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Original Article

Molecular Characterization and Clinical Evaluation of Dengue Outbreak in 2002 in Bangladesh

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SUMMARY: During the febrile illness epidemic in Bangladesh in 2002, 58 people died out of the 6,132 affected. Two hundred hospitalized patients were analyzed clinically, serologically and virologically to determine the features of this dengue infection. Among the 10- to 70-year-old age group of the 200 clinically suspected dengue patients, 100 (50%) were confirmed as dengue cases by virus isolation and dengue IgM-capture ELISA. Of the 100 dengue-confirmed cases, the mean age was 29.0 (±12.4). The possible dengue secondary infection rate determined by Flavivirus IgG-indirect ELISA was 78% in 2002. Eight dengue virus strains were isolated, representing the first dengue virus isolation in the country, and all of the strains were dengue virus type-3 (DEN-3). Sequence data for the envelope gene of the DEN-3 Bangladeshi isolates were used in a phylogenetic comparison with DEN-3 from other countries. A phylogenetic analysis revealed that all 8 strains of DEN-3 were clustered within a well-supported independent sub-cluster of genotype II and were closely related to the Thai isolates from the 1990s. Therefore, it is likely that the currently circulating DEN-3 viruses entered Bangladesh from neighboring countries.

INTRODUCTION

Dengue is considered to be one of the most notable viral infections that may appear in the form of an endemic or epidemic febrile illness. It is transmitted by *Aedes aegypti*/*Aedes albopictus* mosquitoes, which are present in most tropical and subtropical countries of the world (1,2). The clinical manifestations of symptoms due to an infection with this mosquito-borne virus vary from mild flu-like symptoms to dengue fever (DF), fulminating dengue hemorrhagic fever (DHF), or dengue shock syndrome (DSS). DHF/DSS is characterized by plasma leakage, thrombocytopenia and hypovolemic shock caused by any of four antigenically distinct serotypes, known as dengue virus types 1-4 (DEN) (3,4). At present, around 50 - 100 million people worldwide become infected with dengue virus annually. Since 1958, more than 60,000 children have died as a result of DHF (5). DF/DHF has recently emerged as a global health problem due to the greater convenience of air travel (5-7). It is believed that the global dengue pandemic began in the Asian and Pacific regions during and after World War II. Ecological changes occurring at that time probably favored the geographic expansion of the vectors and their density. Various factors influence the emergence and re-emergence of dengue in a given country. Of these, factors such as overpopulation, unplanned urbanization, poverty and health inequalities are considered to be the most important ones for the rapid spread of the disease within a community (7).

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The dengue virus is a single-stranded positive-sense RNA of about 10,700 bases that contains a single open reading frame (8). The viral genome encodes for three structural (C, capsid; PrM, premembrane [M, membrane]; and E, envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (9,10). The structure of the virus E protein confers the infectivity and host-immune responses of the virus (10-12). A phylogenetic analysis of E gene sequence data supports five subdivisions of the dengue-3 virus genotype (13).

Although dengue has been an endemic and epidemic disease in most tropical and sub-tropical countries of the world for the past 30 years, it has been an emerging viral disease in Bangladesh since 2000. It is rapidly becoming a serious public health threat in both urban and rural areas (14). Bangladesh is one of the tropical countries in Asia, and its closest neighboring countries are India, Nepal, Myanmar and Thailand. The first case of DF, popularly known as “Dacca fever”, in Bangladesh was recorded in the mid-1960s and the second in the mid-1990s. However, dengue infections did not appear as an outbreak until 2000 (15). Because of the rapid spread of dengue infections from Dhaka to other cities, such as Chittagong and other smaller cities, it has now become a serious public health problem in the country (14).

Virus isolation and molecular analysis has been shown to be very important in determining the origin, evolution and geographic distribution of dengue viruses (16-18). This study presents an analysis of clinical symptoms, laboratory confirmation by serological examination and virus isolation and the molecular characterization of the first dengue virus strains isolated from the 2002 outbreak in Bangladesh.

PATIENTS AND METHODS

Patients: During the dengue outbreak in Bangladesh in 2002, 6,132 clinical cases of dengue were reported. One thousand two hundred seventy (20.7%) patients were admitted to the Shaheed Suhrawardi Hospital (SSH). Among them, 200 patients admitted to SSH during the epidemic period from June to October were enrolled in this study. The clinical symptoms of the patients were recorded from the date of admission until the patient was discharged from the hospital. Blood extraction for hemanalysis was done on the date of admission, and the results were recorded for this study. Blood samples for this study were collected 1 to 2 days after admission and were from randomly selected 10 - 70-year-old patients. The required informed consent was obtained from the patients or their legal guardians. After the sample collection, all of the serum samples were stored at −70°C until they were used for analysis.

Flavivirus antigen-detection ELISA: To determine the presence of dengue virus in each of the three successive passages of infected culture fluid (ICF), antigen-detection ELISA (Ag-ELISA) was performed as previously described by Igarashi et al. (19). Optical densities (OD) were measured at a 492 nm wavelength using a Multiscan JX (Model no. 353; Thermolabsystem, Tokyo, Japan).

Dengue IgM-capture ELISA: The IgM titer of the 200 serum samples was determined by in-house IgM-capture ELISA according to the method described by Bundo and Igarashi (20). A tetravalent dengue virus antigen (25 ELISA units/serotype) was used as an assay antigen. A P/N (Sample OD_{492}/Negative control OD_{492}) ratio that was equal to or more than 2.0 was considered to be positive.

Flavivirus IgG-indirect ELISA: The IgG titer of 200 serum samples was determined by in-house Flavivirus IgG-indirect ELISA (IgG ELISA) according to the method described by Bundo and Igarashi (20). Purified Japanese encephalitis virus (JaOArS982 strain) was used as the assay antigen (500 ng/100 μl/well). The OD_{492} of the negative control serum (diluted 1,000 times with PBS-Tween 20 + 10% BlockAce [Yukijirushi, Sapporo, Japan]) was designated as 1:1,000 ELISA units, and a standard curve was prepared using the OD_{492} values of dengue-positive patient serum (initial dilution 1,000 times followed by serial twofold until 2^{12} with PBS-Tween 20 + 10% BlockAce [Yukijirushi]) to determine the sample serum IgG titer. A sample titer equal to or more than 1:3,000 was considered to be IgG positive for Flaviviruses (such as dengue virus and Japanese encephalitis virus).

Virus isolation: *A. albopictus* mosquito cell line C6/36 was used for the isolation of the dengue virus (21). Infected cells were incubated at 28°C in Eagle's Minimum Essential Medium supplemented with 2% fetal calf serum and 0.2 mM of non-essential amino acids. At least three successive passages were conducted for each sample. The ICF was harvested 7 days after the third passage and centrifuged at 3,700 g (3,000 rpm) for 10 min at 4°C, and the supernatant was then stored at −80°C until it was used. The presence of a viral antigen in the ICF was verified by Ag-ELISA and reverse transcription (RT)-PCR (19,22).

RT-PCR: RNA was extracted from ICF using Trizol LS (Invitrogen™; Life Technologies, Carlsbad, Calif., USA) according to the manufacturer's instructions. To screen dengue-virus-positive samples from ICF, RT-PCR was carried out using a random hexamer for cDNA synthesis followed by PCR using 4 sets of dengue serotype specific primers; D1-S (5′-GGACTGCGTATGGAGTTTTG-3′); D1-C (5′-ATGGGT TGTGGCCTAATCAT-3′); D2-S (5′-GTTCGTCTGCAAAC ACTCCA-3′); D2-C (5′-GTGTTATTTTGATTTCCTTG-3′); D3-S (5′-GTGCTTACACAGCCCTATTT-3′); D3-C (5′-TC CATTCTCCCAAGCGCCTG-3′); D4-S (5′-CCATTATGGC TGTGTTGTTT-3′); D4-C (5′- CTTCATCCTGCTTCACTT CT-3′) following the method as described by Tanaka (22).

Nucleotide sequencing and phylogenetic analysis: To compare the nucleotide sequences of the E gene, eight Bangladeshi isolates were sequenced. The sequencing strategy involved nucleotide sequencing using the primer extension dideoxy chain termination method. For each sequencing reaction, 30 - 90 ng of purified PCR product was combined with 3.2 pmol of specific primer and Big Dye Terminator Cycle Sequencing Ready Reaction Mixture containing the four dye-labeled deoxynucleotide terminators (Perkin-Elmer™; Applied Biosystems, Foster City, Calif., USA). The thermal cycle sequencing parameters were used, as described by the manufacturer. The reaction mixture was column-purified (Centri-Sep™; Applied Biosystems), and the DNA was vacuum-dried for 25-30 min. The pellet was then resuspended in 15 μl of Template Suppression Reagent (ABI Prism™; Applied Biosystems), heated at 92°C for 2 min, and kept on ice until it was loaded into the sequencer ABI Prism™ 310 Genetic Analyzer (Applied Biosystems).

The nucleotide sequences of the primers used for RT-PCR amplification and sequencing of the PrM, E and NS1 genes of the dengue virus isolates from Bangladesh are listed in Table 1. A homology search and comparison of all the obtained sequences were done using DNASIS (Mac version 3.6 Software System; Hitachi, Tokyo, Japan). Nucleotide sequence alignments were carried out using CLUSTAL X, version 1.82 (23), and the phylogenetic analysis involved the use of the PAUP program version 4.0b (24). The neighbor-joining method was used to construct the phylogenetic tree with a bootstrap analysis of 1,000 replicates (25).

Table 1. Oligonucleotide primers used for PCR amplification and sequencing

Primers[1)]	Sequence 5′ → 3′	Nucleotide position[1)]	Orientation
DEN-3-1	GGGCTATTAAGGTCTTAAAAGGCTTCAAGAAGGAGATCTC	321-360	Sense
DEN-3-2	CCCAGGGTTTACCATACTAG	821-839	Sense
DEN-3-3	AAAGTGGTGCAACATGAGAA	1316-1335	Sense
DEN-3-4	GTCCGTGGTGAGCATTCTA	1494-1476	Complementary
DEN-3-5	GCCCTATTTAGTGGAGTCT	2282-2301	Sense
DEN-3-6	GGGAGTCTGCTTGAAATTTG	2528-2509	Complementary
DEN-3-7	GGAAGCAAATAGCCAATGAA	2616-2636	Sense
DEN-3-8	TGAGAGGAGGAGCACAAAC	3604-3586	Complementary

[1)]: Primers design and nucleotide sequence positions were determined based on the nucleotide sequence of the previously published DEN-3 strain H87 (GenBank accession no. M93130).

Table 2. Description of the dengue type-3 viruses used for the phylogenetic analysis in this study

Virus strains	Country	Year of isolation	Accession number
H-87	Philippines	1956	L11423
5987	Thailand	1962	L11440
PR6	Puerto Rico	1963	L11433
228761	Indonesia	1973	L11425
CH3489D73-1	Thailand	1973	L11620
1300	Malaysia	1974	L11429
1340	Puerto Rico	1977	L11434
1280	Indonesia	1978	L11426
80-2	China	1980	AF317645
29586	Malaysia	1981	L11427
1326	Sri Lanka	1981	L11431
1416	India	1984	L11424
85-159	Indonesia	1985	L11428
1594	Sri Lanka	1985	L11436
1969	Samoa	1986	L11435
D86-007	Thailand	1986	L11441
MK315	Thailand	1987	L11442
31985KLA	Myanmar	1988	AY145712
D88-303	Thailand	1988	AY145714
260698	Sri Lanka	1989	L11437
2167	Tahiti	1989	L11619
D89-273	Thailand	1989	AY145715
2783	Sri Lanka	1991	L11438
D91-393	Thailand	1991	AY145716
29472	Fiji	1992	L11422
LN5547	Malaysia	1992	AF147457
D93-044	Thailand	1992	AY145719
LN1746	Malaysia	1993	AF147458
D92-431	Thailand	1993	AY145720
LN608	Malaysia	1994	AF147460
D95-0400	Thailand	1994	AY145722
MEX6097	Mexico	1995	AY146763
D94-122	Thailand	1995	AY145725
D96-330	Thailand	1996	AY145727
PhMH-J1-97	Philippines	1997	AY496879
D97-0291	Thailand	1997	AY145730
00-27-1HuNIID	Thailand/Bangladesh	2000	AB111080
68784	Brazil	2000	AY038605
LARD6315	Venezuela	2000	AY146767
LARD5990	Venezuela	2000	AY146764
LARD6007	Venezuela	2000	AY146765
LARD6668	Venezuela	2001	AY146774
LARD6722	Venezuela	2001	AY146775
LARD7110	Venezuela	2001	AY146776
LARD7812	Venezuela	2001	AY146777
LARD7984	Venezuela	2001	AY146778
BDH02-01[1)]	Bangladesh	2002	AY496871
BDH02-02[1)]	Bangladesh	2002	AY496872
BDH02-03[1)]	Bangladesh	2002	AY496873
BDH02-04[1)]	Bangladesh	2002	AY496874
BDH02-05[1)]	Bangladesh	2002	AY496875
BDH02-06[1)]	Bangladesh	2002	AY496876
BDH02-07[1)]	Bangladesh	2002	AY496877
BDH02-08[1)]	Bangladesh	2002	AY496878
Caribbean 495-1(D1)[2)]	Aruba	1985	D00505
DEN2/H/IMTSSA-MART/98-703(D2)[2)]	Martinique	1998	AF208496
MY01-23314(D4)[2)]	Malaysia	2001	AJ428560

[1)]: Bangladeshi isolates presented in this study.
[2)]: Strains used as an out-group for the construction of the phylogenetic tree.

GenBank accession numbers: The GenBank accession numbers of the Bangladeshi isolates and of all other isolates used in this study for the construction of the phylogenetic tree, as well as the strain name, country and year of isolation, are listed in Table 2.

Statistical analysis: All of the data in this study are expressed as the mean ± SD or as frequencies and proportions. The Student's *t* test, the chi-square test with Yate's continuity correction or Fisher's exact test for nominal variables were used to analyze differences in the demographic and clinical data between patients with dengue and non-dengue, as appropriate. A *P* value less than 0.05 was considered to be statistically significant. The statistical software R (version 2.1.1; R Foundation for Statistical Computing, Vienna, Austria) was used for the data analysis.

RESULTS

Age distribution and clinical symptoms of patients: Of the 200 clinically diagnosed dengue cases, virus isolation and IgM-capture ELISA confirmed that 100 (50%) cases were dengue (Fig. 1). Of these 100 dengue positives, 92 (92%) cases were positive only for IgM, and 8 (8%) cases were positive only for the detection of the virus. The remaining 100 cases were found to be negative by all methods. The mean age of the confirmed 100 dengue patients was 29.0 years (±12.4) and the male:female ratio was 2.70. In a comparison between the 100 dengue-confirmed patients and the 100 non-dengue patients, the dengue-confirmed cases showed the following symptoms: high fever, 100%; headache, 96% (P = 0.748); abdominal pain, 83% (P = 0.009); joint pain, 91% (P = 0.012); vomiting, 93% (P = 0.794); jaundice, 1%; thrombocytopenia, 86% (P = 0.669); skin rash, 28% (P = 0.227); gum bleeding, 41% (P = 0.772); gastrointestinal bleeding, 50% (P = 0.888); hemorrhagic sclera, 17% (P = 0.422); epistaxis, 2%; edema and ascitis, 7% (P = 0.064) and blood platelet count, 62,450 (P = 0.737) (Table 3).

Virus isolation and serotype determination: Of 200 serum samples, 8 were detected by Ag-ELISA and serotyped as DEN-3 by RT-PCR. Dengue virus isolated from 8 positive samples was collected within 6 days of the onset of fever. At 7 days after the onset of fever, the virus could not be detected (Fig. 2).

IgM-capture ELISA and IgG-indirect ELISA: Of the 200 clinically suspected dengue cases, 92 (46%) cases were found to be positive by dengue IgM-capture ELISA. Of the 100 dengue-confirmed cases, including 8 cases found to be positive by virus isolation, 94 (94%) cases were also found to be positive by Flavivirus IgG ELISA (titer above 1:3,000). Of the 94 IgG positive cases, 68 cases showed very high IgG titers, in excess of 1:100,000, and 26 cases showed low IgG

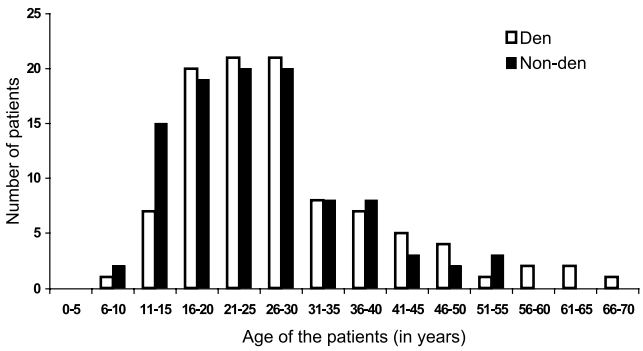

Fig. 1. Age distribution of laboratory confirmed dengue and non-dengue patients in 2002 in Bangladesh. White bars indicate laboratory confirmed dengue cases and black bars indicate non-dengue cases.

Table 3. Clinical profile of the patients during the time of admission to the hospital

Parameters	Confirmed dengue cases (n = 100) (%)	Non-dengue cases (n = 100) (%)	P value
Mean age in years (± SD)	29.0 (±12.4)	25.7 (±10.2)	0.040
Male:Female ratio	2.70	2.13	0.357
General symptoms			
Fever	100 (100)	100 (100)	
Headache	96 (96)	96 (96)	0.748
Abdominal pain	83 (83)	66 (66)	0.009
Joint pain	91 (91)	77 (77)	0.012
Vomiting	93 (93)	91 (91)	0.794
Jaundice	1 (1)	0 (0)	
Hemorrhagic manifestations and DHF related symptoms			
Thrombocytopenia[1)]	86 (86)	89 (89)	0.669
Platelet count (cells/mm^3) (±SD)	62,450 (± 21,564)	64,350 (± 21,444)	0.737
Skin rash	28 (28)	37 (37)	0.227
Gum bleeding	41 (41)	38 (38)	0.772
Gastrointestinal bleeding	50 (50)	48 (48)	0.888
Hemorrhagic sclera	17 (17)	12 (12)	0.422
Epistaxis	2 (2)	0 (0)	
Edema and ascitis	7 (7)	1 (1)	0.064

[1)]: The number and percentage of patients who showed platelet count was less than 100,000/mm^3.

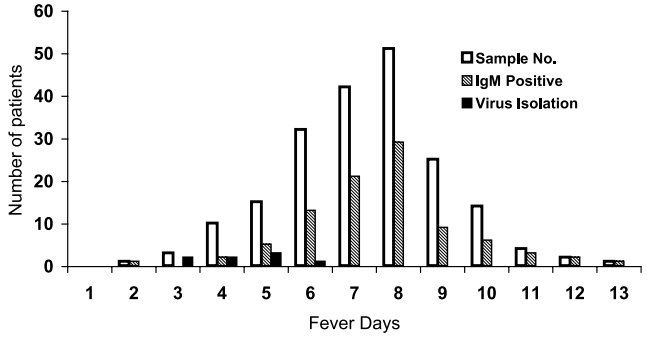

Fig. 2. Relationship between the time of sampling and dengue positivity. White bars indicate number of samples, striped bars indicate number of IgM positive cases and black bars indicate number of positive cases for virus isolation.

Table 4. Serological profile of 100 dengue confirmed patients

Anti-Flavivirus IgG		Anti-DEN IgM		
		Positive	Negative	Total
Positive	High titer[1)]	66	2	68
	Low titer[2)]	20	6	26
Negative[3)]		6	0	6
Total		92	8[4)]	100

[1)]: > 1: 100,000. [2)]: 1:3,000 - 1:100,000. [3)]: < 1:3,000.
[4)]: positive for virus isolation.

titers between 1:3,000 and 1:100,000 (Table 4).

Nucleotide sequence and phylogenetic analysis: The nucleotide sequences of the E gene of the DEN-3 Bangladeshi eight strains were compared with other DEN-3 strains with different geographic origins (Table 2). The phylogenetic tree constructed for the 57 strains is shown in Fig. 3. The tree revealed that all eight Bangladeshi isolates were grouped together in a well-supported distinct cluster of genotype II. In addition, DEN-3 strains from Thailand, Malaysia and Myanmar belong to the same genotype. Genotype I included recent strains from the Philippines, Indonesia, Malaysia, Thailand, Fiji and Tahiti. Genotype III included strains from India, Sri Lanka, Samoa, Thailand and Malaysia as well as strains that are prevalent in Central American countries such as Mexico, Venezuela and Brazil. Genotype IV comprised one old isolate from the Philippines and one from China. Genotype V was composed of two strains from Puerto Rico. The cluster formed by the isolates from Bangladesh was distinct within genotype II due to two unique amino acid (aa) changes in the E gene, E81(I-T) and E140(I-T).

DISCUSSION

Of 200 clinically suspected dengue cases, 100 were laboratory confirmed as dengue by virus isolation and IgM-capture ELISA (Fig. 1 and Table 4). Patients of all age groups, except for the 11 - 15-year-old age group, showed a nearly 50% dengue positivity. Of 100 dengue confirmed cases, the most susceptible age group for a dengue virus infection in this study was the 16 - 30-year-old age group because it comprised 62% of the confirmed cases, followed by the over-30 age group with 30% and the 6 - 15 age group showing only 8% in 2002. On the other hand, in the previous outbreak in 2000, the 5 - 15-year-old age group showed the highest rate at 49.1%, followed by the 16 - 29-year-old age group at 30.9% (26). During the outbreak in 2002, pediatric patients below the age of 10 years were instructed to be admitted to another children's hospital, Shisu Hospital (SH), in Dhaka city. As a result, this age group was not included in our study because our study was done in SSH in Dhaka city, which was designated a special hospital for dengue patients who were above the age of 10 years. The absence of this youngest age group might affect the study with respect to the age distribution of dengue patients. However, Fig. 1 indicated that the 16 - 20, 21 - 25, and 26-30-year-old age groups showed more positive cases and a higher positive ratio than the 11 - 15-year-old age group. This suggests that higher age group subjects were most likely the major target of dengue virus infections in 2002.

The other 100 clinically suspected dengue cases were found to be negative by laboratory examination. One of the possi-

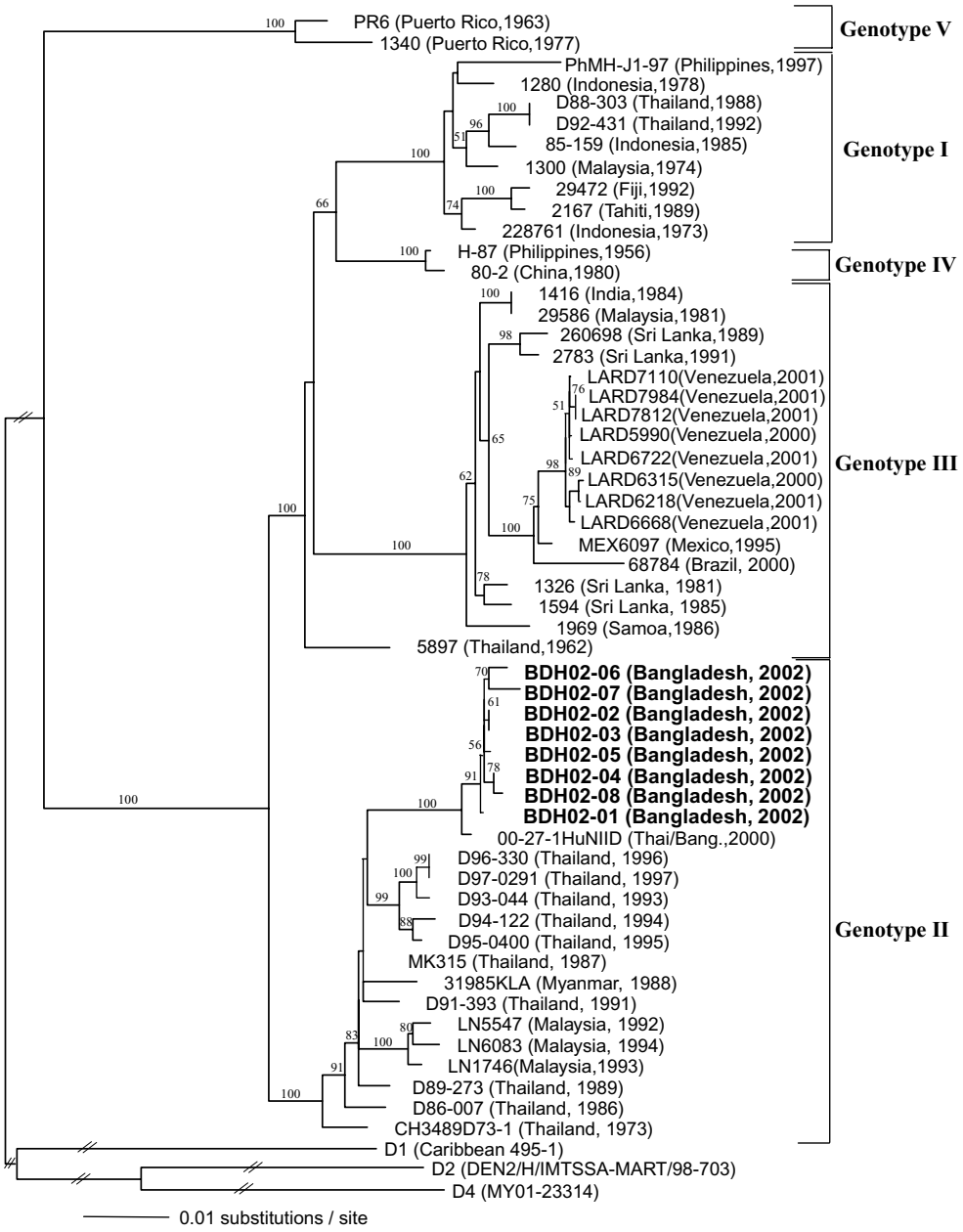

Fig. 3. Phylogenetic analysis of the E gene of DEN-3 viruses. The NJ tree was constructed using the PAUP version 4.0b software. The tree was rooted using DEN-1, DEN-2 and DEN-4 virus sequence information. Bootstrap values for 1,000 replicates are indicated on each branch. The scale at the bottom indicates the number of nucleotide substitutions per site. The eight Bangladeshi isolates are indicated in bold letters.

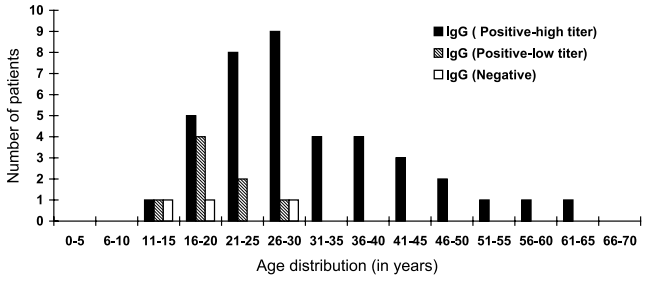

Fig. 4. Flavivirus IgG titers of 50 dengue confirmed cases in which samples were collected 8 days after the onset of fever. The IgG (positive-high titer) indicates titer above 1:100,000 (black bars), IgG (positive-low titer) indicates titer between 1:3,000 and 1:100,000 (striped bars), IgG (negative) indicates titer below 1:3,000 (white bars).

bilities for such a low positivity rate was the single sampling in the acute phase within 7 days from the onset of a fever (Fig. 2). Multiple samplings in the acute and convalescent phases might increase the number of positive cases detected by laboratory examinations. Interestingly, 97 cases, which were sampled 8 days after the onset of the fever, showed only 51% dengue positivity (Fig. 4). This suggests the possibility of the co-occurrence of other febrile infections, the clinical symptoms of which are similar to a dengue virus infection. Examples of such infections are chikungunya fever, influenza, measles and Japanese encephalitis (27) as viral diseases, leptospirosis and typhoid as bacterial diseases, rickettsiosis and malaria.

The major general symptoms in 80% of the confirmed dengue cases were fever, headache, abdominal pain, joint pain and vomiting (Table 3). These symptoms were also observed among non-dengue cases. Hemorrhagic manifestations such

as skin rash, gum bleeding, gastrointestinal bleeding, hemorrhagic sclera and epistaxis were observed in 72 and 77% of dengue and non-dengue confirmed cases, respectively. Among the confirmed dengue cases, the proportion with hemorrhagic manifestations was very high (72%) in 2002 in comparison with the other dengue outbreaks (23 to 25%) in Bangladesh in 2000 (14) and in India (56.4%) (28). This higher percentage of hemorrhagic symptoms suggests an increase in the severity of the disease in 2002 in Bangladesh. Regarding the increase in the severity of clinical symptoms between 2000 and 2002 and the increase in the anti-DEN IgG between 2000 and 2002, the IgG-positive ratio also increased from 73.7 to 94.0%. This finding suggests that more patients might have a history of previous dengue virus infections or other Flavivirus infections. If a previous dengue virus infection had occurred, it might cause more severe symptoms in a subsequent for a dengue virus infection, i.e., DHF due to the mechanism of the antibody-dependent enhancement-risk hypothesis.

Edema and ascitis (7%) are consequences of plasma leakage, one of the major symptoms in DHF cases. Therefore, based on the clinical symptoms detected in this study, we speculate that at least 7%, and up to 72%, of the confirmed dengue cases were DHF.

Some notable features of the clinical symptoms among the patients in the dengue-positive groups were the mean age of the group ($P = 0.040$), frequency of abdominal pain ($P = 0.009$), and frequency of joint pain ($P = 0.012$), all of which were significantly higher than the patients of the non-dengue group.

The results of Flavivirus IgG ELISA showed that 68 (68%) of the 100 dengue-confirmed cases were above 1:100,000, and 26 (26%) showed titers between 1:3,000 and 1:100,000. They were confirmed as Flavivirus IgG positives (Table 4). However, 50 of these 100 dengue-confirmed cases were extracted during the acute phase. Therefore, to conduct a more precise analysis of dengue infections, 50 positive cases, in which samples were extracted 8 days after the onset of fever, were classified into three groups: IgG (positive-high titer), indicating a titer above 1:100,000; IgG (positive-low titer), indicating a titer between 1:3,000 and 1:100,000; and IgG (negative), indicating a titer below 1:3,000 (Fig. 4). Thirty-nine (78%) of the 50 cases were classified as IgG (positive-high titer), 8 (16%) as IgG (positive-low titer) and 3 (6%) as IgG (negative). These results indicated that 78% of the cases were probably secondary dengue infections. If so, the number of secondary dengue cases increased from 65% in the previous outbreak in 2000 to 78% in 2002 (26). The majority of IgG (positive–high titer) cases belonged to the 21-25-year-old and 26-30-year-old age groups, which coincided with the peak age groups for dengue-confirmed cases (Fig. 1 and Fig. 4). The peak for the IgG (positive-low titer) cases was in the 16-20-year-old age group, and we speculate that this could have been the major age group for primary dengue infections in 2002. The age gap between the peak for a probable primary infection (16-20 years old) and a probable secondary infection (21-30 years old) was 5 to 10 years. This age gap could explain the interval between the dengue outbreaks in 2002 and previous outbreaks prior to 2000 in Bangladesh. Hossain et al. (26) reported on the possibility of dengue epidemics in Bangladesh before 1996, and our serological data supported this possibility.

In this study, we describe the first dengue virus isolation and molecular characterization in Bangladesh. All eight dengue virus strains were isolated from samples collected within 6 days after the onset of fever, in good agreement with Rocco et al. (29) and Perret et al. (30).

Aziz et al. (14) reported that among the three dengue serotypes circulating in Bangladesh (DEN-2, -3 and -4), DEN-3 was the predominant one in 2000. Our eight isolates were all DEN-3, suggesting that DEN-3 might be a recent predominant serotype in this country.

Our eight recent strains were clustered in genotype II, together with isolates from Myanmar, Thailand and Malaysia, but in a well-supported independent sub-cluster of the genotype (Fig. 3). Phylogenetic analysis revealed that the Thai isolates from the 1990s were closely related to our strains. In addition, a multiple alignment of aa sequences showed that all of our eight isolates had two aa changes in the E gene, E81(I-T) and E140(I-T), and shared an aa change at position E172(I-V) with 3 out of the 5 mentioned Thai DEN-3 strains isolated in the 1990s. Therefore, we speculate that the currently circulating DEN-3 viruses might have entered Bangladesh from neighboring countries.

To monitor serotype shifts and genotype shifts in dengue virus infections in Bangladesh, further virus isolation and molecular analyses are needed. Continuous surveillance and detailed clinical analysis of dengue-confirmed patients would provide a further understanding of the impact of this disease in the country.

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