RESEARCH ARTICLE

PCR Based Genotypic Characterization of Dengue Virus in Children from a Subset of Local Infected Population

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ABSTRACT

Pakistan is one of the developing countries that had been severely infected with Dengue virus in past years. In order to assist the clinicians, studies were required to characterise the extent and type of dengue virus infection and this lapse result in the huge loss of lives within 3 years of infection. This study elucidated the infection status of children with fever at various day of presentation utilising PCR based qualitative and genotypic assays. A total of 83 children were evaluated, out of which only ten were found to be PCR positive. This detection was based on antibody and antigen status of the patient using RT-PCR. Moreover, all the PCR positive patients were found to be infected with serotype 2 (DENV-2). All these subjects belonged to the group of children that were sampled in first five days of their fever. It is therefore concluded that conventional PCR may serve as an efficient tool for diagnosis of dengue but at a very earlier stage of the disease. Further studies are needed to provide a better screening of the prevalent type of serotypes.

Keywords: Dengue virus, PCR-based detection, NS1, DENV-2.

Dengue is one of the most important arthropod-born diseases in the world, posing a threat to one-third of the human population globally (1). Due to the increased incidence and geographical distribution of dengue in the last 50 years, dengue is becoming increasingly recognized as one of the world's major infectious diseases (2). Dengue is transmitted by the bite of an *Aedes* female mosquito with any one of these four types of dengue viruses. Dengue viruses (DENV) belong to the *Flavivirus* genus of the *Flaviviridae* family.

The flavi viruses are insect-transmitted, icosahedral enveloped RNA viruses that infect vertebrates and frequently cause serious, sometimes fatal, infections in humans. There are four strains of the virus, called as serotypes and are referred to as DENV-1, DENV-2, DENV-3 and DENV-4. All four serotypes can cause severe type of disease (3). Infection with one serotype is believed to produce lifelong immunity to that serotype but only short term protection against the others (4).

The symptoms of Dengue viruses appear between 3-14 days after the infective bite of mosquito. There are

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two types of infections caused by this virus; primary and secondary infection. In primary infection, only milder illness is observed but in secondary infection it become severe (5). Dengue viruses cause a wide spectrum of disease that ranges from dengue fever to the potentially fatal dengue shock syndrome (2). Dengue fever is febrile illness that affects infants, young children and adults. Symptoms range from a mild fever, to incapacitating high fever, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), with severe headache, pain behind the eyes, muscle and joint pain, and rash (6). There are three phases of DHF clinical course: (i) febrile phase lasts for 2–7 days, (ii) critical or leakage phase takes 24–48 hours, and (iii) convalescence phase continues to 2–7 days. In the febrile phase, only supportive and symptomatic treatment is conducted. At the end of the febrile phase, plasma leakage begins. In severe cases, there is a need of hospital admission and proper intravenous replacement (7).

There is currently no effective vaccine or chemotherapy for the disease. Dengue viruses are detected in highly equipped microbiological laboratories where different parameters are conducted for testing and diagnosis (8). The detection tools of Dengue viruses are of two type; direct and indirect methods. This can be done by, viral antigen detection or specific antibodies (serology) direct method comprises of different serological techniques in which IgG ELISA, IgM ELISA and NS1 antigen detection are included. In contrast, PCR detective techniques comprises of virus isolation in cell culture, nucleic acid detection by PCR are listed in indirect method (1). Virus isolation and nucleic acid detection are more accurate than antigen detection, but these tests are not widely available due to their greater cost. These laboratory tests are only of diagnostic value during the acute phase of the illness with the exception of serology. Considering the importance of nonstructural protein 1 (NSP1) and glycoprotein E1 (E1) genes in dengue virus infection, a reverse transcription-polymerase chain reaction (RT-PCR)

was developed for the detection of *virus infections* based on the (9).

Traditionally, dengue has been diagnosed by virus isolation or serological methods, but with recent advances in molecular techniques and in rapid detection technology, a range of novel diagnostic tests will soon be commercially available that will improve case management and aid disease control efforts. The nested RT-PCR assay has routinely been used by some laboratories for the identification of dengue viruses. This comprises a two-step PCR reaction involving an initial reverse transcription and amplification step using universal dengue primers followed by a second amplification that is serotype specific.

The products of these reactions are separated by electrophoresis on an agarose gel, and the different-sized bands observed are compared with a standard marker for the relative molecular mass of nucleic acids. Dengue serotypes are identified by the size of their bands. The real-time RT-PCR assay is a one-step assay system using primer pairs and probes that are specific to each dengue serotype. The use of a fluorescent probe enables the detection of the reaction products in real time without need for electrophoresis. Many real-time RT-PCR assays have been developed either as 'single plex' or 'multiplex' (6). The four plex real-time RT-PCR assays are often less sensitive than nested RT-PCR assay but are faster. An advantage of this assay is the ability to determine viral load in a given sample, which is believed to be important in determining the severity of dengue disease (10).

The present research work was aimed at improving the PCR based detection of all four types of dengue virus and its correlation with the severity of the disease.

Materials and Methods

All the analysis was performed at the Centre for Research in Molecular Medicine (CRIMM), The University of Lahore.

Sample Collection

During 2011 dengue epidemic in Lahore, Punjab, Pakistan, 83 children with fever were suspected of being infected with dengue virus and their blood was collected for the descriptive, prospective and retrospective questionnaire based study. Samples were collected from Dengue Ward, Children Hospital, Ferozpur Road, Lahore. Detailed physical examination was performed and all the available blood tests reports were taken. Questionnaires were duly filled with bio data of the child, clinical presentation of the illness, complete blood count (CBC) record, along with available additional investigative information. Children with diseases like diabetes, cirrhosis, CVD or kidney disease were not included in the study. Clinical history of the patients indicating the results of ELISA based IgM and IgG were also recorded.

1. RNA isolation:

RNA was isolated using Qiagen RNA extraction kit. The extraction was done according to the manufacturer's instructions. Briefly, 100 µl of serum sample was mixed with kit buffers and vortexed. Tubes were kept on ice and centrifuged at 14000 rpm for 10 seconds. Supernatant was collected into new sterile tubes and were mixed with 500 µl of 2-propanpl. After centrifugation at 14000 rpm for 10 seconds, RNA pellet was precipitated, resuspended in ethanol and air-dried for 5-10 minutes.

2. cDNA synthesis

5 µl of extracted RNA (20-50ng) was used for cDNA synthesis using cDNA mix, RT enzyme and RNAse inhibitor in 0.2ml PCR reaction tubes. Respective positive and negative control assays were done in parallel. The thermal cycler conditions are mentioned in Table 1.

Table 1. Cycling conditions of cDNA synthesis

Number of cycles	Conditions
One Cycle	37°C for 50 minutes
One Cycle	94°C for 3 minutes
One Cycle	20°C for 2 minutes

3. First round PCR

3 µl of cDNA was used for the first round PCR using SeroReg Mix. Amplification was done using conditions mentioned in Table 2.

Table 2. Cycling program of first round PCR

Cycle	Conditions
One Cycle:	94°C for 2.0 minutes
30 Cycles:	94°C for 45 seconds
	56°C for 45 seconds
	72°C for 1.5 minutes
One Cycle:	72°C for 10 minutes

4. Type-specific PCR

Reaction mix was prepared using recipe mentioned in Table 3.

Table 3. Enzyme concentration for Mix-A and Mix-B

Reagent	One reaction	10 Reactions
TS Mix-A or TS Mix-B	11.7 µl	117.0µl
PCR Enzymes	0.30µl	3.0µl

3 µl of First Round PCR Amplicons was mixed with 12ul of each Mix-A and Mix-B, and the reaction was conducted using PCR reaction conditions stated in Table 4.

Table 4. Cycling program of type-specific PCR

Cycle	Conditions
One Cycle:	94°C----------2.0 min
30 Cycles:	94°C----------45 sec
	50°C----------45 sec

	72°C----------1.3 min
One Cycle:	72°C---------10 min

Table 5. Oligonucleotide sequences used in reactions

Sr. No.	Primer Name	5'-3' Sequence	Size of amplified product (bp)
1	1 D1-D	TCAATATGCTGA AACGCGWGAGAA ACCG	511
	2 D2-D	TTGCACCARCART CWATGTCTTCWG GYTC	
2	TS1-F	AGGACCCATGAA ATTGGTGA	411
	TS1-R	ACGTCATCTGGTT CCGTCTC	
3	TS2-F	AGAGAAACCGCG TGTCAACT	403
	TS2-R	ATGGCCATGAGG GTACACAT	
4	TS3-F	ACCGTGTGTCAA CTGGATCA	453
	TS3-R	CAGTAATGAGGG GGCATTTG	
5	TS4-F	CCTCAAGGGTTG GTGAAGAG	401
	TS4-R	CCTCACACATTTC ACCCAAGT	

Agarose gel electrophoresis

PCR products were resolved on agarose gel electrophoresis and observed under ultra violet (UV) light. The DENV Serotype-specific bands were determined by identifying the specific cDNA band comparing with 100-bp or 50-bp DNA ladder, used as a DNA size marker.

Results

Gender based frequency of subjects

Total number of patients included in the study were 83. Frequency of male subjects was 44 making 53% of total whereas; female subjects were 47% of the total. The male to female ratio came out to be 1.17:1.

Table 6. Gender based frequency of patients

n: 83	No. of patients (f)	Percentage frequency (%)
Males	44	53
Females	39	47

Age wise distribution

Mean age of patients under investigation was 9.42±0.41 (10) with a range of 1 to 16 years. Among these, male subjects had a mean age of 9.17±0.58 (10) and the average age of female subjects was 9.71±0.58 (10).

Table 7. Mean age of patients according to gender

n = 89	
Mean age cumulative (yrs)	9.42±0.41 (10)
Mean age males (yrs)	9.17±0.58 (10)
Mean age females (yrs)	9.71±0.58 (10).

All the subjects included in the study were divided into three groups. In 0-5 year group, cumulative frequency of patients was 18 which was 22% of the total. In this group, frequency of male subjects was 12 which was 66.67% of total; whereas female subjects were 33.33% of the total with the representative frequency of 6. In 6-10 year group, cumulative frequency of patients was 29 which was 35% of the total. In this group, frequency of male subjects was 16 which was 55.17% of total; while female subjects were 44.83% of the total with the representative frequency of 29. Maximum (43%) patients belonged to the third group with the age ranging from 11 to 16 years. The number of male subjects in this group was 16 which were 44.44% of total; while there were 20 female subjects which amounted to 55.56% of total.

Table 8. Age distribution of patients

Age In years	n	Distribution	Males f	%	Females f	%
0-5	18	18(22%)	12	66.67	06	33.33
6-10	29	29(35%)	16	55.17	13	44.83
11-16	36	36(43%)	16	44.44	20	55.56
Total	83	100%	44	53.0	39	47.0

PCR based detection

Among the total 83 samples, only ten were found to be PCR positive making a percentage of 33.3 of the total NS1 positive samples. The remaining samples were PCR negative highlighting the unavailability of the viral genome in the sample.

Table 9. Percentages of PCR based detection of dengue virus in suspects

n=83	f	%	PCR +ve	%	PCR -ve	%
ELISA NS1+ve	30	36.1	10	33.3	20	66.6
ELISA NS1 -ve	49	48.1	0	0	49	100
ELISA NS1 eq.	4	4.81	0	0	4	100

Distribution of subjects according to the day of fever and PCR results

Majority of the patients reported their fever on day 3 followed by day 4, 5 and 7. PCR positive samples were that of early days of fever i.e. day 1-5. There was no sample PCR positive after day 7. A detailed account of the respective day with the PCR status is described below.

Table 10. Table depicting the distribution of patients according to their day of fever and PCR based detection

Day of fever	Total patients (f)	f (%)	PCR +ve	f (%)	PCR -ve	f (%)
1	5	6	1	20	4	80
2	5	6	3	60	2	40
3	15	18	3	20	12	80
4	12	14.45	0	0	12	100
5	14	16.8	2	14.2	12	85.7
6	7	8.40	0	0	7	100
7	12	13.20	1	8.33	11	91.7
8	2	2.4	-	-	2	100
10	2	2.4	-	-	2	100
11	1	1.2	-	-	1	100
12	3	3.6	-	-	3	100
14	2	2.4	-	-	2	100
20	1	1.2	-	-	1	100
30	2	2.4	-	-	2	100
Total	83	-	10	-	73	-

### Comparative status of ELISA and PCR

All the samples were pretested for NS1, IgG and IgM at the diagnostic laboratory. These samples presented different PCR status primarily
depending on the development of IgM and NS1. However, IgG clearly indicated secondary infection as shown in table 11.

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Table 11. Comparative status of ELISA and PCR based dengue virus in patients

| | | CATEGORY | | | |
	IgM	IgG	NS1	PCR	Interpretation
PCR +ve	-	-	-	0	Pure Dengue – ve
	-	+	-	0	Dengue – ve with previous infection
	-	+	+	1	Dengue +ve with secondary infection
	+	+	E	0	Dengue –ve because of IgM development. Secondary infection also.
	-	-	+	8	Early detection Dengue +ve
	+	-	-	0	Dengue –ve because of IgM development.
	+	+	+	0	Dengue –ve because of IgM development.
	+	+	-	0	Dengue –ve because of IgM development.
	+	-	+	1	Dengue +ve possibly because of initial recovery phase (IgM+)
PCR –ve	-	-	-	15	Pure Dengue – ve
	-	+	-	1	Previous infection as indicated by presence of IgG
	-	+	+	1	Previous infection, possible ↓viral load (as NS1 is positive)
	+	+	E	2	Dengue –ve because of IgM development, Secondary infection.
	-	-	+	1	Dengue – ve, ↓ viral load
	+	-	-	4	Dengue –ve because of IgM development
	+	+	+	8	IgM developed
	+	+	-	31	IgM developed, PCR detection not possible because of Recovery
	+	-	+	9	Dengue –ve because of IgM development

Genotypic infection

All the PCR positive samples were genotyped and found to be positive for serotype 2 as shown in figure 1.
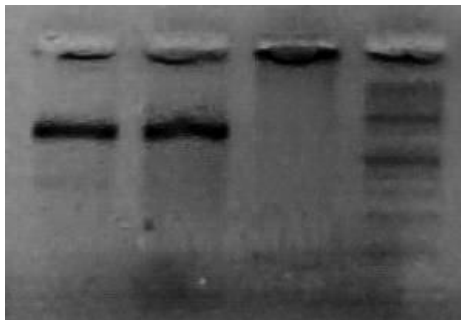
Figure 1. Agarose gel showing genotype 2 of dengue virus through a band of 402 bp along with a 100 bp ladder

Discussion:

In view of the urgent need of the genotypic characterization of the virus infecting the population, the present study targeted the younger population of Lahore. Children included in the study had a mean age of 9 years and ranged from 1 to 16 years. Among the samples, 53% were male and 47 % female subjects. Male population was regarded at high risk of dengue infection because of their proportional number (male: female ratio 1.1:1). All the samples included for analysis were already screened for NS1, IgG and IgM by ELISA. The PCR assay used in this study was designed to detect genotypic configuration if sample is found positive for dengue infection. The area of genome amplified was according to previous

study in Pakistan (11). In order to study the efficiency of PCR assay to diagnose infection, the results were compared with the antigenic antibody profile of the subject.

Out of total 83 children, 30 were NS1 +ve, 49 were NS1 –ve and 4 were equivocal cases: among the 30 NS1 +ve, only 10 were detected as PCR positive, making a percentage of 33.3 of the total. The remaining 20% were different in their antibody profile, as will be discussed below. However, NS1 –ve patients, irrespective of their antibody profile were found PCR negative. The 4 equivocal cases were also negative. This indicated that NS1 protein was an indicator of circulating viral genome, representing active replication of the virus. Moreover, NS1 was a 100% predicator of infection especially if the IgG and IgM are negative.

The subjects with NS1 positive and IgG positive were only two in number, out of which one was positive and the second one was negative. The positive subject in this category did not develop IgM and had a secondary infection as indicated by IgG whereas having similar situation the second subject (PCR -ve) might have a very low viral load which made undetectable in PCR but positive for NS1 ELISA. All the samples with IgM positive ELISA were PCR negative indicating the recovery of patient and eradication of the virus through immune system.

In addition to these observations, it was also noticed that PCR was highly sensitive when the patient was in early days of fever. The majority of the positive patients belonged to the 1-5 days of fever. No samples were detected by PCR after 8 days of fever. Also IgM was a limiting factor indicating that the patient might had recovered from the fever and there are no circulating genomes present in the body fluids.

The study had various limitations including the small sample size, heterogeneous pool of sample with various stages of clinical presentation. A detailed account of the patient and repeated tests at various days of fever of the same patient may elucidate the detailed diagnostic efficiency of PCR

and ELISA tests. It may be of prime importance to the clinicians to evaluate all the tests at various stages so that an efficient treatment strategy may be developed for the patient.

Conclusion

The study efficiently characterized the PCR based dengue detection status of the patients with fever presentation. Also, it was observed that viral genome may only be detected at the earlier days of fever. NS1 was found quite predictive of the positive status of the patient. However, this was limited by IgM. The serotype present in the positive samples was DENV2. A larger study is important for the further evaluation of the prevalence of various serotypes in the population of Punjab which will help the increased patient's survival in case any future breakout of viral infection.

Acknowledgement

Conflict of Interest

The authors declare no conflict of interest.

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