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Genetic and phenotypic characterization of sylvatic dengue virus type 4 strains

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ABSTRACT

Four serotypes of dengue virus (DENV 1–4) currently circulate between humans and domestic/peridomestic *Aedes* mosquitoes, resulting in 100 million infections per year. All four serotypes emerged, independently, from sylvatic progenitors transmitted among non-human primates by arboreal *Aedes* mosquitoes. This study investigated the genetic and phenotypic changes associated with emergence of human DENV-4 from its sylvatic ancestors. Analysis of complete genomes of 3 sylvatic and 4 human strains revealed high conservation of both the 5′- and 3′-untranslated regions but considerable divergence within the open reading frame. Additionally, the two ecotypes did not differ significantly in replication dynamics in cultured human liver (Huh-7), monkey kidney (Vero) or mosquito (C6/36) cells, although significant inter-strain variation within ecotypes was detected. These findings are in partial agreement with previous studies of DENV-2, where human strains produced a larger number of progeny than sylvatic strains in human liver cells but not in monkey or mosquito cells.

Introduction

There is growing concern about the potential emergence of new pathogens, particularly arthropod-borne viruses (arboviruses), from animal reservoirs into humans (Weaver and Reisen, 2010; Wilder-Smith and Gubler, 2008). To gain insight into this process of emergence, it is particularly instructive to study viruses that have completed the trajectory from an enzootic into a human reservoir, such as the four serotypes of dengue virus (DENV-1–4, genus *Flavivirus*, family *Flaviviridae*). These viruses originated in a sylvatic cycle between nonhuman primates, and possibly other enzootic hosts, and arboreal *Aedes* (*Ae.*) mosquitoes. Each serotype emerged independently into a human transmission cycle, wherein humans now serve as the exclusive reservoir and amplification hosts for the endemic/epidemic lineages (Vasilakis et al., 2011). In this human cycle, DENV-1,-2,-3, and -4 are transmitted by domestic and peridomestic *Aedes* mosquitoes, primarily *Ae. aegypti aegypti* and *Ae. albopictus* (Halstead, 1997; Halstead et al., 1964; Rosen et al., 1954; Sabin, 1952; Simmons et al., 1931). The human DENV cycle is presently found in nearly all urban and peri-urban environments throughout the tropics and subtropics. In recent decades, DENV transmission among humans has intensified due to increased travel, uncontrolled urbanization and lack of effective and sustainable vector control programs (Guzman et al., 2010). By current estimates, DENV infects approximately 100 million people each year in over 100 countries.

Unlike the ancestors of many other human viruses, the ancestral sylvatic cycle of DENV remains extant and has been documented in two foci: one in West Africa involving arboreal *Aedes spp.* (e.g. *Ae. furcifer, Ae. luteocephalus)* and primates including patas monkeys (*Erythrocebus patas*), African green monkeys (*Chlorocebus sabaeus*), and Guinea baboons (*Papio papio*) (Cordellier et al., 1983, Diallo et al., 2003, 2005; Hervy et al., 1984; Rodhain, 1991; Saluzzo et al., 1986a; Vasilakis et al., 2008c) and the other in peninsular Malaysia involving *Ae. niveus s. l.* and primates including cynomolgus macaques (*Macaca fascicularis*), pig-tailed macaques (*Macaca nemestrina*) and silvered leaf monkeys (*Presbytis cristata*) (Rudnick, 1986). The continued circulation of sylvatic DENV provides an opportunity to perform comparative studies to elucidate the virus attributes that promote arboviral emergence. However these sylvatic viruses also pose a considerable threat, because they may retain the capacity to re-emerge even as efforts to control circulation of human dengue intensify (Vasilakis et al., 2011), in a manner analogous to urban yellow fever.

Although sylvatic and human DENV strains show substantial genetic differences, our previous studies of DENV-2 demonstrated that such differences do not constitute an adaptive barrier to emergence into the human transmission cycle. Specifically sylvatic DENV-2 showed no detectable deficit relative to human DENV-2 in replication kinetics in

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cultured human or *Ae. albopictus* cells (Vasilakis et al., 2008b), in replication in two proxy measures for human infection, monocyte-derived dendritic cells and SCID mice engrafted with human hepatoma cells (Vasilakis et al., 2007b), or in infection of *Ae. aegypti* and *Ae. albopictus* in vivo (Hanley and Vasilakis, unpublished data). Furthermore, some sylvatic *Aedes* species sympatric with sylvatic DENV are capable of transmitting these viruses and may act as bridge vectors when they move between forest and human habitations. For example in West Africa a highly susceptible vector of sylvatic DENV, the forest-dwelling *Ae. furcifer,* (Diallo et al., 2005), disperses into villages (Diallo et al., 2003), while in Southeast Asia, *Ae. albopictus* disperses from the forest into surrounding agricultural settlements (Smith, 1956). Both species bite humans, resulting in potential transmission of sylvatic DENV to humans. Indeed, several reports have now documented spillover of sylvatic DENV, resulting in infection of individual humans or small outbreaks (Cardosa et al., 2009; Carey et al., 1971; Franco et al., 2011; Monlun et al., 1992, Saluzzo et al., 1986a, 1986b; Vasilakis et al., 2008c). Currently it is not possible to distinguish sylvatic and human DENV infections with antibody-based assays, and thus sylvatic DENV infections may frequently be misclassified as human DENV. Nonetheless two recent reports from Southeast Asia (Cardosa et al., 2009) and West Africa (Franco et al., 2011) reveal that sylvatic DENV infections can result in severe disease.

Collectively, these data indicate that DENV has evolved as an ecological generalist capable of utilizing a broad range of *Aedes* vectors and primate hosts (including humans) and suggest that the public health impact of sylvatic dengue spillover may be substantially greater than is currently appreciated. However to date the vast majority of both experimental (Cox et al., 2011; Mota and Rico-Hesse, 2009, 2011; Vasilakis et al., 2007b, 2008a, 2008b, 2009) and phylogenetic (Vasilakis et al., 2007a) studies of sylvatic DENV have focused exclusively on DENV-2, and it is critical to extend these efforts to encompass the full range of genetic and phenotypic diversities within sylvatic DENV. Thus, we investigated the genetic relationships of sylvatic and human DENV-4 using complete genome sequences from each ecotype. Previous studies have generated phylogenies of DENV-4 that include a only a single sylvatic DENV-4 isolate or a single gene from the all 3 known sylvatic DENV-4 isolates (AbuBakar et al., 2002; Wang et al., 2000); in this study we utilized the complete genome sequences of the three sylvatic DENV-4 isolates as well as 59 human DENV-4 sequences from GenBank that represent the complete genotypic diversity known for this serotype. To better link genetic and phenotypic variations, we also measured the replication kinetics of a subset of human versus sylvatic isolates in both mammalian and mosquito cells in culture.

Results and discussion

Phylogenetic analyses

Complete genome sequences from 59 human DENV-4 isolates that span the genetic, geographic and temporal ranges of DENV-4 diversity (Chen and Vasilakis, 2011; Holmes and Twiddy, 2003; Vasilakis and Weaver, 2008; Villabona-Arenas and Zanotto, 2011; Weaver and Vasilakis, 2009) as well as the only 3 known sylvatic DENV-4 strains (Rudnick, 1986) were used for phylogenetic analysis. Fig. 1 shows a representative phylogenetic tree derived from Bayesian analysis; several consensus trees obtained based on Maximum-likelihood (ML) and Bayesian analyses exhibited similar topologies.

All 3 sylvatic DENV-4 strains were genetically distinct from and basal to human DENV-4 strains. Among the sylvatic strains, P73-1120 and P75-514 were more closely related to each other than to the P75-215 strain, an observation that reflects their history. Strain P73-1120 was isolated from a sentinel silver leaf monkey (*P. cristata*) in the Gunong Besut forest reserve in Malaysia in 1973 (Table 1). Two years later an aliquot of the original serum sample collected from the sentinel monkey was used for experimental infections of silver leaf monkeys, which led to the isolation of P75-514 strain (Rudnick, 1986). Our sequences indicated that, during this single passage, several mutations occurred, which were primarily synonymous and resulted in a 0.1% nucleotide divergence from the parent strain P73-1120 (Table 1). Strain P75-215 was isolated in 1975 from a pool of *Ae. niveus s. l.* mosquitoes collected in the forest canopy of the Gunong Besut forest reserve in late 1974 (Rudnick, 1986).

As shown in previous studies (AbuBakar et al., 2002; Bennett et al., 2003; Foster et al., 2003; Klungthong et al., 2004; Vasilakis and Weaver, 2008; Villabona-Arenas and Zanotto, 2011; Weaver and Vasilakis, 2009), human DENV-4 strains clustered into three major genotypes. Genotype I includes strains from the Philippines, Thailand, Vietnam, Myanmar, Malaysia, Sri Lanka, and India (Cecilia et al., 2011; Dash et al., 2011). The India G11337 strain, which was isolated in 1961 and is therefore one of the oldest DENV-4 strains sampled, is genetically distinct and basal to other isolates of this clade. Genotype II is composed of two distinct sub-lineages: IIa, including strains from Malaysia, Thailand and Taiwan and IIb, comprising strains from the Caribbean and the Americas (Bennett et al., 2003; Foster et al., 2003). Genotype III includes Thai strains sampled between 1997 and 2001 that are distinct from the Thai isolates of genotype II (Klungthong et al., 2004).

Genetic analysis

The DENV genome is comprised of approximately 10.7 kilobases (kb) of single stranded RNA of positive polarity. A single open reading frame (ORF) of 10,164 nucleotides (Table 2) is flanked by untranslated regions (UTRs) at the 5′ and 3′ ends. The 5′-UTR is 101 nt long and is capped with a type I 5′cap (Cleaves and Dubin, 1979; Lindenbach and Rice, 2003), while the 402 nt 3′-UTR lacks the classical polyadenylation site (Wengler et al., 1978) (Table 2).

5′- and 3′- UTRs

The sequences of both the 5′ and 3′ UTRs are highly conserved among the four DENV serotypes (Markoff, 2003). In the DENV-4 strains analyzed, the sequence of both UTRs was highly conserved, and the 5′ UTR had a higher sequence identity between the strains (ranging from 95.2 to 98.0%) than the 3′ UTR (88.7–95.1%) (Table 2; sequence data is presented in Supplemental Data Figs. 1 and 2). The 5′ cyclization sequence (5′CS) located within the coding region of the capsid gene (nt 35–42 after the start codon) (Hahn et al., 1987) was present in all strains analyzed. The flavivirus 3′ UTR comprises three sub-regions: the Variable Region (VR), core, and 3′ terminus (Markoff, 2003). Several previously identified conserved regions within the core and 3′ terminus (RCS2, CS2 and CS1 and CPS (Hahn et al., 1987)) were present in both the sylvatic and human strains (Supplemental Data 3C, D). The highly conserved 3′ terminal dinucleotide of the plus-strand (UC-3′) was present in all DENV-4 sequences for which the 3′ terminal sequence was available, and was complementary to the 5′-terminal dinucleotides (5′ AG), purportedly enabling cyclization of the genome during the early stages of replication (Markoff, 2003; Rice et al., 1985; Wengler and Wengler, 1981). Relative to the sylvatic strains, the VR of human genotypes I–III exhibited 3, 13 or 15 nt deletions, respectively (Supplemental Data 2). A similar accrual of deletions in human strains relative to sylvatic strains has been documented in DENV-2 strains (Vasilakis et al., 2008b), and other investigators (Leitmeyer et al., 1999; Shurtleff et al., 2001) have suggested that these deletions occurred during the evolution of human strains from sylvatic progenitors. Proutski et al. (1999) suggested that the VR region may act as a spacer to protect conserved and structurally important distal regions but the precise effects of these particular deletions in human strains have not been determined.

The predicted RNA secondary structures of representative sylvatic (P73-1120) and human (IndiaG11337) DENV-4 strains were extremely similar at the 5′ and 3′ UTRs (Supplemental Data Figs. 3A–