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# Antibody Avidity Following Secondary Dengue Virus Type 2 Infection Across a Range of Disease Severity

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## Abstract

**Background—**The four dengue virus serotypes (DENV1-4) are responsible for the most prevalent mosquito-borne viral illness in humans. DENV causes a spectrum of disease from self-limiting dengue fever (DF) to severe, life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Antibodies from one infection can contribute to either protection or increased disease severity in a subsequent infection with a distinct DENV serotype. The effectiveness of the antibody response is modulated by both the affinity and avidity of the antibody/antigen interaction.

**Objectives—**We investigated how antibody avidity developed over time following secondary DENV2 infection across different disease severities.

**Study design—**We analyzed sera from 42 secondary DENV2-infected subjects (DF, n=15; DHF, n=16; DSS, n=11) from a pediatric hospital-based dengue study in Nicaragua. IgG avidity against DENV2 virions was measured in samples collected during acute and convalescent phases as well as 3, 6, and 18 months post-illness using a urea enzyme-linked immunosorbent assay.

**Results—**The data show a significant increase in avidity from acute to convalescent phase followed by a decrease from convalescent phase to 3 months post-symptom onset, then a plateau. Linear regression analysis comparing antibody avidity between disease severity groups over time indicate that individuals with more severe disease (DHF/DSS) experienced greater decay in antibody avidity over time compared to less severe disease (DF), and ROC curve analysis showed

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Conflict of Interest

*Competing Interests:* The authors have no conflict of interest with respect to this study or manuscript.

*Ethical approval:* The protocol for this study was reviewed and approved by the Institutional Review Boards of the University of California, Berkeley (CPHS#2010-06-1649), and the Nicaraguan Ministry of Health (CIRE-01/10/06-13.Ver9).

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that at 18 months post-illness, lower avidity was associated with previously having experienced more severe disease.

**Conclusions—**These data suggest that increased dengue disease severity is associated with lower antibody avidity at later time-points post-illness.

### Keywords

Dengue virus; antibody avidity; dengue hemorrhagic fever/dengue shock syndrome; disease severity

## Background

The four dengue virus serotypes (DENV1-4) are responsible for the most prevalent mosquito-borne viral illness in humans, with up to 96 million symptomatic dengue cases annually and 3.6 billion people at risk for infection in tropical and subtropical regions worldwide (1). DENV causes a spectrum of disease ranging from self-limiting dengue fever (DF) to the severe, life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (2). People exposed to primary DENV infections develop long-term serotype-specific neutralizing antibody responses, whereas secondary DENV infections generally result in neutralizing serotype cross-reactive responses (3). Although the majority of secondary DENV infections with a heterotypic serotype result in subclincal or less severe disease (DF), secondary infection is a risk factor for more severe disease (DHF/DSS) (3).

In Nicaragua, the epidemiology of dengue is generally characterized by waves of one dominant serotype followed by another (4, 5). DENV infections in Nicaragua in 2006-2007 were predominantly DENV2 and marked by heightened clinical severity (5). DENV2 has been reported to result in more severe disease than infection with other serotypes (3, 6-9). In addition, a change in the circulating DENV2 clade, together with the host's serotype-specific immunity, contributed to increased disease severity upon DENV2 infection during this period in Nicaragua (5). Here, we examined the anti-DENV2 serum IgG avidity in samples collected in 2006-2007 as part of a pediatric hospital-based study in Managua, Nicaragua. In this study, suspected dengue cases are enrolled at presentation, treated and studied during the acute phase of illness, and followed longitudinally for 18 months, with healthy blood samples collected at 14-21 days ("convalescent"), 3, 6, and 18 months post-illness (10).

The mechanism(s) underlying the potential for increased disease severity in secondary DENV infection are not fully understood but are thought to involve both serotype cross-reactive T cell responses and cross-reactive, poorly-neutralizing antibodies that can participate in antibody-dependent enhancement (ADE), whereby the Fc portion of non-neutralizing antibodies complexes with infecting virions to facilitate virus entry into Fc receptor-bearing target cells (3, 11-14). However, antibodies and T cells also contribute to protection in secondary infections (3). The effectiveness of the antibody response is modulated by both the affinity and avidity of the antibody/antigen interaction, where avidity is the overall strength of antibody binding to its antigen and is influenced by antibody affinity, antibody valency, epitope accessibility, and epitope density (15). What role antibody avidity contributes to protection or pathogenesis during DENV infection has yet to be

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elucidated. A previous study from our laboratory reported an association between anti-DENV3 serum IgG avidity and neutralization during DENV3 secondary infections (16). Utilizing the same modified urea avidity enzyme-linked immunosorbent assay (ELISA), we assessed the association between disease severity and antibody avidity.

## Objectives

The focus of this study was to evaluate anti-DENV2 IgG avidity over time after secondary infection and to examine the association of avidity with disease severity.

## Study Design

### Ethics statement

The protocol for this study was reviewed and approved by the Institutional Review Boards of the University of California, Berkeley, and the Nicaraguan Ministry of Health. Parents or legal guardians of all subjects provided written informed consent, and subjects 6 years of age and older provided assent (10, 16).

### Study population

Study design and execution, subject enrollment and inclusion/exclusion criteria, and classification of disease severity according to the 1997 WHO Guidelines (2) were as previously described (10, 17). Study enrollment occurred in the Nicaraguan National Pediatric Reference Hospital, Hospital Infantil Manual de Jesús Rivera (HIIMJR), from August 1, 2006-January 31, 2008. Subjects were between 6 months and 14 years of age. Serum samples were collected during the acute phase (3 consecutive days after enrollment), convalescence (14-21 days post-symptom onset), and at 3, 6 and 18 months post-onset of illness. DENV infection was confirmed by RT-PCR detection of viral RNA; isolation of DENV on C6/36 cells; and/or seroconversion by IgM ELISA or a 4-fold increase in total antibody titer as measured by Inhibition ELISA in paired acute- and convalescent-phase samples (18-20). Secondary infection was defined by an antibody titer by Inhibition ELISA of !10 in acute samples or !2,560 in convalescent samples (10). Sample sizes were as follows: 15 DF, 16 DHF, and 11 DSS (n=42) samples at acute phase; 15 DF, 15 DHF, and 11 DSS (n=41) at 18 months; and 14 DF, 15 DHF, and 10 DSS (n=39) samples at all 5 time-points (Table 1).

### Viruses and cell line

DENV2 virions were harvested from infected *Aedes albopictus* C6/36 cells (gift from Paul Young, University of Queensland, Australia) as previously described (16). Cell supernatants were concentrated by Amicon filters (100 kDa, 3750 rpm for 30 minutes at 4°C), then virus was pelleted by ultracentrifugation (26,000 rpm for 4 hours at 4°C, no brakes). The virus pellet was then resuspended in PBS and divided into aliquots for storage at −80°C. DENV2 (strain N172, passage 5) was isolated in 2006 and was obtained from the National Virology Laboratory in Managua, Nicaragua.

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### Avidity assay

Serum avidity was measured using a modified ELISA protocol with urea washes (16). Virions purified from Nicaraguan DENV2 N172 clinical isolate were used as antigen. To determine the amount of antigen to coat the plate, an indirect ELISA with pan-DENV mouse monoclonal antibody 4G2 (2 μg/mL) was used. Briefly, serial dilutions of viral antigen were plated, and the dilution of DENV2 antigen that yielded an optical density (OD) of 1 was selected. Ninety-six-well ELISA plates were coated with viral antigen overnight at 4°C and then blocked in 5% non-fat dry milk in PBS for at least 1 hour. Plates were incubated with heat-inactivated patient serum (1:100) for 1 hour, and then treated with either 9M urea or PBS for 10 minutes (16). Next, biotinylated anti-human IgG antibody (1:1,000, donkey anti-human IgG, Jackson ImmunoResearch) was added, followed by a streptavidin-alkaline phosphatase conjugate (1 μg/mL, Invitrogen) and PnPP substrate (1 mg/mL, Invitrogen), and OD was read at 405 nm on a ELx808 ELISA reader (16). Background levels were determined with normal human serum consisting of pooled samples from Oakland Red Cross blood donors (1:100). Serum IgG avidity was calculated as the ratio of the OD of background-adjusted IgG bound to urea-treated wells compared to PBS-treated wells, as follows:

$$\left(\frac{mean_{urea}}{mean_{PBS}}\right) \pm \left(\frac{mean_{urea}}{mean_{PBS}}\right) \times \sqrt{\left[\left(\frac{SD_{urea}}{mean_{urea}}\right)^2 + \left(\frac{SD_{PBS}}{mean_{PBS}}\right)^2\right]}.$$

A positive control consisting of pooled DENV-immune Nicaraguan sera from donors to the Nicaraguan National Blood Bank was included on each plate. Acceptable ranges for the positive control were determined with a positive quality control plate, treating half the plate with 9M urea and the other half with PBS. Experimental plates were included only if the background absorbance was <0.2 OD405nm, positive control absorbance >5X background OD, and experimental positive control within one standard deviation of the positive quality control plate.

### Statistical analysis

Longitudinal analysis of avidity measurements was performed by one-way, repeated-measures non-parametric ANOVA (Friedman test) followed by Dunn's multiple comparison *post hoc* tests to determine differences between time-points. Raw OD values from PBS-treated wells in the IgG ELISA across time-points were analyzed by two-way Friedman test. Linear regression of avidity data over time was performed for each disease severity group with a deviation-from-zero test followed by computation of $r^2$ of the best-fit line. Association of avidity with progression to more severe dengue disease was analyzed by generating ROC curves with avidity data separated into less or more severe disease from samples collected 18 months post-illness. A p-value of $<0.05$ was accepted as statistically significant. Statistical calculations and graphing were performed in GraphPad PRISM 5.0 (La Jolla, CA).

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## Results

Serum IgG avidity was evaluated in samples from 42 secondary DENV infections (Table 1) at the acute phase, convalescence, and 3, 6, and 18 months post-illness by 9M-urea avidity ELISA (Fig. 1 and Supplementary Table 1). We observed a significant increase in serum IgG avidity from the acute to convalescent phase, followed by a significant reduction in serum IgG avidity from the convalescent phase to 3 months post-illness, followed by a plateau – similar to our previous avidity data with secondary DENV3 infections (16). The distribution of the magnitude of avidity among individuals increased over time from 3 to 18 months post-illness with DENV2, also consistent with observations in secondary DENV3 infections (16).

Comparison of antibody avidity between DF and DHF/DSS at each time-point showed a significant difference at 18 months post-illness ($p=0.0019$, Supplemental Table 2). However, antibody avidities were not significantly different at convalescence, 3 and 6 months post-illness and only slightly significant at the acute-phase time-point ($p=0.049$, Supplementary Table 2). To determine whether the observed decay rate of antibody avidity over time was associated with disease severity, individuals were divided into less severe (DF, n=14) and more severe (DHF, n=15; DSS, n=10) disease groups. We examined the decay rate in serum IgG avidity between the acute phase and 18 months post-illness by linear regression (Fig. 2). Individuals with less severe disease following secondary DENV2 infection maintained constant levels of antibody avidity over time. In contrast, linear regression analysis of individuals with more severe disease indicated that both DHF and DSS were significantly associated with decreased avidity by 18 months post-illness.

To test whether decreased antibody avidity was associated with severity of recent DENV infection, we performed Receiver Operating Characteristic (ROC) curve analysis of our secondary DENV2 infection data at 18 months post-illness (Fig. 3). The 18-month time-point post-illness was the only time-point where initial analysis yielded a significant enough difference between disease severity groups to warrant further analysis (Supplementary Table 2). The ROC analysis revealed an inverse relationship between avidity and disease severity in terms of likelihood ratios (LR: 1.73 to 8.08 [avidity range   80.00% to   65.16%], $p=0.00178$, AUC=0.796).

Finally, to determine whether differences in antibody avidity between disease severity groups might be related to the amount of DENV2-specific IgG, we compared the mean OD obtained by ELISA in PBS-washed wells between DF and DHF/DSS at each time-point (Fig. 4 and Supplementary Table 3) and found no significant differences by the Friedman test. Additionally, subject matching for repeated measures revealed that within-subject variability in the OD of DENV2-specific IgG across time-points was not significant. Thus, the differences in antibody avidity were not a function of anti-DENV2 IgG levels.

## Discussion

In this study, we sought to determine whether anti-DENV antibody avidity changed over time following secondary DENV2 infection and whether antibody avidity correlated with

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disease severity. We analyzed serum IgG avidity to DENV2 in longitudinal samples from 42 patients with DENV2 infection of varying disease severity. Longitudinal analysis of antibody avidity showed that avidity increased between acute and convalescent time-points then decreased by 3 months and remained constant to 18 months post-illness, similar to our previous report following secondary DENV3 infections (16). Additionally, the spread of avidity measurements between individuals increased over time. Comparisons of DENV-specific avidity between individuals with less severe and more severe disease across time-points revealed a significant difference at 18 months post-illness. Although statistically significant (p=0.49), the avidity values between severity groups at the acute phase were so close that the biological significance is unclear. To determine whether the observed differences in avidity according to disease severity were due to the amount of overall DENV-specific IgG responses, we compared the OD values from PBS-washed wells at each time-point and found no differences in the amount of DENV-specific IgG between the disease severity groups.

To further evaluate the relationship between antibody avidity and disease severity, we performed linear regression analysis, which indicated that individuals with more severe disease exhibited an increased rate of decay in antibody avidity up to 18 month post-illness. Finally, to address the observed association between decreased avidity and more severe disease, we generated ROC curves. This analysis indicated that lower antibody avidity can be a retrospective indicator of previous DENV infection severity.

Several possible explanations exist for the association of avidity with severity at 18 months post-illness. One possibility is that because the initial anti-DENV antibody response during secondary infection is often predominately directed against the previously infecting serotype(s) (8, 17), generation of memory B cell and long-lived plasma cells against the secondary DENV serotype in some individuals may be impaired and may not be detectable until the immune response has matured. Additionally, differences in avidity across dengue severity groups could be the result of suboptimal immune responses and affinity maturation in disrupted germinal centers in the lymph node and spleen during acute severe infection (DHF/DSS), as previous literature analyzing histopathological changes in DHF cases in post-mortem examinations suggests that “immunodepression may be an integral part of the pathophysiology of DHF” (21, 22). During the acute phase, inflammatory mediators from innate immune and T-cell responses differ from patient to patient and could affect disease outcome, such that antibody characteristics alone do not predict disease outcome or associate directly with disease severity. Additionally, the host response may be inherently different due to other confounding factors, such as host genetics, nutrition, and physiology. Finally, viral titers and individual differences in immune competence may affect the ability to prime cellular immune responses during infection, and this could further exacerbate differences in avidity outcomes between disease severity groups. A recent study reported that higher viremia at early time-points post-symptom onset induces $CD14^{+}CD16^{+}$ monocytes, which can stimulate B cell plasmablast differentiation following *in vitro* DENV infection (23). While higher viremia may result in a greater B cell response during DENV infection, how that relates to the quality of the antibody response is unknown. Regarding the increased spread of avidity measurements among individuals at later time-points, one cause may be increased decay of avidity seen in those who had more severe disease when

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compared to those with less severe disease. Another possible explanation could be boosting of avidity by subsequent homotypic or heterotypic DENV infections. However, this study was not designed to follow all medical episodes of the participants, as in a cohort study; thus, we cannot ascertain the occurrence of subsequent infection(s).

Strengths of the study include the well-characterized samples in our hospital-based study, with longitudinal samples collected from the acute phase through 18 months post-illness. In addition, we have detailed documentation of the circulation of DENV serotypes in Managua, and since one serotype tends to dominate each season's dengue epidemic, we were able to focus on a 2-year period where DENV2 was the dominant serotype. Limitations of the study include that due to the small sample volume collected from pediatric cases, the number of DENV serotypes and assays/conditions that could be investigated was restricted. Additionally, acute-phase viremia data and DENV-specific IgG titers at late convalescent time-points for these individuals were not available.

Thus, these data show that the kinetics of serum IgG avidity over time in secondary DENV2 infections parallels the development of avidity in secondary DENV3 infections (16). Additionally, the data reveal differential avidity decay rates between less severe (DF) and more (DHF/DSS) disease groups from the acute phase to 18 months post-illness and an inverse linear relationship between avidity and days post-illness in more severe but not less severe DENV2 infections. Finally, these data indicate that lower antibody avidity and increased disease severity show an association at later time-points post-illness. However, whether disease severity influences antibody avidity or vice versa is not known yet and is a subject of on-going studies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Highlights

- We studied antibody avidity kinetics relative to disease severity post-secondary DENV2 infection
- Avidity increased between acute and convalescence and stabilized from 3 to 18 months post-illness
- Sera from DHF/DSS cases had lower avidity at 18 months post-illness than DF cases
- A faster rate of decay in avidity was observed in DHF/DSS compared to DF cases
- Severe dengue was associated with lower antibody avidity at later times post-illness

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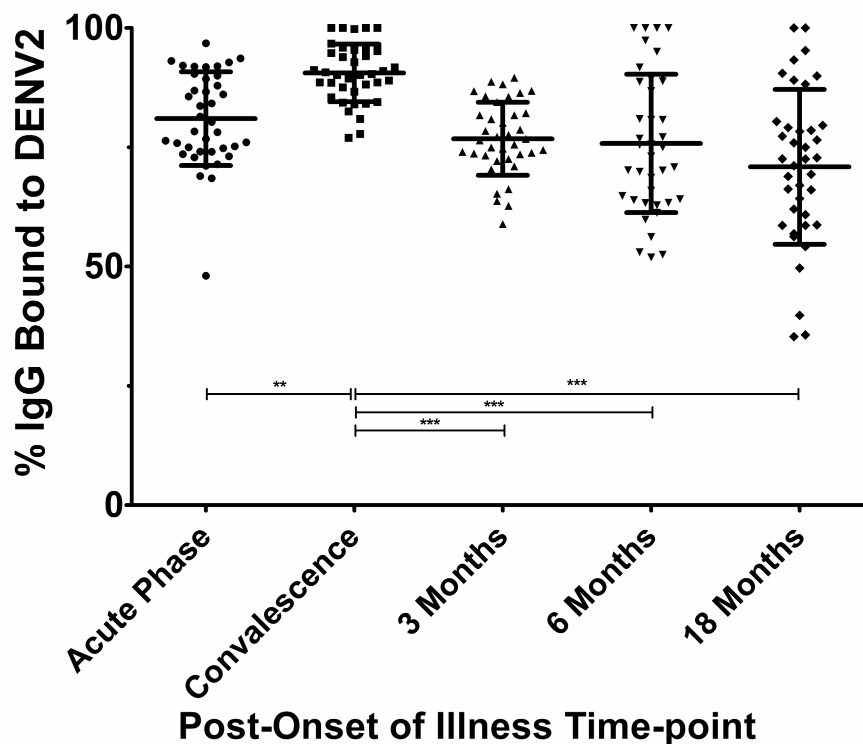


**Figure 1. Serum IgG avidity against DENV2 in longitudinal samples following secondary DENV2 infection**

Serum IgG avidity against DENV2 over time was measured using a 9M-urea avidity ELISA; each point represents one patient, matched across time (n=39). Error bars represent the mean ± standard deviation (SD). Avidity data were analyzed for significant differences using repeated-measures, non-parametric one-way ANOVA (Friedman test) ($p<0.0001$). Dunn's multiple comparison *post hoc* tests were performed to compare avidities between time-points (acute to convalescent phase, $p<0.01$; convalescent to 3 months, 6 months, and 18 months, $p<0.001$, respectively).

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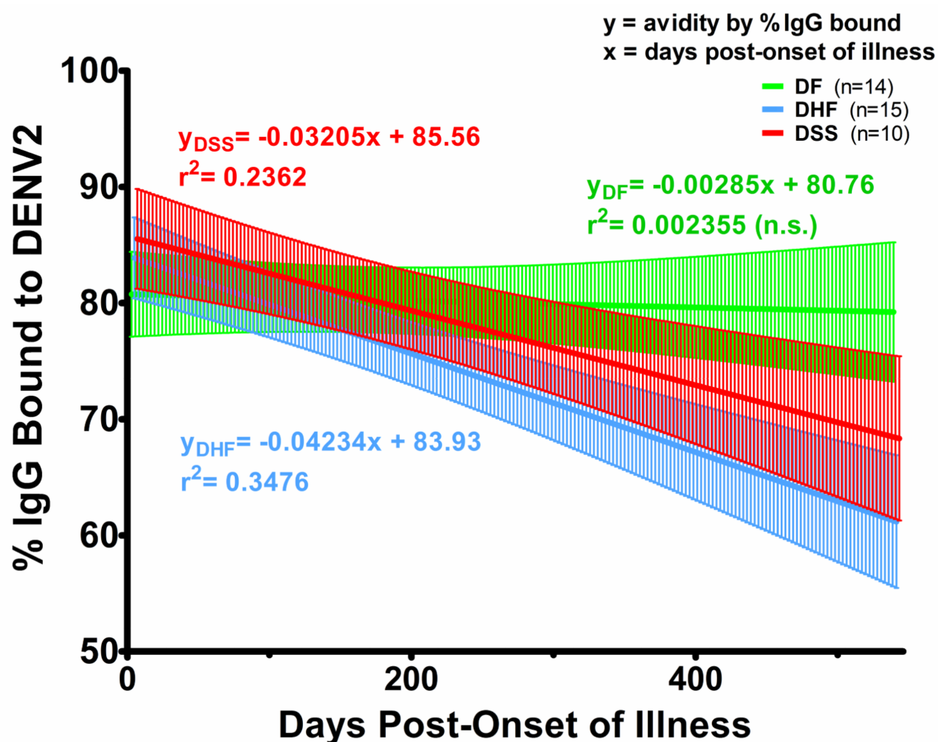


**Figure 2. Linear regression modeling of serum avidity decay rate according to disease severity**

To examine the long-term implications of avidity in relation to disease severity, longitudinal avidity measurements were separated by disease severity, with n=14 (DF), n=15 (DHF), n=10 (DSS). After converting each of the 5 time-points into days post-onset of illness, avidity was plotted against time in days to examine the linear kinetics of serum avidity within each disease severity group, with the 95% confidence interval shown. Data were analyzed by deviation-from-zero tests followed by computation of a linear model with accompanying goodness-of-fit $r^2$ values. Patients with less severe disease exhibited no significant change in avidity over time, whereas patients with more severe disease had significant rates of decay in serum avidity (deviation-from-zero test: DF, p=0.690 (n.s.); DHF, p<0.0001; DSS, p=0.0003; linear regression $r^2$: DF, 0.0024 (n.s.); DHF, 0.348, DSS, 0.236).

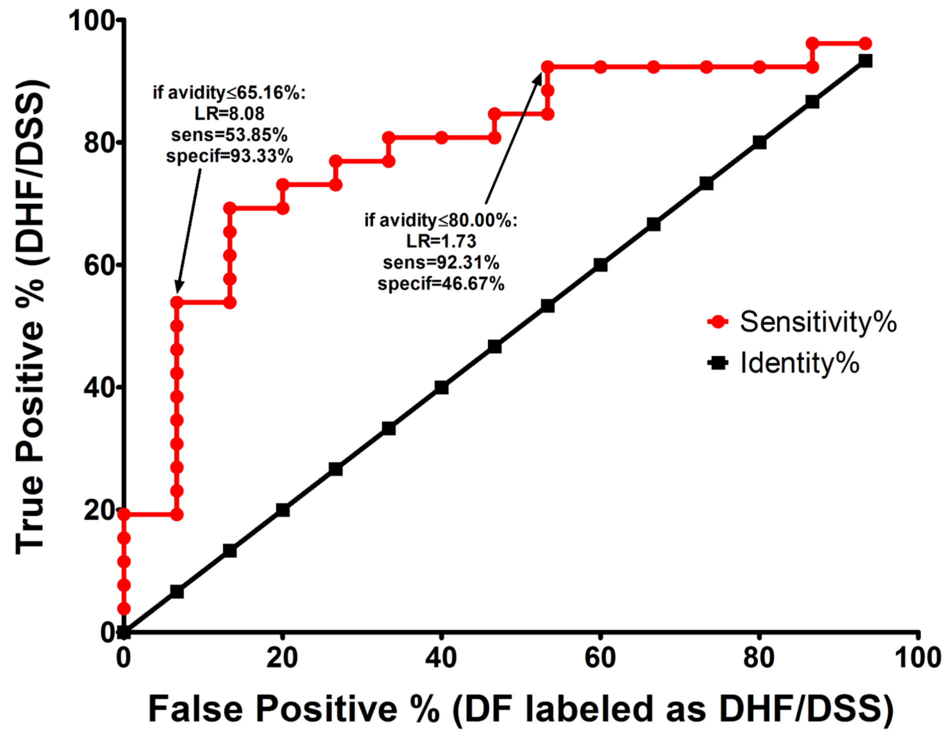


**Figure 3. ROC curve analysis of anti-DENV2 serum avidity 18 months post-illness**

Avidity data were separated into less severe and more severe disease groups, with n=15 (DF) and n=26 (DHF/DSS), for ROC curve analysis. P-values were determined by identifying the null hypothesis as a ROC curve with an area-under-the-curve (AUC) of 0.5 and computing a z-ratio for the area of the generated ROC curve in PRISM GraphPad 5.0 for comparison. At the 18-month time-point, the ROC curve was a good indicator of the severity of the previous DENV infection (AUC=0.796; p=0.00178; LR: 1.73 to 8.08 [avidity range 80.00% to 65.16%]).

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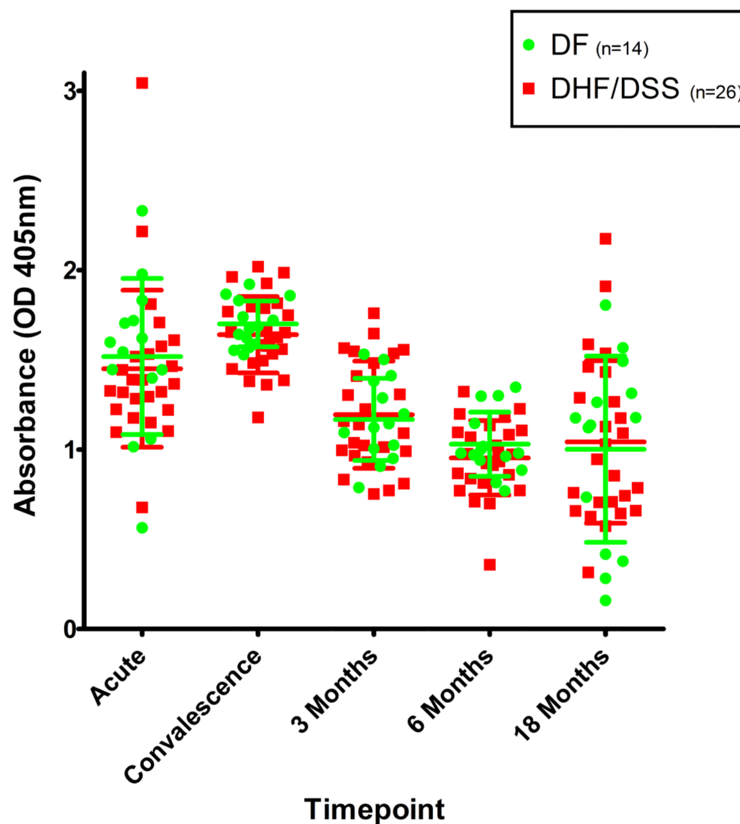


**Figure 4. OD absorbance values from PBS-treated wells, grouped by disease severity**

Mean (± 1 SD) DENV2-specific IgG OD absorbance values from background-adjusted PBS-treated wells of sera at a 1:100 dilution, grouped by disease severity (DF vs. DHF/DSS), across time were plotted. Each dot represents one individual with complete avidity data matched across time (n=39). An increase in mean relative titer occurs from acute phase to convalescent phase, and decreases thereafter. A two-way, non-parametric, repeated measure ANOVA (Friedman test) showed no significant differences in anti-DENV2 IgG between DF and DHF/DSS at any time-point, and matching patients to disease severity by anti-DENV2 IgG bound over time was not significant.

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**Table 1**

Demographic data about study participants

| Parameter | N | % |
|---|---|---|
| General | | |
| Female | 19 | 45.2 |
| Male | 23 | 54.8 |
| Age (mean, S.D.) | | |
| Participant age (years) | 9.5 | 3.8 |
| Disease classification | | |
| Dengue Fever (DF) | 15 | 35.7 |
| Dengue Hemorrhagic Fever (DHF) | 16 | 38.1 |
| Dengue Shock Syndrome (DSS) | 11 | 26.2 |
| Immune status | | |
| Secondary infection | 42 | 100 |