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Antibody cross-reactivity and evidence of susceptibility to emerging Flaviviruses in the dengue-endemic Brazilian Amazon

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ABSTRACT

Objectives: Several Flaviviruses can co-circulate. Pre-existing immunity to one virus can modulate the response to a heterologous virus; however, the serological cross-reaction between these emerging viruses in dengue virus (DENV)-endemic regions are poorly understood.
Methods: A cross-sectional study was performed among the residents of Manaus city in the state of Amazonas, Brazil. The serological response was assessed by hemagglutination inhibition assay (HIA), enzyme-linked immunosorbent assay, and neutralization assay.
Results: A total of 74.52% of the participants were immunoglobulin G-positive (310/416), as estimated by lateral flow tests. Overall, 93.7% of the participants were seropositive (419/447) for at least one DENV serotype, and the DENV seropositivity ranged between 84.8% and 91.0%, as determined by HIA. About 93% had antiyellow fever virus 17D-reactive antibodies, whereas 80.5% reacted to wild-type yellow fever virus. Zika virus (ZIKV) had the lowest seropositivity percentage (52.6%) compared with other Flaviviruses. Individuals who were DENV-positive with high antibody titers by HIA or envelope protein domain III enzyme-linked immunosorbent assay reacted strongly with ZIKV, whereas individuals with low anti-DENV antibody titers reacted poorly toward ZIKV. Live virus neutralization assay with ZIKV confirmed that dengue serogroup and ZIKV-spondweni serogroup are far apart; hence, individuals who are DENV-positive do not cross-neutralize ZIKV efficiently.
Conclusion: Taken together, we observed a high prevalence of DENV in the Manaus-Amazon region and a varying degree of cross-reactivity against emerging and endemic Flaviviruses. Epidemiological and exposure conditions in Manaus make its population susceptible to emerging and endemic arboviruses.

Introduction

Emerging arboviruses are a growing health problem in Brazil and worldwide. During the last decade, Brazil has endured the zika virus (ZIKV), chikungunya virus, and yellow fever virus (YFV) epi-

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demics. These pathogens have not only been associated with morbidity and mortality but also with additional health care cost. Arboviruses, such as dengue virus (DENV) and YFV, have been endemic in Brazil, whereas YFV is endemic/enzootic for centuries being introduced during the slave traffic. DENV was introduced recently and has been endemic for the last 50 years. However, the recent YFV epidemic in Southeast Brazil demands a better understanding on the transmission dynamics and disease distribution in general population [1].

The introduction of ZIKV in Brazil caused a huge epidemic in some regions of Brazil, which was associated with microcephaly in the newborns [2,3] and Guillain-Barré syndrome and a few fatal cases in adults [4,5]. The high density of *Aedes* mosquitoes in several cities was responsible for the high transmission rates; however, little is understood about the interaction between DENV and ZIKV and the underlying cause of the magnitude of the ZIKV epidemic in Brazil.

Previous DENV infection increases the risk of future dengue with warning signs and severe dengue. Non-neutralizing antibodies against the envelope (E) protein induced during the primary DENV infection can cross-react with other DENV serotypes or heterologous Flaviviruses. These DENV cross-reactive antibodies can facilitate subsequent secondary or tertiary heterologous DENV infection of myeloid cells due the antibody-dependent enhancement (ADE) and can increase dengue disease severity in humans [6]. Similarly, DENV serotypes and other Flaviviruses share peptides that can modulate cluster of differentiation (CD; CD4+ and CD8+) T cell responses upon an infection with a heterologous Flavivirus [7].

In vitro and mouse challenge studies have shown that antibodies raised against DENV can enhance ZIKV infection [8,9]. However, previous DENV infection was not associated with ZIKV viremia or cytokine expression in experimentally challenged macaques [10–12] or in humans [13–15] nor with fetal demise or congenital Zika syndrome in pregnant women [16,17]. Emerging evidence suggests that previous DENV infection may not enhance noncongenital Zika disease, but whether previous ZIKV infection increases future dengue disease in humans remains unknown. In this study, we evaluated the antibody cross-reactivity against Flaviviruses in a dengue-endemic population to understand the role of pre-existing antibodies in modulating secondary heterologous Flavivirus infection. Here, we also estimated the seropositivity rates against 10 different endemic and emerging Flaviviruses in Manaus, capital of the Amazonas state.

Methods

Study population and sample and data collection

The study population comprised healthy individuals accessing services at the Centro de Controle de Zoonoses, Manaus-Amazonas. A total of 450 consecutive participants were recruited between January 2015 and December 2015. The study included individuals of both sexes aged ≥18 years who agreed to participate. Study participants signed the written informed consent form and answered an epidemiological questionnaire. A total of 4 ml of venous blood was drawn from each participant using ethylenediaminetetraacetic acid tubes (BD Vacutainer). Soon after blood collection, the collection tubes were centrifuged, and plasma was separated and stored at -80°C until further analysis.

Sample size calculation

A sample size of 398 individuals was calculated using an estimated prevalence of 50% and 95% confidence interval for a large population with a desired precision of 0.01 [18]. To achieve an adequate sampling without significant dropouts due to incomplete questionnaires and subsequent reduction in statistical power, we recruited 450 individuals. However, three questionnaires were incomplete and removed from the analysis. Sample size calculation was performed using the Epi Info software version 7.2.

Maps and socioeconomic indicators from Brazil

Maps were created using the QGIS Software version 2.18.26 for macOS. Graphs displaying the Human Development Indexes and Sanitation Indicators were extracted from public database, Atlas Brasil from the 2010 Census (https://atlasbrasil.org.br) and the National System of Sanitation Information/Trata Brasil from 2018 (http://www.snis.gov.br), respectively.

Dengue rapid test

Lateral flow point of care (POC) DENV antibodies detection test was performed using the BioPix® Dengue IgM/IgG Rapid test (Wama Diagnóstica, São Paulo, Brazil) at the Laboratory of Emerging Viruses at the University of Campinas (LEVE - Unicamp). The plasma samples were assayed to qualitatively detect immunoglobulin (Ig)G and IgM antibodies against all four DENV serotypes. The assay had a sensitivity of 99% and specificity of 98% as declared by the manufacturer. The detection of IgG in this assay was developed to detect low levels of IgG.

Hemagglutination inhibition test

To assess the overall Flavivirus reactivity, hemagglutination inhibition assay (HIA) was performed at the Evandro Chagas Institute, Belém. The plasma samples collected were subjected to an HIA and adapted to the microplate technique, with a titration cut-off point of 1/20, as previously described [18]. The plasma samples were tested for antibodies against the YFV strain (YFV-17D) and 10 endemic or emerging arboviruses of the Flavivirus genus in the Brazilian Amazon: YFV, DENV serotypes 1-4 (DENV-1, DENV-2, DENV-3, and DENV-4), ZIKV, Saint Louis Encephalitis virus (SLEV), West Nile virus (WNV), Ilheus virus (ILHV), and Rocio virus (ROCV).

Cloning, expression, and purification of recombinant DENV2 and ZIKV envelope domain III proteins

The DENV2 envelope domain III protein sequence (E-DIII, residues 577-674, GenBank number HQ026763) was cloned and expressed using the pET28b vector (Silvia Beatriz Boscardin, University of São Paulo), as previously described [19]. The ZIKV envelope amino acid sequences from different isolates were obtained from GenBank, and multiple sequence alignments were performed to identify an amino acid consensus sequence using MEGA software version 7.0.26. A polyhistidine (6x-His) tag was added at the N-terminal to facilitate downstream purification. Consensus ZIKV E-DIII sequence was codon-optimized and inserted into prokaryotic expression vector pGS21-a (GenOne Biotechnologies, Brazil).

Enzyme-linked immunosorbent assay (ELISA) to detect anti-DENV and ZIKV E-DIII-specific IgG antibodies

We standardized an indirect in-house ELISA to detect anti-DENV and anti-ZIKV IgG antibodies in plasma samples using recombinant E-DIII proteins as antigens, as previously described [20].

ZIKV neutralization test

Focus reduction neutralization test (FRNT) was performed in collaboration with the Laboratory of Emerging Viruses at University of Campinas, as previously described [21]. In brief, the plasma

samples were heated at 56°C for 30 minutes to inactivate the complement system. Four-fold plasma samples diluted with Earle minimum essential medium (Sigma-Aldrich) were incubated with 100 focus forming units of ZIKV (strain BeH823339-Asian). Virus antigen was identified by an indirect immunoassay, and focus forming units were counted for each plasma dilution and the results were tabulated.

Statistical analysis

Descriptive statistics was used to describe the sociodemographic features and mosquito preventive measures of the study population. The missing values were excluded from the calculations. Bubble charts showing the percentages of seropositives with HIA antibody titers were created with Microsoft Excel 2019 software. The 95% confidence intervals were computed using the Blaker method. All statistical analyses were performed using the GraphPad Prism software version 9.1.2. A paired *t*-test was used to evaluate the ELISA results for DENV- and ZIKV-E-DIII. The linear correlation between variables was determined using Pearson correlation. *P*-values $\leq$0.05 were considered statistically significant.

Results

High dengue prevalence in urban Manaus

A random convenience sampling strategy enrolled 450 individuals in Manaus; this noninterventional cross-sectional study between January and December 2015 (Supplementary Figure 1). A total of 447 healthy individuals of both sexes and aged $\geq$18 years were included in the final analysis; three questionnaires were incomplete and removed from the analysis. The demographic features of the study population are described in Table 1. We oversampled females (70.5%) and the median age of our study population was 38 years. A total of 30% of the study participants self-declared that they were unemployed in the last 12 months and 61.70% had completed high school (Table 1). A total of 37.5% and 13.2% of the study participants self-reported a previous dengue and malaria infection, respectively. A total of 80.8% reported vaccination for YFV and 75.9% use SUS-Brazil's public health system. A majority (94.9%) reported performing at least one preventive measure against mosquitoes: avoiding standing water and properly disposing of trash, followed by personal mosquito repellents, which were the most popular measures adapted to prevent mosquitoes (Table 2).

Manaus is a tropical metropolis in Brazil with about 2.5 million inhabitants in the middle of the Amazon rainforest (Figure 1a). Manaus, the capital of the Amazonas state, compared with other Brazilian capitals, shows a low sociodemographic and human development index. In Manaus, the lack of sanitation is also widespread, with wastewater facilitating vector proliferation and waterborne diseases, which affect the poorest residents (Figure 1b). Figure 1c compares our study population age distribution compared with the 2010 census data.

Next, we assessed the prevalence of anti-DENV antibodies using lateral flow POC rapid tests and HIA. A total of 74.52% of participants were IgG-positive (310/416) and 1.68% (15/416) had anti-DENV IgM antibodies, as estimated by POC tests. Overall, 93.7% of participants were seropositive (419/447) for at least one DENV serotype and the DENV seropositivity ranged between 84.8% and 91.0%, as determined by HIA (Figure 1d and Table 3). We observed that most of our participants aged over 20 years had anti-DENV antibodies (Figure 1e and Supplementary Figure 2).

Cross-reactivity between principal endemic and emerging Flaviviruses

We performed HIA, a cell-based assay, to assess the overall cross-reactivity by performing serial dilution of the plasma samples; the results are summarized in Figure 2 and Table 3. YFV is endemic in the Amazon region and vaccination is compulsory. Here, we observed that 92.8% of individuals tested have anti-YFV-17D reactive antibodies; although, only 80.3% self-reported receiving YFV vaccine (Table 2) compared with 80.5% toward the wild-type YFV (Table 3). A total of 37.2% self-reported previous DENV infection, whereas we detected 93.7% of individuals with anti-DENV antibodies against at least one DENV serotype. A chi-square test found a significant difference between the observed (laboratory-confirmed) and expected (self-reported) dengue infection (P <0.0001, χ^2 test, data not shown). Among the DENV serotypes, DENV-2, followed by DENV-1, DENV-3, and DENV-4 had the highest seropositivity (Figure 2 and Table 3). Individuals with high DENV titers correspondingly had higher reactivity to other Flaviviruses (Figure 2a). All samples were collected before or during the ZIKV epidemic in Manaus, and we observed that ZIKV, among all viruses tested, had the lowest seropositivity percentage (Figure 2b). The percentage of individuals reactive toward Flaviviruses classified into serogroups is presented in Table 3. Overall, antibody reactivity was the lowest among the 18-19 years age group and increased rapidly with age for all arboviruses tested (Table 3, Figure 2, and Supplementary Figure 2).

Low cross-reactivity between preexisting anti-DENV antibodies and ZIKV

Next, we evaluated the cross-reactivity to ZIKV among individuals who were DENV-positive using HIA, ELISA, and live virus neutralization assay. First, modified bubble plots compared DENV-1, DENV-2, or ZIKV HIA titers and their distribution for each virus to the reference virus. Upon comparing the HIA titers, we observed that most of the individuals who were DENV-positive with low titers failed to react with ZIKV. Whereas only individuals with elevated titers for DENV-1 or DENV-2 reacted with ZIKV in the HIA assay (Figure 3).

E-DIII is an important target for neutralizing antibodies among Flaviviruses; hence, we performed an ELISA using E-DIII proteins for DENV-2 or ZIKV and assessed antibody reactivities. Similarly, in HIA, we observed that anti-DENV2 E-DIII IgG levels were elevated compared with anti-ZIKV E-DIII IgG antibodies (Figure 4a). A paired analysis of the samples showed a significant decrease in the antibody reactivities between DENV and ZIKV (Figure 4b-c). As expected, anti-DENV E-DIII antibody levels estimated by ELISA significantly increased with age (Figure 4d); and stratified by sex, males showed the highest reactivity toward DENV-2 envelope protein domain III (Figure 4e). Splines depict the relationship between HIA antibody titers and ELISA reactivity (Figure 4f).

Furthermore, to understand the DENV antibody reactivity and its relationship to a possible neutralizing antibody response to ZIKV, we performed an FRNT with live ZIKV in a subset (n = 57) of DENV-positive samples chosen at random. Figure 5a compares DENV-2 or ZIKV ED-III ELISA results with ZIKV FRNT50 values; samples were divided as higher or lower than the median of reactivity toward the DENV-2 ED-III antigen. Primarily, we observed that individuals with anti-DENV E-DIII OD >median (red) neutralized ZIKV more efficiently than individuals with anti-DENV E-DIII OD <median (green). The percentage relative infection of ZIKV was used to calculate neutralizing antibody titers; each patient is represented by one curve (Figure 5b-d). Overall, the DENV-2 E-DIII antibody levels are inversely proportional to the ZIKV FRNT values, and individuals with high DENV reactive antibodies had a potential neutralizing activity against ZIKV. Collectively, these results indi-

Table 1
Sociodemographic features of study population- Manaus, Amazonas.

Characteristic		Value %
Age (years)	18-19	7.80
	20-29	23.10
	30-39	24.70
	40-49	23.60
	≥50	20.80
Sex	Female	70.50
Family size	1	3.50
	2	14.80
	3	24.20
	4	25.60
	≥5	31.90
Number of children in house	0	52.90
	1	29.00
	2	11.30
	3	3.90
	≥4	2.90
Occupation[a]	Unemployed	31.40
	Domestic service workers and manual workers	14.00
	Professionals with university education	10.60
	Professionals or technicians	10.10
	Administrative service workers	10.10
	Poorly specified occupations of informal work	9.60
	Service and commercial workers	8.70
	Member of the armed forces, police and military firefighters	2.50
	Senior government officials or senior corporate officials	1.60
	Art Professional	1.40
Family Income	Minimum wage	22.60
	Two minimum wages	29.80
	Three minimum wages	20.50
	Four minimum wages or more	27.10
Education level	Illiterate	1.30
	Literate	7.40
	Elementary school	9.80
	High school	61.70
	Graduate	19.80

[a] Last 12 months

Table 2
Mosquito prevention practices reported by study participants.

Characteristic		Value %
Dengue infection[a]	Yes	37.5
Malaria infection[a]	Yes	13.2
Vaccinated against Yellow fever[a]	Yes	80.8
Use Sistema Único de Saúde (SUS)[b]	Yes	75.9
Neighbors with dengue fever	Yes	43.3
Mosquito problem in neighborhood	Yes	43.1
Perform preventive measures against mosquitoes	Yes	94.9
Mosquito prevention measures[c]:		
	Avoid standing water	64.30
	Disposing trash correctly	62.50
	Personal mosquito repellents: cream and spray	54.90
	Personal and household hygiene	33.70
	Fans	28.10
	Close windows and doors	21.80
	Mosquito coil, mats and liquid vaporizers	12.60
	Mosquito net for door and window	10.90
	Protective clothing	9.40
	Mosquito bed nets	5.80
	Others	4.30

[a] Self-reported
[b] Brazilian public funded health care system
[c] Multiple responses

cate that both the quantity and quality of DENV-specific responses are necessary to neutralize ZIKV.

Discussion

Endemic and emerging arboviruses are a huge public health concern worldwide not only due to their unexpected clinical manifestations and potential complications but also due to the lack of appropriate diagnostic assays and vaccines [22–24]. In this study, we report one of the highest dengue seropositivity in Manaus-Brazil [18]; 74.52% by POC and 93.7% by HIA. This confirms that most residents of the region have likely been infected with DENV, if not with other arboviruses at least once. Evidence of varying degree of antibody cross-reactivity with principal emerging Flaviviruses depends on the virus serogroup and pre-existing level of antibody titers. Thus, individuals with the highest anti-DENV an-

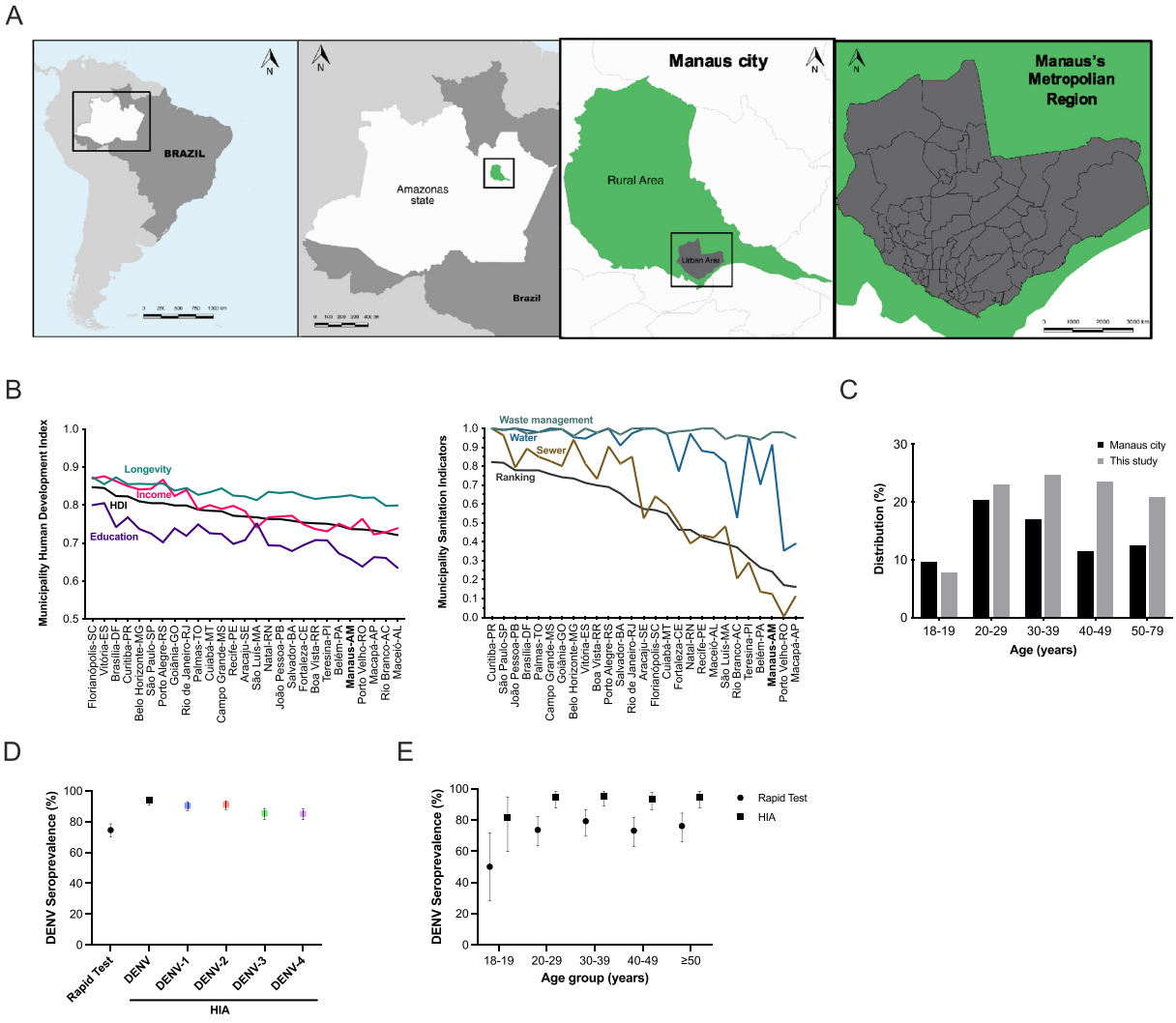

Figure 1. Elevated dengue seropositivity rates in urban Manaus.
(a) Maps depict location of Manaus. (b) HDI (left) and Sanitation indicators (right) for the 27 Brazilian capitals were obtained from the 2010 census and a 2018 survey, respectively. (c) The study population distribution is compared to 2010 Census data obtained from the Brazilian Institute of Geography and Statistics (IBGE). (d-e) Plasma samples were tested for anti-DENV antibodies using BioPix® Dengue Rapid Test IgG/IgM (n = 416) and HIA (n = 447) and seropositivity percentages with 95% confidence interval were plotted. BioPix® Dengue Rapid Test IgG/IgM was qualitative. HIA was quantitative and samples with HIA titer ≥20 units were considered positive for the test virus.
DENV, dengue virus; HDI, Human Development Index; HIA, hemagglutination inhibition assay.

Table 3
Prevalence of anti-Flavivirus antibodies.

Serogroup	Virus[c]	Total[a] %	Age (years)				
			18-19	20-29	30-39	40-49	≥50
Yellow **f**ever **v**accine	YFV/17D	92.8 (415/447)	67.6 (23/34)	98.0 (99/101)	95.4 (103/108)	93.2 (96/103)	92.3 (84/91)
YFV Group	YFV	80.5 (360/447)	61.8 (21/34)	77.2 (78/101)	83.3 (90/108)	79.6 (82/103)	86.8 (79/91)
DENV Group	DENV[b]	93.7 (419/447)	79.4 (27/34)	95.0 (96/101)	95.4 (103/108)	94.2 (97/103)	95.6 (87/91)
	DENV-1	90.6 (405/447)	76.5 (26/34)	93.1 (94/101)	91.7 (99/108)	88.3 (91/103)	93.4 (85/91)
	DENV-2	91.0 (407/447)	82.3 (28/34)	93.1 (94/101)	92.6 (100/108)	88.3 (91/103)	92.3 (84/91)
	DENV-3	85.5 (382/447)	82.3 (28/34)	86.1 (87/101)	87.0 (94/108)	84.5 (87/103)	83.5 (76/91)
	DENV-4	84.8 (379/447)	64.7 (22/34)	87.1 (88/101)	88.0 (95/108)	84.5 (87/103)	84.6 (77/91)
Spondweni Group	ZIKV	52.6 (235/447)	32.3 (11/34)	48.5 (49/101)	55.5 (60/108)	54.4 (56/103)	59.3 (54/91)
Japanese Encephalitis Virus Group	WNV	81.9 (366/447)	55.9 (19/34)	84.2 (85/101)	88.0 (95/108)	78.6 (81/103)	84.6 (77/91)
	SLEV	86.3 (386/447)	64.7 (22/34)	87.1 (88/101)	89.8 (97/108)	84.5 (87/103)	90.1 (82/91)
Ntaya Virus Group	ILHV	85.7 (383/447)	64.7 (22/34)	85.1 (86/101)	90.7 (98/108)	83.5 (86/103)	89.0 (81/91)
	ROCV	75.8 (339/447)	70.6 (24/34)	74.3 (75/101)	72.2 (78/108)	79.6 (82/103)	78.0 (71/91)
Negatives		3.4 (15/447)	11.8 (4/34)	4.0 (4/101)	0.9 (1/108)	3.9 (4/103)	2.2 (2/91)

[a] Hemagglutination Inhibition titer ≥1:20
[b] Positive for at least one DENV serotype
[c] *Flaviviridae* (Flavivirus genus): YFV, DENV serotypes 1 to 4 (DENV-1, DENV-2, DENV-3 and DENV-4), ZIKV, WNV, SLEV, ILHV, ROCV.DENV, dengue virus; ILHV, ilheus virus; ROCV, rocio virus; SLEV, Saint Louis Encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus; ZIKV, zika virus.

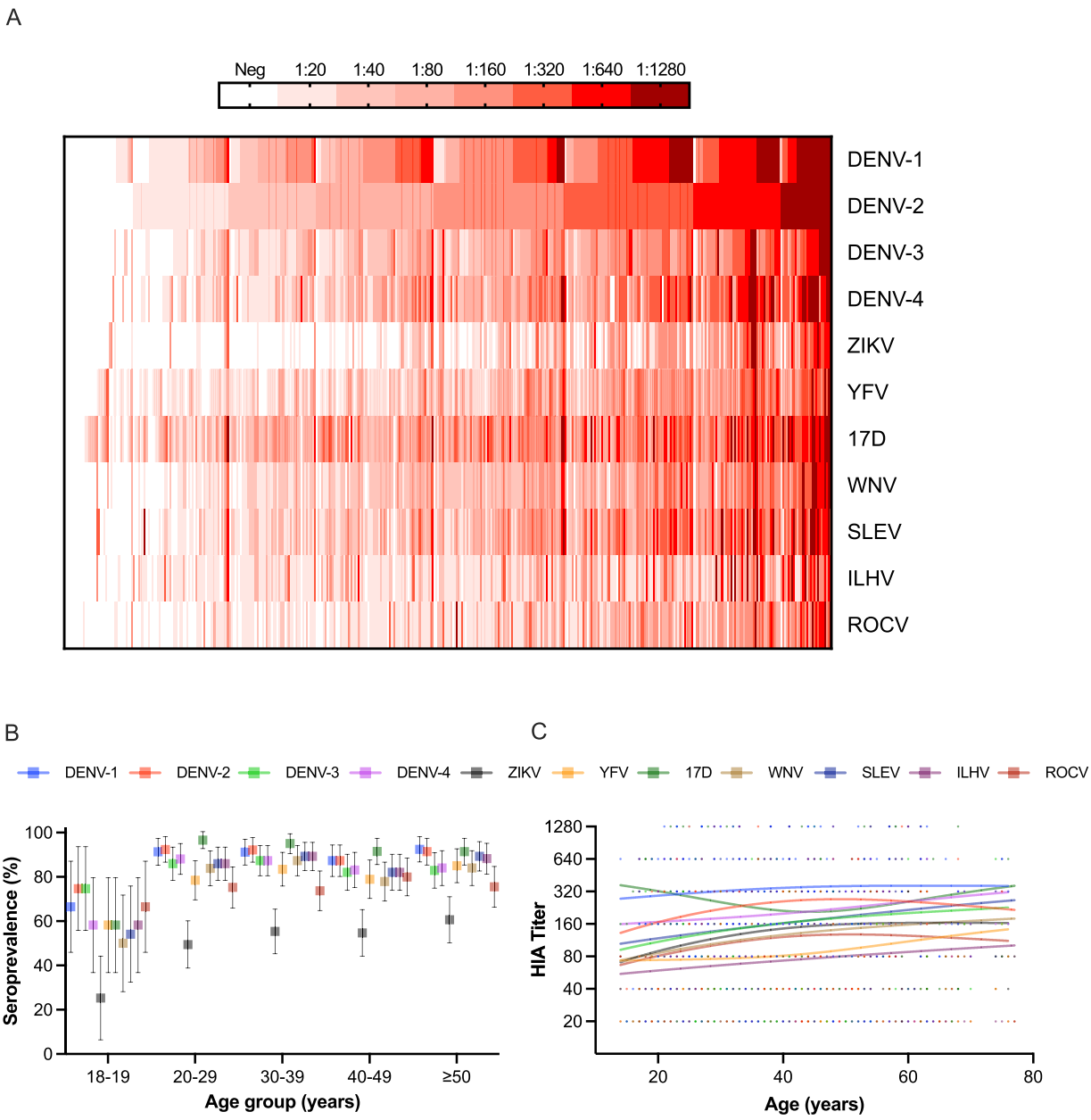

Figure 2. Antibody reactivity profile against emerging and re-emerging human Flaviviruses.
Samples (n = 447) were tested for antibodies against 10 different endemic and emerging Flaviviruses and yellow fever vaccine strain using the HIA. (a) Colors depict the HIA titers, each column represents one patient, and each row is one test virus on the heat map. Samples described in the heatmap were sorted as per DENV-2 HIA titers. (b) Samples with HIA titer ≥20 units were considered positive and plotted as percentage positives among the study age groups. (c) HIA titers were compared to participant age. Solid lines are splines for each test virus.
DENV, dengue virus; HIA, hemagglutination inhibition assay; ILHV, ilheus virus; ROCV, rocio virus; SLEV, Saint Louis Encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus; ZIKV, zika virus.

tibody titers could cross-neutralize ZIKV more efficiently than individuals with low anti-DENV titers. Elevated dengue prevalence in Manaus and the Amazon region does not undermine the threat from other endemic and emerging arboviruses.

Dengue is endemic in Brazil for at least 50 years, and without adequate prevention and treatment, the number of reported cases of dengue has increased in Brazil and other countries in the Americas [25,26]. In our previous study, we observed an increase in seroprevalence in several Brazilian cities in the last decades [18]. On the contrary, dengue incidence levels have been the same over the last decade in most regions; nevertheless, there has been a huge increase in severe dengue disease cases and deaths [25–28]. Three of four Brazilian municipalities are heavily infested with the mosquito *Aedes aegypti*, which highlights the staggering epidemiological and economic burden in the endemic regions [22,29]. Thus, the prospects of controlling dengue or other arbovirus diseases are not promising. All four DENV serotypes have been detected in Manaus, with the late introduction of DENV-3 and DENV-4 [18,30,31]. Our serological results demonstrate a lower seropositivity toward DENV-3 and DENV-4 than DENV-2 and DENV-1. The high DENV prevalence in urban Manaus observed in this study is in line with our previous observations from other Brazilian cities

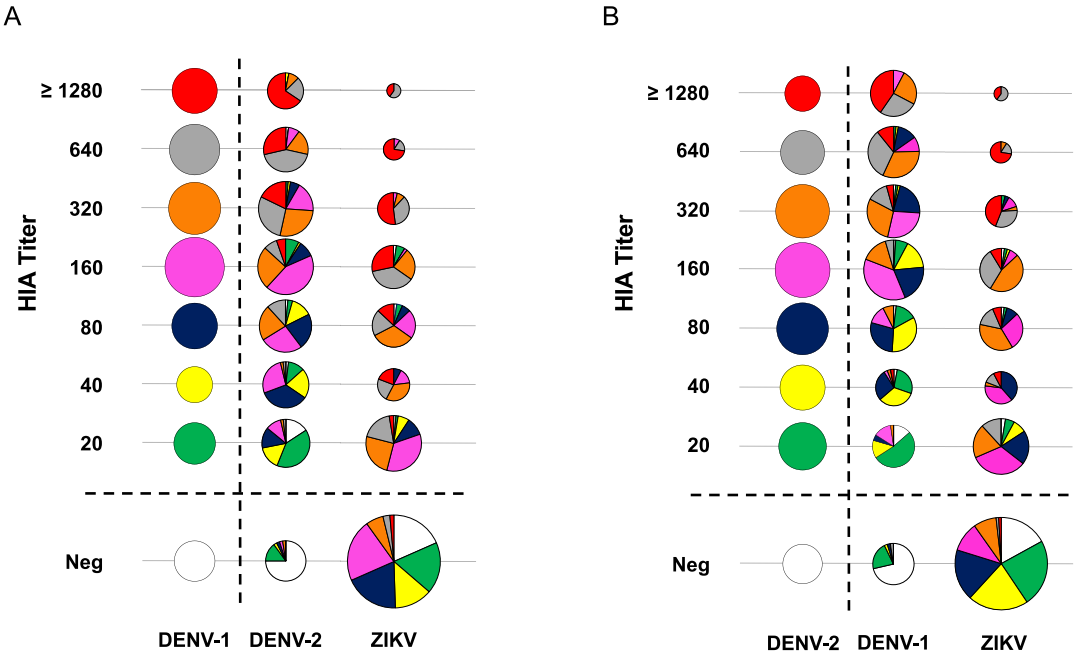

Figure 3. Low cross-reactivity between pre-existing antidengue antibodies and ZIKV.
Modified bubble charts compare cross-reactivity (n = 447), (a) DENV-1 with DENV-2 or ZIKV, and (b) DENV-2 with DENV-1 or ZIKV. All samples with HIA titer ≥20 units were considered positive. Bubble size is proportional to the percentage of individuals with the corresponding HIA titer, and each column adds up to 100%. HIA titers are color coded for the first reference virus column for comparison. Each pie chart represents the composition of HIA titer for the reference test virus.
DENV, dengue virus; HIA, hemagglutination inhibition assay; ZIKV, zika virus.

[18]. A total of 37.2% of individuals self-reported a previous DENV infection compared with over 75% individuals with detectable antibodies in this study. This disparity between the observed and expected numbers could be largely related to asymptomatic or mild cases that have gone unnoticed and highlights the need for more laboratory diagnosis of febrile cases.

Yellow fever vaccination is compulsory in Manaus and in the Amazon region, and 92.8% of the study participants had anti-YFV-17D reactive antibodies, whereas 80.5% had anti-YFV reactive antibodies against the wild-type virus. ILHV and ROCV have been endemic in Brazil; however, their spread in the Amazon region and prevalence studies are lacking. In this current study, the Ntaya virus group, represented by ILHV and ROCV, had a seropositivity rate of 85.7% and 75.8%, respectively. Recently, WNV [32,33] and SLEV [34,35] cases have been reported among horses in Brazil, but no human cases have been reported. The co-circulation of these arboviruses that share B and T cell epitopes provides critical insight into the role of pre-existing immunity essential to assess

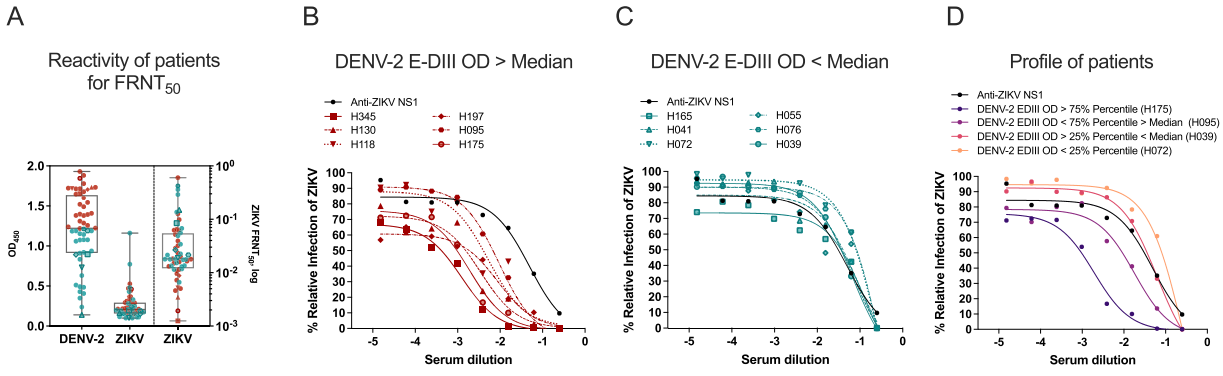

Figure 5. High titers of DENV reactive antibodies neutralize ZIKV.
Focus reduction neutralization test (FRNT) for ZIKV was performed to estimate neutralizing antibodies in patient plasma samples (n = 57). (a) DENV-2 or ZIKV E-DIII protein specific immunoglobulin G reactivities are depicted in first two columns. Box and whiskers indicate the median lines, 25 and 75- interquartile and min-to-max points. Red dots denote samples with anti-DENV-2 E-DIII reactivity values greater than the median value and green dots denote samples displaying anti-DENV-2 reactivity less than the median value. Third column in the graph represents the 50-percent FRNT (FRNT50) values for ZIKV. Right y-axis is FRNT50 values in log for ZIKV. ZIKV antibody neutralization dose-response curves were plotted with (b) high (OD450 > median) or (c) low (OD450 < median) DENV-2 E-DIII antibody reactivity. Each curve represents one patient sample diluted serially. Anti-ZIKV NS1 monoclonal antibody was used as control. (d) Representative neutralization dose-response curves were plotted based on DENV-2 E-DIII antibody reactivity.
DENV, dengue virus; E-DIII, envelope protein domain III; FRNT, focus reduction neutralization test; OD_{450}, optical density, 450 nm; ZIKV, zika virus.

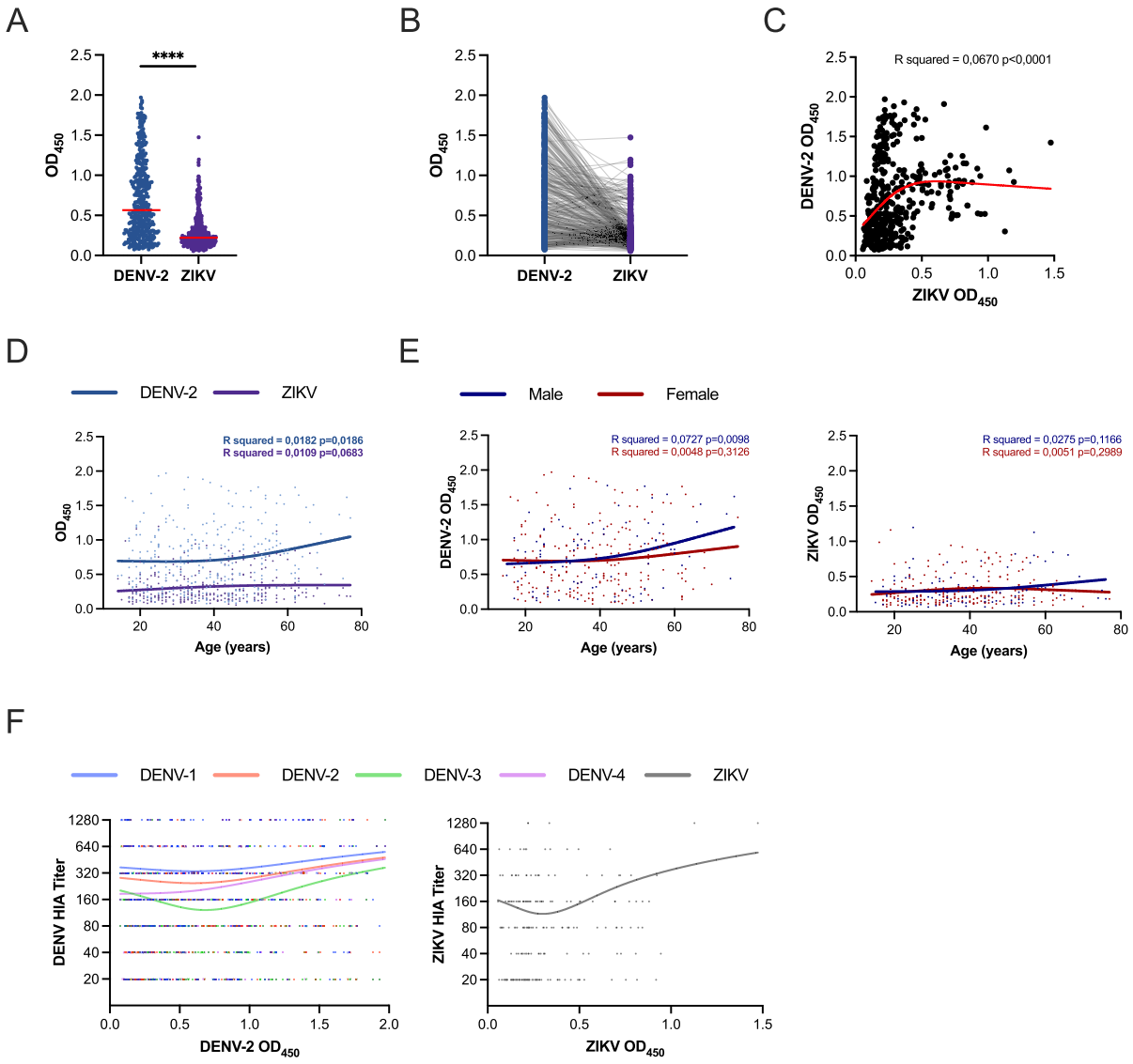

Figure 4. Reactivity between DENV and ZIKV envelope domain III proteins.
ELISA plates were coated with DENV-2 or ZIKV envelope domain III (E-DIII) proteins to detect immunoglobulin G antibodies in human plasma samples (n = 416). (a) Range of antibody reactivity toward E-DIII antigens is depicted. The red horizontal lines denote median OD (450 nm) values. (b) Pairwise comparison denotes the reactivity toward DENV-2 and ZIKV. Mann Whitney or paired *t*-test, **** $P < 0.0001$. (c) DENV-2 and ZIKV anti-E-DIII antibody levels measured by ELISA were compared, red line in the graph represents the spline fitting for the distribution. (d) DENV-2 or ZIKV E-DIII reactivity was compared to participant (d) age or (e) sex. Solid lines represent splines fit for the antibody distribution. Pearson's correlation and *P*-values are denoted in the graphs. (f) HIA tiers for dengue virus serotypes are compared between DENV-2 or ZIKV E-DIII reactivity determined by ELISA. Each solid line is color coded and represents one test virus. Each dot represents one sample in all the graphs.
DENV, dengue virus; E-DIII, envelope protein domain III; ELISA, enzyme-linked immunosorbent assay; HIA, hemagglutination inhibition assay; OD_{450}, optical density, 450 nm; ZIKV, zika virus.

the clinical implications of the disease. Infection with DENV can protect against the onset of apparent signs and severe illness after subsequent JEV, SLEV, and WNV infections [36–38]; ZIKV infection also confers protection against subsequent WNV infection and associated symptoms [39]; JEV and SLEV infections can protect against severe disease and mortality after WNV and DENV infections [40–43]; and ILHV and SLEV can elicit cross-protection against a lethal ROCV challenge [44]. Compulsory YFV vaccination and the high prevalence of DENV in Manaus may partially explain the lack of evidence for co-circulation of other Flaviviruses. This cross-protection may partly also explain why YFV is not present in India and other Asian countries, which are endemic to WNV, JEV, DENV, and other Flaviviruses [45]. On the other hand, the recent ZIKV outbreak in India demonstrates that cross-protection might depend on the residual concentration of cross-reactive antibodies, suggesting that individual antibody titer set point rather than waning could indicate disease risk [46,47].

The samples used in this study were collected before or during the ZIKV epidemic in Brazil. In this current study, 52.6% had anti-ZIKV antibodies, the lowest seropositivity rate among all the viruses tested by HIA. Also, E-DIII ELISA demonstrated a weak reactivity toward ZIKV among individuals who were DENV-positive. Thus, a substantial proportion of participants showed evidence of exposure to DENV but not ZIKV [48]. Moreover, individuals who were DENV-positive with low HIA titers failed to react with ZIKV and only high-titer DENV plasma samples reacted with ZIKV. The role of previous DENV infection in ADE has been controversial; nevertheless, there is little evidence of severe disease after ZIKV in-

fection in individuals who were DENV-positive [8–17]. The patterns of antibody cross-neutralization suggest that ZIKV lies outside the DENV serocomplex [49]; similarly, we observed that HIA was able to distinguish ZIKV from DENV infections when all viruses were analyzed simultaneously. We observed a lack of durable cross-neutralizing antibody response against ZIKV from individuals who were DENV-positive, as previously reported [50]. In this study, patients with the highest anti-DENV antibody titers neutralized ZIKV more efficiently, which is in line with a previous report demonstrating that individuals with elevated DENV titers due to repeated exposure to dengue virus elicit robust cross-neutralizing antibodies against ZIKV [51].

In Manaus, a high prevalence of DENV serotypes and compulsory YFV vaccination together may provide a strong increase in homologous and heterologous cross-neutralizing antibodies. Zika vaccination in an individual who had DENV has been shown to boost pre-existing Flavivirus immunity and elicit protective responses against both ZIKV and DENV [52]. On the other hand, tick-borne encephalitis virus vaccination in YFV vaccinated individuals caused a significant reduction in the tick-borne encephalitis-specific neutralizing antibodies [53]. T cell responses seem to be robust between Flaviviruses and may, at least in part, explain the cross-protection seen against ZIKV from DENV infection [54]. Nevertheless, the durability of humoral and cellular response needs to be assessed in longitudinal studies to determine the role of the pre-existing immunity in protection or pathology.

Due to many positive samples, we were unable to perform a statistical analysis to identify epidemiological risk factors. Our convenience sampling oversampled females which impede calculation of true dengue prevalence for Manaus city; however, our results demonstrate that urban Manaus DENV prevalence is high; most of the individuals get infected before attaining age 36 years and the male sex had higher antibody titers. We used DENV2 ED-III protein in ELISA to compare with ZIKV ED-III response and did not test with other dengue serotypes; nevertheless, we believe that a similar relationship will be observed because DENV and ZIKV belong to distinct serogroups. Although DENV-neutralizing serotype-specific antibodies are mainly against E-DIII, there might be a sustainable antibody response against other targets, such as domains I/II of the envelope protein, which were not evaluated in this study. We also did not have history of hospitalization or severe disease caused by dengue among our study participants to correlate with the humoral response. A longitudinal study in Nicaragua comparing DENV and ZIKV infection observed that primary infection increases the cross-reactive antibodies, which decline slowly over time; however, postsecondary infection the decay rate is much slower [46]. In addition, secondary infection with a different DENV serotype or ZIKV infection can alter the antibody kinetics and levels [49]. In this cross-sectional study, we were able to map the cross-reactivity for emerging and endemic Flaviviruses. However, a longitudinal study would be necessary to understand the persistence of antibody titers after viral infection and the role of reinfection to maintain or increase immune response to the exposed pathogens.

In conclusion, we observed a low cross-protection among individuals who were DENV-positive to ZIKV. However, further longitudinal studies are needed on ADE and the possible role of cross-reactive anti-DENV antibodies in Flavivirus pathogenesis. ZIKV and other emerging Flaviviruses will become increasingly prevalent in DENV-endemic regions, raising the possibility that pre-existing immunity to one virus could modulate the response to a heterologous virus; although, whether this would be beneficial or detrimental remains unclear and could vary with the combination of the viruses prevalent in the region. The COVID-19 pandemic has negatively affected mosquito control measures; in addition, financial resources have been diverted toward the pandemic, making control measures more difficult. Currently, Brazil and the Amazonian region face a complex epidemiological scenario characterized by simultaneous circulation of several arboviruses and high mosquito density; hence, a functional vector control program and febrile syndrome surveillance are essential to identify unforeseen epidemiological threats.

Declaration of competing interest

The authors have no competing interests to declare.

CRediT authorship contribution statement

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Ethical approval

This observational and cross-sectional study was approved by the research ethics committee of the Universidade Federal do Amazonas, with approval number CAAE 96171218.7.0000.5020, in accordance with the Brazilian law, which complied with the Declaration of Helsinki. All study participants gave oral and written informed consent before enrollment.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2023.01.033.

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