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Added value of IgA antibodies against Zika virus non-structural protein 1 in the diagnosis of acute Zika virus infections

Jens M. Warnecke*, Erik Lattwein, Sandra Saschenbrecker, Winfried Stöcker, Wolfgang Schlumberger, Katja Steinhagen

Institute for Experimental Immunology, Euroimmun AG, Seekamp 31, D-23560, Lübeck, Germany

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ABSTRACT

Zika virus (ZIKV) is a mosquito-borne flavivirus posing a public health threat due to its association with neurological complications in newborns and adults. In flavivirus-endemic areas, coming mosquito seasons will require the differentiation of primary versus secondary and acute versus past ZIKV/flavivirus infections. This is complicated by two major difficulties: [i] secondary infections often present with low or undetectable titres of specific IgM and with early-positive IgG, [ii] previous flavivirus infection(s) or vaccinations cause elevated cross-reactivities. Here, we analysed the anti-ZIKV IgA, IgG, and IgM responses at different stages of infection in an endemic setting, scrutinising the diagnostic relevance of specific IgA. Anti-ZIKV antibodies were measured by ELISA based on ZIKV non-structural protein 1 (NS1) in paired sera from 31 patients with suspected primary or (flavivirus-primed) secondary ZIKV infection. The control panel comprised samples from 136 DENV-infected patients. Among ZIKV samples collected 8–16 days after symptom onset, ELISA sensitivities for detecting anti-ZIKV NS1 IgA, IgG, and IgM were 93.5%, 100%, and 48.4%, respectively. The proportion of cases with negative IgM but positive IgA was higher in suspected secondary (61.9%) than in primary (30.0%) ZIKV infections. Combined IgA/IgM detection yielded a sensitivity of 100% at a specificity of 97.1%. In conclusion, at time points after PCR can detect the virus, the determination of anti-ZIKV NS1 IgA may improve the accuracy in diagnosing acute ZIKV infection in flavivirus-endemic regions in the context of both primary and secondary infection, especially when IgM is undetectable.

1. Introduction

Since the 2007 outbreak of Zika virus (ZIKV) on Yap Island, ZIKV epidemics have occurred throughout the South Pacific Islands and the Americas (Baud et al., 2017). Although a decline in ZIKV cases and associated disorders has been reported since the end of 2016, ZIKV has re-emerged recently at Jaipur in Rajasthan (India) and remains a severe public health concern (Pan American Health Organization, World Health Organization, 2017; Saxena et al., 2018; World Health Organization, 2017). ZIKV is an enveloped positive-sense RNA virus of the *Flaviviridae* family, which also includes dengue (DENV), Japanese encephalitis (JEV), yellow fever (YFV), and West Nile virus (WNV). It is primarily transmitted via bites of *Aedes* spp. mosquitos, but also through sexual exposure, blood transfusion, and mother-to-child transmission (Baud et al., 2017). ZIKV infection can be inapparent or may result in clinical disease which is usually mild and self-limiting with symptoms such as fever, rash, myalgia, arthralgia, and conjunctivitis. However, there is compelling evidence linking ZIKV to Guillain-Barré syndrome amongst adults and "congenital Zika syndrome" (Cao-Lormeau et al., 2016; Rasmussen et al., 2016). The clinical manifestations of ZIKV fever are often indistinguishable from that caused by co-endemic viruses, especially DENV and chikungunya virus (CHIKV). Therefore, and given the differences in disease course and outcome (e.g., ZIKV teratological sequelae, DENV hemorrhage, CHIKV persistent arthralgia), specific laboratory diagnostics are required to correctly distinguish between the infectious agents and to provide adequate clinical management. This is of exceptional importance as regards the detection of acute ZIKV infection in pregnant women

Abbreviations: CDC, Centers for Disease Control and Prevention; CI, confidence interval; DENV, dengue virus; dpso, days post symptom onset; ELISA, enzyme-linked immunosorbent assay; FFNT, fluorescent focus neutralisation test; IFA, indirect immunofluorescence assay; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; JEV, Japanese encephalitis virus; MAC, IgM antibody capture; NS1, non-structural protein 1; PRNT, plaque reduction neutralisation test; RT-PCR, reverse transcription-polymerase chain reaction; WNV, West Nile virus; YFV, yellow fever virus; ZIKV, Zika virus

* Corresponding author.
E-mail address: j.warnecke@euroimmun.de (J.M. Warnecke).

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because the gestational stage of maternal infection may affect the severity of fetal outcome (Brasil et al., 2016; Szaba et al., 2018).

Selection of the appropriate diagnostic assay depends on the time point of specimen collection and the clinical scenario (Landry and St.George, 2017). During viraemia, direct virus detection is accomplished using reverse transcription-polymerase chain reaction (RT-PCR). However, as acute viraemia resolves usually within the first week of illness and as many patients show only mild symptoms, RT-PCR is often negative by the time a patient seeks medical consultation. In semen, urine, or saliva, viral RNA may be detectable for a longer period (Bingham et al., 2016). A negative RT-PCR result does not exclude infection and should be followed by detection of virus-specific antibodies as of four days post symptom onset (dpso) (Centers for Disease Control and Prevention, 2016). Interpretation of the ZIKV immune response, however, is complicated especially in flavivirus-endemic settings given the peculiarities of secondary infections and due to cross-reactivity resulting from structural homology among flaviviruses (Rabe et al., 2016).

In primary flavivirus infections, specific IgM antibodies become detectable within the first week of illness, typically persisting for several weeks. This is rapidly followed by the development of specific IgG antibodies, which constitute the major share of neutralising antibodies and persist for years. By contrast, in secondary infections specific IgM is often found at low or undetectable titres, while the synthesis of specific IgG is immediately stimulated, as is a boost in antibodies against the first infecting flavivirus ("original antigenic sin"). Shortly after secondary exposure, the high IgG titre then levels off and is indistinguishable from IgG titres in convalescent infections (Halstead et al., 1983; Rabe et al., 2016; Sa-Ngasang et al., 2006). Knowing whether a patient contracted a primary or secondary flavivirus infection is crucial for patient management, as preexisting DENV immunity may enhance ZIKV infection and vice versa ("antibody-dependent enhancement") (Bardina et al., 2017; Kawiecki and Christofferson, 2016).

The problem of cross-reactivity applies in particular to areas where a significant portion of the population harbours DENV-reactive antibodies or has been vaccinated against YFV or JEV (Rabe et al., 2016). Serologically, cross-reactive and cross-neutralising antibodies bear the risk of false-positive test results and misdiagnosis, but clinically they may confer cross-protection (Priyamvada et al., 2016; Swanstrom et al., 2016). Compared to primary ZIKV infections, secondary (i.e., flavivirus-primed) ZIKV infections are associated with more extensive cross-reactivity of IgM and of neutralising antibodies (Lanciotti et al., 2008). Due to cross-reactivity, serological assays utilising inactivated whole-virus antigen, such as the CDC Zika MAC-ELISA, cannot reliably distinguish between ZIKV and DENV infections, requiring the validation by plaque reduction neutralisation testing (PRNT) of samples with positive, equivocal, or inconclusive MAC-ELISA results (Rabe et al., 2016). PRNT is the current gold standard to confirm and specify flavivirus antibodies, and it is commonly able to identify the infectious agent in primary flavivirus infection. However, for the reasons described above, PRNT may fail to establish a definite diagnosis in secondary infections (Centers for Disease Control and Prevention, 2016; Rabe et al., 2016). Furthermore, it is time-consuming, labor intensive, unavailable in many laboratories, and therefore of limited use for routine diagnostics. Assays based on ZIKV non-structural protein 1 (NS1) have proven to be highly specific (Cleton et al., 2015; Steinhagen et al., 2016a), but their usefulness in flavivirus-endemic regions is under debate, mainly because data on IgM sensitivity contradict utility for initial screening (L'Huillier et al., 2017).

As many inhabitants of flavivirus-endemic regions now have a history of ZIKV and/or other flavivirus (e.g., DENV) infections, the coming mosquito seasons will require reliable identification and differentiation of secondary flavivirus infections by means of highly sensitive and specific ZIKV serology. A solution to the aforementioned problems could be derived from recent reports suggesting that anti-ZIKV IgA is helpful in the diagnosis of ZIKV patients (Bozza et al., 2019; Steinhagen et al., 2016b; Zhang et al., 2017; Zhao et al., 2018). For DENV infections, the diagnostic value of virus-specific IgA has been thoroughly investigated (Balmaseda et al., 2003; Groen et al., 1999). In the present study, we examined the prevalence and kinetics of ZIKV IgA, IgG, and IgM antibodies in the early viraemic and acute stage of RT-PCR-confirmed ZIKV cases, and we scrutinised the relevance of IgA in the diagnosis of acute ZIKV infection.

2. Methods

2.1. Human serum samples

We studied sera (purchased from BocaBiolistics, Pompano Beach, FL, USA) from 31 patients (five males, 26 females of whom ten were pregnant; mean age 35y; age range 25-65y) collected between April and July 2016. These patients were residents of the Dominican Republic and experienced signs and symptoms of arboviral infection. From each patient, two serum samples were collected: early viraemic (2–5 dpso) and acute (8–16 dpso). All early viraemic samples were RT-PCR-positive for ZIKV (LightMix Modular Zika Virus Assay, TIB MOLBIOL, Berlin, Germany), but RT-PCR-negative for DENV and CHIKV (Altona Diagnostics, Hamburg, Germany).

For longitudinal analysis, serum samples were taken at several time points from three Colombian patients (1 male, 2 females; mean age 39y; age range 18–51y) and five Dominican patients (1 male, 4 females; mean age 37y; age range 29–52y) with ZIKV infection confirmed by RT-PCR (Trioplex Real-time RT-PCR Assay, CDC, Atlanta, GA, USA; LightMix Modular Zika Virus Assay). Samples were purchased from Allied Research Society, Inc. (Miami, FL, USA), Biomex GmbH (Heidelberg, Germany), and BocaBiolistics.

The control panel (purchased from SeraDiaLogistics, Munich, Germany) comprised 139 sera from 95 DENV-infected Vietnamese patients (50 males, 45 females; mean age 35y; age range 6–63y) that had been hospitalised with mild to severe fever symptoms. Serum samples were collected 1–19 dpso and classified into three categories: viraemic (1–7 dpso, n = 105), acute (8–16 dpso, n = 22) and late acute (17–19 dpso, n = 12). All control cases were diagnosed using DENV RT-PCR, or DENV NS1 antigen detection (SD BIOLINE Dengue Duo, Panbio Dengue Early ELISA, Alere Inc., Waltham, MA, USA), and serological analyses (Serion ELISA classic Dengue Virus IgG/IgM, Virion/Serion, Würzburg, Germany; Anti-Dengue Virus ELISA IgA/IgG/IgM, Euroimmun, Lübeck, Germany; SD BIOLINE Dengue Duo).

Sera were stored at −20 °C. Serological analyses were performed blinded to clinical data. Individual and ethical approval was not mandatory for this study as samples were used anonymously.

2.2. Enzyme-linked immunosorbent assay (ELISA)

Anti-Zika Virus IgA, IgG, and IgM ELISA kits (Euroimmun) were applied according to the manufacturer's instructions. These assays are based on microplates coated with recombinant ZIKV NS1 antigen (Steinhagen et al., 2016a). Ratio values ($extinction_{sample}$/$extinction_{calibrator}$) < 0.8 were considered negative, ≥0.8 to < 1.1 borderline, and ≥1.1 positive.

2.3. Indirect immunofluorescence assay (IFA)

The Arbovirus Profile 3 IgM (Euroimmun) enables simultaneous detection of IgM antibodies against ZIKV, DENV 1-4, CHIKV, WNV, JEV, YFV, and tick-borne encephalitis virus. The microscope slides provide two reaction fields per analysis, containing a total of ten biochips, each one coated with cells infected with a different virus. The assay was performed according to the manufacturer's instructions. Antibody titres were determined based on a dilution series starting from

Table 1
Sensitivity of anti-ZIKV NS1 IgA, IgG, and IgM detection using ELISA in 31 Dominican patients with ZIKV infection confirmed by RT-PCR.

Suspected ZIKV infection status	Patient	2-5 days post symptom onset				8-16 days post symptom onset				
		Anti-ZIKV-NS1 ELISA (ratio)[a]				Anti-ZIKV-NS1 ELISA (ratio)[a]				ZIKV NT (reciprocal titre)[b]
		IgA	IgG	IgM	IgA/IgM	IgA	IgG	IgM	IgA/IgM	
Primary	1	0.0	0.4	0.1	neg	0.7	**4.9**	**1.4**	**pos**	≥ **2650**
(n = 10)	2	0.1	0.1	0.1	neg	**4.0**	**4.4**	**1.2**	**pos**	≥ **2650**
	3	0.1	0.1	0.0	neg	**5.1**	**3.4**	**1.7**	**pos**	≥ **2650**
	4	0.0	0.3	0.1	neg	0.7	**4.8**	**1.3**	**pos**	≥ **2650**
	5	0.1	0.7	0.7	neg	**2.1**	**4.8**	***1.0***	**pos**	≥ **2650**
	6	0.1	0.7	0.1	neg	**4.3**	**5.0**	0.6	**pos**	≥ **2650**
	7	0.1	0.2	0.0	neg	**3.2**	**4.8**	0.2	**pos**	≥ **2650**
	8	0.1	0.7	0.0	neg	**7.0**	**5.6**	**2.1**	**pos**	≥ **2650**
	9	0.1	0.6	0.1	neg	**2.0**	**5.3**	0.6	**pos**	≥ **2650**
	10	0.1	0.3	0.2	neg	**2.3**	**4.4**	***0.9***	**pos**	≥ **2650**
Secondary	11	0.7	**1.4**	0.1	neg	**6.2**	**5.1**	0.7	**pos**	**1280**
(n = 21)	12	0.3	**2.4**	0.0	neg	**5.1**	**5.9**	**1.5**	**pos**	≥ **2560**
	13	0.2	**1.8**	0.1	neg	**3.6**	**5.6**	***0.9***	**pos**	≥ **2560**
	14	0.1	***0.8***	0.2	neg	**3.2**	**5.3**	**2.5**	**pos**	**40960**
	15	0.1	**1.1**	0.1	neg	**5.5**	**5.1**	0.3	**pos**	≥ **2560**
	16	0.2	**1.4**	0.0	neg	**1.2**	**4.3**	0.2	**pos**	≥ **81920**
	17	0.1	**1.1**	0.0	neg	**2.7**	**5.1**	***0.8***	**pos**	≥ **2560**
	18	0.3	**1.5**	0.1	neg	**7.9**	**5.1**	***0.9***	**pos**	≥ **2560**
	19	0.1	**1.2**	0.1	neg	**3.9**	**5.5**	***1.0***	**pos**	≥ **2560**
	20	0.0	***0.9***	0.0	neg	**4.2**	**5.2**	0.1	**pos**	≥ **2560**
	21	0.1	**1.1**	0.0	neg	**1.8**	**5.8**	0.3	**pos**	≥ **2560**
	22	0.7	**3.8**	0.4	neg	**4.2**	**5.0**	**1.4**	**pos**	≥ **2560**
	23	0.1	**1.1**	0.1	neg	**7.3**	**5.6**	0.7	**pos**	≥ **2560**
	24	0.1	**2.2**	0.0	neg	**3.7**	**5.5**	0.7	**pos**	≥ **2560**
	25	0.2	**1.5**	0.1	neg	**3.8**	**5.1**	**1.2**	**pos**	**10240**
	26	0.1	**3.1**	0.1	neg	**1.8**	**5.9**	0.6	**pos**	≥ **2560**
	27	0.2	**1.2**	0.0	neg	**3.1**	**5.1**	0.1	**pos**	≥ **2560**
	28	0.1	**2.1**	0.2	neg	**2.1**	**5.6**	0.4	**pos**	≥ **2560**
	29	0.1	**1.9**	0.0	neg	**3.0**	**5.6**	0.4	**pos**	≥ **2560**
	30	0.1	***1.0***	0.0	neg	**2.6**	**5.3**	0.4	**pos**	≥ **2560**
	31	0.1	***1.0***	0.0	neg	**1.7**	**4.4**	0.6	**pos**	≥ **2560**
Sensitivity[b]										
Total	1-31	0/31	21/31	0/31	0/31	29/31	31/31	15/31	31/31	31/31
(95% CI)		0.0% (0.0-13.1%)	67.7% (50.0-81.5%)	0.0% (0.0-13.1%)	0.0% (0.0-13.1%)	93.5% (78.3-99.2%)	100% (86.9-100%)	48.4% (32.0-65.2%)	100% (86.9-100%)	100% (86.9-100%)
Primary	1-10	0/10	0/10	0/10	0/10	8/10	10/10	7/10	10/10	10/10
(95% CI)		0.0% (0.0-32.1%)	0.0% (0.0-32.1%)	0.0% (0.0-32.1%)	0.0% (0.0-32.1%)	80.0% (47.9-95.4%)	100% (67.9-100%)	70.0% (39.2-89.7%)	100% (67.9-100%)	100% (67.9-100%)
Secondary	11-31	0/21	21/21	0/21	0/21	21/21	21/21	8/21	21/21	21/21
(95% CI)		0.0% (0.0-18.2%)	100% (81.8-100%)	0.0% (0.0-18.2%)	0.0% (0.0-18.2%)	100% (81.8-100%)	100% (81.8-100%)	38.1% (20.7-59.2%)	100% (81.8-100%)	100% (81.8-100%)

CI: confidence interval; ELISA: enzyme-linked immunosorbent assay; neg: negative; NS1: non-structural protein 1; NT: neutralisation test; pos: positive; ZIKV: Zika virus.

[a] Positive results are highlighted in bold, borderline results are indicated in bold italics.

[b] For the calculation of sensitivity, borderline results were considered positive.

1:10. IgM titres ≥1:10 were considered positive. For samples showing reactivity with more than one virus, endpoint titration was implemented to determine the infectious agent.

2.4. Neutralisation test

ZIKV neutralising antibodies were detected using an in-house fluorescent focus neutralisation test (FFNT). In brief, sera were heat-inactivated at 56 °C for 30 min, diluted in culture medium in a geometric series (1:20 to ≥1:2560) and pipetted in triplicate into 96-well microplates. A suspension (10^3 infectious particles/milliliter) of ZIKV strain MR 766 was mixed with each diluted sample and incubated at 37 °C for 60 min. Vero E6 cells (ATCC, Bethesda, MD, USA) were then added and incubated for 48 h (37 °C, 5% CO_2). Known anti-ZIKV antibody positive sera were used as positive control. In addition, identical amounts of uninfected Vero cells or cells with ZIKV but without sample (negative control) were incubated in parallel. After acetone fixation, an antibody raised against recombinant ZIKV NS1 (see Section 2.5.) and a fluorescein isothiocyanate-labelled secondary antibody were allowed to react. Evaluation was performed using an inverted fluorescence microscope. After triplicate determinations, the titre of ZIKV-neutralising antibodies was defined as the sample dilution where at least two wells showed no infection focus. Titres ≥1:20 were considered positive. For example, no visible specific fluorescence in all dilutions corresponded to a titre ≥1:2560 of neutralising antibodies in the patient sample. Infection foci with a specific fluorescence in all wells indicated the

absence of neutralising antibodies (titre < 1:20).

2.5. Generation of chimeric antibodies against ZIKV NS1

Chimeric anti-ZIKV NS1 was obtained in-house by immunising three chickens with recombinant ZIKV NS1. Immunised chickens were sacrificed to gather the spleens. Shock frozen organs were homogenised in a ball mill, followed by RNA extraction with a NucleoSpin RNAII-Kit (Macherey-Nagel, Dueren, Germany) and cDNA synthesis with a Protoscript II Reverse Transcriptase (NEB, Ipswich, USA). VH and VL cDNA was amplified by PCR using primers derived from van Wyngaardt et al. (van Wyngaardt et al., 2004), and used to generate M13 phage scFv expression libraries. Isolation of NS1-specific clones was carried out by two rounds of selection employing antigen-coated NUNC MaxiSorp™ wells with extensive wash steps and elution by tryptic digestion. The chicken-derived scFv region of NS1-specific phage clones was sequenced and used to generate chimeric IgG comprising human C lambda and CH1 − CH3 domains.

2.6. Statistics

Statistics were performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA) and SigmaPlot 13.0 (SSI, San Jose, CA, USA). Sensitivity was calculated as the proportion of ZIKV samples identified as positive/borderline by the respective assay. Specificity was calculated as the proportion of negative results among control samples. Confidence intervals (95% CI) were calculated according to the modified Wald method. Differences between the medians of two groups were analysed by Mann-Whitney rank sum test with P-values < 0.05 considered significant.

3. Results

3.1. ELISA sensitivities for the detection IgA, IgG, and IgM against ZIKV NS1

Anti-ZIKV NS1 antibodies were determined by ELISA in paired serum samples from 31 patients with RT-PCR-confirmed ZIKV infection. All of the 31 initial samples (2–5 dpso) were negative for anti-ZIKV NS1 IgA and IgM, whereas IgG was detected in 21/31 cases, corresponding to a sensitivity of 67.7%. Among follow-up samples (8–16 dpso), the ELISAs had sensitivities of 93.5% (29/31), 100% (31/31), and 48.4% (15/31) for the detection of IgA, IgG, and IgM, respectively. The presence of neutralising antibodies was confirmed by positive FFNT titres in the follow-up sample of each patient (Table 1).

3.2. Comparison of IgM reactivity against ZIKV NS1 versus antigens presented by ZIKV-infected cells

To find out whether the 16 anti-ZIKV NS1 IgM negative patients had antibodies reactive with other ZIKV antigens, the samples (8–16 dpso) were analysed with a multiparametric IgM IFA mosaic, which provides a broader antigen spectrum of different arboviruses. 12/31 (38.7%) samples were exclusively reactive with ZIKV-infected cells at titres between 1:10 and ≥1:320, seven of which were also positive or borderline by NS1-based IgM ELISA (mean ratio 1.3). 17/31 (54.8%) samples contained polyspecific antibodies against different arboviruses, including eight cases (25.8%) where endpoint titration allowed identification of ZIKV as the infectious agent (≥1:320). Out of these 17 samples, seven were reactive in NS1-based IgM ELISA (mean ratio 1.4), but ten were negative (mean ratio 0.5). Overall, IgM testing by means of virus-infected cells confirmed acute ZIKV infection in 20/31 patients, either directly (38.7%) or via endpoint titration (25.8%), resulting in a sensitivity of 64.5%. Nine patients were classified as having acute flavivirus infection without further differentiation, and two patients remained IgM negative (Table 2).

3.3. Seroconversion in suspected primary and secondary flavivirus infections with ZIKV

Based on descriptions for DENV (Cordeiro et al., 2007) which are also very likely to also apply to other flavivuruses, secondary infection can be assumed if virus-specific IgG is present in the early serum sample together with a positive RT-PCR, while IgM becomes positive at a later stage or, occasionally, remains undetectable. Pursuant to these criteria, 21 patients were classified as having suspected (flavivirus-primed) secondary ZIKV infection. In these patients' follow-up samples, seroconversion for IgA was demonstrated in all (100%) and for IgM in 8/21 (38.1%) cases. The remaining ten patients were assigned to the group of suspected primary ZIKV infection based on positive ZIKV RT-PCR in the absence of ZIKV-specific antibodies in the early viraemic samples. Within two weeks, seroconversion of IgA, IgG, and IgM was detectable in eight (80.0%), ten (100%), and seven (70.0%) cases, respectively (Table 1, Fig. 1).

3.4. Added value of combined anti-ZIKV NS1 IgA/IgM detection

16/31 (51.6%) samples collected 8–16 dpso were anti-ZIKV NS1 IgM negative, and all of them were positive for anti-ZIKV NS1 IgA. Among the IgM-reactive samples, 13/15 (86.7%) were IgA positive and 2/15 (13.3%) IgA negative. Combined IgA/IgM testing resulted in a sensitivity of 100%, increasing the detection rate of acute ZIKV infection in the serological time window by 30.0% (primary infection), 61.9% (secondary infection) and 51.6% (total) compared to the detection of IgM only (Table 1). Time course analysis of anti-ZIKV NS1 IgA in eight patients revealed, in the majority of cases, an increase in IgA titres in the acute and subacute phase of infection followed by a relatively fast decline to low positive or negative levels. Inter-individual variability in antibody kinetics are exemplified in Figure S1.

3.5. Specificity of anti-ZIKV NS1 IgA and IgM detection

We focused on potential cross-reactivity with DENV antibodies, the most prevalent flavivirus antibodies in ZIKV-endemic areas. Among 139 samples from DENV-infected patients, anti-ZIKV NS1 reactivity was below the cut-off in 132 (IgA) and 135 (IgM) cases, corresponding to a specificity of 95.0% (IgA) and 97.1% (IgM). There was no overlap between anti-ZIKV IgA and IgM positive individuals (Supplemental Table 1). In the subset of samples obtained in the acute phase (8–16 dpso), where serological testing is most commonly used for diagnosing ZIKV infections, specificity amounted to 95.5% (IgA) and 100% (IgM). Referring to only those control samples with positive anti-DENV IgA or IgM reactivity (8–16 dpso), specificity was 93.3% (IgA) and 100% (IgM) (Table 3). IgA and IgM antibody levels in the control group were significantly lower than in ZIKV patients (Fig. 2).

4. Discussion

We here provide evidence that ELISA-based measurement of ZIKV-specific IgA (in addition to IgG and IgM) supports the diagnosis of acute ZIKV infection. To this end, we compared the diagnostic efficiency of anti-ZIKV NS1 IgA, IgG, and IgM testing in Dominican patients with RT-PCR-confirmed ZIKV infection.

For the detection of IgM, the classical marker of acute infection, the use of virus-infected cells (IFA) yielded 93.6% sensitivity for the total of ZIKV-specific and polyspecific antibodies. In contrast, the NS1-based ZIKV IgM ELISA provided a sensitivity of 48.4%. In combination with anti-ZIKV NS1 IgA testing, ELISA sensitivity was increased to 100% at a specificity > 97%, and this increase was more pronounced in secondary than in primary infections. Among the ZIKV cases, 16 were IgA positive/IgM negative by NS1-based ZIKV ELISA, two were IgA negative/IgM positive, and 13 were IgA positive/IgM positive. Consequently, IgA and IgM represent mutually complementing markers of acute ZIKV

Table 2
Comparison of IgM reactivity (8–16 dpso) against ZIKV NS1 (ELISA) versus virus-infected cells (IFA) in 31 Dominican patients with ZIKV infection confirmed by RT-PCR.

Suspected ZIKV infection status	Patient	Anti-ZIKV NS1 ELISA IgM (ratio)[a]	IFA mosaic: Arbovirus Profile 3 IgM (reciprocal titre)										
			ZIKV	DENV1	DENV2	DENV3	DENV4	CHIKV	WNV	JEV	YFV	TBEV	Result IFA
Primary (n = 10)	1	**1.4**	**≥ 320**	0	0	0	0	0	**100**	0	0	0	polyspecific (ZIKV)[b]
	2	**1.2**	**≥ 320**	0	0	0	0	0	0	0	0	0	ZIKV
	3	**1.7**	**320**	**320**	**32**	**100**	0	0	0	0	0	0	polyspecific
	4	**1.3**	**≥ 320**	0	0	0	0	0	0	0	0	0	ZIKV
	5	***1.0***	**100**	0	0	0	0	0	0	0	0	0	ZIKV
	6	0.6	**10**	0	0	0	0	0	**10**	0	**100**	0	polyspecific
	7	0.2	**32**	0	0	0	0	0	0	0	0	0	ZIKV
	8	**2.1**	**320**	0	0	**100**	0	0	**10**	0	0	0	polyspecific (ZIKV)[b]
	9	0.6	**≥ 320**	**100**	**100**	**100**	**10**	0	**100**	**10**	**100**	**10**	polyspecific (ZIKV)[b]
	10	***0.9***	0	0	0	0	0	0	0	0	0	0	negative
Secondary (n = 21)	11	0.7	**100**	**100**	0	0	0	0	0	0	0	0	polyspecific
	12	**1.5**	**≥ 320**	0	0	0	0	**≥ 320**	0	0	0	0	polyspecific
	13	***0.9***	**≥ 320**	0	**10**	**100**	0	0	0	0	0	0	polyspecific (ZIKV)[b]
	14	**2.5**	**100**	0	0	0	0	0	0	0	0	0	ZIKV
	15	0.3	**10**	0	0	0	0	0	0	0	0	0	ZIKV
	16	0.2	**≥ 320**	**100**	**≥ 320**	**100**	0	0	**10**	0	0	0	polyspecific
	17	***0.8***	**320**	0	0	0	0	0	0	0	0	0	ZIKV
	18	***0.9***	**320**	**≥ 320**	0	0	0	0	0	0	0	0	polyspecific
	19	***1.0***	**320**	0	0	0	0	0	0	0	0	0	ZIKV
	20	0.1	0	0	0	0	0	0	0	0	0	0	negative
	21	0.3	**100**	**10**	0	**100**	0	0	0	0	0	0	polyspecific
	22	**1.4**	**≥ 320**	**320**	**≥ 320**	**320**	**320**	0	**320**	**320**	**≥ 320**	0	polyspecific
	23	0.7	**100**	0	0	0	0	0	0	0	0	0	ZIKV
	24	0.7	**320**	**100**	**100**	**10**	0	0	0	0	0	0	polyspecific (ZIKV)[b]
	25	**1.2**	**100**	0	0	0	0	0	0	0	0	0	ZIKV
	26	0.6	**100**	0	0	0	0	0	0	0	0	0	ZIKV
	27	0.1	**≥ 320**	0	0	0	0	0	0	0	0	0	ZIKV
	28	0.4	**≥ 320**	**100**	**320**	**10**	**100**	0	0	**10**	**100**	0	polyspecific (ZIKV)[b]
	29	0.4	**≥ 320**	0	**100**	0	**100**	0	0	0	0	0	polyspecific (ZIKV)[b]
	30	0.4	**10**	0	0	0	0	0	**32**	0	0	0	polyspecific
	31	0.6	**≥ 320**	**10**	**10**	**10**	**10**	0	**32**	**32**	**10**	0	polyspecific (ZIKV)[b]

CHIKV: chikungunya virus; DENV: dengue virus; dpso: days post symptom onset; ELISA: enzyme-linked immunosorbent assay; IFA: indirect immunofluorescence assay; JEV: Japanese encephalitis virus; NS1: non-structural protein 1; TBEV: tick-borne encephalitis virus; WNV: West Nile virus; YFV: yellow fever virus; ZIKV: Zika virus.

[a] Positive results are highlighted in bold.

[b] Sera containing polyspecific IgM antibodies against different arboviruses, where endpoint titration allowed to identify ZIKV as the infectious agent.

infection. This finding is consistent with the results of Bozza et al., who reported increased sensitivity using the anti-ZIKV NS1 IgA ELISA (53%) compared to the NS1-based IgM ELISA (33%) (Bozza et al., 2019). The high prevalence of IgA among patients with secondary infections proves its value for diagnosing acute ZIKV infections in flavivirus-endemic regions, where numerous patients have a history of flavivirus infection, and where IgG and IgM testing might be insufficient to differentiate acute (secondary) from past infections. Interpretation of IgA negativity in the presence of IgM should include that class switching may not have occurred yet, as well as the possibility of selective IgA deficiency.

Conforming to the case report by Zhao et al. (Zhao et al., 2018), our analysis of antibody kinetics in eight patients from flavivirus-endemic regions revealed a time course of IgA that frequently resembled that of classical IgM. Five of these cases demonstrated absence of ZIKV-specific IgM coupled with early IgG and positive RT-PCR, probably presenting secondary ZIKV infections. These data support the suitability of NS1-specific IgA as an additional marker of acute ZIKV infection, both in the presence and absence of specific IgM. To confirm these findings and their diagnostic significance, antibody kinetics will have to be studied in more ZIKV patients and longer follow-up. However, similar characteristics of the IgA response have been observed in DENV infections (Balmaseda et al., 2003; Groen et al., 1999).

Coinciding with the antibody prevalences in our ZIKV panel, Shu et al. showed that significant levels of DENV NS1-specific IgG were induced in 100% of DENV-infected patients, whereas DENV NS1-specific IgA and IgM were produced in 60.0% and 75.0% of primary DENV infections and in 90.0% and 40.0% of secondary DENV infections, respectively (Shu et al., 2000). Depending on the infection phase and assay type, even greater differences in anti-DENV IgA positivity among primary versus secondary cases were observed by Groen et al. (16.7% versus 62.1%) and Vázquez et al. (23.8% versus 85.4%) (Groen et al., 1999; Vazquez et al., 2005), indicating the relevance of this Ig class particularly for the diagnosis of secondary-infected patients.

We measured sensitivities of the anti-ZIKV NS1 ELISAs of 67.7% for IgG and 0.0% for IgM in samples collected 2–5 dpso, increasing to 100% for IgG and 48.4% for IgM in samples collected 8–16 dpso. Several groups also compared these assays against RT-PCR and/or PRNT and found sensitivities in the range of 66.7–88.2% for IgG and 48.1–79.0% for IgM, depending on the time of sample collection (Kadkhoda et al., 2017; Lustig et al., 2017; Steinhagen et al., 2016a). L'Huillier et al. reported 28.9% IgM sensitivity of the NS1-based ELISA, when evaluated against the CDC MAC-ELISA. This poor positive agreement might result from the lower specificity of the full-virus-based ELISA compared to the NS1-based ELISA, as demonstrated against PRNT (L'Huillier et al., 2017).

In our DENV panel, the NS1-based ZIKV ELISAs yielded specificities of 95.0% for IgA and 97.1% for IgM. Anti-ZIKV NS1 IgA and IgM reactivity was detected in seven and four DENV-infected Vietnamese patients, respectively, and may result from ZIKV/DENV co-infection (true-positive) or from cross-reactivity (false-positive). As ZIKV is not endemic in Vietnam, co-infection would most likely apply to returning travellers among the DENV-infected patients. Five of the seven patients with a positive IgA result also tested positive by anti-ZIKV-NS1 IgG ELISA and/or FFNT (data not shown), providing evidence for a recent

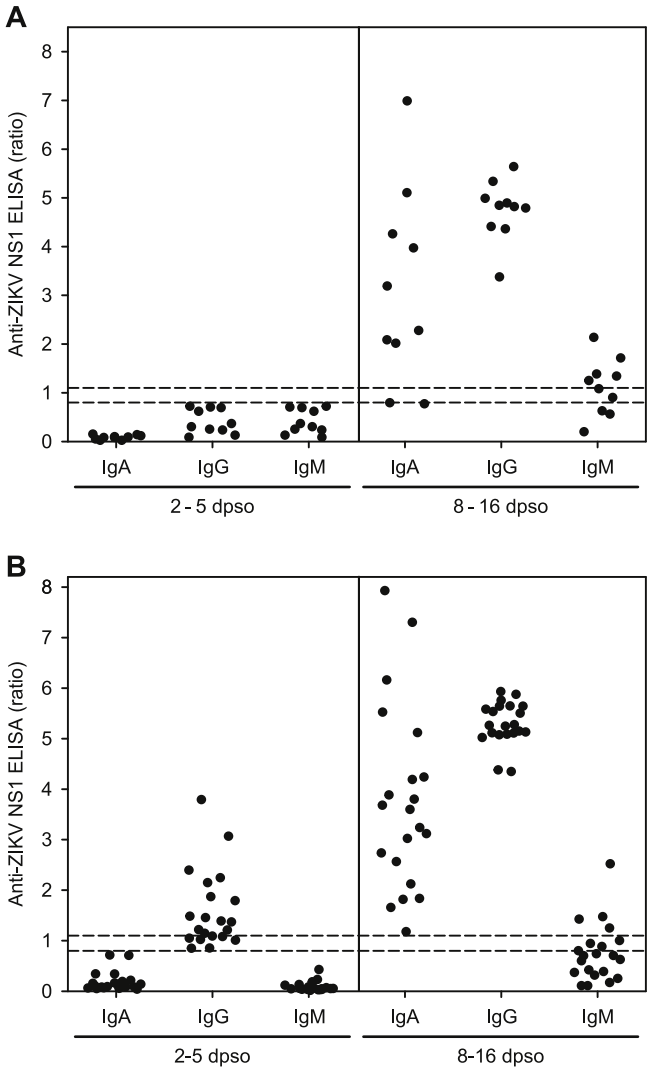

Fig. 1. Anti-ZIKV NS1 IgA, IgG, and IgM reactivity as determined by ELISA in patients with suspected primary (A) or secondary (B) ZIKV infection. 31 Dominican patients with RT-PCR-confirmed ZIKV infection were classified as having a suspected primary (n = 10) or secondary (n = 21) infection according to Cordeiro et al. (Cordeiro et al., 2007) From each patient, a serum pair was collected, representing the early viraemic phase (left panel) and acute phase (right panel) of ZIKV infection. Dashed horizontal lines represent the cut-off ratios for borderline and positive results.

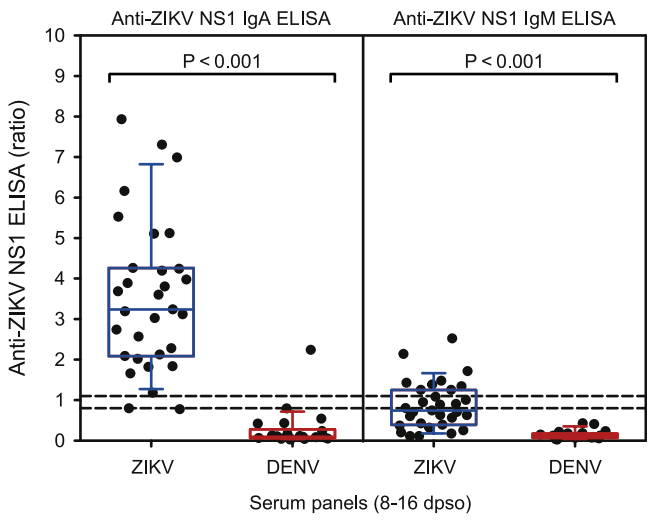

Fig. 2. Anti-ZIKV NS1 IgA and IgM reactivity in ZIKV-infected patients compared to DENV-infected patients. Anti-ZIKV NS1 IgA and IgM antibodies were measured by ELISA in serum samples obtained 8–16 dpso from Dominican patients with confirmed ZIKV infection (n = 31) and Vietnamese patients with confirmed DENV infection (n = 22). Dashed horizontal lines represent the cut-off ratios for borderline and positive results. Boxes indicate the median, 25th, and 75th percentile. The whiskers extend from the 10th to 90th percentile. The median values of two groups were compared using the Mann-Whitney rank sum test, with P values < 0.05 indicating statistically significant differences.

co-infection. In regards to the NS1-based ZIKV IgM ELISA, several studies have also demonstrated a high specificity (≥98.7) using panels of potentially cross-reactive specimens and/or healthy controls (Borena et al., 2017; Huzly et al., 2016; Kadkhoda et al., 2017; Steinhagen et al., 2016a), while others reported elevated IgM cross-reactivity with DENV-positive specimens (L'Huillier et al., 2017).

The strengths of this study include the use of well-characterised, RT-PCR-confirmed ZIKV cases. In addition, paired samples were available and had been collected at approximately equal time intervals, allowing for consistent result interpretation. As a limitation, records of the ZIKV patients' history were not available, so that their categorisation into primary and secondary infections could not be verified anamnestically. This is relevant as it cannot be excluded that some anti-ZIKV IgG positive/borderline results in samples obtained 2–5 dpso were due to cross-reactivity, even though specificity of the anti-ZIKV NS1 IgG ELISA exceeds 99% (Borena et al., 2017; Huzly et al., 2016; Steinhagen et al., 2016a). Another shortcoming is the lack of convalescent samples from the Dominican ZIKV patients and the limitation of kinetic analyses to only eight cases. Finally, the NS1-based IgA ELISA was not subject to

Table 3
Specificity of anti-ZIKV NS1 IgA and IgM detection using ELISA in Vietnamese patients with confirmed DENV infection.

Group (sampling)	DENV-infected patients	n	Anti-ZIKV NS1 ELISA IgA				DENV-infected patients	n	Anti-ZIKV NS1 ELISA IgM			
			Pos	Bdl	Neg	Specificity (CI 95%)[a]			Pos	Bdl	Neg	Specificity (CI 95%)[a]
Viraemic	Total	105	4	0	101	96.2% (90.3-98.8%)	Total	105	2	1	102	97.1% (91.6-99.4%)
(1-7 dpso)	Anti-DENV IgA[b]	57	4	0	53	93.0% (82.8-97.7%)	Anti-DENV IgM[c]	62	2	1	59	95.2% (86.2-89.9%)
Acute	Total	22	1	0	21	95.5% (76.5-99.9%)	Total	22	0	0	22	100% (82.5-100%)
(8-16 dpso)	Anti-DENV IgA[b]	15	1	0	14	93.3% (68.2-99.9%)	Anti-DENV IgM[c]	19	0	0	19	100% (80.2-100%)
Late acute	Total	12	2	0	10	83.3% (54.0-96.5%)	Total	12	1	0	11	91.7% (62.5-99.9%)
(17-19 dpso)	Anti-DENV IgA[b]	12	2	0	10	83.3% (54.0-96.5%)	Anti-DENV IgM[c]	10	1	0	9	90.0% (57.4-99-9%)
All groups	Total	139	7	0	132	95.0% (89.8-97.7%)	Total	139	3	1	135	97.1% (92.6-99.1%)
(1-19 dpso)	Anti-DENV IgA[b]	84	7	0	77	91.7% (83.5-96.2%)	Anti-DENV IgM[c]	91	3	1	87	95.6% (88.9-98.6%)

bdl, borderline; CI, confidence interval; DENV, Dengue virus; dpso, days post symptom onset; ELISA: enzyme-linked immunosorbent assay; neg, negative; NS1, non-structural protein 1; pos, positive; ZIKV, Zika virus.

[a] For the calculation of specificity, borderline results were considered positive.

[b] DENV-infected patients with anti-DENV IgA positive or borderline results according to Anti-Dengue Virus ELISA IgA (Euroimmun).

[c] DENV-infected patients with anti-DENV IgM positive or borderline results according to Anti-Dengue Virus Type 1–4 ELISA IgM (Euroimmun).

systemic analysis of specificity in regards to different DENV serotypes, other flaviviruses, or healthy controls.

In conclusion, this study provides evidence that the determination of anti-ZIKV NS1 IgA can contribute to the diagnosis of acute primary and secondary ZIKV infection at time points when viremia has decreased to an extent that RT-PCR can no longer detect the virus. In flavivirus-endemic areas, where secondary flavivirus infection with ZIKV are becoming more and more frequent, the detection of IgA may compensate for the occasional absence of IgM. Thus, combined IgA/IgM testing improves the efficiency of ZIKV serology, in particular when only a single serum sample is available. Due to high specificity, NS1-based assays may reduce the requirement for PRNT, thus shortening the diagnostic turnaround time. Our results are of particular importance for DENV-endemic countries after introduction of ZIKV, since ZIKV-specific serology is essential for differentiation of (secondary) DENV infection. In addition, the ELISA-based approach may be more manageable for routine laboratories than previously reported ZIKV IgA/IgG testing on a nanostructured platform (Zhang et al., 2017).

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Declaration of interest

EL and KS are designated as inventors on international patent applications WO/2017/144173 and WO/2017/144174. JMW, EL, SS, and KS are employees of Euroimmun. WSt and WSch are board members of Euroimmun.

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Appendix A. Supplementary data

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