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Review

The origin, emergence and evolutionary genetics of dengue virus

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Abstract

Dengue is one of the most important emerging viruses, posing a threat to one-third of the global human population. Herein we show how the comparative analysis of gene sequence data has shed light on the origin and spread of dengue virus, as well as on the evolutionary processes that structure its genetic diversity. This reveals that dengue virus has a relatively recent evolutionary history, with the four serotypes originating approximately 1000 years ago and only establishing endemic transmission in humans in the last few hundred years. However, its place of origin remains uncertain as does the extent of genetic and phenotypic diversity present in the sylvatic (primate) transmission cycle. Although there is some evidence that viral strains differ in key phenotypic features such as virulence, and for positive selection at immunologically important sites, it seems likely that stochastic processes also play a major role in shaping viral genetic diversity, with lineage extinction a common occurrence. A more complete understanding of the evolution and epidemiology of dengue virus, particularly with respect to the aetiology of severe disease, will require large-scale prospective studies and the comparative analysis of complete genome sequences.
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1. Introduction—the biology and epidemiology of dengue virus

Dengue is the most common vector-borne viral disease of humans, with over 50 million cases in tropical and subtropical regions each year, although the majority of infections may be asymptomatic (Burke et al., 1988). Where infection does result in overt disease, the most common outcome is an acute febrile illness similar to influenza (dengue fever, DF). However, in a minority of cases this progresses to spontaneous haemorrhaging (dengue haemorrhagic fever (DHF)) and, most seriously, to dengue shock syndrome (DSS), characterised by circulatory failure. In addition, neurological symptoms associated with dengue have also been reported (Kho et al., 1981; Solomon et al., 2000). There are perhaps 500,000 cases of DHF/DSS of sufficient severity each year to require hospitalisation, with case-fatality rates as high as 5% depending on the availability of treatment (WHO, 2002). As well as imposing a major disease burden, the economic impact of dengue is considerable, in medical care, mosquito control measures, the loss of working hours and reduced tourism. Given the increasing size and mobility of the human population, and the current lack of an effective vaccine, it is likely that dengue in all its forms will represent an important public health problem for many years to come.

The agent of these diseases, dengue virus, is a single-stranded, positive-sense, RNA virus with a genome of approximately 11 kb. The virus is a member of the genus *Flavivirus* (family *Flaviviridae*), which contain a number of important human pathogens, usually vector-borne, such as Japanese encephalitis virus (JEV), West Nile virus (WNV) and yellow fever virus (YFV). Dengue virus is particularly notable in that it exists as four antigenically distinct serotypes (denoted DENV-1–4), within which there is considerable genetic variation in the guise of phylogenetically defined "genotypes" (reviewed in Holmes and Burch, 2000). Humans are the major host of dengue virus, with *Aedes* mosquitoes the principal vectors, particularly *Aedes aegypti* which also transmits YFV. *A. aegypti* is closely associated with human habitation and larvae are often found in artificial water containers such as discarded tyres, buckets, and water storage jars. Other mosquito species are involved in a sylvatic (or "jungle") transmission cycle, in which forest-dwelling mosquitoes transmit the virus between non-human primates, which experience asymptomatic infections. Sylvatic cycles have been demonstrated in Asia, associated mainly with *Macaca* and *Presbytis* sp. monkeys, with mosquitoes of the genus *Ochlerotatus* acting as the

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principal vectors and *Aedes albopictus* transmitting the virus in peridomestic regions, and in Africa, with *Erythrocebus patas* monkeys and various sylvatic vectors including *Aedes taylori-furcifer*, *Aedes luteocephalus* and *Aedes opok* (Gubler, 1998; Rodhain, 1991; Peiris et al., 1993; Wang et al., 2000).

There is no consensus on when dengue first appeared in human populations, largely because its symptoms are often not diagnostic. The earliest record suggested is from a Chinese medical encyclopaedia dating to 992 A.D. (Gubler, 1998). However, it is generally agreed that by the late 18th century a disease bearing a strong resemblance to dengue was causing intermittent epidemics in Asia and the Americas, and that by the late 19th and early 20th centuries the virus was probably widespread in the tropics and subtropics (Hayes and Gubler, 1992; Monath, 1994). Shortly after World War II, a new dengue-associated disease was reported in endemically infected areas of Southeast Asia. This had a far more pronounced impact than DF, since the primary targets were children (Gubler, 1998). The first well documented outbreak of what came to be known as dengue haemorrhagic fever took place in Manila in 1953/54, and was followed by a larger outbreak in Bangkok in 1958 (Halstead, 1980). Since this time DHF/DSS have become endemic in all countries in Southeast Asia, with dramatic increases in case numbers, so much so that dengue is considered an archetypal "emerging" disease. At the same time the geographic range of DHF/DSS has expanded considerably and these diseases have now been reported in over 60 countries (WHO, 2002). In particular, despite the intensive mosquito control measures employed during the 1960s and 1970s, DHF/DSS now represents a significant health problem in the Americas, as typified by a large epidemic of DHF/DSS in Cuba in 1981 (Guzmán et al., 1984a,b). Although major dengue epidemics are less commonly reported in Africa than in other tropical/subtropical regions, surveillance here is poor so that the true prevalence of dengue on this continent is unclear.

2. The origin and emergence of dengue virus

Although antigenic studies have long shown that dengue should be classified as a flavivirus, it has required more detailed molecular phylogenetic analysis to shed light on the evolutionary history of the virus (Zanotto et al., 1996; Kuno et al., 1998; Billoir et al., 2000; Gaunt et al., 2001; Jenkins et al., 2001). However, even with these higher resolution studies, some aspects of dengue's past remain elusive.

All analyses undertaken to date show that the four serotypes of dengue virus are phylogenetically distinct, and often to the same degree as different "species" of flaviviruses (Kuno et al., 1998). A phylogeny of sequences of the nonstructural-5 (*NS-5*) gene from 70 flaviviruses including dengue is shown in Fig. 1. The tree is distinctive in that three major groups of viruses can be distinguished which generally correlate with transmission mode: viruses that are transmitted by ticks, viruses transmitted by mosquitoes, and viruses with no known vector. Although the clustering of the four dengue serotypes within the mosquito-borne clade is well supported, the closest relatives of dengue virus cannot be identified with any certainty, with weak bootstrap support for the critical nodes. Resolution of this part of the flavivirus phylogeny may require the analysis of complete genome sequences which have yet to be undertaken on a large scale. Most phylogenies also show the same branching order among the four viral serotypes, with DENV-4 the first to diverge, followed by DENV-2, and the final split between DENV-1 and DENV-3. However, there is some variation. For example, in the NS-5 tree depicted in Fig. 1 DENV-2 groups with DENV-3, although with weak bootstrap support, and in other parts of the genome DENV-2 clusters with DENV-4 (E. C. Holmes, unpublished observations). Whether this variation in branching order is entirely stochastic, or the signal of ancient inter-serotype recombination, is unclear.

Although the lack of resolution in the flavivirus tree makes it difficult to accurately reconstruct the origin of dengue viruses, some inferences can be made. The most clear-cut involves the identification of the ultimate animal reservoir of dengue. The key observations here are that sylvatic transmission cycles involving monkeys have been identified in Asia and West Africa (Rudnick, 1978; Rodhain, 1991; Wolfe et al., 2001), and that in DENV-2 and DENV-4 these sylvatic strains fall basal to the human dengue viruses within their respective serotype (this may also be true of DENV-1, although there is little resolution in the phylogeny of this serotype) (Wang et al., 2000; Fig. 2). Although no sylvatic strains have yet been identified in DENV-3, the presence of DENV-3 antibodies in Malaysian monkeys suggests that a sylvatic cycle also exists for this serotype (Rudnick, 1984). Hence, there is strong evidence that dengue was originally a monkey virus and that cross-species transmission to humans has occurred independently in all four serotypes.

What is less clear is where dengue originated. Gaunt et al. (2001) suggest an African origin, principally because many of the most divergent mosquito-borne flaviviruses circulate exclusively in Africa and often infect primates, implying that this clade as a whole originated in Africa. Further, *A. aegypti* is believed to have originated in Africa, although this species is only likely to have been adopted as a vector for human transmission in the relatively recent past. Conversely, the presence of all four serotypes in both humans and monkeys from Asia, and particularly the deep phylogenetic position of the Asian sylvatic strains (Wang et al., 2000), suggests that the virus has an Asian rather than an African origin. Although the high prevalence of dengue in this region also supports this hypothesis, only a small number of African samples (from humans and monkeys) are available for analysis. Clearly, resolution of the place of origin of dengue virus will require a wider sampling of sylvatic strains and more precise molecular phylogenies.

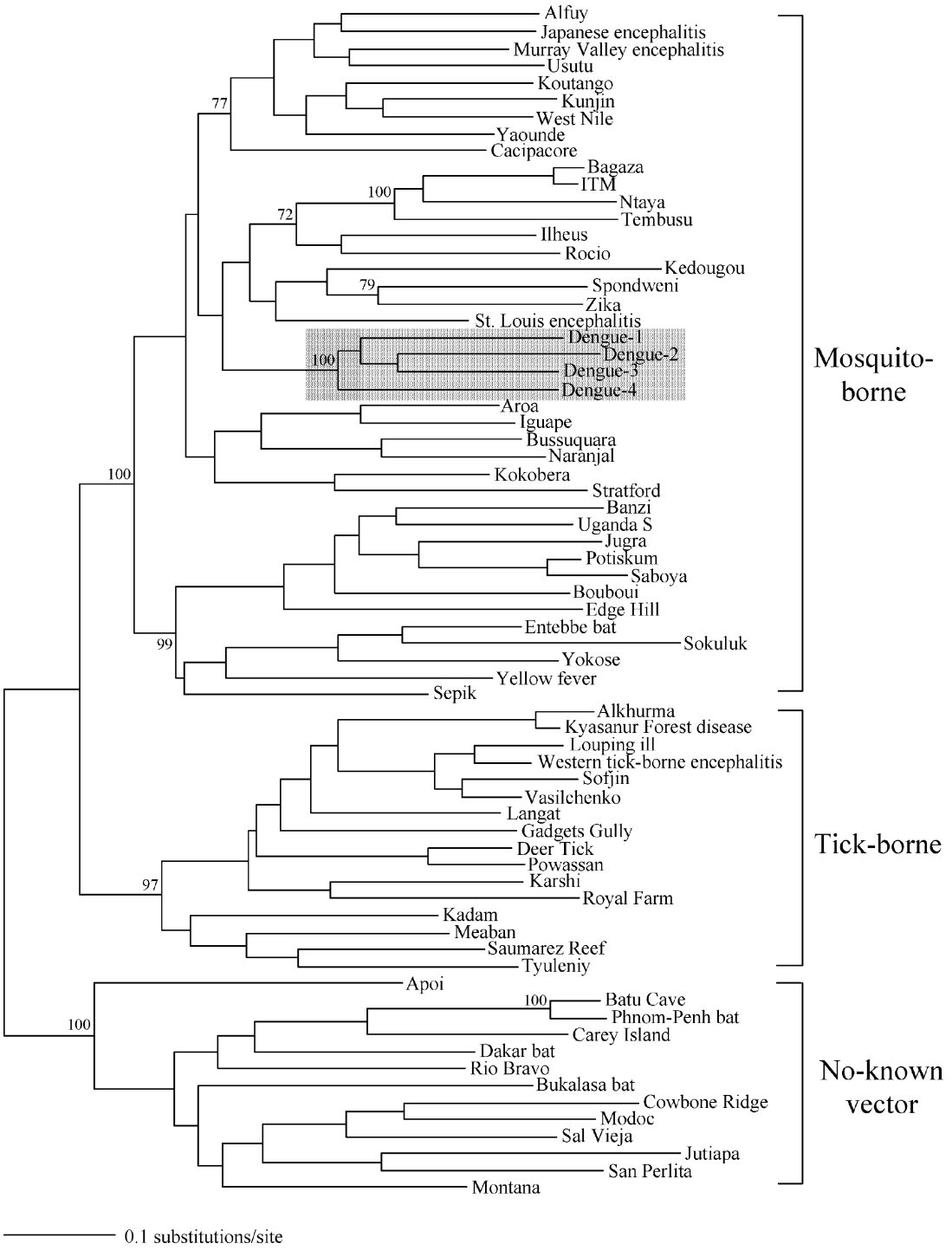

Fig. 1. Maximum likelihood (ML) phylogeny of *NS-5* gene sequences (990 bp) from 70 viruses of the genus *Flavivirus*. Because of major differences in base composition between the three major groups of viruses (tick-borne, no-known vector, vector-borne—Jenkins et al., 2001), the trees for these groups were estimated separately and then joined in a final phylogeny in which all branch lengths were re-optimised. A high variable region of 18 amino acids where the alignment was uncertain was also removed prior to analysis. In all cases the general time reversible (GTR) model of nucleotide substitution was used allowing a different rate of change for each codon position (parameter values available for the authors on request). Bootstrap values (derived from 1000 replicate neighbor-joining (NJ) trees estimated under the ML substitution model) are shown for key nodes where >70%. All analyses were undertaken using the PAUP* package (Swofford, 2002). The tree is mid-point rooted for purposes of clarity only and all horizontal branch lengths are drawn to scale. The four dengue viruses are shaded. Abbreviations are as follows; ITM, Israel-Turkey meningoencephalitis.

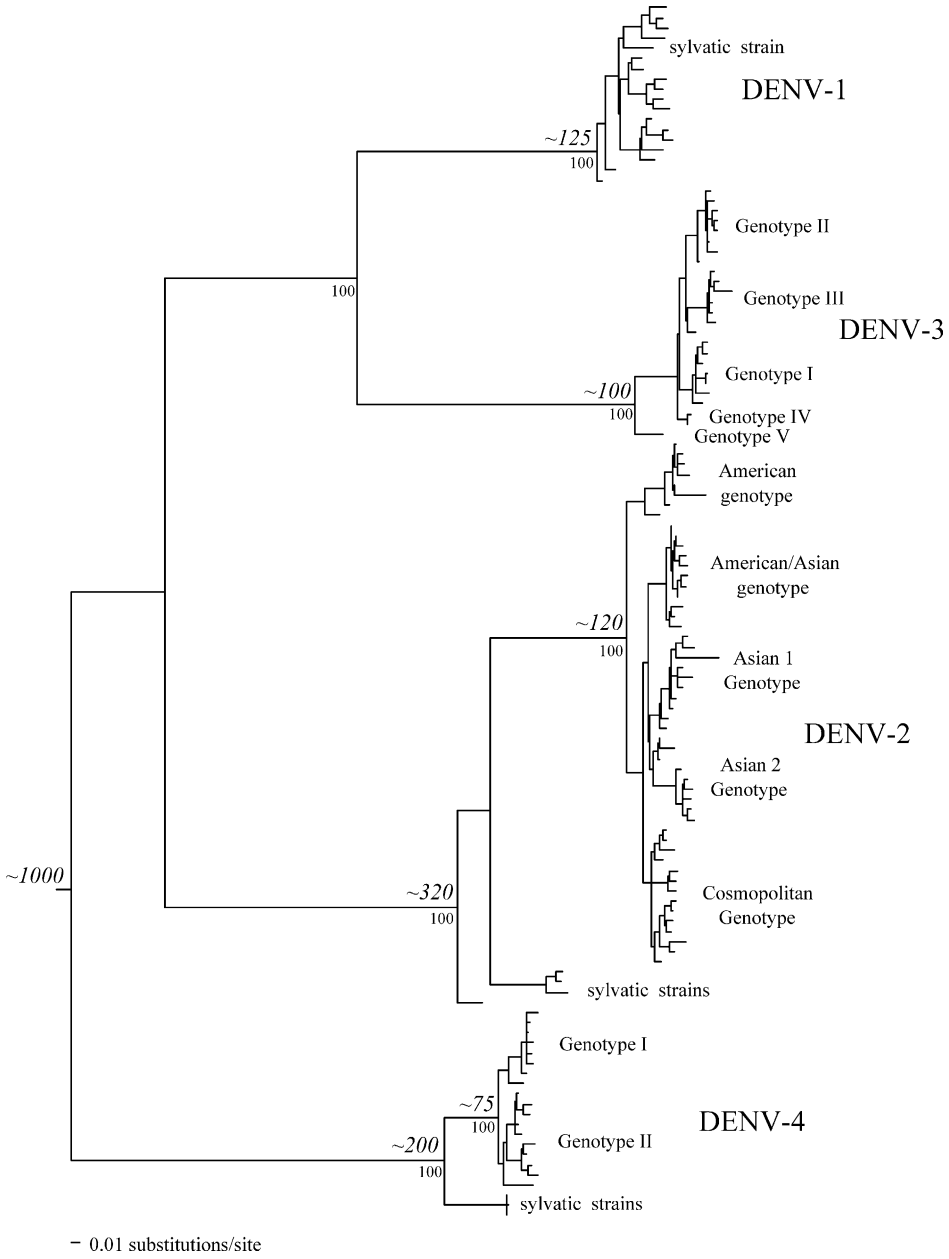

Fig. 2. Maximum likelihood phylogeny of 120 *E* gene sequences (1485 bp) representing the genetic diversity in dengue virus. In all cases the different serotypes and genotypes of dengue virus are identified and the (approximate) divergence times (see text) are shown in italics above key nodes. The GTR model of nucleotide substitution was used allowing a different rate of change for each codon position (parameter values available for the authors on request). Bootstrap values (derived from 1000 replicate NJ trees estimated under the ML substitution model) are shown below selected nodes. All analyses were undertaken using the PAUP* package (Swofford, 2002). The tree is rooted on DENV-4 sequences, as these are usually the first to diverge in dengue phylogenies, and all horizontal branch lengths are drawn to scale.

Insights into the history of dengue virus can be also obtained by reconstructing a molecular time-scale of its evolution. This was recently achieved by estimating rates of nucleotide substitution using a maximum likelihood method that analyses the amount of evolutionary change that has occurred between viruses sampled at different times (Rambaut, 2000). Employing this method to a large number of envelope (*E*) gene sequences revealed that dengue virus evolution often conforms to a molecular clock, although some lineage-specific rate differences were observed; mean substitution rates (for all sites) ranged from 4.55×10^{-4} (DENV-1) to 9.01×10^{-4} substitutions per site, per year (DENV-3) (Twiddy et al., 2003b). These rates are similar to those reported in other vector-borne RNA viruses, although slightly lower than those in RNA viruses transmitted by other mechanisms (Jenkins et al., 2002), most likely because of the fitness trade-offs, and hence selective constraints, inherent when replicating in hosts as divergent as insects and mammals. Using these substitution rates, the origin of dengue virus (that is, the deepest split in the divergence of

the four serotypes) was found to be remarkably recent, at approximately 1000 years ago (Twiddy et al., 2003b), and thus corresponds roughly to the first reports of dengue-like disease. Furthermore, the dates of cross-species transmission from monkeys to humans only occurred between ∼320 (DENV-2) and ∼125 (DENV-1) years ago, and most of the genetic diversity currently observable within each dengue serotype is estimated to have appeared almost simultaneously and only during the past century.

The evolutionary history of dengue virus therefore appears to be a recent one, and only a little older than the human immunodeficiency virus type-1 (HIV-1) which may have emerged in humans within the last 70 years (Korber et al., 2000). As such, we may conclude that up until a few hundred years ago dengue was primarily a sylvatic disease, only causing sporadic outbreaks in humans and resembling the pattern still shown by yellow fever virus today. Human outbreaks would occur following encroachment into the forest habitat and where sufficient numbers of susceptible human hosts were present. However, these epidemics would eventually burn-out when the supply of susceptibles dwindled below a critical threshold level. It was not until the rapid increase in human population size, initiated by widespread urbanisation and the development of modern transportation methods in the recent past, that sufficient numbers of hosts were available on a regular enough basis to enable sustained transmission in humans. At this point dengue virus effectively broke-free of its sylvatic cycle and established itself as the endemic human disease we see today. The early evolution of dengue virus was therefore characterised by continual lineage extinction, as the virus jumped to humans but then burnt out because of a lack of susceptible hosts. The establishment of a human transmission cycle for dengue virus was probably aided by its relatively benign nature compared to YFV, such that fewer hosts were needed to sustain viral transmission. Such a recent evolutionary history also implies that humans did much to disperse dengue virus on a global scale.

One final question to address regarding the origin of dengue is why the virus exists as four distinct serotypes? This can be explained in one of two ways. The most simple is that dengue virus became separated into distinct lineages because of geographic (allopatric) or ecological partitioning in different primate populations, so that the four serotypes evolved independently. Alternatively, dengue viruses may have evolved in sympatry (within a single population) because the presence of four antigenically distinct serotypes facilitated viral transmission through the phenomenon of antibody-dependent enhancement (see further). Under this model, natural selection favours viruses with the degree of antigenic dissimilarity that maximises immunological enhancement, thereby aiding their mutual transmission (Ferguson et al., 1999). At present, most evidence favours the independent evolution hypothesis. In particular, the initial lineage splits would have produced viruses most likely sufficiently antigenically similar for there to be virtually complete cross-protection between them. Furthermore, if antibody-dependent enhancement were the major force shaping dengue genetic diversity then it might be expected that the virus would be subject to continual (immune) selection pressure. The studies of natural selection in dengue virus undertaken to date show that this not the case (see further, Twiddy et al., 2002a,b). Consequently, rather than being a long-term evolutionary strategy to aid viral transmission, antibody-dependent enhancement is more likely the result of recent contact between four viruses that have evolved in isolation for an extended period of time and by chance have a level of antigenic dissimilarity that allows immune enhancement.

3. The evolutionary genetics of dengue virus

RNA viruses show prodigious genetic variability, due to the intrinsically high mutation rate associated with RNA-dependent RNA polymerase (Drake and Holland, 1999), their rapid rates of replication, and their immense population sizes. In dengue virus, this genetic variability is most obviously manifest in the existence of four antigenically distinct serotypes. Before gene sequence data was available, it was known that genetic variation also existed within each serotype (Trent et al., 1983; Monath et al., 1986). However, with the advent of comparative gene sequence analysis, it has been possible to dissect the genetic structure of dengue virus populations and to reveal the processes governing viral evolution.

The landmark analysis of intra-serotype variation in dengue virus was conducted by Rico-Hesse (1990) who used a 240 bp fragment from the *E/NS1* gene region to measure genetic diversity in DENV-1 and DENV-2. With this, she recognised a number of distinct “genotypes”, arbitrarily defined as “a group of dengue viruses having no more than 6% sequence divergence”. Since this time the scope of genotyping studies has greatly expanded, although most studies now rely on complete *E* gene sequences (∼1485 bp) as a phylogenetic marker, and there is still relatively little sequence data available for DENV-1 and DENV-4 (Lewis et al., 1993; Lanciotti et al., 1994, 1997; Chungue et al., 1995; Rico-Hesse et al., 1997, 1998; Twiddy et al., 2002a). A phylogenetic tree showing the extent of genetic variation within dengue virus, as well as key divergence times (inferred by Twiddy et al., 2003b), is shown in Fig. 2. From this it is clear that distinct clusters of sequences, which can be regarded as genotypes, are visible in all serotypes, although these have yet to be formally named in DENV-1. The extent of genetic diversity is best studied for DENV-2 for which most sequence data exists. In this case, the current sample of viruses fall into six genotypes, one of which contains all the sylvatic strains. The genetic variation within DENV-2 is also typical of dengue virus as a whole for another reason, namely that genotypes often have differing geographical distributions. In particular, two genotypes are only found in Asian populations (denoted “Asian 1” and “Asian 2”, respectively),

while a "Cosmopolitan" genotype has a distribution covering much of the tropical world (Twiddy et al., 2002a). That genotypes frequently harbour viruses sampled from very different geographical locations is a powerful indicator of how far infected hosts and vectors can spread the virus.

A question of more importance to evolutionary biologists is whether the difference in geographical distribution shown by dengue genotypes has any sort of selective basis? In other words, do some genotypes have more "epidemic potential" than others (Gubler et al., 1981) which makes them more likely to seed outbreaks in disparate geographical locations? At present there is no clear answer to this fundamental question. Using a maximum likelihood method that measures rates of synonymous (d_S) and nonsynonymous (d_N) substitution codon-by-codon, Twiddy et al. (2002a,b) found sporadic positive selection in the *E* gene sequences of DENV-3 and DENV-4, in some genotypes of DENV-2, but not in DENV-1. Since the E protein is the major antigenic determinant of dengue virus, it was not surprising that the majority of these selected sites were located in or near B- or T-cell epitopes, suggesting that the selection pressure was related to immune evasion. Also of note was that similar (and generally very low) d_N/d_S ratios were observed in the human and sylvatic strains of DENV-2. At face value this implies that there was no major change in selection pressure, or host-specific adaptations in the *E* gene, as the virus changed transmission cycles. If this observation is repeated when a larger sample of monkey-associated strains are analysed, the implication is that present day sylvatic viruses will have little trouble spreading in human populations should the opportunity arise.

Perhaps the most intriguing result of studies of selection pressure is that the widely distributed Cosmopolitan genotype appears to be subject to stronger positive selection pressure than other DENV-2 genotypes, which may correlate with its dispersal ability (Twiddy et al., 2002a). Furthermore, viruses in the Cosmopolitan and American genotypes have characteristic mutations at amino acid E-390 in the envelope gene, which has been identified as a key virulence determinant in experimental studies (Sanchez and Ruiz, 1996). Therefore, these studies tentatively imply that viral genotypes might differ in fitness. This area clearly merits intensive study in the future, not least as it may allow us to identify and monitor the spread of those viral strains that are most likely to pose a serious threat to human health.

Another possible signature for the action of natural selection in dengue virus are instances of strain extinction and replacement. For example, in Thailand there has seemingly been a turnover of DENV-2 strains between 1980 and 1987 (Sittisombut et al., 1997) and of DENV-3 strains in the 1990s (Wittke et al., 2002). Such strain replacement would be expected if the viruses in question differ in fitness, although this not been formally demonstrated. Indeed, this pattern of extinction and replacement could also be explained by entirely stochastic processes, such as population bottlenecks following a decline in mosquito numbers during inter-epidemic years. Large-scale variation in vector population sizes would also mean that genetic drift plays a major role in the evolution of dengue virus, so that the fate of a virus in a population will not always reflect its fitness. Whatever the mechanism, it is likely that strain extinction, perhaps even involving entire genotypes, has been a regular occurrence in viral evolution, especially at times when the numbers of susceptible hosts or mosquitoes were low.

As well as extinction, there have also been attempts to analyse the rates at which dengue virus populations have grown. In the first such study, Zanotto et al. (1996) plotted the number of lineages in a tree of all four dengue serotypes against their time of appearance and observed that the beginning of rapid cladogenesis occurred simultaneously in all serotypes, and that this coincided with the period when the virus became established endemically in humans. A recent study using more sophisticated coalescent methods confirmed that dengue virus has experienced exponential population growth in the recent past, but that growth rates differ between serotypes (Twiddy et al., 2003a). In DENV-1 there appears to have been a single phase of exponential population growth, while both DENV-2 and DENV-3 have a demographic history comprising two-stage exponential population growth; a low growth rate increased dramatically at approximately 50 years ago for both viruses, coinciding with the post-World War II upheavals that are thought to have been instrumental in the rise of DHF/DSS. Insufficient data were available for DENV-4, and in all cases it is important to consider the effects of biased sampling on estimates of population growth rate.

Finally, it is clear that dengue genotypes are not fixed entities as there is now evidence that recombination can occur among them (Holmes et al., 1999; Worobey et al., 1999; Toulou et al., 2001; Uzcategui et al., 2001; AbuBakar et al., 2002; Twiddy and Holmes, 2003). Although recombination has been documented within all four serotypes, it has not been observed between them, as expected given their extensive genetic divergence. As well as dengue, recombination has now been demonstrated in other members of the *Flaviviridae*—hepatitis C virus (Kalinina et al., 2002), GBV-C (Worobey and Holmes, 2001) and pestiviruses (Becher et al., 2001)—indicating that this family of viruses does not always evolve in a clonal manner. As in all RNA viruses where recombination has been detected (other than those with segmented genomes which recombine through a process of segment reassortment), recombination in dengue most likely occurs through a copy-choice mechanism in which the polymerase switches between parental viral molecules during replication (Lai, 1992). Given the huge numbers of infected hosts and vectors, the fact that *A. aegypti* is known to engage in multiple feeding, and the evidence for mixed infections (although only of different serotypes—Lorono-Pino et al., 1999), it is not surprising that there is evidence for recombination in dengue virus. However, it is less clear how important recombination has been in shaping dengue virus evolution in the long-term.

Although recombination will enable the virus to explore adaptive landscapes more effectively, and also to remove deleterious alleles, the relatively low level of recombination observed to date suggests that it is more of a sporadic occurrence, at the mercy of ecological opportunity, rather than a selectively determined trait that increases viral fitness.

4. Viral genetic variation and clinical outcome

One area where studies of dengue virus evolution have been of particular importance is with respect to the aetiology of DHF/DSS, a question that has dominated dengue research for over 30 years. Most attention has been directed toward three hypotheses, although these are not mutually exclusive.

4.1. Differences in host susceptibility

Some individuals may have a genetically determined predisposition or resistance to DHF/DSS, possibly mediated by differences in human leucocyte antigen (HLA) haplotype (Chiewsilp et al., 1981; Zeng et al., 1996; Halstead et al., 2001; Loke et al., 2001). Other possible host factors include age, sex, and pre-existing chronic diseases. However, there have been relatively few studies in this area so that the role of host genetics in determining disease outcome is unclear.

4.2. Antibody-dependent enhancement (ADE)

One of the most important clinical aspects of DHF/DSS is that these syndromes often occur in patients experiencing a secondary infection. The theory of antibody-dependent enhancement proposes that this is so because antibody from a previous dengue infection induces a complex immunologic reaction during a second infection with a different serotype, and that this can result in haemorrhage or shock (Halstead, 1988). Support for this model has come from a number of epidemiological studies (Sangkawibha et al., 1984; Burke et al., 1988; Kliks et al., 1988; Thein et al., 1997; Kourí et al., 1998), which generally show higher levels of DHF/DSS in secondary than in primary infections. Progress has also been made in determining the precise immunopathological basis by which ADE might lead to DHF/DSS (Kurane and Ennis, 1994; Bielefeldt-Ohmann, 1997; Kurane and Takasaki, 2001). Although still controversial, most available data suggests that immune enhancement does play some role in the aetiology of DHF/DSS.

4.3. Strain variability

It is possible that strains of dengue virus, either serotypes or genotypes within serotypes, differ in their capacity to cause DHF/DSS. Early evidence for a strain basis to variable clinical outcomes following dengue infection were observations of viruses consistently associated with mild dengue (as in a 1974 outbreak in Tonga (Gubler et al., 1978)), of DHF/DSS in primary infection (Scott et al., 1976; Reed et al., 1977; Rosen, 1977), and more recently of major DF epidemics with few cases of DHF/DSS (Watts et al., 1999).

The most persistent calls for a strain basis to variable disease outcome concerns the “American” genotype of DENV-2 (the earliest sampled representative of which is the Trinidad/53 strain) which is not associated with DHF/DSS, implying that it constitutes a genotype of intrinsically low virulence (Rico-Hesse et al., 1997; Leitmeyer et al., 1999). For example, during a 1995 DENV-2 outbreak in Iquitos, Peru, it was estimated that there were approximately 50,000 secondary infections. Based on rates of progression to DHF/DSS in Thailand, this should have resulted in 900 to over 10,000 cases of DHF/DSS, yet none were reported (Watts et al., 1999). As a comparison, the 1981 epidemic of DENV-2 in Cuba resulted in ∼300,000 infections and some 30,000 cases of severe disease with 158 deaths (Kourí et al., 1983). Furthermore, American genotype viruses show characteristic differences from Asian viruses that have the capability to cause DHF/DSS (Leitmeyer et al., 1999). Most notably, these viruses differ at amino acid E-390, a known virulence determinant, in their ability to replicate in monocyte-derived macrophages (Pryor et al., 2001), and in the sequence (and hence RNA secondary structure) of the 3′ untranslated region (UTR) which has been shown to correlate with virulence in both dengue (Proutski et al., 1999) and yellow fever virus (Proutski et al., 1997). It has also been proposed that American genotype viruses are less able to replicate in *A. aegypti* than viruses of Asian origin, so that the latter may be more transmittable (Armstrong and Rico-Hesse, 2001).

Although intriguing, the idea that American genotype DENV-2 viruses are universally of low virulence is open to question. For example, Shurtleff et al. (2001) showed that an American genotype virus from Venezuela had a predicted 3′ UTR secondary structure typical of that genotype, but was associated with DHF, and the lack of DHF/DSS in the Iquitos outbreak may be due to the neutralising effect of pre-existing antibody to DENV-1 (Kochel et al., 2002). Also, primary infection with viruses thought to be of the American genotype may have caused DHF/DSS on some Pacific islands (Barnes and Rosen, 1974). Lastly, virulence in dengue is usually associated with high viral load (Vaughn et al., 2000), which would also aid transmission, so that it might be expected that a low virulence American genotype would soon be out-competed by more aggressive Asian strains (Rico-Hesse et al., 1997). However, during the Iquitos epidemic, the overall infection rate was over 86% (Watts et al., 1999), which indicates that American genotype viruses can have great transmission success and hence may not suffer out-competition and ultimately extinction.

On a more localised scale, most analyses of individual genotypes or single populations have provided little evidence that isolates taken from DHF/DSS patients are distinct from those of patients with DF (Chungue et al., 1993; Rico-Hesse

et al., 1997, 1998; Uzcategui et al., 2001). However, there are exceptions. For example, Lanciotti et al. (1994) found that genotype IV of DENV-3 was only associated with mild disease. Similarly, Pandey and Igarashi (2000) divided strains of DENV-2 from Thailand into three subtypes, one of which, subtype II, was more likely to cause DHF/DSS in ADE, but not in primary infection, while subtype III was never associated with DHF/DSS even during secondary infections. Furthermore, Kliks (1990) demonstrated that strains associated with severe dengue had greater monocyte infectivity than those causing DF, while experiments by Diamond et al. (2000) showed that strains of DENV-2 differed in their ability to infect human cells.

Overall, the evidence that different strains of dengue virus are associated with different clinical outcomes is tentative at best, but obviously requires further investigation. Not only will such information be important in the development of future intervention strategies, but it also has a major bearing on the evolution of virulence in dengue virus, a subject that has received little attention to date. For example, in most phylogenetic analyses the American genotype of DENV-2 is pictured as the first human genotype to diverge (Leitmeyer et al., 1999), although the bootstrap support for the critical nodes on the tree are low and in some trees Asian strains diverge first (Twiddy et al., 2002a). If this pattern of divergence is correct, then the early divergence of the sylvatic strains, followed by low-virulence American strains, implies that DENV-2 did not initially usually cause DHF/DSS and that virulence has increased following sustained transmission through humans, perhaps because high virulence strains are also the most transmittable. If true, this observation clearly has a major bearing on the future direction of virus evolution.

5. A perspective

We have learnt a great deal about the evolutionary biology of dengue virus over the last 10 years, most notably concerning the extent and causes of genetic diversity, and the reasons behind the emergence of the four viral serotypes. However, there are still fundamental gaps in our knowledge. Most notably, very little is known about the sylvatic cycle of dengue virus, even though it is likely that sylvatic viruses harbour important phenotypic variation, including strains with altered virulence or transmission potential, and that these viruses may eventually emerge in humans. Not only will the analysis of sylvatic viruses shed light on the geographical origin of dengue, but the in-depth study of a form of the virus not associated with disease will doubtless aid our understanding of the mechanisms under-pinning virulence in humans. The study of sylvatic dengue in Africa merits particular attention; although the presence of sylvatic cycles of all four dengue serotypes has been established in Asia, as yet only DENV-2 sylvatic transmission has been demonstrated in Africa (Rodhain, 1991).

Similarly, little is known about the genetic diversity of dengue viruses within individual hosts, either humans or mosquitoes. Although dengue is a short-term acute infection, the high mutation rate of RNA viruses means that dengue virus populations within individuals will be polymorphic, an expectation confirmed in the few studies undertaken to date (Wang et al., 2002; Wittke et al., 2002). What is less clear is whether these individual populations contain viruses that differ in important phenotypic properties, and what proportion of dengue genomes are defective because of sporadic deleterious mutations. Again, this information will assist in the development of future control strategies as individual virus populations may contain viruses that differ in virulence or which naturally contain vaccine escape mutations. On a broader scale, we need to know how the genetic structure of dengue virus is shaped by antibody-dependent enhancement; in particular, it is critical to determine at which level of genetic divergence immunological cross-protection is lost and enhancement can occur. Such information is important in order to predict whether new serotypes of dengue virus could emerge if genetic diversification in the virus continues unabated and how cross-protective future dengue vaccines will need to be.

Finally, it is clear that a great deal more work is required to understand the precise aetiology of DHF/DSS. In particular, for the relative contributions of the various risk factors for DHF/DSS to be determined, including the role played by differences in virulence between virus strains, several large, long-term prospective studies need to be carried out in regions where dengue is endemic, and it is important that these include comparisons of whole genome sequences. Critically, such studies should involve not only patients suffering clinical disease, but also those in the study group with no overt symptoms, as well as local vector populations. Only in this way will the viral genetic component to dengue be fully elucidated, and with this the study of this important virus will enter a new age.

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