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Short Communication

Comparative Analysis of Immunological and Genomic Outcomes of Dengue Virus Outbreak in Pakistan

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ZF and MI conceived of the study and helped others in technicalities. MS, IA, SA, ZF and LA wrote the manuscript and carried out the experiments and calculated the results.

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ABSTRACT

The mortality and morbidity due to dengue fever is increasing worldwide at a frightful rate. Emergence and re-emergence of dengue fever in previous epidemics has been documented globally, including Pakistan. We conducted a comprehensive study and compared the immunological and genomic status of dengue outbreak in Pakistan through ELISA and PCR. In this study 720 samples were collected from different diagnostic centre's/laboratories of Pakistan for ELISA and PCR tests. Out of 703 dengue positive samples, 93.5% were anti-dengue IgM positive, 71.5% were anti-dengue IgG positive and 81.3% were anti-dengue NS1 positive. Among these seropositive samples, 39.66%, 40.15% and 36.88% were PCR positives, respectively. This immunological and genomic study reveals that there were more seropositive patients than RNA positive ones. Serotype-2 remained the main causative serotype of the outbreak of dengue viral infection, started in the commencement of year 2013 and continues to infect Pakistani population till now.

In terms of morbidity and mortality, dengue fever is a major devastating threat to human health worldwide. The frequency distribution of dengue viral infection has grown dramatically around the world in recent decades, especially in Pakistan. Many of its cases are misclassified as well as the actual number of them may remain ill-reported. Recently, it is estimate that around 390 million dengue infections occur per year (95% credible interval 284–528 million), of which 96 million (67–136 million) are symptomatic infections (Bhatt *et al*., 2013). Another estimate is that 3.9 billion people in 128 countries are at risk of dengue infection (Brady *et al*., 2012). Together with other countries, Pakistan has suffered an increased burden of this infection in last two decades (Fatima *et al*., 2011, 2013; Akram *et al*., 2015). It becomes important to understand the trend of this infection in order to understand virus genetic evolution, spread, disease consequences and control of dengue infection in this region.

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Currently both the methods of polymerase chain reaction (PCR) and Enzyme Linked Immune Sorbent Assay (ELISA) are used to detect positivity by dengue virus in a patient (Qureshi *et al*., 2019). PCR is considered as most sensitive and reliable method for the detection of different serotypes of Dengue virus. The major advantage of PCR assay is the ability to detect dengue RNA in seropositive individuals up to 10 days of post infection (Gurukumar *et al*., 2009). Immunoglobulin tests by ELISA are used for the measurement of different immunoglobulins/antibodies like IgM and IgG in the blood. These antibodies fight against different antigens like viruses, bacteria or some toxic materials. Human IgM and IgG ELISA detection are commonly used for presence of dengue IgM/IgG antibodies during an acute phase of infection from 3-7 days. However, IgM antibodies may stay positive for about 6 months or so. If IgM is found negative or low and on the other hand IgG ELISA is positive, it indicates that the patient had a previous history of infection. Since the immunoglobins circulate even after the virus is cleared from blood, it can give false positive results through ELISA. Presence of anti-dengue IgM may not always

confirm a present infection because they can be detected in blood till 2-3 months post-infection (Marinho *et al*., 2017).

Another assay known as NS1 antigen test (non-structural protein 1) by ELISA permits an early detection of Dengue infection even on the first day of fever and up to day 9. NS1 is highly conserved glycoprotein for all dengue serotypes and it is detectable in the presence of IgM antibodies and even when RT-PCR shows negative results (Anand *et al*., 2016). Even before the appearance of antibodies which can be detectable in blood normally at about day 5 of infection or later, NS1 can be detectable earlier.

Although several dengue viral outbreaks has been reported from different towns, cities and provinces of Pakistan in the last 20 years but the current study was designed to study both the immunological and genomic status and map their difference in dengue outbreak of 2013.

Materials and methods

A total of 720 suspected blood serum samples were collected along with information on demographic characteristics, dengue specific symptoms, area, hematological and biochemical features and duration of onset of disease from commencement of September till end of December 2013 at Division of Molecular Virology, CEMB, University of the Punjab Lahore for the molecular characterization and detection of different serotypes of dengue virus from Pakistan. An informed consent was taken from all hospitals and enrolled patients to conduct the study in accordance with the Declaration of Helsinki, 1964 and Directions for Virtuous Medical Research Practice in Pakistan.

All of the suspected patients having characteristic dengue symptoms were screened for the existence of dengue specific NS1, IgG and IgM antibodies according to the manufacturer code of practice. 100 µl of samples was used to detect dengue IgG and IgM using kits from Calbiotech USA according to manufacture's protocol. Both positive and negative controls were run alongside the samples.

Viral nuclear RNA was isolated from 150 µl of blood serum sample and dengue virus was confirmed by RT-PCR as described earlier (Fatima *et al*., 2013). cDNA was synthesized using Moloney Murine Leukiemia Virus (MMLV) Reverse Transcriptase (RTse) (Invitrogen Biotechnology, USA). Dengue detection by using nested PCR procedure as developed by our laboratory (Fatima *et al*., 2011) was done and the oligonucleotide sequences used to amplify C-prM gene junction of dengue virus were 5'TCAATATGCTGAAACGCGWGAGAAACCG3'and 5'TTGCACCARCARTCWATGTCTTCWGGYTC3'. After performing 30 cycles of PCR amplification, product was run at 2% agarose gel stained with ethidium bromide and visualized under UV light. The size of amplified PCR product was 511bp.For further serotyping analysis of Dengue the primers and procedure was followed as mentioned earlier (Fatima *et al*., 2011). SPSS V.17 software was used for Statistical analysis and all data were presented as number of subjects and percentage. P-value was adjusted at 0.05.

Results

Out of our 720 collected samples, 703 were positive for dengue virus through ELISA and/or PCR detection methods and were further used for our study. We evaluated the incidence of infectivity in both males and females, 70% of samples were obtained from males and 30% from females, suggesting a higher incidence in males than females. The higher number of samples from young patients suggests a greater susceptibility to infection in 16-35 years old age group (Table I), whereas, the two parameters (infectivity and age/gender) were not correlated. Age did not tend to have any specific correlation with IgM positivity (p=0.55), IgG positivity (p=0.23) and NS1 positivity (p=0.89). Similarly, gender could not be associated with IgM positivity (p=0.49), IgG positivity (p=0.89) and NS1 positivity (p=0.72).

Out of 703 positive samples, 658 (93.5%) were positive to IgM and only 261 (39.66%) RNA positives were identified by PCR. In these 261 PCR positive subjects, 2 (0.76%), 145 (55.5%) and 42 (16%) were infected with any one dengue serotype- 1, 2 and 3, respectively. On the other hand 72 (27.58%) IgM positive patients had concurrent infection with serotypes 2 and 3.

Five hundred and three (71.55%) samples were identified dengue positive through ELISA in which 202 (40.1%) were dengue virus RNA positive. From these PCR positive samples 112 (55.4%) and 34 (16.8%) had infections of either serotype 2 or 3, respectively. Only 56 (27.72) IgG positive subjects had concurrent infection with both serotypes 2 and 3.

From 703 studied serum samples 572 (81.7%) exhibited NS1antigen and 211 (36.8%) of these NS1 positive subjects had RNA positivity by PCR. Out of the 211 PCR positive subjects 2 (0.74%), 122 (57.8%) and 37 (17.5%) had serotype 1, 2 and 3 infections, respectively, but 50 (23.69%) NS1 positive subjects had concurrent viral infection with both serotypes 2 and 3 (Table I). The 2:1 male: female ratio in studied population remains the same as already reported (Fatima *et al*., 2013). As 39.66% of anti-dengue IgM positive samples were dengue viral RNA positive, so these results confirm a current infection because viral RNA can be isolated from day one of post-infection till 7-8 days (Marinho *et al*., 2017).

Table I.- Comparison of immunological and genomic results from 2013 outbreak in Pakistan.

Parameters	No. of positive samples	No. of IgM positive	No. of RT-PCR positive*	No. of IgG positive	No. of RT-PCR positive*	No. of NS1 positive	No. of RT-PCR positive*
No. of participants	703	658 (93.5%)	261 (39.66%)	503 (71.55%)	202 (40.15%)	572 (81.3%)	211 (36.88%)
Gender							
Male	489 (69.6%)	462	186	353	144	397	146
Female	214 (30.4%)	196	75	150	58	175	65
City							
Rawalpindi	446	446	183	446	183	437	178
Lahore	241	196	66	45	8	131	31
Faisalabad	2	2	1	1	0	1	1
Multan	3	3	0	0	0	2	0
Swat	11	11	11	11	11	1	1
Age in years							
0-15	82	68	22	55	14	63	20
16-25	210	200	96	148	79	174	78
26-35	228	218	92	168	75	189	75
36-45	97	94	28	71	19	77	20
46-55	31	28	7	21	5	25	7
56-65	21	20	7	16	4	16	3
65-100	34	30	9	24	6	28	8

*RT-PCR positive samples in given group.

Discussion

Some of the important findings and limitations of this study are described here. Firstly, anti-dengue IgM was performed, IgM may stay in blood of patient till 2-3 months after infection and the antigens used for anti-dengue IgM ELISA are derived from the envelope protein of the virus. One of the limitation of this test is the cross reactivity between other circulating flaviviruses. This limitation must be considered when working in regions where multiple RNA viruses co-circulate. Anti-dengue IgM detection may not be 100% useful for dengue serotype determination due to cross-reactivity of the antibody. The result with negative anti-dengue IgM and positive with other methods may be due to compromised or low sensitivity of the ELISA based test which was used to perform IgM detections. According to the Pan American Health Organization (PAHO) guidelines 80% of all dengue cases have detectable IgM antibody by day five of illness, and 93-99% of cases have detectable IgM by day six to ten of illness, which may then remain detectable for over 90 days (Marinho *et al*., 2017).

Secondly, we have performed anti-dengue IgG ELISA on our collected samples. This information of anti-dengue IgG can be used to detect primary and secondary infections by using simple algorithms (Marinho *et al*., 2017). For this early sampling must be done in acute and convalescent phases, but under the settings of our society it may become difficult to collect samples twice because most of the patients, if not admitted to the hospital, do not prefer to give second blood samples. As dengue fever can present a range of symptoms so patients take painkillers to relieve themselves of the pain till the symptoms are gone.

Thirdly, the use of anti-dengue NS1 is considered specific and reliable in identifying current infections as NS1 levels can be detected from day one of post infection with specificity among different flaviviruses. According to CDC laboratory protocol the PCR can give positive results up to 5 days post infection and NS1 can give positive results up to 18 days post infection. So the difference noted in our studied samples may be due to the difference in post infection days at the time of testing (Marinho *et al*., 2017).

The present study suggests that many of the infections were few months previous or not current ones. As dengue fever has a range of symptoms so during an epidemic, few of the symptoms if not due to dengue virus, may lead the doctors to checking for dengue virus detection. The treatment of dengue fever is prophylactic so all of the medical care depends upon laboratory diagnosis *i.e.* ELISA or PCR detection tests.

Another important finding of this study was that the serotype-2 remains the main causative serotype of this outbreak of dengue fever as reported in the previous

outbreaks in Pakistan (Shahid *et al*., 2017).

Conclusion

After performing IgM, IgG antibodies, NS1 and PCR detection, this immunological and genomic study reveals that there are more seropositive patients than RNA positive ones during the study. So we concluded that the detection of RNA through PCR, and NS1 detection are considered more sensitive and specific methods for current infection of dengue virus. Hence they should be incorporated more in detecting the circulating virus in blood of patient.

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Conflict of interest statement

We declare that we have no conflict of interest.

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