Medical Science Journal for Advance Research

Vol.7, No. 1, March 2026
e-ISSN: 2774-4892

The Study on Preparation of Japanese Encephalitis DNA Vaccine

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ABSTRACT

The major immune antigen gene (JEV E) of JEV was synthesized by RT-PCR and cloned into the eukaryotic expression vector, pVAX1 to prepare recombinant plasmid pVAX-JEV E that could be used as a japanese encephalitis DNA vaccine. In mice (immune groups) vaccinated twice im at 2 weeks intervals with 10 or 50㎍ of JEV DNA vaccine, the antibody titers were 1:160-1:320 and survival rate were 42.8~83.3%. A few mild adverse events were observed in volunteers vaccinated twice with 0.5 mg of JEV DNA vaccine and the antibody titer in vaccines were 1:80 to 1:160 at 2~4 weeks after immunization.

Keywords: Japanese Encephalitis Virus (JEV), Envelope (E) Gene, DNA Vaccine, Pvax1 Vector, Recombinant Plasmid, Immunization.

Article Information

Received: January 06, 2026; Revised: February 10, 2025; Online: March 2026

INTRODUCTION

Japanese encephalitis virus (JEV) is a serious mosquito borne flavivirus that causes disease in human central nervous system [3, 7, 8]. JEV has a wide distribution in Asia, including Japan, China, Taiwan, Philippines, far-eastern Russia, all of Southeast Asia, India, Papua New Guinea, and the Torres Straits of Australia [7]. About 50,000 cases and 10,000 deaths by Japanese encephalitis are recognized annually throughout worldwide. According to WHO report, a population of over 2 billion peoples are living in JEV epidemic area [3, 7]. In 1935, the prototype strain Nakayama was recovered from the brain of a human in Tokyo. The genomes of JEV strains GP78, JaGAr-01, and JaOArS982 were 10,976 nucleotides in length, and contained an ORF encoding 3,432 amino acids [5, 7].

The single stranded positive sense RNA genome of JEV encodes a polyprotein that is proteolytically cleaved into at least 10 proteins within infected cells. These include three structural proteins: an envelope (E), a membrane (M or precursor M (PrM)) and a capsid (C) protein and at least seven non-structural proteins NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 [3, 5, 7]. Both inactivated and live-attenuated JE vaccines have been used as first generation vaccines in many countries [26]. However, adverse effects of mouse brain-derived JEV vaccine, including allergic reactions [28] have become less tolerated in recent years. Another major

https://doi.org/10.46966/msjar.v7i1.347

problem associated with the use of inactivated JEV vaccine is the lack of long-term immunity which makes the immunization program costly due to required repeated immunizations. Therefore, efforts to improve or to develop new JEV vaccines have been rekindled internationally [2, 18].

Recently Naked DNA vaccines have been developed as a third generation of vaccines and examined for a variety of viral pathogens [6, 9, 20, 23, 24, 27, 29, 30, 31, 32, 35] including JEV [1, 4, 13, 14, 15, 16, 19, 33, 34]. In JEV infection, E protein of JEV was found to play the major role in inducing immunity against JEV infection [13, 14, 16, 17]. Recent development of prime-boosting strategies in which priming with DNA followed by boosting with recombinant proteins appears to be a better protocol for immunizing against viral and parasitic antigens [10, 21, 23].

According to this protocol, high levels of Th1 type cytokine, enhanced Th1-cell proliferative response and high-titered and long-lasting antibody responses are induced [22, 23, 25]. In addition, the nanoparticle-based nucleic acid vaccines are promising for their development [11, 12]. In this study, we constructed recombinat plasmid DNA vaccine vector, pVAX-JEV E containing the full length sequence encoding the envelope (E) proteins of JEV, examined the protective effect of pVAX-JEV E through JEV challenge in murine model and surveilled bio-safety and adverse effects post inoculation in human volunteers.

MATERIALS AND METHODS

First, we designed specific primers for synthesis of JEV E gene on molecular biologist'support program, DNA star5.02, using NCBI database. Then a major immunoantigenic gene of JEV, envelope (E) gene, were synthesized by RT-PCR from RNA extract of JEV (SA14-14-2 vaccine strain) using following primers (JEV-1 5'-CGAATTCATGGTGGTATTCACCATCCT C-3', JEV-2 5'-AACTGCAGATT AGCATGCACATTGGTCGCTA-3' and JR-1877 5' CGGAATTCATAGGTTGTGCCTTTCAG - 3'). The upstream primer (JEV-1) contain a *Eco*RI site and an ATG start codon, and downstream primer (JEV-2) containe a TTA stop codon and an *Pst*I site to facilitate cloning. The internal primer, JR-1877 was designed to sequence amplification fragment. RNA of JEV were extracted using rapid RNA extract kit (QIAGEN) following manufacter's protocol. The envelope (E) amplification fragment purified from RT-PCR products were then identified by enzyme *Rsa*I digestion and DNA sequencing. The RT-PCR products digested with restriction enzymes *Eco*RI and *Pst*I were inserted into eukaryotic expression vector, pVAX1 (Invitrogen, San Diego) which were digested with same enzymes by T4 DNA ligase reaction to produce recombinant plasmid DNA, pVAX-JEV E. The eukaryotic expression vector pVAX1 contains CMV early promoter and polyadenylation sequences and splicing signal from bovine growth hormone (BGH). Plasmid DNAs (pDNA) were purified from transformed *E. coli* (DH5α) and stored -30℃ as pellets.

The pVAX-JEV E was reconstituted in sterile saline at a concentration of 1 mg/ml for experimental use. We carried out this study in strict accordance with the national guidelines for the care and use of laboratory animals. All animal studies were approved by the Ethics Committee of Pyongyang University of Medical Sciences. Female BALB/c mice (6–8 weeks old) were used in the experiments. For intramuscular (im) pVAX-JEV E immunization, all mice were pre-treated with 100μL of 10% sacarose in each quadricep muscle 1 week before the first pDNA immunization. Groups of five mice were anesthetized and then injected im at 2 week intervals with 50μg of pVAX-JEV E bilaterally

into each quadriceps muscle, respectively. The various groups of immunized mice were challenged ip with JEV (P3 strain) at a dose of 10 times the LD_{50}. The JEV challenged mice were observed daily for symptoms of viral encephalitis for a total period of 30 days. The trial in volunteers was approved by the Institutional Ethics Committee of Pyongyang University of Medical Sciences and was conducted according to the principles of the Declaration of Helsinki (2008) and complied on Good Clinical Practice guidelines. A total of four volunteers were enrolled in this study. All volunteers received two doses of the pVAX-JEV E vaccine (0.5 mg in 0.5 ml for each dose) administered by conventional needle and syringe intramuscularly (IM) at 0, 2 weeks.

Safety of vaccination was assessed by monitoring vaccination site reactions and recording the incidence of local and systemic adverse events during the study. All subjects were evaluated for vaccine safety and local tolerability at the time of vaccination, and at 2 h post-vaccination. All subjects were further evaluated for local tolerability until 30 days post-vaccination. Immunogenicity was assessed by measuring serum hemagglutination-inhibiting (HAI) antibody titers against JEV in blood samples collecting on day 0 (pre-immunization), and at 15, and 30 days post-immunization. Antibody responses to JEV were evaluated using a goose hemagglutination inhibition assay.

RESULTS AND DISCUSSIONS

Design of specific primers for synthesis of JEV E gene

We designed specific primers for synthesis of JEV E gene on molecular biologist'support program, DNA star5.02 using genomic sequences of different JEV strains in NCBI database. These primers were designed to amplify full-length envelope (E) gene of JEV. The upstream primer (JEV-f) contain an *Eco*RI site and an ATG start codon, and downstream primer (JEV-r) contain a TTA stop codon and a *Pst*I site to facilitate cloning. The predicted size of amplification fragment is 1,566bp.

Synthesis and identificaton of JEV envelope (E) gene

Envelope (E) gene of JEV was amplified using RNA extracts isolated from JEV strain as template and above designed primers by one step RT-PCR kit. The RT-PCR products were electrophoresed on 1% agarose gels and were visualized expected amplification fragment by ethidium bromide (EtBr) staining, and UV transillumination **(fig. 1).** After EtBr staining, amplification band of the predicted size, ~1500bp, was detected on agarose gel. when amplification fragment purified from RT-PCR products were then digested by restriction enzyme *Rsa*I and electrophoresed on agarose gel, two fragments (875bp and 691bp) of size expected in design were visualized **(fig. 2)**. Therefore, we determinated that amplification fragment may be correct envelope (E) gene of JEV.

The PCR products were cloned into cloning vector, pGEM-T (Invitrogen, San Diego) and screened and identified recombinant colonies by PCR using specific primers for JEV and restrictic digestion using restriction enzyme EcoRI. As result, 2 colonies predicted with JEV E gene, recombinant plasmid pT-JEV E were be screened primarily. One colony of the primarily selected two pT-JEV E were be analyzied. DNA sequencing of JEV envelope (E) gene fragment was conducted using ALF autocycle™ sequencing kit (Amersham pharmacia) and specific primers in ALF express II sequencer. Dideoxy chain stop method using fluororescence Dye (Cy5 Dye) Labelling ddNTPs was provided for DNA sequencing. Therefore, we correctly confirmed full-length sequences of JEV envelope (E) gene with ATG start codon and TAA stop codon. Phylogenetic analysis with

published sequences of JEV strains isolated previously in different countries show that synthesized DNA fragment is the correct JEV envelope (E) gene (Table 1)

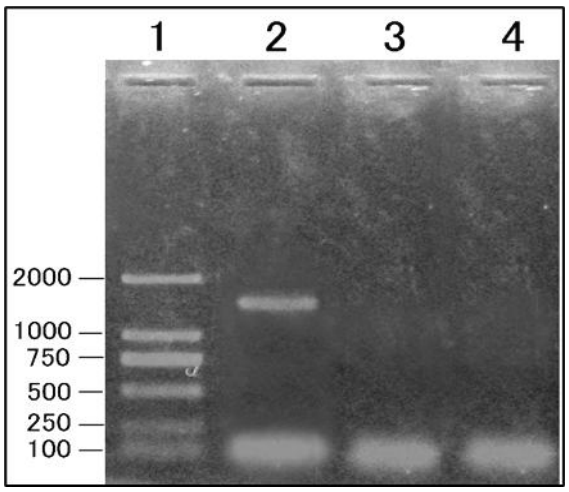

Fig. 1. RT-PCR result for synthesis of JEV E gene (1% AGE): lane 1 molecular marker(DL2000); lane 2 RT-PCR product specific JEV E gene; lane 3 and 4; reagents and sample controls.

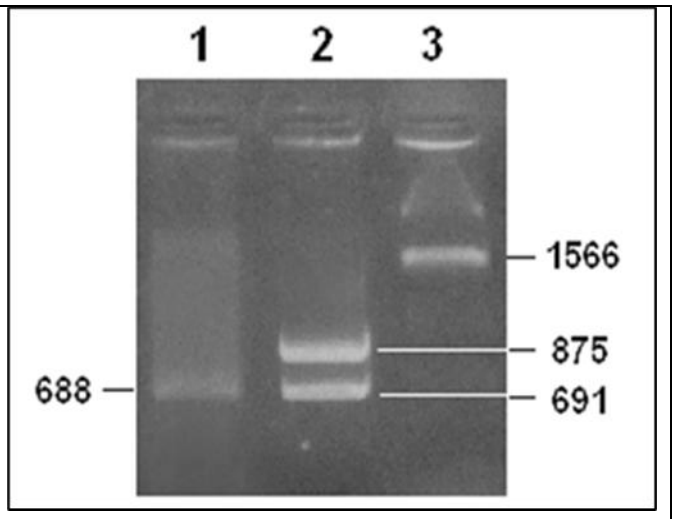

Fig. 2. *Rsa*I restrictic analysis of amplification fragment of JEV E gene (2.5% AGE): lane 1 PCR product known as size (688bp); lane 2 RT-PCR product cleavaged restriction enzyme *Rsa*I; lane 3 RT-PCR product .

Table 1. Identification of JEV E gene by phylogenetic analysis.

Strains	Sequence consistency (%)
Beijing-1 strain	**96.8**
Vaccine strain (JaOH0566)	**97.4**
Wild strain (SA-14-2-8)	**99.0**
attenuated strain SA14-12-1-7	**99.1**

Numbers of anorexia patients cases with different factors

Construction of recombinant plasmid DNA(pVAX-JEV E)

Then, recombinant plasmid pT-JEV E were digested with restriction enzymes *Eco*RI and *Pst*I and inserted into eukaryotic expression vector pVAX1 (Invitrogen, San Diego) which were digested with same enzymes by T4 DNA ligase reaction to produce pVAX-JEV E **(fig. 3)** . *E. coli* (DH5 α) were transfected with recombinant plasmid pVAX-JEV E using transformation protocol and then, we were selected the colonies inserted correctly JEV envelope (E) gene through agarose gel electrophoresis, restrictic analysis (*Eco*RI/*Pst*I) and PCR **(fig. 3, 4, 5)** .

The confirmed *E. coli* (DH5 α) with recombinant plasmid pVAX-JEV E were grown in LB broth containing kanamycin (50μg/mL). Plasmid DNA were isolated by the alkaline lysis method from transformed *E. coli* (DH5 α) and purified using plasmid Giga kits (Qiagen Inc., Chatsworth, CA). Plasmid DNA was reconstituted in sterile saline at a concentration of 1 1mg/mL for experimental use and stored at −20 ◦C.

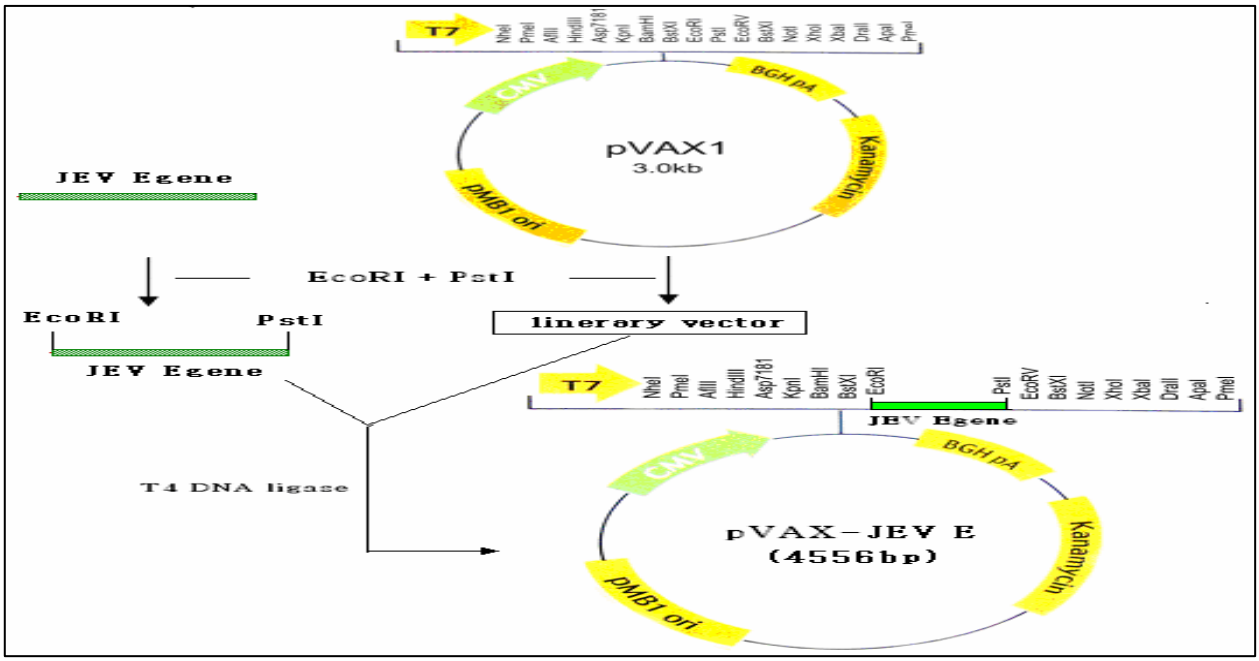

Fig. 3. Construction of pVAX-JEV E plasmid.

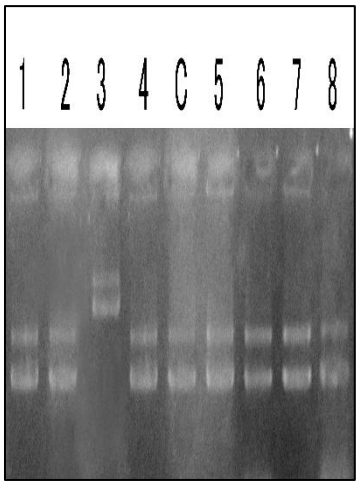

Fig. 3. Primary selection of pVAX-JEV E plasmids. (1%AGE): lane 1-8 recombinant plasmids .

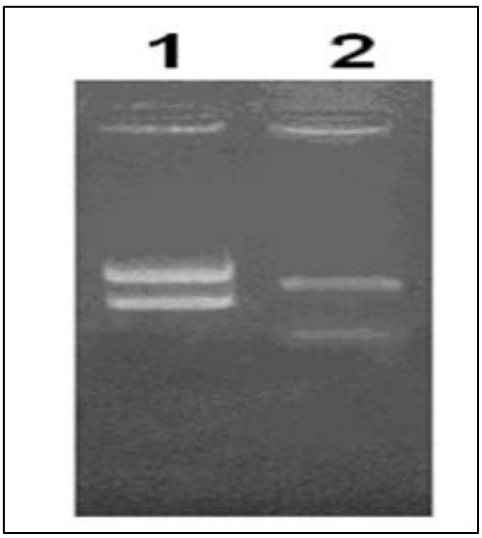

Fig. 4. Identification of recombinant pVAX-JEV E plasmids by restriction enzymes *Eco*RI and *Pst*I digestion (2.5% AGE): lane 1 pVAX-JEV E plasmids; lane 2 pVAX-JEV E plasmids cleavaged *Eco*RI and *Pst*I restriction enzymes .

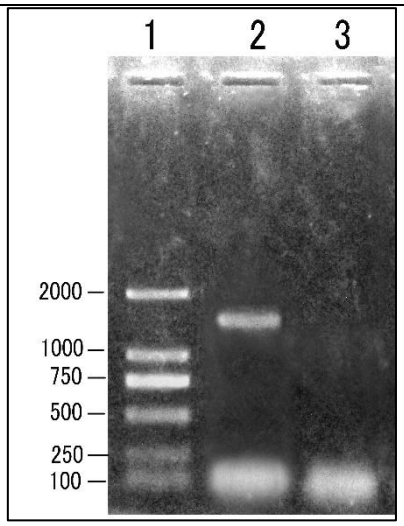

Fig. 5. Identification of recombinant pVAX-JEV E plasmids by PCR (1% AGE): lane 1 molecular marker (DL2000); lane 2 PCR product used pVAX-JEV E plasmids as template; lane 3 control .

DNA immunization and protection from JEV challenge in mice model.

To examine the protective efficacy of JE DNA vaccine, mice were immunized with recombinant plasmid pVAX-JEV E and challenged with JEV. Group of five mice (4 weeks) were immunized via intramuscular (i.m.) route with 10 or 50μg dose of recombinant plasmid DNAs and PBS and lived attenuated JEV vaccine(SA-14-14-2) (controls). A booster dose were administered 14 days later. One week after the administration of the last dose, mice were challenged i.p. with $10LD_{50}$ of neurovirulent JEV P3 strain and the survival of mice was monitored daily up to 14 days post-challenge. The results obtained from experiments are presented in **(Table 2)**.

In contrast of the mean survival rates and antibody titres of mice groups immunized with PBS and lived attenuated vaccine(SA-14-14-2) were 0 and 75.0~85.0 and 0 and 1:40~160, the mean survival rates and antibody titres of mice groups immunized with 10 and 50μg dose of recombinant plasmid DNAs were 42.8~71.4% and 75.0~83.3% and 1:160~320 and 1:160~320. This results demonstrated that after immunization by JEV DNA vaccine based on the envelope protein gene of JEV, immune response against the JEV infection are induced and antibodies against JEV are formed in immunized mice. Although the protective effect of our JEV DNA vaccine is lower than that of the live attenuated virus vaccine, our results indicated that the protective effect of JEV infection is comparable to that of live attenuated virus vaccines.

Table 2: DNA immunization and protection from JEV challenge in mice model.

Group		Dose, times	Survival percent (%)	Antibody titer
Control	PBS(control)	0.2mL, 2	0	0
Experimental	SA-14-14-2	1,000ID, 1	75.0~100.0	1:40~160
	pVAX-JEV E	10μg, 2	42.8~71.4	1:160~320
	pVAX-JEV E	50μg, 2	75.0~83.3	1:160~320

mice number in each inoculation groups: 25

Reactogenicity and antibody production after JEV DNA vaccination in volunteers

After given two doses of 0.5 mg of JEV DNA vaccine, all adverse events (AEs) related to vaccination are shown in **(Table 3)**. Mild vaccination-related local adverse events were reported by all 4 subjects dosed. These included pain and pruritus. All four vaccines (100%) were observed to have mild injection site pains lasting at 1 to 2 hours, even within 20~30 mins, and two of the vaccinees (50%) had mild pruritus lasting 1 to 2 days, even within a few hours. In addition to, mild vaccination related systemic adverse events were reported in 1 subject. These included headache, dizziness and fatigue. He had mild transient headache and dizziness lasting 1.5 h and mild weakness fatigue lasting 1 day, with no anorexia, nausea, vomiting, fever, or other adverse events. Also, the antibody titers against JEV before vaccination with JEV were 1:10-40 in all vaccinees, but 1:80-160 at 15-30 days after vaccination, which increased 4-8 times compared with the pre-vaccination. This finding demonstrated that immunization by JEV DNA vaccine is safe in vaccines and after immunization by JEV DNA vaccine, neutralizing antibodies that can neutralize the JEV are formed in bodies of vaccines.

Table 3. Local and Systemic AE reactogenicity 14 days post vaccination in 4 subjects.

Reactogenicity		Severity	
		None N (%)	Mild N(%)
Local	Pain		4 (100)
	Erythema	4 (100)	
	Warmth		
	Pruritus	2(50)	2(50)
	Induration		
Systemic	Headache	3(75)	1(25)
	Dizziness	3(75)	1(25)

Reactogenicity		Severity	
		None N (%)	Mild N(%)
	Fatigue		
	anorexia		
	Nausea		
	Vomiting		
	Shivering		
	Arthralgia		
	fever		

CONCLUSION

In this study, we synthesized full length cDNA gene encoding *japanese encephalitis* virus (JEV) envelope (E) protein by RT-PCR and constructed recombinant plasmids for JEV DNA vaccines in which this gene was cloned to eukaryotic expression vector, pVAX1. Analysis of the protective efficacy of JEV DNA vaccine constructs in a murine JEV challenge model indicates that they confers about 80% level of protection and 1:160~320 neutralization antbody titre. Therefore, we demonstrate that intramuscular immunization with plasmid DNA vaccine encoding JEV E gene provided protection against JEV challenge in mouse model as official JEV vaccine(SA-14-14-2). We also demonstrated that JEV vaccination was relatively safe in vaccinees and that neutralizing antibody titers after two vaccinations were 4-6 times higher than before vaccination. These results show that the JEV DNA vaccine is a safe and efficient vaccine candidate with immense potential for medical application in the future.

Abbreviation

JEV- Japanese Encephalitis Virus, **DNA**- deoxyribonucleic acid, **E**- envelope, **M**- membrane,

PrM- precursor membrane, **C**- capsid, **NS**- non-structural protein, **NCBI**- National Center of BioInformation, **RT- PCR**- Reverse transcription-polymerase chain reaction, **IM**- intramuscularly, **HAI**- hemagglutination-inhibiting, **AE**- adverse event.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Funding

His research received no external funding.

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