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Insights into the molecular evolution of *Dengue virus* type 4 in Puerto Rico over two decades of emergence

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ABSTRACT

Dengue has emerged globally as a major human health problem since the 1950s and is now the most important arboviral disease of humans, infecting nearly 400 million people annually. While some cases are asymptomatic, others can develop a febrile illness (dengue fever) or even progress to severe and fatal dengue. Dengue is caused by any of 4 closely related but distinct viruses, known as *Dengue virus* serotype 1 to 4 (DENV-1 to DENV-4) which are maintained in endemic transmission to humans in large urban centers of the tropics by *Aedes* mosquitoes. Since the early 1960s, Puerto Rico, a major metropolitan center in the Caribbean, has experienced increasingly larger and clinically more severe epidemics following the introduction of all four dengue serotypes. The first dengue hemorrhagic fever epidemic in 1986, and a particularly severe outbreak in 1998 were dominated by novel DENV-4 strains that evolved in Puerto Rico, replacing earlier strains and spreading throughout the region. Sequence characterization of 54 complete DENV-4 genomes and their comparative evolution against 74 previously published viral sequences from the region over several decades shows that DENV-4 strains from these periods were genetically distinct based on unique changes in the envelope and non-structural genes. Their replacement of earlier strains in Puerto Rico progressed rapidly, suggesting that strong natural selection played a role in their fixation. This study confirms that DENVs evolve through rapid lineage turnover driven in part by natural selection and genetic drift.

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1. Introduction

Dengue virus (DENV), a mosquito-borne pathogen of humans, causes an estimated 390 million infections annually throughout the tropics (Bhatt et al., 2013). Two vector species, principally *Aedes aegypti* and to a lesser extent *Ae. albopictus*, are responsible for most of the transmissions. DENV is a fast-evolving single-stranded, positive sense RNA virus of the genus *Flavivirus* in the family Flaviviridae. Evidence suggests that the four distinct serotypes of DENV (DENV-1 to DENV-4) originally diverged in canopy-dwelling mosquitoes and nonhuman primates in the rainforests of Asia, before colonizing humans several hundred years ago, each serotype undergoing a burst of evolutionary change associated with adaptations to new vectors and/or humans, and their demographic expansions (Wang et al., 2000; Twiddy et al., 2002a,b; Vasilakis et al., 2008; Gubler, 2014).

DENV have continued to evolve in humans, diversifying into genotypes or subtypes within each serotype, accompanied by lineage extinctions and replacements within a country, or sometimes due to exchange from other regions, that have been correlated with epidemic activity and/or disease severity (Gubler, 1988, 1998; Gubler et al., 1978, 1981; Rico-Hesse et al., 1997; Messer et al., 2002, 2003; Bennett et al., 2003, 2006; Steel et al., 2010).

Infection with any DENV serotype can result in a range of disease syndromes from asymptomatic to fever with rash. A small proportion of cases can progress to more severe disease characterized by

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a variety of syndromes ranging from hemorrhagic to neurologic disease; a vascular leak syndrome, with or without hemorrhage, is the most common form of severe dengue. Case fatality rates vary across populations and average about 5%. Many factors contribute to the risk for severe disease, including whether the patient has been previously infected by another serotype (Halstead et al., 1970; Kurane et al., 1991; Green and Rothman 2006; Midgley et al., 2011; Rothman 2011), the genetic background of the individual (Sierra et al., 2007; García et al., 2011Khor et al., 2011; Alagarasu et al., 2013), and the genetic makeup of the virus (Rosen 1977; Gubler et al., 1978, 1981; Gubler, 1988, 1998; Rico-Hesse et al., 1997; Leitmeyer et al., 1999). Control of dengue remains problematic, relying on vector control since there are as yet no vaccines available.

Dengue has emerged as the most important arboviral disease of humans. Historically, epidemic DENV transmission involved a single serotype in specific geographic regions (Gubler, 1998). However, beginning in the 1940s with World War II, DENV began to expand geographically with the movement of mosquito vectors and people. During the past few decades in particular, with increasing globalization and unprecedented urban growth, all DENV serotypes have spread throughout the tropics and now co-circulate in more than 100 countries with increasing frequency and magnitude of epidemics (Gubler, 2002, 2011; Kroeger and Nathan, 2006; Ranjit and Kissoon, 2011).

Puerto Rico exemplifies this expansion, beginning with early circulation of only one serotype at a time, followed by the establishment and co-circulation of multiple serotypes and mounting disease burden. DENV became sequentially re-established in Puerto Rico and the rest of the new world in the 1970s, following the cessation of the hemispheric *Ae. aegypti* eradication program. Early dengue activity in Puerto Rico was characterized by periodic outbreaks of one or at most two serotypes: 1963 (DENV-3), 1969 (DENV-2), 1972/73 and 1975/76 (DENV-2), 1977 (DENV-2 and DENV-3), 1978 (DENV-1), 1981/82 (DENV-1 and DENV-4) (Gubler and Trent, 1993; Dietz et al., 1996). The 1981/1982 outbreak was the first involving DENV-4, after which co-circulation of multiple serotypes became the norm, along with epidemics of increasing size and disease severity (Gubler and Trent, 1993; Dietz et al., 1996). DENV-1 was introduced in late 1977 and caused a major epidemic of classical dengue fever in 1978, remaining the predominant serotype during 1984/85 and 1991–93; DENV-2 (Jamaican/Asian-American genotype) was introduced in 1984, but did not cause an epidemic until 1994, dominating from 1988 to 1990 and 1994 to 1996, while DENV-4 was the predominant serotype during three large epidemics in 1982, 1986/87 and 1998. The emergence of dengue hemorrhagic fever in Puerto Rico occurred during the 1986 epidemic (Gubler and Trent, 1993; Dietz et al., 1996), and the 1998 outbreak was the most clinically severe outbreak up to that time (Bennett et al., 2003, 2010). In the following years, DENV-4, DENV-1 and DENV-2 declined dramatically and DENV-3 became the dominant serotype from 1999 to 2003, supplanted briefly by DENV-2, and then re-emerged in 2007 (McElroy et al., 2011). From 2007 to 2010, all four DENV serotypes were reported in Puerto Rico (Santiago et al., 2012).

Because DENV-4 has played a significant role in the epidemiology of dengue in Puerto Rico for 20 years, and was responsible for causing three of the four epidemics during that period, it has served as a model for understanding how virus evolution correlates with epidemic potential and severity. DENV-4 sequence characterization over two decades based on partial genomes (<40%) indicated that strong adaptive evolution in the nonstructural gene 2A (NS2A) correlated with the 1998 epidemic (Bennett et al., 2003). However, the impact of changes in other important genes, such as the polymerase (NS5), remains uncharacterized. Whole-genome characterization will complete our understanding of the genetic basis of lineage formation across epidemic and non-epidemic periods. The aim of this work was to provide a comprehensive phylogenetic analysis based on full genome sequences of DENV-4 in Puerto Rico from its first appearance in 1981 through the large outbreak in 1998. Our study revealed additional amino acid substitutions in nonstructural genes NS1 and NS5, as well as nucleic acid changes in non-translated regions (NTR), associated with the changing epidemic dynamics and lineage turnover in DENV evolution in Puerto Rico.

2. Materials and methods

2.1. Virus collection

Viruses were originally isolated from human serum samples of dengue cases collected since 1981, by inoculation of *Ae. aegypti* or *Toxorynchities amboinensis* mosquitoes or C6/36 *Ae. albopictus* cells, and archived, by the Dengue Branch of the U.S. Centers for Disease Control and Prevention (CDC), in San Juan, Puerto Rico, as part of its surveillance program (Gubler et al., 1984). We randomly subsampled, stratified by year group, 21 archived strains of DENV-4 for whole-genome sequencing guided by the previously partially characterized 82 specimens (Bennett et al., 2003) spanning the period of dengue emergence in Puerto Rico from 1986 to 1998. In addition, full genome sequencing of five randomly sampled DENV-4 viruses stratified by year group from five Puerto Rico municipalities was conducted by the CDC in collaboration with the BROAD Institute. Table 1 documents all of the viruses sampled and included in this study by year and municipality. Viral stocks were produced in *A. albopictus* C6/36 cells and the total number of passages was kept to below three to minimize *in vitro* evolution. This work has been performed under Institutional Review Board approvals at the Centers for Disease Control and Prevention, the University of Hawaii, and the BROAD Institute.

2.2. RNA extraction, RT-PCR and nucleotide sequencing

Viral RNA was extracted using the QIAamp viral RNA mini kit and reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen). For each strain, the entire genome was amplified by polymerase chain reaction (PCR) using Pfu Ultra II Fusion HS DNA polymerase (Stratagene), a high-fidelity enzyme, and primer pairs of our own design. Amplicons represented overlapping fragments of approximately 400 bp covering the entire 10,700 nucleotide genome, including the viral structural genes capsid (C), precursor membrane (prM) and envelope (E), the seven non-structural genes (NS1, NS2A/B, NS3, NS4A/B and NS5), and the NTR at the 5′ and 3′ ends of the open reading frame (ORF). Each amplicon was confirmed by gel electrophoresis and purified using ExoSAP-IT PCR Clean-up Kit (GE Healthcare). Purified DNA products were sequenced with primers of our own design targeted for both strands, to generate 2x to 6x high-quality coverage in both directions. Sequencing services were performed by the UH Manoa Advanced Studies in Genomics, Proteomics and Bioinformatics Facility using an Applied Biosystem 3730XL DNA Analyzer. Primer sequences are provided as Supplementary material.

2.3. Phylogenetic analysis

Sequences were trimmed, cleaned and assembled using Sequencher 5.0 (Gene Code Corp). Complete assembled genomes were aligned in TranslatorX (http://translatorx.co.uk/) and verified in Se-Al 2.0 (http://tree.bio.ed.ac.uk/software/seal/), along with publicly available sequences, both whole and partial genomes, covering DENV-4 circulation in Puerto Rico. Alignments were analyzed using RAxML to generate a maximum-likelihood (ML) tree with

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