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Performance of nested-RT-PCR assays for Zika and Dengue detection

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ABSTRACT. Arboviruses represent a public health problem in developing countries. Accurate diagnosis has been challenging in Dengue virus endemic countries, especially since the introduction of the Zika and Chikungunya viruses. The aim of this study was to determine the sensitivity and specificity of nested Reverse Transcription Polymerase Chain Reaction (nested-RT-PCR) in a Zika virus epidemic setting. A total of 179 serum samples from patients with acute febrile syndrome reported between 2015 and 2016 were analyzed by nested-RT-PCR using real-time PCR (RT-qPCR) as reference test. The nested-RT-PCR assay demonstrated moderate agreement (kappa = 0.49) in identifying Dengue virus, resulting in 40.00% positive and 99.42% negative predictive values. Zika virus detection showed fair agreement (kappa = 0.29), with 100% positive and 92.61% negative predictive values (p-value < 0.05, Fisher's exact test). The diagnostic accuracy rates for the nested-RT-PCR assays were 97.76% for Dengue virus and 92.74% for Zika virus. The sensitivity and specificity of nested-RT–PCR for detecting Dengue virus were 66.66% and 98.29%, respectively; Zika virus RNA detection had 18.75% sensitivity and 100% specificity with the nested-RT-PCR protocol. In conclusion, the RT-qPCR assay is the most reliable method for diagnosing suspected cases of dengue and zika fever, providing adequate, timely and accurate laboratory responses to the proper management of patients, despite the high overall analytical accuracy of nested-RT-PCR.

Keywords: arboviruses; diagnosis; PCR, sensitivity and specificity.

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Introduction

Arboviral diseases represent a challenge for public health in developing countries. Brazil has been impacted socially and economically by dengue outbreaks for decades, and the introduction of zika and chikungunya has worsened this scenario.

Orthoflavivirus denguei (DENV) and *Orthoflavivirus zikaense* (ZIKV) belong to the *Flaviviridae* family and the *Orthoflavivirus* genus, whereas *Alphavirus chikungunya* (CHIKV) belongs to *Togaviridae* family and the *Alphavirus* genus. These arboviruses are single-stranded positive-sense RNA viruses transmitted to humans mainly through the bite of arthropod hosts (arthropod-borne viruses), especially those from the *Aedes* genus, which maintain the transmission cycle on a large scale in urban environments (Lanciotti et al., 1992; Lanciotti et al., 2008; Lanciotti & Lambert, 2016). These viruses are widely spread throughout the world, especially in countries with tropical climates, such as Brazil (Bronzoni et al., 2005; Weaver et al., 2018).

The recent geographic expansion and introduction of ZIKV and CHIKV, which are associated with their simultaneous occurrence with DENV in Brazil, challenge clinical diagnosis, since these arboviruses are responsible for acute febrile syndromes with similar clinical signs and symptoms (Fahsbender et al., 2020; Weaver et al., 2018).

For these reason, molecular diagnosis plays an essential role due to its ability of arboviruses simultaneous detection. Although, molecular assays implementation for epidemiological surveillance programs represents a significant challenge, especially in countries with scarce resources and low investment in arbovirus control (Wei et al., 2024; Zhang et al., 2024).

The development of accessible techniques and protocols to detect and distinguishing various arboviruses has been widely studied in recent years, especially after the widespread dissemination of ZIKV and its association with the emergence of congenital syndrome (Roma et al., 2019).

However, the state of art of arboviral molecular diagnosis requires diagnostic improvement, which could be reached with the combination of molecular techniques such as RT-LAMP, CRISPR biosensors and molecular sequencing. In this context, new molecular diagnosis strategies may enhance disease control and arbovirus spreading (Cardona-Trujillo et al., 2022; Razavi et al., 2024).

It is important to highlight that the occurrence of ZIKV, DENV, and CHIKV in Brazil has increased mortality rates due to atypical manifestations, especially those related to neurological syndromes, and other impairment for human health (França et al., 2016; Nunes et al., 2015; Nunes et al., 2019). Therefore, molecular diagnosis combined with sensitivity and specificity techniques, is essential for epidemiological control, and especially for an adequate, timely and accurate laboratory response to the proper management of patients.

Thus, this study aimed to evaluate the sensitivity and specificity of a molecular diagnostic technique involving nested-RT-PCR for the detection of ZIKV and DENV in the context of a ZIKV epidemic scenario, with RT–qPCR used as a reference test.

Material and methods

Sample collection

A total of 179 serum samples from patients with acute febrile syndrome and suspected arboviral disease with ⩽ 5 days of symptom onset were molecularly investigated. All the samples were tested for ZIKV, DENV and CHIKV via molecular biology protocols. The samples were collected from August 2015 to August 2016 and coincided with the period of ZIKV and CHIKV introduction in Mato Grosso State, Midwest Brazil. Sera were separated and stored at -80°C until use. Molecular investigations were performed with both nested-RT-PCR and RT-qPCR methods to evaluate the sensitivity, specificity, and accuracy of the molecular diagnosis of this infectious disease. This study was approved by the Research Ethics Committee (Protocol n° 10767319.8.0000.8088).

RNA extraction

Briefly, viral RNA was extracted from 150 μL of serum via the silica membrane method with NucleoSpin® RNA Virus (Macherrey-Nagel-Dueren/GER) according to the manufacturer's instructions. Internal control from the RT-qPCR assay and positive samples of the three arbovirus targets were included in this step of the study.

RT-PCR assay

Initially, a reverse transcription (RT) reaction was performed from viral RNA with a mixture containing 10 μL of RNA template, M-MuLV transcription buffer and 200 U of M-MuLV RT (New England BioLabs® - Ipswich, Massachusetts/USA), 8 U of RNasin® Ribonuclease Inhibitor (Promega® - Madison, Wisconsin/USA), 0.5 mM of each deoxynucleotide triphosphate (dNTP), 0.6μg of random primer (Invitrogen® - Waltham, Massachusetts, USA) and ribonuclease-free water to achieve a final reaction volume of 20 μL. The mixture was incubated at 25°C for 5 min., followed by incubation at 42°C for 60 min. and storage at -20°C until use. A single PCR for the *Orthoflavivirus* genus followed by a multiplex nested PCR protocol (Bronzoni et al., 2005), including the specific set of primers for all four DENV serotypes, was subsequently performed.

ZIKV RNA detection was performed via single PCR (Balm et al., 2012) followed by nested PCR. This single PCR was carried out with a mixture containing DNA polymerase buffer and 1.25 U of Taq DNA Polymerase (Uniscience® - Osasco, São Paulo, Brazil), 5 mM each dNTP, 0.5 μmol L^{-1} and 0.8 μmol L^{-1} forward and reverse ZIKV primers, respectively (Table 1), 6 μL of cDNA template from RT and ribonuclease-free water to achieve a final reaction volume of 25 μL. The mixture was incubated at 94°C for 15 min. for initial denaturation, followed by 45 amplification cycles at 94°C for 15 s., 57°C for 25 s. and 72°C for 20 s. Nested PCR was performed with 1 μL of products from single PCR using 0.5 μmol L^{-1} ZIKVF9027 (Balm et al., 2012) and 0.15 μmol L^{-1} FG2 (Fulop et al., 1993) oligonucleotides as forward and reverse set primers, respectively (Table 1), resulting in a 156 base pair (bp) amplicon size. The mixture contained the same concentrations of reagents from single PCR and was incubated at 94°C for 5 min. followed by 25 amplification cycles at 94°C for 1 min. 53°C for 1 min. 72°C for 2 min., and 72°C for 5 min. as the final extension step. The primer set selected for DENV and ZIKV targeted the conserved NS5 nonstructural protein.

CHIKV RNA detection was carried out with a single PCR using a primer set targeting the nsP1 nonstructural protein (Hasebe et al., 2002). The mixture contained PCR buffer and 1.25 U of Taq DNA Polymerase (Uniscience® - Osasco, São Paulo, Brazil), 5 mM of each dNTP, 0.1 μmol L^{-1} of forward and reverse CHIKV primer (Table 1), 6 μL of cDNA template and ribonuclease-free water to complete the final reaction volume of 25 μL, was incubated at 94°C for 3 min. as a denaturation step followed by 35 cycles at 94°C for 30 s., 54°C for 30 s. and 72°C for 30 s., and 72°C for 5 min. Positive and negative controls were included in all nested-RT-PCR assays, and the reactions were performed in a T100™ Thermal Cycler (Bio-Rad - Hercules, California/USA).

Table 1. Nucleotide sequences of primers used to DENV, ZIKV, and CHIKV RNA detection by RT-PCR.

Assay	Primer code	Target region	Sequence (5' - 3')	Genome position**	Amplicon (bp)
PCR to *Orthoflavivirus* genus	FG1 (+)	NS5	TCAAGGAACTCCACACATGAGATGTACT	8270–8297	958
	FG2 (-)		GTGTCCCATCCTGCTGTGTCATCAGCATACA	9228–9258	
Multiplex Nested-PCR to DENV	nDEN1 (-)	NS5	CGTTTTGCTCTTGTGTGCGC	8653–8673	472
	nDEN2 (-)		GAACCAGTTTGTTTDRTTTCATAGCTGCC	8488–8516	316
	nDEN3 (-)		TTCCTCGTCCTCAACAGCAGCTCTCGCACT	8822–8851	659
	nDEN4 (-)		GCAATCGCTGAAGCCTTCTCCC	8394–8415	222
Single PCR to ZIKV	ZIKVF9027 (+)	NS5	CCTTGGATTCTTGAACGAGGA	9121–9141	192
	ZIKVR9197 (-)		AGAGCTTCATTCTCCAGATCAA	9290–9312	
Nested-PCR to ZIKV	ZIKVF9027 (+)	NS5	CCTTGGATTCTTGAACGAGGA	9121–9141	156
	FG2 (-)		GTGTCCCATCCTGCTGTGTCATCAGCATACA	9246–9276	
Single PCR to CHIKV	CHIK/nsP1-S (+)	nsP1	TAGAGCAGGAAATTGATCCC	228–247	354
	CHIK/nsP1-C (-)		CTTTAATCGCCTGGTGGTAT	562–582	

(+): Forward; (-): Reverse; bp: base pair; **NCBI Reference Sequence: nDEN1 (NC 001477.1); nDEN2 (NC 001474.2); nDEN3 (NC 001475.2); nDEN4 (NC 002640.1); ZIKV (NC 012532.1); CHIKV (NC 004162.2).

Two percent agarose gel electrophoresis with ethidium bromide was carried out with 5 μL of RT-PCR products. The amplicon size was determined via comparison with a read-to-use 100 bp DNA ladder (Promega® - Madison, Wisconsin/USA) under ultraviolet light. All positive samples were purified via NucleoSpin® Gel and PCR Clean-up (Macherrey-Nagel - Dueren/GER) according to the manufacturer's instructions, and nucleotide sequencing was performed with forward and reserve primers via the Sanger method via a 3500 Genetic Analyzer® Applied Biosystems™ (Waltham, Massachusetts, USA). The inspection and alignment of the sequencing chromatograms were conducted via MEGA software version X, and the nucleotide sequences were queried against the NCBI database via BLAST.

Real Time RT-qPCR assay

Viral RNA was amplified via a one-step real-time assay based on the hydrolysis probe method using the ZDC Multiplex RT-PCR Assay® (Bio-Rad - Hercules, California/USA) with the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad - Hercules, California/USA), which targets the *ns4b* gene to ZIKV, the 5'UTR sequences to DENV, and the *nsP2* gene to CHIKV. The mixture was incubated at 50°C for 15 min. for reverse transcription and at 94°C for 2 min. for initial denaturation, followed by 45 amplification cycles at 94°C for 15 s., 55°C for 40 s. and 68°C for 30 s., following the manufacturer's instructions; reactions with cycle threshold (C_t) values lower than 40 were considered positive.

Statistical analysis

To evaluate the performance of both molecular methodologies, statistical analysis was based on the positive predictive value (PPV), negative predictive value (NPV), accuracy, sensitivity and specificity via GraphPad Prism® software (California, USA). RT-qPCR was used as the reference assay, and the nested-RT-PCR assay was used as the index test. Statistical significance was measured via two-tailed Fisher's exact test between the number of samples that tested positive for DENV and ZIKV via nested-RT-PCR and RT-qPCR. A kappa coefficient test was carried out to assess the agreement between the assays.

Results

In this study, we evaluated the specificity and sensitivity of nested-RT-PCR (index test) compared with RT-qPCR (reference test). A total of 179 serum samples from patients with acute febrile syndrome and a

clinical diagnosis frequently associated with dengue, zika or chikungunya viruses were considered. Samples were collected during the first outbreak of ZIKV in Mato Grosso State between 2015 and 2016.

For RT-qPCR, 3/179 (1.67%) samples were positive for DENV, and 16/179 (8.94%) were positive for ZIKV. With respect to nested-RT-PCR, DENV RNA was detected in 5/179 (2.79%) samples, all of which were characterized as the DENV-2 serotype, and ZIKV RNA was detected in 3/179 (1.67%) samples. Coinfection or CHIKV RNA was not detected.

The sensitivity and specificity of nested RT-PCR for detecting DENV RNA were 66.66% and 98.29%, respectively. With respect to ZIKV RNA detection via the nested protocol, 18.75% sensitivity and 100% specificity were observed.

The nested-RT-PCR assay showed moderate agreement (kappa = 0.49) for DENV, with positive predictive value (PPV) and negative predictive value (NPV) values of 40.00% and 99.42%, respectively (Table 2). The nested-RT-PCR protocol for ZIKV detection showed fair agreement (kappa = 0.29), with 100% PPV and 92.61% NPV (Table 3).

Table 2. Performance of nested-RT-PCR assay to DENV RNA detection in 179 serum clinical samples.

		ZDC Multiplex RT-PCR Assay® (Reference Test)			Kappa	PPV %	NPV %	p-Value
		P	N	Total				
DENV Nested-RT-PCR (Index Test)	P	2	3	5	0.49	40.00	99.42	0.0019
	N	1	173	174				
	Total	3	176	179				

P, Positive; N, Negative; PPV, positive predictive value; NPV, negative predictive value.

Table 3. Performance of nested-RT-PCR assay to ZIKV RNA detection in 179 serum clinical samples.

		ZDC Multiplex RT-PCR Assay® (Reference Test)			Kappa	PPV %	NPV %	p-Value
		P	N	Total				
ZIKV Nested-RT-PCR (Index Test)	P	3	0	3	0.29	100	92.61	0.0006
	N	13	163	176				
	Total	16	163	179				

P, Positive; N, Negative; PPV, positive predictive value; NPV, negative predictive value.

The diagnostic accuracies of nested-RT-PCR assays for DENV and ZIKV RNA detection were 97.76% and 92.74%, respectively.

All nested-RT-PCR positive samples were confirmed by nucleotide sequencing (data not shown). Figure 1 shows the DENV and ZIKV amplicons obtained via nested-RT-PCR.

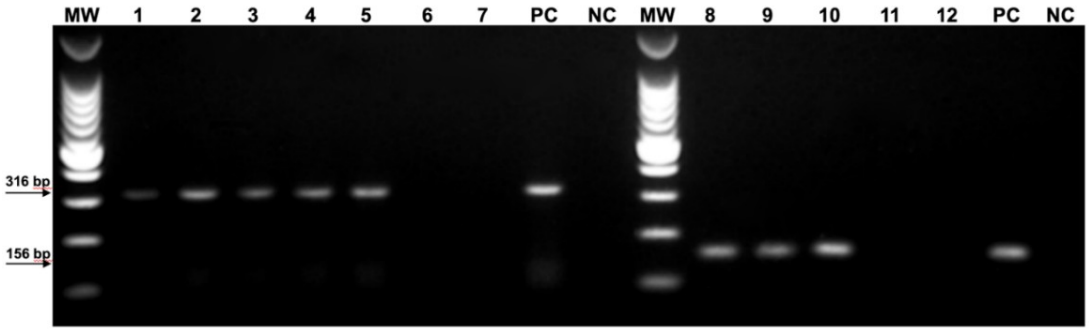

Figure 1. Agarose gel electrophoresis of RT-PCR amplicons from DENV and ZIKV. Lanes 1 to 5, samples positive for DENV-2 (316 bp). Lanes 8 to 10, samples positive for ZIKV (156 bp). Lanes 6, 7, 11 and 12, negative samples. MW, Molecular Weight (DNA Ladder, 100 bp). NC, Negative Control. PC, Positive Control.

Discussion

The introduction of ZIKV and CHIKV in the Americas and its worldwide spread (Campos et al., 2015; Nunes et al., 2015) associated with the high prevalence of DENV represents a public health concern. Differential diagnosis among these arboviruses has been challenging for clinicians and has become a serious public health issue in Brazil, especially because of the similarity of their clinical signs and symptoms to those of other arboviruses or current febrile illness etiologies (Fahsbender et al., 2020).

Laboratorial diagnosis of these arboviruses is performed by primary health care of the Brazilian public health system. Currently, diagnosis is mostly based on the detection of NS1 antigens for DENV or IgM serology for these arboviruses. Compared with viral isolation and reverse transcriptase (RT) PCR assays, this latter method contributes to the occurrence of cross-reaction and false-positive results (Lanciotti et al., 2008). Accurate differential diagnostic tests with an adequate and fast laboratory response can improve the clinical management of patients during outbreaks.

The ZDC Multiplex RT-PCR Assay® (Bio-Rad - Hercules, California, USA) provides simultaneous detection of epidemic arboviruses; however, the sample set used in this study did not present coinfection results. In contrast, molecular diagnostic investigations in Pernambuco and Ceará reported a coinfection rate of 2.6% and 2.3%, respectively (Pessôa et al., 2016; Frota et al., 2023). Similarly, two studies carried out in São Paulo in 2016 reported a rate of 0.9% (Colombo et al., 2017; Estofolete et al., 2019). Furthermore, other study demonstrated the possibility of simultaneous transmission of DENV, ZIKV, and CHIKV (Vogels et al., 2019). The conducted assays did not detect CHIKV RNA, possibly due to the low incidence of virus infection within the nineteen municipalities comprising the study area throughout the sampling period (Brasil, 2016).

Importantly, the ZDC multiplex RT-PCR assay® (Bio-Rad, Hercules, California, USA) is widely used to detect arboviruses in the research field, although there are no reports in the literature about specificity and sensitivity studies compared with nested RT-PCR. Compared with other RT–qPCR kits, the manufacturer's evaluation increases only the efficiency and demonstrates the limit of viral RNA copy number. (Ribeiro et al., 2022; Frota et al., 2023; Ma et al., 2019)

The reference test revealed 16 positive ZIKV RNA samples, while only three of those were detected by nested-RT-PCR. Thus, the molecular diagnosis of ZIKV via hydrolysis probe assays is considerably more sensitive than the index test. Single RT-PCR for ZIKV detection has failed, even when highly specific primers are used, highlighting the limitations of such techniques and the importance of choosing RT-qPCR assays for the diagnosis of this arbovirus. These results corroborate with other studies, suggesting that patients infected with ZIKV present low levels of viral copies in the blood, which would require highly sensitive molecular assays for accurate diagnosis (Balm et al., 2012; Colombo et al., 2018; Judice et al., 2018). Persistence for longer periods of ZIKV and higher levels of viral copies in other human biological samples, such as urine, saliva, and semen, increase the detection window even after the viremic period (Bingham et al., 2016; Judice et al., 2018; Matheron et al., 2016). This fact could affect the molecular diagnosis, as all the samples in this study were represented by serum. However, despite its low sensitivity, the overall accuracy of nested-RT-PCR (92.74%) for diagnosing ZIKV infection highlights its utility as a valuable tool for confirming viral genome analysis through sequencing.

Similarly, compared with RT-qPCR, nested-RT-PCR has relatively low sensitivity and high specificity for DENV RNA detection. Colombo et al. (2019) reported that the sensitivity of the nested-RT-PCR assay for DENV RNA detection can be greater than that of the gold standard RT-qPCR diagnostic test. Three DENV-positive samples detected by nested-RT-PCR were not amplified by the reference test, but confirmatory genetic sequencing revealed DENV-2 RNA in these serum samples. This finding coincides with the observed rapid increase in the circulation of DENV serotype 2 in Brazil since 2014 (Colombo et al., 2019; Jesus et al., 2020).

Despite the high overall analytical accuracy and relatively lower cost compared with RT-qPCR, nested-RT-PCR protocols may be unfeasible for routine diagnosis, especially during epidemic outbreaks, when large numbers of biological samples are processed. In addition, improvements in RT-qPCR, including the simultaneous detection of these three arboviruses and dengue serotyping, have resulted in timely and accurate laboratory responses (Li et al., 2019; Silva et al., 2019; Vieira et al., 2019). Nevertheless, nested-RT-PCR protocols may be fundamental tools for molecular epidemiological surveillance studies and research.

However, it is important to address some limitations of the study, such as the absence of positive samples for CHIKV. In addition, different infectious etiologies should be considered since the survey was designed only for the molecular detection of ZIKV, DENV, and CHIKV. Moreover, the samples were collected between 2015 and 2016, and the molecular analyses were performed only from 2019-20, indicating the possibility of viral RNA degradation.

Conclusion

In conclusion, our results corroborate that molecular diagnosis via RT-qPCR is the most reliable method for the differential diagnosis of suspected cases of dengue and zika fever, especially in countries with concomitant

circulation of these arboviruses. Thus, the continuous development and enhancement of molecular methods for arbovirus diagnosis are highly important. Considering that, the data gathered in this study serve as a reference point for comparing and selecting the most appropriate molecular diagnostic method, while no efficient epidemiological surveillance program is able to control the spread of this disease worldwide.

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