A systematic evaluation of IgM and IgG antibody assay accuracy in diagnosing acute Zika Virus infection in Brazil; lessons relevant to emerging infections

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RUNNING TITLE: Evaluation of Zika Virus antibody assays in Brazil

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Abstract

Accurate diagnostics underpin effective public health responses to emerging viruses. For viruses, such as Zika virus (ZIKV), where the viremia clears quickly, antibody-based (IgM or IgG) diagnostics are recommended for patients who present seven days after symptom onset. However, cross-reactive antibody responses can complicate test interpretation among populations where closely related viruses circulate.

We examined the accuracy (proportion of samples correctly categorized as Zika-positive or negative) for antibody-based diagnostics among Brazilian residents (Rio de Janeiro) during the ZIKV outbreak. Four ZIKV ELISAs (IgM and IgG Euroimmun, IgM Novagnost and CDC MAC), two dengue ELISAs (IgM and IgG Panbio), and the ZIKV plaque reduction neutralization test (PRNT) were evaluated. Positive samples were ZIKV PCR confirmed clinical cases collected in 2015-2016 (n=169); Negative samples (n=236) were collected before ZIKV was present in Brazil (≤2013).

Among serum samples collected ≥7 days from symptom onset, PRNT exhibited the highest accuracy (93.7%), followed by the Euroimmun IgG ELISA (77.9%). All IgM assays exhibited lower accuracy (<75%). IgG was detected more consistently than IgM among ZIKV cases using Euroimmun ELISAs (68% versus 22%). Anti-DENV IgM ELISA was positive in 41.1% of confirmed ZIKV samples tested.

The Euroimmun IgG assay, although misdiagnosing 22% of samples, provided the most accurate ELISA. Anti-ZIKV IgG was detected more reliably than IgM among ZIKV patients, suggesting a secondary antibody response to assay antigens following ZIKV infection. Antibody ELISAs need careful evaluation in their target population to optimise use and minimise misdiagnosis, prior to widespread deployment, particularly where related viruses co-circulate.

Introduction

Zika virus (ZIKV) is an arthropod-borne flavivirus. A public health emergency of international concern (PHEIC) was declared by the World Health Organization (WHO), in response to the large Zika epidemics in South and Central America in 2015-2016 (1). Although transmission has declined, over 85 countries across South and Central America, Asia, West Africa, Caribbean and the Pacific Islands have current or previous transmission of ZIKV and another 61 have established mosquito vectors and remain at risk for Zika infection (2-4). Moreover, autochthonous transmission of ZIKV was demonstrated in Europe with, at least, 3 cases of vector-borne transmission in Southern France in 2019 (5). Accurate diagnostics are essential to guide appropriate clinical management of suspected patients. Both false negative and false positive diagnosis may trigger catastrophic consequences, especially among pregnant women (6).

The first-line diagnostic test for ZIKV detection is direct molecular detection using PCR. However, the time-frame for accurate virus detection following exposure using this method is short. Consequently, the WHO issued laboratory diagnostic algorithms recommending anti-ZIKV antibody-based testing in patients presenting seven or more days after symptom onset (7, 8). Nevertheless, accurate detection of ZIKV antibodies is challenging because antibody-based assays are susceptible to cross-reactivity from related viruses. This is a particular issue in regions such as Latin America where extensive circulation of multiple flaviviruses has occurred in the population over the last 30 years. In Brazil, all four serotypes of dengue virus (DENV) circulate and yellow fever virus (YFV) vaccination is widespread in many regions (9). Previous studies have demonstrated that the vast majority of adults within Rio de Janeiro have been exposed to Dengue, with Dengue IgG sero-prevalence estimated at 90.9% in 2012 (10) . Similarly, yellow fever vaccination has been reported to have reasonable coverage in most regions of Brazil and

coverage is likely to have increased following the vaccination campaigns mounted in response to recent yellow fever outbreaks (11, 12).

The WHO highlighted the important need for field validation of available Zika serological assays in flavivirus exposed populations (13). A range of different antibody-based assays have been developed (14, 15), but evaluation of the assay's performance in local populations lagged behind their use.

To date, there are very few published studies on the performance of commercial ZIKV antibody-based assays using well-characterized samples from South American populations and no systematic evaluation from Brazil. Evaluations based on sera from European travelers who had visited countries with ZIKV circulation found a high sensitivity and specificity for the commercial immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assays (ELISAs) that use a recombinant ZIKV NS1 antigen (16-19). Other commercial assays, such as the IgM μ-capture ELISA, have also been approved for use in the Americas, despite initial reports of low test specificity among travelers (20). The Centers for Disease Control and Prevention (CDC) in the USA, recommends the use of an IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) which is licensed under the CDC FDA-emergency-use-authorization protocol (21). However, reports from Nicaragua and the USA have shown this assay to have relatively low specificity and it is no longer recommended for screening. Further local population evaluations have been recommended (22, 23).

We conducted a systematic evaluation of four antibody-based methods for ZIKV diagnosis: the IgM and IgG NS1 Anti-ZIKV ELISAs (Euroimmun), the IgM μ-capture ZIKV ELISA (Novagnost) and the CDC MAC-ELISA. We also re-evaluated two DENV antibody assays, the IgM and IgG ELISA assays (Panbio) in this new context. We compared these ELISAs against the ZIKV plaque reduction neutralization test (PRNT), which is currently considered the "gold standard" by the WHO for the confirmatory diagnosis of Flavivirus infections. The assays were evaluated using well-characterized sera

from residents of Rio de Janeiro, Brazil, classified by clinical and laboratory testing, as confirmed ZIKV (cases with both clinical evidence of ZIKV infection and positive detection of ZIKV RNA by RT-PCR in at least one specimen) or non-ZIKV cases (sera collected in 2013 or before, prior to the arrival of ZIKV in Rio de Janeiro). Our aim was to examine assay accuracy among the Brazilian population, to investigate the time window of detection of anti-ZIKV antibodies and the biological variability of antibody responses.

Materials and Methods

Ethics Statement

The sera and patient data were used in accordance with the ethical standards of the Instituto Nacional de Infectologia Evandro Chagas and the Instituto Oswaldo Cruz. The study protocol was approved by its Research Ethics Committee (reference CAAE 0026.0.009.000–07 and CAAE 71405717.8.0000.5248). Specimens were given laboratory numbers and so anonymized to testers to ensure patient confidentiality.

Study population and sample selection

ELISA evaluations were performed at the Flavivirus Reference Laboratory, Institute Oswaldo Cruz in Rio de Janeiro (Brazil). Plaque reduction neutralization tests (PRNT) were performed at the Aggeu Magalhães Institute in Recife (Brazil).

Four-hundred and five serum samples in total were tested (Table 1). All sera were collected from residents (n=307) of the State of Rio de Janeiro. Only samples stored at -20ºC or below, and with no history of repeated freeze-thaw were used in the study. Due to limited availability of reagents and serum volumes, not all samples were tested on all assays.

Panels of sera were assembled for the evaluation: 1) to assess assay sensitivity, samples from ZIKV confirmed cases were used (Set1: n=169) from subjects (n=71) with rash-fever symptom and positive detection of ZIKV RNA by RT-PCR. Samples were collected in 2015 or 2016, coinciding with the peak of the ZIKV epidemic in Rio de Janeiro (1). Most subjects (66 out of 71) had two or more sequential samples collected (see Table S1 for further details on serum collections). 2) To assess assay specificity, non-ZIKV samples, collected from individuals during, or before, 2013 were used (Set2 and Set3; n=236). These were collected 2 years before the ZIKV outbreak in Rio de Janeiro began in 2015 (24). Set2 (n=184) included samples from subjects with confirmed dengue (clinical presentation and PCR or IgM positive; n=90), measles or rubella infection (n=40), who had received yellow fever vaccination (n=19), or other population samples (n=35). Set3 samples were collected from patients attending a hepatitis clinic at Fiocruz, Rio de Janeiro (n=52).

Diagnostic Assays

The IgM and IgG ZIKV NS1 ELISA assays (Euroimmun, Lubeck, Germany) use recombinant Zika non-structural protein 1(NS1) as the ZIKV antigen. Assays were performed following the manufacturer's instructions. IgM and IgG results were determined based on optical density (OD) ratio of human sample/calibrator sample. A result was classified as: positive if ratio ≥1.1; indeterminate between ≥0.8 and 1.1; negative ≤0.8.

The Novagnost Zika Virus IgM μ-capture ELISA (NovaTec Immunodiagnostica GmbH, Germany) uses ZIKV NS1 antigen. Assays were conducted according to the manufacturer's instructions. Results were classified based on the OD ratio of human/calibrator sample as per manufacturer's guidelines (ratio ≥1.1 classified as positive).

The CDC ZIKV MAC-ELISA (FDA CDC-designed IgM antibody capture ELISA) utilizes inactivated whole virus antigen. The assays were performed as previously described using the US Centers for Disease Control and Prevention (CDC) emergency use authorization protocol (25). Results were based on the Positive to Negative ratio (P/N). P is obtained as the mean OD of the test serum, reacted on the Zika antigen, which is divided by N, the mean OD of the normal human serum reacted on the Zika antigen. Results were reported as recommended: positive if P/N≥3; indeterminate if P/N≥2 but <3 and negative if P/N<2.

For the detection of DENV IgM and IgG antibodies, the commercial dengue IgG Indirect and IgM Capture ELISAs (Panbio, Alere, United Kingdom) were used. Samples were interpreted as positive for previous or recent dengue infection according to the standard protocols of the manufacturer.

All the ELISA assays used the same volume of patient serum (10 µl, 1:101 in their respective sample buffer). All assays were stored at 2-8°C prior to use.

ZIKV PRNTs were performed following a published method previously employed among the Brazilian population (26). It is an adaptation of an established PRNT protocol (27). PRNT was performed using a Zika virus strain isolated in Northeast Brazil (ZIKV, BR-PE243/2015). The optimal cut-off for PRNT positivity was defined based on a 50% reduction in plaque counts ($PRNT_{50}$). ZIKV neutralizing antibody titers were estimated using a four-parameter non-linear regression. Serum samples were considered positive when antibody titers were >1:100 (log2).

RT-PCR

Individuals with clinical presentation consistent with acute ZIKV infection (during 2015 and 2016) were tested for ZIKV nucleic acid material. RNA was extracted using the Qiamp Mini Elute (Qiagen, Brazil).

Reference ZIKV and DENV RT-PCR (for suspected ZIKV and DENV cases) were performed as previously described (28, 29).

Statistical analyses and calculation of ROC Curves, sensitivity and specificity

Diagnostic performance of the ELISAs was assessed by calculating accuracy (classified as true positive and true negative / all cases tested); sensitivity (classified as positive / all reference positives tested) and specificity (classified as negative / all reference negatives tested), using MedCalc Statistical Software, version 16.2.0 (MedCalc Software, Ostend, Belgium). Figures were generated using STATA v16 and PRISM GraphPad v7 (GraphPad Software, La Jolla, CA). Samples with indeterminate or borderline results were re-tested (if enough specimen was available). Samples with indeterminate results a second time were considered negative according to the instructions for use for all kits and methods used.

This prospective cross-sectional diagnostic accuracy study is reported according to the Standards for Reporting of Diagnostic Accuracy Study (STARD) (30) (Checklist S1, Flow chart S1, Flow chart S2).

Results

Age distribution was unimodal with a median age of 24.5 years (range 1-80y). 55.4% of all subjects were female. Among the confirmed ZIKV samples, the median time of sample collection after symptom onset was 7 days (range 1 - 276). Full details are provided in Table 1.

Diagnostic assays performance

A detailed breakdown of accuracy, sensitivity and specificity for all assays tested are presented in Table 2 and Table 3.

Summary of overall accuracy

Initially, we examined test accuracy among all samples, including samples collected within 7 days of ZIKV symptom onset. The highest accuracy was exhibited by PRNT, followed by ZIKV IgG NS1 Euroimmun, MAC-ELISA, IgM NS1 Euroimmun, and IgM μ-capture Novagnost ELISAs (78.8, 71.2, 63.0, 59.2 and 56.0% respectively - Tables 2 and 3).

Although not designed to detect ZIKV antibodies, we also assessed the performance of the dengue assays (IgG and IgM) among the same samples. The proportion of samples from confirmed ZIKV cases (collected any time from symptom onset) classed as positive by DENV IgM or IgG assays were 32.5% and 85.7% respectively (Table 3).

The WHO recommends that samples collected up to 7 days from symptom onset are tested using a ZIKV specific RT-PCR (31). Analysis was repeated excluding ZIKV samples collected less than 7 days from symptom onset. The accuracy of all ZIKV assays improved. Again, the highest accuracy was exhibited by PRNT followed by the IgG NS1 Euroimmun ELISA (93.7 and 77.9 respectively). The ZIKV IgM (NS1 Euroimmun, μ-capture Novagnost and CDC MAC) ELISAs continued to exhibit lower accuracy (74.1, 72.5 and 71.9% respectively).

PRNT exhibited both good sensitivity (90.9% [80/88]) and specificity (96.1% [98/102]). The major weakness of the Euroimmun IgM and Novagnost IgM ELISAs was poor sensitivity (32.6% [29/89] and 48.3% [43/89] respectively). The CDC MAC-ELISA exhibited reasonable sensitivity but poor specificity (87.7% [50/57] and 63.6% [70/110] respectively; Tables 2 and 3). Assay sensitivity improved in all the assays among samples collected ≥7 to 13 days post symptom onset. Assay sensitivity decreased for specimens collected ≥14 days in the IgM ZIKV Euroimmun and IgM Novagnost assays. The proportion of confirmed ZIKV cases with a positive IgM DENV assay result was also reduced.

Comparison of anti-ZIKV IgG and IgM antibody responses

We compared IgG and IgM anti-ZIKV antibody titers over time from symptom onset (measured using the ZIKV NS1 Euroimmun ELISAs). Based on measuring a single sample, anti-ZIKV IgG was more consistently detected than anti-ZIKV IgM among sera from confirmed ZIKV positive subjects (68% [114/168] vs. 22% [37/167] respectively). Among paired samples, anti-ZIKV IgG more frequently exhibited a rise in antibody titers than IgM (IgG; rise 95% [56/59]; ≥2 fold 75% [44/59]; IgM; rise 81% [46/57]; ≥2 fold 61% [35/57]; Figure 1). Anti-ZIKV IgG also exhibited a higher median fold increase compared to IgM among paired sera (3.5 versus 2.6-fold increase [p=0.001]; taken 7 days apart [median]; Figure 2). Overall, there was a more sustained rise in anti-ZIKV IgG compared to IgM over time from symptom onset (Figure 3).

Anti-DENV IgM and IgG responses in confirmed ZIKV cases

Both IgM and IgG anti-DENV ELISAs gave positive results among sera collected ≥7 days from PCR confirmed ZIKV cases (41.1% [37/90] and 87.8% [79/90] respectively - for dengue IgM these are likely to represent false positive IgM results - Table 3). In an attempt to distinguish between the dengue IgG response in ZIKV patients who had previously been exposed to DENV and acute ZIKV patients exhibiting a false positive cross-reaction against DENV IgG antibodies, we looked at sera collected < 7 days from symptom onset. We hypothesized that within this shorter time from illness onset, acute cross-reactive antibodies had less time to develop and DENV IgG positivity was more likely to reflect past dengue exposure. A high proportion of confirmed ZIKV cases were still DENV IgG positive (83.3% [65/78]). However, a significant correlation between anti-ZIKV and anti-DENV antibody titers was observed when the same sera were measured by IgG NS1 Euroimmun ZIKV and IgG Panbio DENV assays (IgG r^2=0.258; p<0.0001; Figure 4a). A similar, but less significant correlation, was observed between ZIKV Euroimmun IgM NS1 and Panbio DENV IgM assays (IgM r^2=0.015; p=0.03; Figure 4b).

When examining samples collected >7 days from symptom onset a similar positive correlation between anti-ZIKV and anti-DENV IgG antibody titers was also observed.

Diversity of anti-ZIKV IgM antibody responses

To describe the variation in duration and magnitude of anti-ZIKV antibody responses, we compared Zika antibody patterns in four PCR confirmed ZIKV patients (A-D, who had sera collected at ≥5 different time-points after symptom onset (range: 0-276 days). Antibody titers were measured by the IgM NS1 and μ-capture assays (Figure 5). Antibody patterns were diverse in both magnitude and duration of response. Interestingly, in patient A, despite an initial rise in IgM titers, this quickly fell. IgM was below the Euroimmun NS1 ZIKV assay's positive threshold by day 22. We then looked at all samples from confirmed ZIKV subjects. At 14, 27 and 90 days post symptom onset only 28.6% (14/49), 8% (4/27), and 0% (0/14) samples had detectible IgM responses when measured in the ZIKV Euroimmun NS1 IgM assay. Similarly, only 44.9% (22/49), 25.9% (7/27) and 7.1% (1/14) samples were positive, at 14, 27 and 90 days, when the same samples were measured using the IgM μ-capture Novagnost assay.

Improving the accuracy of the IgG NS1 ELISA

The accuracy of the best performing ZIKV ELISA (i.e. the Euroimmun IgG NS1 ELISA) could be improved by modifying the cut-off used to classify a positive result. The modified cut-off was identified using receiver operator curve (ROC) analyses, defining the point at which the cut-off gave the highest likelihood ratio (32) using all sera from non-ZIKV subjects (n=204) and sera collected ≥7 days among confirmed ZIKV subjects (n=90). Using a cut-off of 1.5, which provided the maximum likelihood ratio (>4.4), the ELISA exhibited an accuracy of 81.0% (previously 77.9%). Sensitivity and specificity were 78.9 and 82.2% respectively (Figure 6; Table S3).

Discussion

ZIKV was a viral infection of significant international public health concern that affected over 148 countries during 2015-2019 (5, 33). Pregnant women are still advised not to travel in Brazil and other South American countries. Among people with suspected ZIKV presenting seven or more days from symptom onset serological antibody testing remains the recommended diagnostic approach (31). The majority of ZIKV antibody tests employed in the field have not been validated in their target populations. Despite the ZIKV outbreak in Brazil triggering WHO to declare a public health emergency, there has been no systematic evaluation of the commercial ZIKV antibody assays among Brazilians residents.

The Euroimmun IgG NS1 assay gave the most accurate diagnostic performance among the ELISAs tested. Accuracy could be improved to 81% by modifying the cut-off (from that suggested by the manufacturer). Our results indicate that approximately 1 in 5 subjects are falsely classified by the Euroimmun IgG ELISA when testing a single serum sample. One accepted weakness of employing an IgG based ELISA is that a positive result from a single sample does not discriminate recent from past infection. Akin to other IgG based tests used to diagnose acute infection (34), one option for improving the sensitivity of acute ZIKV diagnosis may be to collect and test serial (paired) samples. During testing of ZIKV PCR positive cases, 95% of paired sera exhibited a rise in antibody levels when measured via the Euroimmun NS1 IgG ELISA (collected seven days apart).

All of the anti-ZIKV IgM ELISAs (Euroimmun NS1, μ-capture and MAC) exhibited lower accuracy (<75%). The ELISAs tended to exhibit low sensitivity for detecting PCR confirmed ZIKV cases, even when serum was collected ≥7 days post symptom onset.

We did not expect the Euroimmun IgG ELISA to give higher sensitivity than the IgM based ELISAs. Over time from symptom onset, the Euroimmun IgG ELISA exhibited more consistent and sustained detection of anti-ZIKV antibody compared to its counter-part anti-ZIKV IgM ELISA. These patterns of IgG and IgM response suggest a secondary immune response to infection (Figures 3 and 7), with an anamnestic boosting of prior immune response most likely due to dengue that cross-reacts in the anti-ZIKV IgG antibody assay (Figure 7). Given our patients presented with their first reported ZIKV infection, their antibody responses suggest they had previously been exposed to a similar virus (i.e. to dengue or another flavivirus). We suggest the prominent IgG response and poor specificity of the anti-Zika IgG assays observed in this study reflect an anamnestic cross-reactive IgG antibody response among local Brazilians who have previously been exposed to other flaviviruses. This warrants further investigation and has implications in the design of both future diagnostic tests and vaccines against ZIKV and DENV in flavivirus exposed populations as it is recognized with dengue.

Our findings contrast markedly with published studies conducted using sera from travelers. Such studies largely tested people visiting but not living in ZIKV or other flavivirus exposed countries. These latter studies reported much higher sensitivity (>90%) for the commercial IgM assays (16, 17, 19) and higher specificity for the IgG NS1 assay (>90%) (20). The CDC MAC-ELISA exhibited reduced accuracy and sensitivity when tested among Nicaraguan and Colombian residents compared to “traveler” subjects. Our findings were consistent with this [16,17].

This disparity is likely to reflect the different flavivirus exposure between visitors and local residents. ZIKV positive visitors are likely to experience their first exposure to flavivirus infection. In contrast to residents of Brazil, who are likely to have been exposed to ZIKV and other circulating flaviviruses in the past (e.g. DENV and/or YFV). If past flavivirus exposure triggers an anamnestic antibody response leading to more IgG than IgM production, this could, in part, explain the poor sensitivity of IgM based

296 ZIKV ELISAs seen here, as it has been shown previously for other flaviviruses (35). Our findings
297 indicate that validation of diagnostic assays should be performed in the population it will be used for.

298 The high rate of DENV IgM assay positivity (41.1% among samples from confirmed ZIKV cases
299 collected >7days from symptom onset) was not anticipated (36). The significant correlation between
300 antibody titers for DENV and ZIKV, when tested via IgG or IgM based ELISAs, indicate there is a
301 degree of cross-reaction both ways following DENV and ZIKV infection detected in these ELISAs.
302 These findings highlight the diagnostic challenges ahead as outbreaks of both DENV and ZIKV have
303 been forecast to re-occur in overlapping geographical regions.

304 Our study findings for the ZIKV diagnostic antibody tests are pertinent to all emerging epidemics,
305 including the current SARS-CoV-2 pandemic. Our results highlight that confirming the accuracy of a
306 diagnostic assay in the target population is imperative to control and manage false positive or negative
307 results across different settings. Such validation should be advocated by governments, national public
308 health agencies and the WHO prior to test deployment. Our results also highlight the need to re-evaluate
309 the accuracy of established tests when a closely related emergent pathogen is introduced in a region or
310 the population changes. In the case of ZIKV, we recommend the re-evaluation of DENV and YFV
311 assays' performance in Brazil. However, for newer threats, such as SARS-CoV-2, re-evaluation of
312 Severe Acute Respiratory Syndrome (SARS) coronavirus antibody tests should be considered among
313 Saudi Arabians and other populations with a history of transmission for closely related viruses.

314 Based on the assays we have assessed, ZIKV PRNTs provide the most accurate assay to diagnose
315 exposure to ZIKV among Brazilian residents in samples collected ≥7 days post symptom onset.
316 Performing PRNTs requires specialized training, sophisticated laboratories and the assays are labor
317 intensive; they are therefore unlikely to be widely used for diagnosis outside of reference laboratories
318 (37). As recommended by the WHO, we support their use as a 'gold standard' reference test for

319 flavivirus diagnosis, including ZIKV, if used with an appropriate cutoff to exclude low level cross-
320 reactions (8).

321 The Euroimmun IgG NS1 ELISA provided the most accurate ELISA test to diagnose exposure to ZIKV
322 among Brazilian residents in samples collected ≥7 days post symptom onset. In order to assess whether
323 exposure is acute, we would recommend taking paired samples (7 days apart) and looking for a rise in
324 antibody titers. The best time of collecting for these samples has not been systematically assessed in this
325 study. Based on our observational data, samples collected on days 2 and 9 post symptom onset were
326 associated with the highest fold-changes. However, this was assessed during the first waves of infection
327 with a newly introduced virus and once significant population exposure has occurred interpreting the
328 significance of a positive Zika IgG for acute diagnosis will be even more challenging.

329 In our study, the ZIKV IgM based ELISAs exhibited poor accuracy (Euroimmun, Novagnost and MAC-
330 ELISA). Similarly, the Panbio DENV ELISAs (particularly IgM) relatively high positivity rate among
331 acute ZIKV cases was a concern. DENV antibody-based serological assays continue to be needed to
332 complement dengue PCR testing. Our findings highlight the need for careful interpretation of existing
333 dengue ELISA results. As more accurate tests are developed, their accuracy in PCR confirmed ZIKV
334 and DENV exposed residents should be comprehensively assessed.

335 Our study has limitations. Serial samples among ZIKV PCR positive subjects were collected non-
336 systematically as convenience samples. Consequently, we cannot confirm the best time to collect paired
337 samples post symptom onset. Similarly, serial samples were not available for our non-ZIKV subjects, so
338 we were unable to assess the specificity of paired samples testing in the Euroimmun NS1 IgG ELISA.
339 Not all samples were tested in all assays due to limited specimen volumes and assay kits available.
340 There remains potential for a false positive rise in titers in acutely ill non-ZIKV subjects due to antibody
341 cross-reaction. The ZIKV PCR positive subjects were not tested for DENV by PCR, so we are unable to

confirm that the positive DENV IgM results are false. Nevertheless, limited acute dengue circulation was reported during the study period in the Rio de Janeiro region. As the PRNT specificity was tested in the context of a non-ZIKV exposed population, future studies will be needed to establish accuracy of the ZIKV PRNT in populations where both DENV and ZIKV have circulated previously (38).

In conclusion, this is a systematic evaluation of antibody-based ZIKV assays in Brazil. Among ZIKV patients, anti-ZIKV IgG was detected more consistently than IgM, suggesting a secondary antibody response to infection. ZIKV PRNT exhibited the highest accuracy of all assays tested if used with an appropriate cut-off. All ZIKV IgM based ELISAs exhibited low accuracy. The Euroimmun NS1 IgG ELISA exhibited the best ELISA accuracy. Nevertheless, when testing a single serum sample, it misdiagnosed 1 in 5 cases. Testing paired samples via ZIKV IgG based ELISA, may offer a more sensitive method of diagnosing acute ZIKV exposure. Our findings highlight that diagnostic antibody assay use and interpretation needs careful assessment in the target population, particularly when deployed among populations exposed to multiple closely related viruses. Clinical symptoms must always be taken into account when arriving at a final diagnosis.

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Tables and Figures:

Table 1 Characteristics of the study population

		No. of patients	No. of samples	% of patients	Year.
Total		307	405	-	2002-2016
Sex	Female	164 (296)*	-	55.4%	-
ZIKV Positive samples (Set1)					
	Total	**71**	**169**	**23.1 (71/307)[a]**	**2015-2016**
	1 Serum	5	5	7.0 (5/71)[b]	2015-2016
	2 Serum	55	110	77.5(55/71)[b]	2015-2016
	3 or more samples	11	54	15.5(11/71)[b]	2015-2016
Controls – ZIKV Negative (Set2)					
	Total	**184**	**184**	**59.9(184/307) [a]**	**2002-2013**
	DENV1-4 (Total)	90	90	82.6 (90/109)[d]	2002-2013
	DENV 1	21	21	23.3(21/90)[c]	2010-2011
DENV	DENV 2	17	17	18.9(17/90)[c]	2008,2010,2011
	DENV 3	21	21	23.3(21/90)[c]	2002,2007,2008
	DENV 4	31	31	34.4(31/90)[c]	2012,2013
Yellow fever		19	19	17.4(19/109)[d]	2003-2007
Measles or Rubella		40	40	21.7(40/184)[e]	2011-2012
Other non-flavivirus infections		35	35	19.0(35/184)[e]	2011-2012
General population (Set3)	Total Set3	52	52	16.9(52/307) a	2013

Abbreviations: No., number, Year, Year of sample collection, *Sex was not documented for 18 patients.

The population data for the subgroups denoted by roman superscript letters a through e add up to 100%:

[a]. represents patients in Set1, 2 and 3. [b]. Patients in Set1. [c]. Dengue Positive patients. [d]. Flavivirus positive subjects. [e]. Non-flavivirus infections subjects.

506 **Table 2** Sensitivity and specificity for the four anti-ZIKV antibody ELISAs

	IgM Euroimmun NS1 ELISA			IgG Euroimmun NS1 ELISA			CDC Zika MAC-ELISA			IgM μ-capture Novagnost		
	Tested (n)	**% Sens. (95%CI)**	**% Spec. (95% CI)**	**Tested (n)**	**% Sens. (95%CI)**	**% Spec. (95% CI)**	**Tested (n)**	**% Sens. (95%CI)**	**% Spec. (95% CI)**	**Tested (n)**	**% Sens. (95%CI)**	**% Spec. (95% CI)**
Zika Positive	167	22.2(15.8-28.4)		168	67.8(60.8-74.9)		125	62.4(53.9-70.9)		167	31.7(24.7-38.8)	
Zika (1-6 Days)	78	10.3(3.5-16.9)		78	46.2(39.1-53.2)		68	41.2(32.7-49.7)		78	12.8(5.8-19.9)	
Zika (≥7-13 Days)	40	37.5(22.5-52.5)		41	80.5(73.4-87.5)		34	85.3(76.8-93.8)		40	52.5(45.4-59.6)	
Zika (≥14 Days)	49	28.5(15.9-41.2)		49	91.8(84.7-98.9)		23	91.3(82.8-99.8)		49	44.9(37.8-52)	
Zika (≥7 Days)	89	32.6(22.9-42.3)		90	86.7(79.6-93.7)		57	87.7(79.2-96.2)		89	48.3(41.3-55.4)	
Non ZIKV (Flavivirus and Non-flavivirus)	166		96.3 (93.5-99.2)	204		74.0(67.4-79.9)	110		63.6 (54.7-72.6)	110		92.7 (87.9-97.6)
DENV (all)	89		100.0(100.0-100.0)	89		76.4(67.6-85.2)	88		61.3 (51.2-71.5)	79		92.4 (86.6-98.25)
DENV1	21		100.0(100.0-100.0)	21		95.2(86.1-100.0)	21		52.3(31.0-73.7)	16		100.0(100.0-100.0)
DENV2	17		100.0(100.0-100.0)	17		76.4(56.3-96.6)	17		35.3(12.6-58.0)	16		100.0(100.0-100.0)
DENV3	21		100.0(100.0-100.0)	21		42.9(21.7-64.0)	21		61.9(41.1-82.7)	17		88.2(72.9-103.55)
DENV4	30		100.0(100.0-100.0)	30		86.6(74.5-98.8)	29		82.8(69.0-96.5)	30		86.6 (74.5-98.8)
Yellow fever	19		100.0(100.0-100.0)	19		84.2(67.8-100.6)	14		57.1 (31.2-83.1)	16		87.5(71.3-103.7)
Non-flavivirus	58		89.7(81.8-97.5)	44		77.2(64.9-89.7)	8		100.0(100.0-100.0)	15		100.0(100.0-100.0)
Measles & Rubella	40		87.5(77.3-97.8)	17		94.1 (82.9-105.3)	8		100.0(100.0-100.0)	1		100.0(100.0-100.0)
Hepatitis	13		92.3(77.8-100.0)	13		69.2(44.2-94.3)	-			13		100.0(100.0-100.0)
Other	5		100.0(100.0-100.0)	14		64.3(39.2-89.4)	-			1		100.0(100.0-100.0)
General Population (Set3)	-			52		63.5(48.9-76.4)						
Overall (all available samples)	**333**	**22.2(15.8-28.4)**	**96.3 (93.5-99.2)**	**372**	**67.8(60.8-74.9)**	**74.0(67.4-79.9)**	**235**	**62.4(53.9-70.9)**	**63.6 (54.7-72.6)**	**277**	**31.7(24.7-38.8)**	**92.7 (87.9-97.6)**
PPV		86.1(72.8-93.4)			68.3(62.5-73.5)			66.1(59.5-72.1)			86.9(76.6-93.1)	
NPV		55.2(53.0-57.3)			73.7(68.9-78.0)			59.8(53.3-66.0)			47.2(44.4-50.1)	
Accuracy		59.2(53.7-64.5)			71.2(66.4-75.8)			63.0(56.5-69.2)			56.0(49.9-61.9)	
Overall (≥7 days)	**255**	**32.6(22.9-42.3)**	**96.3 (93.5-99.2)**	**294**	**86.7(79.6-93.7)**	**74.0(67.4-79.9)**	**167**	**87.7(79.2-96.2)**	**63.6 (54.7-72.6)**	**199**	**48.3(41.3-55.4)**	**92.7 (87.9-97.6)**
PPV (≥7 days)		82.9(67.6-91.8)			59.5(53.5-65.3)			55.6(48.9-62.0)			84.3(72.7-91.6)	
NPV (≥7 days)		72.7(69.7-75.6)			92.6(88.1-95.5)			90.9(83.1-95.3)			68.3(63.6-72.6)	
Accuracy (≥7 days)		74.1(68.3-79.4)			77.9(72.7-82.5)			71.9(64.4-78.5)			72.5(65.6-78.6)	

Table 2 - Sensitivity and specificity of the IgM and IgG Euroimmun NS1 commercial ELISA, the MAC-ELISA and IgM µ-capture Novagnost assays with sera from confirmed ZIKV (Set 1) and the Control non-ZIKV group (Set 1 and Set 2). Specificity values were calculated for each assay based on Set 2 and 3. Data from ZIKV-positive cases served only for determining the sensitivity and was not used for the specificity calculation. Similarly, data from ZIKV-negative cases serve only for determining the specificity and were not used for calculating the sensitivity. Overall sensitivity, PPV, NPV and accuracy were calculated with both a) all the ZIKV Positive samples and b) only the ZIKV samples collected ≥7 days post symptom onset. Note: Sens., Sensitivity; Spec., Specificity; CI, Confidence Interval; PPV, Positive Predictive Value; NPV, Negative Predictive Value; ZIKV, Zika virus; DENV, Dengue virus; Days, number of days the sample was collected after symptom onset. Indeterminate results were considered negative for the calculation. Non-flavivirus includes measles & rubella, hepatitis and general population samples. DENV (all) includes all DENV samples (DENV 1-4).

Table 3 Sensitivity and specificity for the two anti-DENV antibody ELISAs and the ZIKV PRNT

	IgM DENV Panbio ELISA			IgG DENV Panbio ELISA			ZIKV PRNT		
	Tested (n)	**% Sens. (95%CI)**	**% Spec. (95% CI)**	**Tested (n)**	**% Sens. (95%CI)**	**% Spec. (95% CI)**	**Tested (n)**	**% Sens. (95%CI)**	**% Spec. (95% CI)**
Zika Positive	169	32.5(25.5-39.6)		168	85.7(80.4-91.0)		153	67.3(59.9-74.8)	
Zika (1-6 Days)	79	22.8(15.7-29.8)		78	83.3(75.1-91.6)		65	35.4(23.8-47.0)	
Zika (≥7-13 Days)	41	51.2(44.2-58.3)		41	85.4(74.5-96.2)		40	87.5(77.3-97.7)	
Zika (≥14 Days)	49	32.7(25.6-39.7)		49	89.8(81.3-98.3)		48	93.8(86.9-100.0)	
Zika (≥7 Days)	90	41.1(34.0-48.2)		90	87.8(81.0-94.5)		88	90.9(84.9-96.9)	
Non ZIKV (Flavivirus and Non-flavivirus)	159		42.1(34.5-49.8)	135		28.2(20.6-35.7)	102		96.1 (96.0-96.1)
DENV (all)	90		16.7(9.0-24.4)	88		18.2(10.1-26.2)	71		94.4 (94.3-94.4)
DENV1	21		4.8(0.0-13.9)	21		47.6(26.3-67.0)	14		100 (100-100)
DENV2	17		0(0-0)	16		0(0-0)	12		100 (100-100)
DENV3	21		4.76(0-13.9)	20		15.0(0.0-30.65)	16		75.0 (74.8-75.2)
DENV4	31		41.94(24.6-59.3)	31		9.7(0.0-20.1)	29		100 (100-100)
Yellow fever	19		84.21(67.8-100.6)	18		50.0(26.9-73.1)	15		100 (100-100)
Non-flavivirus	50		72.0(59.6-84.5)	29		44.8(26.7-62.9)	15		100 (100-100)
Measles & Rubella	29		65.5(48.2-82.8)	12		91.7(76.0-107.3)			
Hepatitis	12		66.7(40.0-93.3)	13		15.4(0.0-35.0)	16		100 (100-100)
Other	9		100(100-100)	4		0(0-0)			
Overall (all available samples)	**328**	**32.5(25.5-39.6)**	**42.1(34.5-49.8)**	**303**	**85.7(79.4-90.6)**	**28.2(20.8-36.5)**	**256**	**67.3(59.9-74.8)**	**96.0 (96.0-96.1)**
PPV		37.4(31.7-43.5)			59.8(56.8-62.7)			96.3(90.7-98.5)	
NPV		37.0(32.3-42)			61.3(50.0-71.5)			66.2(60.9-71.2)	
Accuracy		37.2(32.0-42.7)			60.1(54.3-65.6)			78.8(73.3-83.7)	
Overall (≥7 days)	**249**	**41.1(34-48.2)**	**42.1(34.5-49.8)**	**225**	**87.8(82.5-93.1)**	**28.2(20.6-35.7)**	**191**	90.9(84.9-96.9	**96.1 (96.0-96.1)**
PPV (≥7 days)		28.7(20.9-36.5)			44.9(41.7-48.1)			95.2(88.4-98.1)	
NPV (≥7 days)		61.9(53.8-69.9)			77.6(65.1-86.5)			92.4(86.3-96.0)	
Accuracy (≥7 days)		45.9(39.9-51.9)			52.0(45.3-58.7)			93.7(89.2-96.7)	

Table 3 - The sensitivity and specificity of the IgM and IgG Panbio DENV commercial ELISA and ZIKV PRNT with the ZIKV panel (Set 1) and the Control non-ZIKV group (Set 2). Specificity values were calculated for each assay based on Set 2. Data from ZIKV-positive cases served only for determining the sensitivity and was not used for the specificity calculation. Similarly, data from ZIKV-negative cases serve only for determining the specificity and were not used for calculating the sensitivity. Overall sensitivity, PPV, NPV and accuracy were calculated with ZIKV Positive samples collected ≥7 days post symptom onset.

Note: Sens., Sensitivity; Spec., Specificity; CI, Coefficient Interval; PPV, Positive Predictive Value; NPV, Negative Predictive Value; ZIKV, Zika virus; DENV, Dengue virus; Days, number of days the sample was collected after symptom onset. Indeterminate results were considered negative for the calculation. Non-flavivirus includes measles & rubella, hepatitis and general population samples. DENV (all) includes all DENV samples (DENV 1-4). The sensitivity of IgG and IgM ELISAs in correctly detecting DENV samples was >81% (Table S2).

Figure Legends

Figure 1 Zika antibody detected in serum samples collected during the acute (1-6 days after onset), early-convalescent phase (7-13d) and late convalescent-phase (≥14d) from PCR positive Zika cases measured by IgM (A) or IgG (B) NS1 anti-ZIKV ELISAs (Euroimmun). C) IgM NS1 anti-ZIKV ELISA measurements for acute (1-6 days after onset) and convalescent (≥7 days) samples from PCR positive ZIKV cases. D) IgG NS1 anti-ZIKV antibody levels in paired serum samples from PCR positive ZIKV positive cases. Dotted horizontal lines represent the cut-off value used in each assay. Data points above the cut-off are considered positive. Trend-line in C) and D) represent the median antibody levels for acute and convalescent samples. Statistically significant differences between two groups were measured by Mann Whitney U test (*** p=0.0001). Figure shows antibody Ratios* calculated as per manufacturers' instructions (Antibody Ratio = OD Sample/OD Calibrator).

Figure 2 The change in in A) IgM and B) IgG NS1 Euroimmun anti-ZIKV antibody levels between paired serum samples from PCR positive Zika cases by day of collection (days post symptom onset) of the first (acute) sample. Based on the first sample (collected 0 -7 days) and second sample (median interval between samples was 7 days). The highest IgG fold change (change in antibody level between 1st and 2nd samples) was observed among paired samples collected on days 2 and 7 post symptom onset.

Figure 3 Anti-ZIKV antibody levels in sequential serum samples collected from Zika PCR positive cases on different days post symptom onset (0-54 days) measured in A) IgM

555 Euroimmun NS1 and B) IgG Euroimmun NS1 anti-ZIKV ELISAs. Dotted line shows assay cut-
556 offs. The figure shows more consistent detection of anti-Zika antibodies (level above the cut-off)
557 among convalescent samples when measuring IgG compared to IgM. Ratios calculated as per
558 manufacturers' instruction; 1st collection (acute sample [closed circles]); 2nd collection
559 (convalescent samples [open squares]); 3rd collection (late convalescent samples [closed
560 triangles])

561

562 **Figure 4** Correlation between anti-ZIKV and anti-DENV antibody levels in individual sera
563 samples. A) IgG anti-DENV ELISA (Panbio) versus IgG anti-ZIKV NS1 (Euroimmun) ELISA.
564 Anti-DENV and anti-ZIKV IgG antibody levels showed a positive correlation. When a patient
565 exhibited a relatively high anti-DENV IgG antibody response they also tended to exhibit a
566 relatively high anti-ZIKV IgG antibody response ($p<0.001$; $r2=0.258$; $n=168$); B) IgM DENV
567 ELISA and IgM ZIKV NS1 ELISA antibody levels. Again, the measurements showed a positive
568 correlation ($p=0.015$; $r2=0.015$; $n=166$). Dotted lines show assay cut-offs. Dashed line shows the
569 best-fitting line (Spearman rank correlation [r2]). Correlation was more significant between
570 anti-ZIKV and anti-DENV IgG antibody measurements. The correlation in antibody
571 measurement between ZIKV and DENV ELISAs suggests a degree of overlap in patient
572 responses and/or cross-reaction in antibody detection.

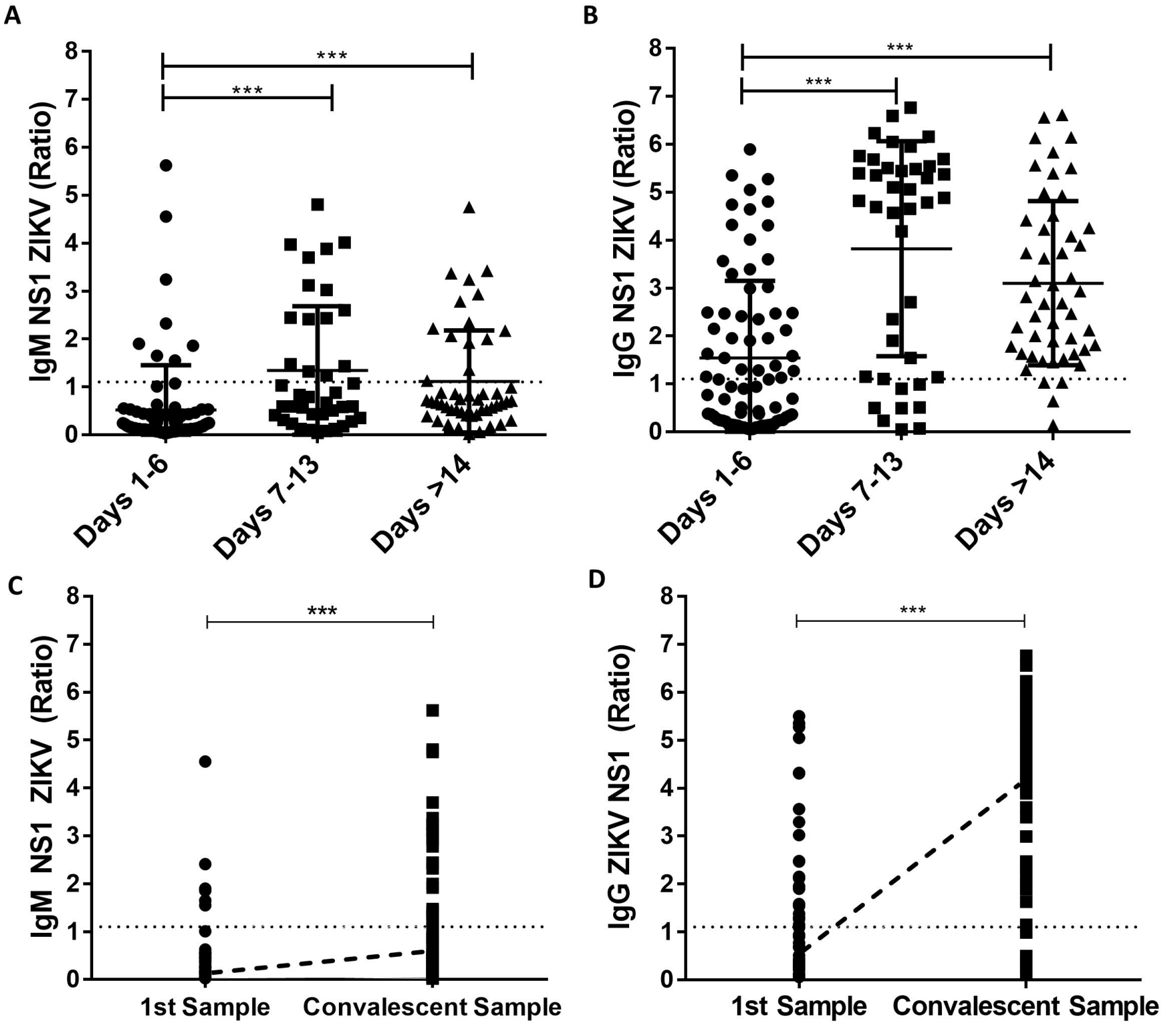
573

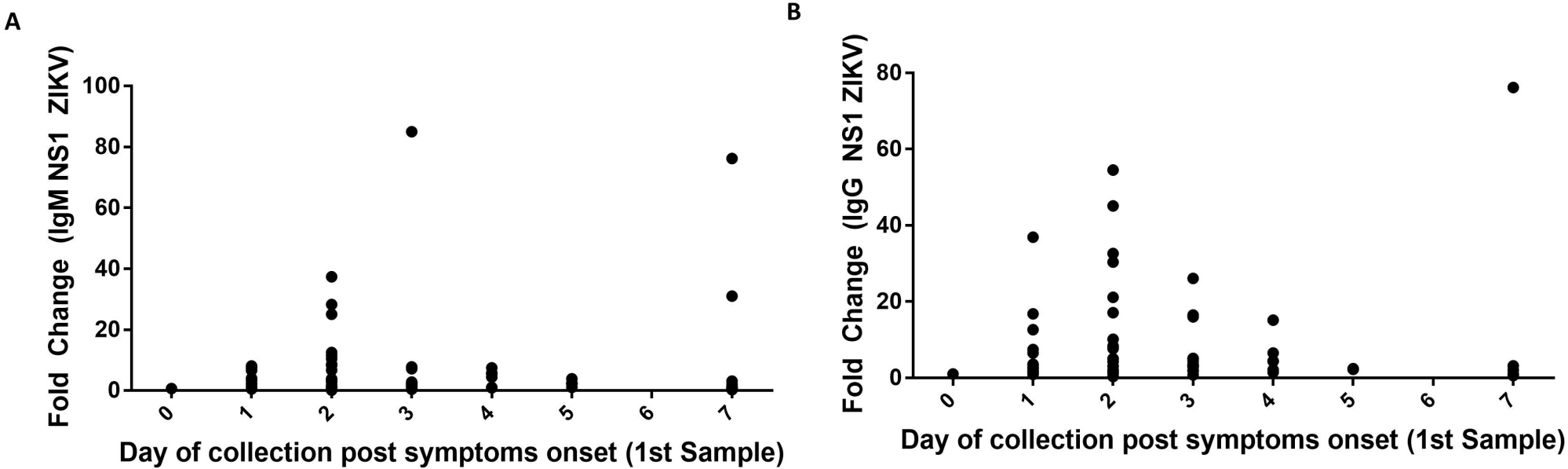
574 **Figure 5.** The change in anti-ZIKV IgM antibody levels by day post symptom onset in
575 sequential sera from ZIKV PCR positive cases (n=4). Plots A-D represent 4 different patients.
576 Each patient had at least five sequential sera samples collected. Anti-ZIKV IgM was measured

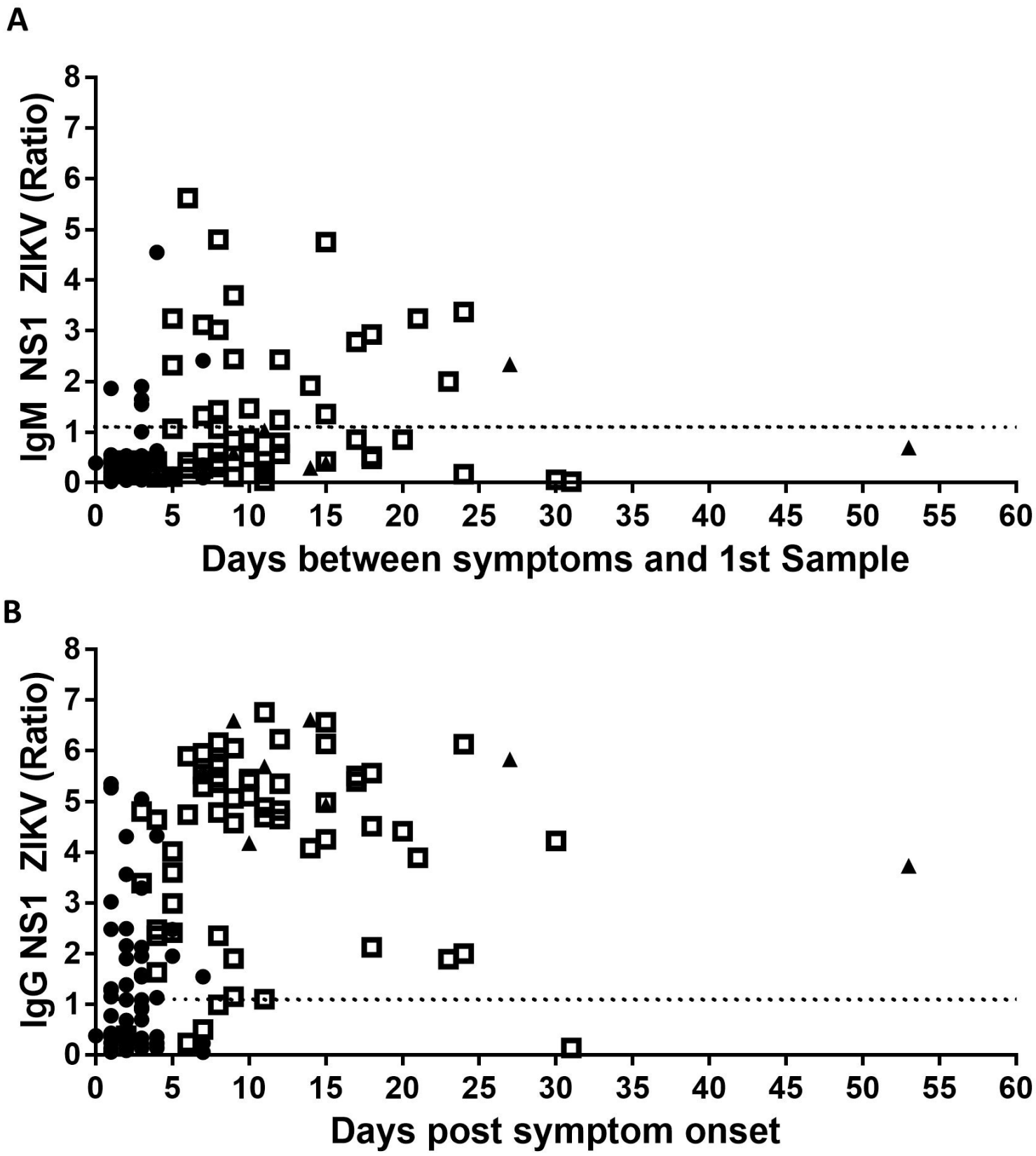
by NS1 (Euroimmun; shown as squares) and μ-capture N (Novagnost; shown as circles) ELISAs. Dotted lines represent cut-off values for each assay. Ratios calculated as recommended by the manufacturers. The plots display a unique pattern of ZIKV IgM antibody response over time for each patient.

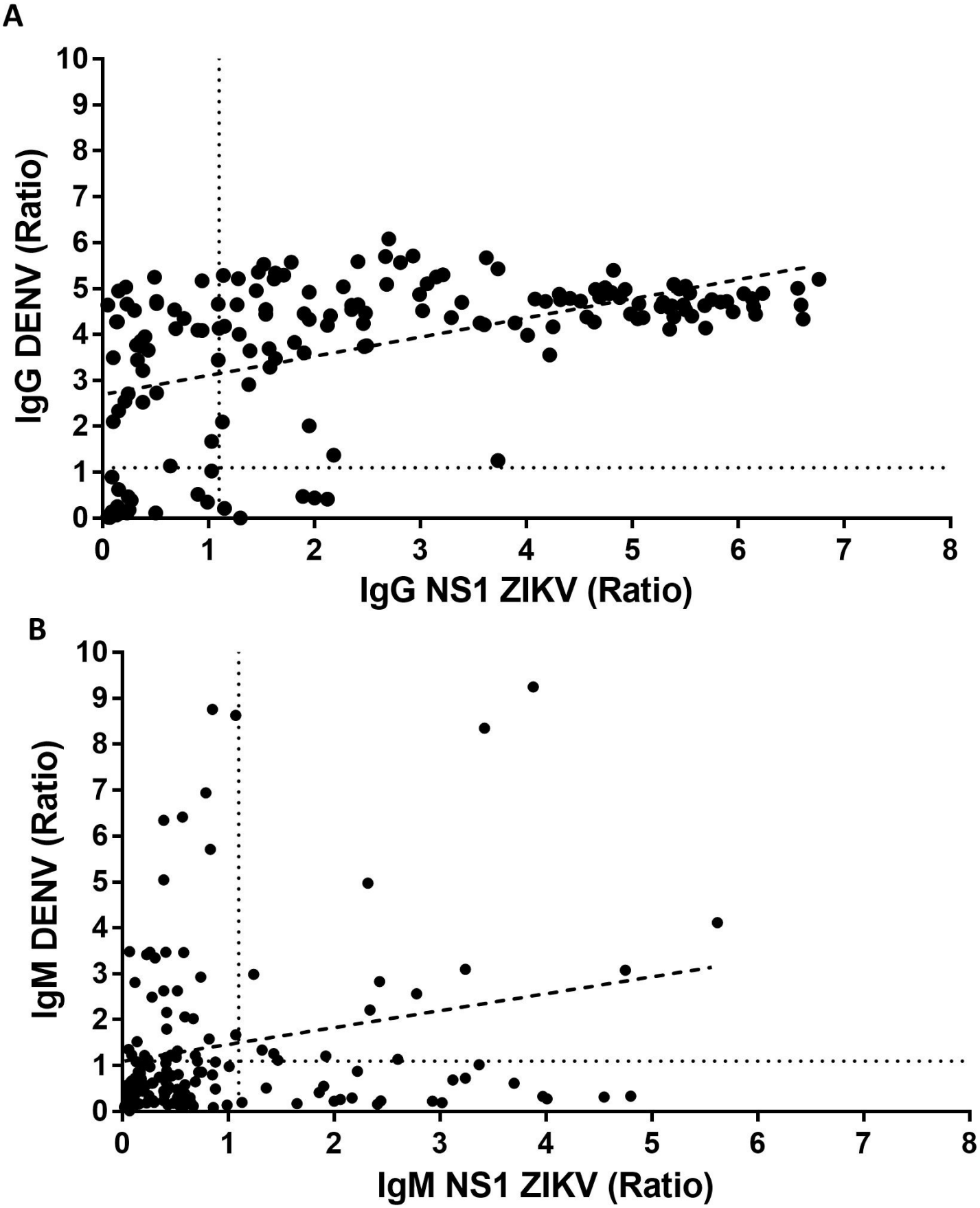
Figure 6 Receiver Operating Characteristic (ROC) curve comparing sensitivity and specificity at different cut-off values for the anti-ZIKV IgG NS1 ELISA (n=294 sera); A) ROC Curve; B) Specificity and Sensitivity at each cut-off. The dotted line in B indicates the cut-off recommended by the manufacturer (ratio of 1.1). The accuracy of IgG NS1 ELISA was 77.9% using the manufacturer's cut-off. Higher accuracy was observed when the cut-off was increased to 1.5 (where the curves intersect on plot B). Using this cut-off, the ELISA had an accuracy of 81.0%. Sensitivity and specificity were 78.9 and 82.2% respectively.

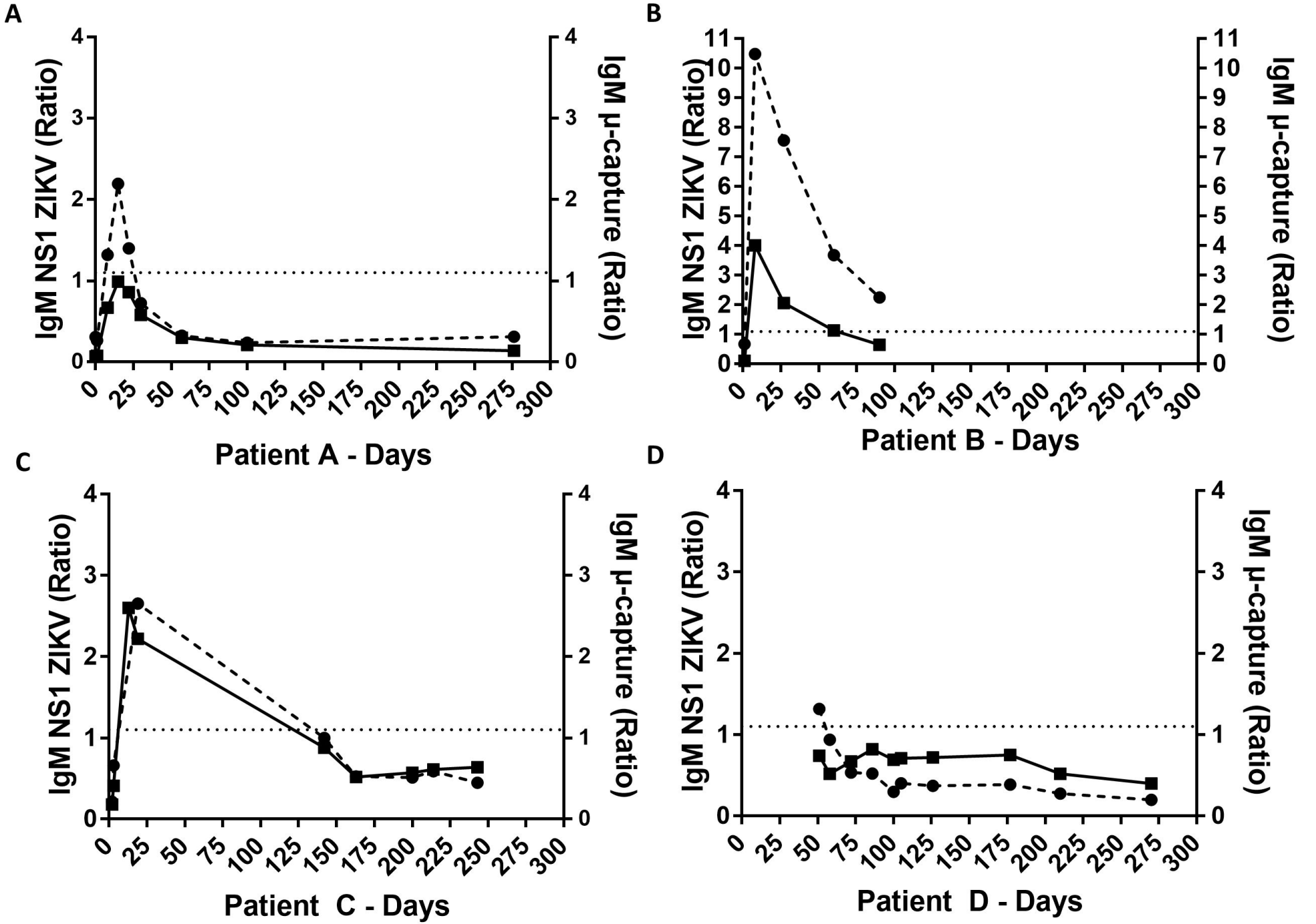
Figure 7 Diagram representing the different patterns of anti-viral IgG and IgM antibody responses and viral RNA detection observed in sera from flavivirus infected individuals over days from symptom onset among (A) virus naïve and (B) previously exposed individuals. The cartoon exhibits a more prominent IgG response compared to IgM among individuals previously exposed to the virus. In our current study, we observed a more prominent anti-ZIKV IgG compared to IgM response (see Figure 3**).**

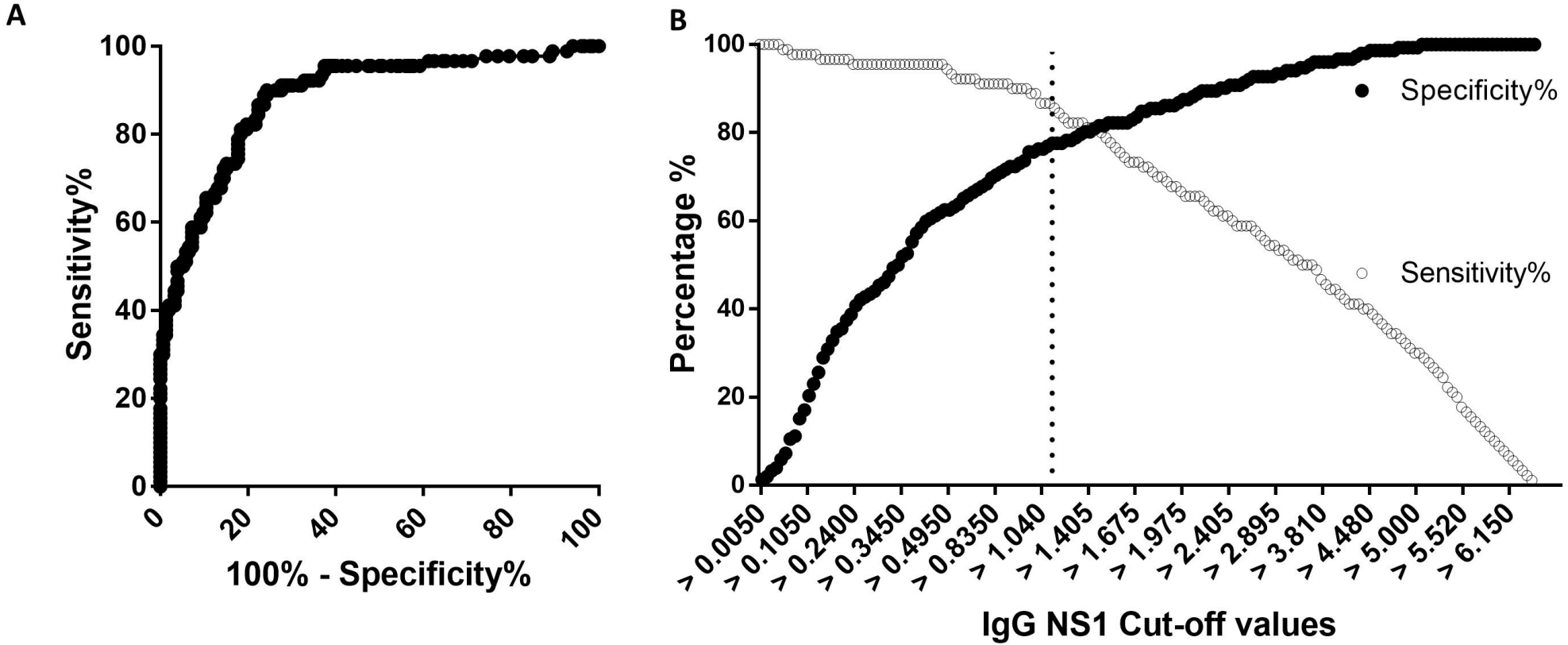

A
IgM NS1 ZIKV (Ratio)
***
***
Days 1-6
Days 7-13
Days >14
B
IgG NS1 ZIKV (Ratio)
***
***
Days 1-6
Days 7-13
Days >14
C
IgM NS1 ZIKV (Ratio)
***
1st Sample
Convalescent Sample
D
IgG ZIKV NS1 (Ratio)
***
1st Sample
Convalescent Sample

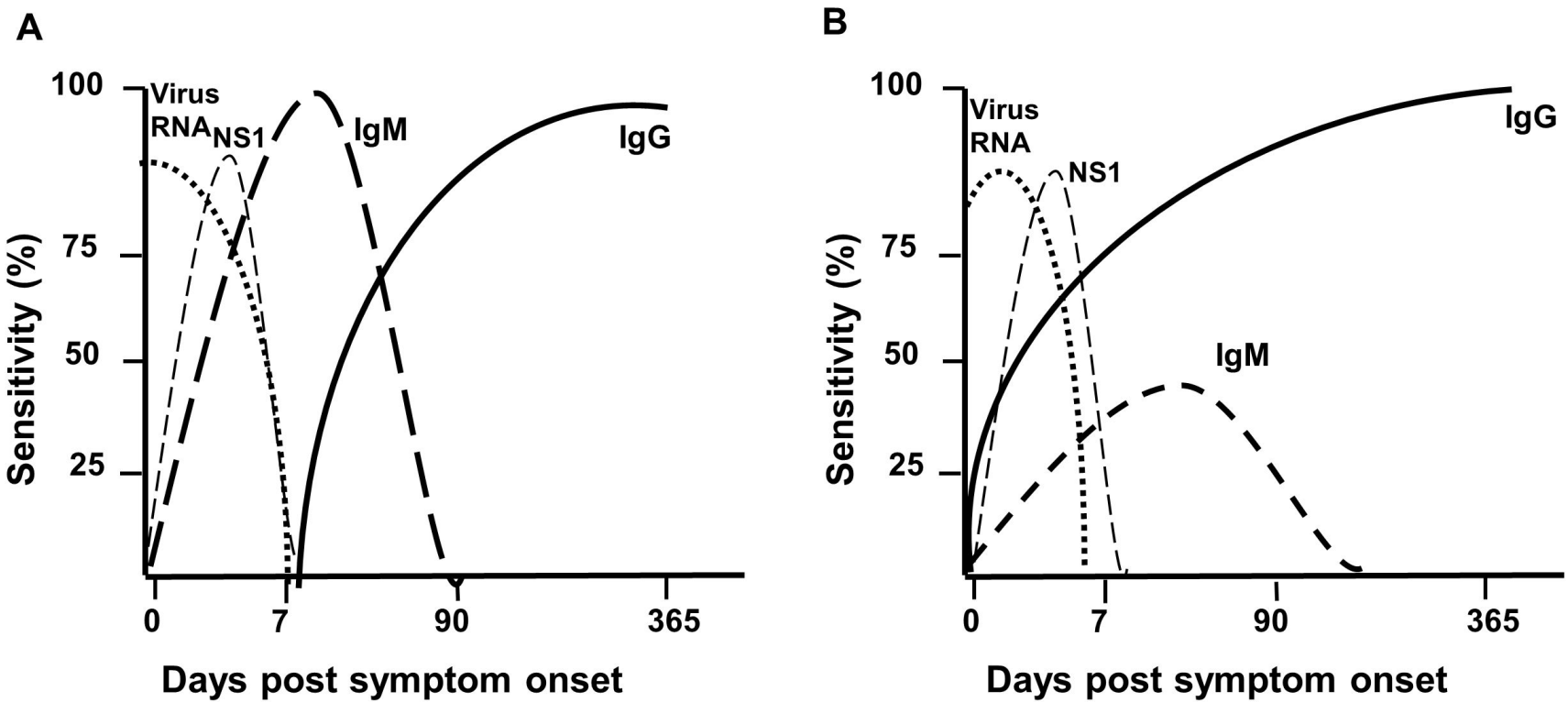

A
Fold Change (IgM NS1 ZIKV)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Day of collection post symptoms onset (1st Sample)
B
Fold Change (IgG NS1 ZIKV)
80
60
40
20
0
0
1
2
3
4
5
6
7
Day of collection post symptoms onset (1st Sample)


A
IgM NS1 ZIKV (Ratio)
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
30
35
40
45
50
55
60
Days between symptoms and 1st Sample
B
IgG NS1 ZIKV (Ratio)
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
30
35
40
45
50
55
60
Days post symptom onset


A
IgG DENV (Ratio)
IgG NS1 ZIKV (Ratio)
B
IgM DENV (Ratio)
IgM NS1 ZIKV (Ratio)


A
IgM NS1 ZIKV (Ratio)
IgM μ-capture (Ratio)
Patient A - Days
B
IgM NS1 ZIKV (Ratio)
IgM μ-capture (Ratio)
Patient B - Days
C
IgM NS1 ZIKV (Ratio)
IgM μ-capture (Ratio)
Patient C - Days
D
IgM NS1 ZIKV (Ratio)
IgM μ-capture (Ratio)
Patient D - Days


A
Sensitivity%
100
80
60
40
20
0
0
20
40
60
80
100
100% - Specificity%
B
Percentage %
Specificity%
Sensitivity%
> 0.0050
> 0.1050
> 0.2400
> 0.3450
> 0.4950
> 0.8350
> 1.040
> 1.405
> 1.675
> 1.975
> 2.405
> 2.895
> 3.810
> 4.480
> 5.000
> 5.520
> 6.150
IgG NS1 Cut-off values


A
100
75
50
25
Sensitivity (%)
Virus
RNA
NS1
IgM
IgG
0
7
90
365
Days post symptom onset
B
100
75
50
25
Sensitivity (%)
Virus
RNA
NS1
IgM
IgG
0
7
90
365
Days post symptom onset