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Dengue virus RNA quantification through PCR: what is the best cost-effective approach?

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Genet. Mol. Res. 22 (1): gmr19075
Received July 07, 2022
Accepted December 18, 2022
Published February 01, 2023
DOI http://dx.doi.org/10.4238/gmr19075

ABSTRACT. Detection via PCR is a fast, sensitive, and highly specific method. However, the cost for testing through this technique is quite high, mainly because of the costs of the kits that are used. We looked for the best cost-effective alternative for Dengue virus (DENV) detection via PCR through the evaluation, optimization, and comparison of RT-PCR (Reverse Transcription - PCR) and RT-qPCR (Reverse Transcription–qPCR) detection kits. The biological material was samples of blood serum collected from 40 Brazilian patients suspected of DENV infection. Two reaction final volumes were tested for diagnosis via RT-PCR, 12.5 µL and 25 µL, and diagnosis via RT-qPCR was performed using the two-step approach with the Sybr Green detection system. An analysis of the associated cost for each approach was also made. Analysis via RT-PCR allowed viral RNA amplification from 27 samples, independent of the final reaction volume tested. Diagnosis via RT-qPCR enabled virus identification from 33 samples. The costs per reaction for the RT-PCR technique were US$ 2.91 and US$ 2.41 American dollars for the final reaction volumes of 25 µL and 12.5 µL, respectively. For the RT-qPCR technique, the reaction cost was found to be US$ 2.30. The comparison between the techniques showed that RT-qPCR was more sensitive, allowing virus detection in a larger number of samples. However, results indicated that RT-PCR (12.5 µL) can be used as a screening method, considering its lower reaction cost. The cost analysis showed that RT-

qPCR had the best cost-benefit ratio, since it allowed virus detection from a larger number of samples with a cost similar to RT-PCR. We also found that optimization of the cDNA (complementary DNA) synthesis step can significantly affect the final diagnosis cost for both techniques.

Key words: RT-PCR; RT-qPCR; Molecular diagnosis; Flavivirus; Viral RNA

INTRODUCTION

Flaviviruses, commonly known as arboviruses, are viruses transmitted by mosquitoes, such as *Aedes aegypti* and *Aedes albopictus* (Mansuy et al., 2018). The Dengue virus (DENV) is an arbovirus transmitted, mainly, by the same vectors that transmit other flaviviruses, such as the Zika virus (ZIKV) and Chikungunya (CHIKV), constituting a growing worldwide problem for public health (Pabbaraju et al., 2016; Mansuy et al., 2018). DENV has four serotypes, named DENV-1, DENV-2, DENV-3 and DENV-4 (Simmons et al., 2012). Nowadays, all serotypes circulate in Brazil, though certain serotypes can be more prevalent in some states than in others (Fares et al., 2015).

Similar to other parts of the world, Brazilian DENV incidence is a threat to public health, considering the high number of cases that are registered in the country. According to a Brazilian Health Ministry report, until the 34th epidemiologic week (12/30/2018 to 08/24/2019), 1,438,471 probable cases of dengue were registered in the country. In 2018, during the same period of time, the registered number of probable cases reached only 205,791, showing that a significant increase (599.5%, when compared to 2018) in the number of probable cases registered in 2019. Regarding the incidence rate of probable cases of DENV, 690.4 cases/100 thousand inhabitants have been registered in 2019, with a predominance of serotype 2 (Brasil, 2019).

DENV infections are characterized by symptoms such as fever, maculopapular rash, musculoskeletal pain, headache, and conjunctivitis, which are often confused with of other flaviviruses infections (ZIKV and CHIKV) due to the similar signs and symptoms (Gubler, 1998; Musso and Gubler, 2016). The difficulty in distinguishing DENV infection from other flaviviruses through the analysis of the symptoms, combined with the problems caused by their infection, encourages the performance of confirmatory laboratory tests. In addition, viral diagnosis becomes even more necessary when considering the most severe cases of the disease, such as its hemorrhagic forms. In cases of dengue hemorrhagic fever (DHF), determining if individuals have been previously infected, through a safe and fast form, is important, since warning signs (abdominal pain, drop in blood pressure, dizziness, bleeding, among others) may only late appear. Thus, this knowledge is essential for understanding the DHF pathophysiology and can allow better caring and improved patient monitoring (Barreto and Teixeira, 2008).

DENV diagnostic test is usually performed through three different procedures, Polymerase Chain Reaction (PCR), virus isolation, and serological assays (Peeling et al., 2010; Honda et al., 2012; Bäck and Lundkvist, 2013), with PCR being the method of choice recommended by the Brazilian Health Ministry for DENV diagnosis up until the 5th day from the disease onset (Brasil, 2016). The detection test via PCR is carried out with blood samples collected during the disease initial phase, usually until the 5th day from symptoms onset, since the viremia period is usually short (Peeling et al., 2010; Simmons et al., 2012;

Brasil, 2016). Virus isolation is considered an outstanding method for dengue diagnosis, but the low sensitivity and the requirement of seven to ten days for virus multiplication in cell lines reduces the usage of this approach (Lai et al., 2007). The serological tests consist in immunoglobulin detection (usually IgM and IgG), which are present in the serum, through ELISA (Enzyme-Linked Immunosorbent Assay) (Peeling et al., 2010). However, due to its detection principle, cross-reaction between different flaviviruses may occur, what is the main limitation of its application as a diagnostic method (Simmons et al., 2012; Mansuy et al., 2018).

PCR detection is a fast, sensitive, and highly specific method, with the advantages over the serology detection of not showing cross-reaction between different flaviviruses (Mansuy et al., 2018) and viral isolation due to the shorter response time (Lai et al., 2007). However, due to the cost of the detection kits that are commonly used, overall cost for performing diagnostic tests through this technique are still quite high, what makes evaluation and optimization of this procedure important for reducing its cost and increasing its diagnosis accessibility. In this way, considering DENV diagnosis through RT-qPCR (Reverse Transcription – quantitative Real Time PCR), several studies, aiming its optimization and analysis of the variables associated to the technique, have been carried out (Waggoner et al., 2013; Pabbaraju et al., 2016; Ambrose et al., 2017; Shukla et al., 2017; Santiago et al., 2018; Lura et al., 2019). However, to our knowledge, studies conducted with the aim of assessing and comparing DENV detection kits via RT-PCR and RT-qPCR are still scarce and thus this study will enable the recommendation of the best cost-benefit methods.

In this context, in addition to the limitations associated to each diagnostic method, high cost is certainly the main drawback for testing through PCR. The high cost of its detection kits makes PCR DENV detection impractical to the vast majority of the population. Thus, this study aimed to determine the best cost-effective alternative of the *Dengue virus* diagnostic test via PCR through the evaluation, optimization, and comparison of RT-PCR and RT-qPCR detection kits.

MATERIAL AND METHODS

Biological material

The biological material used in this study was obtained from the Central Laboratory of Public Health of the Federal University of Tocantins (UFT) and the experiment was conducted at the Laboratory of Molecular Analysis from the same institution. Samples consisted of 1 mL of blood serum from 40 Brazilian patients suspected of dengue infection, which were collected until the seventh day after symptoms onset. Once collected, samples were stored at -80°C until RNA extraction.

RNA extraction and cDNA synthesis

Viral RNA extraction was performed using the Bio Gene DNA/RNA Viral Extraction kit (Quibasa/Bioclin), following manufacturer's instructions. The quantity and purity of the RNA were assessed with the aid of a NanoDrop® spectrophotometer, and only high-quality samples (OD260/280 and OD260/230 > 1.8) with suitable quantities (>50

ng/µL) were used for the diagnosis via RT-PCR and RT-qPCR. 700 ng of viral RNA were reverse transcribed into cDNA (final reaction volume = 20 µL), on a BioRad thermocycler (T100), using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, USA), according to manufacturer's protocol.

DENV detection

The primer pair used for DENV detection (serotype 2; DENV-2) via RT-PCR and RT-qPCR, was obtained from a previous study (Santiago et al., 2013) and is listed on Table 1. Primer synthesis was performed by the Thermo Fisher Scientific company.

Table 1. Primer pair sequence for DENV-2 detection via RT-PCR and RT-qPCR. Fw = forward, Rv = reverse.

Virus	Target gene	*Primers* 5' – 3'	Amplicon size (bp)
DENV-2	Envelope protein (E)	Fw: CAGGCTATGGCACYGTCACGAT Rv: CCATYTGCAGCARCACCATCTC	78

Conventional PCR (RT-PCR)

RT-PCR reactions were carried out using the Go Tap Hot Start Master Mix kit (Promega), following the manufacturer's instructions, except for the final reaction volume. Two different reaction final volumes were performed: 25 µL (manufacturer's recommendation) and 12.5 µL (optimized). For the two different volumes, a fixed volume of cDNA was used, and cycling conditions were as follow: enzyme activation with 5 min at 95 °C, then 35 cycles of 95 ºC for 45 s, followed by 45 s at 59 ºC, and completed by 30 s at 72 ºC. A final extension step (5 min at 72 ºC) was added to allow extension of the ends from all amplified fragments. Results were visually analyzed in 1% agarose gels, using 10 µL of PCR products and 6 µL of the molecular weight marker of 100 bp.

Real-time PCR (RT-qPCR)

RT-qPCR reactions were carried out using the GoTaq qPCR Master Mix (Promega), following manufacturer's instructions, except for the final reaction volume, which was optimized by half (10 µL) of the recommended manufacturer's reaction volume (20 µL). A fixed cDNA volume of 1 µL per reaction was used and the amplification procedure was performed on a ABI PRISM 7500 Fast Real-Time PCR thermocycler (Applied Biosystems), with the following reaction conditions: initial enzyme activation with 2 min at 95 °C, then 40 cycles of 95 °C for 15 s (denaturation), followed by 30 s at 60 °C (primer annealing and extension). The results obtained were stored in the 7500 Fast Software (Version 2.1). In addition to the visualization of the results through the 7500 Fast Software, results were visually analyzed in 1% agarose gels, using 10 µL of PCR products and 6 µL of the molecular weight marker of 100 bp.

Cost analysis

The cost analysis of the kits used in this study was based on a price survey carried out directly on their manufacture's websites. According to the prices obtained and the total reaction number from each kit, the cost per reaction was estimated. The price survey was performed in July 2019, and values were given here according to the dollar quotation at the time of the survey. In order to indicate the best cost-benefit methodologies, a comparison between the cost and the results obtained was performed.

Ethical aspects

This study was approved by the Research Ethics Committee at the Federal University of Tocantins, according to the CAAE Opinion Number 13000819.5.0000.5519. The study met and respected the guidelines and regulatory standards of resolution 466/12 (Brasil, 2013).

RESULTS AND DISCUSSION

Conventional PCR (RT-PCR)

Analysis through RT-PCR allowed the amplification of viral RNA from 27 samples (Figure 1), independently of the final reaction volume used (Figure 1 A and B). The only difference between the two different volumes tested was the intensity of the bands visualized on agarose gels. Reactions with the final volume of 25 µL showed bands from DENV-2 amplification with higher intensity (Figure 1B) when compared to those observed for the final volume of 12.5 µL (Figure 1A). DENV diagnosis through RT-PCR has been commonly reported in the literature (Deubel et al., 1990; Eldadah et al., 1991; Lanciotti et al., 1992; Seah et al., 1995; Raengsakulrach et al., 2002; De Paula et al., 2004; Yong et al., 2007: Sasmono et al., 2014). In the study developed by Yong et al. (2007), DENV detection from 210 samples through RT-PCR enabled the amplification of the viral fragment in 134 (63.8%) samples. A similar result was obtained in this study, considering the percentage of similar detection (67.5%). However, it should be noted that the detection percentage obtained may vary in response to several factors, such as the detection kit being used. For instance, DENV diagnosis by Sasmono et al. (2014) was possible only in 53 (28.8%), out of 184 samples. This 50% difference observed on virus detection can be directly linked to the detection kit that was used, since all samples, including the ones from this experiment and those from these two previously detailed studies, were collected during the acute phase of the disease. Accordingly, De Paula et al. (2004) and Najioullah and Viron (2014) assessed and compared five and four different detection kits, respectively, and concluded that results can drastically change depending on the kit that is used.

Results from studies with RT-PCR assays indicate the efficiency of this method in detecting DENV and in the differentiation of its four serotypes through a rapid, sensitive, and specific manner, mainly for samples obtained during the acute phase of the disease (Lanciotti et al., 1992; Yong et al., 2007). Therefore, the result found here is important, since it corroborates with those described in the literature. Thus, the analysis of the different final volumes for RT-PCR reactions conducted in this study was essential, since it proved that the same DENV detection percentage could be achieved by using half of the

manufacture's recommended reaction volume, leading to a significant reduction in the final cost of the analysis (Table 4).

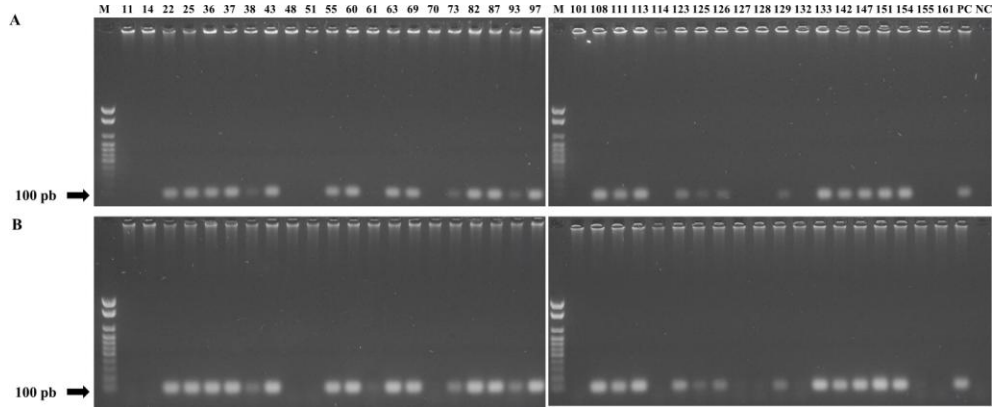

Figure 1. Agarose gels of the amplified products through RT-PCR. An agarose gel with amplified products using 12.5 µL as final reaction volume. B Agarose gel with amplified products using 25 µL as final reaction volume. The numbers on each gel refer to the identification of the biological samples. M = molecular weight marker (100 bp); PC = positive control; NC = negative control

Real time PCR (RT-qPCR)

DENV diagnosis through RT-qPCR allowed the detection of 33 infected samples (Figure 2). This represent a detection percentage of 82.5% (Table 2), which is in accordance with the high detection capacity of RT-qPCR from other diagnosis studies (Mansuy et al., 2018; Santiago et al., 2018; Mun et al., 2019; Tsai et al., 2019), contributing for the use of this technique.

Table 2. Dengue virus detection through RT-PCR and RT-qPCR for the 40 samples evaluated in this study.

Diagnosis method	Result		
	Positive	Negative	Total
RT-PCR (25 µL)	27	13	40
RT-PCR (12.5 µL)	27	13	40
RT-qPCR	33	7	40

Diagnosis via RT-qPCR can be performed through the one-step or two-step methods and, about the detection chemistry, Sybr Green or hydrolysis probes can be used as fluorophores (Gomes-Ruiz et al., 2006; Chen et al., 2015). Therefore, it is important to highlight that, in our analysis, DENV diagnosis through RT-qPCR was performed using the two-step method and Sybr Green as detection chemistry. This information is relevant, considering that the two-step method and the fluorophore Sybr Green are cheaper than the one-step method and the hydrolysis probes.

The one-step method for RT-qPCR is characterized by the cDNA synthesis and the fragment amplification reactions occurring in the same reaction tube. On the other hand, the two-step RT-qPCR differs from the one-step method basically by the fact that cDNA

synthesis and fragment amplification occur in different tubes, or different steps (Wacker and Godard, 2005). The differences related to the RT-PCR and RT-qPCR methods led to the development of studies that aimed, above all, to evaluate the interferences of each method in the final result (Evander et al., 1992; De Paula et al., 2004; Wacker and Godard, 2005).

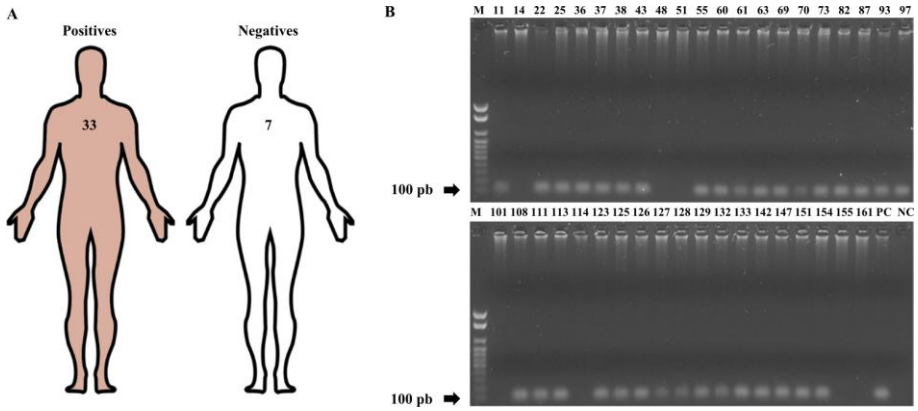

Figure 2. Diagnostic result performed through RT-qPCR. A Representation of the positive and negative results obtained via RT-qPCR. B Agarose gels of the amplified products for the diagnosis via RT-qPCR. Number represents the identification of each biological sample. M = molecular weight marker (100 bp); PC = positive control; NC = negative control

Using the RT-qPCR approach and the commercial kit SuperScript III (Invitrogen), Wacker and Godard (2005), working with reference genes, showed that results can significantly change depending if the one-step or two step methods is used. Variation in the results can also be observed when RT-PCR is used. For DENV detection, great differences in detection capacity has been found depending of the kits that are used (De Paula et al., 2004). Two-step kits showed low detection percentages (<37%) when compared to one-step kit (>88%) (De Paula et al., 2004). For this reason, viral diagnosis studies commonly use the one-step method for both RT-PCR and RT-qPCR techniques (Brasil, 2016; Mansuy et al., 2018; Santiago et al., 2018; Mun et al., 2019).

Regarding the detection chemistry, the use of Sybr Green and hydrolysis probes are widely disseminated in the literature (Chutinimitkul et al., 2005; Yong et al., 2007; Chen et al., 2015; Salles et al., 2017; Colombo et al., 2019; Mun et al., 2019) and, considering the possibility of using both fluorophores, studies comparing these chemistries have been performed in order to determine the one that is most suitable for DENV detection (Gomes-Ruiz et al., 2006; Paudel et al., 2011).

In the study developed by Gomes-Ruiz et al. (2006), DENV-3 detection was performed through Sybr Green and hydrolysis probe chemistries. The results obtained by these authors showed that the number of positive samples was the same for both methods. However, Sybr Green was indicated as the standard chemistry for DENV-3 detection due to its lower price (about 50%) when compared to the hydrolysis probe kit. Paudel et al. (2011) have also evaluated RT-qPCR detection chemistries and concluded that their sensitivity and specificity were very similar. However, Sybr Green was equally sensitive for primary and

secondary infections, differently from hydrolysis probes, which were less sensitive for secondary infections.

The result obtained here for DENV detection through one-step RT-qPCR is interesting since the percentage of positive samples (82.5%) resembles those found in the literature. This similarity is important since it may suggest a possible equivalence among the methods reported in other studies and the one used in our analysis. In addition, the use of Sybr Green makes the result even more advantageous, considering that it showed a high sensitivity level and a lower cost, when compared to hydrolysis probes. Finally, the result obtained by the two-step RT-qPCR with Sybr Green indicates the potential of the technique as a DENV diagnosis method, in addition to representing a reduction in cost, since the two-step approach is cheaper, and the final reaction volume has been reduced by half from the manufacture's recommendation.

Results from studies with RT-PCR assays indicate the efficiency of this method in detecting DENV and in the differentiation of its four serotypes through a rapid, sensitive, and specific manner, mainly for samples obtained during the acute phase of the disease (Lanciotti et al., 1992; Yong et al., 2007). Therefore, the result found here is important, since it corroborates with those described in the literature. Thus, the analysis of the different final volumes for RT-PCR reactions conducted in this study was essential, proving that the same detection percentage of DENV can be achieved by using half of the manufacture's recommended reaction volume, leading to a significant reduction in the analysis final cost (Table 4).

RT-PCR versus RT-qPCR

The comparison of the results obtained through the RT-PCR and RT-qPCR techniques shows that the detection capacity of DENV-2 varied depending on the technique used. RT-PCR allowed the detection of 27 positive samples, independently of the reaction final volume. On the other hand, viral RNA amplification was detected in 33 samples via RT-qPCR. The comparison of both approaches indicates an increase of positive cases of 22% when RT-qPCR is used. It is worth mentioning that the negative samples obtained via RT-qPCR were also negative for the RT-PCR technique.

The higher DENV detection obtained via RT-qPCR was expected, since the sensitivity of assays that use fluorophores as Sybr green is about 100-2,000 times greater than RT-PCR assays (Prada-Arismendy and Castellanos, 2011). The lower sensitivity of the RT-PCR technique was also observed by Poersch et al. (2005), where 50 samples were analyzed for DENV detection via RT-PCR and RT-qPCR and 5 and 17 positive samples were detected for each technique, respectively. Similarly, Chakravarti et al. (2016) evaluated both techniques for DENV detection and showed RT-qPCR was capable of DENV detection from samples with low viral concentrations, what was not observed for RT-PCR.

Our analysis through RT-PCR allowed to diagnose the viral infection in 67.5 % of the samples, indicating the importance of this technique for viral detection. When compared to RT-qPCR, the lower detection (22%) of positive samples by RT-PCR does not impede its use as a molecular diagnosis method. In addition, another point that must be considered is the RT-PCR cost that, as previously mentioned by Poersch et al. (2005), can be up to four times cheaper than RT-qPCR. Therefore, we believe that RT-PCR could be used as a

screening method for samples during the acute phase of the disease, that is, the detection test of the virus would be performed and, in case of a negative result, RT-qPCR would be used to confirm or refute the result.

Cost analysis

The cost analysis of the reagents used in each approach (Table 2) analyzed in this study allowed to determine the real reaction cost and to indicate which method presents the best cost-benefit ratio. Thus, the reaction cost for the RT-PCR technique using the 25 µL and 12.5 µL final reaction volumes are US$ 2.91 and US$ 2.41, respectively, and the reaction cost for the RT-qPCR technique is US$ 2.30 (Table 3).

Table 3. Cost analysis for each Dengue virus diagnosis kit used in this experiment.

Name	Company	Price (US$)	Reaction volume (µl)	Nº of reactions	Price/Reaction
Bio Gene DNA/RNA Viral Extraction kit	Bioclin	234.4	-	50	4.68
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher	384.1	20	200	1.92
GoTaq Master Mix	Promega	99.5	25	100	0.99
GoTaq Master Mix	Promega	99.5	12.5	200	0.49
GoTaq qPCR Master Mix	Promega	379.7	10	1,000	0.38

The comparison of the final cost from each methodology showed a significant difference among them, especially when large numbers of diagnostic tests are considered (Table 4). RT-PCR final cost for the 12.5 µL reaction volume was about 17% lower than the one obtained for the 25 µL volume. This finding means that, for 100 reactions with the final volume of 25 µL, the cost is equivalent to approximately 117 reactions with the final volume of 12.5 µL (Table 4). This difference becomes even more important when considering, for instance, the public health system, which has huge amounts of samples to be analyzed.

Table 4. Final cost per reaction for the tested methodologies to detect Dengue virus.

Name	Description	Price/Reaction
RT-PCR (25 µL)	Bio Gene DNA/RNA viral extraction kit + High-Capacity cDNA Reverse Transcription Kit + GoTaq® Master Mix	7.59
RT-PCR (12.5 µL)	Bio Gene DNA/RNA viral extraction kit + High-Capacity cDNA Reverse Transcription Kit + GoTaq® Master Mix	7.09
RT-qPCR (10 µL)	Bio Gene DNA/RNA viral extraction kit + High-Capacity cDNA Reverse Transcription Kit + GoTaq® qPCR Master Mix	6.98

The comparison between RT-qPCR and RT-PCR showed that the reaction cost for RT-qPCR is lower and higher to those found for RT-PCR with final reaction volumes of 25 µL and 12.5 µL, respectively (Table 4). Thus, among the RT-PCR volume variations, the best cost-benefit ratio was found to be reactions with the final volume of 12.5 µL. Between RT-qPCR and RT-PCR, the use of the first one was found to be advantageous, since it allowed the virus detection from a higher number of samples, with a cost very close to the

one obtained for RT-PCR. It is important to highlight that the largest portion of the reaction final cost is represented by the cDNA synthesis step (Table 3). Therefore, considering that the final reaction volume (cDNA) is 20 µL and only 1 µL is used for diagnostic test through these techniques, the optimization of this step can radically impact the diagnosis final cost.

CONCLUSIONS

The analysis performed in this study showed that both techniques, RT-PCR (final reaction volumes of 12.5 and 25 µL) and RT-qPCR, were capable of detecting DENV in most samples (>67%). The comparison between the techniques showed that RT-qPCR was more sensitive, allowing virus detection in a larger number of samples. However, the results indicated that RT-PCR (12.5 µL) can be used as a screening method, considering its lower reaction cost. The cost analysis showed that RT-qPCR had the best cost-benefit ratio, since it allowed virus detection from a larger number of samples with a cost similar to RT-PCR. The cost analysis also showed that optimization of the cDNA synthesis step can significantly affect the final diagnosis cost for both techniques. To conclude, obtaining high percentages of virus detection in reactions with optimized volumes indicates that the analysis and optimization of DENV diagnosis via RT-PCR and RT-qPCR can enable the development of efficient assays with reduced cost.

ACKNOWLEDGMENTS

We thank the “Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)”, the “Fundação de Amparo à Pesquisa do Estado do Tocantins (FAPT) - Programa Pesquisa para o SUS: Gestão Compartilhada em Saúde PPSUS/TO (Process number 017957/2018)”, the “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)”, the “Rede de Biodiversidade e Biotecnologia da Amazônia Legal (Bionorte)”, the “Fundação Escola de Saúde Pública de Palmas (FESP)”, and the “Universidade Federal do Tocantins (Propesq/UFT)” for the financial support. The aforementioned institutions have not contributed to the design, sampling, data analysis, publication, or writing of this manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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