Cross-Protection of Dengue Virus Infection against Congenital Zika Syndrome, Northeastern Brazil

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The Zika virus outbreak in Latin America resulted in congenital malformations, called congenital Zika syndrome (CZS). For unknown reasons, CZS incidence was highest in northeastern Brazil; one potential explanation is that dengue virus (DENV)–mediated immune enhancement may promote CZS development. In contrast, our analyses of historical DENV genomic data refuted the hypothesis that unique genome signatures for northeastern Brazil explain the uneven dispersion of CZS cases. To confirm our findings, we performed serotype-specific DENV neutralization tests in a case–control framework in northeastern Brazil among 29 Zika virus–seropositive mothers of neonates with CZS and 108 Zika virus–seropositive control mothers. Neutralization titers did not differ significantly between groups. In contrast, DENV seroprevalence and median number of neutralized serotypes were significantly lower among the mothers of neonates with CZS. Supported by model analyses, our results suggest that multitypic DENV infection may protect from, rather than enhance, development of CZS.

The 2015–2016 Zika epidemic in Brazil was associated with congenital malformations summarized as congenital Zika syndrome (CZS) (*1*–*6*). As a consequence, abortion requests and pregnancy delays increased dramatically in Brazil and all of Latin America (*7*,*8*). For unknown reasons, CZS incidence was highest in northeastern Brazil (*1*,*4*,*9*,*10*). In Asia and Africa, where Zika virus circulated for much longer than it did in the Americas, Zika virus infections have not been consistently linked to CZS development; only sporadic cases have been reported (*11*). Thus, CZS development might be affected by ≥1 cofactor (*9*,*12*). The hypothetical list of cofactors affecting CZS development includes maternal vaccination history (*12*), exposure to larvicides (*5*), or socioeconomic factors (*1*,*13*).

Similar to the ubiquitous dengue virus (DENV), which occurs as 4 distinct serotypes, Zika virus is a flavivirus (*14*). Secondary DENV infections can be more severe than primary infections because of antibody-dependent enhancement (i.e., heterotypic subneutralizing antibodies enhancing virus entry into poorly susceptible cells) (*15*,*16*). Zika virus infection can also be enhanced by DENV antibodies in vitro (*17*,*18*) and in mice (*19*). Thus, DENV-mediated antibody-dependent enhancement may be a major cofactor of CZS development in humans (*19*–*21*). However, antibody-dependent enhancement was not observed in experimentally Zika virus–infected nonhuman primates (*22*) and during pivotal epidemiologic studies from Brazil that assessed neither individual DENV serotypes nor microcephaly cases (*5*,*9*,*23*). In addition, DENV is ubiquitous in all regions of Brazil (*20*). Therefore, to explain the accumulation of CZS cases in northeastern Brazil, a hypothetical DENV-mediated effect enhancing CZS development would require region-specific differences in past DENV exposure. To investigate the role that preexisting DENV immunity has in CZS development, we conducted serologic testing in a nested case–control framework and analyzed historical DENV genomic data from Brazil.

Materials and Methods

Study Population

We compared 29 mothers of children born with CZS (cases) and 108 mothers of children born without CZS (controls) from Salvador, northeastern Brazil. All mothers had

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evidence of past Zika virus exposure, determined by use of ELISAs and plaque-reduction neutralization tests (PRNTs) as described previously (*13*). Samples were collected consecutively at the time of delivery from May 2015 through December 2016 at the University of Bahia Climério de Oliveira maternity ward (Appendix Figure, https://wwwnc.cdc.gov/EID/article/25/8/19-0113-App1.pdf). The study was approved by the Institutional Research Ethics Board under protocol no. 1.408.49, and all women delivering during that period accepted participation in the protocol. Age distributions of cases and controls did not differ significantly (median age 26 years for cases, interquartile range 22.0–33.5; median age 29 years for controls, interquartile range 23.3–34.0; p = 0.26 by *t*-test).

Diagnosis of CZS

CZS was diagnosed by attending gynecologists. Lead symptoms of CZS, as defined by Moore et al. (*24*), included microcephaly and other neurologic birth defects (e.g., intracranial calcifications, ventriculomegaly, dysgenesis of the corpus callosum, Dandy-Walker–like malformations, hydranencephaly, porencephaly, hydrocephalus, severe intracranial calcifications, and decreased brain tissue) (*13*). Microcephaly was identified when the measurement of the cephalic circumference was 2 SDs below that of neonates of the corresponding gestational age, according to intergrowth charts from the World Health Organization in addition to clinical and imaging data.

PRNTs

For the serotype-specific PRNT for DENV, we used 3 µL of heat-inactivated serum (56°C, 30 min) diluted by using Dulbecco modified Eagle medium, supplemented with 1% fetal calf serum at 1:50, 1:150, 1:450, 1:1,350, 1:4,050, and 1:12,150. We split serum dilutions into 4 equal aliquots and incubated them separately in 96-well plates with 60 PFUs of DENV serotypes 1–4 (Appendix Table 1) for 60 min at 37°C. Next, we incubated the virus/serum mixtures for 90 min at 37°C in 5% CO_2 on Vero cells grown in 24-well plates, followed by a methylcellulose/minimum essential medium overlay (2% fetal calf serum, 1.2% final methylcellulose concentration). After incubation for 4 days (DENV-1, -3, and -4) or 5 days (DENV-2), we performed formaldehyde fixation, crystal violet staining, and plaque counting. We calculated neutralizing antibody titers by using the built-in variable slope model in GraphPad Prism 6 (GraphPad Software, LLC, https://www.graphpad.com). Any titer ≥1:10 that reduced DENV PFU by ≥90% compared with control titers was considered positive. PRNT is the standard for flavivirus serology. DENV vaccine studies commonly rely on 50% plaque reduction to determine DENV serotype-specific antibody responses (*25*). To minimize the effect of potential cross-reactivity between DENV serotypes on our results, we selected a less sensitive but highly specific 90% PRNT ($PRNT_{90}$).

Phylogenetic Analyses

For phylogenetic analyses, we retrieved all DENV sequences available from GenBank as of June 15, 2018, that contained information on year and place of isolation. We constructed neighbor-joining trees in MEGA7 (*26*) by using a percentage distance method, a pairwise deletion option, and 1,000 bootstrap replicates. We analyzed either the junction of the envelope and the nonstructural protein 1 (NS1) encoding regions (polyprotein gene positions 2215–2454) or a fragment of 561 nt within the NS1 region (polyprotein gene positions 2650–3210). For clarity of presentation, we excluded sequences of <0.5% mutual nucleotide sequence distance. To show different genotypes, we included selected reference strains (Appendix Table 2). All DENV genome positions given within this article refer to a DENV prototype strain available in GenBank under accession no. KC294223.

Confirmation of DENV Strains Used for PRNT

We confirmed the designation and serotype of DENV strains applied for PRNT by Sanger sequencing of the prM-C domains using strain-specific oligonucleotide primers. These primers are available upon request.

Statistical Analyses and Visualization of PRNT Results

To plot PRNT results, we used GraphPad Prism 6. All p values result from 2-tailed tests. For power calculations, we used OpenEpi version 3 (*27*) for 2-sided 95% CIs. Regression lines were calculated by using a least squares (ordinary) fit method.

Model Testing

To compare the effects of different factors on CZS formation, we tested mathematical logistic regression models. Each model considered 1 defined variable to predict the binary outcome as CZS case or control. We included for testing binary predictor variables such as presence or absence of DENV-1 neutralization, as well as ordinal (e.g., number of neutralized DENV serotypes) or continuous predictor variables, such as DENV-1 PRNT titers. Cases were coded as 1 and controls as 0. We fitted 15 models by using the generalized linear model function of R version 3.5.2 (https://www.r-project.org). To compare different models, we calculated the Akaike information criterion (AIC), the difference between a given and the best-supported model in AIC, and the Akaike weights by using the bbmle package version 1.0.20 in R. To show which models allow significant CZS case prediction, we calculated likelihood ratio tests for each model, and to show the effect strength of the models, we calculated odds ratios.

Results

After the reinfestation of Brazil with the main DENV vector, *Aedes aegypti* mosquitoes, in 1976 (*28*), DENV-1 was introduced in 1986 (*29*), DENV-2 in 1990 (*30*), and DENV-3 in 2000 (*31*); DENV-4 reemerged in 2007 after an absence of 25 years (*32*) (Figure 1). At most, 4 years after their first detection in other regions of Brazil, all 4 DENV serotypes were found in northeastern Brazil. In the databases, we identified 992 unique DENV sequences from Brazil that we used to analyze genomic DENV signatures hypothetically segregating the northeast and other regions in Brazil. Analyses of the envelope-NS1 junction, which is frequently used for genome-based serotyping (*33*), revealed high genetic identity of DENV strains from the northeast and other regions of Brazil during 30 years (Figure 2, panel A). A single DENV-4 clade apparently was found uniquely in northeastern Brazil during 2011–2015 (Figure 2, panel A). Nonetheless, these DENV-4 strains were closely related to strains from other regions when a different, larger partial NS1 region was analyzed (Figure 2, panel B). In summary, our analyses showed no phylogenetic evidence for a unique DENV signature segregating northeastern Brazil from other regions.

Low DENV antibody titers have been shown to be a risk factor for severe disease with heterotypic DENV infection (*15*). Therefore, we analyzed the magnitude of DENV antibody titers. Overall median reciprocal $PRNT_{90}$ titers within this study were 56.5 (95% CI 42.0–79.0) for cases and 61.4 (95% CI 54.3–73.1) for controls. Serotype-specific titers were 68.7 (95% CI 51.2–83.2) for DENV-1, 102.8 (95% CI 79.6–130.6) for DENV-2, 44.8 (95% CI 35.3–55.8) for DENV-3, and 52.6 (95% CI 41.9–66.6) for DENV-4. DENV titers did not differ significantly between cases and controls or between serotypes (Figure 3, panel A). However, we have previously shown that Zika virus antibody titers are significantly higher among mothers of neonates with CZS than among mothers of neonates without evidence of CZS (*34*), hypothetically affecting DENV antibody titer estimates. In our cohorts, Zika virus titers did not correlate with DENV titers (Figure 3, panel B) or with the number of neutralized DENV serotypes ($p = 0.8459$ by analysis of variance) (Figure 3, panel C), suggesting robustness of our results irrespective of individual Zika virus PRNT titers.

Strikingly, the overall DENV seroprevalence was significantly lower among cases, at 65.5%, than among controls, at 91.7% ($p = 0.0003$ by χ^2 test; power 90.4%). For each DENV serotype, seroprevalence was also consistently higher among cases than controls (Figure 4, panel A). The relatively lower seroprevalence of DENV-3 and DENV-4 compared with DENV-1 and DENV-2 among study participants is consistent with the shorter circulation of these viruses in Brazil (Figure 1), again suggesting robustness of our data. Last, the median number of neutralized DENV serotypes was significantly lower among cases than among controls ($p<0.0004$ by Mann-Whitney U test; power 94.8%) (Figure 4, panel B). Only 27.6% of cases, compared with 50.9% of controls, had neutralizing antibodies against all 4 serotypes. Predominance of multitypic DENV exposure among controls over cases was consistently observed among participants in all age groups (Figure 4, panel C).

We conducted generalized linear model analyses to compare the effects of various factors on CZS formation. For model analysis, we considered factors that differed significantly between cases and controls in bivariate comparisons and factors that did not differ significantly. Factors included the presence and titers of neutralizing antibodies against specific DENV serotypes and the overall number of neutralized serotypes. We created 15 logistic regression models, each considering 1 factor potentially affecting CZS development (Table). With our data applied, the models considering neutralization of ≥2 DENV serotypes (AIC = 130.4) or the cumulative number of neutralized DENV serotypes showed the highest support (AIC = 130.6).

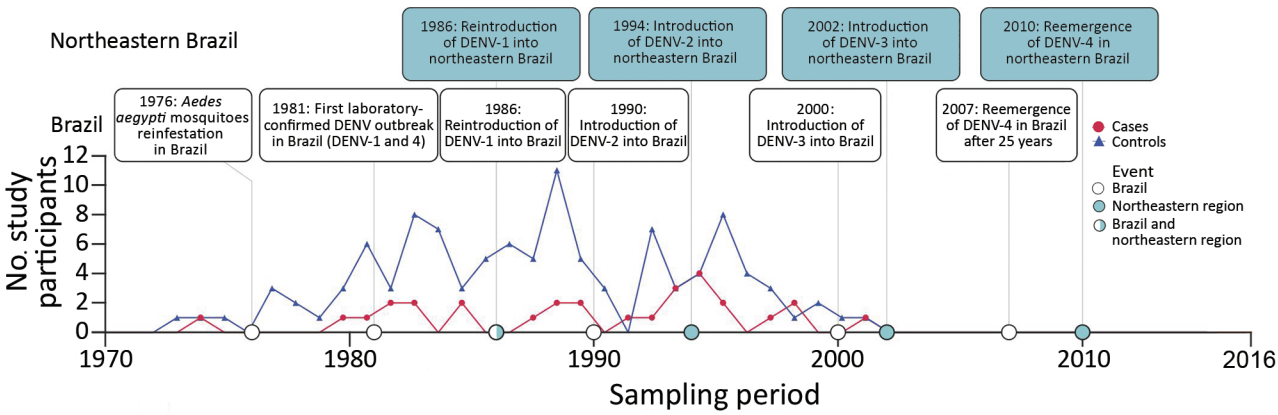

Figure 1. Timeline of dengue virus introduction in Brazil and birth years of participants in study of dengue virus cross-protection against congenital Zika syndrome, northeastern Brazil. DENV, dengue virus.

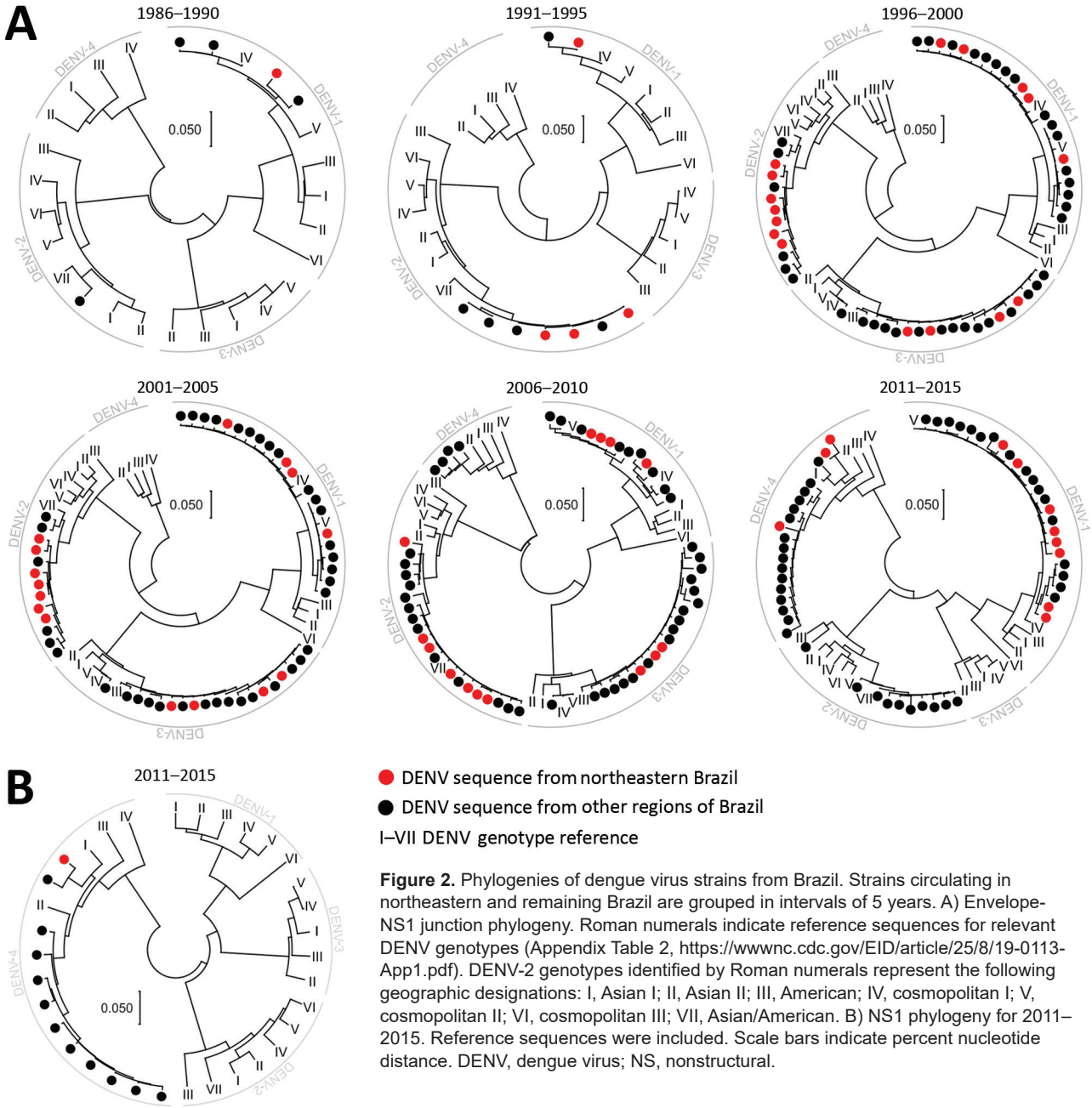

Figure 2. Phylogenies of dengue virus strains from Brazil. Strains circulating in northeastern and remaining Brazil are grouped in intervals of 5 years. A) Envelope-NS1 junction phylogeny. Roman numerals indicate reference sequences for relevant DENV genotypes (Appendix Table 2, https://wwwnc.cdc.gov/EID/article/25/8/19-0113-App1.pdf). DENV-2 genotypes identified by Roman numerals represent the following geographic designations: I, Asian I; II, Asian II; III, American; IV, cosmopolitan I; V, cosmopolitan II; VI, cosmopolitan III; VII, Asian/American. B) NS1 phylogeny for 2011–2015. Reference sequences were included. Scale bars indicate percent nucleotide distance. DENV, dengue virus; NS, nonstructural.

Regarding the presence of serotype-specific neutralizing antibodies, models considering DENV-4 (AIC = 131.4) and DENV-2 (AIC = 131.5) neutralization showed the best support. Models considering age or antibody titers showed relatively lower support with AICs >140. Of all tested models, the model considering neutralization of ≥2 DENV serotypes showed the highest reduction of CZS risk by 84.2% (95% CI 60.5%–93.8%). Of those models considering nonbinary factors, the cumulative number of neutralized DENV serotypes showed the highest reduction of CZS risk by 42.3% (95% CI 23.7%–56.8%) per increase of neutralized serotype. The model considering neutralization of 1 DENV serotype only as a risk factor was not supported statistically or by AIC.

Discussion

Contrary to a large body of in vitro data, our epidemiologic data strongly suggest cross-protection from CZS development by multitypic DENV immunity. The protective effect was observed in bivariate comparisons and in model analyses. Our interpretation is consistent with anecdotal evidence reporting near-complete lack of DENV

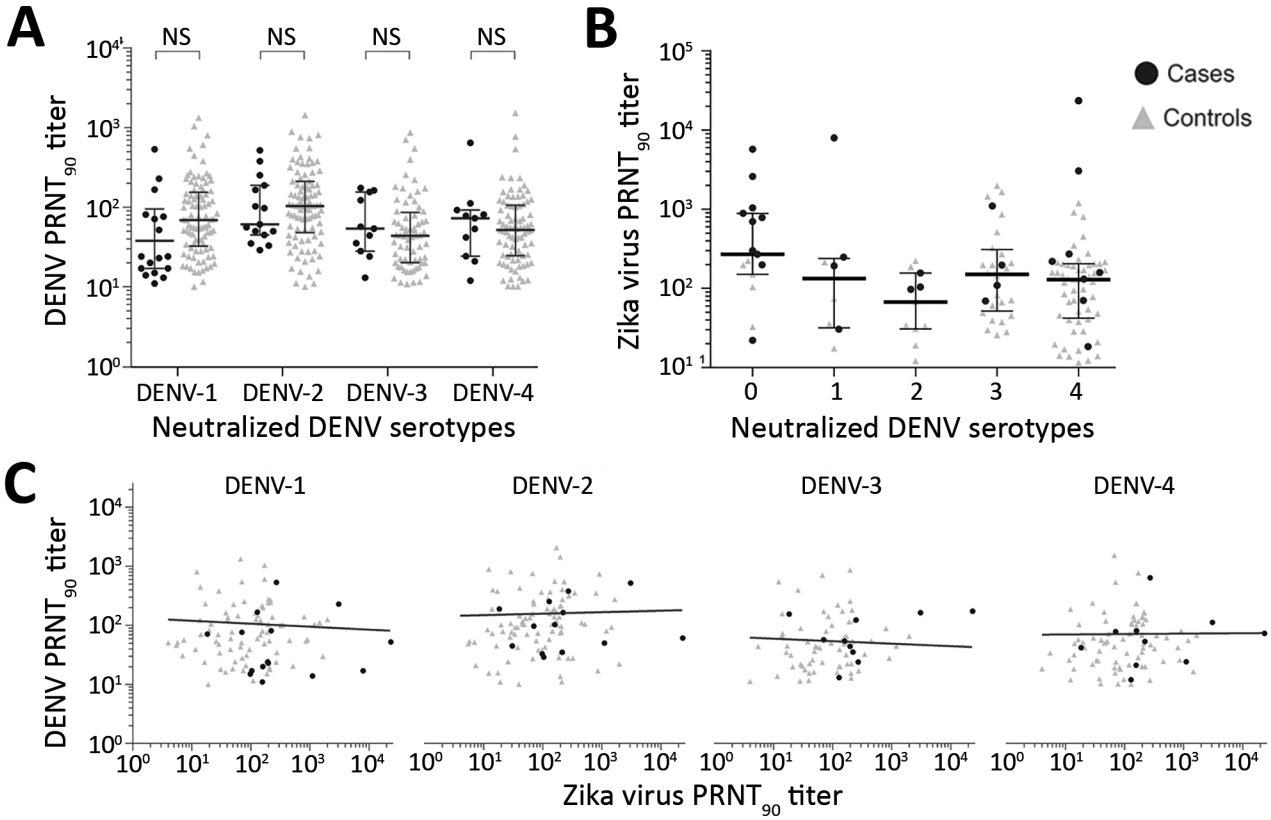

Figure 3. Serologic test results from participants in case–control study of cross-protection of dengue virus infection against congenital Zika syndrome, northeastern Brazil. A) Serotype-specific $PRNT_{90}$ titers for cases and controls. Statistical significance ($p<0.05$) was calculated by the Mann–Whitney U test; no significance was found. B) Zika virus neutralizing antibody titers as a function of the number of neutralized DENV serotypes. C) Correlation of DENV and Zika virus titers. Statistical significance ($p<0.05$) was calculated by Pearson correlation; no significance was found. DENV, dengue virus; NS, not significant; $PRNT_{90}$, 90% plaque reduction neutralization test.

activity during the Zika virus epidemic, hypothetically resulting from cross-protection induced by previous Zika virus exposure (*35*). Moreover, in experimentally infected nonhuman primates, preexisting DENV immunity caused relatively faster clearance of Zika viremia (*22*). Strong support for our interpretation is provided by 2 recently published epidemiologic studies from Brazil and Nicaragua (*36*,*37*). In both studies, preexisting DENV immunity significantly reduced the risk for symptomatic Zika virus infection. Although those studies did not examine the effect of serotype-specific antibodies, in several epidemiologic studies, multitypic DENV immunity was cross-protective for postsecondary DENV infections (*15*,*38*,*39*). In our study, the relatively stronger cross-protection by neutralization of DENV-2 and DENV-4 may suggest that recent DENV infection boosts cross-protection against CZS because both serotypes reportedly were the predominant serotypes in northeastern Brazil before the Zika virus outbreak (*40*).

Antibody protection against DENV is related to antibody titers, and low titers are a risk factor for severe dengue (*15*). In our study, putative DENV-mediated cross-protection against CZS was apparently not linked to antibody titers. Thus, cross-protection from CZS may be mediated by immune responses (*41*) other than cross-protective antibodies. In humans, preexisting DENV immunity has been shown to boost $CD4^+$ and $CD8^+$ T-cell responses during Zika virus infections (*42*–*44*). In pregnant mice, DENV cross-reactive $CD8^+$ T cells have been shown to be a key component of protection from fetal injury or demise during Zika virus infection (*45*,*46*). Of note, $CD8^+$ T cells form a part of the placental barrier that protects the fetus from vertically acquired infections. DENV-primed CD8+ T cells might provide cross-protection from CZS at the placental barrier (*45*). As T-cell–mediated DENV cross-protection wanes over time (*39*,*45*), consecutive heterotypic DENV infections might have afforded relatively stronger and prolonged cross-protection from CZS in controls.

Our study was limited by the absence of longitudinal samples, thereby preventing definite assessments of identical DENV serostatus at the time of congenital

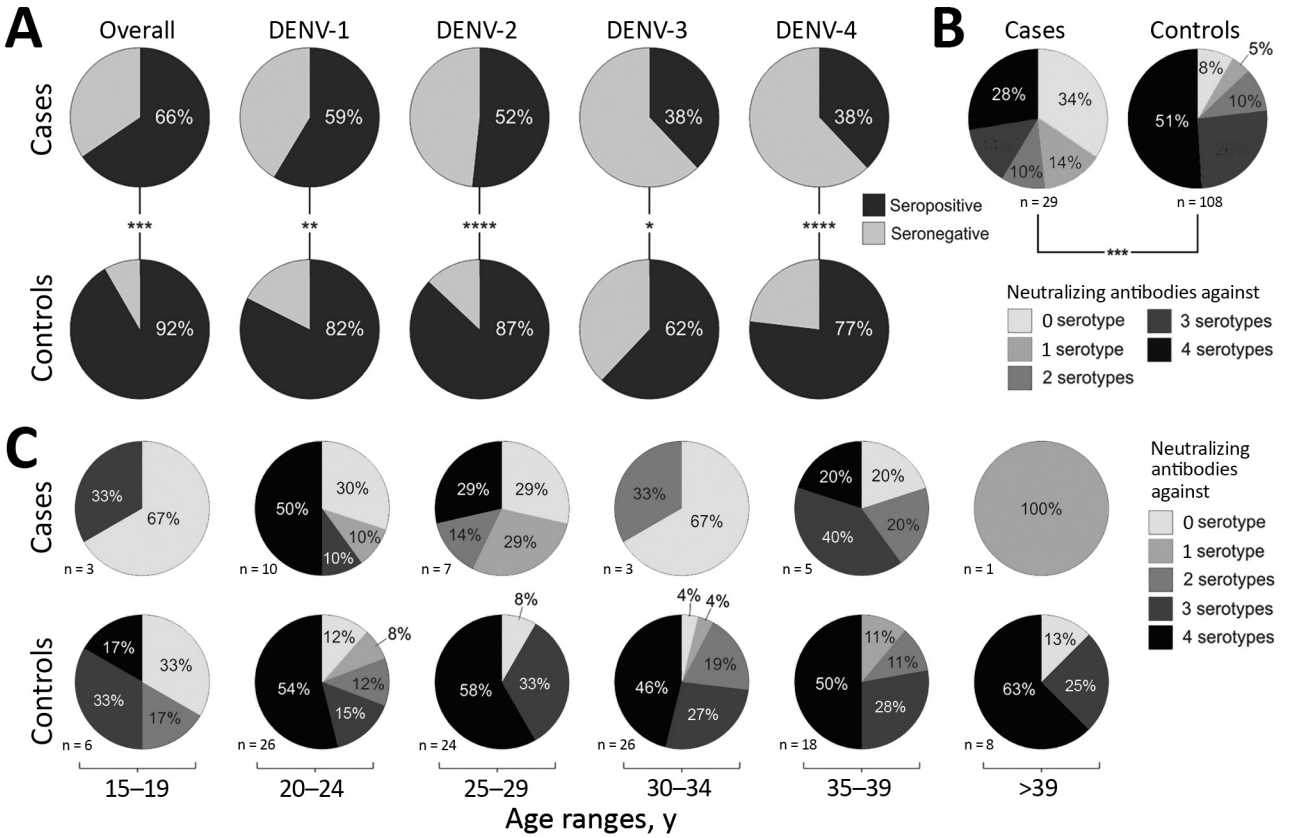

Figure 4. Dengue virus prevalence and neutralization among participants in case–control study of cross-protection of dengue virus infection against congenital Zika syndrome, northeastern Brazil. A) Serotype-specific DENV seroprevalence in cases and controls. Statistical significance was calculated by χ^2 test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. B) Multitypic DENV neutralization in cases and controls. Statistical significance for the numbers of neutralized DENV serotypes was calculated by using the Mann–Whitney U test. ***p = 0.0004. C) Multitypic DENV neutralization in cases and controls in different age groups. DENV, dengue virus. A color version of this figure is available online (http://wwwnc.cdc.gov/EID/article/25/8/19-0113-F4.htm).

Zika virus infection compared with the time of testing at delivery. Nonetheless, the uniformity of our results and low DENV activity during the Zika epidemic (*1*,*35*) speak against putative DENV exposure of mothers after the time of congenital Zika virus infection. Because sampling was conducted at delivery, we could not assess

Table. Comparison of models used to identify factors affecting development of congenital Zika syndrome in study of dengue virus cross-protection against congenital Zika syndrome, northeastern Brazil*

Model	Predictor scale	AIC	ΔAIC	AW	Odds ratio (95% CI)	p value
Neutralization of ≥2 DENV serotypes	Binary	130.4	0	0.2812	0.158 (0.062–0.395)	<0.0001
No. neutralized serotypes	Ordinal, 5 ranks	130.6	0.2	0.2590	0.577 (0.432–0.763)	<0.0001
DENV-4 neutralization	Binary	131.4	0.9	0.1750	0.192 (0.078–0.449)	<0.0001
DENV-2 neutralization	Binary	131.5	1.1	0.1657	0.170 (0.068–0.423)	<0.0001
Neutralization of ≥3 DENV serotypes	Binary	133.0	2.6	0.0771	0.210 (0.086–0.492)	<0.0001
Neutralization of ≥1 DENV serotypes	Binary	134.8	4.4	0.0319	0.170 (0.060–0.477)	<0.0001
DENV-1 neutralization	Binary	139.1	8.6	0.0038	0.298 (0.122–0.733)	0.009
DENV-3 neutralization	Binary	140.3	9.9	0.0020	0.368 (0.154–0.844)	0.018
Neutralization of 4 DENV serotypes	Binary	140.5	10.1	0.0018	0.361 (0.139–0.852)	0.020
Anti-DENV-2 $PRNT_{90}$ titer	Continuous	141.8	11.4	<0.001	0.998 (0.990–1.000)	0.043
Anti-DENV-1 $PRNT_{90}$ titer	Continuous	142.8	12.4	<0.001	0.997 (0.990–1.000)	0.079
Neutralization of 1 DENV serotype	Binary	143.2	12.8	<0001	3.328 (0.776–13.477)	0.101
Age of mothers when giving birth	Continuous	143.2	12.8	<0.001	0.953 (0.896–1.010)	0.136
Anti-DENV-3 $PRNT_{90}$ titer	Continuous	144.2	13.8	<0.001	0.996 (0.990–1.000)	0.195
Anti-DENV-4 $PRNT_{90}$ titer	Continuous	144.9	14.5	<0.001	0.998 (0.990–1.000)	0.326

*p values were calculated by likelihood ratio tests of the different models. Models are sorted by AIC, which is an estimator of the model's quality; models with lower AIC values are superior to models with higher AIC values. The scale of predictor variables must be considered when comparing ORs of different models. AIC, Akaike information criterion; AW, Akaike weight; DENV 1–4, dengue virus types 1–4; OR, odds ratio; $PRNT_{90}$, 90% plaque reduction neutralization test; ΔAIC, difference between a given and the best-supported model in AIC.

the time of maternal and potential congenital Zika virus infection, which affects CZS development (*2*). Because the dates of deliveries were similar among cases and controls sampled continuously within Salvador during the peak of the Zika outbreak (*1*), it seems plausible that cases and controls acquired Zika virus infection at similar stages of pregnancy (i.e., cases were probably not exclusively infected during the first trimester of pregnancy, which is most critical for CZS formation, compared with putatively later times of maternal infection in controls). Because of the small sample sizes, we could not perform PRNT for other endemic flaviviruses (e.g., yellow fever virus) that may also affect CZS development (*19*). However, northeastern Brazil has not consistently implemented yellow fever vaccination, and samples were collected before the large yellow fever outbreak that struck Brazil in the aftermath of the Zika epidemic (*47*). The comparison of historic DENV circulation in northeastern Brazil and other regions of the country is limited by incomplete genome coverage, sampling biases, and resolution of the phylogenetic trees. Nevertheless, our results match the cornerstones of DENV circulation in Brazil and the dataset is larger than other virus databases. The strengths of our study include the combination of highly specific serotype-discriminating DENV $PRNT_{90}$ for examination of preexisting DENV immunity with serologically well-characterized samples from the most relevant persons (i.e., cases and controls sampled during the same time and in the same region) (*13*,*34*), model selection analyses, and an analysis of historical DENV exposure in Brazil.

Our data do not exclude the possibility of sporadic enhancement of CZS development by monotypic DENV immunity or subneutralizing antibodies from nonrecent exposure to DENV depending on the combination (*48*) and the chronologic sequence (*49*) of previous flavivirus infection and the time since previous flavivirus infections (*50*). However, our study strongly suggests a complex interaction between Zika virus and DENV immunity and a protective effect of strong preexisting multitypic DENV immunity of the mother on CZS development in the fetus during the Zika virus outbreak in northeastern Brazil.

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Cross-Protection of Dengue Virus Infection against Congenital Zika Syndrome, Northeastern Brazil

Appendix

Appendix Table 1. Dengue viruses used for PRNT

Serotype	Strain	Genotype	Origin
Dengue virus 1	16007	II	Thailand
Dengue virus 2	16681	Asian I	Thailand
Dengue virus 3	H87	V	Philippines
Dengue virus 4	H241	I	Philippines

Appendix Table 2. Strains used as references for phylogenetic analyses

Serotype	Genotype	Accession number
Dengue virus 1	I	AB074760
Dengue virus 1	II	AF180818
Dengue virus 1	III	AB074761
Dengue virus 1	IV	AF226685
Dengue virus 1	V	GU131863
Dengue virus 1	VI	KR919820
Dengue virus 2	Asian I (I)	GQ868591
Dengue virus 2	Asian II (II)	JF730050
Dengue virus 2	American (III)	AF100467
Dengue virus 2	Cosmopolitan I (IV)	FJ882602
Dengue virus 2	Cosmopolitan II (V)	GQ398258
Dengue virus 2	Cosmopolitan III (VI)	JF327392
Dengue virus 2	Asian/American (VII)	KC294223
Dengue virus 3	I	AY648961
Dengue virus 3	II	EU367962
Dengue virus 3	III	FJ562107
Dengue virus 3	IV	AY146762
Dengue virus 3	V	AF317645
Dengue virus 4	I	AF289029
Dengue virus 4	II	JX024758
Dengue virus 4	III	AY618988
Dengue virus 4	IV	EF457906

Appendix Figure. Dates of birth for controls and cases.