Complete genome sequencing and comparative analysis of three dengue virus type 2 Pakistani isolates

Madiha Akram¹ · Muhammad Idrees²

Received: 27 July 2015 / Accepted: 9 September 2015 / Published online: 18 January 2016
© Indian Virological Society 2016

Abstract Dengue is currently one of the most important arthropod borne human viral diseases caused by a *flavivirus* named as dengue virus. It is now endemic in Pakistan since many dengue fever outbreaks have been observed in Pakistan during the last three decades. Major serotype of dengue virus circulating in Pakistan is serotype 2. Complete genome sequences of three Pakistani dengue virus serotype 2 isolates were generated. Analysis of complete genome sequences showed that Pakistani isolates of dengue virus serotype 2 belonged to cosmopolitan genotype. This study identifies a number of amino acid substitutions that were introduced in local dengue virus serotype 2 isolate over the years. The study provides a significant insight into the evolution of serotype 2 of dengue virus in Pakistan. This is the first report of complete genome sequence information of dengue virus from the most recent outbreak (2013) in Punjab, Pakistan.

Keywords Dengue virus · Outbreak · Genome · Sequence analysis · Serotype · Genotype

Introduction

Dengue is an emerging health problem in tropical and subtropical areas of the world. It is one of the most important mosquito-borne human viral diseases. It belongs to the genus *Flavivirus* of family *Flaviviridae*. About 390 million dengue infections occur worldwide annually and 96 million of which are symptomatic [2]. Four distinct serotypes of dengue virus have been reported. It possesses single stranded RNA genome of positive polarity. Approximately 11,000 nucleotides of dengue virus genome encodes three structural proteins (Capsid, Membrane and Envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [3]. There are also RNA secondary structures at 5' and 3' ends represented by non-coding regions of 100–400 nucleotides respectively [15, 18].

Dengue infection was first documented in Pakistan in 1982 [7]. Since then many outbreaks have been reported in different parts of the country [1, 4, 8–11]. Among other serotypes, serotype 2 (DENV-2) of dengue was dominant in Pakistan during the years 2007–2009 [6]. In the year 2011, an outbreak of dengue was witnessed in Lahore city of Pakistan. There were more than 15,000 dengue cases with high mortality rate during this outbreak [19]. In 2013, another dengue outbreak was witnessed with 2165 dengue cases in Pakistan [25].

Few phylogenetic and epidemiological studies have assigned dengue viruses circulating in Pakistan with their respective types and subtypes using partial as well as complete genome sequences [6, 12, 13]. As serotype 2 is

Electronic supplementary material The online version of this article (doi:10.1007/s13337-015-0279-3) contains supplementary material, which is available to authorized users.

✉ Muhammad Idrees
idreeskhan@cemb.edu.pk

¹ Division of Molecular Virology, National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore 53700, Pakistan

² Centre for Applied Molecular Biology, University of the Punjab, 87-West 14 Canal Bank Road Thokar Niaz Baig, Lahore 53700, Pakistan

the predominant serotype in most of the outbreaks in Pakistan, therefore a detailed characterization of dengue virus serotype 2 circulating in Pakistan was needed. Study of amino acid substitutions across the entire genome of DENV-2 Pakistani isolates will provide an insight into genetic changes that have appeared over the course of time in locally circulating DENV-2 strains. This will be helpful in evolutionary study of this virus.

In the current study we were able to sequence entire genome of three patient derived DENV-2 viruses isolated from 2010, 2011 and 2013 outbreaks in Punjab Pakistan and characterize them in detail for amino acid substitutions.

Material and methods

Sample collection

DENV-2 strains used in the current study were obtained from suspected dengue patients during 2010, 2011 and 2013 dengue outbreaks in Pakistan. Serum samples from dengue infected patients were obtained from Molecular Epidemiology and Molecular Virology laboratory of Dengue Research Group, University of the Punjab Lahore Pakistan. The study was conducted in accordance with the 1964 Declaration of Helsinki and Guidelines for Good Clinical Research Practice in Pakistan. Institutional Review Board (IRB) of Post Graduate Medical Institute Lahore approved the studies. All the samples were DENV-2.

Primer designing

A set of 53 oligonucleotide primers was synthesized using the alignment of full length DENV-2 Sri-Lankan DENV-2/LK/BID-V2421/2003 strain (GQ252676), to amplify viral genome in overlapping fragments of varying sizes. List and sequences of primers is given in S1 Table.

RNA extraction and cDNA synthesis

Viral RNA was extracted by using a Nucleospin Viral RNA Extraction Kit (Macherey–Nagel, Germany) according to the manufacturer's instructions. A cDNA copy of a portion of the viral genome was produced in a reverse transcriptase reaction using M-MLV Reverse Transcriptase Enzyme (Invitrogen Biotechnologies USA) in the presence of anti-sense primer. The cDNA produced was then used for the nested PCR amplification of DENV specific regions using the type-specific primers and the DNA products were evaluated using 2 % agarose gel.

Sequencing

Amplified viral DNA was purified from gel and sequenced in both directions by using the ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Amplicons were sequenced from both strands to get consensus sequences. Reference sequences were obtained from GenBank/EMBL/DDBJ online nucleotide sequence databases.

Sequence analysis

Alignment was done using Clustal W multiple alignment tool. MEGA 5 programme was used for computing evolutionary analyses [23]. Phylogenetic tree was constructed by using statistical neighbor-joining (NJ) Method and Maximum Composite Likelihood model was used for evolutionary distances analysis [22]. Bootstrap value of 1000 was used as a test of phylogeny.

Results

Complete genome sequence

Complete genome sequences of three DENV-2 viruses isolated from Pakistan during 2010 (DENV-2/PK/2010), 2011 (DENV-2/PK/2011) and 2013 (DENV-2/PK/2013) were generated and submitted in GenBank under accession numbers, KF360005, KJ010185 and KJ010186 respectively. Figure 1 shows the amplified non structural gene 3 (NS3) of all three isolates.

The full length RNA genome of DENV-2/PK isolate was 10,629 ntd long which codes for 3391 amino acids with non coding regions of 64 nucleotides at N-terminal and 392 nucleotides at C-terminal. The genome size of

Fig. 1 PCR amplification of dengue virus serotype 2 genome fragment containing non structural gene 3 (NS3) of three Pakistani isolates

Fig. 2 Phylogenetic tree of full-length genome sequences of Pakistani DENV-2 isolates sequenced in this study and of 33 other DENV-2 strains obtained from GenBank databases. Complete genome sequences of DENV-1, 3 and 4 strains were also retrieved from GenBank and used to root the tree. Each strain is represented by

its accession number followed by country and year of isolation. Pakistani isolates sequenced in this study are marked. The phylogenetic analysis was conducted in MEGA5 programme using neighbour joining method. Bootstrap values are shown next to the branches

DENV-2 isolated in India was reported to be 10,670 nt long which codes for 3391 amino acids [5].

Evolutionary analysis

To study the evolutionary history of this virus a phylogenetic tree was constructed (Fig. 2). To construct this phylogenetic tree, total 27 DENV-2 complete genome nucleotide sequences from other parts of the world and 6 from Pakistan were downloaded and aligned with three DENV-2 complete genome sequences generated in this study. In addition, Serotype 1, 3 and 4 complete sequences were used to root the tree. Phylogenetic tree of DENV-2 grouped DENV-2 sequences in five genotypes as indicated in the tree. DENV-2 Pakistani isolates clustered together with DENV-2 cosmopolitan genotype (IV) sequences. DENV-2 genotype IV further divided into two distinct clades in which Pakistani isolates clustered together in a South Asian clade of genotype IV along with sequences from India (DQ448231.2), Sri Lanka (GQ252676.1, GQ252677.1) and China (AF359579.1). This study was in accordance with recent study on DENV-2 in India where Indian DENV-2 isolates grouped with three Sri Lankan and a Chinese isolate [5]. Pakistani isolates shared 96–99 % nucleotide similarity and 98–99 % amino acid similarity with India/2009 and Sri-Lanka/2003–2004 isolates. They shared Maximum homology with SriLankan 2003 and 2004 isolates (99 %). Nucleotide and amino acid percentage similarity was also analyzed between eight Pakistani isolates including three isolates sequenced in this study.

Nucleotide percentage similarity ranged between 96.4 and 99.9 % and amino acid percentage similarity ranged between 98.2 and 99.9 %. Most divergent sequence of them all was PK/2008 which shared 96.4–96.8 % nucleotide and 98.2–98.8 % amino acid similarity with rest of the Pakistani isolates.

Amino acid substitutions analysis

In order to study the sequence variations that have occurred over the years (2008–2013) across the genome of DENV-2 Pakistani isolates, all the complete amino acid sequences of Pakistan DENV-2 strains sequenced in this study PK/2010, PK/2011 and PK/2013 and Pakistan DENV-2 strains from previous years (PK/51/2008, PK/78/2009, PK/4/2011, PK/3/2011 and PK/23/2011) were aligned together with DENV-2 prototype strain New Guinea-C (NGC) sequence using Clustal W multiple alignment tool [24].

This alignment showed many amino acid substitutions scattered throughout the genome covering all the important regions (C, Prm, Env, NS1, NS2A, NS2B, NS3, NS4A/K, NS4B and NS5). Post 2008, amino acids were substituted at 15 sites (C = M104 V, L108 V, Prm = R130 K, Env = T506I, G640E, I602 V, Ns1 = T862S, Ns2A = M1243T, I1160 V, V1301A, Ns5 = A2687T, T2891A, S3122G, I3132 V, K3352R). These substitutions remained stable post 2008. Some of these amino acids were substituted by amino acids of different properties such as in Envelope region T506I, where a polar threonine is replaced by a hydrophobic Isoleucine. Similarly other sites (Env = G640E, NS2A = M1243T,

Table 1 Amino acid substitutions introduced in Pakistani DENV-2 isolates post 2008

No.	Region	Polyprotein position	Gene position	NGC	PK/2010	PK/2011	PK/2013	PK/51/2008	PK/78/2009	PK/209/2009	PK/4/2011	PK/3/2011	PK/23/2011
1	C	104	104	M	<i>V</i>	<i>V</i>	<i>V</i>	–	<i>V</i>	<i>V</i>	<i>V</i>	<i>V</i>	<i>V</i>
2	C	108	108	L	<i>R</i>	–	–	–	–	–	–	–	–
3	Prm	130	16	R	<i>K</i>	<i>K</i>	<i>K</i>	–	<i>K</i>	<i>K</i>	<i>K</i>	<i>K</i>	<i>K</i>
4	E	506	226	T	<i>I</i>	<i>I</i>	<i>I</i>	–	<i>I</i>	<i>I</i>	<i>I</i>	<i>I</i>	<i>I</i>
5	E	602	322	I	<i>V</i>	<i>V</i>	<i>V</i>	–	<i>V</i>	<i>V</i>	<i>V</i>	<i>V</i>	<i>V</i>
6	E	640	360	E	–	–	–	G	–	–	–	–	–
7	NS1	862	87	T	<i>S</i>	<i>S</i>	<i>S</i>	–	<i>S</i>	<i>S</i>	<i>S</i>	<i>S</i>	<i>S</i>
8	NS2A	1160	33	I	<i>V</i>	<i>V</i>	<i>V</i>	A	<i>V</i>	<i>V</i>	<i>V</i>	<i>V</i>	<i>V</i>
9	NS2A	1243	116	I	<i>T</i>	<i>T</i>	<i>T</i>	M	<i>T</i>	<i>T</i>	<i>T</i>	<i>T</i>	<i>T</i>
10	NS2A	1301	174	V	<i>A</i>	<i>A</i>	<i>A</i>	–	<i>A</i>	<i>A</i>	<i>A</i>	<i>A</i>	<i>A</i>
11	NS5	2687	196	A	<i>T</i>	<i>T</i>	<i>T</i>	–	<i>T</i>	<i>T</i>	<i>T</i>	<i>T</i>	<i>T</i>
12	NS5	2891	400	T	<i>A</i>	<i>A</i>	<i>A</i>	–	<i>A</i>	<i>A</i>	<i>A</i>	<i>A</i>	<i>A</i>
13	NS5	3122	631	S	<i>G</i>	<i>G</i>	<i>G</i>	–	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>
14	NS5	3132	641	I	<i>V</i>	<i>V</i>	<i>V</i>	–	<i>V</i>	<i>V</i>	<i>V</i>	<i>V</i>	<i>V</i>
15	NS5	3352	861	K	<i>R</i>	<i>R</i>	<i>R</i>	–	<i>R</i>	<i>R</i>	<i>R</i>	<i>R</i>	<i>R</i>

Sequences obtained in this study (italicized) are compared with other Pakistani DENV-2 strains and prototype DENV-2 strain (NGC). Polyprotein positions where amino acid are replaced by amino acid of different properties are highlighted

Table 2 Amino acid substitutions unique to DENV-2 PK/2010, PK/2011 and PK/2013 Pakistani isolates

Region	Poly protein position	Gene position	PK/2010	PK/2011	PK/2013	PK/51/2008	PK/78/2009	PK/209/2009	PK/4/2011	PK/3/2011	PK/23/2011
C	<i>108</i>	108	<i>R^c</i>	<i>L</i>	<i>L</i>	L	L	L	L	L	L
ENV	<i>344</i>	64	<i>K</i>	<i>K</i>	<i>R</i>	K	K	K	K	K	K
ENV	<i>378</i>	98	<i>G^c</i>	<i>D</i>	<i>D</i>	D	D	D	D	D	D
ENV	<i>380</i>	103	<i>D^b</i>	<i>N</i>	<i>N</i>	N	N	N	N	N	N
ENV	<i>522</i>	242	<i>N</i>	<i>N</i>	<i>K^b</i>	N	N	N	N	N	N
ENV	<i>540</i>	260	<i>L</i>	<i>M</i>	<i>M</i>	M	M	M	M	M	M
ENV	<i>557</i>	277	<i>L</i>	<i>P</i>	<i>L</i>	L	L	L	L	L	L
ENV	<i>604</i>	324	<i>I</i>	<i>I</i>	<i>V</i>	V	V	V	V	V	V
NS1	<i>784</i>	9	<i>R</i>	<i>K</i>	<i>K</i>	K	K	K	K	K	K
NS1	<i>786</i>	11	<i>K</i>	<i>K</i>	<i>T^b</i>	K	K	K	K	K	K
NS1	<i>787</i>	12	<i>E</i>	<i>E</i>	<i>A^c</i>	E	E	E	E	E	E
NS1	<i>796</i>	21	<i>I</i>	<i>F</i>	<i>I</i>	I	I	I	I	I	I
NS1	<i>835</i>	60	<i>V</i>	<i>I</i>	<i>V</i>	V	V	V	V	V	V
NS1	<i>839</i>	64	<i>E</i>	<i>D</i>	<i>E</i>	E	E	E	E	E	E
NS1	<i>872</i>	97	<i>M</i>	<i>M</i>	<i>L</i>	M	M	M	M	M	M
NS1	<i>903</i>	128	<i>P</i>	<i>P</i>	<i>S^a</i>	P	P	P	P	P	P
NS1	<i>917</i>	142	<i>E</i>	<i>Q^b</i>	<i>E</i>	E	E	E	E	E	E
NS2B	<i>1412</i>	67	<i>V</i>	<i>I</i>	<i>I</i>	I	I	I	I	I	I
NS3	<i>1480</i>	5	<i>S^a</i>	<i>W</i>	<i>W</i>	W	W	W	W	W	W
NS3	<i>1481</i>	6	<i>A^c</i>	<i>D</i>	<i>D</i>	D	D	D	D	D	D
NS3	<i>1483</i>	8	<i>P</i>	<i>P</i>	<i>R^c</i>	P	P	P	P	P	P
NS3	<i>1485</i>	10	<i>P</i>	<i>P</i>	<i>Y</i>	P	P	P	P	P	P
NS3	<i>1558</i>	83	<i>R^c</i>	<i>W</i>	<i>W</i>	W	W	W	W	W	W
NS3	<i>1609</i>	134	<i>A^a</i>	<i>T</i>	<i>T</i>	T	T	T	T	T	T
NS3	<i>2075</i>	600	<i>I</i>	<i>I</i>	<i>T^a</i>	I	I	I	I	I	I
NS4B	<i>2373</i>	130	<i>T</i>	<i>S</i>	<i>T</i>	T	T	T	T	T	T
NS4B	<i>2390</i>	147	<i>A</i>	<i>V</i>	<i>V</i>	V	V	V	V	V	V
NS4B	<i>2418</i>	170	<i>V</i>	<i>I</i>	<i>V</i>	V	V	V	V	V	V
NS4B	<i>2450</i>	208	<i>S</i>	<i>N</i>	<i>N</i>	N	N	N	N	N	N
NS5	<i>2508</i>	17	<i>L</i>	<i>V</i>	<i>L</i>	L	L	L	L	L	L
NS5	<i>2509</i>	18	<i>N</i>	<i>S</i>	<i>N</i>	N	N	N	N	N	N
NS5	<i>2592</i>	101	<i>R</i>	<i>K</i>	<i>K</i>	K	K	K	K	K	K
NS5	<i>2618</i>	127	<i>Q</i>	<i>L^a</i>	<i>Q</i>	Q	Q	Q	Q	Q	Q
NS5	<i>2619</i>	128	<i>S</i>	<i>T</i>	<i>S</i>	S	S	S	S	S	S
NS5	<i>2621</i>	130	<i>V</i>	<i>A</i>	<i>V</i>	V	V	V	V	V	V
NS5	<i>2687</i>	191	<i>T</i>	<i>T</i>	<i>T</i>	A	T	T	T	T	T
NS5	<i>2744</i>	253	<i>E</i>	<i>D</i>	<i>E</i>	E	E	E	E	E	E
NS5	<i>2783</i>	292	<i>W</i>	<i>R^c</i>	<i>W</i>	W	W	W	W	W	W
NS5	<i>2965</i>	474	<i>V</i>	<i>I</i>	<i>I</i>	I	I	I	I	I	I
NS5	<i>3091</i>	600	<i>S</i>	<i>G</i>	<i>G</i>	G	G	G	G	G	G
NS5	<i>3093</i>	602	<i>G</i>	<i>V</i>	<i>G</i>	G	G	G	G	G	G
NS5	<i>3101</i>	610	<i>N</i>	<i>G^a</i>	<i>N</i>	N	N	N	N	N	N
NS5	<i>3262</i>	771	<i>G^c</i>	<i>D</i>	<i>D</i>	D	D	D	D	D	D
NS5	<i>3334</i>	843	<i>G^c</i>	<i>E</i>	<i>E</i>	E	E	E	E	E	E
NS5	<i>3360</i>	869	<i>R^b</i>	<i>Q</i>	<i>Q</i>	Q	Q	Q	Q	Q	Q
NS5	<i>3369</i>	878	<i>G^c</i>	<i>E</i>	<i>E</i>	E	E	E	E	E	E
NS5	<i>3381</i>	890	<i>R</i>	<i>G^c</i>	<i>R</i>	R	R	R	R	R	R
NS5	<i>3385</i>	894	<i>D</i>	<i>E</i>	<i>E</i>	E	E	E	E	E	E

Pakistani isolates sequenced in this study are italicized. Amino acid substitutions unique to PK/2011, PK/2011 and PK/2013 are highlighted

^a Hydrophobic to polar switching and vice versa

^b Uncharged to charged switching and vice versa

^c Both a, b

I1160 V, NS5 = A2687T, T2891A, S3122G and K3352R) were also subjected to polar to hydrophobic and hydrophobic to polar amino acids switching (Table 1).

A total of 21 Amino acids substitutions unique to PK/2010 were confined in Capsid, Env, NS2B, NS3, NS4B and NS5 regions. Out of these 21 sites, hydrophilic to hydrophobic and neutral to charged switching was positive at 11 sites (C = L108R, Env = D378G, N380D, Ns3 = W1480S, D1481A, W1558R, T1609A, Ns5 = D3262G, E3334G, Q3360R, E3369G). 18 unique sites were present in PK/2011 where amino acids were replaced. Out of these 18 substitutions, one (E917Q) and four (Q2618L, W2783R, N3101G, R3381G) sites in NS1 and NS5 regions respectively were subjected to changes in hydrophobicity, polarity and charge. In most recent DENV-2 sample, PK/2013, total 9 amino acid substitutions were observed, 6 of them (Env = N522 K, NS1 = K786T, E787A, P903S, NS3 = P1483R, I2075T) were subjected to changes in hydrophobicity, polarity and charge (Table 2).

Discussion

Dengue outbreaks in Pakistan in the year 2011 and thereafter were the main dengue events in Punjab province of Pakistan where more than 15,000 dengue cases were recorded with a high mortality rate [19]. The dengue serotype 2 dominated all the major outbreaks in Pakistan including 2011 and post 2011 outbreaks. The main objective of this study was to identify amino acid substitutions that have appeared in the locally circulating dengue serotype 2 virus over the course of time.

The analysis of full length nucleotide sequences of three Pakistani DENV-2 strains isolated from 2010, 2011 and 2013 outbreaks revealed that DENV-2 strain circulating in Pakistan during these years is most closely related to Sri-Lankan 2003 and 2004 isolates in South Asian clade of Cosmopolitan genotype (IV). This result is in accordance with other studies from this region [12, 13].

DENV-2 Complete genome sequences of Pakistani strains were aligned with DENV-2 prototype NGC, to scan the sites of amino acid substitutions. Post 2008 many amino acid substitutions were observed. Few were in Envelope region of dengue genome which is a major antigenic region exposed on the surface of dengue virus. Envelope protein includes three distinct domains (I, II and III) [16]. Most of the antigenic sites are located in domain III of Envelope gene [21]. One of the amino acid substitutions in Envelope region (G640E), unique to DENV-2 2008 (PK/51/2008) strain was observed where a hydrophobic, neutral Glycine amino acid was replaced by a polar, negatively charged glutamic acid. This substitution remained consistent in all Pakistani isolates post 2008. This

amino acid is localized in C-terminal of domain III of Envelope gene and is therefore a possible determinant of antigenicity and virulence. Apart from it most of the amino acid substitutions in Envelope region were observed outside Domain III, in discontinuous Domain I and Domain II of Envelope, which indicate that these substitutions were not affecting antigenicity or virulence of the virus. Another amino acid substitution NS2A, (M1243T), was also consistent post 2008. At this site a hydrophobic amino acid was replaced by a polar, surfaced amino acid. This amino acid site is located in the transmembrane region, which is important for virus replication [14]. This substitution therefore might affect virus assembly and RNA synthesis.

Over the years (2008–2013), this locally circulating strain of DENV-2 virus has undergone several amino acid substitutions. A number of amino acid substitutions that were unique to 2010, 2011 and most recent 2013 strains sequenced in this study were observed. Few among them were significant with changes in physiochemical properties. It has been said that such small changes in genetic makeup of a virus can bring about a lineage or virus subtype change resulting in a more virulent virus which can cause a bigger epidemic in future [17, 20].

We can therefore infer that either a single subtype of DENV-2 virus has been circulating from 2008 to 2013 and has undergone changes in its genetic makeup over the time or there were multiple introductions of the virus in this region as suggested by different points of origin in the phylogenetic tree. Therefore a much detailed analysis of DENV-2 origin and evolution in Pakistan is needed.

Acknowledgments We are thankful to all the clinicians/consultants/doctors from various clinics and hospitals that provided us with serum samples for laboratory confirmation of dengue infection.

References

1. Akram DS, Igarashi A, Takasu T. Dengue virus infection among children with undifferentiated fever in Karachi. *Indian J Pediatr*. 1998;65(5):735–40.
2. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. *Nature*. 2013;496(7446):504–7. doi:10.1038/nature12060.
3. Chambers TJ, Hahn CS, Galler R, Rice CM. Flavivirus genome organization, expression, and replication. *Annu Rev Microbiol*. 1990;44:649–88. doi:10.1146/annurev.mi.44.100190.003245.
4. Chan YC, Salahuddin NI, Khan J, Tan HC, Seah CL, Li J, et al. Dengue haemorrhagic fever outbreak in Karachi, Pakistan, 1994. *Trans R Soc Trop Med Hyg*. 1995;89(6):619–20.
5. Dash PK, Sharma S, Soni M, Agarwal A, Parida M, Rao PV. Complete genome sequencing and evolutionary analysis of Indian isolates of dengue virus type 2. *Biochem Biophys Res Commun*. 2013;436(3):478–85. doi:10.1016/j.bbrc.2013.05.130.
6. Fatima Z, Idrees M, Bajwa MA, Tahir Z, Ullah O, Zia MQ, et al. Serotype and genotype analysis of dengue virus by sequencing

- followed by phylogenetic analysis using samples from three mini outbreaks-2007–2009 in Pakistan. *BMC Microbiol.* 2011;11:200. doi:[10.1186/1471-2180-11-200](https://doi.org/10.1186/1471-2180-11-200).
7. Hayes CG, Baqar S, Ahmed T, Chowdhry MA, Reisen WK. West Nile virus in Pakistan. 1. Sero-epidemiological studies in Punjab Province. *Trans R Soc Trop Med Hyg.* 1982;76(4):431–6.
 8. Humayoun MA, Waseem T, Jawa AA, Hashmi MS, Akram J. Multiple dengue serotypes and high frequency of dengue hemorrhagic fever at two tertiary care hospitals in Lahore during the 2008 dengue virus outbreak in Punjab, Pakistan. *Int J Infect Dis.* 2010;14(Suppl 3):e54–9. doi:[10.1016/j.ijid.2009.10.008](https://doi.org/10.1016/j.ijid.2009.10.008).
 9. Jamil B, Hasan R, Zafar A, Bewley K, Chamberlain J, Mioulet V, et al. Dengue virus serotype 3, Karachi, Pakistan. *Emerg Infect Dis.* 2007;13(1):182–3. doi:[10.3201/eid1301.060819](https://doi.org/10.3201/eid1301.060819).
 10. Khan E, Siddiqui J, Shakoor S, Mehraj V, Jamil B, Hasan R. Dengue outbreak in Karachi, Pakistan, 2006: experience at a tertiary care center. *Trans R Soc Trop Med Hyg.* 2007;101(11):1114–9. doi:[10.1016/j.trstmh.2007.06.016](https://doi.org/10.1016/j.trstmh.2007.06.016).
 11. Khan E, Hasan R, Mehraj V, Nasir A, Siddiqui J, Hewson R. Co-circulations of two genotypes of dengue virus in 2006 outbreak of dengue hemorrhagic fever in Karachi, Pakistan. *J Clin Virol.* 2008;43(2):176–9. doi:[10.1016/j.jcv.2008.06.003](https://doi.org/10.1016/j.jcv.2008.06.003).
 12. Khan MA, Ellis EM, Tissera HA, Alvi MY, Rahman FF, Masud F, et al. Emergence and diversification of dengue 2 cosmopolitan genotype in Pakistan, 2011. *PLoS ONE.* 2013;8(3):e56391. doi:[10.1371/journal.pone.0056391](https://doi.org/10.1371/journal.pone.0056391).
 13. Koo C, Nasir A, Hapuarachchi HC, Lee KS, Hasan Z, Ng LC, et al. Evolution and heterogeneity of multiple serotypes of dengue virus in Pakistan, 2006–2011. *Virol J.* 2013;10:275. doi:[10.1186/1743-422X-10-275](https://doi.org/10.1186/1743-422X-10-275).
 14. Kummerer BM, Rice CM. Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. *J Virol.* 2002;76(10):4773–84.
 15. Markoff L. 5'- and 3'-noncoding regions in flavivirus RNA. *Adv Virus Res.* 2003;59:177–228.
 16. Modis Y, Ogata S, Clements D, Harrison SC. A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc Natl Acad Sci USA.* 2003;100(12):6986–91. doi:[10.1073/pnas.0832193100](https://doi.org/10.1073/pnas.0832193100).
 17. OhAinle M, Balmaseda A, Macalalad AR, Tellez Y, Zody MC, Saborio S, et al. Dynamics of dengue disease severity determined by the interplay between viral genetics and serotype-specific immunity. *Sci Transl Med.* 2011;3(114):114ra28. doi:[10.1126/scitranslmed.3003084](https://doi.org/10.1126/scitranslmed.3003084).
 18. Proutski V, Gritsun TS, Gould EA, Holmes EC. Biological consequences of deletions within the 3'-untranslated region of flaviviruses may be due to rearrangements of RNA secondary structure. *Virus Res.* 1999;64(2):107–23.
 19. Rai MA. Epidemic: control of dengue fever in Pakistan. *Nature.* 2011;479(7371):41. doi:[10.1038/479041d](https://doi.org/10.1038/479041d).
 20. Steel A, Gubler DJ, Bennett SN. Natural attenuation of dengue virus type-2 after a series of island outbreaks: a retrospective phylogenetic study of events in the South Pacific three decades ago. *Virology.* 2010;405(2):505–12. doi:[10.1016/j.virol.2010.05.033](https://doi.org/10.1016/j.virol.2010.05.033).
 21. Sukupolvi-Petty S, Austin SK, Purtha WE, Oliphant T, Nybakken GE, Schlesinger JJ, et al. Type- and subcomplex-specific neutralizing antibodies against domain III of dengue virus type 2 envelope protein recognize adjacent epitopes. *J Virol.* 2007;81(23):12816–26. doi:[10.1128/JVI.00432-07](https://doi.org/10.1128/JVI.00432-07).
 22. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci USA.* 2004;101(30):11030–5. doi:[10.1073/pnas.0404206101](https://doi.org/10.1073/pnas.0404206101).
 23. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol.* 2011;28(10):2731–9. doi:[10.1093/molbev/msr121](https://doi.org/10.1093/molbev/msr121).
 24. Thompson JD, Gibson TJ, Higgins DG. Multiple sequence alignment using ClustalW and ClustalX. *Current protocols in bioinformatics/editorial board, Andreas D Baxevanis [et al].* 2002; Chapter 2: Unit 2.3. doi:[10.1002/0471250953.bi0203s00](https://doi.org/10.1002/0471250953.bi0203s00).
 25. Wasim A, Kazi AN, Hussain SA. 2013: rise of the deadly dengue fever in Pakistan. *J Infect Public Health.* 2014;7(5):453–4. doi:[10.1016/j.jiph.2014.05.001](https://doi.org/10.1016/j.jiph.2014.05.001).