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Evaluation of Japanese encephalitis virus vaccine strains currently used in pigs by molecular characterization

Jeong Ah Lee, Dong-Kun Yang*, Ha-Hyun Kim, Sun-Young Kim, Jin-Ju Nah, Soo-Dong Cho, Jae-Young Song

Animal, Plant and Fishery Quarantine Inspection and Agency, MIFAFF, Anyang 430-757, Korea

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Abstract

Japanese encephalitis virus (JEV) is one of the main causes of viral encephalitis in human and animals. For over 30 years, a live attenuated JEV vaccine strain has been used in the veterinary field, and it is required to conduct quality evaluation studies on the commercial vaccines. For the quality control of live attenuated JEV vaccine, we investigated the nucleotide sequence similarity of prME gene derived from five JEV vaccines commercially available in pigs in Korea. The Vero cells infected with JEV vaccines showed specific cytopathic effect, which was characterized by rounding and detached cells. In the phylogenetic analysis, all of the vaccine strains showed a close relationship with the original vaccine seed strain (Anyang 300) and clustered into the genotype 3. In comparison of the nucleotide and deduced amino acid sequences of prME genes with the original strain, all JEV vaccine strains showed high amino acid similarity ranging from 98.9% to 99.5%, but had several point mutations, probably due to high mutation rates of viral RNA polymerase by several virus passages. Even though the current JEV vaccine strains have been maintained and produced for a long period of time, the genetic characterization of them have been rarely changed. However, since the mid 1990's, molecular epidemiology of JEV has been changed sharply from genotype 3 to genotype 1 in Korea, further studies on new vaccine strains to genotype 1 is required for more effective prevention in the field.

Key words : Japanese encephalitis virus, Vaccine, prME

INTRODUCTION

Japanese encephalitis (JE) is one of the most important viral encephalitis caused by Japanese encephalitis virus (JEV) in human and animals. JEV is particularly dangerous to domestic animals such as swine, horses, dogs, chickens, ducks and reptiles (Ghosh and Basu, 2009; Gulati et al, 2012). After an incubation period of about 6 to 12 days, severe infection of JEV can cause febrile headache, aseptic meningitis, or encephalitis (Yang et al, 2005).

JEV is a member of the *Flaviviridae* family and *Flavivirus* genus (Yun et al, 2010). RNA genome of JEV contains a single open reading frame (ORF) encoding a polyprotein approximately 11kb in length. The polyprotein is cleaved into three structural proteins - capsid (C), precursor to membrane (prM), envelope (E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Sumiyoshi et al, 1987; Yang et al, 2004; Yang et al, 2005). The E protein (54 kDa) is the major envelope glycoprotein of virion as well as a determinant of viral neurovirulence and neuroinvasiveness (Nitayaphan et al, 1990; Yang et al, 2004; Yang et al, 2005). It has a number of neutralization epitopes that mediate attachment to a host cell, along with a receptor-binding domain that induces immune response. The prM protein interacts with E protein to form prME heterodimers, which are important for formation of immature virion and play an important role in maintaining

*Corresponding author: Dong-Kun Yang, Tel. +82-31-467-1783, Fax. +82-31-467-1797, E-mail. yangdk@korea.kr

E protein (Nitayaphan et al, 1990). JEV is maintained in a zoonotic cycle involving pigs as major amplification/reservoir hosts, water birds as carriers, mosquitoes as vectors and human as dead end hosts (Unni et al, 2011). According to epidemiological investigations, every year at least 67,000 clinical JEV cases occurred worldwide and approximately 15,000 of which have resulted in death. The JEV has spread throughout Asia and as far as northern Australia (Deng et al, 2011; Solomon, 2006; Wang et al, 2010). Since pigs serve as reservoirs and amplifiers of the virus, development of JEV vaccines for pigs could help prevent epidemics among human.

In order to protect sow from reproductive disorders, a live-attenuated JEV vaccine (Anyang 300 strain) was developed by continuous passage in chicken fibroblast cells in 1980 in Korea (Yang et al, 2004). Although attenuated JEV vaccine has been produced and applied to pigs over 30 years in Korea, commercial veterinary vaccine strains have not properly been investigated until now. Therefore, it is necessary to evaluate JEV vaccine strains based on the molecular characterization.

In this study, we identified JEV vaccine strains using cytopathic effects (CPE) in cells, indirect fluorescence assay (IFA) test, reverse transcription - polymerase chain reaction (RT-PCR) technique and investigated nucleotide sequence analysis of prME gene of JEV among five commercial vaccines produced by veterinary pharmaceutical companies in Korea.

MATERIALS AND METHODS

Vaccines

Five Korean commercial JEV vaccines produced by different Korean animal vaccine companies were used in this study as follows: Greencross® JE (Greencross Co., Korea), Daesung® JE (Daesung Co., Korea), SuiShot® JE (ChoongAng Co., Korea), Komipham® JE (Komipharm Co., Korea), Himmvac® JE (KBNP Co., Korea). All JE vaccines have been licensed in Korea for the prevention of JE in pigs.

Identification of JEV in vaccine

Each commercial JEV vaccine was inoculated into Vero cells grown in alpha minimum essential medium (α-MEM) with 10% fetal bovine serum. The cells were incubated in a CO_2 incubator at 37°C for 7 days until CPE was observed and viral titer was checked under microscopy by the Reed-Muench method. Subsequently the cells were fixed with 80% cold acetone and incubated with JEV-specific monoclonal antibody at 37°C for 1 h. Then they were stained with FITC-conjugated anti-mouse IgG (KPL, USA). The cells were then washed in phosphate buffer saline (pH 7.2) and examined under fluorescence microscopy.

RNA extraction and RT-PCR

Viral RNA was extracted from 5 commercial JEV vaccines using an RNA extraction kit (Qiagen, USA) according to the manufacturer's indications. The RNA samples were then amplified by RT-PCR using 4 kinds of specific primer pairs of prME genes of JEV (Table 1). The reaction mixture contained 10 μl of denatured RNA, 1 μl of each primer (50 pmol), 10 μl of 5× buffer (12.5 mM $MgCl_2$), 2 μl of enzyme mix of One-step RT-PCR kit (Qiagen, USA), 2 μl of 10 mM dNTP, and 24 μl of distilled water, for a 50 μl final volume. The

Table 1. List of oligonucleotide primers used in RT-PCR for JEV

Primer designation	Oligonucleotide sequence (5'-3')	Nucleotide position	JEV gene	Size of amplicon (bp)
JEM F	ATCATGTGGCTCGCAAGCTT	1059~1079	M	630
JEM R	CTCCCTTCTAACACCAGATC	1669~1689		
JEE1 F	GTCGCTCCGGCTTACAGTTT	1250~1270	E	543
JEE1 R	GAAGGAGVATTTGGAGTTAC	1772~1793		
JEE2 F	GCGTCTCAAGCAGCAAAGTT	1458~1479	E	565
JEE2 R	GTCATGTCGTTTAAACTCGCGAC	2000~2023		
JEE3 F	CCTGTAAAATTCCAGTTGT	1978~1996	E	523
JEE3 R	GATGTCAATGGCACAGCCAGT	2479~2501		

RT-PCR condition was as follows: cDNA synthesis at 42°C for 30 min; 35 cycles with denaturation at 95°C for 45 sec; annealing at 50°C for 45 sec and extension at 72°C for 45 sec; final extension at 72°C for 5 min. The PCR products were detected on 1.5% agarose gel containing ethidium bromide. The purified PCR products were ligated with pGEM-T® Easy Vector (Promega, USA) and used to transform into competent cells (DH5α). The plasmid DNA was isolated from *Escherichia coli* and identified by *Eco*RI enzyme digestion.

Sequencing and phylogenetic analysis

After cloning the prM and E genes of JEV, the sequences of the purified plasmids were analyzed using an MJ Research PTC-225 Peltier Thermal Cycler and ABI PRISM® BigDye™ Terminator Cycle Sequencing kits with AmpliTaq DNA polymerase (FS enzyme; Applied Biosystems, USA) according to the manufacturer's protocols. The prME genes of JEV vaccine strains were compared with those of the other known JEV submitted to NCBI and seed strain (Anyang 300). The sequences employed were obtained from the NCBI GenBank database [accession numbers: AY303795 (CC27-L1), AY303798 (CC27-S8), AB551990 (JaTAn1/75), M18370 (JaOArS982), EF543861 (SH0601), AF416457 (SA14-12-1-7), M55506 (SA14), AF254452 (CH1392), AY303792 (T1P1-L4), AF069076 (JaGAr01), AB551991 (JaTAn1/90), FJ185036 (B58), EF571853 (Nakayama), L48961 (Beijing-1), L78128 (Ling), EU880214 (XJ169), EU693899 (XJP613), AF045551 (K94P05), AY316157 (KV1899), GQ902062 (4790-85), AF217620 (FU)]. Nucleotide sequence similarity calculations were conducted using the DNASIS® (Hitachi Software, Japan) software. Individual sequences were initially aligned using BioEdit and Clustal X 1.81. Phylogenetic reconstructions were generated using the neighbor-joining method by the computer program DNAstar. Phylogenetic trees were reconstructed on aligned nucleotide sequences using ClustalW (version 2.0.12; European Bioinformatics Institute, UK).

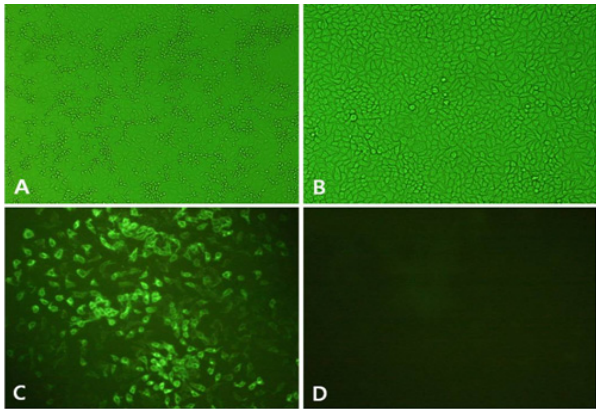

Fig. 1. Confirmation of cytopathic effects (CPE) and JEV-positive cells in the Vero cells infected with JEV vaccines. (A) CPE was shown in Vero cells infected with a JEV vaccine. (B) No CPE was shown in the mock-infected Vero cells. (C) Many JEV-positive cells were detected in the JEV vaccine-infected Vero cells using indirect immunofluorescence assay (IFA). (D) No JEV-positive cells were detected in the mock-infected Vero cells. IFA was performed with monoclonal antibodies against JEV.

RESULTS

The Vero cells inoculated with five Korean commercial JEV vaccines showed specific CPE, which was characterized by the rounding and detachment of cells (Fig. 1A). The viral titers of the vaccines ranged from $10^{5.3}$ to $10^{6.0}$ $TCID_{50}$/ml. Also specific cytoplasmic fluorescence was identified in the JEV vaccine strain-infected Vero cells using IFA with JEV specific monoclonal antibody (Fig. 1C). No CPE and JEV-positive cells were detected in the mock-infected Vero cells (Fig. 1B). The prM and E genes from five vaccine strains

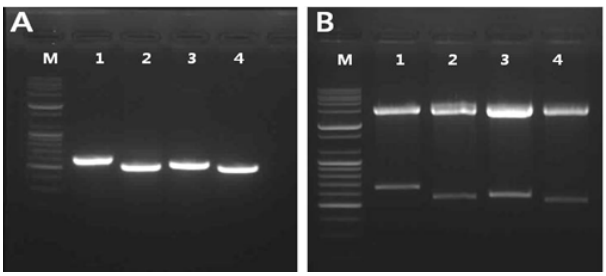

Fig. 2. Amplification and confirmation of JEV prM, E1, E2 and E3 genes. (A) The prM, E1, E2 and E3 genes of JEV were amplified using RT-PCR. (B) The prM, E1, E2 and E3 genes of plasmid DNA were confirmed by EcoR I enzyme digestion. M: 100 bp ladder, Lane 1: prM gene, Lane 2: E1 gene, Lane 3: E2 gene, Lane 4: E3.

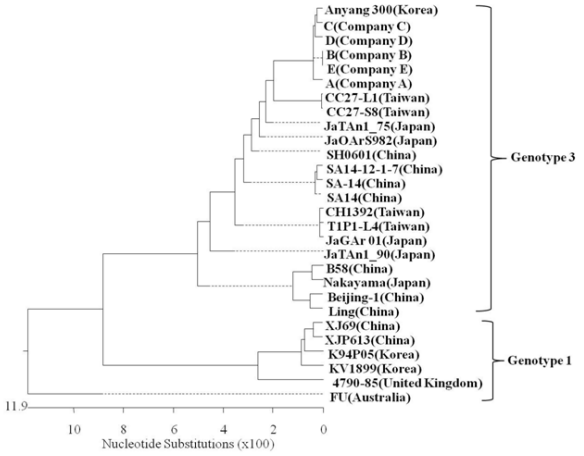

Fig. 3. Phylogenetic tree based on prME genes of JEV vaccine strains with another known sequences obtained from GenBank database to show genetic relationships.

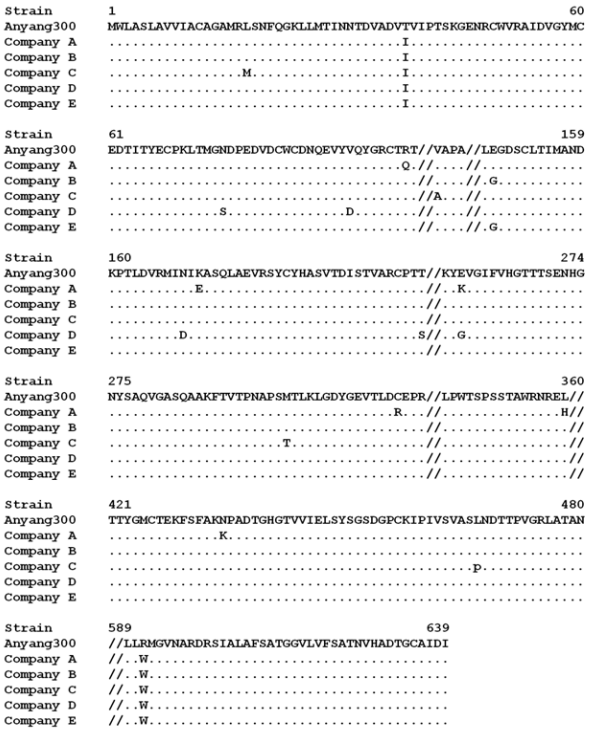

Fig. 4. Comparison of deduced amino acid sequences among five commercial JEV vaccine strains and Anyang 300 strain. The dots and slashes indicate identical amino acids and omissions of amino acid sequences, respectively.

were successfully amplified and cloned using RT-PCR with each gene-specific primer (Fig. 2).

Phylogenetic analysis was conducted using prME genes of JEV vaccine strains with other nucleotide sequences obtained from GenBank database. Most of the Korean field strains have been divided into genotypes 1 or 3. Five commercial vaccine strains including the Anyang 300 strain were grouped in genotype 3, together with JEV strains isolated in China, Japan and Taiwan. Other Korean strains such as KV1899 and K94P05 were clustered into genotype 1 together with strains isolated in China, the United Kingdom, and Australia (Fig. 3). In comparison of the deduced amino acid sequences of prME genes with the original strain, all JEV vaccine strains showed high amino acid similarity ranging from 98.9% to 99.5%, but had several point mutations (Fig. 4).

DISCUSSION

JE is one of the most important viral encephalitis in Asia, especially in rural and suburban areas where rice culture and pig farming coexist (Campbell et al, 2011). JEV is transmitted by a zoonotic cycle involving pigs as major amplification/reservoir hosts, water birds as carriers, mosquitoes as vectors and human as dead end hosts (Unni et al, 2011). As pigs were considered amplification/reservoir hosts of JEV in zoonotic cycle, it was important to immunize pigs for the purpose of blocking the cycle. Therefore, a live-attenuated JEV vaccine (Anyang 300 strain) for pigs was developed by continuous passage in several cells including chicken fibroblast cells in Korea in 1980 (Yang et al, 2004). The JEV vaccines manufactured by biologic companies in Korea have been applied to pigs for the prevention of JE epidemic in human and pigs. Developments of JEV vaccine based on the molecular biology have been reported by several scientists (Beasley et al, 2008; Hong et al, 1998; Yun et al, 2010; Zhang et al, 2011). We conducted biological methods on JEV vaccine and obtained that JEV in the vaccines was identified by specific CPE and IFA in the Vero cells, indicating that five different Korean animal vaccine companies have produced JEV vaccine without changing original seed.

Phylogenetic analyses based on nucleotide sequences of prME, NS1 and full length genes revealed that JEVs worldwide were classified into 5 genotypes (genotype 1,

2, 3, 4 and 5) (Solomon et al, 2003; Nitatpattana et al, 2008; Uchil and Satchidanandam, 2001). In this study, phylogenetic analysis based on the nucleotide sequences of the prME genes indicated that five commercial vaccine strains showed a close relationship with the Anyang 300 strain grouping the genotype 3. All JEV vaccine strains showed high amino acid similarity ranging from 98.9% to 99.5%, but had several point mutations, probably due to high mutation rates of viral RNA polymerase by several virus passages. RNA viruses have a high mutation rate during replication due to both the lack of proofreading and post-replication error correction by RNA polymerase (Steinhauer and Holland, 1987). It also assumed that the current JEV vaccine strains have been produced for a long period of time and passaged in several kinds of primary cells in order to propagate high titer without strict management. On the basis of our results, the strict standards for the live vaccines including JEV vaccines are needed to be imposed for quality control. On the other hand, since molecular epidemiology of JEV has been changed sharply from genotype 3 to genotype 1 in Korea from the mid 1990's, further studies on new vaccine strains to genotype 1 is required for more effective prevention in the field.

In conclusion, five Korean commercial JEV vaccines contained original JEV seed strain but showed some point mutations in prME gene. The biological and molecular methods could be useful for identifying vaccine strains in attenuated JEV vaccines. Especially, the prME-based molecular method might be useful for detection of genetic similarity and variation among JEV vaccine strains.

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