

SHORT REPORT: ORIGIN OF DENGUE TYPE 3 VIRUSES ASSOCIATED WITH THE DENGUE OUTBREAK IN DHAKA, BANGLADESH, IN 2000 AND 2001

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Abstract. Dengue and dengue hemorrhagic fever re-emerged in Bangladesh in 2000 and 2001 and nearly all viruses isolated were dengue type 3. Phylogenetic analyses of the envelope genes of examples of these viruses indicated that they were most closely related to recently emerged dengue type 3 viruses from neighboring Thailand and Myanmar but distinct from those from India and Sri Lanka. Since this strain of dengue virus type 3 had not been associated with unusual patterns of disease in Thailand or Myanmar, it suggested that the outbreak in Bangladesh was due to local factors after the introduction of viruses from countries to the east rather than to the evolution of an unusually virulent strain of virus in Bangladesh.

Dengue viruses (dengue virus type 3 [DENV-3]) were isolated for the first time from patients in East Pakistan (Bangladesh) in 1964.¹ Subsequent reports^{2–4} suggested that dengue fever may have been occurring sporadically in Bangladesh between 1964 and the outbreak that began in 2000⁵ during which predominantly DENV-3 was recovered from patients.⁶ The dengue epidemic in Bangladesh in 2000 raised a number of questions. Why had outbreaks not been occurring regularly in Bangladesh as they had in neighboring Myanmar⁷ and Thailand?⁸ Was the outbreak in 2000 due to the appearance of a new strain of DENV-3 as a result of evolution of local strains or to the introduction of strains of DENV-3 from outside Bangladesh?

All investigations were reviewed and approved by the relevant Institutional Research/Ethics Committees. Sera obtained from 18 dengue patients at two hospitals in Dhaka, Bangladesh in 2000 and 2001 and from employees with dengue from a recreation club in Dhaka where there had been a focus of intense transmission in 2001⁹ were added to 25-cm² monolayers of C6-36 mosquito cells and incubated at 30°C for seven days. Cells were recovered and examined by indirect immunofluorescence¹⁰ with monoclonal antibodies to flavivirus, dengue virus, and dengue virus serotypes¹¹ for evidence of infection with dengue virus. DENV-3 was recovered from six specimens. Two additional DENV-3 isolates recovered from patients in Bangladesh in 2000 were provided by the U.S. Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand. Contemporary strains of DENV-3 were isolated from patients in Sri Lanka (D3.SriLanka.D39.1999), Myanmar (D3.Myanmar.46881.2002), Cambodia (D3.Cambodia.K0520128.2000), and Vietnam (D3.Vietnam.11598.1998) using similar methodology. Supernatant from the cultures of infected C6-36 cells was used as a source of virus.

Sequencing of envelope (E) protein genes of these viruses was performed as previously described.^{12,13} Briefly, RNA was extracted from the supernatant of cultures of infected C6-36 cells using a commercial kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was generated from the RNA by heating a mixture of random hexamer

primers (1 nmol; Boehringer, Mannheim, Germany) and RNA for 10 minutes at 72°C and cooling the mixture on ice. Reverse transcription was carried out at 55°C for 10 minutes followed by 45°C for 60 minutes with Expand reverse transcriptase (Roche, Mannheim, Germany). The cDNA was amplified by a polymerase chain reaction (PCR) using oligonucleotide primers¹³ composed of nucleotide sequences in the pre-membrane and non-structural protein 1 genes of DENV-3 and Expand DNA polymerase (Roche). After an initial denaturation of the cDNA at 92°C for 2 minutes, 30 cycles at 92°C for 40 seconds, 55°C for 40 seconds, and 68°C for up to 2 minutes were used. cDNA for sequencing was obtained by excising the band of interest after electrophoresis of the PCR product on 1.5% agarose/Tris-acetate-EDTA gels and purifying it using a commercial kit (High Pure Gel Extraction Kit; Roche) according to the manufacturer's instructions.

Approximately 100 ng of cDNA was sequenced using 3.2 pmol of oligonucleotide primer^{12,13} and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Wellesley, MA) according to the manufacturer's instructions. The product was purified using Dye-EX Spin Columns (Qiagen) and analyzed at the Australian Genome Research Facility (Brisbane, Queensland, Australia) on a 3730XL Nucleotide Sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were aligned and phylogenetic analyses were performed with EclustalW, Ednapars, Ednadist, Ednakitsch, and Econsense software from the Australian National Genome Information Service (Sydney, New South Wales, Australia). Bootstrap values were derived from 1,000 iterations. Nucleotide sequences of the E genes of additional DENV-3 were obtained from GenBank. The sequences of the Bangladesh viruses have also been deposited in this database (Accession numbers AY656669–AY656674).

The Bangladesh DENV-3 isolates showed little evidence of independent evolution. The consensus nucleotide sequences of the eight DENV-3 isolates from Bangladesh varied at only 18 of 1,479 sites in the E gene. Four of these changes resulted in amino acid changes (E81, Ile→Thr; E134, Asn→Ile; E140, Thr→Ile; and E301, Leu→Ser). The Bangladesh DENV-3 isolates formed a distinct phylogenetic lineage with recent DENV-3 isolates from Thailand (D3.Thailand.HuNII00-27-1.2000) and Myanmar (D3.Myanmar.46881.2002) (Figure 1). Two strains of DENV-3 from Thailand (D3.Thailand.

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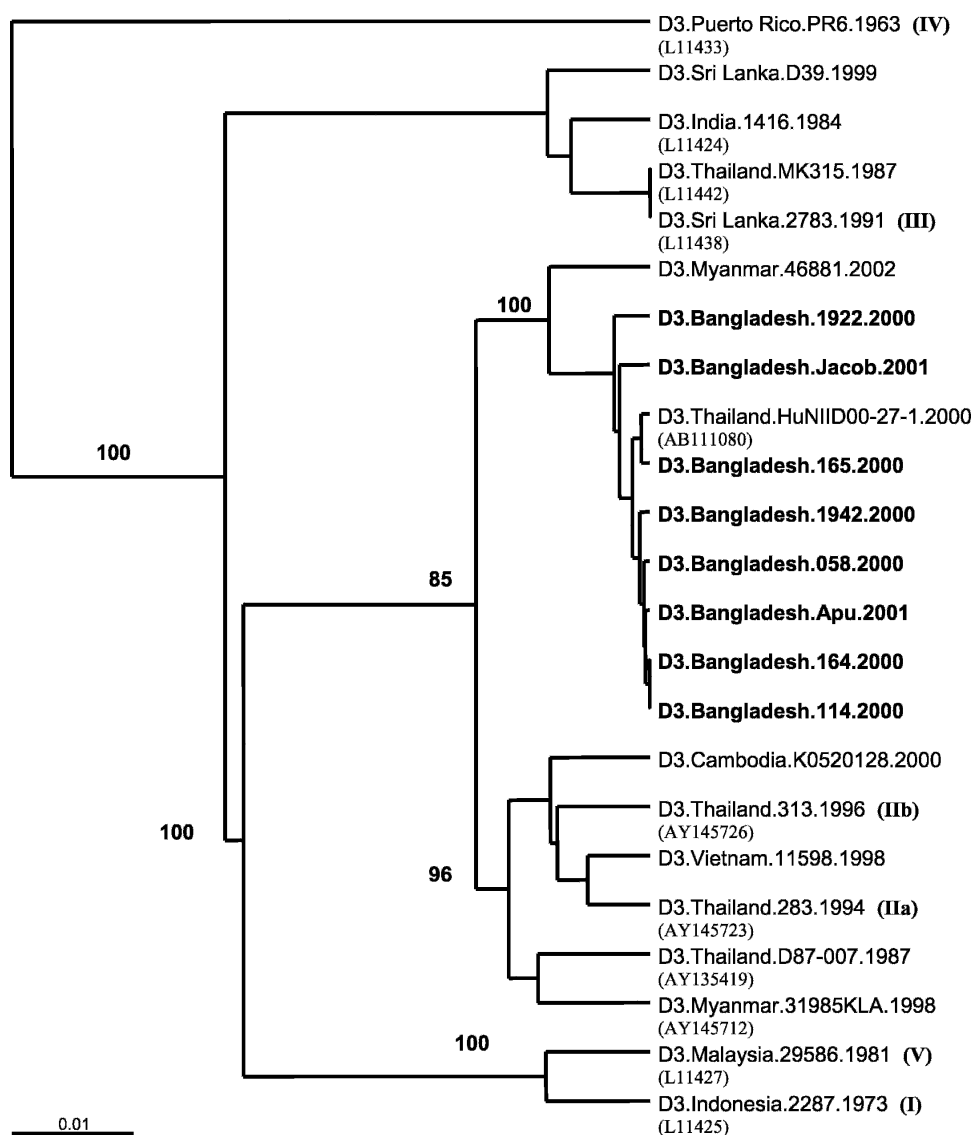


FIGURE 1. Phylogenetic relationships between the nucleotide sequences of the envelope protein genes of dengue virus type 3 (DENV-3) isolated in Bangladesh (shown in **bold**) and neighboring countries determined by neighbor-joining. Bootstrap values for 1,000 replicates are shown on major nodes of the tree. Representatives of each DENV-3 genotype (I, IIa, IIb, III, IV, and V) are identified. The accession numbers of nucleotide sequences derived from GenBank are shown under the corresponding viruses. The horizontal bar at the lower left shows nucleotide substitutions per site.

313.1996 and D3.Thailand.283.1994) belonged to genotypes IIa and IIb, respectively, of DENV-3 that had replaced an earlier clade of DENV-3 in Thailand in 1992.¹² This replacement preceded two consecutive large annual dengue outbreaks in Thailand in 1997 and 1998⁸ that involved predominantly DENV-3. However, strains of DENV-3 from this period were distinct from strains subsequently isolated in Thailand (D3.Thailand.HuNII00-27-1.2000) and Bangladesh. The DENV-3 isolates isolated in Bangladesh in 2000 and 2001 were distinct from viruses from India and Sri Lanka. This was of interest because the spread of genotype III DENV-3 west from the Indian subcontinent to Africa and then to Latin America has been reported.¹⁴

The close genetic relationship between DENV-3 isolates from Bangladesh, Thailand, and Myanmar and the lack of diversity between the viruses from Bangladesh suggested that the recent dengue outbreak in Bangladesh was associated

with the introduction of DENV-3 from countries to its east, rather than to the evolution of a virulent strain of DENV-3 *in situ*. Although this strain of DENV-3 was associated with cases of dengue fever and dengue hemorrhagic fever in Myanmar and Thailand, it was not the most frequently isolated serotype from patients in these two countries before or during the dengue outbreak in Bangladesh. It is unclear why this strain of virus was associated with an outbreak of dengue in Bangladesh, but not in the countries from which it appeared to originate. It also is notable that at the time of the outbreak of DENV-3 infection in Bangladesh, neighboring Myanmar was having its largest dengue outbreak during which 95% of the viruses recovered from patients were DENV-1.⁷ It may be useful to compare the competence of mosquitoes from India, Bangladesh, and Myanmar in transmitting genotype III and the Thai/Myanmar/Bangladesh strains of DENV-3.

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