

Establishment of a Multiplex RT-PCR for the Sensitive and Differential Detection of Japanese Encephalitis Virus Genotype 1 and 3

Dong-Kun Yang*, Ha-Hyun Kim, Hyun-Ye Jo, Sung-Suk Choi and In-Soo Cho

Viral Disease Division, Animal and Plant Quarantine Agency, Gyeongsangbuk-do, Korea

Japanese encephalitis (JE) is a zoonosis that affects the nervous system of humans and other animals. The genotype of JE virus (JEV) has shifted recently from genotype 3 (G3) to genotype 1 (G1) in Asia, including Korea. Thus, a rapid differential assay is required to make an accurate diagnosis of JEV genotype. In this study, we designed common and differential primer sets for JEV G1 and G3 to detect the JEV envelope (E) gene. The specific primer sets for JEV G1 and G3 specifically amplified the target gene. The detection limits of the three primer sets were $10^{1.0}$, $10^{2.0}$, and $10^{2.0}$ TCID₅₀/reaction, respectively. No cross-reactivity was detected with non-JEV reference viruses. The multiplex reverse transcription-polymerase chain reaction (RT-PCR) assay specifically differentiated JEV G1 from G3. Thus, a one-step multiplex RT-PCR assay was established to rapidly and differentially detect JEV. This assay will be useful for confirming JEV infections in animals and checking the JEV genotype in veterinary biological products.

Key Words: Japanese encephalitis virus, Multiplex RT-PCR, Genotype

INTRODUCTION

Japanese encephalitis (JE) is a reemerging zoonosis associated with mosquito activity, global warming, and climate change. JE is caused by JE virus (JEV), which is mediated by mosquitoes. The mosquito species mediating JEV in Korea and Japan is the small red house mosquito *Culex tritaeniorhynchus* (1). Other mosquitoes mediating JEV in Asian countries include *Culex vishnui*, *Culex orientalis*, *Culex pipiens*, *Culex annulirostris*, and *Anopheles* spp. (2).

JEV can cause encephalitis in humans and horses through

infected mosquito bites. JE cases are traditionally identified in Asian countries, but 1.3% of blood donors in French Polynesia were seropositive for JEV between 2011 and 2013 (3), and JEV RNA was detected in *C. pipiens* mosquitoes in northern Italy in 2012 (4). Most adults acquire subclinical JEV infections, but children and seniors infected with JEV may develop fever, headache, consciousness disorders, coma, and death. Pigs, horses, cattle, sheep, goats, and wild animals, including pigeons, herons, bats, and reptiles are susceptible to JEV (2, 5). Most susceptible animals represent a subclinical symptom, but pregnant sows naturally infected with JEV cause reproductive failure such as stillbirth or abortion (2). Pigs are an important public health immune target animal

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*Corresponding author: Dong-Kun Yang, PhD, DVM. Viral Disease Division, Animal and Plant Quarantine Agency, 177 Hyeoksin 8-ro, Gimcheon-si, Gyeongsangbuk-do, 39660, Korea.

Phone: +82-54-912-0785, Fax: +82-54-912-0812, e-mail: yangdk@korea.kr

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because pigs, including wild boars, amplify JEV.

JEV belongs to the genus *Flavivirus* in the family *Flaviviridae*; it is an enveloped virus with a positive single-stranded RNA genome encoding ten viral proteins, including three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins form the viral component, while the non-structural proteins are involved in viral replication. Among the structural proteins of JEV, the glycosylated envelope (E) protein is a major component of the virion surface; thus, E protein is an important part of receptor binding, erythrocyte hemagglutination, major neutralizing antibody induction, virus-specific membrane fusion in acidic endosomes, and viral assembly (6). JEV has been divided into five genotypes (G1-G5) based on the nucleotide sequence of the prM or E gene (7, 8). Among these genotypes, G3 was identified in most Asian countries by 1990 (1, 9). However, since the 1990s, the dominant JEV genotype has changed to G1 in Japan, Korea, and China (2, 10, 11). G1 viruses have been reported in *Culex* mosquitoes, swine blood, and cerebrospinal fluid from Korean patients, indicating that JEV G1 is circulating in Korea (12~14).

There are several serological techniques for detecting JEV antibodies in animals. A significant rise in the antibody titer of paired sera can be measured using the hemagglutination inhibition test, latex agglutination test, complement fixation test, virus neutralization (VN) test, and plaque reduction neutralization test to make a laboratory diagnosis (13). These serological tests are useful for sero-surveillance in vaccinated animals and for determining incidence rates in animal populations. Although it is essential to isolate JEV from suspected samples for a definitive diagnosis, virus isolation requires a cell culture system, time, and skilled personnel. Reverse-transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR assays have been developed for rapid and specific detection of the JEV genome (15).

The molecular epidemiology of JEV has revealed a genotype shift from G3 to G1 in Asian countries, including Korea. Differential diagnostic results obtained from the naturally infected samples can offer epidemiological data to veterinary authorities and may provide basic information for develop-

ment of new JE vaccine. In addition, the nucleotide sequence similarity between JEV G1 and G3 is 87%. Therefore, differential RT-PCR for an accurate diagnosis of JEV genotypes is required. In this study, we established a multiplex RT-PCR assay for the rapid and differential detection of JEV based on the E gene.

MATERIALS AND METHODS

Cells and viruses

Vero cells (African green monkey kidney cell line, ATCC: CCL81) and BHK-21 (baby hamster kidney cell line, ATCC: CCL10) cells were maintained in α -minimum essential medium (Gibco BRL, Grand Island, NY, USA) containing 5% (v/v) fetal bovine serum (Gibco BRL), penicillin (100 IU/ml), streptomycin (100 μ g/ml), and amphotericin B (0.25 μ g/ml) at 37°C under 5% (v/v) CO₂. JEV, KV1899 strain, was used as the standard virus for detecting JEV G1, while JEV, Anyang 300 strain was used as positive control virus to detect JEV G3 by multiplex RT-PCR. Strains KV1899 and Anyang 300 were propagated in Vero cells of a 25 cm² tissue culture flask. The flasks were frozen at 5 days post-infection. After being thawed and frozen three times, the JEV strains were titered in a 96-well microplate (10-fold dilution) and the viral titer was determined by an indirect fluorescent assay calculated using the method of Reed and Muench. A field JEV, K95 strain (NCCP No. 41320), was isolated from mosquitoes in 1995 and was delivered officially from the Centers for Disease Control and Prevention of Korea. Classical swine fever virus (CSFV: LOM strain), porcine parvovirus (PPV: PV9 strain), encephalomyocarditis virus (EMCV: K3 strain), Aujeszky's disease virus (ADV: YS strain), porcine reproductive and respiratory virus (PRRSV: VR2332 strain), epidemic diarrhea virus (PEDV: SM98 strain), and getah virus (GETV: QIAGE9301 strain) were used as negative control for RT-PCR specificity testing. Live attenuated JE vaccines for swine may be one of good reference strains for the application of RT-PCR. Five commercial live JEV vaccines produced by Korean animal vaccine companies were used in this study: Greencross® Porcine JE (Greencross Co., Seoul, Korea); Daesung JE pig vac (Daesung Microbiological Labs

Co., Uiwang, Korea); Suishot® JE (ChoongAng Co., Daejeon, Korea); Provac® JE (Komipharm Co., Ansan, Korea); and Himmvac® JE (KoreaBNP Co., Yesan, Korea). All JE vaccines were licensed in Korea for domestic pigs > 3 months of age or sows.

Design of the primer sets and RNA/DNA extraction

One common primer and two differential primer sets were designed based on E gene sequence data from the Anyang 300 (unpublished) and KV1899 (GenBank accession no. AY316157) and K94P05 strains (AF045651). To optimize the RT-PCR procedure, oligonucleotides within the common primer sets (designated as JEcomFR) were adjusted by replacing nucleotides. Two primer sets, JEG1FR and JEG3FR, were selected on the non-identical region of the JEV E gene between strains Anyang 300 and KV1899. The sequences and nucleotide positions of the primers are given in Table 1. Viral RNA was extracted from several JEV strains (Anyang 300, KV1899, K95 and vaccines) using an RNA extraction kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. The RNA was eluted in 50 µl of RNase- and DNase-free water. Viral DNA was extracted from two viruses (PPV and ADV) using a genomic DNA extraction kit (Bioneer).

RT-PCR and multiplex RT-PCR

RT-PCR was performed in a reaction mixture containing 5 µl of denatured RNA, 1 µl of each primer (50 pmol), 10 µl of 5× buffer (12.5 mM MgCl₂), 2 µl of a dNTP mix, 2 µl of an enzyme mix (reverse transcriptase and Taq polymerase), and 25~29 µl of distilled water (Qiagen, Hilden, Germany). The cycling profile consisted of cDNA synthesis at 42°C for 30 min, followed by 35 cycles of 95°C for 45 s, 57°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 5 min. RT-PCR for JEV was carried out to determine the correct annealing temperatures and conditions. Multiplex RT-PCR was carried out in the same mixture; however, the amounts of each primer and distilled water were adjusted. JEV strains KV1899 and Anyang 300 were used as positive samples; seven viruses (CSFV, PPV, EMCV, ADV, PRRS, PEDV, and GETV) were used to test the internal negative

control of the assay. DNA extracted from PPV and ADV was added to the RT-PCR reaction mixture instead of RNA. RT-PCR was carried out under the conditions mentioned above. The PCR products were separated by electrophoresis and visualized on 1.8% agarose gels containing ethidium bromide.

Detection limit of primer set

Detection limit of multiplex RT-PCR was carried out using 10-fold dilutions of extracted RNA of JEV G1 and G3. The JEV culture supernatant (KV1899: 10^{6.0} TCID₅₀/ml, Anyang 300: 10^{6.0} TCID₅₀/ml) was subjected to a sensitivity test. The eluted RNA was serially diluted 10-fold to 10⁻⁵ and then applied to multiplex RT-PCR. The sensitivity of the multiplex RT-PCR was expressed as infectivity titer equivalent.

RESULTS

Analysis of the primer design

Three JEV-specific primer sets were designed for the multiplex RT-PCR assay: the JEcomFR primer set detected JEV G1 and G3, the JEG1FR primer set detected JEV G1, and the JEG3FR primer set detected JEV G3. The homology of nucleotide sequence in primer sites for JEV G1 (KV1899 strain; AY316157, K94P05 strain; AF045651, and Ishigawa strain; AB051202) and JEV G3 (Anyang 300 strain; unpublished, Beijing-1 strain; L48916, K87P39 strain; U34927) did not show a significant difference. However, the homology in JEV E gene between JEV G1 and G3 exhibit only around 87% (Data not shown). The expected sizes of the multiplex RT-PCR products according to the common, G1, and G3 primer sets were 306, 565, and 451 bp, respectively. As shown in Fig. 1, three primer sets were selected for the same E gene, but three oligonucleotides in the JEcomFR primer set were replaced with mixed bases such as Y (C or T) and R (A or G). The sequence of the G1-specific primer set located in the E gene was identical across JEV G1, but the discrepancy rate of the JEG1R primer against JEV G3 was 28% (7/25). The oligonucleotide sequence of the G3-specific primer set was identical across JEV G3, but the discrepancy rate between JEV G3 and JEV G1 in the primer

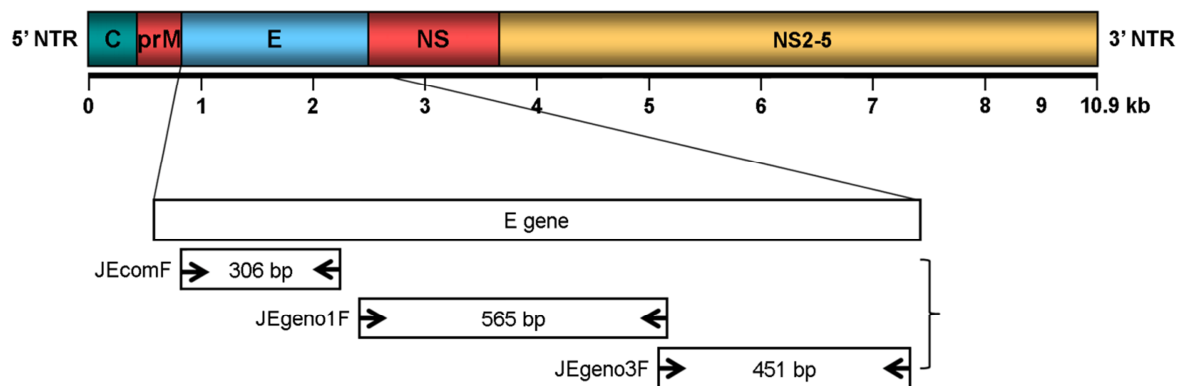


Figure 1. The amplification sites of JEV gene for multiplex RT-PCR in JEV full genome.

Table 1. The design of the three JEV specific primer sets for differential detection of JEV in multiplex RT-PCR

Name of primer	Genotype	Oligonucleotide sequences (5' - 3')	Discrepancy rate (%)	Position in E gene
JEcomF	G1	CCAACACTAGATGTCCGCATGA	3/22 (13.6)	115~135
	G3	CCAACATTGGACGTCCGCATGA		
	Primer	CCAACAY*TR**GAYGTCCGCATGA		
JEcomR	G1	CATCAAGTACAAGGTTGGC	2/19 (10.5)	402~420
	G3	CATCAAATACGAAGTTGGC		
	Primer	CATCAAYTACYAYGTTGGC		
JEG1F	G1	GCGTCTCAAGCAGCAAAGTTTACT	4/24 (16.7)	481~504
	G3	GCGTCCCAGGCGGCAAAGTTTACA		
	Primer	GCGTCTCAAGCAGCAAAGTTTACT		
JEG1R	G1	TGTCTCAGTCGCGAGTTTAAACGAC	7/25 (28.0)	1017~1043
	G3	CGTCTCCGTTGCGAGCCTCAATGAC		
	Primer	TGTCTCAGTCGCGAGTTTAAACGAC		
JEG3F	G1	CCGATTGTCTCAGTCGCGAGTT	5/22 (22.7)	1012~1033
	G3	CCGATCGTCTCCGTTGCGAGCC		
	Primer	CCGATCGTCTCCGTTGCGAGCC		
JEG3R	G1	CCTGGCTTTTCTGGCCACGG	7/20 (35.0)	1443~1462
	G3	TTTGGCCTTCTTAGCCACAG		
	Primer	TTTGGCCTTCTTAGCCACAG		

*: Y; C or T, **: R; A or G.

position of the E gene was 35.0% (7/25). Importantly, the first two nucleotides of the JEG3R primer were not identical to those of JEV G1 at the same E gene position (Table 1).

Optimization of annealing temperature

The annealing temperature should be set up around 50~65 °C for 30~40 seconds to allow for hybridization of the

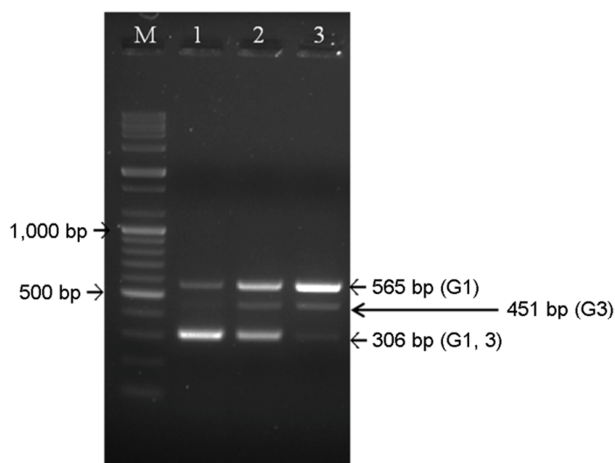


Figure 2. Setting up annealing temperature to optimize temperature condition for detection of JEV genotypes. After conducting RT-PCR at three annealing temperature conditions (lane 1: 50°C, lane 2: 57°C, and lane 3: 62°C), the PCR products were visualized on the 1.8% agarose gel. JEV G3 cannot be amplified by RT-PCR of annealing temperature at 50°C and common parts of JEV G1 and G3 cannot be amplified distinctly at 62°C. Annealing temperature at 57°C turned to be reliable for detection of two JEV genotypes.

primer to the target DNA. If the temperature is too low or high, the primer could bind incompletely or not bind. Therefore, setting up annealing temperature plays a key role in developing a multiplex RT-PCR system. RT-PCR was carried out at three temperature conditions such as 50, 57, and 62°C. As shown in Fig. 2, an annealing temperature of 57°C led to the distinct amplification of JEV G1 and G3.

Detection limit of each primer set

Ten-fold dilutions of extracted RNA of JEV G1 and G3 were used for RT-PCR with the designed primer sets to check their detection limits (Fig. 3). The detection limit of the JEcomFR primer set for JEV G1 and G3 was $10^{1.0}$ TCID₅₀/reaction, while the detection limits of the JEG1FR and JEG3FR primer sets were identical at $10^{2.0}$ TCID₅₀/reaction.

Primer specificity

To evaluate the specificity of the three primer sets, JEV

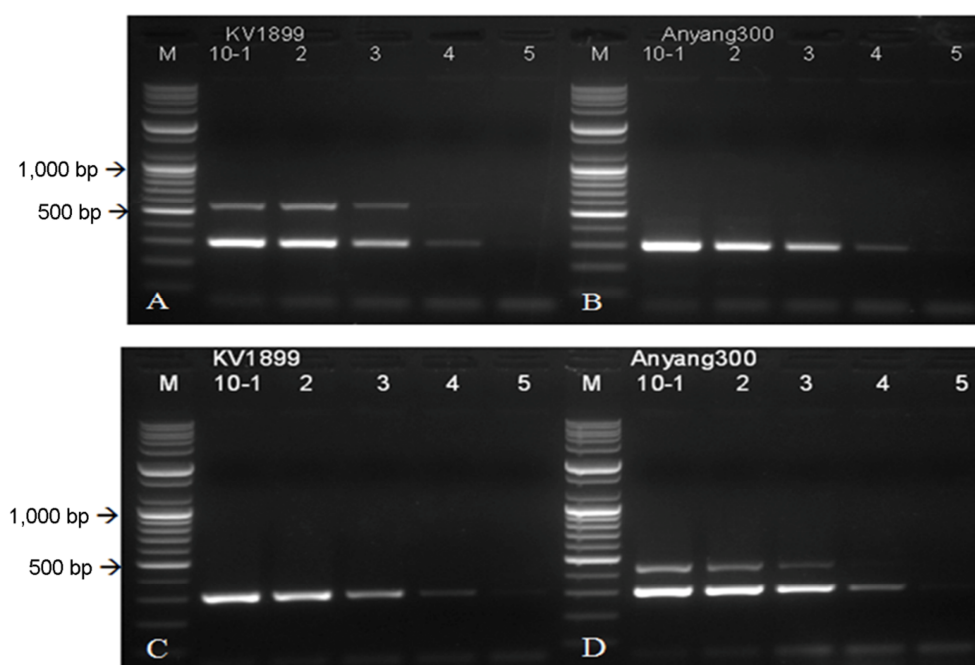


Figure 3. Sensitivity of the specific primer sets for detection of genotypes of JEV. Differential RT-PCR between JEV G1 and G3 (A, B, C and D). Sensitivity of primer sets for common and genotypes was set up based on the annealing temperature. M; 100 bp DNA ladder, lane 1-5; 10-fold serial dilutions of extracted RNA of JEV ($10^{6.0}$ TCID₅₀/ml).

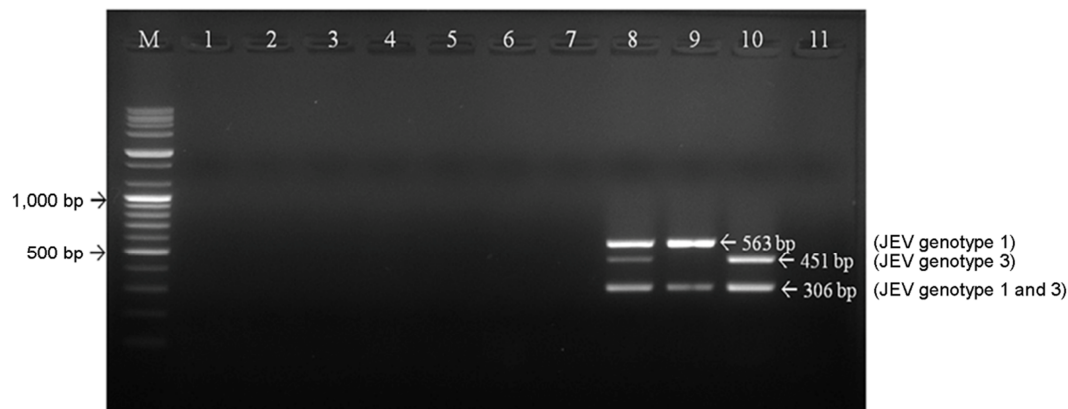


Figure 4. Specificity of differential JEV RT-PCR using three kinds of primer sets. The RT-PCR detected only genotype of JEV and did not have positive signal for any other viral pathogens. The seven viruses showing titer of $10^{5.0}$ TCID₅₀/ml or over were used to extract RNA or DNA. M; 100 bp DNA ladder, lane 1; classical swine fever virus, lane 2; porcine parvovirus, lane 3; encephalomyocarditis virus, lane 4; Aujeszky's disease virus, lane 5; transmissible gastroenteritis virus, lane 6; porcine epidemic diarrhea virus, lane 7; porcine reproductive and respiratory syndrome virus, lane 8; JEV G1 and 3, lane 9; JEV G1, lane 10; JEV G3, lane 11; distilled water (negative control).

G1, JEV G3, five abortion-related viruses in swine (CSFV, PPV, ADV, EMCV, and PRRSV), one mosquito-borne alpha-virus (GETV), and one diarrhea-related virus (PEDV) were subjected to amplification by multiplex RT-PCR with the three primer sets (Fig. 4). Titers of the negative control viruses mentioned above were over $10^{5.0}$ TCID₅₀/ml. The reactions using JEV G1 and G3 produced two or three DNA bands of 306, 451, and 565 bp with the three primer sets; in contrast, no DNA bands were produced from the seven porcine-related viruses. JEV G1 was amplified by the JEcomFR and JEG1FR primer sets, yielding products of 306 and 565 bp. JEV G3 was also amplified by the JEcomFR and JEG3FR primer sets, yielding products of 306 and 451 bp.

Application of multiplex RT-PCR to commercial JE vaccines and a field isolate

The JEV field strain and five commercial JE vaccines produced in Korea were applied to the established multiplex RT-PCR. Two DNA bands (306 and 565 bp) were identified in the KV1899 and K95 strains isolated from swine blood and mosquitoes in 1999 and 1995, respectively, indicating JEV G1 and two DNA bands (306 and 451 bp) were detected in Anyang 300 strain and five commercial JE vaccines, indicating JEV G3 (Fig. 5).

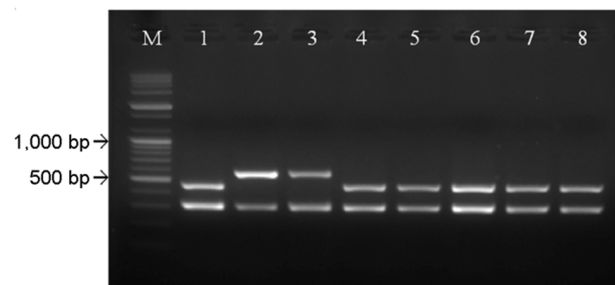


Figure 5. Application of multiplex RT-PCR to commercial JE vaccines. Five JE vaccines and one Korean field strain (K95) showed positive reactions in the multiplex RT-PCR kit. M; 100 bp DNA ladder, lane 1; Anyang 300 strain, lane 2; KV1899 strain, lane 3; K95 strain, lane 4; Greencross® Porcine JE, lane 5; Dacsung JE pig vac, lane 6; Suishot® JE, lane 7; Provac® JE, lane 8; Himmvac® JE.

DISCUSSION

Fluctuations in the incidence of vector-borne diseases such as JE and West Nile virus are related to extreme climatic change (16). A JEV genotype shift from G3 to G1 has been reported in Asian countries over the last 20 years (10, 14, 17). It is assumed that global warming is associated with this genotype shift. Differences in antigenicity and pathogenic ability between JEV G1 and G3 were described using the

VN test (9, 18). However, a live attenuated JEV vaccine (strain Anyang 300) originated from JEV G3 has been used in veterinary medicine in Korea since 1980 (10). We recently reported the development of an inactivated JE G1 vaccine for swine in accordance with the replacement of JEV G3 by G1 (19). In addition, it is not easy to isolate JEV from the naturally infected animals because of short period of viremia. Serological monitoring has mainly focused on supporting epidemiological information to veterinary authorities.

Therefore, a diagnostic method for delicately detecting JEV genotypes is required in Korea. In this study, we designed three specific primer sets for JEV G1 and G3 and established a multiplex RT-PCR assay to simultaneously identify and differentiate JEV G1 from G3 in a single reaction.

Multiplex RT-PCR is rapid and useful for detecting JEV in tissues of the infected swine and simultaneously differentiating JEV genotypes in mosquitoes (12, 20). Critical factors in developing a multiplex RT-PCR assay include the proper annealing temperature, specificity of the primer sets, detection limit, and sizes of the PCR products (21). Chen *et al.* selected a T_m value of around 50 °C and different DNA size to optimize sensitivity and specificity of the multiplex RT-PCR (20). In this study, three oligonucleotides in each forward and reverse primer set (JEcomFR) were substituted for mixed bases and detected JEV G1 and G3. The difference in the first two nucleotides and the low similarity with the JEV G3 reverse primer set (JEG3R) did not allow amplification of JEV G1 in the multiplex RT-PCR assay. This strategy for designing primer sets made it possible to differentiate JEV in a single reaction. JE viral RNAs obtained from strains KV1899 and Anyang 300 were subjected to the multiplex RT-PCR assay to test primer set sensitivity. Although the sensitivity of JEG1FR and JEG3FR was ten times lower than that of JEcomFR, the detection limit of the multiplex RT-PCR assay was $> 10^{2.0}$ TCID₅₀/reaction. It was reported that variable detection limits were shown in multiplex RT-PCR assays for Dengue virus serotypes (22). It has been assumed that these differences in detection limit of the multiplex RT-PCR are due to differences in the proportion of infectious virus to non-infectious RNA transcripts or the

degree of degeneracy against the template viral RNA (7, 23). We evaluated the annealing temperature to determine the optimal condition, which was 57 °C.

In addition, the multiplex RT-PCR assay showed good specificity, as no positive results were found in the distilled water and negative control samples, which included CSFV, PPV, EMCV, ADV, PRRSV, PEDV, and GETV assayed under the same experimental conditions. A rapid and differential detection tool for JEV at a veterinary biological company is important because JEV genotypes cannot be differentiated by distinct cytopathic effects seen in cell culture. In this study, we evaluated the multiplex RT-PCR assay with two JEV genotypes among flaviviruses and applied it to five commercial JEV vaccines. JEV G5 was detected recently in Korean *C. orientalis* and *C. pipiens* (24). Although the results were acceptable and reliable, further study is required to determine how our RT-PCR assay performs with various strains within flaviviruses. Besides application to flaviviruses, results from mosquito samples are needed.

In conclusion, our multiplex RT-PCR assay, which was sensitive and specific for the detection of JEV genotypes, will provide a more reliable way to confirm a diagnosis of JEV infection in swine and to confirm JEV genotypes in veterinary biological products.

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