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Simultaneous Detection of Three Arboviruses Using a Triplex RT-PCR Enzyme Hybridization Assay*

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Abstract: Arboviruses represent a serious problem to public health and agriculture worldwide. Fast, accurate identification of the viral agents of arbovirus-associated disease is essential for epidemiological surveillance and laboratory investigation. We developed a cost-effective, rapid, and highly sensitive one-step “triplex RT-PCR enzyme hybridization” assay for simultaneous detections of Japanese Encephallitis virus (JEV, Flaviviridae), Getah virus (GETV, Togaviridae), and Tahyna virus (TAHV, Bunyaviridae) using three pairs of primers to amplify three target sequences in one RT-PCR reaction. The analytical sensitivity of this assay was 1 PFU/mL for JEV, 10 PFU/mL for GETV, and 10 PFU/mL for TAHV. This assay is significantly more rapid and less expensive than the traditional serological detection and single RT-PCR reaction methods. When “triplex RT-PCR enzyme hybridization” was applied to 29 cerebrospinal fluid (CSF) samples that were JEV-positive by normal RT-PCR assay, all samples were strongly positive for JEV, but negative for GETV and TAHV, demonstrating a good sensitivity, specificity, and performance at CSF specimen detection.

Key words: Japanese Encephalitis virus (JEV); Getah Virus (GETV); Tahyna Virus (TAHV); Multiplex RT-PCR; Enzyme Hybridization

Arboviruses are transmitted via blood-sucking arthropod vectors that bite susceptible vertebrate hosts[27]. There are more than 550 species listed in the international catalog, of which more than 128 are known to infect humans and livestock[16]. Since the 1950s, four arborvirus-related diseases have been reported [Japanese encephalitis (JE), tick-borne encephalitis (TBE), Xingjiang hemorrhagic fever (XHF), and Dengue fever (DEN)] to be endemic to China[14]. In recent years, some emerging and reemerging arboviruses, such as GETV[28], TAHV[17], and JEV[13], have been newly identified in mainland China, which threaten public health and agriculture;

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this has resulted in an urgent need for diagnosis and monitoring methods. However, current diagnostic and detection methods (cell culture, enzyme-linked immunosorbent assay [ELISA], reverse transcription polymerase chain reaction [RT-PCR]) are either time-consuming, or require an adequate quantity of specimen[18] and trained operators, or exhibit a low sensitivity and specificity[20]. The better diagnostic/ monitering method(s) which reduces the hands-on time and obtains greater rapidity and higher sensitivity is largely needed. One-step multiplex RT-PCR can achieve these aims, decrease the risk of contamination, and has been applied to the diagnosis of several other viral diseases[1,2,5,8]. The triplex RT-PCR enzyme hybridization assay developed in the present study can practically screen for the arboviruses JEV, GETV, and TAHV in one reaction. When compared with traditional methods, this new assay is more rapid, requires less specimen and inexpensive facilities, and displays a high sensitivity and specificity. Additionally, the high-throughput potential of our novel assay requires less hands-on time and provides a new option for the diagnosis of emerging and reemerging viral diseases.

MATERIALS AND METHODS

Virus strains

Japanese encephalitis (P3, AF036918), Getah (GETV, EU015063), and Tahyna (TAHV, EU622820) viruses used in this study were maintained in the Viral Encephalitis Department of the Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (Beijing, China).

Samples

Twenty-nine cerebrospinal fluid (CSF) samples were collected from the Liupanshui, Zuiyi, Tongren, Southern Qianxi, Bijie, and Anshun regions of Guizhou Province in 2006 (China), and they were tested positive for JEV by traditional RT-PCR were selected for clinical feasibility testing of the one-step triplex RT-PCR enzyme hybridization assay.

Cell line

BHK-21 (Baby hamster kidney) cells were maintained in the Viral Encephalitis Department of the Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (Beijing, China).

Cell culture, virus amplification and titration

BHK-21 cells were cultured in 25 cm^2 flasks with Eagle’s medium (prepared by our institute) that included 10% fetal bovine serum (Hangzhou evergreen, China), 7.5% sodium bicarbonate, 2 mmol/L glutamine (Gln), and penicillin/streptomycin (100 U/mL). Supernatants containing the arboviruses JEV, GETV, and TAHV were inoculated onto BHK-21 cell monolayer (70-80% confluence) [24] and incubated at 37℃ with 5% CO_2 for viral replication. Cells were observed for cytopathic effects (CPE) daily from days one to seven post-inoculation.

Cells were freeze-thawed three times after the occurrence of CPEs in at least 80% of cells, and then harvested by centrifugation at 4℃ and 12,000 rpm for 20 min. Virus titers in supernatants were determined by the plaque count method[25], and supernatants were then aliquoted, and stored at -80℃.

Primers and probes

All JEV, GETV, and TAHV sequences were downloaded from the public database of the National Center for Biotechnology Information(NCBI) and combined sequences kept in our laboratory were used

to design primers and probes. Multiple sequence alignments were performed using ClustalX 2.1[22] and BLAST result analyses were performed using CLC Free Workbench[3]. These data were used to identify the most conserved gene regions for designing specific primers and probes. Specific annealing of all primers and probes to the conserved region of three arboviruses was confirmed by BLAST searching of public databases (http://www.ncbi.nlm.nih.gov. BLAST/ BLAST.cgi).

Sequences and other information on the primers and probes are shown in Table 1. Reverse primers with biotin modification at the 5' end were synthesized by the Shanghai Bioengineering Corporation (China) and probes labeled with horseradish peroxidase (HRP) were produced by BioNexus Inc. (Oakland, CA. USA).

One-step triplex RT-PCR

Ten-fold serial dilutions of the three arboviruses in Eagle's medium without serum were prepared. RNA was extracted using the QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. The primer mix contained three equivalent pairs of primers (0.25 μmol/L forward and 0.5 μmol/L reverse) and was used for a single step RT-PCR using the One-Step RT-PCR Kit (Qiagen) according to the manufacturer's instructions. The amplification mixture contained 1.5μL Primer Mix, 10μL 5×buffer, 2μL dNTP, 2 μL enzyme mix, and 34 μL template (extracted RNA). After reverse transcription at 50℃ for 30 min

Table 1. Information of primer and probe applied in this study

Family	Organism	Target	Primer/Probe Name	Sequence 5'-3'	Length (bp)	Total sequence (nt)	Hits (bp)	Coverage (%)	Product length (bp)
Flaviviridae West Nile Complex	JEV	C	JEV_F_82_20	GTGGGAGTGAAG AGGGTAGT	20	219	218	99.5	192
			JEV_R_252_22	GAGTGTTCCAAG TTCTCGTTTG	22	219	213	97.3	
			JEV_probe_102_32	ATGAGCTTGTTGG ACGGCAGAGGG CCAGTACG	32	219	217	99.1	
Togaviridae Alphavirus	GETV	NS1	GETV_F_149_22	TTGAGCAAGAGG TTCCAACAGG	22	13	13	100	193
			GETV_R_319_23	GGACACATTCTT GTCTAGCACAG	23	13	13	100	
			GETV_probe_276_34	TGGCGAATTACG CTCGAAAGCTGG CGAAAGCATC	34	13	13	100	
Bunyaviridae California Group	TAHV	S	TAHV_F_49_2	GAGATACCGAGA GGAATAACTTG	23	27	27	100	158
			TAHV_R_185_22	GGAAGATCCTAA CGGAATGGAG	22	27	27	100	
			TAHV_probe_123_38	ATGGATTTGATC CTGATGCAGGGT ATGTGGACTTCTGT	38	27	27	100	

and denaturation at 95℃ for 15 min, amplification proceeded with two cycles at 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s, and 38 cycles of 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 30 s; samples were then held at 72℃ for 10 min.

Enzyme hybridization assay

PCR products were purified using a PCR Purification Kit (Qiagen). Purified products were denatured at 95℃ for 5 min and kept on ice. Each HRP-labeled probe solution (65 μL) was added to the wells of a 96-well avidin-coated microtiter plate (Pierce) and then 5 μL denatured PCR product was added to each well. Positive (PCR products of a 10^2 PFU/mL virus dilution) and negative (PCR products of simple Eagle's medium) controls were set up simultaneously. The microplate was then placed in an incubator for 30 min at 42℃ for the capture and hybridization reactions. Each well was washed with phosphate buffered saline (PBS) solution 10 times and then 200 μL of TMB substrate Reagent A&B was added (BD OptEIA™). After 10 min, the reaction was stopped by addition of 50 μL H_2SO_4. The optical density (OD) of each well was measured at 450 nm using a Microplate Reader (BIO-RAD). The positive cutoff value was calculated to be two times greater than the average value of the negative control and OD ≥0.5.

Sensitivity analysis

Analytical sensitivities were determined by making 10-fold serial dilutions of arbovirus supernatant with Eagle's medium (10^{-2}-10^2 PFU/mL) and detected using a One-Step Triplex RT-PCR enzyme hybridization assay. The highest virus dilution that was positive (OD ≥ 0.5) was considered to be the LOD (limit of detection) of the assay.

Cross-reactivity between three PCR products and species-specific probes

Purified PCR product from suspensions of each arbovirus with a titer 100 times greater than the LOD (10^2 PFU/mL) was hybridized with probes specific for the other two viruses.

Clinical samples

Twenty-nine clinical CSF samples that were confirmed JEV-positive by traditional RT-PCR were selected for clinical feasibility testing of the one-step triplex RT-PCR enzyme hybridization assay. RNA was extracted from 140 μL aliquots of each CSF sample using a QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Extracted RNA was then subjected to our one-step triplex-RT-PCR enzyme hybridization assay. The sensitivity and specificity of this assay were obtained by comparing the results with those of standard RT-PCR.

RESULTS

Primer and probe design

All known sequences (in GenBank as well as tested in our laboratory) were collected, including 219 JEV sequences (covering gene type Ⅰ-Ⅳ), 13 GETV sequences, and 27 TAHV sequences. Primers and species-specific probes targeted the most conserved region of the structural capsid gene for JEV, NS1 for GETV and S for TAHV (Table 1). The length of primers used was 20-23 base pairs (bp); that of probes was 32-38 bp. PCR products were putatively 158-193 bp. The coverage rates (i.e. rates of matching specific sequence) of all primers were 97.3-100%, and 99.1-100% for species-specific probes.

Development and sensitivity analysis of the one-step

triplex RT-PCR enzyme hybridization assay

Based on both the literature[9,12,15,23] and the experience in our laboratory, we used a forward to reverse primer (biotin-modified) ratio of 1:2. Optimum primer concentrations were 0.25 mmol/L (forward) and 0.5 mmol/L (reverse). Sensitivity testing of the 10-fold serial dilutions for the three arboviruses JEV, TAHV, and GETV showed that this assay could detect 10^0 PFU/mL JEV, 10^1 PFU/mL TAHV, and 10^1 PFU/mL GETV (Table 2).

Cross-reactivity between PCR products and species-specific probes

Cross-reactivity was assessed by hybridizing high titer amplicons (10^2,10^1 PFU/mL) of each arbovirus with the probes of the other two viruses. All results (Table 3) were negative, which demonstrates no cross-reactivity between PCR amplicons and probes of the other viruses.

Detection of clinical specimens

All 29 CSF samples that were JEV-positive by standard RT-PCR were strongly-positive for JEV, but negative for the other two viruses (GETV and TAHV) (Table4) when tested using the one-step triplex-RT- PCR enzyme hybridization assay developed in this study.

Table 2. Limit of detection (LOD) of one-step triplex RT-PCR-EHA assay

Organism	10^2 PFU/mL	10^1 PFU/ mL	10^0 PFU/ mL	10^{-1} PFU/ mL	10^{-2} PFU/ mL	NC (Eagle's solution)
JEV	OD: 2.491 +	OD: 2.484 +	OD: 1.300 +	OD: 0.048 –	OD: 0.042 –	OD: 0.060 –
TAHV	OD:2.419 +	OD: 2.088 +	OD: 0.150 –	OD:0.038 –	OD: 0.043 –	OD: 0.022 –
GETV	OD: 2.696 +	OD: 2.082 +	OD: 0.144 –	OD:0.066 –	OD: 0.061 –	OD: 0.057 –

Set the cutoff value to be 0.5. positive results (+):OD value higher than two times average value of negative control group or OD≥0.5; suspective results (+-):0.5＞OD>0.4; negative results (-):OD<0.4.

Table 3. Cross-reactivity results between amplicons (produced by Triplex RT-PCR) and species specific probes

Template \ Probe	JEV	TAHV	GETV	NC (Eagle's solution)	JEV (positive control)
JEV(100 PFU/ mL)	OD:2.736 +	OD:0.236 –	OD:0.330 –	OD:0.135 –	OD:2.611 +
JEV(10 PFU/ mL)	OD:2.729 +	OD:0.278 –	OD:0.321 –	ND	ND
TAHV(100 PFU/ mL)	OD: 0.110 –	OD:2.664 +	OD:0.340 –	OD:0.117 –	ND
TAHV(10 PFU/ mL)	OD:0.090 –	OD:2.333 +	OD:0.215 –	ND	ND
GETV(100 PFU/ mL)	OD:0.068 –	OD:0.043 –	OD:2.941 +	OD:0.046 –	ND
GETV(10 PFU/ mL)	OD:0.060 –	OD:0.050 –	OD:2.327 +	ND	ND

Set the cutoff value to 0.5. Positive results(+):OD≥0.5; Negative results(-):OD<0.4; ND(not detection): did not test this result.

Table 4. Detection of CSF samples by one-step triplex RT-PCR-EHA assay

Sample No.	JEV(OD)	TAHV(OD)	GETV(OD)
1	＋(0.667)	－(0.103)	－(0.069)
2	＋(0.531)	－(0.090)	－(0.053)
3	＋(0.598)	－(0.070)	－(0.054)
4	＋(0.660)	－(0.116)	－(0.039)
5	＋(1.066)	－(0.070)	－(0.052)
6	＋(0.707)	－(0.074)	－(0.057)
7	＋(0.864)	－(0.046)	－(0.055)
8	＋(0.553)	－(0.058)	－(0.050)
9	＋(1.210)	－(0.058)	－(0.052)
10	＋(0.929)	－(0.090)	－(0.078)
11	＋(0.620)	－(0.074)	－(0.053)
12	＋(0.745)	－(0.040)	－(0.047)
13	＋(0.936)	－(0.074)	－(0.050)
14	＋(0.907)	－(0.059)	－(0.051)
15	＋(0.570)	－(0.072)	－(0.055)
16	＋(1.389)	－(0.087)	－(0.046)
17	＋(1.308)	－(0.086)	－(0.062)
18	＋(0.751)	－(0.089)	－(0.076)
19	＋(0.750)	－(0.067)	－(0.052)
20	＋(0.879)	－(0.059)	－(0.073)
21	＋(0.746)	－(0.122)	－(0.052)
22	＋(0.717)	－(0.062)	－(0.056)
23	＋(0.732)	－(0.069)	－(0.052)
24	＋(0.751)	－(0.057)	－(0.048)
25	＋(2.216)	－(0.066)	－(0.072)
26	＋(1.118)	－(0.133)	－(0.058)
27	＋(1.332)	－(0.136)	－(0.063)
28	＋(0.627)	－(0.051)	－(0.041)
29	＋(0.591)	－(0.148)	－(0.053)

Positive results(＋):OD≥0.5; Negative results(－):OD<0.4.

DISCUSSION

At present, arboviruses are important pathogens associated with serious public health and agricultural loss in China[13]. Recent reports of virus isolation and antibody detection suggest the existence of many arboviruses in mainland China[27]. This leads to the calls for urgent investigations and the development of new diagnostic assays.

Virus isolation by cell culture is recognized as the "gold standard"; however, it is slow and requires almost a week for results. Antigen detection can provide rapid results, but often exhibits a low sensitivity and sometimes requires further confirmation by cell culture. Traditional molecular diagnosis methods, such as monospecific PCR[7] and some types of revised monospecific PCR (nested PCR[26] and half-nested PCR[11]) assays are potentially expensive, resource-intensive, and can test for only one pathogen in each reaction. These disadvantages impede their application to arbovirus diagnosis, especially for those viruses that cause identical clinical syndromes. Recently, a new generation of molecular diagnostic methods such as real-time PCR[4], multiplex PCR[6], and microarrays[19], which offer rapid and sensitive results, have emerged, but these require expensive equipment, reagents, and trained technicians. To date, these new techniques have been mainly used in research laboratories and rarely in field studies. To overcome these disadvantages, we developed a nucleic acid-based assay that combines a new generation molecular technique (multiplex RT-PCR[21]) with conventional testing methods(enzyme hybridization similar to ELISA). This assay showed a high sensitivity when testing CSF samples compared to standard RT-PCR. In addition, it requires only about four hours to complete all procedures, thus markedly decreases hands-on time. Use of nucleic acid hybridization enhances the detection signal, leading to improved sensitivity. Additionally, this assay could be applied to other pathogens and has been adopted in many fields, such as screening for pathogens involved in bioterrorism [24], detection of respiratory pathogens [15],

and analysis of enzyme activity associated with carcinoma[10]. This combined one-step triplex RT-PCR enzyme hybridization assay possesses the advantages of both conventional methods and advanced molecular detection, thus providing rapid and highly-sensitive and -specific results with low contamination rates. Further, it exhibits ease-of-use and requires little sample. Normal facilities (PCR amplifier, high-speed centrifuge, and microplate reader) are needed, and technicians trained in ELISA and traditional PCR are capable of performing the combined assay. In addition, the number of primers and probes can be altered depending on the pathogens to be detected.

Due to sample limitations, we did not test GETV- or TAHV-positive clinical samples. However, testing of JEV-positive CSF clinical samples demonstrated that our assay has good sensitivity and specificity compared to standard RT-PCR. In conclusion, we developed a sensitive and specific one-step triplex RT-PCR enzyme hybridization assay that has potential as a field diagnostic tool for the detection of encephalitis pathogens in clinics and diagnostic laboratories.

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