Fast Duplex One-Step RT-PCR for Rapid Differential Diagnosis of West Nile and Japanese Encephalitis Viruses

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Abstract

The aim of this study was to develop a highly sensitive and specific one-step duplex RT-PCR assay for the simultaneous and differential detection of West Nile (WNV) and Japanese encephalitis (JEV) viruses. Bioinformatics analysis of published sequences of WNV and JEV revealed conserved regions not targeted by previously reported primers. A total of thirteen primers were designed based on these regions to detect all of the WNV and JEV lineages, and to discriminate between the two viruses by the generation of 482 and 241 base pair cDNA products, respectively. The results indicate that single-tube duplex PCR using these primers is a useful technique for the detection and differentiation of WNV and JEV in plasma or brain tissue. The novel duplex RT-PCR described in this study enables the early diagnosis of these two encephalitic flaviviruses. In addition, this technique may be useful as part of a testing regimen for human patients, horses, and other susceptible animal species as it is rapid (less than 3.5 h from RNA extraction), sensitive and specific, and may enable differential diagnosis of clinical samples.

Introduction

Species within the Flavivirus genus can cause public health problems around the world. For example, the increasing number of Dengue and Japanese encephalitis virus (JEV) infections in Asia, frequent outbreaks of Yellow fever in Africa and South America, and the ongoing spread of West Nile virus (WNV) throughout the Americas exemplify the geographical burden of flavivirus diseases. Global flavivirus incidence has grown in the past twenty-five years, and this increase is primarily due to unprecedented population growth and migration, uncontrolled urbanization, and lack of
effective mosquito control (9).

WNV and JEV are arthropod-borne flaviviruses ("arborviruses") that can emerge or re-emerge in many regions due to climate change and increased travel (1, 5, 11, 26, 35, 38). WNV and JEV are members of the Flaviviridae family and are arboviruses associated with both human and equine encephalitis worldwide. WNV circulates in Africa, West Asia, Southern Europe, and Australia. It emerged in North America in 1999, leading to an extensive and ongoing outbreak (40). Similar to the emergence and rapid spread of WNV in North America, it is possible that WNV could be introduced into Far East Asia from endemic continents through infected birds, travelers, or insect vectors. If a human pathogen like WNV emerges or re-emerges in a given country, very strict epidemiological regulations need to be immediately implemented. To monitor infection and prevent rapid viral spread in these cases, methods are required for rapid viral detection in suspect cases and potential vectors.

In areas where JEV is endemic, such as Korea and Japan, distinguishing between WNV and JEV is critical for the detection of WNV invasion because JEV is a mosquito-borne flavivirus from the JEV serocomplex that causes encephalitis in humans and horses, and is widespread throughout most of Asia (12, 27). However, Japanese encephalitis serocomplex flaviviruses cross-react antigenically with WNV and thus, are not readily differentiated by serology (10). Molecular diagnostic methods are therefore preferred, and reverse transcriptase-polymerase chain reaction (RT-PCR) methods have been used to develop sensitive and specific assays for the identification of WNV (13, 24). Recently, more sensitive assays, such as fluorogenic real-time (TaqMan) PCR, SYBR Green-based real-time PCR, and loop-mediated isothermal amplification
(LAMP), have been developed for diagnostic detection of WNV genes (16, 21-22). However, these diagnostic methods were only designed to detect strains of WNV isolated in the United States that are closely related to lineage I, and thus, may not detect lineage II strains associated with Africa (2) and more recently, with Europe (8) (17). In areas where JEV is endemic and WNV is absent, it is possible that other WNV lineages may emerge (i.e., lineage 2). Therefore, it is necessary to design specific assays that recognize all lineages of WNV and can distinguish between JEV and WNV in order to make a definitive diagnosis. Two molecular diagnostic methods that simultaneously discriminate between strains of WNV and JEV have been previously reported, one which uses RT-PCR and RFLP analysis, and another that uses fluorogenic real-time PCR (TaqMan) (32-33). In this study, our aim was to develop a more rapid molecular diagnostic method that could detect and distinguish between WNV and JEV using a conventional RT-PCR format in a single-tube duplex platform with a primer mixture specific to JEV strains and all of the lineages of WNV.

**Materials and Methods**

**Viruses**

WNV strains NY385-99 and B956 (American Type Culture Collection, ATCC) were used in this study. The NY385-99 strain (lineage I) was isolated from a snowy owl in New York during the 1999 outbreak (36), and the B956 strain (lineage II) was isolated from a woman in Uganda in 1937 (34). Anyang 300 (39), an attenuated JEV strain, was also used in this study. WNV and JEV were also used in specificity assays to evaluate primer sets for cross amplification. Because the envelope gene was the target gene
selected for differential diagnosis of WNV and JEV in the multiplex RT-PCR, the envelope genes of Dengue virus type 1 to 4 and tick-borne encephalitis viruses were synthesized to demonstrate specificity of the assay among viruses belonging to the flaviviruses family. The genes were cloned into the SacI/XhoI site in the pBluescript II SK (+) vector from New England Biolabs (UK), as live viruses were not available due to their biosecurity status. The following related viruses, which cause neurological conditions in animals or humans, were also employed for specificity assays: Akabane virus, Aino virus, equine herpesvirus 1 (EHV-1), encephalomyocarditis virus (EMCV), bluetongue virus, and Western equine encephalitis virus. The RNAs of following viruses were also included: RNAs extracted from the Cephalovac® VEWT vaccine containing inactivated Eastern, Western, and Venezuelan equine encephalitis viruses (EEEV, WEEV, and VEEV). All the viruses used in this study are listed in Table 1.

Virus cultures and quantitation of viral RNA

The flaviviruses, Akabane virus, Aino virus, EMCV, and WEEV were grown in Vero cells (ATCC CCL-81) in alpha minimum essential medium (αMEM) (GibcoBRL, Invitrogen Corporation, Carlsbad, California, US) containing 10% fetal bovine serum (FBS) (GibcoBRL, Invitrogen Corporation, Carlsbad, California, US) and an antibiotic-antimycotic mixture (Invitrogen) at 37°C in a humidified 5% CO₂ environment. WNV manipulations were performed in a biosafety level 3 (BSL3) containment research laboratory at the National Veterinary Research and Quarantine Service (NVRQS) in accordance with the regulations of the Korean government. EHV-1 was grown in RK13 cells (ATCC CCL-37) and the bluetongue virus was grown in BHK cells. Non-infected Vero, RK13 and BHK cell line cultures were used as controls in the specificity assays.
The detection limit of the assay was evaluated by both plaque forming units (PFUs) and RNA copy numbers. For the titration of WNV and JEV infectivity, a plaque assay was performed according to the method described by Payne et al (23). The viral titer was calculated and expressed as PFUs per milliliter. One PFU represents a circumscribed area of cellular degeneration initially produced by one virion. To calculate RNA copy numbers, viral RNAs of WNV and JEV were quantified by the method of Shi et al. (30) and Santhosh et al. (29).

**Primer design**

Bioinformatics analysis of published sequences of WNV and JEV revealed conserved regions not targeted by previously reported primers. These conserved viral genome regions were chosen as the best candidates for the generation of specific primers. A total of thirteen primers were designed within these regions for duplex RT-PCR to detect WNV and JEV based on the generation of 482 and 241 base pair (bp) cDNA products, respectively (see Table 2).

**Optimization and performance evaluation**

To optimize the reaction conditions, preliminary assays were performed to test different concentrations of each primer set in the duplex RT-PCR. Positive control plasma and brain homogenates were prepared by spiking samples with WNV and JEV cultured from cells, as positive brain and plasma samples were not available. Negative plasma and brain homogenate controls were also used. Plasma was harvested from live chickens and horses, and brain tissues were collected from carcasses of wild birds and horses. Each assay was performed in parallel with the corresponding uniplex RT-PCR.
for each viral and total RNA dilution. Finally, the combination of primer concentrations that yielded the best results for the target flaviviruses was selected and is shown in Table 2. Various amplification tests using the thirteen primers were performed in several reactions, and incubation profile conditions were investigated to select the best type of DNA polymerase and to establish the optimal reaction protocol for the duplex RT-PCR assay. Experiments were performed in triplicate with known titers (or RNA copy numbers) to determine the detection threshold for each virus.

**RNA extraction and RT-PCR**

Total nucleic acids were extracted from 400 µL of plasma and from 10% brain homogenate supernatants spiked with WNV and JEV. Automated extraction was performed using a BioRobot® M48 workstation apparatus (Qiagen, GmBH, Hilden, Germany) with a MagAttract® Virus Mini M48 kit (Qiagen, GmBH, Hilden, Germany). Nucleic acids were recovered in 50 µL of elution buffer. Eluted RNA was stored at -70°C until use, and 10-fold serial dilutions were prepared with the same diluent. The duplex RT-PCR was performed using a one-step RT-PCR kit (Qiagen, GmBH, Hilden, Germany). The reactions were prepared in a volume of 25 µl containing 2 µl RNA, 1× Buffer [Tris-Cl, KCl, (NH₄)₂SO₄], 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 µM each of the specific primers for uniplex RT-PCR or the primer mixture for duplex RT-PCR, 5 U RNase Inhibitor (Intron Biotechnology, Korea), and 1 µl Enzyme Mix (Omniscript and Sensiscript Reverse Transcriptases, HotStartTaq DNA polymerase, Qiagen, GmBH, Hilden, Germany). Reverse transcription-amplification was accomplished in one step with the following optimized incubation program: 30 min at 50°C, 15 min at 95°C, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 1 min at 72°C. RT-PCR
amplifications were performed using an Eppendorf Mastercycler gradient thermal cycler (Eppendorf, Germany). RT-PCR amplification products (5 µl) were analyzed by gel electrophoresis on a 3% agarose gel containing 0.5 µg/ml of ethidium bromide.

**Determination of intra- and inter-assay reproducibility**

Spiked samples were prepared with different concentrations of the respective virus by diluting the virus containing cell supernatant in plasma and 10% brain homogenate. The lowest viral titer (or RNA copy number) with a 100% detection rate was considered the detection threshold. Samples were extracted and amplified three times in the same run to evaluate intra-experimental reproducibility and in eight different runs to evaluate inter-assay reproducibility.

**Results**

**Specificity**

The simplex and duplex RT-PCR for WNV and JEV were confirmed to be specific (see Fig. 1 and Fig. 2). In addition, the primer sequences used to detect WNV are common to all WNV lineages, and all primers were designed using the most recent WNV and JEV sequences published in Genbank. All WNV or JEV positive samples tested using the duplex RT-PCR assay amplified specifically according to the virus present in the sample, indicating that the assay was 100% specific for both viruses. The thirteen selected primers amplified 482 and 241 bp PCR products from WNV and JEV, respectively, and products were not amplified from negative control virus samples. To test whether the amplified PCR fragment corresponded to the expected virus, the PCR
product was run on a gel, and the band was excised and sequenced. Sequencing data confirmed the amplification of the expected product. In addition, all control plasma and brain homogenate samples also tested negative, indicating that the assay was completely specific for WNV and JEV.

**Sensitivity**

To evaluate the sensitivity of this method, three separate duplex RT-PCR experiments were performed on serial 10-fold dilutions of plasma and 10% brain homogenate suspensions containing a known titer of each target virus. WNV isolates NY385-99 and B956 were detected at a minimum titer of $10^{2.2}$ PFU/ml (corresponding to $10^{4.5}$ copies of RNA) and $10^{1.8}$ PFU/ml (corresponding to $10^{3.7}$ copies of RNA), respectively, in brain homogenates and in plasma. Experiments comparing the sensitivity of uniplex RT-PCR and duplex RT-PCR indicated that the duplex assay was 10-fold more sensitive for both lineage I and II WNV, while for JEV the sensitivity was similar for both reactions ($10^{2.4}$ PFU/ml (corresponding to $10^{4.1}$ copies of RNA) in brain homogenate and plasma) as seen in Fig. 3.

**Intra- and inter-assay reproducibility**

Different dilutions of the reference solutions were used as controls to assess the precision and reproducibility of the assay. The coefficient of variation was determined based on the values obtained from ten replicates (intra-assay variation) and between experiments (inter-assay variation). The intra-assay coefficient of variation ranged from 7% to 9% for WNV and from 3% to 7% for JEV. The coefficient of inter-assay variation ranged from 5% to 7% for WNV and from 2% to 11% for JEV in one-step single-tube
duplex RT-PCR. This analysis was conducted in triplicate in eight independent experiments.

**Discussion**

Molecular techniques are more rapid and sensitive than culture-based techniques (particularly when immunocomplexes are formed) for detecting viruses and do not require a BSL3 laboratory. To our knowledge, the RT-PCR assay described in this study is the first one-step single-tube duplex RT-PCR assay developed that allows simultaneous detection of WNV and JEV. Under laboratory conditions, automated nucleic acid extraction (55 min) followed by RT-PCR amplification and gel electrophoresis (150 min) provides a diagnostic result in approximately 3.5 h. In addition to diagnosis, the method described here may be useful for epidemiological surveillance and screening blood donors, and thus could be used during outbreak periods. This method is also cost-effective, as two flaviviruses can be detected in a single assay from a single extract. Duplex RT-PCR might also be useful for identifying viruses in coinfected mosquitoes and measuring their relative abundance in areas where targeted arboviruses circulate.

This study shows that WNV and JEV can be detected using the same plasma extract or brain homogenate through a novel duplex RT-PCR assay, enabling early diagnosis of these flaviviruses. This is of particular interest because these viruses can produce similar symptoms and there is a risk of overlooking exotic WNV cases in a JEV endemic area. To date, a WNV outbreak has not been reported in Far East Asia. However, some reports have documented the potential risk of WNV introduction into
this region (20, 28, 37). Recent increases in travel enhance the chance that arboviral
diseases may emerge or re-emerge in tropical regions, as demonstrated by the recent
chikungunya outbreak in India (25), the emergence of dengue in Hawaii (6), and the
extensive West Nile fever outbreak in the USA (4). In addition, non-tropical areas are
also at risk due to climate change (15), as shown by WNV cases in Europe and the
Mediterranean basin (40), and sporadic chikungunya cases in Italy (7). Arboviral
diseases are also endemic in some regions, such as in Brazil (3), where dengue virus
outbreaks are recurrent. To prevent these outbreaks, there is a need for methods to
rapidly detect viruses in suspect cases and determine the presence of viruses in vectors.
Epidemiological surveillance is essential for outbreak monitoring and disease control,
and should ideally involve diagnostic tools such as the duplex assays described herein.

The development of a rapid, specific and sensitive duplex one-step RT-PCR assay
for detection of all WNV lineages and JEV described in this study allows for the
detection and differentiation of WNV and JEV in a single run from a single extract.
Thus, this assay has the potential for use in clinical diagnosis and epidemiological
surveillance. It is also cost-effective compared to corresponding simplex assays. This
novel single-tube duplex PCR may also be useful as part of the testing regime for horses
and human patients with viral encephalitis, or for surveillance of birds or mosquitoes.

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supported by a grant from the National Veterinary Research and Quarantine Service,
References


34. Smithburn, K., T. Hughes, A. Burke, and J. Paul. 1940. Neurotrophic virus isolated from


Table 1. The neurological viruses and flaviviruses used in this study

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<th>Family</th>
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<th>Species *</th>
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*WNV, West Nile virus; JEV, Japanese encephalitis virus; DENV, dengue virus; TBEV, tick-borne encephalitis virus; EH-1, equine herpesvirus 1; EMCV, encephalomyocarditis virus; BTV, bluetongue virus; WEEV, Western equine encephalitis virus; EEEV, eastern equine encephalitis virus; VEEV, Venezuelan equine encephalitis virus. * RNAs extracted from the Cephalovac® VEWT vaccine. * E, encephalitis (encephalomyelitis or meningoencephalitis); AS, abortion or still-births; M, myocarditis; CA, Congenital abnormalities of the central nervous system; HC, hydranencephaly, cerebral cysts or cerebellar hypoplasia; HF, (hemorrhagic) fever. * NVRQS, National Veterinary Research Quarantine Service, Anyang, Republic of Korea; ATCC, American Type Culture Collection, Manassas, VA, US; IAH, Institute for Animal Health, Pirbright, UK; BI, Boehringer Ingelheim Vetmedica, Missouri, US. * GenBank accession number.
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†Abbreviation for mixed base code. Y = c, t (pyrimidine); K = g, t (keto); R = a, g (purine).
Figure Legends

Figure 1. Gel electrophoresis of the uniplex RT-PCR products. JEV is indicated by PCR product of 241 bp and WNV by PCR product of 482 bp. M, 1kb DNA molecular weight marker (100bp DNA ladder, Bioneer); Lane J, Anyang300 strain of JEV; Lane WN, NY385-99 strain of WNV; Lane WB, B956 strain of WNV, respectively.

Figure 2. Multiplex RT-PCR amplification of WNV and JEV. M, 1kb DNA molecular weight marker (100bp DNA ladder, Bioneer); Lane JEV, Anyang300 strain of JEV; Lane WNV, NY385-99 strain of WNV; Lane WNV and JEV, WNV and JEV in a single tube, respectively.

Figure 3. Detection limit of multiplex RT-PCR assay for the detection of JEV (a) and WNV (b, NY385-99 and c, B956) using primer mixtures designed in this study. Values are plaque forming units (PFU) per ml. WNV isolates NY385-99 and B956 were detected at a minimum titer of $10^{2.2}$ PFU/ml (corresponding to $10^{4.5}$ copies of RNA) and $10^{1.8}$ PFU/ml (corresponding to $10^{3.7}$ copies of RNA), respectively, while for JEV the sensitivity was $10^{2.4}$ PFU/ml (corresponding to $10^{4.1}$ copies of RNA) in brain homogenate and plasma.
Figure 1.
Figure 3.