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Detection and differentiation of dengue virus serotypes by one-step multiplex reverse transcription PCR assays

Detecção e diferenciação de sorotipos do vírus da dengue por ensaios de PCR com transcrição reversa multiplexada em uma etapa

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ABSTRACT
Background: Dengue infections are a severe public health problem in Brazil. The Ministry of Health recommends an immunosorbent assay (ELISA) for the capture of IgM (MAC-ELISA) to diagnose dengue. However, it detects antibodies that cross-react with other flaviviruses and requires confirmation in reference laboratories. Methods: One-step multiplex RT-PCR assay was used to amplify RNA of 197 serum from patients with clinical suspicion of dengue infection. The samples had been screened with the IgM ELISA kit in the Central Public Health Laboratory of the State of Maranhão. Results: Of the 197 samples evaluated by IgM ELISA, 135 were positive; of these, 96 (71.1%) were from patients in the acute phase of the infection. The one-step multiplex RT-PCR detected viral RNA in 88 (91.7%) of this serum. Among the 62-negative serum by ELISA, 29 samples (46.8%) were amplified using the molecular method. Conclusions: One-step multiplex RT-PCR was sensitive in the detection of viral particles from the first day until the sixth day after the onset of the feverish period. Moreover, it was specific and 100% reproducible. Based on these results, we recommend the use of this molecular assay to diagnose and differentiate the DENV serotypes in the acute phase of the disease.

Keywords: Molecular diagnostics, dengue infections, MAC-ELISA, One-step multiplex RT-PCR assay.

RESUMO
Also known as tagging or labeling theory, the labeling approach is a facet of criminological knowledge that helps us understand the selectivity of the criminal justice system and criminalization processes. Supported by the epistemological bases of this theoretical framework, this paper assumes that the repressive activity of the judicial police is also riddled with asymmetries that produce and

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reproduce inequalities, a hypothesis put to the test through a case study developed within the Betim homicide precinct. This academic article sought to empirically analyze how the work of this police unit competes with the selectivity of the criminal justice system. The data collection technique used semi-structured interviews, which were submitted to five police officers working in the Betim homicide police station, focusing on aspects of the professional practice of these public security workers. The analysis of the empirical data reveals some mechanisms by which selectivity operates in concrete reality, namely, the panorama of epidemic violence that created a quantity of almost two thousand investigations of open murders in the city, the scarcity of material and human resources, the social reaction of sectors of communities that support homicidal action as a form of conflict resolution, the dependence on investigations of witness evidence and, finally, a managerial rationality that tends to accentuate bureaucratic formalism to the detriment of a democratic criminal policy whose the end in itself is the defense of the fundamental rights and guarantees of the citizen.

Palavras-chave: Labeling approach. Civil Police of Minas Gerais. Selectivity.

1 INTRODUCTION

Dengue fever is caused by dengue virus (DENV), an arbovirus of the Flaviviridae family and genus *Flavivirus*, which includes four different immunological serotypes, DENV-1–4. A DENV-5 serotype is related, but human infections with this serotype have not been reported; however, it may circulate among nonhuman primates, such as the macaques living in the forests of Borneo[1,2]. DENV infections are transmitted through the bites of infected vectors, mainly female *Aedes aegypti* or *Aedes albopictus*[2,3]. DENV mosquitoes are geographically distributed in more than 100 countries, constituting a severe global public health problem[4,5], particularly in countries located in tropical and subtropical regions[3-5].

Human DENV infections cause a wide spectrum of various signs and symptoms, such as simple fever, classical dengue fever (DF), hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which is potentially fatal[6-8]. The World Health Organization (WHO) estimates that approximately 390 million cases of DENV occur annually worldwide in 100 countries, of which almost 500,000 cases are DHF; many of these cases have progressed to severe dengue, resulting in more than 20,000 deaths[9,10].

The first major outbreak of dengue occurred in Rondônia-RO in 1982. Since then, DENV and its main vector *A. aegypti* have spread in all Brazilian regions. According to data obtained from epidemiological reports, the number of dengue cases monitored by the Ministry of Health from 1990 to the end of 2018 reached astronomical proportions, with approximately 12,939,314 cases[11] and 6.403 deaths confirmed to be caused by DENV[12].

Despite the high rates of DENV infection, a single test is currently unavailable, and the diagnosis is usually based on various criteria encompassing the patient's clinical history, epidemiological aspects, serological tests, and the isolation of virus-infected cells[13].

Regarding diagnostic methods, serological tests are the most frequently used methods in the laboratory to routinely confirm dengue virus infections and to differentiate primary from secondary infections[14].

The ELISA technique is an important tool used to confirm the clinical diagnosis and for epidemiological studies, but it does not represent the current situation of dengue in the population[8,14]. In patients with primary infections, an ELISA is able to detect the titers of IgM antibodies between 3–5 days after the onset of disease, followed by the increase in the titer of IgG antibodies within 5–7 days[13]. In patients with secondary infections, the titers of specific IgGs are the first to increase, followed by the titers of IgM antibodies. Although lower IgM titers are detected compared with primary DENV infections, IgM may not be detected in some patients[13-17].

Molecular techniques may play an essential role in the laboratory diagnosis of DF in patients with different stages of presentations since they detect the virus in the acute phase of the disease and even at the beginning of the convalescent period[13]. Molecular methods, whose the main objective is the detection of the viral genome, play an important role in the diagnosis of dengue infections, because they are able to identify the virus in serum samples from patients in the acute phase of the disease[18].

In recent years, several molecular techniques have been tested in research laboratories around the world to detect and/or identify DENV infections. Among these techniques, the most frequently used methods have been RT-PCR and nucleic acid hybridization[14]. A modification in the heminested RT-PCR assays previously described by Lanciotti et al.[19] allowed the development of a single-step multiplex real-time RT-PCR assay that has been adopted worldwide[14].

Although PCR-based methods are fast, sensitive and specific, they are performed in a laboratory with specialized equipment and trained staff[14]. In Brazil, to date, no molecular test has been adopted by the Ministry of Health as a standard for the diagnosis dengue infections. Thus, we propose to evaluate a *one-step multiplex reverse transcriptase PCR assay* to detect and differentiate dengue serotypes in serum samples from patients with a clinically suspected infection in the present study. This assay does not require specialized equipment or probes and it does not require a computational analysis.

2.MATERIAL AND METHODS

2.1. CLINICAL SAMPLES AND VIRAL CULTURES USED AS POSITIVE CONTROLS

One hundred ninety-seven serum samples from patients with a clinical suspicion of dengue infection were randomly chosen from the LACEN-MA serum bank. These samples were collected from January 2015 to January 2016 and stored at -80 °C. All of these samples had been previously

tested for antibodies against DENV by LACEN-MA technicians with the Dengue IgM capture ELISA kit (E-DEN01M/E-DEN01M05, Panbio, Australia).

The following data were collected from patients and recorded in the LACEN system GAL (Laboratory Environment Manager) database: sex, age, date of collection of the blood sample, time of onset of clinical signs and symptoms, and the IgM ELISA results.

Culture aliquots from the four lineages belonging to the reference serotypes (DENV-1, Hawaii; DENV-2, ThNH7/93; DENV-3, PhMH-J1-97, and DENV-4, SLMC 318) were used as positive controls, and 30 ELISA IgM-negative serum samples obtained from individuals who never had dengue served as negative controls.

2.2 TOTAL RNA EXTRACTION AND ONE-STEP MULTIPLEX RT-PCR ASSAYS

Total RNA was extracted from all serum samples and the reference dengue strains (DENV-1 to DENV-4) using the VIRAL RNA QIAMP Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. The RNA concentrations were quantified using a NanoDrop spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA, USA), and samples were stored at -80 °C until use.

The primers used in the one-step multiplex RT-PCR assays for each serotype were described in a previous study [20]. The primers for DENV-1 were Forward 5′-CACCGGCTAATGTCAGCTGC-3′ and Reverse 5′-CCTGCAGAGACCATTGACTTA-3′ primers that amplify a fragment of 518 base pairs (bp). The primers for DENV-2 were Forward 5′-AGGAATCATGCAGGCAGGAA-3′ and Reverse 5′-TCCACAGTCCTCAGTCACCAC-3′, which produced a 595 bp fragment. The primers for DENV-3 were Forward 5′-CATAATAGACGGACCAAACACAC-3′ and Reverse 5′-TGTGGTTGTGTTGCGAGATAG-3′, which amplify a fragment of 372 bp. The primers for DENV-4 were Forward 5′-GACCAAAGGCAAGAGAGCACT-3′ and Reverse 5′-GCATCTGGCTTTCCAGCACT-3′, producing a 434 bp fragment.

The one-step multiplex RT-PCR assay was performed to simultaneously amplify viral RNA from the reference DENV-1-4 lineages using the four primer pairs in a single tube. Each reaction was performed in a final volume of 50 µL consisting of 100 ng of total RNA (DENV-1 to DENV-4), 20 picomoles of each specific primer pair, 10 µL of 5x QIAGEN OneStep RT-PCR Buffer, 10 µL of Q-Solution, dNTP Mix containing 10 mM of each dNTP, 5 units of the RNasin® Ribonuclease Inhibitor (Promega Corporation, Madison, USA), 1 µL of QIAGEN OneStep RT-PCR Enzyme Mix and RNase-free water.

The amplifications were performed using a Mastercycler (Eppendorf, Germany) with an initial reverse-transcription step of 50 °C for 30 min and denaturation at 94 °C for 15 min (HotStarTaq

DNA Polymerase activation), followed by 35 cycles of 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, and a final extension step at 72 °C for 10 min.

2.3 ELECTROPHORETIC ANALYSIS OF ONE-STEP MULTIPLEX RT-PCR PRODUCTS

The one-step RT-PCR profiles were verified by separating the products on 2% agarose gels. Eight microliters of the product were mixed with 2 μL of Blue Orange 6x Loading Dye buffer (Promega Corporation, Madison, USA) and applied to the gel. Electrophoresis was performed using 0.5x TBE (Tris-borate-EDTA) buffer containing 100 mM Tris base, a 2.0 mM EDTA solution (pH 8.0) and 50 mM boric acid at 85 V for 1 h and 30 min. After the run, gels were stained with 5 μg/mL ethidium bromide for 15 min and observed using the ultraviolet transilluminator[21].

2.4 ONE-STEP MULTIPLEX RT-PCR ASSAYS WITH RNA EXTRACTED FROM SERUM SAMPLES OBTAINED FROM PATIENTS WITH CLINICALLY SUSPECTED DENGUE

After the optimization process with the RNA from the DENV reference strains, the one-step multiplex RT-PCR assays were used to detect and differentiate infectious serotypes in the genetic material extracted from clinical samples, according to previously established conditions. The total RNA from reference strains served as positive and negative controls.

2.5 SENSITIVITY AND SPECIFICITY OF ONE-STEP MULTIPLEX RT-PCR ASSAYS

Serial dilutions of 100 ng were prepared with the total RNA of each reference serotype, DENV-1, DENV-2, DENV-3, and DENV-4, which had been previously quantified using a NanoDrop spectrophotometers, to analyze the sensitivity. One-step multiplex RT-PCR assays were performed to amplify each specific serotype in a final volume of 50 μL consisting of QIAGEN OneStep RT-PCR Kit specific reagents, as described above, along with 20 picomoles of each primer pair. The first reaction tube contained 100 ng of total RNA as the template, and the other tubes contained 50, 25, 12.5, 6.25, 3.12 and 1.56 ng. The amplification assays were performed individually for DENV-1–4 after the sensitivity tests with each of the reference serotypes.

The total RNA extracted from the Yellow fever and the Saint Louis viruses, both of which are members of the family Flaviviridae and the genus *Flavivirus*, was used to verify the specificity of the primers and the multiplex one-step RT-PCR assay. The amplification reactions designed to verify the specificity of the primers were performed under the same conditions described above for DENV.

2.6 STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS version 20 (IBM) Statistics[22]. The nonparametric Chi-square test was performed to evaluate the association of the variables, time after the onset of clinical signs of dengue and the results of the serological (ELISA-IgM) and one-step multiplex RT-PCR analysis, for independent confirmation. The level of significance of the tests was 5%, namely, a difference was considered significant when $p < 0.05$.

2.7. ETHICS STATEMENT

The study was approved by the Research Ethics Committee of the University CEUMA, with opinion consubstantiated number 28024. Informed consent was waived because the serum samples were provided by Central Public Health Laboratory of the State of Maranhão (LACEN-MA).

3. RESULTS

3.1 SERUM SCREENING AT LACEN-MA

The results of the screen performed by LACEN technicians to detect IgM antibodies against DENV revealed that 135 of the 197 samples (68.5%) were positive and 62 (31.5%) were negative. Of the IgM ELISA-positive samples, 88 (65.2%) were obtained from patients who were in the acute phase of the disease, which occurs one to six days after the onset of clinical signs and symptoms of dengue (Table 1).

3.2 OPTIMIZATION OF ONE-STEP MULTIPLEX RT-PCR ASSAYS USING THE RNA FROM EACH DENV REFERENCE SEROTYPE AND FROM CLINICAL SAMPLES

The results of the optimization of one-step multiplex RT-PCR assays using the RNA from each DENV reference serotype as templates produced fragments with sizes of 518 bp for DENV-1, 595 bp for DENV-2, and 372 bp for DENV-3; for DENV-4, two fragments were observed, one at 434 bp and the other at 190 bp (Figure 1). These experiments were repeated three times in triplicate on alternate days using thermal cyclers from different manufacturers.

The one-step multiplex RT-PCR assays using *RNA from clinical samples* detected and differentiated DENV serotypes in serum samples from all patients at four to five days after infection and amplified 50% of serum samples collected from patients at 6 days after the infection. The remaining sera were obtained from patients in the chronic phase, between six to thirty days after infection, and all were negative according to the molecular method.

Interestingly, of the 62 ELISA-negative serum samples, 29 (46.8%) were positive in the one-step multiplex RT-PCR assays, and all these sera were obtained from patients within one to three days of the onset of symptoms. According to the data present in the GAL system, these patients went to the hospital in the first three days of the first clinical signs and symptoms of dengue (Table 1).

Of the 197 samples analyzed by one-step multiplex RT-PCR, serotype 4 was the most prevalent, as it was positive in 115 (85.2%) samples. Figure 2A and 2B show the profiles of some samples. The second most frequently detected serotype was serotype 1, which was detected in 13 (9.6%) samples, followed by serotype 3 detected in 5 samples (3.7%) and serotype 2 detected in only 2 (1.5%) serum samples (Figure 3A, 3B, and 3C).

3.3 SENSITIVITY AND SPECIFICITY OF THE PRIMERS AND ONE-STEP MULTIPLEX RT-PCR ASSAYS

The results of the sensitivity tests confirmed the ability of the one-step multiplex RT-PCR assay to detect the smallest amount of viral RNA from each reference serotype (DENV-1 to DENV-4) present. For all serotypes, the detection limit was 3.12 ng of viral RNA (Figure 4A, 4B, 4C, 4D). No amplification was observed in the specificity tests performed using the RNA from two members of the Flaviviridae family, Saint Louis encephalitis virus and yellow fever, as templates. The tests were conducted in triplicate on different days, and amplification was not detected (data not shown).

3.4 STATISTICAL ANALYSIS

The Kappa (k) concordance index was used for the statistical analysis, and the performance of the molecular test was analyzed at different stages of dengue infection, while considering the different principles of ELISA and the one-step multiplex RT-PCR. The Kappa index provided the level of agreement between the ELISA test and the one-step multiplex RT-PCR assays using the samples collected from patients up to the fifth day after the onset of symptoms. All the analyzed results were considered significant using a probability of significance of less than 5% ($p < 0.05$). Therefore, at least 95% confidence in the conclusions was obtained.

4. DISCUSSION

In recent years, several molecular techniques have been used in research laboratories around the world to detect or identify the DENV serotypes involved in infections. Among these methods, reverse transcription reactions followed by specific PCR amplification have been widely used because of their speed, sensitivity, and specificity[14,23].

In the present study, a one-step multiplex RT-PCR assay was evaluated and detected DENV infection in 91.7% (88 /96) of serum samples from patients in the acute phase of the disease that were positive according to the IgM ELISA. Using this molecular technique, we also detected that 46.8% of ELISA IgM-negative samples were positive for viral RNA. This finding is important because antibodies against DENV generally begin to appear after the fifth day of the onset of clinical signs and symptoms of the disease[13,24].

In a study conducted by Ferraz et al.[25], a real-time multiplex-PCR assay with Pandenv primers exhibited a sensitivity rate of 89% for DENV isolates in C636 cells. However, the authors observed a rate of 76.2% positivity for serum samples obtained from patients within a mean of three days after the onset of the febrile period, which corresponds to the acute phase of dengue when the virus is still present in the bloodstream.

Regarding our results, several molecular biology techniques, such as heminested RT-PCR[19], multiplex RT-PCR[26], semi-nested PCR[27], real-time RT-PCR[28,29], one-step TaqMan RT-PCR Real-time[30,31], and TaqMan probe-based real-time reverse transcriptase-polymerase chain reaction[32], have been used in research laboratories around the world to detect and/or identify the serotypes involved in dengue infections during the early viremic phase[28].

The real-time PCR assay is considered a sensitive method for virus detection; it permits a safe, fast, and reliable evaluation, it does not require viable particles and it has been widely adopted for the diagnosis of DENV infections[30-32-34]. However, this technique presents some limitations, primarily in developing countries, including Brazil. It requires expensive equipment, specialized personnel, expensive reagents, and the synthesis of specific probes[35].

The prevalence of DENV serotype 4 was lower than the value reported in the State of Rio Grande do Norte, which is also located in northeastern Brazil, where the DENV-4 infection was confirmed in 100.00% (150/150) of patients when this serotype was introduced in that state in 2012[36]. In Manaus-AM, only 16 of the 94 samples analyzed (17%) were positive, and DENV-3 was the only viral serotype detected[37]. The high rate of infection by DENV-4 observed here is probably related to the introduction of serotype 4 in the State of Maranhão, which occurred only at the end of 2011[38].

A potential explanation for the profile obtained for serotype 4 shown in Figure 1A is the formation of primer dimers during the simultaneous amplification process using the one-step multiplex RT-PCR with the four primer pairs. However, this phenomenon was repeated in all assays, even with different annealing temperatures ranging from 55 to 60 °C.

The additional band observed for serotype 4 may function as an internal control for the reaction. When subjected to electrophoresis, the amplified products always differentiated the four DENV serotypes, even after increasing the annealing temperature of the primers. Additionally, the negative control was not amplified.

The ages of dengue-infected patients ranged from 1 to 85 years. Descriptive statistics performed using Student's t-test revealed an average age of 32.3 years. However, the most frequently affected age group was aged 21 to 38 years. These data were similar to the results described in São Paulo in the period between 2008 and 2015, where the age group ranging from 20 to 39 years was the most frequently affected. This age range corresponds to the economically active population, who works or studies during the day[39].

Most patients with a suspicion of dengue, 143 (72.6%), resided in an urban area, and only 54 (27.4%) resided in rural areas. This result reinforces the hypothesis that dengue is an urban disease that is favored by ecological, demographic, political, socioeconomic, and cultural conditions that contribute substantially to its occurrence [40-43].

Sensitivity tests were performed individually for each DENV serotype using the one-step RT-PCR (data not shown) and for the one-step multiplex RT-PCR. The detection threshold was the same in all tests: 3.12 ng of viral RNA. Additionally, the viral RNA of the reference serotypes was amplified in all multiplex one-step RT-PCR assays, confirming the reproducibility of the tests.

The specificity of the PCR is directly related to the choice of region to be amplified, and the target DNA segment must characterize the particular organism or group of selected species. Thus, knowledge of the nucleotide sequence is indispensable for the success of the amplification reaction[44]. The correct design of the primers promotes the high specificity and efficiency of the amplification reaction[45]. The primers used in this study presented these characteristics. They originated from the nucleotide sequences of the NS1 protein, a glycoprotein that is highly conserved in genus *Flavivirus* and functions as a potential diagnostic marker for early detection of the infections caused by DENV and other viruses of the genus *Flavivirus*[46,47].

The results obtained from the one-step multiplex RT-PCR assays were consistent with the guidelines of the World Health Organization[48], which considers the detection of viral RNA by molecular techniques as the ideal diagnosis for dengue cases. Furthermore, the genetic material was never amplified from serum samples that were not reactive in IgM and IgG ELISAs.

A significant association was observed between the criteria (2 McNemar p = 0.0001) and good understanding (0.7122) by the Kappa test between the molecular and immunological assays. Therefore, even the tests with different principles produced consistent results.

Based on the results obtained in the present study, to obtain a molecular diagnosis of dengue, the patients must seek the health services in the initial phase of the symptoms, which usually coincides with the onset of the febrile period and the circulation of viral particles in the blood[49]. The hallmark of the one-step multiplex RT-PCR assay optimized here is that it is able to rapidly detect and differentiate between the four DENV serotypes using a method that is simple to execute, sensitive, specific, and does not require skilled labor or expensive equipment. This approach only requires a thermal cycler and an electrophoresis device.

5 CONCLUSIONS

In Brazil, molecular methods have not yet been established by the Ministry of Health to diagnose dengue infection. The real-time reverse transcription-polymerase chain reaction (RT-PCR) with specific probes is commonly used in some countries with this propose, but here is not routinely used for the diagnosis of dengue. In Brazil, this technique has a high cost and is generally performed in reference laboratories. Here, the number of patients with dengue infections is extremely high, which represents very high economic expenses. On the other hand, the methodology optimized here

proves to be sensitive, specific, and reproducible; it does not require expensive equipment or reagents, and it does not need specialized personnel. The results obtained in the present study suggest that the molecular assay evaluated here presents advantages that allow its use in routine clinical analysis laboratories for the diagnosis and identification of dengue serotypes.

Author Contributions: MMFS, BOM, AKSC and JPPN extracted the RNA from the serum samples and did molecular biology assays. SGM and ACPC performed the statistical analyzes of the results. CMFSP and LMSSC selected the records of patients with clinical suspicion of dengue infection and separated the serum samples in the freezers -80 ºC stored in the LACEN-MA. RJGT prepared excel spreadsheets for the analysis of the results. MMT and MRQB designed and conducted the study, analyzed the data and drafted the manuscript. In addition to the activities already mentioned, all authors participated in the analyzes of the results and the writing of the manuscript.

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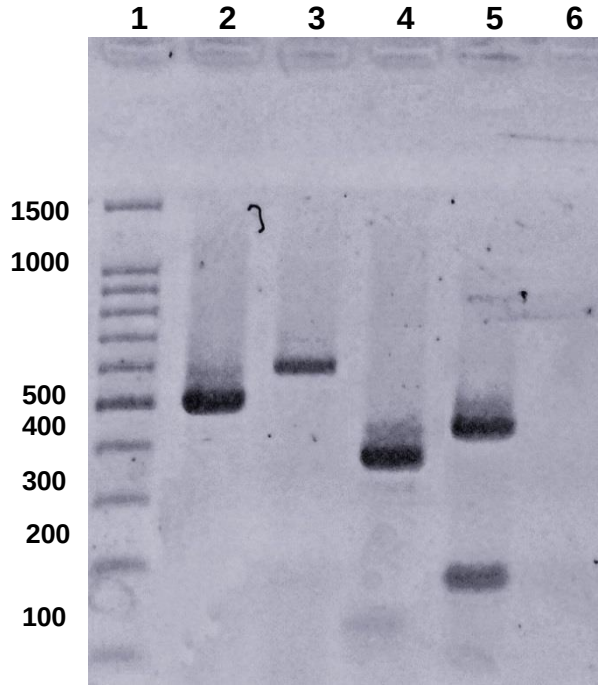
Figure 1- Profiles of DENV serotypes obtained during the *optimization of the one-step multiplex RT-PCR assays.*



Captions: Agarose gel showing the electrophoretic profiles obtained with total RNA from DENV-1 to DENV-4. Lane 1 represents the molecular marker (100 bp DNA ladder, Promega, USA); lanes 2 to 5 represent the profiles of DENV-1 (518 bp), DENV-2 (595 bp), DENV-3 (372 bp), and DENV-4 (434 bp and 190 bp), respectively. Lane 6 represents the negative control.

Figure 2A and 2B - Profiles of clinical samples obtained using the one-step multiplex RT-PCR assays.

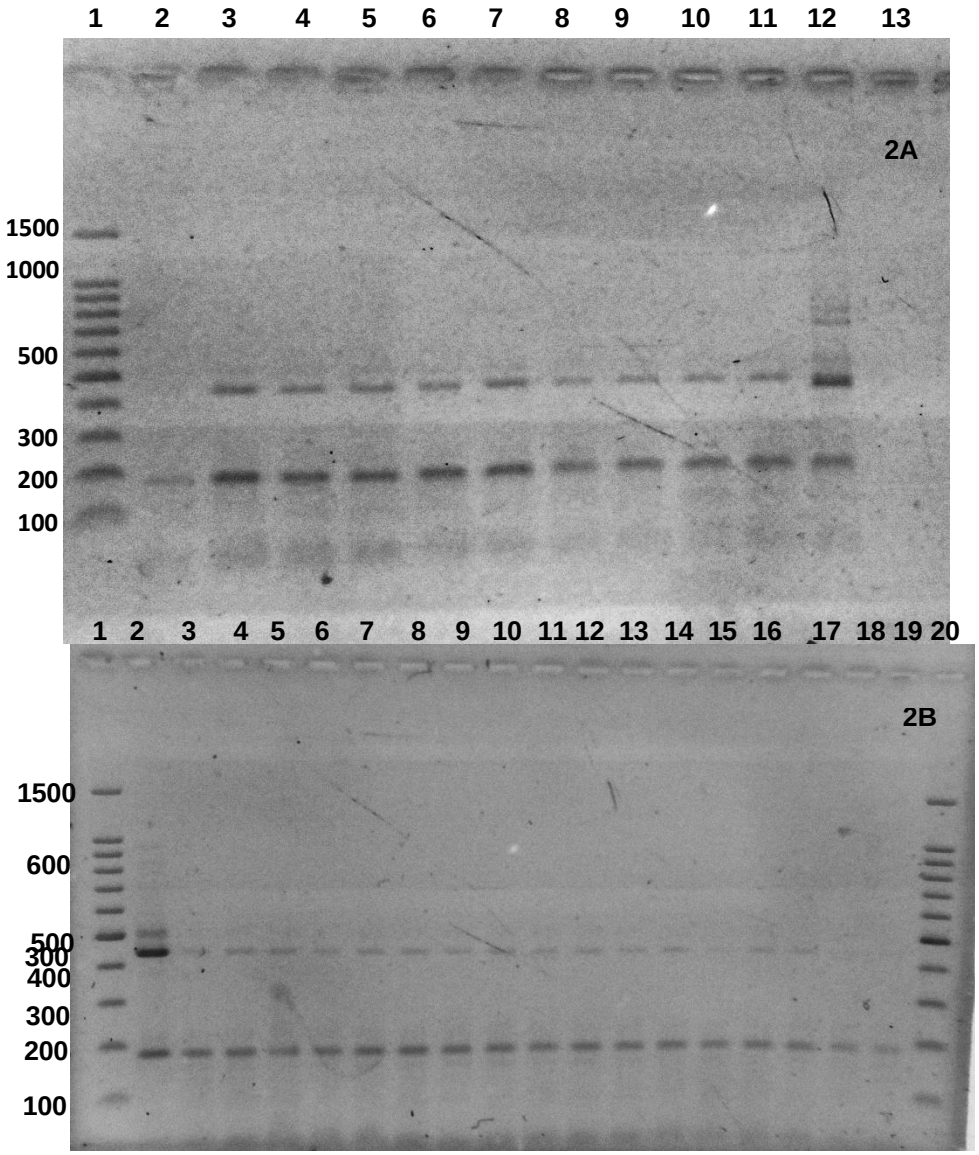


Figure 2A- Electrophoretic profiles obtained with total RNA from clinical samples and separated on agarose gels stained with ethidium bromide. Lane 1 represents the molecular marker (100 bp DNA ladder, Promega, USA). Lanes 2 to 11 represent the profiles obtained from clinical samples. Lane 12 represents the positive control (DENV-4, 434, and 190 bp). Lane 13 represents the negative control. Figure **2B-** Lanes 1 and 20 represent molecular markers (100 bp DNA ladder, Promega, USA). Lanes

3 to 19 represent profiles from clinical samples. Lane 2 represents the positive control (DENV-4, 434 and 190 bp).

Figure 3A to 3C - Agarose gel stained with ethidium bromide showing profiles of dengue serotypes from clinical samples amplified using one-step multiplex RT-PCR assays.

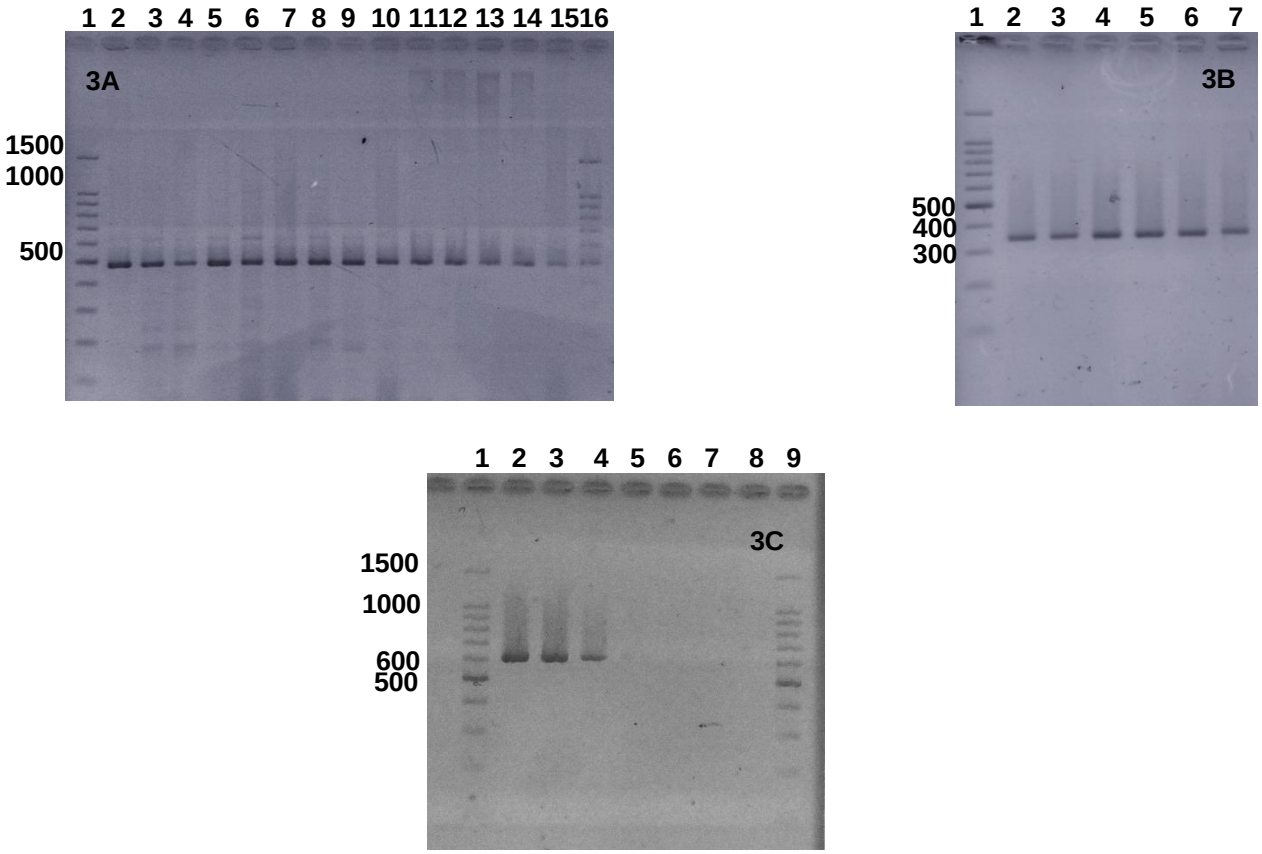
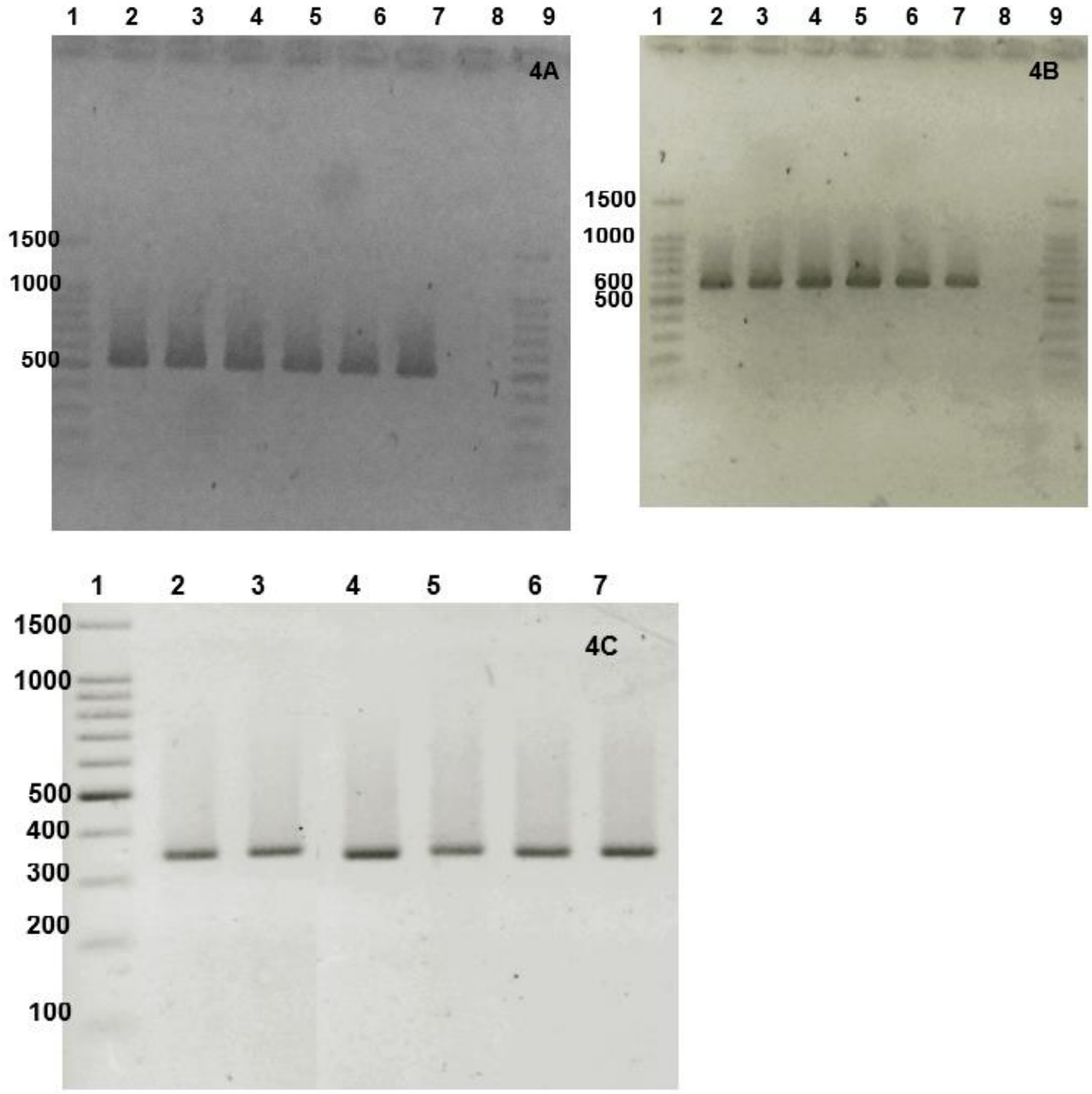


Figure 3A: Lanes 1 and 16 represent the molecular markers (100 bp DNA ladder, Promega, USA), lane 2 represents the positive control (DENV-1, 518 bp), and lanes 3 to 15 represent profiles from clinical samples collected from patients infected with DENV-1. **Figure 3B:** Lane 1 represents the molecular marker (100 bp DNA ladder, Promega, USA), lane 2 represents the positive control (DENV-3, 372 bp), and lanes 3 to 7 represent the profiles of clinical samples from patients infected with DENV-3. **Figure 3C:** Lane 1 represents the molecular marker (100 bp DNA ladder, Promega, USA), lane 2 represents the positive control (DENV-2, 595 bp), and lanes 3 and 4 represent the profiles of clinical samples from patients infected with DENV-2.

Figure 4 - Profiles of the sensitivity tests of DENV-1 to DENV-4 serotypes amplified using multiplex one step RT-PCR assays.



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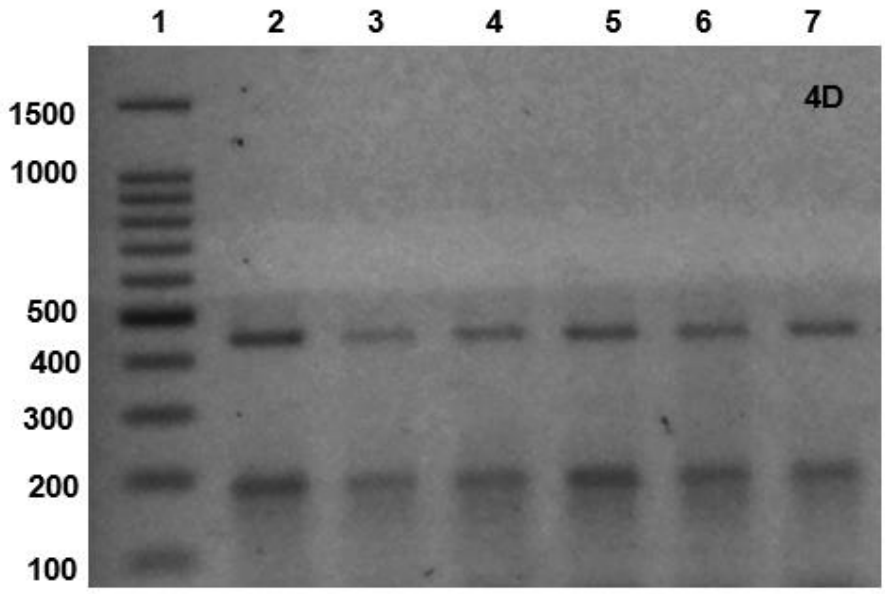

Figure 4A: Electrophoretic profiles obtained from the sensitivity tests using serial dilutions of RNA from DENV-1, 518 bp. Lanes 2 to 7 show the profiles of assays performed using 100, 50, 25, 12.5, 6.25 and 3.12, and 1.56 ng of RNA, respectively. Lane 8 profile of negative control. Lanes 1 and 9 show the profiles of molecular marker (100 bp DNA ladder, Promega, USA). **Figure 4B:** Electrophoretic profiles of sensitivity tests performed with serial dilutions of RNA from DENV-2, 595 bp. Lanes 2 to 7 show the profiles of assays performed using 100, 50, 25, 12.5, 6.25 and 3.12, and 1.56 ng of RNA, respectively. Lane 8 profile of negative control. Lanes 1 and 9 show the profiles of molecular marker (100 bp DNA ladder, Promega, USA). **Figure 4C:** Electrophoretic profiles of sensitivity tests performed with serial dilutions of RNA from DENV-3. Lanes 2 to 7 show the profiles of assays performed using 100, 50, 25, 12.5, 6.25 and 3.12, and 1.56 ng of RNA, respectively. Lane 1 profile of molecular marker (100 bp DNA ladder, Promega, USA). **Figure 4D:** Electrophoretic profiles of sensitivity tests performed with serial dilutions of RNA from DENV-4, 434 and 190 bp. Lanes 2 to 6 show the profiles of assays performed using 100, 50, 25, 12.5, 6.25 and 3.12 ng of RNA, respectively. Lane 1 molecular marker (100 bp DNA ladder, Promega, USA).

Table 1 Number of days after the onset of signs and symptoms suggestive of a DENV infection, according to the data obtained from the GAL system of the LACEN-MA.

IgM-ELISA positive concerning the number of days after the onset of signs and symptoms of dengue/ratio (%)			IgM-ELISA positive, and one-step multiplex RT-PCR positive (%)
	21 (15.6)	4	21 (100.0)
	59 (43.7)	5	59 (100.0)
135 (68.5)	16 (11.8)	6	8 (50.0)
	10 (7.4)	7	0 (100.0)
	9 (6.7)	8	0 (100.0)
	3 (2.2)	9	0 (100.0)
	17 (12.6)	10 to 30 days	0 (100.0)
IgM-ELISA negative concerning the number of days after the onset of signs and symptoms of dengue/ratio (%)			IgM-ELISA negative and one-step multiplex RT-PCR positive (%)
	6 (9.7)	1	6 (100.0)
62 (31.5)	23 (37.1)	2	20 (86.9)
	18 (29.0)	3	3 (16.7)
	15 (24.2)	4 days	0 (100.0)

Caption: (%), percentage, GAL, Laboratory Environment Manager database, LACEN-MA, Central Public Health Laboratory of the State of Maranhão.

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