Letter to the Editor

Real-time RT-PCR Assay for the Detection of *Culex flavivirus*[*]

CAO Yu Xi[1,^], HE Xiao Xia[1,^], FU Shi Hong[1,2], HE Ying[1,2], LI Hao[1], GAO Xiao Yan[1,2], LIANG Guo Dong[1,2], and WANG Huan Yu[1,2,#]

Based on the *Culex flavivirus* (CxFV) E gene sequences in GenBank, CxFV-specific primers and probes were designed for real-time reverse transcription-polymerase chain reaction (RT-qPCR). The specificity test revealed that CxFV could be detected using RT-qPCR with the specific CxFV primers and probes; other species of arboviruses were not detected. The stability test demonstrated a coefficient of variation of <1.5%. A quantitative standard curve for CxFV RT-qPCR was established. Quantitative standard curve analysis revealed that the lower detection limit of the RT-qPCR system is 100 copies/μL. Moreover, RT-qPCR was used to detect CxFV viral RNA in mosquito pool samples. In conclusion, we established a real-time RT-PCR assay for CxFV detection, and this assay is more sensitive and efficient than general RT-PCR. This technology may be used to monitor changes in the environmental virus levels.

The *Culex flavivirus* (CxFV) has a positive-sense single-stranded RNA virus and belongs to the *Flavivirus* genus of the Flaviviridae family[1-2]. The full length of the genome is approximately 11 kb, containing an open reading frame encoding three structural proteins, nucleocapsid protein (C), membrane protein precursor (prM/M), and envelope protein (E), along with seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5)[1]. *Culex flavivirus* is a typical insect virus that was first isolated from mosquitoes in Japan in 2003[3]. This virus was subsequently isolated in the United States[4] and Uganda[5]. The main hosts of CxFV are *C. pipiens*, *C. tritaeniorhynchus*, and *C. quinquefasciatus*. In China, CxFV was isolated from *C. pipiens* in the Shandong Province in 2006[6], then from mosquitoes in the Liaoning Province and Gansu Province in 2011[7-8]. The virus was also isolated from mosquitoes in Taiwan in 2013[2].

At present, RT-qPCR is an *in vitro* amplification technology for the qualitative and quantitative analysis of nucleic acids by detecting dynamic changes in the fluorescence intensity in the system[9]. In the present study, a specific, fast, sensitive, and efficient molecular method for CxFV detection was established using RT-qPCR, which was applied preliminarily in the analysis of mosquito vectors.

Fifty representative strains of CxFV isolated from mosquitoes in different years and areas[6] were selected for the comparison of the E gene sequences of the strains (data from GenBank) using bioinformatics analysis software ClustalX (1.8). Next, the most conservative regions of the sequences were selected as target gene fragments for amplification[10]. Primer and probe sequences were designed using Primer Express v3.0 software (Applied Biosystems, Foster City, CA), and BLAST analysis was conducted to verify the broad spectrum and specificity of the E gene. CxFV-specific primers and probes were synthesized for RT-qPCR. The length of the amplified fragment was 82 bp. The probes were labeled with FAM fluorescent reporter (5′) or TAMRA fluorescence quenching (3′) (Table 1).

Viral RNA extraction was conducted using a Viral RNA Mini Kit (QIAGEN, Dusseldorf, Germany), and real-time RT-PCR was conducted using an AgPath-ID™ One-step RT-PCR Kit (ABI Foster City, CA), as per the manufacturers' instructions. Each RT-qPCR system (25 μL) included 2× reaction buffer (12.5 μL), enzyme mix (1 μL), upstream and downstream primers (10 μmol/L) and probe (5 μmol/L), each 1 μL, RNA template (1 μL), and the final volume was made

doi: 10.3967/bes2015.019

[*]This work was supported by grants from the Development Grant of State Key Laboratory of Infectious Disease Prevention and Control (2012SKLID204, 2015SKLID505); the Ministry of Science and Technology of People's Republic of China (No. 2013ZX10004101).

1. State Key Laboratory of Infectious Disease Prevention and Control (SKLID), Department of Viral Encephalitis, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; 2. Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou 310003, Zhejiang China

up with diethylpyrocarbonate-treated water. The reaction conditions were reverse transcription at 45 °C for 10 min, initial denaturation at 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. A negative extraction control for the assay consisted of supernatant from an uninfected C6/36 cell cultured in EMEM media. A positive extraction control consisted of supernatant from CxFV infected C6/36 cell. A no-template control consisted of DD H_2O. The standard strain of CxFV used in this study was SDDM06-11[6], which was isolated in the Shandong Province in 2006. A battery of arboviruses (a total of 11 strains belonging to three genera and seven species), including Dengue virus (serotypes 1-4), West Nile virus, Japanese encephalitis virus (genotypes 1 and 3), tick-borne encephalitis virus, Tahyna virus, Batai virus, and Getah virus, were selected for specificity verification of RT-qPCR. Only CxFV was successfully amplified and a cycle threshold (Ct) value (Ct=24.2) obtained using the specific primers and probes, and none of the other viruses produced a positive amplification signal.

RNA extracted from the CxFV strain SDDM06-11 was diluted 10^{-1}-10^{-4}-fold. Four parallel detections were performed using these RNA template samples, and the experimental Ct values obtained were statistically analyzed to calculate the mean and coefficient of variation (CV) of the Ct values of all test samples to evaluate the stability of the detection system. The mean, standard deviation (SD), and CV of the Ct values were calculated (Table 2). The CV was <1.5%, indicating that the detection system has good stability.

Target fragments in the cloning plasmid (PGEM-T Easy) were recovered and purified after general RT-PCR amplification. The DNA templates recovered were counted using an UV nucleic acid protein quantization instrument (Eppendorf, Germany). *In vitro* transcription of RNA was performed using a RiboMAX Large Scale RNA Production System (T7) kit (Promega). After the removal of the DNA template, the transcribed RNA was quantified. The RNA copy number was calculated according to the equation $(6.02\times10^{23})\times$ (concentration of transcript ng/μL)/(molecular weight of transcript)=copies/μL, and 5 sample concentrations (10^1-10^5 copies/μL) were obtained by serial dilution. A standard curve was drawn using the copy number and the Ct of samples at the different concentrations to construct a quantitative analysis model for the viral gene copy number. The resulting standard curve equation for the detection system was as follows: y=−3.913x+42.93, R^2=0.998. The lower detection limit for the method is 100 copies/μL (Figure 1).

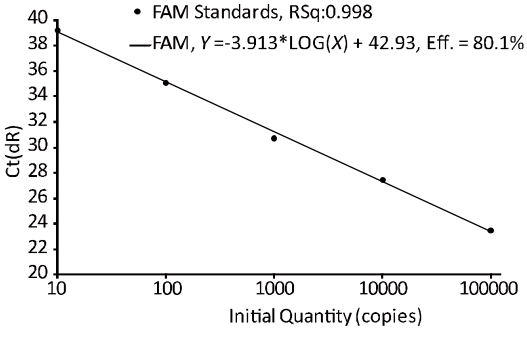

Figure 1. Standard curve for genomic copy quantification.

Table 1. Primers and Probe Used for RT-qPCR

Primer/probe	Sequence (5′-3′)	Coding Position	Length (nt)	Product Size (bp)
CxFV-F	CACGCCGAACGGACTTCT	1031-1048	18	
CxFV-R	TCCATTGGCCGCCATATATC	1092-1111	20	82
CxFV-Pro*	TTTCGCACCGGAGCAGCCG	1053-1071	19	

Note. *5′ end labeled with FAM, 3′ end labeled with TAMRA.

Table 2. Reproducibility of RT-PCR for CxFV Detection

CxFV RNA	Ct_1	Ct_2	Ct_3	Ct_4	Mean	SD	CV (%)
10^{-1} diluted	19.47	19.13	19.20	19.54	19.34	0.20	1.03
10^{-2} diluted	23.40	23.29	22.76	23.24	23.17	0.28	1.22
10^{-3} diluted	26.67	26.96	26.35	26.60	26.65	0.25	0.94
10^{-4} diluted	29.84	30.23	29.75	30.72	30.14	0.44	1.47

In total, 5303 mosquitoes from three genera were collected using UV-light trap (Hubei Lucky Star Environment Protection Co., Ltd.) in Hanzhong City, Weinan City, and Yan'an City, China in 2012 and sorted into 124 pools as per the species, location, and collection date. The pools contained approximately 50 mosquitoes each. The nucleic acids were extracted after grinding the mosquitoes[6]. The sensitivity of the detection system was compared and evaluated between general RT-PCR detection and RT-qPCR detection. In 124 batches of mosquito samples, 4 samples with positive nucleic acid results for CxFV were detected using the general RT-PCR method. However, 9 samples with positive nucleic acid results (Ct<35) were detected using the RT-qPCR method, including 4 samples identified as positive by the general RT-PCR method. This indicates that the CxFV-specific RT-qPCR detection system has a higher sensitivity than the general RT-PCR system (Table 3).

For the RT-qPCR detection method developed in this study, a minimum time of 5 h is needed from the grinding of mosquito samples to the determination of the results, and the outcome obtained is identified as CxFV. The steps and methods required for virus isolation are more cumbersome and time consuming; however, the virus strains obtained may be used for further research. As compared with the general RT-PCR methods, the method established in this study does not require electrophoresis. Real-time RT-PCR provides a new technology for epidemiological investigations of CxFV viral infection in China, particularly when combined with monitoring data. This technology may be used to dynamically monitor changes in viruses with the seasonal fluctuation of mosquitoes and to provide early warning analysis for epidemiological trends in diseases.

Table 3. Sensitivity of RT-qPCR and General RT-PCR Assay for the Detection of CxFV

Samples	Mosquitoes	General RT-PCR	Taqman PCR	
		Int	(Ct)	Int
S1	*Culex pipiens pallens*	Pos	23.35	Pos
S2	*Culex pipiens pallens*	Pos	22.61	Pos
S3	*Culex pipiens pallens*	Pos	23.93	Pos
S4	*Culex pipiens pallens*	Pos	22.31	Pos
S5	*Culex tritaeniorhynchus*	Neg	24.49	Pos
S6	*Culex tritaeniorhynchus*	Neg	34.63	Pos
S7	*Culex tritaeniorhynchus*	Neg	34.43	Pos
S8	*Culex tritaeniorhynchus*	Neg	33.07	Pos
S9	*Culex tritaeniorhynchus*	Neg	34.96	Pos

Note. Int: interpretation; Pos: positive; Neg: negative.

^These authors equally contributed to this manuscript.

#Correspondence should be addressed to WANG Huan Yu, E-mail: rainoffall@yahoo.com

Biographical notes of the first authors: CAO Yu Xi, male, born in 1977, PhD, majoring in biosafety and molecular epidemiology investigation of arbovirus; HE Xiao Xia, female, born in 1986, assistant researcher, major in establishing the laboratory detection system for arbovirus.

Received: September 6, 2015;

Accepted: December 9, 2015

REFERENCES

1. Fields Virology. Edited by: Fields BN, Knipe DM, Howley PM. Lippincott Williams, 2007; 5, 1154-228.
2. Chen YY, Lin JW, Fan YC, et al. First detection of the Africa/Caribbean/Latin American subtype of Culex flavivirus in Asian country, Taiwan. Comp Immunol Microbiol Infect Dis, 2013; 36, 387-96.
3. Hoshino K, Isawa H, Tsuda Y, et al. Genetic characterization of a new insect flavivirus isolated from Culex pipiens mosquito in Japan. Virology, 2007; 359, 405-14.
4. Blitvich BJ, Lin M, Dorman KS, et al. Genomic sequence and phylogenetic analysis of Culexflavivirus, an insect-specific flavivirus, isolated from Culexpipiens (Diptera: Culicidae) in Iowa. J Med Entomol, 2009; 46, 934-41.
5. Cook S, Moureau G, Harbach RE, et al. Isolation of a novel species of flavivirus and a new strain of Culex flavivirus (Flaviviridae) from a natural mosquito population in Uganda. J Gen Virol, 2009; 90(Pt 11), 2669-78.
6. Wang HY, Wang HY, Fu SH, et al. Isolation and identification of a distinct strain of Culex Flavivirus from mosquitoes collected in Mainland China. Virol J, 2012; 9, 73.
7. An SY, Liu JS, Ren Y, et al. Isolation of the Culex Flavivirus from Mosquitoes in Liaoning Province, China. Chinese Journal of Virology, 2012; 28, 511-6. (In Chinese)
8. Zha B, Yu DS, Fu SH, et al. Investigation of mosquitoes and arboviruses in Hexi Corridor of Gansu province, China in 2011. Chinese Journal of Vector Biology and Control, 2012; 23, 424-7. (In Chinese)
9. Ouyang SY, Yang D, Ouyang HS, et al. Real-time Fluorescent Quantitative PCR Technology and Its Application. Chemistry of Life, 2004; 24, 74-6.
10. Livak KJ, Flood SAJ, Marmaro J, et al. Oligo nueleotides with fluorescentdyes at opsite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization PCR. Methods Applicat, 1995; 4, 357-62.