



## Evaluation of immunoglobulin M-specific capture enzyme-linked immunosorbent assays and commercial tests for flaviviruses diagnosis by a National Reference Laboratory

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### ABSTRACT

Zika and Dengue viruses present considerable immunological cross-reactivity, resulting in a troublesome sero-diagnosis due to occurrence of false positive results. Due to Brazil's wide variety of circulating flaviviruses we aimed to access the use of in house serological tests adapted by National Reference Laboratory for Arboviruses in Brazil and evaluate commercial tests available. We evaluated in house IgM ELISAs for the individual detection of anti-ZIKV, -DENV, and -YFV IgM, against a panel of samples positive for dengue, zika, yellow fever, Rocio, Ilheus, Saint Louis encephalitis, West Nile and chikungunya. We also evaluated two commercial kits for dengue and zika IgM detection recommended by the Brazilian Ministry of Health in 2015. The sensitivity and specificity for the in house ZIKV IgM ELISA was 60.0 % and 88.6 % and for the in house DENV IgM ELISA was 100 % and 82.2 %, respectively. The in house YFV IgM ELISA presented 100 % for both sensitivity and specificity. The Novagnost Zika Virus IgM test presented a sensitivity of 47.3 % and specificity of 85.3 % and the Serion ELISA classic Dengue Virus IgM, 92.8 % and 58.9 %, respectively. Overall, both in house ELISAs for ZIKV and DENV adapted and evaluated here, presented better performances than the commercial kits tested.

### 1. Introduction

All flaviviruses are antigenically related and many of them, such as Zika virus (ZIKV) and Dengue virus (DENV) co-circulate in many tropical and subtropical regions worldwide (Liu et al., 2019). The first confirmed ZIKV infection in the Americas was reported in the Northeast region of Brazil in 2015, however the virus may have been introduced late 2013, more than one year before the epidemic reported in the continent (Faria et al., 2016).

Most ZIKV infections are asymptomatic, however, when symptomatic is usually a one-week mild illness characterized, in most cases by rash, low fever, arthralgia, myalgia, and conjunctivitis. An infected person usually recovers completely and despite rare, complications and deaths may occur. In fetuses and babies from infected mothers, zika-related congenital syndrome may occur (Duffy et al., 2009; Musso et al., 2019). Moreover, it has been shown that prior dengue exposure

and preexisting dengue antibodies reduces rather than enhances the risk of ZIKV infection and disease (Rodriguez-Barraquer et al., 2019; Gordon et al., 2019).

ZIKV and DENV are not only transmitted by the same vector *Aedes aegypti* (Krammer and Lim, 2019), but also share similar clinical manifestations and structural similarities in the viruses' capsid (C), envelope (E) and precursor of membrane (prM) proteins as they belong to the same family. Moreover, a 45–55 % protein homology is shared between ZIKV and DENV, and during natural infections, antibodies are induced mainly against E protein, but also against NS1, NS3 and NS5 (Heinz and Stiasny, 2017). Therefore, considerable immunological cross-reactivity is observed (Priyamvada et al., 2017; Tsai et al., 2017). Since the clinical manifestation of acute ZIKV infection is nonspecific, a definitive diagnosis relies on molecular or serologic testing (Musso et al., 2019).

According to WHO recommendations, the diagnosis is based on detection of the ZIKV genome by PCR, serology, and neutralization

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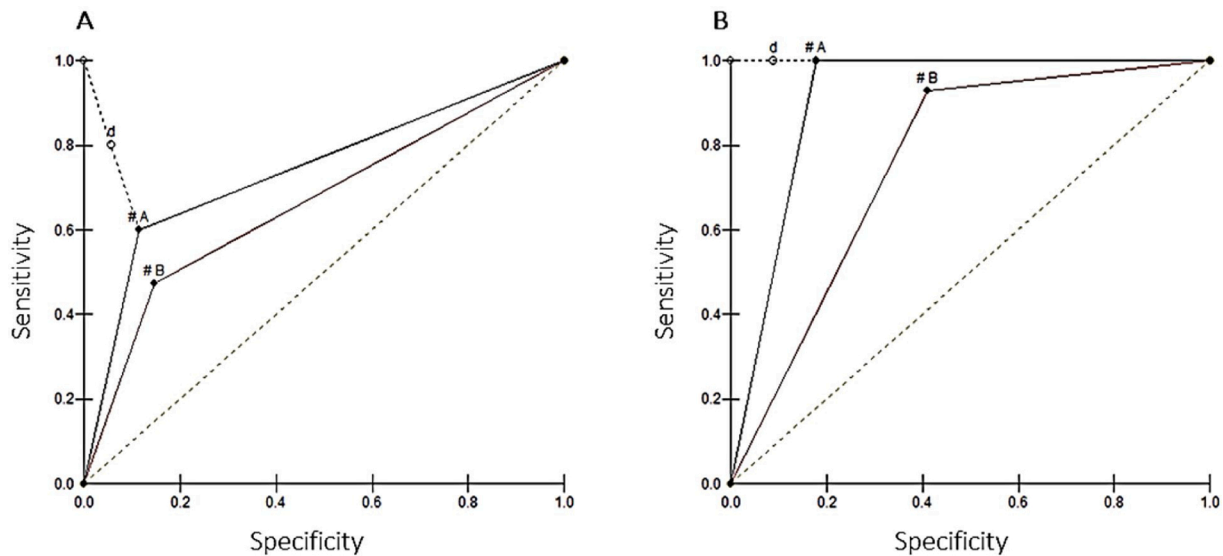
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**Fig. 1.** The ROC (Receiver Operating Characteristic) analysis. Comparison between (A) the *in house* DENV IgM ELISA (#A) and the Serion ELISA *classic* Dengue Virus IgM kit (#B) and (B) *in house* ZIKV IgM ELISA (#A) and Novagnost Zika Virus IgM kit (#B). The smaller line “d” distance indicates the best performance. CI 95 %: Confidence interval 95 % (area).

assays, such as plaque-reduction neutralization tests (PRNT). Although a ZIKV RNA detection provides conclusive evidence of an infection, a negative result does not rule out the diagnosis (WHO, World Health Organization, 2016).

Despite widely used, serodiagnosis may be troublesome due to cross-reactive false positive results where other flavivirus co-circulate. The level of anti-DENV and anti-YFV IgM and IgG have been well established in the past and documented for both primary infection and sequential infections (WHO, World Health Organization and TDR, Special Programme for Research and Training in Tropical Diseases, 2009), however for Zika, the use of IgM and IgG ELISAs is still a challenge, but strongly needed, especially for the investigation of probable congenital and neurological disease associated to the virus infection, which in most cases are observed after the viremic period.

Since twelve different flaviviruses have been isolated in Brazil and with the ZIKV emergence, here we aimed to evaluate Flavivirus IgM capture ELISAs to access the real effectiveness of the use of the in-house serological tests in a flavivirus immune population.

The use of paired samples, such as the ones used here, is known to be the gold standard approach for reliable serological investigations however, those are not commonly and easily available to many laboratories. The Evandro Chagas Institute is a National Reference Laboratory for the Brazilian Ministry of Health, established in 1970 and it has a wide variety of diagnostic tools, infrastructure and expertise in investigating arboviruses. Moreover, it has reported hundreds of arboviruses over the years and due to its role in the country, suspected cases all over the country are received, as convenience samples for differential diagnosis and stored in a biobank.

## 2. Material and methods

### 2.1. Ethical statement

The specimens analyzed in this study were received as convenience samples from an ongoing Project approved under the number CAAE 06213119.1.0000.0019 by the Ethical Committee from the Evandro Chagas Institute, National Reference Laboratory for arboviruses, Ministry of Health, Brazil.

### 2.2. Samples

A panel of serum samples ( $n = 73$ ) (supplementary table 01) positive for YFV ( $n = 7$ ), DENV ( $n = 14$ ), ZIKV ( $n = 25$ ), *Rocio virus* (ROCV,  $n = 1$ ), *Ilheus virus* (ILHV,  $n = 2$ ), *Saint Louis Encephalitis virus* (SLEV,  $n = 2$ ), *West Nile virus* (WNV,  $n = 4$ ), *Chikungunya virus* (CHIKV,  $n = 5$ ) and negative cases ( $n = 13$ ) was used for the in house IgM ELISA evaluation. Sera were collected after the 5th day of symptom onset (serological phase), but were selected based on their results in the diagnostic reference test (RT-qPCR) during the viremic phase (maximum five days after symptom onset) and / or the results of the Plate Reduction Neutralization Test (PRNT). For the commercial kits evaluations, three YFV, one WNV and one negative sample were included to the panel ( $n = 78$ ) (supplementary table 02). Overall, the sample panel was composed by 53.8 % (42/78) of females and 46.2 % (36/72) of males and, a median age of 37.6 years old.

### 2.3. In house IgM ELISA

We adapted the in house IgM ELISA described by Martin et al., 2000 for the individual detection of anti-ZIKV (*in house* ZIKV IgM ELISA), -DENV (*in house* DENV IgM ELISA), and -YFV IgM (*in house* YFV IgM ELISA), by using mouse brain antigens produced by sucrose-acetone method (Beatty et al., 1995) in optimal dilutions as established by antigen titration, and the use of the 6B6C-1 peroxidase-conjugated monoclonal antibody (CDC, Atlanta, USA) and TMB substrate (KPL, Maryland, USA). The optical density (OD) reading was taken with a spectrophotometer at a wavelength of 450 nm and results were considered negative when  $OD < 0.2$ , undetermined when OD was between 0.2 to 0.3 and positive when  $OD > 0.3$ . A cross-reactive sample was only considered positive when presenting at least a 2-fold increased titer, otherwise the result was considered as a cross-reaction among flaviviruses (Kuno et al., 1987).

### 2.4. Commercial IgM assays

We further evaluated two commercial kits recommended by the Brazilian Ministry of Health (MoH) during the 2015 epidemic for dengue and zika IgM detection. The Novagnost Zika Virus IgM (Novatec Immundiagnostica, Dietzenbach, Germany) and the Serion ELISA Classic Dengue Virus IgM (Serion, Würzburg, Germany) were performed

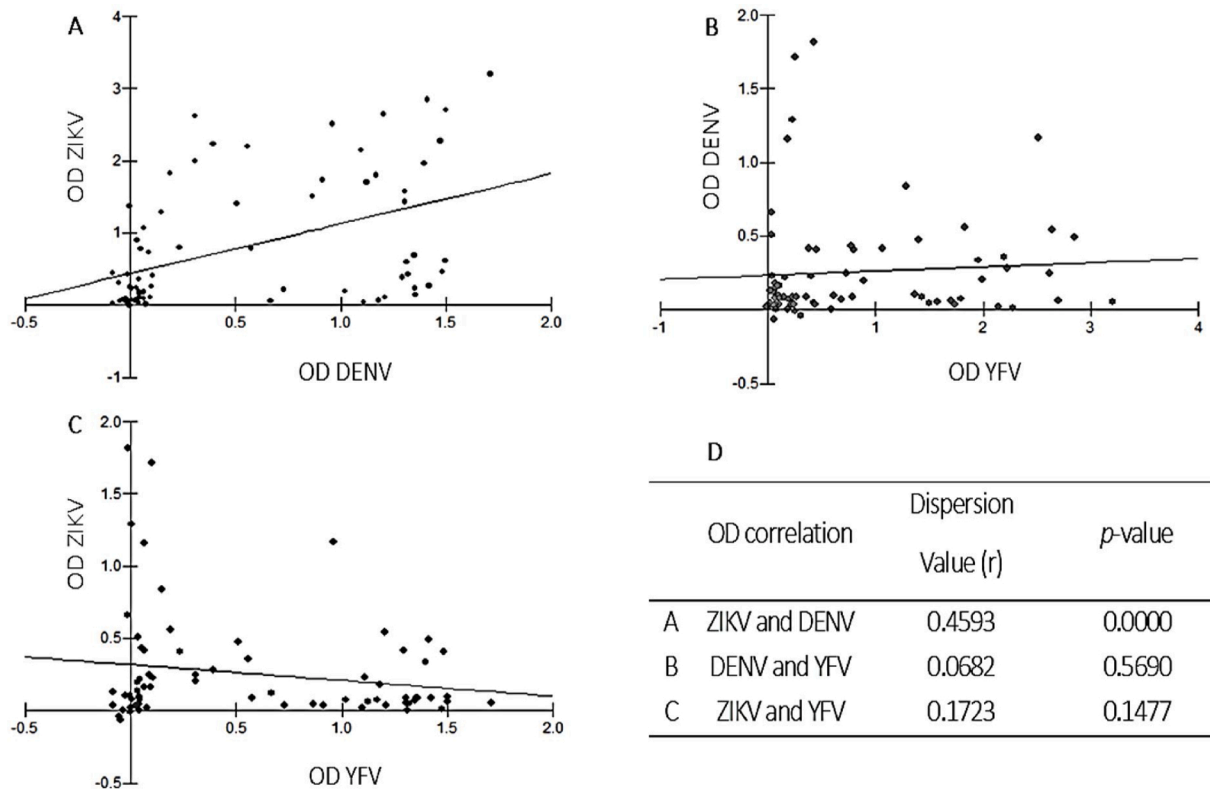


Fig. 2. Correlation analysis between the *in house* ELISAs for Flaviviruses. OD values' correlation between (A) ZIKV and DENV, (B) DENV and YFV, (C) ZIKV and YFV and (D) *p*-values for correlations between assays.

according to the manufacturer's protocols.

### 2.5. Statistical analysis

The sensitivity and specificity, as well the ROC curve and linear correlations were accessed between the viruses by using the BioEstat program, version 5.0 (Ayres et al., 2007). The statistical analysis was performed using GraphPad Prism software, version 8.0 (GraphPad Software Inc., California, USA). Values of  $p < 0.05$  were considered significant for all statistical analysis.

### 3. Results

The sensitivity and specificity of the *in house* ZIKV IgM ELISA was 60.0 % (IC<sub>95</sub> 35.75–80.18) and 88.6 % (IC<sub>95</sub> 76.02–95.05) respectively, and for the *in house* DENV IgM ELISA was 100 % (IC<sub>95</sub> 72.25–100) and 82.2 % (IC<sub>95</sub> 68.67–90.71), respectively. The *in house* YFV IgM ELISA presented 100 % for both sensitivity and specificity (IC<sub>95</sub> 64.57–100 and IC<sub>95</sub> 94.08–100). The Novagnost Zika Virus IgM test presented a sensitivity of 47.3 % (IC<sub>95</sub> 27.33–68.29) and specificity of 85.3 % (IC<sub>95</sub> 71.56–93.12). The Serion ELISA Classic Dengue Virus IgM test showed a sensitivity of 92.8 % (IC<sub>95</sub> 68.53–98.73) and specificity of 58.9 % (IC<sub>95</sub> 45.88–70.83). Based on the ROC curve, it was observed that both *in house* ELISAs for ZIKV and DENV presented better performances than the commercial kits tested, exhibiting cutoff points of 0.60 and 0.89 for sensitivity and specificity, respectively for ZIKV and 1.0 and 0.82 for DENV (Fig. 1).

The correlation between the OD for ZIKV and DENV was statistically significant, presenting a moderate positive correlation ( $r = 0.4593$ ,  $p = 0.0000$ ). The correlation between the OD of YFV and DENV showed that the two variables did not depend linearly on each other ( $r = 0.0682$ ), whereas the OD for YFV and ZIKV had a weak negative correlation, although both analyzes were not statistically significant (Fig. 2).

### 4. Discussion

The DENV and YFV *in house* IgM-ELISAs demonstrated a good performance with satisfactory sensitivities and specificities, however, the same was not observed for the ZIKV one that presented a low sensitivity (60.0 %). This unsatisfactory sensitivity may be related to the pattern of immune response of the population studied, possibly pre-exposed to other flaviviruses and/or presenting antibodies due to YFV vaccination.

Of the six ZIKV true positive samples and with a non-reagent result by the *in-house* IgM ELISA, two did not present specific IgM for ZIKV at all and four, despite the anti-ZIKV IgM detection, they were also reagent for other flavivirus with OD values two-fold higher. Similar results were also observed by our group (Silva and Gonçalves, 2018), in a study with non-human primates (NHP) (*Clorocebus aethiops*) experimentally infected with DENV-2 and re-infected after 13 months with ZIKV.

Although we confirmed the NHP's susceptibility to ZIKV infection, anti-ZIKV IgM production was not observed after secondary infection. The reduced sensitivity observed in the test might be due to cross-protection (heterologous antibodies) caused by DENV. It is known that specific neutralizing antibodies to DENV produced during a primary infection, would protect partially to ZIKV infection, and the production of ZIKV specific antibodies is abruptly shortened and/or is produced in a threshold below cut-off of this methodology. In DENV secondary infections, IgM levels may be significantly lower or undetectable (Kuno et al., 1991; Falconar et al., 2006).

False positive results for VDENV were observed in four ZIKV samples and those presented two to three times higher levels of IgM (OD) to DENV than to ZIKV. Moreover, this may be due to the antigenic similarities shared by those viruses, as well by the possible production of specific anti-DENV IgM, as memory cells produce high levels of IgG, but may also elicit other classes of immunoglobulins (Scroferneker and Pohlmann, 1998; Murphy et al., 2010). According to the Pan American Health Organization (1994) guideline, the specificity and sensitivity of

**Table 1**  
Performance of the Flaviviruses MAC-ELISAs considering different cut-off values.

	OD cut-off	Statistical indexes						
		Sensitivity (%)	Specificity (%)	True Positive	False Positive (%)	True Negative	False Negative (%)	Accuracy (%)
<i>in house</i> ZIKV IgM ELISA	≥ 0.1	92.0	64.5	40	35.4	33	8	73.9
	≥ 0.2	92.0	72.9	36	27.0	37	8	79.4
	≥ 0.3	92.0	75.0	35	25.0	38	8	80.8
	≥ 0.3*	92.0	87.5	29	12.5	44	8	98.0
<i>in house</i> DENV IgM ELISA	≥ 0.1	100	32.2	54	67.8	0	0	45.2
	≥ 0.2	100	45.7	46	54.2	27	0	56.1
	≥ 0.3	100	57.6	39	42.3	34	0	65.7
	≥ 0.3*	100	88.1	21	11.8	52	0	90.4

Notes: \*Considering a 2-fold increase in the OD.

the DENV *in-house* IgM ELISA are of 98.3 % and 90 % respectively, using the protocol described by Kuno et al., 1987. The protocol by Martin et al. (2000) has been established in the SAARB/IEC and it is currently in use for the serological diagnosis of arboviruses.

In this evaluation, we used mouse brain antigens produced by the SAARB/IEC, and as result, we observed a low OD on the negative control, favoring false positive results when the P/N ratio interpretation is used, as recommended by the authors. Currently, public health laboratories in Brazil performing *in-house* IgM ELISA for flaviviruses do use antigens produced and provided by the SAARB/IEC and therefore, follow the results interpretation adopted here.

We evaluated the cut-off values for the anti-DENV and anti-ZIKV IgM ELISAs and accessed the results' interpretation established by the SAARB/IEC. A screening test was performed to evaluate several cut-off values ( $\geq 0.100$ ,  $\geq 0.200$  and  $\geq 0.300$ ) that results in the assays' interpretation, as well as for the analysis of the samples considered reagent when the mean OD is at least, twice as high for one flavivirus as for the other.

For ZIKV *in-house* IgM ELISA, the use of the cut-off  $\geq 0.100$  resulted in 35.4 % of false-positives, decreasing to 25 %, when the cut-off  $\geq 0.300$  was considered. Moreover, if we consider the OD titles for DENV and YFV, we observe a even greater decrease in false positive results, down to 12.5 % (Table 1). Similarly, for DENV *in-house* IgM ELISA we observed 42.3 % of false-positives when using the cut-off value of  $\geq 0.300$  (Table 1). The percentage of false-positives was then significantly reduced (11.8 %) after comparing the mean OD values with those obtained for the other flaviviruses (Table 1). Taken together, these data demonstrate the importance of concomitantly analyzing the same sample for other flaviviruses in epidemic settings where two or more flavivirus co-circulate.

The protocol changes performed by Martin et al. (2000) for the *in house* IgM ELISA increased the test sensitivity and established a gray zone where values are determined as false positive ones.

We also observed increased post-standardization sensitivity for the DENV and YFV assays, however, the lower specificity observed in comparison to Kuno et al. (1987) protocol for DENV can be explained by the use, in this evaluation, of a wider panel of samples from individuals exposed to several flaviviruses.

The *in house* DENV IgM ELISA showed 17.0 % (7/41) of cross-reactivity with other flaviviruses, and 57.1 % of those samples (4/7) were ZIKV positive ones. However no cross-reactivity with DENV positive samples was observed on the *in house* ZIKV IgM ELISA. Both *in house* DENV IgM and ZIKV IgM ELISA presented a moderate positive correlation ( $r = 0.4593$   $p = 0.0000$ ).

Both anti-DENV and anti-ZIKV IgM ELISA evaluated here, demonstrated better performances when compared to the IgM ELISA kits tested (Fig. 1) and, as observed for the ZIKV *in house* IgM ELISA, the specificity presented by the Novagnost Zika Virus IgM kit was much higher than the sensitivity.

A previous study compared the Euroimmun IgM and IgG commercial tests to a MAC-ELISA and reported suboptimal sensitivities of 29.8 % and 23.7 %, respectively (L'Huillier et al., 2017). A multi-laboratory

study, analyzed three commercial kits, the ZIKV Detect™ IgM Capture ELISA (InBios International Inc., Seattle, USA), the Novalisa® Zika IgM  $\mu$ -capture ELISA (Novatec Immundiagnostica, Dietzenbach, Germany) and the combined Anti-Zika Virus IgM and IgG ELISA (Euroimmun, Lübeck, Germany) against the CDC Zika MAC-ELISA. The InBios commercial kit was the most sensitive compared to reference protocol, however, the sensitivity was poor for samples with low positive results in those collected within 6 days after onset of symptoms (Basile et al., 2018).

Recently, a study in Colombia analyzed the Zika Virus IgG/IgM antibody rapid test (Biocan Diagnostics, Coquitlam, Canadá) and reported a sensitivity of 71.4 %, but a specificity of 23.3 %. Positive and negative predictive values were 53.8 % and 36.6 %, respectively. The IgM detection in patients with 3–7 days of symptoms was 50 %, but in the convalescent-phase was of 91.6 % (Arrieta et al., 2019).

For dengue diagnosis, several commercial kits are currently available and have been evaluated over the years, presenting distinct sensitivities and specificities (Groen et al., 2000; Blacksell et al., 2007; Lima et al., 2010; Andries et al., 2016; Lee et al., 2019). In this study, the commercial kit's specificity was much lower than the *in house* assay, showing 42.2 % of cross-reactivity with other flaviviruses and 20 % non-specific reaction with negative samples.

The results obtained here show that emergence of other flaviviruses in the country can directly influence the immunological response of the exposed population and, therefore, serological tests. Moreover, the possible booster of flavivirus-specific antibodies responsible for a previous infection would directly influence the decrease of the IgM ELISA's specificity for the virus responsible for the previous infection as well as the decrease in the IgM ELISA's sensitivity to the flavivirus responsible for the recent one.

In conclusion, both *in house* DENV IgM ELISA and YFV IgM ELISA adapted and evaluated by the SAARB/IEC were reliable and are useful for routine diagnosis. Moreover, the *in house* DENV IgM ELISA and ZIKV IgM ELISA presented better performances when compared to the commercial kits evaluated. It has been shown that improved serological assays and studies focusing the antibodies kinetics are still needed (Van den Bossche et al., 2019). Furthermore, the data presented here demonstrate the importance of using serological tests that present good performance for all flaviviruses co-circulating simultaneously, mainly in endemic areas, such as the one we have in Brazil.

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## Declaration of Competing Interest

The authors declare no conflict of interest exists.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jviromet.2020.113976>.

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