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Analysis of real time PCR amplification efficiencies from three genomic region of dengue virus.

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Keywords: dengue virus, qPCR amplification, acute phase diagnosis, NS5 gene region.

Abstract. Early diagnosis of dengue virus (DENV) infection represents a key factor in preventing clinical complications attributed to the disease. The aim of this study was to evaluate the amplification efficiencies of an in-house quantitative real time-PCR (qPCR) assay of DENV, using the non-structural conserved genomic region protein-5 (NS5) versus two genomic regions usually employed for virus detection, the capsid/pre-membrane region (C-prM) and the 3'-noncoding region (3'NC). One-hundred sixty seven acute phase serum samples from febrile patients were used for validation purposes. Results showed that the three genomic regions had similar amplification profiles and correlation coefficients (0.987-0.999). When isolated viruses were used, the NS5 region had the highest qPCR efficiencies for the four serotypes (98-100%). Amplification from acute serum samples showed that 41.1% (67/167) were positive for the universal assay by at least two of the selected genomic regions. The agreement rates between NS5/C-prM and NS5/3'NC regions were 56.7% and 97%, respectively. Amplification concordance values between C-prM/NS5 and NS5/3'NC regions showed a weak (κ= 0.109; CI 95%) and a moderate (κ= 0.489; CI 95%) efficiencies in amplification, respectively. Serotyping assay using a singleplex NS5-TaqMan® format was much more sensitive than the C-prM/SYBR Green® I protocol (76%). External evaluation showed a high sensitivity (100%), specificity (78%) and high agreement be-

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tween the assays. According to the results, the NS5 genomic region provides the best genomic region for optimal detection and typification of DENV in clinical samples.

Análisis de las eficiencias de amplificación por PCR en tiempo real de tres regiones genómicas del virus dengue.

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Palabras clave: virus dengue, amplificación por qPCR, diagnóstico en fase aguda, Región génica NS5.

Resumen. El diagnóstico precoz de la infección por el virus dengue (DENV) constituye un elemento clave para la prevención de las complicaciones clínicas propias de la enfermedad. El objetivo del estudio fue evaluar la detección de DENV mediante un ensayo cuantitativo de PCR-tiempo real (qPCR), desarrollado localmente, utilizando la región no-estructural-5 (NS5), versus dos regiones tradicionalmente empleadas para la detección del virus, la región cápside/pre-membrana (C-prM), y la región noncodificante-3' (3'NC). Se recolectaron 167 muestras de suero de pacientes en fase aguda de la enfermedad. Las tres regiones génicas tuvieron perfiles de amplificación/coeficientes de correlación similares (0,987-0,999). Sin embargo, la región NS5 tuvo la eficiencia de amplificación más elevada para los cuatro serotipos (98-100%). Durante el proceso de validación, 41,1% (67/167) de las muestras de suero resultaron positivas para DENV al menos por dos de las regiones genómicas empleadas. Los valores de concordancia entre las regiones NS5/C-prM y NS5/3'NC fueron de 56,7% y 97%, respectivamente. La concordancia fue débil entre las regiones NS5/C-prM (κ= 0,109; CI 95%), sin embargo, fue moderada entre las regiones NS5/3'NC (κ= 0,489; CI 95%). El ensayo de tipificación uniplex en formato NS5/TaqMan® mostró alta sensibilidad (100%) que el protocolo C-prM/SYBRGreen®-I (76%). La validación externa del ensayo mostró una alta sensibilidad (100%), especificidad (78%) y acuerdo alto entre los ensayos utilizados. De acuerdo a los resultados obtenidos, la región NS5 ofrece la mayor opción para la detección y serotipificación del DENV en muestras clínicas.

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INTRODUCTION

From the clinical and epidemiological standpoint, dengue is the most important arthropod-borne viral infection (1). Worldwide, an estimated 2.5 billion people are at risk of infection, just about 975 million of whom live in urban areas in tropical and sub-tropical countries in Southeast Asia, the Pacific and the Americas (2, 3). The diseases is caused by any of the four antigenically related, but genetically distinct viruses named dengue viruses 1, 2, 3 and 4 (DENV-1 to DENV-4) transmitted to humans mainly by mosquitoes from the genus *Aedes*, being *Aedes aegypti* the main

vector (4, 5). Infection with any of the four DENV can derive in a wide spectrum of clinical presentations, ranging from apparent infection, or an influenza-like disease known as dengue, to a severe disease characterized by massive plasma-leakage or hemorrhagic manifestation that may eventually lead to shock and death, known as severe dengue as per the WHO/TDR classification(3, 6-8). Infection by a given serotype induces a lifelong protective immunity against the homologous serotype, but only a transient and partial protection against the three other serotypes (9). Indeed, secondary infection with another serotype is considered to be a major risk factor for developing severe dengue, presumably due to a phenomenon called antibody-dependent enhancement (10, 11) and immune response and inflammation (12, 13).

Like other members of the *Flaviviridae* family, DENV has a positive-sense single-stranded RNA genome of approximately 11kb in length, surrounded by a nucleocapsid and covered by a lipid envelope (14, 15). The RNA genome contains a single open reading frame flanked by 5' and 3' non-coding regions (5'NC and 3'NC), that encodes a polyprotein which is cleaved co- and post-translationally by serine-proteases to produce three structural proteins (C-prM/M-E) and seven nonstructural proteins (5'-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3') (14, 16).

Confirmatory diagnosis of dengue virus infection can only be made by laboratory techniques (17-20). Isolation of virus in cell culture remains the gold standard method, however, it requires long processing time, and biosafety laboratory procedures which result impractical in most situations (21). Detection of circulating antigens like NS1 is an excellent tool for early diagnosis, but does not reveal the infected serotype (22). Finally, the serological test based on anti-dengue IgM or IgG detection by enzyme-linked immunosorbent assay (ELISA), is the most commonly used criteria for presumptive diagnosis of DENV infections, however it has a major limitation because it requires serum samples of patients with at least 5 days after the fever onset(18, 23-25). Thus, there is a need for rapid and sensitive methods for detection of DENV early in the course of infection, for a better patient management (26).

In the past 10 years, several amplification methods, based upon reverse transcriptase polymerase chain reaction (RT-PCR) for detection and serotyping DENV in serum have been reported (19, 20). The most widely used test is the Nested RT-PCR developed by Lanciotti *et al*. (27). Real time PCR (qPCR) is the most recent format of this assay with many advantages including short-time consumption, low-cross contamination and quantitative possibilities (28). Several authors have developed RT-qPCR for detection of all four serotype of DENV in clinical samples using diverse regions of the genome (29-31). Nevertheless, the continuing evolution of the viral RNA has caused difficulties to reach consensus sequences for a molecular diagnostic assay.

The aim of this study was to evaluate the efficiencies of amplification of an in-house qPCR assay using a portion of NS5 versus two genomic regions usually employed for virus detection, C-prM and 3'NC. Assays to evaluate amplification efficiencies among the C-prM/NS5/3'NC regions were formulated using a one-step RT-qPCR SYBR Green® I protocol. Two RT-qPCR protocols were used for the validation process in human serum specimens, the first one corresponded to a one-step RT-qPCR SYBR Green® I assay using C-prM/NS5/ 3'NC regions, and the second one consisted in a RT-qPCR based on TaqMan® technology to amplify the NS5

region. Serotyping assay was performed using conventional RT-qPCR SYBR Green® I protocol for C-prM region and a RT-qPCR TaqMan® protocol for NS5 region.

METHODS

Virus strains

Strains of DENV (DENV-1/23644-07/16007-06, DENV-2/24344-04/16681-06, DENV-3/21429-04/1728-06 and DENV-4/8887-07/10377-06) were kindly provided by Dr. Guillermo Comach (Laboratorio Regional para el Diagnóstico e Investigación del Dengue y otras Enfermedades Virales, LARDIDEV, Maracay, Aragua state, Venezuela). Viruses were propagated and maintained in the mosquito cell line *Aedes albopictus* C6/36 and the infection was confirmed by indirect immunofluorescence (18). Viruses were quantified by plaque formation and viral title expressed as a plaque forming units per milliliters (PFU/mL) (32, 33). Several aliquots of all viruses were stored at –70°C until use.

Serum samples

One hundred sixty seven (n=167) samples were collected from patients clinically diagnosed as having an acute dengue infection (8). The serum samples were collected during two periods of the disease: 1-4 days after onset of symptoms for virological tests and 5-7 days after onset of illness, for serological tests. The sera were separated, aliquoted and stored at –70°C. Individuals and parents/guardian consents were obtained from each participant following the Helsinki's Declarations and WHO ethics principles in human research (34). The study was approved by the ethical committee of Universidad de Los Andes as well. Samples were processed at the Laboratorio de Microbiología y Salud Pública of Mérida state, Venezuela (LSPM).

RNA extraction

Viral RNA was extracted from 140 μL of infected C6/36-cell culture supernatant or serum samples using the QIAamp® Viral RNA Mini kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions (21). The RNA was eluted with 60 μL of the corresponding elution buffer and stored in –70°C until use. For quantitative assays, a 10-fold-dilution series containing a known infectivity of target viral RNA was used for RNA extraction. RNA quantification and purity (A260/A280 ratio of 1.8-2.1) was assessed by an UV spectrophotometer (UV1101/1101T-Biotech, Cambridge, UK).

Dengue virus RNA standard curve

Standard curves were generated from each virus serotypes in a five-point ten-fold dilutions for RT-qPCR quantification of viral load. Viral RNA titer was expressed in PFU/mL. The original titles determined in LARDIDEV by double-overlay plaque assay were DENV-1 8×10^3 PFU/mL; DENV-2 7.8×10^6 PFU/mL; DENV-3 5.8×10^3 PFU/mL and DENV-4 84×10^3 PFU/mL.

Primer and probes design

Specificities of primers were evaluated comparing complete genomes sequences of DENV-1, DENV-2, DENV-3 and DENV-4 available in GenBank. Sequences were aligned using the CLUSTAL X software (Program Molecular Evolutionary Genetics Analysis - MEGA version 4.0, Biodesign Institute, Tempe, Arizona, USA)(35). Three sets of primers targeting homologous region of all DENV were used for detection assay. For the serotype assay using C-prM region, a combination of the consensus sequence mD1_F as a sense primer and serotype-specific anti-sense sequences primers were used as described somewhere else (30) (Table I).

TABLE I
CHARACTERISTICS OF PRIMERS FOR DETECTION AND SEROTYPING ASSAY

Detection assay		
Genomic region	Primer designation	Nucleotide sequence (5'- 3')
C-prM	mD1_F	TCA-ATA-TGC-TGA-AAC-GCG-AGA-GAA-ACC-G
	D2_Rv	TTG-CAC-CAA-CAG-TCA-ATG-TCT-TCA-GT-TC
NS5	DUNS5_F	GGT-TAG-AGG-AGA-CCC-CTC
	DUNS5_Rv	GAG-ACA-GCA-GGA-TCT-CTG
3'NC	3'NC_F	TTG-AGT-AAA-CYR-TGC-TGC-CTG-TAG-CTC
	3'NC_Rv	GGG-TCT-CCT-CTA-ACC-TCT-AGT-CCT
Serotyping assay		
C-prM	rTS1_Rv	CCC-GTA-ACA-CTT-TGA-TCG-CT
C-prM	mTS2_Rv	CGC-CAC-AAG-GGC-CAT-GAA-CAG-TT
C-prM	TS3_Rv	TAA-CAT-CAT-CAT-GAG-ACA-GAG-C
C-prM	rTS4_Rv	TTC-TCC-CGT-TCA-GGA-TGT-TC

The NS5 protocol utilizes two Flaviviral consensus primers (mFU1 and CFD2) (TAC-AAC-ATG-ATG-GGA-AAG-CGA-GAG-AAA-AA/GTG-TCC-CAG-CCG-GCG-GTG-TCA-TCA-GC) and four dengue virus serotype-specific TaqMan® fluorogenic probes labeled at their 5'-ends with a reporter and a BHQ at 3'-ends (30). We modified three of all 5'-reporter probes according to our calibration systems (VIC-D2P/BHQ1; NED-D3P/BHQ1; JOE-D4P/BHQ1). Primer pairs and probes were commercially synthesized (Applied Biosystems, Foster City, California, USA) and had no homology with other Flavivirus members.

RT-qPCR SYBR Green® I assay

Detection and serotyping of DENV was performed using a one-step SYBR Green® I protocol in a 25 μL reaction containing 2X QuantiFast SYBR Green® I Buffer (QIAGEN, GmbH, Hilden, Germany), 20 pmol/μL of each primer, nuclease-free water and 100 ng of RNA. The amplification involved a reverse transcription at 50°C for 30 min; one cycle of initial denaturation of the reverse transcriptase and activation of the HotStartTaq Plus polymerase at 95°C for 5 min and 36 cycles at 95°C for 15 s, and 60°C for 31 s. The threshold cycle (Ct) value, at which fluorescence was detected above the threshold, was determined by a mathematical algorithm. A sample was defined as positive if the Ct value was above cycle 15 and below cycle 36, based on background cross-reactivity of the primers in non-template control reactions. For all four serotypes of DENV, viral load in dengue-positive patient's serum was determined using five-point ten-fold dilutions of serotype-specific viral RNA extracted from quantified virus stocks and expressed in PFU/mL. Since SYBR Green® I dye intercalates nonspecifically with any double-stranded DNA generated during PCR, a melting curve analysis was performed following amplification to confirm the identity of the amplified product by its specific melting temperature (Tm) profile (18). Tm curve analysis included one cycle of denaturation at 94°C for 1 min, followed by 78.5°C for 10 s and a ramp to 94°C at a rate of 0.1°C/10 s with

continuous fluorescence measurement. The Tm data was analyzed using the Sequence Detection software version 1.4 (Applied Biosystems, supporting program) and CFX Manager Software version 1.6 (Bio-Rad Laboratories, Hercules, California, USA). Amplification was performed in either ABI7500 (Applied Biosystems, Foster City, California, USA) and CFX96 (Bio-Rad Laboratories, Hercules, California, USA) systems.

DENV positive and negative samples were randomly selected for external, double-blind evaluation by a reference laboratory (Laboratorio-Biología de Virus, Instituto Venezolano de Investigaciones Científicas, IVIC, Miranda state, Venezuela) involving cell culture, end-point-RT-PCR (C-prM region) and NS1 protein detection.

RT-qPCR TaqMan® Assay

In order to improved uniplex/multiplex and sensitivity capabilities of dengue assay, a NS5/TaqMan® assay for serotyping DENV was performed. Reaction mixture (25 μL) was prepared using a One-step RT-qPCR kit (QuantiFast® Probe RT-PCR Kit QIAGEN, Hamburg, Germany) containing 2X QuantiFast Master Mix, 100 pmol/μL of each primer and 25 pmol/μL of each probes, 100 ng of RNA and nuclease-free water. The amplification involved a reverse transcription at 50°C for 30 min; one cycle of initial denaturation of the reverse transcriptase and activation of the HotStartTaq Plus polymerase at 95°C for 5 min and 40 cycles at 95°C for 15 s, and 60°C for 31 s. The data was analyzed using the Sequence Detection software version 1.4 (Applied Biosystems, supporting program) and CFX Manager Software version 1.6 (Bio-Rad Laboratories, Hercules, California, USA).

Dengue IgM ELISA

Serum samples were tested using the Dengue IgM Capture MicroELISA (UMELISA® Dengue IgMPlus, La Habana, Cuba) according to the manufacturer's instructions (36, 37).

Statistical analysis

Patient information was collected in a data collection questionnaire, designed "ad hoc". Data base and statistical analyses were performed using the EPI Info 2008, version 3.5 (Center for Disease Control and Prevention, Atlanta). The agreement rate, squared-Chi, Fischer test *p*-value (a measure of the imbalance in the distribution of discordant pairs), and Kappa value, were calculated. Depending upon the concordance force, a qualitative scale was used, <0-20: poor; 0.21-0.40: weak; 0.41-0.60: moderate; 0.61-0.80: good; 0.81-1.00: very good. Calculation was performed in a 95% of interval co-efficiency (IC).

RESULTS

One-step RT-qPCR SYBR Green® I assay

RNA isolated from a DENV panel was amplified using the appropriate universal primer sets for C-prM, NS5 and 3'NC genomic regions. Assays were optimized using identical RT-qPCR cycles and post-amplification analyses. The standard curves for the four serotypes of DENV had very narrow slope variation ranging from –3.2 to –4.2. Although all regions had similar amplification profiles (Ct/Tm) and correlation coefficients (0.987-0.999), NS5 had the highest qPCR efficiencies of 100%, 98%, 99.1% and 99.8% for DENV-1,-2,-3,-4, respectively compared with C-prM and 3'NC regions. Serials dilutions of viral RNA extracted from quantified virus stocks were used to evaluate the limit of detection (LOD). Detection sensitivity was highest for DENV-2 and DENV-4 when NS5 region was used (Table II).

One hundred sixty seven (n=167) acute phase serum specimens were used to evaluate the performance of the assay in de-

TABLE II
PERFORMANCE OF RT-qPCR ASSAY for ISOLATED DENGUE VIRUS USING c-prM, NS5 AND 3'NC REGIONS

Viruses	C-prM			NS5			3'NC		
	Ct (Tm)	E (%)-C	LOD (PFU/mL)	Ct (Tm)	E (%)-C	LOD (PFU/mL)	Ct (Tm)	E (%)-C	LOD (PFU/mL)
DENV-1	31.5 (83.6°C±0.26)	45.2-0.991	0.01	23.8 (84.9°C±0.31)	100.0-0.996	0.01	27.6 (83.9°C±0.61)	86.2-0.993	0.1
DENV-2	15.1 (83.3°C± 0.29)	46.0-0.987	0.1	14.0 (84.6°C±0.27)	98.0-0.999	0.001	19.7 (82.6°C±0.34)	85.0-0.981	0.01
DENV-3	13.7 (83.9°C±0.63)	53.1-0.998	0.1	14.0 (86.2°C±0.33)	99.1-0.998	0.1	18.6 (84.2°C±0.28)	85.0-0.996	0.1
DENV-4	24.1 (82.9°C±0.25)	47.3-0.997	0.1	24.6 (85.2°C±0.44)	99.8-0.999	0.001	27.2 (83.6°C±0.26)	92.1-0.989	0.1

C-prM, capside pre-membrane protein; NS5, non-structural protein 5; 3'NC, 3' non coding region. Ct: threshold cycle values. Tm = Melting temperature. E: Amplification efficiency calculated from the slope of Ct values and expressing as a percentage (%). C: Correlation Coefficient calculated based on the equation of the line R2. LOD: Limit of detection expresed in PFU/mL. DENV1 8×10^3 PFU/mL. DENV2 7.8×10^6 PFU/mL. DENV3 5.8×10^3 PFU/mL. DENV4 84×10^3 PFU/mL.

tecting and serotyping viral RNA. The results showed that 65.8% (110/167) of the samples tested were positive for DENV infection. Among the positive cases, amplification was observed in 60.9% (67/110) of the cases for DENV RNA using at least two of selected genomic regions. Out of these samples, 87.2% (96/110) were positive for dengue IgM on a second sample collected after 6 days of the disease onset and 45.4% (50/110) of serum samples collected within 4 days after the onset of illness were positive for both methods (serological and virological test). The agreement between NS5/C-prM and NS5/3'NC regions were 59.7% and 97% respectively. Kappa values showed a weak concordance between NS5/C-prM (κ= 0.109; CI 95%) and moderate between NS5/3'NC (κ= 0.489; CI 95%). Statistical analysis based on the score numbers for NS5/C-prM indicated that no significant difference was observed between the assays (p = 0.263). However, NS5/3'NC assays showed a better correlation (p < 0.0001) (Table III).

Twenty three serum samples positive for DENV infection by RT-qPCR SYBR Green® I assay using the NS5 region, were tested by RT-qPCR TaqMan® assay and analyzed for the agreement between both protocols, as shown in Table III. The results showed a a significant concordance between the RT-qPCR assays (95.5%, κ = 0.646; CI 95%).

For all four serotypes of DENV, viral load in dengue-positive patient's serum was determined using five-point ten-fold dilutions of serotype-specific viral RNA extracted from quantified virus stocks and expressed in PFU/mL. All four serotypes showed an excellent correlation between Ct value and infectious-virus titer over a broad dynamic range. The Ct value of each sample was plotted against a $\log_{10}$ virus titer of the corresponding DENV. All four DENV serotypes generated amplification curves using SYBR Green® I protocol, showing a 100% sensitivity at 95%IC (data non-shown).

When serotyping assay was analyzed, the TaqMan®/NS5 region was much more sensitive (100%) than the SYBR Green®

TABLE III
VALIDATION OF UNIVERSAL RT-qPCR SYBR GREEN® I AND TAQMAN® ASSAY IN SERUM SAMPLES

Genome region		NS5/SYBR Green I		Total/n (%)
		Positive/n (%)	Negative/n (%)	
C-prM/SYBR Green I	Positive/n (%)	37 (55.2)	0 (0.0)	37 (55.2)
	Negative/n (%)	27 (43.2)	3 (4.47)	30 (44.7)
	Total/n (%)	64 (95.5)	3 (4.47)	67 (100)
3'NC/SYBR Green I	Positive/n (%)	64 (95.5)	0 (0.0)	64 (95.5)
	Negative/n (%)	2 (2.98)	1 (1.49)	3 (4.47)
	Total/n (%)	66 (98.5)	1 (1.49)	67 (100)
		NS5/TaqMan		Total/n (%)
		Positive/n (%)	Negative/n (%)	
NS5/SYBR Green I	Positive/n (%)	21	0	21
	Negative/n (%)	1	1	2
	Total/n (%)	22	1	23

Agreement SYBR Green® I Assays: NS5/C-prM= 59.7%, κ= 0.109; CI 95%, p=0.263; NS5/3'NC= 97%, κ= 0.489; CI 95%, p< 0.0001. Agreement SYBR Green® I/NS5versus TaqMan®/NS5 Assay: 95.5% κ = 0.646; CI 95%

I/C-prM region (76%). Additionally, evaluation of assay for NS5 region showed amplification in 91% (61/67) of the patient's samples to one DENV serotype and 8.95% (6/67) to two serotypes simultaneously. This DENV co-infection was confirmed by a double-blind protocol in a reference laboratory, involving cell culture and End-point PCR. The agreement between both regions was 85% for DENV-1, 70% for DENV-2, 80% for DENV-3 and 57.1% for DENV-4. Kappa value showed a weak concordance (κ= 0.019; CI 95%) between NS5/C-prM regions for all four serotypes (Table IV).

One-step RT-qPCR TaqMan® Assay

In order to improve uniplex/multiplex and sensitivity capabilities of dengue RT-qPCR assay, an attempt for a TaqMan®/NS5 protocols for serotyping DENV was performed. Serotyping assay was performed using the specific primer/probe set for each DENV serotype (singleplex) independently; or one-tube combinations of two, three or four serotype-specific primer/probe sets (multiplex) protocol (30). The singleplex NS5/TaqMan® protocol generated amplification curves for all four DENV serotypes. However, when all four serotype-specific primers/probes sets were combined in one-tube format, during the multiplex assay, there were no amplification curves for DENV-1 and DENV-4. Therefore, even when the multiplex NS5/TaqMan® protocol offers the advantage of a one-tube reaction with real-time capability, comparison of assay sensitivity profile between singleplex (either SYBR Green® I or NS5/TaqMan®) and multiplex formats showed that there is a higher amplification efficiency when each serotype is performed separately (100%) than when combination of primer/probe sets are placed in one-tube (50% of sensitivity).

The standard curve obtained by plotting five-point ten-fold dilutions of RNA extracted from quantified virus stocks, indicated that the efficiencies of amplification (correlation coefficients) in the singleplex NS5/TaqMan® assay reached values of 84.7 (0.996), 76.0 (0.989), 73.1 (0.990) and 92.2 (0.997) for DENV-1, DENV-2, DENV-3 and DENV-4, respectively. Quantification assay estimated sensitivities of detection (in PFU/mL) ranges of 0.33-1.44 to DENV-1; 0.28-0.78 to DENV2; 0.52-0.81 to DENV-3 and 1.12-1.19 to DENV-4 (data non-shown).

TABLE IV
PERFORMANCE OF SEROTYPING DENGUE USING SYBR GREEN® I/C-PRM AND SINGLEPLEX TAQMAN®/NS5 ASSAY

Viruses	C-prM n/Positive Total (%)	NS5 n/Positive Total (%)	Agreement Rate (%)
DENV-1	12/67 (17.9)	13/67 (19.4)	85.0
DENV-2	21/67 (31.3)	30/67 (44.7)	70.0
DENV-3	8/67 (11.9)	10/67 (14.9)	80.0
DENV-4	5/67 (7.46)	8/67 (11.9)	57.1
Co-Infection	5/67 (7.46)	6/67 (8.95)	83.3
Non-determined	16/67 (23.8)	0 (0.00)	-
TOTAL*	67/67 (100)	67/67 (100)	-

C-prM (Capside/pre-membrane protein), NS5 (non-structural 5 protein). SYBR Green® I(C-prM)/TaqMan® (NS5) κ= 0.019; CI 95%. Co-Infection detected DENV-1/DENV-2; DENV-1/DENV-4; DENV-2/DENV-3; DENV-2/DENV-4.

Double-blind evaluation from an external reference laboratory

Ninety two randomly selected acute serum samples and isolated viral RNA from DENV positive and negative's patient samples were externally evaluated by a reference Laboratory (IVIC). Viral cell culture and NS1 determination were performed in some of the samples due to the lack of enough material for all assays. Serum samples were inoculated as viral seeds in C6/36 cell system. The PCR amplifications were performed by semi-nested end-point RT-PCR format as previously published (22).

Agreement between universal assays, RT-qPCR SYBR Green® I/NS5 and Nested-RT-PCR/C-prM, showed a true positive result by both assays in 61.3% (57/93), true negative in 30.1% (28/93), differences in positivity in 8.6% (8/93), and in negativity in 0% of the cases, resulting in a significant difference (p=0.00001), high sensitivity (100%) and specificity (78%) and κ= 0.811. The nucleic acid amplification of C-prM gene area by end-point RT-PCR showed the presence of dengue virus in 61.3% (57/93) of samples, where DENV-2 was also the most frequently amplified in 26.3% (15/57), followed by DENV-4, DENV-1 and DENV-3 (15.7%, 12.3% and 8.7%) respectively. In 17.5% (10/57) of cases, co-infection was observed, where DENV-1/DENV-2 was the most common. Using the same gene area by RT-qPCR, DENV-2 was amplified in 18.8% (12/64) of the cases, followed by DENV-1, DENV-3 and DENV-4 (18.8%, 7.8% and 7.8%) respectively. In 9.4% (6/64) of cases, co-infection was observed, where DENV-1/DENV-2 was also the most common (Table V). In both centers, even

TABLE V
VIRAL ASSAYS FOR EXTERNAL EVALUATION OF THE IN HOUSE RT-qPCR SYBR GREEN® I/NS5 AMPLIFICATION

	LSPEM	Reference Laboratory (IVIC)		
Universal assay	RT-qPCR/NS5 (n=96)(%)	Nested-RT-PCR/C-prM (n=93) (%)	Viral Culture (n=38)(%)	NS1 (n=87) (%)
Positive	64 (66.6)	57 (61.3)	15 (13.2)	41 (47.1)
Negative	32 (33.3)	36 (38.7)	23 (60.5)	46 (52.8)
Serotyping	RT-qPCR/NS5 (n=64)(%)	Nested-RT-PCR/C-prM (n=57)(%)		
DENV-1	12 (18.8)	7 (12.3)	3 (20)	-
DENV-2	24 (37.5)	15 (26.3)	1 (6.6)	-
DENV-3	5 (7.8)	5¥ (8.7)	1¥ (6.6)	-
DENV-4	5 (7.8)	9 (15.7)	7 (46.6)	-
Serotype combinations	6*(9.4)	10*(17.5)	3 (20)	-
Non-determined Serotype	12**(18.8)	11**(19.2)	-	-

LSPEM: Laboratorio de Microbiología y Salud Pública, Mérida state, Venezuela; IVIC: Laboratorio Biología de Virus, Instituto Venezolano de Investigaciones Científicas, Miranda state, Venezuela.
*Serotype combinations: LSPEM: DENV-1/DENV-2=3; DENV-1/DENV-4=1; DENV-2/DENV-3=1; DENV-2/DENV-4=1. IVIC: DENV-1/DENV-2=4; DENV-1/DENV-3=1; DENV-1/DENV-4=1; DENV-1/DENV-2=1; DENV-2/DENV-3=3. ¥= one of the DV3 resulted positive by viral culture and Nested-PCR, however several bands were observed on the gels. Using RT-qPCR/NS5 amplification was positive for DV1. **Serum samples were positive by the universal PCR assay, however serotype were not determine. Analysis RT-qPCR SYBR Green® I/NS5 versus Nested-RTPCR/C-prM: p=0.00001. Sentitivity=1; Specificity=0.78; VPP=0.88; VPN=1. κ = 0.811.

though universal assay was able to amplify DENV in clinical samples, in 18.8% (12/64) for LSPEM and 19.2% (11/57) for IVIC serotyping was not determined.

DISCUSION

Early diagnosis of DENV infection is essential to early management decisions, to avoid clinical complications and also to develop epidemiological control strategies (17, 30, 38). Several qPCR based methods for detection of DENV have been reported in the last ten years (12-17). These assays have targeted different genomic regions such as envelope protein (E), pr-M protein, NS3, NS5 and 3'NC gene sequences (29, 30, 39, 40). A major concern in the design of RT-qPCR for DENV is the genetic variation in the nucleotide sequences of the viruses. One of the strategies to resolve the lack of amplification had been the use of degenerated primers to encompass all possible permutations (27). The aim of our study was to evaluate the amplification efficiencies of NS5, one of the most conserved regions in DENV genome compared with traditional areas of amplification such as C-prM and 3'NC regularly used for detection/serotyping of DENV in clinical samples. Targeting a gene in the most conserved region of the genome such as NS5, which encodes the viral RNA-dependant-RNA-polymerase will result in the ability to detect DENV with high specificity. Within this region, the degree of variation is relatively low (41). Studies where the NS5 region had been used, showed a good sensitivity and specificity of amplification for all virus serotypes when mosquito's and clinical samples were used (41-43). Therefore, an assay based on the NS5 region provides a rapid and accurate test for diagnosis of the disease.

Our data showed that the C-prM viral region have a low amplification efficiency (<60%) for all four DENV serotypes and a weak concordance compared with NS5, in comparison with similar publications (30, 44). The nature of dengue genomic variation is mainly attributed to a codon degeneracy phenomenon, especially for the polypeptide coding regions of capside, pre-membrane and envelope (39). Unpredictable mismatched sequences within the C-prM target genome may result in assay variations for DENV isolates of various origins (39, 45). The SYBR Green®-I assay using 3'NC region, had a better amplification efficiency (>80%) for all four DENV serotypes and a moderate concordance with NS5. The sequence of 3'NC region used in this study ranging from 10418 to 10564 nucleotides including at least 52 conserved nucleotides within the distal region allowing to distinguish among different DENV serotypes. Our results using this genomic region are consistent with previous reports by others (30, 39, 45). The existence of multiple primer sets all apparently capable to amplifying DENV, leads to the question as to which primer set is better, therefore according to our conditions and local endemnicity situation, the NS5 genomic region appears to be the best option for dengue virus detection followed by the 3'NC region with a specificity of 100% and amplification efficiencies over 90% for each DENV serotypes. The assay was also able to detect viral RNA in acute samples (45.5%) with detectable anti-dengue IgM in the second sample during the critical stage of the diseases (>4 days of fever onset). It is known that a specific antibody can neutralize DENV and subsequently prevents viral replication. Nevertheless, we had serum samples in acute phase (1-4 days after the onset the fever) of the disease with detectable viremia and immune response (anti-dengue IgM). Similar results have been published by other authors (46). This fact could be explained by two reasons, patients did not

clearly indicate the day the onset the symptoms; or local high-endemnicity with sequential infections and early rise of IgM antibodies.

The sensitivity of the RT-qPCR in terms of PFU equivalents/mL using NS5 region was highest for DENV-2 and DENV-4 (0.001 PFU/mL) followed by DENV-1 and DENV-3 (0.01-0.1 PFU/mL). Sensitivity differences between patients may be due to the degree of infection, IgM immune-complexes and replication rate (47). Therefore, viremia could be affecting the capability of diagnosis, such as viral culture, PCR amplification and other viral assays, during the acute stages of the diseases.

Although the multiplex assay offers the advantage of a one-tube reaction with real-time capability, in this study the multiplex TaqMan®/NS5 assay was not able to amplify the four serotypes of DENV simultaneously. Similar difficulties had been reported by other investigators (29, 30). Furthermore, amplification can be affected by a competition phenomenon between the different virus serotypes, primers and probes present in the same sample or lack of a suitable stretch of conserved nucleotides for designing the conventional TaqMan® probes, earlier observed by Laue *et al*. (47). Johnson *et al*. (31) developed in 2005 a single tube multiplex RT-qPCR TaqMan® assay for DENV detection using four different serotype-specific sets of primers/probes; however, this assay does not use consensus genomic regions of DENV, which eventually does not favor the detection of DENV serotypes from different geographic regions(31). In spite of the difficulties encountered by the multiplex format of the TaqMan®-NS5 protocol, the singleplex assay was more sensitive (100%) than the SYBR Green® I-C-prM protocol (76%).

The external evaluation of the in-house universal assay, using NS5 resulted in a high sensitivity compared with C-prM region. It is important to highlight those difficulties in the serotyping, characterized by undetermined DENV amplification, combinations and genotypes in the C-prM assays. The results of this study support the hypothesis that the distal portion of the NS5 region is a serotype-specific and well-conserved region for DENV isolates for DENV circulating in America, specifically in Venezuela. It is recommended to perform an initial screening of acute samples with a SYBR Green I/NS5 region assay and subsequently serotyping with a TaqMan-NS5 assay. Therefore, NS5- based assays could be an excellent tool for routine laboratory diagnosis of acute DENV infection and consequently, to evaluate the association of the viral load with the disease severity in different dengue endemic areas.

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