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A Luminex-based single DNA fragment amplification assay as a practical tool for detecting and serotyping dengue virus

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A B S T R A C T

Dengue is a mosquito-borne viral infection that can evolve from subclinical to severe forms of disease. Early recognition during initial primary and secondary infections correlates with a reduced case-fatality rate in susceptible groups. The aim of this study was to standardize a DNA hybridization assay based on the Luminex technology for detecting and serotyping dengue virus (DENV). Reference DENVs representing the four different serotypes were used as controls to standardize the test. For validation, 16 DENV isolates obtained from a reference laboratory were analyzed in a double-blind manner to validate the test. Sixty blood samples from patients suspected of having dengue fever were used to evaluate the methodology after the validation step, and the results were compared with the reference semi-nested RT-PCR. Additionally, five human samples of each Zika and Chikungunya confirmed patients were used for specificity analysis. The Luminex-based assay correctly identified all 16 DENV isolates. In the evaluation step, the results of the RT-PCR/Luminex assay showed a concordance of 86.7% with those of the semi-nested RT-PCR. None of other virus infection samples was amplified. This is the first description of a hybridization assay that can discriminate the four DENV serotypes using probes against a single DENV sequence. The results indicated that the RT-PCR/Luminex DENV assay designed and evaluated in this study is a valuable additional tool for the early and rapid detection and serotyping of DENV, which could, in the future, be applied to new targets such as the Zika and Chikungunya viruses.

1. Introduction

Dengue fever (DF) is an arboviral disease that affects humans with high percentages of morbidity and mortality worldwide. According to the World Health Organization (WHO), Dengue virus (DENV) infection has a wide geographical distribution, with a higher prevalence in tropical countries. DF has an estimated occurrence of 50–100 million cases per year, and an estimated 500,000 individuals per year require hospitalization due to severe manifestations of the disease, 2.5% of whom die, mostly children (Weaver and Reisen, 2010; WHO, 2009). Dengue virus belongs to the genus *Flavivirus*, included in the *Flaviviridae* family. The genus *Flavivirus* consists of more than 70 viruses, including DENV, yellow fever virus, West Nile virus, tick-borne encephalitis virus and Japanese encephalitis virus. DENV is one of the most significant viral pathogens transmitted to humans through the bite of the female mosquito *Aedes aegypti* and *Aedes albopictus*. Four DENV serotypes (DENV-1, DENV-2, DENV-3, DENV-4) have been described, based on plaque reduction neutralization tests (Russell and Nisalak, 1967). Recently, the existence of a fifth DENV serotype has been suggested (Mustafa et al., 2015) but additional studies are needed to confirm if it is really a new serotype or a new variant of one of the already know serotypes (da Silva Voorham, 2014).

Early identification of DENV infection is of paramount importance to guide the selection and implementation of measures aimed

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to prevent morbidity and mortality. Two assays, the NS1- and DENV-specific IgM and IgG detection, are the diagnostic methods most commonly used independently or in association. The NS1 antigen is detectable within the first 5 days of infection, but the sensitivity of the commercial kit is variable. If used as a single assay, the sensitivity can be lower than 60%, while combination with a IgM detection assay improves sensitivity, which scales up to 85.70–88.65% (Duong et al., 2011; Wang and Sekaran, 2010). Furthermore, discrimination of the DENV serotypes is not possible by these methods (Anderson et al., 2014; Peeling et al., 2010). Virus isolation using cell culture remains the "gold standard" for the detection of DENV, but this method has a low sensitivity and it is time-consuming, as it requires additional steps for viral identification by indirect immunofluorescence using monoclonal antibodies against the four DENV serotypes (Peeling et al., 2010; Shu and Huang, 2004). The molecular detection of DENV genomic RNA offers a high degree of sensitivity, and it is more rapid and easier to perform than viral isolation for the diagnosis of DENV infection. In fact, due to their higher sensitivity, molecular techniques have gradually replaced the traditional virus isolation method as the new standard for DENV detection in acute-phase serum samples (Guzman et al., 2010; Sasmono et al., 2014). However, molecular methods still present some disadvantages, such as the need for the use of agarose gels and the possible risk of sample cross-contamination. For real-time PCR techniques, the main disadvantage is the restriction in the number of fluorescence detection filters, which limits the number of targets in a multiplex analysis (Binnicker and Espy, 2013; Gray and Coupland, 2014; Smith et al., 1998). New multiplex methodologies have been developed for the detection of different pathogens and, in this context, RT-PCR/Luminex-based assays may constitute useful alternatives for multiplex diagnosis (Wootton et al., 2016).

Luminex xMAP technology was developed in the late 1990s and has become an advanced system for multiplexed biological assays. Currently, many applications for this technology exist, such as immunoassays, DNA hybridization, studies on enzymatic activity, and the number of applications using this technology is increasing (Wessels et al., 2014; Wu et al., 2014). Additionally, the use of this technology in association with biomolecular reagents, fluorescent beads, flow cytometry and optical and digital processing has been shown to provide powerful methodologies for the diagnosis of infectious diseases and protein quantification in blood samples (Dunbar, 2006; Fulton et al., 1997; Pickering et al., 2002; Prabhakar et al., 2002; Pride et al., 2012; Santos et al., 2013; Wu et al., 2014).

In recent years, the search for early and faster diagnosis of DENV infections has been pursued extensively, by using different methods and combinations of various techniques to achieve improved sensitivity and specificity. The objective of the present study was to develop a Luminex-based methodology for the identification and differentiation of DENV serotypes, offering potential use in the early diagnosis of DENV infection. The RT-PCR/Luminex assay showed a high efficiency during the standardization and evaluation process by detecting and differentiating DENV serotypes in the samples used in this study. The optimization of a system based on a multiplexed biological assay, which might represent a more rapid and powerful method for detecting and differentiating DENV serotypes in a single reaction, is a promising tool that can aid the early and rapid diagnosis of DENV infection.

2. Materials and methods

2.1. Virus isolates and clinical specimens

Reference DENV representative of all four serotypes recognized to date were used as positive controls to standardize the methodology. Additionally, a total of 16 blinded DENV isolates were used to evaluate the reproducibility of the assay. The viral isolates corresponded to low-passage viral strains cultured in C6/36 cells. The viral isolates were kindly provided by the WHO/Pan American Health Organization Reference Center for Arbovirus Reference and Research at the Department of Arbovirology and Hemorrhagic Fevers, Instituto Evandro Chagas, Brazilian Ministry of Health, Ananindeua, Brazil. DENV isolates were identified by the letters A to P.

Human whole-blood samples were obtained from 60 patients clinically suspected of having dengue fever, within 0–5 days from the time of symptoms onset, at the University Hospital Clementino Fraga Filho/Federal University of Rio de Janeiro (HUCFF/UFRJ), Rio de Janeiro State, Brazil. All samples were tested in accordance with protocols approved by the ethics in research committee of the HUCFF/UFRJ, and were identified with codes to maintain the anonymity of the patients. Human whole-blood samples were collected and immediately stored with a solution of RNA Later (Ambion, USA), as recommended by the manufacturer, to inhibit degradation of the RNA samples.

Table 1
List of primers and probes used in this study.

	Sequence (5′ – 3′)
Primers	
DENVF	GGTTAGAGGAGACCCCTCCC
DENVR	BIOTIN – GAGACAGCAGGATCTCTG
Probes	
DENV1	AmC12 – ACACCATGGGAAGCTGTACCTTG
DENV2	AmC12 – GAGATGAAGCTGTAGTCTCACTGG
DENV3	AmC12 – TGAGGGAAGCTGTACCTCCTTGCA
DENV4	AmC12 – CCCGAAGCCAGGAGGAAGCTGTACTC
DEN GEN	AmC12 – GGACTAGAGGTTAGAGGAGACCCC

2.2. Primer and probe designing

Twenty-four DNA DENV sequences deposited in GenBank (genetic sequence database at the National Center for Biotechnical Information (NCBI)) were analyzed for primer and probe design. The accession numbers for the sequences are AF311957, AF311958, AF513110, EF025110, AY277659 and DQ672564 for DENV-1; AF359579, AY702038, AY702040, EU179858, EU081177 and EU056812 for DENV-2; AF317645, AY858037, AY858040, GQ466079, EF643017 and FJ644564 for DENV-3; and AF326573, AY762085, AY947539, KC333651, NC_002640 and JQ822247 for DENV-4. These sequences were aligned using the CLC Sequence Viewer software v.7.0.2 (CLC Bio, Denmark). After alignment, a fragment of approximately 159 bp was selected to design the primers and five probes. Among the five probes, four were serotype-specific and one was common to all DENV serotypes. The primers showed 100% consensus between the 24 aligned sequences. The probes were analyzed for potential cross-hybridization with other microorganisms using the Basic Local Alignment Search Tool (BLAST). The reverse primer was biotinylated at the 5′ end, and the probes were synthesized with a 5′ end amino-modified group and linked to a 12-carbon linker. When designing the probes, regions prone to secondary structures were avoided (Table 1).

2.3. RNA extraction

Viral RNA was extracted from 250 μL of cell culture supernatants or human blood samples using the TRIZOL LS Reagent (Ambion, USA) according to the manufacturer's recommendations. Extracted RNA was stored at −70 °C until be used.

2.4. Two-step RT-PCR

2.4.1. Reverse transcription

cDNA was synthesized from clinical and culture samples using the ImProm-II Reverse Transcriptase kit (Promega, USA) according to the manufacturer's recommendations.

2.4.2. Amplification of DENV cDNA

The PCR was performed using a pair of primers (DENVF and DENVR, Table 1) common to all four serotypes of DENV and 10 μL cDNA. The reverse primer DENVR was synthesized with biotin at the 5′ end. Each PCR mixture, in a final volume of 50 μL, contained 20 mM Tris-HCl (pH 8,4), 500 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of the four deoxynucleotide triphosphates (dNTPs), 0.2 μM of each primer DENV-F and DENV-R, 1.25 U Taq DNA Polymerase (Thermo Fisher Scientific, USA), and 5 μL aliquot of cDNA under testing. PCR amplifications were carried out in a Veriti 96-well thermal cycler (Applied Biosystems, USA) programmed for initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 1 min and primer extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min.

2.5. Luminex methodology

2.5.1. Probe coupling

The coupling procedure was performed with capture probes that were amine-modified at the 5′ end and magnetic carboxylated microspheres (MagPlex® microspheres, Luminex Corp, USA) using a protocol described by the manufacturer with some modifications. Briefly, 2.5×10^6 of the individual stock microspheres were transferred to a 1.5 mL microcentrifuge tube after being homogenized and sonicated for 30 s. The microcentrifuge tube was then placed on a magnetic separator for 2 min. The supernatant was discarded and the magnetic microspheres were suspended in 200 μL 0.1 M MES (2-*N*-morpholino-ethanesulfonic acid; Sigma, USA), pH 4.5, by vortexing and sonicating for approximately 30 s. The tube was again placed on a magnetic separator for 3 min, the supernatant was removed, and the precipitated microspheres were again washed with 200 μL 0.1 M MES pH 4.5, by vortexing and sonicating for approximately 30 s. The tube was placed in the magnetic separator for 3 min, and 175 μL of the supernatant were removed. Then, 1 mM of each capture probe was added to a set of magnetic microspheres, and 3 μL 30 mg/mL freshly prepared *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbonate (EDC) (Thermo Fisher Scientific, USA) was immediately added to each microsphere–probe mixture and incubated for 30 min at room temperature in the dark to allow the amine-modified probe to attach to the carboxylated microspheres. The incubation was repeated using 3 μL of freshly prepared 30 mg/mL EDC solution. Then, the coupled magnetic microspheres were washed once with 1 mL of 0.02% Tween-20 (polyxyethylenesorbitan monolaurate) (Sigma, USA) by vortexing, and the tube was placed in the magnetic separator for 3 min. The supernatant was removed and the precipitated coupled magnetic microspheres were suspended in 1 mL of 0.1% sodium dodecyl sulfate (SDS) (Sigma, USA) by inversion. The mixtures were again placed in the magnetic separator for 3 min. The supernatant was discarded and the coupled microspheres were resuspended and stored in 50 μL of TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA) (Sigma, USA) in the dark at 4 °C.

2.5.2. Hybridization with serotype-specific magnetic microsphere-capture probes

Complementary 5′ biotin-labeled PCR amplicons were hybridized with the serotype-specific magnetic microsphere-capture probes. The assay was performed in a 96-conical-well plate (BioRad, USA). Firstly, a working solution containing five magnetic microsphere sets previously coated with the respective capture probes was prepared. For the preparation of the working solution, each coupled microsphere set was diluted to contain 84 microspheres/μL in 1.5 × TMAC buffer (4.5 M tetramethylammonium chloride, 75 mM Tris–HCl, pH 8.0, 6 mM EDTA, and 0.15% sarkosyl), and a volume of 33 μL of working solution was used per reaction. The total reaction volume was 50 μL, which included 33 μL of working microsphere solution and 17 μL of TE buffer for the blank control well or 14 μL of TE buffer and 3 μL of labelled PCR amplicons for the test wells. The plate was placed in a thermocycler (Veriti 96-well thermal cycler; Applied Biosystems, USA), and the amplicons were denatured at 95 °C for 5 min, followed by incubation at different temperatures (42 °C, 45 °C, 48 °C and 50 °C) for 40 and 60 min. After incubation, the reaction plate was placed on a magnetic stand for 3 min. Then, using a pipette, 25 μL were carefully removed from each well. The plate was then removed from the magnetic stand, and 75 μL of 5, 6.7 or 10 μg/mL streptavidin- R-phycoerythrin conjugated (SAPE) (Molecular Probes, USA) in 1 × TMAC buffer (3 M tetramethylammonium chloride, 50 mM Tris–HCl, pH 8.0, 4 mM EDTA, and 0.1% sarkosyl) were added to each well, followed by gentle mixing with a pipette. The plate was placed in the thermocycler and incubated for 10 min at the same hybridization temperature and was then analyzed in the MAGPIX Luminex® platform.

The analysis of the data was performed using Luminex xPonent® for MAGPIX® software v. 4.2 (Luminex Corp, USA). To each reaction well, the MAGPIX Luminex® platform emits the median fluorescence intensity (MFI) to identify the microspheres and the SAPE conjugates in the samples. The MFI values for the samples were corrected by subtracting the MFI value of the blank control.

2.5.3. Determination of the limit of detection of the RT-PCR/Luminex assay

We determined the limit of detection (LOD) of the assay by using 10-fold serial dilutions of RNA copies of each reference DENV serotype. Viral RNA was obtained from cell culture supernatants infected with each one of the four DENV serotypes and their concentrations were measured using an UV–vis SpectraMax 190 Microplate Reader (Molecular Devices, USA).

2.5.4. Analysis of specificity against other arboviruses

The specificity of the RT-PCR/Luminex assay was evaluated by using RNA obtained from clinical samples collected from ten patients with acute febrile infection symptoms, that were confirmed by real time RT-PCR amplification being five for Zika virus and five for Chikungunya virus.

2.6. Semi-nested RT-PCR

Semi-nested RT-PCR was performed as described by Lanciotti et al. (1992). In brief, the first round was performed using a primer pair (D1 and D2) common to all four serotypes of DENV and 10 μL of cDNA. A second round of reaction was initiated with 5 μL of diluted material (1:50 in sterile distilled water) from the first-round reaction. Primer D2 was replaced with the DENV serotype-specific primers TS1, TS2, TS3 and Den-4. The amplified products were fractionated by electrophoresis in a 2% agarose gel, stained with GelRed™ 1X (Biotium) and visualized on an ultraviolet transilluminator. The size of each amplified product was specific for each serotype analyzed.

3. Results

3.1. Evaluation of the primer pair designed for the RT-PCR

The designed primers amplified a 159 bp fragment of the viral genome common to all four DENV serotypes. All four DENV control samples supported the amplification of this fragment when RT-PCR was performed. The amplified fragments were used as targets for multiplex hybridization reactions in the Luminex assay.

3.2. Hybridization assay standardization

The results of tests performed at different hybridization temperatures (42 °C, 45 °C, 48 °C, 50 °C), incubation times (40 and 60 min), and SAPE concentrations (5, 6.7 and 10 μg/mL) were evaluated to determine the optimal hybridization assay conditions necessary to discriminate the different DENV serotypes. Hybridization condition assays with control samples of each DENV serotype showed optimum hybridization signals at 42 °C for 60 min. By using these conditions, the Luminex-based assay was able to distinguish between the four DENV serotypes, with no cross-reactions. However, the MFI values obtained using hybridization temperatures of 45 °C and 48 °C for 60 min could not discriminate between the DENV serotypes, although the MFI values of the control samples were higher than those of the blank and the cut-off values. Increasing the hybridization temperature to 50 °C for 60 min, led to MFI values for DENV-1 and DENV-3 serotypes that were below the cut-off MFI values and therefore, were unsatisfactory. The evaluation of different SAPE conjugate concentrations, revealed very high MFI values for a concentration of 10 μg/mL, including the blank control. Therefore, two additional concentrations were evaluated, and a SAPE concentration of 6.7 μg/mL was found to show the best MFI signal with a minimum MFI value for the blank, still retaining the ability to detect and differentiate the DENV serotypes (Fig. 1). The MFI values of the control samples and negative controls were corrected by subtracting the blank MFI value of the respective probe. The cut-off MFI value was calculated from the measurement data obtained for six negative controls. The highest isolate values of 3 standard deviations above the mean negative control were 104, 109, 158, 101, and 471 for the DENV-1, DENV-2, DENV-3, DENV-4 and DENV GEN capture probes, respectively.

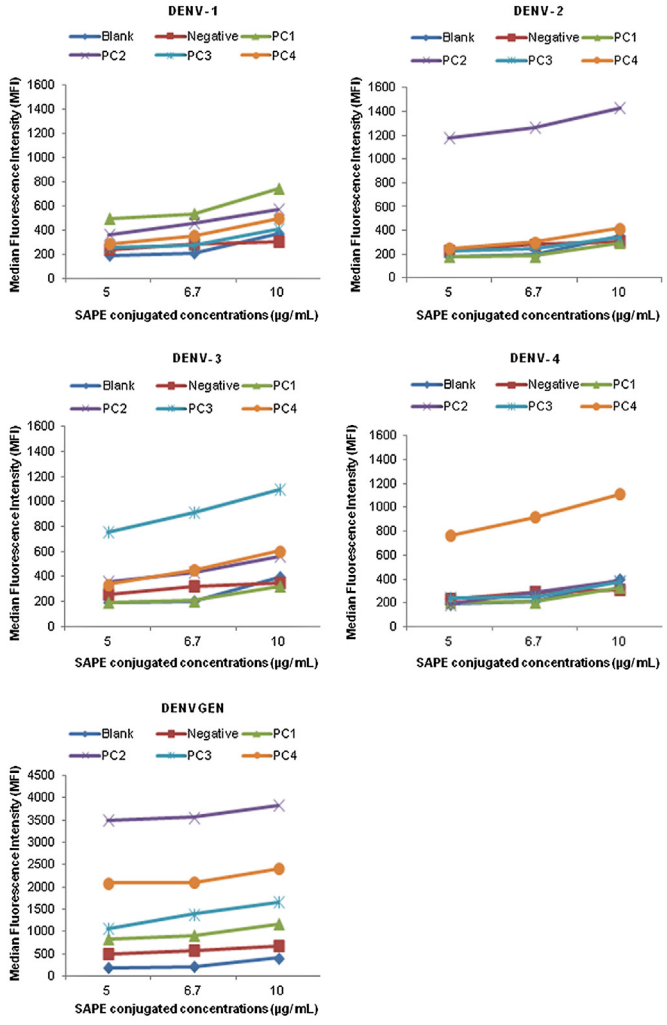

Fig. 1. Graphic representation of the hybridization signals obtained from biotinylated PCR products using different SAPE conjugated concentrations for each of the five fluorescent magnetic microspheres sets assessed. Three concentrations of the SAPE conjugated (5, 6.7 and 10 μg/mL) were evaluated for each of the four serotype-specific capture probes (DENV-1 to DENV-4) and the capture probe common to all four serotypes (DENV GEN). NC = Negative control; PC1 = Positive control for DENV-1; PC2 = Positive control for DENV-2; PC3 = Positive control for DENV-3; PC4 = Positive control for DENV-4.

3.3. Oligonucleotide coupling confirmation

During the standardization using the serotype-specific and DENV GEN capture probes, we observed that the DENV-1 capture probe, specific for DENV serotype 1, presented the MFI value closest to the cut-off value. Therefore, we decided to evaluate the coupling efficiency between the serotype-specific DENV-1 probe and the fluorescent magnetic microspheres. Different concentrations (5 fmol, 10 fmol, 20 fmol, 50 fmol, 100 fmol and 200 fmol) of a biotin-labelled complementary oligonucleotide were used. The evaluation showed that the hybridization was satisfactory and demonstrated efficient coupling of the DENV-1 capture probe with the fluorescent magnetic microspheres, because the MFI values increased in relation to the concentration of the complementary biotin-labelled oligonucleotide up to a saturation point (Fig. 2).

3.4. Determination of LOD and analysis of specificity

RT-PCR/Luminex assays were performed with different dilutions (10^{-1}–10^{-8}) of the viral RNA extracted from cell culture

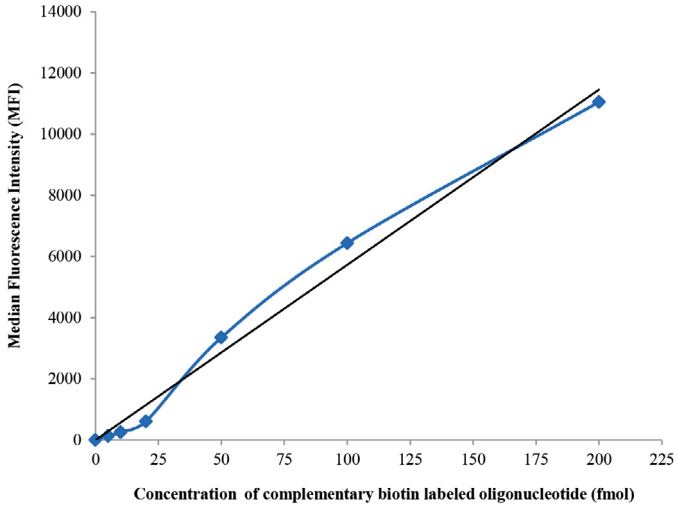

Fig. 2. Result of analyzing of the coupling efficiency between the serotype-specific capture probe DENV-1 and the fluorescence magnetic microsphere. The complementary biotin labeled oligonucleotide was added at different concentrations and analyzed in the MAGPIX Luminex® equipment.

Table 2
Results of double-blinded evaluation of the Luminex assay designed in the present study by testing cDNA from 16 viral isolates representative of different DENV serotypes.

Samples/controls	Capture Probes				
	DENV-1[a]	DENV-2[a]	DENV-3[a]	DENV-4[a]	DENV GEN[a]
BLANK	0	0	0	0	0
Neg[b]	8	9	4	23	314
D1[c]	**551**	46	65	82	**2390**
D2[d]	31	**752**	24	30	**2120**
D3[e]	65	25	**800**	56	**1977**
D4[f]	76	81	154	**662**	**1449**
A	0	**254**	0	0	**646**
B	44	23	**470**	33	**749**
C	81	80	82	**308**	**705**
D	44	35	38	**436**	**541**
E	**306**	0	0	30	**2146**
F	35	19	**660**	32	**1805**
G	**242**	18	23	35	**900**
H	**349**	33	48	57	**868**
I	0	**304**	0	0	**624**
J	**350**	34	45	49	**813**
K	75	**374**	67	72	**786**
L	23	20	17	**188**	**545**
M	20	**384**	18	16	**683**
N	36	33	33	**271**	**481**
O	28	13	**466**	28	**772**
P	83	73	**534**	82	**964**

Bold number means positive reaction.
[a] Cut-off values were 104, 109, 158, 101, 471 for DENV-1, DENV-2, DENV-3, DENV-4 and DENV GEN capture probes, respectively.
[b] Negative control.
[c] Positive control for DENV serotype 1.
[d] Positive control for DENV serotype 2.
[e] Positive control for DENV serotype 3.
[f] Positive control for DENV serotype 4.

supernatants infected with each of the four DENV serotypes. The LODs found for each DENV serotype were 3 pg/μL, 3 pg/μL, and 1 pg/μL and 1.5 pg/μL to DENV-1, DENV-2, DENV-3 and DENV-4, respectively.

Analysis of the specificity of the reactions against different arboviruses, has revealed no reactivity of clinical samples obtained from patients with suspected acute febrile infections and with confirmation in real time RT-PCR testing by amplification of viral RNA to Zika virus and Chikungunya virus. Thereby, the RT-PCR/Luminex assay was showed to be 100% specific for DENV.

3.5. RT-PCR/Luminex assay evaluation

A double-blinded analysis of a panel of 16 viral isolates representing different DENV serotypes was performed, employing the best parameters for the RT-PCR/Luminex DENV assay previously determined. All 16 isolates amplify a biotinylated fragment of 159 bp that were used in the Luminex-based assay. All 16 isolates were reactive with the probes used in the Luminex assay. A positive result in this assay was considered when two reactions were positive, one with a serotype-specific capture probe (DENV-1, DENV-2, DENV-3 and DENV-4) and the other with the capture probe common to all four DENV serotypes (DENV GEN). The results are shown in Table 2.

The semi-nested RT-PCR and RT-PCR/Luminex methodologies were compared to evaluate the efficiency of the RT-PCR/Luminex methodology. For this comparison, a panel of 60 human whole blood samples was used. Both methodologies analyzed showed a concordance of 86.7%. Eight samples were identified and serotyped by both methodologies, but six samples were identified and serotyped only by the RT-PCR/Luminex assay (1 for DENV-1; 4 for DENV-3 and 1 for DENV-4) and one sample was only amplified by Semi-nested RT-PCR for DENV-4. Forty-four human whole blood samples were not identified by the RT-PCR/Luminex methodology and were not amplified by the Semi-nested RT-PCR (Table 3).

4. Discussion

Rapid and accurate diagnosis of DENV infection is fundamental for implementation of more efficient clinical treatments, contributing to reduce the risk of developing severe manifestations of the disease as well as the morbidity and mortality associated with this infection. In this context, laboratory confirmation of DENV infection plays an important role in distinguishing it from other febrile illnesses such as malaria, leptospirosis, infection by Chikungunya virus and Zika virus, among others (Capeding et al., 2013; Rowe et al., 2014).

Luminex-based methods have usually been used to detect pathogens or protein markers implicated in several pathological conditions (Appanna et al., 2012; Dunbar, 2006; Gray and Coupland, 2014; Santos et al., 2013; Wu et al., 2014), but studies involving nucleic acid detection are still relatively limited. This might be due, at least in part, to difficulties in the standardization of these tests, especially multiplex formats, which are complex and depend on well-defined algorithms. Even with improvements in the stability of the DNA molecules in solution, predictions concerning the hybridization affinity between the probes and their respective targets in microarrays remain empirical (Liu et al., 2003; Naiser et al., 2009; Zhang et al., 2007). The optimal hybridization temperature is empirically determined through a process of measuring the melting temperature of the probes. For application in a multiplex system, TMAC buffer is added to eliminate the dependence of the dissociation temperature on the GC content of the probe, and thus, a multiplex probe can be used under a single condition. In addition, the selection of probes with secondary structure and other characteristics should be avoided (Diaz, 2007).

In this study, five different DNA probes were used in a RT-PCR/Luminex-based assay (Table 1), which were coupled to five different fluorescent magnetic microsphere sets: each probe was used to sensitize a different microsphere set. The sizes of the probes ranged from 22 to 27 nucleotides, within the range usually recommended for hybridization assays (Dunbar, 2006). Probes ranging in size from 8 to 19 nucleotides have also been used in microarray-based techniques, but shorter fragments do not allow stringent optimum conditions due to a low hybridization temperature, tending, therefore, to entail some loss of probe specificity. Although probes larger than 50 nucleotides increase the sensitivity of the test, their specificity is usually lower, with increasing chances of forming secondary structures. A suitable-sized fragment for a probe would is 20–30 nucleotides. Thus, the size of the probe in the hybridization reaction is directly related to the sensitivity of the test and inversely related to its specificity (Diaz, 2007; Dunbar, 2006). In addition, the size of the amplified fragment analyzed in this study (159 bp) is also within the range recommended in the literature, because most of the amplified fragments used in other studies have ranged from 90 bp to 417 bp, to minimize the potential for allosteric impediments that affect the efficiency of hybridization (Dunbar, 2006; Fitzgerald et al., 2007).

At the completion of this study, no description of a RT-PCR/Luminex assay for the detection and differentiation of DENV serotypes, using only a fragment common to all four DENV serotypes was available in the literature. The advantage of the assay is that it can test for the presence of a broad panel of DENV genotypes and can provide rapid and reliable results; therefore, it can be used as a marker in early infection.

Luminex is a methodology that has been growing in its diagnostic applications, because it allows the inclusion of additional molecular targets according to the epidemiological background

Table 3
Comparison of the results obtained with the Luminex assay and Semi-Nested RT-PCR methodologies for the different DENV serotypes in sixty blood samples from patients suspected of having dengue fever.

		LUMINEX					
		NA[a]	DENV-1	DENV-2	DENV-3	DENV-4	Total
Semi-Nested RT-PCR	NA[a]	**44**	1	0	4	1	**50**
	DENV-1	0	**2**	0	0	0	**2**
	DENV-2	0	0	**0**	0	0	**0**
	DENV-3	0	0	0	**2**	0	**2**
	DENV-4	1	0	0	1	**4**	**6**
	Total	**45**	**3**	**0**	**7**	**5**	**60**

Bold number means concordant results.
[a] Not amplified.

of the area under study (Munro et al., 2013). Therefore, the use of the RT-PCR/Luminex technique might lead to significant improvements in the early diagnosis and treatment of acute DENV infections. However, because of the possibility of having different targets in different stages of infection, the diagnosis of DENV infection must still be performed by combining methods: considering the nature of the NS1 antigen, the presence of viral particles in the blood and the levels of IgM and IgG antibodies against DENV (Peeling et al., 2010; Shu and Huang, 2004). Low viral loads might be related to the absence of reactivity as shown in other studies (Munro et al., 2013). Besides, different genotypes within each of the four serotypes have been described and might lead to changes in the sequence of RNA that interfere with probe hybridization (Carneiro et al., 2012; De Simone et al., 2004; Weaver and Vasilakis, 2009). However, nested PCR has been used with the same aim, and changes in the recognition of the amplified fragments have not been described to date.

The results indicate that the RT-PCR/Luminex assay developed in this study might constitute a new robust technique for the early diagnosis of DENV infections, contributing to meeting the increasing demand for accurate differential diagnosis of DF and helping to reduce the morbidity and mortality caused by this infection. The RT-PCR/Luminex assay might also be a valuable tool to offer surveillance and assistance to the public health system, to implement interventions through the detection of DENV serotypes in a single reaction. In addition, new targets can be added to the multiplex assay by Luminex methodology, in the future, to obtain differential diagnosis and more accurate detection of other etiologic agents, such as Chikungunya and Zika viruses.

5. Conclusion

The RT-PCR/Luminex DENV assay showed a high efficiency during the standardization and evaluation procedures by detecting and differentiating DENV serotypes in the samples used in this study. Further optimization and validation of the RT-PCR/Luminex assay are necessary to broaden the application of this methodology as an aid for the early diagnosis of DENV infections in different populations.

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