Original Article

Multiplex SYBR Green real-time RT-PCR assays for the improved detection of viral pathogens in horses: equine infectious anemia, West Nile and Japanese encephalitis viruses

Sunutcha Suntrarachun[1*] Surasak Akesowan[1] Suchitra Khunsup[1]

Abstract

Equine infectious anemia virus (EIAV), West Nile virus (WNV) and Japanese encephalitis virus (JEV) are mosquito-borne viruses that are of great concern for the equine industry worldwide. These viruses produce similar clinical signs and veterinarians need to be able to differentiate between them quickly. In this study, multiplex known as duplex and triplex SYBR Green real-time RT-PCR assays were developed to detect these three viruses and to discriminate between them based on differences in the melting temperatures (Tm) of their amplification products. Three melting peaks, generated simultaneously at temperatures of 78.5±0.5°C, 83.5±0.5°C and 85.0±1.0°C, indicated the presence of EIAV, JEV and WNV, respectively. The optimal melting rate for duplex was 0.5°C/sec and a slower rate of 0.1°C/sec for triplex to improve resolution of the melting temperature. The optimal combinations of primers for multiplex were 600 or 900 nM for EIAV and WNV and 300 or 600 nM for JEV detection. The assays all demonstrated high sensitivities, ranging from $3.29X10^{-2}$ to $3.29X10^{6}$copies/µl which was similar to those obtained based on agarose gel analysis of the RT-PCR products. No cross-reaction was observed with the other closely related viruses, which indicated a high specificity of these assays. Less than 0.5% of the coefficient of variation (CV) of intra-specific assay and inter-specific assay were considered acceptable to generate reproducible results. These results indicated that the multiplex, duplex and triplex, SYBR Green real-time RT-PCR assays described in this paper could be used for the detection of EIAV, WNV and JEV and would provide a valuable addition to the methods currently available for the routine diagnosis and surveillance of these zoonoses which are listed by the World Animal Health Organization (OIE) as important for international trade.

Keywords: SYBR Green, Real-Time Reverse Transcription – Polymerase Chain Reaction (Real-time RT-PCR), EIAV, WNV, JEV

[1]*Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok 10330, Thailand*
***Correspondence**: sunutcha@yahoo.com* (S. Suntrarachun)
Received December 3, 2021
Accepted October 14, 2022
https://doi.org/10.14456/tjvm.2022.77

Introduction

Viral infection is a permanent threat to the equine industry worldwide. Great care should be taken to prevent it in equines including horses, donkeys, mules and ponies. Three mosquito-borne viruses that are of great concern for the equine industry are equine infectious anemia virus (EIAV), West Nile virus (WNV) and Japanese encephalitis virus (JEV). However, WNV and JEV produce similar clinical signs, ranging from asymptomatic or a mild flu-like illness to clinical encephalitis (Chao *et al*., 2007). It is important to discriminate these three viruses to prevent transmission of diseases in equines, especially in the horse industry.

EIAV, a member of the genus *Lentivirus* of the *Retroviridae* family, is distributed worldwide and has only been reported in equids (Cook *et al*., 2002; Spyrou *et al*., 2003). The virus is transmitted through blood-sucking insects such as mosquitoes, horse flies and deer flies (Nagarajan and Simard, 2001). Many horses have very mild or inapparent signs but may develop severe and fatal symptoms. EIAV was first recognized in Thailand in 1983 but could have been present earlier. A serological survey in the northern area (Lampang province) in 2001 and 2006 showed that 4.17% and 6.9%, respectively, of the horses were EIAV positive (Akesowan and Suntrarachun, 2007; Suntrarachun *et al*., 2010). A sero-diagnostic assay, which detects antibodies against the major core protein of EIAV (p26), is the agar gel immunodiffusion assay (AGID) or Coggins test. Although this assay is convenient and useful for large-scale screening, its most obvious limitation is the inability to detect early stages of infections when anti-EIAV antibodies are either absent or undetectable leading to a false negative result (Nagarajan and Simard, 2001). RT-PCR assays which target the gag region of the virus genome can detect the viral RNA in the plasma of animals at early stages of infection long before coming EIAV-seropositive but pose a considerable transmission risk. However, molecular diagnosis of EIAV-seropositive animals can be problematic as the virus genome is integrated into the host cell genomic DNA and remains latent. The integrated proviral DNA with a low level of viral replication leads to low levels of viral RNA in the plasma which is not readily detectable (Cook *et al*., 2002; Quinlivan *et al*., 2007).

WNV and JEV are mosquito-borne flaviviruses that are associated with both human and equine encephalitis worldwide (Spyrou *et al*., 2003; Chao *et al*., 2007). They cause similar symptoms in humans and horses, ranging from asymptomatic to lethal encephalitis (Yeh *et al*., 2010; Barros *et al*., 2013). Serological methods can be used to detect for the presence of WNV and JEV antibodies in infected hosts. However, cross-reactivity of antibodies with related non-target flaviviruses often occur in serology assays (Chao *et al*., 2007). Nested RT-PCR, multiplex RT-PCR and real-time RT-PCR assays using virus-specific primer pairs are used to differentiate WNV and JEV from other flaviviruses in tissue, blood and CSF (Shirato *et al*., 2003; Shirato *et al*., 2005; Yeh *et al*., 2010; Barros *et al*., 2013; Vazquez *et al*., 2016).

Real-time PCR with SYBR Green melting curve analysis is a simple and reliable technique that has been effective for the detection and identification of various pathogens. SYBR Green based detection technique is reliable for detecting nucleic acid targets characterized by sequence variability. The SYBR Green dye will bind non-specifically to double stranded DNA by intercalation and/or minor groove binding. Specific identification may be achieved by melting curve analysis that can be identified at the species level or even identification of strains of a virus pathogen (Varga and James, 2006). Therefore, improved assays are required for screening for viral infections in equines, especially horses used for producing pharmaceutical products. This study aimed to develop the multiplex, duplex and triplex SYBR Green real-time RT-PCR assays, for the simultaneous detection and discrimination of EIAV, JEV and WNV in horses before receiving snake immunization for antivenom production.

Materials and Methods

Primer Design: Oligonucleotide primers were designed using Primer3 software for both the RT-PCR and SYBR Green real-time RT-PCR assays and were based on nucleotide sequences retrieved from the NCBI GenBank (Table 1). The nucleotide sequences were aligned using Clustal W to search for conserved virus genome regions. As shown in Table 1, two pairs of EIAV primers, selected from gag gene, were used for the nested RT-PCR in two amplification steps. First a 498 bp product was amplified using an outer primer pair P26-F and P26-R. The second amplification, using nested pair P26-FN and P26-RN, produced a 246 bp PCR product. The JEV primers (JE-F and JE-R) amplified a 381 bp sequence of the polyprotein region of the genome. The primer pairs of WNV, selected sequences of the polyprotein genes, were designed for semi-nested RT-PCR in two amplification steps. The first primer pair, WN1-F and WN-R, produced a 247 bp using RT-PCR. The second amplification, using semi-nested pair WN2-F and WN-R, produced a 239 bp. However, a specific primer pair (WNGC-F and WNGC-R) of WNV used for SYBR Green real-time PCR was modified with GC content added due to the similar melting temperatures between JEV and WNV. All the primers were checked by BLAST analysis against sequences of EIAV, WNV, JEV and related lentiviruses and flaviviruses from the NCBI database to ensure their specificity.

A synthetic RNA fragment: A 1,126 bp synthetic RNA fragment containing a composite of partial sequences of the EIAV, WNV and JEV genomes was synthesized for use as the RNA template for developing and optimizing the SYBR Green real-time RT-PCR assays. The RNA template was synthesized by Integrated DNA Technologies, USA. It contained a 498 bp sequence of the gag region (nucleotide position 528-1,025) of the EIAV genome, a 381 bp sequence of the polyprotein region (nucleotide position 5,739-6,119) of the JEV genome and a 247 bp sequence of the polyprotein region (nucleotide position 385-632) of the WNV genome (Table 1.).

Construction of a standard curve for virus quantification: A 400-ng preparation of the synthetic composite RNA template was calculated for the number of RNA copies based on its size of 1,126 bp. A dilution series of the RNA template, ranging from $3.29X10^{-4}$ to $3.29X10^{6}$copies/µl was then prepared and used in the SYBR Green real-time RT-PCR assay in order to construct a standard curve for quantifying the three viruses in the assays.

Nested and multiplex RT-PCR assays: First strand cDNA synthesis (Thermo Scientific RevertAid First Strand cDNA Synthesis, Waltham, Massachusetts, USA) was carried out in 12 µl reaction volumes of 1 µg RNA template and random hexamer on a thermocycler (MWG Biotech, USA) at 65°C for 5 mins. Then, the components of 5XReaction buffer, 10 mM dNTP mix, 20U RNase Inhibitor, and 200U RevertAid MMLV RT were added to the random hexamer primed synthesis. The reaction was incubated 25°C for 5 mins, followed by 60 mins at 42°C. The product of the first strand cDNA synthesis was used in 50 µl of the first PCR reaction (Vivantis Technologies Sdn Bhd, Selangor Darul Ehsan, Malaysia) which consisted of 2XTaq Master mix, 50 mM $MgCl_2$, and 50 pmol/µl of each primer (P26-F and P26-R for EIAV, JE-F and JE-R for JEV, and WN1-F and WN-R for WNV). The reaction was carried out at 94°C/55°C/72°C 1 min each, for a total of 35 cycles. Nested RT-PCR (Vivantis Technologies Sdn Bhd, Selangor Darul Ehsan, Malaysia) was performed under the same conditions as indicated previously for the first PCR and carried out with 5 µl from the first reaction product as a template with inner primers (P26-FN and P26-RN for EIAV, and WN2-F and WN-R for WNV). The second amplification cycle conditions consisted of initial denaturation at 94°C 2 mins followed by 94°C 1 min, 56°C 30 sec, 72°C 1 min for 35 cycles and a final extension of 72°C 7 mins. Multiplex, duplex and triplex, RT-PCR assays were carried out using the same RT-PCR conditions, allowing assays to be performed in a single run. However, the primers were optimized by testing concentrations between 100 to 300 nM for 200 bp and 400 to 1000 nM for 300 bp. Similarly, $MgCl_2$ concentrations were optimized by testing concentrations between 3 mM and 5 mM. Annealing temperatures and times were optimized by testing temperatures of 50°C to 60°C, and between 1 and 4 mins, respectively, for the specific bands. The final nested and multiplex RT-PCR products were analyzed on a 2% agarose gel containing ethidium bromide in 1XTAE buffer along with appropriate molecular size markers.

Simplex and multiplex SYBR Green real-time RT-PCR assays: A simplex SYBR Green real-time RT-PCR was carried out with CFX 96 Touch™ Real-Time PCR) using iTaq Universal SYBR Green One-Step kit (Biorad, CA, USA) for EIAV gag gene and polyprotein genes of JEV and WNV. Five microliters of the synthetic RNA templates were added to 15 µl of reaction mixture including 2XiTaq universal SYBR Green reaction mix, iScript reverse transcriptase, 10 pmol of each primer (P26-F and P26-R for EIAV, JE-F and JE-R for JEV, and WNGC-F and WNGC-R for WNV) and nuclease-free water. The reaction was performed under the following conditions: 95°C 3 mins followed by 40 cycles of 95°C 10 secs, 52°C 10 secs and finally 72°C 30 secs. In order to determine the optimal temperature of the primers, gradient tests were run from 50°C to 60°C for the annealing step and from 70°C to 75°C for elongation temperature. Following amplification, melting curve analysis was performed. This was to verify the specificity of the RT-PCR products by looking at melting temperatures. The melt curve protocol was followed by 10 secs of 95°C and then 5 secs each at 0.1 or 0.5°C increments between 65°C to 95°C. Duplex and triplex SYBR Green real-time RT-PCR assays were performed under the same conditions described above for simplex SYBR Green real-time RT-PCR. However, the primer concentrations were optimized using different concentrations among 300, 600 and 900 nM.

Sensitivity, specificity, intra-assay (repeatability) and inter-assay (reproducibility) of the SYBR Green real-time RT-PCR assays: To determine the sensitivity of the agarose gel-based RT-PCR and the SYBR Green real-time RT-PCR assays, a standard curve was constructed using serial 10-fold dilutions of the synthesis RNA templates, from 10^{9} to 10^{-4}copies/µl. Triplicate was tested in the same assay and the limit of detection was calculated for each standard. The specificity was evaluated using viral RNA from a panel of Human Immunodeficiency Virus, Yellow Fever Virus and Dengue Virus. To confirm the accuracy and reproducibility of SYBR Green real-time RT-PCR assays, the intra-assay precision (repeatability) was determined in three repeats within one run. The inter-assay variation (reproducibility) was investigated in three different experimental runs performed in 5 days (Santhosh *et al*., 2007; Rodriguez-Sanchez *et al*., 2008).

Assessment of the efficacy of the SYBR Green real-time RT-PCR assays: The synthetic RNA template was used to assess the efficacy of the SYBR Green real-time RT-PCR assays for clinical samples. Aliquotes (200 µl) of a virus-negative horse plasma preparation were spiked with a serial dilution of the RNA template and RNA was then extracted from the spiked plasma samples with 300 µl of Trizol (Molecular Research Center, Inc, Cincinnati, Ohio, USA), according to the manufacturer's instruction. The concentration and purity of the extracted RNA were determined by measuring the absorbance at 260 nm and 280 nm. A ratio of 260 nm/280 nm of between 1.8 and 2.0 was considered of acceptable purity. The RNA concentration in µg/ml was calculated using the formula OD_{260}Xdilution factorX40. SYBR Green simplex, duplex and triplex, real-time RT-PCR assays were also performed. The animal protocol for collecting horse plasma sample was reviewed and approved by the Queen Saovabha Memorial Institute Animal Care and Use Committee (QSMI-ACUC-02-2017).

Table 1 Primer sequences and the characteristics of EIAV gag region and the polyproteins of JEV and WNV generated by the agarose gel-based RT-PCR and SYBR Green real-time RT-PCR assays.

Primer	Sequences (5′–3′)	Genomic target region	Amplicon size (bp)	Average Tm (°C)	NCBI Accession Number
JE-F	AGAGCGGGGAAAAAGGTCAT	5,739 - 6,119	381	83.50	AB551990
JE-R	TCCATTGGGCATGTGTATGT (RT-PCR, multiplex RT-PCR, and SYBR Green real-time RT-PCR)				JN381869
P26-F	AATGAATGCATTTTTGGATGT	528 - 1,025	498	78.50	NC001450
P26-R	CATGCATACATTTTCTCTTCT				AF327877
P26-FN	ACAAGGGCCTATTCCCATGACA	660 - 905	246		AF247394
P26-RN	GGATGTCCCTCACTTTTTATC/TT (Nested RT-PCR, multiplex RT-PCR, and multiplex SYBR Green real-time RT-PCR)				
WN1-F	CGGCGGAGCTCAAAACAAAAGA	385-632	247	83.00	NC001563
WN-R	GCACTGGGCATTCATAAGTGATAGT				NC009942
WN2-F	CTCAAAACAAAAGAAAAGAGGAGG	394-633	239		
WN-R	GCACTGGGCATTCATAAGTGATAGT (Nested RT-PCR and multiplex RT-PCR)				
WNGC-F	GGGCCCCCCGGGGGGGGGCCCGGCGGAGCTCAAAA CAAAAGA	385 - 632	287	85.00	NC001563 NC009942
WNGC-R	GGGCCCCCCGGGGGGGGGCCGCACTGGGCATTCAT AAGTGATAGT (Simplex and multiplex SYBR Green real-time RT-PCR)				

Results

The standard curve for virus quantification: The standard curve for virus quantification using SYBR Green real-time RT-PCR generated from the Ct values obtained against the log of known RNA copies (dilution series of 10^2 to 10^7copies/µl) (Fig 1.). The correlation coefficient (R^2) of three viruses, 0.9843 (JEV), 0.9819 (EIAV) and 0.9774 (WNV), also suggested a good correlation between Ct values and known copy numbers of RNA transcripts. The inter-assay coefficient of variation (inter-assay CV) of less than 5%, which is considered acceptable, indicated that this assay was able to generate reproducible results. Turnaround time for the real-time RT-PCR assay was approximately 3 hours inclusive of melt curve analysis while completion of agarose gel-based RT-PCR assays including that of agarose electrophoresis analysis and gel staining requires about 6 hours.

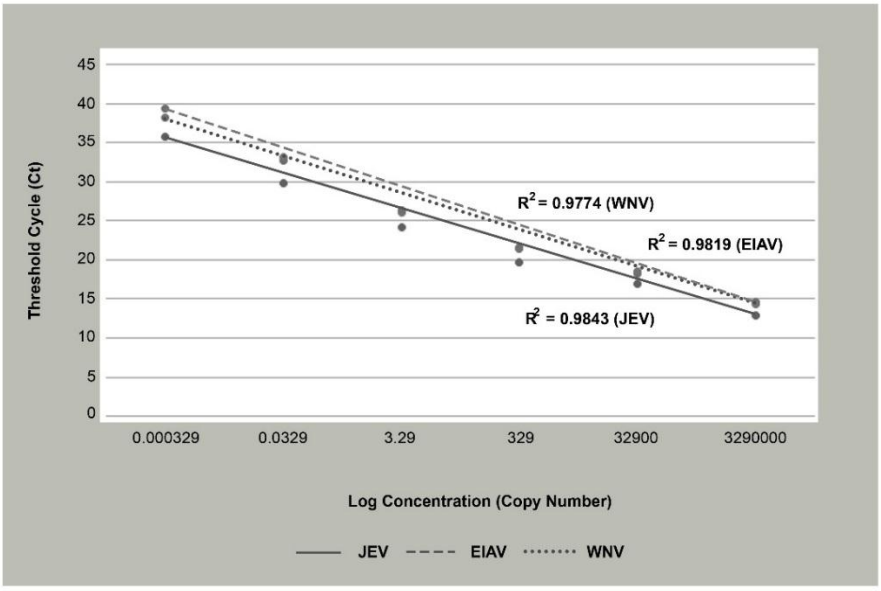

Figure 1 The standard curve generated from the detection of three viruses by SYBR Green real-time RT-PCR. Each point represented the mean of six determinations. A linear relationship between threshold cycle and serially diluted *in vitro* RNA transcripts from 10^7 to 10^2copies/µl. SYBR Green real-time RT-PCR efficiency for three viruses was 98.43% (JEV), 98.19% (EIAV) and 97.74% (WNV).

Nested and multiplex RT-PCR assays: The final optimal conditions for nested RT-PCR components were 1,000 nM primers, 4 mM $MgCl_2$ and annealing temperature 55°C. The optimized conditions of duplex and triplex RT-PCR were 300 nM for primers of EIAV and WNV and 600 nM for JEV, 4 mM $MgCl_2$ and annealing temperature 56°C for 1 min. The RT-PCR products of these viruses were shown in a 2% agarose gel electrophoresis from $3.29x10^7$ to $3.29x10^{-6}$copies/µl

(Fig. 2A, 2B, and 2C). The lower limit of detection for JEV was $3.29x10^{1}$copies/µl. The minimum detection limit for EIAV and WNV was $3.29x10^{-1}$ and $3.29x10^{-2}$copies/µl, respectively. However, multiplex, duplex and triplex, RT-PCR products, especially between EIAV (246 base pair) and WNV (239 base pair), were difficult to separate due to close band sizes (Fig. 2D). A nonspecific band (500 bp) was observed due to large amounts of DNA template from the first round of EIAV.

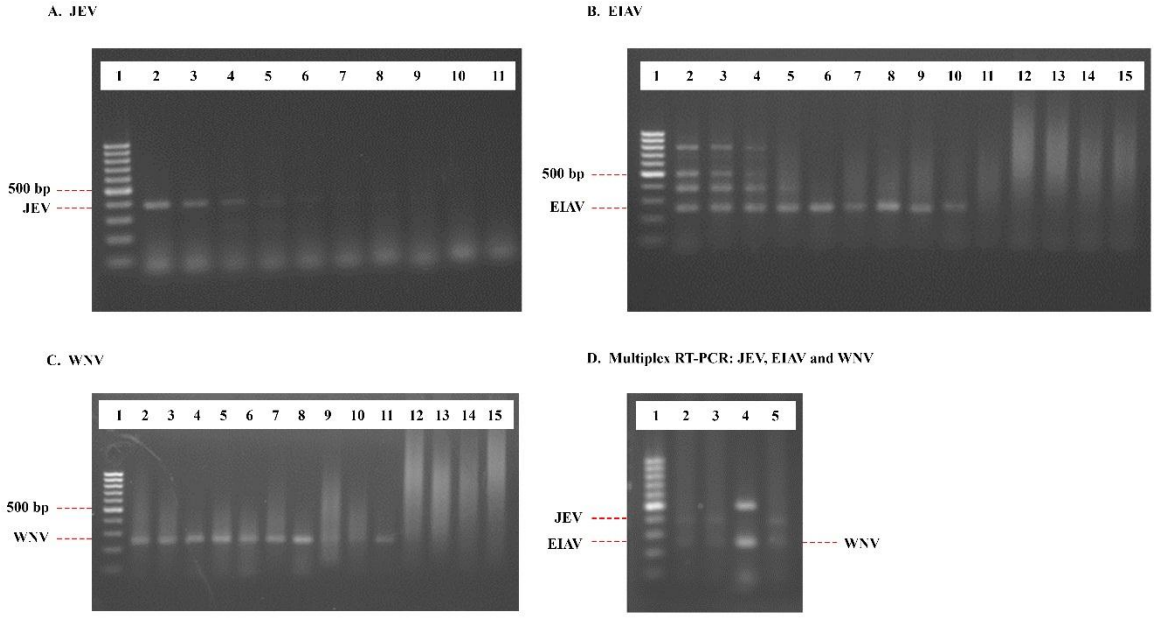

Figure 2 Agarose gel analysis of the RT-PCR products of (A) JEV using 10-fold serial dilutions of the RNA template with concentrations of $3.29X10^{7}$ to $3.29x10^{-2}$copies/µl; (B) EIAV and (C) WNV at template concentrations of $3.29X10^{7}$ to $3.29X10^{-6}$copies/µl. (D) Duplex RT-PCR assays: Lane 2, EIAV (246 bp) and JEV (381 bp); Lane 3, WNV (239 bp) and JEV (381 bp); lane 4, EIAV (246 bp) and WNV (239 bp). Triplex RT-PCR assays: lane 5, EIAV (246 bp), WNV (239 bp) and JEV (381 bp). Lane 1 in (A) to (D): GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific, Sweden).

Simplex SYBR Green real-time RT-PCR: The primers used for simplex SYBR Green real-time RT-PCR were identical to those described previously in the agarose gel-based RT-PCR except WNV. A specific primer for WNV was modified with GC content added due to the similar melting temperatures between JEV (83.0°C) and WNV (83.5°C). The modified primer used for SYBR Green real-time RT-PCR would increase the melting temperature of WNV from 83.5°C to approximately 85.0°C (84.0 to 86.0°C) of melting peak. These primers allowed the species-specific amplification of JEV, EIAV and WNV in a CFX 96 Real-Time System (Bio-Rad, Hercules, USA). The optimal condition of primer concentration was 500 nM and annealing temperature was 52°C 1 min. The melt curve protocol was followed by 10 secs of 95°C and then 5 secs each at 0.5°C increment between 65°C to 95°C. Three melting peaks, generated simultaneously at temperatures of 78.5±0.5°C, 83.5±0.5°C and 85.0±1.0°C, indicated the presence of EIAV, JEV and WNV, respectively. The primer dimer produced much lower fluorescence at 75.0±0.5°C (Fig. 3).

Multiplex SYBR Green real-time RT-PCR: The optimal conditions of primer concentrations came from different combinations for multiplex, duplex and triplex, real-time RT-PCR assays, including 600 or 900 nM for EIAV, 300 or 600 nM for JEV and 600 or 900 nM for WNV. The gradient with annealing temperatures ranging from 50°C to 60°C was performed and the finalized annealing temperature was 52°C for both duplex and triplex real-time RT-PCR. Moreover, at the end of the amplification cycles, melting rates had been optimized between 0.1 to 0.5°C when SYBR Green duplex and triplex real-time RT-PCR assays were performed. The optimal melting rate to increase the melt peak resolution for duplex was 0.5°C/sec up to 95°C. However, a slow melt rate using 0.1°C/sec, resulting in higher Tm resolution and improved discrimination of the melt peaks was suitable for a triplex.

SYBR Green is a double-strand DNA binding molecule that does not recognize specific double-strand DNA targets. Hence, the different amplicons were distinguished by the melting curve analysis. The melting temperature (Tm) was defined as the peak of the curve, and if the highest point was a plateau, then the mid-point was identified as the Tm. The melting curves of EIAV and JEV in multiplex SYBR Green real-time RT-PCR, duplex and triplex, were similar to the simplex SYBR Green real-time RT-PCR. However, the melting temperature of WNV in multiplex SYBR Green real-time RT-PCR was highly variable, 85.0±1.0°C, ranging from 84.0 to 86.0°C (Fig. 4 and Fig. 5).

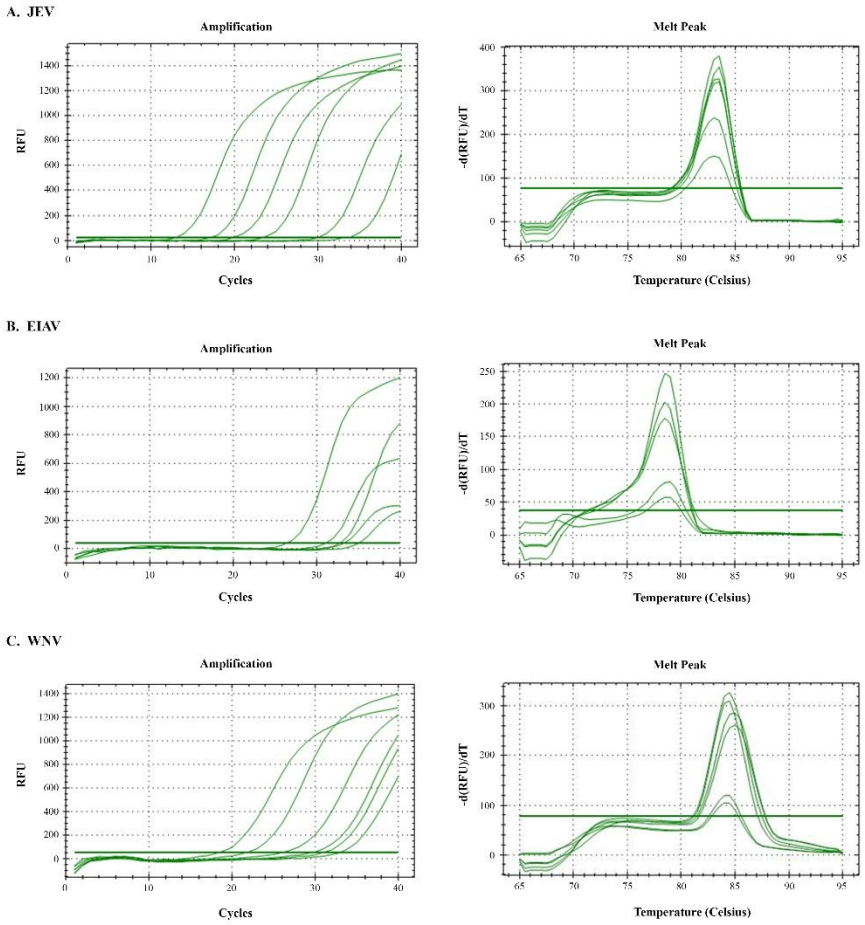

Figure 3 Sensitivity of the simplex SYBR Green real-time RT-PCR assays determined with 10-fold serial dilution of *in vitro* RNA transcripts from 10^6 to 10^{-2}copies/µl. Amplification plots and melt peaks based on SYBR Green were 83.5±0.5°C, 78.5±0.5°C, and 85.0±1.0°C for JEV (A), EIAV (B), and WNV (C), respectively.

Sensitivity, specificity, intra-assay (repeatability) and inter-assay (reproducibility) of SYBR Green real-time RT-PCR: The sensitivity, specificity, repeatability and reproducibility were also evaluated using serial 10-fold dilutions of the synthetic RNA templates, from 10^9 to 10^{-4}copies/µl. In this context, EIAV, JEV and WNV were detected in 100% at 3.29X10^{-2}copies/µl. However, showing the different sensitivity displayed in duplex and triplex SYBR Green real-time RT-PCR assays, higher concentrations of 3.29X10^6copies/µl were detected when compared with simplex SYBR Green real-time RT-PCR. Ct values were plotted as a function of virus concentration to ensure goodness of fit and linearity of slope and to determine the levels of detection. Multiplexing of the reagents for the concurrent testing for three viral RNA species in the same reaction was associated with some reduced the sensitivity but did not affect the specificity of the assay. No cross-reaction was observed with the other closely related viruses, which indicated the high specificity of the assay. No amplification could be seen when non-template control was used. The intra-assay (repeatability) and the inter-assay (reproducibility) of SYBR Green real-time RT-PCR are shown in Table 2. The repeatability assay ranged from 0.34% to 0.36% and the range of the reproducibility was 0.34% to 0.36%. The mean coefficient of variation (CV) of intra-assay variation and inter-assay variation of the crossing points was < 0.5%. The mean CV of < 0.5% is considered acceptable (Ong *et al*., 2007).

Assessment of the efficacy of the SYBR Green real-time RT-PCR assays: SYBR Green real-time RT-PCR was validated with horse plasma spiked samples. Diluted series of horse plasma spiked samples were prepared for EIAV, JEV and WNV with four serial 10 fold dilutions to check the recovery of these viruses. The limit of detection of simplex, duplex and triplex SYBR Green real-time RT-PCR assays using horse plasma spiked samples was found to be 3.29X10^6copies/µl (Fig. 6).

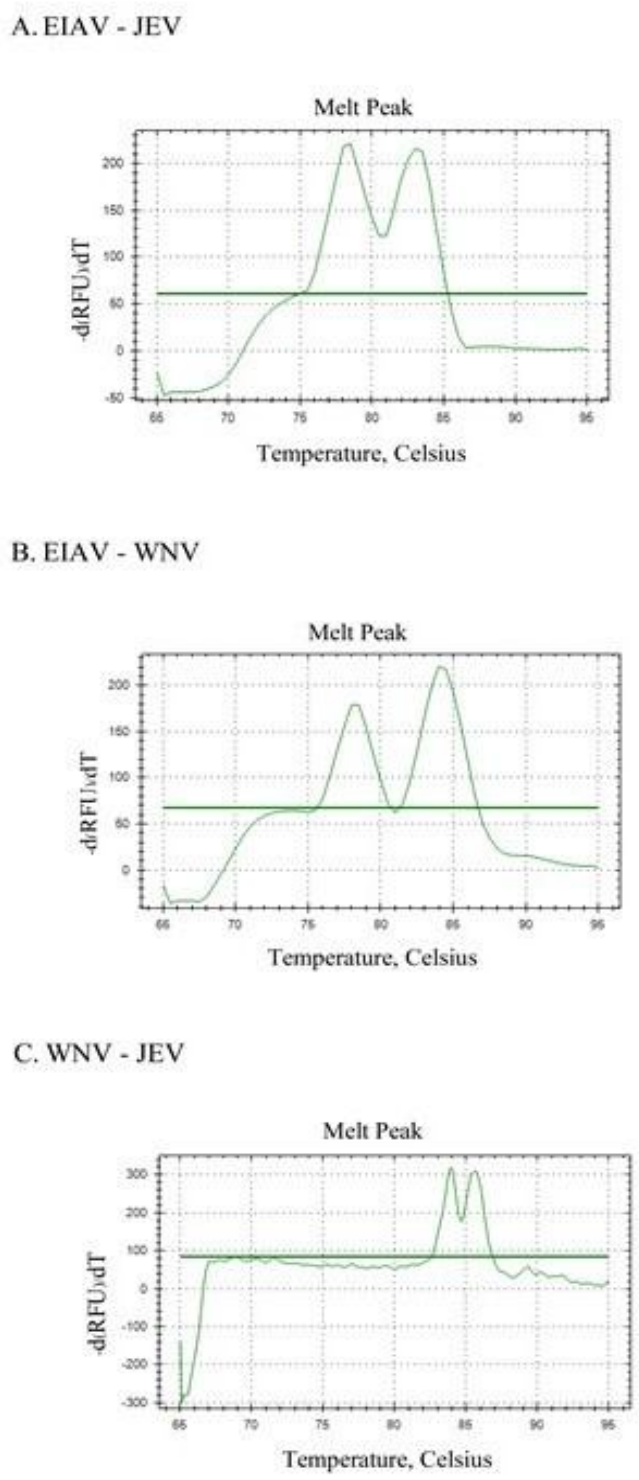

Figure 4 Melting peaks in the duplex SYBR Green real-time RT-PCR assay for each pair were well separated, indicating that they not only were able to detect EIAV (78.5±0.5°C), JEV (83.5±0.5°C) and WNV (85.0±1.0°C) but also had better compatibility in terms of similar annealing temperature and a low tendency of forming heterodimers.

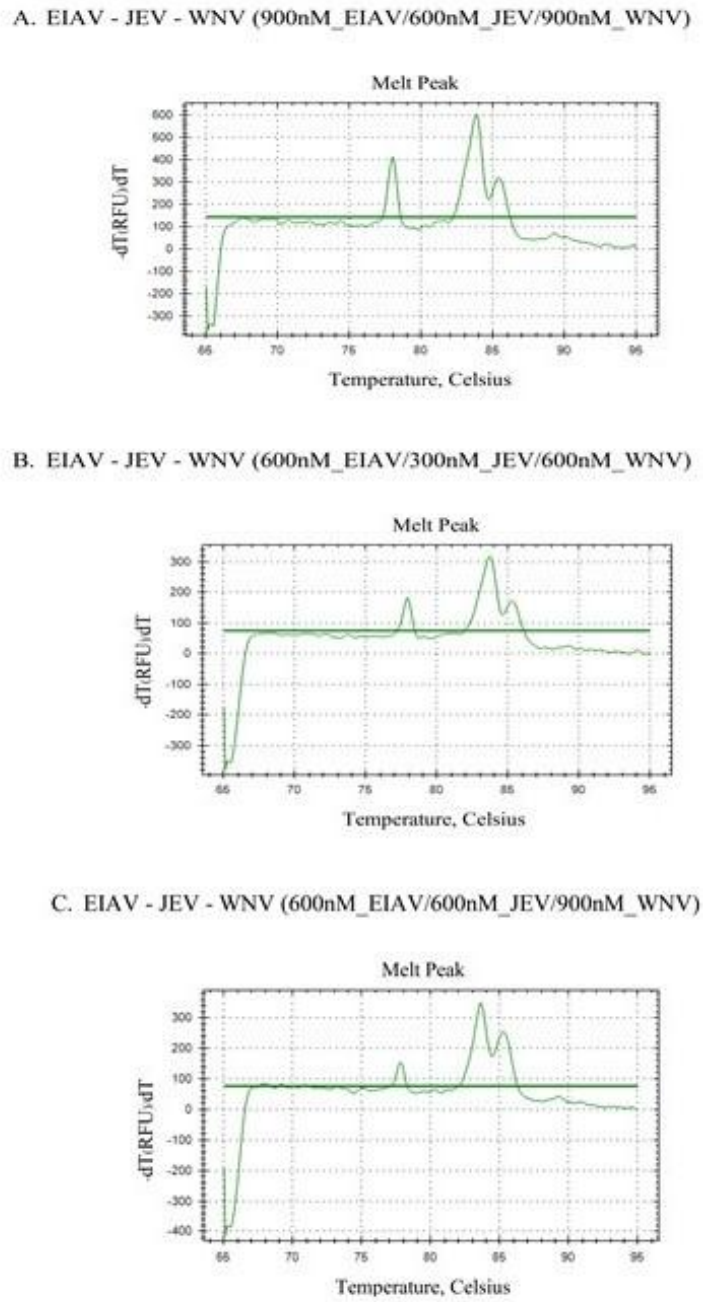

Figure 5 Melting curves in the triplex SYBR Green real-time RT-PCR for 3 viruses: EIAV (78.5±0.5°C), JEV (83.5±0.5°C), and WNV (85.0±1.0°C) were well separated in the different combinations of the primer concentrations: A. 900/600/900 nM B. 600/300/600 nM C. 600/600/900 nM, respectively.

Table 2 The intra-specificity and inter-specificity assays of the SYBR Green real-time RT-PCR assays to determine the repeatability within runs and the reproducibility between runs.

Intra-specific assay (Repeatability)				Inter-specific assay (Reproducibility)			
	Tm values of amplicon	Mean	CV%	Tm values of amplicon		Mean	CV%
JEV	83.00 83.00 83.50	83.17	0.34	JEV	83.00 83.50 83.50 83.00 83.00	83.20	0.33
EIAV	78.00 78.50 78.00	78.17	0.36	EIAV	78.00 78.50 78.00 78.50 78.00	78.20	0.35
WNV	84.50 84.50 85.10	84.70	0.41	WNV	84.50 84.50 85.10 85.20 85.20	84.90	0.43

CV% = (Standard deviation/mean) x 100; Tm: melting temperature

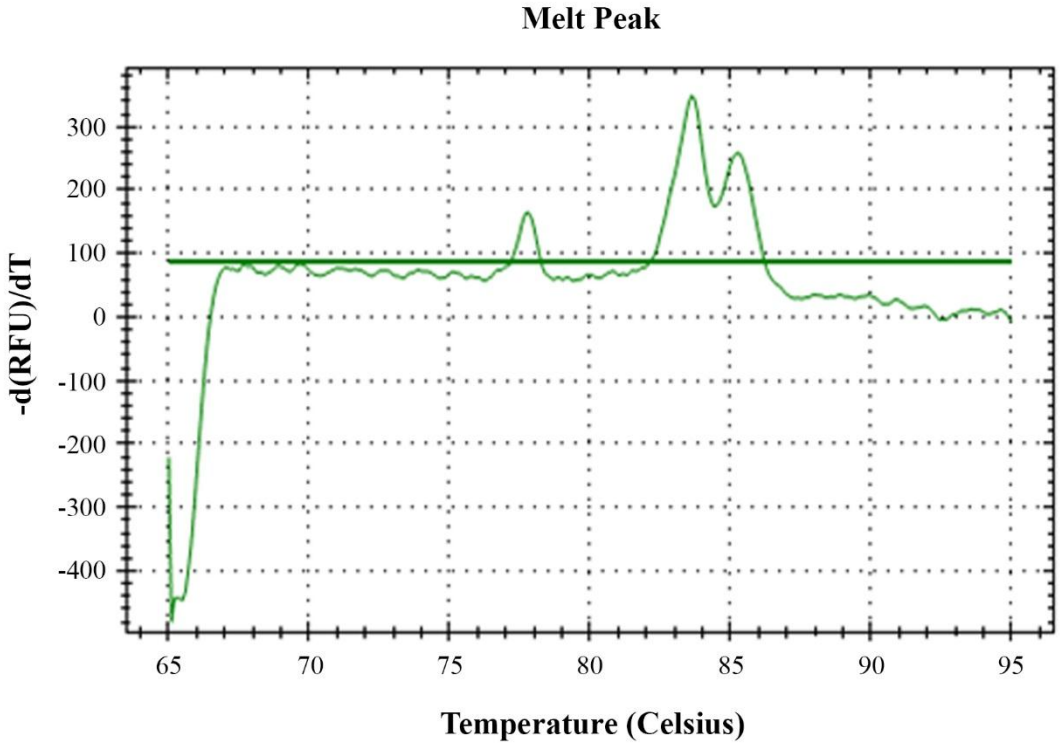

Figure 6 Horse plasma spike sample was detected using triplex SYBR Green real-time RT-PCR at the concentrations 3.29X10^{6}copies/µl.

Discussion

The most widely used, with real-time RT-PCR is SYBR Green I-based fluorescence method which can be applied directly to any gene without the need to design and synthesize fluorescent-labeled target-specific probes (Kleiboeker *et al*., 2004; Chao *et al*., 2007; Naze *et al*., 2009). The SYBR Green real-time RT-PCR method that binds preferentially to double-stranded DNA, has been widely used in the qPCR because it is cheaper than hydrolysis probes. It is simple in primer design and fewer steps are required in the protocol. It may be an excellent alternative for the detection of individual genes (Yong *et al*., 2007). The high number of sequences used in this study to select the primers provides an upgraded method for the detection of EIAV, JEV and WNV. It was presumed that the EIAV, JEV and WNV specific real-time RT-PCR primers designed will work with all strains of these viruses. Following amplification, a melting curve analysis was performed to verify the correct product by their specific melting temperature (Tm) (Parida, 2008; Brault *et al*., 2015). It has been found that the optimization of primer concentration is critical in preventing primer-dimer and nonspecific amplification of other unrelated gene products (Santhosh *et al*., 2007; Wan *et al*., 2016).

Melt curve analysis not only increased the size of the melt peaks but also affected the resolution of Tm's (Varga and James, 2006). A loss of peak resolution occurred with a ramp rate of 0.5°C/s when the Tm's of the two amplicons were close. This is related to the shift in Tm from low to high which is associated with a low to high shift in melt rate, as well as the machine's optics and/or software interpretation of fluorescence data. The delicate balance of the three melting peaks observed in this assay relied mainly on the precision of primer sets concentrations ratio and selection of dual annealing temperatures which required extensive optimization (Ong *et al*., 2007; Crignis *et al*., 2010). Selection of primer sets with Tm of at least 2°C apart also contributed significantly to the development of this assay A slower melt rate at 0.1°C/s melt rate, melt peaks were distinct giving an increased resolution of peaks. This is useful for improving the resolution of multiple targets such as EIAV, JEV, and WNV in a single reaction. However, actual Tm derived from SYBR Green melt curve analysis is dependent on many interacting factors such as GC content, primer length, primer melting, sequence composition, SYBR Green concentration as well as machine capabilities and melt run settings (Chen *et al*., 2015).

We developed a strategy to improve the Tm value of WNV amplicon by introducing 20-mer GC-rich oligonucleotide into 5' end of the specific primers for duplex and triplex SYBR Green real-time RT-PCR. Under the new primer sets, the melting curve-based multiplex SYBR Green RT-PCR assay can well detect and distinguish WNV from EIAV and JEV. However, the GC-modified WNV primers generated the amplicon with unstable Tm value, 85.0±1.0°C, in the range of 84.0°C to 86.0°C which was higher than obtained with the original WNV primer Tm 83.5°C. The variable SYBR Green pattern associated with melt curve analysis of multiple targets in multiplex reaction and may be caused by imperfect binding of the dyes to the DNA, especially the GC-rich content and length of a specific primer of WNV (Varga and James, 2006).

To improve the sensitivity of the multiplex system, the concentrations of primers were optimized. The assay was conducted with different primer concentrations from 300 nM to 900 nm (Kang *et al*., 2010; Simmons *et al*., 2016). The optimal conditions of primer concentration for duplex SYBR Green real-time RT-PCR came from different combinations between forward and reverse, at 600 or 900 nM for EIAV and WNV and 300 or 600 nM for JEV detection. Moreover, the triplex SYBR Green real-time RT-PCR for 3 viruses: EIAV, JEV and WNV were well separated in the different combinations of the primer concentrations, especially at 900/600/900, 600/300/600 and 600/600/900 nM. Nevertheless, it seems likely that duplex and triplex, SYBR Green real-time RT-PCR assays based clinical samples often have low sensitivity or even false-negative results caused by inhibition of RT-PCR in plasma samples. Thus, primer optimization, improved RNA/DNA extraction techniques and automated RNA/DNA extraction machines should be applied to increase the efficiency of RNA/DNA purification and sensitivity of real-time RT-PCR (Waggoner *et al*., 2018). The sensitivity and specificity of RT-PCR assays and simplex SYBR Green real-time RT-PCR assays for 3 viruses were similar to the results of previous findings. The lower limit of detection of these assays was less than 20 copies/µl. No cross-reactivity was observed between these viruses and other flaviviruses & retroviruses (Nagarajan and Simard, 2001; Cook *et al*., 2002; Quinlivan *et al*., 2007; Santhosh *et al*., 2007; Naze *et al*., 2009; Yeh *et al*., 2010; Vazquez *et al*., 2016). However, multiplex SYBR Green real-time RT-PCR assays for these viruses have never been reported.

In summary, the findings of the present study indicated that multiplex, duplex and triplex, SYBR Green real-time RT-PCR assays described here are relatively simple and utilize SYBR Green with a melting curve analysis of a single fragment of each virus with unique Tm. Three melting peaks indicated the presence of EIAV, JEV and WNV, 78.5±0.5C, 83.5±0.5°C and 85.0±1.0°C, respectively. Unstable or variable melting curve analysis of WNV could be associated with the GC-rich content and length of a specific primer of WNV modified for multiplex SYBR Green real-time RT-PCR. Melt rate affects target detection in melt curve analysis, with a slower ramp rate, 0.1°C/sec, producing a more reliable and improve Tm resolution. These assays improve the current diagnostic capability and can be easily implemented for screening a large number of samples in a rapid and sensitive way for the purpose of horse immunization for antiserum production.

Acknowledgements

This work was supported by a grant from the Thai Red Cross Society, Bangkok, Thailand under the operating expense of 2561. The CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, USA) was sponsored by the Chonkolneenithi Foundation. We thank the staff of Horse Farm & Laboratory-Animal Breeding Center, Queen Saovabha Memorial Institute, The Thai Red Cross Society for collecting samples. The authors declare no conflict of interest.

References

Akesowan S, and Suntrarachun S. 2007. Equine Infectious Anemia (EIA). J. Thai Vet. Pract. 19: 25-38.

Barros, S., Ramos, F., Ze-Ze, L., Alves, M. J., Fagulha, T., Duarte, M., Henriques, M., Luis, T. and Fevereiro, M. 2013. Simultaneous detection of West Nile and Japanese encephalitis virus RNA by duplex TaqMan RT-PCR. J. Virol. Methods. 193: 554-557.

Brault, A. C., Fang, Y. and Reisen, W. K. 2015. Multiplex qRT-PCR for the detection of western equine encephalomyelitis, St. Louis encephalitis, and West Nile viral RNA in mosquito pools (Diptera: culicidae). J. Med Entomol. 52: 491-499.

Chao, D. Y., Davis, B. S. and Chang, G. J. J. 2007. Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes. J. Clin. Microbiol. 45: 584-589.

Chen, H., Parimelalagan, M., Lai, Y. L., Lee, K. S., Koay, E. S-C., Hapuarachchi, H. C., Ng, L. C., Ho, P. S. and Chu, J. J. H. 2015. Development and evaluation of a SYBR green-based real-time multiplex RT-PCR assay for simultaneous detection and serotyping of dengue and chikungunya viruses. J. Mol. Diagn. 17: 722-728.

Cook, R. F., Cook, S. J., Li, F., Montelaro, R. C. and Issel, C. J. 2002. Development of a multiplex real-time reverse transcriptase-polymerase chain reaction for equine infectious anemia virus (EIAV). J. Virol. Methods 105: 171-179.

Crignis, E. D., Re, M. C., Cimatti, L., Zecchi, L. and Gibellini, D. 2010. HIV-1 and HCV detection in dried blood spots by SYBR green multiplex real-time RT-PCR. J. Virol. Methods 165: 51-56.

Kang, X., Li, Y., Liu, H., Lin, F., Cai, X., Sun, T., Chang, G., Zhu, Q. and Yang, Y. 2010. A duplex real-time reverse transcriptase polymerase chain reaction assay for detecting western equine and eastern equine encephalitis viruses. Virol. J. 7: 284.

Kleiboeker, S. B., Loiacono, C. M., Rottinghaus, A., Pue, H. L. and Johnson, G. C. 2004. Diagnosis of West Nile virus infection in horses. J. Vet Diagn. Invest. 16: 2-10.

Nagarajan, M. M. and Simard, C. 2001. Detection of horses infected naturally with equine infectious anemia virus by nested polymerase chain reaction. J. Virol. Methods 94: 97-109.

Naze, F., Roux, K. L., Schuffenecker, I., Zeller, H., Staikowsky, F., Grivard, P., Michault, A. and Laurent, P. 2009. Simultaneous detection and quantitation of Chikungunya, Dengue virus typing using high resolution melting. J. Virol. Methods 162: 1-7.

Ong, W. T., Omar, A. R., Ideris, A. and Hassan, S. S. 2007. Development of a multiplex real-time PCR assay using SYBR green 1 chemistry for simultaneous detection and subtyping of H9N2 influenza virus type A. J. Virol. Methods 144: 57-64.

Parida, M. M. 2008. Rapid and real-time detection technologies for emerging viruses of biomedical importance. J. Biosci. 33: 617-628.

Quinlivan, M., Cook, R. F. and Cullinane, A. 2007. Real-time quantitation RT-PCR and PCR assays for a novel European field isolate of equine infectious anemia virus based on sequence determination of the *gag* gene. Vet. Rec 160: 611-618.

Rodriguez-Sanchez, B., Fernandez-Pinero, J., Sailleau, C., Zientara, S., Belak, S., Arias, M. and Sanchez-Vizcaino, J. M. 2008. Novel gel-based and real-time PCR assays for the improved detection of African horse sickness virus. J. Virol Methods 151: 87-94.

Santhosh, S. R., Parida, M. M., Dash, P. K., Pateriya, A., Pattnaik, B., Pradhan, H. K., Tripathi, N. K., Ambuj, S., Gupta, N., Saxena, P. and Lakshmana Rao, P. V. 2007. Development and evaluation of SYBR Green I-based one step real-time RT-PCR assay for detection and quantitation of Japanese encephalitis virus. J. Virol methods. 143: 73-80.

Shirato, K., Miyoshi, H., Kariwa, H. and Takashima, I. 2005. Detection of west nile virus and Japanese encephalitis virus using real-time PCR with a probe common to both viruses. J. Virol methods. 126: 119-125.

Shirato, K., Mizutani, T., Kariwa, H. and Takashima, I. 2003. Discrimination of West Nile virus and Japanese encephalitis virus strains using RT-PCR RFLP analysis. Microbiol Immunol 47: 439-445.

Simmons, M., Myers, T., Guevara, C., Jungkind, D., Williams, M. and Houng, H. S. 2016. Development and validation of a quantitative, one-step, multiplex, real-time reverse transcriptase PCR assay for detection of dengue and chikungunya viruses. 54: 1766-1773.

Spyrou, V., Papanastassopoulou, M., Psychas, V., Billinis, Ch., Koumbati, M., Vlemmas, J. and Koptopoulos, G. 2003. Equine infectious anemia in mules: virus isolation and pathogenicity studies. Vet. Microb. 95: 49-59.

Suntrarachun, S., Akesowan, S., Tirawatnapong, T. and Khow, O. 2010. Detection of Equine Infectious Anemia viral nucleic acid in asymptomatic carrier horses in Thailand by nested PCR. Asian Biomed. 4: 1-5.

Varga, A. and James, D. 2006. Real-time RT-PCR and SYBR green I melting curve analysis for the identification of Plum pox virus strains C, EA, and W: Effect of amplicon size, melt rate, and dye translocation. 2006. J. Virol. Methods. 132: 146-153.

Vazquez, A., Herrero, L., Negredo, A. and Hernandez, L. 2016. Real time PCR assay for detection of all known lineages of west nile. J. Virol. Methods. 236: 266-270.

Waggoner, J. J., Rojas, A., Mohamed-Hadley, A., de Guillen, Y. A. and Pinsky, B. A. 2018. Real-time RT-PCR for Mayaro virus detection in plasma and urine. J. Clin. Virol. 98: 1-4.

Wan, Z., Zhang, Y. N., He, Z., Liu, J., Lan, K., Hu, Y. and Zhang, C. 2016. A melting curve-based multiplex RT-qPCR assay for simultaneous detection of four human coronaviruses. Int. J. Mol. Sci. 17: 1880.

Yeh, J. Y., Lee, J. H., Seo, H. J., Park, J. Y., Moon, J. S., Cho, I. S., Lee, J. B., Park, S. Y., Song, C. S. and Choi, I. S. 2010. Fast duplex one-step reverse transcriptase PCR for rapid differential detection of West Nile and Japanese encephalitis viruses. J. Clin. Micro. 48: 4010-4014.

Yong, Y. K., Thayan, R., Chong, H. T., Tan, C. T. and Sekaran, S. D. 2007. Rapid detection and serotyping of dengue virus by multiplex RT-PCR and real-time SYBR green RT-PCR. Singapore Med J. 48: 662-668.