5.1 Virus isolation

Isolation of CHIKV was attempted in sera collected from clinically suspected CHIKF subjects during the 2006 epidemic in Port Blair. Acute patients serum samples were inoculated into a monolayer of an *Aedes albopictus* clone C6/36 cell lines and were incubated at 28°C in Mitsuhashi Maramorosch media (Mitsuhashi and Maramorosch 1964; Sivaram *et al.*, 2010). Subsequently the culture supernatant was subjected to blunt passage.

The tissue culture supernatant collected from flask containing C6/36 monolayer was inoculated into the monolayer of Vero cell line to observe for CPE under inverted microscope. CPE development was observed between 48–96 h post inoculations on Vero cell line monolayer. The CPE was characterized by rounding of cells, increased granularity and vacuolation, followed by cell death and disruption of the monolayer by detachment of the dead cells (Figure 5a) but no cytopathic effect was observed on uninfected (control) Vero cell monolayer (Figure 5b).

5.1.1 Indirect immunofluorescence (IFM) assay

Virus specific fluorescence as apple green colour was observed in 9 out of 9 tissue culture supernatant inoculated on C6/36 cells grown on cover glass. But there were no such fluorescence of uninfected control cells, alternatively red colour of counter stain (Evans blue) was observed. This was presumptively confirmed the presence of CHIKV in those corresponding serum inoculated tissue culture supernatant.

5.1.2 Reverse transcription PCR assay

RNA isolation and RT-PCR amplification from the culture supernatant further confirmed the presence of the virus. In RMRC, Port Blair 9 (37.5 %) CHIKV isolates were obtained out of 24 clinical samples from subjects showing clinical symptoms particularly 20 cases of severe arthropathy and 4 cases of acute flaccid limb weakness of CHIKF and 6 isolates were obtained out of 17 (35.2 %) suspected patients sera samples at NIV, Pune. Unfortunately, none of the isolate was obtained from the cases with acute flaccid paralysis during the 2006 outbreak in Port Blair.
Figure 5: a) Control Vero cell monolayer; b) Vero cell monolayer with CHIKV specific cyto-pathic effect.
**Figure 6:** Immunofluorescent microscopic view of CHIKV infection in C6/36 cell line.

**Figure 7:** Amplicons of non-structural Protein (nsP1) and Envelope-1 (E1).
<table>
<thead>
<tr>
<th>S.No</th>
<th>Patient ID</th>
<th>Location (Port Blair)</th>
<th>Age &amp; sex</th>
<th>Duration of illness</th>
<th>IgM</th>
<th>Signs and symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>JG-03</td>
<td>Junglighat</td>
<td>29/F</td>
<td>3 days</td>
<td>Negative</td>
<td>High grade fever, limitation of movements and joint pain.</td>
</tr>
<tr>
<td>2</td>
<td>JG-05</td>
<td>Jungleghat, Prem nagar</td>
<td>27/M</td>
<td>3 days</td>
<td>Negative</td>
<td>High grade fever, sore throat, tenderness over joints, joint pain and muscle tenderness.</td>
</tr>
<tr>
<td>3</td>
<td>JG-07</td>
<td>Jungleghat, Prem nagar</td>
<td>15/M</td>
<td>2 days</td>
<td>Negative</td>
<td>High grade fever, sore throat, tenderness over joints and joint pain.</td>
</tr>
<tr>
<td>4</td>
<td>DF-02</td>
<td>Dairy Form</td>
<td>30/F</td>
<td>2 days</td>
<td>Negative</td>
<td>High grade fever and joint pain.</td>
</tr>
<tr>
<td>5</td>
<td>H-8 (H-18)</td>
<td>Haddo</td>
<td>34/F</td>
<td>2 days</td>
<td>Negative</td>
<td>High grade fever, sore throat, tenderness over joints, joint pain, maculopapular rashes, muscle tenderness, fatigue and itching.</td>
</tr>
<tr>
<td>6</td>
<td>DEL-17</td>
<td>Dilanipur</td>
<td>41/M</td>
<td>3 days</td>
<td>Negative</td>
<td>High grade fever, sore throat, tenderness over joints, joint pain and muscle tenderness.</td>
</tr>
<tr>
<td>7</td>
<td>DEL-02</td>
<td>Dilanipur</td>
<td>34/F</td>
<td>2 days</td>
<td>Negative</td>
<td>Fever, tenderness over joints, joint pain and muscle tenderness.</td>
</tr>
<tr>
<td>8</td>
<td>DEL03</td>
<td>Middle point</td>
<td>37/M</td>
<td>2 days</td>
<td>Negative</td>
<td>High grade fever and joint pain.</td>
</tr>
<tr>
<td>9</td>
<td>H-06</td>
<td>Haddo, Lillypur</td>
<td>27/M</td>
<td>3 days</td>
<td>Negative</td>
<td>High grade fever, sore throat, tenderness over joints, joint pain, maculopapular rashes, redness over joints and muscle tenderness.</td>
</tr>
</tbody>
</table>

Table 3: Details of patient's serum positive for *Chikungunya virus* isolation (at RMRC, Port Blair).
5.1.3 Phylogenetic analysis

The cDNA from RT PCR assay for envelope1 (E1) gene 294 bp and non-structural protein1 (nsP1) gene 354 bp corresponding to all isolates was sequenced. The phylogenetic analysis was performed using DLST concept to understand the evolutionary relationships between various genotypes of CHIKV with the sequences of isolates from the 2006 outbreak, resulting in atypical manifestations viz., acute flaccid limb weakness and severe chronic arthropathy in these islands.

The analysis of Andaman CHIKV isolate was done using DLST concept along with the representative sequences from various part of the world. CHIKV isolates sequences from the Andaman Islands grouped with single cluster along with the recent outbreak isolates on the mainland India as well as Réunion isolates (figure 8).

The pairwise mean genetic distance (Kimura 2-parameter) in-between CHIKV isolates of Andaman was 0.00 percentage. The percentage of mean genetic distance (Kimura 2-parameter) between the cluster of CHIKV isolates from Andaman and that of East Central and South African (ECSA) strains was 0.011, which was smaller than Asian genotypes (0.042), West African genotypes (0.130) and other closely related species O’Nyong-Nyong virus (0.273) from Uganda.

Comparison of DLST Phylogenetic analysis (Figure 8) with phylogenetic tree using whole genome sequences from various part of the world (whole genome sequence of Andaman isolate is not included) (Figure 9) showed an identical clustering pattern. Whereas the evaluation of sensitivity as well as specificity of DLST in contrast to SLST of E1 (Figure 10) and nsP1 (Figure 11) revealed that the measure of the reliability was high in DLST.
Figure 8: Neighbour joining tree based on DLST analysis using E1 as well as nsP1 partial gene of Andaman CHIKV isolates along with reference sequences from different part of the world including different genotypes.
Figure 9: Neighbour joining tree using whole genome sequences of CHIKV from various part of the world including different genotypes.
Figure 10: Neighbour joining tree using E1 partial gene sequences of Andaman CHIKV isolates along with reference sequences from various part of the world including different genotypes.
Figure 11: Neighbour joining tree using nsP1 partial gene sequences of Andaman CHIKV isolates along with reference sequences from various part of the world including different genotypes.
5.1.4 Molecular evolution of DEL-03 CHIKV isolate

The major protein of CHIKV viz., nsP1, nsP2, nsP3, nsP4 Capsid, E1 and E2 coding partial gene sequences were attempted on the first isolate (CHIKV DEL03) isolated from the 2006 outbreak in Andaman. Major proteins coding gene sequences of DEL03 CHIKV isolate are furnished in Table 2, which was obtained following PCR amplification and sequencing. It was identified that the CHIKV sequence DEL03 having nucleotide changes in three different positions which has not been reported earlier in the sequences available in NCBI Genbank. The unique nucleotide polymorphisms were identified in the following positions viz., 2134 Cytosine to Guanine, 5134 Cytosine to Thiamine and 7707 guanine to Adenine.

The ORF of the major genes of DEL-03 CHIKV isolate was done using the NCBI ORF Finder. The output was received as deduced amino acid sequence; subsequently the output of the analysis and comparison with substantiation of CHIKV protein sequences in NCBI Genbank also was done. Finally, it was confirmed that the Long ORFs candidate protein coding regions in the Forward DNA sequence of the frame +3 as well as frame +2.

In continuation of the analysis the assembled sequences of CHIKV were further examined using various complete protein sequences for the presence of unique amino acid change corresponding to the positions of unique nucleotide change in DEL03 isolate. Overall analysis confirmed that there are two amino acid corresponding to the nucleotide polymorphism positions 2134 (Leucine to Valine) and 7707 (Valine to Isoleucine), these changes were not reported earlier in the CHIKV sequences available in the NCBI Genbank. But the nucleotide polymorphism in the position 5134 did not change the amino acid.

This observation further confirmed that the amino acid change in the position 2134 (Lucine to Valine) was in the non-structural polyprotein, especially in nsP2 region of Chikungunya virus. Similarly, another amino acid change in the position 7704 (Valine to Isoleucine) was in the structural polyprotein that is in capsid region.
Phylogenetic analysis: The phylogenetic tree using sequence of DEL03 CHIKV isolate and the isolates from different states of India, and different part of the world was done using MEGA4.1. The analysis was done using nsP1, nsP2, nsP3, nsP4, Capsid, E1 and E2 coding partial gene sequences following SLST as well as MLST showed the DEL-03 CHIKV isolate grouping in ECSA genotype. Subsequent analysis of phylogenetic tree generated based on SLST indicated that nsP1, nsP2, nsP4, capsid, E1 and E2 partial sequences has significance in clustering with specific genotypes. But in the case of nsP3, partial gene sequence showed less significance in genotype analysis.
Figure 12: (a) Sequence data explorer describing the presence of unique nucleotide polymorphism in the position 2134 cytosine to guanine of DEL-03 CHIKV isolate of Andaman. (b) Describes the change of the amino acid Leucine to Valine corresponding to the nucleotide polymorphism in the position 2134 cytosine to guanine of DEL-03 CHIKV isolate of Andaman.
Figure 13: (a) Sequence data explorer describing the presence of unique nucleotide polymorphism in the position 5134 Cytosine to Thiamine of DEL-03 CHIKV isolate of Andaman. (b) Describes there is no change of amino acid corresponding to the nucleotide polymorphism in the position 5134 Cytosine to Thiamine of DEL-03 CHIKV isolate of Andaman.
Figure 14: (a) Sequence data explorer describing the presence of unique nucleotide polymorphism in the position 7707 Guanine to Adenine of DEL-03 CHIKV isolate of Andaman. (b) Describes the change of amino acid Valine to Isoleucine corresponding to the nucleotide polymorphism in the position 7707 Guanine to Adenine of DEL-03 CHIKV isolate of Andaman.
Figure 15: Neighbour joining tree using E1 partial gene sequences of Andaman CHIKV DEL-03 isolates along with reference sequences from various part of the world including different genotypes.
Figure 16: Neighbour joining tree using E2 partial gene sequences of Andaman CHIKV DEL-03 isolates along with reference sequences from various part of the world including different genotypes.
Figure 17: Neighbour joining tree using Capsid protein partial gene sequences of Andaman CHIKV DEL-03 isolates along with reference sequences from various part of the world including different genotypes.
Figure 18: Neighbour joining tree using nsP4 partial gene sequences of Andaman CHIKV DEL-03 isolates along with reference sequences from various parts of the world including different genotypes.
Figure 19: Neighbour joining tree using nsP3 partial gene sequences of Andaman CHIKV DEL-03 isolates along with reference sequences from various part of the world including different genotypes.
Figure 20: Neighbour joining tree using nsP2 partial gene sequences of Andaman CHIKV DEL-03 isolates along with reference sequences from various parts of the world including different genotypes.
Figure 21: Neighbour joining tree using nsP1 partial gene sequences of Andaman CHIKV DEL-03 isolates along with reference sequences from various part of the world including different genotypes.
Figure 22: Neighbour joining tree MLST concept analysis of Andaman CHIKV DEL-03 isolates along with reference sequences from various part of the world including different genotypes.