

Dengue virus-like particles serotype-3 production in silkworm larvae and its capability eliciting a humoral immune response in mice model

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Abstract

To develop monovalent dengue virus-like particle for serotype 3 (DENV-LP/3), two kinds of structural polyprotein constructs, DENV-3 Capsid-premembrane-Envelope (DENV-3CprME) and premembrane-Envelope (DENV-3prME), have been prepared and expressed using silkworm and Bm5 cells. The expressed PA-tagged 3CprME and 3prME polypeptides were partially purified by PA-tag affinity chromatography and found the molecular weights of 85 and 75 kDa, respectively. Expressed proteins were verified using the anti-PA tag antibody, DENV premembrane polyclonal antibody, and DENV envelope polyclonal antibody, as primary antibody separately. Transmission electron microscopy (TEM) image revealed these DENV-3CprME and 3prME formed a rough spherical shaped dengue virus-like particles (DENV-LP/3CprME and DENV-LP/3prME), respectively, with a diameter of 30–55 nm. The heparin-binding assay demonstrated that these DENV-LPs contain Envelope Domain (ED) III on the surface of each DENV-LP. Both DENV-LPs showed the affinity to sera from human dengue patients and immunized-mice. Immunization of DENV-LP/3prME to mice induced a significantly higher of antibodies level to itself than that of DENV-LP/3CprME. These results indicate that DENV-LP/3prME is suitable as a vaccine candidate compared to DENV-LP/3CprME.

Key Points

- *Dengue virus-like particles for serotype 3 (DENV-LPs/3) were produced in silkworm.*
- *Heparin-binding assay showed that DENV-LPs/3 contain Envelope Domain III.*
- *DENV-LPs/3 showed affinity to sera from human dengue patients and immunized-mice.*

Introduction

DENV has been the primary epidemic of arboviral disease in the world, mostly in the tropical and subtropical countries of the Americas, Asia, Africa, and the Pacific. The number of cases of dengue has been registered to the World Health Organization (WHO) by 2016, and gradually increased to over 3.34 million from an estimate of fewer than 1,000 cases worldwide a year in the 1950s. It was estimated that DENV infections occur annually at 390 million, while another study suggested that 3.9 billion people were at risk for infection in 128 countries. Dengue fever alone cost 8.9 billion US dollars per year worldwide. Dengue cases globally distributed as 18% hospital admitted, 48% outpatient, and 34% non-medical (Añez et al. 2016; Shepard et al. 2016; Yuan et al. 2020).

DENVs are categorized into four antigens distinct serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), and human infection can present as undifferentiated fever, dengue fever, and dengue hemorrhagic fever (Halsey et al. 2012). Primary infection by each of the four serotypes leads to a long periodic serotype-specific immunity and a six-month partial immunity to other serotypes. After it decreases, the person becomes vulnerable to another three DENV serotypes, and the secondary infection can result in the Antibody-dependent Enhancement (ADE)/clinical manifestations (Murrell et al. 2011).

DENV serotype 3 (DENV-3) is one of four DENV serotypes that are geographically distributed. DENV-3 has 5 distinct genotypes (I – V). The emergence of DENV-3 strains and lineages is increasingly reported (Waman et al. 2017). In some countries, such as Thailand, Brazil, Pakistan, and India, DENV-3 and DENV-2 serotypes are prevalent in the region and are associated with a more serious clinical profile than other serotypes (de Araujo et al. 2012; Fried et al. 2010; Mehta and Shah 2018; Yousaf et al. 2018). There were also re-emergence of DENV-3 infections in Brazil in 2007, in China in 2009–2010, and Gabon in 2016–2017, which can increase the risk of repeated DENV infections in a certain area (Abe et al. 2020; Liang et al. 2013; Rodriguez-Barraquer et al. 2011).

Virus particles (VLPs) are self-assembled particles which consist of viral structural proteins and can imitate the conformation of a genuine native virus without genomic DNA or RNA, and now become a viable option to live-attenuated vaccines (Urakami et al. 2017). Dengue encodes three structural proteins: Capsid (C), Membrane (M), or premembrane (prM), and Envelope (E). DENV C protein is a highly simple protein with a size of around 11 kDa. It helps with the assistance of RNA interaction in nucleocapsid assembly. Membrane-associated protein is an approximately 26 kDa M glycoprotein that can promote the development of E protein as a mature virus particle and help to distinguish the immune response to different flaviviruses. The E protein is a surface protein with a large constituent of around 55 kDa of virus particles and is responsible for the virus being bound and fused to the host cell membrane (Cardosa et al. 2002; Ma et al. 2004; Modis et al. 2004). The expression of prM-E or the co-expression with the C-prM-E structural proteins was the effective approaches for the development of recombinant flavivirus VLPs. The presence of capsid protein can stabilize the VLPs assembly, although this is not necessary for particle formation (Krol et al. 2019).

Silkworms have a strong potential for recombinant protein processing and can produce therapeutic human proteins. Moreover, silkworms simplify and cheaply increase the ability to produce protein, and several studies support insect cells as helpful in manufacturing affordable antigens and recombinant vaccines. Furthermore, large-scale VLP development is manufactured by insect larvae, including silkworms (Vipin Kumar Deo 2012).

In this study, we prepared DENV-LPs consisting of Capsid-premembrane-Envelope (3CprME) and premembrane-Envelope (3prME) polypeptides, which were expressed with *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid in the silkworms. We observed the formation of VLPs and verified in a mouse model for the elicitation of antibody production.

Materials And Methods

Construction of recombinant BmNPVs

The C-prM-E with the deletion on the anchor region of the capsid and prM-E polypeptide coding sequences (GenBank: KU050695, Genewiz, New Jersey, USA) were used for this study. A linker sequence (GGGGSGGGGS) and PA tag sequence (EGGVAMPGAEDDVV) were fused with at its C-terminus was amplified using a template (the synthetic gene described above) by polymerized chain reaction (PCR)

with a set of primers (3CprME-F and 3CprME-R-EcoRI, Table 1) and as a template for the coding sequence of DENV-3 C-prM-E. DENV-3 prM-E primers set (3prME-F and 3prME-R-EcoRI, Table 1) was used to isolate the DENV-3 prM-E coding sequence. The PCR steps began with an initial denaturation at 98°C for 10 sec, followed by 98°C for 10 sec 35 cycles, and 55°C for 5 sec, then 72°C for 20 sec, with 72°C for 3 min for the final extension. A thermal cycler (TaKaRa, Kyoto, Japan) has been used to perform the PCR process. Each construct was ligated into pFastbac1 (Thermo Fisher Scientific K. K., Tokyo, Japan) and the resulting vector was introduced into *Escherichia coli* BmDH10bac CP⁻ Chi⁻ (Karimi et al. 2013). The recombinant products, BmNPV/3CprME and BmNPV/3prME bacmids were extracted from white colonies, respectively. Each recombinant BmNPV bacmid was mix with chitosan (Sigma-Aldrich, Tokyo, Japan) and injected into fifth instar silkworm larvae (Ehimesansyu, Ehime, Japan). The hemolymph was collected from the larvae 6–7 days-post-injection (dpi) and mix with 1-phenyl-2-thiourea at 1 mM and its aliquots of hemolymph and was kept at –80°C before use (Karimi et al. 2013).

Expression and purification of 3CprME and 3prME polypeptides in silkworm larvae

Fifth instar silkworm larvae (Ehimesansyu) was injected with 100-fold diluted hemolymph in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4), and raised on an artificial diet (Silkmate S2, Nosan Co., Yokohama, Japan). The larvae's leg was cut to collect the hemolymph. The fat body was collected by dissecting the larvae, and for 0.1 g fat body, 1 ml Tris-buffered saline containing 0.1% Triton X-100 (TBST) was added, and sonicate 5 min within 20-sec intervals with 10-sec break using a sonicator (Vibra Cell VC 130PB, Sonics & Materials Inc., Newtown, USA), and centrifuged (Kubota 3700, Tokyo, Japan) at 12,000 × g, 4°C for 10 min. The soluble fraction of the silkworm fat body was mixed with 200 µL of anti-PA tag antibody beads (FUJIFILM Wako Pure Chemical, Osaka, Japan) at 4°C for 2 h. The mixed beads were collected, and five times washed with TBS buffer (20 mM Tris-HCl, 150 mM NaCl) four times of bead volumes. A 0.1 mol/L glycine-HCl (pH 3.0) was added for making the elution and collected in 5 fractions to recover PA tagged-target proteins. The Amicon ultra centrifugal filters (Merck Japan, Tokyo, Japan) was used to concentrate the eluents by ultrafiltration, and the concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific K. K.).

As comparisons, 3CprME and 3prME constructs also expressed in BM5 cells and silkworm pupae (Ehimesansyu). Bm5 cells were gifted by Prof. K. S. Boo (Insect Pathology Laboratory, School of Agricultural Biotechnology, Seoul National University, Seoul, Korea). Sf-9 and Bm5 cells were maintained at 27 °C in Sf-900II Serum-Free Medium (SFM; Thermo Fisher Scientific K.K.) supplemented with 1% fetal bovine serum (Thermo Fisher Scientific K.K.) and Antibiotic-Antimycotic (Thermo Fisher Scientific K.K.).

SDS-PAGE and western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% or 12% was used to separate the proteins, and subsequently subjected for western blotting by blotting the proteins trapped in acrylamide gel onto an Immobilon-P PVDF (polyvinylidene fluoride) membrane (Merck Japan) using the

Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). After blotting, the membrane was blocked in 5% skim milk (FUJIFILM Wako Pure Chemical) in TBST (pH 7.6), then incubated in a 10000-fold diluted rat anti-PA tag antibody (FUJIFILM Wako Pure Chemical). Alternatively, the primary antibody was the 3000-fold diluted rabbit anti-DENV E antibody (GeneTex, Irvine, CA, USA), or 3000-fold diluted mouse anti-DENV prM antibody (GeneTex). It was incubated for 1 h in 10000-fold diluted horseradish peroxidase (HRP)-conjugated anti-rat IgG antibody (FUJIFILM Wako Pure Chemical), after the membrane was washed with TBST three times. Immobilon Western Chemiluminescent HRP substrate (Merck Japan) was used for detection. On a Fluor-S MAX Multi-Imager (Bio-Rad) the specific bands were found.

Transmission electron microscopy and immunoelectron microscopy

Transmission electron microscopy (TEM) and immunoelectron microscopy (IEM) was carried out as previously described (Utomo et al. 2019) with modifications. The purified antigen sample was added to the Cu-Grid TEM (Nisshin EM Co., Ltd., Tokyo) and incubated for 30 sec at room temperature, washed by 30 μ l of PBS and incubated for 30 sec, repeated three times over. For IEM observation, 30 μ l of 2% v/v BSA was used for blocking after adding a distilled antigen sample and washed three times with PBS. The Cu-grid was washed sequentially. Anti-E rabbit polyclonal antibody (FUJIFILM Wako Pure Chemical) diluted 30 times and gold nanoparticles goat anti-rabbit IgG-conjugated (FUJIFILM Wako Pure Chemical) diluted 50 times were used as the first and secondary antibodies, separately. Phosphotungstic acid 2% was applied to the Cu-grid and analyzed using the TEM apparatus.

Heparin-binding assay

The heparin-binding assay was carried out as previously described (Utomo et al. 2019), with modifications. Six ng/ml of diluted Biotin-labeled heparin (Sigma-Aldrich Japan) and 1.8 ng of heparin were immobilized into the blocking-less type avidin plate (Sumitomo Bakelite, Tokyo, Japan) wells and washed with PBS three times. For negative control, 2 μ g of BSA has been used. At each quantity, purified proteins with different concentrations (0.5, 1, 5, and 10 μ g/ml) were added into wells, incubated at room temperature for 1 h, then washed with PBST. Rat anti-PA tag antibody (FUJIFILM Wako Pure Chemical) 1000-fold diluted was added as primary and followed with a secondary antibody with 1000-fold diluted HRP-conjugated anti-rat IgG antibody (FUJIFILM Wako Pure Chemical) after serial washing. For following was adding 100 microliters of substrate 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine (TMB) in 100 mM sodium acetate (CH_3COONa), pH 6.0 to each well with 0.2% (v/v) of 30% hydrogen peroxide, and 50 μ l of 1 N H_2SO_4 was added to stop the reaction. The absorbance was estimated at 450 nm.

VLP antigenicity by Enzyme-linked immunosorbent assay (ELISA)

Direct ELISA method had been performed to detect an interaction between antigens, 3CprME, 3prME, and mock fat-body of silkworm (negative control) and sera. Two kinds of sera were used, mice sera immunized with DENV tetravalent DNA vaccine (mice-Ab) (Putri D.H., personal communication, June 2017), and dengue patient sera (human-Ab) [NS1(+), RT-PCR (+)] which collected by Dengue community

study from March 2010 until December 2011 and ethical approval was given by Research Ethical Committee Faculty of Medicine, Universitas Indonesia, No. 71/PT02.FK/ETIK/2009. The human-Ab RT-PCR confirmed based on the Lanciotti method (Lanciotti et al. 1992). Both of the sera were stocks from the Department of Microbiology, Faculty of Medicine, Universitas Indonesia.

Each diluted sample, 100 ml of 20 ng/ml in coating buffer (0.05 M Carbonate-bicarbonate, pH 9.6), was applied into a 96 well ELISA plate followed by incubation at 4°C overnight. After incubation, the coating solution was discarded and a 100 ml blocking solution (5% skim milk in PBS) was then added into each well followed by 1 h incubation at 37°C. The plates were then washed serially with PBST buffer followed by the addition of 100 ml of 1:5000 diluted mice-Ab or human-Ab in PBS. Plates were then incubated at 37°C for 1 h and washed three times with washing buffer after it, followed by the addition of 100 ml of 1:5000 goat anti-mouse or anti-human IgG-HRP conjugated antibody. Plates were then incubated at 37°C for 1 h, and washed sequentially, and 50 ml of TMB substrate was applied and incubated 10 min, and 50 ml of 1 M H₂SO₄ was added to stop the reaction. The absorbance was read at 450 nm.

Mice immunization

A total of 12 BALB/c mice, age 4–6 weeks were used in this study which divided into 4 groups: i) negative control (immunized with PBS), ii) immunized with 3CprME, iii) immunized with 3prME and iv) immunized with alhydrogel as an adjuvant. All mice were housed in a temperature-controlled, light cycled room, and divided into four groups according to the immunogen. Each mouse was immunized three times intraperitoneally with 50 mg purified 3CprME and 3prME proteins with alhydrogel adjuvant, within two weeks interval. Blood samples were collected on 0, 16 and 30 d, via the tail vein, and sera were isolated and stored at –80°C for further analysis. All animal procedures were conducted in compliance with established guidelines from the Animal Laboratory of Center of Pharmaceutical and Medical Technology, Agency for Technology Assessment and Application (BPPT), Indonesia. Animal experiment protocols were reviewed and approved by Research Ethical Committee Faculty of Medicine, Universitas Indonesia, No. KET-476/UN2.F1/ETIK/PPM.00.02/2019.

Results

Expression of 3CprME and 3prME polypeptides in silkworm

The structural proteins of DENV consist of a C and two membrane proteins, prM and E, which are translated in the order C-prM-E at the beginning of the polyprotein. The constructed BmNPV/3CprME (Fig. 1a) and /3prME (Fig. 1b) bacmids were injected into silkworm larvae and, hemolymph and fat body of silkworm were collected at 5 dpi. The expression of 3CprME and 3prME was confirmed in fat body samples, showing their molecular weights were 85 and 75 kDa, respectively, which were corresponded to the estimated one (Fig. 2a). Also, both constructs were expressed in silkworm pupae (Fig. 2b) and Bm5 cells (Fig. 2c), and their molecular weight was the same as the estimated one. To analyze the cross-reactivity of these proteins to antibodies of DENV serotype 2, anti-DENV-2 prM and anti-DENV-2 envelope

antibodies were used. The specific bands for 3CprME and 3prME were not detected using these antibodies (Fig. 2d). These results indicate no cross-reactivity between DENV-2 specific antibodies with both recombinant 3CprME and 3prME expressed in silkworm.

Purification of 3CprME and 3prME polypeptides

The purified 3CprME and 3prME polypeptides using affinity chromatography were confirmed by western blot using anti-PA-Tag antibody showing bands with size ~85 kDa for 3CprME in elution 1–4 and ~75 kDa for 3prME in elution 1–4 (Fig. 3a, b). To confirm whether the purified 3CprME and 3prME polypeptides contain the prM and E proteins, western blot was carried out using anti-prM or anti-E antibodies. The bands approximately ~85 kDa for 3CprME and ~75 kDa for 3prME were confirmed (Fig. 3c, d). These results confirm that the purified 3CprME and 3prME polypeptides contained the prM and E proteins.

Morphology of 3CprME and 3prME polypeptides

TEM and IEM were performed to confirm the morphology of the 3CprME and 3prME polypeptides. Spherical structures were observed with the size around 30–55 nm (Fig. 4a, b) and it is also strengthened by the data DLS (Fig. 4e, f). The IEM observation shows there were lipid bilayer-structured spherical and some immunogolds were bonded to the particle surface (Fig. 4c, d). The binding of gold nanoparticles on the surface of spherical structures indicates that the particles contain dengue E protein. These results suggest that 3CprME and 3prME polypeptides expressed in silkworm can generate virus-like particles of Dengue 3 (DENV-LP/3CprME and /3prME).

Heparin-binding assay of the DENV-LPs/3CprME and /3prME

The heparin-binding assay was performed to confirm the expression of E protein domain III (EDIII) on the surface of the DENV-LPs. In this assay, the binding assay of the purified 3CprME and 3prME polypeptides was carried out using the heparin-immobilized microtiter plates. Absorbance in ELISA was increased proportionally to the presence of E protein compared to BSA (Fig. 5). These results suggest that the 3CprME and 3prME contain the EDIII domain on the surface of its DENV-LPs.

Antigenicity characterization of DENV-LPs/3CprME and /3prME

Purified DENV-LPs/3CprME and /3prME were characterized for its antigenicity toward mice-Ab and human-Ab by direct ELISA. As shown in ELISA analysis, 3CprME and 3prME showed reactivity to either mice-Ab and human-Ab compare to the negative control (Fig. 6a). This result indicates that DENV-LPs/3CprME and /3prME can be used to discriminate against the sera from DENV3-infected humans.

DENV-LPs/3CprME and /3prME elicited virus-specific IgG

BALB/c mice were immunized intraperitoneally three times at 3-week intervals with 50 µg of DENV-LPs/3CprME and /3prME with an alhydrogel adjuvant. Mice serum titers were measured by ELISA after

two weeks from the last immunization. Immunization of DENV-LPs/3CprME and /3prME were induced the antibody generation. The anti-3CprME Ab and anti-3prME Ab were analyzed using their antigens to see the specific binding. The result showed that both of the specific antibodies show a low affinity to the DENV-LPs/3CprME (Fig. 6b). On the contrary, the anti-3CprME Ab, and anti-3prME Ab can be recognized by the DENV-LPs/3prME (Fig. 6c). This indicates that to elicit the generation of specific antibodies, the DENV-LPs/3prME is suitable.

Discussion

Our previous study that the DENV-LP serotype 2 (DENV-LP/2) was expressed in silkworm larvae's hemolymph (Utomo et al. 2019). In this study, DENV-3 structural proteins 3CprME were expressed by removing the anchor sequence of capsid protein between C and prM polypeptides. The purpose of anchor capsid deletion was to increase the protein expression amount, but it caused the protein cannot be released into the hemolymph of the silkworm. The expressed 3CprME and 3prME polypeptides were detected in fat body soluble fraction, not in the hemolymph.

It has been shown that recombinant flaviviruses VLPs can be efficiently generated by the expression of proteins prM and E, with or without C proteins (Sangiambut et al. 2013). The absence of prM and/or C did not affect the self-assembly of the envelope in the VLP-forming (Chang et al. 2001; Mani et al. 2013; Putnak et al. 2003). As indicated by heparin-binding, the EDIII domain was shown on the surface of the VLPs, because the heparin in the cell membrane was known to bind to interact with EDIII, the putative receptor-binding domain in the flavivirus E protein crystal structure. It also contains epitopes that block viral adsorption and are targeted by many antibodies, including serotype-specific neutralizing monoclonal antibodies (Chen et al. 2016; Frei et al. 2018; Han et al. 2018; Hidari et al. 2013; Yang et al. 2016). With the EDIII displayed on the surface of the VLPs, the mediated antibody is expected to give increased viral neutralization activity so it may reduce the viral load in normal DENV infection. A neutralization assay is recommended for further study.

Both 3CprME and 3prME also showed reactivity to both mice-Ab and human-Ab, but human-Ab showed higher affinity than mice-Ab in the direct ELISA. Since mice-Ab was obtained from tetravalent DNA vaccine-immunized mice, it had low immunogenicity compared to human-Ab that was originally from DENV-infected patients. Mix sera of Dengue patients can react to many types of non-specific DENV epitopes. DENV tetravalent DNA vaccine itself has shown the vaccine to be well tolerated and capable of generating good anti-dengue interferon-gamma (IFN γ) T-cell responses (Danko et al. 2018).

The DENV-LPs/3CprME and /3prME were capable of generating the IgG antibody levels, although the addition of alhydrogel adjuvant did not give any specific binding. The detected IgG total responses in mice immunized with the 3prME showed a better profile compare to 3CprME. These differences of the response to the antigens might be caused by the deletion of anchor domain at C region and lowering its antibody response, even though the capsid protein is not exposed on the viral surface. Each DENV serotypes carry the conserved antibody epitope incorporated in N and C terminal regions of C protein and

efficiently recognized by dengue patients who exposed to primary and secondary infections from other serotypes. The C-protein central region has an epitope of the peptide, primarily targeting by serotype-specific antibodies (Alves et al. 2016; Nadugala et al. 2017; Rana et al. 2018). It is recommended for further analysis with antibodies response to other serotypes of a structural protein and also to the different DENV serotypes.

Declarations

Ethical approval and consent to participate

Research Ethical Committee Faculty of Medicine, Universitas Indonesia approved the collection of dengue patient sera (human-Ab) [NS1(+), RT-PCR (+)] which performed by Dengue community study from March 2010 until December 2011 (No. 71/PT02.FK/ETIK/2009). Animal experiment protocols were reviewed and approved by Research Ethical Committee Faculty of Medicine, Universitas Indonesia (No. KET-476/UN2.F1/ETIK/PPM.00.02/2019). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent for publication

Not applicable.

Availability of data and material

All the data and materials have been provided in the main manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DISU was the main researcher for this study including the experiment design and operation. SP and FS participated in the animal experiment and provided resources, respectively. TK polished the language for the manuscript. EYP provided ideas, the fund for the research, revising the manuscript, and supervising this study. All authors read and approved the manuscript.

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Tables

Table 1 Used primers

Name	5–3
3CprME-F	TAA <u>TGG ATC CAT</u> GAA TAA CCA GCG CAA GAA
3CprME-R-EcoRI	TAA <u>TGA ATT CTC</u> AGA CTA CGT CGT CTT CCG C
3prME-F	TAA <u>TGG ATC CAT</u> GTT TCA TCT CAC TTC CCG TGA TGG C
3prME-R-EcoRI	TAA <u>TGA ATT CTC</u> AGA CTA CGT CGT CTT CCG CAC
pFastBac1-F	TAT TCC GGA TTA TTC ATA CC
pFastBac1-R	ACA AAT GTG GTA TGG CTG ATT
pUC/M13-F	CCC AGT CAC GAC GTT GTA AAA CG
pUC/M13-R	AGC GGA TAA CAA TTT CAC ACA GG

Underlines indicate restriction enzyme cleaving sites.

Figures

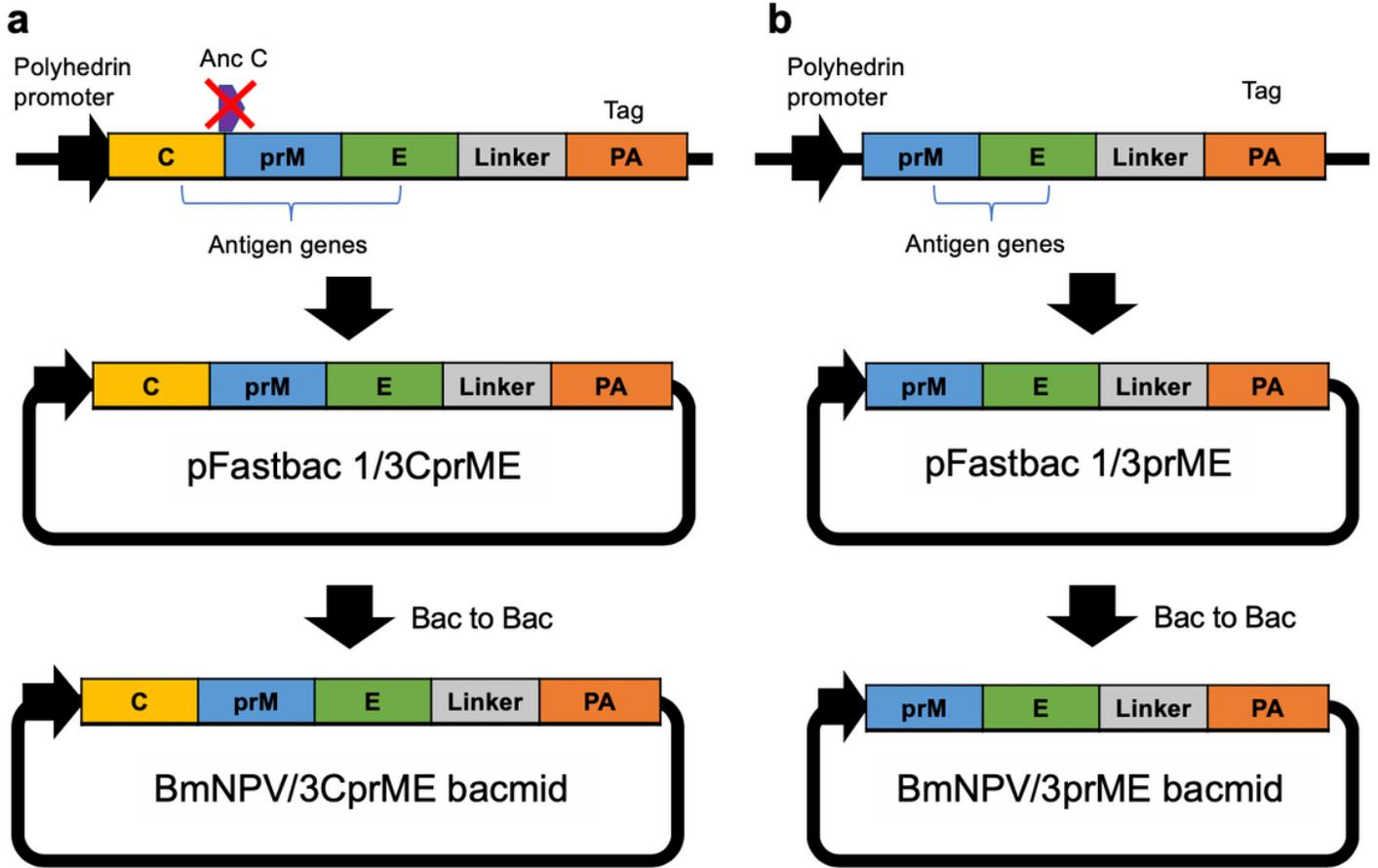


Figure 1

Construction of recombinant dengue virus structural proteins expressed in this study. a 3CprME with the deletion of the anchor C region and b 3prME polypeptides of DENV-3 was expressed in silkworms, pupae, and Bm5 cells as a fusion protein with PA tag.

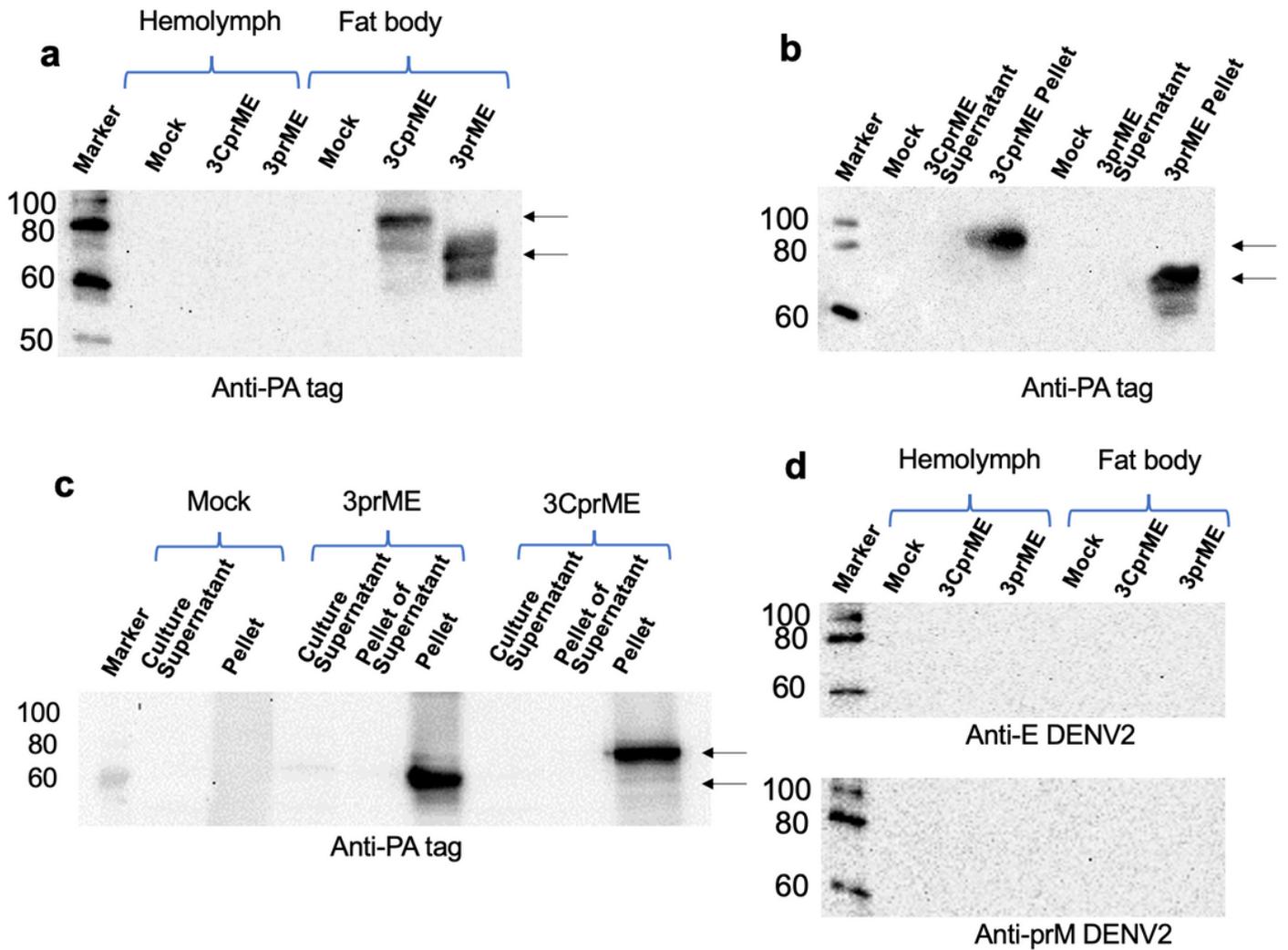


Figure 2

Expression of 3CprME and 3prME polypeptides in silkworm larvae (a), pupae (b), and Bm5 cells (c). In the case of silkworm larvae, hemolymph and fat body were collected after recombinant BmNPV infection. The homogenate of each sample was prepared according to the protocol described in Materials and methods. Expressed 3CprME and 3prME polypeptides were detected by western blot using rat anti-PA tag as a primary antibody. d Hemolymph and fat body from silkworm larvae were verified for cross-reaction using mouse anti-E antibody (anti-E DENV2) and mouse anti-prM antibody (anti-prM DENV2) of DENV serotype 2.

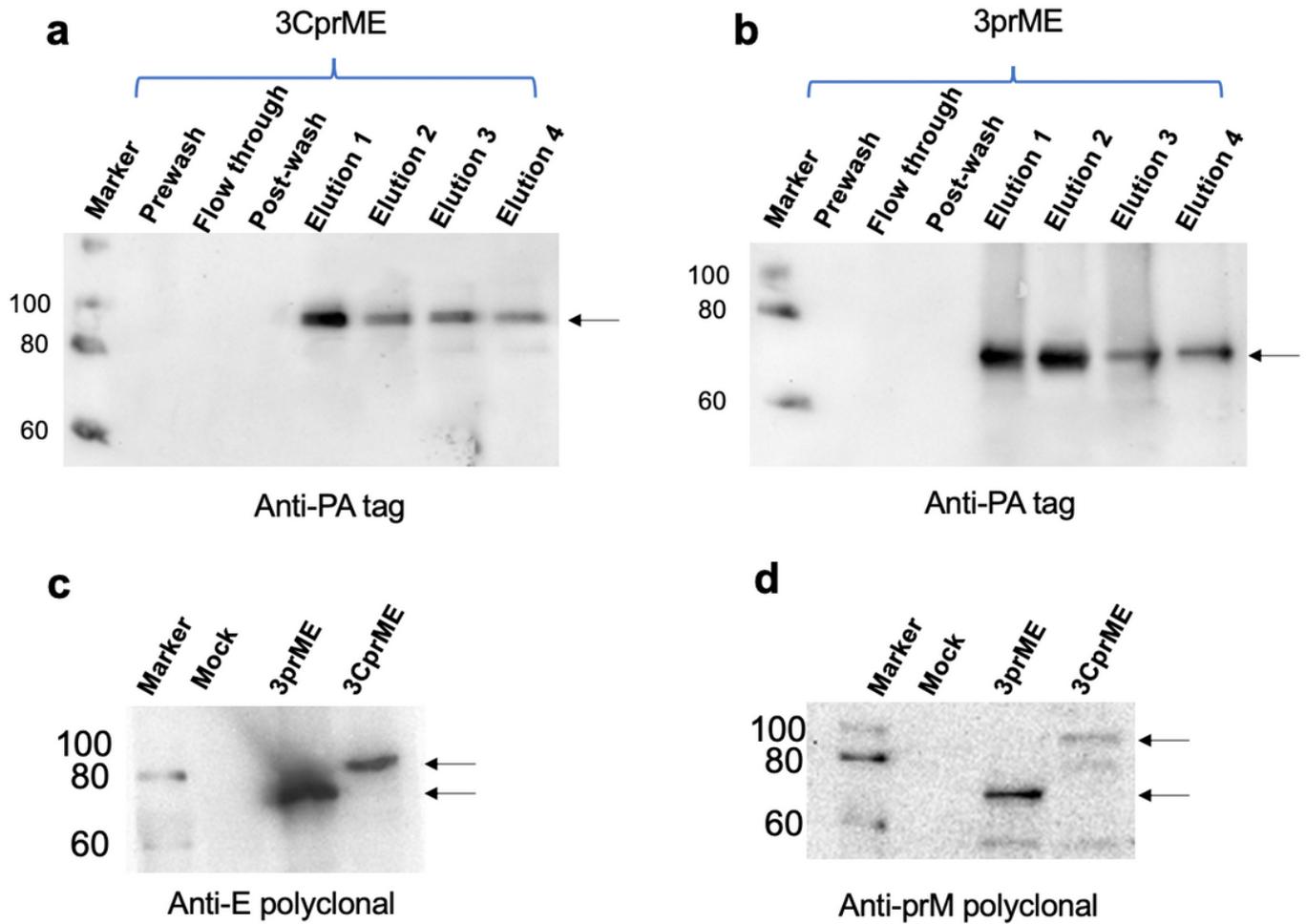


Figure 3

Western blot of purified 3CprME (a) and 3prME (b) polypeptides. Each protein was purified from silkworm hemolymph using PA-tagged protein purification gel column chromatography. The E and prM were verified using rabbit anti-E protein polyclonal (c) and rabbit anti-prM protein polyclonal (d) antibodies.

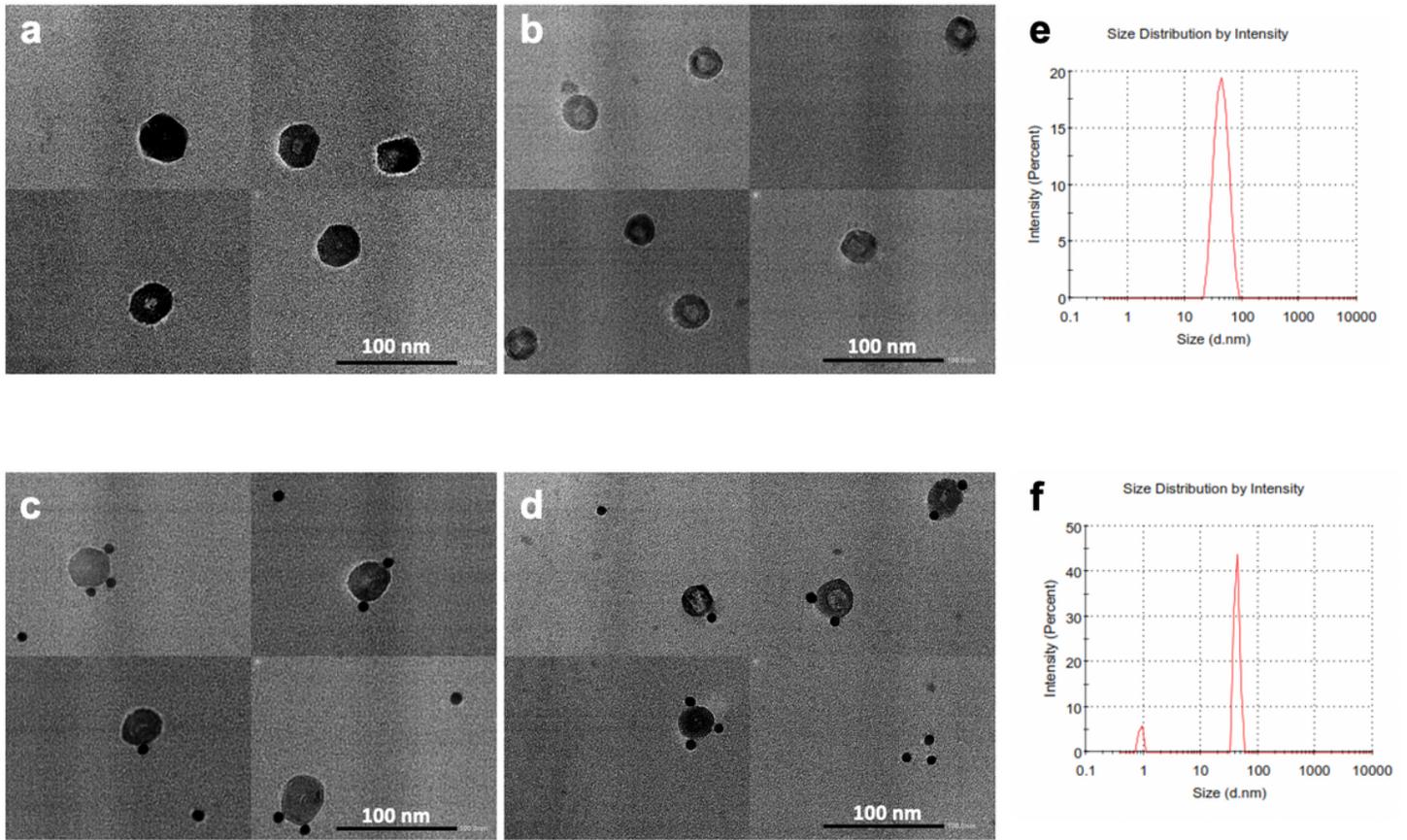


Figure 4

TEM analysis of purified 3CprME (a) and 3prME (b) polypeptides. The purified 3CprME (c) and 3prME (d) polypeptides were immunogold labeled using an anti-E polyclonal antibody and analyzed on IEM. Black spots in c and d indicate immunogold label. DLS size distribution by intensity analysis for 3CprME (e) and 3prME (f) polypeptides.

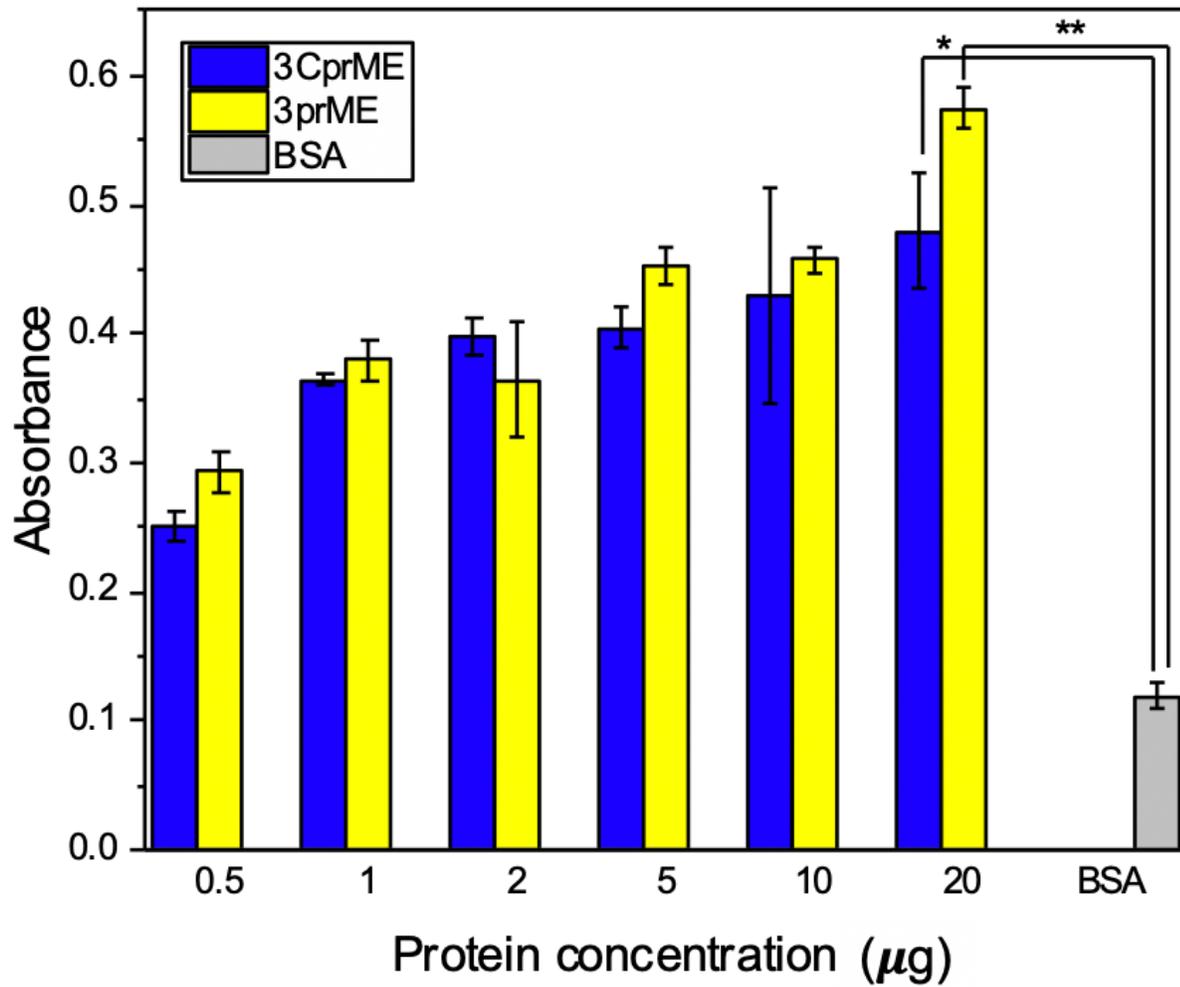


Figure 5

Binding assay of DENV-LPs/3CprME and /3prME to heparin using ELISA. Heparin (1.8 ng) was coated onto each well of an ELISA plate and each amount of purified each protein was used for this ELISA which was carried out according to the protocol described in Materials and methods.

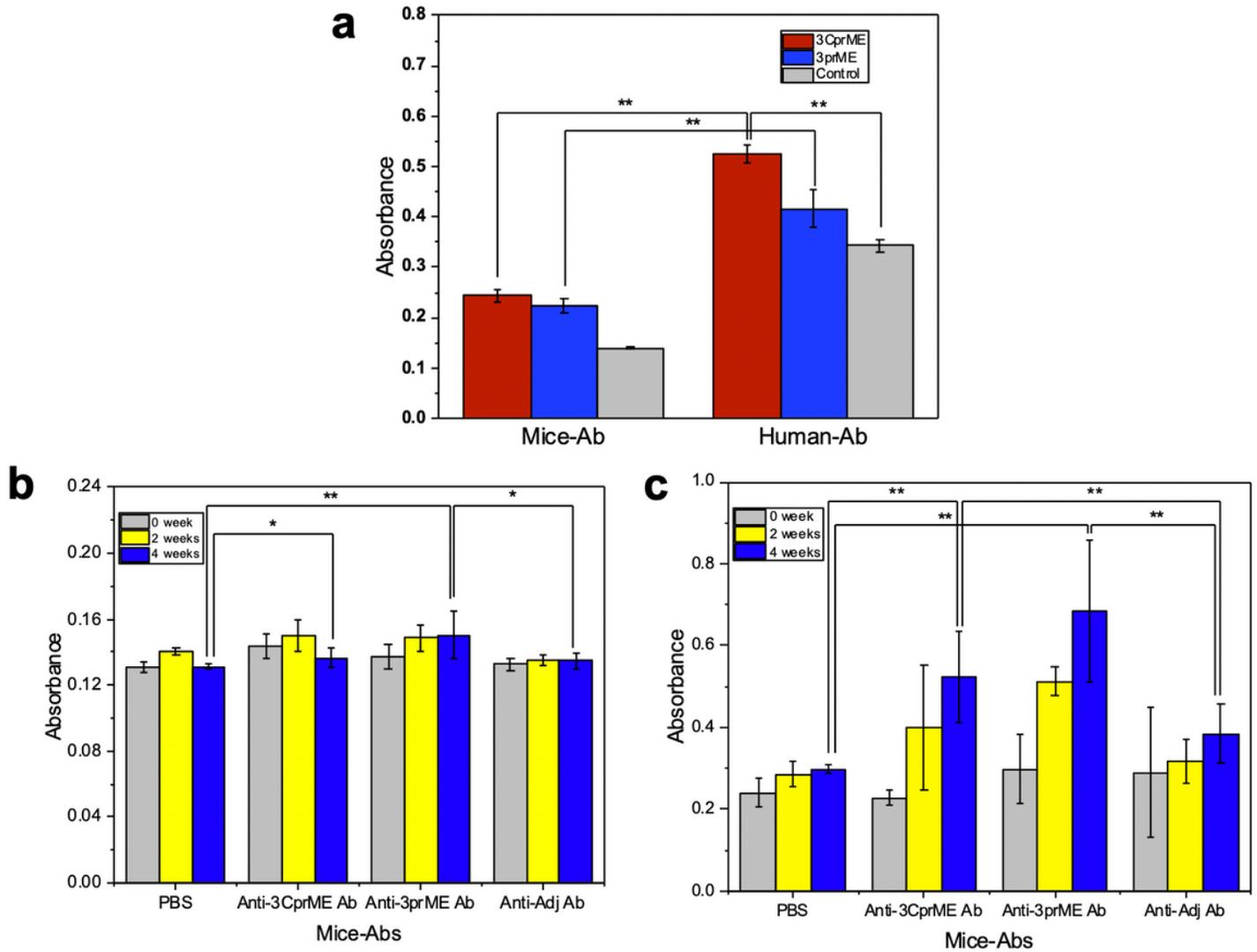


Figure 6

a An interaction of DENV-LPs/3CprME and /3prME with mice sera immunized with DENV tetravalent DNA vaccine and mixed sera from patients [NS1(+), RT-PCR (+)] were investigated. Both of binding reactions were analyzed using direct ELISA as described (Welch t-test, $**p < 0.01$). Specific IgG generation by DENV-LPs/3CprME (b) and /3prME (c). BALB/c mice were intraperitoneally immunized with 50 μ g monovalent DENV-LPs/3CprME and /3prME. At 2 and 4 weeks, sera were collected, and used to test for binding the specific IgG (Welch t-test, $*p < 0.05$, $**p < 0.01$).