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Serological Differences after Acute Zika Virus Infections between Children and Adults: Implication for Use of a Serological Test

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Abstract. Information is limited regarding differential serological responses after acute Zika virus (ZIKV) infections and prevalence of cross-reactivity with anti-dengue virus (DENV) assays comparing children and adults. Early convalescent sera from a cohort of suspected mild DENV cases between December 2016 and September 2018 at Bamrasnaradura Infectious Diseases Institute in Thailand were tested for nonstructural protein 1 (NS1)–based anti-ZIKV IgM and IgG ELISAs (Euroimmun), and in-house anti-DENV IgM- and IgG-capture ELISAs. ZIKV cases were identified by positive real-time reverse transcriptase-polymerase chain reaction on urine. Sera from 26 (10 children and 16 adults) ZIKV and 227 (153 children and 74 adults) non-ZIKA cases collected at the median duration of 18 days (interquartile range [IQR] 18,19) post-onset of symptoms were tested. Comparing pediatric ZIKV to adult ZIKV cases, the mean anti-ZIKV IgM ratio was higher (2.12 versus 1.27 units, respectively; $P = 0.07$), whereas mean anti-ZIKV IgG ratio was lower (3.13 versus 4.24 units, respectively; $P = 0.03$). Sensitivity of anti-ZIKV IgM and specificity of anti-ZIKV IgG in pediatric ZIKV were higher than in adult ZIKV cases (80.0% versus 43.7% and 79.1% versus 43.2%, respectively). No cross-reactivity with anti-DENV IgM- and IgG-capture ELISA were reported in pediatric ZIKV cases in our study, whereas 25% and 12.5% were found in adult ZIKV cases, respectively. Age-related ZIKV serological differences have been observed. Positive NS1-based anti-ZIKV IgM and IgG ELISA at the early convalescent phase could be useful for ZIKV diagnosis in children, even in a dengue endemic setting.

INTRODUCTION

Zika virus (ZIKV), a single-stranded RNA virus belonging to the *Flavivirus* genus and *Flaviviridae* family, is primarily transmitted through infected *Aedes* species mosquitoes, and other routes include sexual contact, mother-to-child transmission, blood and blood products transfusions, and organ transplantation.[1] Since its identification in 1947, the first large ZIKV outbreak occurred on the Island of Yap in 2007, and during 2013–2017, ZIKV spread across the Pacific and Americas, causing rare but serious consequences, including congenital microcephaly and Guillain–Barré syndrome.[2] Since then, ZIKV has become the global public health threat.[3] Zika virus is a member of the mosquito-borne flaviviruses, whose phylogenetic analysis was closely related to dengue virus (DENV).[4] Immunological cross-reactivity between different type of flaviviruses have raised concerns for serological diagnosis, especially between DENV and ZIKV.[4,5] Currently, specific molecular testing by real-time reverse transcriptase polymerase chain reaction (RT-PCR) is the preferred method to confirm acute ZIKV infection[6]; however, it is costly and not routinely performed in clinical settings.

Most acute ZIKV infections are asymptomatic; only 20% manifest symptoms.[7] Zika virus disease may mimic non-severe forms of DENV disease, which makes accurate diagnosis even more difficult, especially in areas endemic for arboviruses.[8] Recent studies have also revealed varied clinical features of ZIKV disease according to their age; which pediatric ZIKV cases reported milder symptoms than adult ones.[9–11] Hence, most pediatric ZIKV cases were not properly diagnosed under the current World Health Organization's ZIKV case definitions because their symptoms were nonspecific, particularly in young children.[10,11] No clear explanation for this age-dependent clinical features has been documented; and information regarding age-specific serological responses following ZIKV infections has also been lacking. To date, controversy still exists regarding whether a previous infection by ZIKV or DENV protects against or enhances a secondary infection by a heterologous flavivirus.[12,13]

At present, several ELISA platforms are available to detect serological responses following specific flavivirus infections, and these vary in sensitivity, specificity, and degree of immunological cross-reactivity between different populations in different geographic regions.[14–16] Our understanding of the serological response to ZIKV infection and cross-reactivity with DENV assays remains very limited, particularly in children. We aimed to study the serological responses after acute ZIKV infection in a dengue endemic area comparing children and adults, and the cross-reactivity with the serological diagnostic assays for DENV infection.

MATERIALS AND METHODS

The serum samples were collected from a cohort of patients enrolled in a prospective study of ZIKV disease among suspected non-severe DENV cases during December 2016 to September 2018 at the Bamrasnaradura Infectious Diseases Institute, Nonthaburi, Thailand.[11] The patient-enrollment criteria were all children (aged $\leq$ 15 years) and adults (aged $>$ 15 years) both presenting with acute illness suspected of DENV disease without evidence of plasma leakage (no rising of hematocrit $\geq$ 20% over baseline). Zika virus cases were identified by positive PCR (RealStar® Zika Virus RT-PCR Kit 1.0; Altona Diagnostics, Hamburg, Germany) on urine, collected within 7 days of symptoms onset.

The serum samples were collected on days 18 $\pm$ 2 post-onset of symptoms and were sent to the Thai NIH for anti-ZIKV and anti-DENV serologic assays. The anti-ZIKV serological assays were performed using commercial ELISA kit (EUROIMMUN, Lübeck, Germany), whereas the anti-DENV serological assays were performed using in-house IgM- and IgG-capture ELISA. All participants provided written informed consent with assent forms from children aged $>$ 7 years.

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TABLE 1
Comparisons of serological response between ZIKV vs. non-ZIKV cases in each age group at a median 18 (IQR 18,19) days post-onset of symptoms

	Children (N = 163)			Adults (N = 90)		
	ZIKV (N = 10)	Non-ZIKV (N = 153)	P value	ZIKV (N = 16)	Non-ZIKV (N = 74)	P value
Female, n (%)	6 (60)	61 (39.9)	0.32	10 (62.5)	50 (67.6)	0.77
Age, mean (SD), y	10.0 (3.19)	6.73 (4.49)	**0.02**	36.19 (10.06)	36.04 (13.93)	0.97
ZIKV IgM,* mean (SD), cutoff ratio	2.12 (1.30)	0.27 (0.33)	**< 0.01**	1.27 (0.99)	0.18 (0.18)	**< 0.01**
ZIKV IgG,* mean (SD), cutoff ratio	3.13 (0.99)	0.87 (1.18)	**< 0.01**	4.24 (1.29)	1.92 (1.71)	**< 0.01**
DENV IgM,† mean (SD), cutoff unit	7.10 (3.99)	46.81 (47.05)	**< 0.01**	19.13 (25.52)	45.45 (44.95)	**0.03**
DENV IgG,† mean (SD), cutoff unit	31.50 (28.63)	37.24 (44.25)	0.69	53.06 (39.67)	62.54 (56.76)	0.53

DENV = dengue virus; IQR = interquartile range; NA = not analyzed; ZIKV = Zika virus. Categorical variables were test by Fisher's exact test. Continuous variables were tested by unpaired t-test. Significant P-values at the < 0.05 level are in bold font.
* Positive ZIKV IgM or IgG were defined as cutoff ratio $\geq$ 1.1.
† Positive DENV IgM or IgG were defined as cutoff value $\geq$ 40 units and $\geq$ 100 units, respectively.

Euroimmun anti-ZIKV serological assays. The Euroimmun anti-ZIKV IgM and IgG ELISAs are indirect ELISAs based on the ZIKV nonstructural protein 1 (NS1) antigen. The results were reported semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator. A specimen was considered positive if the ratio was $\geq$ 1.1, equivocal if the ratio was $\geq$ 0.8–$<$ 1.1, and negative if the ratio was $<$ 0.8.

In-house anti-DENV serological assays. Anti-DENV IgM and IgG ELISA were performed by in-house IgM- and IgG-capture ELISA using a tetravalent of dengue antigen derived from tissue cultivation as previously described[17] with some modifications. The cutoff values for IgM and IgG were 40 and 100 units, respectively. Interpretations for single serum collected at day $\geq$ 18 post-onset of symptom were 1) acute DENV infection, primary; IgM $\geq$ 40 units, IgM/IgG ratio $\geq$ 1.8 and 2) acute DENV infection, either primary or secondary; IgM $\geq$ 40 units, IgM/IgG ratio $\leq$ 1.8.

Statistical analysis. Descriptive data are presented as mean (SD) for normally distributed continuous variables and median (interquartile range [IQR]) for non-normally distributed continuous variables. Categorical variables are presented as frequency and percentage. Proportions were compared using the χ^2 test or Fisher's exact test as appropriate, and differences in means were compared using the unpaired t-test or unpaired Mann–Whitney U test as appropriate. A $P < 0.05$ was considered statistically significant. The sensitivity and specificity of the Euroimmun anti-ZIKV IgM and IgG assays against the PCR-confirmed ZIKV cases and the prevalences of the in-house anti-DENV IgM and IgG assays cross-reactivity were calculated. Analysis was undertaken using IBM SPSS Statistics for Windows, Version 23.0 (Armonk, NY).

RESULTS

Two hundred and fifty-three sera were available for tested from 163 pediatric and 90 adult cases with a mean age of 17.4 years (SD 16.5). The mean ages for pediatric and adult cases were 6.93 (SD 4.49) and 36.07 (SD 13.3) years, respectively. Sixty-seven (41.1%) pediatric cases and 60 (66.7%) adult cases were female. The median duration of illness at sera collection was 18 (IQR 18,19) days. Overall, ZIKV cases (10 pediatric and 16 adults) revealed higher cutoff ratios of anti-ZIKV IgM (mean 1.60 [SD 1.17] versus 0.24 [SD 0.29], respectively; $P < 0.001$) and anti-ZIKV IgG (mean 3.81 [SD 1.29] versus 1.21 [SD 1.46], respectively; $P < 0.001$) compared with non-ZIKV cases (153 pediatric and 74 adults). Euroimmun anti-ZIKV IgM revealed a sensitivity of 57.7% and a specificity of 98.7%, whereas IgG revealed a sensitivity of 100% and a specificity of 67.4%. The combined positivity of anti-ZIKV IgM and IgG revealed a sensitivity of 57.7% and specificity of 99.1%. The prevalences of cross-reactivity of the in-house anti-DENV IgM- and IgG-capture ELISA for ZIKV cases were 15.4% and 7.7%, respectively. Comparison of serological responses between ZIKV and non-ZIKV cases by age group are as shown in Table 1.

Comparisons of serological responses between pediatric ZIKV (10 cases) and adults ZIKV cases (16 cases) are as shown in Figure 1. Pediatric ZIKV cases revealed a higher ratio of anti-ZIKV IgM (mean 2.12 [SD 1.30] versus 1.27 [SD 0.99]; $P = 0.07$) but significantly lower ratio of anti-ZIKV IgG (mean 3.13 [SD 0.99] versus 4.24 [SD 1.29]; $P = 0.03$) than adult ZIKV cases. Lower mean values of in-house anti-DENV IgM- and IgG-capture ELISA were found in pediatric ZIKV than in adult ZIKV cases.

Performances of Euroimmun anti-ZIKV IgM and IgG assays against PCR-confirmed ZIKV cases for each age group are shown in Table 2.

Anti-ZIKV IgM yielded higher sensitivity among pediatric ZIKV than adult ZIKV cases (80.0% versus 43.7%, respectively) while specificity was high in both age groups (98.7% versus 98.6%, respectively). Anti-ZIKV IgG revealed 100% sensitivity, but low positive predictive value (PPV) in both age groups (23.8% versus 27.6%, respectively). Combined positivity of anti-ZIKV IgM and IgG did not reveal any increase in sensitivity in both age groups compare with positive ZIKV IgM alone.

Prevalences of cross-reactivity of the in-house anti-DENV IgM and IgG captured ELISAs among ZIKV cases are shown in Table 3.

No cross-reactivity of anti-DENV assays were found among pediatric ZIKV cases, whereas adult ZIKV cases revealed prevalences of anti-DENV IgM and IgG cross-reactivity of 25% and 12.5%, respectively. No cross-reactivity of Euroimmun anti-ZIKV IgM and IgG were found among 30 (24 children and six adults) non-ZIKV cases, who we identified as acute primary DENV infection on the basis of the DENV ELISA interpretation.

DISCUSSION

To the best of our knowledge, our study is the first to characterize the serological responses and evaluate the performance of the commercial NS1-based anti-ZIKV IgM and IgG assays (Euroimmun) among pediatric ZIKV cases, compared

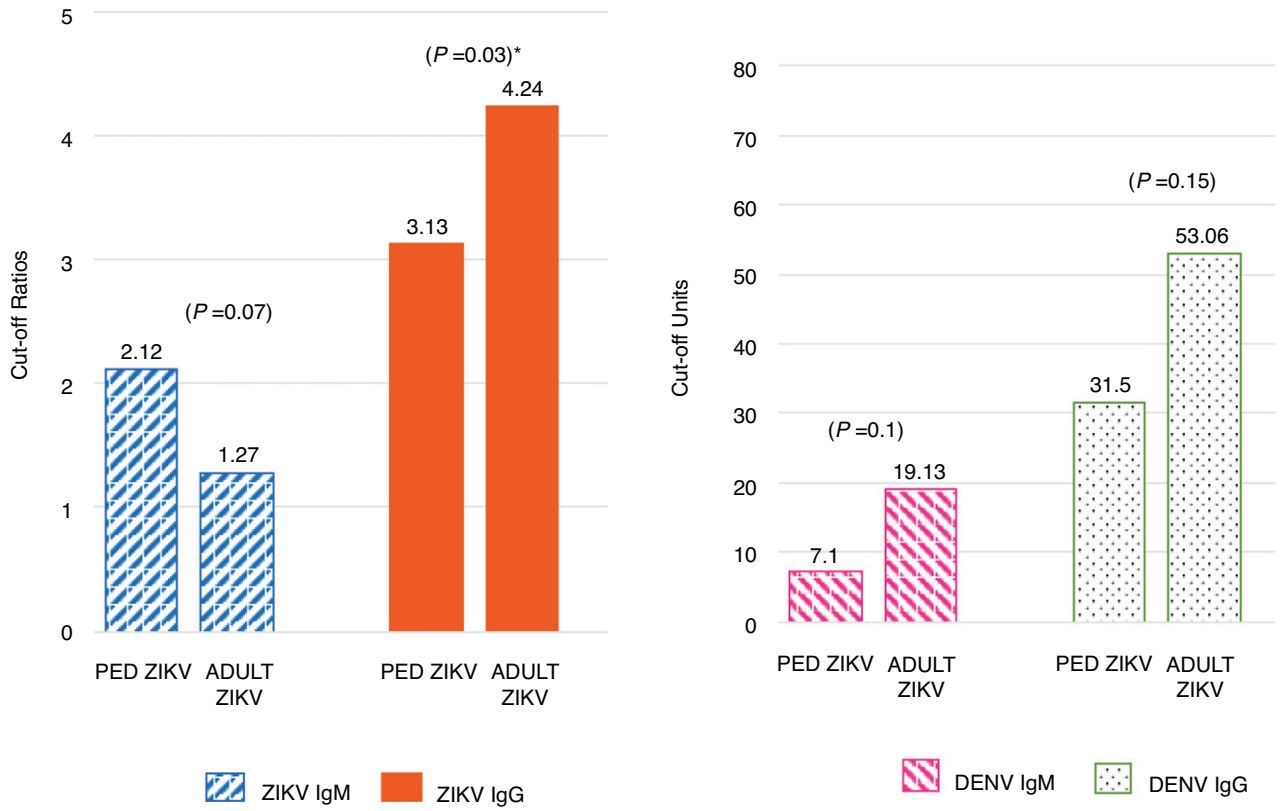

FIGURE 1. Comparisons of nonstructural protein 1 (NS1)-based anti-Zika virus (ZIKV) (left) and anti-dengue virus (DENV) capture ELISAs (right) between pediatric ZIKV and adult ZIKV cases, at median 18 (interquartile range [IQR] 18,19) days post-onset of symptoms. This figure appears in color at www.ajtmh.org.

with adult ZIKV cases, in a dengue endemic setting. Euroimmun anti-ZIKV IgM and IgG assays are widely used with various reports of sensitivities and specificities depending on dates of sera collections post-onset of symptoms and background exposure to flaviviruses among different populations. In our study, the sera were collected once at the early convalescent phase (day 18 post-onset of symptoms), which serological responses after acute flavivirus infections should be presented. Suboptimal overall sensitivity (57.7%) of ZIKV IgM in our study was comparable to that of a previous study performed in an arbovirus-endemic area (49%), which might be explained by the larger proportion of secondary flavivirus infections in the cohort.[15]

However, in our study, when analyses were undertaken for each age group; a higher sensitivity of anti-ZIKV IgM (80%), together with a higher level of anti-ZIKV IgM but a lower level of anti-ZIKV IgG cutoff ratios in pediatric ZIKV, compared with adult ZIKV cases, supported the pathogenesis of primary flavivirus infection for most of our pediatric ZIKV cases. This finding is also compatible with a previous study demonstrating that DENV-naive ZIKV cases presented with a considerably higher rate of ZIKV IgM positivity (86%) at around 7–10 days of illness when compared with DENV-exposed ZIKV cases (33%).[16] Another NS1 based anti-ZIKV ELISA study also revealed high IgM ratio values among ZIKV-infected travelers returning from endemic areas, while

TABLE 2
Performance of EUROIMMUN anti-ZIKV IgM and IgG ELISA at the median 18 (IQR 18,19) days post-onset of symptoms

	Children (*N* = 163)						Adults (*N* = 90)					
	ZIKV (*N* = 10)	Non-ZIKV (*N* = 153)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	ZIKV (*N* = 16)	Non-ZIKV (*N* = 74)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Anti-ZIKV IgM												
Positive*	8	2	80.0% (49.0, 94.3)	98.7% (95.4, 99.6)	80.0% (49.0, 94.3)	98.7% (95.4, 99.6)	7	1	43.7% (23.1, 66.8)	98.6% (92.7, 99.8)	87.5% (52.9, 97.8)	89.0% (80.4, 94.1)
Negative†	2	151					9	73				
Anti-ZIKV IgG												
Positive*	10	32	100% (72.2, 100)	79.1% (71.9, 84.1)	23.8% (13.5, 38.5)	100% (96.9, 100)	16	42	100% (80.6, 100)	43.2% (32.6, 54.6)	27.6% (17.7, 40.2)	100% (89.3, 100)
Negative†	0	121					0	32				

IQR = interquartile range; NPV = negative predictive value; PPV = positive predictive value; ZIKV = Zika virus.
* Positive = cutoff ratio ≥ 1.1.
† Negative = cutoff ratio < 1.1 (equivocal ratio of ≥ 0.8– <1.1 included).

TABLE 3
Cross-reactivity of in-house anti-DENV IgM/IgG captured ELISA following ZIKV infection at the median 18 (IQR 18,19) days post-onset of symptoms

	Children (N = 163)				Adults (N = 90)			
	ZIKV (N = 10)	Non-ZIKV (N = 153)	P value	No. of cross-reactivity among ZIKV cases (%)	ZIKV (N = 16)	Non-ZIKV (N = 74)	P value	No. of cross-reactivity among ZIKV cases (%)
Anti-DENV IgM								
Positive*	0	72	< 0.01	0/10 (0%)	4	34	0.17	4/16 (25%)
Negative†	10	81			12	40		
Anti-DENV IgG								
Positive*	0	19	0.61	0/10 (0%)	2	29	0.05	2/16 (12.5%)
Negative†	10	134			14	45		

DENV = dengue virus; IQR = interquartile range; ZIKV = Zika virus.
* Positive = cutoff value ≥ 40 unit (DENV IgM), ≥ 100 unit (DENV IgG).
† Negative = cutoff value < 40 unit (DENV IgM), < 100 unit (DENV IgG).

majority of endemic area residents who had ZIKV infection revealed high IgG ratios.[18]

Similar characteristic of the serological responses were also found with DENV infection, as previous DENV serological studies revealed that specific anti-DENV IgM responses (percentage of positivity and mean ELISA titers) were higher in primary DENV than secondary DENV infections, whereas anti-DENV IgG responses were higher in secondary DENV than in primary DENV cases.[19,20]

We also observed 100% positivity of ZIKV IgG among all of our ZIKV cases at day 18 post-onset of symptoms, which is similar to the previous finding that all ZIKV patients presented IgG seroconversion after 21 days of disease onset.[16]

No cross-reactivity of in-house anti-DENV IgM and IgG capture ELISA were found among our pediatric ZIKV cases, whereas 25% and 12.5% were found among adults ZIKV cases. Previous study revealed lower percentage of falsely positive for anti-DENV IgM in DENV- naïve ZIKV cases (14%) than in DENV-exposed ZIKV cases (44%).[16] Interestingly, we also found no cross-reactivity of Euroimmun anti-ZIKV IgM and IgG ELISA among the cohort of serologically proven as acute primary DENV infection. Our findings support the use of specific NS1-based anti-IgM response among the pediatric population, whom most ZIKV cases revealed nonspecific symptoms, particularly in young children.

There were some limitations in this study. First, we did not perform molecular testings for DENV, and no serologic tests for neutralizing antibodies against ZIKV or DENV had been done, so we could not be certain whether the cross-reactivity with in-house anti-DENV serological assays be the results from previous or coinfections with DENV, and how correlate of the values of NS1 anti –ZIKV ELISA ratio with the neutralizing activities against ZIKV, though a previous study revealed 83% sensitivity of the combined Euroimmun IgM/IgG against neutralizing tests.[21] Second, we collected only single specimen at the early convalescent phase, so kinetic of serological responses since acute phase could not be demonstrated, given the persistence of anti-ZIKV IgM had been reported.[22]

In conclusion, differential serological responses after acute ZIKV infection between children and adults have been observed, further research is needed to better understand the underlying immunologic mechanisms. Nonetheless, NS1-based anti-ZIKV IgM and IgG ELISA performed once during early convalescent phase (around day 18 post-onset of symptoms) could be helpful in making ZIKV diagnosis in children even though living in a dengue endemic area.

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