Chapter – 4

Materials and Methods
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The study was conducted from April 2011 to December 2014. The study was approved by the Ethical committee, BLDE’s shri. B. M. Patil medical college, hospital and research centre, Vijayapura. Chikungunya suspected cases with clinical symptoms of high fever (>38.5° C) and multiple joint pains of 5 to 8 days were collected from Government primary health centers (PHC’s), community health centres (CHC’s), taluk and district hospitals of Vijayapura district. Informed consent was obtained from all the patients prior to sample collection. A total of 500 blood samples were collected. A set of questions were asked for the patients before sample collection and format is given in annexure.

4.1. Sample collection

2 to 5 ml of venous blood was collected aseptically in sterile clean, screw-capped, labeled, vacutainer without anticoagulant. The specimen was allowed to clot at ambient temperature for 30 minutes and transported to lab in vaccine carrier. The sample was centrifuged at 3000rpm for 10 min and serum was separated. The serum samples were aliquoted into two sterile, leak proof cryovials in duplicate. (one vial for serological assay and other for molecular assay) Serum was stored at -70°C.

4.2. Sample processing

The serum samples were tested by serological methods and Seropositive samples were subjected to further testing by molecular methods.
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**Figure no.8 : Study Design**

1. Community wise distribution of study subjects
2. Total Number of Subjects Included (500)
3. Total Number of Blood Samples Collected (500)
4. Samples tested for serological Identification - IgM ELISA (500)
5. Sero Positive Samples Were Further Tested by Molecular Methods - RTPCR (33)
6. Statistical Analysis
7. Samples were proceeded for molecular characterization (9)
   - Amplification of RNA
   - Purification of Amplified Gene
   - Sanger Sequencing
     - Phylogenetic Analysis
     - Mutational Analysis
     - Protein Modelling
     - Mutation Mapping
4.2.1. Serological identification:

IgM - Enzyme Linked Immuno Sorbent Assay (ELISA) test was performed. Standard diagnostics (SD) Chikungunya IgM ELISA kit was used. The test was performed strictly as per the manufacturer’s instructions for optimal results.

Microplate wells were coated with concentration 0.2±0.04µg/well recombinant CHIK antigen. Sample dilution of 1:100 was made by adding sample diluents to negative control, positive control and samples. 990µl of sample diluent and 10µl of sample. 100µl of diluted negative control in triplicate wells, positive control in duplicate wells, single well of Internal Quality Control (IQC) and samples were added to wells. Microtitre plate was covered with adhesive tape sealer, mixed well and incubated at 37±1°C for 30 minutes. After incubation wells were aspirated completely and washed with 350µl of 20X working wash buffer solution for 5 times with 10 seconds soak time. 100µl of 101X working conjugate solution was added to all wells. Microtitre plate was covered with adhesive tape sealer and incubated at 37±1°C for 30 minutes. After incubation wells were aspirated completely and washed with 350µl of 20X working wash buffer solution for 5 times with 10 seconds soak time. 100µl of 1:1 working TMB substrate solution was added to all wells. The plate was incubated in dark at room temperature for 10 minutes. Finally 100µl of stop solution was added and read absorbance of the wells (OD) through bi-chromatic spectrophotometer (450-630nm wavelength)
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**Calculation:**

Mean absorbance of negative controls = NC-1+NC-2+NC-3/3

Cut off = Mean negative control+0.300

**Interpretation:**

- Negative Result: The samples with OD less than the cutoff.
- Positive Result: The samples with OD more than or equal to the cutoff.
- Borderline (gray result): The samples with OD around cutoff, for eg. cut off of 0.300 sample OD near 0.300 these samples were retested, if the same result was found such samples always need to be verified using a confirmatory test.

4.2.2. Molecular confirmation:

Molecular confirmation was carried out for seropositive samples at RAS Life sciences Pvt. Ltd, Hyderabad. Viral Ribonucleic Acid (RNA) was extracted and purified and Real Time polymerase Chain Reaction (RTPCR) was performed.

4.2.2.1. Extraction and purification of Viral RNA:

CHIKV RNA was extracted by using QIAamp Viral RNA mini extraction kit. The protocol is mentioned below.

Aliquoted 5.6 µl carrier RNA and added 560µl of buffer AVL in Nuclease free 1.5ml microcentrifuge tube. 140µl serum/plasma was added to the microcentrifuge tube and mixed well by pulse-vortexing for 15 sec and incubated at room temperature (15-25ºC) for 10 min. The tube was centrifuged for few
seconds at lower rpm to remove drops from the inside of the lid. 560µl ethanol (96-
100%) was added to incubation mixture and mixed by pulse-vortexing for 15 sec.
Then tube was centrifuged for few seconds at lower rpm to remove drops from the
inside of the lid. 630µl of solution was transferred into QIAamp mini column. Cap
was tightened and centrifuged at 8000rpm for 1 min. The QIAamp mini column
was replaced into a clean 2ml collection tube and tube containing the filtrate was
discarded. After that Carefully opened the QIAamp mini column and added 630µl
of solution into QIAamp mini column. The tube was centrifuged at 8000rpm for 1
min. QIAamp mini column was replaced into a clean 2ml collection tube and tube
containing the filtrate was discarded. QIAamp mini column was opened carefully
and transferred 500µl buffer AW1. The cap was tightened and tube was
centrifuged at 8000 rpm for 1 min. QIAamp mini column was replaced into a clean
2ml collection tube and tube containing the filtrate was discarded.

After that QIAamp mini column was opened carefully and transferred
500µl buffer AW2. The cap was tightened and tube was centrifuged at 14000 rpm
for 3 min. QIAamp mini column was replaced into a new 2ml collection tube. Old
column tube with filtrate was discarded. The tube was centrifuged at full speed for
1 min. QIAamp mini column was replaced into a new clean 1.5ml microcentrifuge
tube and old collection tube containing filtrate was discarded. To elute viral RNA
50µl elution buffer AVE equilibrated at room temperature. The cap was tightened
and incubated at room temperature for 1 min. The tube was centrifuged at 8000
rpm for 1 min. The microcentrifuge tube contains viral RNA was stored at -80°C
for further analysis.
4.2.2.2. Reverse transcriptase Real time Polymerase Chain Reaction (RT-PCR):

RT-PCR was carried out using Amplisure® Chikungunya RT-PCR kit. Non structural protein (nsp) gene was amplified and detected in the assay. The assay was performed using ABI 7500 thermo cycler. Good laboratory practices followed to avoid cross contamination. RNA extraction was carried out in sample preparation room, PCR setting up in PCR room and PCR performance in instrumentation room. Each step is restricted to the respective area to avoid contamination. Strict adherence to kit protocol was followed for optimal results.

The reaction volume was set to 30µl by adding 15µl of RAS q RNA PCR MIX, 7µl of RAS RT mix (Reverse transcriptase), 2µl of RAS CHK PPM (Primer-probe mix), 5µl of RNA/positive control/Negative control and 1µl of RAS Internal control mix. Internal control was used to check for possible PCR inhibition. In RT-PCR machine slots were labelled as per strip tube in the same order as striptube kept in the machine. For each slot type of sample (unknown/positive control/Negative control) was selected. Cycling conditions were reverse transcription step at 42°C for 15 min; followed by 40 cycles of thermo cycling which includes denaturation step at 95°C for 1 min, annealing step at 94°C for 15 sec and extension step at 60° for 1 min. Data was analyzed after completion of run.

Cycle amplification plot was checked to observe the amplification signal generated by different samples in run. The threshold was set manually just above the background signal of the negative control and negative samples by referring to cycle amplification plot. The values for unknown samples appeared in the result...
column in Ct in FAM channel. Only Sample results showing no amplification signal in FAM channel and showing amplification in Yakima yellow channel were considered.

**Interpretation:** Interpretation of the values of unknown samples was based on observation as described in the following table.

**Table –7: Interpretation of RTPCR test results**

<table>
<thead>
<tr>
<th>Observation</th>
<th>Interpretation</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification signal detected in CHK channel (FAM) and in internal control channel (Yakima yellow)</td>
<td>Chikungunya RNA detected</td>
<td>Proceed for further analysis</td>
</tr>
<tr>
<td>Amplification signal detected in CHK channel (FAM) and no signal in internal control channel (Yakima yellow)</td>
<td>Chikungunya RNA detected</td>
<td></td>
</tr>
<tr>
<td>Amplification signal not detected in CHK channel (FAM) but detected internal control channel (Yakima yellow)</td>
<td>Chikungunya RNA not detected</td>
<td></td>
</tr>
<tr>
<td>No amplification signal detected in CHK channel (FAM) as well as internal control channel (Yakima yellow) in unknown samples</td>
<td>Possible inhibition of PCR</td>
<td>Dilute the RNA sample (1:100) and repeat assays</td>
</tr>
</tbody>
</table>
4.2.3. Molecular characterisation:

RTPCR positive serum samples were further subjected for molecular characterisation. Sequencing was carried out by Di-deoxy Sanger sequencing method at RAS Lifesciences Pvt. Ltd, Hyderabad by using the commercial facility.

4.2.3.1. Sequencing:

4.2.3.1.1. Amplification of RNA:

The RNA of E1 gene between genome positions 10.389 to 10.943 (555 base pairs) in CHIKV genome was amplified using E1-F1 (GCTCCGCGTCCTTTAC) and E1-R1 (ATGGCGACGCCCCAAAGTC) primers [table no-8] (Kudukkil P Niyas et al., 2010 and Jatin Shrinet et al., 2012). PCR was performed by using genomic DNA template in 25 µl reactions. Taq DNA polymerase (0.5 unit), 0.2mM deoxy-nucleotide triphosphates, and 0.2mM of each primer on ABI7500 thermo cycler with a reverse transcription step at 42°C for 15 min; followed by 40 cycles of thermo cycling which includes denaturation step at 95°C for 1 min, annealing step at 94°C for 15 sec and extension step at 60° for 1 min.

4.2.3.1.2. Purification of amplified Gene product (Gel Extraction):

To check the size and quantity, amplified products were electrophoresed on 1.5 % agarose gel. The agarose gel containing the corresponding band was excised, weighed and buffer was added in three time volume to 1 volume of gel (100mg=100µl). To the sample 200µl of iso-propanol was added and mixed well. A Q/A quick spin column (QIA quick, Quigen) were placed in a 2ml collection
tube. The sample was applied to the Q/A quick column and to bind the DNA it was centrifuged for 1 min. Flow through was discarded and Q/A quick column was kept back into the same tube and washed with buffer PE. 30µl of elution buffer was added to the center of Q/A quick membrane and column was centrifuged for 1 min. The elute was used as purified gene product.

4.2.3.1.3. Cycle sequencing of DNA:

E1 partial gene sequencing was carried out by Sanger sequencing method (Di-deoxy sequencing) for 9 samples. Sequencing PCR was performed by using Big dye terminator (BDT) cycle sequencing ready reaction kit version 3.1 (applied biosystems, Foster city, CA) as per kit protocol. Sequencing was done by using DNA Sequencer (ABI 3130 xl GA) instrument. Both the strands were amplified as per the manufacturer’s protocol. Briefly, to 1µl of ready reaction mixture, 15µl of buffer, 30ng of template DNA, 3.2pmol of primer was added and the final volume was made to 10 µl using deionized water. Amplification of the PCR products were done in a thermocycler with the initial denaturing step by rapid thermal ramp to 96°C for 1 min then following cycling parameters of 25 cycles: Denaturation with rapid thermal ramp 96°C for 10 sec, annealing for 5 sec at 50°C, and elongation for 4 minute at 60°C. The reactions were kept at 4°C until ready for the purification. PCR product was purified using Ethanol, EDTA and sodium acetate precipitation protocol described by the manufacturer. Pellet was suspended in 2µl of Hi Di formamide. The reaction was made ready for the sequencing by giving a denaturation at 95°C for 5 min followed by snap chill.
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**Figure -9:** Target sequences in E1 gene for sequencing.

**Table -8:** Details of Primers used for PCR amplification in the study

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequences 5’-3’</th>
<th>Genome position</th>
<th>Amplicon size bps</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1-F1</td>
<td>GCTCCGCGTCCTTTAC</td>
<td>10389-10943</td>
<td>555</td>
</tr>
<tr>
<td>E1-R1</td>
<td>ATGGCGACGCCCCCAAGTC</td>
<td>10943</td>
<td></td>
</tr>
</tbody>
</table>

4.2.3.2. Phylogenetic analysis:

For the Phylogenetic tree construction, sequence search was performed at the National Centre for Biotechnology Information (NCBI) website, Basic Local Alignment Search Tool (BLAST) programme was run. Both the nucleotide sequence and predicted translated amino acid sequence were used in BLAST to identify the reference strains which can be considered for further analysis based on the geographical locations. The accession numbers used for the study are given below along with the positions matching with the samples.
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Chikungunya sequences were aligned using Clustal W2 software. The unrooted tree was constructed using Neighbor-Joining method. (Saitou N et al., 1987) The optimal tree with the sum of branch length = 0.2448 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) is shown next to the branches (Felsenstein J. et al., 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the Phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura K et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 502 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura K et al., 2013). The accession numbers used for the study are given below along with the positions matching with the samples.
Table -9: Reference strains with nucleotide and protein accession number

<table>
<thead>
<tr>
<th>Nucleotide Sequence Accession Numbers with position</th>
<th>Protein Sequence Accession Numbers (Uniprot)</th>
<th>Strain</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF369024.2:10427-10941</td>
<td>Q8JUX5</td>
<td>S27 (ECSA)</td>
<td>1952</td>
</tr>
<tr>
<td>DQ309336.1:223-737</td>
<td>A0SE38</td>
<td>Reunion 223/05 (ECSA)</td>
<td>2005</td>
</tr>
<tr>
<td>AM258992.1:10376-10890</td>
<td>Q1W367</td>
<td>Reunion (ECSA)</td>
<td>2006</td>
</tr>
<tr>
<td>EF027139.1:10428-10942</td>
<td>A6MH23</td>
<td>INDIA-00-MH4 (Asian)</td>
<td>2007</td>
</tr>
<tr>
<td>HM045811.1:10410-10924</td>
<td>D7R978</td>
<td>Tanzania (ECSA)</td>
<td>1953</td>
</tr>
<tr>
<td>HM045797.1:10410-10916</td>
<td>D7R952</td>
<td>RSU1 (Asian)</td>
<td>1985</td>
</tr>
<tr>
<td>HM045817.1:10412-10926</td>
<td>D7R990</td>
<td>Senegal (West Africa)</td>
<td>2005</td>
</tr>
<tr>
<td>HM045816.1:10412-10926</td>
<td>D7R988</td>
<td>Senegal (West Africa)</td>
<td>1966</td>
</tr>
</tbody>
</table>

4.2.3.3. Mutational analysis:

It was carried out for 8 out of 9 samples, because Sample Ck 403 couldn’t be translated.

4.2.3.4. Protein modelling:

Template search with Blast and HHBlits has been performed against the SWISS-MODEL template library. (SMTL, last update: 2015-12-03, last included
PDB release: 2015-11-27) The target sequence was searched with BLAST (Altschul et al., 1997) against the primary amino acid sequence contained in the SMTL. A total of 20 templates were found. An initial HHblits profile has been built using the procedure outlined in, (Remmert, et al., 2012) followed by 1 iteration of HHblits against NR20. The obtained profile was then searched against all profiles of the SMTL. A total of 14 templates were found.

For each identified template, the template's quality has been predicted from features of the target-template alignment. The templates with the highest quality have then been selected for model building i.e 3n43 was used as a template for model building.

Models are built based on the target-template alignment using Promod-II. Coordinates which are conserved between the target and the template are copied from the template to the model. Insertions and deletions are remodelled using a fragment library. Side chains are then rebuilt. Finally, the geometry of the resulting model is regularised by using a force field. In case of loop modelling with ProMod-II (Guex, et al., 1997) does not give satisfactory results, an alternative model is built with MODELLER (Sali, et al., 1993).

The global and per-residue model quality has been assessed using the QMEAN scoring function (Benkert, et al., 2011). For improved performance, weights of the individual QMEAN terms have been trained specifically for SWISS-MODEL.
4.2.3.5. Mutation mapping:

Positions of three observed mutations in E1 protein was mapped in current isolates by using PyMOL software. The sequence positions were labelled according to reference strain S-27.

4.3. Statistical analysis

The data was compiled and analysed using Statistical Package for Social Services (SPSS vs 21). The categorical data was presented using frequencies and percentages. Chi-square test was used a test of significance. The quantitative variables were presented by using measures of central tendency. Independent sample t test was used as test of significance. A p value of less than 0.05 was considered statistically significant result. (Bryman et al., 2011)