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MAJOR ARTICLE

Virus and Antibody Dynamics in Travelers With Acute Zika Virus Infection

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(See the Major Article by Plennavaux et al on pages 1164–72 and the Editorial Commentary by Simmons et al on pages 1181–3.)

Background. To improve our understanding of the natural history of Zika virus (ZIKV) infection in humans, we described the dynamics of ZIKV RNA shedding in different body fluids and antibody responses in patients with acute infection.

Methods. Twenty-nine adults with travel-associated infection and 1 case of sexual transmission were enrolled and followed up with weekly ZIKV RNA testing in blood, urine, saliva, and semen samples and antibody testing.

Results. ZIKV RNA was detected in plasma, urine, and saliva of 57%, 93.1%, and 69.2% of participants, with estimated median times to clearance of 11.5 days (interquartile range [IQR] 6–24 days), 24 days (IQR, 17–34), and 14 days (IQR, 8–31), respectively. In 2 pregnant women, ZIKV RNA persisted in blood until delivery of apparently healthy infants. ZIKV RNA was detected in semen of 5 of 10 tested men; median time to clearance was 25 days (IQR 14–29), and the longest time of shedding in semen was 370 days. In flavivirus-naive patients, the median times to detection of ZIKV nonstructural protein 1 (NS1)–specific immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies were estimated as 8 days (IQR, 5–15 days) and 17 days (IQR, 12–26 days), respectively. ZIKV NS1 IgM antibodies were undetectable in patients with previous dengue.

Conclusions. Prolonged viremia and ZIKV RNA shedding in urine, saliva, and semen occur frequently in patients with acute ZIKV infection. At the time of diagnosis, about half of patients are ZIKV IgM negative. ZIKV NS1 IgM antibodies remain undetectable in patients with previous dengue. Estimates of the times to viral clearance and seroconversion are useful to optimize diagnostic algorithms.

Keywords. Zika virus; semen; follow-up; persistence; clearance.

Zika virus (ZIKV) is a mosquito-borne flavivirus, which was not considered a relevant human pathogen until the recent large outbreaks in the Pacifica area and the Americas, which highlighted its association with Guillain-Barré syndrome and fetal microcephaly [1, 2]. Studies in animal models and in humans have described the dynamics of ZIKV infection and characterized host adaptive immune response, highlighting viral persistence in body fluids such as blood, urine, saliva, semen, and vaginal secretions [3, 4] and in organs, such as the brain, testes, and the gastrointestinal tract [5]. Infection elicits humoral and cellular adaptive immune responses with activation and proliferation of ZIKV-specific $CD4^+$ and $CD8^+$ T lymphocytes and production of ZIKV neutralizing antibodies, which play a key role in the control of ZIKV infection [6, 7]. Due to the genetic similarity among flaviviruses [8], antibodies induced after ZIKV infection are broadly cross-reactive and may pose problems to the differential diagnosis, especially in countries where different flaviviruses co-circulate [9]. Thus, diagnosis of acute ZIKV infection relies on detection of viral nucleic acids in blood and other body fluids [1]. In this context, aim of this follow-up study was to describe clinical features and dynamics of ZIKV RNA shedding in different body fluids and serum antibody responses in a series of adult travelers with acute ZIKV infection. The results of this study provide useful information to understand ZIKV disease and to improve diagnostic algorithms.

METHODS

Study Population

Patients recruited in this follow-up study were referred between January 2016 and January 2017 to the Microbiology and Virology Unit of Padova University Hospital, Padova, and the Molecular Virology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, where laboratory testing confirmed a recent ZIKV infection according to the criteria of the European Centre for Disease Prevention and Control. These criteria comprise at least 1 of the following: detection of ZIKV RNA in a clinical specimen; virus isolation from a clinical specimen; detection of ZIKV specific immunoglobulin M (IgM) antibodies in serum and confirmation by neutralization test; seroconversion or

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4-fold increase in the titer of ZIKV-specific antibodies in paired serum samples. Follow-up evaluation consisted of weekly collection of blood, urine, saliva, and semen samples for ZIKV RNA and antibody testing. Some participants agreed to collect daily urine and saliva samples. Follow-up was continued until ZIKV RNA tests become negative and ZIKV-specific IgM/immunoglobulin G (IgG) antibodies, confirmed by a neutralization test, were detectable in serum. The protocol was approved by institutional ethics committees, and written informed consent was obtained from study participants.

Laboratory Tests

ZIKV RNA was detected in plasma, whole blood, urine, saliva, and semen samples by using an in house real-time reverse-transcription polymerase chain reaction (RT-PCR) assay based on the primer and probe set 1086/1162c/1107-FAM designed by Lanciotti et al [9] and by pan-flavivirus PCR and sequencing according to Scaramozzino et al [10], as previously described [11]. ZIKV nonstructural protein 1 (NS1)–specific IgM and IgG antibodies were detected by enzyme-linked immunosorbent assay (ELISA) ZIKV IgM and IgG (Euroimmun AG, Luebeck, Germany). Titers of ZIKV neutralizing antibodies were determined by virus microneutralization assay using Vero cells seeded in 96-well cell culture microplates. ZIKV was isolated from body fluids on Vero E6 cells, as described previously [11, 12]. Sequencing of the ZIKV genome from body fluids was performed by the Sanger method.

Data Collection and Statistical Analysis

Data were summarized and reported as median, interquartile range (IQR), range, and 95% confidence interval (CI). The time to ZIKV RNA clearance from body fluids and the time to ZIKV IgM and IgG antibody detection were estimated by using Weibull regression models. The time until ZIKV RNA clearance and antibody detection were defined for each participant as the number of days between the onset of symptoms (or the day of arrival in Italy for asymptomatic travelers) and, respectively, the first negative PCR result or antibody detection by ELISA. In patients with intermittent ZIKV RNA shedding, the interval was calculated until the first negative PCR result after the last recorded positive PCR result. Data were censored for the participants who still had a positive PCR result or negative ELISA result at the time of the analysis. Correlations between ZIKV RNA load in different body fluids were evaluated by linear regression analysis. Agreement between ZIKV RNA positivity in different body fluids was assessed by κ statistic. All statistical analyses were performed by using Dell Statistica software, version 13.1 (Dell Inc, Round Rock, Texas).

Table 1. Background Characteristics of 30 Travelers With Acute Zika Virus Infection

Characteristic	No.
Age, median, IQR, (range)	41 y, 30–50 y (18–63 y)
Sex, female/male	13/17
Nationality, Italian/other	25/5
Previous flavivirus infection or vaccination	Dengue (5), yellow fever (2)
Visited country	Brazil (2), Dominican Republic (11), Venezuela (4), Haiti (2), Jamaica (3), Cuba (2), Virgin Islands (1), Martinique (1), El Salvador (1), Bahamas (1), French Antilles (1), none (probable sexual transmission) (1)
Comorbidity	0
Pregnancy	2

Abbreviation: IQR, interquartile range.

RESULTS

Characteristics of the Study Population

Demographic characteristics of study subjects are summarized in Table 1 and Figure 1A. The study population consisted of 26 patients who developed symptoms after returning from a geographic area with known ZIKV transmission in Central America and the Caribbean, 3 asymptomatic individuals who sought testing because they traveled with other patients who developed symptomatic ZIKV disease, and a woman without travel history who developed symptoms 13 days after having unprotected sexual intercourse with her husband, who had also symptoms and 4 days before had returned from the Dominican Republic. Testing of a semen sample collected 24 days after symptom onset from this man demonstrated high ZIKV RNA load. In symptomatic patients, symptoms were mild and resolved within 2–8 days, without requiring hospitalization. Laboratory diagnosis of recent ZIKV infection was based on the detection of viral RNA in body fluids in 29 patients, and by detection of IgM followed by IgG antibodies, confirmed by neutralization assay, in 1 case. Two cases were previously described [11, 13], and updated follow-up information are reported here.

Kinetics of Zika Virus RNA in Body Fluids

The median duration of the interval between the time of symptom onset (or the day of arrival for asymptomatic individuals) and the first laboratory evaluation was 5 days (IQR, 3–7.5 days; range, 1–34 days). Twenty-four of the 30 patients attended follow-up visits. The number of follow-up visits and the duration of follow-up varied among patients (mean duration of follow-up, 38 days [95% CI, 22–54 days]; range, 0–480 days]). Quantitative RT-PCR analysis of ZIKV RNA was done in a total of 80 plasma, 188 urine, 201 saliva, and 95 semen specimens.

Figures 1–3 show the kinetics and load of ZIKV RNA in plasma, whole blood, urine, saliva, and semen and the antibody response; Table 2 summarizes key data on ZIKV RNA load in body fluids and time to clearance.

Plasma

ZIKV RNA was detectable in plasma of most of the subjects tested within the first 4 days after onset, but only in 35% of

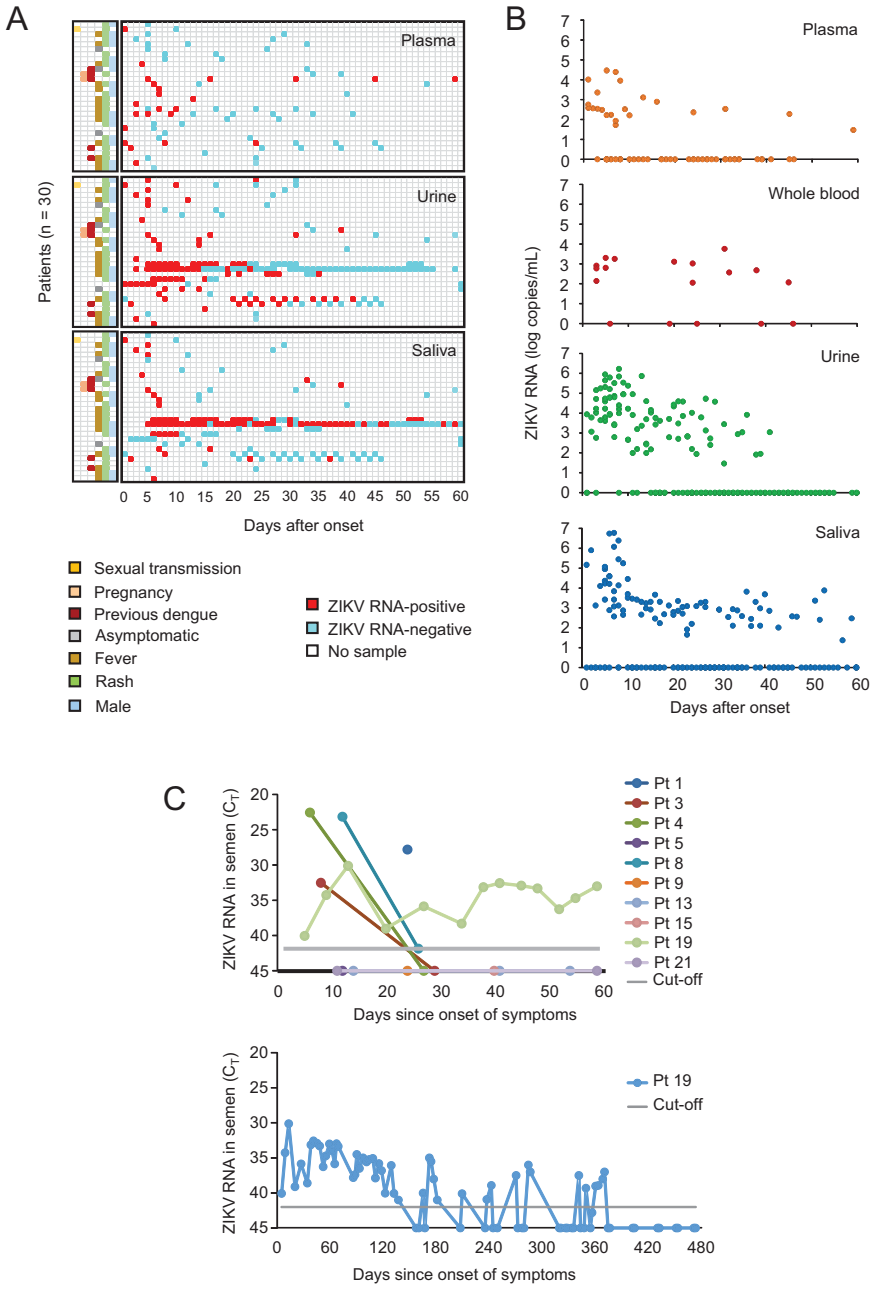

Figure 1. Zika virus (ZIKV) RNA in body fluids during acute infection. *A*, Results of real-time reverse-transcription polymerase chain reaction (RT-PCR) testing for ZIKV RNA in different body fluids (plasma, urine, and saliva) during follow-up in 30 patients with acute ZIKV infection. In the grids, lines represent patients (patients 1–30), and columns represent days after symptom onset (from day 1 to day 60 after onset). ZIKV RNA–positive and –negative samples are highlighted in red and light blue, respectively. *B*, ZIKV RNA load in plasma, whole blood, urine, and saliva determined by real-time RT-PCR in 30 patients with acute ZIKV infection. Data of the first 60 days following onset are shown. *C*, Results, reported as threshold cycle (C_T) values, of ZIKV real-time RT-PCR testing of semen samples from 10 men with acute ZIKV infection. Long-term follow-up results of a patient (patient 19) with persistent ZIKV RNA shedding in semen are shown in the lower panel. Abbreviations: C_T, threshold cycle; ZIKV, Zika virus.

those tested between days 5 and 8 (Figure 1A; Table 2). Viral load in plasma was generally low (about 300–500 copies/mL), but in 4 cases it reached levels >10^4 copies/mL (Figure 1B). The median time to ZIKV RNA clearance from plasma was estimated as 11.5 days (IQR, 6–24 days), on the basis of the Weibull model (Figure 3). Similar results were obtained by Kaplan-Meier curves. In 3 patients in whom ZIKV RNA load was monitored both in plasma and whole blood, ZIKV RNA was detectable in whole blood for a longer time than in plasma (ie, up to 45, 32, and 24 days after onset vs 24, 3, and 2 days after onset, respectively).

Urine

Overall, shedding of ZIKV RNA in urine was demonstrated in 27 of 29 tested patients (93.1%). In particular, ZIKV RNA shedding in urine was observed in 20 of 22 patients (90.9%) tested during

Table 2. Summary of Zika Virus RNA Levels in Body Fluids

	Plasma	Urine	Saliva
Maximum observed ZIKV RNA load			
Median [Log copies/mL]	2.66	5.05	4.94
IQR [Log copies/mL]	2.49–4.01	3.98–5.48	3.42–5.90
Range [Log copies/mL]	2.21–4.47	1.48–6.22	3.12-6.77
No. measurements	10	16	13
ZIKV RNA load (days 1-4 after onset)			
Median [Log copies/mL]	2.56	4.02	3.12
IQR [Log copies/mL]	2.48–2.75	2.76–5.24	0.00–5.16
No. measurements	9	11	9
No. (%) below detection limit	1 (11%)	2 (18%)	3 (33%)
ZIKV RNA load (days 5-8 after onset)			
Median [Log copies/mL]	0.00	4.22	4.21
IQR [Log copies/mL]	0.00–2.09	3.39–5.21	0.00–4.94
No. measurements	20	18	17
No. (%) below detection limit	13 (65%)	0 (0%)	5 (29%)

Abbreviations: IQR, interquartile range; ZIKV, Zika virus.

the first week after onset and in 9 of 12 patients (75%) tested during the second week (Figure 1A). In 4 cases, the duration of ZIKV RNA shedding in urine was >1 month. The median time to ZIKV RNA clearance in urine was estimated as 24 days (IQR, 17–34 days) (Figure 3). Peak ZIKV RNA levels were reached approximately 1 week after onset and ranged from 580 copies/mL to 1.7×10^6 copies/mL (median, 4×10^4 copies/mL) (Figure 1B).

Saliva

Among the 26 patients who were tested for ZIKV RNA in saliva, 18 (69.2%) had at least 1 positive sample (Figure 1A). The median time to ZIKV RNA clearance in saliva was estimated as 14 days (IQR, 8–31 days) (Figure 3). Peak ZIKV RNA levels were reached approximately 1 week after onset and ranged from 1×10^3 to 6×10^6 copies/mL (median, 6×10^4 copies/mL). In 5 patients, ZIKV RNA was detected in saliva for >1 month after onset, although at low titer and intermittently. Infectious virus was isolated in cell culture from saliva of 1 of these cases, as previously reported [13].

Semen

Shedding of ZIKV RNA in semen was demonstrated in 5 of 10 (50%) male patients who provided at least 1 semen sample for testing (Figure 1C). None of the patients reported hematospermia or other signs of genital tract infection. The median time to ZIKV RNA clearance in semen was estimated as 25 days (IQR, 14–29 days) (Figure 3). One of these cases (patient 19), whose follow-up data at 6 month have been already reported [14], had persistent shedding of ZIKV RNA in semen, at low titer and intermittently, up to 370 days after onset (Figure 1C). In 2 patients, in whom ZIKV RNA load in semen was very high (approximate cycle threshold value = 23 by real-time RT-PCR), infectious virus could be isolated in cell culture.

Persistent viral replication in the host may lead to the emergence of genetic variants, especially in the case of RNA viruses such as ZIKV, characterized by RNA polymerase enzymes without proofreading activity. To evaluate if ZIKV RNA persistence was associated with the emergence of genetic changes in the ZIKV genome, we sequenced a region of 2400 nucleotides containing the prM and E genes in semen samples of patient 19 collected 3–4 months after symptom onset. The prM-E genes were chosen for sequencing because they encode for the surface proteins of the virus that determine its tropism for host cells. Only a synonymous nucleotide change (T to C in position 1865) was identified in comparison with the viral genome sequence obtained from a urine sample collected at day 7 after symptom onset (GenBank KX269878), indicating high intrahost genetic stability of ZIKV for months after the initial infection. As control, we sequenced the prM-E region in saliva and urine samples collected at day 5 after onset of symptoms in a patient (patient 5) who had no viral shedding in semen. Also in this case, only a synonymous nucleotide change was identified between the sequences obtained from the 2 samples.

Correlation Between Zika Virus RNA Shedding in Different Body Fluids

Significant correlations were observed between ZIKV RNA load in plasma, urine, and saliva (plasma and urine, linear regression analysis, adjusted $R^2 = 0.13$, $P < .05$; plasma and saliva, adjusted $R^2 = 0.27$, $P < .05$). At variance, no significant association was observed between viremia and detection of ZIKV RNA in semen.

Dynamics of Zika Virus-Specific Antibodies

At the time of first evaluation, 12 (40%) patients were negative for both ZIKV NS1 IgM and IgG antibodies; 7 (23%) had only IgM antibodies, 6 (20%) had both IgM and IgG antibodies, and 5 patients (17%), all with previous dengue virus (DENV) infection, had only IgG antibodies. Flavivirus-naive patients developed ZIKV-specific IgM antibodies followed by IgG antibodies, with median times to detection of ZIKV IgM and IgG antibodies estimated as 8 days (IQR, 5–15 days), and 17 days (IQR, 12–26 days), respectively (Figures 2 and 3). At variance, patients with previous dengue infection had already high-level ZIKV IgG at the time of diagnosis, but did not develop detectable ZIKV IgM after infection (Figure 2). However, in these patients, an increase of ZIKV-neutralizing antibody titer was demonstrated between acute and convalescent sera. Patients vaccinated against yellow fever developed ZIKV IgM antibodies, followed by IgG antibodies, as with flavivirus-naive individuals (Figure 2A).

Infection Dynamics in Pregnant Women

Two women were pregnant. The first pregnant woman developed mild symptoms the day after her return from El Salvador, at 10 weeks' gestation. Plasma, urine, and saliva collected 5 days after symptom onset were positive for ZIKV RNA, and infectious virus was isolated from urine. The second pregnant

woman acquired ZIKV infection during a visit to her family in Santo Domingo at 12 weeks' gestation. Laboratory examination conducted 16 days after symptom onset revealed ZIKV RNA in plasma and saliva. In both cases, amniotic fluid collected by ultrasound-guided transabdominal amniocentesis at 20 weeks' gestation was negative, ZIKV RNA remained positive in plasma until delivery, but the virus was not transmitted to the newborns.

DISCUSSION

In this follow-up study, we described the dynamics of ZIKV RNA in body fluids and antibody response in travelers with acute ZIKV infection. Study participants included both symptomatic and asymptomatic individuals, flavivirus-naive individuals, and those with previous DENV infection or yellow fever vaccination, nonpregnant and pregnant women.

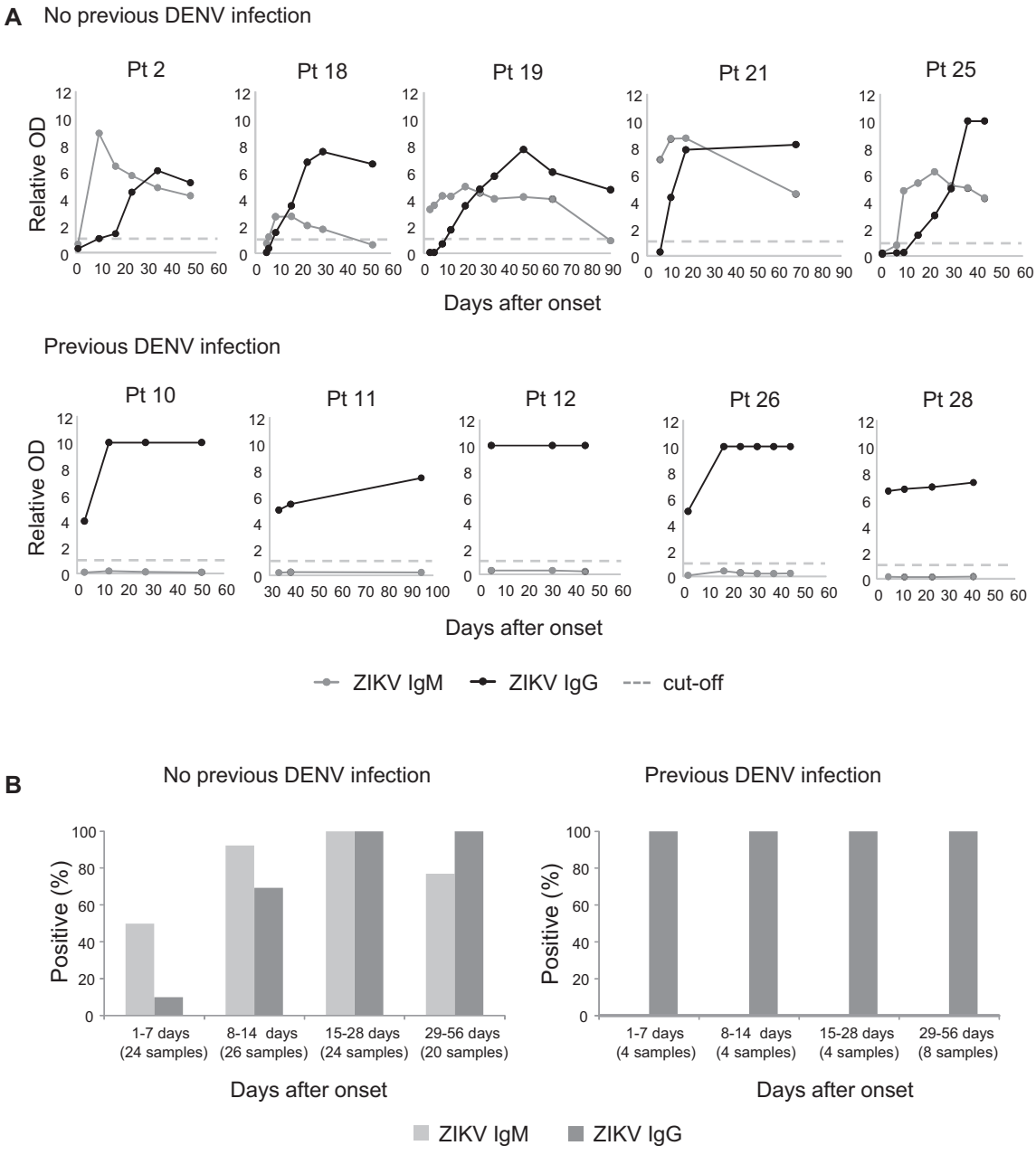

Figure 2. Antibody response to Zika virus (ZIKV) infection. *A*, Anti-ZIKV immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies measured by ZIKV nonstructural protein 1-based enzyme-linked immunosorbent assay during follow-up in patients with acute ZIKV infection. Five representative cases without previous dengue virus (DENV) infection and the 5 patients with a history of previous DENV infection are shown. Patients 19 and 21 were vaccinated against yellow fever. *B*, Percentages of serum samples positive for ZIKV IgM and IgG antibodies obtained during follow-up, at different time intervals after onset, from 30 patients with acute ZIKV infection. Abbreviations: DENV, dengue virus; IgG, immunoglobulin G; IgM, immunoglobulin M; OD, optical density; ZIKV, Zika virus.

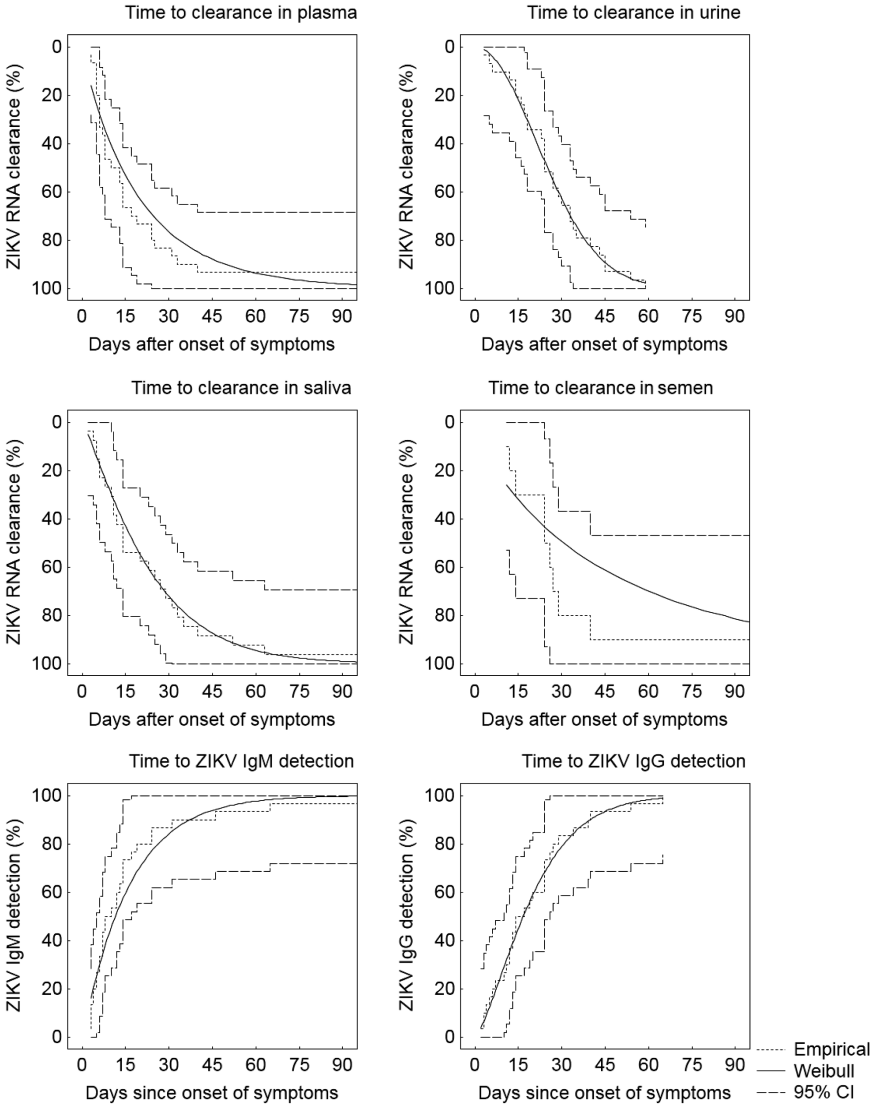

Figure 3. Time to Zika virus (ZIKV) RNA clearance in different body fluids and time to serum ZIKV immunoglobulin M (IgM) and immunoglobulin G (IgG) detection in patients with acute ZIKV infection. Times were estimated by using Weibull regression model, applied to follow-up results. Empirical cumulative distribution function with upper and lower 95% confidence interval and Weibull regression are shown. Times to ZIKV IgM and IgG detection were estimated only in the group of patients without previous dengue virus infection. Abbreviations: CI, confidence interval; IgG, immunoglobulin G; IgM, immunoglobulin M; ZIKV, Zika virus.

ZIKV RNA was detectable in plasma of 57% of participants and the viral load was relatively low. The estimated median time to ZIKV RNA clearance from plasma was 11.5 days (IQR, 6–24 days), but the duration of viremia was longer when ZIKV RNA was tested in whole blood, as observed also by other authors [14, 15]. Notably, 2 pregnant women had persistent low-level viremia until delivery of apparently healthy infants, without evidence of virus transmission. Persistent viremia has been already described in pregnant women, but associated with ZIKV transplacental transmission and fetal microcephaly and hypothesized to result from virus replication in placental-fetal tissues [16, 17]. Both women had a history of previous DENV infection, which has been hypothesized to be a risk factor for ZIKV transplacental transmission and adverse pregnancy outcome [18]. Hoverer, a recent study in a large cohort of pregnant women in Rio de Janeiro did not find any significant association between disease severity, viral load, prior DENV antibodies, and pregnancy outcome [19].

Shedding of ZIKV RNA in urine and saliva (93.1% and 69.2%, respectively) was more frequently detected than viremia and reached peaks with high loads about 1 week after onset of symptoms. Some patients had prolonged viral shedding in urine and saliva, and the median time to clearance was 24 days (IQR, 17–34) and 14 days (IQR, 8–31), respectively. These rates of ZIKV RNA positivity in urine and saliva are higher, while the prevalence of viremia is lower than those reported in a large cohort of patients who were followed up in Puerto Rico [3]. These discrepancies could be explained by differences in

the study population (resident population with high previous exposure to flaviviruses vs travel-associated infection in flavivirus-naive patients) and in the sensitivity of the real-time RT-PCR methods employed. In fact, a study on 53 travelers by the Florida Department of Health, which used the same real-time RT-PCR method of our study, achieved similar results, with detection of ZIKV RNA in 92% of urine specimens, 81% of saliva specimens, and 51% of serum specimens collected on the same day [20].

Finally, shedding of ZIKV RNA was evaluated in semen of 10 participants enrolled in our study, 5 (50%) of whom tested positive and, in some cases, with very high viral load. The estimated median time to ZIKV RNA clearance from semen was 25 days (IQR, 14–29 days). In agreement with our data, other series reported shedding of ZIKV in semen in about 30%–70% of patients with both symptomatic and asymptomatic infection and persistence of the virus in the male genital tract for months after the onset of symptoms [3, 13, 21–25]. A noteworthy finding of our study was the case with detectable ZIKV RNA in semen for 370 days after symptom onset. So far, ZIKV RNA has been detected in semen as late as 188 days (range, 3–188 days) after symptom onset, and infectious virus has been isolated in semen up to 69 days after onset [13, 22, 23]. Cases of late sexual transmission from male to female have been also reported, occurring up to 44 days after the onset of symptoms in the index partner [26]. Considering the low number of reported cases of late sexual transmission, it is conceivable that the risk of sexual transmission of ZIKV beyond 2–3 months after infection is very low. Studies are, however, warranted to determine the replication capacity and viability of ZIKV in the semen of chronically infected patients, to correlate ZIKV RNA load in semen with infectivity, and to evaluate the effect of ZIKV infection on male fertility [25, 27].

Analysis of ZIKV NS1-specific IgM and IgG antibody responses identified different kinetics in flavivirus-naive patients and in those with previous DENV infection: Whereas patients with primary ZIKV infection developed high-titer IgM antibodies at about 8 days after the onset of symptoms, followed by IgG antibodies, patients with previous dengue did not develop detectable ZIKV IgM antibodies, but had already high titer ZIKV IgG antibodies at the time of diagnosis. The absence of IgM antibody response in patients with previous dengue can be ascribed to the high similarities between ZIKV and DENV structural proteins [8], which can lead to cross-reacting antibodies and the so-called "original antigenic sin" phenomenon, according to which a prior exposure to an antigen induces an ineffective or even no response to a related antigen [6, 9]. The relatively low sensitivity of the ZIKV NS1 IgM ELISA used in our study, which has been well documented in validation analyses [28–32], has probably also contributed to the negative IgM results observed in patients with previous DENV infection. Thus, considering the criticalities of DENV and ZIKV serological diagnosis, sensitive and specific antibody tests are needed, especially in areas where these flaviviruses co-circulate [33].

In conclusion, this follow-up study described the dynamics of ZIKV RNA shedding in body fluids and the antibody response in patients with ZIKV infection, improving our understanding of human ZIKV infection and providing useful information to optimize diagnostic algorithms. The small number and heterogeneity of participants, which reduced the power of statistical analyses, are important limitations for this study. This heterogeneity, however, is a good representation of the variable clinical presentation and natural history of ZIKV infection in humans, which can be experienced in real life by physicians involved in the management of patients with suspected ZIKV infection.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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