Development and evaluation of a novel TaqMan fluorescence probe-based real-time reverse transcriptase polymerase chain reaction assay for detection and quantification of West Nile virus

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In order to improve and accelerate the detection of West Nile virus (WNV), a rapid and specific real-time reverse transcription polymerase chain reaction (rtRT-PCR) was established. Primers and probe were designed according to the conservative sequence of capsid protein gene of WNV. Tenfold successive dilutions of positive WNV DNA were used to measure the sensitivity of rtRT-PCR. The amplifying curve showed that this method could successfully amplify 10^3 copies/μl WNV gene, while reference to Japanese encephalitis virus (JEV) and blank control were all negative. The assay system showed high reproducibility with coefficient of variation (CV) < 2%. The detection of WNV can be completed within 2 to 3 h. By detecting cDNA samples (n = 55) with rtRT-PCR and the conventional PCR assay, the established rtRT-PCR showed 96.36% (37 + 16/55) coincidence rate with the conventional PCR. All the results showed that the newly established rtRT-PCR assay was shown to be a rapid, sensitive and specific test for detecting WNV.

Key words: West Nile virus, capsid protein gene, real-time RT-PCR.

INTRODUCTION

West Nile virus (WNV) is an arbovirus (genus Flavivirus; family Flaviviridae) that was first isolated in a fever woman’s blood in Uganda in 1937. WNV has a wide geographical range that includes portions of Europe, Asia, Africa, Australia (Kunjin virus) and North, Central and South America. According to the Centers for Disease Control and Prevention of USA, from 1999 to 2010 there have been over 30,622 human cases of confirmed symptomatic WNV infection in the USA with over 1163 fatalities (De Filette et al., 2012). In the recent years, it also was separated from migratory birds in some regions of Xinjiang Uigur autonomous region of China (Petersen and Roehrig, 2001; Sambol et al., 2009). WNV is transmitted to vertebrates by infected mosquitoes or tick vectors. Mosquitoes of the genus Culex are the main vectors of WNV (Esteves et al., 2005; Figuerola et al., 2007; Lanciotti et al., 2000). Wild birds are the reservoir host of WNV (Braunt et al., 2007; Komar et al., 2003; Wodak et al., 2011). WNV can disseminate through blood transfusion, which brought huge threat towards health safety of humankind (Nicolle et al., 2004).

Neither a vaccine nor an effective therapy has been developed against WNV infection in humans. WNV diagnosis is achieved routinely by serological assays; however, there is cross-reactivity with other flaviviruses. For this reason, plaque reduction neutralization tests (PRNTS) must be done as the reference assay for specific diagnosis. PRNT is a laborious and time-consuming technique that has to be carried out in biosafety level 3 (BSL-3) facilities with the consequent risks for the personnel involved in live virus manipulation.

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Development of an effective real-time reverse transcription PCR with a novel TaqMan probe. The assay should be economical for large-scale routine test of clinical samples and blood products in China.

MATERIALS AND METHODS

Virus strains, virus gene, reagents and sample

The WNV NY99 (AF 202541) strain and WNV cDNA sample were kindly provided by the Department of Haematology of the University of Cambridge. The live vaccine of JEV (Japanese encephalitis virus) was maintained in our lab. The Premis Ex Taq Hot Start version (TaKaRa, Dalian, China) was used in all real-time PCRs. The AMV system, plasmid kit, gel extraction kit and pGEM-T-easy vector were all bought from the Promega Company (Madison, Wisconsin, USA); the DNA/RNA nucleic acid extraction kit was bought from AXYGEN Company (San Francisco, California, USA).

Primers and probe design

Specific real-time primers and probe were designed according to the conservative region of capsid gene of WNV. The oligonucleotide sequences of the primers and the TaqMan fluorescence probe were as follows: Forward Primer (WP1): 5′-CAAGAGGCC GGCTGTCAATA-3′; Reverse Primer (WP2): 5′-CTTCAGGTCAATCGAAGGACAA-3′; TaqMan probe (WP): FAM-ATGCTAAACCGCGGAATGCCCG-CGTGTT-TAMRA. The primers and probe were synthesized by Beijing Sun-bio Company.

Preparation of standard templates of WNV

The RNA extraction of WNV was accorded to the manual of AXYGEN RNA extraction kit. Total RNA was extracted directly from 200 μl of the supernatant of infected cell cultures. The amplified segments were subcloned into pGEM-T easy vectors. Sequencing of the amplified segment was carried out by Beijing Sun-bio Company. Ten-fold successive dilutions of positive WNV DNA (10⁷ to 10³ copies/μl) plasmids were used as standard templates of WNV.

Real-time amplification

The standard templates (10⁷ to 10³ copies/μl) were amplified by real-time. Real-time PCR was performed in a final volume of 20 μl containing 2 μl 10×PCR Buffer, 4mM MgCl₂, 0.25 mM dNTPs, 2 μM CP1, 2 μM CP2, 2 μl WNV plasmid DNA templates, 5U TaqTM, 1 μM TaqMan probe. The RT-PCR mixtures were followed by denaturation at 94°C for 5 min and 40 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 30 s.

Specificity, sensitivity and reproducibility of the real-time PCR assay

The specificity of assaying system was determined by amplified WNV gene segments and JEV gene segments with the same primers, probe and reaction system of WNV. To measure the sensitivity of real-time assay for WNV, the assay was performed using serially diluted standard templates. The reproducibility of intra assay and inter assay was evaluated by Coefficient of Variance (CV).

Comparison between the conventional PCR and real-time PCR in human samples detection

Mimic cDNA samples extracted from human serum were detected with the procedure, and detected with the Conventional PCR assay (Yeh et al., 2010) to confirm the coincidence. 55 cDNA samples and 39 positive cDNA samples from humans infected with WNV were kindly provided by the Department of Haematology of the University of Cambridge, and 16 negative cDNA samples were extracted from human serum maintained in our lab.

RESULTS

Establishment of real-time PCR standard curve

Ten-fold serial dilutions of the standard templates were used in the real-time PCR assay. The Ct values for each dilution were measured in duplicate (Figure 1).

The log-linear regression plot generated from the collected data showed a strong linear relationship (Figure 2). The regressive equation: Y = -2.225X + 46.375; r = 0.967. By using the regressive equation, we were able to quantify the amount of unknown samples.

Specificity of the real-time assay

To determine assay specificity, JEV live vaccine strains and WNV gene were tested in the real-time PCR assay. The detection limit of the assay was determined to be 10³ copies/μl. The result indicated that the assaying system could not amplify the JEV gene segments. Thus, the results indicated a high specificity for assaying WNV.

Reproducibility of the real-time assay

The reproducibility of inter assay was assessed by testing the standard templates in different days. The reproducibility of intra assay was assessed by testing the standard templates in 6 repeats. The assay system showed high reproducibility with CV < 2%.

Detection specimens with the real-time PCR and the conventional PCR

By the real-time PCR and the conventional PCR assay, the cDNA samples (n = 55) were tested respectively. The established real-time PCR showed 96.36% (37+/16/55) coincidence rate with the conventional PCR (Table 1).
**Figure 1.** Establishment of the real-time PCR standard curve. 1, $1 \times 10^7$ copies/μl; 2, $1 \times 10^6$ copies/μl; 3, $1 \times 10^5$ copies/μl; 4, $1 \times 10^4$ copies/μl; 5, $1 \times 10^3$ copies/μl.

**Figure 2.** Standard amplification regression curve.

**Table 1.** Coincidence rate between the real-time PCR and conventional RT-PCR.

<table>
<thead>
<tr>
<th>Conventional RT-PCR</th>
<th>Real-time PCR</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
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<td>0</td>
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<tr>
<td>Negative</td>
<td>2</td>
<td>16</td>
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<tr>
<td>Total</td>
<td>39</td>
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**DISCUSSION**

Although virus isolation is still considered as the “gold standard”, it needs high-security laboratories and many days to obtain results. The novel real-time RT-PCR assays for the detection of WNV, which are faster and more sensitive than virus isolation, have been developed in recent years. Real time fluorescence quantitative PCR was developed based on conventional PCR, and it has several advantages over conventional PCR. Real-time is more sensitive and yields more information. Detection, classification and quantitation take place within one tube. Real-time have been extensively used for detection of many kinds of pathogens (Wacharapluesaddee et al., 2011; Jansen et al., 2011; Bennett et al., 2011).

TaqMan probe method is to use the 5’ end with fluorescent substances (such as: FAM), 3’-end with fluorescence quenching the material (such as: TAMRA). The fluorescent probes in TaqMan assay are known to be target specific and sensitive to mismatches. The real-time PCR assay permits the simultaneous detection and quantification of DNA. It is useful for understanding the pathogenesis of the disease and the mechanisms of virus
transmission by enabling the investigation of viral dynamics (Yang et al., 2009; Mackay et al., 2002). Compared to the conventional RT-PCR assay, this real-time RT-PCR was more sensitive and could detect 10^1 copies/μl WNV gene whereas the conventional RT-PCR had a detection limit of 10^3 copies/μl WNV gene. Lanciotti et al. (2000) developed a TaqMan qRT-PCR for rapid detection of WNV in human clinical specimens, this TaqMan RT-PCR could detect less than 1 PFU of virus. Compared with others, the first limitation of this present study is that the number of samples is small. However, even with this small number of samples, it also revealed that the developed real-time RT-PCR assay could be useful for rapid and sensitive detection of WNV. Secondly, restricted by policy of China, we only detected the cDNA samples. Finally, this study only detected WNV in human samples. Further evaluation of this assay to detect WNV in mosquitoes, birds and horses is needed.

With the increase of human movement, the migration of migratory birds, and the input of foreign blood products, the risk of WNV emergence in China is eminent. So, it was necessary to establish an assay method to detect WNV in China. The amplified segments in this study chose the most conservative capsid area of WNV genes. The development of the real-time RT-PCR assay described earlier represents a new step towards the control of exotic infectious diseases in China. The real-time RT-PCR described here for detection and quantitation of WNV has been shown to be sensitive and specific. It could be used for monitoring in WNV outbreak areas or as a screening method for WNV eradication strategies.

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REFERENCES


