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Short communication

Specificity of NS1-based immunochromatographic tests for dengue virus with regard to the Zika virus protein

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ABSTRACT

Objectives: This study was performed to determine whether Dengue virus (DENV) immunochromatographic tests can detect and differentiate nonstructural protein 1 (NS1) from each of the four DENV serotypes and do not cross-react with the Zika virus (ZIKV) NS1 protein.
Methods: We compared the specificity of six NS1-based DENV immunochromatographic tests (point of care) in the detection of NS1 proteins from each of the four DENV serotypes and ZIKV. The tests were performed with NS1 proteins produced in mammalian cells. Cross-reactivity was confirmed with a purified recombinant ZIKV NS1 protein and $DENV^+$ or $ZIKV^+$ human serum samples.
Results: Cross-reaction was observed in 2 out of the 6 evaluated tests using cell culture supernatants containing NS1 protein of each tested virus. Cross-reactivity with ZIKV was confirmed with purified recombinant ZIKV NS1 produced in *Escherichia coli*. Further analyses with serum samples collected from $DENV^+$ or $ZIKV^+$ patients confirmed the cross-reactivity with ZIKV protein in 2 tests.
Conclusions: The detection of the NS1 protein is the basis for several commercially available serological DENV diagnostic tests. The present results emphasize the relevance of testing specificity of presently available NS1-based DENV serological tests and the need of adjustments of tests that cross-react with the ZIKV protein. Our results are particularly relevant for regions where both viruses are endemically found, as in the case of Brazil.

1. Introduction

Dengue virus (DENV) is one of the most epidemiologically relevant arboviruses transmitted to humans. This virus has a wide distribution, with an estimated number of 3.9 billion people at risk of infection in 128 countries and approximately 500,000 cases with more severe forms of the disease (World Health Organization (WHO); Bhatt et al., 2013). The clinical symptoms of this infection are easily confused with symptoms induced by other arboviruses. Thus, the use of specific laboratory tests is essential for the correct diagnosis and patient management, especially in endemic regions for these arboviruses.

The detection of the nonstructural protein 1 (NS1) is the basis of several commercially available DENV serological tests (Cuzzubbo et al., 2001). Since the NS1 protein is released by infected cells and accumulates in the blood of DENV-positive patients, it is used as a marker of acute infection and can be detected by immunochromatographic tests. However, with the recent spread of Zika virus (ZIKV) in the world, the specificity of these tests may be compromised by the extensive shared similarities between these viruses, consequently, increasing the risks of false-positive results in areas where both viruses circulate (Muller and Young, 2013; Gyurech et al., 2016; Matheus et al., 2016). Thus, in the present study, we compared the specificities of 6 different commercial DENV immunochromatographic tests (5 are widely available in the Brazilian market, whereas 1 is available in other countries). The tests were performed with NS1 proteins expressed by the 4 DENV serotypes and ZIKV using culture supernatants of infected cells, recombinant proteins

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expressed in *Escherichia coli* and human serum samples collected from DENV$^+$ or ZIKV$^+$ infected subjects.

2. Methods

2.1. Human samples and ethics statement

All procedures involving human serum handling followed the recommendations of the Institutional Review Board of the Hospital das Clinicas, University of São Paulo Medical School (CAPPesq – Research Projects Ethics Committee – 3.731.697). Confirmation of positivity for DENV or ZIKV NS1 was performed by ELISA or qRT-PCR, respectively.

2.2. Immunochromatographic tests

Commercial immunochromatographic tests designed for DENV NS1 capture were evaluated. Immuno-rapid Dengue NS1 (WAMA Diagnostics, Brazil; sensibility: 94.6% and specificity: 98.6%*), Dengue Antigen ECO test (ECO Diagnostics, Brazil; sensibility: 92.9% and specificity: 98.5%*), Orange Lab Check Dengue NS1 (Orange Life, Brazil; sensibility: 92.8% and specificity: 98.4%*), Dengue Duo Rapid Test (INLAB, Brazil; sensibility: 95.8% and specificity: 96.1%*), OnSite Dengue Antigen Rapid Test (CTK Biotech, USA; sensibility: 100% and specificity: 98.8%*) and Asan Easy Test Dengue NS1 Ag (ASAN, Korea sensibility: 42.9% and specificity: 99.2% (Lee et al., 2019)) were tested. Each test was evaluated, according to the manufacturer's instructions, using the secreted NS1 proteins of DENV and ZIKV produced by infected Vero cells or the recombinant ZIKV NS1 protein as samples. * The informed sensitivity and specificity were provided by the manufacturers.

2.3. NS1 protein production from DENVs and ZIKV

Vero cells were infected and cultivated for seven days with DENV1 (GenBank access number: JX669467), DENV2 (GenBank access number: AHG97599.1), DENV3 (GenBank access number: KC425219), DENV4 (GenBank access number: GU289913.1) or ZIKV (GenBank access number: ALU33341). The supernatant was harvested and clarified by centrifugation. The recombinant ZIKV NS1 protein produced by recombinant a *Escherichia coli* strain was obtained as previously described (Kanno et al., 2020) and used at different concentrations (15 or 50 μg/mL in phosphate-buffered saline (PBS)) to evaluate the test. The NS1 proteins were not purified or quantified in the supernatants, only confirmed by dot blot assays.

3. Results

Six dengue NS1 immunochromatographic tests were evaluated with culture supernatant of cells infected with each of the four DENV serotypes or with ZIKV. All 6 tests positively reacted with the samples derived from DENV-infected cells with no detected difference regarding the DENV serotype (Table 1). When tested with culture supernatants of ZIKV-infected cells, two DENV tests showed positive reactions (Table 1). The same result was confirmed using recombinant purified ZIKV NS1 tested at two different amounts (1.5 μg or 5.0 μg) (Table 1).

To further confirm the results generated with proteins secreted in cell culture supernatants, we repeated the tests with six serum samples collected from DENV-infected patients, previously characterized for a positive NS1 response (data not shown). Despite the high sensitivity alleged by manufacturers, at least three DENV NS1$^+$ samples did not react with the tested kits (Table 2). Only 3 serum samples were detected by all tested assays, while from the 3 remaining serum samples, 2 gave positive results only with the IN SITE test and 1 serum sample the WAMA test, which indicated differences in the sensitivity among the tested immunochromatographic tests. In addition, we tested three serum samples collected from a single patient with positive qRT-PCR response but at different time points (Table 2). The same two tests that have previously shown to cross-react with the ZIKV-protein also reacted with the third collected sample from the ZIKV$^+$ patient (the INLAB and ON-SITE DENV immunochromatographic tests) (Table 2), which could indicate the accumulation of NS1 during the course of the infection.

4. Discussion

The cross-reaction of DENV NS1-based diagnostic tests with ZIKV$^+$ serum samples is a concern, particularly in regions where the two viruses co-circulate endemically. In this study, two out of six (33%) DENV commercial kits designed to detect DENV NS1 reacted with ZIKV NS1 present in cell culture supernatants. Cross-reactivity of DENV NS1 tests with ZIKV NS1 was also confirmed using a recombinant ZIKV NS1 protein at concentrations usually found in the blood of DENV-infected patients (Young et al., 2000; Muller and Young, 2013). In addition, analyses based on serum samples of DENV- or ZIKV-infected subjects confirmed the results observed with *in vitro* produced NS1 proteins. These results emphasize the relevance of validation of presently available NS1-based DENV diagnostic tests and clearly indicate that some of the tests presently available should be optimized in order to improve sensitivity and, particularly, specificity with regard to ZIKV$^+$ serum samples.

During ZIKV infections cumulative mutations occur in virus subpopulations in different organs of the same infected person (Oliveira et al., 2018). Despite the lack of evidences that the natural diversity expected to occur in both ZIKV and DENV would impact the sensitivity or specificity of presently available serological diagnostic tests, these constant genetic changes may be followed by antigenic modifications that can affect their sensitivity and specificity. Under the tested conditions, *in vitro* cultured cells employed to propagate the virus and to generate the NS1 protein lack selection forces that may impose a genetic drift into the tested proteins. Similarly, proteins accumulated in cell culture supernatants do not interact with immunoglobulin or proteins that may hinder the detection of soluble NS1 at the blood current (Blacksell, 2012). Thus, the experimental conditions adopted in the present study avoided the interference of host proteins and/or selection factors that may reduce the sensitivity of NS1-detection assays.

Our observations complemented a previous publication based on the detection of DENV-specific IgG or IgM (Lee et al., 2019) and demonstrated that tests based on the detection of NS1 do not differentiate the 4 DENV serotypes. Nonetheless, differences in sensitivities, regarding the detection of free soluble NS1 in serum samples of infected subjects, was clearly demonstrated.

The present results showed that some of the presently available DENV serological tests based on the detection of the NS1 protein cross-react with the ZIKV NS1 protein. Such information emphasizes the need of further optimizations of these tests. In addition, even though we tested only a small fraction of the present available DENV-specific serological tests, our results, together with previous observations (Buathong et al., 2015; Dejnirattisai et al., 2016; Felix et al., 2017; Zaidi et al., 2020), indicate that cross-reactivity of DENV and ZIKV serological tests is still an unmatched issue. Therefore, an extensive evaluation of the cross-reactivity of DENV serological diagnostic tests with ZIKV$^+$ samples shall be extensively applied. Similarly, the data reinforce the need of technological developments that may improve the sensitivity and specificity of DENV serological tests in order to monitor the immunological

Table 1
Reactivity of immunochromatographic tests with native NS1 expressed by DENV- or ZIKV-infected cells or recombinant purified NS1.

Sample[a]	DENV – diagnostic tests					
	WAMA	Eco Diagnóstica	Orange Life	INLAB	ON-SITE	ASAN
DENV1 culture	+	+	+	+	+	+
DENV2 culture	+	+	+	+	+	+
DENV3 culture	+	+	+	+	+	+
DENV4 culture	+	+	+	+	+	+
ZIKV culture	–	–	–	+	+	–
rNS1 ZIKV 15 μg/mL	–	–	–	+	+	–
rNS1 ZIKV 50 μg/mL	–	–	–	+	+	–

[a] Culture supernatants were harvested after 7 days of infection with each DENV or ZIKV. Recombinant proteins were produced by *Escherichia coli* and purified through Niguel affinity chromatography.

Table 2
Reaction of ZIKV$^+$ or DENV$^+$ human serum samples with the tested DENV-NS1 immunochromatographic tests.

Human sera[a]	DENV – diagnostic tests					
	WAMA	Eco Diagnóstica	Orange Life	INLAB	ON-SITE	ASAN
DENV sample 1	+	+	NP[b]	+	+	NP
DENV sample 2	–	–	NP	–	+	NP
DENV sample 3	+	+	NP	+	+	NP
DENV sample 4	–	–	NP	–	+	NP
DENV sample 5	+	+	+	+	+	NP
DENV sample 6	+	–	NP	–	–	NP
ZIKV sample 1A	–	–	NP	–	–	NP
ZIKV sample 1B	–	–	NP	–	–	NP
ZIKV sample 1C	–	–	–	+	+	NP
Negative control	–	–	NP	–	–	NP

[a] 6 DENV NS1 ELISA$^+$ samples (DENV sample 1–6) and 3 sequentially collected ZIKV qRT-PCR$^+$ from the same patient (ZIKV sample 1A–1C) were evaluated.
[b] Non-performed.

status of populations living in endemic areas were both viruses circulate.

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Conflict of interest

No conflict of interest to declare by any of the authors.

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