4.1 Ethical considerations
The study was approved by the Institutional Ethics Committee of the Regional Medical Research Centre (Indian Council of Medical Research), Dollygunj, Port Blair, Andaman and Nicobar Islands, India.

4.2 Outbreak investigations
Blood specimens from patients suspected with CHIKF during July – September 2006 were collected. Serum were separated by centrifugation at 3000 rpm for 10 minutes and transferred into sterile cryovial, transported in cold chain to the serum bank of the RMRC (ICMR), Port Blair. The collected serum samples were kept frozen at -80°C until further processing. Isolation of CHIKV was attempted at National Institute of Virology (NIV) ICMR, Pune, India and RMRC (ICMR) from serum samples, collected during 2006 outbreak and stored in RMRC serum bank.

Since there were no systematic follow up study and non-availability of chronic clinical samples from 2006 outbreak due to difficulties in access of study subjects in the geographical nature of Andaman and Nicobar Islands. Therefore, to fulfil the study to understand the natural history of acute as well as chronic CHIKV infection, the outbreak of CHIKV infection near Mangalore in Dakshina Kannada District of Karnataka, India was followed systematically. The following section would describe the studies undertaken in “acute” and “chronic” stages of the infection.

4.3 Isolation and molecular characterization of Chikungunya virus
Isolation of CHIKV was attempted from acute sera of patients showing clinical manifestations of CHIKF during the 2006 outbreak in Port Blair. Isolation of CHIKV was simultaneously attempted at NIV (ICMR) as well as RMRC (ICMR), Port Blair.

C6/36 cell line: C6/36 cell line is a continuous cell line, derived from Aedes albopictus mosquitoes (Igarashi 1978). In the present study C6/36 cell lines were maintained in the form of monolayers in T-75 cm² flasks (Nunc clone, Germany) with Mitsuhashi Maramorosch medium (Appendix 1.5) supplemented with 10% fetal bovine serum (Appendix 1.2) and antibiotics (Penicillin 200U/ml and Streptomycin 20ug/ml) (Appendix 1.1). To passage the cell line, the cell line monolayer was washed with
Trypsin–PBS-Versene-Glucose (TPVG) (Appendix 1.4). TPVG was completely removed and the flask was kept at 37°C for 2 to 3 minutes. The detached cells were re-suspended in Mitsuhashi Maramorosch medium and seeded in to new 75-cm² flasks with split ratio of 1:4 or 1:6 with sealing density, which having 1 million cells/ml and incubated at 28°C. The cells grown in a similar manner in T-25 cm² flasks were used for the propagation of Chikungunya virus.

Sample preparation: Sample preparation was done as 1 ml of 1 in 10 (100 µl serum with 900 µl of maintenance medium) dilution using MM medium with 2% FBS to inoculate in C6/36 cell line. The complete sample preparation procedures were performed under cold chain using ice tray. The virus isolation procedures were held in Class-II B2 Biosafety cabinet in Bio Safety Level-3 (BSL-3) facilities.

Inoculation of specimens into cell culture: Isolation was attempted in C6/36 cell line, the confluent monolayer was grown in T-25 cm² flask. The diluted sample in 1 ml maintenance medium was inoculated into the C6/36 monolayer of T-25 cm² flask, after removing the existing growth medium and incubated in horizontal position at 28 (C for 2 hours with intermittent shaking of the flask. At the end of primary incubation 3 ml of maintenance medium were added additionally and the incubation continued at 28°C. Cell culture fluid was collected on 5th day after inoculation and serial blunt passage was also carried out to increase the sensitivity for the isolation of Chikungunya virus.

4.3.1 Observation of cytopathic effect (CPE)
Culture supernatant was inoculated into confluent monolayer of Vero cell line. The sample preparation was done as 1 ml of 1 in 10 (100 µl sample + 900 µl of maintenance media) dilution Minimum Essential Medium (MEM) with 2% FBS to inoculate. The diluted sample in 1 ml of maintenance medium (MEM with 2% fetal bovine serum) were inoculated into the Vero cell monolayer of T-25 cm² flask after removing the existing growth medium and incubated in horizontal position at 37 (C for 2 hours by shaking the flask in between, at the end of primary incubation approximately 5 ml of maintenance medium was added and incubated at 37°C with 5 % CO₂ in maintenance medium. Cell lines were observed under inverted phase contrast microscope for CPE from day one post inoculation and cells were observed daily for evidence of typical
CHIKV CPE. Serum inoculated tissue culture fluids, which showed positive for cytopathic effect in Vero cell lines were subjected to indirect immunofluorescence assay.

### 4.3.2 Indirect immunofluorescence (IFM) assay

C6/36 cells were seeded on sterile 18 mm X 19 mm coverslip which was kept in the well of 6 well tissue culture plates and incubated overnight at 28°C. Cell monolayer was infected with the tissue culture infected cell supernatant after the preparation of 1 ml of 1 in 10 dilution using MM medium with 2% FBS. The inoculated C6/36 cell monolayer was incubated at 28°C for 3-4 days. The coverslip was removed, rinsed with PBS and fixed with ice cold acetone (Appendix 10) for 15 minutes. The cover-slip was washed in PBS and blocking was done using PBS with 1% bovine serum albumin for 1 hour at 37°C. After 3 washes in PBS for 15 minutes, the cells on the coverslip were incubated at 37°C for 1 hour with the specific antibody (1 in 100 dilution) against CHIKV developed in mouse (received from Dr. Muthumani, University of Pennsylvania, Philadelphia, U.S.A.). Coverslip was washed with PBS for thrice and further incubated with anti-mouse IgG detection antibody tagged with Fluorescein Iso-thiocyanate (FITC) (SIGMA) at 37°C for 1 hour.

Then the coverslip was washed once again in PBS. Finally the coverslip containing cells were counter stained with Evan’s blue (Appendix 3) for 5 – 10 minutes. Then the coverslip was washed two times in PBS and rinsed once in distilled water, dried and mounted with 1:1 PBS (Appendix 12) and glycerol mix on a glass slide. The cells were visualized under a fluorescence microscope (AX40, Zeiss) and readings were recorded. Apple green fluorescence observed under the microscope was considered as test positive. All cell culture fluids, which showed positive for indirect immunofluorescence microscopy assay, was subjected to further confirmation by RT-PCR.

### 4.3.3 RT-PCR assay

**RNA extraction and RT-PCR:** Genomic viral RNA was extracted from 140 μL of cell culture supernatant using the QIAamp Viral RNA Mini Kit (Qiagen), following the manufacturer's instructions, protocol in brief; 560 μl of buffer AVL with 5.6 μl of carrier RNA was pipetted into a 1.5 ml micro centrifuge tube and then 140 μl of serum
sample was added into the same tube. The tube was mixed for 15 seconds, incubated at room temperature for 10 minutes, then 560 µl of ethanol (96-100%) was added to the sample and mixed by pulse vortex for 15 seconds. Subsequently 630 µl of above prepared lysate was transferred to the RNA extraction column and centrifuged at 8000 rpm for 1 minute. A new 2 ml collection tube was placed to the column after discarding the collection tube with flow through. The step was repeated until the lysate was consumed.

Washing of column was performed by adding 500 µl of buffer AW1 and centrifuged at 8000 rpm for 1 minute and again a new collection tube was placed and discarded the old tube containing the filtrate. Subsequently second washing was carried out by 500 µl of Buffer AW2 added into the column and centrifuged at full speed (15000 rpm) for 3 minutes. The empty spin was carried out at full speed for 1 minute after placing a new 2 ml collection tube. As final elution step the column was fixed into a new 1.5 ml micro centrifuge tube for collection of RNA. Then 45 µl of buffer AVE was added and incubated at room temperature for 1 minute and centrifuged at 8000 rpm for 1 minute. The eluted RNA was stored at –20°C freezer until further use.

c-DNA Conversion: RNA templates were converted into cDNA using High capacity cDNA reverse Transcription kit (Applied Biosystems) following manufacturer’s instructions, as follows; Preparation of 2X RT master mix was done prepared were; 2.0 µl of 10X RT buffer, 0.8 µl of 25X DNTP, 2.0 µl of 10X Random primer, 1.0 µl of RNase Inhibitor, 1.0 µl of MultiScribe Reverse Transcriptase 50 U/µl and 3.2µl of Nuclease- free water. Along with this 10 µl of RNA was added. The total volume of 20 µl reaction mix containing 10 µl of 2X master mix and 10 µl of RNA templates were mixed and converted into cDNA with ABsystem cDNA kit following manufacturer’s instructions.
Step 1
25°C for 10 minutes

Step 2
37°C for 120 minutes

Step 3
85°C for 5 minutes

Step 4
72°C for 30 seconds

Step 5
72°C for 10 minutes

4°C Hold

Schematic representation of thermal cycle conditions

Polymerase chain reaction: The polymerase chain reaction (PCR) was carried out in 50 μl consisting 5 μl of cDNA, 10X PCR buffer with 1.5mM MgCl2, 2.5mM dNTPs each, 3U Taq polymerase (Bangalore Genei) and Forward and Reverse Primers at 0.5 μM concentration. PCR was carried out in Applied Bio systems AB-2720 Thermal cycler.

A similar protocol for amplification of nsP1 and E1 genes were followed. The previously published (Hasebe et al., 2002) Primer set 1-the genomic position is 228 to 581 (forward: CHIK/NSP1-S 5´-TAG AGC AGG AAA TTG ATC CC-3´ and reverse: CHIK/NSP1-C 5´- CTT TAA TCG CCT GGT GGT AT-3´) was used to amplify the NSP1 region. Primer set 2-the genomic position is 10246 to 10539 (forward: E1S 5´- TAC CCA TTC ATG TGG GGC-3´ and reverse: E1C 5´- GCC TTT GTA CAC CAT T-3´) was used to amplify the E1 region.
**Agarose gel electrophoresis**: Agarose gel electrophoresis is a technique used for the separation of DNA fragments according to size as they migrate through a gel matrix. Agarose is a highly purified polysaccharide isolated from seaweed. Its major constituent is a linear polymer of alternating D-galactose and 3,6-anhydro-L-galactose units. Bromo phenol blue (BPB), a ‘tracking dye’ in a loading buffer is mixed with DNA to make DNA loading easier and DNA migration visible. The phosphate groups in the DNA backbone carry negatively charged oxygen which gives a DNA molecule overall negative charge. In an electric current the negatively charged DNA moves towards the positive pole in the electrophoresis chamber. The DNA fragments were separated by size and visualized by staining with the fluorescent dye Ethedium bromide (EtBr) that intercalate between bases of DNA.

The PCR products obtained after amplification of CHIKV genes were resolved and analysed by electrophoresis using 2% agarose gel (appendix 5) prepared in 1X TAE buffer (Tris Acetate EDTA; Appendix 4.1) containing 3 µl EtBr (appendix 4.2). A 100 bp DNA ladder (1µg/L, Sigma) was used as DNA marker for estimation of size of the PCR products were visualized under UV-transilluminator (Bio-Rad) and photograph of the gels were recorded using gel documentation system (Bio-Rad).

**Gel extraction and purification of PCR products**: All RT-PCR products were excised from the gel and purified using QIAquick gel extraction kit (Qiagen, Germany). The QIAquick system uses spin column technology with the selective DNA binding properties of silica membrane. DNA is absorbed to the silica membrane in the presence of high concentration of salt while contaminants like unused primers, EtBr, molten agarose etc. pass through the column. After removal of impurities, the pure DNA was eluted in 10 µl of nuclease water.

The procedure in brief, three volumes of (eg. 300 µl) of QG (Qiagen) buffer were added to 1 volume (eg. 100 mg of gel slice) of gel containing excised DNA fragment. The incubation was carried out at 56°C for 15-20 minutes or until the gel was completely dissolved in QG buffer. Isopropanol (100 µl) was added in a proportion equal to gel volume (100 mg) and mixed properly. The mixture was applied to the
QIAquick column placed on a collection tube (provided in the kit) and centrifuged at 10,000 rpm for 1 minute at room temperature.

After discarding the flow through, 500 µl QG buffer was added to the column and centrifuged at 10,000 rpm for 1 minute at room temperature. The flow through was discarded and 750 µl of PE buffer were added to the column and incubated at room temperature for 5 minutes. The columns were centrifuged at 10,000 rpm for 1 minutes and the flow-through was discarded. In order to remove PE buffer completely, an additional spin at 13,000 rpm for 1 minute was given. The QIAquick columns were placed on the new collection tube and DNA was eluted in 10 µl of EB (Elution Buffer) by spinning the column at room temperature. The purified DNA was stored at -20°C until utilized.

4.3.4 Nucleotide sequencing
Purified PCR products were sequenced using ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit, V3.1. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with fluorescent dyes of different wavelengths of fluorescence and emission. The Terminator Ready reaction mix (TRRM) contains Adenine-Dye terminator labelled with dichloro (R6G), Guanine-Dye Terminator labelled with dichloro (R110), Cytosine-Dye Terminator labelled with dichloro (ROX), Thiamine-Dye Terminator labelled with dichloro (TAMARA), deoxynucleotide triphosphates (dATP, dCTP, dITP, dGTP, dUTP), AmpliTaq DNA polymerase, MgCl₂ and Tris-HCL buffer (pH 9.0) (Appendix 7).

Thermal cycling of the sequencing reactions creates and amplifies extension products that are terminated by one of the four dideoxynucleotides. In normal condition, the chain elongation takes place when the 5’ carbon of an incoming dNTP is joined to the 3’ carbon at the end of the chain by phosphodiester linkage. A dideoxy NTP lacks 3’ hydroxyl group necessary to form the linkage with an incoming nucleotide. Hence, the incorporation of a dideoxy NTP halts the chain elongation and incompletes the products of various lengths are created. The ratio of deoxynucleotides to dideoxy nucleotide is optimized to produce a balanced population of long and short extension products.
Preparation of cycle sequencing reaction mix: Two cycle sequencing reactions (one with forward and the other with reverse primer) were carried out of each PCR product. The composition of the reaction mix was as follows: 20 μl of reaction mix containing 4 μl of TRRM, 1 μl of forward/reverse primer (0.5 μM concentration), 1-5 μl of Template (Depending on the intensity of the PCR products) and 10 μl of nuclease free distilled water. PCR was carried out under following thermal cycling conditions:

![Thermal cycle conditions diagram]

Post cycle sequencing purification of PCR products: Post cycle sequencing reaction, the PCR products were purified using Ethanol/EDTA/sodium acetate method. The rational for purification was to completely remove unincorporated dye terminators prior to capillary electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with base calling. Ethanol/EDTA/sodium acetate precipitation is recommended to obtain signal from base 1. Sodium acetate (Appendix 6) precipitates the DNA and EDTA helps to stabilize extension products during precipitation, and wash out unincorporated dyes from the completed reaction.

The mix was prepared with 1 - 2 μl of 125 mM EDTA (Appendix 8), 10 μl of nuclease free water, 2–3 μl of 3M Sodium acetate and 50 μl of absolute Ethanol (Appendix 9) to 10 μl of cycle sequenced products, 12 μl of the mix was added and mixed properly. This was followed by addition and proper mixing of 52 μl of mix 2. The mixture was incubated for 15 minutes and centrifuged at 10,000 rpm for 30 minutes at room temperature. The supernatant was discarded and the tubes were centrifuged again at
10,000 rpm for 5 minutes at room temperature. Ethanol was removed completely by air drying the pellet at room temperature for 30 minutes. The pellet was reconstituted in 20 µl of Hi-Di™ formamide (Applied Bio-system, U.S.A). The denaturation of DNA was carried out at 95°C for 2 minutes followed by snap chilling on ice for a minute. The contents were mixed by using vortex mixture, spin and loaded in automated sequencer, ABI 3010 Genetic analyser (Applied Bio-system, U.S.A).

**Nucleotide sequences:** The nucleotide sequences were collected using an automated sequencer, ABI 3010 Genetic analyser (Applied Bio-system, U.S.A). The cycle sequenced products were subjected to capillary electrophoresis. During capillary electrophoresis, the extension products of the cycle sequencing reaction enter in to the capillary as a result of electrokinetic injection. A high voltage charge applied to the buffered sequencing reaction forces the negatively charged fragments into the capillaries. The extension products are separated by size. Shortly before reaching the positive electrode, the fluorescent labelled DNA fragments separated by size, move across the path of a laser beam. The laser beam causes the dyes on the fragments to fluoresce. An optical detection device detects the fluorescence. The data collection software converts the fluorescence signal to digital data and records the data. When excited by the laser each dye emits light at a different wavelength. All four bases labelled with four different colours can be detected and distinguished in one capillary injection.

**Assembling sequences:** The forward and reverse sequences collected from sequencer were checked manually in the electropherograms using the SeqMan II version 5.03 (DNASTAR). The checked and corrected nucleotide sequences of CHIKV strains were adjusted in FASTA format. The nucleotide sequences of same nsP1 and E1 genomic region of CHIKV from world over were retrieved from (www.ncbi.nlm.nih.gov/nuccore) National Centre for Bioinformatics (NCBI) database.

**4.3.5. DLST (Double locus sequence typing)**

The sequences of CHIKV both nsP1 and E1 sequences of each isolates were aligned together following DLST (Double Locus Sequence Typing) concept to increase the
sensitivity of genetic diversity. DLST data could be considered as similar to MLST (Multi Locus Sequence Typing) data (Kuhn et al., 2007).

Sequences aligned with worldwide diverse CHIKV sequences for phylogenetic and molecular evolutionary analyses by ClustalW multiple alignment and pair wise alignment for phylogenetic and subsequently analysed using Kimura two-parameters as a method of substitution and neighbour-joining to reconstruct the phylogenetic tree (MEGA version 4.1) (Tamura et al. 2007). The statistical significance of the relationships obtained was estimated by bootstrap resampling analysis (1000 repetitions).

In order to evaluate the sensitivity as well as specificity of DLST in contrast to SLST (Single Locus Sequence Typing), E1 and nsP1 sequences of Andaman CHIKV isolates with reference sequences was analysed following SLST method. Similarly the whole genome sequences of reference strains belong to various genotypes were also analysed to compare the efficiency of DLST (Whole genome of Andaman isolates was not included).

**Phylogenetic analysis:** Phylogenetic tree is a graphical representation of the evolutionary relationship between taxonomic groups. It is a specific type of cladogram (a branched diagram similar to family trees) in which the branch lengths are proportional to the predicted or hypothetical evolutionary time between organisms or their sequences. The phylogenetic trees depicted the evolutionary relationship between taxonomic groups were generated for all structural as well as non-structural gene sequences using molecular evolutionary genomic analyser software MEGA 4.1 (Tamura et al., 2007). Genetic distances were calculated by using the Kimura 2 parameter (K2P) model at the nucleotide level and phylogenetic trees were constructed by using neighbour-joining method.

The reliability of phylogenetic trees was tested by applying the bootstrap test with 1000 bootstrap replications. Bootstrapping is a method of testing the reliability of the dataset. In phylogenetic analyses non-parameteric bootstrapping is the most commonly used method. The pseudoreplicate dataset are generated by randomly sampling the original
character matrix to create new matrices of the same size as the original. The frequency
with which a given branch is found is recorded as the bootstrap proportion. These
proportions can be used as a measure of the reliability (within limitations) of individual
branches in the optimal tree.

Pairwise (P) distances between Andaman isolates as well as reference identified in the
present study was calculated by using K2P distance and pairwise method in MEGA 4.1
software. The corresponding percentage of homologies were calculated using a K2P
distance converter. The K2P distance was also calculated for whole genome of CHIKV
from different part of the world.

<table>
<thead>
<tr>
<th>S.No</th>
<th>CHIKV isolate</th>
<th>Genotype</th>
<th>Genbank Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>nsP-1</td>
</tr>
<tr>
<td>1</td>
<td>CHIKV JG-05</td>
<td>ECSA</td>
<td>JN578240</td>
</tr>
<tr>
<td>2</td>
<td>CHIKV JG-03</td>
<td>ECSA</td>
<td>JN578241</td>
</tr>
<tr>
<td>3</td>
<td>CHIKV JG-O7</td>
<td>ECSA</td>
<td>JN578242</td>
</tr>
<tr>
<td>4</td>
<td>CHIKV DF02</td>
<td>ECSA</td>
<td>JN578243</td>
</tr>
<tr>
<td>5</td>
<td>CHIKV H-8</td>
<td>ECSA</td>
<td>JN578244</td>
</tr>
<tr>
<td>6</td>
<td>CHIKV DEL-7</td>
<td>ECSA</td>
<td>JN578245</td>
</tr>
<tr>
<td>7</td>
<td>CHIKV DEL-2</td>
<td>ECSA</td>
<td>JN578246</td>
</tr>
<tr>
<td>8</td>
<td>CHIKV DEL-03</td>
<td>ECSA</td>
<td>JN578247</td>
</tr>
<tr>
<td>9</td>
<td>CHIKV H6</td>
<td>ECSA</td>
<td>JN578248</td>
</tr>
</tbody>
</table>

* ECSA: East Central and South African genotype

Table 1: List of CHIKV isolates obtained from 2006 Outbreak in Port Blair
and their NCBI Genbank Accession number.
4.3.6 Molecular evolution of DEL-03 CHIKV isolate

The attempt for molecular analysis of the first CHIKV isolate DEL-03 was made to understand the changes in the genetic makeup of the CHIKV, which was associated with acute flaccid limb weakness as well as severe chronic arthropathy during the first ever confirmed outbreak in these Islands in the urban region of Port Blair during the 2006 outbreak.

Primer design: Primer is a short synthetic oligonucleotide that is used in the molecular techniques such as PCR, DNA sequencing and etc. Different sets of primers for the major proteins such as nsP1, nsP2, nsP3, nsP4, Capsid, E2 and E1 in CHIKV were made. The approximate length of amplicons was ranging 500 - 700 bp. The primers were designed based on the alignment of sequences available in NCBI Genbank. The following parameters were considered during primer designing.

1. Length of the primer : 18 – 24 mer
2. Base composition : G+C content 50 – 60%
3. The optimum range for annealing temperature: 45 -65°C
4. Prevention of primer-dimer synthesis by avoiding complementary nature of 3`- ends of primers
5. Choosing primer end (3`) with G or C, or CG or GC to increase the efficiency of priming. Series of three or more Cs or Gs at the 3`-end of primers may promote mispriming (because of stability of annealing), hence it needs to be avoided.
## Table 2: List of in-house designed primers used for molecular evolution of CHIKV.

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sense</th>
<th>Oligo-nucleotide sequence</th>
<th>Gene Location</th>
<th>Gene</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nsp1f</td>
<td>+</td>
<td>WTGGCTGCGTGAGACACACGT</td>
<td>Nsp1</td>
<td>1-21</td>
<td>750 bp</td>
</tr>
<tr>
<td>Nsp1r</td>
<td>-</td>
<td>AACTTGCTCTCGTCTACCTTCGT</td>
<td></td>
<td></td>
<td>728-750</td>
</tr>
<tr>
<td>Nsp2f</td>
<td>+</td>
<td>TACCTGGTACTCTCCCCGCAGA</td>
<td>Nsp2</td>
<td>1751-1772</td>
<td>730 bp</td>
</tr>
<tr>
<td>Nsp2r</td>
<td>-</td>
<td>TCTGCCTTGGTCTCAACAAGGC</td>
<td></td>
<td></td>
<td>2480-2501</td>
</tr>
<tr>
<td>Nsp3f</td>
<td>+</td>
<td>CGGCTGTGGATATGGCGGAGAT</td>
<td>Nsp3</td>
<td>4701-4722</td>
<td>691 bp</td>
</tr>
<tr>
<td>Nsp3r</td>
<td>-</td>
<td>TGTACGACCGGACACAGCTCTTG</td>
<td>Nsp3</td>
<td>5370-5391</td>
<td>691 bp</td>
</tr>
<tr>
<td>Nsp4f</td>
<td>+</td>
<td>TGTCAGAGACCCCCAAAAGTCCCTAC</td>
<td>Nsp4</td>
<td>5931-5955</td>
<td>760 bp</td>
</tr>
<tr>
<td>Nsp4r</td>
<td>-</td>
<td>ACATTGGGTAAGGAGACGCGGT</td>
<td>Nsp4</td>
<td>6669-6690</td>
<td>834 bp</td>
</tr>
<tr>
<td>Capsidf</td>
<td>+</td>
<td>TCGAGAAGCTCAGAGGACCCGT</td>
<td>Capsid</td>
<td>7452-7473</td>
<td>834 bp</td>
</tr>
<tr>
<td>Capsidr</td>
<td>-</td>
<td>AGGGCTGTACGGGCTCCTCTCAT</td>
<td>Capsid</td>
<td>8264-8285</td>
<td>834 bp</td>
</tr>
<tr>
<td>E2f</td>
<td>+</td>
<td>TGTCACAACAGTGCGGCAACGT</td>
<td>E2</td>
<td>9083-9104</td>
<td>606 bp</td>
</tr>
<tr>
<td>E2r</td>
<td>-</td>
<td>GCTGCCATACCCACCATCGACA</td>
<td>E2</td>
<td>9677-9688</td>
<td>606 bp</td>
</tr>
<tr>
<td>E1f</td>
<td>+</td>
<td>AATGAGCGTGCGGGCCACA</td>
<td>E1</td>
<td>9850-9870</td>
<td>561 bp</td>
</tr>
<tr>
<td>E1r</td>
<td>-</td>
<td>CCTTGGTAAAGGACGGAGGC</td>
<td>E1</td>
<td>10389-10410</td>
<td>561 bp</td>
</tr>
</tbody>
</table>
One step Reverse Transcription Polymerase Chain Reaction (RT-PCR): Single passage virus and high-fidelity PCR enzymes were used to minimize experimentally induced genetic alterations in whole genome analysis. The Qiagen One-Step RT-PCR Kit was used for the amplification of the CHIKV genes. The Qiagen one step RT-PCR Enzyme mix contains an optimized combination of Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase and Hot-start Taq DNA polymerase.

Omniscript and Sensiscript Reverse Transcriptase are new, unique enzymes and are different from the reverse transcriptase of Maloney murine leukemia virus (MMLV) or avian myeloblastosis virus (AMV). Omniscript and Sensiscript Reverse Transcriptase are recombinant heterodimeric enzymes expressed in E. coli. HotStart Taq DNA polymerase is a modified form of a recombinant 94-kDa DNA polymerase (deoxynucleotide-triphosphate: DNA deoxynucleotidyl transferase, EC 2.7.7.7), originally isolated from Thermus aquatics expressed in E. coli.

The Qiagen One-Step RT-PCR Kit provides a blend of Sensiscript and Omniscript Reverse Transcriptases, HotStarTaq DNA Polymerase, Qiagen OneStep RT-PCR Buffer, a dNTP mix, and Q-Solution, a novel additive that enables efficient amplification of "difficult" (e.g., GC-rich) templates. The buffer ensures specific primer annealing over a wide range of temperatures and Mg\(^{2+}\) concentrations; providing robust and highly efficient RT-PCR from any RNA template.

PCR Reaction: In order to amplify the genes of CHIKV, RT-PCR assay was carried out on RNA isolated from the first CHIKV isolate DEL03 using the Qiagen mini preparation kit following the manufacturer`s instructions, (discussed in the section 4.3.3). The RT-PCR mix was prepared as per manufacturer’s instructions.

The composition of the reaction mix was prepared as follows: 50 µl of reaction mix containing 10.0 µl of 5X Qiagen OneStep RTPCR buffer, 2.0 µl of dNTP mix (containing 10 mM of each), 2.0 µl of 10 µM Forward Primer, 2.0 µl of 10 µM Reverse Primer, 2.0 µl of Qiagen OneStep RT-PCR Enzyme mix, RNase-free water (Provided with the kit) 28.0 µl and template RNA or negative control 4.0 µl. The RT-PCR
conditions involved an initial reverse transcription step of 30 minutes at 45°C followed by PCR activation and amplification.

Schematic representation of thermal cycle conditions

Agarose gel electrophoresis: The PCR products obtained after amplification of *Chikungunya virus* genes were resolved and analysed by gel electrophoresis using 2% agarose gel to confirm the amplification of the gene fragment (described in the section 4.3.3).

Gel extraction and purification: Gel extraction and purification of all the PCR products were completed using QIAquick gel extraction kit (Qiagen). The protocol was following the manufacturer's instructions (described in the section 4.3.3). After removal of impurities the pure cDNA was eluted in the elution buffer.

Nucleotide sequencing: Both strands of purified PCR products were sequenced using ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit, following the manufacturers fragment (described in the section 4.3.4). The cycle sequenced products were subjected to capillary electrophoresis and the nucleotide sequences collected using an automated sequencer, ABI 3010 Genetic analyser (Applied Bio-system, U.S.A).

Assembling sequence: The forward and reverse sequences collected from sequencer were checked manually in the electropherograms using the chromaspro software as well as MEGA4.1 (Tamura *et al*. 2007). With the help of reference sequences of
CHIKV ECSA genotype from various part of the country India as well as Re union isolates the CHIKV isolate DEL03 was checked and corrected.

Identification of unique nucleotide polymorphism: In order to understand the molecular characters of CHIKV isolate obtained from 2006 outbreak was responsible for acute flaccid limb weakness and chronic arthropathy, critical analysis was made along with the reference sequences, which coding respective major proteins (nsP1, nsP2, nsP3, nsP4, Capsid, E2 and E1) to find the presence of unique nucleotide change in the sequence of Chikungunya virus. The pairwise and multiple alignments was done using the ClustalW alignment algorithm of Mega 4.1. The nucleotide sequencer was observed for the presence of unique nucleotide change if any in the first CHIKV isolate from Andaman which was not reported earlier.

Open Reading Frame (ORF) finding: The ORF of the major proteins such as nsP1, nsP2, nsP3, nsP4, Capsid, E2 and E1 genome of DEL-03 CHIKV isolate was found using NCBI ORF Finder, which is a graphical analysis tool which finds all open reading frames of a selectable minimum size in a user's sequence or in a sequence already in the database. This tool is helpful to identify all open reading frames using the standard or alternative genetic codes. The output was received as deduced amino acid sequence, which can be saved in various formats and searched against the sequence database using the NCBI BLAST server. Further the proper coding region of the sequence was confirmed in comparison with CHIKV protein sequences of NCBI Genbank.

Identification of unique aminoacids: Similarly the assembled sequences coding nsP1, nsP2, nsP3, nsP4, Capsid, E2 and E1 of DEL03 CHIKV isolate was further analysed for the presence of unique amino acid change if any, which has not been reported in the sequences available at NCBI Genbank. Using various protein sequences (ORFs) of CHIKV in MEGA 4.1 alignment explorer the existing unique amino acid changes corresponding to the positions of unique nucleotide change in DEL03 isolate was explored to confirm the mutation of the first CHIKV isolate in Andaman Islands.

Phylogenetic Analysis: The phylogenetic analysis was done following single locus sequence typing as well as multi locus sequence typing protocols of DEL03 CHIKV
isolate with the reference sequences of CHIKV available from different part of the country as well as the world. The analysis was performed using MEGA4.1, following the pairwise and multiple alignment using the tool ClustalW and phylogenetic tree was made. The analysis was done using nsP1, nsP2, nsP3, nsP4 Capsid, E1 and E2 coding partial gene sequences following SLST as well as MLST concepts to understand the significance and also to observe the measures of reliability of various genes and its uniqueness in molecular evolution of CHIKV.

4.4 Follow up study to understand clinical progression
As described in the earlier section (4.2), natural history could not be studied particularly the severity of the infection in acute as well as the chronic stage of the illness. Therefore, to accomplish understanding the natural history of acute as well as chronic CHIKV infection, the outbreak in Dakshina Kannada District of Karnataka, India (Manimunda et al., 2010) were systematically followed with different cohorts.

The outbreak of chikungunya fever started from January 2008 in Dakshina Kannada district, Karnataka, India (Manimunda et al., 2010). The detailed information regarding number of suspected cases of CHIKF reported to the primary health centre (PHC) during the period of January to September 2008 was obtained from the records of PHC.

A cross-sectional survey was covered among the people residing in the jurisdictional area of a PHC in Adyanadka, Dakshina Kannada District of Karnataka state to estimate the magnitude of the epidemic and the proportion of CHIKV infections that remained clinically in-apparent (http://www.kar.nic.in/zpdk/district_profile.htm).

Presences of various symptoms were recorded and the CHIKV infections were confirmed by IgM Capture ELISA. The follow up study was carried out with the new cohort having confirmed subjects. The study duration was from June 2008 to April 2009 at this PHC (Adyanadka) level. The Adyanadka PHC jurisdiction has two villages. Based on PHC statistics, about 2,000 people had suffered from suspected CHIKF by mid-August. In addition to X-ray, MRI and serological investigations viz., IgM capture ELISA, cytokine profile and anti-Cyclic Citrullinated Peptide test (anti-CCP), Erythrocyte Sedimentation Rate (ESR) and Rheumatoid Factor (RF) were
carried out. The confirmed CHIKV infected subjects serum sample from acute, chronic as well as recovered subjects were included in the study to assess the role of various cytokines and chemokines.

4.4.1 Enrolment of patients and inclusion criteria
The recruitment was carried over 5 days: 1 day at the PHC and 1 day at each sub-centre, during the third week of June 2008. Any patient aged 10 years or more reporting to the health facility with fever or joint pain or both was eligible to be included in the study. Informed consent was taken from all the patients and parents/guardians of children. The proportion of persons suspected to have suffered CHIKF (age- and sex-specific attack rate of suspected CHIKF) was calculated for each age and sex strata.

4.4.2 Follow-up during first month of illness and confirmation of diagnosis
The number of suspected cases of CHIKF reported to the PHC during the period between January and September 2008 by month of reporting was obtained from the records of PHC. A cross-sectional survey was carried out among a sample population of 1,174 living in 300 households drawn from all the four sub-centre areas (75 households in each sub centre area) of the PHC. In each sub-centre area, a systematic sampling method was followed. Each household obtained from the enumeration list maintained at PHC served as sampling frame. The first house to be surveyed in each sub-centre area was selected at random from the sampling frame and then, every 10th house was included in the sample. Those who suffered from fever, joint pain, or both during the epidemic period were considered as suspected cases of CHIKF. The study population was stratified into five age strata [i.e., < 13 years (children), 13–19 years (teenagers), 20–29 years, 30–44 years, and ≥45 years (elderly)].

Structured questionnaire: A structured questionnaire was used for eliciting necessary information. A clinician interviewed the patients on the first day of illness at the health facility and on subsequent days of follow-up (days 2, 3, 7, 14, 21, 30) at their houses. Temperature was recorded on all these days. Details regarding personal identification and demographic details, pre-existing joint pain and its nature, other pre-existing diseases, onset, evolution and duration of various symptoms and the joints involved were taken. The symptoms other than systemic/non-systemic which has not been included in the structured part of the questionnaire were also recorded.
Specimens: Blood specimens were collected from these subjects between July to September 2008. Serum was separated by centrifugation and transferred into sterile cryo-vial. The collected serum samples were transported in cold chain to the RMRC serum bank and kept frozen at -80°C until processed. The presence of anti-CHIKV immunoglobulin M (IgM) antibody was tested by the IgM-capture enzyme-linked immuno-sorbent assay (ELISA) method using the kit developed by the National Institute of Virology (NIV, Pune, India).

Laboratory Diagnosis of CHIKV: Initially all the collected serum specimens were subjected for the laboratory diagnosis to confirm the CHIKV outbreak in Mangalore. Blood samples were tested for CHIKV specific IgM antibodies using IgM Capture ELISA kit obtained from NIV Pune, India (Hundekar et al., 2002; Yergolkar et al., 2006).

Test protocol in brief, the sera of patients were diluted with sample dilution buffer in to 1:100 dilutions. The anti-human IgM coated wells were washed thrice with 200 µl of wash buffer and transferred 50 µl of diluted samples to the appropriate wells. The positive control and negative control were also included. The plate was kept in a humidified box (A bread box with a soaked cotton/tissue paper) and incubated the plate at 37°C for 1 hour.

At the end of incubation, the plate was washed five times with 300 µl of wash buffer and added 50 µl of CHIKV antigen to each well. The plate was kept in a humidified box and incubated the plate at 37°C for 1 hour. At the end of incubation, the plate was washed five times with 300 µl of wash buffer and added 50 µl of CHIK-B Monoclonal antibody to each well. The plate was again kept in a humidified box and incubated the plate at 37°C for 1 hour. At the end of incubation, the plate was washed five times with 300 µl of wash buffer and added 50 µl of Avidin-HRP to each well and again kept the plate in a humidified box and incubated the plate at 37°C for 30 minutes.

The washing step was repeated thrice subsequently 100 µl of substrate (TMB/H₂O₂) added to each well. The plate was incubated in dark at room temperature for the
development of colour, which normally develops in 10± 1 minutes and the reaction was stopped with 100 µl of 1N H₂SO₄ and measured the absorbance at 450 nm within 10 minutes (Yergolkar et al., 2006; Hundekar et al., 2002). A known positive (P) and a known negative (N) serum control were used in every test. A test was considered positive when the test optical density (OD) was ≥ 2.1 times the negative control OD. The in-house validation of this assay was carried out at NIV which showed a high specificity of 96.5% (A. Sudeep, personal communication). The acute sera were stored for cytokine analysis.

4.5 Investigations to study the joint pathology

4.5.1 X-ray and MRI

During the tenth month of illness all the laboratory-confirmed patients with CHIKV infection were examined by the same clinician. The persisting chronic symptoms and signs were recorded. Between the second and ninth month the patients were under the care of the PHC medical officer.

A subpopulation of those who were still suffering from joint pain following CHIKV infection were chosen for X-ray and MRI imaging of joints. The selection criteria were joint pain of grade more than II in at least one joint of the body and the willingness to undergo the procedures (D’Cruz 2002). In each patient one joint having severe pain was selected and X-ray and MRI was done on the same joint.

4.5.2. Erythrocyte sedimentation rate

The Erythrocyte Sedimentation Rate of all the patients who were suffering from chronic joint pain following CHIKV infection was estimated by the Westergren method. Protocol in brief: The blood sample with sodium citrate were collected from the chronic patients and tested for the erythrocyte sedimentation rate. Samples were tested within two to six hours after collection of blood and it was diluted with 31.3 gm/l trisodium citrate (Appendix 11) in the proportion of one volume of citrate to four parts of blood. Blood was mixed properly and filled into a clean dry Westergren tube. Tube was placed in a stand in a vertical position without leakage and undisturbed for 60 minutes. After one hour the reading was taken above the upper margin of the column of sedimenting cell to the nearest millimetre. Report was made with the measurement as the ESR (Westergren) in units of mm in 1 hour (Perkins et al., 1999).
4.5.3. Anti-cyclic citrullinated peptide (anti-CCP)

Among all the patients for whom X-ray and MRI were taken, the presence of anti-cyclic citrullinated peptide (anti-CCP) antibody was tested by ELISA to rule out the presence of rheumatoid factor (RF). The DIASTAT™ Anti-CCP test is qualitative enzyme-linked immunosorbent assay (ELISA) was used for the detection of the IgG class of auto antibodies specific to cyclic citrullinated peptide (CCP) in serum samples.

Samples were diluted in 1:100 in sample dilution buffer; 100 µl of diluted sample were added into the 96 well flat bottom micro-titre plated coated with a highly purified synthetic cyclic peptide containing modified arginine residues along with the standards (with our any dilution as per the manufacture's instructions) along with positive and negative control provided with the kit. Plate was incubated for 60±10 minutes at 18-25°C, followed by washing the plate with wash buffer for 3 times to remove the unbound material in the well.

The 100 µl conjugated antibody was added to each well and incubated for 30±5 minutes at 18-25°C, followed by washing the plate 3 times with the wash buffer, then 100 µl of substrate provided with the kit was added and incubate for 30±5 minutes at 18-25°C. Subsequently 100 µl of stop solution to each well and read the absorbance at 450 nm. The amount of conjugate bound was measured in absorbance units along with the reference controls. The concentration of anti-CCP autoantibody was estimated by interpolation from a dose-response curve based on the Standards. Calculations were made with the absorbance value (Optical Density) ratio for the Positive and Negative Controls and for each sample <0.95 considered as Negative >1.0 Positive as per the manufactures instructions.

4.5.4 IgM antibodies among follow-up patients

Samples collected during follow up study of CHIKV infected chronic as well as recovered patients were also tested for anti-CHIKV IgM antibodies to know the persistence of IgM antibody in chronic patients from 30th day, 60th day, 90th day and up to 300 days using IgM capture ELISA kit developed by NIV Pune, India.
Data analysis: During the tenth month of illness, the prevalence of various symptoms and 95% CIs were calculated for all laboratory-confirmed patients during the first and the tenth month of illness as well as for a proportion of patients from the original cohort suffering from any symptoms/signs attributable to CHIKV infection. During the first month of illness the prevalence of symptoms was calculated among age subgroups of patients and statistical significance of the difference in the prevalence of symptoms in different age groups was tested by \( \chi^2 \) test. The median day of appearance of each symptom and the median duration of the presence of various symptoms that subsided within a month were calculated.

4.6 Assessment of various cytokine in acute and chronic CHIKF patients

In continuation with the above study, to further understand the immunobiology behind the chronicity that leads to bony erosion in patients with CHIKV infection, attempts were made to assess the role of various cytokines in the pathogenesis of CHIKV arthropathy by studying the levels of these cytokines in the serum of patients who had different clinical outcomes after CHIKV infection.

Study subjects: Patients were grouped into different cohorts with varying severity and chronicity of symptoms. Twenty-two patients from this cohort were included in the present study and were divided into three groups based on their stage of illness and chronicity of symptoms. Six patients who were having acute illness formed group 1 and another six patients who had acute illness but had recovered without chronic manifestations formed group 2. Group 3 included 10 patients with chronic joint pain that persisted up to 10 months post-infection. In addition, six apparently healthy individuals negative in CHIKV IgM ELISA were included as controls.

4.6.1. Multiplex cytokine assay

The concentration of 30 parameters including cytokines, chemokines and growth factors present in the serum samples were estimated using Human Cytokine 30 plex Kit (Invitrogen, Catalogue no: LHC6003, USA) following the procedure prescribed by the kit manufacturer. Beads with defined spectral properties conjugated to analyte (cytokine)-specific capture antibodies and 25 μl of 1:2 diluted serum samples were pipetted into the wells of a micro-titre plate supplied with the kit.
Standards of known analyte concentrations were included for each parameter assayed. The plate was incubated for 2 hours to allow binding of the capture antibodies to the specific cytokine. After washing the beads, analyte-specific biotinylated detector antibodies were added and incubated with the beads for 1 hour. During this period, the analyte-specific biotinylated detector antibodies recognized their epitopes and bound to the appropriate immobilized analyte.

After the removal of excess biotinylated detector antibodies, streptavidin conjugated to the fluorescent protein, R-phycoerythrin (Streptavidin-RPE), was added and incubated for 30 minutes to form a four-member solid phase sandwich. The levels of cytokines were analysed using the Luminex 100™ instrument. The cytokines that were assayed included GM-CSF, IL-1β, IL-1 RA, IL-6, IL-8, TNF-α, IFN-γ, IL-2, IL-2 R, IL-4, IL-5, IL-10, IFN-α, IL-7, IL-12p40/p70, IL-13, IL-15, IL-17, Eotaxin, IP-10, MCP-1, MIG, MIP-1α, MIP-1β, RANTES, EGF, HGF, FGF-basic, G-CSF and VEGF.

Statistical analysis: The distributions of the parameters between the four groups of study subjects were studied using the dot plots with median values. The difference in the median values of the parameters between pairs of groups was tested using non-parametric test (Mann Whitney U Test). P values <0.05 were considered statistically significant. Data presented as the mean± SEM calculated from triplicate samples from each experimental group.