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Zika virus infection among symptomatic patients from two healthcare centers in Sao Paulo State, Brazil: prevalence, clinical characteristics, viral detection in body fluids and serodynamics

Tania Regina Tozetto-Mendoza^{1,*}, Vivian lida Avelino-Silva^{1b 2,*}, Silvia Fonseca³, Ingra Morales Claro², Anderson Vicente de Paula¹, Anna Sara Levin^{1,2}, Ester Cerdeira Sabino^{1,2}, Maria Cassia Mendes-Correa^{1,2}, Walter Manso Figueiredo⁴, Alvina Clara Felix¹, Nathalia C. Santiago Souza¹, Angela Aparecida Costa⁴, Marta Inenami⁴, Rosangela M. Gasparetto da Silva⁴, José Eduardo Levi¹, Camila Malta Romano¹, Glaucia Paranhos-Baccalà^{1,5}, Aluisio Cotrim Segurado^{1,2}, Philippe Mayaud^{1,6}

¹Universidade de São Paulo, Instituto de Medicina Tropical de São Paulo, São Paulo, São Paulo, Brazil

²Universidade de São Paulo, Faculdade de Medicina, Departamento de Moléstias Infecciosas e Parasitárias, São Paulo, São Paulo, Brazil

³Hospital São Francisco, Ribeirão Preto, São Paulo, Brazil

⁴Universidade de São Paulo, Faculdade de Saúde Pública, Serviço Especial de Saúde de Araraquara, Araraquara, São Paulo, Brazil

⁵Medical Diagnostic Discovery Department (MD3), bioMérieux SA, Marcy l'Etoile, France

⁶London School of Hygiene and Tropical Medicine, Faculty of Infectious and Tropical Diseases, London, United Kingdom

*These first authors contributed equally to this article

Correspondence to: Vivian lida Avelino-Silva

Universidade de São Paulo, Instituto de Medicina Tropical de São Paulo, Av. Dr. Eneas de Carvalho Aguiar, 470, CEP 05403-000, São Paulo, SP, Brazil
Tel: +55 11 30617347

E-mail: viviansilva87@gmail.com

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ABSTRACT

Zika virus (ZIKV) clinical presentation and frequency/duration of shedding need further clarification. Symptomatic ZIKV-infected individuals identified in two hospitals in Sao Paulo State, Brazil, were investigated regarding clinical characteristics, shedding in body fluids, and serodynamics. Ninety-four of 235 symptomatic patients (Site A: 58%; Site B: 16%) had Real-Time PCR-confirmed ZIKV infection; fever, headache and gastrointestinal symptoms were less frequent, and rash was more frequent compared to ZIKV-negative patients. Real-Time PCR in serum had worse performance compared to plasma, while urine had the highest sensitivity. Shedding in genital fluids and saliva was rare. IgM positivity was the highest <14 days after the symptoms onset (86%), decreasing >28 days (24%); IgG positivity increased >14 days (96%) remaining positive in 94% of patients >28 days. ZIKV prevalence varied importantly in two neighboring cities during the same transmission season. Urine Real-Time PCR can improve diagnostic sensitivity; serum testing is less useful. Accurate serological tests are needed to improve diagnosis and surveillance.

KEYWORDS: Zika virus. Differential diagnosis. Kinetics. Body fluids. Polymerase chain reaction. Real-Time Polymerase Chain Reaction. Serology. Brazil.

INTRODUCTION

In 2016, the World Health Organization (WHO) declared Zika virus (ZIKV) infection-related microcephaly and other neurological disorders a Public Health Emergency of international concern¹. Since early 2015, the geographic spread of ZIKV has been reported to increase in Brazil, with the potential for broader dissemination due to the lack of pre-existing exposure of populations, absence of effective preventive methods and widespread distribution of the transmitting *Aedes* mosquito vector²⁻⁶. Many reports have also shown that the infection can be transmitted from person to person through sexual contact, which could further increase the potential for ZIKV spreading⁷⁻¹³. However, while some studies have

explored the frequency, further characterization of viral load and duration of viral shedding in urine, saliva and genital fluid of ZIKV-infected patients, could improve the understanding of potential transmission dynamics^{14,15}.

Infection with ZIKV is commonly asymptomatic or associated with mild febrile symptoms and pruritic skin eruption¹⁶. Rare neurologic complications of acute ZIKV infection in adults, such as the Guillain-Barre syndrome, myelitis and encephalitis have also been described¹⁷⁻¹⁹. Further characterization of ZIKV clinical case definition is still necessary in the context of co-circulating Dengue (DENV) and Chikungunya (CHIKV) infections, frequently observed in Central and South America.

Ribeirao Preto and Araraquara are medium-sized cities in the countryside of Sao Paulo State, Brazil, with an estimated population of 682,302 and 230,770 inhabitants respectively²⁰. Both municipalities have documented an alarmingly high incidence of DENV in 2016, accumulating almost 60,000 reported DENV cases²¹. During the same period, 1,376 ZIKV infections and 195 CHIKV infections were reported^{22,23}.

In this study, we identified individuals with ZIKV infection in a cohort of patients who sought medical care with acute arboviral symptoms in Araraquara and Ribeirao Preto in 2016. We described the clinical characteristics and results from the etiologic investigation at the time of enrollment, and prospective measurements of ZIKV serodynamics and ZIKV Real-Time polymerase chain reaction (RT-PCR) in blood, urine, saliva and genital fluid samples for participants with confirmed ZIKV infection.

METHODS

Study population and settings

We enrolled consecutive patients aged 16 and older attending the emergency department at Serviço Especial de Saude in Araraquara, Brazil (Site A) and Sao Francisco Hospital in Ribeirao Preto, Brazil (Site B) between January and September 2016. Patients were considered eligible when presenting with acute (<14 days) symptoms including at least two of the following: fever (>37.8 °C axillary temperature), maculopapular rash, headache, myalgia, arthralgia, fatigue, bleeding or conjunctivitis. We excluded participants with febrile symptoms considered to be related to other infections.

Study procedures

Eligible participants were interviewed for the assessment of demographic characteristics, current symptoms and medical comorbidities. Possible past DENV exposure

was ascertained through self-reporting of participants. A peripheral blood sample was collected for ZIKV testing by RT-PCR for all participants. For participants allocated in Site B, baseline investigation also included DENV and CHIKV testing by RT-PCR. For participants allocated in Site A, ZIKV was also tested by RT-PCR in urine samples. Results were communicated to healthcare providers and participants. Participants with confirmed ZIKV underwent three additional visits (<14, 15-28 and >28 days following symptoms onset) during which trained nurses collected a blood sample, while urine, saliva and cervicovaginal fluid samples were self-collected. . Whole saliva samples were self-collected by spontaneous salivation. Cervicovaginal fluid was self-collected using a cotton swab. One nursing mother provided breast milk samples at 20 and 23 days after symptoms initiation. We were also able to collect late cervicovaginal fluid samples using a brush (Evalyn Brush – Rovers Medical Devices BV, Oss, The Netherlands) one year after the baseline for ZIKV RT-PCR. Semen samples were obtained by masturbation from six participants, one year after the baseline, with complete analysis reported in a separated manuscript²⁴.

Laboratory methods

All samples were maintained under refrigeration (4-8 °C) up to 72 h until delivery in a local laboratory. The genital swab was plunged in 500 µL of DNase free water and slowly homogenized for 30 s. Then, they were immediately stored in aliquots of 500 µL at -80 °C until analysis. Nucleic acid extraction was performed using EasyMag automated system (bioMérieux®, Paris, France) according to the manufacturer's instructions. The one-step multiplex RT-PCR assay for CHIKV/DENV1-4 and ZIKV was performed according to the protocols and primer sets described previously²⁵⁻²⁸ using the TaqMan® Fast Virus 1-Step Master Mix (Life Technologies®, Austin, USA). Detection of anti-ZIKV IgM and IgG was performed by a commercial ELISA kit (Anti-Zika Virus ELISA, EUROIMMUN, Lübeck, Germany)²⁹, according to the manufacturer's instructions.

Statistical analysis

Baseline characteristics and the presence of each symptom at the time of enrollment and follow-up visits were described by using counts, proportions, medians and interquartile ranges (IQR), as appropriate. We used the Wilcoxon rank-sum test, the Chi-square or the Fisher's exact test for comparisons between ZIKV-positive and -negative groups (categorical variables) and the Student's t-test

for continuous quantitative variables. All analyses were performed by the Stata software, version 13.1 (StataCorp. College Station, TX: StataCorp LP).

Ethical aspects

The institutional Ethics and Research Committee of the University of Sao Paulo Medical School, as well as local ethical committees, approved the study protocol (Process N° 1.489.145/2016 and 25706913.6.0000.0066/2014). All participants signed an informed consent form.

RESULTS

A total of 235 patients with a median age of 41 years (IQR, 31-54) were enrolled. Women comprised 69% of the sample, 24 (11%) of whom were pregnant, 23% of participants had at least one chronic comorbidity and 31% reported prior DENV infection (Table 1). Etiologic investigation of the arboviral syndrome at the time of admission included ZIKV testing only for the 134

participants of Site A, and ZIKV, DENV and CHIKV testing for the 101 participants of Site B. Overall, 94 participants (40%) had confirmed ZIKV infection by RT-PCR in either plasma, serum or urine samples, 58% (95%CI: 49-67%) in Site A and 16% (95%CI: 9-24) in Site B. In addition, in site B, prevalence of RT-PCR-positive DENV was 10%, while CHIKV or coinfections were not detected. ZIKV-positive patients were slightly older (44 versus 38 years, $p=0.001$) and included a higher (although not statistically significant) proportion of female participants compared with the ZIKV-negative group (76 versus 64%, $p=0.058$). At the time of enrollment, the median time since symptoms onset was 3 days (IQR, 1-4) for ZIKV-positive participants and 4 days (IQR, 2-5) for ZIKV-negative participants ($p<0.001$). Clinical presentation was broadly similar for ZIKV-positive and ZIKV-negative patients, although ZIKV-positive patients reported significantly more episodes of fever, headache, cutaneous rash, while gastrointestinal symptoms (abdominal pain, nausea, vomiting, and diarrhea) were less frequent, compared to ZIKV-negative patients.

Table 1 - Demographic and clinical characteristics of the study participants according to Zika virus RT-PCR results at the time of enrollment.

Patient characteristics	All participants N=235	Zika-positive N=94 (40%)	Zika-negative N=141 (60%)	p-value
Age	41 (31-54)	44 (36-59)	38 (29-52)	0.001
Female sex (%)	161/235 (69)	71/94 (76)	90/141 (64)	0.058
Pregnant (%)	18/159 (11)	7/70 (10)	11/89 (12)	0.641
Chronic comorbidities (%)	53/226 (23)	24/86 (28)	29/140 (21)	0.215
Prior dengue infection (%)	70/229 (31)	29/92 (32)	41/137 (30)	0.797
Symptoms at enrollment				
Days with symptoms	4 (2-5)	3 (1-4)	4 (2-5)	<0.001
Fever (%)	121/232 (52)	40/92 (43)	81/140 (58)	0.032
Rash (%)	201/235 (86)	89/94 (95)	112/141 (79)	0.001
Pruritus (%)	145/213 (68)	61/93 (66)	84/120 (70)	0.494
Petechiae (%)	12/131 (9)	8/75 (11)	4/56 (7)	0.555
Bleeding symptoms (%)	11/100 (11)	4/16 (25)	7/84 (8)	0.073
Conjunctivitis (%)	25/231 (11)	9/91 (10)	16/140 (11)	0.713
Arthralgia (%)	152/234 (65)	62/94 (66)	90/140 (64)	0.793
Articular edema (%)	35/134 (26)	25/78 (32)	10/56 (18)	0.065
Headache (%)	157/234 (67)	56/94 (60)	101/140 (72)	0.045
Weakness (%)	27/134 (20)	16/78 (21)	11/56 (20)	0.901
Myalgia (%)	69/129 (53)	40/75 (53)	29/54 (54)	0.967
Sore throat/cough (%)	42/129 (33)	27/75 (36)	15/54 (28)	0.326
Gastrointestinal symptoms* (%)	87/164 (53)	33/80 (41)	54/84 (64)	0.003

Continuous variables are presented as medians and interquartile ranges. RT-PCR: real-time polymerase chain reaction. *Abdominal pain, nausea, vomit, diarrhea

Given the uncertainties on the ideal specimen for ZIKV diagnostic testing at the time this study was carried out, samples collected for testing at the time of enrollment included plasma, serum and urine samples in different combinations as described in [Figure 1](#). Among participants who had a positive ZIKV RT-PCR in at least one of the specimens tested, RT-PCR was negative in 31% (24/78, 95% CI: 21-42%) of plasma samples, 46% (12/26, 95% CI: 27-67%) of serum samples, and 5% (4/78, 95% CI: 0-13%) of urine samples.

ZIKV-positive participants were followed-up during three additional visits with medians of 13 (IQR, 11-14), 17 (IQR, 16-20) and 47 (IQR, 31-52) days after symptoms onset. Patients were invited to provide additional blood, urine, genital fluids, saliva and breast milk (if breastfeeding) samples for ZIKV RT-PCR. ZIKV was detected at follow-up visits 1 and 2 more frequently in urine than in plasma and serum samples ([Table 2](#)). On visit 3, ZIKV was still detectable in plasma samples of two patients (11%), in

serum of one patient (50%) and in urine samples of three patients (19%).

Ten women were tested at least once for ZIKV in genital fluids, with only one woman having a positive RT-PCR result. She was a 26-year-old non-pregnant participant who was enrolled three days after the onset of rash, headache and arthralgia, and had a cervicovaginal sample collected 18 days after the initial symptoms. The vaginal fluid was her only ZIKV positive sample besides a plasma sample. There were no data available on her sexual partner's symptoms or infection status. Besides, genital fluid samples were collected from 16 women with confirmed ZIKV infection with a median of 12 months (IQR, 11-14) after enrollment and none tested positive for ZIKV RT-PCR (data not shown).

As shown in [Table 2](#), saliva was tested in a small number of patients across two follow-up visits with no viral detection. One nursing mother who had ZIKV symptoms onset at week 36 of gestation provided breast milk samples 20, 23 and 30 days after symptoms onset. The sample

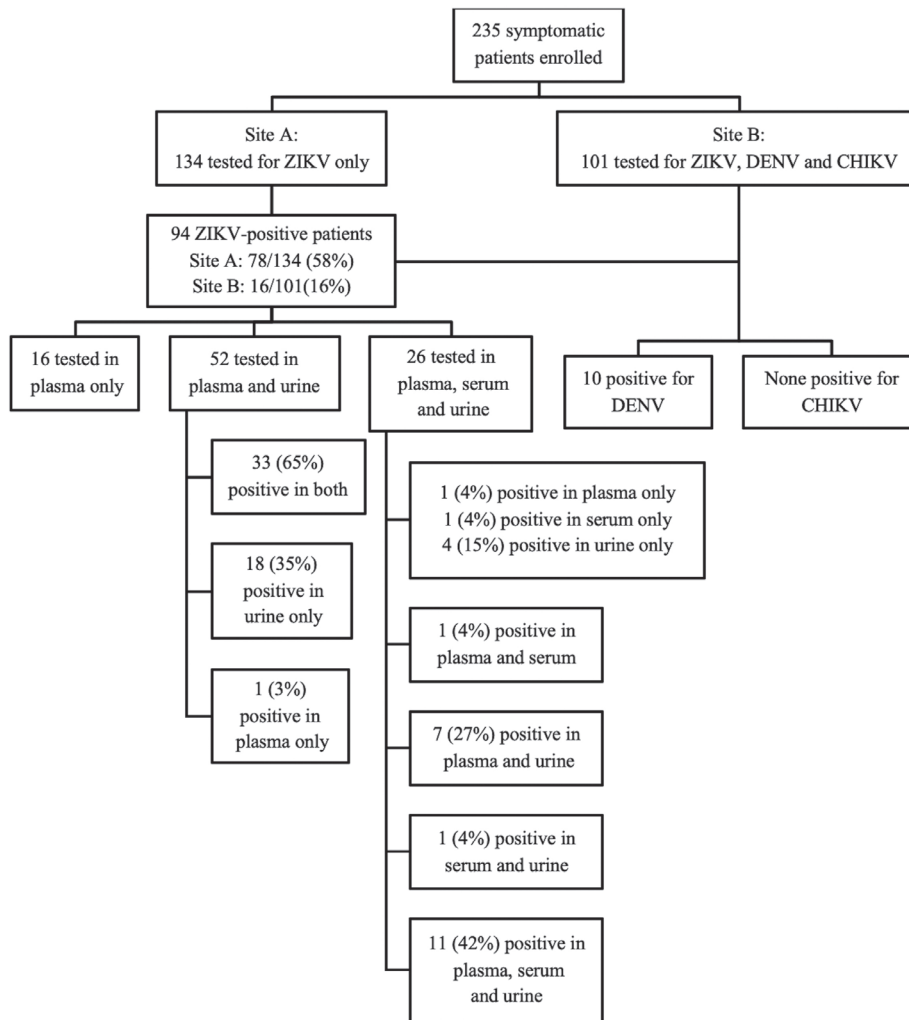


Figure 1 - Investigation of arboviral diseases at enrollment.

Table 2 - Zika virus RT-PCR detection in body fluids during follow-up of 94 patients with confirmed Zika virus infection at the time of enrollment.

Visit	Median days since symptoms onset (IQR)	Zika virus in plasma (%)	Zika virus in serum (%)	Zika virus in urine (%)	Zika virus in genital fluids (%)	Zika virus in saliva (%)	Zika virus in breast milk (%)
Baseline	3 (1-4)	70/94 (74)	14/26 (54)	74/78 (95)	-	-	-
Visit 1 (≤14d)	13 (11-14)	2/20 (10)	0/9 (0)	12/18 (67)	-	-	-
Visit 2 (15-28d)	17 (16-20)	3/53 (6)	2/4 (50)	13/42 (31)	1/5* (20)	0/6 (0)	1/1 [‡] (100)
Visit 3 (>28d)	47 (31-52)	2/18 (11)	1/2 (50)	3/16 (19)	0/10 (0)	0/12 (0)	0/1 (0)

IQR: interquartile range; RT-PCR: real-time polymerase chain reaction; *ZIKV positive in vaginal swab 28 days after symptoms onset; [‡]ZIKV positive in breast milk 20 days after symptoms onset

collected 20 days after symptoms onset tested positive for ZIKV RT-PCR, as well as in Vero cells culture. We tested cord blood, saliva and urine samples from the infant at birth, and re-tested another urine sample one month after birth: all infant samples were negative for ZIKV RT-PCR. Unfortunately, blood samples from this infant were not tested by ZIKV serology.

ZIKV serology results at the time of enrollment and follow-up visits are presented in Table 3. At the time of enrollment, four out of 72 participants with RT-PCR-positive ZIKV infection (6%) had positive ZIKV IgM and 11 (15%) had positive ZIKV IgG results; two (3%) were positive for both IgM and IgG. Among 85 participants with negative baseline ZIKV RT-PCR, five (6%) had positive ZIKV IgM, 21 (25%) had positive ZIKV IgG and one (1%) was positive for both, IgM and IgG. Serology was performed in subsequent visits only for participants with positive baseline ZIKV RT-PCR. On visit 1, only seven participants were tested and six (86%) had positive results for both, IgM and IgG. On visit 2, 65% of participants had positive IgM and 96% had positive IgG, while on visit 3, only 4 (24%) of those tested remained IgM positive and

94% were IgG positive. On follow-up visits, most patients with positive IgM were also positive for IgG.

Pregnancy outcomes for ZIKV-positive pregnant participants were known for six out of seven cases. One participant with first-trimester pregnancy had a miscarriage at gestational week seven, 22 days after the enrollment and 26 days after symptoms onset. She was hospitalized for curettage and discharged without additional medical complications. No investigation was performed on the fetus or on miscarriage products. Four participants with third-trimester pregnancies and one participant with a second-trimester pregnancy at the time of acute ZIKV infection gave birth to children without apparent signs of Congenital Zika Syndrome according to the initial evaluation. Eighteen to 21 months after birth, all five children were still under neuropsychiatric follow-up and had no signs or symptoms of neurological or developmental abnormalities.

DISCUSSION

In this hospital-based study, we enrolled 235 patients from two cities in the Sao Paulo State, Brazil, for whom

Table 3 - Zika virus serology among 94 patients with confirmed Zika virus infection and among 85 patients with negative Zika virus RT-PCR at the time of enrollment.

Visit	Median days since symptoms onset (IQR)	Positive IgM (%)	Positive IgG (%)	IgM/IgG dual positive (%)
Patients with confirmed Zika virus infection (N=94)				
Baseline	3 (1-4)	4/72 (6)	11/72 (15)	2/72 (3)
Visit 1 (≤14d)	13 (11-14)	6/7 (86)	6/7 (86)	6/7 (86)
Visit 2 (15-28d)	17 (16-20)	32/49 (65)	47/49 (96)	30/49 (61)
Visit 3 (>28d)	47 (31-52)	4/17 (24)	16/17 (94)	4/17 (24)
Patients with negative Zika virus RT-PCR at enrollment (N=85)				
Baseline	4 (2-5)	5/85 (6)	21/85 (25)	1/85 (1)

IQR: interquartile range; RT-PCR: real-time polymerase chain reaction

investigation of acute ZIKV infection was performed by blood or urine RT-PCR. We found an overall prevalence of 40% of ZIKV-positive patients, who presented with cutaneous rash, fever, headache and less frequently with gastrointestinal symptoms when compared to ZIKV-negative patients. Few patients had detectable ZIKV RNA in plasma samples (n=2 or 11%), serum (n=1 or 50%) and urine (n=3 or 19%) up to the last follow-up visit >28 days after enrollment. We reported the presence of ZIKV in genital fluids of one female patient. We have also observed the presence of ZIKV in breast milk, and, although we were able to isolate ZIKV in culture, we cannot confirm the potential viral transmission of breast milk.

These findings corroborate those of more detailed viral kinetics studies performed in Puerto Rico after the follow-up of 150 RT-PCR-confirmed ZIKV infection patients from whom serum, urine, saliva, semen and vaginal secretion specimens were analyzed until all specimens became RT-PCR negative. They were able to determine the median time of viral shedding, which was highest for semen samples (34 days), followed by serum (14 days) and urine (8 days) samples. Interestingly, only one among 50 women tested positive in vaginal fluid¹⁵. Persistence of ZIKV in genital fluids has also been described in a smaller study conducted in Spain, which identified RT-PCR-positive samples in one of four women and one of five tested men, with viral shedding detected between days 37 and 69 for vaginal fluids and between days 23 and 107 for semen³⁰.

Although Araraquara (Site A) and Ribeirao Preto (Site B) are only 70 km apart and enrollment in both cities took place in the same year, ZIKV prevalence was strikingly different, of 58% and 16%, respectively. Our findings and conclusions are also quite similar to those of a study conducted in another part of the Sao Paulo State³¹ and this disparity between ZIKV prevalences highlights the potential impact of different investigation protocols on results. Furthermore, it is essential to understand the local ZIKV epidemiology based not only on clinical definitions, which may be less useful in settings with lower epidemic intensity, but also on laboratory-based surveillance of standardized samples and time points. In 2016, 236 and 1,140 ZIKV cases were reported in Site A and Site B, respectively, corresponding to an incidence rate of 102.3 and 167.1/100,000 inhabitants, respectively²². However, it is likely that data presented in official reports are an underestimate of the actual incidence given the low availability of diagnostic tools and the frequency of asymptomatic infections.

ZIKV can be identified by RT-PCR from different body fluids and the best specimens for diagnosis are still to be defined. Our results suggest that RT-PCR testing in urine is more sensitive than in plasma, while serum was

the specimen less likely to be positive. ZIKV RT-PCR testing in whole blood, has shown to be the most frequently positive fluid in a study conducted in Guadeloupe³², but was unfortunately not tested in our study. Among ten women tested for ZIKV detection in genital fluids on visits 2 or 3, only one was positive 18 days after symptoms onset, which is slightly longer timeframe than the ones previously reported³³⁻³⁵. Among 16 women tested 12 months after the initial infection, none had detectable ZIKV suggesting that ZIKV detection in female genital fluids is probably rare and short-lived, but this has not been systematically characterized hitherto^{33,34}. In addition, probable ZIKV female-to-male sexual transmission has also been reported³⁶. The quantification of ZIKV transmission risk through sexual intercourse is still to be determined, and this would represent important information to characterize the potential trajectory of the ZIKV epidemic³⁷.

Serological dynamics of ZIKV infection were analyzed in the 94 patients with confirmed ZIKV infection. We observed that 6% of patients were IgM positive and 15% were IgG positive at the time of enrollment, a median of 3 days after symptoms onset; as expected, IgM positivity increased between 14 and 28 days and decreased sharply after that, while IgG positivity increased around 14 days and remained positive in the majority of tested patients. Interestingly, among 85 patients with negative ZIKV RT-PCR at the baseline who underwent serology, 27 (32%) were positive for IgM, IgG or both. We were not able to discriminate whether these were true ZIKV infections with negative RT-PCR or false positive serological results. Although our study was not explicitly designed to address the performance of current serological tests, our results corroborate the findings from other studies that suggested a lower sensitivity of the Euroimmune IgM compared to IgG³⁸⁻⁴⁰ antibodies detection

Our study had some limitations. Because this study was conducted in the context of real-life routine clinical settings, we had missing observations and heterogeneous testing of specimens in different time points. Data on prior DENV infection was self-reported by participants, a procedure that is more prone to patients' memory bias. It is possible that patients with no previously reported DENV had been exposed to the virus with asymptomatic or mild disease presentation. Unfortunately, it was not possible to perform DENV serology. This information would have been useful in the interpretation of ZIKV serological dynamics, as has been highlighted by our group and others⁴¹. Information on yellow fever vaccination was not collected from study participants and could also have potentially influenced ZIKV serology interpretation. Moreover, potential subjectivity in the identification of eligible participants by excluding those with other suspected

infections based on clinical evaluation could have impacted our findings. Despite these limitations, our study provides useful information on some of the viral and serological kinetics of ZIKV in the context of an epidemic.

In conclusion, our study found critical differences in ZIKV prevalence among symptomatic patients presenting with similar arboviral syndromes in two neighboring cities in the Sao Paulo State, Brazil. In addition to a heterogeneous occurrence, nonspecific symptoms and overlapping clinical definitions for ZIKV, DENV, and CHIKV infections, highlights the need for accurate and accessible diagnostic tests, that should be timely performed using the specimens more likely to be positive. Our findings confirm the results from previous studies showing that urine ZIKV RT-PCR testing in the acute phase of infection can improve the sensitivity of diagnosis, while testing serum is probably less useful despite more sustained shedding in some cases. Higher sensitivity and specificity serological tests are needed for the diagnosis of patients who seek medical attention after the period of ZIKV detection by RT-PCR and for surveillance purposes in settings of high DENV background infection.

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