

Sensitive and Specific Detection of Strains of Japanese Encephalitis Virus Using a One-Step TaqMan RT-PCR Technique

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A rapid, sensitive, and accurate laboratory diagnostic test is needed for distinguishing Japanese encephalitis virus (JEV) from other diseases featuring similar clinical symptoms and also for preventing potential outbreaks. In this study, a TaqMan reverse transcription (RT)-polymerase chain reaction (PCR) assay was developed for rapid detection and quantification of the viral RNA of various JEV strains. A consensus JEV NS3 region was chosen to design the primers and the TaqMan probe. The JEV TaqMan assay used the EZ-*rTth* RT-PCR system featuring advantages such as a one-step, high-temperature RT reaction modality and preventing carry-over contamination. The sensitivity of the JEV TaqMan assay for detecting in vitro-transcribed JEV NS3 RNA was estimated to be one to five copies of RNA per reaction. For cultured JE virions, less than 40 plaque forming unit (PFU)/ml of virus load (corresponding to 0.07 PFU/test) could be detected. In addition, the JEV TaqMan assay could detect all seven strains of JEV tested, but provided negative results for nine other flaviviruses and encephalitis viruses tested. The JEV TaqMan assay demonstrated greater sensitivity and specificity than traditional RT-PCR methods as has been previously reported. The application of the JEV TaqMan assay herein has been shown to the sensitive detection of the JEV from both mosquito pools and also JEV-spiking human blood. The assay should be of use in diagnostic laboratory conduct and could be used to replace or complement time-consuming viral-culture methods, thus achieving more rapid, sensitive, and highly specific identification of JEV infection. **J. Med. Virol.** 74:589–596, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: JEV; NS3; TaqMan assay; real-time PCR; encephalitis; flavivirus

INTRODUCTION

Japanese encephalitis virus (JEV) is a member of the family Flaviviridae, genus *Flavivirus*, and its transmission to humans has frequently been reported to result in severe encephalitic and neurological disease manifestation [Burke and Leake, 1988]. The virus circulates in natural transmission cycles involving primarily *Culex tritaeniorhynchus* mosquitoes and swine, with human beings as incidental hosts [Rosen, 1987]. The virus is epidemic in many parts of India, China, and South-east Asia [Solomon et al., 2000]. During epidemic periods, fatality rates as high as 20% have been noted, particularly amongst the immunologically naive and those at the extremes of the age spectrum [Tsai, 2000]. Numerically, it is the most important cause of epidemic encephalitis; its geographical area of influence is expanding despite the availability of vaccines [Solomon and Mallewa, 2001].

The flaviviruses possess a single-stranded plus-sense RNA genome of approximately 11,000 nucleotides [Sumiyoshi et al., 1987]. This 11 kb molecule comprises 5'- and 3'-untranslated regions (UTRs), between which lies a single open reading frame carrying genes for three structural proteins (capsid [C], membrane [M], and envelope [E]) and seven nonstructural (NS) proteins [Chambers et al., 1990]. The JEV serocomplex includes JEV, St. Louis encephalitis (SLE) virus, West Nile virus, and Murray Valley encephalitis virus [Murphy et al.,

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1995]. With limited nucleotide sequencing, five genotypes of JEV have been identified [Chen et al., 1992; Uchil and Satchidanandam, 2001] and 18 strains have been sequenced fully [Solomon et al., 2003]. Amongst these 18 strains, 14 strains are Genotype III, the most frequently isolated and widely distributed strains of JEV. Such strains include isolates from Japan, China, Taiwan, the Philippines, Indonesia, Malaysia, Thailand, Cambodia, Vietnam, India, Sri Lanka, and Nepal [Ni et al., 1994]. Other genotypes are isolated relatively rarely, especially Genotypes IV and V [Solomon et al., 2003].

In South-east Asia, outbreaks of severe febrile encephalitis associated with human fatality and symptoms similar to the symptoms of JEV have been reported previously [Chua et al., 1999]. Nipah virus has been identified as the etiological agent of an outbreak of severe encephalitis in Malaysia in 1998 [Chua et al., 2000]. Enterovirus 71 was the cause of an outbreak of encephalitis causing rapid clinical deterioration and death amongst young children in Taiwan since 1998 [Wang et al., 2002]. For these reasons, the development of a rapid, sensitive, and specific diagnostic method for accurate identification of JEV strain(s) infection is important for preventing of the potential outbreak of Japanese encephalitis.

Currently, the diagnosis of JEV infection is made by detection of specific antibodies or virus isolation in animals and mosquito cells. Various serological tests have been used routinely for the diagnosis of JE viral infection including the hemagglutination inhibition (HAI) test, the plaque reduction neutralization test (PRNT), and the IgM antibodies capture ELISA (MAC-ELISA) assay. It is noteworthy, however, that serological methods reflect inherently limited levels of sensitivity and specificity and may not diagnose accurately infection at a sufficiently early stage for optimal disease management. JEV can be isolated frequently from the blood of affected patients during the early phase of acute encephalitis illness, when IgM antibodies may not be detectable [Monath and Heinz, 1996]. However, virus isolation and identification with a JEV specific monoclonal antibody requires the use of cell-culture assays that are technique-dependent and time-consuming [Gubler et al., 1984]. The development of a more rapid diagnostic assay for JEV detection with high sensitivity and specificity will be useful for the management and treatment of infected patients and for epidemiological surveillance.

Molecular diagnostic systems using reverse transcriptase-PCR (RT-PCR) based techniques for detecting viral RNA have been shown to be more rapid assays than cell culture-based techniques and are highly effective for diagnosing infection of RNA viruses [Lanciotti et al., 1992]. A number of studies using RT-PCR employ a time-consuming two-step nested amplification approach to achieve increased sensitivity for the detection of the JEV [Murakami et al., 1994; Sun et al., 2000]. Such a method, however, also increases the likelihood of false-positive reactions due to carry-over contamination

of PCR products in the laboratory [Henke et al., 1997]. Several diagnostic assays using fluorescent DNA probes in a 5' exonuclease (TaqMan) assay [Lyamichev et al., 1993; Livak et al., 1995] have been developed for a variety of pathogens [Martell et al., 1999; Lanciotti et al., 2000; van Elden et al., 2001; Warrilow et al., 2002; Dosten, 2003]. These TaqMan detection assays offer the advantage over traditional RT-PCR techniques of increased sensitivity, specificity, decreased contamination, and absolute quantification [Bustin, 2000].

A one-step TaqMan-based quantifiable real-time RT-PCR technique is described for rapid, sensitive, and specific detection of virtually all strains of the JEV from different tissue samples. This JE-NS3 TaqMan RT-PCR technique is more sensitive for the detection of JEV than the conventional RT-PCR assays, it can detect a broad range of different JE strains and distinguish these strains from a host of other flaviviruses and encephalitis viruses. Further, this assay can generate results within 3 hr, and does not require a time-consuming sample set-up, a secondary PCR amplification step, or any post-PCR gels. The closed-tube detection technique and the AmpErase uracil-*N*-glycosylase (UNG) treatment process eliminate the potential sources of carry-over contamination and reduce the false-positive rate associated with more conventional RT-PCR techniques [Kwok and Higuchi, 1989]. The assay also provides a quantitative viral RNA assay result simultaneously. The development of this assay provides a powerful diagnostic tool for the identification of JEV infection for subsequent patient management and rapid vector control.

MATERIALS AND METHODS

Virus Stocks and Plaque Assay

All viruses including all strains of the JEV tested were obtained from the virus bank maintained at the Institute of Preventive Medicine, National Defense Medical Center, Taipei, Taiwan. JEV strains were propagated in mosquito cells (C6/36) incubated in RPMI 1640 medium containing 2% FCS at 28°C for 5–7 days. The culture fluids were clarified by centrifugation and stored at –70°C. The viruses used were titrated in BHK-21 cells by standard plaque assay [Beatty et al., 1989].

Viral RNA Isolation and In Vitro Transcription

The culture fluids of JEV strains were diluted 10-fold with PBS. Viral RNAs were extracted from 140 µl of different diluents by using the QIAamp viral RNA kit (Qiagen, Valencia, CA) and eluted from the Qiagen columns in a final volume of 100 µl of elution buffer and then stored at –70°C in aliquoted. For preparation of positive JEV RNA controls, the plasmid pGEM/NS3 containing 1.5 kb NS3 fragment of JEV NT113 strain was purified by QIAprep Spin Miniprep kit (Qiagen) and then linearized by restriction enzyme *Sal* I, and transcribed in vitro using T7 RNA polymerase (T7 transcription kit, Promega, Madison, WI). The RNA transcripts

were treated with RNase-free DNase and extracted with phenol/chloroform, and then precipitated by ethanol. Subsequent to further purification with the RNeasy mini kit (Qiagen), the RNA was eluted in DEPC-treated water, and the concentration was determined by spectrophotometric reading. The RNAs were 10-fold diluted from 10^{12} to 10^0 copies in DEPC-treated water containing 20 $\mu\text{g/ml}$ of carrier RNA (poly[A]; Roche Molecular Biochemicals, Mannheim, Germany) and stored at -70°C until used.

One-Step RT-PCR and TaqMan EZ-*rTth* RT-PCR Assays

The one-step RT-PCR was carried out with the OminiscriptTM reverse transcriptase and SensiscriptTM reverse transcriptase one-step RT-PCR kit (Qiagen) by using 2 μl of RNA and 600 nM of each primer in a 50 μl total reaction volume by following the manufacturer's protocol with the following cycling times and temperatures: one cycle of 50°C for 30 min and 94°C for 15 min and 40 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min and a final 72°C for 10 min. After the RT-PCR procedure had been completed, a 10 μl product was analyzed by agarose gel electrophoresis on a 3% agarose 1000 gel (Gibco-BRL, Rockville, MD) and visualized under UV light following ethidium bromide staining.

For the TaqMan EZ-*rTth* assay, 2 μl of RNA were combined with 900 nM of each primer and 200 nM of the FAM- and TAMAR-labeled probe in a total reaction volume of 25 μl by using a single-tube, single-enzyme-*rTth* system, TaqMan EZ-*rTth* RT-PCR Kit (Applied Biosystems, Foster City, CA). RT-PCR amplification began with an initial of 50°C for 2 min, then 60°C for 30 min followed by denaturation at 95°C for 5 min, and then 45 cycles of 94°C for 20 sec and 62°C for 1 min. Each sample and non-template control (NTC) was tested in triplicate. The ABI Prism 7700 Sequence Detection System instrument (Applied Biosystems) was used to analyze the emitted fluorescence during RNA amplification. The complete procedure of TaqMan EZ-*rTth* RT-PCR lasts about 2½ hr. The RT-PCR cycle number at which fluorescence increases above an inter-assay-calibrated threshold value is defined as threshold cycle number (C_T). The C_T value for the NTC was 45.

Detection of JEV in Mosquito Pools and Human Sera

To evaluate the sensitivity of the TaqMan assays for mosquito-pool specimens, dilution of a JEV-positive mosquito pool and negative control pool were prepared. Twenty female *Aedes albopictus* mosquitoes were intrathoracically inoculated with 0.1 μl of JEV NT113 as previously described [Weng et al., 1997]. Three mosquitoes from the inoculated group were immediately frozen at -70°C to serve as the inoculated control, whilst the remaining were incubated at 28°C for 6 days. Fifty *Aedes albopictus* were used as the negative-control pool. Pools of mosquitoes were placed in polypropylene, round-bottom tubes and were ground with a glass bar.

An adequate volume of RPMI 1640 medium was added and the homogenates subjected to filtration and centrifugation, and subsequently, using the negative mosquito homogenate as a diluent, serial 10-fold dilutions of the virus-positive mosquito suspension were prepared. The specimens obtained from these mosquito pools were tested for virus presence by using a plaque assay, a RT-PCR, and a TaqMan RT-PCR technique.

In order to evaluate the sensitivity of the TaqMan assays for human sera specimens, serial 10-fold dilutions of JEV were used for the "spiking" of six individual human bloods. These samples were used so as to mimic blood samples corresponding to viremia stages from 4×10^6 , 4×10^4 , 4×10^2 to 4×10^0 virions/ml blood. These "viremia" serum specimens were incubated at room temperature for 1 hr followed by centrifugation 8,000 rpm for 10 min. Viral RNAs were then isolated from these specimens and analyzed by TaqMan RT-PCR. The human blood samples were simultaneously assayed to detect for the presence of JEV-specific neutralization antibody, as measured by the plaque-reduction neutralization assay [Lin et al., 1996].

RESULTS

JEV NS3 TaqMan Primer/Probe Design

To design the TaqMan assay that can detect most strains of the JEV, the NS3 sequences from several fully sequenced JEV strains were aligned by DNASTAR Lasergene software (Windows 3.08, DNA Star, Madison, WI). NS3 is a highly conserved region among flaviviruses [Westaway and Blok, 1997], and plays an important role in viral RNA replication [Utama et al., 2000]. To avoid the cross detection with other flaviviruses, we did not choose the more conserved regions-NS5, which are the target regions for performing flavivirus-common molecular detection [Scaramozzino et al., 2001]. The relatively highly conserved structure and function of the NS3 makes it to be a logical region for developing a consensus PCR assay which could be used for detection of most JE strains. The JEV NS3 primers and TaqMan probe were designed to match the TaqMan condition using the Primer Express Oligo Design software (Version 1.5, Applied Biosystems). The optimal primers and probe set which were suitable for most JEV strains were thus selected (Table I). The sequences of primer and probe were checked by Blast web service (National Center for Biotechnology Information) to assure no non-specific detection with other host and/or pathogen genes. According to a computer search, the sequences of JEV NS3 TaqMan primers and probe could be annealed perfectly with the sequences of many strains of JEV.

Sensitivity of JEV NS3 TaqMan Assay

The sensitivity of the JEV NS3 TaqMan assay was first evaluated by testing 10-fold dilutions of JEV NT-113 culture that had been quantitated previously for viral presence by plaque titration. The JEV NS3 TaqMan primer-probe combination can detect 40 PFU

TABLE I. Oligonucleotide Primers and Probe Used in the JEV TaqMan Assay

Primer/probe ^a	Sequence (5'–3')	Location	T _m (°C)
JENS3F	AGAGCACCAAGGGAATGAAATAGT	5357–5380	58
JENS3R	AATAGGTTGTAGTTGGGCACTCTG	5452–5427	59
JENS3probe	^{FAM} CCACGCCACTCGACCCATAGACTG ^{TAMRA}	5393–5417	67

^aThe primers and probe were 100% identity with JE strains, CH2195LA, JaOArS982, SA14, CH1392, RP-9, JaGAR, HVI, TC, SA(V), SA(A), SA14-12-1-7, CH2195SA, RP-2, SA14-14-2, 99–96% identity with GP78, Ling, P20778, Beijing, P3 Strains and 87% identity with Fu (genotype2), Ishikawa, K94P05 (genotype1).

per ml of virus culture, corresponding to 0.07 PFU per reaction (Fig. 1A; Table II). The relative sensitivity of the JEV NS3 TaqMan assay proved to be even more substantial than the one-step RT-PCR assay using the most sensitive sensiscript RT (Table II). The relative sensitivities of the two assays were also compared by detecting the *in vitro*-transcribed JEV NS3 RNA (Fig. 2A) which could serve as the standard positive control. The JEV NS3 TaqMan assay yielded a 100% detection rate for five copies of NS3 RNA and a 66.7% detection rate for one copy (Figs. 1B and 2B; Table II), while the one-step RT-PCR assay revealed approximately a five-to-ten-fold lower sensitivity (Fig. 2B; Table II). The traditional nested RT-PCR primer assays [Murakami et al., 1994] were also used to detect these samples in order to compare with these one-step JEV NS3 RT-PCR and JEV NS3 TaqMan RT-PCR assays. The sensitivity of this nested PCR technique did not differ greatly from that of the JEV NS3 TaqMan RT-PCR assay; however, the nested PCR technique is relatively complex, time-consuming, and frequently, difficult for interpretation of results (data not shown).

Specificity of JEV NS3 TaqMan Assay

The JEV NS3 TaqMan assay was designed for specific detection of JEV strains and for distinguishing such strains from other viruses. To evaluate the specificity of the JEV NS3 TaqMan assay, viral culture supernatants of seven different strains of JEV (JaGAR01; HVI; TL; TC; Nakayama; NT109; RP-9) were collected and subjected them to analysis using the JEV NS3 TaqMan assay. The data revealed that the JEV NS3 primers and TaqMan probe were sufficiently sensitive to detecting all JE strains tested. The relative sensitivity of the assay to

most strains of JEV appeared to be quite similar to that of the NT 113 strain (Table III), although the sensitivity of the assay for detecting the JE NaKayama strain appeared to be about 10-fold less than for the other strains. The detection rate for the NaKayama strain was 100% for 10 PFU per reaction, however.

In addition, the JE NS3 primer and probe could be used successfully to distinguish different viral RNA extracted from JEV and two serotypes of dengue viruses (DV1, DV2) which are also mosquito-borne flavivirus known to circulate in Southern Asia (Fig. 3). The primer/probe set was also tested for specificity with six serologically related flavivirus samples (Yellow fever virus, Saint Louis Encephalitis virus, West Nile virus, Murray Valley Encephalitis virus, DV 1 and DV 2), and three arthropod-borne viruses (Eastern Equine Encephalitis virus, Western Equine Encephalitis virus, Venezuelan Equine Encephalitis virus) that induced viral encephalitis similar to the JEV. The C_T value for each of these viruses was 45, i.e., the same as the value for the no-template control (Table III). No fluorescent signal was generated with any of the flaviviruses or encephalitis viruses tested. Such results clearly demonstrate that the JEV NS3 TaqMan assay with highly specificity to detect various strains of JEV, but not other viruses.

Detection of JEV RNA From Mosquito Extracts

To evaluate the sensitivity and application of the JEV NS3 TaqMan assay for virus-infected mosquitoes, mosquitoes were inoculated intrathoracically with JEV. After incubation at 28°C for a period of 6 days, the mosquito-pool specimens were homogenated and tested for the presence of JEV RNA by plaque titration,

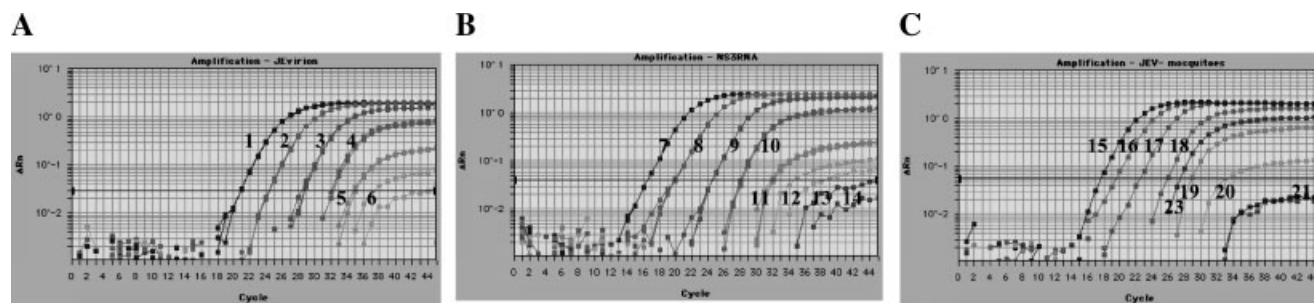


Fig. 1. Amplification plots of Japanese encephalitis virus (JEV) NS3 TaqMan RT-PCR for detecting the RNAs from cultured JEV NT113 virions (A), *in vitro*-transcribed JEV NS3 RNA (B), JEV-inoculated mosquitoes (C). The numbers marked in A, B, and C correspond to the sample no. type, quantity and results listed in Table II.

TABLE II. Sensitivity Test of JEV NS3 TaqMan RT-PCR for Different Specimens

Sample no.	Type	Quantity plaque of RNA per test		TaqMan RT-PCR		
				C _T ^a	Int ^b	RT-PCR
JE virions						
1	10 ⁻¹ dilution	7,000	PFU	20.7	Pos.	Pos.
2	10 ⁻² dilution	700	PFU	24.3	Pos.	Pos.
3	10 ⁻³ dilution	70	PFU	28.5	Pos.	Pos.
4	10 ⁻⁴ dilution	7	PFU	32.0	Pos.	Pos.
5	10 ⁻⁵ dilution	0.7	PFU	35.1	Pos.	Pos.
6	10 ⁻⁶ dilution	0.07	PFU	40.7	Pos./Neg. ^c	Neg.
NS3 RNA						
7	10 ⁻⁶ dilution	10 ⁶	Copies	18.2	Pos.	Pos.
8	10 ⁻⁷ dilution	10 ⁵	Copies	21.7	Pos.	Pos.
9	10 ⁻⁸ dilution	10 ⁴	Copies	25.1	Pos.	Pos.
10	10 ⁻⁹ dilution	10 ³	Copies	28.4	Pos.	Pos.
11	10 ⁻¹⁰ dilution	10 ²	Copies	31.8	Pos.	Pos.
12	10 ⁻¹¹ dilution	10 ¹	Copies	34.2	Pos.	Pos.
13	5 × 10 ⁻¹² dilution	5	Copies	36.3	Pos.	Neg.
14	10 ⁻¹² dilution	1	Copy	43.5	Pos./Neg.	Neg.
JEV inoculated mosquitoes, 28°C incubation for 6 days						
15	Undilution	154,000	PFU	17.9	Pos.	Pos.
16	10 ⁻¹ dilution	25,900	PFU	19.8	Pos.	Pos.
17	10 ⁻² dilution	2,275	PFU	22.7	Pos.	Pos.
18	10 ⁻³ dilution	315	PFU	26.1	Pos.	Pos.
19	10 ⁻⁴ dilution	26.25	PFU	29.1	Pos.	Neg.
20	10 ⁻⁵ dilution	1.13	PFU	33.1	Pos.	Neg.
21	10 ⁻⁶ dilution	0.131	PFU	45.0	Neg.	Neg.
22	10 ⁻⁷ dilution	0	PFU	45.0	Neg.	Neg.
23	Non-incubation	45.5	PFU	27.6	Pos.	Pos.

^aThe C_T values presented are the means of C_T values from duplicate intra-assays.

^bThe interpretation (Int) of TaqMan RT-PCR assay is made according to the CT value, ΔRn and multicomponent. The C_T value <45, ΔRn >0 and an increased multicomponent value can be interpreted as a positive (Pos.) result.

^cNeg., negative result.

the TaqMan assay, and the one-step RT-PCR assay described above. The JEV NS3 TaqMan assay proved to be an appropriate RNA-detection technique that was able to provide rapid, sensitive, and quantifiable results. This assay system could detect as little as one JE virion

in the mosquito specimen (Figs. 1C and 2C; Table II). The quantitative data appeared to correlate well with the corresponding data derived from the plaque assay, which is a laborious and time-consuming technique revealing a fairly broad standard deviation for grouped

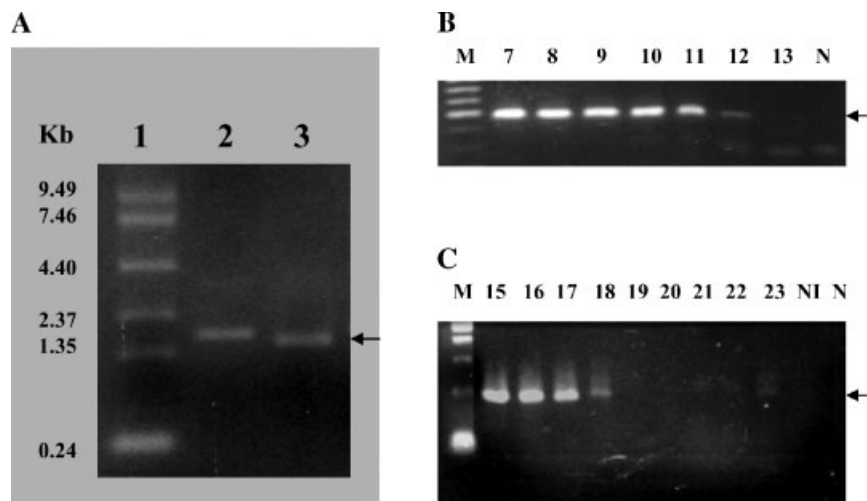


Fig. 2. **A:** Denatured gel electrophoresis of in vitro-transcribed JEV NS3 RNA. **Lane 1:** RNA molecular-weight marker; **lane 2:** 1,800 bases in vitro-transcribed control RNA; **lane 3:** 1,561 bases JEV NS3 RNA. **B:** One-step RT-PCR analysis of 10-fold serial diluted JEV NS3 RNA. **Lane M** (Marker): DNA molecular-weight marker; **lanes 7–13** correspond to the samples listed in Figure 1B and Table II; **lane N**

(negative): non-template control for TaqMan assay. **C:** RT-PCR analysis of RNA derived from JEV-inoculated mosquitoes. **Lane M:** DNA molecular-weight marker; **lanes 15–22** correspond to the samples listed in Figure 1C and Table II; **lane 23:** JEV-inoculated but non-incubated mosquitoes that serve as inoculated control; **lane N:** non-inoculated mosquitoes control; **lane N:** non-template control.

TABLE III. Specificity Test of the JEV NS3 TaqMan RT-PCR Assay for Different JEV Strains and Viruses

JEV strains ^a	Quantity	C _T	JEV strains ^a	Quantity	C _T	Other viruses ^b	Quantity ^c	C _T
JaGAR01	2,567 PFU	18.9	HVI	3,888 PFU	18.1	YFV	N.D.	45
	257 PFU	22.3		389 PFU	21.4	SLEV	N.D.	45
	26 PFU	25.7		39 PFU	24.8	WNV	N.D.	45
TL	7,000 PFU	17.7	TC	4,239 PFU	18.4	MVEV	N.D.	45
	700 PFU	21.1		424 PFU	21.8	DV1	N.D.	45
	70 PFU	24.4		42 PFU	25.2	DV2	N.D.	45
Nakayama	10,888 PFU	22.5	NT109	22,167 PFU	17.9	EEEV	N.D.	45
	1,089 PFU	25.5		2,217 PFU	21.2	WEEV	N.D.	45
	109 PFU	28.6		222 PFU	24.5	VEEV	N.D.	45
	11 PFU	34.4		22 PFU	27.3			

^aGenBank accession number: JaGAR01 (AF069076); HVI (AF098735), TL (AF098737), TC (AF098736), Nakayama (S75726), NT109 (parent strain of RP9), RP-9 (AF014161, data not shown).

^bYFV, Yellow fever virus; SLEV, Saint Louis Encephalitis virus; WNV, West Nile Virus; MVEV, Murray Valley Encephalitis Virus; DV1, Dengue virus serotype1; DV2, Dengue virus serotype2; EEEV, East Equine Encephalitis Virus; WEEV, West Equine Encephalitis Virus; VEEV, Venezuelan Equine Encephalitis Virus.

^cThe virus titer for most of these viruses has not been well quantified before JEV NS3 TaqMan assay; however, these viral RNAs have been checked by their virus specific primer pairs as described [Dennis et al., 1992; Linssen et al., 2000]. All of these viruses have clear and distinct bands of RT-PCR product with expected sizes.

results (Table II). Further, it proved to be somewhat difficult to derive clear results following the application of the one-step RT-PCR assay, it being approximately 100-fold less sensitive than the TaqMan assay for detecting the presence of the JEV in mosquito pools (Fig. 2C; Table II). The process of testing the mosquito specimens for the presence of JEV revealed dramatic differences in convenience, sensitivity, and accuracy between the TaqMan RT-PCR and traditional RT-PCR assays.

Detection of JEV RNA From Human Specimens

To evaluate the sensitivity and application of the JEV NS3 TaqMan assay for human sera specimens, six human blood samples (three JEV antibody positive and three negative) were spiked with different quantities of culture-derived JE virions, viral RNA being isolated

from the blood samples and analyzed using the JEV NS3 TaqMan RT-PCR technique. The detection rates for 4×10^2 PFU/ml viremia samples were 100%, which corresponded to 4.48 virions/test (Fig. 4). The detection rates for 4×10^1 PFU/ml (data not shown) and 4×10^0 PFU/ml (Fig. 4) viremia samples were 87.5 and 58.3%, respectively. The sensitivity and detection rate did not appear to have been influenced by the presence/absence of the JE antibody in serum. Therefore, the potential application and the relative sensitivity of the JEV NS3 TaqMan assay for detecting the presence of JE virions in human sera matched the results for cultured JE virions and purified, in vitro-transcribed JE RNA. Thus, the rapid and highly specific characterization of JEV NS3 TaqMan assay makes it an excellent alternative to the conventional two-step nested RT-PCR for diagnosis of infection by various strains of the JEV.

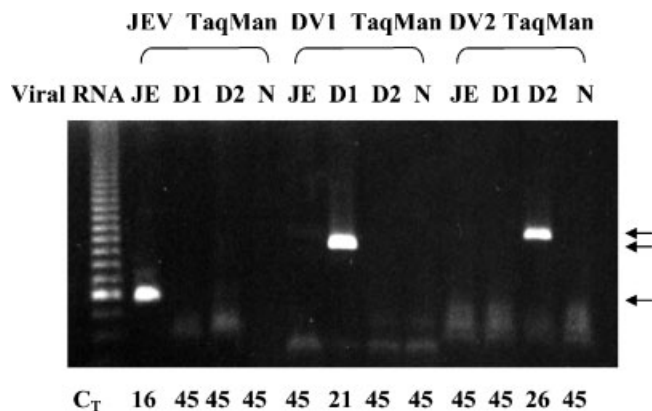


Fig. 3. Differential diagnosis of JEV and DV. JEV, DV1, and DV2 viral RNAs were analyzed by the JEV NS3 TaqMan RT-PCR assay described herein, and DV TaqMan RT-PCR described by Laue et al. [1999]. The TaqMan RT-PCR products were analyzed by gel electrophoresis and the C_T values derived from real-time amplification plots were scored below.

DISCUSSION

A rapid, highly sensitive, and highly specific technique was developed for the detection of all JE strains tested. This technique can be applied to both mosquito and human specimens for the detection of the presence of JEV viral RNA. To the best of our knowledge, this report is the first published paper pertaining to the application of real-time RT-PCR for the detection of many JEV strains.

Accurate diagnosis of the responsible pathogen of suspected encephalitis infection is important for optimal patient management; however, it is difficult to distinguish encephalitis caused by JEV from other viral infections such as enterovirus, Nipah virus, HSV, and mump virus according to clinical symptoms. Clearly, appropriate identification of the infecting pathogen must rely upon laboratory diagnosis. The average time required for the laboratory confirmation of JEV is approximately 7 days in Taiwan. A rapid and accurate

laboratory diagnostic procedure for JEV infection is needed for optimal patient management, efficient vector control. In Taiwan and Hong Kong, real-time TaqMan assay techniques have replaced the traditional nested RT-PCR techniques for early diagnosis of severe acute respiratory syndrome (SARS)-related specimens in the SARS-Coronavirus outbreak during the early summer of 2003 [Poon et al., 2003]. The JEV TaqMan assay described herein could be appropriately applied to obtaining highly specific and sensitive JEV strains identification, eliciting quantitative results within less than 3 hr.

Some earlier studies have reported that the RT-PCR assay may be used to detect JEV presence, and such a procedure seems to be more suitable for epidemiological survey than specific virus isolation [Sun et al., 2000], although such studies did apply conventional RT-PCR and nested RT-PCR assays, many of which exhibit some limitations of PCR. The TaqMan RT-PCR technique described above allows for the rapid, sensitive, and highly specific diagnosis of all JEV strains that were tested. The one-step TaqMan RT-PCR procedure is certainly time-saving and less complicated than other RT-PCR methods used for such a purpose. The use of AmpEraseUNG and closed-tube detection systems, as described above, minimizes the chances of laboratory cross-contamination of samples. The relative sensitivity of the one-step TaqMan RT-PCR system described above (0.07 PFU) would appear to be substantially greater than previously reported sensitivities of conventional RT-PCR techniques, e.g., 64 PFU, 100 PFU, and 1 pg JEV RNA, respectively [Murakami et al., 1994; Paranjpe and Banerjee, 1998; Sun et al., 2000].

The JEV TaqMan assay was tested with different specimens, such as cultured virion, in vitro-transcribed NS3 RNA, mosquito extract and human sera, and the relative sensitivity for such different specimen types seemed to vary only slightly. To our knowledge, the purified in vitro-transcribed NS3 RNA can serve as the appropriate standard control for absolute quantitation. The fact that we did observe a greater level of sensitivity for cultured JE virions than for NS3 RNA (0.07 PFU vs. five copies of RNA) may result from the presence of defective virions in cultured JE virions which may thus contribute to the observed sensitivity difference between molecular diagnosis and plaque assay techniques. The sensitivities of JEV-infected mosquitoes (100% detection rate at 1.13 PFU/test) and JEV-spiked human sera (100% detection rate at 4.48 PFU/test) demonstrated that a variety of dirty specimens could be analyzed in our assay system without apparent inhibitors. The enterovirus spike experiments demonstrate that the real-time TaqMan PCR assay can be applied to different clinical specimens, such as plasma, serum, CSF, and NT swab, providing timely and accurate diagnosis of viral infection status without apparent inhibition [Nijhuis et al., 2002]. The level of this inhibition effect in mosquito specimens (Sakallah et al., unpublished data) appeared to be notably lower for JEV EZrTth RT-PCR TaqMan assay than for other RT-PCR and real-

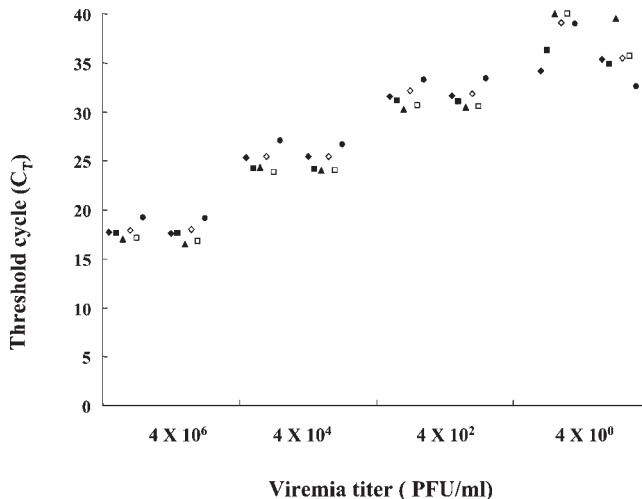


Fig. 4. The JEV NS3 TaqMan RT-PCR assay for detecting JE viral RNA from JEV-spiked human sera. The defined titers of JE virions were used for spiking JEV-negative human sera in order to generate the JEV viremia sera suite with 4×10^6 – 4×10^0 PFU/ml (horizontal axis). The sample qualities correspond from 4,480 to 0.0448 PFU per test. The C_T results of the TaqMan RT-PCR assay of six different human sera each featuring four different virus loadings are shown on the vertical axis. The detection rates are 100% for JEV viremia sera with 4×10^6 , 4×10^4 , 4×10^2 PFU/ml. There seemed to be no difference in detection rate between JEV Ab positive (●, ■, ◆) and negative (▲, □, ◇) sera.

time RT-PCR assays. In this study, the primers were used for real-time, one-step SYBR green RT-PCR assay for the samples shown in Tables II and III. This assay avoids the usage of TaqMan probe, which is more expensive and labile. The SYBR green RT-PCR assay has shown similar sensitivity but less specificity (i.e., less cross-reactivity with West Nile virus and Dengue virus serotype 2) than JEV NS3 TaqMan assay. The national JEV vaccination policy has been implemented since 1968 in Taiwan, and as a consequence, it would appear that JE and associated outbreaks are well controlled [Wu et al., 1999]; and thus, the assays described in this report including JEV NS3 TaqMan and SYBR green assays will likely have more application for JEV diagnosis and surveillance in other JEV-endemic areas (Fig. 4).

We also believe that the technique will constitute a tool for the evaluation of anti-JEV drugs [Wu et al., 1999; Chen et al., 2002] and control of vector-virus transmission.

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