Serological & Molecular Diagnosis of Chikungunya in and around the Region of Bijapur (Vijayapura-North Karnataka)

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Annexure-I

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Dedicated to my parents

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LIST OF ABBREVIATIONS

AP – Andra Pradesh

ARBO- Arthropod born

CDR- Crude Death Rate

CHC- Community Health Centers

CHIK-Chikungunya

CHIKF- Chikungunya fever

CHIKI- Chikungunya infection

CHIKV-Chikungunya virus

Ct- Cycle threshold

DEN- Dengue

DF- Degree of Freedom

DNA-De-oxy Ribonucleic acid

ECSA- East central South African

EIA- Enzyme immune assay

ELISA- Enzyme linked immune sorbent assay

IEC - Information Education and Communication

IFA- Indirect fluorescent antibody

Ig G – Immunoglobulin G

Ig M - Immunoglobulin M

IHR- International Health Regulations

IQC – Internal Quality Control

JE- Japanese encephalitis

KFD- Kyasunur forest disease

MOHSA -Ministry of health and social affairs

NC- Negative control

NIV- National institute of virology.

nSP - Non structural proteins

NVBDCP- National vector born disease control programme

ONN-O'nyong-nyong

PAHO - Pan America Health organisation

PC- Positive control

PCR- Polymerase chain reaction

PHC- Primary Health Centers

RNA- Ribonucleic acid

RPM- Revolution per minute

RTPCR- Real time polymerase chain reaction

SFA- Semliki forest antigen

SFV - Semliki forest virus

SNP- Single nucleotide polymorphism

SP – Structural proteins

SS RNA- Single stranded RNA

TN – Tamilnadu

VBD – Vector born diseases

WA- West African

WHO - World Health Organisation

WN- West nile

χ^2–Chi Square

%- Percentage

ABSTRACT

Background-

Chikungunya fever is an arthropod borne debilitating non-fatal viral fever. It was originally distributed in several parts of Africa, South Asia and Southeast Asia. Chikungunya is an RNA virus. Chikungunya virus is an Arthropod borne virus, which spreads from one person to other person by the bite of vector mosquitoes – Aedes genus (*Aedes aegypti* and *Aedes albopictus*). Chikungunya fever presents with High fever, severe joint pain and rashes. The mortality and morbidity of Chikungunya were drastically increased after 2005-06 outbreaks in Indian-ocean islands and India due to mutations and increased host range.

Aim-

To find-out the prevalence of Chikungunya disease, genotype responsible for and mutations in E1 gene and correlation of protein structure, infectivity, virulence and clinical manifestations with observed mutations.

Materials and Methods-

Clinical samples (500 blood samples) were collected from Chikungunya suspected cases from primary health centers, community health centers, taluk hospitals and district hospital of Vijayapura district, Karnataka. Chikungunya IgM antibody ELISA test was performed on all serum samples. Chikungunya IgM antibody positive samples were further tested for Chikungunya RNA by RTPCR test. Molecular characterisation studies like Phylogenetic analysis, mutational analysis, protein modeling and mutations mapping studies were conducted to find-out the genotype prevalent in the area and mutations in E1 gene.

Statistics - Chi square test is done.

Result-

Out of 500 serum samples tested, 33 samples were found positive by Chikungunya IgM antibody ELISA test. Chikungunya RNA was detected in 31 of 33 seropositive samples. The current strains clustered with ECSA genotype and are closely related to Reunion strains. **E1K211E, E1M269V and E1D284E,** three observed amino acid mutations were present in all strains. All the three mutations observed in E1 gene lie in the area of the major secondary structure. Novel mutation E1A226V was absent in current strains.

Conclusion-

We have found that, Chikungunya prevalence in and around the region of Vijayapura district was 6.2% and Statistical analysis concluded that there was no significant difference between suspected and confirmed cases with respect to year, sex, age and taluk. The ECSA strains of current study may have evolved from ECSA Reunion islands strains. From the study, we have concluded that the observed mutations in E1 gene couldn't alter the protein structure and ECSA strain circulating in Vijayapura district are comparatively less virulent.

Chapter – 1
Introduction

Health is defined as a state of complete physical, mental and social well-being and not merely the absence of disease. The Disease is a state of pathological disruption in structure and functions of human body organs. Diseases can be classified into Infectious and non-infectious diseases. Infectious diseases are caused by infection of Microorganisms. World Health Organisation (WHO) reported, of all infectious diseases, more than 17% of diseases are of Vectorborne diseases (VBD) and results in 1 million deaths annually. VBD are diseases that are transmitted to humans by the bite of living organisms called vectors. These vectors transmit infections between humans or from animals to humans. Blood sucking insects like mosquitoes, ticks, flies and fleas serve as vectors. Infected mosquitoes can cause Malaria, Dengue, Chikungunya (CHIK), Japanese encephalitis, Rift valley fever, Yellow fever and zika diseases. VBD are also called as Arthropod Born (ARBO) Viral diseases.

Among VBD, CHIKF was the neglected disease in Asia and other continents till 2005-06 due to sharing similar clinical manifestations of dengue and limited diagnostic facility. Vijayapura district, Karnataka state reported 80,000 clinically suspected cases annually during 2006-2008 outbreak and still a statistically significant number of cases are reported in current years. This study was conducted to find out the prevalence of CHIK in and around Vijayapura district. Clinically suspected CHIK cases should be confirmed by laboratory diagnosis. CHIK IgM ELISA test is the most widely used diagnostic tool for the diagnosis. False positive serology results are reported due to antigenic cross-reactivity of CHIK with other Alphavirus. (semliki forest antigen complex) To overcome this limitation molecular

confirmation was carried out. Real-time polymerase chain reaction (RTPCR) is the gold standard test for the diagnosis of CHIKF.

Three genotypes were observed in CHIKV, namely East Central South African (ECSA), West African and Asian. The nomenclature of genotype is based on its first isolation in the respective continent. Previously (before 2000) the genotypes were reported only from respective continent viz ECSA in east central and South Africa, West African in West Africa and Asian in Asia. Transcontinental movement of genotypes has been observed in last decade. ECSA genotype was responsible for 2005-06 outbreak in India and Indian ocean and currently Asian genotype is causing outbreaks in Europe and America. So the study was conducted to find out the genotype prevalent in the area.

Phylogenetic studies are important to trace the origin of the virus, follow the pattern of disease spreading, genetic relatedness of current strains with previous isolates as well as to discriminate between the strains among different geographical area. Hence we want to correlate strains from our study within themselves and between previously reported strains.

It was documented that a single point mutation in E1 gene E1A226V alters host range, host adaptability, virulence and infectivity of CHIKV and results in the large spread of disease and severe clinical manifestations. The observed mutations may alter the protein structure. So the study was conducted to find out the mutations in E1 gene to correlate infectivity, virulence and clinical manifestations.

Chapter – 2
Review of Literature

An extensive survey of literature related to the research topic has been carried out and reviewed under the fallowing headings.

2.1. History:

Chikungunya (CHIK) is an arthropod-borne debilitating viral fever. The name CHIK derived from word kungunyala in Makonde language (local language) which means “become contorted or folded” due to an arthritic symptom of the disease. (WHR Lumsden, 1955) Retrospective studies suggested that CHIK epidemics experienced as early as 1779, but it was misdiagnosed as dengue outbreak. (Carey DE, 1971). CHIK originated from Africa and was first time reported in an outbreak from Newala and Masasi Districts of Southern Province, Tanganyika in July 1952. (WHR Lumsden, 1955).

2.2. Epidemiology:

CHIK was originally distributed in several parts of Africa, South Asia, and Southeast Asia. (Bharath M D et al., 2015) After a severe outbreak reported from Reunion Island it spread to other continents of the world. During current years different countries of Europe and American continents are experiencing CHIK cases in the large population.

2.2.1. Geographical distribution of Chikungunya in Africa:

CHIK was reported for the first time during the year 1952 and 1953 from Newala and Masasi Districts of Southern Province, Tanganyika. The first case was identified in Mchichira on the south-eastern fringe of the plateau in July 1952. Authors postulated that the disease may have originated from Ruvuma valley and

through travellers spread to plateau. The outbreak spread rapidly and reached a peak in January 1953. 49 of 62 localities were affected and crude incidence rate ranged from 13 to 95%. (WHR Lumsden, 1955) Thereafter in November 1958 CHIK suspected cases were reported from Belgian, Democratic Republic of Congo (DRC). (Posterrieth et al., 1960) Between May and June 1959, 4 out of 13 cases were identified of CHIK etiology from Luanshya, Northern Rhodesia. (L.M. Rodger et al., 1961). After 7 years in October 1966 suburbs of Dakar and other interior places in Senegal reported CHIK cases. Results showed that 11 out of 27 samples and 49 out of 53 samples were positive for CHIKV isolation and serology respectively. (Roche S et al., 1967) Thereafter CHIK cases were reported from Launda, Angola in 1971-1972. 80 serum samples were tested and 50 cases were identified as CHIK etiology. (Armindo R filipe et al., 1973) CHIK outbreaks were reported in the year 1996 and 1997 from different regions in Senegal kaffrine and Niakhar respectively. The incidence rate was 35.3% (Thonnon j et al., 1999). Thereafter CHIKF cases were reported from the democratic republic of Congo. CHIKF cases were identified from May 1999 and February 2000 from matete and Kingabwa quarters of Kinshasa. (capital city of the democratic republic of congo) 76 serum samples were collected, out of which 44 samples were found to be positive for IgM antibodies. The Virus was isolated from 9 out of 21 samples. (B pastorino et al., 2004)

During 2004, CHIKV caused an outbreak in Lamu Island, Kenya. Large numbers of individuals were affected in July 2004. (around 1300) A sero-prevalence study was conducted in October 2004 to find out the magnitude of transmission. A total of 288 sera consistent with CHIK fever clinical

manifestations were tested. IgM and or IgG antibodies were detected in 75% of tested samples with a higher titer of IgG antibodies. They have estimated that of 18000 population with an attack rate of 75% around 13500 individuals may have experienced CHIK fever during July 2004. (Kibet Sergon et al., 2008) After 3 years CHIK cases were reported from Mashi, northern Tanzania between 17 September 2007 to 31 August 2008. 7.9% of tested cases were confirmed as CHIK etiology. (Julian T Hertz et al., 2012) After one year 8 travellers returned from Senegal to different parts of the world were identified as CHIKV infection. So in the year 2009 CHIKV was actively circulating in the Senegal area (Thierry piston et al., 2009) The Ministry of Health and Social Affairs (MOHSA) Senegal reported CHIK cases from Kedougou, senegal on 9th September 2015. Out of 14 samples tested 10 cases were identified as CHIK etiology at institute Pasteur of Dakar. (Chikungunya–senegalhttp:/www.who.int/csr/don/14-september-2015-chikungunya/en)

2.2.2. Geographical distribution of Chikungunya in Europe:

Pioneer autochthonous transmission of CHIK outbreak in Europe was reported from Italy in 2007. A large number of cases with arthralgia symptoms were reported from two villages Castiglione di Cervia and Castiglione di Ravenna in Emilia-Romagna (E-R) region in the year July to September 2007. The index case was a traveller from Kerala and the first autochthonous case was identified in the first week of September. During this outbreak, 217 laboratory confirmed cases were identified. The other towns like Cervia, Cesena, Ravenna and Remini also experienced the same cases. Current Strains clustered with ECSA genotype with similar mutations found in Indian-ocean island isolates. (G Rezza et al., 2007 and

Giancarlo marialiumbuno et al., 2008). Then after one year 105 clinically suspected CHIK cases were reported from three villages of Kumba- northwest region of Cameroon i.e Ngehndzen, Ndzeru and Tasai. Out of 105 samples tested 54 cases (51.4%) were laboratory confirmed. (Maurice Demanou, 2010) In the year 2010 two autochthonous cases of CHIK were reported from France during September 2010. Two 12-year-old female children were affected, studying in the same high school in Frejus with no travel history and resident of the neighborhood of 7 year old children returning from Asia with a laboratory confirmation of CHIK. (E A Gould et al., 2010) National IHR focal point of France and Spain reported four and one laboratory confirmed autochthonous transmission cases of Chikungunya fever in Montapellier, France and Gandia city Spain during 21st October 2014 and 3rd august 2015 respectively. The affected 4 cases in France belonged to the same family with no travel history. [Chikungunya Spain (update). http://www.who.int/csr/don/17-september-2015-chikungunya/en/]

2.2.3. Geographical distribution of Chikungunya in America:

America experienced first CHIK outbreak in 2013. During mid-November 2013 CHIK cases were suspected clinically and on 6th December 2013 at saint martin, Caribbean islands reported first autochthonous laboratory confirmed case of CHIK. Current strains belongs to Asian genotype. Netherland overseas territories also reported autochthonous cases. (W. van Bortel et al., 2014) The statistics of CHIK cases reported from different islands is mentioned in table – 1.

Table – 1: Chikungunya cases reported from different islands of America as on march 2014

Territories	Island	Suspected cases	Confirmed cases
Caribbean french overseas territories	Saint martin	2750	784
	Saint barthelemy	435	134
	Martinique	9340	1207
	Guadeloupe	2270	734
Netherland overseas Territories	Saint martin	-------	234
	Saint martin	-------	784
UK overseas territories		-------	14

In the same year, Brazil also experienced CHIK outbreak. It was started in April 2014 and the first autochthonous case was identified in Oiapoque, Amapa State. Totally 41 importations and 27 autochthonous cases were identified. The genotypic analysis revealed that both Asian and ECSA genotypes circulate in Brazil. (Marico Roberto Teixeiranuses et al., 2015) By October 18, 2014 Brazilian Ministry of Health notified 682 CHIK autochthonous cases. Pan America Health organization (PAHO) reported yearly statistics of CHIK cases in America. In the year 2015 overall 6.07.961 clinically suspected cases and 35.019 laboratory-confirmed cases were identified from different parts of America.

The incidence rate was 64.9/10.000 population and 77 suspected deaths may have been caused by CHIK. (Chikungunya fact sheet) http://www.who.int/mediacentre/factsheets/fs327/en/)

2.2.4. Geographical distribution of Chikungunya in Asia:

CHIK cases were first time reported in Asia from Bangkok, Thailand in the year 1958. (Hammon WM et al., 1960) Then after three decades consecutive outbreaks were reported from different regions of Thailand, viz Khon Kaen, Nakhon Si Thammarat and Nongkhai provinces during July 1991, July 1995 and August 1995 respectively. (Thaikruea L et al., 1997) After 6 years CHIK re-emerged in Indonesia. Different regions of Indonesia experienced 24 episodes of clinically suspected outbreaks between September 2001 to march 2003, out of which 11 outbreaks were laboratory confirmed. Two outbreaks were studied in detail, Bogar city and Bekas regency reported attack rate of 2.8/1000 and 6.7/1000 population respectively. (Kanti Laras et al., 2005)

In the year 2007 CHIK cases were reported from Malaysia. 11 clinically suspected cases were identified, out of which 8 cases were confirmed of CHIK etiology and Asian genotype was identified. (Sazaly AbuBakar., 2007). In the same year, Southern Sri Lanka also experienced CHIK outbreak. 797 cases were clinically suspected out of which 28 cases were laboratory confirmed with a positivity rate of 3.5% and ECSA genotype was identified. (Megan E Reller et al., 2013) Then after Singapore reported 13 CHIK cases between January and February 2008. (Yee S et al., 2009) After that Thailand was affected by a large-scale outbreak. 1756 samples were collected from different regions of southern Thailand

viz Narathiwat, pattern, Yala and Songkhla between 1st January 2008 to 30thJune 2009. 964 cases were confirmed of CHIK etiology with a relative infection rate of 54.4% and 55.6% in 2008 and 2009 respectively. (Usavadee Thavara, 2009) In the same year between months, April to June 45 CHIK laboratory confirmed cases were reported from the hospital of south Thailand. The strains belonged to ECSA genotype. (Sarunyou chusri et al., 2014 Bureau of epidemiology, department of disease control, the ministry of public health, Thailand summarized yearly statistics of CHIK cases in Thailand. They have reported 2433 cases in 2008 and 49069 cases in 2009 and 1533 cases in 2010. (Sonthaya Tiawsirisupet al., 2011)

In the year 2011 (May to December) 24 Laboratory confirmed CHIK cases were reported from provinces of Cambodia. (Veasna Duong et al., 2012) Then after for the first time CHIK cases were reported from Bhutan, south Asia in 2012. A total of 215 CHIK suspected cases were reported from south western districts hospitals (Samtse, Chukha, and Thimphu) and strains belonged to ECSA genotype. (Sonam Wangchuk et al., 2013) An epidemiological surveillance study conducted in Bandung, Indonesia during non-outbreak season depicted that 7.1% of tested samples were identified as CHIK etiology and they belonged to Asian genotype. (Herman Kosasih et al., 2013)

2.2.5. Geographical distribution of Chikungunya in Indian Ocean Island:

A severe epidemic of CHIK was reported from Indian Ocean Island, Reunion Island during the year 2005-2006. Reunion Island is a French overseas district located in the southwestern Indian ocean, east of Madagascar.

On 16th march 2005 WHO intimated alert to global alert and response network regarding CHIK outbreak in Reunion Island. Surveillance system and sentinel network were set up for monitoring of cases. The first case of CHIK was identified in mid-April 2005 and a total of 16.050 laboratory confirmed cases have been identified. By April 2006 Surveillance system estimates overall 2.44.000 cases with an attack rate of 35% including 123 severe cases and 203 deaths. This was the most mortal and morbid epidemic so far caused by CHIKV since its occurrence. (Philippe Renault et al., 2007) In the same year other islands, Mayotte and Madagascar were also affected by the massive outbreak. A seroprevalence study conducted in pregnant women concluded that in October 2005 1.6% tested woman's were affected and drastically raised to 26% during march-April 2006. A cross-sectional clinical community survey was also conducted during the current epidemic between January to May 2006. A total of 2235 individuals were examined and noted that 26% of population were consistent with CHIK fever symptoms with an attack rate of 249.5 cases per 1000 population. (Daouda Sissoko et al., 2008)

2.2.6. Geographical distribution of Chikungunya in India:

Pioneer outbreak of CHIK in India was reported from Kolkata, West Bengal in 1963. Nearly 200 individuals were affected with the majority in children. (Sarkar J K et al., 1964) Subsequent outbreaks were reported from different cities of three southern states like Tamilnadu, Andhra Pradesh, and Maharastra. Cities like Chennai, Pondicherry and Vellore in 1964, Vishakapatnam, Rajahmundry,

Kakinada and Nagpur in 1965 and Barsi Maharashtra in 1973 were affected. (Jupp PG et al., 1988)

After 32 years of quiescence, CHIK re-emerged in Indian ocean Island and spread to India in 2006. Andhra Pradesh (AP) reported the first laboratory confirmed case in mid-April 2006. Southern states like Andhra Pradesh, Maharashtra and Karnataka were affected in large scale. In 2006, a total of 1938 serum samples were collected from three states and 33.5 to 41.7% of tested clinical samples were declared positive for CHIK IgM antibodies. In 2006 the state governments of AP, Maharashtra & Karnataka declared >25.000, >65.000 and >36.000 clinically suspected cases respectively and causing strains belonged to ECSA genotype. (Prasanna N. Yergolkar et al., 2006) Then after CHIK spread to another southern state Tamil nadu. During June 2006 Malela village, Kadapa Dist, Andhra Pradesh and Gowripet area Avadi, Chennai, Tamilnadu reported 90 and 5 laboratory confirmed cases respectively. (Prabhdeep kaur et al., 2008)

A multi-centric hospital based study was conducted by NIV, Pune in three zonal centers from 1st June 2008 to 31st may 2009. Karnataka institute of medical sciences (KIMS), Hubli, Karnataka identified as the south zone center, Sawai man Singh medical college (SMS) Jaipur Rajasthan as the west zone center and the All Indian institute of medical sciences (AIIMS) Delhi as the north zone centre. Overall positivity rate was 25.37%, highest in KIMS-49.36% than SMS-16.28% and AIIMS-0.56%. (Prathima Ray et al., 2012) During 2009 Chittoor district, Andhra Pradesh alone contributed 520 confirmed cases between January to October with a positivity rate of 68.1% (C.V.M Naresh Kumar et al., 2013) Karnataka was the worst affected state during that outbreak. Bellary district,

Karnataka experienced outbreak during 2009-2011. A total of 1386 serum samples were tested, out of which 343 (24.75%) cases were laboratory confirmed. The prevalence was 23.07%, 28.40% and 19.05% in 2009, 2010 and 2011 respectively. (Narayan shrihari et al., 2012) Then after Gwalior, Madhya Pradesh reported 15 laboratory confirmed cases between october to November 2010. (Manisha soni et al., 2013) After 2006 as per National Vector Borne Disease Control Programme (NVBDCP) CHIK became endemic in India and currently causing epidemic and sporadic cases all over India. NVBDCP reported state wise yearly statistics of CHIK cases from India (http://nvbdcp.gov.in/Chikun-Guidelines.html)

Table –2: Statistics of Chikungunya cases from 2006 to 2008 (adopted from NVBDCP website- http://nvbdcp.gov.in/chik-cd.html)

	Year								
	2006			2007			2008 (till30/07/2008)		
State	Clinically suspected cases	No. of samples referred to NIV/NIC	No. of confirmed cases	Clinically suspected cases	No. of samples referred to NIV/NIC	No. of confirmed cases	Clinically suspected cases	No. of samples referred to NIV/NIC	No. of confirmed cases
Andra Pradesh	77535	1224	248	39	39	11	5	2	1
Karnataka	762026	5000	298	1705	641	133	41227	1442	478
Maharashtra	268333	5421	786	1762	297	135	343	44	19
Tamil nadu	64802	648	116	45	13	10	3	0	0
Madya Pradesh	60132	892	106	0	0	0	0	0	0

Gujarath	75419	1155	225	3223	238	122	139	28	3
Kerala	70731	235	43	24052	4732	909	22815	888	220
A &N Island	1549	0	0	0	0	0	0	0	0
Delhi	560	560	67	203	203	22	0	0	0
Rajasthan	102	44	24	2	2	2	0	0	0
Puducherry	542	52	9	0	0	0	0	0	0
Goa	287	75	2	93	93	18	16	14	3
Orissa	6461	171	34	4065	423	90	0	0	0
West Bengal	21	21	21	19138	1135	347	0	0	0
Lakshadweep	35	0	0	5184	10	10	0	0	0
Uttar Pradesh	4	4	4	4	4	4	0	0	0
Haryana	0	0	0	20	20	13	0	0	0
Total	1390322	15961	2001	59535	7850	1826	64548	2418	724

Table –3: Clinically suspected Chikungunya fever cases from 2009 to 2016 * (adopted from NVBDCP website - http://nvbdcp.gov.in/chik-cd.html)

Sl no	State	2009	2010	2011	2012	2013	2014	2015 (prov.)	2016
1	Andra Pradesh	591	116	99	2827	4827	1359	817	934
2	Arunachal Pradesh	0	0	0	0	0	0	35	239
3	Assam	0	0	0	0	742	0	0	38
4	Bihar	0	0	91	34	0	0	3	566
5	Goa	1839	1429	664	571	1049	1205	561	308
6	Gujarat	1740	1709	1042	1317	2890	574	406	2920
7	Haryana	2	26	215	9	1	3	1	5336
8	Jarkhand	0	0	816	86	61	11	21	47
9	Karnataka	41230	8740	1941	2382	5295	6962	20763	13506
10	Kerala	13349	1708	183	66	273	272	175	124
11	Madya Pradesh	30	113	280	20	139	161	67	2215
12	Meghalaya	0	16	168	0	0	0	78	248
13	Maharashtra	1594	7431	5113	1544	1578	1572	391	7354
14	Orissa	2306	544	236	129	35	10	81	51
15	Punjab	0	1	0	1	0	2	80	4314
16	Rajasthan	2056	1326	608	172	76	50	7	1686

17	Tamilnadu	5063	4319	4194	5018	859	543	329	72
18	Telangana	0	0	0	0	0	1687	2067	611
19	Tripura	0	0	0	0	0	34	180	283
20	Uttar Pradesh	0	5	3	13	0	4	0	2299
21	Uttarkhand	0	0	18	0	0	0	0	35
22	West Bengal	5270	20503	4482	1381	646	1032	1013	1071
23	A and N Island	0	59	96	256	202	161	68	14
24	Chandigarh	0	0	1	0	1	0	1	1315
25	D and N haveli	0	0	0	100	2	0	0	0
26	Delhi	18	120	110	6	18	8	64	12221
27	Lakshadweep	0	0	0	0	0	0	0	0
28	Puduchery	0	11	42	45	146	399	245	327
Total		73288	48176	20402	15977	18840	16049	27553	58136

Note: * Provisional till 31st December 2016

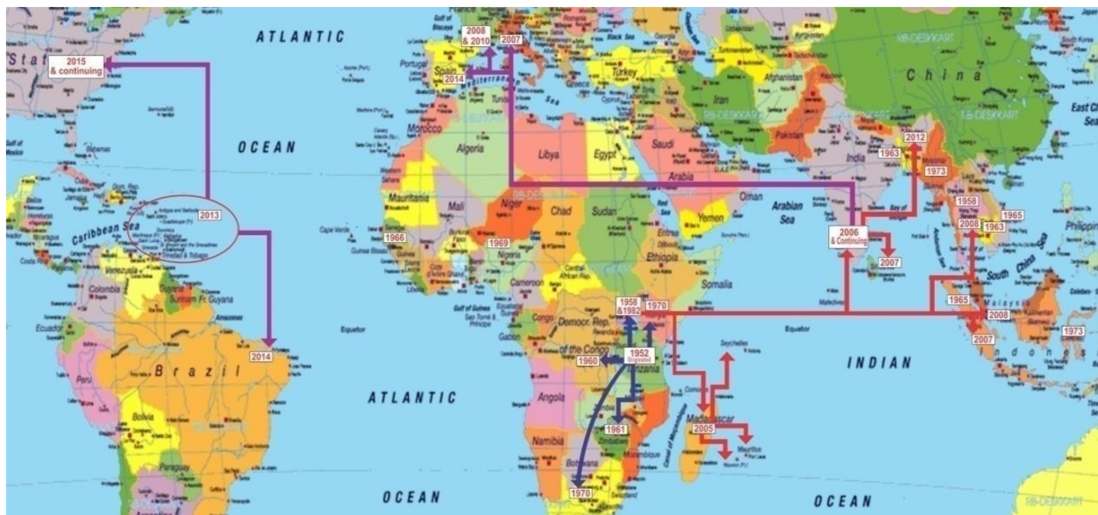


Figure – 1: Predicted dispersal pattern of Chikungunya virus in the world

2.3. Virion structure:

CHIKV is a RNA virus, belongs to the family Togaviridae and genus alphavirus. CHIKV is a spherical shaped and enveloped virus. RNA is linear, positive sense and single stranded with an approximate length of 11.8 Kbp. (Jawetz, Medical Microbiology). Alphavirus has 8 antigenic complexes and each complex has many species. (Simizu B et al., 1984 and Weaver S. C et al., 2005)

Table – 4 : Alphavirus species and antigenic relationships.

Antigenic complex	Species
Barmah forest	Barmah forest virus (BFV)
Eastern quine encephalitis	Eastern equine encephalitis virus (EEEV)
Middelburg	Middelburg virus (MIDV)
Ndumu	Ndumu virus (NDUV)
Semliki forest	Bebaru virus (BEBV) **Chikungunya virus (CHIKV)** Getah virus (GETV) Mayaro virus (MAYV) O'nyongnyong virus (ONNV) Ross River virus (RRV) Semliki Forest virus (SFV) Una virus (UNAV)

Venezuelan equine encephalitis	Venezuelan equine encephalitis virus (VEEV) Mosso das pedras virus (MDPV) Everglades virus (EVEV) Mucambo virus (MUCV) Tonato virus (TONV) Pixuna virus (PIXV) Cabassou virus (CABV) Rio Negro virus (RNV)
Western equine encephalitis	Aura virus (AURAV) Sindbis virus (SINV) Whataroa virus (WHAV) Fort Margen virus (FMV) Highlands J virus (HJV) Western equine encephalitis virus (WEEV)
Unclassified	Trocara virus (TROV) Salmon pancreas disease virus (SPDV) Southern elephant seal virus (SESV)

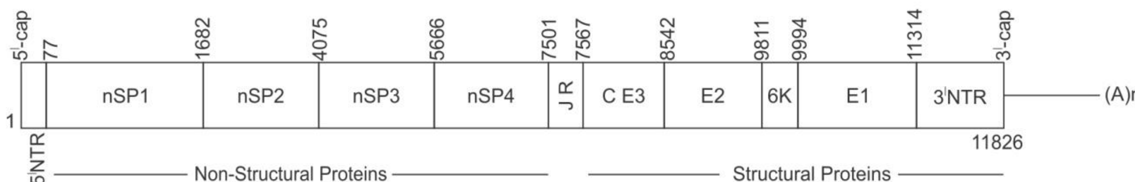


Figure -2: Structure of Chikungunya virus genome

2.3.1. Genomic organisation:

CHIKV genome consists of two open reading frames, one code for non-structural poly-proteins (2474 aa) and another code for structural proteins (1244 aa). Genomic organization of CHIKV is 5'cap-nsp1-nsp2-nsp3-nsp4-(junction 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 and one third towards 3'end consists of structural proteins (Figure - 1). The 5' NTR has 76 nt, 3' NTR has 526 nt and internal poly-A region has 68 nucleotides. 3' end has internal poly-adenylation site and repeated sequence elements (RSEs). (Bharath M D et al., 2016)

2.3.2. Chikungunya proteins:

CHIKV has non-structural and structural proteins.

2.3.2.1. Non-structural proteins: CHIKV has four non-structural proteins (nsP) namely nsP1, nsP2, nsP3 and nsP4 with length of 7425 nucleotides .

2.3.2.2 Structural proteins: CHIKV has five structural proteins namely Envelope proteins (E) E1, E2 and E3, Capsid protein (C) and 6K with the length of 3735 nucleotides. (Bharath M D et al., 2016)

2.4. Mode of transmission:

CHIKV is a vector born virus and is transmitted to humans by the bite of infected vector-Aedes mosquitoes and so it's called an arboviral disease. Primarily it was transmitted by *Aedes aegypti* and Indian tiger mosquito *Aedes albopictus* was identified as a competent vector during 2005-06 in an outbreak reported from Indian ocean island and India (Xavier de Lamballerie et al., 2008). CHIKV can also be transmitted through infected blood and blood products (Codrel H et al., 2006) and from mother to neonate (intrapartum infection) by vertical transmission (Patrick Ge´rardin et al., 2008 and Ramful D et al., 2007)

Aedes mosquitoes are two winged insects belongs to the order Diptera, family Culicidae and subfamily Culicinae.

2.4.1. Life cycle of Aedes mosquitoes:

Mosquito life cycle completes in around 8-10 days. To complete the immature and adult stages, mosquitoes require two different environments. The immature stages. Eggs, larvae, and pupae require an aquatic environment whereas adult mosquitoes require aerial and terrestrial environment. The stages of development are as follows.

2.4.1.1.Egg:

In Genus Aedes, the eggs are laid separately often in dry hollows or manmade/artificial containers which contain mostly moist and damp environment. It can hold viability for a long period without water.

2.4.1.2.Larvae:

The eggs are hatched into larvae within 2-3 days. There are four stages of larvae known as instars and larvae emerge to pupae within 4-5 days.

2.4.1.3.Pupae:

The pupae are a non-feeding stage and undergo morphological and physiological changes and emerge into adult mosquitoes within 2-3 days.

2.4.1.4.Adult mosquitoes:

The adult mosquito emerges from pupal skin, rest for few minutes and finally expands its wings and flies out. (Mosquito life cycle *Aedes aegypti*. Fact Sheets http://www.cdc.gov/.pdf)

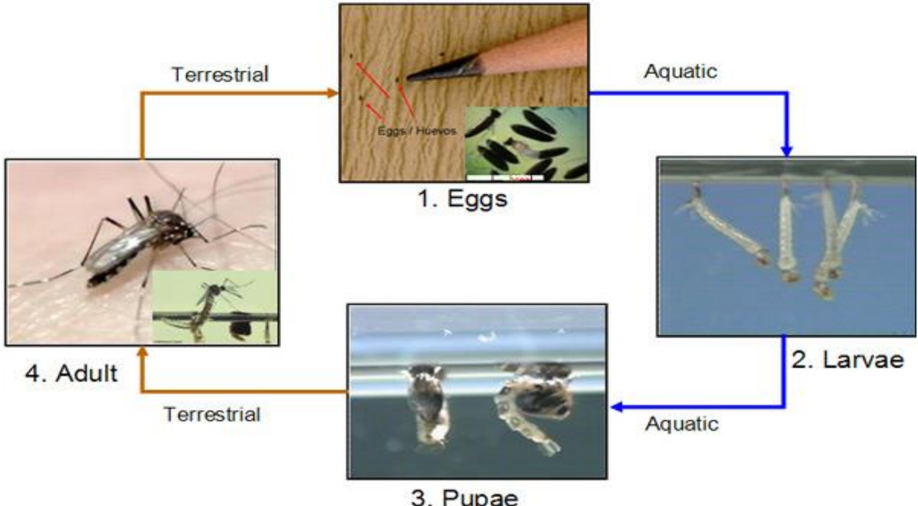


Figure –3: Life cycle of Aedes mosquitoes adapted from Centers for disease control and prevention website

(http://www.cdc.gov/dengue/entomologyecology/m_lifecycle.html)

2.4.2. Survival of Chikungunya virus in Nature:

CHIKV is maintained in nature in two different cycles.

2.4.2.1. Enzoonotic Sylvatic mosquito cycle:

CHIKV is maintained in Africa by Sylvatic cycle. It takes place in the forest. Forest dwelling mosquitoes like *Aedes furcifer* principle vector*, Aedes vittatus, Aedes fulgens, Aedes luteocephalus, Aedes dalzieli, Aedes vigilax and Aedes camptorhynchites* serves as vectors (Diallo M thonnon et al., 1999, Peyrefitte C.N et al., 2007 and Peyrefitte C.N et al., 2008) and Non-human primates like monkeys, Chimpanzees, Horse, pigs water buffaloes, cattle, dogs, rodents, birds are reservoirs or amplifying host. (Weinbren M.P et al., 1958) Through vectors people living nearby forest usually get an infection.

2.4.2.2. Endemic/Epidemic urban cycle:

CHIKV is maintained in Asia by urban cycle. It takes place in urban and peri-urban areas. *Aedes aegypti* and *Aedes albopictus* mosquitoes serve as vectors and no amplifying host has been recognized. People living in the urban areas usually get infected (Weaver S C et al., 2012).

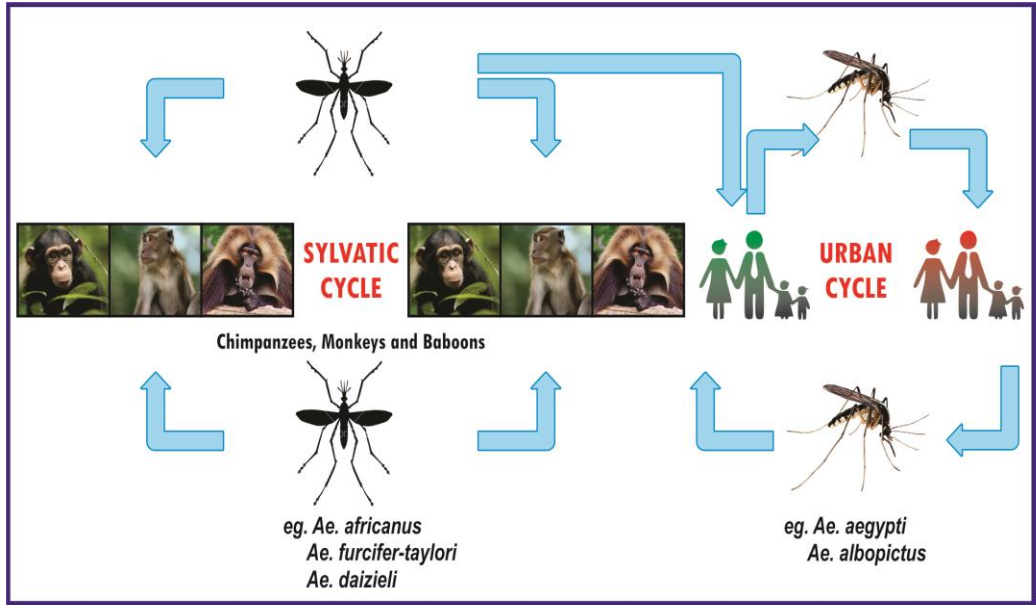


Figure – 4: Life cycle of CHIKV showing the relationship between Sylvatic cycle and Urban cycle occurs in Africa adopted from Michelle M. Thiboutot et al., 2010.

2.5. Pathogenesis:

After mosquito bite, CHIKV enters into subcutaneous capillaries, in which susceptible immune cells like macrophages or fibroblasts and endothelial cells are infected. Local viral replication occurs and CHIKV further moves to secondary lymphoid organs which are nearer to the site of inoculation. Through blood CHIKV either in free form (virions) or in infected monocyte, moves to near secondary lymphoid organs like liver, spleen, muscle and joints. Macrophage and mononuclear cell infiltration occurs and results in replication. Usually, Subclinical infection occurs in liver (hepatocyte apoptosis) and lymphoid organs (adenopathy). Whereas in muscles and joints it causes severe pain and may result in persisting arthritis**.** (Laurence Dupuis et al., 2012)

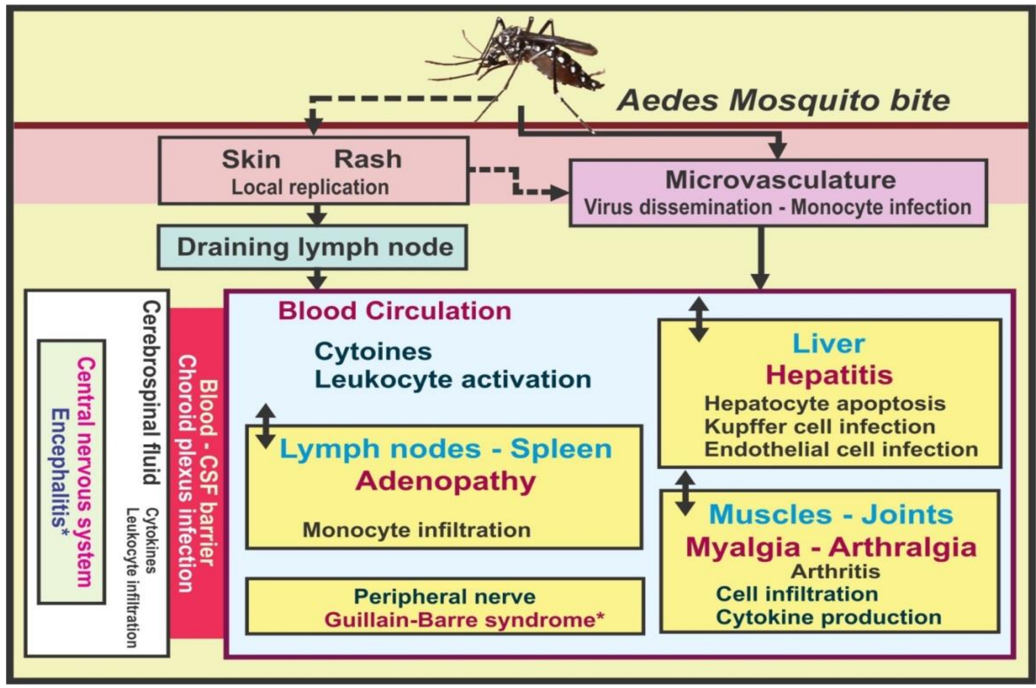


Figure – 5: Virus dissemination to target organs adopted from Laurence Dupuis et al., 2012. (doi:10.1371/journal.pntd.0001446.g001)

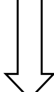
2.5.1. Chikungunya Replication:

CHIK viral replication takes place in host fibroblast, endothelial and epithelial cells and stepwise cellular events are mentioned in the below flow chart.

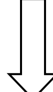
Virus enters into fibroblasts, endothelial and epithelial cells through P^H mediated receptor dependent endocytosis- prohibitin on Microglial cells (Bernard, E et al., 2010 and Wintachai P et al., 2012)

⇩

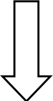
Due to acidic nature of endosome E1 dissociated from E2 protein. (Li L. Jose et al., 2010)



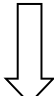
Viral membrane fuses with endosome, viral cytoplasm exposes and results in the release of SS RNA genome, which is converted into mRNA by reverse transcriptase enzyme. (Li L. Jose et al., 2010)



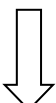
Translated polyprotein precursor of 2474 amino acid is synthesized. (Li L. Jose et al., 2010)



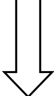
Poly protein precursor is lysed and replication complex comprises of non-structural proteins nsP1-nsP4 is formed (Li L. Jose et al., 2010)



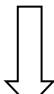
Full length negative strand RNA intermediate is formed. (Li L. Jose et al., 2010)



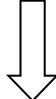
RNA intermediate serves as template for synthesis of sub-genomic RNA (26S) and genomic RNA (49S) (Jose J et al., 2009)



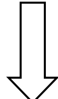
Sub-genomic RNA (26S) is translated into structural polyprotein precursor (1244 aa) C-E3-E2-6K-E1.(Jose J et al., 2009)



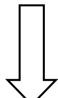
Due to auto-proteolytic serine protease activity capsid protein (C) is detached from structural polyprotein precursor. (Jose J et al., 2009)



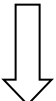
E3 protein has a signal sequence which translocates E3-E2-6K-E1 complex (spike protein) into the endoplasmic reticulum. (Jose J et al., 2009)



Endoplasmic reticulum signalase cleaves polyprotein into PE2 (E2-E3), 6K and E1 proteins. (Jose J et al., 2009)

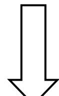


PE2 (E2-E3) and E1 proteins arranged in heterodimer and oligomerize to form non-fusogenic spikes. (Jose J et al., 2009)



Host cell furin or furin-like protease cleaves E2-E3 to E2 and E3. (Jose J et al., 2009)

Translocation of non-fusogenic spikes from endoplasmic reticulum through golgi bodies and plasma membrane occurs. (Jose J et al., 2009)



Capsid protein binds to RNA and facilitates the arrangement of envelope glycoprotein's (E1, E2 and E3) on envelop and makes complete virus. (Jose J et al., 2009)

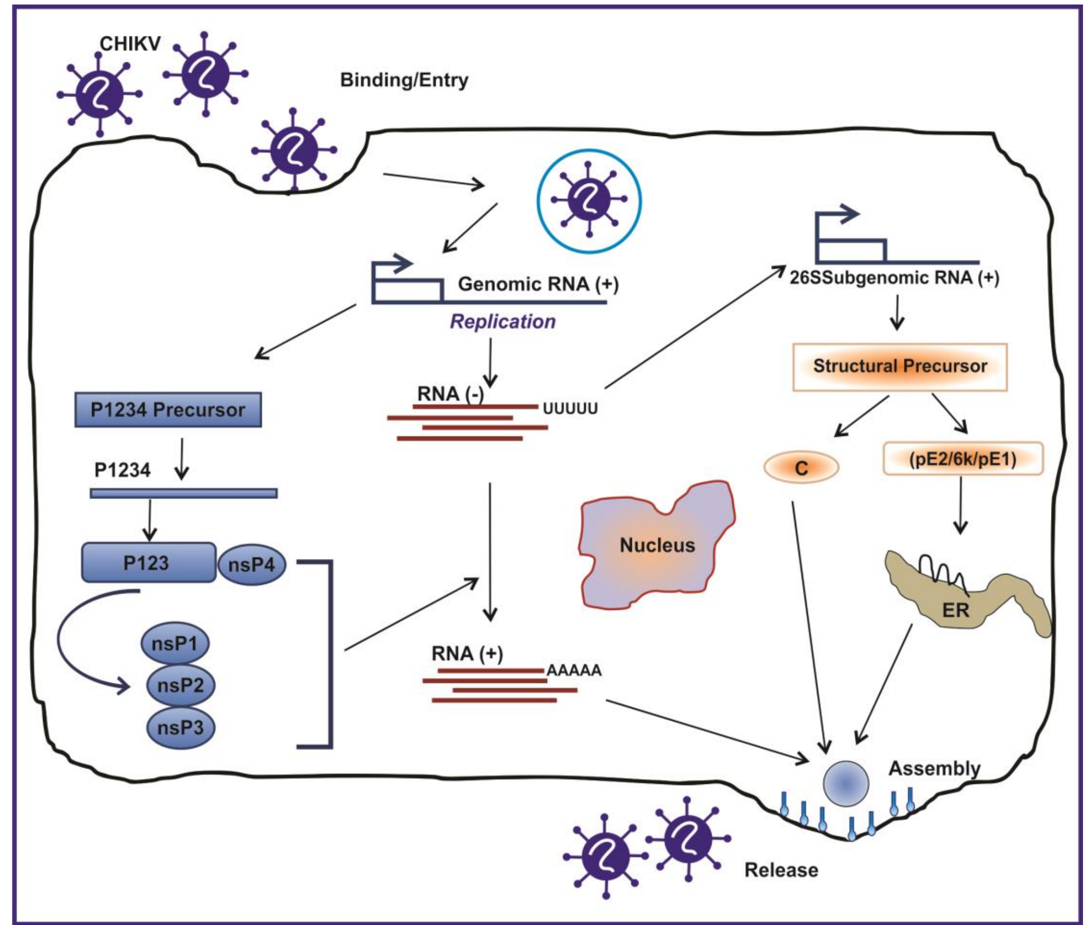


Figure –6: Life cycle of Chikungunya virus inside the infected cells adopted from Michelle M. Thiboutot et al., 2010.

2.5.2. Immunological pattern of CHIK in Cynomolgus macaques animal model.

CHIK immunological disease pattern was studied in an immuno-competent Cynomolgus macaques animal model. Results depicted that CHIKV persists mainly in mononuclear cells predominantly macrophages, to lesser extent in endothelial cells. Viremia was observed in muscular tissue, lymphoid organs and liver for 3, 2 and 1.5 months respectively. Long-term CHIKV persistence was observed in joints, muscles, lymphiod organs and liver. During chronic phase of

disease, macrophages act as reservoir of virus and may be responsible for long-lasting arthralgia. (karine Labadie et al., 2010).

2.5.3. Mechanism of Chikungunya persistence and tissue inflammation in chronic disease

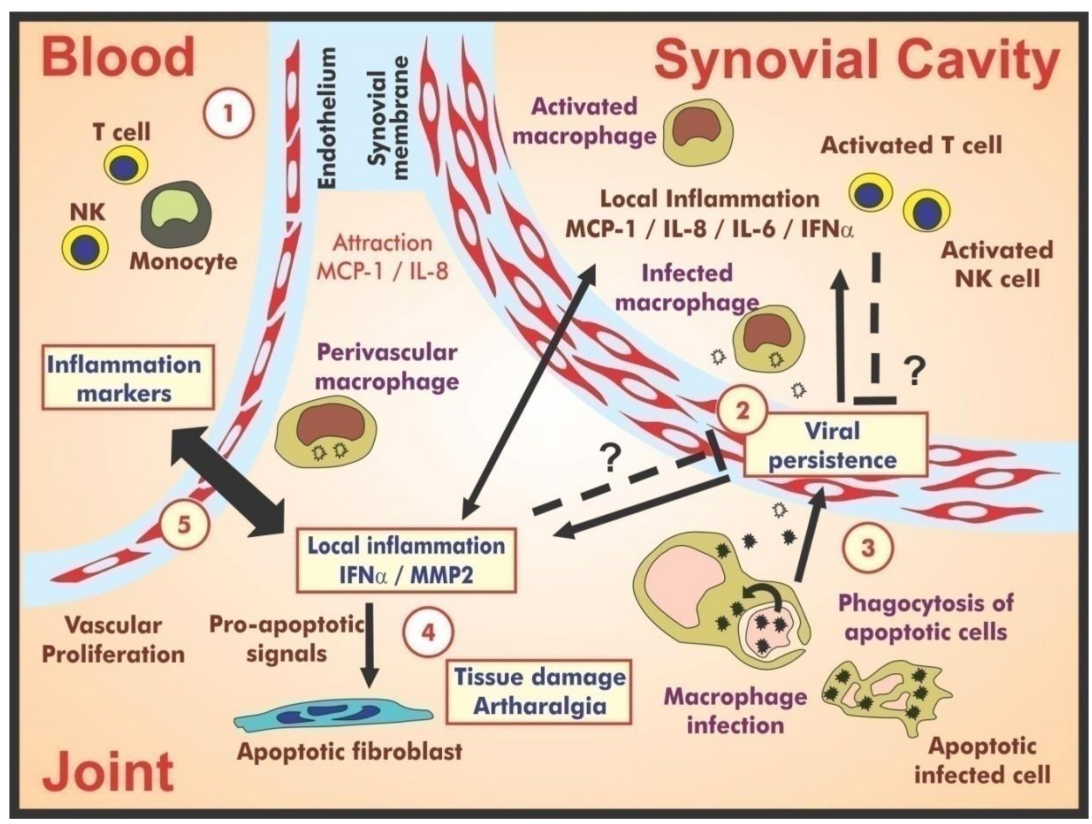


Figure – 7: Mechanism of Chikungunya persistence and tissue inflammation in chronic disease adopted from Laurence Dupuis et al., 2012.

In chronic infection (>30days) monocytes (macrophages), T-cells and natural killer cells are attracted towards inflamed joint and gets activated. Infected macrophages cause local inflammation and release cytokines, chemokines and pro-inflammatory effectors such as IFN-alpha, MCP-1/CCL-2, IL-8, IL-6 and MMP-2. The Phagocytosis of apoptic bodies from infected cells occurs and may contribute

to viral persistence. The beneficial or deleterious effect of local inflammation on viral persistence is still unclear. Fibroblast apoptosis and cartilage destruction result in tissue damage, pain and arthritis. The coordination between local inflammation and systemic activation driven by inflammatory markers in plasma and blood cells is unclear. (Hourau JJ et al., 2010)

2.5.4. Bone pathology in Chikungunya disease

The main effecter cells affected by CHIKV are bone cells. In humans, Bone homeostasis is maintained by coordination of bone cells i.e osteoblast (bone forming cells) and osteoclast (bone resorbing cells). Osteoclastogenesis is the formation of osteoclast and is regulated by osteoblastic and non-osteoblastic cells like T-cells through Receptor activator of nuclear factor kB ligand (RANKL) and osteoprotegerin, a soluble decoy receptor for RANKL. Alteration in ratio of the RANKL to osteoprotegerin results in Rheumatoid arthritis and osteoporosis. (Hofbauer L C et al., 2004) In case of infection with arthritogenic alpha viruses like Ross River Virus, the primary osteoblastic cells produce more RANKL to osteoprotegerin ratio which results in osteoclastogenesis. (Chen W et al., 2014) Author strongly suggested that there must be a mechanism through which arthritogenic viruses like Ross River viruses and CHIKV directly infect osteoblast and results in osteoclastogenesis. They postulated that arthritogenic virus infection increases susceptibility to rheumatoid arthritis and bone pathologies. The individuals with prior bone diseases are more prone (double than normal people) to go for severe bone pathology. (Felicity burt et al., 2014)

2.5.5. Immune mediators in Chikungunya infection

Immune mediators like Pre-existing natural antibodies, CHIK specific induced antibodies, B cells, CD4 TH cells with cytokines IFN-Ɣ and TNF play a very important role in control of CHIK infection. TH cells play important role in viral suppression (Fok-moon Lum et al., 2013). During infection neutralizing Immunoglobulin IgG (class IgG3) was the predominant antibody and IgG3 antibodies in acute infection facilitate more severe symptoms and result complete recovery. IgG3 antibodies produced in the later stages of infection shall develop mild symptoms and results chronic disease. Approximately 30% of late IgG3 responders develop persistent arthralgia. (Yiu-wing kam et al., 2012) The role of cytokineswas studied in detail. In Early acute phase (0-5 days after infection) predominant Cytokines are Interferon’s (IFN) alpha, beta gamma, Interferon gamma induced protein-10 (CXCL-10/IP-10) and Interleukins 1 beta. In Extended symptomatic phase (15-30 days after infection) Cytokines Tumor necrosis factor (TNF) alpha, Monocyte chemo-attractant protein (MCP-1) Interleukins IL-4, IL-6 and IL-10 are prominent. Low IgM titre with High TNF-alpha, IL-6 and IL-8 were observed in recovered individuals. High TH-2 cells, IgM and low IgGtiter were observed in Persistent arthralgia cases. (Anuradha Venugopalan et al., 2014)

2.6. Clinical manifestations:

Majority of the infections are subclinical, ratio of subclinical (asymptomatic) and clinical (symptomatic) infections are 4.6:1. A significant age wise difference in the ratio was observed. Children of less than 5 years of age ratio was 2:1 and in adults of more than 50 years of age it was 12:1. (In-KyuYoon et al.,

2015) The acute stage of disease lasts for 3 to 10 days. (Borgherini G et al., 2007) In adults CHIK usually presents with High fever 102-105^{0} F continued for 1-6 days, Severe joint pain in interphalangeal joint of fingers, knee, ankle and shoulder especially during morning time, Maculopapular rash in trunk and extensor surface of limbs, Headache and Tachycardia. (Lumsden W.H.R. et al., 1955)

In Neonates CHIK usually presents with high fever, generalised edema, tenderness, poor feeding, pain, distal edema, paradoxical cry and various skin manifestations. Less frequently seizures, meningoencephalitis, and echo-cardiographic abnormalities were also observed. (Hariharan Gopakumar et al., 2012 and Sebastian M R et al., 2009)

Severe atypical clinical manifestations were observed during recent CHIK outbreak of Indian ocean island and India 2006. System wise clinical features are mentioned in table - 5. (Philippe Renault et al., 2007, S.D. Suryawanshi et al., 2009, Chandak NH et al., 2003 and Rajapakse S et al., 2010)

Table –5: Atypical Clinical manifestations of Chikungunya

Sl no	System	Symptoms
1.	Neurological	Meningoencephalitis, encephalopathy, seizures, Guillain-Barré syndrome, cerebellar syndrome, paresis, palsies, neuropathy
2.	Ocular	Optic neuritis, iridocyclitis, episcleritis, retinitis, uveitis

3.	Cardiovascular	Myocarditis, pericarditis, heart failure, arrhythmias, hemodynamic instability
4.	Dermatological	Photosensitive hyperpigmentation, intertriginousaphthous-like ulcers, vesiculobullous dermatosis
5.	Renal	Nephritis, acute renal failure
6.	Other	Bleeding dyscrasias, pneumonia, respiratory failure, hepatitis, pancreatitis, syndrome of inappropriate secretion of antidiuretic hormone (SIADH), hypoadrenalism

2.6.1. Persistent arthralgia:

The hall mark feature of CHIK disease is Persistent arthralgia. Usually, after two weeks of infection acute stage symptoms resolve and patient gets complete recovery. In a significant number of cases individuals will proceed to the chronic stage of disease characterized by persistent arthralgia. (Manimunda S P et al., 2010) CHIK induced Persistent arthralgia is consequence of immuno-pathological reaction. A strong association of CD4 T (TH) cells with arthritis have been observed. (Yee suan poo et al., 2014) Macrophage acts as viral reservoirs in affiliated joints which were protected from neutralization action of IgG3 antibodies. (Yiu-wing kam et al., 2012) The persistence of CHIKV in macrophage of joints may lead to local inflammation and pain. As observed in HIV, local

inflammation may contribute to sustained replication and viral persistence in macrophages of tissue. So imperfectly resolved inflammation may result in persistent arthralgia. The regulatory mechanism is responsible for the CHIK viral clearance from local joints and results in recovery from persistent arthralgia. Any defect in the regulatory mechanism may also lead to persistent arthralgia (Chow A et al., 2011 and Hourau J J et al., 2010). In people of age ≥45 years, severe joint pain during initial stages of disease and comorbid osteoarthritis are risk factors for persistent arthralgia. ~55-65% of infected cases experiences Persistent arthralgia. (Gianandrea Borgherini et al., 2008 and Daouda sissoko et al., 2009)

Table –6: Number of cases affected with Persistent arthralgia associated with Chikungunya disease

Author	Place of study	Number of cases affected with persistent arthralgia	Percentage affected
Hourau JJ et al., 2010	La Reunion island	12	~50%
Manimunda SP et al., 2010	Dakshinakannada	10	49%
Chow et al., 2011	Singapore	3	13%
Kelvin AA et al., 2011	Emilla-Romagna	6	70%
		12	32%

2.7. Complications

Complications due to CHIK were very rarely reported before Indian ocean island outbreak. During Reunion epidemic especially in old age (≥65 years) and children following serious complications were observed. Respiratory failure, cardiovascular decompensation, meningoencephalitis or other CNS problems, Severe acute hepatitis, severe cutaneous effects, sensorineural deafness and kidney failure. (Philippe Renault et al., 2007, S.D, Suryawanshi et al., 2009, Chandak NH et al., 2003 and Rajapakse S et al., 2010)

2.8. Mortality

CHIK mortality was not observed till 2005. For the first time during 2005 reunion island reported 203 deaths. (Philippe Renault et al., 2007) During the peak of CHIK outbreak in 2006 Ahmadabad and Mauritius cities reported a comparatively high number of deaths i.e 2944 and 743 respectively. But it was not confirmed as deaths due to CHIK etiology. (Dileep mavalanker et al., 2008 and Sanjay Beeson et al., 2008)

2.9. Laboratory diagnosis:

CHIKV can be diagnosed in the laboratory by fallowing methods.

2.9.1 Virus isolation.

2.9.2 Reverse transcription polymerase chain reaction.

2.9.3 Serological tests.

2.9.1. Virus isolation:

This method is the gold standard for confirmation of Chikungunya etiology and to be carried out in Bio-safety level 3. Blood samples are collected in less than 4 days of symptoms and serum is separated and transported to the laboratory in the cold chain. The sample is inoculated into Vero, BHK-21, Hela cell lines or Sucking mice. Cell lines were observed for cytopathic effect within 3 days. Culture supernatant or brain suspension is used to confirm the Chikungunyaetiology by immuno fluorescence assay (IFA) or RTPCR. (Pyndiah MN et al., 2012)

2.9.2. Reverse transcription polymerase chain reaction (RTPCR):

Reverse transcriptase polymerase chain reaction is sensitive and specific method for detection of CHIK RNA in clinical sample. Blood samples are collected from cases between 1 to 8 days of symptoms and samples are immediately transported to laboratory in cold chain, care should be taken that as CHIKV is RNA virus and RNA free materials should be used during sample collection and transportation. In the assay RNA is converted into DNA by Reverse transcriptase enzyme and same will be amplified and detected. The steps of RTPCR are: a reverse transcription step at 42°C for 15 min; fallowed 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. (Bharath M D et al., 2016) The advantage of Real time PCR is that the amplified product is detected in same time during the assay where as in conventional PCR methods genetic material is detected only after the end of the assay. (Hasebe F et al., 2002)

2.9.3. Serological tests:

CHIK specific IgM antibodies are detected in the serum by IgM Enzyme linked immuno sorbent assay (ELISA), Plaque reduction neutralization testing (PRNT) and Hemagglutination inhibition (HI) tests. Serum samples are separated, transported in cold chain and stored at 2-8°C for further analysis. PRNT detects CHIK specific fourfold rise in acute/convalescent serum samples. Acute serum samples are collected immediately after onset of symptoms and convalescent sample after 10-14 days after acute sample. The most widely used test is IgM ELISA for the detection of CHIK specific IgM antibodies. Anti CHIK IgM antibody starts appearing on day 4, peaks on day 7 and decreases rapidly and maintained in sustained level through day 14. IgG antibody starts appearing on day 5 and increases till day 24. (Sarunyou Chusri et al., 2014) Serum samples should be collected after 5 days of onset of symptoms. Antigenic cross-reactivity was observed between Alphavirus of semliki forest virus antigen complex. So during interpretation of results, geographical distribution of Alphavirus should be considered. (Sergio oliveira De paula et al., 2004)

2.10. Therapeutics and vaccines:

No antiviral therapy is prescribed, treatment is solely symptomatic and supportive. Adequate fluid intake must be administered. Paracetamol or nonsteroidal anti-inflammatory drugs (NSAIDS) may be used for fever and pain relief. (Topley & Wilson’s 1997) Chloroquine phosphate at a dosage of 250mg/day for 20 weeks can be used to treat Persistent arthralgia cases in CHIK confirmed patients. ~50% of the patients improved with morning stiffness and other articular

symptoms. Future studies need to be carried out with large sample size to find out the role of Chloroquine phosphate in the treatment of Persistent arthralgia. (Brighton S. W. et al., 1984)

2.11. Prevention and Control:

Control of mosquitoes is one of the most effective, feasible and economical ways of controlling mosquito born diseases. In mosquito born diseases vector control plays a very important role by reducing the vector population. Mosquito control methods are classified into

2.11.1. Protective measures against mosquito bites

2.11.2. Control of mosquito breeding sites

2.11.2.1. Mechanical destruction of mosquito breeding sites

2.11.2.2. Larvicidal Control of Mosquitoes

2.11.2.3. Surveillance

2.11.2.4. Information, Education and Communication Activities

2.11.1 Protective measures against mosquito bites:

The mosquito man contact can be reduced by fallowing methods

2.11.1.1 Wearing full sleeved cloths.

2.11.1.2 Usage of insecticide treated bed nets.

2.11.1.3 Usage of mosquito repellents.

2.11.1.4 Installation of mosquito proofing for windows and doors. (Andreas Schoepke et al., *1998*)

2.11.2 Control of mosquito breeding sites:

Mosquito breeding sites can be destroyed by fallowing methods

2.11.2.1 Mechanical Destruction Of Mosquito Breeding Sites:

Aedes mosquitoes breeds in clean and clear stagnant water, which is available in all domestic storage containers like drums, barrels, earthen pots and also in other favourable breeding sources like tyres, plastic cups, coconut shells, flower pots, grinding stones. To control domestic mosquito breeding, all domestic storage containers should be covered with airtight fitting lids. Complete source reduction can be achieved by destructing the egg stage attached to the inner walls of the container and this is done by rubbing the inner wall of the containers by using brush with soap and water. Peri-domestic mosquito breeding sources like solid wastes (discarded tyres, plastic cups, coconut shells, flower pots and grinding stones etc) should be segregated and discarded in such a way that, there should be no further chance for water collection and in turn of becoming a breeding source for mosquitoes.

2.11.2.2 Larvicidal Control of Mosquitoes:

The earliest chemical control of mosquitoes was directed against the larval stage. A larvicide is used to destroy the larvae stage of mosquitoes. In olden days crude kerosene and distilled petroleum oil were used as Larvicidal agents. Presently the most widely used Larvicidal agent is Temephos (abate). This chemical compound is widely used because of its low mammalian and fish toxicity, low cost and lower dosage needed. Temephos is used in areas of standing water, shallow ponds, swamps, marshes and intertidal zones by using backpack sprayers and fixed-wing aircraft in liquid form.

Preparation of working Temephos solution: Dilute 10 ml 1 PPM abate solution in 490ml distilled water, formulated solution is used in the field as 1ml in 10 LTR of stored water.

Biological insecticides such as *Bacillus sphaericus* and *Bacillus thuringiensisisraelensi*s can also be used as the microbial larvicides.

2.11.2.3 Surveillance:

Routine entomological surveillance is carried out to determine geographical distribution of vector, density of vector and vector population in an area and to evaluate the efficacy of control programs. To monitor immature *Aedes* mosquito population following indices are routinely used, House index (HI), Container index (CI) and Breatue index (BI). House index is the percentage of houses infested with larvae or pupae. Container index is the percentage of water holding containers infested with larvae and pupae. Breatue index is a number of positive container per 100 houses inspected.

2.11.2.4 Information Education and Communication (IEC) activity:

The only way to tackle the outbreak of CHIK is to strengthen the IEC activity. This can be achieved by addressing or creating awareness to the public in mass. Effective IEC methods are

2.11.2.4.1 Display of long lasting san board posters in all public places and hospitals.

2.11.2.4.2 Awareness campaign by conducting jathas/rallies involving college/nursing students.

2.11.2.4.3 Educating children at school and college level.

2.11.2.4.4 Involvement of media, NGO's and school health education for all awareness programms.

2.11.2.4.5 Creating awareness among all elected representatives from gram panchayath to national level.

Awareness through wall writing at any strategic public places where people congregate. (India fights Dengue. Strategy and plan of action for effective community participation in prevention and control of dengue-Version-1. http://www.nvbdcp.gov.in/Doc/-.pdf.)

Chapter – 3
Aims and Objectives

3.1. Aims and Objectives:

3.1.1 To carry-out the Sero-epidemiological surveillance of Chikungunya in and around Vijayapura district.

3.1.2. To carry-out the Molecular confirmation of Sero positive Chikungunya cases.

3.1.3. To carry-out Molecular characterisation of Chikungunya strains.

3.1.3.1 To find out the genotype prevalent in the area and to assess the Phylogenetic relation and homology of current strains with previous isolates.

3.1.3.2. To find out the Mutations in E1 gene of current strains and correlation of its effect on protein structure, virulence, infectivity and host adaptability.

3.2. Hypothesis:

Chikungunya may be endemic in the Vijayapura region. The isolates circulating in the Vijayapura region may belongs to East Central South African (ECSA) genotype and may be closely related to ECSA strains of Re-union islands. The isolates may have novel mutation in E1 gene E1A226V which is responsible for host adaptability with respect to *Aedes albopictus* mosquitoes and elevated infectivity and virulence characters of Chikungunya virus. The other mutations in E1 gene may also present and which may contribute to the increase in infectivity, virulence and host adaptability and alteration in protein structure of Chikungunya virus.

Chapter – 4
Materials and Methods

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 (>38.5^0 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.

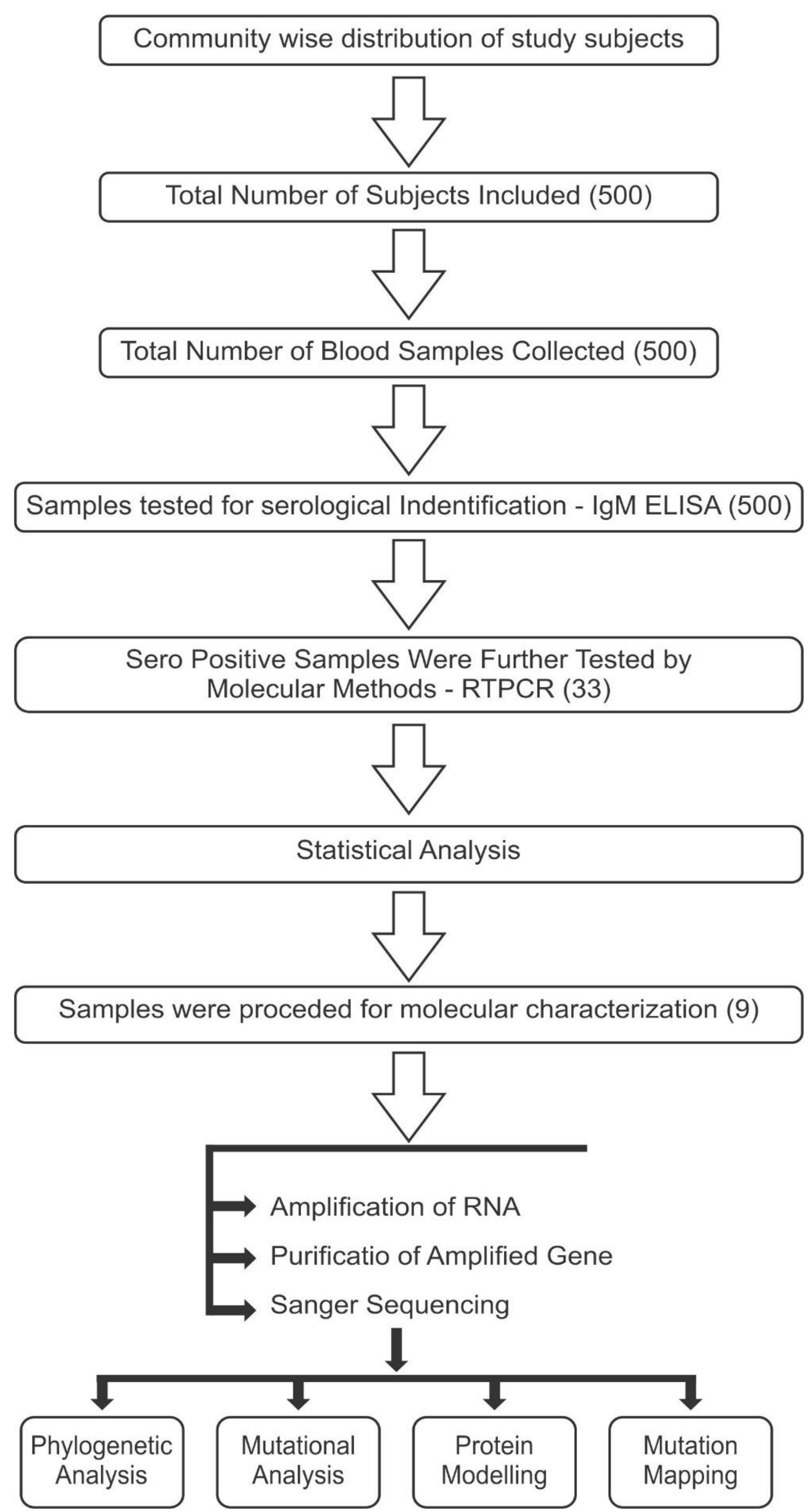
4.1. Sample collection

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.

Figure no.8 : Study Design



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)

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 (15-25^{0}C) 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^{0}C for further analysis.

4.2.2.2. Reverse transcriptase Real time Polymerase Chain Reaction (RTPCR):

RTPCR was carried out using Amplisure® Chikungunya RTPCR 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 RTPCR machine slots were labelled as per strip tube in the same order of 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

Observation	Interpretation	Conclusion
Amplification signal detected in CHK channel (FAM) and in internal control channel (Yakima yellow)	Chikungunya RNA detected	Proceed for further analysis
Amplification signal detected in CHK channel (FAM) and no signal in internal control channel (Yakima yellow)	Chikungunya RNA detected	
Amplification signal not detected in CHK channel (FAM) but detected internal control channel (Yakima yellow)	Chikungunya RNA not detected	
No amplification signal detected in CHK channel (FAM) as well as internal control channel (Yakima yellow) in unknown samples	Possible inhibition of PCR	Dilute the RNA sample (1:100) and repeat assays

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 (ATGGCGACGCCCCCAAAGTC) 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.

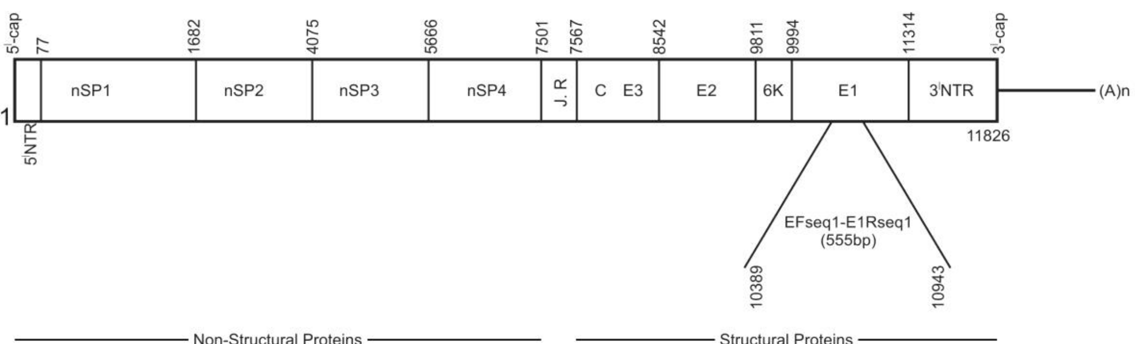


Figure -9: Target sequences in E1 gene for sequencing.

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

Primer Name	Sequences 5'-3'	Genome position	Amplicon size bps
E1-F1	GCTCCGCGTCCTTTAC	10389-10943	555
E1-R1	ATGGCGACGCCCCCAAAGTC		

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.

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

Nucleotide Sequence Accession Numbers with position	**Protein Sequence Accession Numbers (Uniprot)**	**Strain**	**Year of isolation**
AF369024.2|:10427-10941	Q8JUX5	S27 (ECSA)	1952
DQ309336.1|:223-737	A0SE38	Reunion 223/05 (ECSA)	2005
AM258992.1|:10376-10890	Q1W367	Reunion (ECSA)	2006
EF027139.1|:10428-10942	A6MH23	INDIA-00-MH4 (Asian)	2007
HM045811.1|:10410-10924	D7R978	Tanzania (ECSA)	1953
HM045797.1|:10410-10916	D7R952	RSU1 (Asian)	1985
EU192143.1|:2861-3367	B2BZX4	Indonesia (Asian)	2007
HM045817.1|:10412-10926	D7R990	Senegal (West Africa)	2005
HM045816.1|:10412-10926	D7R988	Senegal (West Africa)	1966

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)

Chapter – 5
Results

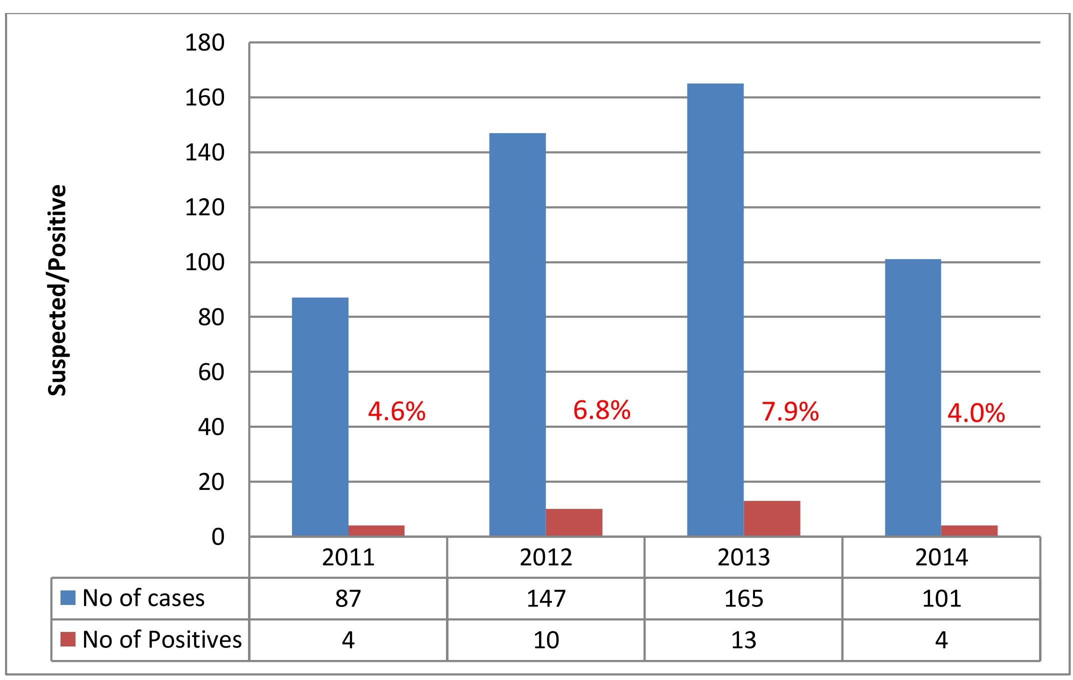
5.1. Serological Identification:

Of the 500 serum samples tested 33 serum samples were found to be positive for Chikungunya IgM antibodies.

5.2. Molecular Confirmation:

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

Figure –10: Year wise distribution of Chikungunya clinically suspected and confirmed cases (2011 to 2014)



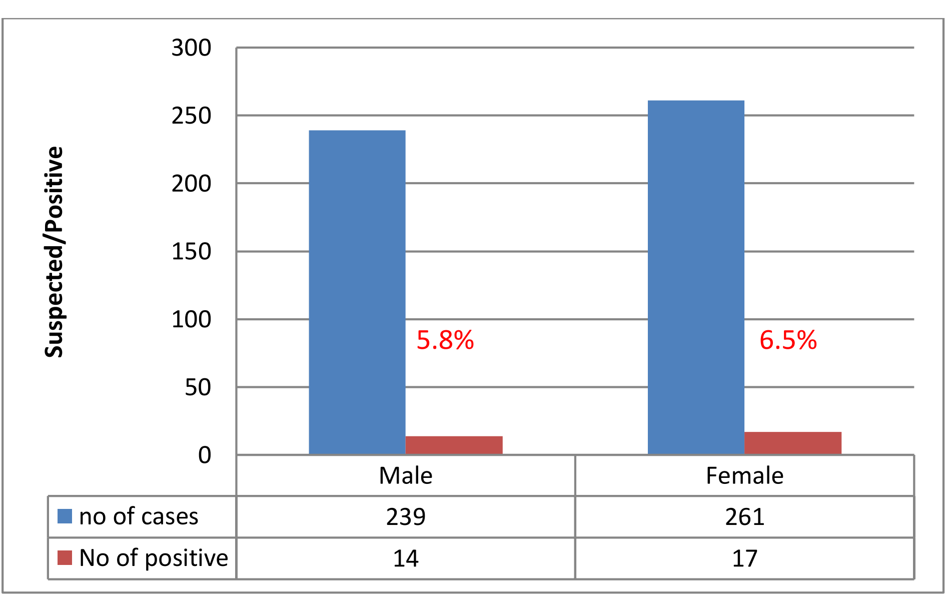
	2011	2012	2013	2014
No of cases	87	147	165	101
No of Positives	4	10	13	4

χ^2 Value= 2.147 DF=3 P value=0.543, Not significant

Figure – 10 explains that in the year 2011, 87 CHIK cases were tested, out of which 4 cases were confirmed of CHIK etiology with positivity rate of 4.6%. Similarly in the year 2012 out of 147 CHIK suspected cases, 10 cases were positive for CHIKV with positivity rate of 6.8%. In the year 2013, 13 of 165

suspected cases were confirmed as CHIK etiology with positivity rate of 7.9%. Lastly in the year 2014, out of 101 suspected cases, 4 cases were positive for CHIKV with positivity rate of 4%.

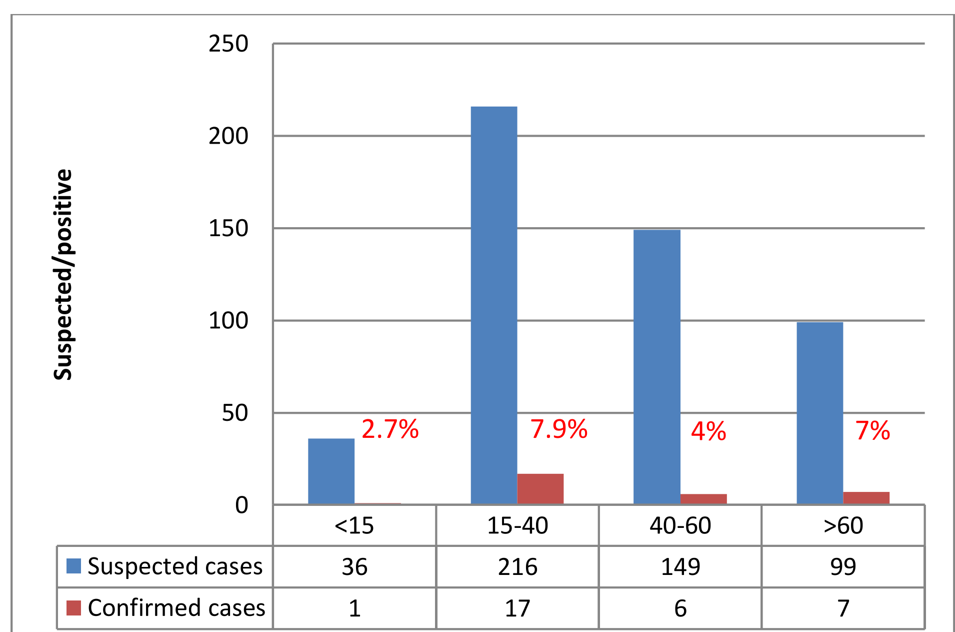
Figure -11: Sex wise distribution of clinically suspected and laboratory confirmed cases.



χ^2 Value= 0.092 DF=1 P value=0.764 , Not significant

Figure – 11 reflects that among 500 suspected cases, 239 were male and 261 were female. In case of males 14 were confirmed of CHIK etiology with positivity rate of 5.8% and in females 17 cases were positive for CHIKV with positivity rate of 6.5%.

Figure -12: Age wise distribution of clinically suspected and confirmed cases.

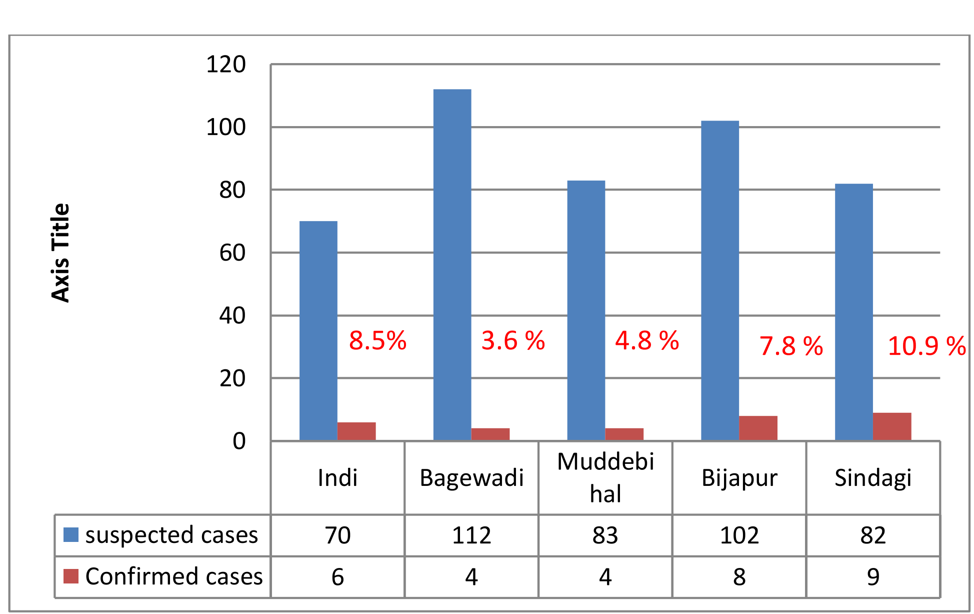


	<15	15-40	40-60	>60
Suspected cases	36	216	149	99
Confirmed cases	1	17	6	7

χ^2 Value= 3.1 DF=3 P value=0.376, Not significant

Figure – 12 signifies age wise distribution of CHIK cases. In people with age group below 15 years, 36 cases were suspected, out of which 1 case was confirmed CHIK etiology with positivity rate of 2.7%. In case of people of age group 15 to 40 years, 17 of 216 were confirmed as CHIK etiology with positivity rate of 7.9%. In case of age group between 40 to 60, out of 149 suspected cases 6 cases were identified as CHIK etiology with positivity rate of 4%. In people of age group more than 60 years, 99 cases were suspected out of which 7 cases were positive for CHIKV with positivity rate of 7%.

Figure –13: Taluk wise distribution of clinically suspected and laboratory confirmed cases.



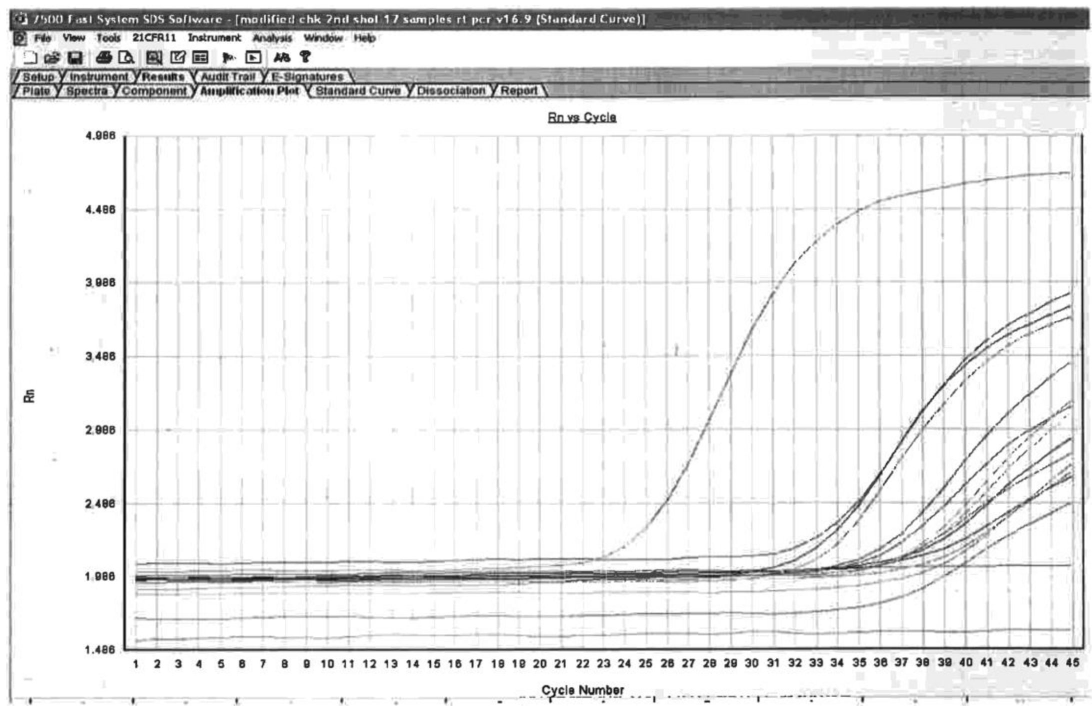
	Indi	Bagewadi	Muddebi hal	Bijapur	Sindagi
suspected cases	70	112	83	102	82
Confirmed cases	6	4	4	8	9

χ^2 Value= 5.054 DF= 4 P value=0.282, Not significant

Figure – 13 interprets taluk wise distribution of CHIK cases. Vijayapura district has 5 taluks namely Indi, Bagawadi, Muddebihal, Bijapur (Vijayapura) and Sindagi. In Indi taluk, 6 out of 70 suspected cases were positive for CHIKV with positivity rate of 8.5%. In Bagewadi taluk, 112 cases were suspected out of which 4 cases were confirmed as CHIK etiology with positivity rate of 3.6%. In case of Muddebihal taluk 4 of 83 suspected cases were positive for CHIKV and in Vijayapura taluk 8 of 102 suspected cases were positive for CHIKV. Lastly in Sindagi taluk 82 cases were suspected, out of which 9 cases were positive for CHIKV. The positivity rates in Muddebihal, Vijayapura and Sindagi taluks were 4.8%, 7.8% and 10.9% respectively.

Chikungunya samples run rt pcr:

Samples from ck/235-ck/380 target (chikungunya):Amplification plot



Samples from ck/235-ck/380 Internal control: amplification plot

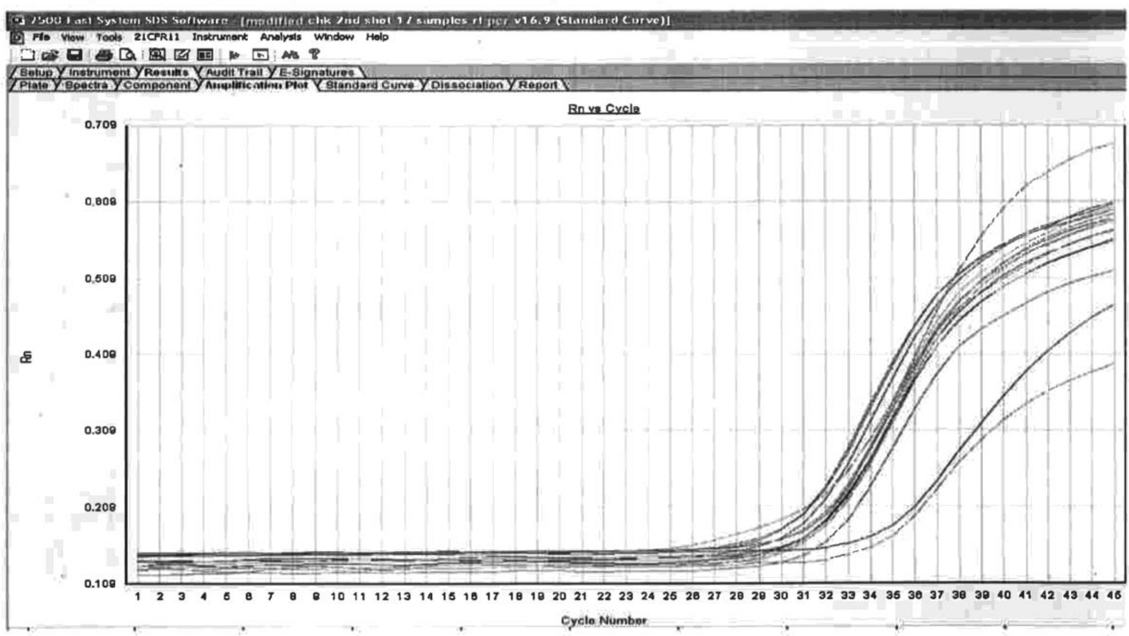


Figure –14: Graphical Representation of Real time PCR data.

Figure – 14 is the graphical representation of real time PCR data of our study. Rn is the fluorescence of the reporter dye devided by fluorescence of passive reference dye, i.e Rn is the reporter signal normalized to the fluorescent signal. In this diagram Rn is plotted against PCR cycle number. So amplification plot concluded that only one sample among 31 samples had cycle threshold (Ct) value of 23 and remaining samples had Ct value of more than 30. The details are mentioned in the table - 10

Table –10.1: Ct Values of RTPCR Samples

S.No	SAMPLE ID	CT
1	CK-53	36.5
2	CK-54	38.1
3	CK-76	37.9
4	CK-77	36.2
5	CK-94	-
6	CK-147	34.7
7	CK-150	39.1
8	CK-156	39.7
9	CK-158	38.7
10	CK-191	39.9
11	CK-193	39.3
12	CK-216	41.2
13	CK-217	28.6
14	CK-218	35.1
15	CK-219	35.2

Table –10.2: Ct Values of RTPCR Samples

S.No	SAMPLE ID	CT
1	CK-235	36.9
2	CK-237	37.2
3	CK-281	32.6
4	CK-282	34.9
5	CK-294	38.2
6	CK-297	36.5
7	CK-314	37.5
8	CK-315	36.7
9	CK-323	-
10	CK-327	38.1
11	CK-344	32.4
12	CK-345	35.4
13	CK-379	36.2
14	CK-380	23.3
15	CK-403	30.5
16	CK-405	33.8
17	CK-471	35.6
18	CK-476	33.6

5.3. MOLECULAR CHARACTERIZATION:

Among 31 cases, 9 representative strains were sequenced (Due to low viral load remaining strains couldn't be sequenced). The sequences were compared with standard strains, sequence homology of > 99% were confirmed as CHIKV.

Figure -15: Sequence alignment of CK- 235 with reference strain HPCK0573.

```
Chikungunya virus isolate HPCK0573 polyprotein gene, partial cds
Sequence ID: gb|KT218525.1|  Length: 568  Number of Matches: 1

Range 1: 47 to 564  GenBank  Graphics                    ▼ Next Match  ▲ Pr

Score             Expect   Identities      Gaps          Strand
929 bits(503)     0.0      513/518(99%)    0/518(0%)     Plus/Plus

Query  8    TACCTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCAAATTCATTGTGG  67
            || |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  47   TAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCAAATTCATTGTGG  106

Query  68   GGCCAATGTCTTCAGCCTGGACACCTTTCGACAACAAAATTGTGGTGTACAAAGGTGACG  127
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  107  GGCCAATGTCTTCAGCCTGGACACCTTTCGACAACAAAATTGTGGTGTACAAAGGTGACG  166

Query  128  TCTATAACATGGACTACCCGCCTTTTGGCGCAGGAAGACCAGGACAATTTGGCGATATCC  187
            |||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct  167  TCTATAACATGGACTACCCGCCCTTTGGCGCAGGAAGACCAGGACAATTTGGCGATATCC  226

Query  188  AAAGTCGCACACCTGAGAGTGAAGACGTCTATGCTAATACACAACTGGTACTGCAGAGAC  247
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  227  AAAGTCGCACACCTGAGAGTGAAGACGTCTATGCTAATACACAACTGGTACTGCAGAGAC  286

Query  248  CGGCTGCGGGTACGGTACACGTGCCATACTCTCAGGCACCATCTGGCTTTAAGTATTGGC  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  287  CGGCTGCGGGTACGGTACACGTGCCATACTCTCAGGCACCATCTGGCTTTAAGTATTGGC  346

Query  308  TAAAAGAACGCGGGGCGTCATTGCAGCACACAGCACCATTTGGCTGCCAAATAGCAACAA  367
            |||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||
Sbjct  347  TAAAAGAACGCGGGGCGTCACTGCAGCACACAGCACCATTTGGCTGCCAAATAGCAACAA  406

Query  368  ACCCGGTAAGAGCGGTGAACTGCGCCGTAGGGAACATGCCCATCTCCATCGACATACCGG  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  407  ACCCGGTAAGAGCGGTGAACTGCGCCGTAGGGAACATGCCCATCTCCATCGACATACCGG  466

Query  428  AAGCGGCCTTCACTAGGGTCGTCGACGCGCCCTCTTTAACGGACATGTCGTGCGAGGTAC  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  467  AAGCGGCCTTCACTAGGGTCGTCGACGCGCCCTCTTTAACGGACATGTCGTGCGAGGTAC  526

Query  488  CAGCCTGCACCCATTCCTCAGACTTTGGGGCGGTCGCC  525
            ||||||||||||||||||||||||||||||  ||||||
Sbjct  527  CAGCCTGCACCCATTCCTCAGACTTTGGGGGCGTCGCC  564
```

Figure -15 represents the pair-wise nucleotide sequence alignment of current strain with HPCK0573 polyprotein partial coding region. From the table it can be observed that the E-value (Expectation value is 0.0) of the alignment proves to be significant. There are no gaps introduced in the alignment providing the information that during the process of evolution, there are no insertions or deletions occurred but only the substitutions or the mutation at the positions C10A, T150C, T328C, G519C are found to be significant. From the local sequence alignment it can be observed that the region of similarity is starting from the eigth nucleotide of the query sequence which is matching from the forty seventh position of the subject or reference strain HPCK0573 with 99% identity.

Figure -16: Amino acid Multiple sequence alignment in Clustal W2 software

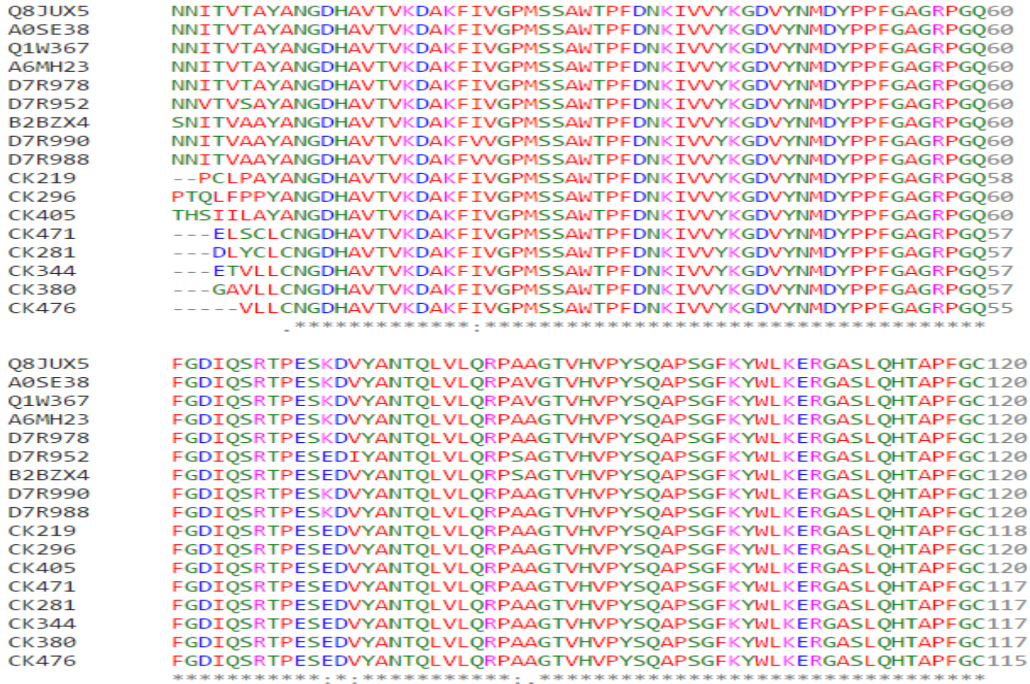


Table -11 shows the multiple sequence alignment (MSA) of reference sequences Q8JUX5, A0SE38, Q1W367, A6MH23, D7R978, D7R952, B2BZX4, D7R990, D7R988 along with CK219, CK296, CK405, CK471, CK281, CK344, CK380 and CK476. From the alignment it can be observed that most part of the MSA is highly conserved. The initial 5 columns of the alignment are not considered for the analysis due to insertion of the gaps introduced in CK471, CK281, CK344, CK380 and CK476 for the optimal alignment. A significant mutation from Tyrosine to Alanine was observed at ninth position. A partially conserved mutation from Valine to Isoleucine was observed at twenty third positions. At seventy second position a mutation from Lysine to glutamate which is partially conserved charged amino acid was observed. Both the mutations observed at eighty six and eighty seven are also semi conserved and partially conserved mutations.

5.3.1. Phylogenetic analysis:

The 9 representative strains confirmed as CHIKV compared with standard strain sequences with NCBI viz ECSA, west African and Asian genotypes. The unrooted Phylogenetic tree shows strains are closely related to Reunion strains and distantly related to S27 Tanzanian strain and made sister group of ECSA. So strains belongs to ECSA genotype. Strains were even more divergent to Asian and West African genotypes. Comparative nucleotide and amino acid homology analysis reveal that strains are 94.9±5.1% nucleotide homology and 97.1±2.9% at amino acid homology with S-27 strain.

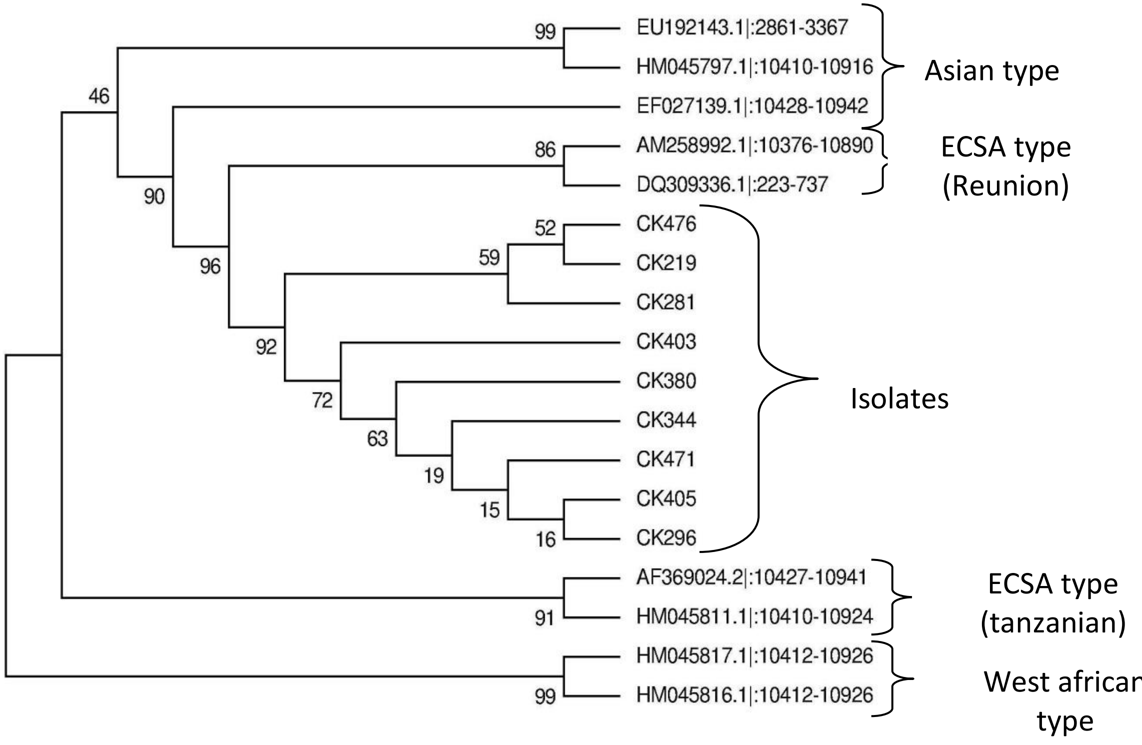


Figure -17: Phylogenetic analysis based on Partial E1 nucleotide sequences and relationship with reference strains. Gene bank accession number for reference strain labelled.

Table –11: Distance matrix of Chikungunya strains of our study compared with reference strains.

	HM045817.1	HM045816.1	HM045797.1	EU192143.1	AF369024.2	HM045811.1	EF027139.1	CK219	CK281	CK476	CK296	CK403	CK344	CK380	**DQ309336.1**	**AM258992.1**	CK405	CK471
HM04581 7.1	0.0000	0.0129	0.1913	0.1974	0.1604	0.1634	0.1655	0.1843	0.1887	0.1774	0.2051	0.1866	0.1861	0.1889	0.1768	0.1768	0.1910	0.1832
HM04581 6.1	0.0129	0.0000	0.1937	0.1998	0.1684	0.1715	0.1685	0.1873	0.1918	0.1804	0.2021	0.1836	0.1832	0.1859	0.1798	0.1798	0.1880	0.1802
HM04579 7.1	0.1913	0.1937	0.0000	0.0133	0.0529	0.0505	0.0703	0.0627	0.0653	0.0606	0.0653	0.0657	0.0606	0.0606	0.0602	0.0602	0.0655	0.0630
EU19214 3.1	0.1974	0.1998	0.0133	0.0000	0.0628	0.0603	0.0703	0.0677	0.0703	0.0656	0.0704	0.0708	0.0656	0.0656	0.0652	0.0652	0.0706	0.0680
AF369024. 2	0.1604	0.1684	0.0529	0.0628	0.0000	0.0022	0.0379	0.0422	0.0472	0.0379	0.0540	0.0426	0.0402	0.0424	0.0309	0.0309	0.0424	0.0378
HM04581 1.1	0.1634	0.1715	0.0505	0.0603	0.0022	0.0000	0.0356	0.0399	0.0449	0.0356	0.0516	0.0402	0.0378	0.0401	0.0286	0.0286	0.0400	0.0355
EF027139. 1	0.1655	0.1685	0.0703	0.0703	0.0379	0.0356	0.0000	0.0263	0.0310	0.0220	0.0424	0.0288	0.0288	0.0310	0.0197	0.0197	0.0310	0.0265
CK219	0.1843	0.1873	0.0627	0.0677	0.0422	0.0399	0.0263	0.0000	0.0326	0.0322	0.0371	0.0344	0.0346	0.0282	**0.0152**	**0.0152**	0.0347	0.0321
CK281	0.1887	0.1918	0.0653	0.0703	0.0472	0.0449	0.0310	0.0326	0.0000	0.0170	0.0304	0.0259	0.0236	0.0260	**0.0198**	**0.0198**	0.0328	0.0280
CK476	0.1774	0.1804	0.0606	0.0656	0.0379	0.0356	0.0220	0.0322	0.0170	0.0000	0.0393	0.0277	0.0192	0.0258	**0.0109**	**0.0109**	0.0325	0.0277
CK296	0.2051	0.2021	0.0653	0.0704	0.0540	0.0516	0.0424	0.0371	0.0304	0.0393	0.0000	0.0346	0.0283	0.0285	**0.0264**	**0.0264**	0.0365	0.0258
CK403	0.1866	0.1836	0.0657	0.0708	0.0426	0.0402	0.0288	0.0344	0.0259	0.0277	0.0346	0.0000	0.0127	0.0213	**0.0153**	**0.0153**	0.0323	0.0169
CK344	0.1861	0.1832	0.0606	0.0656	0.0402	0.0378	0.0288	0.0346	0.0236	0.0192	0.0283	0.0127	0.0000	0.0084	**0.0131**	**0.0131**	0.0237	0.0169
CK380	0.1889	0.1859	0.0606	0.0656	0.0424	0.0401	0.0310	0.0282	0.0260	0.0258	0.0285	0.0213	0.0084	0.0000	**0.0153**	**0.0153**	0.0238	0.0212
DQ30933 6.1	0.1768	0.1798	0.0602	0.0652	0.0309	0.0286	0.0197	0.0152	0.0198	0.0109	0.0264	0.0153	0.0131	0.0153	0.0000	0.0000	0.0152	0.0109
AM25899 2.1	0.1768	0.1798	0.0602	0.0652	0.0309	0.0286	0.0197	0.0152	0.0198	0.0109	0.0264	0.0153	0.0131	0.0153	0.0000	0.0000	0.0152	0.0109
CK405	0.1910	0.1880	0.0655	0.0706	0.0424	0.0400	0.0310	0.0347	0.0328	0.0325	0.0365	0.0323	0.0237	0.0238	**0.0152**	**0.0152**	0.0000	0.0127
CK471	0.1832	0.1802	0.0630	0.0680	0.0378	0.0355	0.0265	0.0321	0.0280	0.0277	0.0258	0.0169	0.0169	0.0212	**0.0109**	**0.0109**	0.0127	0.0000

The table 11 shows the distance matrix generated for the Chikungunya virus CK219, CK281, CK476, CK296, CK403, CK344, CK380, CK405 and CK471 by considering the reference sequences from Chikungunya virus isolate Reunion 223/05 structural polyprotein gene, partial cds (DQ309336.1) and Chikungunya virus gene for non-structural polyprotein, gene for non-structural polyprotein short and long variant and gene for structural polyprotein, genomic RNA, strain 06-021 (AM258992.1).

From the distance matrix it can be observed that the strain CK296 has the highest distance log odd value representing it is distantly related to the reference sequences. The lowest distance observed is with CK476 and CK471 showing the closely related sequences.

5.3.2. Mutational analysis: Table-12 explains few random nucleotide changes in the partial E1 region. Three amino acid mutations were observed. E1K211E, E1M269V and E1D284E in all isolates.

Table-12: Amino acid Mutational analysis of isolates with respect to positions.

Position			Reference strains with uniprot ID									Isolates							
Nucleotide	Polypeptide	Protein	Q8JUX5-S27	A0SE38-Reunion	Q1W367 Reunion	A6MH23-INDIA-00-MH4	D7R978-Tanzania	D7R952-RSU1	B2BZX4-Indonesia	D7R990-Senegal	D7R988Senegal	219	281	296	344	380	405	471	476
A10624G	1020	211	K	.	.	.	.	E	E	.	.	E	E	E	E	E	E	E	E
A10798G	1078	269	M	V	V	V	.	.	.	I	V	V	V	V	V	V	V	V	V
T10845A	1093	284	D	E	E	.	.	.	.	.	.	E	E	E	E	E	E	E	E

5.3.3. Molecular modelling: Homology modelling with mutations has been projected to three dimensional structure [Fig-18]. All the three observed mutations lies in the area in major secondary structure.

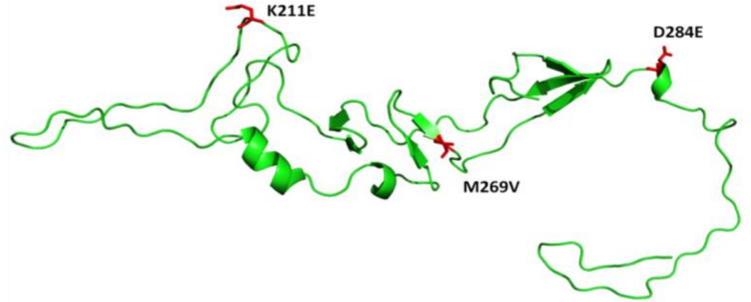


Figure -18: Mapping of mutations positions in E1 gene.

Chapter – 6

Discussion

Previously CHIK was ignored despite causing millions of cases in Africa, Asia and mis-diagnosed as dengue due to similar clinical features and limited diagnostic facilities, a massive outbreak reported in Reunion island with increased mortality and morbidity changed the picture. This CHIK outbreak spread in a fast pace to larger areas with uncharacteristic symptoms and severe complications resulting in serious economic and social impact. From 2005 to 2016 CHIK affects large number of population in many countries in Asia. A survey conducted in India reported that approximately more than 1.4 billion individuals affected by CHIK during 2005- 2006 explosive outbreak. The two states Karnataka and Kerala, southern India experienced maximum number of cases. During 2006 Karnataka state alone reported 762026 number of CHIK suspected cases and and 27 districts reported over 54.74% of the total suspected cases from India. Several districts of the state such as Vijayapura, Gulbarga, Tumkur, Bidar, Raichur, Dharwad, Bellary, Chitradurga, Davangere and Kolar have recorded large number of Chikungunya fever cases (Vijayapura district over 80000 cases) (Manimunda S. P. et al., 2010 and Talawar A. S. et al., 2010)

During 2009-2011 a survey was conducted to find-out the prevalence of CHIK infection in and around Bellary district, Karnataka. Same study reported that overall CHIK positivity rate was 24.75%, in decreasing order of positive cases from 2010, (28.04%) 2009 (23.07%) and 2011 (19.05%) Male to female ratio was 0.98 (Narayan S et al., 2012). Whereas in Vijayapura district which is 250km distant from Bellary, overall CHIK positivity rate was 6.2%, in decreasing order of cases from 2013 (7.9%), 2012 (6.8%), 2011 (4.6%) and 2014 (4%). Compared to males (5.8%)

more number of females (6.5%) were affected. The majority of cases affected were in age group of 15 to 40 (7.9%).Vijayapura district contains five taluks, more number of confirmed cases were reported from Sindagi taluk (10.9%) and least number of positive cases Bagewadi taluk (3.6%). The taluks Indi, Vijayapura and Muddebihal reported 8.5%, 7.8% and 4.8% positive cases respectively.

Chikungunya RNA couldn't be detected from two Sero-positive samples (Ck-94 & Ck-323). The probable reason for molecular negativity may be due to very low viral load. These two samples were collected on day 7 after onset of illness. For Molecular diagnosis (RTPCR) clinical samples should be collected less than 8 days after onset of illness. The cases which produce strong antibody mediated and cell mediated immune response experience short period of viremia (median 6 days) (Poo Y. S. et al., 2014). Other probable reason for molecular negativity may be antigenic cross-reactivity. Antigenic cross-reactivity was observed between Chikungunya and other alpha viruses like Onyong nyong, Ross River, Mayaro and Sindbis viruses, which also causes similar clinical manifestations (Chanas A. C. et al., 1976).

The long term persistence of arthralgia associated with CHIK infection was noted in significant proportion of cases. A study conducted in Reunion island 2006 reported 63.8% affected individuals experienced persistent arthralgia after 18 months of acute phase of CHIK disease. (Gianandrea Borgherini et al., 2008) Other study reported 87.9% affected individuals are devoid of persistent arthralgia after 3-5 years of infection (Brighton SW et al., 1983). A study conducted in France stated that 48% travellers returning from Indian ocean island were presenting with persistent arthralgia after 6 months of infection (Simon F et al., 2007) In our study,

65% and 42% of CHIK confirmed cases experienced persistent arthralgia after 12 and 18 months of the acute phase of infection respectively. The Majority of affected individuals belonged to old age (more than 45 years). Among old age individuals 92% cases experienced arthralgia symptom.

As the name itself indicates that among alpha viruses previously only Eastern equine encephalitis and Western equine encephalitis were causative agents of encephalitis. But during re-emergence in Reunion islands, 123 cases experienced encephalitis. (Philippe Renault et al., 2007) In Sindbis virus the replacement of Threonine with Leucine at position 538 and 18 nucleotide deletions in nsP3 together claimed to affect neuro-virulence in other alpha viruses. (Heise MT et al., 2003 & Suthar MS et al., 2005). But in the present study no encephalitis cases were reported.

In C6/36 (*Aedes albopictus*) cell lines comparatively, ECSA genotype infected early and maintained significant higher titre than Asian genotype, where as in mammalian vero, RD cells and CCL-125 cell lines both genotypes replicated equally. In countries like India where both Asian and ECSA genotypes coexist and *Aedes albopictus* mosquitoes are prevalent, this kind of evolved adaptation may result in displacement of Asian genotype strains. (I-Ching Sam et al., 2012) The infection pattern of prototype S-27 (Tanzanian) and 2006 (south indian) strains has been assessed by inoculating strains on mammalian cell lines. Reunion strains demonstrated increased cytotoxicity, larger plaque formation, early release of progeny viruses (growth kinetics) and higher protein expression level results in high replication rate than S-27 strains. Reunion strains have higher infectivity rate in vectors than S-27 as well. So due to faster replication rate and higher infectivity rate,

Reunion strains somehow escaped from host immune response and results in high pathogenicity and virulence. (Abhishek Kumar et al., 2014)

In the present study Phylogenetic analysis concluded that current Chikungunya viral strains belongs to ECSA genotype and has a close relation with Reunion strains than prototype tanzanian strain (S-27). It indicates that these strains may have evolved from ECSA genotype reported from Reunion Island during 2005-2006 (subtype Indian ocean lineage-IOL).

It was confirmed that a very less number of mutations were acquired between 2001 to 2005 and maximum number acquired before 2001. (Abhishek Kumar et al., 2014) A study was conducted in Kozhikkode district of northern Kerala during the year 2009 reported two important mutations A226V and V291I in E1 gene (Niyas K. P. et al., 2010). Other study conducted in new Delhi during the year 2010 reported number of mutations in E1 gene namely K211E, M269V, D284E, V179A, S234P, R196K and R247C (Jatin shrinet et al., 2012). But in the present study we have found only three mutations K211E, M269V and D284E.

Previously it was demonstrated that E1A226V mutation increases the midgut infectivity and viral dissemination to secondary organs and intern enhance Chikungunya virus transmissibility by *Aedes albopictus* mosquitoes. Similarly, it was postulated that E1A226V mutation decreases cholesterol dependence in target cell and increases fitness of Chikungunya virus on *Aedes albopictus* mosquitoes. So E1A226V mutation increases viral infectivity, transmissibility and fitness of CHIKV to *Aedes albopictus* mosquitoes and results in increase host range (Konstantin A et

al., 2007). It was demonstrated that in Semliki forest virus (SFV) the replacement of Proline to Serine amino acid at 226 position in E1 gene causes decreased growth requirement of cholesterol in insect cells. (Vashishtha M et al., 1998) E1A226V mutation started appearing after September 2005 in Reunion Island strains and results in increased transmission rate and severe clinical symptoms. (Santosh S. R et al., 2008) This mutation was absent in our current strains, indicates less infectivity and transmissibility. In SFV the position 226 in E1 protein is located in IJ loop, which fuses with fusion peptide. This region is predicted to interact with the target membrane. The amino acid substitution may have a selectively neutral effect or due to genetic drift may alter characteristics of CHIKV. (Isabelle Schuffenecker et al., 2006)

Between 2005-2011, E1A226V mutation was present in 53% strains and absent in 47% of isolates, indicates circulation of both isolates in India and other affected countries. So more than half of the isolates even though in the absence of E1A226V mutation are highly infective and caused epidemics. E1A226V substitution may cause synergistic effect and result in increased transmission potential in short span of time. (Abhishek Kumar et al., 2014) A study was conducted to find-out the effect of E1A226V mutation on adaptation of virus to *Aedes albopictus* mosquitoes. One strain having E1226A and other strain having E1226V were individually inoculated on different C6/36 cell lines. It was observed that both strains resulted in equal and higher titre than prototype. (S-27) It indicates that there are some un-identified mutations in ECSA lineage responsible for adaptation of ECSA to *Aedes albopictus* mosquitoes. These mutations might affect the

displacement of Asian lineage by ECSA lineage in India where both genotypes exist. (Wikan N et al., 2012).

It was established that E1K211E mutation was positively selected (new non-synonymous advantageous mutations) site with a posterior probability of >75% (Arankalle V A et al., 2007) 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. (Tsetsarkin K. A. et al., 2009).

The Position 284 in E1 gene was highly conserved region and replacement of aspartate to glutamic acid amino acid have dual effects on CHIKV. First was the amino acid position at 284 was exactly located at the interface between E1 protomers on Virion structure, which functions as participating in contact that makes up the icosahedral E1 scaffold and may affect the protein structure. (Isabelle Schuffenecker et al., 2006). The second one was aspertate amino acid position in contact with the main chain amide group of E1 promoter hydrogen bond is formed. When aspertate is replaced by glutamic acid, which has longer side chain (extra CH_2 side group compared to aspertate) may result in slight distortion at the contact sites. So intern result either in less efficient of new particles or more efficient particle disassembly during invasion in new cells or combination of both. So it’s interesting to assess the effect of aspertate/glutamic acid change by site directed mutagenesis studies. (Isabelle Schuffenecker et al., 2006).

The observed mutations which lie in predicted T cell epitopes will alter the immunogenicity of CHIKV. The unique mutations E2K577Q and E1A226V lie in

functionally significant domains of structural proteins may alter the protein structure. (Sreekumar. E et al., 2009) But in the present study the observed mutations lie in primary structure, so couldn't affect protein structure.

The mutations in non-structural protein (T128K, T376M, T1670I, T2117A) have predicted effect on phosphorylation potential and mutations in both structural protein (V27I, E637N, V756I & D1093E) and non-structural protein (S589N, A1328Y, Y1550H, T1670I & T1938A) have predicted effect in binding to important domains of host proteins. It was speculated that these mutations may influence the stability of the viral proteins or their binding partners during infection. (Abhishek Kumar et al., 2014) So genetic mutations in CHIK viral genome alters phenotypic characters of the virus.

Overall comparatively Reunion strains showed a number of mutations throughout the genome. nSP3 and E2 proteins showed a greater degree of variations in nSP and SP respectively. (Isabelle Schuffenecker et al., 2006)

Table -13: Comparison of amino acid substitution reported in Indian isolates.

Author	Gene		Mutations
Santosh S. R. et al., 2008	Non-structural protein		M326V, Q488R, S589N, A132V, Y1550H, P1661Q, T1607I, P1691S, R1768C, A1771V, T1782M, L1794P, P1804S, T1938S & T2117A.
	Structural protein	E2	V487A, I536T, T637M, M643V, S700T, V702I & V711A.
		E1	I828V, D1093E & T1186A.
		6K	V756I
Abhishek Kumar et al., 2014	Non-structural protein	nsP1	T128K, M314L, T376M & Q488R
		nsP2	S589N & A1328V
		nsP3	Y1550H, T1670I, P1804S
		nsP4	T1938A and T2117A
	Structural protein	Capsid	P23S & V27I
		6K	V756I
		E1	D1093E & V1167A
		E2	T637M, S700T & V711A
Sreekumar E et al., 2010	Non-structural protein	nsP1	T128K, T376M and Q488R,
		nsP2	S54N AND A793V
		nsP3	I376T, L461P and P471S
		nsP4	T75A and T254A
	Structural protein	Capsid	P23S and V27I
		6K	V8I
		E1	A226V and D284E.
		E2	K252Q, S375T and V386A.
Isabelle Schuffenecker et al	Structural protein	E1	A226V and D284E
Kudukkil P niyas et al	Structural protein	E1	A226V and V291I
Jatinshrinet et al	Structural protein	E1	K211E, M269V, D284E, V179A, S234P, R196K and R247C
Current study	Structural protein	E1	K211E, M269V and D284E

Before 2005 CHIK was last time reported from India during 1973. It was quiescent for 32 years. Few studies suggested that it had disappeared from the country, but no surveillance studies were conducted to prove the disappearance. (Pavri K et al., 1986 & Burke DS et al., 1985) A Serological survey conducted in 1995 from Calcutta reported that children's and young adults had very less or no antibodies against CHIKV. So there was no effective immunity in these population, Immunologically naive vulnerable population were at high risk. (Neogi DK et al., 1995) Paul R Epstein postulated that global warming results in drought, the soil became caked and CHIKV got scattered in animal carcasses (sylvan reservoir). Finally, CHIKV escaped from animal carcasses and re-emerged in Kenya in 2004.

During present years CHIK epidemic occurred in America and Europe continents. There are different ways of CHIK spreading across the world. First is human mobility. CHIK spread from one place to other by travellers, after a period of time vectors infect new host and results in autochthonous transmission. So tourism spread the infection worldwide. (Katherine R. Harter et al., 2014 & Philippe Parola et al., 2006) Second is the transportation of tyres. Tyres are the potential mosquito breeding sites. The tyres transported from one place to another carry these infected mosquito eggs and spread the infection. (Rezza G. 2012)

During 2004 ECSA genotype caused an outbreak in Lamu island Kenya (Democratic Republic of Congo). It was postulated that the same ECSA strains from Kenya may spread to Reunion island. (Kibet Sergon et al., 2008). But ECSA genotype was first time isolated in India from mosquito during 2000 (Yawat strain) The origin of yawat strain was not clear and the ECSA genotype has caused

outbreaks in Indian ocean Island, India and presently causing sporadic and epidemic cases in India. A full length genome analysis was conducted to find out the origin and spread of ECSA genotype in India. The study demonstrated that ECSA Reunion island strains showed 99.9% nucleotide homology with Indian strains. So same strains were responsible for the epidemic in both Reunion island and India and it spreads from Reunion island to India. A very important finding of the study which could explain probable origin and spread of CHIK was the yawat strain had 99.62% homology with Uganda strains in 1982, intern the same Uganda strain was having 98.2% homology with 2006 indian strain than S-27 prototype (97.3%). So it's clear that the parental strain responsible for spread was yawat strain and it was introduced to India before 5 years epidemic. ECSA and Asian genotypes have been circulating in India after 2000, so there was a suspect that current strains may be a result of recombination of both genotypes. But it's proved that current strain was not result from recombination of prototype ECSA (S27) and Asian genotypes and it was under purifying selection and may have evolved due to random neutral and non-synonymous mutations (Arankalle V.A. et al., 2007). Another study also showed evidence that ECSA genotype was introduced to India later than Asian strain by the independent introduction of CHIK strain. (Cherian SS et al., 2009)

Here we list out probable reasons for CHIK resurgence during 2005-06. The virus strain acquires more virulence due to virus evolution (mutations), large immunologically vulnerable population in the country, increased host range, lack of surveillance activities, lack of diagnostic facilities, misdiagnosis as dengue due to similar clinical symptoms and lack of awareness, globalisation of *Aedes* vectors,

human mobility and rapid urbanisation (Kumar NP et al., 2008, Santosh SR et al., 2008 and Sreekumar E et al., 2010). There may be sporadic cases during the period, due to lack of awareness and no active surveillance activities which are under reported. During deforestation (rapid urbanization) vectors move from forest to urban and rural area and intern increases vector density in the area. When it gets infected with virus chances of transmitting infections to humans are more. Before 2005 ECSA genotype was restricted only to East and central Africa. Due to transcontinental movement of ECSA genotype, a new genotype of CHIKV reached India. Chances of epidemics are more due to lack of immunity against the particular genotype and altered virulence of particular genotype. Increased host range of ECSA genotype with respect to *Aedes albopictus* mosquitoes was also proved during the same epidemic.

Chapter – 7

Summary and Conclusion

7.1. Summary and Conclusion

7.1.1. The purpose of the study was to carry out the epidemiological surveillance in and around Vijayapura district and to find out the prevalence of CHIK disease, genotype responsible for, mutations present in E1 gene and their effect on protein structure, infectivity, virulence and host adaptability to *Aedes albopictus* mosquitoes.

7.1.2. A total of 500 blood samples from fever and arthralgia of 5 to 8 days were collected and tested for CHIK IgM antibodies by SD CHIK IgM ELISA kit.

7.1.3. Out of 500 samples 33 were positive for CHIK IgM antibodies.

7.1.4. 33 Sero-positive samples were selected for molecular confirmation and RTPCR was performed by using Amplisure ® CHIK RTPCR kit.

7.1.5. 31 out of 33 samples were positive for CHIK RNA.

7.1.6. The prevalence rate of CHIK was 6.2% in and around the Vijayapura district.

7.1.7. Statistical analysis concluded that there was no significant difference between suspected and confirmed cases with respect to year, sex, age and taluk.

7.1.8. E1 partial gene sequencing was performed for 9 RTPCR positive samples.

7.1.9. Phylogenetic analysis study revealed that the current strains belongs to ECSA genotype and which may evolved from Re-union island strains.

7.1.10. Three amino acid mutations E1K211E, E1M269V and E1D284E were consistently present and E1A226V mutation was absent in current strains.

7.1.11. The observed mutations didn't affect the CHIK viral protein structure.

7.1.12. From the findings, we conclude that the current strains circulating in Vijayapura district may be less infective, less virulent and less adaptable to *Aedes albopictus* mosquitoes than Re-union island strains.

7.2. Limitations of the Study:

7.2.1. RTPCR was only performed to Sero-positive cases, which was the limitation of the study. CHIK IgM antibodies starts appearing 5 days after onset of illness and CHIK RNA can be detected till 8 days after onset of illness. So samples were collected between 5 to 8 days after onset of illness. So that samples should be positive for both CHIK IgM antibodies and CHIK RNA.

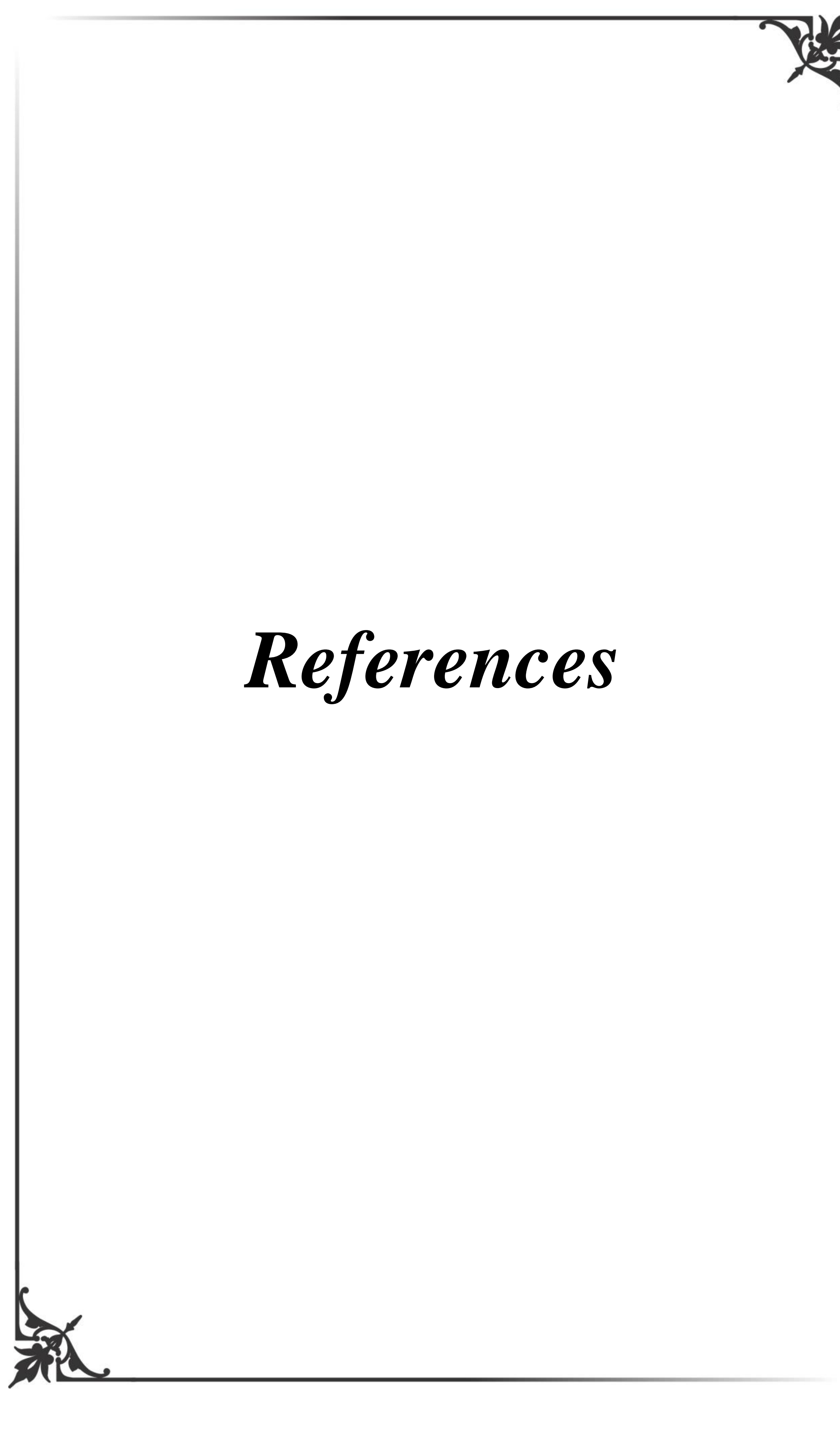
7.2.2. Genetic characterization was carried out only for 9 samples, due to low viral load we couldn't get sequences from remaining samples.

7.3. Future Directions:

7.3.1. Future research is required to find out the mutations in whole genome.

7.3.2. Future studies to be carried out to check the effect of these mutations on viral characteristics.

7.3.3. Future research is required to find out the reasons for the mysterious behaviour of dramatic outbreaks, periods of prolonged absence, factors triggering outbreaks and replacement of Asian strains by ECSA strains.



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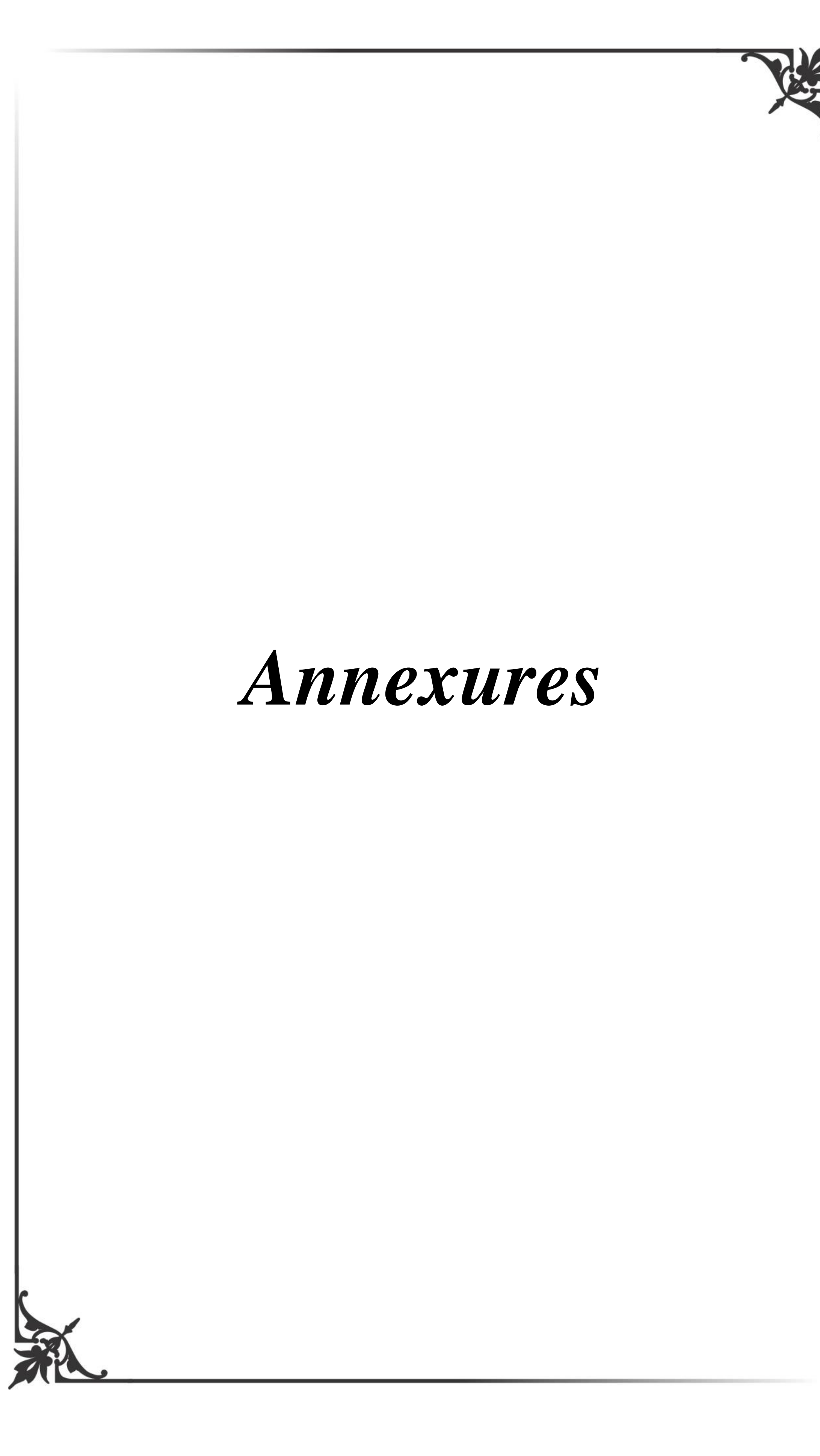

Annexures

ANNEXURE

Sample Collection proforma

Sl No.	Sample No.	Name of the patient	Address	Taluk	age	sex	Date of onset of illness	Type of sample	Date of Sample Collection

List of publications to Ph. D thesis

Original articles:

1. Bharath Mudurangaplar and B V Peerapur. Sero-epidemiological survey of Chikungunya in and around the regions of Bijapur (Vijayapura - North Karnataka). Journal of Clinical and Diagnostic Research. 2015 May;-9(5):DC01-DC02.
2. Bharath Mudurangaplar and B V Peerapur. Molecular characterisation of clinical isolates of Chikungunya Virus: A study from tertiary care hospitals in southern india. Journal of Clinical and Diagnostic Research. 2016 Mar;10(3): DC14-DC17

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Seroepidemiological Survey of Chikungunya in and Around the Regions of Bijapur (Vijayapura - North Karnataka)

BHARATH MUDURANGAPLAR[1], BASAVARAJ V. PEERAPUR[2]

ABSTRACT

Background: Chikungunya is a debilitating, non-fatal, mosquito borne viral fever caused by Chikungunya virus (CHIVA). The disease is transmitted to humans by the bite of *Aedes aegypti* and *Aedes albopictus* mosquitoes. Severe outbreaks of Chikungunya have been reported in several countries of Africa and Asia. Chikungunya fever is characterized by fever with sudden onset, arthralgia, rash, headache and myalgia. However, arthralgia is painful and long-lasting, affecting primarily the peripheral joints.

Objectives: To find out the prevalence of Chikungunya fever in and around the regions of Bijapur district.

Materials and Methods: 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-epidomological surveillance is needed for the control of Chikungunya fever.

Keywords: Arboviral, IgM ELISA, Viral fever

INTRODUCTION

Chikungunya is a "debilitating non-fatal viral fever". 'Swahili' is a language spoken in East Africa. The meaning of Chikungunya in Swahili language is 'that which bends up' in reference to the stooped posture developed as a result of the arthritic symptoms of the disease. The disease was first documented in 1952, following an outbreak on the Makonde Plateau, along the border between Tanganyika (Tanzina) and Mozambique [1]. Chikungunya fever is an arboviral disease caused by Chikungunya virus (CHIVA). It is a member of family Togaviridae and genus alphavirus. It is an enveloped ribonucleic acid virus. RNA is single-stranded, linear, positive-sense of approximately 11.8 kb [2].

The disease is transmitted to humans by the bite of the female *Aedes aegypti* and *Aedes albopictus* mosquitoes. Chikungunya fever has been originally distributed in several parts of Africa, South Asia and Southeast Asia. In India, well-documented outbreaks occurred in 1963 and 1964 in Kolkata and southern India respectively. A small outbreak of Chikungunya (CHIK) was Reported from Solapur district, Maharashtra in 1973. The virus disappeared for three decades and re-emerged in French island of Reunion in the Indian Ocean in 2005. In 2006, a large outbreak occured in India Andhra Pradesh, Andaman & Nicobar Islands, Tamil Nadu, Karnataka, Maharashtra, Gujarat, Madhya Pradesh, Kerala and Delhi were badly affected. An outbreak of Chikungunya was reported from Italy in 2007 and Thailand and south India in 2009 [3-7]. Recently in 2014, Chikungunya reached United States and cases have been reported from Florida by Centers for Disease Control and Prevention [8].

The Chikungunya fever presents with triad of symptoms fever, arthralgia and rashes. A very important feature of Chikungunya fever is a debilitating and prolonged arthralgia that primarily affects the peripheral small joints. While the acute febrile phase of the illness normally resolves within a few days, the pain associated with CHIKV infection of the joints typically persists for weeks to months or years together in chronic cases [9,10].

In the present study clinically suspected cases were tested by ELISA for Chikungunya IgM antibodies. Disease in general, serological diagnosis and the prevalence of Chikungunya in and around Bijapur has been described. Recently in 2014 Bijapur name has been changed to Vijayapura by Government of India.

MATERIALS AND METHODS

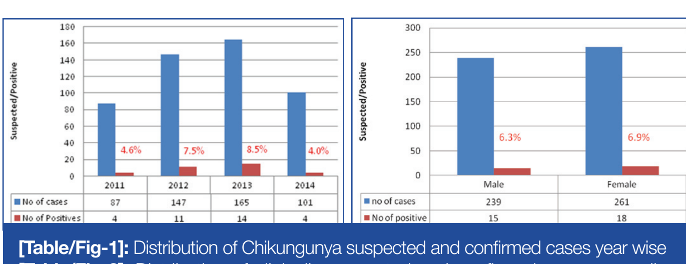
The study was conducted from April 2011 to December 2014. A total of 500 blood samples from suspected Chikungunya cases were included in the study. Blood samples from clinically suspected Chikungunya patients, i.e. pyrexia of more than >38.5°C and polyarthralgia were collected from BLDE's hospital, Bijapur 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 was separated by centrifugation and labelled with patient identification number and stored at -70°C. Serum samples were tested for 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

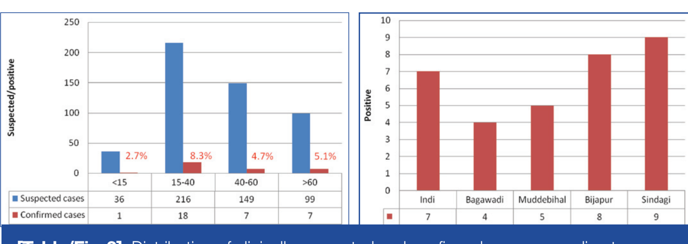
Cut-off was calculated by sum of mean negative control to 0.300. The samples of absorbance less than cut-off was considered as negative and samples of absorbance more than cut-off was considered as positive. Out of 500 samples tested, 33 samples were found positive for Chikungunya IgM antibodies. The 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



[Table/Fig-1]: Distribution of Chikungunya suspected and confirmed cases year wise
[Table/Fig-2]: Distribution of clinically suspected and confirmed cases according to gender



[Table/Fig-3]: Distribution of clinically suspected and confirmed cases according to age
[Table/Fig-4]: Chikungunya confirmed cases taluk* wise *Taluk is an administrative division in India

as Bijapur, Gulbarga, Tumkur, Bidar, Raichur, Dharwad, Bellary, Chitradurga, Davangere and Kolar have recorded large number of Chikungunya fever cases (Bijapur district over 80,00 cases) [6,12,13]. Chikungunya fever presents with similar symptoms to other arboviral diseases. Proper aetiological identification (serological diagnostic tests) is necessary for the better clinical management of disease.

Narayan Shrihari et al., conducted a study on prevalence of Chikungunya arboviral infection in and around Bellary district, Karnataka from 2009-2011. Sero positivity rate of Chikungunya was 24.75%. The number of positive cases were more in 2010 (28.04%), than 2009 (23.07%) and subsequently decreased in 2011 (19.05%). Male to female ratio was 0.98 [14].

Present study was conducted in Bijapur, which is 250km distant from Bellary. This study included cases with strong suspicion of CHIVA infection. Inclusion criteria were cases with fever and arthralgia. In this study we have noticed the prevalence of Chikungunya was 6.6% in and around regions of Bijapur. More number of cases affected were in 2013 (8.5%) in decreasing order 2012 (7.5%), 2011 (4.6%) and 2014 (4.0%) [Table/Fig-1]. Females (6.9%) were more affected compared to males (6.2%) [Table/Fig-2]. The majority 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.

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

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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 evolvement 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 belongs 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.

Keywords: E1 gene, Phylogenetic analysis, Mutational analysis

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 and single stranded. Genomic organisation of CHIKV is 5′cap-nsp1-nsp2-nsp3-nsp4-(junction 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, Maharastra, 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

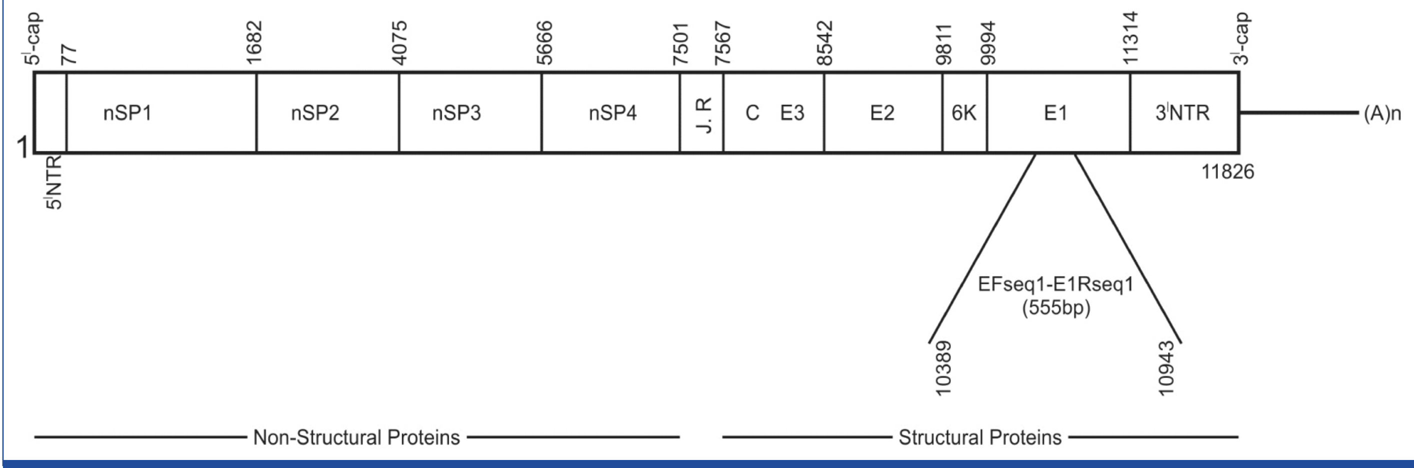
RNA was isolated from 33 serum samples using QIAamp viral RNA mini kit (Qiagen) according to manufacturer instructions [11].

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° 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



[Table/Fig-1]: Structure of Chikungunya virus whole genome with arrangement of structural and non structural genes. The location primers marked in E1 gene.

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.

Primer Name	Sequences 5'-3'	Genome position	Amplicon size bp
E1-F1	GCTCCGCGTCCTTTAC	10389bp–10943pb	555
E1-R1	ATGGCGACGCCCCCAAAGTC		

[Table/Fig-2]: Details of primers used for PCR amplification in the study.

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].

Nucleotide Sequence Accession Numbers with position	Protein Sequence Accession Numbers (Uniprot)	Strain	Year of isolation
AF369024.2|:10427-10941	Q8JUX5	S27 (ECSA)	1952
DQ309336.1|:223-737	A0SE38	Reunion 223/05 (ECSA)	2005
AM258992.1|:10376-10890	Q1W367	Reunion (ECSA)	2006
EF027139.1|:10428-10942	A6MH23	INDIA-00-MH4 (Asian)	2007
HM045811.1|:10410-10924	D7R978	Tanzania (ECSA)	1953
HM045797.1|:10410-10916	D7R952	RSU1 (Asian)	1985
EU192143.1|:2861-3367	B2BZX4	Indonesia (Asian)	2007
HM045817.1|:10412-10926	D7R990	Senegal (West Africa)	2005
HM045816.1|:10412-10926	D7R988	Senegal (West Africa)	1966

[Table/Fig-3]: Reference strains with nucleotide and protein accession number.

Mutational Analysis

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

Protein 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.

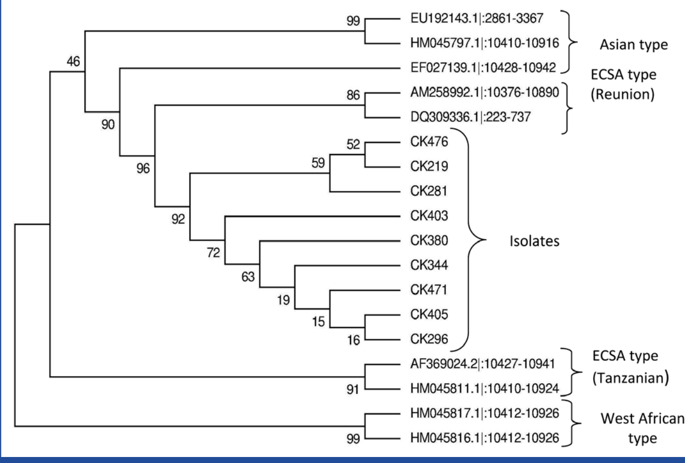
Mutation Mapping

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.



[Table/Fig-4]: Phylogenetic analysis based on Partial E1 nucleotide sequences and relationship with reference strains. Gene bank accession number for reference strain labelled.

Position			Reference strains with uniprot ID									Isolates							
Nucleotide	Polypeptide	Protein	Q8JUX5-S27	A0SE38-Reunion	Q1W367 Reunion	A6MH23-INDIA-00-MH4	D7R978-Tanzania	D7R952-RSU1	B2BZX4-Indonesia	D7R990-Senegal	D7R988Senegal	219	281	296	344	380	405	471	476
A10624G	1020	211	K	.	.	.	.	E	E	.	.	E	E	E	E	E	E	E	E
A10798G	1078	269	M	V	V	V	.	.	.	I	V	V	V	V	V	V	V	V	V
T10845A	1093	284	D	E	E	.	.	.	.	.	.	E	E	E	E	E	E	E	E

[Table/Fig-5]: Amino acid Mutational analysis of isolates with respect to positions.

Phylogenetic Analysis [Table/Fig-4]

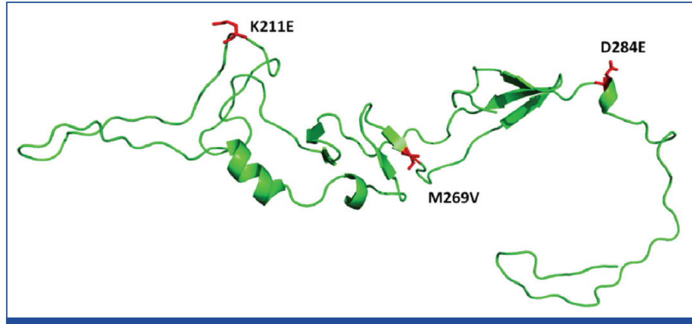
Unrooted Phylogenetic tree shows isolates are closely related to Reunion strains and distantly related to S27 Tanzanian strain and made sister group of ECSA. So, isolates belong to ECSA genotype. Isolates were even more divergent to Asian and West African genotypes. Comparative nucleotide and amino acid 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.



[Table/Fig-6]: Mapping of mutations positions in E1 gene.

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 Sero-positive 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].

Niyas KP et al., reported two important mutations A226V and V291I in E1 gene [13]. Shrinet J et al., reported number of mutations in E1 gene namely, K211E, M269V, D284E, V179A, S234P, R196K and R247C [14]. But, in the present study we have found only three mutations K211E, M269V and D284E [Table/Fig-7].

Author	Mutations
Isabelle Schuffenecker et al., [7]	A226V and D284E
Kudukkil P Niyas et al., [13]	A226V and V291I
Jatin Shrinet et al., [14]	K211E, M269V, D284E, V179A, S234P, R196K and R247C
Current study	K211E, M269V and D284E

[Table/Fig-7]: Comparison of E1 gene amino acid substitution reported in Indian isolates.

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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