Original article

Analytical and clinical performance of molecular assay used by the Brazilian public laboratory network to detect and discriminate Zika, Dengue and Chikungunya viruses in blood

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ABSTRACT

In response to the Zika epidemics in Brazil, the ZDC molecular assay (Bio-Manguinhos) was developed and registered at the Brazilian Regulatory Agency of Health Surveillance - ANVISA. The circulation of Zika (ZIKV) Dengue (DENV) and Chikungunya (CHIKV) viruses and their clinical similarities are challenges to correctly diagnose these viruses. The simultaneous detection of ZIKV, DENV and CHIKV is an important tool for diagnosis and surveillance. Here, we present the analytical and clinical performance evaluation of ZDC molecular assay (Bio-Manguinhos) at the public health laboratories three years after its registration at ANVISA. The clinical performance demonstrates the ZDC molecular assay (Bio-Manguinhos) has 100% sensitivity and 100% specificity to detect and discriminate ZIKV, CHIKV, and DENV from clinical plasma samples. The ZDC molecular assay (Bio-Manguinhos) results were highly reproducible and no cross-reactivity was seen during testing with a panel of other infectious agents. In conclusion, the ZDC molecular assay (Bio-Manguinhos) is an accurate and reliable tool to monitor Zika, dengue and chikungunya infections in countries like Brazil with simultaneous circulation of the three viruses.

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Introduction

The circulation of Zika (ZIKV) Dengue (DENV) and Chikungunya (CHIKV) viruses are responsible for significant epide-
bite of infectious mosquitoes, there are alternative means of
human-to-human ZIKV transmission. ZIKV has been detected
in urine and saliva, and there are case reports of sexual and
perinatal transmission.1,3

ZIKV, DENV and CHIKV have similar clinical manifesta-
tions such as fever, myalgia and headaches at early stage of
infection.2,10 The viral infection can be laboratory confirmed
by viral RNA recognition or identification of serum specific
antibodies.2,8,9,11 Virus culture and isolation is a very sensitive
method, but not feasible to be used as a routine diagnostic tool
because it is time-consuming and can require biosafety level 3
laboratory to reduce the risk of viral transmission. The cross-
reactivity of antibodies between Flaviviruses, such as dengue,
Zika, or yellow fever, limits the use of serology.12,13 Molecular
tests are the most sensitive and discriminatory diagnostic
tools for ZIKV, DENV or CHIKV,9,11 but the presence of nucleic
acids in body fluids may be short-lived.14 It is recommended
that real-time RT-PCR testing to be done within the first six
days of the onset of illness.12,14

In 2016, in response to the ZIKV epidemics in Brazil,
the ZDC molecular assay (Bio-Manguinhos) was developed
and registered at the Brazilian Regulatory Agency of Health
Surveillance - ANVISA. Clinical similarities and co-circulation
of these arboviruses are challenges to correctly diagnose
these viruses. The simultaneous detection of ZIKV, DENV
and CHIKV is an important tool for diagnosis and vigilance
through the public health laboratories. Transmission of the
arboviruses ZIKV, CHIKV and DENV, in Brazil, can occur dur-
ing the whole year, due to its tropical weather. Because of the
concurrent arbovirus epidemics, and the overlapping endemic
regions,10,15 the differential diagnosis must always include
ZIKV, DENV and CHIKV infection.

Herein, we present the analytical and clinical performance
evaluation of ZDC molecular assay (Bio-Manguinhos) three
years after its registration at ANVISA and implementation in
Brazilian public health laboratories.

Material and methods

ZDC molecular assay (Bio-Manguinhos)

The ZDC molecular assay (Bio-Manguinhos) is a real-
time nucleic acid amplification, with an internal control
(IC), designed to detect and discriminate in one reaction
Zika/Chikungunya/IC and in the other reaction Dengue/IC, in
samples previously submitted to nucleic acid extraction. The
ZDC molecular can detect all serotypes of DENV, but do not
differentiate among them. Primers and MGB-probes target NS1
gene of ZIKV (VIC), NS1p gene of CHIKV (FAM), and 3′-NCR gene
of DENV (VIC – DENV 2 and FAM – DENV 1, 3, 4), e IC (Dye3). The IC
is a virus like particle protected by patent (P0600715-5). The IC
eliminates false-negative results and controls all steps of the
reaction, from extraction to amplification, without interacting
with the virus present in the plasma of infected patients. Pos-
itive controls supplied by the manufacturer are run in every
batch. Additionally, an internal quality control (ICQ) from Bio-
Manguinhos or a known positive sample of each laboratory
of analysis is added to each run. The ICQ is a sample known
to be positive for ZIKV, CHICK and DENV. The amplification

is carried out on the Applied Biosystems 7500 Real-Time PCR
System (Thermofisher) with the following conditions: 51 °C for
30 min, 95 °C for 10 min, and 40 cycles at 95 °C for 30 s and 58 °C
for 30 s. A result was considered positive if any curve crossed
this threshold prior to cycle 38 to Zika/Chikungunya and 33 to
Dengue. Dengue results with Ct values between 33 and 37 are
inconclusive, and the samples must be tested again.

Limit of detection

ZIKV, CHIKV and DENV 1, 2, 3 and 4 RNA from virus culture
were kindly provided by LAVIMOAN (Laboratório de Virolo-
gia Molecular, UFRJ) and LATEV (Laboratório de Tecnologia
Viral Bio-Manguinhos) and were used to determine the LOD
of the assay. Samples from the following reference panels
were also used: (i) 1st World Health Organization International
Standard for Zika virus RNA for Nucleic Acid Amplification
Techniques (NAT)-Based Assays (PEI code 11468/16); (ii) 1st
World Health Organization International Standard for Chikun-
gunya virus RNA for Nucleic Acid Amplification Techniques
(NAT)-Based Assays (PEI code 11785/16); (iii) Dengue Early
Infection Accuset™ Performance Panel (Seracare 0845-0050).
LOD was determined in eight independent assays at two-fold
dilutions ranging 2.00E+03 IU/mL to 2.50E+01 ZIKV, 5.00E+03
IU/mL to 8.00E+00 to CHIKV and 3.90E+04 to 1,22E+01to
Dengue.

Additionally, LOD was also performed with a synthetic RNA
that was synthesized by IDT (Integrated DNA Technologies,
USA) to ZIKV and CHIKV containing the target sequence of
the assay.

The software IBM SPSS Statistics, version 26, was used
to perform the PROBIT analysis and determine LOD (95% confi-
dence interval - 95% CI).

Specificity studies

The specificity was evaluated by testing the detection of target
and non-target viral RNA. Forty-four positive plasma
samples previously tested by Trioplex real-time RT-PCR assay18
were used. Eleven samples were positive for ZIKV, seven for
DENV and 26 for CHIKV. Ninety-six plasma samples previously
tested by serological and/or molecular assays, and with neg-
ative results for Zika, Chikungunya and Dengue were used.
Cross-reactivity with non-target was evaluated with samples
previously tested and with positive results by serological
and/or molecular assays to HIV, HCV, HBV, HTLV, Chagas dis-
ease, Yellow fever, Mayaro, and Syphilis.

Briefly, viral RNA was isolated from 500 µL of human
plasma samples in the molecular biology workstation
(BioRobot MDx, Qiagen) using the QIAamp one-for-all nucleic
acid kit (Qiagen) in accordance with the manufacturer’s proto-
col. The internal control (IC) was added to each sample before
extraction.

Clinical performance

In order to evaluate the clinical performance of ZDC molecu-
lar assay (Bio-Manguinhos) 269 clinical samples were selected
from the Ministry of Health’s surveillance network. Forty-
one plasma samples were from Instituto Evandro Chagas (IEC),
positive to ZIKV (n = 19), CHIKV (n = 13) and DENV (n = 9), previously tested by an in-house protocol; and 228 plasma samples were from Feira de Santana, a city from Bahia state, previously collected from febrile cases in 2015 and without molecular diagnosis.

Reproducibility

A replicate panel was made spiking virus stock into truly negative plasma, to reach final Ct of 28 to ZIKV, CHIKV and DENV. Replicate tests were performed one week apart with different operators.

Results

The assessed results of analytical sensitivity with 95% and 50% positivity detection limits are summarized in Table 1. The specific target detection was evaluated with plasma samples from febrile patients previously tested and confirmed ZIKV, DENV, CHIKV infections and negative (Table 2). No false positive was detected.

Results of the clinical performance with ZDC molecular assay (Bio-Manguinhos) showed 100% positive agreement when testing IEC samples, with medium Ct of 33 to ZIKV, 22.7 to CHIKV and 22.5 to DENV. The evaluation of Feira de Santana samples have found 44 samples positive to CHIKV with mean Ct of 28.4, and six samples positive to ZIKV with mean Ct of 32.6.

The reproducibility within the laboratory was tested with a replicate panel with 100% agreement. All positive samples were detected and there was no false positive result. The mean Ct (+ SD) was 28.28 (+ 0.32) to ZIKV, 27.16 (+0.87) to CHIKV, 25.47 (+0.71) to DENV1, 28.21 (+0.31) to DENV2, 25.79 (+0.63) to DENV3, and 26.92 (+0.48) to DENV4.

Discussion

Due to the current epidemiological situation in Brazil, with introduction of Chikungunya and Zika, since 2014 and 2015, respectively, and the continuous challenge of dengue, the ZDC molecular assay (Bio-Manguinhos) proved to be a useful tool for accurate detection, discrimination and epidemiological surveillance of these endemic diseases with similar clinical manifestation.

The performance of the ZDC molecular assay (Bio-Manguinhos) was verified using virus culture, international/commercial panels and synthetic RNA, each of them having results in different units makes a comparison difficult, especially because of the non-comparable unit pfu/mL. However, the virus culture from ZIKV and CHIKV came from LA VIMOAN, the same laboratory that provided the material during the development and validation of the ZDC molecular assay (Bio-Manguinhos). So, the same quantification protocol was used. Comparing the results of this study with the product insert, the same sensitivity is reported to CHIKV and an improved sensitivity was found to ZIKV.

One limitation of this study was the lack of synthetic RNA from DENV. Unfortunately, the material ordered arrived degraded to our laboratory. When the commercial panel was used, DENV results have shown an improved sensitivity. Since the product registration at ANVISA until now, some improvements have been made to the product (data not shown). This is part of a strategy of continuous improvement and it will culminate with a new version of the ZDC molecular assay (Bio-Manguinhos) which will soon incorporate the typing of DENV in the assay.

It is worth highlighting that the IC from ZDC molecular assay (Bio-Manguinhos) added to each sample before the nucleic acid extraction step is fundamental to control the effectiveness of the assay, since the extraction step is the user's responsibility.

The clinical performance of ZDC molecular assay (Bio-Manguinhos) showed 100% sensitivity and 100% specificity to detect and discriminate ZIKV, CHIKV and DENV from clinical plasma samples. Clinical similarities of these arboviruses make their diagnoses a challenge for clinicians and health authorities. The co-circulation and possibility of co-infections reinforce the importance of a diagnostic tool that includes

Table 1 – Analytical sensitivity of ZDC molecular assay (Bio-Manguinhos).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sample tested</th>
<th>LOD 95%</th>
<th>LOD 50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZIKV</td>
<td>Virus culture</td>
<td>12.9 pfu/mL</td>
<td>2.36 pfu/mL</td>
</tr>
<tr>
<td></td>
<td>Paul Erlich Panel</td>
<td>357.3 IU/mL</td>
<td>93.1 IU/mL</td>
</tr>
<tr>
<td></td>
<td>Synthetic RNA</td>
<td>105.3 copies/mL</td>
<td>29.8 copies/mL</td>
</tr>
<tr>
<td>CHIKV</td>
<td>Virus culture</td>
<td>50.0 pfu/mL</td>
<td>10.6 pfu/mL</td>
</tr>
<tr>
<td></td>
<td>Paul Erlich Panel</td>
<td>2889.5 IU/mL</td>
<td>218.5 IU/mL</td>
</tr>
<tr>
<td></td>
<td>Synthetic RNA</td>
<td>195.8 copies/mL</td>
<td>24.5 copies/mL</td>
</tr>
<tr>
<td>DENV</td>
<td>Serumac panel</td>
<td>1.60 copies/mL</td>
<td>0.958 copies/mL</td>
</tr>
<tr>
<td></td>
<td>Virus culture DENV1</td>
<td>221.5 pfu/mL</td>
<td>47.3 pfu/mL</td>
</tr>
<tr>
<td></td>
<td>Virus culture DENV2</td>
<td>4.1 pfu/mL</td>
<td>1.3 pfu/mL</td>
</tr>
<tr>
<td></td>
<td>Virus culture DENV3</td>
<td>23.8 pfu/mL</td>
<td>6.6 pfu/mL</td>
</tr>
<tr>
<td></td>
<td>Virus culture DENV4</td>
<td>9.2 pfu/mL</td>
<td>3.0 pfu/mL</td>
</tr>
</tbody>
</table>

Table 2 – Performance of ZDC molecular assay (Bio-Manguinhos) with clinical plasma samples true positive and true negative.

<table>
<thead>
<tr>
<th>Specimen category</th>
<th>Tested</th>
<th>ZIKV positive</th>
<th>DENV positive</th>
<th>CHIKV positive</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zika</td>
<td>11</td>
<td>11/11</td>
<td>0/11</td>
<td>0/11</td>
<td>100% (71.5–100%)</td>
<td>100% (97.2–100%)</td>
</tr>
<tr>
<td>Dengue</td>
<td>7</td>
<td>0/7</td>
<td>7/7</td>
<td>0/7</td>
<td>100% (59–100%)</td>
<td>100% (97.3–100%)</td>
</tr>
<tr>
<td>Chikungunya</td>
<td>26</td>
<td>0/26</td>
<td>0/26</td>
<td>26/26</td>
<td>100% (86.8–100%)</td>
<td>100% (96.2–100%)</td>
</tr>
<tr>
<td>Negative</td>
<td>96</td>
<td>0/96</td>
<td>0/96</td>
<td>0/96</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A not applicable.

detection of all three viruses and differentiates among them. The ZDC molecular assay (Bio-Manguinhos) results were highly reproducible (100%) with no cross-reaction when testing a panel of other infectious agents.

In conclusion, this broad evaluation demonstrated the capacity of ZDC molecular assay (Bio-Manguinhos) to detect ZIKV, DENV and CHIKV RNA, and discriminate among them, in plasma specimen, with high sensitivity and specificity. The ZDC molecular assay (Bio-Manguinhos) is an accurate and reliable tool to monitor Zika, dengue and chikungunya infections in countries like Brazil with simultaneous circulation of the three viruses.

Ethical approval

This study was performed in accordance with The Declaration of Helsinki and the Nuremberg Code, complying with the rules for medical research involving human subjects of the National Health Council and ethical principles. Ethical approval from Institutional Boards were not required for this study because all molecular testing were conducted in addition to the official diagnostic procedures carried out at Central Laboratories (LACENs) in Brazil.

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Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES