Letter to the Editor

Real-time RT-PCR Assay for the detection of Tahyna Virus*

LI Hao, CAO Yu Xi, HE Xiao Xia, FU Shi Hong, LYU Zhi, HE Ying,
GAO Xiao Yan, LIANG Guo Dong, and WANG Huan Yu#

A real-time RT-PCR (RT-qPCR) assay for the detection of Tahyna virus was developed to monitor Tahyna virus infection in field-collected vector mosquito samples. The targets selected for the assay were S segment sequences encoding the nucleocapsid protein from the Tahyna virus. Primers and probes were selected in conserved regions by aligning genetic sequences from various Tahyna virus strains available from GenBank. The sensitivity of the RT-qPCR approach was compared to that of a standard plaque assay in BHK cells. RT-qPCR assay can detect 4.8 PFU of titrated Tahyna virus. Assay specificities were determined by testing a battery of arboviruses, including representative strains of Tahyna virus and other arthropod-borne viruses from China. Seven strains of Tahyna virus were confirmed as positive; the other seven species of arboviruses could not be detected by RT-qPCR. Additionally, the assay was used to detect Tahyna viral RNA in pooled mosquito samples. The RT-qPCR assay detected Tahyna virus in a sensitive, specific, and rapid manner; these findings support the use of the assay in viral surveillance.

Tahyna virus (TAHV) is spread by mosquitoes and can cause fever, headache, joint pain, loss of appetite, and other clinical symptoms in humans. Neurological symptoms can be found in severe cases[1]. TAHV was first isolated from a specimen of *Aedes caspius* collected in Czechoslovakia in 1969[2]. It was subsequently isolated in Europe and other countries[1,3]. Isolation of TAHV in China began in 2006, when LV Zhi isolated the virus from *Culex* collected in Kashi Prefecture, Xinjiang[4]. In 2007, LV Zhi isolated two viral strains in Weili County, 900 kilometers from Kashi[5]. Subsequently, WANG and others isolated the virus from *A. detritus* collected in Qinghai, and Cao and others isolated two strains from *A. dorsalis* and *C. modestus* collected in Inner Mongolia[6-7]. A seroepidemiological survey conducted in Xinjiang, Qinghai, revealed antibodies to TAHV in apparently normal individuals and in patients with fever, which suggested the viral exposure and/or infection[8-9].

Detection methods for monitoring mosquito and human populations infected with TAHV are mainly focused on virus isolation, neutralization tests, IFA, etc. These methods are time consuming and laborious, and cannot meet the demand for rapid detection and monitoring[6,9-10]. Real-time PCR (qPCR) techniques exhibit high specificity, sensitivity, and reproducibility, and are simple to operate[11]. This study established RT-qPCR detection methods based on S gene segments of TAHV isolated from China, applied to monitor the condition of the mosquitoes carrying the virus.

TAHV, a segmented, negative-strand RNA virus, belongs to the Bunyaviridae family. The S, M, and L segment sequences of TAHV encode the viral nucleocapsid protein, the glycoprotein, and the RNA-dependent RNA polymerase, respectively[12]. Studies have shown that the S and L fragments of TAHV isolated from Xinjiang are relatively conserved, while the M gene segment displays significant variability[5]. Complete S fragment sequences have been identified for Inner Mongolian and Qinghai isolates, and these reveal extensive conservation[6-7]. Due to this sequence conservation and in order to ensure the sensitivity and specificity of our RT-qPCR detection techniques, we chose the S fragment as an amplification target. Sequence data from 30 TAHV strains acquired from different regions deposited in GenBank were used as the reference, and bioinformatics software Clustal X (version 1.83, www.clustal.org) was used for multiple sequence alignment of S fragments. Primer Express 3.0 software (Applied Biosystems, Foster City, CA) was used for primer and probe sequence design, and

doi: 10.3967/bes2015.052

*This work was supported by the National Science and Technology Major Project of the Ministry of Science and Technology of China (No.2013ZX10004-101).

National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China

BLAST analysis was used to verify the specificity of the amplified S gene fragments. Details of the RT-qPCR primers and probes designed to detect TAHV are shown in Table 1. The amplified target fragment length was 74 bp.

This study used the TAHV NM08003 strain isolated from *A. dorsalis* collected in 2008 from Inner Mongolia as a reference standard to establish the methods and conditions. For qPCR, RNA was extracted from virus and mosquito samples using QIAGEN RNeasy Mini kit (QIAGEN, Dusseldorf, Germany) according to the manufacturer's instructions. Both reverse transcription and cDNA amplification were performed in a 25 μL TaqMan reaction mixture containing 1 μL of 10 μmol/L sense and antisense primers, 1 μL of 5 μmol/L probe, 12.5 μL of 2× reaction buffer, 1 μL Enzyme Mix (ABI, AgPath-ID™ One-step RT-PCR Kit, Foster City, CA), 1 μL RNA, and 8.5 μL of DEPC-treated water. PCR was performed using the Mx3000P Real Time System (STRATAGENE, Santa Clara, CA). Thermal cycling conditions were as follows: 45 °C, 2 min; 95 °C, 10 min; and then 40 cycles of 95 °C, 15 s and 60 °C, 1 min. The cutoff cycle threshold (Ct) value for a positive sample was set at 35 cycles. Negative extraction control for the assay consisted of supernatant from uninfected BHK cells cultured in EMEM. Positive extraction control consisted of supernatant from TAHV-infected BHK cells. No template control was from DD H_2O.

To evaluate the sensitivity of the RT-qPCR assay, TAHV strain NM08003 was used to conduct plaque formation assays to determine viral titer. Ten-fold dilution series were employed to measure the sensitivity of the experimental systems. When the virus was diluted 10^{-5}, viral nucleic acid could be detected (Table 2). Six other TAHV isolates from mosquito samples collected in China were tested (Supplementary Table). In addition, we tested other common mosquito-borne arboviruses from China, including Batai virus, Japanese encephalitis virus, dengue virus, Sindbis virus, Getah virus, Banna virus, and Liaoning viruses (Supplementary Table). The assay detected TAHV viral RNAs extracted from all strains of TAHV found in China, with no other common arboviruses generating positive results (Table 2). All the results verified the assay specificity of our RT-qPCR primers and probes for TAHV.

The titrated TAHV (NM08003 strain) was diluted across a 10^{-1}-10^{-4} gradient, and four different virus concentrations were obtained that were used as templates to conduct four replicates of parallel repeated experiments. Statistical analysis was conducted using the obtained Ct values; the mean, standard deviation (SD), and coefficient of variation (CV) of the Ct values for each sample in four separate experiments were calculated to evaluate the reproducibility of the detection systems. The SDs for the Ct values for each sample in parallel repeated experiments ranged from 0.15 to 0.30. CV values for the assay ranged 0.62%-1.46%, indicating that assay reproducibility was robust (Table 3).

The mosquitoes used for the assay were obtained from 19 batches collected in Tuoketuo County, Hohhot, Inner Mongolia in 2008. After conventional grinding, the centrifugal supernatant was used to evaluate the detection systems in comparison with the results of viral isolation and culture[7]. There were positive results in two batches, which is consistent with the results of virus isolation using BHK assays (Table 4).

A nested PCR method was used to detect TAHV infection status in Golmud, Qinghai Province, also employing the S segment as a target; two cases of TAHV infection were found[9]. Compared with conventional PCR, the RT-qPCR method established in this study needed no electrophoretic analysis. Our approach can be used for high-throughput, laboratory-based case surveillance and epidemiological surveys for many samples. Currently, qPCR technology is mainly used in scientific research and diagnostics[13]. RT-qPCR detection methods can be completed within 5 hours from sample receipt to results. The steps required for virus isolation are more complicated and time consuming, but viral strains can be obtained so that further research can be

Table 1. Primers and Probe Used for RT-qPCR

Primer/probe	Sequence (5′-3′)	Coding Position	Length (nt)	Product Size (bp)
TAHV-S-F	CCATTCCGTTAGGATCTTCTTCCT	186-209	24	
TAHV-S-R	CCTTCCTCTCCGGCTTACG	241-259	19	74
TAHV-S-P*	AATGCCGCAAAAGCCAAAGCTGC	211-233	23	

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

Table 2. Sensitivity and Specificity of RT-qPCR Assay for Tahyna Virus

Viruses	Standard Plaque Assay Quantity (PFU/mL)	RT-qPCR	
		Ct	Int
Titrated Tahyna virus			
NM08003 (10^{-1} diluted)	48,000	20.5	Pos
NM08003 (10^{-2} diluted)	4,800	24.3	Pos
NM08003 (10^{-3} diluted)	480	27.3	Pos
NM08003 (10^{-4} diluted)	48	30.9	Pos
NM08003 (10^{-5} diluted)	4.8	34.0	Pos
NM08003 (10^{-6} diluted)	0.48	37.6	Neg
NM08003 (10^{-7} diluted)	0.048	NA	Neg
Tahyna virus strains			
NM08010	ND	21.2	Pos
QH07029	ND	23.2	Pos
QH07060	ND	24.3	Pos
XJ0708	ND	20.6	Pos
XJ0710	ND	19.2	Pos
XJ0625	ND	20.0	Pos
Other viruses			
Batai virus (92-4)	ND	NA	Neg
JEV (HN11084)	ND	NA	Neg
JEV (LN0828)	ND	NA	Neg
Dengue virus(M110)	ND	NA	Neg
Sindbis virus (YN87448)	ND	NA	Neg
Getah virus (ES61)	ND	NA	Neg
Banna virus (NM0706)	ND	NA	Neg
Liaoning virus (NE31)	ND	NA	Neg

Note. Int: interpretation; ND: not done; NA: no amplification; Pos: positive; Neg: negative.

Table 3. Reproducibility of RT-qPCR for TAHV Detection

TAHV-NM08003	Ct_1	Ct_2	Ct_3	Ct_4	Mean±SD	CV (%)
48000PFU	20.3	20.5	20	20.7	20.4±0.29	1.46
4800PFU	24.1	24.3	24.1	24.4	24.2±0.15	0.62
480PFU	27.2	27.3	26.8	27.5	27.2±0.30	1.08
48PFU	30.8	30.9	30.6	31.2	30.9±0.25	0.81

Table 4. Detection of Tahyna Virus in Mosquito Pools by RT-qPCR and Cell Culture Assays

Samples	No. of Mosquitoes	Cell Culture	Real-time PCR	
			Ct	Int
NM08001	25	Neg	NA	Neg
NM08002	25	Neg	NA	Neg
NM08003	25	Pos	27.2	Pos
NM08004	16	Neg	NA	Neg
NM08005	25	Neg	NA	Neg
NM08006	25	Neg	NA	Neg
NM08007	25	Neg	NA	Neg
NM08008	40	Neg	NA	Neg
NM08009	25	Neg	NA	Neg
NM08010	30	Pos	31.5	Pos
NM08011	25	Neg	NA	Neg
NM08012	25	Neg	NA	Neg
NM08013	30	Neg	NA	Neg
NM08014	30	Neg	NA	Neg
NM08015	30	Neg	NA	Neg
NM08016	30	Neg	NA	Neg
NM08017	30	Neg	NA	Neg
NM08018	30	Neg	NA	Neg
NM08019	30	Neg	NA	Neg

Note. Int: interpretation; NA: no amplification; Pos: positive; Neg: negative.

conducted[6]. qPCR has a very wide dynamic range and is also highly sensitive, specific, and reproducible. It can be easily, rapidly, and accurately used for the quantitative analysis of viral loads and can further be employed to analyze the relationships between changes in viral load and the occurrence and development of disease after TAHV infection. The methods described here present improved technology and refined confidence for the epidemiological investigation and diagnosis of TAHV infection. More importantly, our technology can be used to dynamically monitor viral changes and distributions in the seasonal fluctuations of mosquitoes, as well as provide early warning for disease trends.

All authors declared no conflicts of interest.

#Correspondence should be addressed to WANG Huan Yu, MD, Tel: 86-10-58900843, E-mail: rainoffall@yahoo.com

Biographical note of the first author: LI Hao, male, born in 1981, assistant researcher, majoring in establishing the laboratory detection system and molecular epidemiology investigation of rabies and arbovirus.

Received: February 15, 2015;
Accepted: March 30, 2015

REFERENCES

1. Hubalek Z. Mosquito-borne viruses in Europe. Parasitol Res, 2008; 103, S29-S43.
2. Bardos V, Medek M, Kania V, et al. Isolation of Tahyna virus from the blood of sick children. Acta Virol, 1975; 19, 447.
3. Lundstrom JO. Mosquito-borne viruses in Western Europe: A review. J Vector Ecol, 1999; 24, 1-39.
4. Lv Z, Lv XJ, Fu SH, et al. Tahyna virus and human infection, China. Emerg Infect Dis, 2009; 15, 306-9.
5. Lv Z, Fu SH, Wang FT, et al. Circulation of Diverse Genotypes of Tahyna Virus in Xinjiang, People's Republic of China. Am J Trop Med Hyg, 2011; 85, 442-5.
6. Li WJ, Wang JL, Li MH, et al. Mosquitoes and Mosquito-Borne Arboviruses in the Qinghai-Tibet Plateau-Focused on the Qinghai Area, China. Am J Trop Med Hyg, 2010; 82, 705-11.
7. Cao YX, Fu SH, Tian ZF, et al. Distribution of Mosquitoes and Mosquito-Borne Arboviruses in Inner Mongolia, China. Vector Borne Zoonotic Dis, 2011; 11, 1577-81.
8. Lv Z, Fu SH, Wang FT, et al. Investigation of Tahyna Virus Infection among unknown Fever Cases in Xinjiang, China. Chinese Journal of Virology, 2011; 27, 71-4. (In Chinese)
9. Li WJ, Cao YX, Fu SH, et al. Tahyna Virus Infection, a Neglected Arboviral Disease in the Qinghai-Tibet Plateau of China. Vector Borne Zoonotic Dis, 2014; 14, 353-7.
10. Hubalek Z, Zeman P, Halouzka J, et al. Antibodies against mosquito-borne viruses in human population of an area of Central Bohemia affected by the flood of 2002. Epidemiol Mikrobiol Imunol, 2004; 53, 112-20.
11. Sun YL, Liang MF, Qu J, et al. Early diagnosis of novel SFTS bunyavirus infection by quantitative real-time RT-PCR assay. J Clin Virol, 2012; 53, 48-53.
12. Richard SB, Anthony KG, Murphy BR, et al. Tahyna virus genetics, infectivity, and immunogenicity in mice and monkeys. Virol J, 2011; 8, 135.
13. Korobe nikova AS, Nafeev AA, Skvortsova TM. Specific markers for the detection of circulation of Tahyna, Inko and Batai viruses (Bunyaviridae, Bunyavirus) in humans, mosquitoes, ticks and cattle of the Ulianovsk region. Vopr Virusol, 2003; 48, 45-6.

Supplementary Table. Virus Strains used in this Study

No.	Viruses		Strains	Location of Isolation	Isolation Date	Source
	Species	Family				
1	Tahyna virus	Bunyaviridae	NM08003	Inner Mongolia, China	2008	mosquito
2	Tahyna virus	Bunyaviridae	NM08010	Inner Mongolia, China	2008	mosquito
3	Tahyna virus	Bunyaviridae	QH07029	Qinghai, China	2007	mosquito
4	Tahyna virus	Bunyaviridae	QH07060	Qinghai, China	2007	mosquito
5	Tahyna virus	Bunyaviridae	XJ0708	Xinjiang, China	2007	mosquito
6	Tahyna virus	Bunyaviridae	XJ0710	Xinjiang, China	2007	mosquito
7	Tahyna virus	Bunyaviridae	XJ0625	Xinjiang, China	2006	mosquito
8	Batai virus	Bunyaviridae	92-4	Yunan, China	1998	mosquito
9	JEV	Flaviviridae	HN11084	Hunan, China	2011	mosquito
10	JEV	Flaviviridae	LN0828	Liaoning, China	2008	mosquito
11	Dengue virus	Flaviviridae	M110	Yunnan, China	1989	mosquito
12	Sindbis virus	Togaviridae	YN87448	Yunan, China	1987	CSF
13	Getah virus	Togaviridae	ES-61	Hubei, China	2010	mosquito
14	Banna virus	Reoviridae	NM0706	Inner Mongolia, China	2007	mosquito
15	Liaoning virus	Reoviridae	NE31	Jilin, China	1997	mosquito