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Dynamics of Dengue Disease Severity Determined by the Interplay Between Viral Genetics and Serotype-Specific Immunity

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Abstract

The rapid spread of dengue is a worldwide public health problem. In two clinical studies of dengue in Managua, Nicaragua, we observed an abrupt increase in disease severity across several epidemic seasons of dengue virus serotype 2 (DENV-2) transmission. Waning DENV-1 immunity appeared to increase the risk of severe disease in subsequent DENV-2 infections after a period of cross-protection. The increase in severity coincided with replacement of the Asian/American DENV-2 NI-1 clade with a new virus clade, NI-2B. In vitro analyses of viral isolates from the two clades and analysis of viremia in patient blood samples support the emergence of a fitter virus in later, relative to earlier, epidemic seasons. In addition, the NI-1 clade of viruses was more virulent specifically in children who were immune to DENV-1, while DENV-3 immunity was associated with more severe disease among NI-2B infections. Our data demonstrate that the complex interaction between viral genetics and population dynamics of serotype-specific immunity contribute to the risk of severe dengue disease. Furthermore, this work provides insights into viral evolution and the interaction between viral and immunological determinants of viral fitness and virulence.

INTRODUCTION

Dengue virus (DENV) is a mosquito-borne, enveloped flavivirus, with an RNA genome of 10.7 kb that is translated into 3 structural (C, prM/M, and E) and 7 nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4A, NS5) proteins. DENV infection results in a range of disease severity, from asymptomatic infection to dengue fever (DF), a debilitating but self-limited febrile illness, to the more severe, potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DHF and DSS are characterized by increased vascular permeability, low numbers of circulating platelets (thrombocytopenia) and hemorrhagic manifestations; DSS ensues when plasma leakage results in low blood pressure–induced shock that can lead to death (1). DENV infections frequently occur in the context of pre-existing immunity in the host to another one of the four DENV serotypes (2). Immune responses to prior DENV infection play an important role in determining the outcome of subsequent *heterologous* infection with a serotype distinct from the previous infection (3–9). Studies in human volunteers (10) and in non-human primates (11) have shown that, after an initial infection with one DENV serotype, the individual is protected for a period of time from dengue disease and/or high viral load when infected with a heterologous DENV serotype. However, this immunity is short-lived, and infection with a heterologous DENV serotype after longer time intervals leads to increased risk of a more severe clinical phenotype, a phenomenon termed *enhancement* (3–6, 9, 12, 13). The precise time intervals of cross-protection and enhancement during heterologous DENV infection are not well defined. This enhanced disease can result from antibody-dependent enhancement (ADE) whereby subneutralizing concentrations of antibodies directed to a DENV serotype from a previous infection result in increased viral replication in Fc γ receptor (Fc γ R)–bearing myeloid cells (14–17) and/or from potentially harmful T cell responses that are directed

primarily toward the prior rather than the current infecting serotype (18–20). Mathematical modeling and analysis of dengue incidence data in endemic populations have suggested both cross-protection from and enhancement of infection by heterologous DENV strains as the main drivers of periodic fluctuations in the incidence of different DENV serotypes within a given geographical location across time (21–24). However, the risk of severe disease upon DENV infection cannot be explained completely by a misdirected host immune response to a prior infecting serotype (3, 5–7, 10, 25–29); rather, disease severity appears to be determined by a combination of multiple host (30–37) and viral factors, including the genetics of the infecting viral strain (38–48).

Each of the four DENV serotypes is composed of several genotypes, which in turn consist of various clades. DENV evolution is characterized by lineage turnover in which an entire clade of circulating viruses is replaced by a new clade (49–52). Certain genotypes within DENV serotypes and certain clades within genotypes have been shown to be more frequently associated with severe disease outcomes (53–55). Fitter viruses (*fitness* is defined here as the ability to replicate better in a given environment (56)) that replicate more efficiently are often hypothesized to be more pathogenic in the host (53). In support of this concept, high levels of virus in patient sera (*viremia*) have been associated with severity of dengue disease (57, 58). Consistent with a model in which fitter viruses are more virulent, DENV-2 viruses of Asian origin are capable of more robust replication *in vitro* relative to the less virulent American DENV-2 genotype viruses (45, 59–64). Further, Asian DENV-2 viruses that are more frequently associated with severe disease are less sensitive than American genotype DENV-2 viruses to antibody-mediated neutralization by sera from individuals previously infected with DENV-1 (65), suggesting that mutations that may alter the neutralization profile of certain DENV genotypes may lead to increased fitness relative to other strains of the same serotype.

The spread of dengue over the past several decades has made this disease a major public health concern worldwide. Previous work has clearly shown that the risk of severe dengue disease is higher in a secondary DENV infection, especially for DENV-2 (3, 4). Further, it has been shown that particular genotypes of DENV increase risk of severe disease (53). However, it is not well understood how the specific interplay between serotype-specific immunity and viral genetics affects risk of severe dengue disease. Such an understanding is not only essential for advancing our understanding of DENV epidemiology and pathogenesis but is also important for the development of safe and effective dengue vaccines. Here we took advantage of a rare observation in two population-based studies in Nicaragua in which a clade replacement in the context of DENV-2 infection occurred concurrently with an increase in dengue disease severity. We combine epidemiological, phylogenetic, virologic, serological, and clinical analyses to provide a more precise understanding of the contributions of DENV genetics and pre-existing immunity to dengue disease severity.

RESULTS

Association of Prior DENV-1 Exposure with Increased Dengue Severity in Later Seasons

Here we report our findings from two independent studies in Managua, Nicaragua (one hospital-based study, termed here the *Hospital* study and one prospective cohort study, termed here the *Cohort* study) from the 2004/5 to the 2008/9 epidemic seasons. Children in the *Hospital* study were enrolled upon presentation to the hospital with relatively more severe disease, allowing us to examine factors related to severe dengue (defined as DHF/DSS according to the WHO 1997 guidelines (1)); in contrast, children in the community-based prospective Cohort study presented earlier and demonstrated a lower rate of severe disease. In addition, annual healthy blood samples collected throughout the Cohort study provided a rich source of material with which to investigate questions related to prior DENV immunity (see Materials and Methods for detailed study descriptions; see Table 1 for demographic information).

In our Hospital study, 29% of DENV-2 hospitalized cases were classified as DHF/DSS in the 2005/6 season, and this proportion increased to 63% across the 2006/7–2008/9 seasons ($P = 0.001$; Fig. 1A). We observed a similar trend over the same time-frame among symptomatic DENV-2 infections in our Cohort study ($P = 0.05$; Fig. 1B). In both studies, DENV-2 was the dominant circulating serotype (Fig. 1C, Hospital study; Fig. 1D, Cohort study), which mirrors the serotype circulation dynamics reported for Managua by the Ministry of Health as a part of the Nicaraguan National Surveillance System (fig. S1). Furthermore, most symptomatic DENV-2 infections occurred in children already immune to another DENV serotype (85% of Hospital cases; 65% of Cohort cases; Fig. 1E, Table 1), including the majority of severe cases in both studies (91% of Hospital DHF/DSS cases; 89% of Cohort DHF/DSS cases).

Prior to the circulation of DENV-2 in the epidemic seasons described here, DENV-3 (1994–8), DENV-2 (1999–2002) and DENV-1 (2002–5) circulated in Nicaragua, as did very low levels of DENV-4 (48, 66–71) (Fig. 2A). We used this serotype timeline to estimate risk of exposure of children in both studies to specific heterologous serotypes by birth year, as children from the same birth year share a common risk of DENV exposure over time. In the Cohort study, we were able to estimate DENV seroprevalence for the entire cohort for each birth year from the annual samples collected prior to each season of dengue transmission. These data showed that children born before 1999 had higher rates of exposure to DENV than children born in 1999 or later (Fig. 2B). Referring to the serotype timeline, we inferred that children born before 1999 in our studies would likely have been exposed to DENV-3 (“DENV-3 era”), whereas children born in 1999 or later (“post-DENV-3 era”) would have been exposed to either DENV-2, which circulated 1999–2002 (“DENV-2 era”), or DENV-1, which circulated in 2002–2005 (“DENV-1 era”). We tested a panel of DENV-immune sera from our Cohort study to determine the serotype of primary infection prior to subsequent DENV-2 infection and, of the 81% of samples for which we could make a determination, we found that indeed most DENV-immune children born before 1999 had been exposed to DENV-3, while all children born in 1999 or after had been exposed only to DENV-1 (Fig. 2C).

We also found that the proportion of secondary DENV-2 Hospital cases in “post-DENV-3-era” children, as compared to “DENV-3-era” children, increased significantly after the 2005/6 season (from 14% to 40%; $P = 0.02$; Fig. 3A). Thus, risk for symptomatic infection changed over time, such that DENV-1-immune children contributed increasingly to Hospital cases in later seasons (2006/7–2008/9), the same epidemic seasons in which we observed increased severity (Fig. 1, A and B). These data suggest that after DENV-1 exposure, cross-reactive immunity decreased the risk of symptomatic infection in the 2005/6 season, but increased the risk of severe disease in later seasons as immunity waned. In the same study, we did not observe a significant increase in severity across seasons in the predominantly DENV-1 immune group (“post-DENV-3-era”; $P = 1.00$; Fig 3B), although the extremely small number of cases ($n = 4$) in the 2005/6 season may be masking an increase in severity in later seasons in this group. However, in this study we also noted an increase in severity across seasons in the predominantly DENV-3 immune group (“DENV-3-era”; i.e., children born before 1999) from 28% to 71% ($P = 0.0005$; Fig. 3C), suggesting that waning DENV-1 immunity did not entirely explain the increase in severity observed across seasons. Therefore, an additional factor, such as viral genetics, must have also played a role.

Association of Increased Viral Fitness with Clade Replacement in Nicaraguan DENV-2 Infection

We derived complete coding-region and partial untranslated-region (UTR) consensus sequences from patients infected with DENV-2 in both the Hospital and Cohort studies from the 2004/5 season through the 2008/9 season, as well as from viruses previously isolated during DENV-2 epidemics in Nicaragua (1999–2002). Through phylogenetic analysis of these complete coding-region sequences, we found that in the context of the American, Asian 1, and Asian/American genotypes, Nicaraguan sequences formed a unique clade (NI) within the Asian/American genotype (Fig. 4A). Of the viruses circulating in the 2004/5 and 2005/6 seasons, the majority formed a single clade defined as NI-1 that was replaced after the 2005/6 season by a related, yet independently derived clade of viruses, NI-2 (Fig. 4, A–C; fig. S2A). NI-2 was composed of two subclades, NI-2A and NI-2B, with NI-2A contributing relatively few sequences and NI-2B rapidly coming to dominance after the 2005/6 epidemic season (Fig. 4, A–C), supported by an increase in relative genetic diversity at the time of clade replacement (fig. S2, B and C). On the basis of nucleotide ancestral node reconstructions, which represent the hypothetical ancestral sequence of a clade as determined by extant sequences, we observed a total of 36 amino acid residues at which Nicaraguan virus sequences showed nonsynonymous variation that altered the encoded amino acid as compared to the American, Asian 1, and Asian-American DENV-2 ancestral sequences (table S1). The NI-1 to NI-2B clade replacement event was associated with nine nonsynonymous mutations, as well as 4 mutations in the viral UTRs that together distinguished NI-1 from NI-2B viruses (Table 2). Three mutations from the ancestral NI sequence occurred in the NI-1 lineage (R97K in capsid (C), K94R in nonstructural protein 1 (NS1) and P245T in nonstructural protein 3 (NS3)), one mutation occurred in the evolution of all NI-2 viruses (N245S in NS4B), and five mutations occurred during the evolution of the NI-2B clade (M492V in envelope (E), L279F in NS1, and K200Q, T290I and R401K in nonstructural protein 5 (NS5); bold in Table 2). Notably, assessment of all publicly available full-length DENV genomes ($n=2,660$) and available E gene sequences ($n=1,650$) revealed

that several of the nine nonsynonymous mutations that occurred during the evolution of NI-2B viruses have previously been observed only rarely in sampled DENV-2 sequences; specifically, the NI-2B mutations N245S in NS4B and K200Q/T290I in NS5 are novel, with only a single previous observation of T290I in any DENV serotype (table S2). In addition, several different methods were implemented to test for positive selection (see Supplemental Methods), but in all site and branch-site models, log likelihoods of the alternate and null models indicated no significant evidence of selection.

To determine whether a fitness increase in clade NI-2B viruses in either the mosquito vector or human host could explain its dominance in later epidemic seasons, we assessed the relative replication of low-passage NI-1 and NI-2B viral isolates in vitro. We adapted a direct viral competition assay (72), in which the cells were infected with an equal proportion of NI-1 and NI-2B viruses, and the relative proportion of each virus in culture supernatant was measured by direct nucleic acid sequencing and analysis of relative peak height with polySNP software (73), which was used to derive a relative fitness value (ω) for each competition (72). In C6/36 mosquito cells, NI-2B viruses had higher median relative fitness values than did NI-1 viruses in multiple pairwise comparisons (Fig. 4D and fig. S3A). Replication of NI-1 and NI-2B viruses was next assayed in primary human monocyte-derived immature dendritic cells (iDCs), in which the NI-2B viruses again demonstrated higher mean relative fitness (Fig. 4E and fig. S3B). Likewise, in the human monocytic cell line U937/DC-SIGN, which expresses the DENV attachment factor DC-SIGN, viral competition assays revealed higher median relative viral fitness of NI-2B viruses as compared to NI-1 (fig. S3 C and D), and similar results were also obtained in a viral replication assay in the K562 hematopoietic cell line, which requires antibody-mediated entry of DENV (fig. S3E).

In addition to this in vitro evidence of relative NI-2B fitness, we found in vivo evidence from Hospital study serum samples collected during acute DENV infection. Analysis of virus levels in these serum samples (*viremia*) showed a significant relation among clade identity and disease severity and hospital day ($P = 0.0003$), such that, among DF cases early in the course of infection, NI-2B viruses reached higher viremia compared to NI-1 and NI-2A viruses (fig. S4). Among DHF/DSS cases, all clades exhibited high viremia (fig. S4). Thus, our in vitro and in vivo data indicate that enhanced replicative fitness of NI-2B viruses might have contributed to the displacement of NI-1 viruses by NI-2B viruses in later dengue epidemic seasons.

Contribution of Viral Genetics to Increased Dengue Severity in the Context of Serotype-Specific Immunity

We next analyzed data from our Hospital study to determine whether the arrival of NI-2B viruses into the population could explain the increased severity in later seasons. However, the observed increase in disease severity across seasons was not simply a direct effect of clade replacement, as we saw the same trend of increased severity among those infected with either NI-1 or NI-2B viruses (Fig. 5A). Specifically, 9/26 (35%) NI-1 infections were severe (DHF/DSS) in the 2005/6 season, increasing to 10/14 (71%) in the later seasons ($P = 0.05$), a pattern similar to that of NI-2B infections, for which no cases (0/4) of severe disease

were documented in the 2005/6 season, but 47/77 (61%) NI-2B infections in later seasons were severe ($P = 0.03$) (Fig. 5A). NI-2A also had low rates of severe disease (25%) in the early season followed by high rates (64%) in the later seasons (Fig. 5A), but the difference was not statistically significant ($P = 0.28$), likely due to low numbers of NI-2A-infected patients.

Although a direct correlation of clade and disease severity was not initially observed, we found that clade in the context of serotype-specific immunity impacted disease outcome. In the later seasons (2006/7–2007/8), the proportion of all secondary NI-1/NI-2A infections that occurred in “post-DENV-3-era” children (“1999+”; Fig. 5B) increased from 16% to 58% as compared to “DENV-3-era” children ($P = 0.01$), suggesting an interplay between NI-1/NI-2A viruses and DENV-1 immunity. The effect in “post-DENV-3-era” children appears to be specific to the most severe form of dengue, DSS, as 5/9 (56%) NI-1 and 4/6 (67%) NI-2A cases, as compared to 4/30 (13%) NI-2B cases, were classified as DSS in later seasons (2006/7–2008/9) ($P = 0.003$) (Fig. 5C). No significant difference in the rate of severe disease was observed when DHF and DSS were analyzed together ($P = 0.54$; Fig. S5). In further support of the increased severity of NI-1/NI-2A infections as compared to NI-2B infections, we observed that NI-1 cases developed DSS significantly earlier in the course of illness than did NI-2B cases ($P = 0.001$) (Fig. 5D) and that NI-1 infections in the later seasons resulted in lower platelet counts (a sign of more severe disease (69, 74)) when compared with NI-2A and NI-2B infections (Fig. 5E; $P = 0.05$). Finally, multivariate analysis showed an association between NI-1 infections with DSS and platelet counts

50,000 cells/mL in later seasons (table S3) (74). Therefore, NI-1/NI-2A infections are at higher risk of more severe disease, particularly DSS, in “post-DENV-3-era” children in later seasons. These observations suggest that there is a specific interaction between NI-1/NI-2A viruses and DENV-1 immunity such that NI-1/NI-2A viruses efficiently infected and caused severe disease in DENV-1-immune children after a period of protection had passed.

Next, we observed that patients with NI-2B infections were distributed differently across birth groups compared to NI-1 or NI-2A infections. Specifically, NI-2B Hospital cases came disproportionately from children born before 1999 (“DENV-3-era”), with the “1994–1995”, “1996–1998”, and “<1994” birth groups showing significantly more cases than the “post-DENV-3 era” (“2002–2004” and “1999–2001”) birth groups ($P = 0.005$; Fig. 6A), and with a similar pattern apparent in the distribution of DHF/DSS cases ($P = 0.04$; Fig. 6B). The distribution of NI-2B cases in the Cohort study also followed the same pattern ($P = 0.004$; Fig. 6C). Thus, the relative over-representation of NI-2B infections as compared to NI-1/NI-2A infections in children born before 1999 implies that NI-2B infections were more likely to present as symptomatic cases and as severe cases than were NI-1/NI-2A infections specifically in predominantly DENV3-exposed children.

To empirically test the association of NI-2B and DENV-3 immunity with more severe disease, we measured the virus neutralization capacity of annual serum samples collected from Cohort DENV-2 cases *prior* to infection with NI-2B viruses (most Cohort cases in later seasons resulted from infection with NI-2B viruses). Using a nested case control analysis, we matched 8 DHF/DSS Cohort cases (all from the 2007/8 season except for one from January of the 2006/7 season) with 21 randomly selected DF cases from 2007/8, and

we measured neutralizing antibody titers to DENV-1 and DENV-3 using Reporter Viral Particle (RVP) and Focus Reduction Neutralization Test (FRNT) neutralization assays. Among the NI-2B cases, the severe cases displayed higher neutralizing antibody titers to DENV-3 ($P = 0.003$) but not to DENV-1 ($P = 0.72$), compared to the DF control group (Fig. 6D). Similar results were obtained using the FRNT assay with Nicaraguan viruses, with higher titers among NI-2B severe cases to DENV-3 ($P = 0.0005$) but not to DENV-1 ($P = 0.60$), compared to the DF control group (fig. S6). Therefore, the arrival of NI-2B viruses into the population in later seasons and their higher propensity to cause severe disease in DENV-3-exposed children, compared to NI-1/NI-2A viruses, also contributed to the increase in disease severity observed across seasons. Moreover, the greater contribution of NI-2B viruses, compared to NI-1/NI-2A viruses, to cases in DENV-3 immune children could have resulted in a competitive advantage for NI-2B viruses in the population, as they may have been able to replicate more efficiently in the DENV-3-immune environment.

DISCUSSION

Through an interdisciplinary approach, we have demonstrated that two evolving factors, serotype-specific immunity and viral genetics, both contribute to the dynamics of dengue disease severity across time; specifically, the complex interplay between both factors is a major driver in determining dengue disease outcome. Such a dramatic increase in risk of severe dengue disease across several years of epidemics caused by the same serotype, as we observed in Managua, presented a unique opportunity to assess the role of viral genetics in dengue disease severity. Our study was strengthened by the fact that a single DENV serotype generally dominates each epidemic season in Nicaragua to a greater extent than in other locations where dengue is hyperendemic (75), thereby constraining the immunological background of previous DENV infections in the population. Further, the studies were restricted to a small geographic area, ensuring a constant host genetic background, and to a brief temporal period, restricting viral genetic variation.

The 1999–2002 DENV-2 epidemics in Nicaragua demonstrated a rate (~20%) of severe disease (DHF/DSS) in children who were part of our previous study in the same hospital (69) that was similar to the rate we observed in the relatively mild 2005/6 season analyzed here. This previous DENV-2 period was preceded by DENV-3 in 1994–1998 (66) (see Fig. 2A), suggesting that the sequence of DENV-3/DENV-2 infection does not necessarily produce the high levels of severe disease (~60% DHF/DSS) observed in the later 2006/7–2008/9 seasons of the Nicaraguan DENV-2 epidemics. Therefore, factors that are variable across epidemic seasons, such as an evolving adaptive immune response to prior DENV infections and changes in viral genetics, are key candidates for driving dengue disease outcomes across seasons.

Our data indicate that pre-existing immunity to DENV-1 initially provided protection from dengue disease during a subsequent DENV-2 infection in the 2005/6 season, but then waning cross-protection contributed to an increased risk of DENV-2 disease in the 2006/7–2008/9 seasons. A model consisting of a window of protection from disease after heterologous infection is well-supported by previous observations, particularly when the sequence of infection is DENV-1 followed by DENV-2 (10). Studies in nonhuman primates

of secondary DENV-2 infection immediately following a primary DENV-1 infection also resulted in reduced viremia and protection from disease in these animals (11). However, epidemiological studies in human populations have demonstrated that when DENV-2 infection follows DENV-1 infection after an interval of several years, individuals are at higher risk of severe dengue disease, as observed in both Cuban and Thai populations (3, 76). Our results suggest a window of DENV-1-induced cross-protection against symptomatic dengue disease due to subsequent DENV-2 infection on the order of one to three years and thus help to more specifically define the timing between infections that contributes to disease-protective versus pathogenic immunity.

We demonstrated that a novel, phylogenetically distinct DENV-2 clade (NI-2B) with increased fitness began to circulate in the study populations concurrently with the observed increase in disease severity. Both *in vitro* analyses of viral isolates and viremia levels in patient sera suggest that the NI-2B viruses have increased fitness relative to the previously circulating DENV-2 clade, likely explaining their success in overtaking the NI-1 viruses that were circulating in the Nicaraguan population. Therefore, our data are consistent with a model in which DENV clade-replacement events may be associated with increased viral fitness, rather than stochastic events (49). Fitness of NI-1 and NI-2B viruses *in vivo* in the *Aedes aegypti* mosquito vector are important to confirm our *in vitro* findings and are underway.

Nine amino acid changes distinguish the more fit NI-2B viruses from the replaced clade (NI-1). One or more of these mutations may contribute to the observed increased replicative ability of NI-2B isolates. In particular, we are intrigued by the two mutations in the RNA-dependent polymerase (RdRp) domain of the NS5 viral protein. One RdRp domain mutation (R401K) is adjacent to the RNA-binding groove and may affect the efficiency of RNA replication. The other RdRp mutation (T290I) maps to a region implicated in binding to the viral helicase NS3 and is a candidate for modulating the interaction between the DENV NS3 and NS5 proteins that is required for viral replication (77). The single mutation identified in E (M492V) lies adjacent to the membrane and would not be expected to directly alter antigenicity or function of E, although there is a possibility that this mutation could alter the conformation of E at other sites that could affect function.

Mathematical modeling has suggested that both the patterns of circulation of DENV serotypes across seasons and clade-replacement events are linked to cross-reactive immunity in the population (78). In fact, we found evidence of interplay between clade genetics and specific immune backgrounds. The Nicaraguan clades — for which relative circulation in the population changed across seasons (more NI-1/NI-2A in early seasons; more NI-2B in later seasons) — differentially affected risk of disease in children who were previously exposed to specific DENV serotypes. NI-1 and NI-2A infections were associated with more severe disease (specifically, DSS) than were NI-2B infections in children primarily exposed to DENV-1. Therefore, the few NI-1 and NI-2A viruses circulating in later seasons appear to have contributed to the increase in severity across seasons specifically after protective DENV-1 immunity was lost. In addition, we found that, compared to NI-1 and NI-2A viruses, NI-2B viruses disproportionately caused disease in DENV-3-immune children. Our analysis of the neutralization capacity of sera collected prior to NI-2B infection in the

Cohort study demonstrated that cases of severe disease were significantly more likely to be immune to DENV-3 than a related control group of DF cases from the same season. The observed increase in severity across years after NI-2B infection may result specifically from interactions between NI-2B genetics and the immune response to DENV-3, which is different in infection with NI-1 or NI-2A viruses. Therefore, specific mutations (such as those outlined in Table 2) may contribute not only to the increased fitness of NI-2B viruses relative to NI-1 viruses, but also to the enhanced virulence of NI-2B viruses in DENV-3 immune children. Supporting the potential importance of these mutations in the observed immunological backgrounds is the observation that several of these mutations are unique to the Nicaraguan strains (table S2).

Our study has several possible limitations. First, we were not able to obtain complete DENV genome sequences from all of the patient samples. However, we developed a genotyping assay to distinguish among the Nicaraguan clades and were then able to obtain clade information for ~90% of the viruses studied. Also, our analyses were limited to a subset of cases, from both studies, that had complete clade data and other clinical and demographic information; however, only ~11% of cases were missing clade or other data, and thus we do not believe these omissions significantly affected our results. In addition, the shift in clade occurred completely in tandem with changing epidemic seasons in the Cohort study, preventing association analyses of either variable with disease severity. However, clade shift was not as tightly linked with dengue season in the Hospital study; thus, we were able to perform robust association analyses that demonstrated associations of both clade and dengue season with disease severity. Further, whereas the Cohort study design permitted analysis of samples collected *prior* to DENV-2 infection due to the existence of annual healthy blood samples, this material was not available in the Hospital study. Therefore, we relied on birth year of children in the Hospital study as a proxy measure of infection history; this is a valid approach because of the serotype timeline in Nicaragua wherein one serotype dominates in each dengue season, and we provide experimental support using data from the Cohort study (Fig. 2C). Finally, we cannot rule out unmeasured factors that may have contributed to our observation of increased severity across seasons.

Our study provides insight into the complex dynamics that determine dengue disease outcomes across epidemics. The population-level dynamics of immunity to specific DENV serotypes can be a primary driver of observed fluctuations in disease manifestations and may explain a significant portion of the variation in disease outcomes observed in endemic populations over time (75). We find that changes in the immunological profile of the population and changes in viral genetics, such as those detailed here for both NI-1/2A and NI-2B infections, contributed to the abrupt increase in DENV-2 disease severity that occurred after the 2005/6 season. Our data and samples should enable the deciphering of biological processes and mechanisms that underlie the switch between a protective versus a pathogenic immune response.

Notably, the fact that we did not observe an apparent link between viral genetics and disease severity until the host immunological background was accounted for highlights the importance of phylodynamic studies that combine high-resolution genomic information with associated epidemiological information (79). This interplay between viral genetics and

serotype-specific immunity that determines risk of dengue disease has important implications for our understanding of DENV epidemiology and pathogenesis as well as for dengue vaccine design. In addition, our results strongly support the notion that genetic diversity in RNA viruses, given their rapid evolution and relatively recent common ancestry, is predominantly driven by the immune structure of the host population they infect. Finally, our work addresses fundamental questions in viral evolution and the interaction between viral and immunological determinants of viral fitness and virulence.

MATERIALS & METHODS

Nicaraguan DENV cohort and hospital studies

The protocols for both studies were reviewed and approved by the Institutional Review Boards (IRBs) of the University of California, Berkeley (CPHS 2004-6-178 and CPHS 2005-5-35, respectively) and of the Nicaraguan Ministry of Health (CIRE-02/05/06-008 and CIRE-02/05/06-012, respectively); in addition, the Cohort study protocol was reviewed and approved by the IRB of the International Vaccine Institute in Seoul, South Korea (IRB 2004-010). Parents or legal guardians of all subjects in both studies provided written informed consent, and subjects 6 years of age and older provided assent.

The Nicaraguan Pediatric Dengue Cohort Study (PDCS) (70, 80, 81)—The Cohort study is a community-based prospective study that was initiated in August of 2004 in low- to middle-income District II of Managua, Nicaragua. The cohort population consists of children from 2 to 14 years of age with yearly enrollment of new 2-year olds and withdrawal of 15-year olds to maintain the age structure. The catchment area as well as study design and methods have been described previously (81). Our study has a high case capture rate (82), and we have not observed significant population movement (81). Briefly, participants are encouraged to present at the first sign of illness to the Health Center Sócrates Flores Vivas (HCSFV), in which study physicians use standardized forms to collect data on the signs and symptoms of dengue. Subjects were followed daily during the acute phase of illness. Acute and convalescent (~14 days after onset of symptoms) blood samples were drawn for dengue diagnostic testing (see below), and complete blood count (CBC) and blood chemistry tests were performed as indicated by the physicians. Participants requiring hospitalization were treated at the study hospital (Hospital Infantil Manuel de Jesús Rivera, HIMJR), in which medical data is collected on systematic forms. Each year in July-August, a healthy blood sample was drawn from all subjects to identify silent DENV transmission during the year and to determine dengue immune status.

The Nicaraguan Dengue Hospital Study (69, 83)—This study was based in the Infectious Disease Ward of the Hospital Infantil Manuel de Jesús Rivera (HIMJR), the national pediatric reference hospital in Nicaragua, and has been ongoing since 1998. During the peak dengue season (August–January), children between six months and 14 years of age with suspected dengue disease (acute febrile illness with at least one of the following accompanying symptoms: headache, arthralgia (bone/joint pain), myalgia (muscle pain), retro-orbital (eye) pain, positive tourniquet test, petequiae (small red spots, caused by broken capillaries), signs of bleeding (1)) who presented to the hospital within 7 days of symptom

onset were eligible to participate in the study. Blood samples were collected at enrollment, during the acute phase, and 2 weeks after symptom onset (convalescent phase) for clinical laboratory tests and dengue diagnostic tests. Clinical data, including vital signs, symptoms, fluid balance and treatment, platelet count, hematocrit and ultrasound and/or X-ray results were recorded on standardized data collection forms by a team of study physicians and nurses. Participants who required more intensive therapies were transferred to the intensive care unit (ICU). In both studies, a study physician completed case report forms (CRFs), which were then reviewed by a second physician, after which the CRFs were systematically monitored, and the information was entered into an Access 2003 database by double-data entry, with quality control checks performed daily and weekly.

A suspected dengue case was considered dengue-positive if it met one or more of the following criteria (68): DENV was isolated (66, 84); DENV RNA was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) (68, 85); seroconversion of DENV-specific IgM antibodies was detected in paired acute- and convalescent-phase samples (86, 87); or antibody titer by Inhibition ELISA (67, 88) demonstrated a 4-fold increase in titer between acute and convalescent sera. Primary DENV infections were those in which acute antibody titer was <10 or convalescent antibody titer was $<2,560$ and secondary infections were those in which antibody titers were ≥ 10 (acute) or $\geq 2,560$ (convalescent) as determined by Inhibition ELISA (68). In paired annual blood samples, seroconversion from a titer of $<1:10$ to $1:10$ by Inhibition ELISA indicated a primary DENV infection, while a 4-fold increase in anti-DENV antibody titer indicated a secondary DENV immune response (81).

Genome sequencing and genotyping

The clade of Nicaraguan DENV-2 sequences derived from patient samples was assigned based on either full-length genome sequencing ($N=159$) or a clade genotyping assay ($N=107$) (see <http://www.broadinstitute.org/annotation/viral/Dengue/GlobalPopulationStructure.html> for a description of Nicaraguan sample sets). For genome sequencing, viral RNA was isolated (QIAmp viral RNA mini kit, Qiagen) from serum or first-passage cell culture supernatant (C6/36) and reverse-transcribed to cDNA using Superscript III RT (Invitrogen), random hexamers (Roche), and a specific oligonucleotide that targeted the 3' end of the target genome (5'-AGAACCTGTTGATTCAACAGCAC-3'; nucleotides 10701-10723 for DENV-2). cDNA was then amplified using a high-fidelity DNA polymerase (pfu Ultra II, Stratagene) and a pool of specific primers to produce 14 overlapping amplicons of 1.5 to 2 kb in size, for a physical coverage of 2X. Amplicons were then generated using primer panels consisting of 96 specific primer pairs tailed with M13 forward and reverse primer sequences, which produced 500- to 700-bp amplicons from the target viral genomes. These amplicons were sequenced in the forward and reverse directions using M13 primers, with a total post-sequencing coverage of 8-fold. Resulting sequence reads were assembled *de novo* using the Broad Institute's AV454 assembly algorithm, followed by genome annotation using the Broad Institute's in-house viral annotation algorithm and quality-checking using the annotation and supporting reads to resolve any base call ambiguities and frame-shifts. Resulting genome sequences were deposited in GenBank (see accession numbers in References and Notes). To establish a more complete data set beyond that for which we had complete genome sequence, we used an RT-PCR

genotyping assay that enabled classification of DENV-2 strains as NI-1, NI-2A or NI-2B. Addition of the RT-PCR genotyping information to the complete genome data permitted identification of clade in ~90% of DENV-2 infections across both studies. Viral RNA was extracted (QIAmp Viral RNA Mini) and nested RT-PCR targeting either of 2 regions (one in NS2A and another in NS3; see table S4 for primer sequences) was performed. Reverse transcription and PCR were performed with 0.2µL AMV-RT (Invitrogen), 0.25µL GoTaq (Promega), 5X GoTaq Buffer, 0.5µL 10mM dNTPs, 2.5µL 5M betaine, 0.75µL 1M tetramethylammonium chloride (TMAC), 1.25µL each 10µM primer, 1µL RNaseOUT (Invitrogen), 1.25µL 1mM DTT and 5µL viral RNA. Cycling conditions were 50°C for 60 minutes (min) followed by 30 cycles of 94°C for 30 seconds (sec), 54°C for 30 sec and 72°C for 1 min, followed by a final extension at 72°C for 3 min. First-round product was diluted 1:10 and 5µL amplified by a second, nested PCR including 0.125µL GoTaq, 5µL 5X GoTaq Buffer, 2.5µL 5M betaine, 0.75µL TMAC, 2.5µL each 10µM primer and 0.5µL 10mM dNTPs by cycling at 95°C for 2 min, 30 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min, followed by a final extension at 72°C for 3 min. Amplification of PCR products was verified by gel electrophoresis, and amplicons were sequenced directly using an internal primer (table S4) for each amplicon. Between 3 and 6 positions (3659, 3672, 3704, 5667, 5685 and 5757; numbering starting at AUG initiation codon) distinguishing NI-1, NI-2A and NI-2B were evaluated for each viral RNA, allowing for the DENV-2 clade to be determined.

Phylogenetic analysis

Maximum likelihood (ML) and Bayesian Maximum Clade Credibility (MCC) phylogenetic trees were inferred from the coding sequences of 184 complete DENV-2 genome sequences. Twenty-five additional sequences representing the American, Asian 1, and Asian/American genotypes of DENV-2 were also included in the analysis (table S1). UTRs were not used for phylogenetic reconstructions in order to eliminate alignment artifacts resulting from unconfirmed indels in these regions. Sequences were aligned using ClustalW 1.83 (89) and manually curated. ML trees were generated using RAxML 7.0.4 (90) and PhyML 2.2 (91) incorporating the GTR+Γ4 model of nucleotide substitution, as determined by ModelTest v3.7 (92), and bootstrap resampling of 1000 and 100 replications respectively. For PhyML, we invoked the invariant sites. All resulting trees were compared across the two algorithms using in-house scripts to confirm that all major nodes were consistent across the algorithms. For final display, branches with <60% bootstrap support in the final tree were collapsed.

We used the *pamp* module of PAML 4 (93) to infer ancestral states by maximum parsimony for best trees and all bootstrap trees from RAxML and PhyML. We computed final ancestral sequences by comparing the calls from the RAxML and PhyML best trees and the amount of ancestral variation represented in the bootstrap trees for each position and making a decision about which sequence was most probable. Ambiguity codes were assigned where no clear most probable ancestral base existed.

Cells and viruses

C6/36 cells (gift from P. Young, University of Queensland, Australia) were grown in M199 (Invitrogen) with 10% Fetal Bovine Serum (FBS) (Denville Scientific), 100 units/mL penicillin/100 µg/mL streptomycin (P/S), and 1X HEPES at 28°C with 5% CO₂. Immature

monocyte-derived dendritic cells (iDCs) were grown in RPMI/10% FBS plus P/S at 37°C in 5% CO₂. Vero-76 cells (gift from Aravinda de Silva, University of North Carolina, Chapel Hill) were grown in DMEM/10% FBS plus P/S at 37°C in 5% CO₂. iDCs were derived from a Leukopak donor (All Cells, Inc.) as previously described (14). For infection, cells were thawed and plated in 10-cm dishes in 10mL of RPMI/10% plus P/S at 2.5×10^6 cells/mL. Cells were allowed to adhere for 1 hour (h) at 37°C, and non-adherent cells were removed by washing 5–10 times in warm RPMI. Ten mL of RPMI/10% FBS plus P/S was added to each plate, supplemented with 20,000U each of IL-4 (R&D Systems) and GM-CSF (PeproTech). Cytokines were supplemented 4–5 days post-plating with an additional 10,000U of each cytokine. iDCs were harvested at day 6 or 7 and phenotyped by flow cytometry to confirm DC phenotype (FSC/SSC; CD209-PerCP).

Low-passage (P1-P4) stocks of virus isolates from Nicaraguan patient sera were generated by inoculating acute patient sera onto C6/36 cells as previously described (66), serial-passaging in C6/36 cells and storing at –80°C. Re-sequencing of five isolates after 2 passages in C6/36 cells did not reveal the introduction of any spurious mutations (sequences available upon request). The quantity of virus present in each stock (genome equivalents; GE) was determined by quantitative RT-PCR targeting the NS5 gene as described below. The ratio of GE to PFU was not significantly different among clades (fig. S3F).

Viral competition assays

C6/36 cells or iDCs were infected with low-passage (<P4) viral isolates from each clade (5 NI-1 and 5 NI-2B pairwise; total $N=25$) mixed at equal GE ratios (1:1). C6/36 cells were plated at 3×10^5 cells/mL in 125µL in a flat-bottom 96-well plate one day prior to infection with 1.9×10^7 GE/well of each virus isolate (3.8×10^7 total GE/well) in triplicate. Supernatants were removed 3 h post-infection, wells washed 1X in PBS, and 250µL media added back to each well. To establish Day 0 values, 20µL supernatant was removed and pooled from triplicate wells before washing. All supernatant was removed at each time-point, frozen at –80°C, and replaced with 250µL fresh media. For iDC competition experiments, 5×10^4 iDCs were infected with 1×10^8 GE of each virus (2×10^8 GE total) per well. Infected cells were washed 1X in PBS after overnight incubation, and supernatants collected and frozen at –80°C after 48 h. Viral RNA was isolated (QIAamp Viral RNA Mini Kit) and amplified by one-step RT-PCR (One-Step RT-PCR Kit) with primers specific to sites in NS1 conserved among Nicaraguan DENV-2 isolates (Forward 5'-GAATCCCCTTCAAACTAGC-3'; Reverse 5'-ATGTCCGGCTGTGACCAAG-3') to generate a 950-kb amplicon. Two µL of viral RNA was amplified with 4µL 5X Buffer, 1µL dNTPs, 1µL each primer (10µM stock), 0.8µL One-Step RT enzyme mix (Qiagen), and 1µL RNaseOUT (Invitrogen) in a final reaction volume of 20µL. Viral RNA was reverse transcribed for 30 min at 50°C and heat-inactivated for 15 min at 95°C, and cDNA was amplified by 30 cycles of 94°C for 45 sec, 54°C for 45 sec, and 72°C for 1.5 min, followed by a final extension at 72°C for 10 min. Amplification was confirmed by electrophoresis on 1% agarose, and amplicons were directly sequenced by adding 0.5µL PCR product to 0.5µL NS1 sequencing primer (5'-GAGGGCATTGTGGAATCC-3') in a final volume of 13µL, amplifying using the BigDye Terminator method, and sequencing on an ABI3730xl DNA analyzer (UC Berkeley DNA Sequencing Facility). ABI chromatograms were analyzed via

the PolySNP PERL script (73), and the relative proportion of each virus in the dual infection was calculated by averaging the proportions of all valid SNPs. The relative fitness of each virus in each pairwise dual infection was calculated as previously described (72). Briefly, the proportion of each isolate at a given time-point after infection (f_o) was divided by its initial proportion in the inoculum (i_o) to derive a relative fitness (ω) value ($\omega = f_o/i_o$).

Neutralization assays

Reporter viral particles (RVPs) (94) that represented the WHO reference viruses (DENV-1, WestPac-74; DENV-2, S16803; DENV-3, CH53489) were a kind gift of Molecular Integral, Inc. (Philadelphia, PA) (95). RVP neutralization assays were performed by incubating 25 μ L of each RVP with 25 μ L diluted patient serum [starting at a 1:10 dilution (serum + RVP) in RPMI/10% FBS plus P/S, pH 8.0] shaking at room temperature for 1 h at 100 rpm and adding 4×10^4 Raji/DC-SIGN cells (ATCC)/well in a total volume of 100 μ L. After incubation for 48 h at 37°C, Green Fluorescent Protein (GFP)-positive cells were enumerated on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed in FlowJo v. 7.2.5 (TreeStar Software). Relative infection was determined using an RVP-only control. Human dengue-naïve serum (Red Cross) and anti-DENV polyvalent sera (WHO Reference Sera; NIBSC, UK) were used as negative and positive controls, respectively. NT₅₀ values were calculated using nonlinear regression analysis with a variable slope (GraphPad 5.0; Prism). Samples were classified as seropositive for the serotype with the higher NT₅₀ as compared to the other serotypes whenever the NT₅₀ exceeded 40.

Statistical analysis

To analyze disease severity, we classified study participants as DF, DHF or DSS using traditional WHO criteria (1). Secondary outcomes of disease severity included low platelet count ($< 50,000$ cells/mL) (74). Viral clade was assigned as described above. All analyses were restricted to subjects with known gender, age, immune status, season of infection, infecting viral clade, and primary outcome. This resulted in a sample of 136 out of 154 participants from the Hospital study and 95 out of 107 participants from the Cohort study. Fisher's exact test was used to compare proportions. Kruskal Wallis and Mann Whitney/Wilcoxon Rank-Sum tests were used for all nonparametric tests of >2 or 2 groups, respectively. Odds ratios for our primary and secondary outcomes were estimated using logistic regression in univariate and multivariate analyses adjusting for season of infection, immune status (previous DENV exposure), gender, and age.

To compare rates of DSS over the course of illness for each clade, the logrank test was used. For birth year analyses, subjects were classified as "DENV-3-era" if they were born before 1999 and "post-DENV-3-era" if they were born in 1999 or after. We adjusted for the changing representation of birth groups over time using the chi square test with a Rao-Scott correction. To further refine immunological background, children were sub-classified into groups based on birth year: (i) <1994 ("pre DENV-3"), (ii) 1994–1995 ("early DENV-3") and (iii) 1996–1998 ("later DENV-3"), (iv) 1999–2001 ("DENV-2"), (v) 2002–2004 ("DENV-1"), and (vi) 2005–2007 ("recent DENV-2"). The chi square goodness of fit test was used to compare the observed and expected distribution of cases of dengue. The Rao-Scott adjustment was used to adjust for the year span of each birth group, as birth groups

that spanned more years would be expected to have more cases of dengue. We constructed a full mixed model, by clade, of viremia on the log scale as a repeated measure, with disease classification as a fixed effect and hospital day of sample as a random effect. *P*-values ≤ 0.05 were considered to be significant, while *P*-values >0.05 but ≤ 0.1 were considered to be nonsignificant trends. All analyses were performed in SAS version 9.2 (SAS Institute Inc., Cary, NC).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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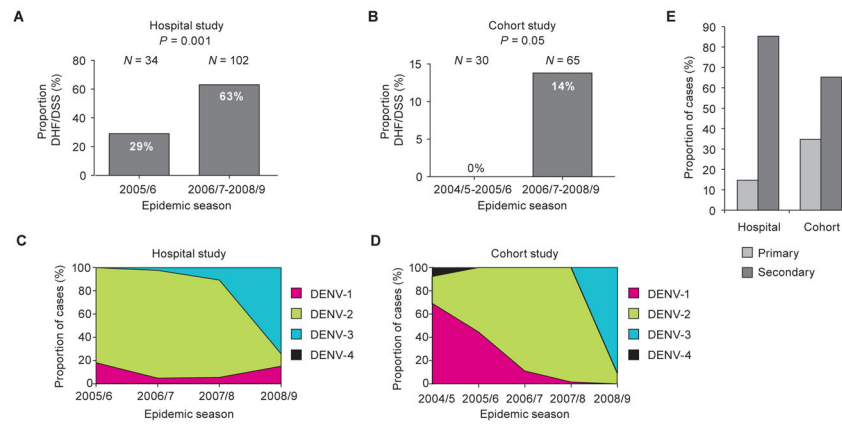


Fig. 1. Increase in severity of DENV-2 infections in two independent studies of pediatric dengue in Nicaragua

(**A, B**) The proportion of DENV-2 cases, including both primary and secondary infections, classified as DHF/DSS in the early as compared to later seasons in the Hospital ($P = 0.001$, Fisher's exact test) (**A**) and Cohort ($P = 0.05$, Fisher's exact test) (**B**) studies. The total number (N) of DENV-2 infections included in the analysis is shown above each graph. (**C, D**) The proportion of each serotype across four epidemic seasons (2005/6–2008/9) in the Nicaraguan Hospital study (**C**) and five epidemic seasons (2004/5–2008/9) in the Nicaraguan Pediatric Dengue Cohort study (**D**). Pink = DENV-1; green = DENV-2; blue = DENV-3; black = DENV-4. (**E**) The proportion of all DENV-2 cases classified as primary (light gray bars) or secondary (dark gray bars) DENV infections in the Hospital and Cohort studies, as defined in Materials and Methods.

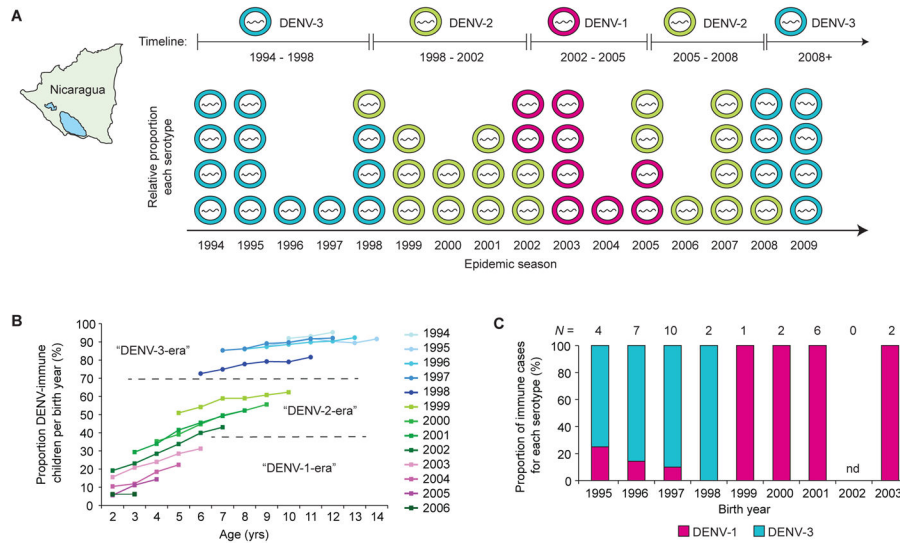


Fig. 2. DENV serotype exposure across seasons in Nicaragua

(A) The relative circulation of DENV serotypes (DENV-1 in pink; DENV-2 in green; DENV-3 in blue) by year in Nicaragua from 1994 to 2009 as derived from various DENV epidemiological studies across these years (48, 66–69). (B) The proportion of children from the Cohort study who were immune to DENV plotted as a function of birth year. Immunity was measured annually by Inhibition ELISA in samples collected prior to each epidemic season. The dotted lines separate “DENV-3-era” (born before 1999; blue), “DENV-2-era” (born between 1999 and 2002; green) and “DENV-1-era” (born between 2003 and 2006; pink) children as analyzed in our study. Birth year color codes correspond to dominant serotype as defined in panel A and are shown to the right of the graph. (C) Proportion of patients in the Cohort study in each birth year immune to DENV-1 (pink) or DENV-3 (blue) as determined by assaying neutralizing antibody titers (NT_{50} determined using RVP assay) to DENV-1 and DENV-3; N is the total number of samples analyzed from each year; the number of samples assayed in each year that did not generate significant titers for either DENV-1 or DENV-3 were 1/5 (1995), 1/8 (1996), 0/10 (1997), 3/5 (1998), 0/1 (1999), 1/3 (2000), 2/8 (2001), 0/2 (2003); nd, not determined.

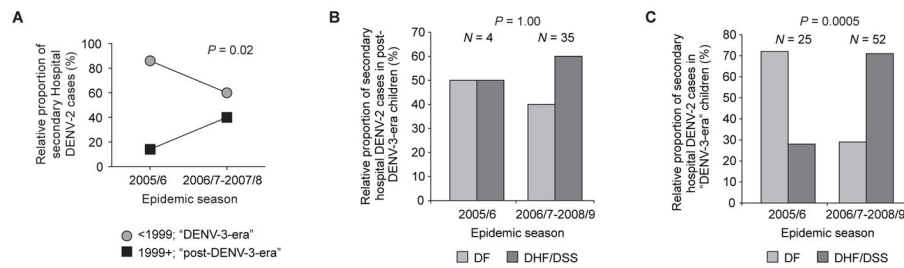


Fig. 3. Higher frequency of DENV-2 infection in DENV-1-immune children in later (2006/7–2008/9), versus earlier (2005/6) seasons

(A) Relative proportion of secondary DENV-2 cases in the Hospital study that occurred across seasons, stratified by birth group (circles, <1999, “DENV-3-era”; squares, 1999+, “post-DENV-3-era”; $P = 0.02$, Rao Scott chi square test, comparing the proportion of secondary DENV-2 Hospital cases from the “post-DENV-3-era” birth group (as opposed to the “DENV-3-era” birth group) in early versus later seasons, adjusting for the decreased representation of children from the older birth group in later seasons). (B) Relative proportion of secondary DENV-2 cases in “post-DENV-3-era” children (1999+) in the Hospital study classified as DF (light gray bars) or DHF/DSS (dark gray bars) ($P = 1.00$; Fisher’s exact test, comparing the proportion of secondary DENV-2 Hospital cases that are DHF/DSS in early versus later seasons among “post-DENV-3-era” children). (C) Relative proportion of secondary DENV-2 cases in “DENV-3-era” children (<1999) in the Hospital study classified as DF (light bars) or DHF/DSS (dark gray bars) ($P = 0.0005$; Fisher’s exact test, comparing the proportion of secondary DENV-2 Hospital cases that are DHF/DSS in early versus later seasons among “DENV-3-era” children).

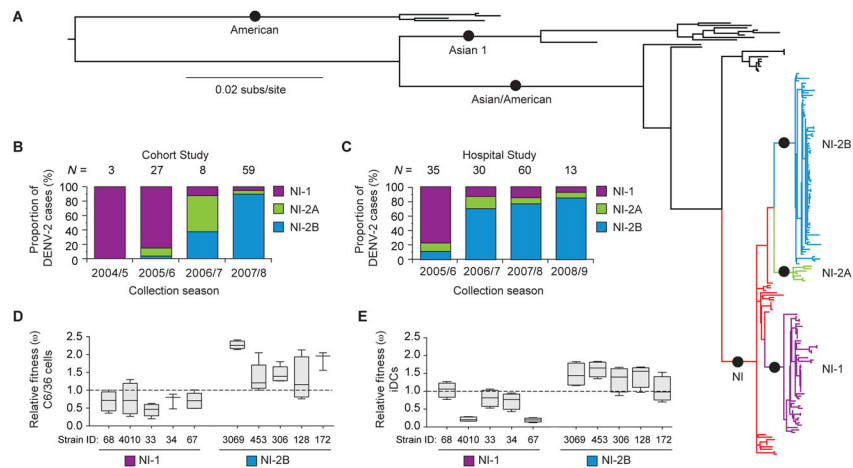


Fig. 4. Association of Nicaraguan DENV-2 clade replacement with increased viral fitness (A) Maximum likelihood tree of 159 complete coding-region nucleotide sequences sampled from the Cohort and Hospital studies, as well as other samples collected in Nicaragua (see Materials and Methods for details) between 1999 and 2008. Twenty-five sequences representing the major genotypes of DENV-2 (American, Asian 1 and Asian/American) were used as outgroup sequences, as they were genetically distinct from the Nicaraguan sequences. Only nodes (circles) that are supported at least 60% of the time by bootstrap re-sampling (1000 replications) of the data are shown. Major Nicaraguan clades are highlighted (NI = red; NI-1 = purple; NI-2A = green; NI-2B = blue). (B, C) Changes over time in the proportion of DENV-2 cases caused by infection with major Nicaraguan clades that occurred during the specified epidemic seasons for virus isolates that were either sequenced or genotyped (B) in the Cohort study (2004/5–2007/8; $N = 97$) or in (C) the Hospital study (2005/6–2008/9; $N = 138$). (D, E) Relative fitness (ω) as determined by dual-infection assays of NI-1 isolates in competition with each NI-2B isolate as assayed in (D) mosquito cells (C6/36) and (E) human monocyte-derived immature dendritic cells (iDCs). The proportion of each isolate present in the cell supernatant at a given time-point after infection (f_o) was divided by its initial proportion in the inoculum (i_o) to derive a relative fitness (ω) value ($\omega = f_o/i_o$) (72); $f_o = 4$ days for C6/36 cells; $f_o = 2$ days for iDCs. A relative fitness value of 1, the neutral expectation given equal fitness of viral isolates, is shown as a dotted line. Box plots show the 25th and 75th percentiles with a line indicating the median relative fitness value and error bars showing the minimum and maximum fitness values. Five NI-1 viruses were competed against 5 NI-2B viruses. Viral strain numbers used in each assay are indicated at the bottom of the graph.

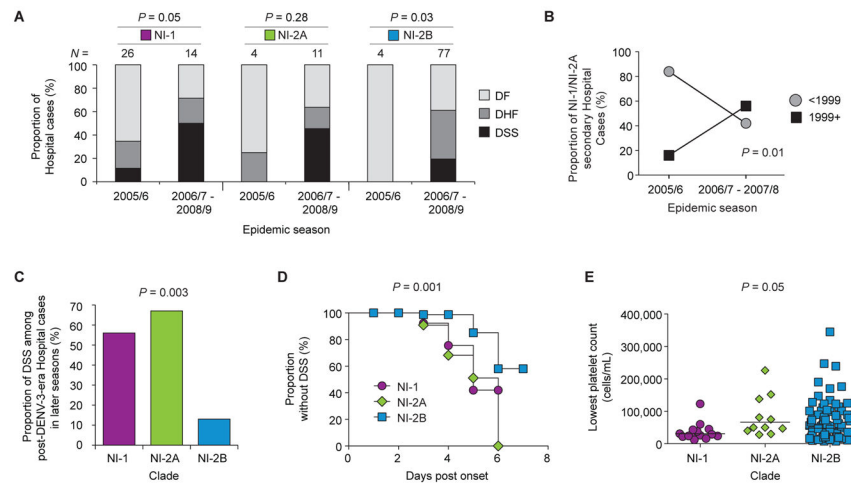


Fig. 5. Association of NI-1 and NI-2A infections with more severe disease outcomes in DENV-1-immune children

(A) The proportion of dengue cases across seasons by clade—NI-1, NI-2A or NI-2B—classified as DF (light gray), DHF (dark gray) or DSS (black) in the Hospital study. An increase in cases of severe dengue disease (DHF/DSS) was observed in all clades (NI-1, $P = 0.05$; NI-2A, $P = 0.28$; NI-2B, $P = 0.03$; Fisher’s exact test, comparing the proportion of DENV-2 Hospital cases that are DHF/DSS in early versus later seasons for each clade). (B) The proportion of cases of secondary NI-1 and NI-2A infections in the Hospital study across seasons for each birth group (circles, <1999; squares, 1999+; $P = 0.01$, Rao Scott chi square test, comparing the proportion of NI-1/NI-2A secondary Hospital cases from the “post-DENV-3-era” birth group in early versus later seasons, adjusting for the expected decrease in representation of children from the older birth group in later seasons). (C) The proportion of cases of secondary DENV infections from the 1999+ birth group (DENV-1-immune) in the Hospital study in seasons 2006/7–2008/9 classified as DSS for each clade (NI-1, NI-2A and NI-2B). The proportion of each clade classified as DSS is compared against the null hypothesis that all three clades are at equal proportion ($P = 0.003$; Fisher’s exact test, comparing the proportion of DENV-2 Hospital cases in the later seasons that are DSS, caused by NI-1 versus NI-2A versus NI-2B, among children from the “post-DENV-3-era” birth group). (D) Kaplan-Meier plot showing the time to DSS critical phase by clade among all cases of DENV-2 infection in the later seasons in the Hospital study plotted as a function of days post-onset of illness. NI-1, circles; NI-2A, diamonds; NI-2B, squares ($P = 0.001$; Logrank test, comparing the time to develop DSS in the later seasons, NI-1 versus NI-2A versus NI-2B, among children in the Hospital study). (E) Lowest platelet count (cells/mL) measured during illness episode from children infected with each of the DENV-2 clade viruses in the Hospital study in later seasons (2006/7–2008/9). NI-1, circles; NI-2A, diamonds; NI-2B, squares. $P = 0.05$; Kruskal-Wallis, comparing the lowest platelet count, NI-1 versus NI-2A versus NI-2B, among DENV-2 Hospital cases in the later seasons. Line indicates the geometric mean. For all panels, clade designations are colored as in Figure 4.

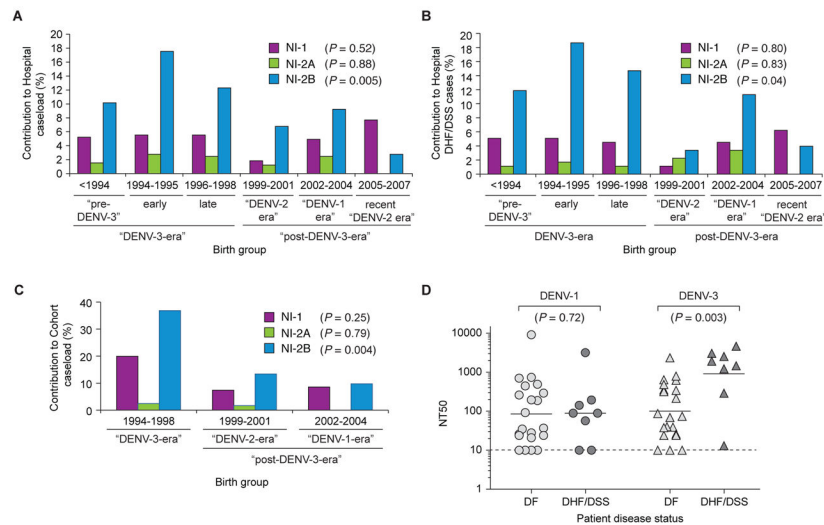


Fig. 6. Association of prior DENV-3 immunity with subsequent DENV-2 NI-2B infection (A–C) Contribution of each clade (NI-1, purple; NI-2A, green; NI-2B, blue) to (A) all dengue cases in the Hospital study, (B) cases of DHF/DSS in the Hospital study, (C) all dengue cases in the Cohort study. The observed distribution of each clade was compared to the expected distribution under the assumption that all children have equal risk of infection. Birth years were grouped into birth groups based on the history of the circulation of particular serotypes in Nicaragua (“pre”, “early” and “late” “DENV-3-era” and “post-DENV-3-era”, including “DENV-2-era”, “DENV-1-era” and “recent DENV-2 era”). Clade NI-2B is not evenly distributed among birth groups (chi square goodness of fit test with Rao Scott correction: (A) $P = 0.005$; (B) $P = 0.04$; (C) $P = 0.004$). (D) Titers of neutralizing antibody (NT_{50}) against DENV-1 (circles) and DENV-3 (triangles) assayed using the RVP neutralization assay in serum samples collected prior to 2006/7 and 2007/8 DENV-2 seasons from DF ($n = 21$) or DHF/DSS cases ($n = 8$). The limit of detection of the assay ($NT_{50} = 10$) is shown with a dotted line. The solid line indicates the geometric mean. DENV-1, $P = 0.72$; DENV-3, $P = 0.003$; Wilcoxon Rank-Sum test, comparing RVP NT_{50} titers in DF versus DHF/DSS cases in a nested case control study within the Hospital study. For all panels, clade designations are colored as in Figure 4.

Table 1

Characteristics of DENV-2 cases, Cohort study, 2004/5–2008/9, and Hospital study, 2005/6–2008/9.

| Demographic Characteristics | Cohort study | | Hospital study | |
|--|--------------------|--------------------|--------------------|--------------------|
| | 2004/5 – 2005/6 | 2006/7 – 2008/9 | 2005/6 | 2006/7 – 2008/9 |
| | N (%) ^a | N (%) ^a | N (%) ^a | N (%) ^a |
| DENV-2 Cases | 30 | 65 | 34 | 102 |
| Sex | | | | |
| Female | 12 (40) | 28 (43) | 16 (47) | 48 (47) |
| Male | 18 (60) | 37 (57) | 18 (53) | 54 (53) |
| Age, years (mean) | 5.2 | 7.8 | 8.3 | 8.2 |
| Day of presentation after onset of symptoms (mean) | 2.0 | 2.1 | 4.1 | 4.7 |
| Immune Status | | | | |
| Primary | 10 (33) | 23 (35) | 5 (15) | 15 (15) |
| Secondary | 20 (67) | 42 (65) | 29 (85) | 87 (85) |
| Birth Group | | | | |
| <1994 | 0 (0) | 0 (0) | 9 (26) | 13 (13) |
| 1994–1995 | 6 (20) | 3 (4) | 7 (21) | 21 (21) |
| 1996–1998 | 11 (37) | 31 (48) | 10 (29) | 23 (23) |
| 1999–2001 | 10 (33) | 17 (26) | 0 (0) | 16 (16) |
| 2002–2004 | 3 (10) | 14 (22) | 5 (15) | 22 (22) |
| 2005+ | 0 (0) | 0 (0) | 3 (9) | 7 (7) |
| WHO Classification | | | | |
| Dengue Fever (DF) | 30 (100) | 56 (86) | 24 (71) | 38 (37) |
| Dengue Hemorrhagic Fever (DHF) | 0 (0) | 6 (9) | 7 (21) | 37 (36) |
| Dengue Shock Syndrome (DSS) | 0 (0) | 3 (5) | 3 (9) | 27 (26) |
| Lowest Platelet Count (cells/mL) (mean) | 249,000 | 174,000 | 129,000 | 66,000 |

^a n = number of patients; % = % of total

Nonsynonymous amino acid mutations and UTR mutations that distinguish Nicaraguan DENV-2 clades^a.

Table 2

| Gene | Position ^b | Annotation ^c | NI ^d (N=159) | NI-1 (N=50) | NI-2A (N=7) | NI-2B (N=80) |
|--------------------|-----------------------|-------------------------|-------------------------|-----------------------------|-----------------------------|-----------------------------|
| 5'UTR ^e | -21 | - | TTTTT- | TTTTT- | TTTTTT ^f | TTTTTT ^f |
| C | 97 | - | R (AGG) | K (AAG) ^f | R (AGG) | R (AGG) |
| E | 492 | - | M (ATG) | M (ATG) | M (ATG) | V (GIG) ^f |
| NS1 | 94 | - | K (AAA) | R (AGA) ^f | K (AAA) | K (AAA) |
| NS1 | 279 | - | L (CTC) | L (CTC) | L (CTC) | F (ITC) ^f |
| NS3 | 245 | P | P (CCA) | T (ACA) ^f | P (CCA) | P (CCA) |
| NS4B | 245 | - | N (AAC) | N (AAC) | S (AGC) ^f | S (AGC) ^f |
| NS5 | 200 | C | K (AAA) | K (AAA) | K (AAA) | Q (CAA) ^f |
| NS5 | 290 | P | T (ACA) | T (ACA) | T (ACA) | I (ATA) ^f |
| NS5 | 401 | - | R (AGA) | R (AGA) | R (AGA) | K (AAA) ^f |
| NS5 | 523 | P | G (GGC) | G (GGC) | S (AGC) ^f | G (GGC) |
| NS5 | 642 | - | A (GCT) | A (GCT) | V (GTT) ^f | A (GCT) |
| 3'UTR ^g | 19 | - | T | T | T | C ^f |
| 3'UTR ^g | 132 | - | A | A | G ^f | G ^f |
| 3'UTR ^g | 145 | - | T | T | C ^f | C ^f |

^a Consensus sequences for each clade, NI-1, NI-2A and NI-2B.

^b Amino acid numbering based on start of each protein.

^c C = charge change; P = polarity change.

^d NI is an ancestral sequence inferred by maximum parsimony.

^e 5'UTR: the base preceding the start AUG is -1.

^f Amino acids or UTR mutations that distinguish NI-1, NI-2A or NI-2B viruses from the Nicaraguan ancestor ("NI") are shown in bold.

^g 3'UTR: the first base of the stop codon is 1.