Dengue Virus Serotype 2 (DEN-2): the Causative Agent of 2011-Dengue Epidemic in Pakistan

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Abstract

Introduction: Dengue virus (DENV) is an arthropod-borne virus that belonged to the Flaviviridae viral family. Four known serotypes DEN-1 through DEN-4 do exist and circulate in diverse geographical regions of the world causing epidemics. The management of dengue patients, and especially dengue hemorrhagic fever (DHF)/Dengue shock syndrome (DSS) cases, has been a challenge in Pakistan now days. *Method:* We have carried out a comprehensive study of the current outbreaks of dengue virus infection on molecular level with the aim to find out the common serotype/s of DENV responsible for this outbreak using PCR, real-time PCR and nucleotide sequencing targeting the *C-prM* gene junction. For this purpose total 1129 serum samples received between from start of August till end of November 2011 from all the major hospitals of Lahore, Punjab at Division of Molecular Virology, National Centre of Excellence in Molecular Biology (CEMB) University of the Punjab Lahore were utilized for the DENV diagnosis and serotypes/genotypes analysis. *Results:* Male female ratio of the suspected dengue patients was 2.4:1. Their mean age were 31.14 $\pm$ 16.03 (SD) years ranging from 9 months to 90 years. Out of these 1129 serum samples, total 930 (82.37%) were found infected with DENV. Out of the 930 DENV RNA positive samples, 893 (96.02%) had DEN-2

and 37 (3.97%) sample had concurrent infection with serotypes 2 and 3. ***Conclusion:*** Based on the results of this study we conclude that DEN-2 is the responsible genotype for the current dengue epidemic that started from the beginning of year 2011 and is continuing till now. The additional serotype detected in the current study was serotype 3 that remained in very low frequency in Pakistan for last several decades.

Keywords: Dengue Virus, Serotype 2, Serotype 3, Concurrent infection, C/prM gene

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1. Introduction

Dengue (DEN) the most common vector-borne viral disease in humans; is currently one of the most significant emerging diseases that challenges the global public health worldwide particularly in tropical and subtropical countries (1, 2). The causative agent of dengue fever is dengue virus (DENV). DENV is a small spherical, single-stranded enveloped RNA virus belonging to viral family Flaviviridae and genus Flavivirus that infects mammalian and vector cells (3). Dengue infection is an emerging pandemic seen in last 30 years and over 70% of the dengue fever occurs in Asia and the Pacific, followed by the Americas, the Middle East and Africa (4). Globally 2.5 billion people living in more than 100 countries are at risk for acquiring dengue virus.

At least four related but antigenically divergent DENV serotypes (each having several genotypes) such as dengue virus-1 (DENV-1), dengue virus-2 (DENV-2), dengue virus-3 (DENV-3), and dengue virus-4 (DENV-4) have been identified from different geographical regions of the world (5, 6). Dengue infection caused by a different serotype/genotype can be resulted in dissimilar outcome in the form of simple dengue fever (DF), dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (7, 8). Firm evidence has been established that patients with DEN-2 infections are more likely to have a severe outcome than patients with DEN-1, DEN-3 and DEN-4 infections. During dengue outbreak several serotypes can be in circulation at the same time. Some dengue serotypes are assumed to induce greater viremia than the others and are transmitted more readily causing large epidemic (9, 10).

Dengue serotypes DEN-1 and DENV-2 appear to have a worldwide distribution and their relative prevalence varies from one geographic area to another (11). DEN-3 and DEN-4 are also found in the same regions of the world. Sometimes all four dengue viruses are circulating simultaneously in areas where about 2/3rd of the world's population lives and are infested with dengue vectors, mostly *Aedes aegypti* (12). At least seven dengue epidemics have been reported from Pakistan in last 20 years (13, 14, 15, 16, 17, 18, 19).

The above introduction signifies the prevalence of four DENV serotypes that has re-emerged radically in recent years along with an increase in viral genetic diversity. The evolution of DENV has had a major impact on dengue epidemiology and their virulence on humans globally. In order to perform disease surveillance and understand virus evolution and its effects on virus transmission and disease outcome, the exact serotype/genotype circulating in a particular region is required. Therefore the present study was designed and initiated to exactly find out the common dengue virus serotype/s and/or genotypes circulating in Pakistan in the current outbreak.

2. Materials and Methods

2.1. Patients and samples

A total of 1129 serum samples were received along with information on demographic characteristics, area, biochemical and hematological characteristics and duration of disease from start of August till end of November 2011 at Division of Molecular Virology, National Centre of Excellence in Molecular Biology (CEMB), University of the Punjab Lahore for the detection and serotyping of Dengue Virus from all the major hospitals of Lahore, Punjab Pakistan. An informed consent was taken from all patients and the study was conducted in accordance with the 1964 Declaration of Helsinki and Guidelines for Good Clinical Research Practice in Pakistan.

2.2. Dengue Qualitative PCR

Initially the diagnosis of DV was based on the presence of DENV RNA by PCR. Only DENV RNA PCR positive samples were included in the study. DENV PCR was done as described previously (1). Briefly, viral RNA was extracted from patients' serum samples using Nucleospin Viral RNA Extraction Kit (Macherey-Nagel, Germany) following RNA isolation procedure given in the kit protocol. Complementary DNA (cDNA) was synthesized using extracted RNA with M-MLV Reverse Transcriptase Enzyme (Invitrogen Biotechnologies USA) in the presence of anti-sense primer. cDNA was subjected to nested PCR for the amplification of DENV specific region that was evaluated using 2% agarose gel post PCR amplification.

2.3. Serotype analysis

DENV serotyping was carried out using type-specific DENV serotyping method as previously described in detail (1). Briefly, about 100 ng of DENV RNA was reverse transcribed to cDNA using 200 U of M-MLV reverse transcriptase enzymes at 37 °C for fifty minutes. The M-MLV was heat inactivated at 95 °C for five minutes. For PCR amplification of *C-prM* gene junction region of DENV in first round, four μl of synthesized cDNA was used. As there were 4 different DENV serotypes that we tried to detect, so the type-specific primers were divided into four groups and four second-round PCRs were performed for each first round PCR sample. First PCR mix contained DEN-1 specific primers, second mix contained DEN-2 specific primers, third mix contained DEN-3 specific primers and fourth mix had DEN-4 specific-primers. The second round PCR products were electrophoresed on a 2% agarose gel to separate type-specific fragment. The gel was stained with ethidium bromide and evaluated under UV transilluminator. A 100-bp DNA ladder (Invitrogen, Corp., California, USA) was run in each gel as DNA size marker and the DENV serotype/s for each sample was determined by identifying the DENV specific PCR band. The amplified product sizes for specific serotypes such as DEN-1, DEN-2, DEN-3 and DEN-4 were 411-bp, 403-bp, 453-bp and 401-bp respectively.

2.4. Sequence analysis of *C-prM* gene junction region of DENV

The *C-prM* gene junction regions of total 20 isolates were sequenced bi-directionally. Briefly, the PCR product was run on 1.2% agarose gel. The specific bands were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Germany). This gel purified amplicons were used for sequencing PCR reactions. Sequencing PCR products analysed on automated genetic analyzer according to the manufacturer's instructions (Big Dye Deoxy Terminators; Applied Biosystems, Weiterstadt, Germany). The identity of the sequences was confirmed by *Basic Local Alignment Search Tool* (BLAST). Sequences that were unique (obtained for the first time) were submitted to GenBank Data Base. The BioEdit v7.0.5 software was used for the translation, the alignment of amino acid and protein sequences. The MEGA version 5 was used for the phylogenetic and molecular evolutionary analyses (20, 21). The phylogenetic tree was drawn by using the Neighbor-Joining (N-J) method with bootstrap analysis of 1000 replicates (22). The sequences of different geographical regions were retrieved from GenBank Database.

2.5. Statistical analysis

SPSS version 10.0 for window was used for data analysis and the summary statistic. The results for all variables were given in the form of rates (%). Chi Square and Fisher's Exact tests were used for categorical variables that measured association among categorical variables. All data are presented as mean values or number of patients. P-values less than 0.05 were considered significant.

3. Results

Figure 1 show the study disposition and exclusion criteria. During the course of this study, total of 1129 suspected dengue positive serum samples were received from tertiary care centers/hospitals (Table 1). The over all results of the study can be seen from table 2. Out of these 1129 sera, total 930 (82.37%) were found infected with DENV by PCR method. Total 199 (17.6%) samples were found negative by DENV nested

and serotyping PCRs. Table 3 demonstrates the serotypes distribution in dengue epidemics 2011 in PCR positive samples. Out of the 930 DENV RNA positive samples, 893 (96.02) had DEN-2 and 37 (3.97%) sample had concurrent infection with serotypes 2 and 3. Male female ratio was 2.4:1. Mean age of patients was 31.14 ± 16.03 (SD) years ranging from 9 months to 90 years old. The distributions of DENV serotypes were similar in male and female patients.

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│ Total of 1129 suspected positive sera│
│ samples for dengue were received     │
│ from tertiary care centers/hospitals │
│ (Table 1)                            │
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                  ├───────▶│ Total 199 (17.6%) samples were │
                  │        │ found negative by DENV nested  │
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│ Total 930 (82.37%) samples were      │
│ found infected with DENV by PCR      │
│ method and were thus selected for    │
│ serotype analysis                    │
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│ Total 893 (96.02)│   │ Total 37 (3.97%) had │
│ had DEN-2        │   │ concurrent infection  │
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│ Total 20 samples were selected randomly for  │
│ sequence analysis and all were found with    │
│ Genotype IV                                  │
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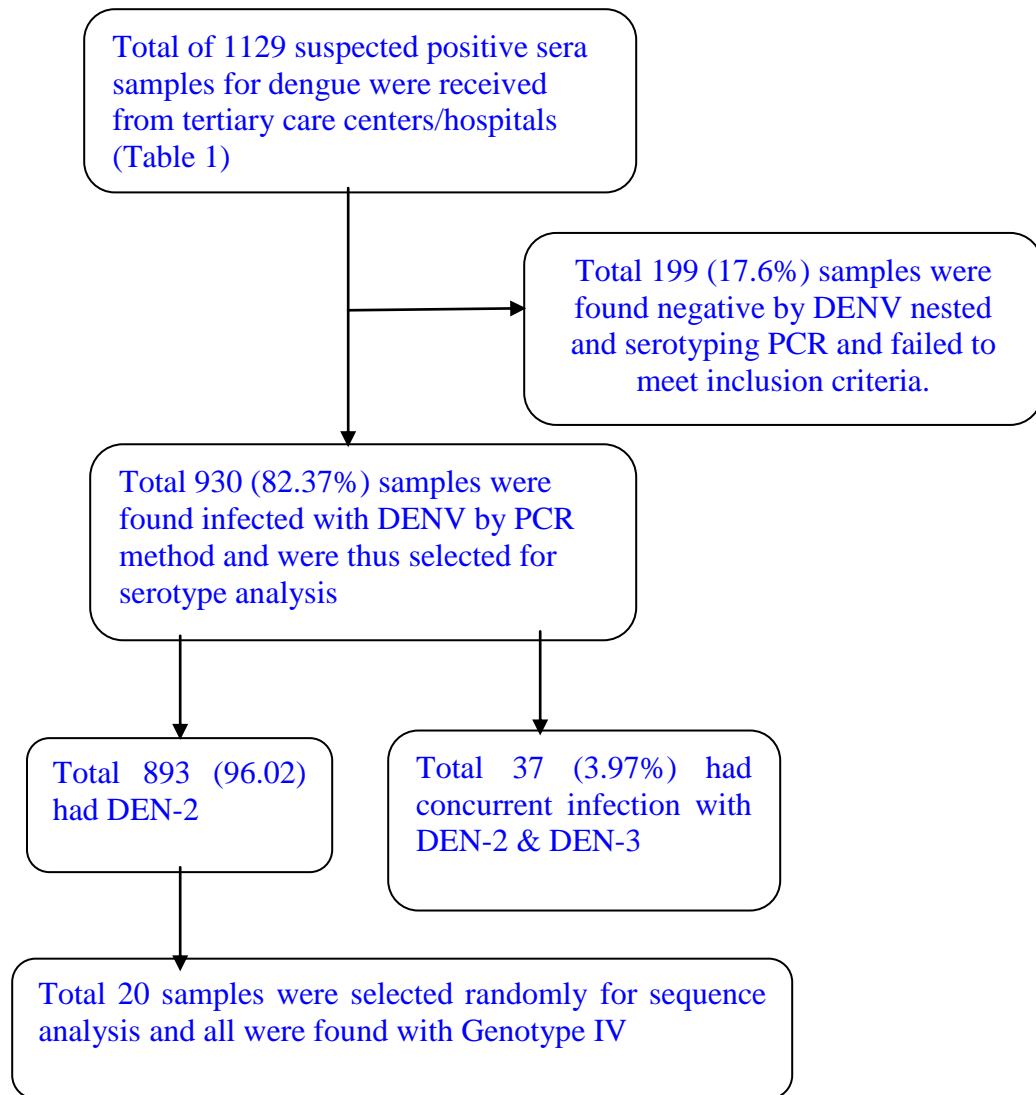
Figure 1 Patients disposition and study enrollments

Total twenty samples selected randomly were sequenced in both directions and consensus sequences were submitted to GenBank Database. All these samples belonged to DEN-2. The assigned Accession Numbers of these 20 sequenced isolates are: JQ390235; JQ390236; JQ390237; JQ390238; JQ390239; JQ390252; JQ390253; JQ390254; JQ390255; JQ390256; JQ390261; JQ390262; JQ390263; JQ390264; JQ390265; JQ390284; JQ390285; JQ390286; JQ390287 and JQ390288. The BLAST results showed that homology of an average of 99% was found between our sequences of serotype 2 and Sri Lankan strain [GenBank: GQ252676] of this serotype. Further the *C-prM* fragment of serotype 2 was found to be rich in AG composition.

Figure 2 illustrates the phylogenetic analysis of the sequenced samples selected randomly from all the 930 DENV RNA PCR samples along with reference sequences that was conducted using the MEGA 4 software and multiple sequence alignment was deduced by using BioEdit software. No insertions or deletions were seen in the sequenced regions. The reference sequences were retrieved from GenBank data base. A 329-bp region (nt194-522 of prototype 2) for DEN-2 was chosen. It is clear from the phylogenetic tree that all the 20 sequenced DENV-2 strains lied in the category of genotype IV. The sequences fall in genotype IV with northern Indian strains.

The findings of the current study clearly show that DEN-2 is the responsible serotype for the current dengue epidemic in Lahore Pakistan starting from March 2011. The sequenced data further clarified that genotype IV of Serotype 2 is the genotype of the current epidemic in Punjab Pakistan. The other serotype isolated from the present epidemic was serotype 3 however in a very low frequency.

Table 1: Samples received from various Hospitals of Punjab Pakistan and analyzed for Dengue virus PCR & Serotyping (N=1129)

Hospitals				
	Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
Lahore General Hospital	493	43.7	43.7	43.7
Institute of Public Health	216	19.1	19.1	62.8
Jinnah Hospital	232	20.5	20.5	83.3
Sheikh Zaid hospital	36	3.2	3.2	86.5
Ghurki trust	64	5.7	5.7	92.2
CEMB	10	.9	.9	93.1
Dow university of medical sciences karachi	73	6.5	6.5	99.6
Services Hospital	4	.4	.4	99.9
Punjab University	1	.1	.1	100.0
Total	1129	100.0	100.0	

Table 2: Over all results of PCR and serotypes of epidemics 2011 (N=1129).

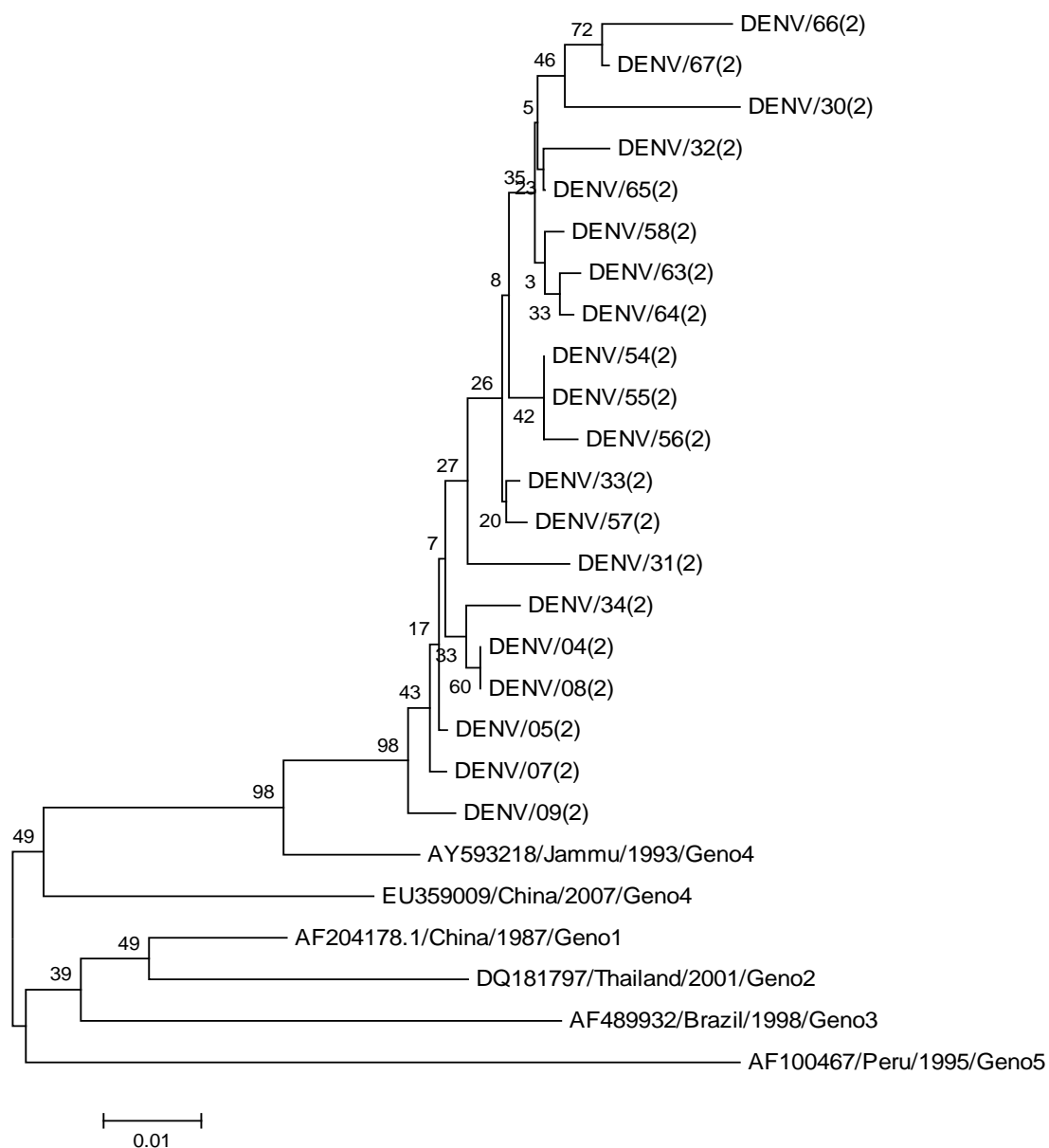| Serotype				
Serotype	**Frequency**	**Percent (%)**	**Valid Percent**	**Cumulative Percent**
Serotype 2	893	79.1	79.1	79.1
Mixed serotype 2 and 3	37	3.3	3.3	82.4
Not Detected	199	17.6	17.6	100.0
Total	1129	100.0	100.0	

Figure 2. Evolutionary relationships of DENV serotypes/genotypes
The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.37623114 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 291 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

4. Discussion

Viral epidemics have become gradually larger in the last 10 years in Asians countries and among these epidemics; dengue is the most important mosquito-borne viral disease upsetting humans (23). Recent decades have seen an extraordinary increase in the incidence and severity of dengue

virus infection that pose a continous global threat to human health (11). According to WHO Dengue infection is the most quickly spreading, vector borne disease in the globe (24, 4) and is endemic in Pakistan, circulating throughout the year with epidemics in the post monsoon period as a serious outbreak of dengue affecting around five million people and killing more than 500 people was experienced just after 2011 monsoon. The main objective of the current study was to characterize serotypes of dengue virus involved in this recent outbreak of dengue in Punjab because in this outbreak, the number of dengue infection cases in Lahore, the capital of Punjab province was unusually high. Dengue infection cases reported in the start of the epidemic were more than 15,000 (25) that increased to more than 50'00'000 by the end of December 2011. According to Health Department's reports, the number of deaths caused by the epidemic remained at 365 in the provincial metropolis (26); however the correct number may be more than 700.

In the current study we were able to diagnosed and serotyped a huge number (N=1029) of samples from the epidemic. We were able to successfully serotype more than 82% of collected samples compared to previous studies that reported only 17.5% positive dengue virus samples (1) as this time samples were collected at second to third day of fever because at this time the infection rate is at its peak. Serotype 2 dominated this outbreak with 100% infection rate. Concurrent infections with serotypes 2 and 3 were 3.8%. Previously we had established DEN-2 and DEN-3 as responsible serotypes for the 2006-2009 dengue out-breaks in Pakistan (1). DEN 2 was found associated with DHF in our previous study. Contrary to our results, others reported three (DEN-1, 2 & 3) or all four serotypes of dengue virus from Pakistan (27, 19, 17, 18). Humayoun and colleagues (19) has reported DEN4, DEN2, and DEN3 serotypes from Pakistan with predominant serotype DEN4. We were unable to detect a single patient infected by DEN-1 and DEN-4 serotypes from Pakistan during the current outbreak. Even our samples belonged to a large number of cities and towns of Punjab and Karachi (Sindh) and the sample size was 5-6 times higher than previously analyzed samples in other studies

from this country on dengue, still we were unable to isolate DEN-1 and DEN-4. The reason of difference may be due to the difference in sensitivity and specificity of methods used for the serotyping of DNV. We utilized the most sensitive real-time and nested PCRs and the most specific method of sequence analysis of randomly selected samples. The results of the current study based on most sensitive and specific methods available in market clearly show that this dengue outbreak was caused by DEN-2 only. However we had also seen fewer cases of DEN-3 infection which was in concurrence with DEN-2. It is assumed that viruses of DEN-3 have been continued in Pakistan in a low transmission rate for years being detected in last few years from this region of the world. According to CDC (28), DEN-3 was re-introduced into American region in 1994 and later on spread to various countries of Latin American and the Caribbean. Serotype DENV-3 has five genetically distinct groups called genotypes (29, 30). Messer and colleagues (31) has already been reported that genotype III of DENV-3 is circulating in the Americas that is believed to be originated in Sri Lanka. This is the same genotype (III) that we were able to isolate in our previous study and had about 99% homology with Sari Lankan DEN-3 (III) strain (1). In the current study the frequency of this (DEN-3) serotype is very low.

The circulation of DEN-2 is alarming two fold. Firstly, this serotype is more virulent as compared to other three serotypes as most outbreaks of dengue related DHF have previously been associated with DEN-2. Secondly, there is more chance of the occurrence of DHF or DSS in patients who have had prior dengue infection with one or more dengue serotypes in the past with secondary infections by different serotypes from the first infection. This occurrence is known as original antigenic sin and in this situation the immune system of the patient is unable to react sufficiently to the stronger infection resulting in more serious outcome of secondary infection. It is predicted that if next year the epidemics come with a dengue serotype other than DEN-2, it will increase the risk for DHF and DSS in this region significantly than we have seen in the current epidemics that was very low.

5. Conclusion

The findings of the current study lead us to conclude that DEN-2 is the causative agent of the 2011 dengue epidemics in Pakistan. Few cases had concurrent infection with DEN-3 and DEN-2. Two serotypes DEN-1 & DEN-4 does not exist in Pakistan.

Acknowledgements

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