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Major Article

Evaluation and optimization of SYBR Green real-time reverse transcription polymerase chain reaction as a tool for diagnosis of the *Flavivirus* genus in Brazil

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Abstract

Introduction: The genus *Flavivirus* includes several pathogenic species that cause severe illness in humans. Therefore, a rapid and accurate molecular method for diagnosis and surveillance of these viruses would be of great importance. Here, we evaluate and optimize a quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) method for the diagnosis of the *Flavivirus* genus. **Methods:** We evaluated different commercial kits that use the SYBR Green system for real-time RT-PCR with a primer set that amplifies a fragment of the NS5 flavivirus gene. The specificity and sensitivity of the assay were tested using twelve flaviviruses and ribonucleic acid (RNA) transcribed from the yellow fever virus. Additionally, this assay was evaluated using the sera of 410 patients from different regions of Brazil with acute febrile illness and a negative diagnosis for the dengue virus. **Results:** The real-time RT-PCR amplified all flaviviruses tested at a melting temperature of 79.92 to 83.49°C. A detection limit of 100 copies per ml was determined for this assay. Surprisingly, we detected dengue virus in 4.1% (17/410) of samples from patients with febrile illness and a supposedly negative dengue infection diagnosis. The viral load in patients ranged from $2.1\times10^7$to 3.4×10^3copies per ml. **Conclusions:** The real-time RT-PCR method may be very useful for preliminary diagnoses in screenings, outbreaks, and other surveillance studies. Moreover, this assay can be easily applied to monitor viral activity and to measure viral load in pathogenesis studies.

Keywords: *Flavivirus*. Arbovirus. Real-time RT-PCR. Diagnosis.

INTRODUCTION

Members of the *Flavivirus* genus, the *Flaviviridae* family, are single-stranded, positive-sense ribonucleic acid (RNA) viruses with a genome approximately 11 kilobases in length that encodes three structural and seven non-structural proteins[1]. Most flaviviruses are maintained in nature via complex cycles that include arthropod vectors and vertebrate hosts. Currently, 57 *Flavivirus* species have been described worldwide; humans serve as the end hosts for most flaviviruses[2]. Many of these viruses pose significant public health problems, including dengue virus (DENV), Zika virus (ZIKV), yellow fever virus (YFV), West Nile virus (WNV), and Saint Louis encephalitis virus (SLEV)[3][4][5].

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Human flavivirus infection can lead to diseases ranging from mild fever to encephalitis and hemorrhagic fevers[1][2]. Therefore, for many flavivirus diseases, early diagnosis is essential for patient management and adoption of preventive measures[2][6].

The diagnosis of flavivirus infections is commonly performed using serological tests such as enzyme-linked immunoabsorbent assays (ELISAs) for immunoglobulin M (IgM)-capture, which are commercially available for a number of viruses[1][2][7]. The diagnosis of flavivirus infection can also be conducted by viral isolation in cell cultures including in C6/36 *Aedes albopictus* cells; however, this method is time-consuming and expensive and runs the risk of contamination. Alternative assays for the diagnosis or detection of flavivirus infection, such as flow cytometry and microarrays, have been reported; however, these assays are not used routinely because of their high costs and complexity[8][9][10]. Thus, the principal molecular method for the diagnosis and detection of *Flavivirus* species is reverse transcription polymerase chain reaction (RT-PCR)

developed for the main specific virus or multiplex causing human flavivirus diseases[11] [12] [13]. Here, we describe the evaluation and optimization of a broad specificity SYBR Green I real-time RT-PCR method for the universal detection of flaviviruses.

METHODS

Real-time RT-PCR

To evaluate the real-time RT-PCR method for the universal detection of flaviviruses previously described by Moureau et al.[11],we tested the following commercial kits: SuperScript III Platinum SYBR Green One-Step (Invitrogen, USA), SYBR Green PCR Master Mix (Applied Biosystems, USA), SYBR Select Master Mix (Applied Biosystems, USA), Power SYBR Green PCR Master Mix (Applied Biosystems, USA), and KAPA SYBR FAST Universal 2X quantitative polymerase chain reaction (qPCR) Master Mix (Kapa Biosystems, USA). All of the kits tested were used according to the manufacturer's recommendations. The assays were performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA).

Flaviviruses, RNA extraction, and cDNA synthesis

The flaviviruses used for evaluation of the real-time RT-PCR were propagated in C6/36 *Aedes albopictus* cells cultured in Leibovitz's-15 medium supplemented with 10% heat-inactivated fetal bovine serum, 50mg/mL of gentamicin, and 2mg/mL of amphotericin B (Vitrocell, Brazil). The infected cells were incubated for 5 to 10 days at 28°C. Next, viral RNA was extracted from the flaviviruses using the QIAamp viral RNA extraction kit (Qiagen, Germany) and converted to double-stranded complementary DNA (cDNA) using Superscript III reverse transcriptase (Invitrogen, USA), according to the manufacturer's instructions.

Standard curve, limit of detection, and specificity of real-time RT-PCR

To enable quantitation using real-time RT-PCR, we introduced a 272bp fragment of the NS5 gene ofthe YFV 17D vaccine strain into the pET28a vector and then transformed it into *Escherichia coli* DH5α cells (Invitrogen, USA), according to the manufacturer's instructions.The insertion was subsequently, confirmed by PCR and plasmid extraction using the QIAquick PCR Purification Kit (Qiagen, Germany). *In vitro* transcription was performed using the MEGAscript T7 Transcription kit (Invitrogen, USA), quantified with a Qubit 2.0 fluorometer (Invitrogen, USA) and stored at -70ºC. The number of RNA copies/ml was determined using the following formula: RNA concentration (g per ml)/the number of nucleotides in the transcript × $340 \times 6.022 \times 10^{23}$.

The standard curve for quantitation of RNA flaviviruses in real-time RT-PCR was created with five decimal serial dilutions of transcribed RNA, as described above. The real-time RT-PCR limit of detection for flaviviruses was determined in triplicate with serial decimal dilutions of *in vitro* transcribed RNA (2.29×10^{11} to 2.29×10^{-1} RNA copies per ml). Additional arboviruses, such as the Venezuelan equine encephalitis virus, Western equine encephalitis virus, Eastern equine encephalitis virus, Mayaro virus, Aura virus, and Mucambo virus were also tested to confirm the specificity of the assay.

Clinical samples

To evaluate the clinical applicability of *Flavivirus* real-time RT-PCR, we tested a total of 410 serum samples from patients with acute febrile illnessesand negative diagnosesfor dengue virususing conventional RT-PCR[14] or microarrays for arbovirus and robovirus (*unpublished data*), as described in **Table 1**. Of these sera samples, 88 were obtained during the dengue outbreak of 2011 to 2013 inthe City of São Jose do Rio Preto in São Paulo, Brazil, 55 were obtained during the dengue outbreak of 2006 to 2008 in Ribeirao Preto in São Paulo and 167 were obtained from patients with suspected dengue infection in 2011 in Sinop in Mato Grosso, Brazil. RNA was extracted from the samples and subjected to real-time RT-PCR.

Ethical considerations

This study was approved by the Human Research Ethics Committee of the School of Medicine of the University of São Paulo, Brazil (Process nr. 2013/164.277).

Nucleotide sequencing

The gene products amplified using our real-time RT-PCR were confirmed by nucleotide sequencing. The amplicons were treated with ExoSAP-IT (Affymetrix, USA) to remove unused deoxynucleotide triphosphates **(**dNTPs**)** and the purified DNA was sequenced using primers PF1S and PF2Rbis and the BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Mixture (Applied Biosystems, USA) following the manufacturer's protocol. The reaction was performed with an ABI Prism 3100 Sequencer Genetic Analyzer (Applied Biosystems, USA). The nucleotide sequences were analyzed using the Basic Local Alignment Search Tool for viral identification.

RESULTS

To evaluate SYBR Green I real-time RT-PCR for the universal detection of flaviviruses, several commercial kits were tested. The KAPA SYBR FAST Universal 2X qPCR Master Mix kit exhibited the maximal levels of sensitivity and specificity for RNA from several flaviviruses as well as *in vitro* transcribed RNAs. Evaluation of the other four commercial real-time RT-PCR kits for *Flavivirus* detection yielded different results; however, all kits had low or no amplification. Non-specific amplification and primer-dimers were also observed (**Figure 1**). Of the commercial kits evaluated using *Flavivirus* stocks, only SuperScript III Platinum SYBR Green One-Step produced viral amplification. Using the SYBR Green PCR Master Mix, SYBR Select Master Mix, and Power SYBR Green PCR Master Mix, it was possible to distinguish between positive and negative samples. However, these kits showed many primer dimers, precluding the use of these kits.

Using the KAPA SYBR FAST Universal 2X qPCR Master Mix, viral amplification was observed after 18 cycles, with the correct melting curve and without primer dimers. The optimal reaction mixture contained 2µl of cDNA template, 1µl of primer PF1S forward and 0.5µl of primer PF2R-bis reverse (at 10pmol/µl), 0.4µl of ROX (2X); 10µl of SYBR buffer (2X), and 6.1µl of diethylpyrocarbonate (DEPC)-treated water in a final volume of 20µl.

TABLE 1 - Positive samples identified in this study using our real-time RT-PCR for *Flavivirus*.

ID	Virus	T_M (ºC)	Viral load (RNA copies per ml)	Previous test
4	DENV type 4	81.76±0.10	1.1×10^6	De Morais Bronzoni, 2005
27	DENV type 1	79.92±0.00*	5.2×10^3	De Morais Bronzoni, 2005
2734	DENV type 3	81.76±0.10	4.4×10^3	Microaarray**
2502	DENV type 3	81.76±0.10	2.0×10^4	Microaarray**
3024	DENV type 3	81.76±0.10	9.0×10^4	Microaarray**
1279	DENV type 3	81.83±0.00*	5.5×10^3	Microaarray**
1290	DENV type 3	81.69±0.00*	5.6×10^3	Microaarray**
2582	DENV type 3	81.46±0.10	1.2×10^4	Microaarray**
2761	DENV type 3	81.84±0.00*	4.9×10^3	Microaarray**
2596	DENV type 3	81.98±0.00*	7.7×10^3	Microaarray**
2564	DENV type 3	81.84±0.00*	4.4×10^4	Microaarray**
1485	DENV type 3	81.77±0.09	5.1×10^3	Microaarray**
1465	DENV type 3	81.62±0.10	1.9×10^4	Microaarray**
327	DENV type 1	80.74±0.11	5.1×10^3	De Morais Bronzoni, 2005
301	DENV type 1	81.11±0.00*	5.0×10^3	De Morais Bronzoni, 2005
2531	DENV type 3	81.61±0.10	2.1×10^7	Microaarray**
1374	DENV type 3	81.47±0.10	3.4×10^3	Microaarray**

RT-PCR: real-time reverse transcription polymerase chain reaction; **ID:** identification**;** T_M: temperature melting; **RNA:** ribonucleic acid; **DENV:** dengue virus. *Standard deviation < 0.001. ** Unpublished.

The was amplification conditions consisted of: 95°C for 10 minutes for Taq polymerase activation and double-stranded cDNA denaturation, followed by 45 cycles of 95°C for 15 seconds for denaturation and 60°C for 1 minute for primer annealing. The amplification the genomes of different *Flavivirus* species was detected at the expected temperatures ranging from 79.92 to 83.49°C (**Table 2** and **Figure 2**). As expected, amplification of the genomes of other RNA arboviruses was not observed. To quantify viral RNA, a standard curve of serially diluted transcribed RNA was generated by plotting the C_T value against the number of copies of RNA per ml (**Figure 3**). The limit of detection was at least 100 copies of RNA per ml.

In our evaluation of real-time RT-PCR for the detection of flaviviruses, we detected *Flavivirus* viral genomes in 4.1% (17/410) of samples from patients with febrile illness anda negative diagnosis for dengue virus (**Table 1**). The amplicons were sequenced and compared with sequences deposited in GenBank, showing that the patients were infected with dengue virus types 1, 3, and 4 with viral loads ranging from 3.4×10^3 to 2.1×10^7 copies of RNA per ml (**Table 1**).

DISCUSSION

Flaviviruses are an important public health concern world wide[5] [6] [15]. Therefore, a fast, efficient, and sensitive method for *Flavivirus* diagnosis is essential for the management of infected patients[2] [16] [17]. In this study, we report the evaluation of real-time RT-PCR for the universal detection of flaviviruses as well as adaptations that allow for the measurement of viral load[11].

The use of real-time RT-PCR for the diagnosis of *Flavivirus* infection has many advantages compared with virus isolation, serological assays, and conventional RT-PCR. Virus isolation is considered to be the *gold standard* for the diagnosis of many *Flavivirus* infections[18]. However, this method has low sensitivity and requires several days for completion. Serological tests can cross-react between flaviviruses or have false negative results[18]. Compared with conventional RT-PCR, real-time RT-PCR has several advantages such as rapidity, low risk of false positive results, high sensitivity, specificity, and the possibility of viral quantification[12] [19] [20]. In fact, a previously described real-time RT-PCR method[11] was able to detect a broad range of flaviviruses found in Brazil[3] [21] [22]. This assay amplified the genomes of 12 mosquito-borne flavivirusesthat are among the principal arboviruses responsible for human disease in Brazil (**Table 2**)[3] [23]. The genomes of the flaviviruses tested were amplified using a melting curve of 79.92°C to 83.49ºC, demonstrating the wide range of detection of this assay. Real-time RT-PCR was specific and no amplification was observed for the genomes of other tested arboviruses, further demonstrating the specificity ofthis assay. Moreover, this primer set used in this study can be used not only for the detection of known flaviviruses but also to discover novel flaviviruses, as previously reported[24] [25] [26].

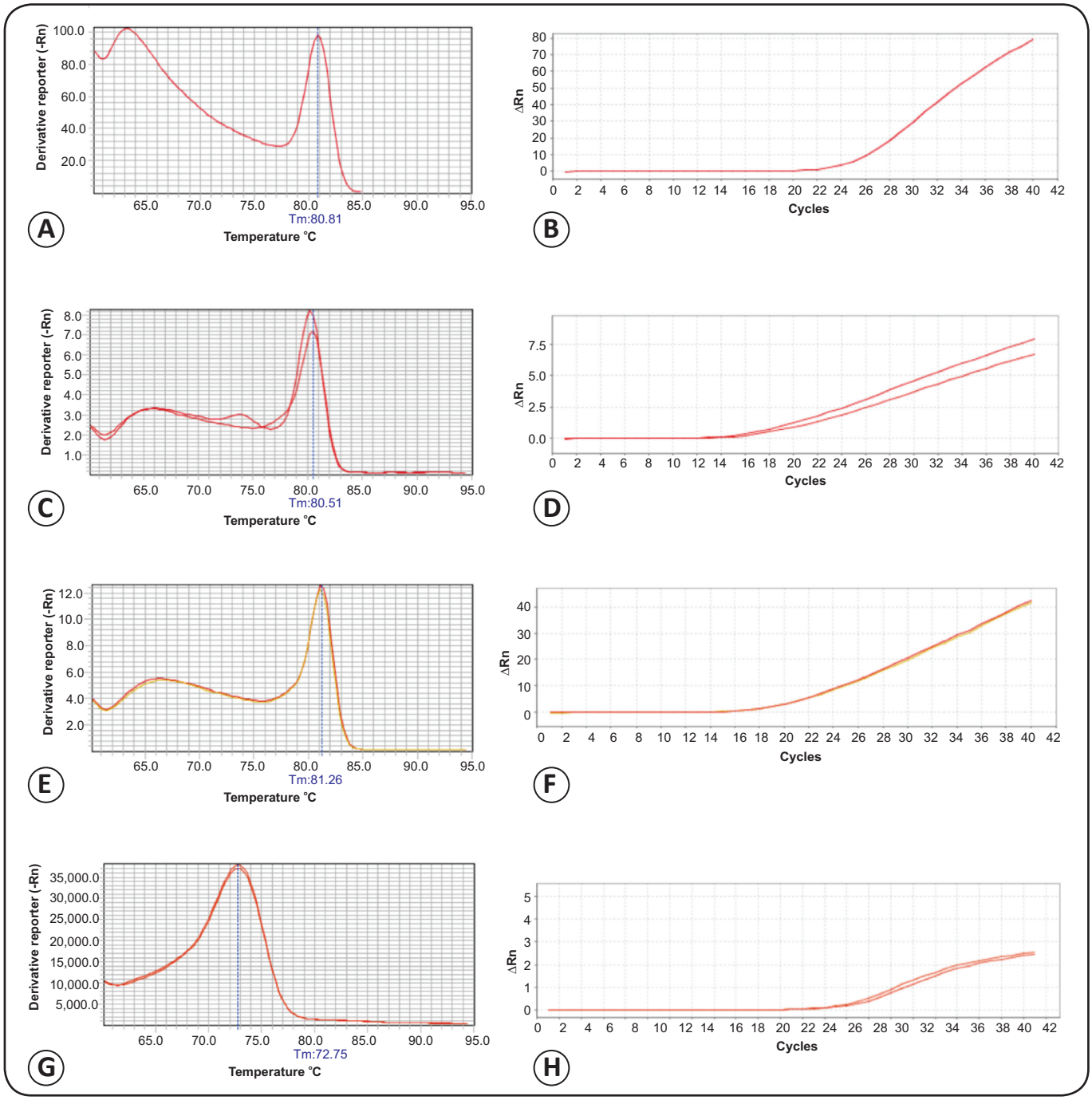

FIGURE 1 - Melting peaks and amplification curves of real-time RT-PCR for mosquito-borne *Flavivirus* using four different kits. A). Amplification curve using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). B). Melting peaks for real-time RT-PCR using Power SYBR Green PCR Master Mix. C). Amplification curve using SYBR® Green PCR Master Mix (Applied Biosystems). D). Melting peaks for real-time RT-PCR using SYBR® Green PCR Master Mix. E). Amplification curve using SYBR® Select Master Mix (Applied Biosystems). F). Melting peaks for real-time RT-PCR using SYBR® Select Master Mix. G). Amplification curve using SuperScript III Platinum SYBR® Green One-Step (Invitrogen, Carlsbad, CA, USA). H). Melting peaks for SuperScript III Platinum SYBR® Green One-Step. RT-PCR: real-time reverse transcription polymerase chain reaction.

TABLE 2 - *Flavivirus* strains detected by real-time RT-PCR.

Virus	Strain	TM (ºC)	Viral load*
DENV type 1	Mochizuki	83.41±0.10	2.0×107
DENV type 2	New Guinea C	82.22±0.10	4.0×106
DENV type 3	H-87	82.37±0.09	1.6×107
Dengue virus type 4	H-241	82.30±0.00**	1.5×106
Yellow fever virus	17D	83.11±0.10	1.3×108
West Nile virus	NY99	83.17±0.03	3.1×106
Rocio virus	SPH 34675	82.36±0.10	2.8×107
Iguape virus	SPAn 71686	83.40±0.00**	1.8×104
Cacipacore virus	BeAn 327600	83.48±0.00**	1.2×106
Ilheus virus	BeH 7445	82.29±0.00**	1.8×104
Bussuquara virus	Ar 41922	83.11±0.10	6.8×103
Saint Louis encephalitis virus	BeH-355964	82.81±0.10	1.2×108

RT-PCR: real-time reverse transcription polymerase chain reaction; **TM**: temperature melting; **DENV:** dengue virus. **RNA:** ribonucleic acid.*RNA copies per ml. **Standard deviation < 0.001.

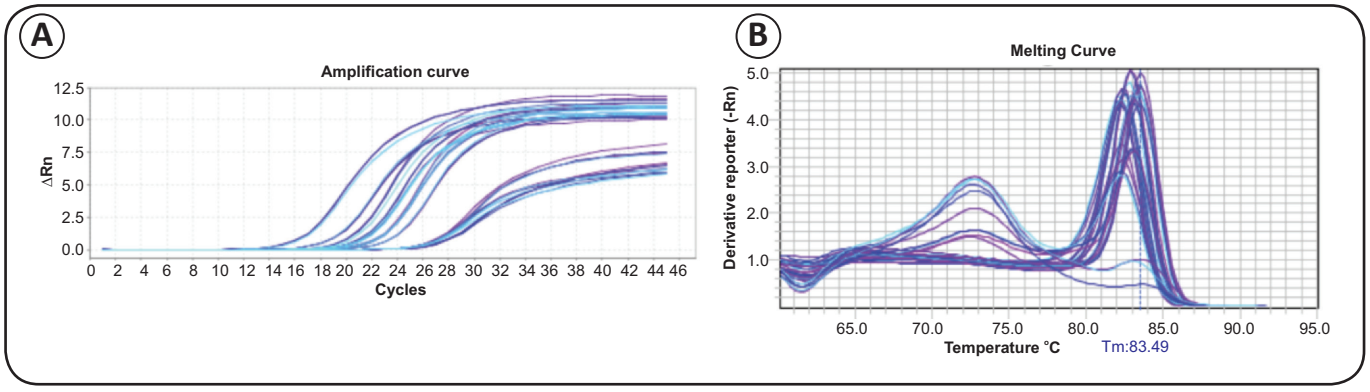

FIGURE 2 - Melting peaks and amplification curves of real-time RT-PCR for mosquito-borne *Flavivirus*. A). Amplification curve obtained for the flaviviruses described in Table 2. B). Melting peaks of real-time RT-PCR obtained for the mosquito-borne flaviviruses described in Table 2. RT-PCR: real-time reverse transcription polymerase chain reaction.

The high sensitivity of this real-time RT-PCR method for *Flavivirus* detection was demonstrated using *in vitro* transcribed RNA showing that 100 copies of viral RNA per ml could be detected. The results from patients with indications of dengue infection presenting a viral load of 1.5×10^4 to 9.9×10^5 copies per ml demonstrate the high sensitivity of our detection limit[27]. Other *Flavivirus* real-time RT-PCRs, such as those for DENV, JEV (Japanese Encephalitis virus), WNV, or *Usutu virus*, showed similar sensitivity (20 to 1,000 copies of viral RNA per ml); however, these assays do not exhibit as broad a range of *Flavivirus* detection as our assay[28] [29] [30] [31].

To evaluate real-time RT-PCR, the sera of patients with a negative diagnosis for the dengue virus were tested as described in the methods section. Our results indicated that 4.1% (17) of samples were *Flavivirus* positive; surprisingly, these positive samples were identified as dengue infection by nucleotide sequencing. However, although our assay exhibits a broad range of *Flavivirus* detection, this assay does not allow for the discrimination of viral species by melting temperature. Nevertheless, the *Flavivirus* amplicon of approximately 272bp obtained with this test allows for distinguishing and identifying different viruses by nucleotide sequencing, as performed in our assay[11]. This ability further enhances the high sensitivity observed using *in vitro* transcribed RNA, suggesting that the real-time RT-PCR for *Flavivirus* is more sensitive than the traditional RT-PCR assay[14].

In summary, the real-time RT-PCR method may be quite useful for preliminary diagnosis in infection screenings, outbreaks, and surveillance of flaviviruses, with Zika virus (ZIKV) in Latin America and especially in Brazil. This assay can also be implemented for monitoring samples from blood banks. In addition,

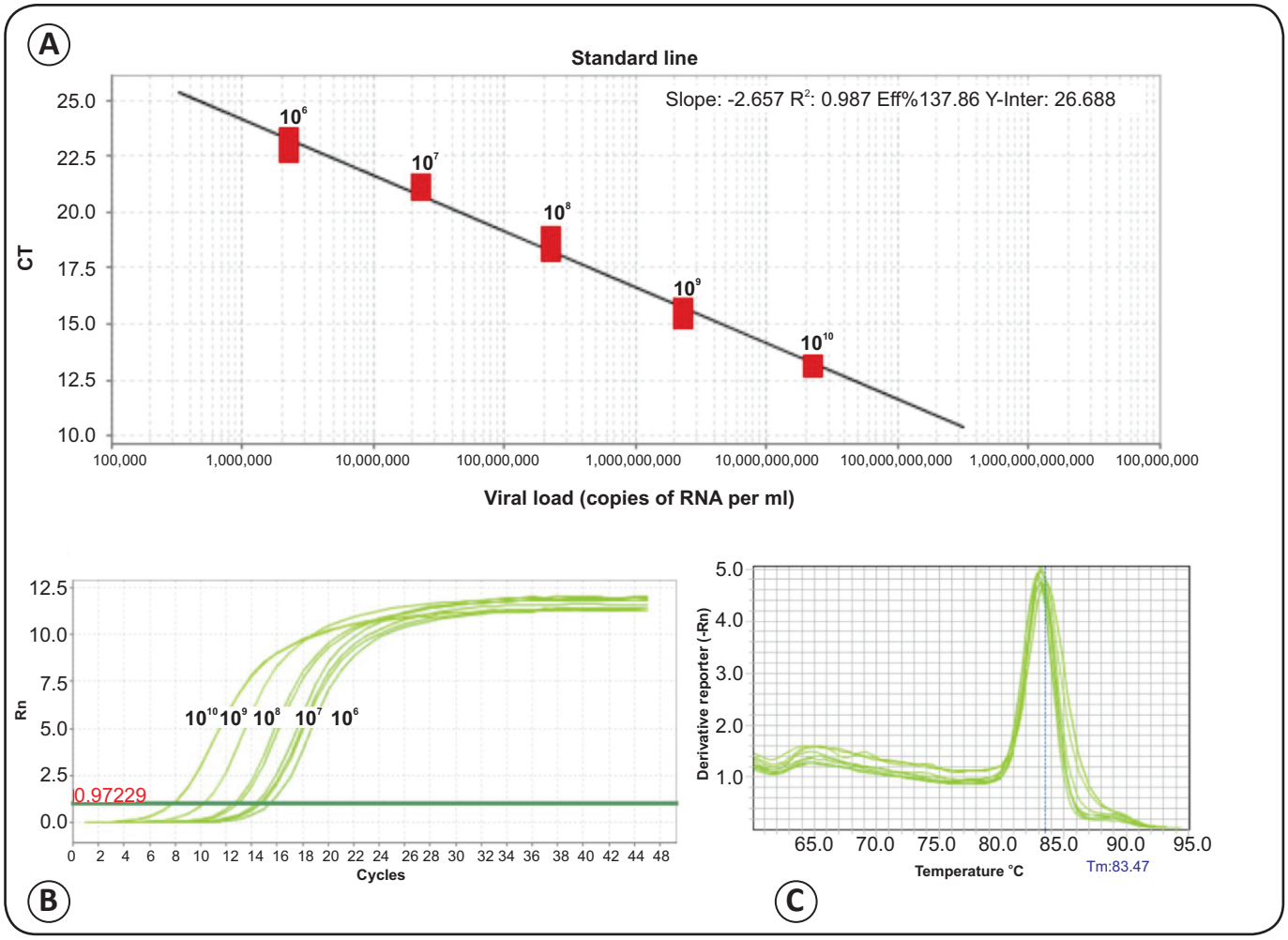

FIGURE 3 - Standard line of real-time RT-PCR with *Flavivirus* transcribed RNA. A). Standard line with serial decimal dilutions of transcribed *Flavivirus* RNA. B). Amplification curve obtained using serial decimal dilutions of transcribed *Flavivirus* RNA. C). Melting peaks for real-time RT-PCR using serial decimal dilutions of transcribed *Flavivirus* RNA. C_T: threshold cycle; **Δ*Rn:*** normalized reporter; **RT-PCR:** real-time reverse transcription polymerase chain reaction; **RNA:** ribonucleic acid.

we improved the assay to enable quantification of the viral genome, thus it can be easily implemented for monitoring viral activity and measuring viral load in pathogenesis studies.

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Conflict of interest

The authors declare that there is no conflict of interest.

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