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Original Article

Analysis of Dengue Virus Genotypes and Further Investigations for Mixed Infections Through RT-PCR in Classical Dengue Fever Patients in Pakistan

Fazal Hanan[1], Jawad Ahmad[2], Shamsul Hadi[3], Ijaz Ali[4], Muhammad Fahim[5], Asif Qayum[6]

[1, 3]Department of Pathology Saidu Group of Teaching Hospital Swat, [2]Department of Microbiology, Institute of Basic Medical Sciences Khyber Medical University Peshawar, [4] Department of Biosciences, COMSATS Institute of Information Technology, [5] Islamia college University University Peshawar, [6] Shin welfare center, Swat.

ABSTRACT

Background: Four different serotypes (DEN1, DEN2, DEN3 and DEN4) are implicated in either Dengue fever (DF), Dengue Hemorrhagic Fever (DHF) or Dengue shock syndrome (DSS). DHF are often a consequence of mixed/double infections with two different genotypes or secondary viral infection. In the past few years, Pakistan has faced several sporadic outbreaks of dengue fever that eventually developed into a significant epidemic in 2011, 2017, 2018 and 2019, with dire consequences for public health. This study was carried out to investigate different dengue virus genotypes in patients with classical dengue fever and further investigate the patients for mixed infection.

Methodology: A total of 200 blood samples from classical dengue fever patients were collected during September-December 2011 and 2019 were used for isolation of sera. Presence of virus was initially investigated through Immuno-Capture ELISA (IC-ELISA). ELISA positive samples were further probed through RNA-based nested RT-PCR. For DEN-genotyping total RNA was extracted from patients' sera and was reverse transcribed into cDNA; serotypes-specific primers were used for validation of various genotypes.

Results: Through RT-PCR, majority (51.85%) of the patients tested positive for serotype 2, followed by serotype 3 (42.59%) and only 5.55% of patients had mixed infection (both DEN2 and DEN3). None of the samples tested positive for DEN1 and DEN4.

Conclusion: It would be interesting to emphasize on cases where patients with Classical dengue fever had double-dengue-virus-genotype-infection exhibit full recovery. We believe investigating such cases would provide clues to devising treatment for sever dengue hemorrhagic disease.

Key words: Dengue, Classical dengue fever, DEN2, DEN-3, Pakistan

Introduction

Dengue infection is one of the major emerging health problems in Pakistan. The disease is caused by Dengue virus (DEN) in Genus flavivirus, family Flaviviridae[1]. Dengue virus (DEN) is responsible for disease in human causing fatalities worldwide with nearly 2.5 billion people at risk of DEN infection. According to World Health Organization (WHO) estimation, the number of infected cases and emergence of new cases is rising as the infection spreads to the different and new regions of the world [2].

CORRESPONDENCE AUTHOR
Fazal Hanan
Department of Pathology
Saidu Group of Teaching Hospital Swat
Email:drfhanan@gmail.com

Every year an outbreak of dengue fever leads to hospitalization of over 55,000 individuals, many of these infected patients are children with a mortality rate of 2.5% estimated during yearly episode, however, the actual number of deaths from the virus may be twice as much[3].

Dengue virus (DEN) is a single-stranded positive-sense RNA molecule with four different serotypes (DEN1, DEN2, DEN3 and DEN4). Virus is transmitted from person to person through female Aedes aegypti and rarely by Aedes albopictus. Disease is mostly endemic to tropical and subtropical countries, where conditions are most favorable for mosquito breeding. Climate change and rise in global temperature may lead to the spread of virus and its vector to new areas. An individual in an endemic area can get infected with up to four different infections in life, because DEN infection with any serotypes only provide immunity

against that species and the other serotypes can severely compromise the immune system. Dengue viral infection can lead to Dengue fever (DF), Dengue Hemorrhagic Fever (DHF) or Dengue shock syndrome (DSS) as a mild, moderate or severe form respectively in which DSS can be life threatening.[4, 5]

DEN infection usually affects different organ system of the body, including the skin, brain, lungs, and gastrointestinal systems. DEN can also affect other organs of the body less frequently like the heart, pancreas, liver and nervous system producing inflammation in the respective organ system. Mostly DEN infection can cause asymptomatic dengue fever although 5% cases may prove fatal and can lead to death.[6-8]

Different genotypes of DEN, can lead to different clinical and epidemiological profiles, to define precisely what are the clinical features associated with different genotypes has been elusive. Several reports showed that severe form of disease was due to DEN2 and DEN3 while DEN4 was associated with mild disease [9-11].

In Pakistan, the first major outbreak of DHF was reported in 1994, in Karachi. Since then DF has been identified as one of the cause of fever in the region, with some cases of DHF and small number of deaths that are directly attributable to DHF / DSS. In the following year (i.e.1995), DEN2 infection was identified in patients from Baluchistan province .[4, 5] Vast majority of patients have been diagnosed on clinical judgment compatible with DF and DHF, while a small number of cases were confirmed in the laboratories of various hospitals.

Virus has become widespread in some parts of the country with a peak season during monsoon rains. Peak incidence of DF was observed from August to October during each outbreak. This pattern is consistent and similar with reports from other affected countries, and correlates well with any hot summer and the monsoon season that provides favorable and fertile conditions for the population buildup of the vector *Aedes aegypti* .[12, 13]

DHF or DSS are often a consequence of secondary or dual infection with a different serotype of DEN. However, there are few reports around the globe that some patients with mixed infections recover to full health without treatment .[10]

Here we report the genotypes circulating among DEN infected people and cases where patients with mixed infection developed only classical symptoms and later recovered to full health, despite the fact that the 2011 outbreak of Dengue in Lahore district was the most severe in terms of morbidity and mortality.

Materials and Methods

Approval was obtained from ethical committee of Advanced Study and Research Board (ASRB) of Khyber Medical University (KMU) Peshawar vide letter No.DIR/KMU-EB/GD/000009.

Out of the total 200 samples, a 100 serum samples were collected (from September to December 2011) as a part of Pakistan Medical and Research Council (PMRC) funded project from patients with clinical manifestations of classical Dengue like symptoms, from Lahore and 100 samples with clinical manifestation of classical dengue like symptom (from August-December 2019) at two different hospitals i.e. LRH and KTH Peshawar Pakistan. Dengue infection in patients was confirmed by Dengue-specific IgM and IgG profiles. About 166 patients without bleeding tendency or shock i.e., classical dengue fever patients, were selected from the total based on Dengue-specific capture-ELISA for identification of various DEN genotypes. These samples were further validated through nested RT-PCR following manufacturer's protocols (Favorgen BiotechCorp, Taiwan).PCR positive samples/patients were further processed at Institute of Biotechnology and Genetic Engineering (IBGE),University of Agriculture, Peshawar, for identification of serotype using serotype-specific primers.

Samples were transported from Lahore and other Hospitals to IBGE, Peshawar in liquid nitrogen and stored at -80^{o}C refrigerator in Molecular Genetics Lab, Health Biotechnology Division at IBGE.

Samples were pooled for optimization of genotyping protocol by taking 20µl of each sample and mixing in a Falcon tube. Mixture was used as a positive control throughout the course of this study.

RNA of Dengue was isolated from serum of patients 150 µl/sample, using Favorgen viral RNA extraction kit (Favorgen BiotechCorp Taiwan) following manufacturer's protocol as; 150µl serum was transferred from each sample to lysis buffer amounting 570µl and incubation of mixture was done after vortexing at room temperature for 10 minutes. Then 570µl of ethanol (100%) was added to the sample mixture.700µl of sample mixture was then loaded to collection tube having VNE column and centrifuged at 8000xg for 1 minute.Column was washed in a new collection tube with wash buffers of 1500µl and centrifuged at 8000X g for 1 minute. Washing was conducted with 570µl of washing buffer 2 to the column and centrifugation was done again at 8000X g for 1 minute. Moreover, drying of the column was conducted by centrifugation at 12000X g for 5 minutes. Finally 50µl of RNAse free water was added carefully to the centre of the column and incubated along with the elution tube at room temperature for 2 minutes thereafter centrifuged at 12000X g for 2 minutes and RNA was eluted and stored at -80^{o}C for short time.

The complementary DNA (cDNA) was made from extracted RNA in 20 µl of reaction mixture containing the chemicals and RNA as; 4 µl 5Xfirst standard RT buffer,

2 µl 10mM dNTPs, 1 µl 50 pM reverse anti-sense primer (D2), 1µl (200 units) M-MLV Reverse transcriptase enzymes (invitrogen biotechnologies USA), 2 µl dH_2O and 10µl (from 20-50 ng) of extracted RNA. Optimized 20µl reaction mixture was incubated at 37°C for 50 minutes followed by 2 minutes of heat activation of M-MLV. Finally, the reaction samples were incubated for two minutes at 22°C.

For serotype analysis, initially the larger fragment of cDNA was amplified by using Sense (D1) and anti-sense (D2) primers in the genome of the samples by adding the reagents used in PCR protocol. For amplification of cDNA 20 µl of reaction mixture was made as; 2 µl 10X PCR buffer, 1.8 µl $MgCl_2$(from 25mM stock), 1.5 µl 200µM dNTPs, 1 µl 50 pmol each primer,1 µl DMSO (1%), 0.5 µl 2.5U *Taq*-DNA polymerase (invitrogen biotechnologies USA), 9.2 µl RNAse free water, and 2 µl cDNA (50-100 ng).Thermal profile for first round was; initial Denaturation at 94 °C for 3 minutes followed by 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 1 minute, extension at 72 °C for 45 seconds and final extension at 72 °C for seven minutes. In the 2nd round PCR, the reverse primer was replaced by four serotype specific primers. D1 and TS1 (Type specific 1) were used for the identification of serotype 1 and the amplification of a 482bp region. Similarly, D1 and TS2 (type specific 2) were used to identify serotype 2 and amplify a region of 119bp, while D1 and TS3 (type specific 3) amplified a region of 290bp and detected serotype 3. A 392bp region of Serotype 4 was amplified by D1 and TS4 (type specific 4). 20µl reaction mixture was made and nested PCR was run on the same thermal profile of first round to find out the serotype. The primer sets (Table 1) used for PCR amplification was described by Lanciotti *et al*., 1992[14].

Table 1: List of primers, expected amplified size and their genome position in DENV.

Primer	Sequence	Genome position	Amplified size
D1	5´-TCAATATGCTGAAACGCGCGAGAAACCG-3´	134-161bp	511bp
D2	5´-TTGCACCAACAGTCAATGTCTTCAGGTTC-3´	616-644bp	511bp
TS1	5´-CGTCTCAGTGATCCGGGGG-3´	568-586bp	482bp(with D1)
TS2	5´-CGCCACAAGGGCCATGAACAG-3´	232-252bp	119bp(with D1)
TS3	5´-TAACATCATCATGAGACAGAGC-3´	400-421bp	290bp(with D1)
TS4	5´-CTCTGTTGTCTTAAACAAGAGA-3´	505-527bp	392bp(with D1)

PCR amplified products were visualized by using 2% agarose gel prepared in TBE buffer (0.5X). A florescent dye ethidium bromide amounting 10 µg/ml was added to the amplified products and electrophoresis was then carried out at 110 V for 30 minutes. Gel was pictured by using Alpha quant (Alpha Innotech,) Gel documentation system. A 50bp of DNA ladder was used as a DNA marker.

Results

A total of 166 patients were initially found DEN-positive based on ELISA. RNA-based PCR of the ELISA positive samples showed that 65% (108/166) samples (were positive, while 35% (58/166) cases (were found negative for dengue virus genome (Figure 1).

Out of 200 samples, 166 samples were found ELISA positive for classical dengue fever patient. These samples were further validated through nested RT-PCR, out of which 108 samples (65.06%) were found

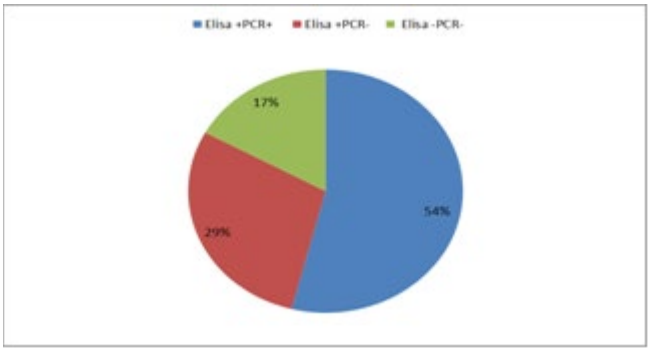

Figure 1: Molecular identification and distribution of DEN:

positive through PCR assay, while 58 cases (34.93%) were found negative.

Among the 108 PCR positive patients, DEN2 was predominant with 56 (51.85%) relative incidence, followed by DEN3 in 46 (42.59%) cases and 6 (5.55%) samples with mixed infection of DEN2 and DEN3 (Figure 2).

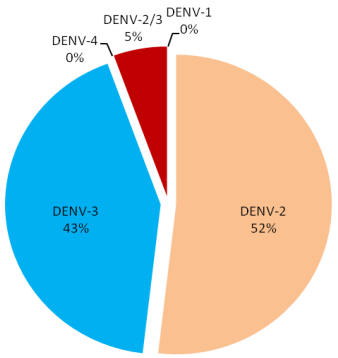

Figure 2: Characterization and Serotyping of DEN

Figure shows that DEN2 (52%) is dominated, followed by DEN3 (43%) while other serotypes are not detected. Only 5% of mixed infection with DEN2+DEN3 is noted. 68.67% of the affected patients were males, while the rest were females (Figure 3). Studied subjects were divided into four different age groups. The 1st age group consisted patients younger than 11 years. The second age group was between 11-20 years, the third age group includes patients aged 21-51 years, while 4th age group consisted of patients > 51 years. Out of the 18 patients in the 1st age group, 12 (66.66%) were males while 06 (33.33%) were females. Second age group consist of 34 patients, out of which 20 (58.82%) were male and 14 (41.17%) were female. Patients in third age group were observed to be more affected. Out of the 94 cases, 64 (68.08%) patients in this group were males and 30 (31.91%) were females. Older age group of over 51 years consisted of 20 patients out of which 18 (90%) were males and 02 (10%) were females (Figure-3).

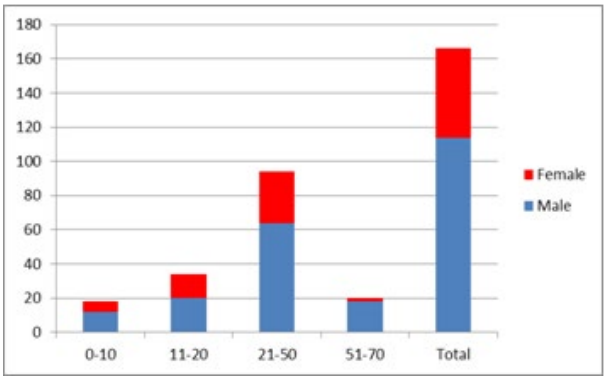

Figure 3: Gender-wise distribution of DEN.

Incidence of DEN is most common in age group 21-50 years old and overall male are more affected than female.

Male and female were affected either by DEN2, DEN3 or co-infection of both serotypes.

Gel photograph was taken by using Alpha quant (Alpha Innotech) Gel documentation system. 20bp and 50 bp marker was used in the study for Gel photograph. The Gel photograph representing bands of 119bp and bands of 292bp are positive for serotype 2 and serotype 3 respectively (Figure 4).

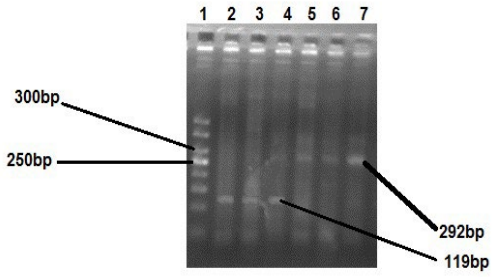

Figure 4: Gel photograph representing serotype 2 and 3 positivity. Key: Well 1: 50bp Ladder Well 2-4: showing band for serotype2 (119bp) Well 5-6: showing bands for serotype3 (292bp).

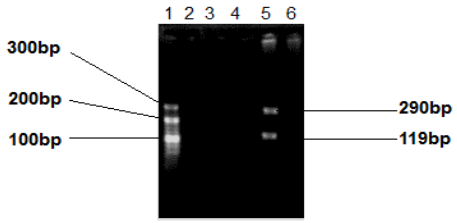

Figure 5: Gel photograph. Well 1: DNA ladder 20 bp. Well 2-4: Negative Control. Well 5: Sample positive for mixed infection (DEN2 and DEN3).Well 6: Negative control

Discussion

Our study have shown that majority of the patients with classical dengue fever were infected by DEN serotype 2 and 3.These findings seems to be similar to those noted during the 2006 outbreak in New Delhi [15], and, the outbreak of Dengue in Karachi in 2006 which was dominated by serotype 2 and 3, and contributed to many deaths among different age groups[16]. Additionally, the serotype 3 in this outbreak was genetically more identical to the serotype 3, which was the major contributor of 2004 New Delhi epidemic.[17] One of the potential explanation for the genotypic similarity could be due to public commutation and spread through Wahga Border, as the Indian capital is located 428 km away from Lahore.[18] However, a thorough sequence analysis is required to identify the first point of entry of DEN into Pakistan.

This study have also shown that the prevalence of different DEN serotypes are not gender specific, and,

can affect males and females equally.

The unique feature of our study is identification of mixed pattern of infection in patients with classic dengue fever in Pakistan. We present six (06) cases with confirmed mixed DEN infection in patient with classic dengue fever. Although, mixed infection with DHF and DSS have been reported in other countries, like Brazil[12,19,20], Taiwan and mainland China[21,22,23], but till date, no studies have reported mixed infection in patient with classic dengue fever. It is of utmost clinical and prognostic importance, to identify this sub group of patients as the disease pattern in such patients is mild and they usually have good long term prognosis. This is in contrast to DEN 2 and DEN 3 infections, which exhibit severe form of the disease as reported by earlier studies.[9-11]

Different genotypes of DEN, can lead to different clinical and epidemiological profiles, define precisely what are the clinical features associated with different genotypes has been elusive. Several reports showed that severe form of disease was due to DEN2 and DEN3 while DEN4 was associated with mild disease.[9-11] Patients who suffer from DEN2 were more susceptible to Dengue hemorrhagic fever (66%) than those infected by other viral serotypes. The severity of disease from DF to DHF was recognized in the DEN2 infected patients, as compared to those affected with DEN1 and DEN3.[11]

There are several possibilities of a person getting dual infection with two different genotypes of DEN2.[23, 24] It is, however, very intriguing fact that some patients with concurrent infection do not develop DHF and fully recover. Either our current understanding of DHF pathogenesis is incomplete or these patients have remarkable immune system that is able to clear out even double infection of DEN genotypes.[25] We found concurrent infection with DEN2 and DEN3 in six patients (out of 108 infections). These patients had mild symptoms and did not develop hemorrhagic fever. Similar study is reported from Brazil, where a patient with Concurrent infection due to DEN-2 and DEN-3 during 2003 epidemic developed only mild symptoms and later recovered to full health. Such cases are not uncommon as several other authors have observed and reported similar cases .[26]

In Pakistan, patients have been reported with severe manifestation of the disease in print media; however, there is no systematic study on association of DEN concurrent infection with these deaths. The mixed infection recognized in the study was due to DEN2 and DEN3, while the other serotypes were found absent. This combination might be due to the prevailing serotypes, requiring a large scale study. These observations, though rare, but still point to the fact that certain cases do not support the hypothesis that double infection with dengue viruses always leads to more severe hemorrhagic disease.[27,28]

Conclusions

It would be really interesting to carry out a detailed sequencing analysis and characterization of immunity pathway of patients who fully recover from concurrent infection of DEN genotypes, as it will further explore the possibility of developing antiviral therapy and definitive vaccine development.

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Competing interests

The authors of the study have no conflict of interest.

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CONTRIBUTION OF AUTHORS	
Author	**Contribution**
Fazal Hanan	A,B,C
Jawad Ahmad	A,C
Shams Ul Hadi	C
Ijaz Ali	C
Muhammad Fahim	C
Asif Qayum	C

KEY FOR CONTRIBUTION OF AUTHORS:

A. Conception/Study/Designing/Planning
B. Active Participation in Active Methodology
C. Interpretation/ Analysis and Discussion