Bradford assay. The results of Western blot and Bradford assay indicate that the recombinant clone has adequately expressed seamless EDIII fusion protein in 293T transfected cells. These results also confirmed predictive findings of our previous bioinformatics study of vaccine construct which showed that vaccine is stable, properly folded and antigenic.[24]

The infected cells were observed daily for CPE. The CPE was observed around day 6 post infection and onward, which consisted of enlarged infected cells fused with the neighbouring infected cells and appearance of vacuoles within cell cytoplasm. $TCID_{50}$-CPE titre was determined by Reed-Muench method using C6/36 cell line and serial dilutions of DENV-1 to 4 serotypes (table 1).

Neutralizing antibody has been believed to provide protection against dengue. The neutralizing antibody titre is considered an important marker of protection in dengue vaccine development. A neutralizing antibody titre of 1:10 or more has been suggested as protective for DENV.[37-39] In our study, neutralizing antibody titres were determined by $TCID_{50}$-CPE inhibition assay (50% serum end-point dilution assay). Neutralizing antibody analysis was carried out by comparing the means between three time points;

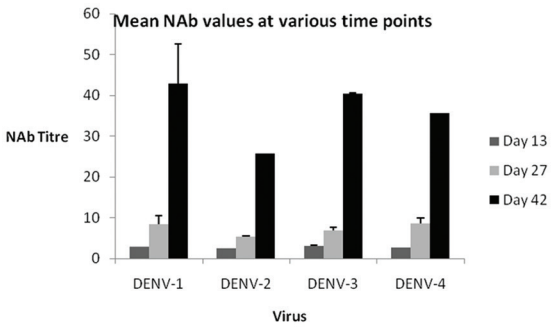

Figure 2: Neutralizing antibody response in vaccinated mice at various time points.

Table 1: $TCID_{50}$-CPE titre

Dengue serotype	$TCID_{50}$-CPE titre
DENV-1	10^6/100 µl
DENV-2	10^5/100 µl
DENV-3	10^4/100 µl
DENV-4	10^4/100 µl

Table 2: Vaccine efficacy in BALB/c mice

DENV serotype	Mean survival time in days		P value (Mann-Whitney test)
	Mean±SD		
	Placebo group N=24 (6 mice/serotype)	Vaccine group N=24 (6 mice/serotype)	
DENV-1	17.5±1.8	20.5±1.2	0.0114
DENV-2	13.3±4.0	17.5±3.9	0.0398
DENV-3	14.2±3.4	20.0±2.4	0.0128
DENV-4	14±3.5	20.2±2.0	0.0125

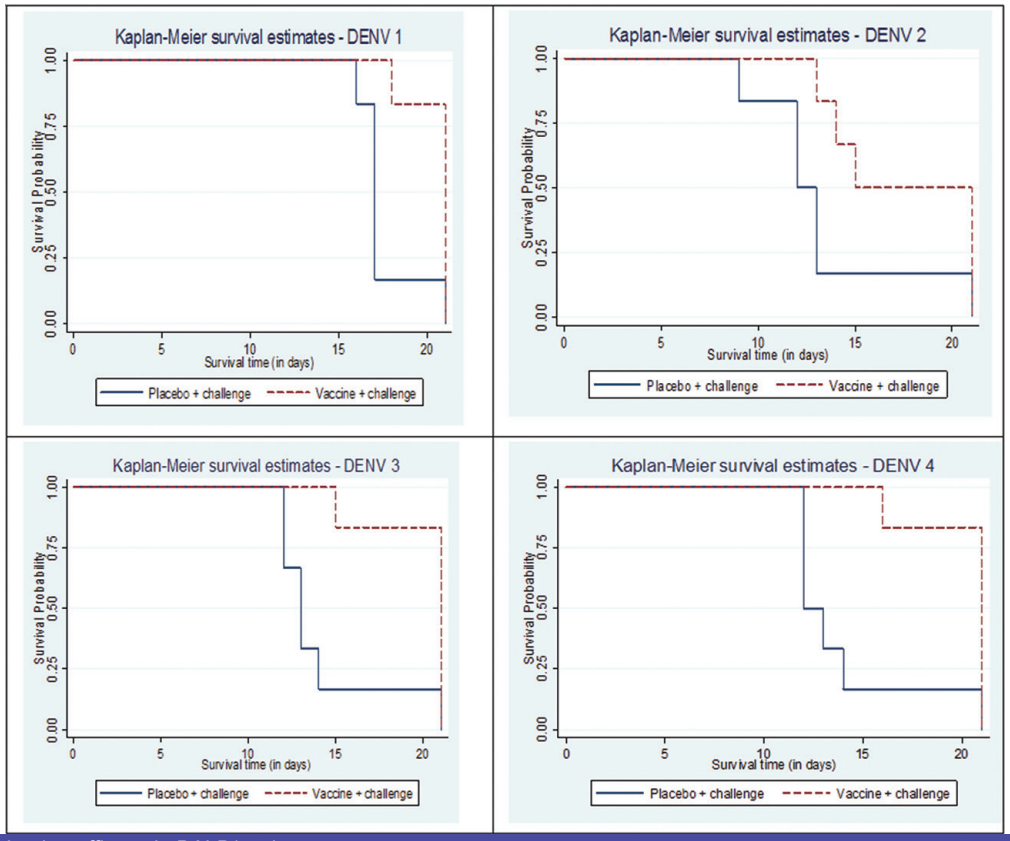

Figure 3: Vaccine efficacy in BALB/c mice.

after 1st dose (day 13), after 2nd dose (day 27) and after 3rd dose (day 42), using one-way repeated measures ANOVA. All DENV serotypes showed statistically significant differences (P<0.01), figure 2. It was observed that the mice immunized with recombinant tetravalent DNA vaccine construct developed neutralizing antibodies against all four DENV serotypes, with a notable increase after subsequent boosters. The vaccine induced protective neutralizing antibody response (1:10 or more) against all DENV serotypes after the last boost of vaccine. These data demonstrated that the recombinant vaccine construct is immunogenic and elicited antibodies that recognized and neutralized all four DENV serotypes at variable levels. Relatively similar neutralizing antibody titres (1:45, 1:29, 1:57 and 1:22 for DENV-1 to 4, respectively) by plaque reduction neutralization test (PRNT) were reported by Zhao et al.[22] While, a lower neutralizing antibody response (1:10) using CPE inhibition assay and DENV-2 was reported by Mota et al.[18]

A significant protection was observed in vaccinated mice challenged with DENV-1, DENV-3, and DENV-4 while a lower protection was observed against DENV-2. In addition, vaccination prolonged the mean survival time compared with placebo-immunized mice by Mann-Whitey test; and was found statistically significant (table 2). The Kaplan-Meier survival estimates of all four DENV serotypes showed early failures in the placebo group and better survival time for vaccinated mice groups challenged with DENV-3 and DENV-4. The median survival time was estimated to be 13 and 21 days for the placebo and vaccine groups, respectively (figure 3). Relatively similar findings were reported by Mota et al.[18] and Zhao et al.[22]

In the present study, $TCID_{50}$-CPE inhibition assay results and efficacy findings indicated that the protective neutralizing antibody titres (cut off) were serotype specific and higher neutralizing antibody titre would be required to neutralize DENV-2. Similar findings were reported in Thai cluster studies conducted in Thai schoolchildren and also in adults by Darunee Buddhari et al.[37] and in another cohort study conducted in Thai school children by Chukiat Sirivichayakul et al.[39]

The present study adds to the existing knowledge on the efficacy of novel DNA recombinant tetravalent dengue vaccine construct in mice and the induction of neutralizing antibody response. However, as a limitation, the present study was carried out using DENV strains procured from NIV (Pune, India) and not using wild circulating strains in the country.

Conclusion

Our results suggest that vaccine is immunogenic and protective. However, further studies are required to improve the immunogenicity, particularly against DENV-2, such as the use of vaccine in conjunction with adjuvants, other vaccine approaches, or using advanced immunization methods like gene gun electroporation etc.

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Conflict of Interest: None declared.

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