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Short communication

Simultaneous detection of West Nile and Japanese encephalitis virus RNA by duplex TaqMan RT-PCR

Silvia C. Barros[a], Fernanda Ramos[a], Líbia Zé-Zé[b], Maria J. Alves[b], Teresa Fagulha[a], Margarida Duarte[a], Margarida Henriques[a], Tiago Luís[a], Miguel Fevereiro[a,*]

[a] INIAV, Rua General Morais Sarmento, 1500-311 Lisboa, Portugal
[b] Center for Vectors and Infectious Diseases Research, National Institute of Health (CEVDI-INSA), Av. da Liberdade 5, 2965-575 Águas de Moura, Portugal

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A B S T R A C T

West Nile virus (WNV) and Japanese encephalitis virus (JEV) are important mosquito-borne viruses of the *Flaviviridae* family, associated with encephalitis, mainly in humans and horses. WNV is also pathogen for many bird species. The incidence of human and animal WNV infections in Europe has risen, mostly in recent years, and JEV was detected in 2011 in mosquitoes collected in Italy and may emerge in Europe in the same way as other flaviviruses had emerged recently (USUTU and Bagaza virus) and should be regarded as a potential threat to public health.

Prompt identification and discrimination between WNV and JEV provides critical epidemiological data for prevalence studies and public and animal health management policies. Here we describe a quantitative one-step duplex TaqMan RT-PCR, targeting non-structural protein 2A gene (NS2A-qRT-PCR), based on only one primer pair and two probes for differential diagnosis of WNV and JEV. Also this assay enables the detection of both WNV lineages (WNV-1 and WNV-2).

To access the specificity of NS2A-qRT-PCR a panel of different arboviruses were used. The assay was shown to be specific for both WNV lineages (WNV-1 and WNV-2), WNV related Kunjin virus and JEV, since no cross-reactions were observed with other tested arboviruses. Sensitivity of the assay was determined using serial dilutions of in vitro-transcribed RNA from WNV and JEV. The duplex NS2A-qRT-PCR assay was shown to be very sensitive, being able to detect 10 copies of WNV and JEV RNA.

This assay is a suitable tool for the diagnosis of WNV and JEV, and provides a valuable addition to the methods currently available for routine diagnosis of these zoonoses and for surveillance studies.

West-Nile virus (WNV) and Japanese encephalitis virus (JEV) are zoonotic mosquito-transmitted flaviviruses belonging to the family *Flaviviridae*, which cause public health problems worldwide. They have a single-stranded positive-sense RNA genome which encodes 10 proteins, three structural (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5).

Japanese encephalitis virus (JEV) causes acute encephalitis with a mortality rate up to one-third of infected patients, and nearly half of the survivors suffer neurological or mental sequelae (Vaughn and Hoke, 1992).

WNV is the most distributed arbovirus in the world (Gould et al., 2003; Kramer et al., 2008), while JEV is prevalent in Southeast Asia, Indonesia, Australia, Papua New Guinea, and Pakistan (Ghosh and Basu, 2009). In Europe, two recent reports were published concerning the detection of JEV in birds and mosquitoes collected in Italy (Ravanini et al., 2012; Platonov et al., 2012), raising the risk that JEV may emerge in Europe, in the same way as other flaviviruses (Usutu and Bagaza virus) have emerged (Vásquez et al., 2011; Gamino et al., 2012).

Phylogenetic studies based in the envelope protein show that WNV can be separated into two major lineages. WNV lineage 1 (WNV-1) is the most widespread, and has been detected for decades in Europe. It was assumed that these viruses are particularly pathogenic for birds, humans and horses (Solomon et al., 2003). WNV lineage 2 (WNV-2) was thought to be confined to sub-Saharan Africa and Madagascar until it was first detected in Europe in 2004 (Bakonyi et al., 2005). Initially it was postulated that WNV-2 was non-pathogenic for horses (Lanciotti et al., 1999) however retrospective studies demonstrated that some variants of WNV-2 can also cause severe symptoms in horses and humans (Botha et al., 2008; Venter et al., 2009).

Real-time RT-PCR methods are essential tools in laboratory diagnosis. Several methods have been developed for the laboratory diagnosis of WNV infection (reviewed by De Filette et al., 2012). Many of these methods were initially designed to detect strains of WNV-1 (Buckley et al., 2003; Lanciotti et al., 2000) but not

* Corresponding author. Tel.: +351 217115288.
E-mail address: miguel.fevereiro@iniav.pt (M. Fevereiro).

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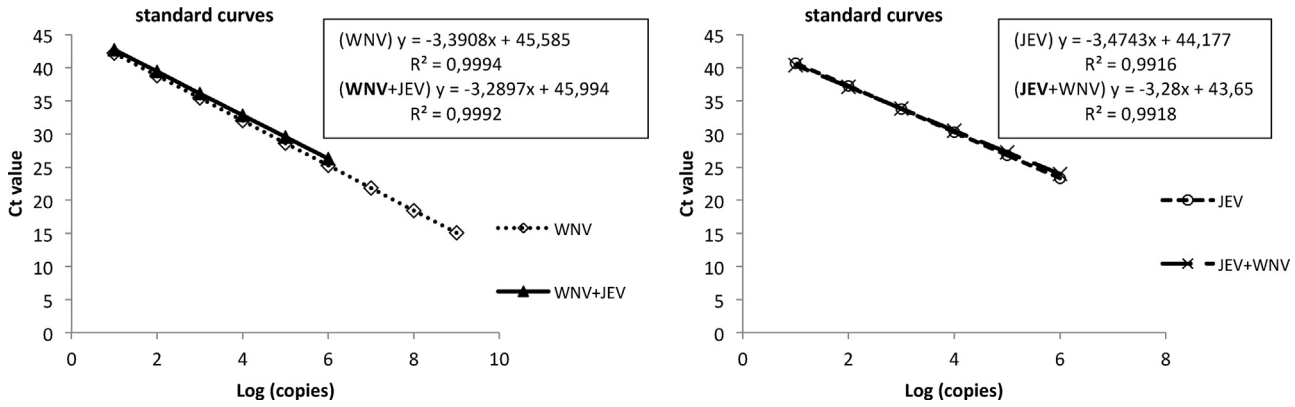

Fig. 1. Standard curves of TaqMan uniplex and duplex NS2A-qRT-PCR assay generated from the Ct values obtained against the log of known WNV and JEV RNA copies (dilution series of 10^1–10^9 WNV copies RNA per reaction and 10^1–10^6 JEV copies RNA per reaction). The regression equations are indicated.

strains of lineage 2, found in Africa and more recently in Europe. However, novel real-time RT-PCRs have been published for detection and discrimination of WNV-1 and WNV-2 (Del Amo et al., 2013; Eiden et al., 2010; Jimenez-Clavero et al., 2006; Linke et al., 2007). Moreover, molecular methods to discriminate between strains of WNV and JEV have been reported previously, using RT-PCR complemented with restriction fragment length polymorphism (RFLP) analysis (Shirato et al., 2003), conventional RT-PCR (Yeh et al., 2010) and real-time RT-PCR based on the use of a single probe and primer sets for both viruses (Shirato et al., 2005). Compared to the conventional RT-PCR, real-time RT-PCR is more sensitive, specific, rapid, and allowing quantification of viral genome; thus progressively replacing the conventional RT-PCR (Mackay et al., 2002).

The aim of the present study was to develop a duplex one-step TaqMan quantitative real-time RT-PCR (qRT-PCR) assay targeting the NS2A gene (NS2A-qRT-PCR), based in only one primer pair and two probes, for rapid and specific detection of both WNV lineages and JEV. This assay was rendered quantitative by using known copy numbers of WNV and JEV RNA transcripts.

Specific primer pair and probes were designed manually based on alignment of WNV and JEV sequences retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/) and potential mismatched bases were taken into consideration (Table 1).

The duplex NS2A-qRT-PCR assay was carried out in 25 μl reactions using the one-step RT-PCR kit (Qiagen, Germany). Each reaction mixture contained final concentrations of 1 μM of each primer, 0.2 μM of each TaqMan probe, 1X one-step RT-PCR buffer, 0.4 mM dNTP mix, 1 μl enzyme mix (including RT and hot-start Taq polymerase) and 1 μl of RNA template. The reaction was performed using the following thermal cycling program: one cycle of RT at 50 °C for 30 min, one cycle at 95 °C for 15 min to activate the hot-start Taq polymerase, 50 cycles of amplification of cDNA with melting at 95 °C for 20 s, annealing at 50 °C for 30 s and elongation at 72 °C for 30 s. The negative control contained PCR-grade water instead of template RNA.

The specificity of NS2-qRT-PCR assay was evaluated by testing WNV and JEV specific probes against RNAs extracted from several arboviruses (Table 2). With the exception of Kunjin and JEV, provided as RNAs by the European reference laboratory (ANSES—Maisons-Alfort, France), all other viruses were grown in VeroE6 cells in minimum essential medium (MEM) (Invitrogen, USA) containing 10% fetal bovine serum (FBS) and an antibiotic-antimycotic mixture (Invitrogen, USA) at 37 °C in a humidified 5% CO_2 environment. Viral RNA was extracted using MagCore® viral nucleic acid extraction kit (RBCbioscience, Taiwan) according to the manufacturer's recommendations.

As a control to monitor the integrity and specificity of RNA, an in-house pan-FLAVI conventional RT-PCR targeting the NS3 region was also performed (primers: 5461F-ATGGATGARGCTCAYTTCAC and 5669R-GTKATCCATYCRTATCCA) and the amplicons were subjected to sequence analysis to confirm their specificity.

The results demonstrated that the assay was completely specific for JEV and WNV RNA, detecting both lineages of WNV (WNV-1 and WNV-2) as well as the WNV-1b Kunjin virus (Table 2). No cross-reaction was observed with the other ten closely related flaviviruses nor with the CHIKV and Toscana arbovirus. Pan-FLAVI conventional RT-PCR assay yielded positive results for all flaviviruses tested, which was confirmed by sequence analysis. No amplification was detected when non-template control was used.

For sensitivity determination, in vitro-transcribed NS2A genes from WNV and JEV were used. A 136 bp portion of WNV-Eg 101 and JEV-Nakayama NS2A genes were amplified by NS2A primers. The amplicons were purified with the Qiaex II gel extraction kit (Qiagen, Germany) and cloned into the pCR2.1 vector using One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen, USA). Plasmid was extracted using the plasmid midi purification kit (Qiagen, Germany). The BamHI restriction enzyme (Biolabs, UK) was used to linearize NS2A-pCR2.1 plasmids prior to in vitro transcription with the MAXIscript kit (Ambion, UK), according to the manufacturer's instructions. Transcribed RNA was treated with RNase-free DNase and purified with the DyeEx 2.0 Spin kit (Qiagen, Germany).

Table 1
Primer pair and TaqMan probes for WNV and JEV detection.

Primer/probe	Sequence (5′ → 3′)	Tm (°C)	Position[d,e]
NS2A-F	CCTTTTCAGY[a]TGGGCCTTCTG	59.5	3548–3568[d] 3555–3575[e]
NS2A-3R	CAGTGTAV[b]GTV[b]ATR[c]CCCCCAA	60.8	3682–3662[d] 3690–3670[e]
WNVpNS2A-3612	FAM-AGCCAAGATCAGCATGCCAGC-TAMRA	61.8	3612–3632[d]
JEVpNS2A-3628	RED-TGACCATTCCTGCGGTTTTGGGG-BHQ	62.1	3628–3650[e]

[a,b,c] Degenerate nucleotides: Y = C or T; V = A, C or G; and R = A or G. Fluorophores: FAM (6-carboxyfluorescein) and RED (Texas red). Quenchers: TAMRA (6-carboxy-tetramethyl-rhodamine) and BHQ-1 (black hole dark quencher 1). [d,e] Numbering according to the sequences of WNV Eg101 (AF260968) and JEV (U15763) respectively.