Annexures
## ANNEXURE

### Sample Collection proforma

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<th>Sample No.</th>
<th>Name of the patient</th>
<th>Address</th>
<th>Taluk</th>
<th>age</th>
<th>sex</th>
<th>Date of onset of illness</th>
<th>Type of sample</th>
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List of publications to Ph. D thesis

Original articles:


The study was conducted from April 2011 to December 2014. Five hundred serum samples were collected from cases with pyrexia and arthralgia. Serum samples were tested for Chikungunya antibodies by Chikungunya IgM ELISA.

Results and Conclusion: Out of 500 samples 33 samples were confirmed positive for Chikungunya IgM antibodies. The prevalence rate of Chikungunya was 6.6% with maximum number of cases in the year 2013 (8.5%) and age group 15 to 40 (8.3%). Females (6.9%) were more affected than males. Thus, continuous sero-epidemiological surveillance is needed for the control of Chikungunya fever.

Keywords: Arboviral, IgM ELISA, Viral fever

Materials and Methods: The study was conducted from April 2011 to December 2014. A total of 500 blood samples from suspected Chikungunya cases were included in the study. Blood samples from clinically suspected Chikungunya patients, i.e. pyrexia of more than >38.5°C and polyarthralgia were collected from BLDE’s hospital, Bijapur and Government PHC, CHC, taluk and district hospitals of Bijapur district. Informed consent was obtained from all the patients prior to sample collection. Two to five ml of venous blood was collected aseptically. The samples were transported to the laboratory in a vaccine carrier. Serum samples were tested for Chikungunya antibodies by Chikungunya IgM antibody with SD (standard diagnostics) Chikungunya IgM ELISA kits. Direct Enzyme linked immune sorbent assay (ELISA) was performed. The tests were performed strictly as per the manufacturers’ instructions. The data thus obtained was presented by using frequencies and percentages.

RESULTS
Cut-off was calculated by sum of mean negative control to 0.300. The samples of absorbance less than cut-off was considered as negative and samples of absorbance more than cut-off was considered as positive. Out of 500 samples tested, 33 samples were found positive for Chikungunya IgM antibodies. The disease was more prevalent in 2013 (8.5%), followed by 2012 (7.5%) [Table/Fig-1]. Females (6.9%) were more affected compared to males [Table/Fig-2]. More number of cases belonged to age group of 15 to 40 (8.3%) [Table/Fig-3].

DISCUSSION
Chikungunya has emerged as a major public health problem in many tropical countries of Africa and Asia. In 2005- 2006 an explosive outbreak of Chikungunya occurred in India affecting more than 1.4 million people in 13 states especially in southern India [11]. Kerala and Karnataka were the worst affected states during 2006 CHIKV outbreak, 27 districts of the Karnataka state reported over 54.74% of the total suspected cases. Several districts of the state such...
of cases affected were in age group of 15 to 40 (8.3%) [Table/Fig-3]. Bijapur has six taluks, more number of confirmed cases were found in Sindagi, in the decreasing order from taluks Bijapur, Indi, Muddebihal and Bagewadi [Table/Fig-4].

CONCLUSION

Bijapur (Northern part of Karnataka) is endemic to Chikungunya, it is necessary to diagnose the disease for the treatment and management. The prevalence rate of Chikungunya in and around regions of Bijapur was 6.6%. IgM antibody detection by ELISA is a very important tool in diagnosis of infection.

REFERENCES


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Molecular Characterisation of Clinical Isolates of Chikungunya Virus: A Study from Tertiary Care Hospitals in Southern India

ABSTRACT

Introduction: Indian ocean islands and India have experienced massive severe Chikungunya outbreak from 2005 up till now and then Chikungunya became epidemic in India. The mutations that occurred in E1 gene were responsible for increased infectivity, virulence and host adaptability. It is important to find out the genotype and its probable evolution and novel mutations in the E1 gene reported during 2006-2009 from the current isolates, which may affect the local protein structure.

Aim: To perform Molecular diagnosis and Molecular Characterisation of Chikungunya virus isolates.

Materials and Methods: A total of 33 samples were included in the study. RNA was isolated from 33 serum samples and real time PCR was carried out. Further, Nested PCR and E1 partial gene sequencing was performed. Phylogenetic analysis, mutational analysis and protein modelling studies were carried out.

Results: Out of 33 samples tested, 31 were found positive for CHIK RNA. Phylogenetic analysis showed that isolates belong to ECSA genotype and E1K211E, E1M269V and E1D284E mutations were observed from all the isolates.

Conclusion: The isolates may have evolved from ECSA Reunion island strains and identified unique mutations in E1 gene were maintained. These mutations have not affected local protein structure.

INTRODUCTION

Chikungunya fever was first documented in 1952 during epidemic in Newala and Masasi Districts of Southern Province, Tanzania. Chikungunya is an arthropod borne virus, transmitted to humans by the bite of Aedes aegypti mosquito [1]. Indian tiger mosquito Aedes albopictus was found to be a competent vector for Chikungunya transmission during 2005-2006 [2]. Chikungunya fever presents with symptoms of fever, polyarthralgia, headache, backache and persistent arthralgia [3]. Severe neurological manifestations were also observed during recent outbreak [4].

Chikungunya virus belongs to family togaviridae and genus alpha virus. It's an enveloped RNA virus and RNA is linear, positive sense genome organisation of CHIKV is 5' cap-nsp1-nsp2-nsp3-nsp4-(juncture region)-C-E3-E2-6K-E1-(poly A/3'cap). The length of RNA is 11805 bp excluding 5' cap nucleotide, 3' cap (I-poly A) tract and 3' poly A tail. Two third of genomic RNA from 5' end consists of non-structural proteins (nsP1, nsP2, nsP3 and nsP4) with length of 7425 nucleotides and one third towards 3'end consists of structural proteins (E1, E2 and E3, 6K and C) with length of 3735 nucleotides [Table/Fig-1]. The 5' NTR has 76 nt, 3' NTR has 526 nt and internal poly A region has 68 nucleotides. 3' end has internal polyadenylation site and repeated sequence elements (RSEs). CHIKV genome consists of two open reading frames, one code for non-structural poly-proteins (2474 aa) and another for structural proteins (1244 aa) [5].

Chikungunya virus has three genotypes namely East Central South African (ECSA), West african and Asian. The genotypes were named according to prior geographical distribution. The ECSA genotype was confined to East, Central and South Africa previously but in the year 2000 same genotype was first time isolated in India from mosquito samples collected from Yawat, Pune district, Maharashtra, India [6]. The same genotype caused explosive outbreak in different regions of Indian ocean island, India, Europe and other parts of the world between 2005-2009 [7,8]. The Mutations in structural and non-structural coding region of viral genome in alpha virus affects infectivity and virulence [9,10].

AIM

In the present study, Molecular diagnosis and Molecular Characterisation of Chikungunya virus isolates, in detail novel mutations in E1 gene responsible for increased host adaptability, infectivity and virulence of Chikungunya virus have been studied.

MATERIALS AND METHODS

Clinical Samples

Total of 33 serum samples with symptoms of fever and arthralgia, which were Chikungunya serodiagnosed (IgM antibody) included in the study. Blood samples were collected from BLDE’s hospital, Bijapur and Government PHC, CHC, taluk and district hospitals of Bijapur district, Karnataka from 2011 to 2014. Serum was separated and stored at -70°C. Informed consent was obtained from all cases before sample collection.

RNA Extraction

RNA was isolated from 33 serum samples using QIAamp viral RNA mini kit (Qiagen) according to manufacturer instructions [11].

Reverse Transcriptase Real Time PCR [Table/Fig-2]

RT-PCR was carried out with 5µl of isolated RNA using Amplisure Chikungunya RTPCR kit on ABI7500 thermo cycler at RAS Lifesciences Pvt Ltd. The pathogen detection was based on amplification of specific regions in NSP gene. The steps of RT-PCR were: 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°C for 1 min. Strict adherence to manufacturer’s instructions was followed for optimal results and to avoid PCR contamination. Kit supplied internal control (IC) was used to identify possible PCR inhibition [12].

Sequencing

Sequencing was performed at RAS Lifesciences Pvt Ltd by using commercial facility. Nested PCR was performed and E1 partial
gene sequencing was carried out by Sanger sequencing method for 9 samples. Approximately, 555 base pairs were amplified from samples using E1F1 & E1R1 primers [13,14]. Sequencing was done by using DNA Sequencer (ABI 3130 xL GA) instrument.

**Table/Fig-2:** 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 bp</th>
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</thead>
<tbody>
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<td>E1-F1</td>
<td>GCTCCGCGTCCTTTAC</td>
<td>10389-10943pb</td>
<td>555</td>
</tr>
<tr>
<td>E1-R1</td>
<td>ATGGCGACGCCCCAAAGTC</td>
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</table>

**Phylogenetic Analysis**

Chikungunya sequences were aligned using Clustal W2 software. The unrooted tree was constructed using Neighbour-Joining method [15]. 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) are shown next to the branches [16]. 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 [17] 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 [18]. The accession numbers used for the study are given below along with the positions matching with the samples [Table/Fig-3].

**Table/Fig-3:** 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>
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<td>AF369024.2:10427-10941</td>
<td>Q8LJX5</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>AM259992.1:10376-10890</td>
<td>Q1W667</td>
<td>Reunion (ECSA)</td>
<td>2006</td>
</tr>
<tr>
<td>EF027139.1:10428-10942</td>
<td>A6MH23</td>
<td>INDA-00-MH4 (Asian)</td>
<td>2007</td>
</tr>
<tr>
<td>HM045811.1:10410-10924</td>
<td>D7RR78</td>
<td>Tanzania (ECSA)</td>
<td>1953</td>
</tr>
<tr>
<td>HM045797.1:10410-10916</td>
<td>D7RR52</td>
<td>RSU1 (Asian)</td>
<td>1985</td>
</tr>
<tr>
<td>HM045817.1:10412-10926</td>
<td>D7RR990</td>
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<td>2005</td>
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<tr>
<td>HM045816.1:10412-10926</td>
<td>D7RR888</td>
<td>Senegal (West Africa)</td>
<td>1966</td>
</tr>
</tbody>
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**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.

**RESULTS**

**RT-PCR**

Chikungunya RNA was detected in 31 (93.9%) samples. Two Sero-positive samples were found negative for Chikungunya RNA.

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**Gene Modelling**

Template search with Blast [19] and HHBlits [20] has been performed against the SWISS-MODEL template library. 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 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 were remodelled using a fragment library. Side chains are then rebuilt. Finally, the geometry of the resulting model is regularized by using a force field. In case loop modelling with ProMod-II [21] does not give satisfactory results, an alternative model is built with MODELLER [22]. The global and per-residue model quality has been assessed using the QMEAN scoring function [23]. For improved performance, weights of the individual QMEAN terms have been trained specifically for SWISS-MODEL.

**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.

**RESULTS**

**RT-PCR**

Chikungunya RNA was detected in 31 (93.9%) samples. Two Sero-positive samples were found negative for Chikungunya RNA.
Phylogenetic Analysis [Table/Fig-4]
Unrooted Phylogenetic tree shows isolates are closely related to Reunion strains and distantly related to S27 Tanzanian strain and made sister group of ECSA. So, isolates belong to ECSA genotype. Isolates were even more divergent to Asian and West African genotypes. Comparative nucleotide and amino acid homology analysis reveals that isolates are 94.9±5.1% nucleotide homology and 97.1±2.9% at amino acid homology with S-27 strain.

Mutational Analysis [Table/Fig-5]
Few random nucleotide changes were observed in partial E1 region. Following amino acid mutations were observed. E1K211E, E1M269V and E1D284E in all isolates.

Molecular Modelling
Homology modelling with mutations has been projected to three dimensional structure [Table/Fig-6]. All the three observed mutations lies in area in major secondary structure. So it couldn’t affect local protein structure.

DISCUSSION
South Indian states Karnataka and Kerala had major Chikungunya outbreaks during 2006-2009. In 2006 Karnataka state reported 7,62,026 number of Chikungunya suspected cases, Bijapur was one among the district experienced huge number of cases [24]. Statistically significant number of Chikungunya confirmed cases (sporadic and epidemic) are being reported in present years. In the present study phylogenetic analysis of Chikungunya virus shows close relation of isolates with Reunion strains than prototype (S 27). It indicates that these isolates may have evolved from Reunion ECSA genotype (subtype Indian ocean lineage-IOL).

Chikungunya RNA couldn’t be detected from two Seronegative samples. The probable reason for molecular negativity may be due to very low viral load. The cases which produce strong antibody mediated and cell mediated immune response experience short period of viremia (median 6 days) [25]. These two samples were collected on day 7, so in the samples viremia may be short. Antigenic cross-reactivity was observed between Chikungunya and other alpha viruses like Ross River, Onyong nyong, Mayaro, and Sindbis viruses, causing similar clinical manifestations [26].

Previously, it has been demonstrated that E1A226V mutation increases the midgut infectivity and viral dissemination to secondary organs and in turn enhances Chikungunya virus transmissibility by Aedes albopictus mosquitoes. Similarly, it was postulated that mutation decreases cholesterol dependence in target cells and increases fitness of Chikungunya virus on Aedes albopictus mosquitoes [27]. This mutation was absent in the current isolates.

First time ECSA genotype was isolated in India from mosquito during 2000 (Yawat strain). Thereafter, ECSA and Asian genotypes have been circulating in India. The ECSA genotype has caused outbreaks in Indian ocean Island, India and presently causing sporadic and epidemic cases in India. A study did conduct to find out the origin and spread of ECSA genotype in India. It was concluded that Reunion ECSA genotype was not resulted from recombination of prototype ECSA (S27) and Asian genotypes. It’s under purifying selection and may evolved due to random neutral and non-synonymous mutations [28].

In a study, infection of C6/36 cell lines by E1226A and E1226V strains, resulted in higher titre than prototype (S27) [29]. It indicates that there are some un-identified mutations in ECSA lineage responsible for adaptation of ECSA to Aedes albopictus mosquitoes [30,31]. These mutations might affect the displacement of Asian lineage by ECSA lineage in India where both genotypes exist [32].

It was established that E1K211E mutation was positively selected (new non-synonymous advantageous mutations) site with a posterior probability of >75% [28,33]. In Bijapur district, Aedes aegypti mosquitoes were predominant species than Aedes albopictus. Mutations present in E2 gene might play a vital epistatic role in E1 gene for the adaptation of Chikungunya virus to Aedes aegypti and Aedes albopictus mosquitoes. However, further studies are required to elucidate on the effect of mutations in whole genome on viral infectivity, epidemiology and vector adaptability.

LIMITATIONS
Genetic characterization was carried out for 9 of 31 samples due to low viral load, as we couldn’t get sequences from remaining samples.
CONCLUSION

Comparative nucleotide and amino acid homology studies reveal that the current isolates may have evolved from Reunion island strains. The mutation A226V which claimed to increase the host adaptability, infectivity and virulence in Chikungunya virus was absent in present isolates. The identified unique mutations in E1 gene K211E, M269V and D284E were still maintained in current isolates. Homology modelling studies concluded that observed mutations have not altered the local protein structure.

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REFERENCES


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