CHAPTER 4

MATERIALS AND METHODS

4.1. Strategy:

The study was conducted in the tertiary care centre after obtaining Institutional Ethics Committee approval (IEC Ref No: IEC-NI/09/APR/09/11).

4.2. Case definition:

Patients suffering from fever for less than 10 days were included in the study. In addition to the suggestive clinical diagnosis, all the samples were tested for dengue and chikungunya by ELISA and RT-PCR. Samples were also collected from healthy individuals those who are not having fever within last one month.

4.3. Study center

This study was conducted at Clinical Virology and Molecular Biology section, Department of Microbiology, Sri Ramachandra Medical College and Research Institute, Porur, Chennai. Written informed consent and assent form were obtained from all study participants.

4.4. Study population:

4.4.1. Chikungunya:

Blood samples were collected from CHIKV suspected cases during two outbreak periods (July, 2008 to September, 2008 &

October and November, 2009) in three Northern districts (Kasaragod, Kozhikode and Kannur) of Kerala and a tertiary care centre in Chennai.

4.4.2. Dengue:

Blood samples (5 ml) of patients with suspected cases of DENV infection were collected during 2009, 2010, 2013 and 2015 in tertiary care centre in Chennai. The study included in-patients and out-patients attending the Medicine and Paediatrics department of Sri Ramachandra Medical College and Research Institute, Chennai.

4.5. Enrolment of the patients:

4.5.1. **Chikungunya and Dengue:**

Patients with acute febrile illness and high index of suspicion of Chikungunya and Dengue infection were enrolled in the study.

4.5.2. Patient Selection:

Patients of different age group with acute febrile illness fulfilling the following criteria were recruited in the study.

4.5.3. Inclusion criteria:

Patients visited hospital or admitted to the medical wards with the history of acute undifferentiated febrile illness.

4.5.4. Exclusion criteria:

Patients with immunosuppressive conditions including HIV infection, haematological malignancy, autoimmune disorders, acute

infection such as urinary tract infections (symptomatic), bacterial meningitis and suppurative abscesses were not included in the study. The demographic profile, clinical characteristics and subsequent laboratory findings were documented during sample collection. The proforma included details such as name, age, sex, occupation, contact details & details of symptoms like, fever, joint pains, maculopapular rash, myalgia, edema of involved joints, headache etc. (Proforma is included in the Annexure)

4.6. Specimen collection:

Bloods (5 ml) was collected in the vacutainer with anticoagulant (Becton &Dickinson, USA), and appropriately labelled with patient name, hospital number and date. Samples were transported to the laboratory at SRMC, Chennai at $4^{o}C$.

4.7. Specimen processing:

Blood was centrifuged at 2000 rpm for 10 minutes at $4^{o}C$ and plasma was collected and aliquoted as 1 ml in sterile vials, labelled with lab ID and stored at – $80^{o}C$.

4.8. Quality measures followed in CHIKV RT-PCR & DENV RT-PCR:

Positive control for uniplex RT-PCR and multiplex RT-PCR for Dengue (serotype 1-4) and chikungunya stock viruses were obtained from National Institute of Virology, Pune and King Institute of Preventive Medicine and Research, Chennai respectively.

To avoid PCR carry-over contamination, negative control (Sterile Milli Q water) was used between three samples and reagent control was included to ensure the reagent quality.

4.9. Detection of Chikungunya virus by RT-PCR:

4.9.1. RNA Extraction:

RNA Extraction was performed using commercial kit (Qiagen mini viral RNA, USA) as per instructions of manufacturer. Viral lysis buffer (560 µl) containing carrier RNA (5.6 µg) (1:100) was added to sterile micro centrifuge. Then the plasma (140 µl) was added to the tube containing lysis buffer, vortexed and incubated for 10 min at room temperature followed by brief spin. Ethanol (500 µl) was added to the sample, vortexed and briefly centrifuged. This mixture was added to QIAmp spin column and centrifuged at 8000 rpm for 1 min. After spin, the collection tube containing the filtrate was discarded & the spin column was transferred to a fresh collection tube. Wash buffer 1 (500 µl) was added to the spin column and centrifuged at 8000 rpm for 1 min. Filtrate was discarded and placed in a new collection tube. Wash buffer 2 was added to spin column & centrifuged at 14,000 rpm for 3 min. Empty spin was done at 14,000 rpm for 1 min. The spin column was then placed in fresh sterile micro centrifuge tube and 50 µl of elution buffer was added and incubated at room temperature for 3 min. Following incubation, spin column with micro centrifuge tube was

centrifuged at 8,000 rpm for 1 min. Eluted RNA was used as template for RT-PCR.

4.9.2. CHIKV RT-PCR:

One step RT-PCR was carried out using Qiagen One step RT-PCR (Qiagen, USA) on thermo cycler (PTC-200, MJ Research, USA).

Chikungunya RT-PCR was performed targeting Envelop protein (E2) (Edwards *et al.*, 2007). The E2 gene was selected as the target region for the RT-PCR as it shows a high degree of divergence among the alpha viruses and harbors virus specific nucleotide stretches suitable for primer design (Edwards *et al.*, 2007) (Table 4.1). PCR amplification mix was prepared with one step RT-PCR kit as per instructions of the manufacturer (Qiagen, USA). The Reverse transcription step is at 50^{o}C for 30 min, initial denaturation and initial activation at 94^{o}C for 10 min followed by 45 cycles of 94^{o}C for 30 sec, 55^{o}C for 30 sec, 68^{o}C for 2 min 20 sec and final elongation at 68^{o}C for 5 min.

Primer (Edwards *et al.*, 2007)	Sequence (5' to 3')	Base pair size (bp)
Forward	TATCCTGACCACCCAACGCTCC	305
Reverse	**ACATGCACATCCCACCTGCC**	

Table 4.1. Primers used for the detection of CHIKV

Primer (Lanciotti *et al.*, 1992)	Sequence (5' to 3')	Base pair size (bp)
Forward	TCAATATGCTGAAACGCGCGAGAAACCG	511
Reverse	TTGCACCAACAGTCAATGTCTTCAGGTTC	

Table 4.2. Primer sequences for diagnosis of DENV

Virus	Sequence (5' to 3')	**Target gene**	Base pair size (bp)
DENV Primers: (Lanciotti *et al.*, 1992)	Forward- TCAATATGCTGAAACGCGCGAGAAACCG	CPrM	511
	Reverse- TTGCACCAACAGTCAATGTCTTCAGGTTC		
CHIKV Primers: (Hasebe *et al.*, 2002)	Forward - TACCCATTCATGTGGGGC	E1	294
	Reverse - GCCTTTGTACACCACGATT		

Table 4.3: Primer sequences used for Multiplex RT-PCR for DENV and CHIKV

4.9.3 Agarose gel electrophoresis:

Submerged agarose gel electrophoresis (SAGE) was used to analyse the amplified product**.** Tris Acetate EDTA (TAE) buffer was used to prepare agarose gel (1%) and these were boiled at Microwave oven till gel solution was clear. The agarose gel was allowed to cool until it reached at 55^{o}C- 60^{o}C and 2 µl of Ethidium bromide (0.2 µg/ml) was added. This mixture was gently added to the gel tray to avoid air bubbles and appropriate comb was placed in the gel. The gel was allowed to settle at room temperature. The comb was displaced from the gel without disturbing the wells and the gel placed in the tank with TAE buffer which related to power pack with anode and cathode end. Loading wells were placed at cathode (black) ends. Loading dye (3 µl) was added onto parafilm and 10 µl of PCR product was mixed together and it was loaded into wells of the gel. DNA ladder (2 µl) (Biolabs, USA), 3 µl of loading dye and 8 µl of distilled water were mixed and added. The electrophoretic tank cover was placed on the tank the samples ran towards the anode (red) end at 120 volts for 30 min. The run was tracked through the dye and stopped after the dye had migrated about two thirds or three quarters of the gel. The amplification product 305bp lies within the gene that codes for viral envelope protein E2. Suitable positive and negative controls were used to analyze the amplified products of test samples.

4.10. Detection of dengue virus by RT-PCR

4.10.1. RNA extraction.

RNA extraction was performed as per the protocol described in **4.9.1.**

4.10.2. DENV RT-PCR

Dengue RT-PCR was performed by targeting CPrM region (511bp) as per Lanciotti *et al*., 1992 (Table 4.2). PCR amplification reagent mix was prepared with one step RT-PCR kit as per kit instruction (Qiagen, USA). MJ-Research Thermal Cycler was used for RT-PCR. The amplification involved the following steps: reverse transcription at 50°C for 30 min; one cycle of initial denaturation and activation of the enzyme mix at 95°C for 15 min, 55°C for 15 sec, and 72°C for 30 sec; 34 cycles at 95°C for 15 sec, 55°C for 15 sec, and 72°C for 30 sec; and final extension at 72°C for 10 min.

4.10.3. Agarose gel electrophoresis

The target (CPrM region) amplicon size is 511bp. RT–PCR amplicon products were run through agarose gel electrophoresis as described in **4.9.3.** using 100bp ladder (Biolabs, USA as a DNA size marker). The amplification product 511 bp lies within the gene that codes for viral envelope protein CPrM. Suitable positive and negative controls were used to analyze the amplified products of test samples.

4.11. Standardization of multiplex RT-PCR assay for detection CHIKV and DENV

4.11.1. RNA extraction:

RNA Extraction was performed as per the protocol described in **4.9.1.**

4.11.2. Optimization of multiplex RT-PCR for CHIKV and DENV

Multiplex RT-PCR targeting CPrM region (Lanciotti *et al.*, 1992) for Dengue and E1 (Hasebe *et.al.* 2002) genes was standardized with specific primers (Table 4.3). Multiplex RT-PCR was optimized with commercial RT-PCR master mix (Genet Bio, Korea) by adjusting the primer concentration and annealing temperature by gradient PCR using stock viruses.

4.11.3. Evaluation of Multiplex RT-PCR for CHIKV and DENV

Uniplex RT-PCR was used as the gold standard for evaluating the Multiplex RT-PCR assay for the detection of CHIKV and DENV. Sixty samples were evaluated of which 30 samples from CHIKV suspected individuals (20 positives and 10 negatives) and 30 samples from Dengue suspected individuals (20 positives and 10 negatives). Ten healthy controls were included in the assay. The sensitivity and diagnostic accuracy were calculated using standard formula.

4.12. Determine the phylogeny of CHIKV positives during the outbreak by sequencing the partial sequences of envelope gene E2 and identification of the prevalence of CHIKV genotypes/clades in this region

4.12.1. Chikungunya RT-PCR was carried out as described in 7.6.2 and 4.9.2

4.12.2. DNA Sequencing

4.12.3. Amplicon purification (Pre cleanup).

Pre clean up was done to remove unused primers and purify the amplified products. PCR products were mixed with nuclease free water and made up to the volume of 100 µl. It was loaded in 96 well Multiscreen $_{HTS}$ PCR plate (Millipore, USA). It was filtered with vaccum manifold for 5-10 min or until wells are dry. Then nuclease free water was added to each well. Each wells were agitated by pipetting and the products were aspirated and they were used for cycle sequencing PCR.

4.12.4. Cycle sequencing PCR

Genomic sequencing was done using the Big dye Terminator kit v3.1 dideoxy- chain termination method (Sanger *et al.*, 1977). All dideoxynucleotides were incorporated in single reaction tube and these dideoxynucleotides were labelled with four different dyes in this newly improved method. The ready reaction mix includes dye terminators, dideoxynucleoside triphosphates, AmpliTaq DNA

polymerase, magnesium chloride and buffer. DNA Sequencing was performed bidirectionally. The reagent mix was prepared as follows: Big-dye Terminator ready reaction mix, 1.0 µl, Template dsDNA, 2.0 µl, 1 µl of forward primer (1 pmol) and the reaction mixture brought to a final volume of 10µl using deionized water. Another similar reaction set up was prepared with reverse primer. Reactions set up were kept in a thermal cycler (Applied Biosystems, USA) by setting up reaction volume to 10 µl and the cycling conditions programmed as follows: twenty-five cycles of rapid thermal ramp to 96^{o}C for 15 sec, 50^{o}C for 20sec and 60^{o}C for 4 min.

4.12.5. Post clean-up

The sequencing mixture was purified again by Montage $_{SEQ96}$ filtration (Millipore, USA) using injection solution to remove the unincorporated dye terminators which will help to avoid ambiguous data during sequencing process and interfere with base calling by the 3730 genetic analyzer.

4.12.6.Separation of cycle sequencing fragments by electrophoresis using Genetic Analyzer (Applied Biosystems 3730)

Distribution of each dyes showing the sequences of the specific nucleotides with respect to the label was analysed by electrophoresis. Sequences were analysed using ABi Prism Big dye Terminator Genetic Analyzer, Applied Biosystems, USA).

Separation of fragments according to their size were performed electrophoretic ally by using automated 48 capillary systems. Separated fragments were detected by an internal charge-coupled device camera (CCD). The CCD camera capture specific fluorescent signal since each dye coupled with specific dye of different wavelength (Peterson, 2001) and output information was received by the analyzing software. Then output information generated as template sequence and data were stored automatically.

4.12.7. Verification of sequences quality

The data was retrieved from the system. Quality of the results were analysed using BioEdit software (Version 7.2.5). Consensus sequences were analyzed using Basic Local Alignment Sequence Tool (BLAST) (link) (Altschul *et al.*, 1997). This software tool aligns the nucleotide or protein sequences with the data base sequence and estimates the statistical significance of similarities. NCBI BLAST was used to analyse the target sequences to identify dissimilarities, deletion or insertion with references to the existing sequences in the database.

BLAST can also be used to describe the functional and evolutionary association among the sequences as well as assist in identifying family members of genes.

4.12.8. GenBank submission

Partial sequence of E2 gene obtained from the study were deposited in GenBank and accession numbers were obtained. Sequin software downloaded from the GenBank website, all the relevant information were entered and FASTA sequences were uploaded. After giving annotations, files were validated and the files were saved with the suffix. sqn. Sequin file which contains our sequence data were mailed to GenBank admin and then GenBank accession numbers were obtained.

4.12.9. Phylogenetic analysis

Over all 360 CHIKV full genome sequence were identified in NCBI GenBank database as of 24/02/2016 and all 360 sequences were retrieved and E2 region alone was taken for analysis along with the study sequences. Sequences were categorized based on the year of isolation, continents, country and genotypes. Consensus sequences were made for each category using Codon Code Aligner (software link or address).

Study sequences and consensus of database sequence were categorized as different files in FASTA format as mentioned earlier. These FASTA sequences were aligned using ClustalW inbuilt with MEGA6 (Molecular Evolutionary Genetics Analysis version 6.0). After performing multiple aligned sequence (MAS), it was converted into MEGA format and saved. The MEGA file was opened with

MEGA and the phylogenetic tree was constructed with aligned sequences using MEGA6 software for each category using a maximum likelihood on partial sequence of E2 gene sequence (305bp). Bootstrap analysis was performed with 1000 replicates to determine confidence values on the clades within trees. The S27 strain (AF369024) prototype strain and O'nyong nyong (out group) were used in the tree (Tamura *et al.*, 2013).

Nucleotide and aminoacid substitutions were analysed using multiple aligned sequences of S27 prototype Chikungunya strain and other sequences from different geographic regions. Sequences were aligned with ClustalW (Thomson *et al.*, 1994). Substitution nucleotides and aminoacid sequences were checked by comparing with database sequence manually.

4.12.10. Selection pressure analysis

Data monkey web interface of the Hyphy (Hypothesis testing using phylogenesis) was used to determine the heterogenous selection pressure in amino acid residues (Pond and Frost, 2005) for statistical valuation. Site by site based analysis was done using this molecular evolution platform with variable. Single likelihood ancestor counting (SLAC), fixed effects likely hood (FEL), internal branch FEL (IFEL), Random effects likelihood (REL) and fast unconstrained Bayesian approximation (FUBAR) are complementary

methods used for detecting evidence of positive selection. All our CHIKV sequences were included for analysis.

4.12.11. Structure prediction analysis:

The nucleotide sequences of partial E2 gene were converted to amino acid sequences using Expasy server amino acid conversion tool (http://web.expasy.org/translate/). CHIKV E2 protein homology models were built, the most similar structural template was used to compare the current CHIKV samples. SWISS MODEL tool was used for structure prediction. The 2XFC structure chain B was used as template and based on it, the 3D structure of all the sequences were predicted. Followed by structure prediction, superimposition was done using PDBeFOLD tool. The super imposed structures were analyzed using RasMol. RMSD (Root Mean Square Deviation) values were <0.1.

4.13. Real time RT-PCR for dengue serotyping

Samples collected in 2015 were subjected to CDC real-time PCR for serotyping and semi quantitation of dengue virus in the patients.

4.13.1. Principle:

The CDC DENV-1-4 Real-Time RT-PCR Assay includes a set of oligonucleotide primers and dual labeled hydrolysis (Taqman®) probes for *in vitro* qualitative detection of DENV serotypes 1, 2, 3 or 4 from serum or plasma collected from human patients with signs

and symptoms consistent with dengue (mild or severe). The fluorescently labeled probes anneal to amplified DNA fragments and the fluorescent signal intensity is monitored by the real time PCR instrument during each PCR cycle. A positive control virus mix is also included, which consists of heat-inactivated DENV-1 Haw, DENV-2 NGC, DENV-3 H87, and DENV-4 H241. To ensure the recovery and integrity of the RNA extraction reagent, an internal control Human Specimen Control (HSC) is used. It is non-infectious cultured human cell material that provides a positive signal in the assay. The CDC DENV-1-4 Real-Time RT-PCR assay is run in multiplex format (the four DENV serotypes are run in the same reaction).

4.13.2. RNA Extraction

RNA Extraction was performed as per the protocol in 4.9.2.

4.13.3. Assay mix for dengue serotyping was prepared as per the table 4.4

4.13.4. Assay mix for human specimen control was prepared as per the table 4.5.

4.13.5. The Reverse transcription step is at 50^{o}C for 30 min, initial denaturation and initial activation at 94^{o}C for 2 min followed by 45 cycles of 95^{o}C for 15 sec and 60^{o}C for 60 sec.

S.NO	Reagents	Volume (20 µL reaction)-1x (in µL)
1	Nuclease free water	2.2
2	2X Premix	12.5
3	Primer D1-F	0.5
4	Primer D1-R	0.5
5	Primer D2-F	0.5
6	Primer D2-R	0.5
7	Primer D3-F	0.5
8	Primer D3-R	0.5
9	Primer D4-F	0.5
10	Primer D4-R	0.5
11	Probes (DENV 1-4)	0.45
12	Taq Mix	0.5

Table. 4.4. Assay mix preparation for dengue serotyping

S.NO	Reagents	Volume - 1X (in µL)
1	Nuclease free water	2.2
2	2X Premix	12.5
3	Primer RP-F	0.5
4	Primer RP-R	0.5
5	Probe	0.5
6	Taq Mix	0.5

Table. 4.5. Assay mix preparation for Human specimen control

4.14. Determination of cytokine profile in Chikungunya and Dengue patients:

4.14.1. Sample collection, processing and storage

Anticoagulant treated blood specimens were collected aseptically from the suspected patients of dengue and chikungunya and 30 blood samples from healthy controls were included in the assay and transported to the laboratory in cold chain. Plasma was separated and stored at -80^{o}C multiple storage vials until used for the study. Freezing and thawing were avoided and the samples were brought to room temperature before the assay. After screening of the samples by CHIKV and DENV RT-PCR, Cytokine analysis was performed for total of 60 sixty samples which included positive samples for CHIKV (n=20) and DENV (n=20) which were confirmed by RT-PCR. Ten samples negative for CHIKV and DENV by RT-PCR were also included in the assay. Additionally, cytokine analysis was performed for fifteen patients with DENV disease both in acute and convalescent phase.

4.14.2. Cytokines

Cytokines such as IL-6, IL-8, IL-10, TNF-α and IFN-γ were measured by using single analyte Qiagen quantitative cytokine ELISA (Qiagen, USA).

4.14.3. Principle

The single analyte ELISArray kit was designed to quantitatively measure amount of individual protein analyte using a standard sandwich enzyme-linked immunosorbent assay (ELISA) technique. A target specific capture antibody has been coated on the plate; the kit also included with the detection antibody, Antigen standard, and a complete set of reagents for colorimetric ELISA.

4.14.4 Preparation of antigen standard dilutions

Assay procedures were similar for all cytokines included in the study. Antigen standard kept on ice for 20 minutes for thawing just before use. Serial dilution of the Antigen Standard (2000, 1000, 500, 250, 125, 61.5, and 31.25 pg/ml) in a set of eight 5 ml (12 x 75) polypropylene tubes was prepared as follows. Appropriate sample dilution buffer (2495 µl) was added to the first tube and 500 µl to the each of the remaining seven tubes. In the first dilution, 2000pg/ml of Antigen Standard was prepared and serial dilutions (1:2) were generated by transferring 500 µl from the first to the second tube, mixed well and then repeated from the second to the third and so forth on up to the seventh tube. No known source of antigen standard was added in eighth tube to determine the background signal. Detection limit of the cytokines IL-6, IL-8, IL-10, TNF-α and IFN-γ were 14.0pg/ml, 16.5pg/ml, 5.9pg/ml, 21.4pg/ml and 39.0pg/ml respectively.

4.14.5 Single analyte cytokine ELISA protocol

All required reagents and samples for ELISA were brought to room temperature before proceeding of ELISA. Standards were diluted from the range 2000 pg/ml to 31.25 pg/ml. Assay buffer and sample dilution buffer were prepared according the manufacturer's instruction (manufacturer's address). Assay (50 µl) was added into each well, 50 µl of serially diluted standard and 50 µl of sample were added to the respective wells. The plate was incubated at room temperature for 1 hr. After incubation, the plate was washed for 3 times with 1x wash buffer. Detection antibody (100 µl) was added and incubated at room temperature for 1 hr. Plate was washed three times. Avidin-HRP (100 µl) was added into each well and incubated for 30 minutes at room temperature. Plate was washed four times. Development solution (100 µl) was added and incubated for 15 min in the dark. After 15 minutes incubation, stop solution (100 µl) was added and the plate was read at 450nm within 30 minutes.

4.14.6 Data Analysis of cytokine ELISA

a. Processing the Raw Data

Typical absorbance values ranged from 0.00 to 2.50. the mean(average) absorbance for each set of replicate standards and experimental samples were calculated. These values were subtracted by the mean (average) background absorbance as determined from assays performed in the absence of antigen.

b. Standard Curve

Corrected mean absorbance of the standards was plotted a standard curve. Best straight-line curve fit was calculated through the points on the graph within the linear dynamic range of the results.

c. Determination of Unknowns

Background-corrected mean absorbance of each dilution of each unknown experimental samples were used and the standard curve to determine the protein amount in the sample.

4.15 Statistical analysis

Performance of multiplex RT-PCR was compared against uniplex One-step RT-PCR as gold standard by contingency table analysis and sensitivity and specificity were reported. For phylogenetic analysis, the evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.

All the data points were entered into MS Excel and double checked with raw data sheets. Statistical Software 'R' version 3.2.5 was used for statistical analysis. Means and Standard Deviations of all measured variables are compared across the groups and t-test and one way ANOVA were applied for related inferential statistics wherever needed. Parametric p-values were also compared against the same from nonparametric methods.