CLONING AND EXPRESSION OF ENVELOPE PROTEIN OF THAI GENOTYPE I STRAIN KE-093 OF JAPANESE ENCEPHALITIS VIRUS

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Abstract. The purpose of this study was to clone and express envelope (*E*) gene of Japanese encephalitis virus (JEV) genotype I, Thai strain KE-093. The *E* gene was amplified by PCR and cloned using the expression vector, pET-15b. Analysis of the insert sequence revealed a point mutation, which was corrected by site directed mutagenesis. The envelope 53 kDa protein expression was generated by *in vitro* coupled transcription translation system. Heterologous expression in *Escherichia coli* Rosetta 2 strain, but not in *E. coli* BL21 (DE3) resulted in 2 immunoreactive bands (13 and 53 kDa) using anti-JEV E protein antibodies, and an additional band (35 kDa) using anti-His antibodies, suggesting that E protein antigenicity is located at the carboxy-terminal region. This is the first report of a successful cloning and heterologous expression of an *E* gene of JEV genotype I. This should prove useful in the application for diagnostics and vaccine development of JEV genotype I strains.

Key words: Japanese encephalitis virus, *E* gene, Thai strain

INTRODUCTION

Japanese encephalitis virus (JEV) causes the disease Japanese encephalitis (JE), which affects the central nervous system. JE is the most important form of viral encephalitis in Eastern and Southern Asia and parts of Western Pacific with 35,000-50,000 cases reported annually (Liang *et al*, 2009; Fischer *et al*, 2010). Nevertheless, the case numbers are likely an underestimate of the real burden of this disease because the diagnostic and the surveillance capacity in several endemic areas are underdeveloped (Fischer *et al*, 2010). There was a recent outbreak of JE in northeastern India and Nepal that caused approximately 1,344 fatalities in September 2005 (Parida *et al*, 2006) and an ongoing outbreak in eastern Uttar Pradesh, India since April 2006. There have been reports that there were 2,675 cases and 577 deaths with case fatality rate of 22 in 2007 in India (Saxena and Dhole, 2008).

The majority of infections are asymptomatic. Overt encephalitis occurs in 1 out

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of every 50-1,000 persons infected. However, 25–30% of encephalitis cases are fatal and 30% of survivors exhibit physiological disorder and neurological sequelae (Lyons *et al*, 2007). In Thailand the disease causes severe morbidity and mortality particularly among children (Poneprasert, 1989).

Immunization against the virus is the single most important control strategy that could reduce dramatically the numbers of JE cases (Nitatpattana *et al*, 2008). Purified formalin-inactivated mouse brain-derived vaccine and an inactivated Vero cell culture-derived vaccine have been licensed in the United States and a live attenuated vaccine (SA 14-14-2) is used in many countries in Asia (Fischer *et al*, 2010; Misra and Kalita, 2010). These 3 commercially available vaccines against JE are based on the Nakayama, Beijing-1, and SA14-14-2 strains, which belong to genotype III (Oya and Kurane, 2007). However, genotype I JEV strains cause local epidemics in Thailand and Cambodia, and genotype I JEV strains, ThCMAr4492, KE-093, and P19-Br, are now spreading in China and Japan (Nerome *et al*, 2007; Saito *et al*, 2007; Wang *et al*, 2007; Nitatpattana *et al*, 2008).

The E protein is an outer membrane glycoprotein of virus that plays an important role for viral infection processes such as attachment, virulence and attenuation. The E protein also serves as the major antigen that could stimulate the immune response and elicit neutralizing antibodies responsible for host protection (Alka *et al*, 2007). The sequence homology of E protein between JE strains range from 80% to 98% and that of genotype III vaccine strains has 90-98.8% homology with the local Thai strains, whereas the corresponding homology within Thai strains is 98.2-100% (Nerome *et al*, 2007; Wang *et al*, 2007). This might explain the occurrence of JEV in Thailand in vaccinated person (Hoke *et al*, 1988). The KE-093 strain shows an outstanding immunological difference from the other strains (Hasegawa *et al*, 1995). There are three amino acid replacements in E protein, E-222, E-327, and E-366, in 12 selected Thai strains that might play a role for antigenic heterogeneity of Thai strains. Neutralization assay has shown that there is a definite antigenic difference between Thai strains and Nakayama and JaGAr01 strains (Ali and Igarashi, 1997). Subin and KE-093 strains are less neutralized by test antisera from the same and different genotypes.

Therefore, we have cloned and expressed the *E* gene from the Thai genotype I KE-093 strain of JEV in order to obtain a better antigenic characterization and to understand the presence, or absence, of cross-neutralizing antibodies in vaccine recipients.

MATERIALS AND METHODS

Bacterial host strains, plasmid vector and plasmid template

Escherichia coli strain BL21 (DE3) was purchased from Stratagen (La Jolla, CA) and Rosetta 2 strain from Novagen (Madison, WI). pET-15b plasmid vector was obtained from Novagen (Madison, WI). Plasmid template containing the whole fragment of *E* of JEV strain KE-093 had been previously constructed (Lorroengsil *et al*, 2008). KE-093 strain of JEV used in this study was originally isolated in 1983 from brain tissues of an encephalitis patient at Kamphaeng Phet Province, Thailand (Harinasuta *et al*, 1984).

Cloning and sequencing

Genomic RNA of JEV strain KE-093 was reverse transcribed to cDNA and the

E gene was amplified (forward primer, *Xho*I_E093; GAT*CTCGAG*TGCACCATGG TCCGTCCGGCTTACA containing *Xho*I site (shown in italics), and reverse primer, *Bam*HI_1550; GCA*GGATCC*TTAGATG TCA ATGGCACATCCAGTGT containing *Bam*HI site (shown in italics)), cloned and sequenced as previously described (Lorroengsil *et al*, 2008). The *E* amplicon was inserted into pET-15b vector between *Xho*I and *Bam*HI restriction site. The recombinant plasmid was delivered to Cybergene Company to perform primer-walking sequencing. The nucleic acid sequences of the inserts were translated to amino acid sequences by ExPASy proteomic server (Swiss Institute of Bioinformatics). The nucleic acid and amino acid sequences were analyzed by sequence alignment with ClustalX 1.81 program (EBI, UK).

***In vitro* mutagenesis**

It was found that a single point deletion mutation occurred in the *E* gene of the transformant, pE1. Thus, the sequence was repaired by Quick Change® Site-Directed Mutagenesis kit (Stratagene, Heidelberg, Germany). Primers were designed to repair the sequence at the mutated region (nucleotides 1,531-1,534) that contained a stop codon (TAG). The forward primer, delGinA_093f (CAATGTGCATGCTGAC ACTGG**TTAG** CCATTGACATCTAAGG) consisted of 41 nucleotides (position 1,509-1,550) of *E* gene and the mutated region that had been altered to become a stop codon (underlined-bold letter) by deleting base G (1,532) and inserting base A in its place in order to repair the sequence and arrange it in-frame. The reverse primer, delGinA_093_r (CCTTAGATGTCAATG G**CTAA**CCAGTGTCAGCATGCACATTG) consisted of 41 nucleotides (1,509-1,550) of *E* gene, which was complementary to the forward primer. Plasmid sequences were determined and analyzed by primer walking using ABI PRISM 3100 Genetic Analyzer and Gene scan analysis program. The nucleic acid sequences were translated to amino acid sequences and the nucleic acid and amino acid sequences analyzed as described above.

Expression of recombinant plasmids by *in vitro* transcription/translation method

Synthesis of ^{35}S-methionine-labelled E protein from pE1 was conducted using TNT™ T7 coupled reticulocyte lysate system (Promega, Madison, WI) according to manufacturer's instruction. pDrive_E093 was used as the template for the positive control reaction. The translation products were separated by 10% SDS-PAGE and the expressed target protein in the dried polyacrylamine gel was visualized by autoradiography on X-ray film (Fuji xBas1000). The exposure time was approximately 48 hours at room temperature.

Heterologous expression in *E. coli*

The recombinant pET-15b plasmid containing the *E* gene was transfected into *E. coli* BL21 (DE3) and Rosetta 2 strains and protein expression was induced using IPTG (Birk *et al*, 1998). Transformed *E. coli* BL21 and Rosetta bacteria were grown in Luria broth containing the suitable antibiotic at 37ºC until OD_{600} of 0.6 and 1.0 was reached, respectively. *E. coli* BL21(DE3) carrying pET-28a vector containing *β-gal* gene (Novagen, Madison, WI) was used as positive control. Non-transformed *E. coli* BL21 (DE3) cells and Rosetta 2 were used as negative controls. Lysed bacteria samples were characterized by 12% SDS-PAGE and Western blotting using anti-JEV E glycoprotein (Abcam, Cambridge, MA) and anti-histidine antibodies (Rotkreuz, Switzerland).

RESULTS

Cloning and sequencing of JEV *E* gene

The full-length JEV *E* gene was inserted into pET-15b plasmid to produce recombinant plasmid, pE1. The size of the full-length *E* gene, E093, was 1.550 kb as expected. The sequence of E093 is shown in Fig 1. E093 contained an A deletion mutation, which was repaired by *in vitro* site-directed mutagenesis. Primers were designed to delete G and insert A to the mutated region that had resulted in a stop codon after the nucleotide position 1,528 of the *E* gene. The stop codon resulted in E protein with four amino acids, A, I, D, and I, lost. It was found that there were transformants containing the correct amino acid sequence.

Analysis of *in vitro* translation protein expression

The corrected *E* gene was analyzed using an *in vitro* transcription/translation system and ^{35}S-methionine-labeling of proteins. The *E* gene from the KE-093 strain encoded the expected 53 kDa protein as shown in Fig 2.

Analysis of protein expressed in *E. coli*

E protein expression in BL21 (DE3) cells could not be induced with 1 mM IPTG (data not shown). However, E protein expression in Rosetta 2 cells showed that there were 2 immunoreactive protein bands (using anti-JEV antibodies) of 53 kDa and 13 kDa (Fig 3). These 2 bands were obtained using anti-His antibodies with an additional band of 35 kDa.

DISCUSSION

In this study, attempts were made to construct recombinant plasmid containing a fragment of full-length *E* gene of JEV Thai strain KE-093 and to investigate the expression of the E protein in order to develop a vaccine against JEV. The strain KE-093 used in this study was isolated from a JE patient during the JE outbreak in 1983 at Kamphaeng Phet Province, Thailand. The serological and genetic properties of this strain were different from the vaccine strains including Nakayama, Beijing-1 and SA 14-14-2 strains (Hasegawa *et al*, 1995). KE-093 strain is separate and distinct from those strains on the basis of hemagglutination inhibition and neutralization test. When the nucleotide sequences of each JEV strain were studied, it was shown that the KE-093 strain can be classified as genotype I, whereas vaccine strains are classified as genotype III (Oya and Kurane, 2007). It has been reported that some JEV particles could escape from the immunity of the vaccinees who had received either Nakayama or Beijing-1 JEV vaccine (Hoke *et al*, 1988). Using the infectious strain to develop candidate JE vaccine could theoretically increase the level of protection compared with current JE vaccines (Holbrook and Barrett, 2002).

The full-length *E* gene of KE-093 strain was cloned and sequenced. The nucleic acid and deduced amino acid sequences were compared with the sequence of pDrive_E093 previously reported (Lorroengsil *et al*, 2008). The cloned *E* gene contained an A deletion resulting in an in-frame stop codon after nucleotide position 1,528. *In vitro* site-directed mutagenesis was used to repair this mutation. This is a convenient method that it is used to make point mutations, switch amino acids, and delete or insert single or multiple amino acids (Erdogan *et al*, 2005).

The corrected recombinant plasmid, pE1, was able to express a protein of 53 kDa in an *in vitro* transcription/translation method. This result agreed with the pre-

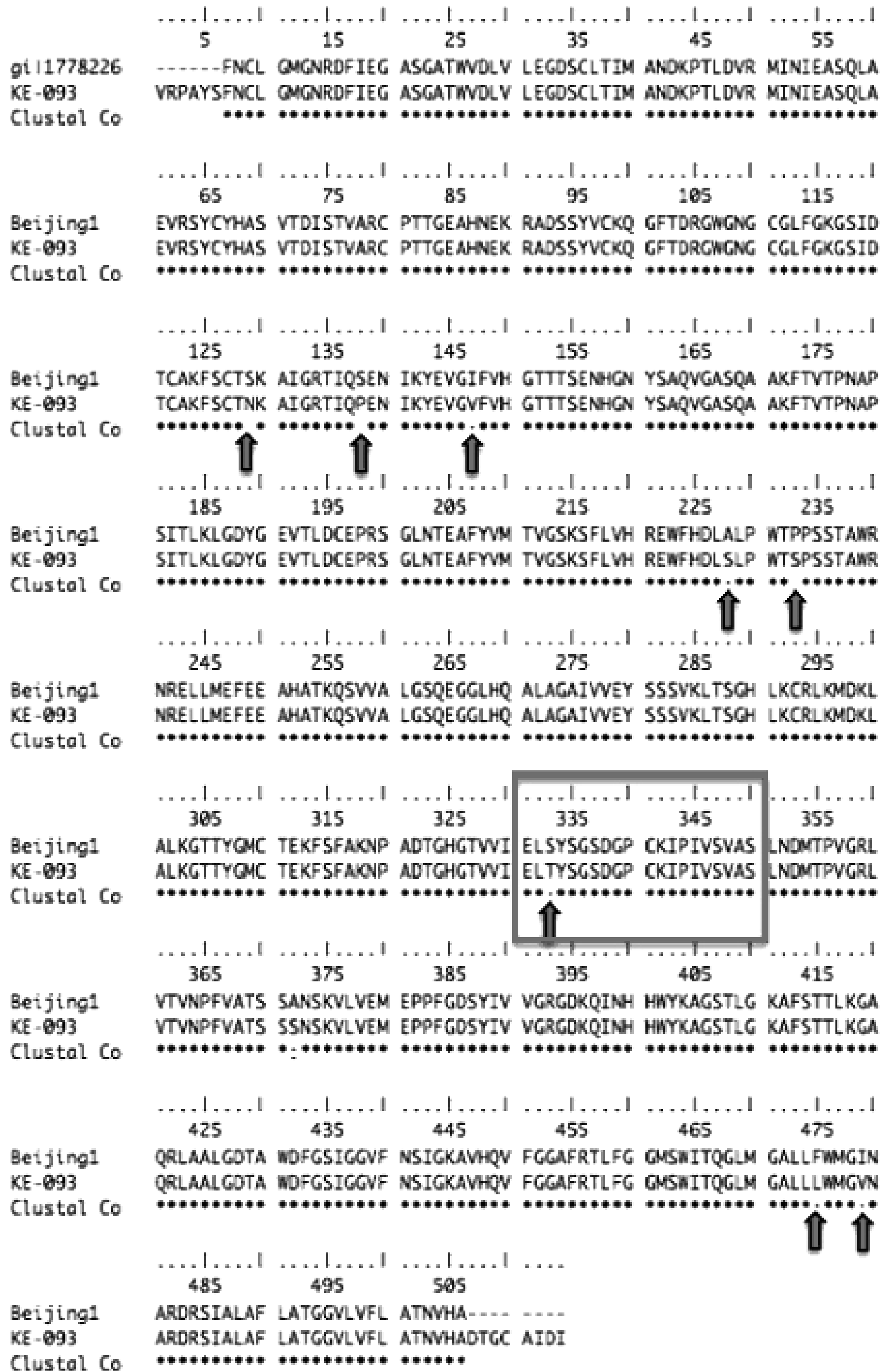

Fig 1–Sequence alignment between the envelope genes of JEV KE-093 and Beijing strains. The arrows indicate discrepancies between the strains and the box indicates the major antibody epitope in domain III.

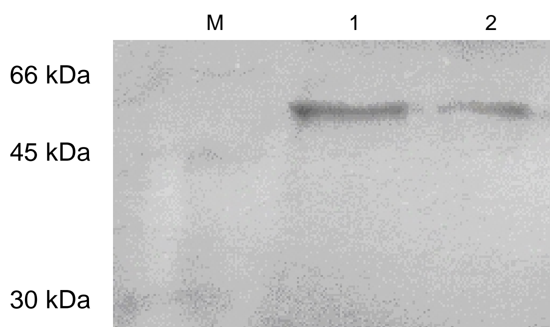

Fig 2–Expression of JEV E protein by *in vitro* transcription/translation system. Lane1, using pDrive; lane 2, using pET-15b plasmid; lane M, Rainbow (CFA756, GE Healthcare, Sweden) size markers.

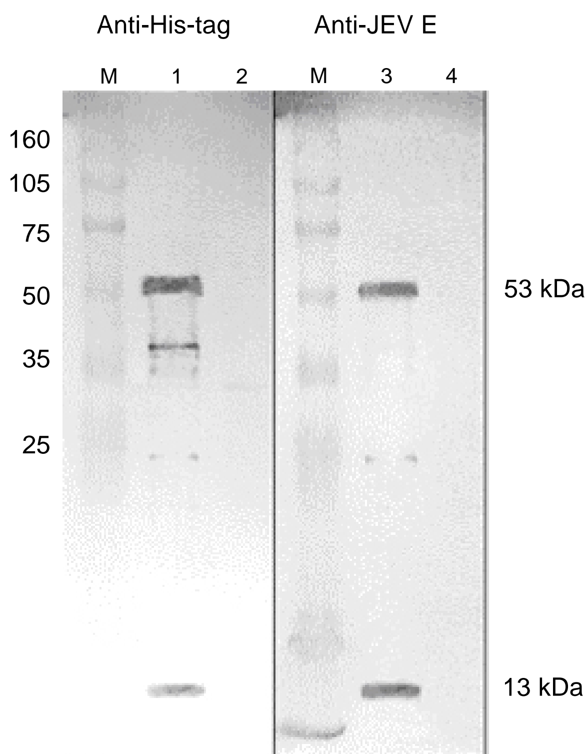

Fig 3–SDS-PAGE analysis of the JEV E protein expressed in *E. coli* Rosetta strain. Following electrophoresis, total cell lysate was immunostained with anti-His antibodies (lanes 1 and 2), or with mAb to JEV E protein (lanes 3 and 4). "M" indicates Rainbow size markers. Lanes 2 and 4 are untransformed Rosetta strain *E. coli* cell lysates.

viously report of JEV strain KE-093 E protein production (Lorroengsil *et al*, 2008) and the structural basis of the flavivirus (Burke and Monath, 2001).

The recombinant plasmid, pE1, was also used to investigate E protein in *E. coli* BL21 (DE3), protease-deficient strain, but was unsuccessful. The result was contrary to the previous studies in expressing different JEV strains in *E. coli* BL strain (Kaur *et al*, 2002). The expression system was checked for the expression of β-gal protein by performing the positive control experiment (data not shown). It has been reported that the rare codon usage could decrease protein expression of some foreign genes in *E. coli* because of the lower abundance of the corresponding tRNAs in the bacterial cells (Chen *et al*, 2004). The *E. coli* strain Rosetta 2 containing pRARE2 plasmid encoding the tRNA genes for the 7 rarely used codons namely, AUA, AGG, AGA, CUA, CCC, GGA, and CGG has been used as a host cell for the expression of subunit A of the *Aedes albopictus* V1VO ATPase (Hunke *et al*, 2007). *E. coli* Rosetta 2 expressed the desired E093 protein, molecular weight 53 kDa, and an additional proteins of 35 and 13 kDa as detected by Western blotting using anti-JEV and anti-His antibodies. Staining of acrylamide gels with silver stain revealed multiple protein bands, some of which corresponded to immunoreactive bands (data not shown).

The rare codons in the full-length *E* gene of JEV strain KE-093 were analyzed. There are six codons

of AGG, the least used in *E. coli* (Novy *et al*, 2001). The other JEV strains, *ie*, GP78, Nakayama, and JaOArS982, contain only four codons of AGG and the E protein from these strains could be expressed in *E. coli* without supplying tRNAs of rare codons (Kaur *et al*, 2002; Alka *et al*, 2007). All of the 8 rare codons (AUA, AGG, AGA, CUA, CCC, GGA, CGA and CGG) in *E* gene of JEV strain KE-093 constituted about 10.4% of all codons, while the percentage in *E* gene of JEV strains GP78, Nakayama, and JaOArS982 was approximately 9.6, 10, and 10.6%, respectively. It is likely that the critical rare codon, AGG coding for Arginine, might play an important role in limiting of E protein expression of JEV strain KE-093 in *E. coli*.

Using antibodies to the 6-His-tag revealed the presence of a weak band of 35 kDa, which was not seen when using anti-JEV E glycoprotein monoclonal antibodies. Since the His-tag is located at the N-terminal of E protein, this indicated that the epitope should be located at the C-terminal region of JEV E protein. Importantly, a major epitope of the JEV E protein has been localized to the C-terminal domain III of the E protein at amino acids 320-335 (Lin and Wu, 2003; Wu *et al*, 2003). Interestingly, one of the differences between the Beijing and Thai JEV strains is located at amino acid 327, one of the contact residues between mAb to JEV and Beijing envelope protein (Wu *et al*, 2003). Thus, some antibodies indeed may be type or strain specific. The KE-093 strain used in this study is antigenically distinct from most other Asian JEV strains, and fails to be effectively recognized by many antibodies (Hasegawa *et al*, 1995). This may partly be explained that KE-093 strain belongs to JEV of genotype I, whereas the two vaccine strains belong to genotype III. Importantly, it has been shown that a recombinant vaccine based on genotype II offers better protection against JEV of genotypes II and III, as compared to genotypes I and IV (Beasley *et al*, 2004). Thus, it is clear that a better knowledge of the antigenicity of the Thai KE-093 strain is needed to better decide if a local Thai vaccine is needed as a complement to the existing vaccines.

In summary, this study revealed that the *E* gene of the Thai KE-093 JEV strain can be effectively expressed by an *in vitro* transcription/translation system to produce the expected 53 kDa protein. However, expression in *E. coli* resulted in the 53 kDa protein being partly cleaved into at least two fragments. As far as we are aware, this is the first envelope protein from JEV genotype I strain that has been expressed in *E. coli* and may prove useful in development of diagnostics and vaccines for JEV genotype I strains.

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