PREDOMINANCE OF THE DEN-3 GENOTYPE DURING THE RECENT DENGUE OUTBREAK IN BANGLADESH

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Abstract. A recent outbreak of dengue in Bangladesh was marked by many fatal complications. As clinical virulence varies among the genotypes of dengue virus, a study was conducted to investigate the molecular genotypes of dengue in Bangladesh. Reverse transcription polymerase chain reaction was used to determine viral genotypes using oligonucleotide generic primers that produce a 511 bp product. The resulting product was typed by nested PCR with strain-specific primers, yielding 482 (DEN-1), 119 (DEN-2), 290 (DEN-3) and 392 (DEN-4), visualized on UV transilluminator after electrophoresis on 2% agarose gel stained with ethidium bromide. Of 45 clinically diagnosed dengue patients (mean age 28 years; male/female 30/15), 19 (42.2%) had detectable viral RNA in their blood. However, during the first 5 days of fever in 30 patients, the frequency was 60% (18/30), implying that the sooner serum is drawn after the fever, the greater the chances of detecting viral RNA. DEN-3 was detected in all except 2 patients who were infected with DEN-2. DEN-2 (two cases) and DEN-4 (one case) were present as co-infections with DEN-3. All of the patients presented with fever, anorexia and vomiting; many had headache and general body ache; a few had a rash. About a quarter had suffered episodes of bleeding, while ascites, pleural effusion and CNS symptoms were found in a few patients. Patients positive for viral RNA were also positive for anti-dengue IgM (p=0.007) in subsequent sampling. The study suggests the predominance of DEN-3 infection with occasional co-infection with other types, during the recent outbreak of dengue in Bangladesh.

INTRODUCTION

Dengue is an acute febrile illness common in the tropics. In humans, dengue infection causes a spectrum of illness ranging from a relatively mild, non-specific viral syndrome (dengue fever) to severe and potentially fatal disease: dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Dengue viruses are transmitted primarily by *Aedes aegypti* and *Aedes albopictus* mosquitos, which are endemic in most tropical areas (Gubler *et al*, 1988). Periodic outbreaks have been reported from both developed and developing countries (Carey, 1973; Gubler *et al*, 1988). In Bangladesh, in the summer of 1964, a febrile illness that became known as 'Dacca Fever' was investigated and found to be due to infection with dengue virus (Aziz *et al*, 1967); subsequent entomological and serological studies have indicated the continued presence of the mosquito vector and dengue virus in the country (Gaidamovich *et al*, 1980; Khan, 1980; Islam *et al*, 1982; Khan and Ahmed, 1986). An outbreak of an acute febrile illness clinically suspected as dengue and DHF occurred in and around Dhaka City during the summer of 1999 (Alam, 2000): serological evidence of dengue virus infection was found in the majority of these cases.

The dengue viral genome is a single-stranded, positive RNA molecule encapsidated

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by a single core protein (Nimmannitya and Cardosa, 1996). The virus replicates in the cells of the macrophage-mononuclear cell lineage and the severity of disease is correlated to the ability of the virus to infect these cells (Halstead, 1988). Dengue virus has four antigenically distinct types, DEN-1, DEN-2, DEN-3 and DEN-4. Infection by one serotype leads to an immune response that confers protection against reinfection by the same serotype; however, a person may go on to be infected by a different serotype, and such secondary infections are implicated in patients who present with dengue hemorrhagic fever and its related complications (Nimmannitya and Cardosa, 1996). It follows that a molecular epidemiology of virus types is important when assessing the relative risk of hemorrhagic complications in the inhabitants of any given region.

The laboratory diagnosis of dengue infection currently depends upon the detection of virus-specific antibodies in patients' sera. Enzyme immunoassay (EIA) detection of dengue virus-specific IgM is useful in the diagnosis of acute infection because the titer begins to rise 7 days after the onset of illness (Bundo and Igarashi, 1985; Saluzzo *et al*, 1986). Reverse transcription PCR (RT-PCR) based genotyping using type specific primers may be used to identify efficiently different types of dengue virus and is reported to be highly sensitive and specific (Mirawati *et al*, 1997; Lanciotti *et al*, 1992). In Bangladesh, the genotyping of dengue virus had not been studied. This study was undertaken to investigate the different types of dengue virus, to determine the exact viremic phase, and to find the genotypes of dengue in Bangladesh.

MATERIALS AND METHODS

Study subjects

The study comprised of 45 patients with clinically-suspected dengue whose fever had lasted for 1-10 days and who were admitted to the Samorita Hospital, Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), or the Bangabandhu Sheikh Mujib Medical University (BSSMU), Dhaka. Of the 45 patients, 30 (66.7%) were male and 15 (33.3%) were female; their mean (±SD) age was 28 (±11) years. Cases were selected and verified by an internist (MN Alam, a member of the reserch team) during the period August to October 2000. The subjects were included only if they had consented to participate in the study. The study protocol was approved by the Ethical Review Committee.

Sample collection

Blood samples from the patients were collected in diethyl pyrocarbonate (DEPC)-treated microcentrifuge tubes; sera were separated and RNA was extracted on the day of blood collection; extracted RNA was stored at -70ºC until RT-PCR; remaining sera were stored at -70ºC.

Virus strains

Prototype DEN-1 was obtained from Dr Pradeep Seth, Professor and Head, Department of Microbiology, AIIMS, New Delhi, India; prototypes of DEN-2, 3 and 4 were obtained from the Armed Forces Research Institute of Medical Sciences, Bangkok,Thailand.

RNA extraction

Viral RNA was extracted using a commercial reagent, TRIZOL LS (Gibco BRL, Maryland, USA): the reagent is a monophasic solution of phenol and guanidine isothiocyanate and has been modified for the single-step RNA isolation method developed by Chomezynski and Sacchi (1987). 750 µl of TRIZOL LS were added to each 250 µl of sample and then mixed several times through a pipette to promote lysis of the cells in the sample. The homogenized sample was incubated for 5 minutes at 15 to 30ºC to permit the complete dissociation of nucleoprotein complexes. 200 µl of chloroform were added to this solution and shaken vigorously for 15 seconds and then incubated at 15-30ºC for 2-15 minutes. The sample was centrifuged at 12,000*g* for 20 minutes at 2-8ºC.

Following centrifugation, the mixture was separated into a lower red phenol-chloroform phase, an interface, and a colorless upper aqueous phase; RNA is found only in the aqueous phase. The aqueous phase was transferred to a clean tube and RNA was precipitated by mixing with isopropyl alcohol. After incubation at 15-30ºC for 10 minutes, the sample was centrifuged at 12,000*g* for 15 minutes at 2-8ºC. The supernatant was discarded and the RNA pellet was washed with 75% ethanol and centrifuged at 7,500*g* for 10 minutes at 2-8ºC. At the end of procedure, the RNA pellet was air-dried for 5-10 minutes and dissolved in RNase-free water.

Oligonucleotide primers

Dengue virus generic primers D1 (5′ TCA ATA TGC TGA AAC GCG CGA GAA ACC G 3′) and D2 (5′ TTG CAC CAA CAG TCA ATG TCT TCA GGT TC 3′) as well as the type-specific primers TS1 (5′ CGT CTC AGT GAT CCG GGG G 3′), TS2 (5′ CGC CAC AAG GGC CAT GAA CAG 3′), TS3 (5′ TAA CAT CAT CAT GAG ACA GAG C 3′), and TS4 (5′ CTC TGT TGT CTT AAA CAA GAG A 3′) were used according to Lanciotti *et al* (1992) and purchased commercially (Gibco-BRL, USA).

Amplification of virus RNA

Target sequence of the virus RNA was converted to a complementary DNA copy (cDNA) using reverse transcriptase (RT) and the dengue virus downstream consensus primer (D2), homologous to the genomic RNA of the four serotypes, prior to enzymatic DNA amplification. Subsequently, amplification of resulting cDNA was performed using the upstream dengue virus consensus primer (DI).

Twelve microliters of extracted RNA were added to the 13 μl of RT mixture containing 5 x RT buffer (250 mM Tris-HCl-pH 8.3; 375 mM KCl; 15 mM $MgCl_2$), 1.25 mM of each dNTP's (Gibco BRL, Maryland, USA), 25 pmol D2 primer, 200 units of Superscript II and 20 units of RNase inhibitor (Promega, Madison, USA). The cDNA was synthesized by incubating this mixture at 37ºC for 60 minutes, followed by enzyme inactivation at 100ºC for 10 minutes.

The first round of PCR was carried out using 50 μl volume. Twelve microliters of cDNA were added to 38 μl of the PCR mixture. The PCR mixture contained 10 x PCR buffer (50 mM KCl; 10 mM Tris-HCl-pH 9.0; 1.5 mM $MgCl_2$ and 0.1% Triton X-100) (Promega, Madison, USA), 2.5 mM of each dNTP's (Gibco BRL, Maryland, USA), 12 pmol D1 primer and 1 unit *Taq* DNA polymerase (Promega, Madison, USA). The reaction was carried out in a Techne (Duxford Cambridge, UK) genecycler at 94ºC for 5 minutes of initial denaturation followed by 35 cycles of denaturation (94ºC for 1 minute), primer annealing (55ºC for 1.5 minute), and primer extension (72ºC for 2.5 minutes) followed by final extension at 72ºC for 7 minutes.

Virus typing by nested PCR

A second amplification reaction was performed with 1.0μl of the amplified product of the first amplification reaction. The reaction mixture contained all the components described for the first amplification reaction with one exception: the primer D2 was replaced with the dengue virus type-specific primers TS1, TS2, TS3 and TS4. After initial denaturation at 94ºC for 5 minutes, the samples were subjected to 30 cycles of denaturation at 94ºC for 1 minute, primer annealing at 58ºC for 1 minute and primer extension at 72ºC for 2 minutes, followed by final extension at 72ºC for 7 minutes. Fifteen microliters of the amplicon were subjected to ethidium bromide stained 2.0% agarose gel (Sigma, Missouri, USA) electrophoresis and the size of resulting DNA band was characterized for each dengue virus type.

Dengue serology

Samples were collected after 7 days of fever for anti-dengue immunoglobulins (IgM and IgG) which were detected by ELISA (Enzyme-linked immunosorbent assay) using commercially available kits (OMEGA, UK).

Statistical analysis

Data were expressed as frequencies or percentages and were analysed (chi-square test) in order to determine the association of antibody status and dengue virus identification as well as the association of IgM and IgG anti-dengue antibodies. P values ≤ 0.05 were considered statistically significant.

RESULTS

Clinical presentation

On presentation, all of the subjects had high fever, anorexia, and vomiting; most of them complained of headache (93.0%) and general body ache (90.9%). Skin rash was a feature in some cases (25%); some patients were bothered by bleeding episodes (23.7%). A few patients developed sonographically detectable ascites (10.5%) and pleural effusion (2.9%) while some developed anemia (12.8%), hepatomegaly (2.7%) and CNS symptoms (5.4%) (Table 1).

Virus genotypes and anti-dengue antibodies

Of the 45 subjects studied, 19 (42.2%) had dengue genomic RNA in their blood; of these positive cases, the most common genotype was DEN-3 (17/19), followed by DEN-2 (4/19). Fourteen RNA-positive patients were found to have only DEN-3, while two had only DEN-2. Two patients were co-infected with DEN-2 and-3, while one patient was infected with DEN-3 and-4. In 26 (57.8%) patients, no viral RNA could be detected. Almost all patients positive for viral RNA were also positive for anti-dengue IgM (18/19) antibody in the samples drawn about one week after the onset of fever; for IgG, the figure was 73.7% (14/19) (Table 2). As shown in Fig 2, the probability of detecting viral RNA was negligible 5 days after the onset of fever. The association between positive genotypes and anti-dengue IgM antiboby was found to be significant (94.7%, p=0.007). Of the 19 positive for viral genome, only one patient (5.3%) was negative for anti-dengue IgM antibody; 15 (57.7%) of the 26

Table 1
Clinical profile of the subjects.

Character (n)	Value (n/N)	Percentage
Fever at presentation	45/45	100
Headache	40/43	93
Anorexia	38/38	100
Vomiting	41/41	100
Several body ache	40/44	90.9
Rash	10/40	25.0
Bleeding episode	9/38	23.7
Ascites	4/34	10.5
Pleural effusion	1/34	2.9
Anemia	5/39	12.8
Jaundice	0/41	0
Enlarged liver	1/37	2.7
Enlarged spleen	0/36	0
CNS symptoms	2/37	5.4

Note: Mean age of subjects in years (±SD) was 27.64 (±11.28).

Table 2
Virus genotypes in relation to serological findings.

Genotypes	n (%)	Positive IgM n (%)	Positive IgG n (%)
Type-2	2 (4.4)	2 (100)	2 (100)
Type-3	14 (31.1)	13 (92.9)	10 (71.4)
Type-2,3	2 (4.4)	2 (100)	2 (100)
Type-3,4	1 (2.2)	1 (100)	0
Negative	26 (57.8)	15 (57.7)	19 (73.1)
Total	45	33	33

Note: Parentheses contain percentages; percentages related to gerotype prevalence are percentages of column totals; percentages related to immunoglobulins are percentages of row totals.

Table 3a
Association between the viral genome and antibody response.

Viral status	Anti-dengue antibody (IgM)		Total
	Positive (%)	Negative (%)	
Positive	18 (94.7)	1 (5.3)	19
Negative	15 (57.7)	11 (42.3)	26
Total	33 (73.3)	12 (26.7)	45

Note: within parentheses are percentages of row totals; χ^2 = 7.704; p = 0.007

Table 3b
Association between anti-dengue IgM and IgG antibodies.

IgM status	IgG status		Total
	Positive	Negative	
Positive	25 (76)	8 (24)	33
Negative	8 (67)	4 (33)	12
Total	33 (74)	12 (26)	45

Note: within parentheses are percentages of row totals. χ^2 = 0.372; p = 0.705

patients without detectable viral RNA were positive for anti-dengue IgM antibody (Table 3a). There was no significant association between the status of IgM and IgG (χ^2 = 0.372; p = 0.705) (Table 3b).

DISCUSSION

In this study, the genotypes of dengue virus in the recent outbreak in Bangladesh were identified: a clear preponderance of DEN-3 was found. Presence of DEN-2 in four patients and a solitary example of DEN-4 suggested the existence of multiple strains in our population. Infection with one type of virus usually confers protection against reinfection by the same serotype; however, if an individual is infected by a different serotype, the chance of developing DHF and DSS is higher and therefore identifying the genotypic status of the virus may be important for management. Severe and life-threatening manifestations of dengue may occur even in the first week of clinical illness when antibody (IgM) status is expected to be negative. RT-PCR can detect and identify the type of dengue virus during this period and may prove helpful in the early diagnosis and proper management of the illness.

The nested RT-PCR method for detecting dengue virus used in the present study has sensitivities of 94% with dengue type 1 virus, 93% with dengue type 2 virus and 100% with dengue type 3 and 4 viruses (Lanciotti *et al,* 1992). The specificity of the method relies upon the ability of the type-specific primers to recognize RNA sequences unique to each dengue virus type. Identification of dengue genotypes was confirmed using the reference prototypes of dengue virus strains obtained from reference laboratories. The possibility of carry-over contamination was checked by using negative controls in each run, by the physical separation of pre-and post-PCR manipulations, and by the use of aerosol-barrier pipette tips.

In this study, 60% of the patients with a fever lasting for 5 days or less were positive for viral RNA. However, the presence of dengue

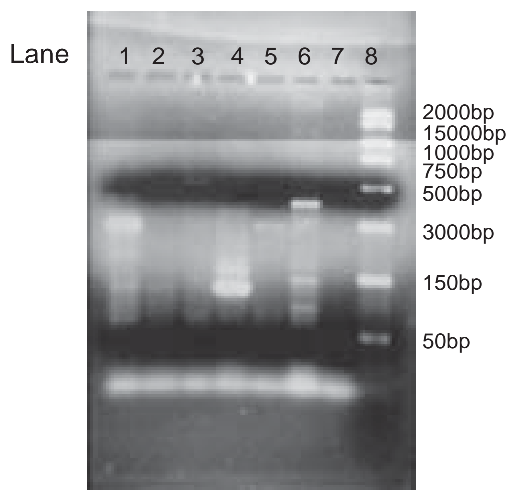

Fig 1–Agarose gel analysis of polymerase chain reaction amplification products.
Lane 1-Sample from patient co-infected with DEN-2 and 3.
Lane 2-Sample from patient with DEN-2.
Lanes 3-6-Dengue prototype 1, 2, 3 and 4.
Lane 7-Negative control.
Lane 8-Molecular weight (MW) markers: DNA sizes are given in base pairs.

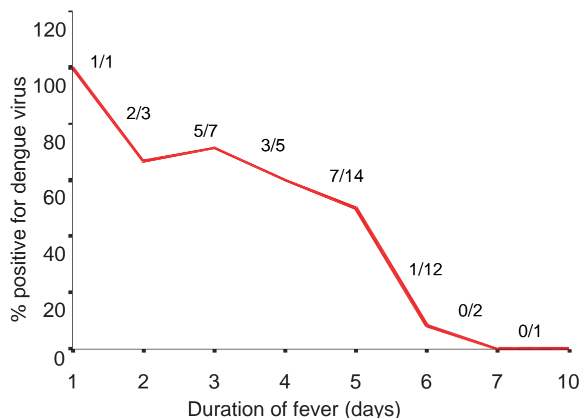

Fig 2–Frequency of viral RNA detected on various days of fever.

IgM antibody in the 15 samples negative for viral RNA (Table 2) indicated that the viremic phase of these patients had passed. The PCR product included occasional non-specific bands at 190bp in addition to the target PCR product. Different annealing temperatures, ranging from 48ºC to 62ºC, were used and it was found that the 190bp product persisted at all annealing temperatures, whereas the target PCR product was not detected at 62ºC. The persistence of a similar 190bp product was also reported in a study by Lanciotti *et al* (1992). In the present study, the presence of viral RNA was significantly correlated (>94%) to the presence of IgM antibody in the serum samples collected one week after the onset of fever, underscoring the usefulness of PCR-based genotyping for the early diagnosis of dengue - although one of the viral RNA positive samples was found to be negative for IgM antibody but positive for IgG antibody (Table 3a). The antibody index of the IgM was 0.71 while that of the IgG was 3.41 (antibody index > 1.0 is considered positive in the kit used for anti-dengue IgM and IgG antibody): it was assumed that this could be indicative of an early phase of secondary dengue infection. In fact, a good proportion of the total number of study subjects were positive for IgG antibody; some of these subjects were also positive for dengue RNA, indicating secondary dengue infection.

In conclusion, recent outbreak of dengue in Bangladesh featured DEN-3 as the predominant genotype; however, co-infection by other types was also found. The population studied was very small and did not represent all areas of the country in which dengue has been reported. A nationwide study of seroprevalence and viral genotypes with sequencing should be conducted in order to aid the detection and control of the disease.

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