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Development of IgM and IgG capture ELISAs for differentiating ZIKV recent and past infection

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by

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Under the Direction of David, Katz, PhD and Julia Hilliard, PhD

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ABSTRACT

Zika virus (ZIKV) was declared as “Public Health Emergency of International Concern” in 2016. Differentiating ZIKV recent infections (< 12 weeks post symptom onset) from past infections (> 12 weeks post symptom onset) is a significant challenge of serological test, which is widely used to detect ZIKV infections. We developed ZIKV NS1 IgM and IgG capture ELISAs and applied four indicators: IgM values, capture IgM/IgG ratios, IgG avidity, combined IgG avidity and capture IgM/IgG ratios, to differentiate ZIKV recent and past infection for a cohort of serum collected in ZIKV epidemic regions of Bogota, Colombia. The sensitivity for differentiating ZIKV recent and past infections using each of the four indicators is: 54.78%, 54.88%, 40.68%, and 31.25%, respectively. The specificity for differentiating ZIKV recent and past infection using each of the four indicators is: 62.50%, 84.21%, 58.33%, and 47.37%, respectively.

INDEX WORDS: Zika Virus, Capture ELISA, IgM values, Capture IgM/IgG ratios, IgG Avidity

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with 8 µg/ml of recombinant ZIKV NS1-His and 1: 100 dilution of tested samples. A: IgG capture ELISA under the condition that NS1 was incubated at 4 °C overnight (O/N) using SeraCare anti-human IgG. B: IgG capture ELISA under the condition that NS1 was incubated at 37 °C for 1 h SeraCare anti-human IgG. C: IgG capture ELISA under the condition that NS1 was incubated at 4 °C overnight using Fisher Sci anti-human IgG. D: IgG capture ELISA under the condition that NS1 was incubated at 37 °C for 1 h using Fisher Sci anti-human IgG. 17

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
ZIKV	zika virus
DENV	dengue virus
IgG AV	IgG avidity
IgM/IgG	capture IgM/IgG ratios
InBios Z-IgM kit	InBios ZIKV Detect 2.0 IgM Capture ELISA kit
Abcam Z-IgM kit	Abcam ZIKV IgM Capture ELISA kit
Abcam Z-IgG kit	Abcam ZIKV IgG capture ELISA kit
RAI	relative avidity index
NHS	normal human sera
HRP	horseradish peroxidase
MAb	monoclonal antibody
recombinant ZIKV NS1-His	recombinant ZIKV NS1-His
anti-his MAb-HRP	anti-6x-histidine MAb-HRP
Z-NS1-IgM-capELISA	ZIKV NS1 IgM capture ELISA
Z-NS1-IgG-capELISA	ZIKV NS1 IgG capture ELISA

INTRODUCTION

Zika virus (ZIKV) was first isolated in Uganda in 1947 and was considered as a minor harmful virus to human for a long time until the first reported disease outbreak in Yap Island in the Federated States of Micronesia in 2007 in French Polynesia in 2013–2014. After its introduction into Brazil in 2015, ZIKV has spread rapidly, and the World Health Organization declared it a "Public Health Emergency of International Concern" in February 2016.[1] ZIKV is an arbovirus (mosquito-borne) from the *Flaviviridae* family, genus *Flavivirus,* which is an enveloped, ss+ RNA virus that can be transmitted to human by *Aedes aegypti or Aedes albopictus* mosqiutoes.[2] ZIKV has three structural proteins (E for envelope, M for membrane, and C for capsid) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).[2] The main route of ZIKV infection was through Aedes mosquitos' bites, but the virus could also have been sexually or vertically transmitted.[3]

Although most of the ZIKV infections were asymptomatic or caused only mild symptoms (low-grade fever, arthralgia, conjunctivitis, and rash),[3] some lead to neurological diseases, such as Guillain-Barré syndrome in adults and congenital birth defects, including microcephaly in the developing fetus of infected mothers.[1] Laboratory diagnostic methods are therefore most important for the management of the ZIKV outbreaks and its related severe diseases.

Real-Time-PCR (RT-PCR) and serological tests are two diagnostic methods for the detection of ZIKV infections. RT-PCR can provide accurate and sensitive diagnosis for ZIKV presence in the tested sample in acute phase (days 1 to 6 post symptom onset).[4, 6] But their use is limited since the window for using RT-PCR to detect ZIKV infection in serum only extends 10 days post symptom onset.[6] Therefore, serological tests are needed since they can detect ZIKV infection even after the virus is cleared. However, there are two significant challenges within the

serological tests: a) how to avoid cross-reactivity between antibodies that are triggered by other flaviviruses, vaccinations or even by other serogroups virus, including but not limited to dengue virus (DENV).[1, 4, 6] b) how to differentiate ZIKV recent (< 12 weeks post symptom onset) and past infection (> 12 weeks post symptom onset).[7]

IgG immunoglobulin avidity or IgM/IgG ratios were used as indicators to differentiate ZIKV recent and past infection based on the immunological characters of IgM and IgG immunoglobulins. After virus infection, IgM will be firstly made and decrease until they disappear within 12 weeks post symptom onset, and IgG will be then made with low avidity and will increase to high avidity IgG finally. So, patients with ZIKV recent infection should have high concentration of IgM and low concentration of IgG with low avidity. And patients with ZIKV past infection should have low concentration of IgM and high concentration of IgG with high avidity. Based on the IgM and IgG immunological features, stand-alone IgM values could be used as an indicator for ZIKV acute infection. [8-10] However, IgM may remain detectable for 2-4 months, or even more than a year in some cases, and it will cause false positive results for the diagnosis of recent infections. [7] Therefore, IgM/IgG ratios was considered as another indicator for differentiating ZIKV recent and past infection because ZIKV recently infected patients would have high IgM/IgG ratios and ZIKV past infected patients would have low IgM/IgG ratios with the high IgG concentration even when IgM would not decrease. [8-11] IgG avidity (IgG AV) was also used as an indicator to differentiating ZIKV recent and past infection because patients would have low IgG AV when recently infected and high IgG AV when past infected.[17]

We thus developed ZIKV NS1 IgM and IgG capture ELISAs and evaluated these indicators (IgM values, capture IgM/IgG ratios, IgG avidity, and combined IgG avidity and capture IgM/IgG ratios) to differentiate ZIKV recent (< 12 weeks post symptom onset) and past infection

(> 12 weeks post infection). The sensitivity and specificity for differentiating ZIKV recent and past infection were calculated by testing a cohort of sera samples from patients in Colombia ZIKV epidemic area, including early convalescent sera (samples were from ZIKV infected patients that were collected within 12 weeks post symptom onset) and late convalescent sera (samples from ZIKV infected patients that were collected more than 2 years post symptom onset). We found that the capture IgM/IgG ratios could be the most accurate indicator for ZIKV recent infection based on the sensitivity and specificity results.

METHODS AND MATERIALS

1. IgM and IgG capture ELISAs in ZIKV-antibody negative sera

An aliquot of 50 µl/well of anti-human IgM (SeraCare, MA) (2.5 µg/ml) and anti-human IgG (SeraCare, MA) (2.5 µg/ml) (diluted in 1X PBS) were coated on each of two 96-well polystyrene plates (Corning Life Science, MA) and plates incubated for 1 hour at 37 °C, followed by 3 times washes with BBST (Borate buffered saline + Tween 20 and blocking using 200 µl/well of Blotto™ for 1 hour at 37 °C. For the IgM capture ELISA, after 3 times washes with BBST, the anti-human IgM-coated wells were incubated (1 hour at 37 °C) after adding 50 µl/well of serial concentrations (6400, 3200, 1600, 800, 400, 200, 100, 50 ng/ml) of purified IgM (Rockland Immunochem, PA) and 50 µl/well of normal human serum (NHS) dilutions (1: 50, 100, 200, 400, 800, 1600, 3200, 6400). For the IgG capture ELISA, 50 µl/well of serial concentrations (6400, 3200, 1600, 800, 400, 200, 100, 50 ng/ml) of purified IgG (Rockland Immunochem, PA) and 50 µl/well of each dilution of NHS (1: 50, 200, 800, 3200, 12800, 51200, 204800, 819200) were added to the anti-human IgG-coated plate, incubated for 1 hour at 37 °C, followed by 3 times washes with BBST. Then, 50 µl/well of diluted anti-Ig (A, G, M) (H+L) (alkaline phosphatase labeled) (SeraCare, MA) (0.5 µg/ml, diluted in Blotto) was added to each plate, which was incubated at 37 °C . After 3 times washes with BBST, 200 µl/well of 1 mg/ml of 10% Diethanolamine + Phosphate (pNPP) (Sigma, MO) was added to each plate as substrate, and plates were then incubated at room temperature (RT) for 25 min. The reactions were stopped by 3N sodium hydroxide (NaOH). Plates were read at $A_{(405\text{-}490)}$ by a microtiter plate reader (BioTek, ,VT).

2. InBios ZIKV Detect 2.0 IgM Capture ELISA kit (InBios Z-IgM kit):

The IgM-capture assay was performed according to the manufacturer's instructions (InBios, WA). An aliquot of 50 µl/well of each control and individual serum (1:100 dilution with sample dilution buffer provided in kit) was added to IgM antibody-coated and blocked strips and incubated at 37 °C for 1 hour. After 3 times washes with BBST, 50 µl/well of ready-to-use (RTU) ZIKV Ag (prM/E), cross-reactive Ag (CCA), and normal cell Ag (NCA) were added and incubated at 37 °C for 1 hour, followed by 3 times washes with BBST. Then, 50 µl/well of RTU anti flavivirus monoclonal antibody (MAb) was added as secondary antibody and incubated at 37 °C for 30 min, followed by 3 times washes with BBST. Subsequently, 50 µl/well of anti-mouse antibody-HRP was then added and incubated at 37 °C for 30 min. After 3 times washes with BBST, 75 µl/well of TMB was added as substrate, and the plates incubated at RT for 20 min. The reaction was stopped by 50 µl/well of stop solution. After the reaction, the strips were read at A_{450} by a microtiter plate reader (BioTek, VT).

3. *Abcam ZIKV IgM and IgG Capture ELISA kits (Abcam Z-IgM and Z-IgG kits):*

The capture assays were performed according to the manufacturer's instructions (Abcam, MA). Aliquots of 50 µl/well of each control and individual serum (1:100 dilution with sample dilution buffer provided in kit) were added to IgM Ab- and IgG Ab-coated and blocked strip wells that were then incubated at 37 °C for 1 hour. After 3 times washes with BBST, 50 µl/well of HRP-labeled ZIKV recombinant NS1 was added. For IgM-capture ELISA, the provided NS1 antigen was incubated at RT for 30 min. For IgG-capture ELISA, the provided NS1 antigen was incubated at 37 °C for 1 h. Following triple washes with BBST, 100 µl/well of TMB was added as substrate and plates incubated at RT for 15 min. The reaction was stopped using 100 µl/well

of stop solution. After the reaction, the strips were read at A_{450} by a microwell plate reader (BioTek, VT).

4. ZIKV and DENV infected cell lysates preparation:

4.1. ZIKV infected cell lysates preparation:

ZIKV stock (Puerto Rico strain, titer $2.8E^{06}$ pfu/ml) was used to infected ten T300 flasks (Celltreat, MA) of Vero cells (ATCC, VA) (passage 131), which were grown in Minimum Essential Media Eagle (MEM) (Mediatech, VA), with 0.5 multiplicity of infection (MOI) and incubated at 37 °C in 5% CO_2. The infected cells were harvested and centrifuged after 3 days post infection. The pellet was resuspended with MEM and stored at -80 °C with 1% of Dimethoxy-4-chloroamphetamine (DOC), which was the ZIKV infected cell lysate. The concentration of the ZIKV infected cell lysate was determined by Bicinchoninic acid (BCA) protein assay according to manufacturer's instruction (ThermoFisher, MA). The titer of the ZIKV infected cell lysate was determined by plaque assay.

4.2. DENV infected cell lysates preparation:

DENV type 1 (DENV-1) stock was used to infected six T300 flasks (Celltreat, MA) of Vero cells (ATCC, VA) (passage 133), which were grown in Minimum Essential Media Eagle (MEM) (Mediatech, VA), with 0.5 multiplicity of infection (MOI). The infected cells were harvested and centrifuged after 3 days post infection. The pellet was resuspended with MEM and stored at -80 °C with 1% of DOC, which was the DENV infected cell lysate. The concentration of the DENV infected cell lysate was determined by BCA protein assay according to manufacturer's instruction (ThermoFisher, MA). The titer of the DENV infected cell lysate was determined by plaque assay.

5. ZIKV IgM capture ELISA using poly-histidine-tagged recombinant ZIKV NS1:

An aliquot of 50 µl/well of anti-human IgM (SeraCare, MA) (2.5 µg/ml, dilute with 1X PBS) was added to each well of the 96-well polystyrene plate (Corning Life Science, MA) and the plate incubated for 1 hour at 37 °C, and subsequent 3 times washes with BBST then blocked with 200 µl/well of casein-blocking buffer (0.5% casein in 0.05% PBST) (Sigma, MO) for 1 hour at 37 °C. After 3 times washes with BBST, 50 µl/well of each serum (1: 100 dilution with casein-blocking buffer) was added to a well and the plate was incubated at 37 °C for 1 hour. After 3 times washes with BBST, 50 µl/well of 8 µg/ml poly-histidine-tagged recombinant ZIKV NS1 (Sino Biological, PA) (diluted with casein-blocking buffer) was added and the plate was incubated at 4 °C for overnight. Wells were subsequently washed 3 times with BBST, and 50 µl/well of anti-histidine MAb-HRP (Fisher Sci, NH), (1:500 dilution in casein-blocking buffer) was added to each well, and the plate incubated at 37 °C for 1 hour, followed by 3 times washes with BBST. Then, 100 µl/well of TMB (Thermo fisher, MA) was added as substrate to each well and the plate was subsequently incubated at RT for 25 minutes. The reaction was stopped by 100 µl/well of 1M sulfuric acid (H_2SO_4). Plates were read at A_{450} using the microtiter plate reader (BioTek, VT).

6. ZIKV IgG capture ELISA using poly-histidine-tagged recombinant ZIKV NS1:

An aliquote of 50 µl/well of anti-human IgG (mouse sera preabsorbed) (Fisher Sci, NH) (9 µg/ml, diluted with 1X PBS) was added to each well of the 96-well polystyrene plate (Corning Life Science, MA) and the plate was incubated for 1 hour at 37 °C, followed by 3 times washes with BBST and blocking with 200 µl/well of casein-blocking buffer (0.5% casein in 0.05% PBST) (Sigma, MO) for 1 hour at 37 °C. After 3 times washes with BBST, 50 µl/well of 8

µg/ml poly-histidine-tagged recombinant ZIKV NS1 (Sino Biological, PA) (diluted with casein-blocking buffer) was added and incubated at 37 °C for 1 hour. Wells were then washed by 3 times with BBST, and 50 µl/well of anti-histidine MAb-HRP (Fisher Sci, NH), (2 µg/ml, diluted in casein-blocking buffer) was added and incubated at 37 °C for 1 hour, followed by 3 times washes with BBST. Then, 100 µl/well of TMB (Thermo fisher, MA) was added as substrate and the plate incubated at room temperature for 25 minutes. The reaction was stopped by 100 µl/well of 1M H_2SO_4. Plates were read at A_{450} by a microtiter plate reader (BioTek, VT).

7. ZIKV IgM/IgG ratios.

ZIKV capture IgM/IgG ratios was calculated by dividing capture IgM $OD_{A450\ nm}$ by capture IgG $OD_{A450\ nm}$.

8. IgG Urea Avidity test.

The test was adapted with modifications as previously described.[8] Briefly, A 96-well polystyrene plate (Corning Life Science, MA) was coated with 50 µl/well of ZIKV infected cell lysate (2 µg/well, diluted in 1X PBS) and incubated at 37 °C for 1 hour, followed by 3 times washes with BBST, and blocking with 200 µl/well of Blotto for 1 hour at 37 °C. Then, 50 µl/well of each serum (1:20 dilution with Blotto) was added to two wells coated with ZIKV infected cell lysates. After incubation for 1 hour at 37 °C, one serum incubated well was exposed to 6 M urea solution and the other serum incubated wells was exposed to phosphate buffer for 15 min. After 3 times washes with BBST, 50 µl/well of anti-human IgG (H+L)-alkaline phosphatase (1:4000 dilution with B

lotto) (Promega, WI) was added and incubated at 37 °C for 1 hour. After 3 times washes with BBST, 200 µl/well of pNPP was added and incubated at RT for 25 minutes. The reactions were stopped by100 µl/well of 3N NaOH and read at A_{450} by a microtiter plate reader (BioTek, VT). The IgG avidity was expressed as the percentage of OD units remaining in the urea-treated wells (relative avidity index: RAI): $OD_{A405\text{-}490\ nm}$ on urea treated wells/$OD_{A405\text{-}490\ nm}$ on untreated wells. Samples with RAIs of $< 60\%$ were regarded as having low avidity, and those with RAIs of $> 60\%$ were regarded as having high avidity.

RESULTS

1. *IgM and IgG concentrations in normal human sera as determined by the developed IgM- and IgG-capture ELISAs.*

The efficacy of the IgM and IgG capture ELISAs was tested by quantification of IgM and IgG immunoglobulin concentrations in normal human sera (NHS), comparing these with published IgM and IgG standard range of concentrations (Figure 1). Two data points from the IgM and IgG linear part of the titration curves were chosen to calculate the IgM and IgG concentrations of the NHS using the linear formula generated from the IgM and IgG standard curves.

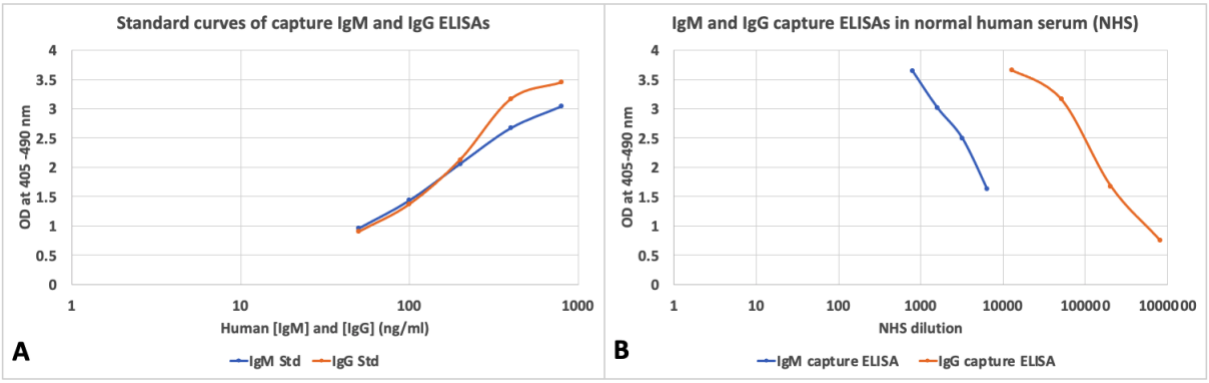

Figure 1. ELISA quantification of Normal Human Sera. A: standard curves of capture IgM and IgG ELISAs generated by serial concentrations of purified human IgM and IgG. B: linear part of capture IgM and IgG ELISAs obtained in normal human serum based on different NHS dilution.

The calculated IgM concentration ([IgM]) in NHS was approximately 1.14 mg/ml, which was in accordance with published IgM immunoglobulin concentrations by the Sigma Aldrich "0.2-2.8 mg/ml",[12] and published paper "0.4-2.3 mg/ml".[13] And the IgG concentration ([IgG]) in NHS was approximately 25 mg/ml after calculation, close to the published value [IgG] by the Sigma Aldrich "7.5-22 mg/ml",[12] and also close to the published paper "7-16 mg/ml".[13] The high range of IgG measured by ELISA might be due to the errors caused by the higher NHS dilution (1:

51200 and 1: 204800) used in IgG-capture ELISA, comparing to the NHS dilution (1: 1600 and 1: 3200) used in IgM-capture ELISA. In general, the IgM and IgG in NHS as determined from IgM and IgG capture ELISAs were within the published range overall, indicating that the immunoglobulin-capture assay is acceptable.

2. ZIKV-specific IgM- and IgG-capture ELISAs.

Development of ZIKV-specific IgM and IgG capture ELISA proceeded based on the data reported in the previous section. The capture anti-human IgM and anti-human IgG antibodies were used to coat microtiter plates, then the human serum samples were added to facilitate capture of the IgM and IgG isotypes in each serum sample. The specific antibody reactivity of the captured isotypes was accomplished for these experiments using a quality assessed ZIKV antigen. There were several options that could be used for the detection of the bound antigen. We first tried to use a horseradish peroxidase (HRP)-labeled ZIKV infected cell lysate (concentration: 6.0 mg/ml, titer: $3.6E^{07}$ pfu/ml) as antigen, and the same ZIKV infected cell lysate in combination with an anti-flavivirus monoclonal antibody (MAb)-conjugate similar to the CDC MAC ELISA kit.[15] These labeled step failed and led us to try ZIKV recombinant antigens either directly labeled with HRP or alkaline phosphatase (AP) or indirectly detected by an anti-flavivirus MAb conjugate.

Then, two of commercially available anti-flavivirus MAb, whose target protein is flavivirus envelope protein, were tested to for their specificity on ZIKV infected cell lysate compared to a DENV infected cell lysate (concentration: 3.8 mg/ml, titer: $1.12E^{06}$ pfu/ml) using a direct ELISA. Neither of the two anti-flavivirus MAb showed reactivity and specificity on ZIKV. The anti-flavivirus MAb-HRP from Hennessy (Hennessy, KS) did not recognize either ZIKV or

DENV perhaps due to insufficient number of specific envelope epitopes with which each monoclonal antibody reacted, and the anti-flavivirus MAb-AP from Sigma (MilliporeSigma, MA) had high background on uninfected cell lysate.

InBios ZIKV IgM ELISA kit (InBios Z-IgM kit) in which the ZIKV antigen is ZIKV recombinant proteins composed of preM and the envelope protein was next evaluated, because our results thus far indicated that either the ZIKV infected cell lysates and the two commercial anti-flavivirus MAbs could not be used in a ZIKV- specific capture ELISA.[16] The kit was used for the analysis of ZIKV infected cell lysate and the anti-flavivirus MAbs by replacing the InBios reagents with them as shown in Table 1 (See "Methods and Materials").

Table 1. Combination of replacement permutations of InBios IgM capture ELISA kit.

	Capture anti-human IgM source	ZIKV Antigen (source)	Anti-flavivirus MAb host/isotype (source)	Conjugate (source)	Results	Possible Reason for not working
A	InBios	rec. ZIKV preM and E (InBios)	mouse/IgG (InBios)	Anti-mouse IgG-HRP (InBios)	Work	
B	Seracare	rec. ZIKV preM and E (InBios)	mouse IgG (InBios)	Anti-mouse IgG-HRP (InBios)	Work	
C	Seracare	rec. ZIKV preM and E (InBios)	mouse/IgG (Sigma)	Anti-mouse IgG-AP (Bethyl)	No work	Sigma anti-flavivirus MAb or the conjugate has high background on uninfected cell lysate
D	Seracare	ZIKV infected cell lysate (GSU)	mouse/IgG (InBios)	Anti-mouse IgG-HRP (InBios)	No work	Inbios anti-flavivirus MAb is against preM/E, and preM/E in ZIKV infected cell lysate Ag is low
E	Seracare	rec. ZIKV preM and E (InBios)	hamster/IgG (Rockland)	Anti-mouse IgG-HRP (InBios)	No work	The target viral protein of the hamsters anti-flavivirus MAb may be not preM and envelope protein
F	Seracare	ZIKV infected cell lysate (GSU)	hamster/IgG (Rockland)	Anti-mouse IgG-HRP (InBios)	No work	Insufficient antigen concentration
G	InBios	rec. ZIKV preM and E (InBios)	hamster/IgG (Rockland)	Anti-mouse IgG-HRP (InBios)	No work	The target viral protein of the hamsters anti-flavivirus MAb may be not preM and envelope protein
H	InBios	ZIKV infected cell lysate (GSU)	hamster/IgG (Rockland)	Anti-mouse IgG-HRP (InBios)	No work	Insufficient antigen concentration

* rec.: recombinant

Results from experiments A and B in Table 1 indicated that the plates that were coated with commercial capture anti-human IgM worked as well as the InBios capture anti-human IgM-coated plates, provided that all other reagents used for detecting the ZIKV-specific IgM were from InBios. Results from experiment C in Table 1 showed that the Sigma anti-flavivirus MAb was not working with the InBios reagents since it had high background on uninfected cell lysate. Results from experiment D in Table 1 showed that the ZIKV-specific IgM capture ELISA using ZIKV infected cell lysate also failed even when all other reagents were from InBios perhaps due to insufficient preM and envelope, which is the target protein of InBios anti-flavivirus MAb, in ZIKV infected cell lysate.

In addition, one hamster anti-flavivirus MAb from Rockland, whose target viral protein was characterized as ZIKV NS1, was used to detect the captured ZIKV-specific IgM antibodies with the antigen in ZIKV infected cell lysate or the InBios recombinant ZIKV preM and envelope antigen (experiments E-H in Table 1). All the IgM capture ELISAs using hamster anti-flavivirus MAb failed, even when all other reagents were from InBios The reason for the negative results using the ZIKV infected cell lysate in the IgM capture ELISA, which was then detected by hamster anti-flavivirus MAb, could be the insufficient NS1 concentration in ZIKV infected cell lysate (experiments F and H in Table 1). The reason for the negative results using the InBios recombinant ZIKV preM and envelope antigen in the IgM capture ELISA, which was then detected by hamster anti-flavivirus MAb could be that the target viral protein of the hamsters anti-flavivirus MAb is NS1, not preM and envelope protein.

Recombinant ZIKV antigen could be the potential antigen that can be applied to the ZIKV-specific IgM and IgG capture ELISAs based on the results from the replacement permutations experiments shown in Table 1.

3. Development of ZIKV IgM and IgG-capture ELISAs using poly-histidine-tagged recombinant ZIKV NS1.

Recombinant ZIKV NS1 was confirmed to be effective for detection of antibody triggered by ZIKV. We previously showed that this antigen had low cross-reactivity with sera collected from patients with non-ZIKV flavivirus infections, the antigen detected antibodies even in serum samples with low levels of ZIKV antibodies.[20] Poly-histidine-tagged recombinant ZIKV NS1 (recombinant ZIKV NS1-His) antigen was detected using anti-6x-histidine MAb-HRP (anti-his MAb-HRP), reacting with the histidine tag of the recombinant protein. In this ELISA format, the

capture anti-human IgM or IgG antibodies captured ZIKV-specific IgM and IgG isotypes from patient serum samples.

3.1. ZIKV NS1 IgM-capture ELISA (Z-NS1-IgM-capELISA) optimization.

Optimal conditions for performing the Z-NS1-IgM-capELISA were investigated in experiments A-F for which the experimental design shown is in Figure 2. Experiments A, B and C were performed to determine the optimal recombinant ZIKV NS1-His concentration to be used in the capture ELISA by comparing the results of three serum samples under different recombinant ZIKV NS1-His incubation conditions. The following sera were tested: a ZIKV antibody-positive serum pool prepared from late convalescent human from Colombia; a ZIKV antibody-negative serum pool; and a human ZIKV IgM positive serum from a recent infection (S3). Experiments D, E, F were performed to determine the serum samples' optimal working dilution by comparing the two different dilutions (1:20 and 1:100) for the three sera tested with the optimal concentration (8 µg/ml) of the recombinant ZIKV NS1-His.

Results indicated that the ZIKV-NS1-IgM-capELISA worked best when recombinant ZIKV NS1-his was incubated at 4 °C overnight, resulting in the highest degree of differentiation between the positive IgM control (S3) serum compared to the negative control serum. Because the difference between the high dilution (1: 100) and low dilution (1: 20) was not big, we have chosen the 1:100 dilution for future serum sample use.

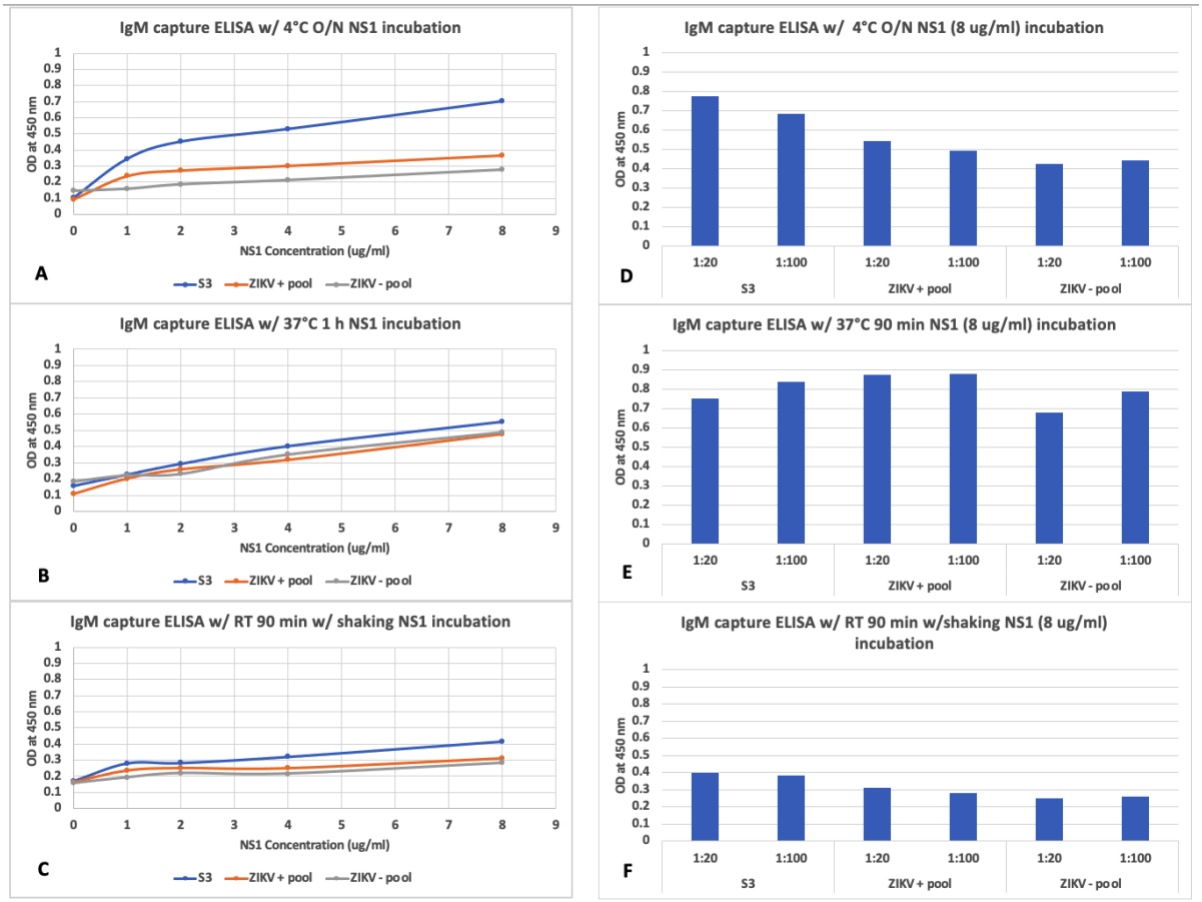

Figure 2. Optimization of the ZIKV NS1 IgM capture ELISA. In A, B, C, serial concentrations of recombinant ZIKV NS1-His (0, 1, 2, 4, 8 µg/ml) were applied for a 1: 100 dilution of tested sera samples. In A, IgM capture ELISA for 1: 100 dilution of sera samples with serial concentrations of NS1 incubated at 4 °C overnight (O/N). B: IgM capture ELISA for 1:100 dilutions of sera samples with serial concentrations of NS1 incubated at 37 °C for 1 h. C: IgM capture ELISA for 1: 100 dilutions of sera samples with serial concentrations of NS1 incubated at room temperature (RT) for 90 min with shaking. In D, E, F, two different dilutions of serum samples (1: 20 and 1: 100) were tested with same concentration of recombinant ZIKV NS1-His (8 µg/ml); D: IgM capture ELISA for 1: 20 and 1:100 dilutions of sera samples with 8 µg/ml of NS1 incubated at 4 °C overnight. E: IgM capture ELISA for 1:20 and 1: 100 dilutions of sera samples with 8 µg/ml of NS1 incubated at 37 °C for 90 min without shaking. F: IgM capture ELISA for 1: 20 and 1:100 dilutions of sera samples with 8 µg/ml of NS1 incubated at RT for 90 min with shaking.

3.2. ZIKV NS1 IgG-capture ELISA (Z-NS1-IgG-capELISA) optimization.

The developed ZIKV IgG-capture ELISAs in which the detecting antigen is poly-histidine-tagged recombinant ZIKV NS1 (Z-NS1-IgG-capELISA) was similar to the Z-NS1-IgM-

capELISA except that the plate was coated with anti-human IgG for the IgG-capture ELISA (see "Methods and Materials"). The Z-NS1-IgG-capELISA (Figure 3, Panels A-D) was performed to determine the optimal recombinant ZIKV NS1-His incubation condition at the previously determined optimal concentration of 8 µg/ml and 1: 100 serum dilution. In these experiments, the following sera were used: a ZIKV antibody-positive serum pool prepared from late convalescent human from Colombia; a ZIKV-antibody negative serum pool; a human IgM positive serum from a ZIKV recent infection (S3); a human IgM positive serum from a recent ZIKV infection (<12 weeks post infection) (#0163 SR1); and four human IgG positive sera post ZIKV infection (#0164 SR1, #0333 SR1, #0326 SR1, and #0309 SR2), which were all from early convalescent human patients.

As shown in Figure 3, experiment A and B were performed to determine the optimal recombinant ZIKV NS1-His incubation condition at the previously determined optimal concentration of 8 µg/ml using SeraCare anti-human IgG, which was not preabsorbed with mouse sera. Results indicated that the SeraCare anti-human IgG have high cross-reaction with the anti-his MAb-HRP (mouse IgG) and caused very high background. To remove the high background caused by the cross reactivity between capture SeraCare anti-human IgG and the anti-his MAb-HRP (mouse IgG), we switched to apply the mouse sera preabsorbed anti-human IgG (Fisher Sci anti-human IgG) to the Z-NS1-IgG-capELISA, which resulted in a lower background. So, experiments C and D were performed to determine the optimal recombinant ZIKV NS1-His incubation condition with determined optimal concentration (8 µg/ml) using the Fisher Sci capture anti-human IgG by comparing the results difference on tested sample controls. Results from experiments C and D indicated that the tested positive and negative control and other sera showed significant differentiation when the recombinant ZIKV NS1-His was

incubated at 37 °C for 1 hour condition than at 4 °C overnight in the developed Z-NS1-IgG-capELISA.

In conclusion, 1: 100 dilution of samples and 8 µg/ml of recombinant ZIKV NS1-His incubated at 37 °C for 1 hour condition were applied to the Z-NS1-IgG-capELISA assay.

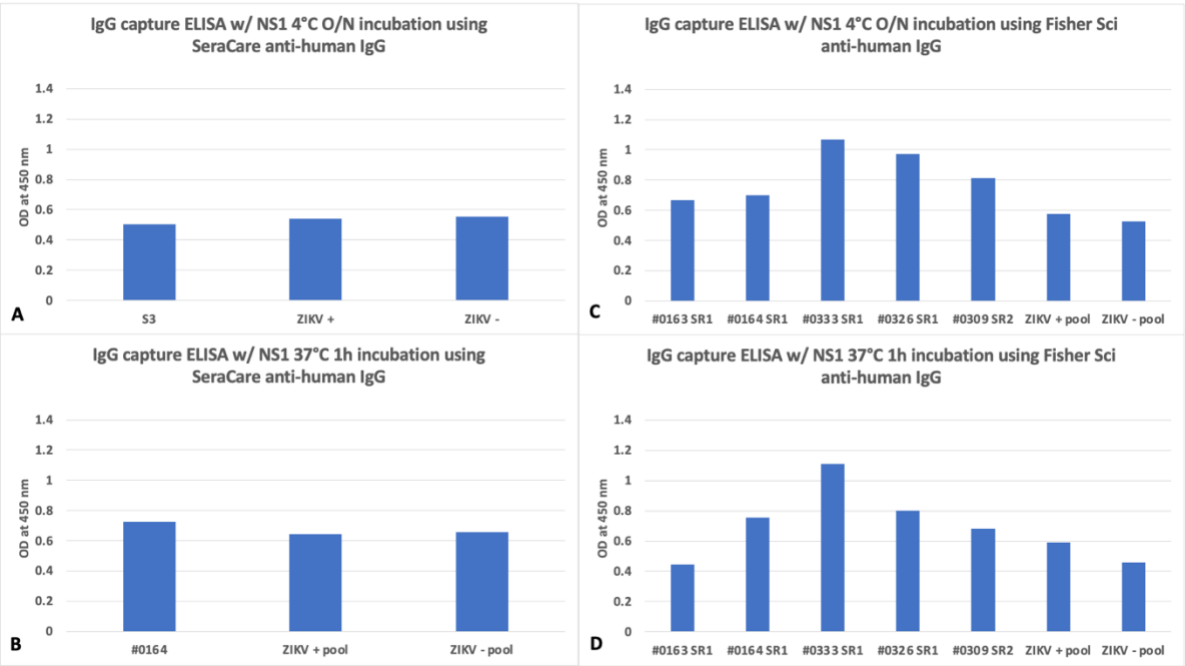

Figure 3. ZIKV NS1 IgG capture ELISA under different conditions, where figure A and B show results using anti-human IgG without mouse sera preabsorbed (anti-human IgG from SeraCare), and figure C and D were show results using the mouse serum preabsorbed anti-human IgG (anti-human IgG from Fisher Sci). All figure A, B, C, D were all applied with 8 µg/ml of recombinant ZIKV NS1-His and 1: 100 dilution of tested samples. A: IgG capture ELISA under the condition that NS1 was incubated at 4 °C overnight (O/N) using SeraCare anti-human IgG. B: IgG capture ELISA under the condition that NS1 was incubated at 37 °C for 1 h SeraCare anti-human IgG. C: IgG capture ELISA under the condition that NS1 was incubated at 4 °C overnight using Fisher Sci anti-human IgG. D: IgG capture ELISA under the condition that NS1 was incubated at 37 °C for 1 h using Fisher Sci anti-human IgG.

4. ZIKV NS1 IgM and IgG capture ELISAs validation.

A cohort of patients from a Colombia ZIKV epidemic region and a set of CDC sera comprising ZIKV IgM-positive sera were tested by the developed ZIKV NS1 IgM- and IgG- capture ELISAs and commercially available ZIKV IgM- and IgG -capture ELISA kits (Table 2).

Table 2. Tests performed for the human sera used in this research.

A cohort of human sera used in this research

	# tested in Abcam ZIKV IgM and IgG capture ELISAs	# tested in InBios ZIKV IgM capture ELISA	# tested in ZIKV NS1 IgM and IgG capture ELISAs	# tested in urea avidity test to calculate IgG AV	# which the IgM/IgG ratios were calculated	# which both IgG AV and IgM/IgG ratios were calculated
Early Convalescent sera (total n=120)	50	17	115	118	82	80
Late convalescent sera (total n=24)	17	3	24	24	19	19
DENV positive sera (total n=3)	3	3	3	3	0	0
ZIKV negative sera (total n=11)	10	3	11	10	0	0
CDC IgM panels (total n=21)	0	19	21	21	21	0

* "#": number of sera
* All tested sera were from Colombia except CDC IgM panels
* Early convalescent sera: human sera that were from Colombia ZIKV epidemic area and were collected within 12 weeks post infection
* Late convalescent sera: human sera that were from Colombia ZIKV epidemic area and was collected more than 2 years post infection
* DENV positive sera: human sera that were from Colombia DENV epidemic area and were collected more than 2 years post infection
* ZIKV negative sera: human sera that were neither ZIKV or DENV infected
* CDC IgM panels: human sera that was ZIKV infected and was collected within 12 weeks post infection

The agreements between the different tests listed in Table 2 were analyzed. We have calculated three agreements, separately combined tested sera results from InBios Z-IgM kit and Z-NS1-IgM-capELISA, Abcam Z-IgM kit and Z-NS1-IgM-capELISA, Abcam Z-IgG kit and Z-NS1-IgG-capELISA, which were 66.13%, 50.00%, and 66.25%, respectively (Table 3). InBios Z-IgM kit was a reliable ZIKV IgM capture ELISA kit that had 100% sensitivity compared with CDC MAC-ELISA with plaque reduction neutralization test (PRNT) confirmation but Abcam Z-IgM kit has only 57% sensitivity compared with CDC MAC-ELISA with PRNT confirmation.14 The developed Z-NS1-IgM-capELISA had high agreement with InBios Z-IgM kit and relatively low agreement with Abcam Z-IgM kit, which indicates that the developed Z-NS1-IgM-capELISA was validated to be a reliable capture ELISA to detect the ZIKV-specific IgM antibody in ZIKV infected patients' sera. The Abcam Z-IgG kit was assumed to be the reliable IgG capture ELISA and served in the experiments as a reference test for comparing the Z-NS1-IgG-capELISA. The fact that the developed Z-NS1-IgG-capELISA had a high agreement with Abcam Z-IgG kit assures the reliability of our newly developed Z-NS1-IgG-capELISA.

Table 3. ZIKV NS1 IgM and IgG capture ELISAs validation by comparing samples results from Abcam ZIKV IgM and IgG capture ELISAs and InBios ZIKV IgM capture ELISA to and ZIKV NS1 IgM and IgG capture ELISAs.

Abcam ZIKV IgM and IgG capture ELISA compared to ZIKV NS1 IgM and IgG capture ELISA

	Abcam and ZIKV NS1 IgG capture ELISA				Abcam and ZIKV NS1 IgM capture ELISA			
	Abcam IgG POS & NS1 IgG POS	Abcam IgG POS & NS1 IgG NEG	Abcam IgG NEG & NS1 IgG POS	Abcam IgG NEG & NS1 IgG NEG	Abcam IgM POS & NS1 IgM POS	Abcam IgM POS & NS1 IgM NEG	Abcam IgM NEG & NS1 IgM POS	Abcam IgM NEG & NS1 IgM NEG
Early Convalescent sera (n=50)	19	13	3	15	3	1	27	19
Late Convalescent sera (n=17)	5	9	2	1	1	0	9	7
DENV infected sera (n=3)	0	0	0	3	0	0	1	2
ZIKV negative sera (n=10)	0	0	0	10	1	0	2	7
Agreement	66.25%				50.00%			

Inbios ZIKV IgM capture ELISA compare to ZIKV NS1 IgM capture ELISA

	Inbios IgM POS & NS1 IgM POS	Inbios IgM POS & NS1 IgM NEG	Inbios IgM NEG & NS1 IgM POS	Inbios IgM NEG & NS1 IgM NEG
Early Convalescent sera (n=34)	3	10	7	14
Late Convalescent sera (n=3)	0	0	1	2
DENV infected sera (n=3)	0	0	1	2
ZIKV negative sera (n=3)	0	1	0	2
CDC IgM panels (n=19)	18	0	1	0
Agreement	66.13%			

* Agreement are calculated by dividing the sum of sera that have same positive or negative results on both compared tests by the sum of total sera number

5. Capture IgM/IgG ratios and IgG avidity could be potential indicators for differentiating ZIKV recent and past infection.

We then tested CDC human sera (n=21), which were all ZIKV IgM positive (were collected < 12 weeks post symptom onset) according to the CDC classification using our IgM and IgG capture ELISAs. Sera in group 1 (n=12) were from patients that experienced a recent infection with a flavivirus (not determined as a specific ZIKV infection), and sera in group 2 (n=9) were from patients that experienced a specific ZIKV recent infection. Our group at the Viral Immunology Center at GSU tested these sera using an in-house developed ZIKV-specific competition ELISA (cELISA), finding that all 21 sera were ZIKV specific. We also analyzed these sera using a urea avidity test in which 60% relative avidity index (RAI) was chosen as the cutoff for differentiating low and high IgG AV.[17,18] (see "Methods and Materials"). We found that all 12 sera from the first group plus 2 of 9 sera from the second group (total 14 sera) were measured as "Low Avidity" and the remaining 7 sera from the second group were measured as "High Avidity".

The different virological and immunological responses that may occur after the onset of a ZIKV infection are shown schematically in Figure 4. Based on the scenario of events described in Figure 4 and our results, we concluded that all 21 sera were from patients that experienced a ZIKV infection. The low avidity sera (n=14) were from patients that experienced a recent infection, and the 7 high avidity sera were from patients that experienced a past ZIKV infection if just based on the avidity results. However, all the 21 sera were collected with 12 weeks post symptom onset and should be considered as recent infection. So, we hypothesized these 7 high avidity sera were from patients that experienced ZIKV reinfection. Currently, there is no evidence that humans or nonhuman primates can be re-infected by ZIKV, however, without more specific patient information for the donors of the seven sera that measured as high avidity sera, it is possible to question whether or not humans can be reinfected by ZIKV.

In general, patients having a recent ZIKV infection would have a relatively high concentration of IgM antibodies and relatively lower concentrations of low avidity IgG antibodies, and patients that experienced a past ZIKV infection would have a low concentration of IgM antibodies and relatively high concentration of high avidity IgG antibodies (Figure 4). However, IgM may remain detectable for a long time up to years in some patients that had a past infection. In these cases, the IgM test will result in a false positive diagnosis. Interestingly, in pregnant women, infection continues until delivery and this may further confound identification of acute versus later infection.

Because of the possibility of false positive results when relying solely on the IgM test, we decided to adopt the analytical strategy described by Prince et al,[10] and combined the results of the IgM test with the results of the IgG test to calculate IgM to IgG ratios. The capture IgM/IgG ratios were considered as a potentially more precise indicator for differentiating ZIKV recent and

past infection because that ZIKV infected patients were expected to have high IgM/IgG ratios early in infection, while ZIKV patients > 12 weeks post infection were expected to have low IgM/IgG ratios even in cases where IgM did not decrease while high IgG levels increased in these patients. If patients indeed can be re-infected with ZIKV, they would be predicted to show high avidity IgG secreted from memory plasmablasts generated during the primary ZIKV infection.[10]

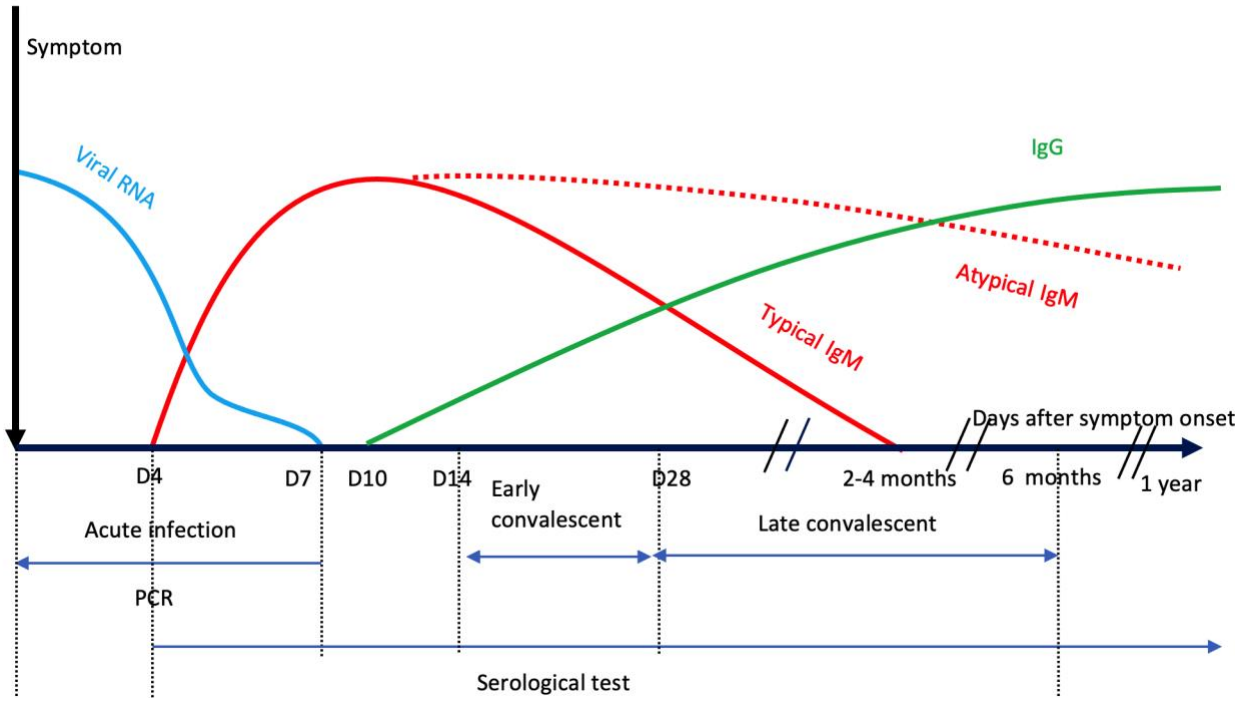

Figure 4. The time course of ZIKV infection, including the stage of viremia and antibody development after the onset of symptoms. Viremia is detected up to 7 days after the onset of symptoms. IgM antibodies can be a few days after symptoms (3- 4 days) to reaching a peak at around Day 10 – Day 14 and then decrease to disappear at around 2 - 4 months after symptoms. IgG antibodies can be detected at around Day 7 – Day 10 and may reach a peak at around 3 months.

The CDC sera (n=21) tested by our ZIKV NS1 IgM and IgG capture ELISAs were all ZIKV IgM positive while 15/21 of these sera showed ZIKV IgG as well (Table 4). Results shown in Figure 5 is the box plot representation of the capture IgM/IgG ratios for the low-avidity CDC sera

comprising Group 1 (n=12) and two from Group 2 compared to the ratios obtained with the 7 high-avidity CDC sera from Group 2. The difference between the mean IgM/IgG ratios of the low-avidity (4.5 Index) and the high-avidity (3.1 Index) groups was calculated to be statistically significant according using the two-tailed Student t-test (same for the following Student t-test) (*p* *p*-value: 0.01565 < 0.05). These results support the hypothesis that the capture IgM/IgG ratios analysis provides an accurate diagnostic tool for differentiation of ZIKV recent infections from past or secondary infections.

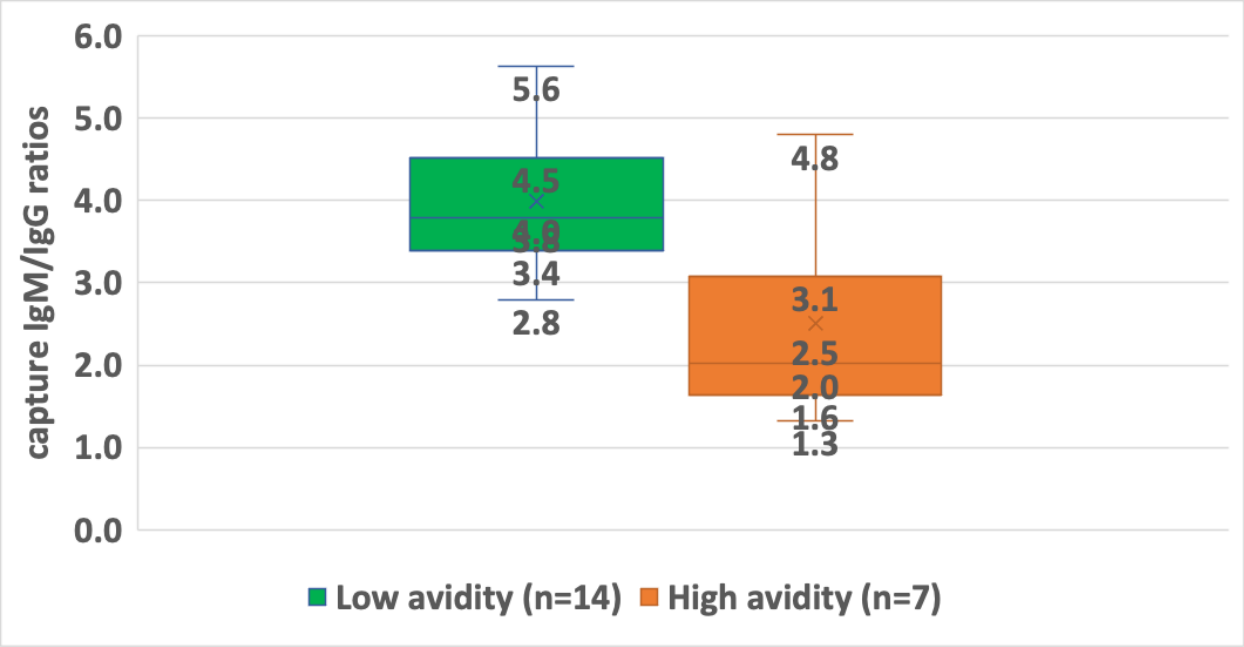

Figure 5. Capture IgM/IgG ratios of CDC IgM panels in low avidity group (n=14) and high avidity group (n=7).

6. Assay of Colombia early and late convalescent sera using the ZIKV NS1 IgM- and IgG-capture ELISAs and analysis of results using four different indicators: IgM values, capture IgM/IgG ratios, IgG avidity, and combined IgG avidity and capture IgM/IgG ratios, for differentiating ZIKV recent and past infection.

The Colombian patient cohort was the source sera samples collected from a ZIKV-epidemic area, including early convalescent sera (samples were collected from ZIKV infected patients < 12 weeks post symptom onset) and late convalescent sera (samples were collected from ZIKV infected patients >12 weeks post infection) (Table 2), were tested by the ZIKV NS1 IgM-and IgG-capture ELISAs according to the protocols described above (see "Methods and Materials"). The cutoff for determining IgM positive results was ≥ 0.51 OD at A_{450} and the cutoff for determining IgG positive results was ≥ 0.1 OD at A_{450}. The cutoffs were based on the calculated mean OD plus 3X standard deviation (SD) using a ZIKV negative-antibody pool of sera assayed using our ZIKV NS1 IgM- and IgG-capture ELISAs (Table 4).

Table 4. ZIKV NS1 IgM and IgG capture ELISAs results for the cohorts of human sera.

	ZIKV NS1 IgM and IgG capture ELISAs						
	IgM POS & IgG POS	IgM POS & IgG NEG	IgM NEG & IgG POS	IgM NEG & IgG NEG	IgM POS percentage	IgG POS percentage	Mean of IgM/IgG ratio
Early Convalescent sera (n=115)	31	32	19	33	54.78%	43.48%	4.56
Late convalescent sera (n=24)	8	1	10	5	37.50%	75.00%	3.27
DENV positive sera (n=3)	0	1	0	2	33.33%	0.00%	not apply
ZIKV negative sera (n=11)	0	2	0	9	18.18%	0.00%	not apply
CDC IgM panels (n=21)	15	6	0	0	100.00%	71.43%	3.5

* IgM/IgG ratios are not calculated for sera that are both IgM and IgG negative
* Mean of IgM/IgG ratio is the average of all calculated IgM/IgG ratios

There is one serum in DENV positive sera group (n=3) that showed IgM antibodies that may be cross-reactive with ZIKV NS1.[19] Alternatively, this serum may have been from a patient that experienced a dual infection resulting in both ZIKV and DENV antibodies according to ZIKV IgG direct ELISA test. Two sera from the ZIKV negative group (n=11) were IgM positive which most probably represented a false positive reaction. Nine sera comprising the Colombian late

convalescent sera were IgM positive, which possibly because of IgM antibodies that persisted for more than 12 weeks after the primary infection.

6.1. Indicator 1: IgM values.

Detection of IgM antibodies for the diagnosis of recent infections was widely used for numerous infectious diseases. Here, we present data obtained with our IgM capture ELISA that was used for detection of IgM antibodies in the early and late convalescent sera from Colombia. Figure 6 is the distribution of IgM values for all early and late convalescent sera. The cutoff for differentiating ZIKV recent and past infection using ZIKV IgM values was 0.51 as mentioned above. The difference of the IgM values between the early and late convalescents sera was statistically significant by Student t-test (p-value: $0.012 < 0.05$).

The Colombia early convalescent sera were assumed as ZIKV recently infected sera according to the sample collection day (within 12 weeks post symptom onset) and in house diagnosis, including but not limited to PCR, IgM test, and IgG test. The Colombia late convalescent sera were assumed as ZIKV past infected sera according to samples collection day (more than 2 years post symptom onset) and in house diagnosis. Results from the Z-NS1-IgM-capELISA indicated that 63/115 of early convalescent sera had IgM values higher than the 0.51 and 15/24 of late convalescent sera have IgM value lower than 0.51. In this analysis, sera that had IgM values higher than 0.51 were considered as ZIKV recently infected and sera that had IgM values lower than 0.51 were considered as ZIKV past infected.

To determine the sensitivity for differentiating ZIKV recent and past infection using each indicator, we tested the early convalescent sera, as classified by our Colombian colleagues (< 12 weeks post symptom onset), and calculated the percent of sera that were indicated as ZIKV

recent infection by our test relative to the total number of early convalescent tested. To determine the specificity of our assay, we tested the late convalescent sera, as classified by our Colombian colleagues (> 12 weeks post symptom onset), and calculated the percent of sera that were indicated as ZIKV past infection by our test relative to the total number of late convalescent tested. According to these analyses, the sensitivity and specificity for differentiating ZIKV recent and past infections using IgM values indicator are 54.78% and 62.50%, respectively (Table 5. A).

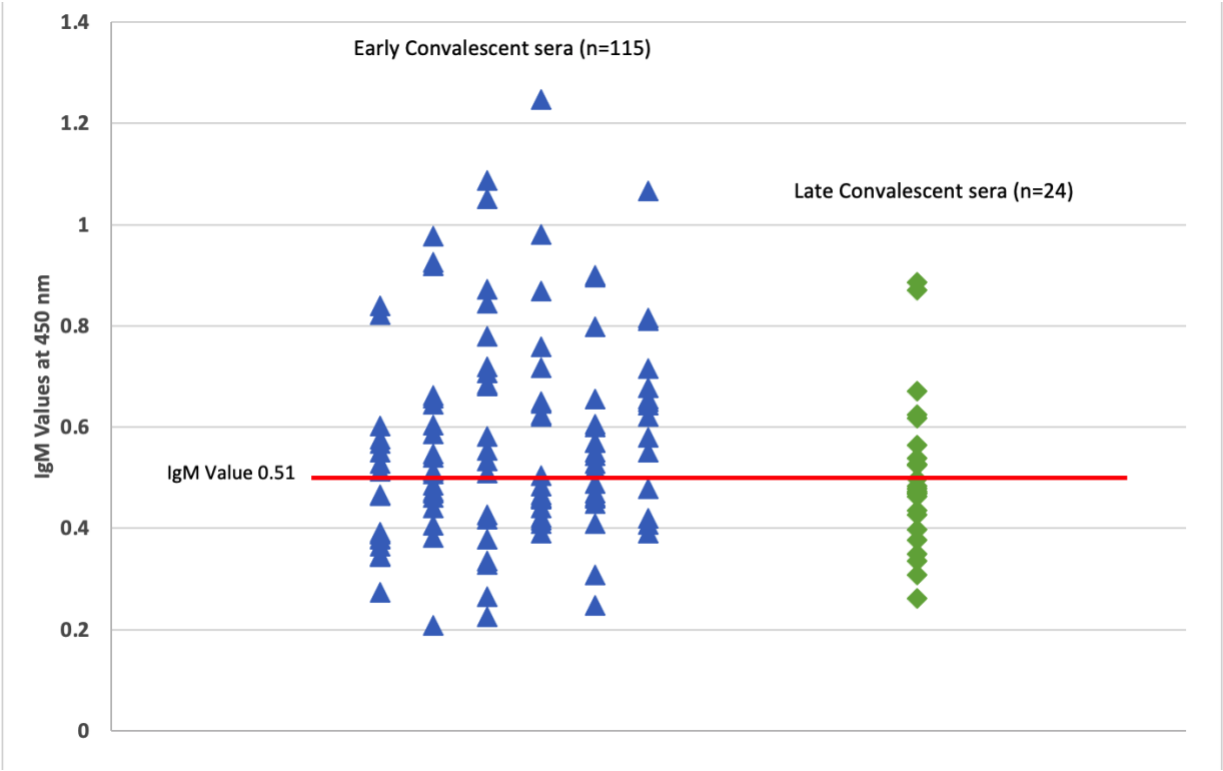

Figure 6. IgM values for Colombia early and late convalescent sera.

6.2. Indicator 2: Capture IgM/IgG ratios (IgM/IgG).

The box plot graphic analysis and Student t-test were performed to compare the difference of the capture IgM/IgG ratios (IgM/IgG) in early and late convalescent sera. Results shown in Figure 7 shows a significant difference between the early and late convalescent sera (*p*-value: 0.0016 <

0.001). This difference of the IgM/IgG ratios between early and late convalescent sera group supported the assumption that the IgM/IgG can serve as an indicator for differentiating ZIKV recent and past infection as discussed above for the 21 IgM positive CDC sera (Figure 5).

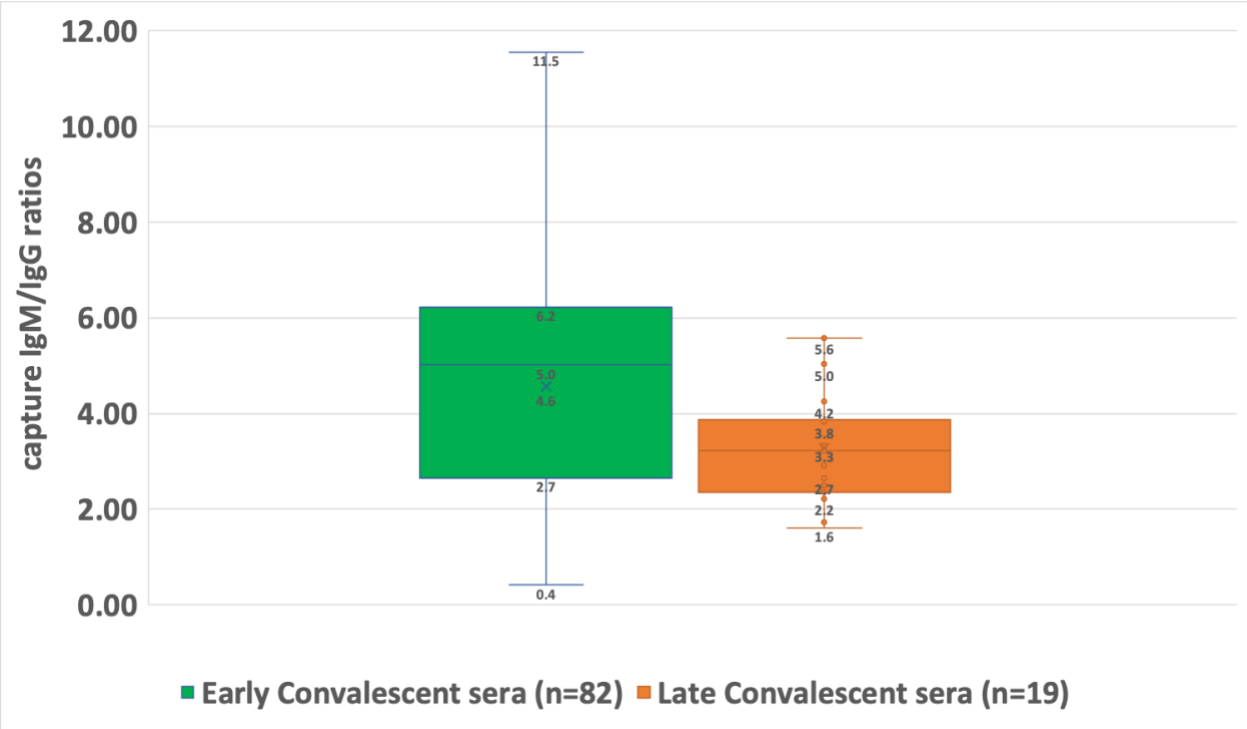

Figure 7. Capture IgM/IgG ratios for Colombia early and late convalescent sera.

Figure 8 shows the distribution of IgM/IgG for all early and late convalescent sera. The 4.5 avidity index-cutoff for differentiating ZIKV recent and past infection using the IgM/IgG indicator was determined by calculating the mean OD at A_{450} plus 3X SD of ZIKV antibody-positive serum pool obtained using the ZIKV NS1 IgM- and IgG-capture ELISA. The IgM/IgG ratios were calculated from the ratio of results from each of the ZIKV NS1 IgM- and IgG-capture ELISA. 45 of 82 of early convalescent sera had IgM/IgG > 4.5 cutoff and 16 of 19 late convalescent sera had IgM/IgG ratios < 4.5 cutoff. In this analysis, sera with IgM/IgG > 4.5 were considered to be from patients with recent ZIKV infection and sera with IgM/IgG < 4.5 were considered to be from patients with past ZIKV infections. The sensitivity and specificity for

differentiating ZIKV recent and past infection using capture IgM/IgG ratios were calculated as described above for the IgM test. The IgM/IgG evaluation resulted in a 54.88% sensitivity and an 84.21% specificity (Table 5. B).

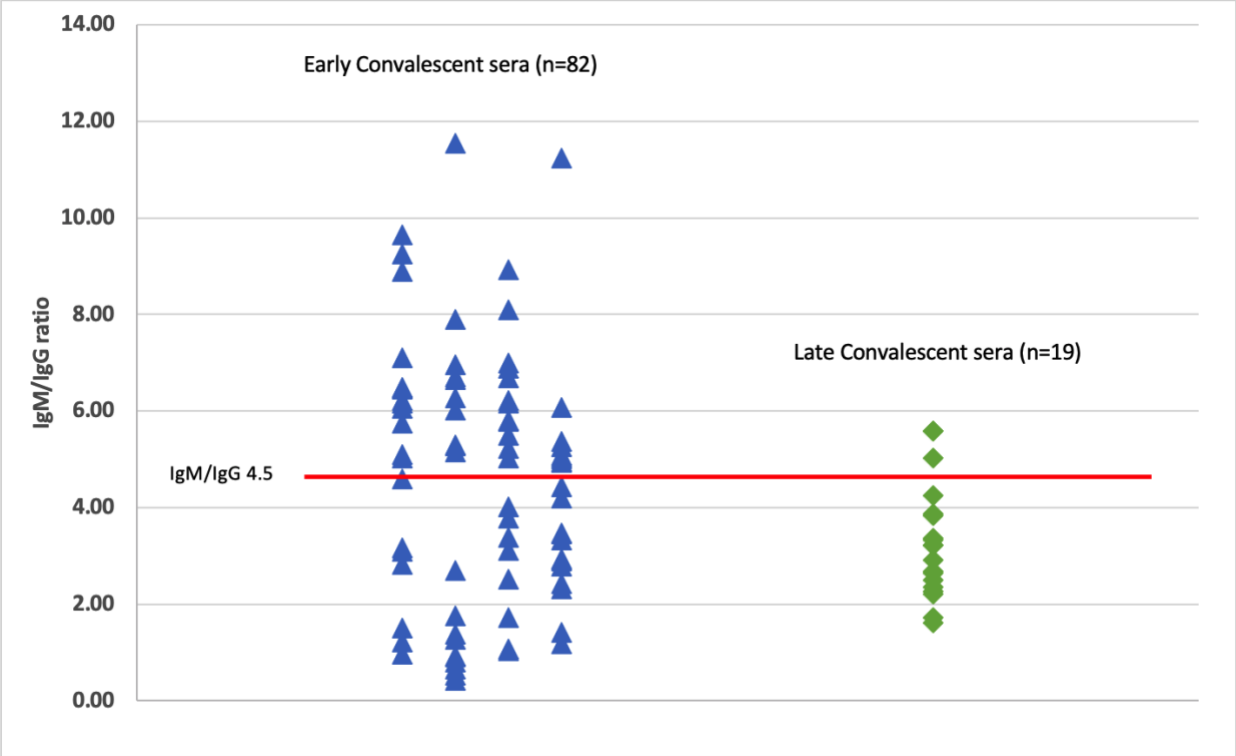

Figure 8. Capture IgM/IgG ratios for Colombia early and late convalescent sera.

6.3. Indicator 3: IgG Avidity (IgG AV).

IgG avidity (IgG AV) is considered to be another indicator for differentiating recent from past infections, because of the low avidity IgG antibodies that develop after infection which undergo affinity maturation later after infection.[21] The cohorts of sera described above were therefore tested by the Urea Avidity Test as described in "Methods and Materials". Figure 9 shows the distribution of IgG AV for all Colombia early and late convalescent sera. The cutoff to differentiate low avidity values (indicate recent infection) and high avidity values (indicate past infection) was 60%.[8]

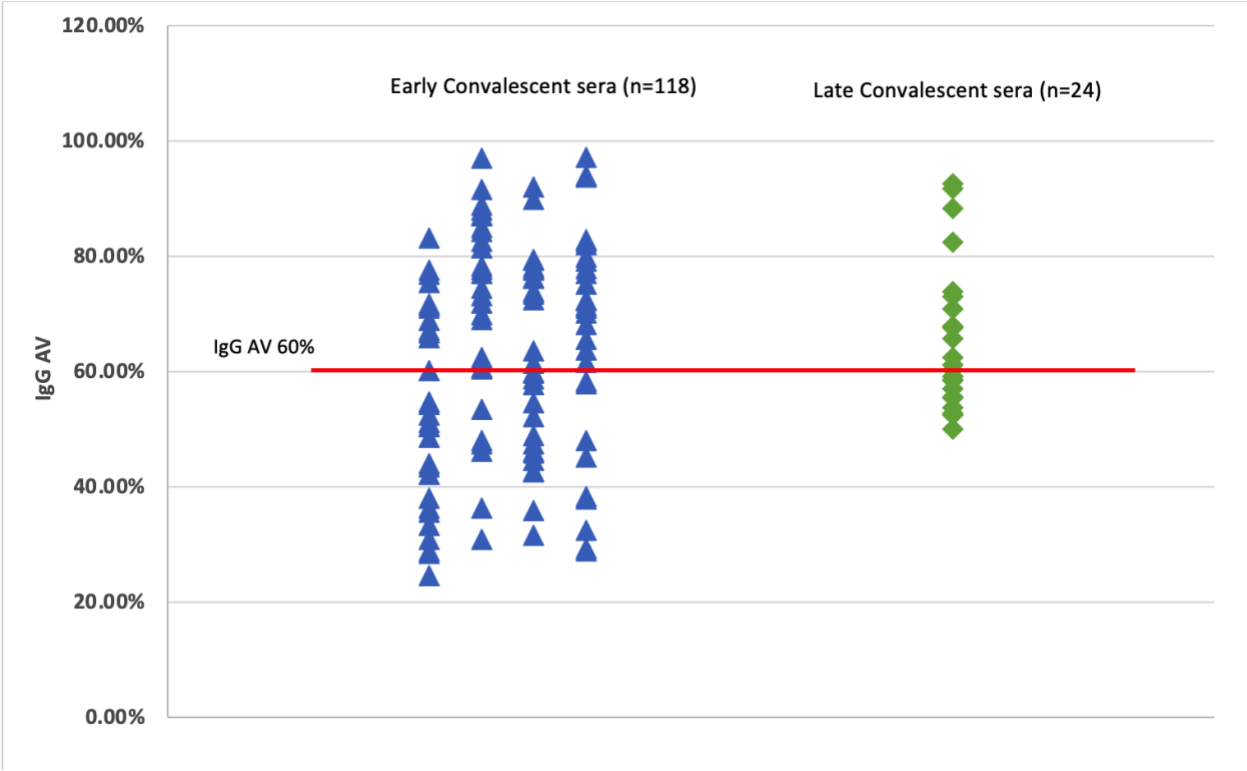

Figure 9. IgG AV for Colombia early and late convalescent sera.

Results indicate that 48 out of 118 of early convalescent sera had IgG AV values equal or lower than 60% and 14 out of 24 of the late convalescent sera had IgG AV values higher than 60%. In this analysis, sera that had IgG AV lower than 60% were considered as ZIKV recently infected and sera that had IgG AV higher than 60% were considered as ZIKV past infected. Based on the calculation method as described above, the sensitivity and specificity for differentiating ZIKV recent and past infection using IgG AV are 40.68% and 58.33%, respectively (Table 5. C).

6.4. Indicator 4: Combined IgG AV and IgM/IgG.

Since the agreement for differentiating ZIKV recent and past infection using the three indicators: IgM values, capture IgM/IgG ratios, IgG AV, were not high, we analyzed the outcome of an

analysis of combined data from the IgM/IgG ratios and from the Urea Avidity Test to differentiate sera from ZIKV recent and past infection. Figure 10 shows the combined distribution of IgG AV and IgM/IgG, indicating visible correlation between IgG AV and IgM/IgG: most sera that has < 60% IgG AV also has > 4.5 IgM/IgG, and most sera that has > 60% IgG AV also has < 4.5 IgM/IgG. Patients with both high IgM/IgG and low IgG AV could have most possible ZIKV recent infection and patient with both low IgM/IgG and high IgG AV could have most possible ZIKV past infection.

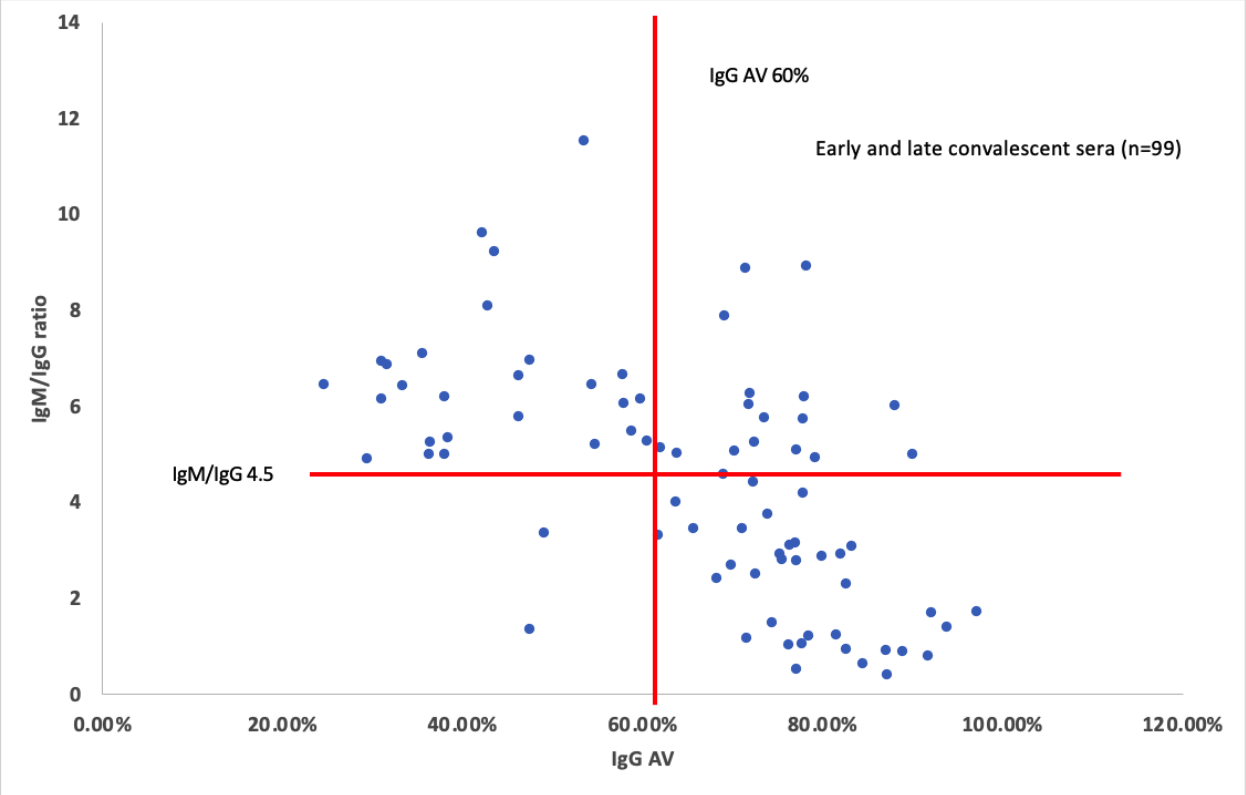

Figure 10. Correlation between capture IgM/IgG ratios and IgG AV.

Firstly, all sera (early and late convalescent sera) were divided into low avidity group and high avidity group based on the Urea Avidity test results. We examined the distribution of the IgM/IgG between the low avidity and high avidity groups. As shown in Figure 11, there was highly significant difference set (*p*-value: $1.36826E^{-07} < 0.001$) between the ratios of IgM/IgG in

the low and high avidity groups from early convalescent sera as calculated using Student t-test. There was, however, no significant difference (*p*-value: 0.69 > 0.05) of IgM/IgG between the low and high avidity group in late convalescent sera found when analyzed using Student t-test. One reason could be that the IgG AV fails to differentiate ZIKV past infection as effectively as IgM/IgG avidity indices. When we combined all early and late convalescent sera (total n=99) together and then separated them all into low avidity (n=35) and high avidity group (n=64), an Student t-test demonstrated a highly significant difference (*p*-value: $1.42256E^{-05}$ < 0.001) between the IgM/IgG in low and high avidity groups, which suggests that the combined IgG AV and IgM/IgG can serve as another potential assay for differentiating ZIKV recent and past infection.

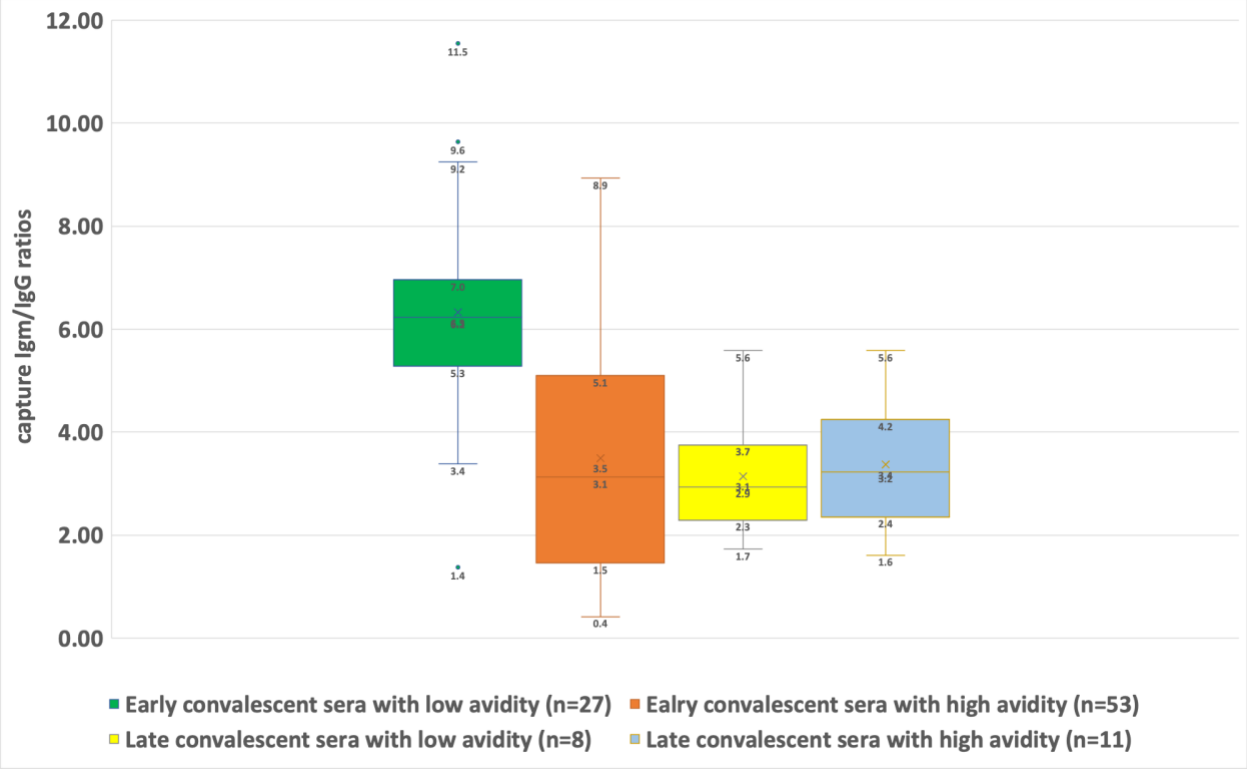

Figure 11. Capture IgM/IgG ratios in low and high avidity group of Colombia early convalescent sera and late convalescent sera.

In this analysis, sera with both low IgG AV (< 60%) and high capture IgM/IgG ratios (> 4.5) were classified to be from patients with recent ZIKV infections. Serum with both high IgG AV (≥ 60%) and low capture IgM/IgG ratios (< 4.5) were classified to be from patients with a past ZIKV infection. The results indicated that 25/80 of early convalescent sera had both < 60% IgG AV and > 4.5 IgM/IgG and 9/19 of late convalescent sera had both > 60% IgG AV and < 4.5 IgM/IgG. Based on the sensitivity and specificity calculation methods defined above, the sensitivity and specificity for differentiating ZIKV recent and past infection using the combined IgG AV and IgM/IgG were calculated, which were 31.25% and 47.37% (Table 5. D).

Table 5. Differentiation ZIKV recent and past infection using all four indicators: A: IgM values, B: capture IgM/IgG ratios, C: IgG AV, and D: combined IgG AV and capture IgM/IgG ratios.

Differentiate ZIKV recent and past infection

A Differentiate ZIKV recent and past infection using IgM values

	Early Convalescent sera (n=115)	Late Convalescent sera (n=24)
IgM values > 0.51	63	9
IgM values < 0.51	52	15
Sensitivity	54.78%	
Specificity	62.50%	

* IgM values > 0.51: indicates ZIKV recent infection
* IgM values < 0.51: indicates ZIKV past infection

B Differentiate ZIKV recent and past infection using IgM/IgG

	Early Convalescent sera (n=82)	Late Convalescent sera (n=19)
IgM/IgG > 4.5	45	3
IgM/IgG < 4.5	37	16
Sensitivity	54.88%	
Specificity	84.21%	

* IgM/IgG > 4.5: indicates ZIKV recent infection
* IgM/IgG < 4.5: indicates ZIKV past infection

C Differentiate ZIKV recent and past infection using IgG AV

	Early Convalescent sera (n=118)	Late Convalescent sera (n=24)
IgG AV < 60%	48	10
IgG AV > 60%	70	14
Sensitivity	40.68%	
Specificity	58.33%	

* IgG AV < 60%: indicates ZIKV recent infection
* IgG AV > 60%: indicates ZIKV past infection

D Differentiate ZIKV recent and past infection using IgG AV + IgM/IgG

	Early Convalescent sera (n=80)	Late convalescent sera (n=19)
IgG AV < 60% and IgM/IgG > 4.5	25	1
IgG AV < 60% and IgM/IgG < 4.5	2	7
IgG AV > 60% and IgM/IgG > 4.5	18	2
IgG AV > 60% and IgM/IgG < 4.5	35	9
Sensitivity	31.25%	
Specificity	47.37%	

* IgG AV < 60% and IgM/IgG > 4.5: indicates ZIKV recent infection
* IgG AV > 60% and IgM/IgG < 4.5: indicates ZIKV past infection

* Sensitivity = number of early convalescent sera that were indicated with ZIKV recent infection according to the diagnosis by the total number of tested Colombia early convalescent sera
* Specificity = number of late convalescent sera that were indicated with ZIKV past infection according to the diagnosis by the total number of tested Colombia late convalescent sera

DISCUSSION/CONCLUSION

Zika virus (ZIKV) caught the world's attention in 2015-16 as a result of its potential to cause a global pandemic. Emerging and re-emerging pathogens often result in the realization of our lack of preparedness for identifying new pathogens rapidly and efficiently. Therefore, laboratory diagnostic methods are most important for the management of the ZIKV outbreaks and other pathogen-related outbreaks. The goal of this study was to evaluate the infection status of patients using a unique collection of sera from a defined Colombian patient cohort. A combination of serological tests were applied to determine whether laboratory information provided an accurate diagnosis and differentiation of recent (< 12 weeks post symptom onset) and past (> 12 weeks post symptom onset) ZIKV infections.

To accomplish this goal, we developed the ZIKV NS1 IgM- and IgG-capture ELISAs using poly-histidine-tagged recombinant ZIKV NS1 as the detecting antigen, which was confirmed to have reduced cross-reactivity with antibodies triggered by other flavivirus.[20] We then examined the possibility that the ratio between capture IgM and IgG OD at A_{450} values may be an effective indicator for differentiation of ZIKV recent and past infections.

To validate the developed capture ELISAs, we tested sera from Colombia cohort living in a ZIKV pandemic area in and around Bogota in an altitude region that included mosquitos and a CDC IgM positive serum panel collected from patients as well as experimentally infected macaques. We compared the results to three commercial ZIKV IgM- and IgG-capture ELISA kits (InBios ZIKV IgM-capture ELISA kit and Abcam ZIKV IgM- and IgG-capture ELISA kits). The results from the developed ZIKV NS1 IgM-capture ELISA showed high agreement (66.13%) with InBios ZIKV IgM-capture ELISA kit and lower agreement (50.00%) with Abcam ZIKV IgM-capture ELISA kit in multiple replicate assays. The InBios Z-IgM kit was confirmed

as a reliable ZIKV IgM-capture ELISA kit that had 100% sensitivity compared with CDC MAC-ELISA with PRNT confirmation, but Abcam Z-IgM kit was reported to be less reliable with a sensitivity of only 57% in comparison with CDC MAC-ELISA with PRNT confirmation.14 The developed ZIKV NS1 IgG-capture ELISA had high agreement (66.25%) with Abcam ZIKV IgG-capture ELISA kit. In conclusion, the inhouse ZIKV NS1 IgM- and IgG-capture ELISAs performance was found to be reliable for detecting ZIKV IgM and IgG antibodies, albeit slightly less sensitive than the InBios and CDC assays. The newly developed, in house ZIKV NS1 IgM- and IgG-capture ELISAs was found to be ZIKV-specific because no observed significant cross-reactivity reactivity between the recombinant ZIKV NS1-His with West Nile polyclonal antibodies (WN), anti-Saint Louis encephalitis (SLE) and anti-DENV antibodies observed when tested by direct ELISA.

We then used the ZIKV NS1 IgM- and IgG-capture ELISA for testing this assay's potential for differentiating ZIKV recent (< 12 weeks post symptom onset) and past infection (> 12 weeks post symptom onset). As depicted in Figure 4, the first antibody isotype induced post infection is IgM, which may last at least a few weeks, but may also endure as previously reported by multiple investigators. The switch to the IgG antibody isotypes, along with subclass-switching occurs generally by 21 days post infection. Following B cell activation, a portion of B cells travel to the germinal center follicles where they develop into naïve memory cells that can be activated to become antibody-secreting plasmablasts within 48-72 hours post re-infection as well as during late stages of primary infection in many virus infections. The increasing avidity of the IgG antibodies results from RAG-recombination of the immunoglobulin supergene family genes encoding the specificity of the B cell receptor (which mirrors the antibodies secreted by the plasmablasts) present on the surface of the memory B cells. Based on this course of events, we

analyzed our results from each of the four assays described for differentiating ZIKV recent from past infections: IgM values, IgM/IgG ratios, IgG avidity and combined IgG avidity and capture IgM/IgG ratios. The following cutoffs were used for each of the assays: 0.51 OD at A_{450} for the IgM test, 4.5 avidity index calculated from antigen-specific IgM/IgG ratios, 60% RAI for the IgG avidity, and the avidity cutoff (60%) and the IgM /IgG cutoff (0.45). The sensitivity and specificity for the detection and differentiation of ZIKV recent and past infections were calculated for each of the four indicators relative to the classification determined by the medical and laboratory teams of our Colombian colleagues who defined one cohort of sera as early convalescent (were collected within 12 weeks post symptom onset) and the other as late convalescent sera (were collected more than 2 years post symptom onset). The CDC utilized a slightly modifies terminology with < 12 weeks post symptom onset and > 12 weeks post symptom onset for the same classifications used by our Colombia colleagues. The sensitivity for differentiating ZIKV recent was calculated for each of the four indicators relative to the classification by the medical team in Colombia of one cohort of sera as early convalescent sera (< 12 weeks post symptom onset). Sensitivity calculated values for the four indicators were: IgM values: 54.78%; capture IgM/IgG ratios: 54.88%; IgG avidity: 40.68%; combined IgG avidity and capture IgM/IgG ratios: 31.25%. The specificity for differentiating ZIKV past infections was calculated for each of the four indicators relative to the classification by the medical team in Colombia of one cohort of sera as early convalescent sera (> 2 years post symptom onset). Specificity was the calculated values for each of the four assays: IgM values: 62.50%; capture IgM/IgG ratios: 84.21%; IgG avidity: 58.33%; combined IgG avidity and capture IgM/IgG ratios: 47.37%. These results indicate that the capture IgM/IgG ratios indicator yielded the highest specificity (84.21%) for charactering ZIKV past infection compared to other three

indicators. Although the stand-alone IgM values indicator and capture IgM/IgG ratios indicator had similar sensitivity for charactering ZIKV recent infection compared to other two indicators, however, relatively low.

To discover the possible reason for low sensitivity for charactering ZIKV recent infection, we checked all tests' results of the early convalescent sera. We found that there were 19 sera out of the Colombia "defined" early convalescent sera had ZIKV NS1 IgM negative results and ZIKV NS1 IgG positive results as well as high avidity results that are not typical for ZIKV recent infected sera. We then found 10 out of these 19 sera also showed IgM negative in InBios IgM test. We therefore hypothesized that at least these 10 sera could have been from patients that experienced ZIKV reinfection after the primary infection cleared, or from possibly long-term infections, which our data can neither confirm nor refute. However, 9 out of these 19 sera showed IgM positive in InBios ZIKV IgM test though they showed IgM negative in ZIKV NS1 IgM test. This discrepancy might be due to the different ZIKV antigen used in the two tests. It could be that these 9 sera that were positive by the InBios IgM test are indeed from ZIKV recently infected individuals and our test was not sensitive enough to detect them. Still, the result seems uncertain because these 9 sera had high avidity which is an indicator for ZIKV past infections.

In conclusion, based on the analysis of the Colombia sera in this study, the capture IgM/IgG ratios could be the best indicator for charactering ZIKV past infection rendering an 84.21% specificity and a 54.88% sensitivity. This sensitivity may not be enough for an accurate diagnosis of recent and past infections. However, we found some discrepancies between the diagnosis provided by the medical staff in Colombia and between our results. We therefore, for further

validation of our capture ELISAs, we would need to continue our study with more sera from other cohorts that experienced ZIKV outbreaks, preferably from different parts of the world.

REFERENCES

1. Charrel, R. N., Leparc-Goffart, I., Pas, S., de Lamballerie, X., Koopmans, M., & Reusken, C. (2016). Background review for diagnostic test development for Zika virus infection. *Bulletin of the World Health Organization*, *94*(8), 574–584D. https://doi.org/10.2471/BLT.16.171207.
2. Keasey, S. L., Pugh, C. L., Jensen, S., Smith, J. L., Hontz, R. D., Durbin, A. P., Dudley, D. M., O'Connor, D. H., & Ulrich, R. G. (2017). Antibody Responses to Zika Virus Infections in Environments of Flavivirus Endemicity. Clinical and vaccine immunology: CVI, 24(4), e00036-17. https://doi.org/10.1128/CVI.00036-17.
3. Coelho, S., Neris, R., Papa, M. P., Schnellrath, L. C., Meuren, L. M., Tschoeke, D. A., Leomil, L., Verçoza, B., Miranda, M., Thompson, F. L., Da Poian, A. T., Souza, T., Carneiro, F. A., Damaso, C. R., Assunção-Miranda, I., & de Arruda, L. B. (2017). Development of standard methods for Zika virus propagation, titration, and purification. *Journal of virological methods*, *246*, 65–74. https://doi.org/10.1016/j.jviromet.2017.04.011.
4. Lazear, H. M., & Diamond, M. S. (2016). Zika Virus: New Clinical Syndromes and Its Emergence in the Western Hemisphere. *Journal of virology*, *90*(10), 4864–4875. https://doi.org/10.1128/JVI.00252-16.
5. Balmaseda, A., Zambrana, J. V., Collado, D., García, N., Saborío, S., Elizondo, D., Mercado, J. C., Gonzalez, K., Cerpas, C., Nuñez, A., Corti, D., Waggoner, J. J., Kuan, G., Burger-Calderon, R., & Harris, E. (2018). Comparison of Four Serological Methods and Two Reverse Transcription-PCR Assays for Diagnosis and Surveillance of Zika Virus

Infection. *Journal of clinical microbiology*, *56*(3), e01785-17. https://doi.org/10.1128/JCM.01785-17.

6. Mishra, N., Caciula, A., Price, A., Thakkar, R., Ng, J., Chauhan, L. V., Jain, K., Che, X., Espinosa, D. A., Montoya Cruz, M., Balmaseda, A., Sullivan, E. H., Patel, J. J., Jarman, R. G., Rakeman, J. L., Egan, C. T., Reusken, C., Koopmans, M., Harris, E., Tokarz, R., … Lipkin, W. I. (2018). Diagnosis of Zika Virus Infection by Peptide Array and Enzyme-Linked Immunosorbent Assay. *mBio*, *9*(2), e00095-18. https://doi.org/10.1128/mBio.00095-18.
7. Centers for Disease Control and Prevention. NEW Zika and DENV Testing Guidance. 2019.
8. de Souza, V. A., Fernandes, S., Araújo, E. S., Tateno, A. F., Oliveira, O. M., Oliveira, R. R., & Pannuti, C. S. (2004). Use of an immunoglobulin G avidity test to discriminate between primary and secondary dengue virus infections. Journal of clinical microbiology, 42(4), 1782–1784. https://doi.org/10.1128/jcm.42.4.1782-1784.2004.
9. Levett, P. N., Sonnenberg, K., Sidaway, F., Shead, S., Niedrig, M., Steinhagen, K., Horsman, G. B., & Drebot, M. A. (2005). Use of immunoglobulin G avidity assays for differentiation of primary from past infections with West Nile virus. Journal of clinical microbiology, 43(12), 5873–5875. https://doi.org/10.1128/JCM.43.12.5873-5875.2005.
10. Prince, H. E., Yeh, C., & Lapé-Nixon, M. (2011). Utility of IgM/IgG ratios and IgG avidity for distinguishing primary and secondary dengue virus infections using sera collected more than 30 days after disease onset. Clinical and vaccine immunology : CVI, 18(11), 1951–1956. https://doi.org/10.1128/CVI.05278-11.
11. de Souza, V. A., Tateno, A. F., Oliveira, R. R., Domingues, R. B., Araújo, E. S., Kuster, G. W., & Pannuti, C. S. (2007). Sensitivity and specificity of three ELISA-based assays for

discriminating primary from secondary acute dengue virus infection. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 39(3), 230–233. https://doi.org/10.1016/j.jcv.2007.04.005.

12. Sigma-Aldrich, Normal Serum, Ascites, and Cell Supernatant Typical Immunoglobulin Concentration Ranges Normal Sera.
13. Gonzalez-Quintela, A., Alende, R., Gude, F., Campos, J., Rey, J., Meijide, L. M., Fernandez-Merino, C., & Vidal, C. (2008). Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. *Clinical and experimental immunology*, *151*(1), 42–50. https://doi.org/10.1111/j.1365-2249.2007.03545.x.
14. Safronetz, D., Sloan, A., Stein, D. R., Mendoza, E., Barairo, N., Ranadheera, C., Scharikow, L., Holloway, K., Robinson, A., Traykova-Andonova, M., Makowski, K., Dimitrova, K., Giles, E., Hiebert, J., Mogk, R., Beddome, S., & Drebot, M. (2017). Evaluation of 5 Commercially Available Zika Virus Immunoassays. *Emerging infectious diseases*, *23*(9), 1577–1580. https://doi.org/10.3201/eid2309.162043.
15. Centers for Disease Control and Prevention. Zika Mac ELISA Instruction for use, 2018.
16. Basile, A. J., Goodman, C., Horiuchi, K., Sloan, A., Johnson, B. W., Kosoy, O., Laven, J., Panella, A. J., Sheets, I., Medina, F., Mendoza, E. J., Epperson, M., Maniatis, P., Semenova, V., Steward-Clark, E., Wong, E., Biggerstaff, B. J., Lanciotti, R., Drebot, M., Safronetz, D., … Schiffer, J. (2018). Multi-laboratory comparison of three commercially available Zika IgM enzyme-linked immunosorbent assays. *Journal of virological methods*, *260*, 26–33. https://doi.org/10.1016/j.jviromet.2018.06.018.

17. Amaro, F., Sánchez-Seco, M. P., Vázquez, A., Alves, M. J., Zé-Zé, L., Luz, M. T., Minguito, T., De La Fuente, J., & De Ory, F. (2019). The Application and Interpretation of IgG Avidity and IgA ELISA Tests to Characterize Zika Virus Infections. *Viruses*, *11*(2), 179. https://doi.org/10.3390/v11020179.

18. Furuya, A., Hunt, D., George, K. S., Dupuis, A. P., 2nd, Kramer, L. D., Shi, P. Y., & Wong, S. (2019). Use of the immunoglobulin G avidity assay to differentiate between recent Zika and past dengue virus infections. *Clinical science (London, England : 1979)*, *133*(7), 859–867. https://doi.org/10.1042/CS20180874.

19. Rönnberg, B., Gustafsson, Å., Vapalahti, O., Emmerich, P., Lundkvist, Å., Schmidt-Chanasit, J., & Blomberg, J. (2017). Compensating for cross-reactions using avidity and computation in a suspension multiplex immunoassay for serotyping of Zika versus other flavivirus infections. *Medical microbiology and immunology*, *206*(5), 383–401. https://doi.org/10.1007/s00430-017-0517-y.

20. Mora-Cárdenas E, Aloise C, Faoro V, Knap Gašper N, Korva M, et al. (2020) Comparative specificity and sensitivity of NS1-based serological assays for the detection of flavivirus immune response. PLOS Neglected Tropical Diseases 14(1): e0008039. https://doi.org/10.1371/journal.pntd.0008039.

21. Mishra, A. K., & Mariuzza, R. A. (2018). Insights into the Structural Basis of Antibody Affinity Maturation from Next-Generation Sequencing. Frontiers in immunology, 9, 117. https://doi.org/10.3389/fimmu.2018.00117.