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Vectors expressing chimeric Japanese encephalitis dengue 2 viruses

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Summary. – Vectors based on self-replicating RNAs (replicons) of flaviviruses are becoming powerful tool for expression of heterologous genes in mammalian cells and development of novel antiviral and anticancer vaccines. We constructed two vectors expressing chimeric viruses consisting of attenuated SA14-14-2 strain of Japanese encephalitis virus (JEV) in which the PrM/M-E genes were replaced fully or partially with those of dengue 2 virus (DENV-2). These vectors, named pJED2 and pJED2-1770 were transfected to BHK-21 cells and produced chimeric viruses JED2V and JED2-1770V, respectively. The chimeric viruses could be passaged in C6/36 but not BHK-21 cells. The chimeric viruses produced in C6/36 cells CPE 4-5 days after infection and RT-PCR, sequencing, immunofluorescence assay (IFA) and Western blot analysis confirmed the chimeric nature of produced viruses. The immunogenicity of chimeric viruses in mice was proved by detecting DENV-2 E protein-specific serum IgG antibodies with neutralization titer of 10. Successful preparation of infectious clones of chimeric JEV-DENV-2 viruses showed that JEV-based expression vectors are fully functional.

Keywords: Japanese encephalitis virus; dengue 2 virus; chimeric virus

Introduction

Flaviviruses belong to the family *Flaviviridae*, which are single stranded positive sense RNA viruses. The genomic RNA is 11 kb long containing single open reading frame (ORF). The organizational pattern of the genome is 5'-NCR-C-PrM/M-E-NS1-S2B-NS3-NS4A -NS4 B-NS5- NCR-3. The E protein encoded by E gene is a viral envelope glycoprotein, which is an important viral antigen binding to cell surface receptor, mediating membrane fusion, and inducing neutralizing antibody. The PrM/M protein encoded by the PrM gene is considered as another important antigen that induces neutralizing antibody. Non-structural gene and non-coding regions (NCR) are related to virus replication and protein translation.

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Abbreviations: CPE = cytopathic effect; DENV-2 = dengue virus 2; IFA = immunofluorescence assay; JEV = Japanese encephalitis virus

Vectors based on self-replicating RNAs (replicons) of flaviviruses are becoming powerful tools for gene expression in mammalian cells and for the development of novel antiviral and anticancer vaccines. Kunjing, Dengue, YF-17D viruses have already been used for replicon vector construction to express foreign proteins (Khromykh, Varnavski *et al*., 1998; Varnavski and Khromykh, 1999; McAllister *et al*., 2000; Varnavski *et al*., 2000; Khromykh *et al*., 2001; Pang *et al*. 2001; Anraku *et al*., 2002; Harvey *et al*., 2003, 2004; Ward *et al*., 2003; Herd *et al*., 2004). There are many advantages for replicon vector applications. Firstly, a relatively small genome size and simple rapid generation of recombinants; secondly, cytoplasmic RNA amplification eliminates nuclear involvement and leads to extremely high levels of gene expression (Khromykh, 2000).

Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus* of the family *Flavividae*. JEV SA14-14-2 strain, used as an attenuated live vaccine developed in China, is a sTable attenuated virus strain derived from virulent strain SA14 passaged on a monolayer of primary hamster kidney cells. More than 200 million people in China were vaccinated

by JEV SA14-14-2 strain vaccine, demonstrating its safety and efficacy.

In this study, a full-length prM/E gene of DENV-2 (1983 bp) and a fragment (1770 bp) of this gene lacking 3′-terminal 213 bp were inserted into the MCS of an expression vector harboring the JEV replicon. *In vitro* RNA transcripts from these recombinant vectors were used for expression of corresponding chimeric JED2V and JED2-1770V viruses in BHK-21 and C6/36 cells. The genome composition of chimeric viruses was confirmed by RT-PCR, IFA and Western blot analysis. The chimeric viruses could be passaged in C6/36 but not in BHK-21 cells, produced CPE and were immunogenic in mice.

Material and Methods

Virus. Dengue virus 2 New Guinea C strain (DENV-2 NGC strain) was introduced by *Institute Pasteur* (France) in 1978 and provided by Chinese National Pharmaceutical & Biological Products Control Institute. JEV SA14-14-2 stain is from Chengdu Biological Product Institute. These strains were passaged no more than 3 times in our laboratory.

Cells and animals. BHK-21 cells and C6/36 cells were from ATCC, stored in our lab. Special pathogen free (SPF) Balb/C mice (6–8-week-old, female) were obtained from Institute of Laboratory Animal Sciences, CAMS&PUMC.

Vector construction. The full-length prM/E gene (DENV-2-prM/E, 1983bp) and 213 bp deletion of 3′-terminal fragment of prM/E gene (DENV-2-1770, 1770bp) were amplified from DENV-2 virus cDNA template by Platinum Pfx DNA polymerase (Invitrogen, USA). The primer sequences for amplification of DENV-2-prM/E are: forward primer 5′-*accggt*TTCCATTTAACCACA-3′ (AgeI restriction site is underlined) and reverse primer 5′-cccgggGGCCTGCACCATGAC-3 (XmaI restriction site is underlined). The primer sequences for amplification of DENV-2-1770 are: forward primer 5'-*accggt*TTCCATTTAACCACA-3′ (AgeI restriction site is underlined) and reverse primer 5′-cccggg GGATCCAAAATCCCAAGCTG-3′ (XmaI restriction site is underlined). The PCR amplification conditions were: denaturation at 94°C for 30 sec and 25 cycles of 94°C 15 sec, 52°C 30 sec, 68°C 2 min, with a final extension at 68°C for 10 min. After PCR amplification of DNA fragment, 1% agarose gel electrophoresis was undertaken to analyze the PCR products. TA clones were designated as DENV-2-prM/E-TA and DENV-2-1770-TA and verified by restriction analyses and sequencing. pFullΔprM/E and pPartialΔprM/E vectors were constructed using Age I and XmaI restriction enzyme digestion. Competent *Escherichia coli* XL1-Blue cells were transformed with the ligation products. The recombinant plasmids were verified by restriction enzyme digestion and designated as $pJED_2$ and $pJED_2$-1770.

RNA in vitro transcription. Plasmids $pJED_2$ and $pJED_2$-1770 were linearized by SalI digestion and purified. *In vitro* transcription, RNA was prepared by RIBOMAX large scale RNA production system (Promega). Transcription was done in an 100 μl reaction mixture containing 5× transcript buffer, 25 mmol/l rATP, rCTP, rUTP each, and 8.33 mmol/l rGTP, 40 mmol/l of m7G cap, 10 U of T7 RNA polymerase (Promega), and 15–25 ng/μl of linearized plasmid. After 4 hr incubation at 37°C, 2 μl of DNase were added to the mixture and the incubation continued for an additional 20 mins at 37°C. The in vitro transcript was purified by RNeasy mini kit (Qiagen) and stored at -70°C

Preparation and passaging of chimeric viruses. BHK-21 confluent monolayer in a T25 flask was harvested by 10% trypsin, resuspended in DMEM with 10% FBS and centrifuged at 400 x g for 5 min. The cells were washed twice with cold PBS, resuspended into 400 μl of PBS, mixed with 10 μg of *in vitro* RNA transcript and then transfected by electroporation using a Biorad Gene Pulser at settings of 850 V, 25 μF capacitance, ∞ resistance and double pulse. The electroporated cells were immediately, after 5 min incubation on ice, transferred to six-well plates with 2.5 ml of DMEM with 10% FBS and incubated at 37°C. After the cells attached, culture medium was replaced with fresh medium and the incubation continued. The mock electroporated BHK-21 cells were used as negative control. The morphology of the electroporated cells was observed each day. When the transfected cells showed CPE, the BHK-21 cells with culture medium were thawed and frozen 3 times and centrifuged at 4,000 x rpm for 10 min. BHK-21 and C6/36 cells in T25 flasks were infected by 0.5 ml of supernatant and incubated at 37°C and 28°C for 1 hr, respectively. The infected cells were cultured in medium containing 2% FBS. The morphology of the infected cells was observed each day. The obtained chimeric virus strains were designated as JED2V and JED2-1770V.

RT-PCR. Total RNA from cells infected with JED2V and JED2-1770V showing CPE was isolated and reverse transcribed to cDNA. PCR was carried out with the cDNA template and with primers: 5′-CGAGAACTTGGAACACTCATTGACG-3′ (forward) and 5′-GCGCTTTGTGGACGATCTTCGCTAG-3′(reverse). The PCR products were verified by sequencing. RNA from non-transfected cells was used as negative control.

Immunofluorescence assay (IFA). After washing twice with PBS, JED2V and JED2-1770V inoculated cells showing CPE were resuspended in PBS. Antigen slides were prepared and fixed with cold acetone for 15 min. Viral antigen levels were detected by recombinant DENV-2 E III protein rabbit antiserum (1:200) and Japanese encephalitis virus NS1 protein rabbit monoclonal antibody (1:400). FITC conjugated goat anti-rabbit serum was used as secondary antibody (1:100). Samples were analysed in the fluorescence microscope (Nicon).

Western blot analysis (WBA). JED2V and JED2-1770V infected cells showing CPE were lysed in cell lysis buffer on ice for 30 min. 40 μl of cell lysates with 5× loading buffer were boiled for 3 min and centrifuged at 12,000 x rpm for 10 min. The supernatants were subjected to SDS-PAGE. The proteins were transferred to a PVDF membrane by a semi-dry transfer apparatus. Viral protein levels were detected by recombinant DENV-2 E III protein rabbit antiserum (1:200) and Japanese encephalitis virus NS1 protein rabbit monoclonal antibody (1:400).

HRP conjugated goat anti-rabbit antiserum was used as secondary antibody (1:100) and proteins were visualized by DAB staining.

Immunization of mice. The supernatant containing chimeric virus was collected from C6/36 cells at the 4th passage and filtered through 0.45 μm filter membrane. The viral titers were measured by performing tissue culture infectious dose 50% endpoint titers ($TCID_{50}$) and adjusted to 10^{-5} by RPMI-1640 culture medium containing 1% FBS. DENV-2 and JEV virus with the same viral titers were prepared as described before. Twenty five SPF grade Balb/C mice were randomly divided into five groups (JED2V, JED2-1770V, DENV-2, JEV, negative control) and each group consisted of five mice. The animals in control group were inoculated with RPMI-1640 culture medium with 1% FBS. Mice were immunized by intraperitoneal injections of 200 μl of homogenized supernatant or negative control culture medium. 4 weeks later, mice sera were collected from femoral artery blood.

ELISA of DENV-2 E-specific antibodies. 96-well plates were coated with 100 ng per well of purified prokaryote-expressed DENV-2 E III protein at 4°C overnight. Plates were washed 3 times with PBST, blocked with 5% non-fat dry milk at 37°C for 2 hr, and washed 3 times with PBST. Collected mice sera were diluted 1:100, 200, 400, 800 in 5% non-fat dry milk respectively. Hundred μl of sera per well were added into 96-well plates and incubated for 1 hr, followed by washing 6 times with PBST. Secondary antibody solution containing HRP conjugated goat anti-mouse IgG (1:500 in 5% non-fat dry milk) was added to the wells at 100 μl per well and incubated at 37°C for 1 hr, followed by washing 6 times with PBST. After washing, the plates were developed in the dark by addition of 50 μl of substrate A and B solution per well for 10 min. Fifty microliters per well of 2N H_2SO_4 were added to stop the reaction. The plates were read at absorbance A_{450} in an ELISA reader (Biorad).

Neutralization titration. BHK-21 cells were seeded into 96-well plates and cultivated until confluent monolayer was formed. All serum samples were heat-inactivated at 56°C for 30 min and filtered with 0.45 μm filter membrane. Two-fold serially diluted sera (starting at 1:5) were incubated with an equal volume of DENV-2 virus (100x $TCID_{50}$) at 37°C for 1 hr. Ninety-six well plates were washed once with maintaining buffer. Two hundred μl of serum were added (in 4 parallel wells) and then incubated at 37°C, in 5% CO_2. Cytopathic effect (CPE)was observed everyday for 7 days. The neutralization titer was defined as the dilution of serum in 4 replicates that showed no CPE.

Statistic analysis. ELISA results of serum antibody levels were shown as absorbance A_{450} ($\bar{x} \pm s$). One-way ANOVA test was used to test the differences. Statistical analyses were performed by SPSS 16.0 software. Differences with $P \leq 0.05$ were considered significant.

Ethical aspects. This study was carried out in strict accordance with the recommendations in the Guide for the care and use of laboratory animals of Institute of Laboratory Animal Sciences, CAMS & PUMC. The protocol was approved by the Committee on the Ethics of Animal Experiments of CAMS (Permit Number: SCXK (Jing) 2004-0001). All surgery was performed under ether anesthesia, and all efforts were made to minimize suffering.

Results

Cytopathogenicity of chimeric viruses

Typical CPE such as cell rounding and detachment was observed in pJED2V and pJED2-1770V *in vitro* transcribed RNA-transfected BHK-21 cells 5–7 days after transfection. This was similar to the CPE observed in BHK-21 cells infected with DENV-2 and JEV (Fig. 1). The virus could not to be detected in BHK-21 cells in 2nd to 5th passage, indicating that the chimeric viruses could not be passaged in BHK-21 cells. Typical cytopathic changes such as cell swelling and aggregation were observed in C6/36 cells infected with chimeric virus JED2V and JED2-1770V 4–5 days after infection. The cytopathic changes were similar to the changes observed in DENV-2 and JEV infected C6/36 cells (Fig. 2).

Identification of chimeric viruses by RT-PCR, IFA and WBA

RNA was extracted from culture supernatant of the third generation of cells containing viral particles and reverse transcribed to cDNA. Primers were designed to amplify the region outside of chimeric virus prM/E gene and synthesized to amplify a 2,100 bp fragment from cDNA template. Non-transfected RNA served as negative control to detect DNA contamination. The sequencing results verified the chimeric gene of DENV-2 prM/E, JEV C and NS1 gene (partial JEV E gene in JED2-1770V). For chimera construction, in order to clone the exogenic gene, we introduced AgeI and XmaI restriction sites into the primer sequence.

Antigen slides were prepared by using the 4th generation of chimeric virus infected C6/36 cells. Recombinant DENV-2 E III protein rabbit immune serum was used as primary antibody to detect DENV-2 E protein. Specific fluorescence proved the expression of DENV-2 E protein. JEV infected C6/36 cells and uninfected C6/36 cells remained negative (Fig. 3). Anti-JEV NS1 monoclonal antibody was used as primary antibody to detect NS1 protein. DENV-2 infected C6/36 cells and uninfected C6/36 cells did not interact with this monoclonal antibody and were negative (Fig. 4).

Western blot analysis was performed by using cell lysates of the chimeric virus infected C6/36 cells. Recombinant DENV-2 E III protein rabbit immune serum was used as primary antibody to detect DENV-2 E protein at molecular weight 60 kDa. Anti-JEV NS1 monoclonal antibody was used as primary antibody to detect NS1 protein at molecular weight 47 kDa as expected (Fig. 5).

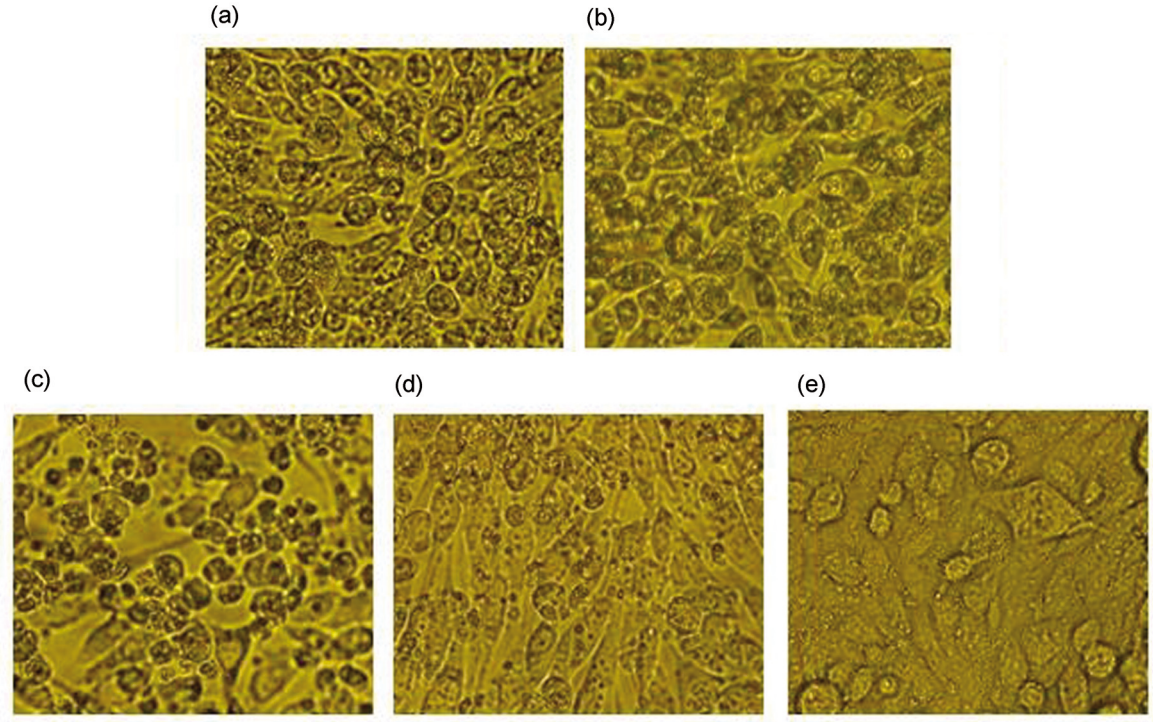

Fig. 1
CPE in BHK-21 cells transfected with vectors expressing chimeric viruses and infected with DENV-2 and JEV
The legend: pJED2 (a), pJED2-1770 (b), DENV-2 (c), JEV (d), and mock (e).

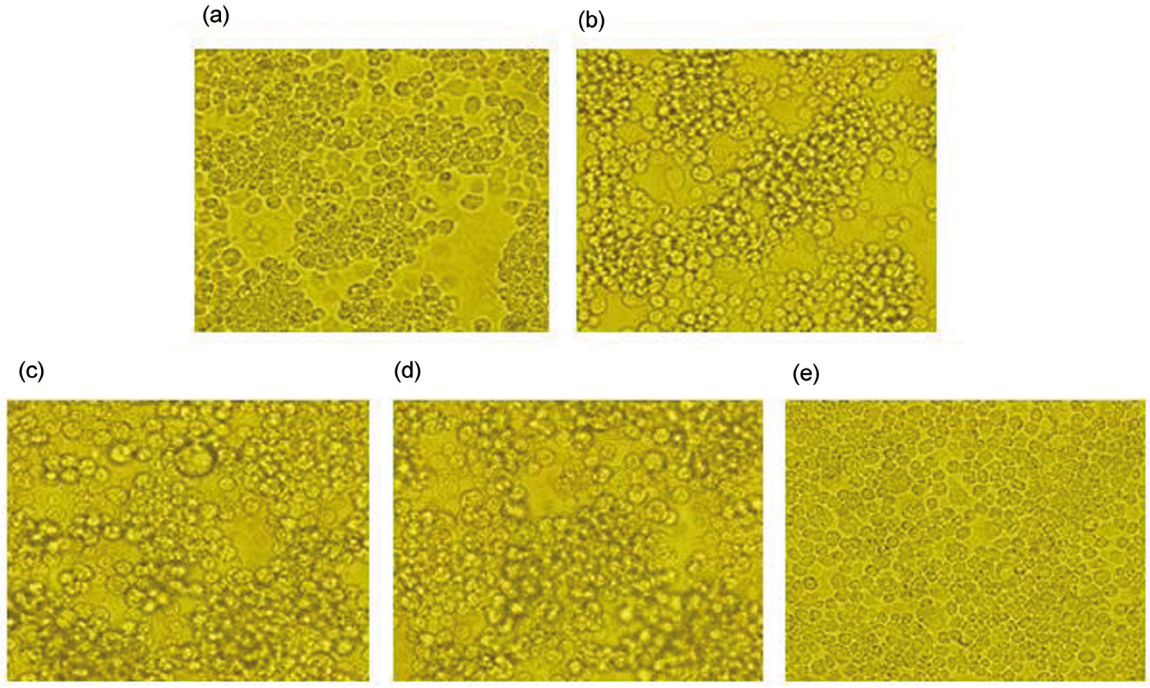

Fig. 2
CPE in C6/36 cells infected with chimeric viruses, DENV-2 and JEV
The legend: JED2V (a), JED2-1770 (b), DENV-2 (c) and JEV (d), and mock (e).

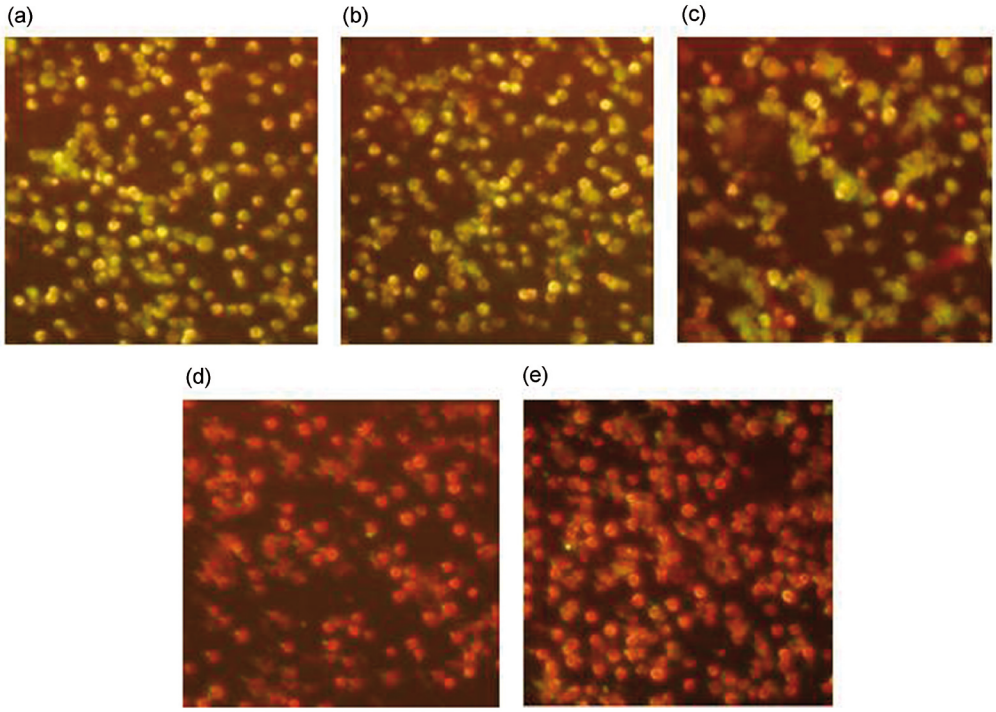

Fig. 3
Expression of DENV-2 E protein of chimeric viruses in C6/36 cells
The legend: IFA. JED2V (a), JED2-1770V (b), DENV-2 (c) and JEV (d), and mock (e).

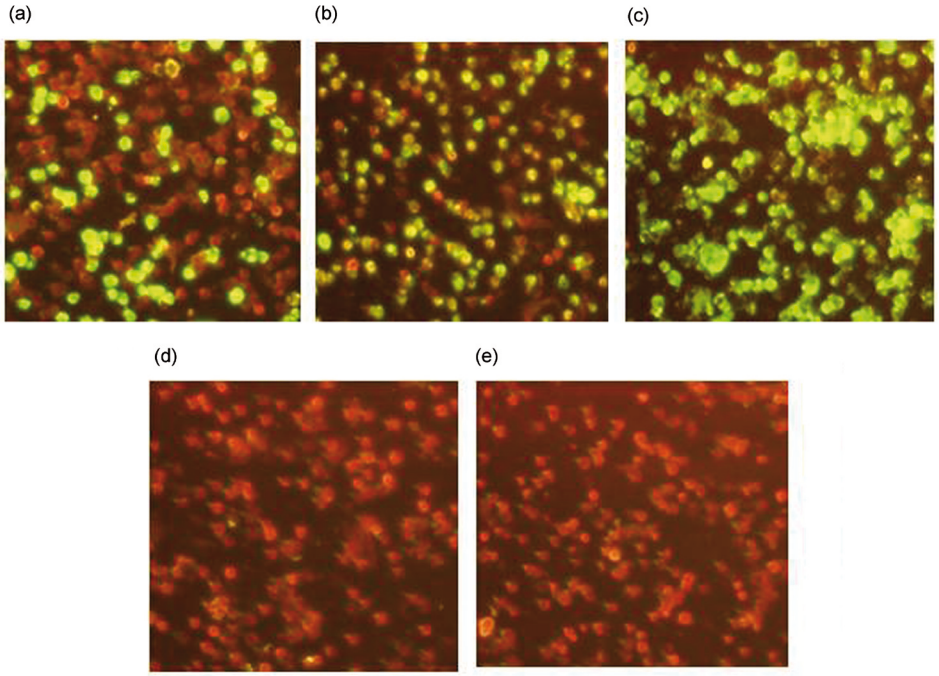

Fig. 4
Expression of JEV NS1 protein of chimeric viruses in C6/36 cells
The legend: IFA. JED2V (a), JED2-1770V (b), DENV-2 (c), and JEV (d), and mock (e).

Immunogenicity of chimeric viruses

To evaluate the immunogenicity of the chimeric virus, we have immunized Balb/C mice with the chimeric virus. Four weeks after immunization, the anti E protein IgG levels of immune sera were detected by recombinant DENV-2 E III protein coated on 96-well plates. The results proved that the A_{450} values of mouse sera from the chimeric virus and DENV-2 immunized groups were significantly higher than those from the JEV immunized and negative control groups at every dilution ($P < 0.05$)(Fig. 6). These results indicate that the mice immunized with the chimeric virus were able to produce a specific anti-DENV-2 E protein IgG antibody.

To evaluate the neutralizing activity of mouse sera of the chimeric virus immunized group, we used the serum dilution neutralization assay to determine the neutralization titer of the serum. The serum antibody titers of these two chimeric virus strains were up to 1:10. The serum antibody titers of the positive control DENV-2 were up to 1:20 as shown in Table 1.

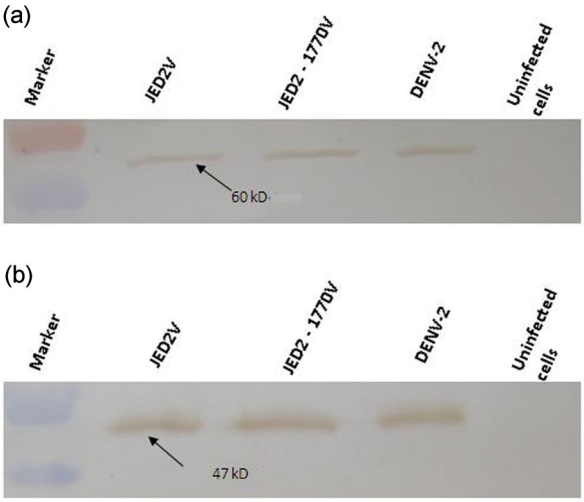

Fig. 5
DENV-2 E and JEV NS1 proteins of chimeric viruses in C6/36 cells
The legend: Western blot analysis. DENV-2 E protein (a) and JEV NS1 protein (b).

Discussions

In our previous study, we established the infectious clone of JEV SA14-14-2 strain and on this basis we have constructed JEV replicon vectors pFullΔprM/E and pPartialΔprM/E with full or partial structural protein gene prM/E (Wei *et al.*, 2009). Based on previous study, we established two chimeric virus clones pJED2 and pJED2-1770 through inserting full length prM/E gene to pFullΔprM/E or C terminal 213 bp deleted DENV-2 prM/E gene to pPartialΔprM/E. After transfecting BHK-21 cells with *in vitro* transcripts of these two chimeric viruses, two Japanese encephalitis/Dengue type 2 chimeric virus strains (JED2V and JED2-1770V) were rescued. The rescued chimeric virus strains have been verified to express the envelope protein of DENV-2 by RT-PCR, IFA, Western blot and exerted cytopathic effects such as cell swelling and aggregation similarly to parent strains. However, the chimeric virus could not be detected in BHK-21 cells after any of five passages. It indicated that the chimeric virus RNA cannot infect BHK-21 cells although with successful virus assembly and exocytosis. Both, BHK-21 and C6/36, cell lines are sensitive to JEV and DENV-2. Each protein of the parent virus strain is conserved after long term evolution in nature. These proteins are able to synergize with viral and cellular parts in viral replication and interact with host cells. The incompatibility of the chimeric virus may be caused by genetic heterogeneity. The change of the natural synergy responds to the change of biological characteristics such as host tropism and some unexpected changes. There were other studies of chimeric viruses with similar results. ChimeriVax-JE chimera constructed by Guirakhoo *et al.*

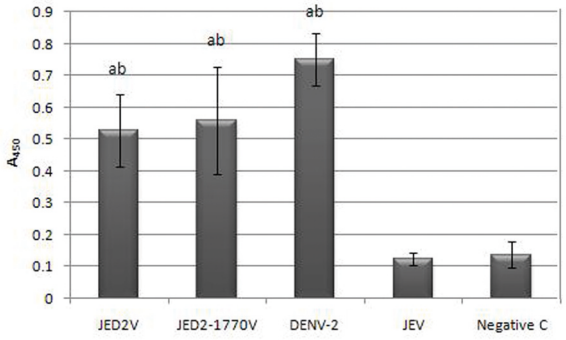

Fig. 6
IgG antibodies to DENV-2 E protein induced in mice by chimeric viruses
The legend: ELISA, sera diluted 1:100. The values differed significantly from those of negative control (NC) (a) and JEV (b), respectively.

Table 1 Neutralization titration of sera of mice immunized with chimeric viruses, DENV-2 and JEV

Immunization with	No. of positive sera/total of sera			
	Serum dilution			
	1:5	1:10	1:20	1:40
JED2V	5/5	3/5	0/5	0/5
JED2-1770V	5/5	4/5	0/5	0/5
DENV-2	5/5	5/5	3/5	0/5
JEV	0/5	0/5	0/5	0/5
Negative control	0/5	0/5	0/5	0/5

(1999) has a similar performance in Vero, LLC-MK and C6/36 cells but has different performance with parent virus strains in MRC-5 and FRh1 cells where CPE was not observed even when infecting with high viral titers (up to 10^6 PFU/ml). The replication ability of chimeric virus of TBEV and DV4 decreased in monkey cells but was impaired in mosquito cells (Pletnev *et al.*, 1992).

Furthermore, we immunized Balb/C mice to determine the immunogenicity of the chimeric virus. Four weeks after immunization, using recombinant DENV-2 E III protein as antigen and serial dilution of mouse serum, the ELISA results showed that A_{450} value of each dilution of mouse sera from the chimeric virus and DENV-2 immunized groups was higher than those of JEV immunized group and negative group ($P < 0.05$). This indicates that the Balb/C mice immunized with chimeric virus can produce specific anti-DENV-2 protein IgG antibody. Neutralization assay showed that the serum antibody titers of these two chimeric virus strains were around 1:10, while the serum antibody titers of the positive control-DENV-2 were up to 1:20. However, prM and E genes from DENV inserted into some other flavivirus replicon vectors (YF-17D, DEN2 PDK-53, rDEN4Δ30) reached 1:40 neutralizing titers or higher (Guirakhoo *et al.*, 2000, 2001; Huang *et al.*, 2003; Durbin, 2006). This is the first time to express the prM/E protein with the SA14-14-2 vector, so much more study is needed to explore the compatibility of SA14-14-2 backbone with other foreign proteins. We hypothesized that lower A_{450} value of ELISA and the lower neutralizing titers of mouse sera from the chimeric virus immunized mice group than from DENV-2 immunized group may relate to the weaker replication ability caused by the genetic heterogeneity.

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