

# Identification of concurrent infection by multiple dengue virus serotypes during an epidemic in 2011 in Pakistan

## Main text

Dengue Virus belongs to the genus *flavivirus* and family *flaviviridae*, with single stranded positive sense RNA genome is a major public health problem in South East Asia. There are four different serotypes of dengue virus DENV1-4.<sup>1</sup> The infection caused by only one serotype is usually a mild and self limiting. The more severe form of dengue infection is dengue hemorrhagic fever DHF and dengue shock syndrome DSS. The DHF and DSS responsible for high morbidity and mortality rate.<sup>2</sup> Dengue infection is transmitted by mosquito and the *Aedes aegypti* is a principal vector and the *Aedes albopictus* also transmit the virus. The *Aedes aegypti* is day biting mosquito and breed in artificial as well as the natural water.<sup>3</sup> More than 100 million cases of dengue virus infection with 25000 estimated deaths have been reported annually. More than 100 countries are at risk of dengue virus infection.<sup>4</sup> Pakistan is a dengue endemic country from many years and many outbreak of dengue virus has been reported previously. In Pakistan for the first time dengue virus infection was detected in 1982 from a serum sample collected in 1968 and 1978 from Punjab Province.<sup>5</sup>

In Pakistan first outbreak of dengue Hemorrhagic fever was detected in 1994 from Karachi caused by dengue virus serotypes DENV-1 and DENV-2.<sup>6</sup> In 1994 the first lab confirmed cases of DENV-2 was reported from Baluchistan Province.<sup>7</sup> The prevalence of dengue virus serotype 3 in Pakistan was detected during the 2005 dengue virus outbreak in Karachi.<sup>8</sup> The co-circulation of DENV-2 and DENV-3 was identified in 2006 during the outbreak of dengue virus in Karachi.<sup>9</sup> In 2008 the co-circulation of three serotypes DENV-2, DENV-3 and DENV-4 was also documented during the outbreak of dengue virus in Lahore.<sup>10</sup> Recently it is reported that the dominant serotypes of dengue virus circulating in Pakistan from 2007-2009 are DENV-2 and DENV-3. The dengue is a major public health problem in Pakistan over the last 20-25 years. In last few years co-circulation of different dengue virus serotypes is identified with mixed infection. From Pakistan only one study in which they reported some cases of mixed infection with DENV-2 and DENV-3. In present study we for the first time reported many cases of mixed infection with DENV-1/ DENV-2, DENV-1, DENV-2 and DENV-3 from different geographic areas of Pakistan. The laboratory detection of dengue virus infection is achieved by virus isolation in cell culture, Serologic diagnosis by detection of IgM/IgG antibodies and more sensitive and rapid technique is the Molecular detection. From August to October 2011 a total of 87 serum samples were received at the department of virology National Institute of Health Islamabad Pakistan during an outbreak. These samples were collected from suspected dengue virus cases along the complete history sheet including the date of onset, age; sex etc. All serum samples were stored at -70 until further processing.

All serum samples were analyzed for the detection of dengue virus IgM antibodies by using IgM capture ELISA (Panbio Tech Co Brisbane, Australia) according to the instructions provided by the

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manufacturer. RNA was extracted from 140ul of ELISA Positive serum samples by using QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to manufacturer instructions. In the present study RNA samples were analyzed by the molecular detection by using the real time PCR assay. Real time PCR is a one step assay using Primer pair and Probes specific to dengue serotypes. In single plex reaction mixture 5ul of RNA template was added with 50pmol of each primer and 9pmol of probe in 50ul total iScript one-step RT-PCR kit for Probes. Each reaction tube contains primers and probe for only one dengue serotype. In this assay one RNA sample was tested against four dengue serotypes in four separate reactions tubes.<sup>11</sup> In this technique the use of fluorescent probe enables the detection of PCR reaction product without need of gel electrophoresis. Another advantage of this assay is the ability to determine viral load in the patient sample which is important to understand the severity of dengue infection.<sup>12</sup>

A total of 87 serum samples from suspected dengue virus infection along with the history sheets were received from the different districts of Punjab, KPK and Azad Kashmir were analyzed for the present study. The study was conducted at the department of virology National Institute of Health Islamabad Pakistan. The Average age of the patients was 30 year (19-66), 80% of cases were Male and 20% were Female. The samples were received in the month of July, August and September 2011 during the outbreak of Dengue virus. All the serum samples were tested by ELISA for the detection of dengue virus IgM antibodies. Out of 87, 49(27.40%) were IgM Positive against dengue virus. All IgM positive and IgM negative samples were tested by Real-time PCR for the detection of dengue virus RNA. Out of 87 the dengue virus RNA was detected from 63(70%) samples. According to the case investigation forms many samples were collected after the 10 to 15 days of onset. Dengue serotype 1 was detected from 17(27%) samples and DENV-2 was identified from 35(55.5%) samples. From 11(27%) samples out of 63 the co-infection cause by multiple dengue serotypes was identified. From 8(81%) samples co-infection caused

by dengue serotype 1 and 2 and from 3 (27%) samples co-infection of dengue serotypes 1, 2 and 3 were identified. Four cases with dengue 1, 2 and only one case with dengue 1, 2 and 3 have bleeding and low platelets count. Dengue virus infection is a major arboviral infection in many regions of the world which supports the breeding of mosquitoes.

Pakistan is a dengue endemic country and had experienced many severe outbreaks since 1994.<sup>6</sup> Some published reports on dengue has been shown that the dengue virus infection in Pakistan has been documented from 1968 and 1978.<sup>5</sup> According to the published data all four serotypes of dengue virus DENV1-4 are prevalent in Pakistan. Dengue virus serotypes 1 and 2 were reported as predominant serotypes in outbreaks of 2007-2009 in Pakistan. If we overview the published data on dengue. Outbreaks in our country an increase in severity and incidence of the disease has been observed. In 2006 the co-circulation of DENV-2 and DENV-3 has been reported during the outbreak in Karachi Pakistan. According to a recently published study dengue virus serotypes 2, 3 and 4 were co-circulating during the outbreak in 2008 in Pakistan.<sup>10</sup>

The results of another study from Pakistan showed that all four serotypes of dengue virus were co-circulating during the outbreak in 2008 in Lahore.<sup>13</sup> For the first time the co-infection caused by multiple serotypes of dengue virus was identified from Puerto Rico in 1982.<sup>14</sup> India reported high percentage (19%) of co-infection during an outbreak of dengue virus in Delhi in 2006. Different percentages of co-infection of dengue serotypes has been reported, Taiwan (9.5%) Indonesia (11%) Mexico Puerto Rico (5.5%).<sup>15</sup> In the present study we are documenting the highest percentage (27%) co-infection caused by multiple serotypes of dengue virus during a huge outbreak in 2011 in Pakistan as compared to the previous studies. More than 20,000 dengue virus confirmed cases along with 300 deaths has been reported during outbreak in 2011 in Pakistan and this outbreak is still continue. A very little information regarding co-infection caused by dengue serotypes has been provided in a recently published study from Pakistan,<sup>16</sup> on the bases of this information the present study was conducted and high percentage of co-infection is identified from the samples collected from the huge outbreak in 2011. Results of our study indicated the simultaneous circulation of different serotypes of dengue virus in different geographical areas of the country. In present study three cases had mixed infection with DENV-1, DENV-2 and DENV-3. The mixed infection caused by same three serotypes has already been reported in Thai Children.<sup>17</sup> The same combination of dengue serotypes has also been detected from a patient in India.<sup>18</sup> The mixed infection caused by DENV-1 and DENV-2 has been detected in many patients from 1998-2003 in Brazil.<sup>19,20</sup> The co-infection with dengue serotype DENV-2 and DENV-3 has been first time documented in China from a patient who have history of travel to Sri Lanka during 2004.<sup>21</sup> The concurrent infection with dengue virus serotype 1 and 2 was detected in four patients by RT-PCR assay in 2008-2009 in Netherland.<sup>22</sup> This is the first documented report of mixed infection with DENV-1/DENV-2 and DENV-1/DENV-2/DENV-3 from Pakistan. The huge numbers of mixed infections with different dengue virus serotypes can be occur in our country where different serotypes have been co-circulating from many Years.

The mixed infection with dengue virus serotypes in human and mosquitoes provide an opportunity for the recombination of viral genome and completely new strain of the virus can be produced which

could change the clinical picture of the disease and make it more severe as well as increase the epidemic potential of Dengue virus. The co-circulation of different dengue serotypes is a major factor responsible for the emergence of severe form of the dengue virus infection like DHF and DSS due to co-infection. There are many possibilities for the mixed infection it may be resulted due to the repeated mosquito feeding or it may be occur due to traveling/migration from the area where one serotype is prevalent to the area where another serotype is circulating. In coming years, close monitoring and, molecular characterization of the dengue virus must be conducted to understand the clinical spectrum and genetics of the dengue virus in the country like Pakistan in which multiple serotypes of dengue virus are prevalent. The development of dengue hemorrhagic fever and dengue shock syndrome is still a subject of debate, but the most reliable and strongest experimental evidence leads to the conclusion that an intense immune response against dengue virus promotes a series of events that lead to the leakage of plasma from blood capillaries. Co-circulation of multiple serotypes can increase the risk for dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).<sup>23</sup>

In Pakistan there is no proper epidemiological/ Field based surveillance system has been established up till now, so there is a hospital based surveillance system working for dengue infection with the help of WHO. Some time the WHO surveillance officer working in the field for the Polio and Measles play role in the dengue surveillance. Our study showed that the Male were infected more as compared to the female because the males go out for their jobs and female mostly stay indoors. The age group infected mostly is in between the age of 06-66 years majority of them are the male patients. As for as the history of dengue vector in Pakistan is concerned the *Aedes aegypti* is the most prevalent vector in urban areas like Karachi, Lahore, Rawalpindi, attock and it also found up to haripur district but in Hilly areas like abbotabad, Mansehra, Kotli AJK the *Aedes albopictus* has also been found during dengue season. Results of present study also showed that during the outbreak of dengue in 2011 in Pakistan many cases showed the mixed infection which may be the reason of high mortality as compared to the previous dengue outbreaks in the country.

The dengue virus is endemic in Pakistan and different dengue virus serotypes found to be circulating throughout the year. In dengue endemic areas the increase in temperature not only favorable for the survival of the mosquitoes but it also plays a role in its replication and maturation as well. In Pakistan an overall increased in temperature has been observed in different parts of the country which provide suitable environment for the survival of dengue vectors, and in winter these vectors migrate into the areas previously free of disease and population of such areas are susceptible to any new infection. The climate of Pakistan is more suitable for the growth of dengue vector including other mosquito's species. The dengue infection mostly has been seen during the month of august–November in different areas of the country. In Pakistan different factors are involved in the spread of dengue epidemics. The most important is the favorable climate for mosquitoes breeding especially during the monsoon season in which the hot and humid conditions available for mosquitoes breeding. In Pakistan others factors like increase in vector population, susceptible human population, and poor urban planning, overcrowding, poor socioeconomic conditions, lack of public health infrastructure also play a vital role in disease outbreaks/epidemics (Table 1).

**Table 1** Multiple dengue serotypes infection detected in patients during the 2011 epidemic in Pakistan

Dengue Virus Real Time PCR Results							
S- No	Lab No	Age/Sex	Province	District	Symptoms	IgM ELISA results	Real-time PCR results
1	548	35/M	ICT	Islamabad	DHF	Positive	D1+D2
2	552	40/M	ICT	Islamabad	DF	Positive	D1+D2
3	567	36/M	Punjab	Lahore	DF	Positive	D1+D2+D3
4	1600	66/M	AJK	Kotli	Unknown	Positive	D1+D2
5	1604	19/F	KPK	Haripur	DHF	Positive	D1+D2
6	1605	10/F	KPK	Haripur	DF	Positive	D1+D2
7	1606	54/M	KPK	Mansehra	DHF	Positive	D1+D2
8	1633	37/M	Punjab	Rawalpindi	DF	Positive	D1+D2
9	1650	22/F	Punjab	Rawalpindi	DHF	Positive	D1+D2
10	1698	40/M	KPK	Abbottabad	DF	Positive	D1+D2+D3
11	1702	20/M	KPK	Abbottabad	DHF	Positive	D1+D2+D3

DF, dengue fever; DHF, dengue hemorrhagic fever

## Conclusion

The results of our study indicated that the multiple serotypes of dengue virus are co-circulating in different geographic areas of Pakistan in which dengue virus serotype 2 was dominant serotype. The incidence of mixed infection with multiple serotypes, could increase the severity of the disease during the future outbreaks in Pakistan. It has been concluded that the co-infection caused by multiple dengue virus serotypes was consider a factor for the emergence of dengue Hemorrhagic fever and dengue shock syndrome as we observed during the dengue outbreak in 2011 in Pakistan.

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## Conflicts of interest

Authors declare that there is no conflict of interest.

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