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TaqMan reverse transcription polymerase chain reaction for the detection of Japanese encephalitis virus

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One step TaqMan reverse transcription polymerase chain reaction (RT-PCR) using TaqMan probe was developed for detection of Japanese encephalitis virus (JEV). Real-time RT-PCR was optimized to quantify JEV using the detection system (Rotor Gene 2000 detector) and dual-labeled fluorogenic probes. The gene specific labeled fluorogenic probe for the 3' non-translated region (3' NTR) was used to detect JEV. When the specificity of the assay using specific JEV primers was evaluated by testing three different JEV strains, other swine viruses and bovine viral diarrhea virus, no cross-reactions were detected with non-JE reference viruses. A single tube TaqMan assay was shown to be 10-fold more sensitive than the conventional two-step RT-PCR method. Detection limits of two step and real-time RT-PCR for JEV were 112 $TCID_{50}$/ml and 11.2 $TCID_{50}$/ml, respectively. Quantification of JEV was accomplished by a standard curve plotting cycle threshold values (C_t) versus infectivity titer. Real-time RT-PCR assay using single tube method could be used as a sensitive diagnostic test, and supplied the results in real time for detection and quantification of JEV. We could detect JEV RNA genome in plasma samples of pigs inoculated with KV1899 strain at 2 days post inoculation, but couldn't in 41 fetus samples. This assay was sensitive, specific, rapid and quantitative for the detection of JEV from laboratory and field samples.

Key words: Real-time RT-PCR, JEV, TaqMan, quantification

Introduction

Japanese encephalitis (JE) is a mosquito-borne viral disease of animal that causes one of the major reproductive disorders in swine industry. JEV has been implicated in periodic outbreaks of encephalitis cases reported in different countries of Asia in human [2]. JEV is a member of the *Flavivirus* genus in the *Flaviviridae* family with a positive sense, and single stranded RNA viruses [2,6,27]. Experimental infection of susceptible pregnant sow causes no clinical signs in dams but results in infection of fetus in uterus and subsequent abnormal farrowings: various numbers of mummified fetus of different sizes, stillborn and weak piglets with subcutaneous edema and hydrocephalus [13,24]. Mosquitoes of *Culex* are the major vectors involved in transmission. Migrating birds such as night heron and cattle egret are thought to be important in the dispersal of JEV to new geographic areas [11]. Among domestic animals, pigs are considered as a principal amplifying host. Consistent development of viremia in pigs ensures a continued supply of infected mosquitoes. It is not easy to control the mosquitoes; therefore immunization policy of pigs with JEV live vaccine has been applied before mosquitoes activate as an applicable control and preventive measurement in Korea.

Several serologic tests, such as hemagglutination inhibition (HI) test, enzyme-linked immunosorbent assay (ELISA), serum neutralization technique, epitope blocking immunoassay and dot enzyme immunoassay, have been used for detection of antibody for JEV infection [3,5,13]. Nevertheless, some of the above techniques require time to set up and are complicate to apply for the diagnostic test. Although isolation of the virus from samples is essential to make a definitive diagnosis, recently RT-PCR has been used to detect *flavivirus* genome in a rapid and specific test [10,15, 16,20,25]. Two-step RT-PCR assay requires agarose gel analysis for the detection of amplicons after PCR cycling. So, the assay is labor-intensive and has a very high risk of contamination.

Recently, real-time RT-PCR technique has been used extensively to detect amplicon that is amplified during the PCR cycling in real time [1,12,19,23]. The development of fluorogenic PCR utilizing 5'-3' nuclease activity of Taq DNA polymerase made it possible to eliminate post PCR processing such as visualization in agarose [9,26]. In

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addition, the real-time RT-PCR technique is shown to give good sensitivity and linear relationship between copy number and cycle threshold (C_t) values. The technique uses oligonucleotide probes labeled with fluorescent dyes, a reporter at the 5' end and a quencher at the 3' end to monitor accumulation of PCR products [4].

In this study, a real-time RT-PCR assay with TaqMan probe was investigated and applied for laboratory detection and quantification of JEV. In addition, applicability of the real time RT-PCR was evaluated for the detection of JEV RNA from plasmas and aborted fetuses of pigs.

Materials and Methods

Viruses and sample preparation

The JEV isolate KV1899, Anyang 300 (attenuated vaccine strain) and Nakayama strain were used as standard virus for detection of JEV by real-time RT-PCR. The KV1899 and Anyang 300 strains were cultured in TF104 cells (a cell line cloned from MA104 cells), and the Nakayama strain was propagated from the infected mouse brain emulsion. The classical swine fever virus (CSFV), transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), porcine rotavirus (PRV), encephalomyocarditis virus (EMCV) and bovine viral diarrhea virus (BVDV) were used as reference strains for specificity test of the real-time RT-PCR.

Four healthy pigs of 5 weeks old, weighing 5-7 kg, were infected intramuscularly with 1 ml of $10^{6.0}$ $TCID_{50}$/ml respective JE viruses (KV1899 strain, which had undergone 37 passages in TF104 cell and Anyang 300 strain, which was an attenuated virus). From 2 days post inoculation, blood samples from pigs inoculated with JEV were collected and plasmas were separated and stored at –20°C until use. Forty one fetuses that aborted before 70 days of gestation were collected from several provinces of Korea for the diagnosis of abortion in 2003 and the internal organs were homogenized with PBS. Viral RNA was extracted using viral RNA extraction kit (Bioneer, Korea). The bound RNA was eluted in 50 µl of diethyl pyrocarbonate (DEPC) treated distilled water. Extracted RNA including RNase inhibitor, RNAsin (Promega, USA) was stored at –70°C until use.

Selection of primers and probes

Three different primers and probes were designed based on conserved regions of 5' NTR, prM and 3' NTR genes from sequence data of KV1899 strain (GenBank accession No. AY316157) by using Beacon designer (Proligo, Singapore). The sequences of primer set for two-step and real-time RT-PCR were selected within highly conserved 3' NTR gene. Besides, 3' NTR primer for TaqMan probe was designed based on sequence data of conserved region for JE virus genotype I to IV. This probe was labeled with a fluorescent reporter dye (FAM: 6-carboxyfluorescein) at 5' end and a quencher dye (TAMRA: 6-carboxyteramethy-rhodamine) at 3' end. Sequences and nucleotide positions of primers and probes are given in Table 1.

Two-step RT-PCR

The extracted RNA samples were denaturated by heating at 95°C for 5 min and 15 µl (10-100 ng/µl) were added to RT-mix for synthesis of cDNA. The RT-mix consisted of 6 µl of 5X Universal buffer, 3 µl of 0.1 µl M DDT, 1 µl of 10 mM dNTP, 1 µl of reverse primer (20 pM), 1 of Superscript reverse transcriptase (50 U/ul), 9 µl of DEPC water and 10 of JE viral RNA to a total volume of 30 µl. Thermocycler conditions for reverse transcription were one hour at 42°C. The PCR mix (Qiagen, Germany) was made up to a volume of 50 µl, containing 25 µl of 2X Universal master mix, 1 µl of forward primer (20 pM), 1 µl of reverse primer (20 pM), 8 µl of DEPC water and 15 µl of cDNA. After 5 min incubation at 95°C, the cDNA was amplified by 45 three-step cycles: 10 s at 95°C, 20 s at 55°C and 20 s at 72°C and 5 min at 72°C for final extension. PCR products were visualized after electrophoresis with 100 volts on 2% agarose gels (Seakem, USA).

Table 1. Primers and probes used for the TaqMan RT-PCR and two step RT-PCR against JEV

Primer & probe	Sequence (5'-3')	Nucleotide position	Genomic region	Length of amplicon
JE1F	AAACCGGGCCATCAATATGC	125-144*		
JE1R	TGATAAGAGCCAGCACGAATCG	228-249	5' NTR	125 bp
Probe1	6Fam-TGCCGTGGGCAACGATCCG-Tamra	220-239		
JE2F	CCATCACGTACG AATGTCCG	620-639		
JE2R	GCACCAGCAGTCCACGTCT	666-684	prM	65 bp
Probe2	6Fam-TGCCGTGGGCAACGATCCGG-Tamra	645-664		
JE3F	GGTGTA AGGACTAGA GGTTAG AGG	10,726-10,750		
JE3R	ATTCCC AGGTGTCAATATGCTGTT	10,848-10,871	3' NTR	146 bp
Probe3	6Fam-CCCGTGGAAACAACATCATGCGGC-Tamra	10,754-10,777		

*Nucleotide sequence position according to KV1899 strain of JEV (GenBank accession number AY316157).

TaqMan RT-PCR

Real-time RT-PCR assays using TaqMan probe were carried out in a micro reaction tube (Corbett Research, Australia). The reaction mixture for each one tube TaqMan reaction mix consisted of 5 µl of Universal 5X buffer, 1 µl of 10 mM dNTP, 1 µl of enzyme mix (Qiagen, Germany), 1 µl of 20 pM forward primer, 1 µl of 20 pM reverse primer, 1 µl of 25 pM fluorogenic FAM labeled JEV probe, and 15 of JE viral RNA sample to a total volume of 25 µl. Thermo-cycling conditions were as follows: 30 min at 50°C for reverse transcription; 5 min at 95°C to activate DNA polymerase and to deactivate reverse transcriptase; 45 or 50 cycles of 10 s at 95°C to denature and 20 s at 55°C to anneal and 20 s at 72°C for extension and 5 min at 72°C for final extension. Reverse transcription and PCR amplification were performed by using the Rotor Gene 2000 real-time thermal cycler (Corbett Research, Australia).

Post PCR analysis

Amplification products from RT-PCR and real-time RT-PCR were visualized by electrophoresis on 2% agarose gels stained with ethidium bromide (0.5 µg/µl). PCR products in volumes of 15 µl were subjected to electrophoresis for 30 min. In real-time RT-PCR, Rotor Gene 2000 detector measured fluorescent signal generated by the sequence-specific probes. The analysis of data was undertaken with version 4.6 Rotor Gene software program. The tube was scanned at 518 nm (FAM) and 582 nm (TAMRA). Normalized fluorescence is the fluorescence signal increase due to template amplification. The amplification plots were generated with normalized fluorescence mean value on the y-axis and cycle number on the x-axis. The threshold cycle (C_t) was defined as the cycle number at which the reporter fluorescence generated by cleavage of the probe passed a fixed threshold above baseline. Standard deviation 10 above base line was used to determine the fixed threshold. For analysis of C_t values, less than 20 cycles threshold need to be adjusted manually.

Results

Selection of primer and specificity

The three primer sets: 5' NTR, prM and 3' NTR were designed and real-time RT-PCR assays were carried out with three different primer and probe sets for specificity (Fig. 1). Results showed that primer set of 3' NTR had good results but the primer sets of prM and 5' NTR didn't. Therefore, primers and probe of 3' NTR for the diagnostic purpose of JEV were selected. The concentration of primers and probe giving the highest fluorescent and the lowest threshold cycle were selected as follows: 20 pM forward and reverse primers, 25 pM probe. JE virus reference strains and field isolate were chosen in order to assess the correct specificity of the real-time RT-PCR assay. Both Anyang 300 and KV1899 strain were cultured in TF104 cells, while Nakayama strain was obtained from brain emulsion of the inoculated mouse. When several reference viruses were screened by the selected TaqMan probe, Anyang 300, Nakayama and KV1899 strains were shown as positive by real-time RT-PCR. As shown in Fig. 2, no cross-reactions were detected with the other viruses (CSFV, TGEV, PEDV, PRV, EMCV, BVDV) and normal TF104 cells.

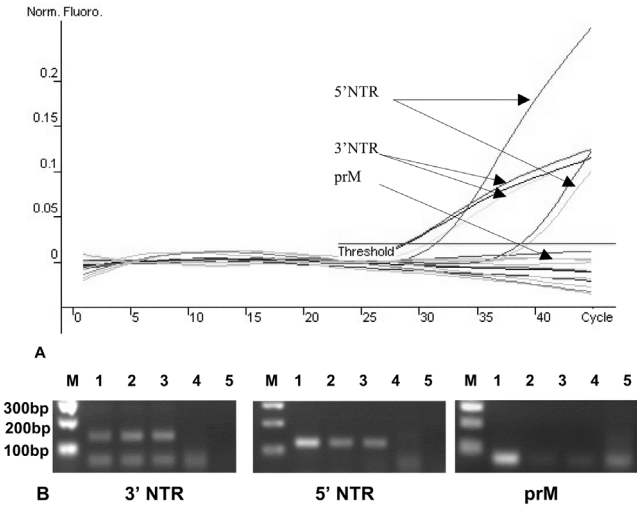

Fig. 1. Comparison of real-time RT-PCR and gel electrophoresis analysis of the product for the detection of JEV RNA. Three different primers and probes were designed based on conserved regions of 5' NTR, prM and 3' NTR genes. A: Real-time profiles of JEV cDNA amplification reaction. Three different curves corresponding to each gene of JEV show PCR amplification profiles of 3 different viruses, respectively. B: Agarose gel electrophoresis of real-time RT-PCR amplified products. M; 100bp ladder, lane 1; KV1899, lane 2; Anyang 300, lane 3; Nakayama, lane 4; TF104 cells, lane 5; no template control in B.

Sensitivity and reproducibility

The sensitivity of real-time RT-PCR and RT-PCR assay for detection of JE virus was compared. As shown in Table 2, the JEV culture supernatants (KV1899; $10^{6.75}$ $TCID_{50}$/ml) were subjected to sensitivity test. The eluted RNA was serially diluted in 10-fold up to 10^{-7} and used for cDNA synthesis. Sensitivity of RT-PCR assay for detection of JEV was expressed as infectivity titer equivalent. The expected fragments of RT-PCR products were detected in infectivity titer equivalent from $2.24 \times 10^{5.0}$ $TCID_{50}$/reaction to $2.24 \times 10^{1.0}$ $TCID_{50}$/reaction. Real-time RT-PCR assay could detect JE RNA ranging from $1.12 \times 10^{6.0}$ to $1.12 \times 10^{1.0}$ $TCID_{50}$/ml. Therefore, detection limits of RT-PCR and real-time RT-PCR were 112 $TCID_{50}$/ml and 11.2 $TCID_{50}$/ml, respectively. In the sensitivity of detection, real-time RT-PCR assay with single tube was 10-fold more sensitive than RT-PCR assay. When the same person performed the real-time RT-PCR three times at the different day, the same results were obtained from all the three trials (Fig. 3B).

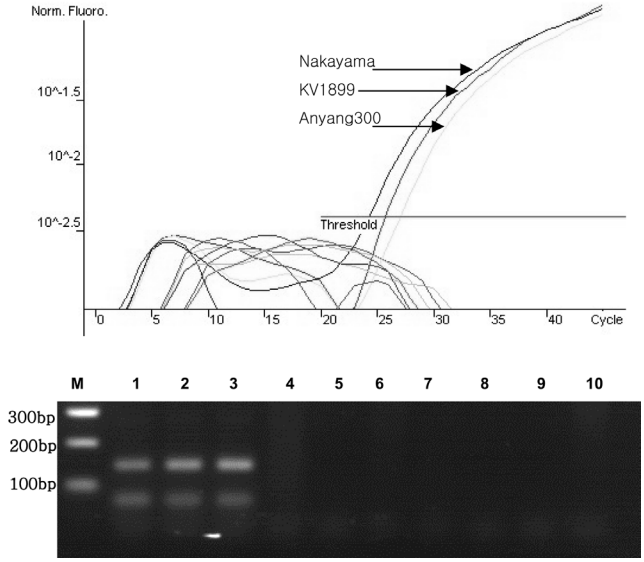

Fig. 2. Real-time RT-PCR amplification plots of RNAs from 3 JEV isolates using primers and probe corresponding to 3' NTR. Gel electrophoresis of the amplicons to test specificity of PCR probe (lower figure). The expected amplicon size is 146 bp. M; 100 bp ladder, Lane 1; KV1899, Lane 2; Anyang 300, Lane 3; Nakayama, Lane 4; TGEV, Lane 5; PEDV, Lane 6; PRV, Lane 7; CSFV, Lane 8; BVDV, Lane 9; EMCV, Lane 10; TF104 cell. Titers of reference viruses ranged from $10^{5.0}$ to $10^{7.0}$ $TCID_{50}$/ml.

Standard curve for quantification of JEV

Real-time RT-PCR amplifications were performed with serial dilutions (10^{-1} to 10^{-7}) of RNA molecules prepared from isolate KV1899 to assess quantification assay. JEV RNA concentration of infectivity equivalent was from $1.1 \times 10^{6.0}$ $TCID_{50}$/ml to 1.1 $TICD_{50}$/ml. Fig. 3A showed the amplification profile with number of cycles versus normalized fluorescence values. A detectable fluorescence signal above the threshold occurred at 24.7 cycles corresponding to $1.1 \times 10^{6.0}$ $TCID_{50}$/ml. The Rotor Gene detection system software generated a standard curve by plotting the C_t values against each standard dilution of known virus concentration. A linear standard curve was obtained from 10^{0} to 10^{-5} per reaction mixture, resulted in C_t values ranging from 24.7 to 46.1 cycles.

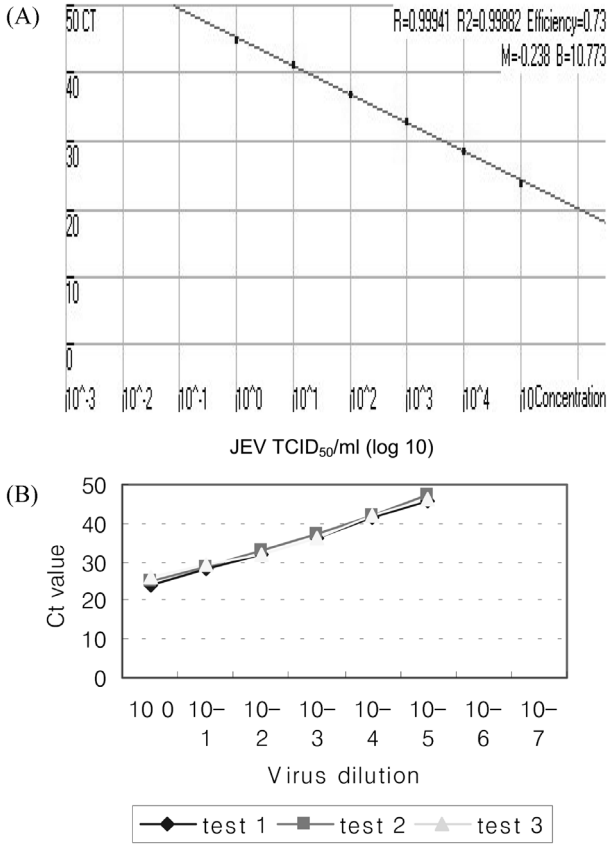

Fig. 3. Real-time RT-PCR standard curve generated from RNA amplification plots. Standard curve was plotted in the sample $TCID_{50}$ on the x-axis and cycle threshold (Ct) on the y-axis. Correlation coefficient was 0.999 (A). Reproducibility of real-time RT-PCR was tested three times at different day (B).

Application of the real-time RT- PCR assay

Blood plasmas from 8 piglets inoculated with JEV and 41 aborted fetuses were examined for JEV by real-time RT-PCR and virus isolation methods. The optimized technique

Table 2. Comparison of RT-PCR and real-time RT-PCR for the detection of JEV in culture supernatant

Virus dilution	Infectivity titer equivalent of JEV RNA extraction		Real-time RT-PCR	RT-PCR
	In isolated RNA*	In cDNA synthesis**		
10^{0}	1,123,682	224,936	+	+
10^{-1}	112,368	22,493	+	+
10^{-2}	11,236	2,249	+	+
10^{-3}	1,123	224	+	+
10^{-4}	112	22.4	+	+
10^{-5}	11.2	2.24	+	-
10^{-6}	1.12	0.24	-	-
10^{-7}	0.11	0.02	-	-
control	0	0	-	-

*$TCID_{50}$/50 μl eluted RNA/ 1 ml virus solution.
**$TCID_{50}$/25 μl RNA reaction.

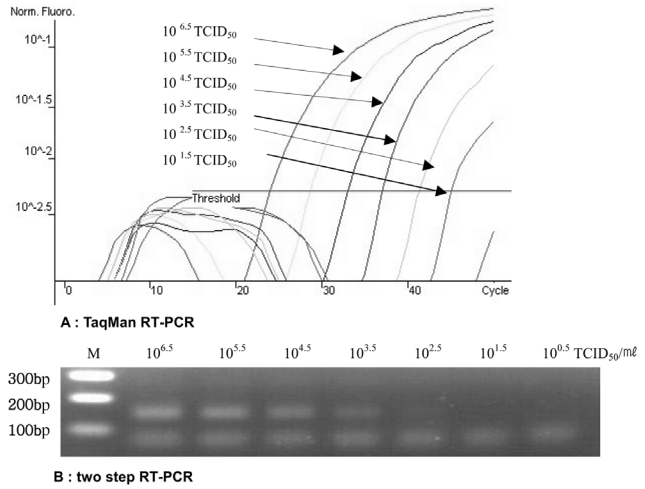

Fig. 4. Sensitivity of real-time RT-PCR assay for quantitative detection of JEV (A) and detection of JEV by RT-PCR (B). One milliliter of JEV culture supernatant ($10^{6.5}$ $TCID_{50}$/ml) was subjected to RNA isolation.

was applied on plasma samples from piglets that were experimentally inoculated with JEV. Two of 8 pigs were positive by real-time RT-PCR for JE virus at 2 days post inoculation (PID). The C_t value for the positive samples was 46.7 and 47.1 (Fig. 5A). Table 3 showed that JEV in two plasmas of pigs was isolated by individual virus isolation method using TF104 cells. The results from real-time RT-PCR and virus isolation were accurately correlated. Therefore, the real-time RT-PCR method could be used to detect and quantify JEV concentration in pigs for the purpose of determining JEV viremia. Forty one fetuses that aborted before 70 days of gestation also were tested by real-time RT-PCR, but did not show any positive reactions for JE viral RNA (Fig. 5B).

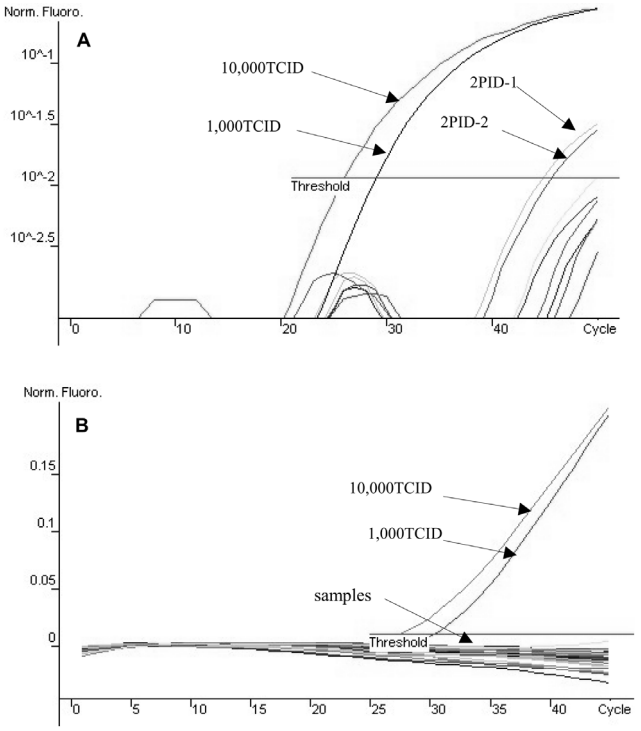

Fig. 5. Application of real-time RT-PCR assay to 9 plasma samples collected at 2 PID (A) and 41 fetus samples (B). JEV culture supernatant ($10^{4.0}$ $TCID_{50}$/ml and $10^{3.0}$ $TCID_{50}$/ml) was subjected to standard virus.

Discussion

In this study, we investigated real-time RT-PCR for laboratory detection of JEV in different samples. Specificity, sensitivity and quantitative range of real-time RT-PCR were also evaluated. When compared to the previous reports for detection of *Flavivirus* [7,16,25], real-time RT-PCR method

Table 3. Detection of JEV by real-time RT-PCR and virus isolation

Virus strain	ID. of pig	Post inoculation day						
		2	4	6	8	15	22	30
KV1899*	1	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	3	+/+	-/-	-/-	-/-	-/-	-/-	-/-
	4	+/+	-/-	-/-	-/-	-/-	-/-	-/-
Anyang300	1	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	3	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	4	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Control	1	-/-	-/-	-/-	-/-	-/-	-/-	-/-

Real-time RT-PCR and virus isolation were performed using blood plasmas of pigs intramuscularly inoculated with JEV.
*: KV1899 strain had undergone 37 passages in TF104 cells.
+/+: Positive results in both virus isolation and real-time RT-PCR.
-/-: Negative results in both virus isolation and real-time RT-PCR.

has several advantages over conventional PCR. First, real-time RT-PCR yields more rapid and sensitive test result than conventional RT-PCR [23,26]. The second advantage of the closed one-tube RT-PCR is that it is less likely to produce false positive by contamination during preparing the sample. In addition, the fluorogenic probes can reduce time consuming postPCR analysis such as gel electrophoresis, because the amplification of a specific PCR product is measured in real time during PCR cycling.

The conserved sequences such as 5' NTR, 3' NTR and NS1 region have been reported for amplifying fragment of JEV [16]. An improper selection of primers could result in failure of the PCR assay [21]. JEV strains isolated from different host animals in diverse geographical locations have successfully been detected by RT-PCR using 3' NTR primers [12,20]. In this study, JEV primers and probes selected from 3' NTR showed reliable specificity for the detection of JEV in real time RT-PCR assay. We also considered the length of amplified fragment, because amplicon size was affected by hybridization of the fluorescent probe. The amplified fragment between 100 to 200 base pairs was known to be reliable and reproducible [12]. Therefore, the primers were designed to get 146 base pairs amplicon in this study.

The comparison of real-time RT-PCR with conventional RT-PCR proved to be useful in assessing the sensitivity of the newly developed method. Ten-fold serial dilutions of the extracted viral RNA from the JEV infected culture supernatants were analyzed to define the sensitivity of tests. The results showed that real-time RT-PCR proved to be 10-fold more sensitive than conventional RT-PCR. The reasons for different sensitivity in two-step and real-time RT-PCR are uncertain, but may include fidelity of enzyme in closed one tube and the usage of highly sensitive TaqMan probe. The detection limit of the real-time RT-PCR was calculated to be 11.2 $TCID_{50}$/ml (Table 2). This result of detection limit was similar to that of the previous study [12].

The conventional method of $TCID_{50}$ determination is laborious, expensive, time consuming and also requires the susceptible cells. Quantification of JEV investigated in this study was rapid and reproducible compared with the conventional culture method. Because primer and probe set is derived from a highly conserved 3' NTR of the genome, the assay can allow rapid quantification of JEV. Although quantification is not required for a diagnostic test, real-time RT-PCR could be useful for several applications such as virus titration within a short period of time.

RT-PCR assay for detection of JEV envelope gene in various biological samples such as infected cell cultures, *Aedes* larvae, mosquitoes and mouse blood had been applied [20]. When mosquito pools for the presence of West Nile virus were tested using real-time RT-PCR in single tube, the high degree of sensitivity and specificity were observed [8, 18]. We also could prove the RNA genome of JEV from the infected tissue culture as well as plasma samples that obtained at 2 days post experimental infection with Korean isolate (KV1899 strain). But, we could not detect JEV genome in blood plasma that collected from four pigs inoculated with live vaccine virus and in 41 aborted fetus samples. It was described that pigs developed a significant viremia that lasted 2 to 4 days following natural infection with JEV [6]. The pigs inoculated with vaccine, Anyang 300 strain, were not positive by real-time RT-PCR, because they might be little viremic with the highly attenuated JEV in chicken fibroblast cells. Our result was in agreement with the previous study that viremia in the infected young adult using attenuated (m) strain could not be detected [14]. For aborted fetuses, abortion might be caused by other viral diseases such as parvovirus, porcine reproductive and respiratory syndrome virus, encephalomyocarditis virus and pseudorabies virus. Moreover, JEV is known to have fragile feature in the physical characteristics. Isolation rate of JEV may have been considerably low [17]. Therefore, the virus might be degraded by autolyzed fetal tissue in uterus. The previous study reported that JE transmission seemed possible for only 3 days post infection [22]. In this study, two of 4 pigs that inoculated with cell cultured passaged KV1899 were positive only at 2 PID in real-time RT-PCR. It was assumed that the KV1899 was passaged 37 times in TF104 cells and the virus might be a little attenuated.

In conclusion, the TaqMan real-time RT-PCR assay described here for detection and quantification of JE virus has been shown to be rapid, easy to handle, sensitive and specific. These features make it an excellent tool for laboratory detection of JEV in tissue cultured samples as well as field samples such as pig plasma. The high degree of sensitivity and specificity observed with the tissue culture propagated virus suggested that the assay should be a useful tool for field investigation of JEV infection.

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