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Circulating Dengue Virus serotypes in 2016 outbreak in kolkata

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ABSTRACT: Dengue is an emerging infectious disease throughout tropical and subtropical regions and is transmitted mainly by Aedes aegypti which expands its habitat across Asia, Africa, Central America, South America, and the Pacific. Dengue virus belongs to family Flaviviridae, having four serotypes- DEN1, DEN2, DEN3 and DEN4.All four DENV serotypes have emerged from sylvatic strains in the forests of South-East Asia.It represents an expanding global health challenge and is the most common arbo -viral infection of humans. The disease ranges from mild undifferentiated acute febrile illness (classical dengue fever, DF) to more life threatening form of the disease, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), is responsible for high mortality rate, especially in children.

In this study the Dengue infection was diagnosed the presence of NS1 antigen in blood by the method PanBio Dengue NS1 antigen ELISA and then dengue serotypes were identified by Nested RT-PCR method. 85 archive blood samples of 2016 outbreak were selected randomly to detect the presence of NS1 antigen of dengue virus. Blood samples that were NS1 antigen positive were further analyzed for serotyping. Out of 85 serum samples,31.76% of the blood samples were NS1 antigen positive. These NS1 (+)ve blood samples were further analyzed by serotyping. We have processed dengue NS1 positive samples which were collected from different hospitals in Kolkata, West Bengal. Out of which 13 (48.15%) were DENV-1, 7 (25.93%) were DENV-2, 5 (18.52%) were DENV-3 and 2 (7.4%) were DENV-4. Among all the serotypes DENV-1 was the most prevalent serotype followed by DENV-2 circulating in Kolkata, West bengal in 2016.

KEYWORDS

bp	BASE PAIR
DENV	DENGUE VIRUS
DHF	DENGUE HAEMORRHAGIC FEVER
DSS	DENGUE SHOCK SYNDROME
E	ENVELOPE
ELISA	ENZYME LINKED IMMUNOSORBENT ASSAY
HRP	HORSE RADISH PEROXIDASE
IG	IMMUNOGLOBULIN
µM	MICRO MOLAR
ml	MILLI LITRE
N	NORMALITY
NS	NON STRUCTURAL
PrM	PRE CORE MEMBRANE
TAE	TRIS ACETATE BUFFER
UTR	UNTRANSLATED REGION
WHO	WORLD HEALTH ORGANIZATION

I. INTRODUCTION

Dengue is anarthropod-borne acute viral infection with potentially fatal complications. Worldwide, an estimated 2.5 billion people are at risk of infection, approximately 975 million of whom live in urban areas in tropical and sub-tropical countries in Southeast Asia [Maria G.Guzman et al, 2007].Transmission also occurs in

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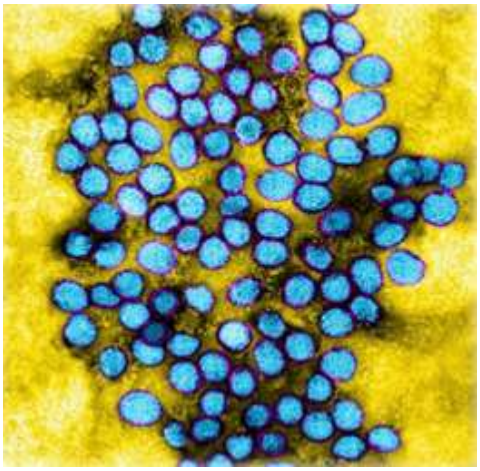
Africa and the Eastern Mediterranean, and rural communities are increasingly being affected. Dengue fever was first referred as "water poison" associated with flying insects. The word "dengue" is derived from the Swahili phrase Ka-dinga pepo, meaning "cramp-like seizure".Dengue viruses (DV) belongs to the family Flaviviridae and genus Flavivirus and there are four serotypes of the virus referred to as DENV-1, DENV-2, DENV-3 and DENV-4[Srivastava et al, 2012].This is a positive-stranded encapsulated RNA virus and is composed of three structural protein genes, which encode the nucleocapsid or core (C) protein, a membrane-associated (M) protein, an enveloped (E) glycoprotein and seven non-structural (NS) proteins. The transmission of dengue to humans occurs through mosquitoes like Aedes aegypti and Aedes albopictus, hence these infections are also referred as arboviral infections and have become a growing public health problem in the tropical and subtropical countries. The mosquitoes are day-biting mosquitoes that prefer to feed on humans and breeds in standing water.

Taxonomic position of the virus follows -

Group: Group IV [(+)ssRNA]
Order: Unassigned
Family: Flaviviridae
Genus: Flavivirus
Species: Dengue virus



II. AIMS &OBJECTIVES.

The Specific Objective ofthis Project Are:

- Investigation of outbreak of any febrile illness in the endemic areas for the evidence of Dengue virus infection
- Early detection of dengue by ns1 antigen testing
- Determination of dengue serotype among ns1 seroreactivesamples through Nested RT Polymerase Chain Reaction

III. MATERIALS AND METHODS

SAMPLE COLLECTION

Patients coming to the outdoor of Infectious Disease and Beliaghata General (ID & BG) hospital during the outbreak of 2016 may be seen similar symptoms with dengue were screened. Demographic characteristics, onset of fever, related illness was recorded for each dengue suspected cases. 85 human serum samples were randomly selected from the archive samples from 2016 outbreak.

DETECTION OF ANTIGEN USING THE PANBIO DENGUE NS1 ELISA

Detection of dengue serotype from 2016 dengue outbreak in Kolkata with the help of Dengue NS1antigen capture ELISAand Serotyping. Serum from the collected blood samples were separated and were stored at -20°C.

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. DENGUE NS1 Ag MICROLISA is designed for in vitro qualitative detection of Dengue NS1 antigen in human serum or plasma and is used as a screening test for testing of collected blood samples suspected of DENGUE. The kit detects all four subtypes: DEN1, DEN2, DEN3 & DEN4 of Dengue Virus.

DENGUE NS1 Ag MICROLISA is a solid phase enzyme linked immunosorbent assay (ELISA) based on the "Direct Sandwich" principle. ELISAs are typically performed in 96-well polystyrene plates, which will passively bind antibodies and proteins. The microwells are coated with Antidengue NS1antibodies with high reactivity for Dengue NS1 Ag. The samples are added in the wells followed by addition of enzyme conjugate (monoclonal anti-dengue NS1 antibodies linked to Horseradish Peroxidase (HRPO)).

A sandwich complex is formed in the well wherein dengue NS1 (from serum sample) is "trapped" or "sandwiched" between the antibody and antibody HRPO conjugate. Unbound conjugate is then washed off with wash buffer. The amount of bound peroxidase is proportional to the concentration of dengue NS1 antigen present in the sample. Upon addition of the substrate buffer and

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chromogen, a blue colour develops. The intensity of developed blue colour is proportional to the concentration of dengue NS1 antigen in sample. To limit the enzyme-substrate reaction, stop solution is added and a yellow colour develops which is finally read at 450nm spectrophotometrically.

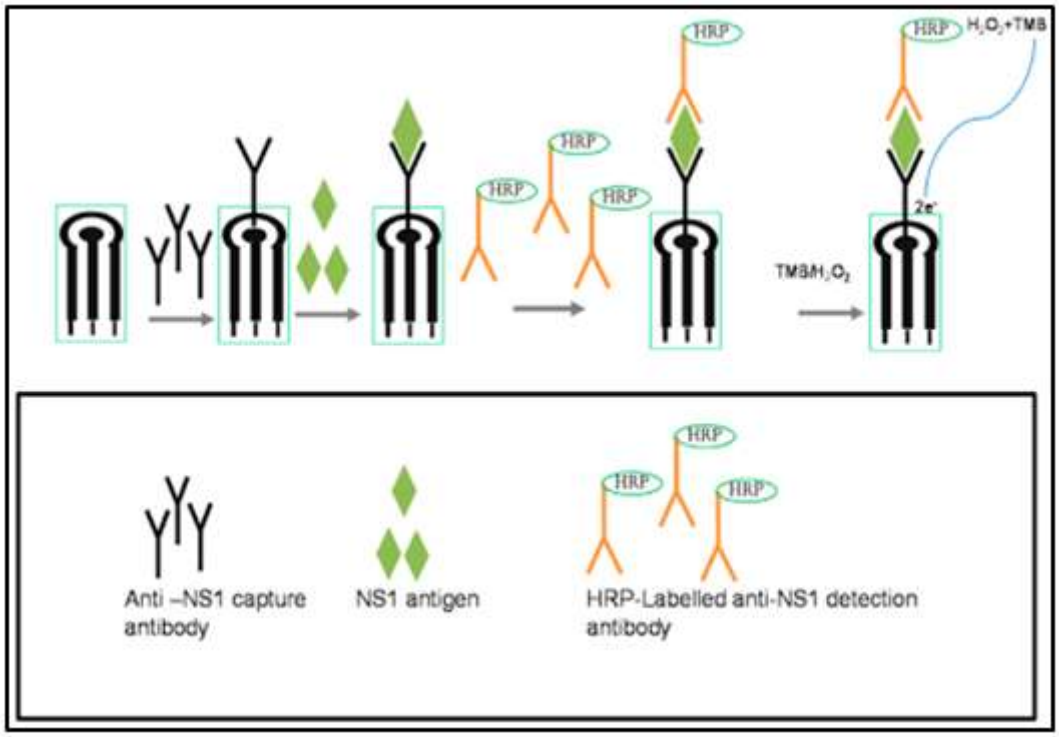

Fig 11: Mechanism of ELISA assay for detection of Dengue NS1 Antigen

MATERIALS REQUIRED

- Microwells
- Diluent
- Enzyme Conjugate (Containing Monoclonal Anti-Dengue NS1 linked to horseradish peroxidase with protein stabilizers)
- Wash Buffer (Concentrated Phosphate buffer)
- TMB Substrate (TMB, to be diluted with TMB Diluent H_2O_2 before use)
- Stop Solution (Ready to use, 1N H_2SO_4)
- Plate Sealers (Adhesive sheets to cover the microwells during incubation)
- microwells during incubation
- Micropipettes and microtips
- Elisa reader
- Elisa washer
- Distilled or deionized water
- Incubator 37°C
- Graduated Cylinders, for reagent dilution
- Vials to store the diluted reagent
- Sodium hypochlorite solution
- Disposable gloves

PROCEDURE:

The procedure according to the kit is as follows-

1. Serum was collected by separating clot from the blood sample with the help of centrifugation.
2. Serum was diluted in 1:100 ratio with serum dilution buffer.
3. Wells coated with anti dengue NS1 antibody were washed thrice by wash buffer.
4. 50µl of diluted samples were transferred to appropriate wells. Equal amount of positive and negative controls&Calibratorwere added to respective wells.
5. The plate was kept in a humified box(A bread box with a soaked tissue paper) and was incubated at 37˚C for 1hour.
6. After incubation, the plate was washed six times with wash buffer. The plate was tapped on a tissue paper after last wash.
7. 100 µl of anti dengue NS1 antibody linked to HRP was added to each well. Then the plate was kept in a humified box for30mins. Step 6 was repeated.
8. Add 150 µl of working substrate (TMB) solution in each well.

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9. Incubate at room temperature (20-30ºC) for 30 min. in dark.
10. Add 100 µl of stop solution
11. The plate was incubated at room temperature for color development which normally takes 10minutes.
12. Optical density was measured at 450nm using an ELIZA reader.

ASSAY PLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A	DV#1250	DV#1257	DV#1261	DV#1264	DV#1269	DV#1275	DV#1278	DV#1282	DV#1284	DV#1287	DV#1291	DV#1293
B	DV#1302	DV#1308	DV#1313	DV#1319	DV#1326	DV#1337	DV#1339	DV#1342	DV#1345	DV#1348	DV#1351	DV#1355
C	DV#1351	DV#1364	DV#1369	DV#1371	DV#1375	DV#1383	DV#1387	DV#1394	DV#1395	DV#1399	DV#1402	CAL-1
D	DV#1417	DV#1421	DV#1428	DV#1435	DV#1449	DV#1453	DV#1454	DV#1456	DV#1458	DV#1464	DV#1473	CAL-2
E	DV#1477	DV#1483	DV#1491	DV#1497	DV#1502	DV#1503	DV#1507	DV#1513	DV#1519	DV#1520	DV#1522	CAL-3
F	DV#1526	DV#1529	DV#1534	DV#1546	DV#1551	DV#1557	DV#1558	DV#1559	DV#1560	DV#1562	DV#1565	+VE
G	DV#1566	DV#1572	DV#1578	DV#1581	DV	DV#1594	DV#1596	DV#1597	DV#1598	DV#1599	DV#1600	-VE

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					#1584							
H	DV#1601	DV#1607	DV#1608	DV#1609	DV#16111	DV#1615						

MOLECULAR DETECTION :

- VIRAL RNA ISOLATION:-

Viral RNA can be purified from plasma (treated with anticoagulants other than heparin), serum, and other cell-free body fluids. In this experiment QIAamp Viral RNA Mini Kit (Spin Protocol) was used. The kit combines the selective binding properties of a silica gel- based membrane with the speed of microspin technology and is suited for simultaneous processing of multiple samples. The sample is first lysed under the highly denaturing conditions provided by Buffer AVL (containing guanidine thiocyanate) to inactivate RNases and to ensure isolation of intact viral RNA. Carrier RNA, added to Buffer AVL, improves the binding of viral RNA buffering conditions are then adjusted to provide optimum binding of the RNA to the QIAamp membrane, and the sample is loaded onto the QIAamp Mini spin column. Carrier RNA improves binding and recovery of low-concentrated viral RNA. The RNA binds to the membrane, and contaminants are efficiently washed away in two steps using two different wash buffers (AW1 and AW2). High-quality RNA is eluted in a special RNase-free buffer, ready for direct use or safe storage.

The use of two different wash buffers, AW1 and AW2, significantly improves the purity of the eluted RNA by removing the contaminations (potential PCR inhibitors) like salts, metabolites and soluble macromolecular cellular components. AW1 contain guanidine hydrochloride, which can form highly reactive compounds when combined with bleach. The elution buffer AVE is RNase-free water that contains 0.04% sodium azide to prevent microbial growth and subsequent contamination with RNases.

MATERIALS REQUIRED

1. Biosafety Cabinet
2. Microcentrifuge tube
3. Centrifuge
4. Micropipette
5. Tips
6. Vortex
7. Eppendorfs
8. Thermomixer
9. Qiagen RNA Extraction Kit

- AVL Buffer (Lysis buffer)
- Carrier RNA (It enhances binding of viral RNA to the membrane in case of low viral titer, as well as limits possible degradation of viral RNA due to any residual RNase activity)
- Ethanol
- QIAamp Mini Column
- Collection tube
- Buffer AW1 (Wash buffer 1)
- Buffer AW2 (Wash buffer 2)
- Buffer AVE (Elution buffer)

PROCEDURE:

The procedure according to the Qiagen RNA extraction kit is as follows-

1. 560 µl of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube.
2. 140 µl plasma, serum, urine, cell-culture supernatant, or cell-free body fluid was added to the Buffer AVL–carrier RNA in the microcentrifuge tube and Mixed by pulse-vortexing for 30s.
3. Incubated at room temperature (15–25°C) for 10 min.
4. The tube was briefly centrifudged to remove drops from the inside of the lid.
5. 560 µl of ethanol (96–100%) was added to the sample, and mixed by pulse-vortexing for 30 s. After mixing, the tube was briefly centrifuged to remove drops from inside the lid.
6. 630 µl of the solution from step 5 was added to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and

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centrifuge at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed into a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

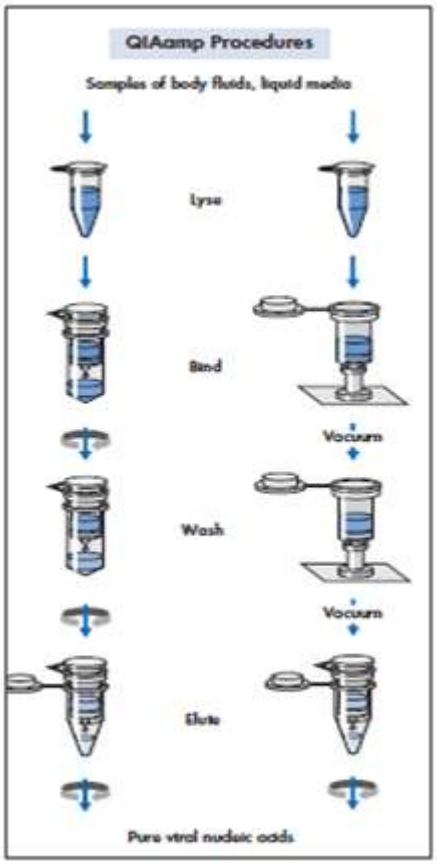
7. The QIAamp Mini column was opened, and step 6 was repeated until whole volume of the samle was passed through the column.

8. The QIAamp Mini column was opened and 500 µl of Buffer AW1 was added. The cap was closed, and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed in a clean 2 ml collection tube (provided), and the filtrate was discarded.

9. The QIAamp Mini column was opened and 500 µl of Buffer AW2 was added. The cap was closed, and centrifuged at 6000 x g (8000 rpm) for 3 min.

10. The QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube. The old collection tube containing the filtrate was discarded. The QIAamp Mini column was opened and 50 µl of Buffer AVE equilibrated to room temperature was added. The cap was closed, and incubated at room temperature for 1min. It was centrifuged at 6000 x g (8000 rpm) for 1 min to obtain the elution with viral RNA.

11. For quantification of RNA we have used the instrument called nanodrop. Then it was stored in -80˚C for future use.



- **REVERSE TRANSCRIPTASE – POLYMERASE CHAIN REACTION**

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. RT-PCR utilizes a pair of primers, which are complementary to a defined sequence on each of the two strands of the cDNA. These primers are then extended by a DNA polymerase and a copy of the strand is made after each cycle, leading to logarithmic amplification.

RT-PCR includes three major steps. The first step is reverse transcription (RT), in which RNA is reverse transcribed to cDNA using reverse transcriptase. This step is very important in order to perform PCR since DNA polymerase can act only

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on DNA templates. The RT step can be performed either in the same tube with PCR (one-step PCR) or in a separate one (two-step PCR) using a temperature between 40°C and 50°C, depending on the properties of the reverse transcriptase used .The next step involves the denaturation of the dsDNA at 95°C, so that the two strands separate and the primers can bind again at lower temperatures and begin a new chain reaction.

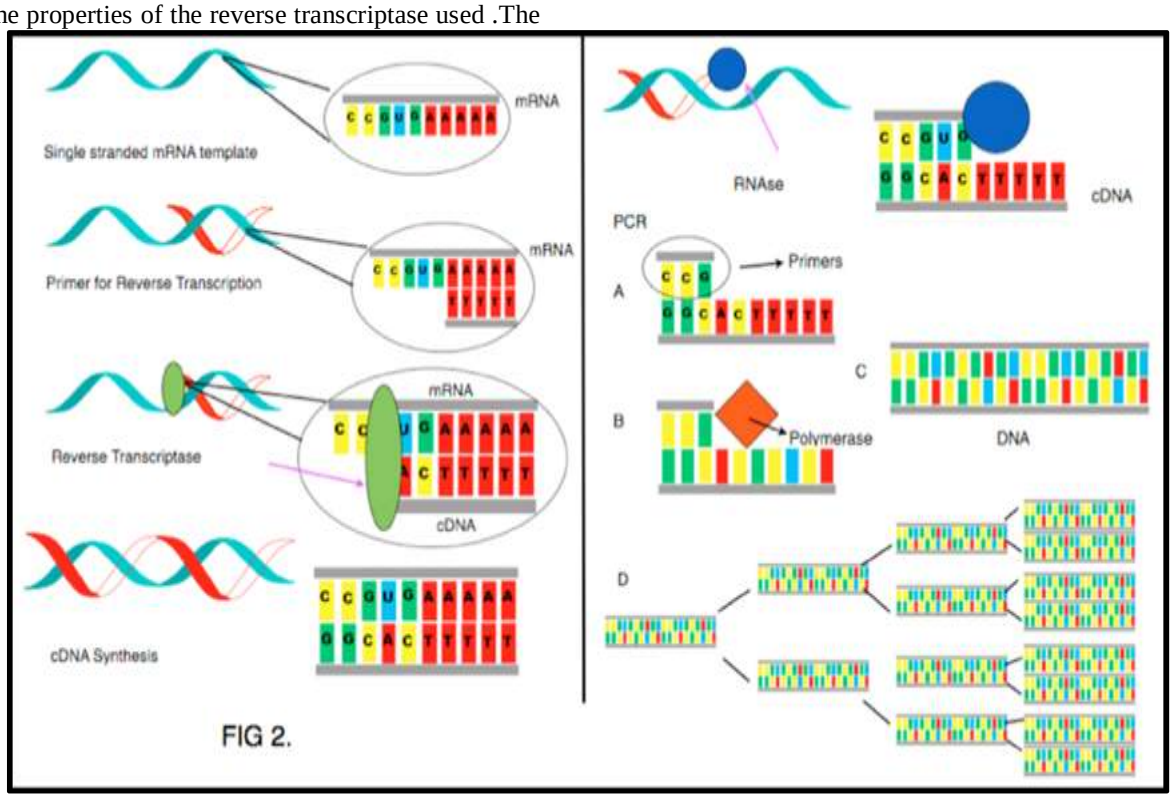

Fig 12: -Mechanism of RT Polymerase Chain Reaction

Then, the temperature is decreased until it reaches the annealing temperature which can vary depending on the set of primers used, their concentration, the probe and its concentration (if used), and the cations concentration. The main consideration, of course, when choosing the optimal annealing temperature is the melting temperature (Tm) of the primers and probes (if used). The annealing temperature chosen for a PCR depends directly on length and composition of the primers. This is the result of the difference of hydrogen bonds between A-T (2 bonds) and G-C (3 bonds). An annealing temperature about 5 degrees below the lowest Tm of the pair of primers is usually used .The final step of PCR amplification is DNA extension from the primers. This is done with thermo stable Taq DNA polymerase, usually at 72°C, the temperature at which the enzyme works optimally. The length of the incubation at each temperature, the temperature alterations, and the number of cycles are controlled by a programmable thermal cycler. The analysis of the PCR products depends on the type of PCR applied. If a conventional PCR is used, the product is analyzed by agarose gel electrophoresis.

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Fig13 : PCR Machine

MATERIALS REQUIRED

1. Previously isolated viral RNA samples
2. PCR tubes.
3. Micropipettes and autoclaved tips, minicooler.
4. PCR machine (Thermo cycler).
5. Biosafety Hood.
6. Mastermix

- Nuclease free water
- 10x RT-PCR buffer
- Forward primer (Primer D1)
- Reverse primer (Primer D2)
- dNTPs(10mM)
- $Mgcl_2$
- AMV Reverse Transcriptase (Promega, Madison, WI, USA)
- Taq DNA polymerase (Fermentas Inc., USA)

MASTER MIXTURE

Components	**Volume for 1 reaction(µl)**
Milipore water	10.3
10 x PCR buffer-II	2
dNTPs (10mM)	1.6
MgCl2 (25 mM)	1.2
Primer D1(10nM)	1
Primer D2(10nM)	1
AMV RT	0.4
Taq Polymerase	0.5
TOTAL	**18**

PROCEDURE

1. Target RNA was amplified in 20 µl volumes for each sample with 18µl master mix containing the following components: 2µl of 10x PCR buffer-II, 1.6µl of 10mM dNTPs (Life Technologies, Foster City, CA), 1µl each of 10mM of forward and reverse primers (D1 and D2), 1.2 µl of 25mM of MgCl2 (Life Technologies), 0.4U/µl of AMV RT (Promega, Madison, WI), 0.5U/µl of Taq Polymerase (Life Technologies) and 2 µl of extracted RNA.
2. Then the reaction mixture was mixed well by a short spin.

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3. After spin reaction tubes were subjected to the PCR machine and the following programme was set for reverse transcription as well as polymerase chain reaction specific for Dengue virus reaction.
4. The reaction conditions were 1 hr at 42°C, initialdenaturation at 95°C for 5 min followed by 35 cycles, 95°C for 30 sec, 55°C for 1 min, 72°C for 2 min followed by final extension at 72°C for 5 min.

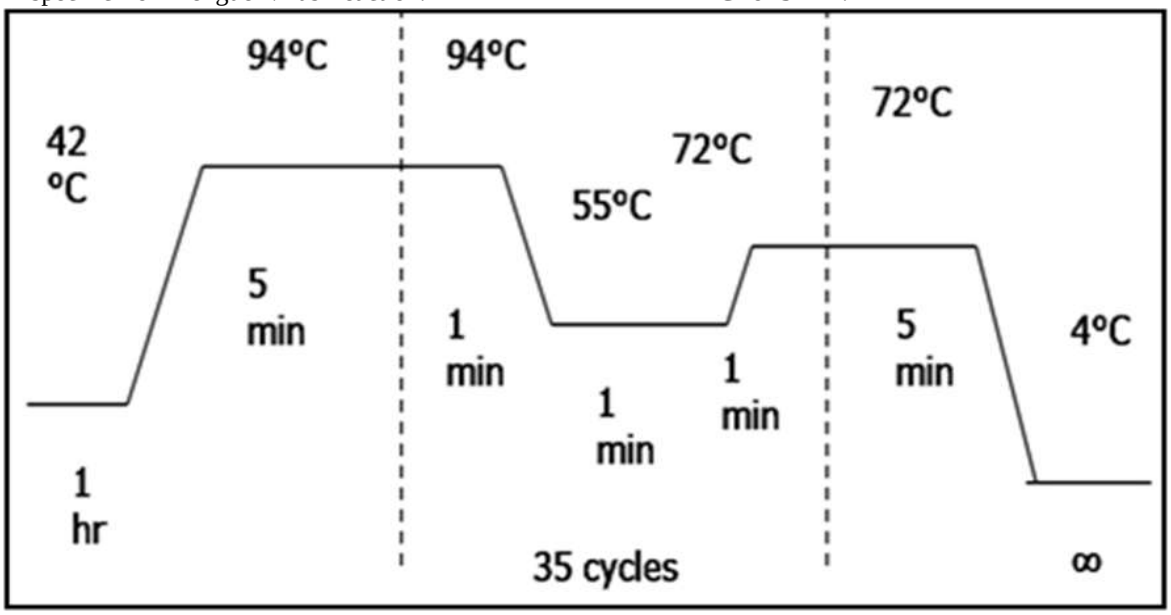

Fig14 : Reaction Condition of Reverse Transcriptase Polymerase Chain Reaction

- **NESTED PCR**

The sensitivity and specificity of PCR can be increased by using nested PCR (n PCR). In n PCR, two separate amplifications are used. The first uses a set of primers that yields a large product, which is then used as a template for the second amplification. The second set of primers anneal to sequences within the initial product producing a second smaller product. The primers for the second round of amplification are either both different from the first set or both located within the amplified DNA region. If only one of the second round primers is located within the amplified region and is used together with one of the first round primers, it is termed as semi-nested PCR. Nested PCR increases the specificity of the reaction because formation of the final product depends upon the binding of two separate sets of primers, which may preclude the need for verification of the PCR product by blotting, restriction digestion or sequencing. The second set of primers also serves to verify the specificity of the first product.

TheNested PCR used for Dengue stereotyping is basically semi nested PCR. Here the forward primer is as same as used in the RT-PCR reaction. But the reverse primers are serotype specific i.e. the TS1 , TS2 , TS3 ,TS4 primers are specific for DEN-1 , DEN-2 , DEN-3 , DEN-4 respectively. All the four primers are used in a single PCR reaction as reverse primer. The 4 primers give the amplification of different sizes. TS1 – 482 base pair (bp) for DEN-1 , TS2 – 119bp for DEN-2 , TS3 – 290bp for DEN-3 , TS4 – 392bp for DEN-4 PCR product.

MATERIALS REQUIRED

1. The product of RT –PCR.
2. Autoclaved dilution tubes.
3. The other requirements are as same as the requirements of RT-PCR reaction.
4. Master mixture for DENV Nested PCR

MASTER MIXTURE

Components	Volume for 1 reaction(µl)
Milipore water	10.37
10 x PCR buffer-II	2.5
dNTPs (10mM)	2

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MgCl2 (25 mM)	2.5
Primer D1(10nM)	1
Primer TS1 (10nM)	1
Primer TS2 (10nM)	1
Primer TS3 (10nM)	1
Primer TS4 (10nM)	1
Taq Polymerase	0.63
TOTAL	**23**

PROCEDURE

1. Serotyping of dengue virus was conducted by nested PCR using 1:100 times dilution of the first round RT-PCR product as the template.
2. The nested PCR was performed in a total volume of 25µl using 2.5µl of 10x PCR buffer-II, 2.0 µlof 10mM dNTPs (Life Technologies), 2.5 µl of 25mM MgCl2 (Life Technologies), 1 µl each of 10mM of forward and reverse primers (D1, TS1, TS2, TS3, and TS4), 0.625U/µl of Taq Polymerase (Life Technologies) and 2 µlof first round diluted product.
3. Then the reaction mixture was mixed well by a short spin.
4. After spin reaction tubes were subjected to the PCR machine and the following programme was set for Nested PCR specific for Dengue virus reaction.
5. The PCR was performed at initial denaturation of 95°C for 5 min followed 95°C for 30 sec, 55°C for 1 min and 72°C for 2 min for 30 cycles with a final extension at 72°C for 5 min.

- **AGAROSE GEL ELECTROPHORESIS**

Agarose gel electrophoresis is performed for the confirmation of the presence of viral RNA in the sample. Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA. Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones (Sambrook & Russel 2001). In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest. Extension of the technique includes excising the desired "band" from a stained gel viewed with a UV transilluminator. In order to visualize nucleic acid molecules in agarose gel ethidium bromide or SYBR Green are commonly used dyes.

Requirements

1. Gel casting apparatus
- Comb
- Gel casting tray
2. Weighing machine.
3. Agarose
4. Spatula.& Micropipette
5. TBE buffer (1X)
6. Beaker & Conical flask
7. Microwave oven.
8. Ethidium bromide.
9. Electrophoresis apparatus
- Tris Borate EDTA Buffer (6X)
- Power supply
10. Gel loading dye (6X)
11. Product of Nested PCR.
12. DNA ladder (100bp)
13. Negative control& Den- 1-4 RNA Positive Control
14. BIO RAD GEL DOC for photography of gel after electrophoresis.

PROCEDURE

1. All the gel casting apparatus were cleaned and fixed in place.
2. Required amount of 1X Tris Borate EDTA(TBE) buffer was prepared from 10X TBE buffer. 1.5% agarose was measured and dissolved in 100ml amount of the prepared 1X buffer.
3. The agarose was dissolved completely in the buffer by heating within Then it was allowed to cool to a certain extent.
4. 1.5µL Ethidium bromide was added to it and mixed gently and poured in the gel casting apparatus(setted previously with a comb) and care was taken from forming any air bubbles.
5. After solidification of gel the comb was removed without damaging the shape of the wells.
6. The gel was then transferred to electrophoretic apparatus and it was sink with 1X TBE buffer.
7. 10-20µl sample are mixed with loading dye and was loaded to respective wells. 100bp DNA ladder, positive and negative control were loaded onto the first three wells respectively.

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8. The power supply was switched on and maintain the current at 150mA. the sample was allowed to travel in the gel for certain distances. After proper migration the current was switched off.

9. Gel was observed in Gel Doc under UV-Translluminatormode.

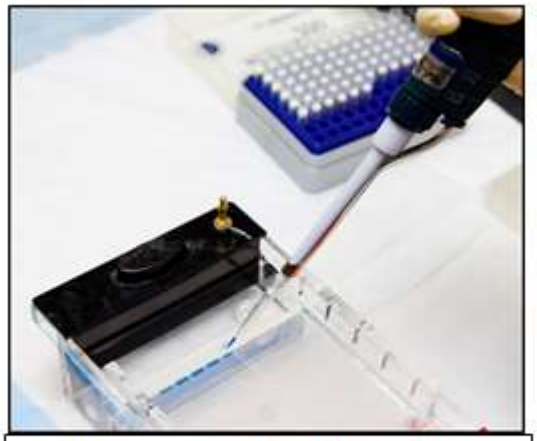

Fig 15: Wells of the gel loaded with sample

IV. RESULT

NS1 ELISA

Optical Density (O.D) values of the test samples at 450 nm with reference of 600-650nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.343	0.074	0.05	0.123	0.241	0.093	0.154	0.06	0.091	0.225	0.106	0.139
B	0.334	0.076	0.028	0.046	0.263	0.082	0.148	0.14	0.432	0.158	0.361	0.112
C	0.207	0.380	0.056	0.21	0.333	0.058	0.531	0.09	0.123	0.178	0.204	0.463
D	0.373	0.257	0.065	0.321	0.162	0.034	0.041	0.217	0.206	0.779	0.285	0.457
E	0.265	0.081	0.255	0.033	0.084	0.089	0.025	0.143	0.076	0.16	0.053	0.451
F	0.118	0.131	0.405	0.125	0.277	0.284	0.206	0.156	0.04	0.335	0.039	0.843
G	0.078	0.265	0.094	0.077	1.563	0.071	0.099	0.369	0.013	0.05	0.517	0.037

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H	0.586	0.244	0.298	0.097	0.636	0.08						

Calculations:

- Cut-off value = Mean absorbance of Calibrator × Calibration Factor.
- **Mean of absorbance of Calibrator =(0.463+0.457+0.451)/3=0.457**
- **Calibration Factor = 0.53**
- **Cut –off value = 0.457 × 0.53 =0.242**

Values greater than cut-off(>0.242) value considered as NS1 Antigen Positive.
Hence, 27 Samples are NS1 antigen positive out of 85 samples.

Nested RT-PCR and DENV Serotype

The Results and the representative photograph of 2 gels are on the following page

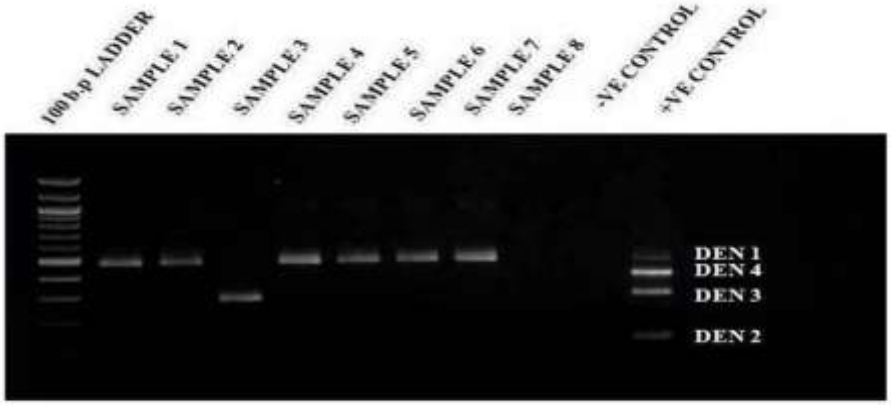

Fig16 : Representative picture of Gel -1

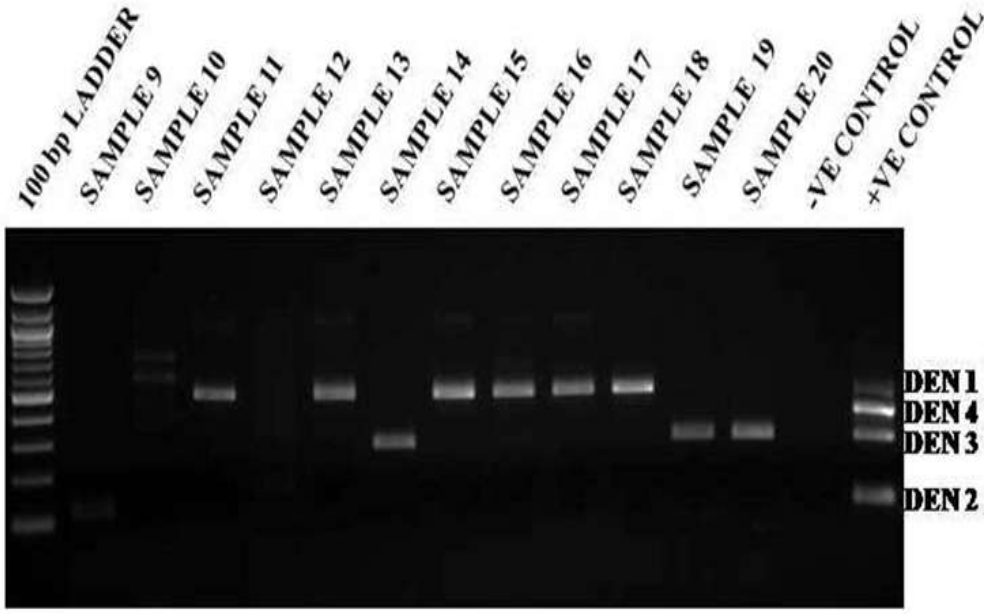

Fig17 : Representative picture of Gel-2

Detection of dengue virus serotypes in NS1 positive samples by nested RT-PCR. In the 1st Representative picture of gel (Fig 14) Lane 1loaded with100 bp DNA molecular size marker; lane 2–9 patients sample lane 11 negative control (-ve); lane 12 dengue positive control (+ve) of four

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serotypes, DEN-1 (482 bp), DEN-2 (119 bp), DEN-3 (290 bp), and DEN-4 (392 bp). Similarly in the 2nd Representative picture of Gel (Fig 15) lane-1 loaded with 100 bp molecular size marker, lane 2-13 patient sample, lane 14 negative control & lane 15 positive controlof four serotype.

Molecular result shows that out of 27 dengue NS1 positive samples, 13 samples gave positive for DEN-1 Serotype, 7 samples gave positive for DEN-2 Serotype, 5 samples gave positive for DEN-3 Serotype, 2 samples gave DEN-4 serotype positive. The positivity of the sample is identified by the detection of the DNA band of size loaded on agarose gel visualized in Geldoc.

Table: Percentage positivity of NS1 ELISA and Nested RT-PCR

NS1 ELISA TESTNESTED-RT PCR											
Sample Tested	No. of Positive Sample	Percentage of Positivity (%)	Sample Tested	Positivity among serotypes							
				DEN-1		DEN-2		DEN-3		DEN-4	
				No	(%)	No	(%)	No	(%)	No	(%)
85	**27**	**31.76**	**27**	**13**	**48.15**	**7**	**25.93**	**5**	**18.52**	**2**	**7.4**

V. DISCUSSION

Outbreaks of Dengue have been recorded in India on several occasions, including Kolkata. In this study the samples were of the year 2016 collected from different areas of Kolkata from the patients, showing similar symptoms of Dengue fever.

We have processed 27 dengue NS1 positive samples which were collected from different hospitals in Kolkata, West Bengal. Out of which 13 (48.15%) were DENV-1, 7 (25.93%) were DENV-2, 5(18.52%) were DENV-3 and 2 (7.4%) were DENV-4. Among all the serotypes DENV-1 was the most prevalent serotype followed by DENV-2 circulating in West Bengal in 2016.

VI. CONCLUSION

From the above study we can conclude that

- The study proves that the Dengue is emerging as a major health problem round the year in Kolkata and is now proving to be an endemic health problem that draws major concern for its fast diagnosis and treatment.
- Serotyping through Molecular technique also solves the problem of cross reactivity and the cases of multiple infection of different Dengue serotypes that causes serious health problem. For that reason, monitoring of dengue virus is important.
- Hence, it needs a continuous surveillance and further future epidemiological data on any other unknown fever outbreak.

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