Development and evaluation of a control plasmid for serotyping and quantification of dengue virus through real time RT-PCR

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SEROTYPING AND QUANTIFICATION OF DENGUE VIRUS THROUGH REAL
TIME RT-PCR

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DEVELOPMENT AND EVALUATION OF A CONTROL PLASMID FOR SEROTYPING AND QUANTIFICATION OF DENGUE VIRUS THROUGH REAL TIME RT-PCR

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ABREVIATIONS

ADE Antibody-dependent enhancement

AMP Ampicillin

CFU Colony-Forming Unit
1. ABSTRACT

*Dengue virus* (DENV) is the causative agent of one of the most important febrile illnesses worldwide. Four closely related serotypes of DENV have been described as responsible for a broad clinical spectrum of the disease. For more than a decade, molecular methods such as RT-PCR and more recently real-time RT-PCR have become important in serotyping because
of their high sensitivity and specificity. For the validation of molecular test results, positive controls are required, consisting of equimolecular mixtures of viral RNA from each of the four serotypes commonly obtained from in vitro cell cultures. In this study, the in silico design of a plasmid with the target sequences to the oligonucleotides and probes for DENV serotyping from Johnson et al (J Clin Microbiol. 2005; 43:4977-83) was performed, to generate a multi-target control RNA. The plasmid pBluescript II KS (+) with the cloned DENV target sequences, was obtained by commercial gene synthesis service, propagated by transformation of E. coli DH5α and verified by restriction analysis. The plasmid was linearized by enzyme digestion and used to obtain large amounts of RNA by run-off in vitro transcription. The transcripts were treated with DNase I, purified and used as a positive control in the serotyping of DENV by qRT-PCR. The control RNA was used successfully as a positive control in the serotyping and quantification of DENV from clinical isolates, with a high control RNA yield from a single in vitro transcription reaction.

Keywords: Serotyping; Real time RT-PCR; Control plasmid; Dengue virus.

2. INTRODUCTION

Dengue is a febrile illness of major public health concern in the tropical and sub-tropical regions of the world (WHO, 2012). In the past 50 years, its spread has had a 30-fold global increase, estimating 50-100 million dengue infections per year. In the 1970’s nine countries around the world accounted for all the cases of dengue virus (DENV) infections that
progressed to severe dengue (SD) disease. Today, SD cases are observed in more than 100 countries in tropical and subtropical regions (Guo et al., 2017). In Colombia, 150,000 cases of dengue occurred in 2010, mild dengue disease accounted for 143,791 cases whereas 6,209 were SD, with 217 fatalities (Velandia & Castellanos, 2011).

DENV belongs to the family Flaviviridae and, the genus Flavivirus. The basic unit of these viruses is a single-stranded RNA molecule with positive polarity (ssRNA+) within an icosahedral nucleocapsid composed of monomers of the capsid protein (C). This set is wrapped in a mosaic of viral envelope (E) and membrane (M) proteins inserted in a lipid bilayer taken from the host cell (Guzman et al., 2010; Kuhn et al., 2002; Usme-Ciro, Gómez-Castañeda, & Gallego-Gómez, 2012). The virus genome encodes three structural proteins (C, prM and E) and seven nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The latter is the RNA-dependent RNA polymerase (RdRp), which belongs to the group of enzymes with the highest error rate (lowest fidelity) of all known RNA polymerases, ranging from $10^{-3}$ to $10^{-5}$ substitutions per nucleotide copied (Lázaro Lázaro & Escarmís Homs, 2002). This explains why these viruses present a higher rate of mutation, favoring its evolution in a short period of time.

Four antigenically distinct but closely related serotypes of DENV (1 to 4) have been found in the American Continent. A primary infection induces permanent protective immunity to the homologous serotype while inducing incomplete non-protective immunity against heterologous serotypes (Sánchez, 2013). Antibodies generated by a primary serotype may cross-react against the secondary serotype inducing virus-antibody complexes, which bind to Fc receptors at monocytes and macrophages, thus enhancing the virus infection of these cells and the spreading of virus in the human body. This process is known as antibody-dependent
potentiation (ADE), being one of the most accepted theories regarding factors involved in SD (Velandia & Castellanos, 2011).

Various techniques have been implemented for the diagnosis of DENV. Depending on the stage of the disease and the purpose for which the test is carried out, the virus can be detected in different ways: Viral isolation, detection of antigens/antibodies (IgM) or detection of viral nucleic acid; are techniques for the diagnosis of DENV infection in the early stages of the disease given that the detection of the virus in plasma, serum or circulating blood cells can only be performed in the first 4-5 days after the onset of the disease.

Serologic detection of DENV IgG antibodies is the diagnostic method used at the end of the acute phase of infection (World Health Organization. & Special Programme for Research and Training in Tropical Diseases., 2009). However, this technique does not provide serotype information.

The viral isolation can be performed if the sample (plasma, serum, peripheral blood mononuclear cells or some tissues) was collected during the first five days of infection.

The most usual method for DENV isolation is the cell culture with confirmation by indirect immunofluorescence test (IFA) for the detection of the envelope antigen or RT-PCR. For this, cell lines derived from Ae. albopictus (C6/36), hamster (BHK21) or monkey (VERO) are commonly used. For the IFA test, serotype-specific monoclonal antibodies and flavivirus group-reactive or dengue complex-reactive monoclonal antibodies are used (World Health Organization. & Special Programme for Research and Training in Tropical Diseases., 2009).

Although this diagnostic technique allows serotyping at low cost, it can take up to 2 weeks and a successful outcome may not be obtained because it depends on the amount of viable virus within the sample (Teles, Prazeres, & Lima-Filho, 2005), which can be affected by transporting and storage conditions (4-8 °C in the first 24 hours, -70 °C for longer periods).
In addition, viral isolation in cell culture is performed only in specialized laboratories (trained staff, equipment and facilities) (Harris et al., 1998).

Another method used is the neutralization test (NT) which allows identification of serotype-specific antibodies. However, just like the IFA, it is not useful in early diagnosis (Alvarez Vera, González Rodríguez, Díaz Morejón, Morier Díaz, & Guzmán Tirado, 2010).

The detection of dengue antigens, such as the NS1 glycoprotein-based assay, is a technique in which antigens are detected as immune complexes in primary and secondary cases of infection; allowing a diagnosis up to nine days after the onset of symptoms. This technique does not require specialized equipment and results are obtained in a few hours. However, it does not allow serotyping, these kits are expensive, and the sensitivity can decrease up to 67% in secondary infections (Hermann et al., 2014) wherein its performance is still under evaluation (World Health Organization. & Special Programme for Research and Training in Tropical Diseases., 2009).

Serological tests have been performed according to the antibody detected:

- **MAC-ELISA** (Detection of IgM antibodies). By means of monoclonal or polyclonal dengue antibodies, specific dengue antigens that are bound to anti-dengue IgM antibodies can be detected. It is a test that has good sensitivity and specificity only if the sample was taken after the fifth day of onset of fever.

- **GAC-ELISA** (Detection of IgG antibodies). It allows the detection of dengue even after 10 months of infection, mainly. It is used for the identification of primary or secondary infections due to dengue. However, it is an uncommon test in laboratories as it is not commercially available.
• MAC/GAC-ELISA (IgM / IgG ratio). It is a common test to distinguish primary infections from secondary, using the relationship between IgM antibodies and IgG antibodies (IgM / IgG > 1.2: Primary infection, IgM / IgG < 1.2-1.4: Secondary infection). The relationship may vary according to the laboratory, which makes this test less specific.

• ACC-ELISA (Detection of IgA antibodies). The capture of IgA anti-dengue antibody, which normally occurs from the eighth day from the onset of fever to day 40, is measured. This test does not allow differentiation of a primary infection from the secondary, so it is not used frequently.

• Haemagglutination-inhibition (HI). It is used in paired sera obtained in the acute and convalescent phase, since they are able to inhibit the agglutination of the erythrocytes used in the test. HI allows the identification of a primary and secondary infections, but does not distinguish between infections caused by closely related flaviviruses.

The serological methods are widely used because they include rapid tests and the collection and handling of samples are simplified (Teles et al., 2005). Nevertheless, all serological tests available in the market have not been carefully evaluated, therefore they are not 100% reliable (World Health Organization. & Special Programme for Research and Training in Tropical Diseases., 2009).

The increase in epidemiological cases and the lack of a vaccine, have led to opt for techniques that allow an early diagnosis and with a high specificity, in order to offer a timely treatment and thus prevent the progression of the disease (Gutiérrez-Ruíz, Quintero-Gil , & Martínez-Gutiérrez, 2012).
Nucleic acid detection techniques have shown excellent results in the identification of viral RNA in a short time and with a sensitivity of 80-100%. Nevertheless, RNA handling and storage should be carefully performed (World Health Organization. & Special Programme for Research and Training in Tropical Diseases., 2009).

The development of polymerase chain reaction (PCR) techniques has facilitated the advancement of different diagnostic assays for virus detection (Lanciotti, Calisher, Gubler, Chang, & Vorndam, 1992). Thus, detection and typing of the virus by reverse transcriptase-PCR (RT-PCR) offers more specific results in a shorter time, and has a greater sensitivity for detection of the viral genome in the acute phase serum sample (Usme-Ciro et al., 2012) or in some tissues (collected from autopsies). In addition, it has been shown that only virus isolation and RT-PCR can detect the DENV serotypes reliably (Shu et al., 2004). In most cases, serotyping has been performed using conventional RT-PCR, however the real-time and quantitative RT-PCR technique (qRT-PCR) is more sensitive and allows estimation of viral load (Gutiérrez-Ruíz et al., 2012).

Johnson et al. (2005) reported the development of a fourplex DENV serotype-specific real-time nucleic acid detection assay, where they designed primers pairs and fluorogenic probes specific for each serotype of DENV. The assay was evaluated with serial dilutions of the virus against previously identified and serotyped DENV serum samples, showing high sensitivity and specificity (Johnson, Russell, & Lanciotti, 2005).

Nonetheless, the validity of the DENV diagnosis and serotyping by qRT-PCR, as with all diagnostic tests, relies on positive and negative controls inclusion. The Centers for Disease Control and Prevention (CDC) have developed a kit to facilitate the use of molecular tests for the diagnosis of dengue; this assay allows the detection of the four DENV serotypes in serum or plasma from patients with symptoms compatible with DENV infection. However,
the positive control for this assay consists of a mixture of the four serotypes which is difficult to obtain due to the need for trained personnel in cell culture management as well as equipment and facilities for this purpose. In addition, this product only serves for a limited number of tests, since each kit contains the required amount of each primer and probe for 200 reactions approximately (CDC, 2013). Constraints for positive control production limit the establishment of in-house molecular protocols of DENV detection, serotyping and quantification.

Therefore, in this study, we evaluated the utility of in vitro-transcribed control RNA containing the target sequences for each of the four DENV serotypes in the validation of a real-time RT-PCR assay. To assess this, the target sequences of primers and probes described by Johnson et al. (2005), were cloned in the plasmid pBluescript KS (+), which was later used for generation of large amounts of a control RNA by in vitro transcription.

Real-time RT-PCR assays allowed the detection of the 4 serotypes from the in vitro-transcribed DENV control RNA. In addition, it was possible to generate standard curves with high efficiency, which allowed the quantification of the viral load within each sample tested.

3. OBJECTIVES

3.1 General Objective

Develop and evaluate the usefulness of a plasmid to generate a positive control RNA in the serotyping of dengue virus by real-time RT-PCR.

3.2 Specific Objectives

- Design a control plasmid for DENV serotyping using bioinformatic tools.
• Perform the synthesis of RNA from the control plasmid by T7 promoter-driven *in vitro* transcription.

• Evaluate the performance of *in vitro*-transcribed RNA as a positive control in the serotyping of DENV from clinical isolates by real-time RT-PCR.

• Quantify the viral load of the clinical isolates for the four DENV serotypes.

4. MATERIALS AND METHODS

4.1 *In silico* design of the control plasmid

The plasmid was designed and developed as a positive control for *in vitro* transcription and subsequent use in the serotyping of DENV by real-time RT-PCR. The control had 4 complementary target sequences to the serotype-specific probes as well as the sequences of
the oligonucleotides used in the amplification of each DENV serotype previously described by Johnson et al. (2005). The SeqBuilder module of the LaserGene® suite (DNASTarLasergene, 2004-2006) was used for the design of the construct. Initially both the forward and reverse primers, in addition to the serotype-specific probes, were compared to aligned sequences from each serotype to evaluate the genetic variability present in those regions. Then, the four fragments were assembled, incorporating restriction sites at the intersections to facilitate future modifications of the target hybridization sequences of oligonucleotides and probes, as a result of the accumulation of genetic variability in the virus over time. The restriction sites used were selected from those present in the cloning vector (pBluescript II KS +) and absent in the four serotype-specific regions included in the construct. The target oligonucleotide and probe sequences (direction 5’ to 3’) were used in the following order: D1-F (caaaaggaagtcgtgcaata); D1-Probe (catgttgtgggagcacgc); D1-R (ctgagtgaattctctctactgaacc); D2-F (caggttatggcactgcacag); D2-Probe (ctctccgagaacggcctcctgac); D2-R (ccatctgcagcaacaccatctc); D3-F (ggaactggcacacgcactca); D3-Probe (acctggatgctcctgaaggagcttga); D3-R (catgtctacacctctctgcctgtct); D4-F (ttgctcctgactctctcc); D4-Probe (ttctactctctcgcattcc) and D4-R (tccacatgtcctcttccca) (Figure 1).
Figure 1. **DENV insert design.** Probes and primers sequences for each serotype of DENV where it shows the cleavage site of *SacI, AflIII, SmaI, BamHI, HindIII, and KpnI* enzymes.

4.2 **Generation of the construct by gene synthesis**

Once the construct was designed, gene synthesis and cloning in the vector *pBluescript II KS+* was performed by the GenScript® company. This vector was chosen as it contained the T7 promoter upstream of the multiple cloning site (MCS), which was necessary for the subsequent *in vitro* transcription to produce control RNA. The new construct, named pBlue-qDENV-Control plasmid was propagated in *E.coli* and used as template for *in vitro* RNA synthesis.

4.3 **Preparation of competent E. coli DH5α cells and transformation with the control plasmid**

4.3.1 **Preparation of competent cells**
For the preparation of the competent cells, the Inoue method was used as previously described (Sambrook & Rusell W., 2001). A single colony (2-3mm diameter) was picked up from a plate that was incubated overnight at 37.5°C. It was transferred to 25ml of Luria Bertani (LB) medium in a 250-ml flask which was incubated by shaking (New Brunswick Environmental Shaker Model G24) for 6-8h at 300 rpm at 37°C. Then, the small inoculum (SI) was transferred to a large inoculum: Two bottles containing 250 ml LB medium were used. To the first one 10 ml of SI was added, and to the second 3ml of the same SI. The flasks were left in the incubator with moderate shaking (180 rpm) overnight. It was not possible to obtain a temperature of 18-22°C as indicated in the guide, the approximate temperature was 23°C. The following day the optical density at 600 nm (OD600) was measured (Thermo Scientific™ GENESYS™ 30) and the bacterial culture that first reached a value of 0.55 was chosen. The chosen flask was incubated on ice 10 min and centrifuged for 10 mins at 2500g, 4°C. The supernatant was discarded; and the cell pellet was allowed to drain on absorbent paper for 2 mins, then re-suspended in 80 ml of ice-cold Inoue transformation buffer. The solution was centrifuged again for 10 mins at the same temperature and speed.

Subsequently, 20 ml of Inoue buffer (2.18g of MnCl₂ 4H₂O, 0.44 g of CaCl₂ 2 H₂O, 3.33g KCl, 2ml PIPES (0.5M, pH 6.7) and H₂O) plus 1.5 ml of DMSO were added, mixed by shaking and stored on ice for 10 mins. 100-μl aliquots were quickly transferred to 1.5-ml sterile tubes previously cooled in an ethanol-dry ice bath. Tubes containing competent cells were stored at -70°C until use.

4.3.2 Dilution of the plasmid

Four (4) μg of pBlue-qDENV-Control plasmid were received from the GenScript company. The plasmid was diluted in 13.33μl of sterile nuclease-free water to achieve a stock
concentration of 300ng/μl. The plasmid concentrations were estimated in the Thermo Scientific™ NanoDrop™ spectrophotometer.

4.3.3 Bacterial Transformation

The competent cells previously stored at -70°C were thawed by holding them in the palm of the hand. The tubes were transferred to an ice bath for 10mins. The cells were transferred to thin-wall 0.5ml PCR tubes for a better heat shock. All tubes were kept on ice, including those of the positive and negative controls. The transformation efficiencies for competent cells prepared from a flask that received 3ml of a starter culture was tested.

One μl (22ng) of pBlue-qDENV-Control plasmid was added per 50μl of competent DH5α bacteria. In the positive control (pcDNA STRUCT-DENV), 5μl (0.5ng) were used for transformation. For the negative control, 150μl of competent cells in the absence of plasmid DNA were subjected to heat shock and seeded in LB.

To calculate the transformation efficiency, the number of Colony Forming Units (CFU) in the petri dishes of transforming competent bacteria with the plasmid pcDNA STRUCT-DENV were counted.

The transformation efficiency was calculated using the following formula:

\[
\frac{\text{Number of colonies}}{\text{ng of DNA}} \times 1000 = \text{CFU/mg}
\]

\[
\frac{\text{Volume used in transformation} \times \text{Plasmid concentration} \times \text{cell suspension}}{\text{Total volume of the reaction}} = \text{CFU/mg}
\]

4.4 Plasmid propagation and purification through miniprep

Five transformed colonies, previously plated in LB+AMP medium, were incubated overnight in 6 ml of LB+AMP medium. Subsequently, 800 μl of each culture were mixed with 200 μl
of pure sterile glycerol and stored at -80°C. The remaining volume of the culture was used for purification. Purification of the 5 colonies grown on LB+AMP medium from both the plasmid pBlue-qDENV-Control and the pcDNA-STRUCT-DENV control plasmid was carried out with the QIAprep Miniprep commercial kit (Qiagen Inc., Chatsworth, CA, USA), following the manufacturer’s recommendations. Briefly, the remaining bacteria content from the previous step was centrifuged at 4000g for 15 mins at 4°C and then resuspended the bacteria pellet in 250 μl Buffer P1; 250 μl of Buffer P2 was added and mixed by inversion. Buffer N3 (350 μl) was added and centrifuged to form a compact pellet. 800 μl of the supernatant obtained in the previous step was added to the QIAprep 2.0 spin column and centrifuged again. A wash was performed adding 0.5 ml of buffer PB and 0.75 ml of buffer PE. Finally, the QIAprep 2.0 column was passed to a new microcentrifuge tube to elute the DNA by adding 50 μl of buffer EB to the center of the column and centrifuged again for 1 min. Plasmid DNA was stored at -20°C.

4.5 Restriction analysis

To establish the identity of the designed plasmid with the insert, the expected fragments following digestion with a restriction endonuclease were confirmed. For the digestion, the BspHI restriction enzyme (New England Biolabs, Inc) was used, which generates three fragments of the plasmid. Briefly, 500 ng of each clone were digested with 5U of the BspHI enzyme (10U/μl). The digestion reaction contained 0.5 μl of the BspHI enzyme + 2 μl of 10X Buffer and the plasmid according to their concentration for a final volume of 20 μl (See Table 1). Reaction was incubated at 37°C for 2h, followed by 65°C for 20mins and finally 4°C until analysis by electrophoresis. To analyze the results obtained in the restriction reaction, DNA fragments were separated through agarose gel electrophoresis. Eight μl of each sample and 2 μl of 5X loading dye, as well as a line for the molecular marker (1kb plus DNA Ladder,
Invitrogen, USA) were seeded. The gel was run for 1.5 hours at 90 volts to clearly visualize the three bands corresponding to each expected fragment.

4.6 Run-off in vitro transcription

To obtain the control RNA containing the sequences of the four serotypes of DENV that could be used as positive control of the reverse transcription and polymerase chain reaction during the real-time RT-PCR, the commercial T7 RiboMAX ™ Express Large-Scale RNA Production System (Promega Corporation) was used following the manufacturer recommendations. The pBlue-qDENV-Control clone 5 was used for the in vitro transcription, since it showed the higher DNA concentration. Before the in vitro transcription, the plasmid was linearized with the endonuclease Sap I, which cuts the plasmid in a region around 391 bp downstream to the DENV 1-4 sequences and generates a 5’ overhang end, which is indispensable to avoid the formation of non-specific transcripts. The volumes for the reaction mixture are shown in Table 2. Two reactions were made with a final volume of 25μl, which were subsequently unified for purification. Subsequently, the linearized plasmid DNA was purified with QIAQuick PCR Purification kit (Qiagen In., Chatsworth, CA, USA). Briefly, 10μl of Ribomax Express T7 2X buffer and 2μl of Enzyme mix T7 Express were mixed with 8μl of linear DNA template. The thermal profile used for incubation was: 37°C for one hour, 65°C for 20mins and 4°C. The elimination of the template DNA and unincorporated rNTPs was carried out following the transcription, using RQ1 RNase-Free DNase (included in the T7 RiboMAX ™ Express Large-Scale RNA Production System kit) to a concentration of 1 unit per microgram of template DNA (0.5μl in this case). The reaction was incubated for 15mins at 37°C. The subsequent purification was performed using the QIAamp® Viral RNA Mini Kit (Qiagen Inc., Chatsworth, CA, USA).
4.7 Standard curve for qRT-PCR testing

Aiming to evaluate the elimination of plasmid DNA and validate the reverse transcription (RT) step, the real-time RT-PCR was performed in One-step and Two-step assays (Tables 3 and 4). The use of both, the DNase and the Reverse Transcriptase enzymes was alternated. Expecting lower CT values for the DNase (-) RT (+) assay, followed by a slightly higher CT in the DNase (-) RT (-) assay, a larger CT in the DNase (+) assay RT (+) and finally no amplification was expected in the DNase (+) RT (-) assay. After verifying the effective removal of the template plasmid DNA, a standard curve was generated from five 10-fold serial dilutions (1x10^{-1} to 1x10^{-5} ng/ul) of the control RNA. A master mix of primers and probes was made as stock for the subsequent tests. Twenty μl (100 μM) of each of the primers DENV-1R, DENV-1F, DENV-3R and DENV-3F, were mixed with 10 μl (100 μM) of each of the primers DENV-2R, DENV-2F, DENV-4R and DENV-4F, and 3.6μl (100 μM) the probes for each serotype. The final volume of the mix was 200μl. Aliquots of this mix were stored at -20°C. Upon obtaining amplification in the samples where it was not expected, it was necessary to perform a new assay with DNase retreatment, adding 2U of RQ1 RNase-Free DNase and 153.6 ng of plasmid RNA in a final volume of 20 μl. It was incubated at 37°C for one hour.

4.8 Evaluation of the in vitro synthetized RNA as positive control and quantification of viral load

For the evaluation of the RNA as positive control, total RNA extracted from C6/36 cell culture supernatants with DENV isolates (obtained from human sera samples referred to the virology group at the INS) were used.

The SuperScript™ III Platinum® Reaction One-Step qRT-PCR Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) was used with the Applied Biosystems® 7500 fast and 7500
Real-Time PCR equipment for the amplification and quantification of the samples by real-time PCR.

For the quantification of the viral load, the calculator to determine the number of copies of a template (designed by Andrew Staroscik 29 January 2004) was used, which is based on the formula shown in figure 2.

\[
\text{Number of copies (molecules)} = \frac{X_{\text{ng}} \times 6.0221 \times 10^{23} \text{molecules/mole}}{(N \times 340 \text{ g/mole}) \times 1 \times 10^9 \text{ng/g}}
\]

**Figure 2.** Formula to convert nanograms to number of copies. \(X\) = Amount of transcribed RNA (ng), \(N\) = length of transcribed RNA. 340 g/mole = average mass of a RNA nucleotide (Staroscik, 2011).

5. **RESULTS**

5.1 **Effective transformation of competent E. coli DH5α with the plasmid pBlue-qDENV-Control**

Transforming colonies were obtained from transformation experiments and negative controls did not show any growth in the ampicillin-resistance selective medium, as expected (Figure 3).
34 CFU were obtained in the Petri dishes, containing transformed cells with pcDNA-STRUCT-DENV plasmid, data that was used in the following formula to estimate the transformation efficiency:

\[ \text{Transformation efficiency: } \frac{5\mu l \times 0.1ng/\mu l \times 150\mu l}{955\mu l} = 0.08 \text{ ng plated DNA} \]
\[
\frac{34 \, \text{UFC}}{0.08 \, \text{ng}} \times 1000 \, \text{ng/\mu l} = 4.35 \times 10^5 \, \text{CFU/\mu g DNA}
\]

5.2 *Restriction analysis demonstrated the integrity of the plasmid construct*

The plasmid construct using the pBlueScript Ks (+) backbone and containing a region of target sequences for molecular detection of the four DENV serotypes is represented in a linearized form in Figure 4.

![Figure 4](image)

*Figure 4.* Cutting sites of the *Bsp* HI enzyme in the plasmid pBlue-qDENV-Control and length of the expected fragments (1008 bp, 1528 bp and 809 bp).

After plasmid miniprep of the pBlue-qDENV-Control clones recovered from the transformation experiment, the five clones of higher concentration were selected for restriction analysis as shown in Table 5.

As the *Bsp* HI enzyme cuts at the 730, 2258 3266 bp positions within the pBlue-qDENV-Control plasmid, three fragments of 1008 bp, 1528 bp, and 809 bp were expected (Figure 4).
Three bands in each sample of the plasmid corresponding to the expected fragments were consistently observed for every analyzed clone (Figure 5).

![Figure 5](image.png)

**Figure 5.** Electrophoresis of the pBlue-qDENV-Control plasmid digested with the Bsp HI enzyme. Three bands corresponding to each fragment (1008 bp, 1528 bp and 809 bp) are shown for each of the plasmid clones (Clone 1-5).

### 5.3 Successful generation of qDENV-Control RNA by run-off in vitro transcription assays

After plasmid purification and linearization with Sap I, pBlue-qDENV-Control clone 5 was used for *in vitro* T7-driven transcription. The linearized plasmid allowed the production of the qDENV-Control RNA.

The qDENV-Control RNA was subsequently treated with DNase I and used for qRT-PCR amplification. Amplification was successful in all assays, even in the assay where the complexes synthetic RNA/DNA template were treated with DNase and without RT enzyme (in which no amplification was expected). Although the CT value for this assay showed to
be highest for DENV1, 3 and 4, the result demonstrated that DNase I treatment was not complete and increasing the amount of this enzyme is required. The CT values are shown in table 6.

The results obtained after double DNase I treatment are shown in the Table 7. The expected lack of amplification was shown after DNase I treatment and the absence of RT enzyme in the assay. The NTC also showed not determined CT values that never crossed the threshold line during the first 38 amplification cycles.

5.4 *The qRT-PCR is a highly efficient method for DENV detection and serotyping*

The standard curve for each serotype was obtained from serial dilutions of the qRT-PCR-DENV-Control RNA (Figure 6). An efficiency of 101% was observed for the standard curve of DENV-1 and R² of 0.974. The standard curve of DENV-2 showed an efficiency of 94.476% and R² 0.988. In the standard curve of DENV-3, an efficiency of 99.482% and R² of 0.98 were obtained. The 101.818% efficiency was shown in the standard curve of DENV-4 and a value of 0.979 was the value of R² for this same curve. These results demonstrate the usefulness of the qRT-PCR-DENV Control RNA for validating every DENV serotype-specific probe, and therefore its usefulness as a positive control for this qRT-PCR technique.
Figure 6. Standard curve of each serotype made from the serial dilutions of the transcribed RNA (Stock concentration 19.2 ng/μl). A) Standard curve DENV-1, B) Standard curve DENV-2, C) Standard curve DENV-3 and D) Standard curve DENV-4
5.5 The qRT-PCR-DENV Control RNA allowed the absolute quantification of DENV RNA genome copies in unknown samples

The use of serial dilutions of the qRT-PCR-DENV Control RNA allowed the generation of a standard curve and the interpolation of the CT values of unknown samples for their absolute quantification. Amplification was observed for all samples derived from culture supernatants of DENV growth curves from each serotype. Table 8 shows the CT values of the number of viral RNA copies on days 1 to 6 and for each serotype.

The relation within serotype, number of copies and the titration curve day are shown in figure 7.

![Relation between serotype and number of RNA copies through the titration curve day.](image-url)
6. DISCUSSION

The widespread use of molecular techniques for virus detection and serotyping during the last decades has led to the development of positive controls for validation of the efficacy of tests, allowing adequate interpretation of results. The use of known samples in the absence of reference strains as positive controls is helpful in specific settings where a novel agent, serotype, genotype or strain is circulating. However, the manipulation of infectious samples, or the repeated PCR amplification of a biological sample as a positive control can increase the probability of cross-contamination and therefore false positives (Acevedo et al., 2009; Lion, 2001).

Commonly, the positive controls used for tests such as RT-PCR for virus detection, are generated from sera from patients infected with the virus; these sera are carefully balanced and validated to be included in commercial kits. Therefore, it is important to have enough clinical samples, equipment and qualified personnel (Golden et al., 2016).

Different companies and researchers that offer kits for real-time RT-PCR tests still depend on “home-made” positive controls, which include positive samples with known sequences (T. Lion, 2001). However, the development of plasmid controls has been an advantage because it facilitates the copy number quantification in a sample by means of the interpolation of a standard curve with known concentrations in the real-time RT-PCR test (Acevedo et al., 2009).

The transformation of competent cells is a technique that has been implemented for the effective propagation and conservation of plasmids including those designed in silico and generated by chemical gene synthesis. The plasmid DNA can be stored in convenient
quantities for a long period of time and can be used in many downstream applications (Ghahri, Shahhosseiny, Moslemi, Zolfaghari, & Shahhosseiny, 2013).

Our results showed that transformation efficiency of the *E. coli DH5α* was lower than expected according to the Inoue method for transformation of competent cells (1-3 × 10⁹ CFU/μg); however, the efficiency was enough to successfully transform the cells with our plasmid construct, obtaining an sufficient number of colonies in a selective medium with AMP (Inoue, Nojima, & Okayama, 1990).

Successful cloning of the insert (*in silico* design of the pBlue-qDENV-Control) within the cloning vector pBluescript II KS+ was perormed by the manufacturer through Sanger sequencing and confirmed by restriction analysis after propagation in *E.coli DH5α*. As seen in Figure 5, digestion with the *Bsp* HI enzyme produced the expected fragments, indicating that the plasmid had the predicted size and that the designed DENV insert is in the appropriate position, between 653-1036 bp.

The last step of the *run-off in vitro* transcription assay consisting of DNase I treatment of the transcribed RNA/plasmid DNA mix, in order to eliminate the plasmid DNA was not sufficient at 0.5U of DNase I per 340.8ng of transcribed RNA/plasmid DNA mix. It was expected that the CT values of the assay DNase (-) RT (+) would be much lower compared to the assays DNase (+) and RT (+) and DNase (-) and RT (-) but the values were found to be very close to each other (See table 6). In the case of the One Step assay it was observed that the CT values were not very different however it was expected that the assay with DNase I treatment would show the higher CT values (Table 7). For this reason, a double treatment with 2U of DNase I was necessary to ensure complete elimination of plasmid DNA and to
warrant that the amplification of DENV sequences was exclusively from the *in vitro*-transcribed pBlue-qDENV-Control RNA.

Having confirmed the complete elimination of plasmid DNA after the double DNase I treatment, the remaining *in vitro* transcribed RNA, allowed to perform the standard curve from serial dilutions and to use it as a positive control for the quantification and serotyping of DENV in culture supernatants from growth curves, as part of the *in vitro* characterization of DENV strains.

For the standard curve, it is necessary to know the range in which a PCR amplicon is generated, also, a high amplification efficiency (measured as a percentage) is an important datum to consider from the standard curve (Ruijter et al., 2009). An efficiency of 100% is reached when the PCR amplicons double in quantity after each cycle during the geometric phase. However, a PCR efficiency of exactly 100% is rarely observed, since there will always be external factors that can influence the amplification such as the quality of the reagents, the precision of the instruments used, the proper handling of the sample, etc. For this reason, an efficiency between 90 and 110% is considered acceptable (Livak KJ, 2001) (Applied Biosystems, 2010). Thus, the standard curves of this study, showing percentages between 94 and 102%, can be considered good amplification efficiency.

The $R^2$ and slop are other parameters that corroborate the efficiency of amplification. The $R^2$ is the measure that indicates how one value can predict another correctly, i.e., the closeness of fit between the CT values of the samples and the regression line. The setting is perfect when the value of $R^2$ is 1.00; therefore, it is expected to obtain values >0.99. Obtaining values <0.99 indicates a high deviation in CT values between the replicates of a sample. For this reason, it is necessary to make at least 3 replicates of each sample.
In this study, unlike recommended, only two replicates were performed per sample because of the lack of sufficient reagents, which explains the $R^2$ values <0.99.

However, the slope values are within the expected range, -3.58 and -3.10, indicating an efficiency between 90 and 100% (Applied Biosystems, 2010), except for the DENV-4 curve that is above these values, which may indicate problems of pipetting during serial dilutions preparation or sample quality (Biosystems, 2004).

As it has been discussed throughout this report, one of the main reasons by which real-time PCR has been one of the most used techniques today is because of its high sensitivity, which means that the concentration and the number of copies at the end of the amplification can be known in problem samples with very low amounts of viral genomes.

The results of the quantification of copy number of genomes in DENV-1 to -4 growth curves were obtained with the unique goal of validating the use of the pBlue-qDENV-Control RNA in DENV serotyping and quantification of samples with different virus concentrations.

DENV-1 showed higher increase in copy numbers from day 1 to day 6, suggesting a higher replication rate. Here, it is not possible to make conclusions about the replication differences between DENV serotypes, because the geographical source, the adaptation to cell cultures, the presence of specific mutations, and other factors can influence the growth dynamics (Shin, Richards, Alto, Bettinardi, & Smartt, 2013).

This study is expected to contribute to the improvement of the dengue diagnosis and surveillance through real-time RT-PCR, allowing to validate the detection and typing of each serotype independently through the use of a unique pBlue-qDENV-Control RNA. The DENV Control RNA developed here allows the reduction of difficulties in manipulating
virus strains in cell culture laboratories with differing biosecurity levels, according to the
country, the associated costs and the need to calibrate each serotype at equimolecular
quantities. It facilitates the establishment of in-house protocols that are the preferred option
in several research laboratories where virus quantification could be the most important
application of the present development.

7. CONCLUSIONS

- A plasmid control with sequences corresponding to the four serotypes of the
  DENV was obtained from an in silico design using cutting-edge bioinformatics
tools.
- Large amount of DENV control RNA was successfully obtained through T7
  promoter-driven in vitro transcription and subsequent template digestion by
  DNase I.
- The in vitro-transcribed DENV control was successfully used as positive control
  in qRT-PCR assays and allowed the generation of a standard curve for absolute
  quantification.
- The qRT-PCR assay in combination with the DENV control RNA, made it
  possible to estimate the copy number of DENV RNA in serum samples from
  suspected dengue patients.

8. FUNDING

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9. ACKNOWLEDGMENT
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10. REFERENCES


DNAStarLasergene. (2004-2006). SeqBuilder © (Version 7.1.0(44)). Madison, WI.


Sánchez, R. M. (2013). *Identificación y serotipificación del virus dengue, densidades y tasa de infección de poblaciones domésticas de Ae. aegypti en Cancún, Quintana Roo, México.* (PhD of Science with accentuation in medical entomology), Universidad Autónoma de Nuevo León, México. Retrieved from http://eprints.uanl.mx/id/eprint/3721


11. ANNEXES

**Table 1.** Volume required for the digestion reaction (final Vol:20μl) in each of the pBlue-qDENV-Control plasmid clones.

<table>
<thead>
<tr>
<th></th>
<th>pB1 Vol (Concentration ng/μl)</th>
<th>pB2 Vol (Concentration ng/μl)</th>
<th>pB3 Vol (Concentration ng/μl)</th>
<th>pB4 Vol (Concentration ng/μl)</th>
<th>pB5 Vol (Concentration ng/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BspHI</td>
<td>0.5 μl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer 10X</td>
<td>2 μl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA (500ng)</td>
<td>7 μl</td>
<td>12 μl</td>
<td>10 μl</td>
<td>8 μl</td>
<td>5 μl</td>
</tr>
<tr>
<td></td>
<td>(7,1 ng/μl)</td>
<td>(42,5 ng/μl)</td>
<td>(49 ng/μl)</td>
<td>(60 ng/μl)</td>
<td>(100,7 ng/μl)</td>
</tr>
<tr>
<td>H2O</td>
<td>10.5 μl</td>
<td>5.5 μl</td>
<td>7.5 μl</td>
<td>9.5 μl</td>
<td>12.5 μl</td>
</tr>
</tbody>
</table>

**Table 2.** Used volume of each reagent in the linearization of the plasmid.

<table>
<thead>
<tr>
<th></th>
<th>1X</th>
<th>10μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBlue-qDENV-Control</td>
<td>10μl</td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td>11.5μl</td>
<td></td>
</tr>
<tr>
<td>Sap I Enzyme</td>
<td>1μl</td>
<td></td>
</tr>
<tr>
<td>Buffer NE10x</td>
<td>2.5μl</td>
<td></td>
</tr>
<tr>
<td>Final Vol</td>
<td>25μl</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Two-step RT-PCR assay, using M-MLV RT (Invitrogen™).

<table>
<thead>
<tr>
<th>ASSAY # 1: Two steps RT-PCR</th>
<th>cDNA synthesis</th>
<th>Expected Results (PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> RNA+ Template plasmid DNA</td>
<td>DNase (+)</td>
<td>RT (+)</td>
</tr>
<tr>
<td></td>
<td>DNase (+)</td>
<td>RT (-)</td>
</tr>
<tr>
<td></td>
<td>DsNase (-)</td>
<td>RT (+)</td>
</tr>
<tr>
<td></td>
<td>DNase (-)</td>
<td>RT (-)</td>
</tr>
</tbody>
</table>

Table 4. One step RT-PCR assay alternating the use of DNase.

<table>
<thead>
<tr>
<th>ASSAY # 2: One step RT-PCR</th>
<th>Expected Results (RT-PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> RNA + Template plasmid DNA</td>
<td>DNase (+)</td>
</tr>
<tr>
<td></td>
<td>DNase (-)</td>
</tr>
</tbody>
</table>

Table 5. Concentration of each of the samples of the purified plasmids: pcDNA STRUCT-DENV and pBlue-qDENV-Control.

<table>
<thead>
<tr>
<th>Clone</th>
<th>pcDNA STRUCT-DENV</th>
<th>pBlue-qDENV-Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36.5 ng/µl</td>
<td>71.7 ng/µl</td>
</tr>
<tr>
<td>2</td>
<td>49.0 ng/µl</td>
<td>42.5 ng/µl</td>
</tr>
<tr>
<td>3</td>
<td>40.8 ng/µl</td>
<td>49.0 ng/µl</td>
</tr>
<tr>
<td>4</td>
<td>40.7 ng/µl</td>
<td>60.0 ng/µl</td>
</tr>
<tr>
<td>5</td>
<td>84.1 ng/µl</td>
<td>100.7 ng/µl</td>
</tr>
</tbody>
</table>
**Table 6.** CT values obtained in the real-time RT-PCR assay using the qRT-PCR-DENV Control RNA.

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>DENV-1</th>
<th>DENV-2</th>
<th>DENV-3</th>
<th>DENV-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNAse (+) RT (+)</td>
<td>14.00</td>
<td>11.53</td>
<td>11.75</td>
<td>8.79</td>
</tr>
<tr>
<td>DNAse (-) RT (+)</td>
<td>13.81</td>
<td>10.69</td>
<td>11.40</td>
<td>8.62</td>
</tr>
<tr>
<td>DNAse (+) RT (-)</td>
<td>18.44</td>
<td>13.79</td>
<td>19.71</td>
<td>19.40</td>
</tr>
<tr>
<td>DNAse (-) RT (-)</td>
<td>15.62</td>
<td>14.30</td>
<td>15.66</td>
<td>16.19</td>
</tr>
<tr>
<td>OS_DNAsa (+)</td>
<td>11.81</td>
<td>12.51</td>
<td>11.75</td>
<td>12.26</td>
</tr>
<tr>
<td>OS_DNAsa (-)</td>
<td>11.05</td>
<td>11.91</td>
<td>11.67</td>
<td>11.87</td>
</tr>
<tr>
<td>NTC</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Table 7.** CT Values obtained in the real-time RT-PCR after double treatment of the qRT-PCR-DENV Control RNA with DNase I.

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>DENV-1</th>
<th>DENV-2</th>
<th>DENV-3</th>
<th>DENV-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNAsa (+) RT (+)</td>
<td>24.52</td>
<td>26.18</td>
<td>25.26</td>
<td>26.03</td>
</tr>
<tr>
<td>DNAsa (+) RT (-)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NTC</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Table 8.** Information corresponding to serotype, CT values of genomes in culture supernatants from days 1-6 and the number of copies of serum samples from patients suspected of DENV.

<table>
<thead>
<tr>
<th>Code</th>
<th>Serotype</th>
<th>Day</th>
<th>CT Value</th>
<th>Number of copies</th>
</tr>
</thead>
<tbody>
<tr>
<td>449308</td>
<td>DENV-2</td>
<td>1</td>
<td>19.26</td>
<td>9.095×10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>16.21</td>
<td>1.319×10^8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>14.98</td>
<td>1.501×10^8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>14.49</td>
<td>1.637×10^8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>14.36</td>
<td>7.276×10^7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>14.08</td>
<td>7.503×10^7</td>
</tr>
<tr>
<td>452018</td>
<td>DENV-2</td>
<td>1</td>
<td>22.99</td>
<td>6.412×10^5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>17.58</td>
<td>1.592×10^7</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>15.90</td>
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</tr>
<tr>
<td></td>
<td>4</td>
<td>15.01</td>
<td>9.095×10⁷</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14.96</td>
<td>9.549×10⁷</td>
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<tr>
<td></td>
<td>6</td>
<td>14.49</td>
<td>1.319×10⁸</td>
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<tr>
<td>449686</td>
<td>DENV-3</td>
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<td>30.27</td>
<td>1.66×10³</td>
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<td>2</td>
<td>27.77</td>
<td>7.23×10³</td>
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<td>26.60</td>
<td>1.444×10⁴</td>
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</tr>
<tr>
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<td>6</td>
<td>20.33</td>
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<td>DENV-1</td>
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<td>29.15</td>
<td>2.888×10⁴</td>
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<td>21.81</td>
<td>4.57×10⁵</td>
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<td></td>
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<td>18.02</td>
<td>2.274×10⁶</td>
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<td>4</td>
<td>14.05</td>
<td>1.364×10⁷</td>
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<td>13.64</td>
<td>5.229×10⁷</td>
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<td>6</td>
<td>11.72</td>
<td>1.728×10⁸</td>
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<td>426553</td>
<td>DENV-4</td>
<td>1</td>
<td>19.77</td>
<td>1.146×10⁶</td>
</tr>
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<td></td>
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<td>16.09</td>
<td>9.095×10⁶</td>
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<td></td>
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<td>15.49</td>
<td>2.956×10⁷</td>
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<td>12.47</td>
<td>5.457×10⁷</td>
</tr>
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<td>6</td>
<td>9.27</td>
<td>7.526×10⁸</td>
</tr>
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</table>