Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Surveillance of chikungunya virus in Andhra Pradesh, Southern India

CVM Naresh Kumar[1], P Sangamithra[1], M Rajasekhar[2], DVR Saigopal[1*]

[1]*Department of Virology, College of Sciences, Sri Venkateswara University, Tirupati-517502, Andhra Pradesh, India*
[2]*Medical Officer, Mobile Medical Unit, District Medical Health Office, Kadapa-516001, Andhra Pradesh, India*

ARTICLE INFO

Article history:
Received 12 July 2010
Received in revised form 27 Angust 2010
Accepted 15 September 2010
Available online 20 November 2010

Keywords:
Chikungunya virus
E1 gene
RT-PCR
Non synonymous mutations
Screening

ABSTRACT

Objective: The study involved survey and screening of areas suspected of chikungunya virus (CHIKV) infection, characterizing the causative agent and identifying the circulating CHIKV genotype. **Methods:** Acute phase samples were screened by the use of RTPCR using primer set DVRChk-F/DVRChk-R whereas convalescent samples were tested by CHIKV IgM strips. **Results:** Two hundred and seventy five acute phase samples were screened by RT-PCR, of which 149(54.18%) showed positivity for CHIKV. Later on 192 convalescent phase samples were tested for CHIKV specific antibodies in which 125(65.10%) samples were found to be positive. Four CHIKV strains were selected and subjected to cloning followed by nucleotide sequencing and were submitted to the Genbank DNA database with the Accession numbers (GQ119362, GQ119363, GQ119364, and FJ225403). The Sequence analysis of "CHIK-Kadapa" strain (GQ119362) with other CHIKV isolates suggested that the present CHIKV strain has (99.23± 0.52) % and 100 % identity with Central East South African isolates (CESA) at nucleotide and amino acid levels respectively. Two unique non synonymous mutations S168L and D183V were depicted in E1 gene of the selected strains of the present study. **Conclusions:** The 14 months survey revealed the circulation of CHIKV in 2008-2009 in Andhra Pradesh and the causative agent is identified to be of Central East South African (CESA) origin. The importance of the non synonymous mutations (S168L and D183V) and their role in the mobility and strength of the E1-E1 and E1-E2 interactions needs further investigations. The study also urges the need for intensifying the epidemiological and entomological surveillance to combat any such CHIKV outbreak in the near future.

1. Introduction

Chikungunya virus (CHIKV), a reemerging arboviral infection belongs to the genus *Alphavirus*, family *Togaviridae* is an enveloped virus containing positive sense single stranded RNA as its genetic material. It is about 11 805 nucleotides in length containing two open reading frames of about 7 422(non structural genes: nsP1-nsP4) and 3 744(Structural genes: C, E3, E2, 6K, E1) nucleotides in length. The *Alphavirus* genus consists of 29 species of arboviruses that have been classified into 7 antigenic complexes: Barmah Forest (BF), Eastern equine encephalitis (EEE), Middelburg (MID), Ndumu (NDU), Semliki Forest (SF), Venezuelan equine encephalitis (VEE), and Western equine encephalitis (WEE). Chikungunya virus belongs to Semiliki Forest virus antigenic complex and is usually characterized by fever, arthralgia and myalgia[1]. Meningo-encephalitis, meningo encephalo myeloradiculitis, myeloradiculitis, myelitis, myeloneuropathy, Guillain-Barre syndrome, external opthalmoplegia, facial palsy, sensorineural hearing loss and optic neuritis are the various neurological complications observed during the recent CHIKV outbreak[2]. It is transmitted by *Aedes aegyptii* and *Aedes albopictus* mosquitoes. *Aedes albopictus* played a pivotal role in transmitting the CHIKV in the recent outbreaks of Reunion islands, North Eastern Italy and India[3-5]. CHIKV epidemic was first reported from Makonde plateau, Tanzania during 1952-1953 followed by numerous outbreaks in South Africa, Congo, Zimbabwe, Uganda, Zambia, Senegal, Nigeria, Angola[6-8]. First outbreak in India of CHIKV was reported in 1963 in Kolkata followed by epidemics in Chennai, Pondicherry and Vellore in 1964; Vishakapatnam, Rajmundry, Kakinada, Nagpur in 1965; and Barsi in 1973[9]. Phylogenetic analysis based on partial E1 gene sequences showed the presence of three distinct CHIKV phylogroups. The first phylogroup contained all

*Corresponding author: Prof. D. V. R. Saigopal, Head, Dept of Virology, SVU College of Sciences, S.V.University, Tirupati, Andhra Pradesh, India.
Tel: +91 877 2289452
Fax: +91 877 2289555
E-mail: profdvrsaigopal@gmail.com

isolates from West Africa; the second phylogroup involved isolates from Central, East and South Africa (CESA); and the third phylogroup contained Asian isolates. Until 2006 these three genotypes were restricted to their geographical areas. Earlier outbreaks in India were due to Asian genotype. The present outbreak in Indian Ocean islands, India were attributed to be of CESA genotype[9-12]. The reemergence of CHIKV in Malaysia provided evidence of co-circulation of different genotypes in the same geographical area[13, 14]. Chikungunya has emerged as an important arboviral disease of public health concern, by which approximately 7.5 million people were victimized over a period of 5 years[15]. It has been declared as a high priority pathogen by NIH[16]. Two hundred and sixteen districts in India were affected by CHIKV infections with an estimate of 1.38 million people being victimized by the end of 2006 and which further declined to an estimated 59 535, 95 091 and 68 245 cases by the end of 2007, 2008 and 2009, respectively [17,18]. Andhra Pradesh, the most affected state was first to report the CHIKV epidemic in December 2005 in India[19]. Blood group, age and gender are found to be associated with the status of CHIKV. It is observed that the Rh positive individuals are more susceptible when compared to their Rh negative counterparts in acquiring CHIKV disease[20]. In some recent studies higher CHIKV infection rates were observed in males compared to their female counterparts and differential exposure to *Aedes albopictus* mosquitoes is supposed to be the plausible reason[20-22]. Mutations in CHIKV genome, enhanced efficiency of mosquitoes to transmit CHIKV, immunologically naive population, rapid means of trade and travel, global warming and lack of public health system are some of the important factors influencing the re-emergence of CHIKV[23].

Due to similarity in symptoms with other viral, bacterial and parasitic diseases differential diagnosis of CHIKV plays an important role[24]. RT-PCR, Real time PCR and RT LAMP are some of the techniques used for early detection of CHIKV during acute phase of infection. In the present study we carried out survey and screening of areas having CHIKV suspected fever. We characterized the causative agent, identified the circulating CHIKV genotype and scrutinized into the mutations prevalent in the circulating CHIKV strains of the present study.

2. Materials and methods

2.1. Study area and sample collection

In March and April of 2008, the Mobile Medical Unit Office, Kadapa district, Andhra Pradesh observed a huge influx of patients to the Primary Health Center (PHC) with crippling arthralgia and fever in Kokkarayapalli village. Similar fever cases with arthralgia were observed in different places of Kadapa district. A survey was carried out from March 2008-April 2009; in different places of kadapa district and blood samples from patients exhibiting symptoms compatible with CHIKV infection were collected. Prior to sample collection 'Informed oral consent' were obtained from all the patients and their parents (in case of minors). The details of the areas surveyed are furnished in Figure 1. Details of the clinical samples processed during the present study are furnished in Table 1. Samples were collected in sterile vials without anticoagulant, transported in coolant packs and stored at 4 ℃ till use. All samples were processed within 24 hours after the collection.

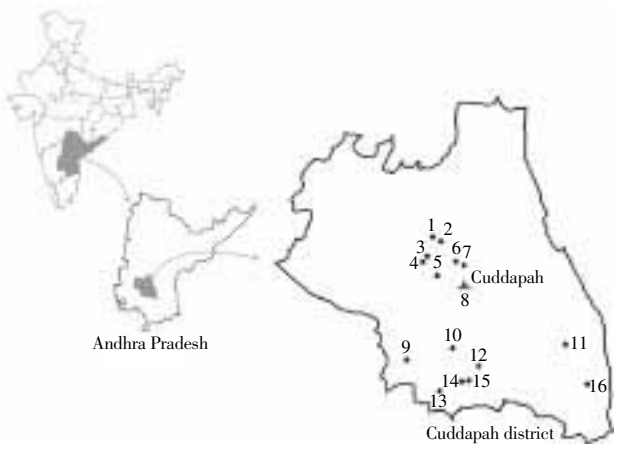

Figure 1. Map of Andhra Pradesh showing the location of sample collection areas (•).
1. Sannapalle, 2. Thudumuladinne, 3. Dadireddipalle, 4. Kamalapuram, 5. Pedda Putha, 6. Kokkarayapalle, 7. Chennur, 8. Cuddapah, 9. Galivedu, 10. Rayachoti, 11. Rajampet, 12. Kattaguttapalle, 13. Chinnamandem, 14. Yeguvarajugaripalle, 15. Diguvarajugaripalle, 16. Kodur.

2.2. RNA extraction and RT-PCR

RNA was extracted from 250 μL of serum samples according to the manufacturer's instructions (TRI Reagent® BD Sigma-Aldrich, USA), the pellets were dissolved in nuclease free water and stored at -20 ℃ till use. cDNA was synthesized in 20 μL reaction volume using Oligo (dT) primer. A primer pair DVRChk-F/DVRChk-R targeting 330 bp fragment of E1 gene was used for screening of CHIKV suspected acute phase blood samples[11]. PCR amplification was carried out in 50 μL of reaction volume containing 3 μL of cDNA, 10 pmol of forward primer (DVRChk-F:5'-ACCGGCGTCTACCCATTCATGT-3'), 10 pmol of reverse primer (DVRChk-R 5'-GGGCGGGTAGTCCATGTTGTAGA-3'), 2 mM $MgCl_2$ (Fermentas, USA), 2.5 Units of Taq DNA polymerase (Fermentas, USA) 0.2 mM each of dNTPs (Fermentas, USA) and nuclease free water. The reaction was carried out using gradient thermal cycler (Corbett Research, Model CG1-96, Australia) and the PCR amplification conditions included an initial denaturation cycle at 94 ℃ for 5 min followed by 35 cycles of denaturation for 45 sec at 94 ℃, annealing for 30 sec at 56 ℃ and extension for 1 min at 72 ℃. A final extension step of 15 min at 72 ℃ was carried out at the end and the PCR products along with the Marker DNA (100 bp DNA ladder, Fermentas, USA) were electrophoresed in 2 % agarose gel.

2.3. CHIK IgM rapid tests

The convalescent phase serum samples were tested for CHIKV specific antibodies using SDBioline Chikungunya IgM Rapid Tests according to manufacturer's instructions.

2.4. Cloning, nucleotide sequencing and sequence analysis

Four of the above study strains were gel extracted using the Qiaquick gel extraction kit (Qiagen, USA) and then cloned into pTZ57R/T vector (Fermentas, USA) according to the Manufacturer's instructions. The resulting recombinant plasmids were transformed into *Escherichia coli* DH5 α cells and the recombinant clones were selected by blue white colony selection. The positive clones were sequenced using M13 universal primers at commercial sequencing facility (Eurofins Genomics India Pvt Ltd, Bangalore). Multiple sequence alignments were done using Bioedit software (USA) and the generated alignments were used to determine the percentage of homologies between the sequences. Neighbour Joining algorithm was used to construct phylogenetic trees by the use of MEGA version 4.0 (bootstrap analysis with 1 000 replication) using O'nyong nyong virus (AF079456) as the out group.

2.5 Statistical analysis

The percentage of CHIKV infection was calculated by dividing the number of CHIKV positive samples by total number of samples. For observing the statistical significance Chi square test (χ^2) has been performed and a P value of < 0.05 was considered significant.

3. Results

In this cohort, patients with severe fever, joint pains, myalgia and headache which are the characteristics features of CHIKV infections were observed during our epidemiological surveys. In total 467 CHIKV suspected samples were collected from various places in kadapa district of Andhra Pradesh during March 2008-April 2009. As malaria is endemic in Eguvarajugaripalli and Diguvarajugaripalli villages, the DMHO staff tested the patients and found that all the samples were negative for *P. falciparum*. Later on these samples were tested for CHIKV. CHIKV specific RNA was detected from clinical samples up to 7th day after the onset of the disease. A total of 149 (54.18%) serum samples were found positive for the presence of CHIKV gene specific 330 bp amplicon (Figure 2). 192 convalescent phase serum samples were screened by CHIK IgM rapid tests for the presence of IgM and IgG anti CHIKV antibodies. The serological investigation revealed the presence of anti CHIK IgM in 104 samples (54.16%) and anti-CHIK IgM and IgG in 21 patients (10.93%). The statistical significance between the CHIKV diagnostic tests (RT-PCR and IgM strip analysis) and CHIKV suspected samples was observed using Chi square test statistic and was found to be statistically significant (Chi-Square: 5.121 2; *P<0.05). Hence it is concluded that the CHIKV diagnostic tests played an important role in confirming CHIKV infections. The possible role of viremic people in these villages coupled with rapid means of transportation facility in spreading CHIKV to neighbouring places cannot be underestimated. Four clinical strains (CHIK-Kadapa; CHIK- Kamalapuram; CHIK-Kattaguttapalli and CHIK-AP-KDP) were selected, cloned, sequenced and deposited to Genbank DNA database under the accession numbers: GQ119362, GQ119363, GQ119364 and FJ225403. In order to determine the phylogroup of the circulating CHIKV strains of the present study a 303 bp region of E1 gene of the present study strains (region 10213 bp-105 15 bp corresponding to AM258990) were compared with 17 other CHIKV isolates corresponding to 3 different CHIKV genotypes. Phylogenetic analysis showed that all the 4 CHIKV strains (Present study) from India, detected during 2008-2009 grouped into CESA genotype and clustered in a distinct lineage (Figure 3). This lineage showed close relationship to the Reunion 2005, 2006 strains (AM258990, DQ443544) showing the possible evolution of the Indian strains from Reunion Outbreak. Comparative homology analysis of "CHIK-Kadapa" strain (GQ119362) revealed (99.23± 0.52) % and 100 % of homology at nucleotide and amino acid level respectively with CESA isolates. Percent homology of CHIK-Kadapa strain (GQ119362) with different CHIKV isolates is furnished in Table 2. The alignment of the common region of the sequences (deduced amino acids) of the present study strains with the reference strains of CHIKV is given in Figure 4(region 10 186 bp -10 515 bp corresponding to AM258990). In total 28 synonymous mutations and 2 non synonymous mutations were observed in the present study strains, details of which are furnished in Table 3. Six mutations, viz. A306G, C384T, T519C, C531T, T552C and C555T found consistently in all the four CHIKV strains of the present study were synonymous mutations. C503T nucleotide change in CHIK-AP-KDP strain (FJ225403) and A548T change in CHIK-Kattaguttapalli strain (GQ119364) resulted in non synonymous mutations S168L and D183V respectively.

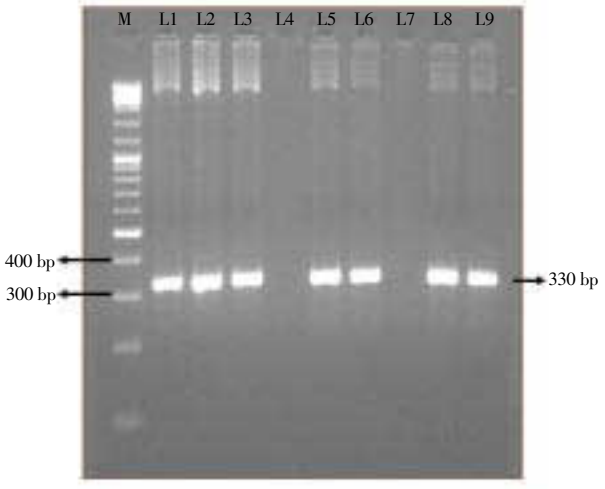

Figure 2. Gel photograph showing 330 bp of E1 gene of Chikungunya virus by reverse transcriptase-polymerase chain reaction.
Lane M: 100 bp marker; Lane 1, 2, 3, 5, 6, 8, 9: 330 bp amplicon of E1 gene from clinical samples; Lane 4, 7 CHIKV negative samples.

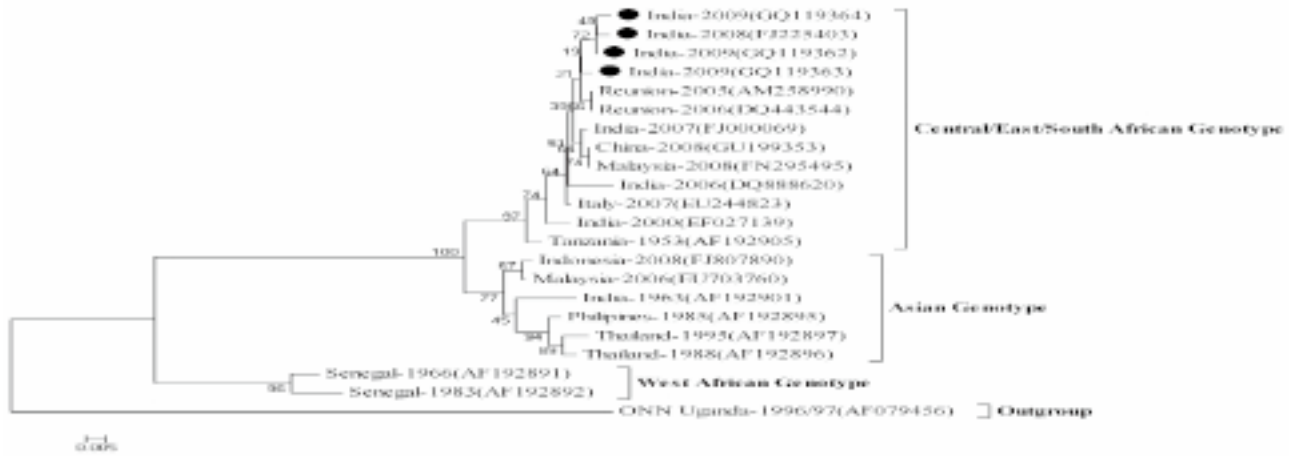

Figure 3. Phylogenetic tree constructed from the alignment of partial E1 gene amino acid sequences of the present study strains with those of other reported CHIKV sequences taking ONN as an out group.

The present study strains grouped with the isolates of Central East South African genotype. Taxa marked in "•" indicate the present study strains. The values at the forks indicate the number of trees that this grouping occurred after bootstrapping the data. The scale bar shows the number of substitutions per base.

Table 1

Details of clinical samples processed during the present study.

Place	Sampling time	CHIKV suspected samples	
		Acute phase samples* (149/275)	Convalescent phase samples# (125/192)
Chennur	March 2008	0/4	0/1
Kokkarayapalli	March 2008	39/55	9/17
Kodur	June 2008	13/27	7/19
Yeguvarajugari palle and Diguvarajugari palle	July 2008	2/3	29/30
Rajampeta	September 2008	18/ 39	9/21
Thudumaladinne	October 2008	11/16	7/8
Sannapalle	October 2008	5/8	2/3
Dadireddy Palle	December 2008	5/7	3/8
Kamalapuram	January 2009	4/9	21/21
Kattagutta Palli	February 2009	3/12	7/7
Kadapa town	February 2009	19/37	8/12
Pedda Putha	March 2009	3/8	4/4
Rayachoti	March 2009	18/35	13/31
Galivedu	April 2009	2/5	3/7
Chinnamandem	April 2009	7/10	3/3

* Screened by RT-PCR, Acute phase samples= No. of samples positive/No. of samples tested; # Screened by CHIK IgM strips, Convalescent phase samples=No. of samples posibive/No. of samples toted.

Table 2

Percent homology of CHIK-Kadapa strain (GQ119362) with different CHIKV isolates at nucleotide and amino acid level

Isolates/Strains	Percent homology with "CHIK-Kadapa" Strain(GQ119362)	
	Nucleotide level	Amino acid level
Present study strains	99.63± 0.29	99.50± 0.58
Central/East/South African isolates(CESA)	99.23±0.52	100.00±0.00*
Asian isolates	95.18±1.89	97.40±1.26
West African isolates	90.83±7.10	98.30±1.53

* Showed 100% homology, Data are expressed as mean±SD, present study strains include: [GQ119363 (CHIK- Kamalapuram) GQ119364 (CHIK-Kattaguttapalli) FJ225403 (CHIK-AP-KDP)] .

Table 3

Nucleotide and amino acid changes observed in CHIKV strains of the present study.

Genbank (Accession No.)	Isolate/Strain	E1 nucleotide position										E1 protein position	
		306	384	402	495	**503**	519	531	**548**	552	555	**168**	**183**
AF369024	Reference strain	A	C	C	A	C	T	C	A	T	C	S	D
*FJ225403	CHIK-AP-KDP	G	T	–	T	**T**	C	T	–	C	T	**L**	–
*GQ119362	CHIK-Kadapa	G	T	–	T	–	C	T	–	C	T	–	–
*GQ119363	CHIK-Kamalapuram	G	T	T	–	–	C	T	–	C	T	–	–
*GQ119364	CHIK-Kattaguttapalli	G	T	–	T	–	C	T	**T**	C	T	–	**V**

*Present study strains, Letters in BOLD indicate changes leading to non synonymous mutations.

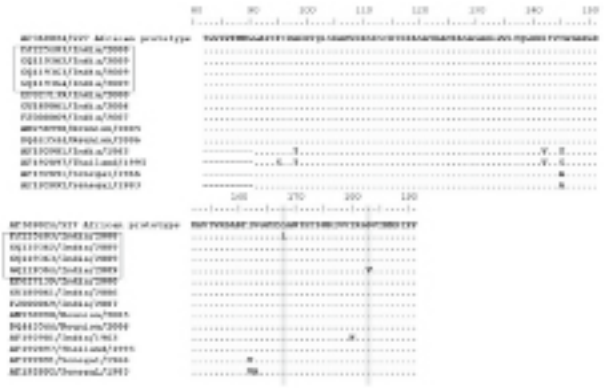

Figure 4. Alignment of the deduced amino acids of CHIKV E1 gene sequences of the present study strains with the reported CHIKV genotypes.

Non synonymous mutations S168L and D183V are highlighted in shaded colour. The present study strains are represented within the Boxes.(AF369024:CHIKV Reference strain); (EF027139, GU189061, FJ000069, AM25899, DQ443544: Central East South African genotype); (AF192901, AF192897: Asian Genotype); (AF192891,AF192892: West African Genotype).

4. Discussion

In India CHIKV epidemic was first reported in 1963 and in Andhra pradesh the CHIKV epidemic was first reported during 1965 in Vishakapatnam, Rajmundry and Kakinada. After a time lapse of 40 years, Andhra Pradesh reported CHIKV epidemic during 2005. Twenty three districts were affected and nearly 77 533 CHIKV cases were suspected by the end of 2006[25]. In a recent study it was estimated that the burden of Chikungunya for Kadapa district was 160 DALYs (Cost: US$ 290 000) and 6 600 DALYs (Cost: US$ 12 400 000) for the state of Andhra Pradesh[26]. CHIK cases have been reported from Anantapur, Chittoor, Guntur, Kadapa, Kurnool, Nalgonda and Prakasam districts of Andhra Pradesh[9,11,19,27,28]. CHIK cases were also reported from Tirupati, Hyderabad and Secunderabad[11,12,20,29-31]. By the end of 2007 CHIKV cases were almost declined and it was thought that the CHIK fever was subsided. CHIKV type fever cases were reported in some PHC during 2008 in Kadapa district of Andhra Pradesh. The focus of the present study was to carry out a thorough survey of the regions having CHIKV suspected fever and characterize the causative agent. Early detection of CHIKV is an essential prerequisite for effective control and prevention of the epidemic and RT-PCR using the primer pair DVRChk-F/DVRChk-R served as a valuable tool for CHIKV detection in the present study as well as in earlier studies in Andhra Pradesh, India and Southern Thailand[8,11,20,32]. The circulating CHIKV genotype was identified to be of CESA origin.

The primary sequence of CHIKV E1 glycoprotein is quite similar to the E1 fusion protein of SFV and hence it is likely that the 3-D folding of these related proteins is similar [1]. In a recent study, Homology based model of CHIKV E1 protein was constructed using glycoprotein E1 from Semiliki Forest Virus as a template[33]. In alphaviruses E1 is responsible for cell fusion and E2 is mainly involved in receptor binding and cell entry[34]. Domains I and II of the E1 protein are involved in E1 trimerization during the viral fusion process. Domain II mediates the E1-E2 interactions during the virus maturation and budding from infected cells[33]. C503T Nucleotide change in CHIK-AP-KDP strain (FJ225403) resulted in a non synonymous mutation (S168L) wherein Serine (hydrophobic amino acid) was replaced by leucine (hydrophilic amino acid). Similarly A548T change in CHIK-Kattaguttapalli strain (GQ119364) resulted in non synonymous mutation (D183V) wherein aspartic acid (hydrophilic amino acid) was replaced by valine(hydrophobic aminoacid). The amino acid changes observed in the present study strains (S168L & D183V) are non conservative and which might affect the mobility and strength of E1-E1 and E1-E2 interactions.

In conclusion our 14-month survey revealed the circulation of CHIKV in 2008-2009 in Andhra Pradesh and the causative agent is identified to be of Central East South African (CESA) origin. We observed that in spite of the proper guidelines framed by the concerned health authorities, CHIKV infections has been underreported due to similarity in symptoms with other arboviral diseases(like dengue) and due to lack of adequate facilities for proper disease diagnosis. It is quite necessary for the physician's to diagnose the disease properly and provide proper medications to the patients. At the same time surveillance of the disease should be intensified so as to prevent the outbreaks in the near future. The movement of viremic individuals in other cohorts may play an important role in spread of CHIKV. However, this cannot be controlled and hence a better mosquito control adoption measures would play a pivotal role in restricting the magnitude of CHIKV outbreaks.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

C.V.M. Naresh Kumar gratefully acknowledges University Grants Commission, New Delhi for financial assistance in the form of UGC-JRF in Research Fellowship in Sciences for Meritorious Students Scheme (RFSMS) and Standard Diagnostics, Korea for providing anti CHIKV IgM kits. Authors also acknowledge the Director, Deputy Director of District Medical Health office, Kadapa; Dr. Arjun, Dr Arun, Medical Officers of the Primary Health Centers, Kadapa district and Bharathi Health Labs, Kadapa, for their help in Sample Collection.

References

[1] Solignat S, Gay B, Higgs S, Briant L, Devaux C. Replication cycle of chikungunya: A re-emerging arbovirus. *Virology* 2009; **393**: 183-97.

[2] Murthy JMK. Chikungunya virus: The neurology. *Neurol India* 2009; **57**: 113-5.

[3] Moro ML, Gagliotti C, Silvi G, Angelini R, Sambri V, Rezza G, et al. Chikungunya virus in North-Eastern Italy: A Seroprevalence study. *Am J Trop Med Hyg* 2010; **82**: 508-11.

[4] Philip Samuel P, Krishnamoorthi R, Hamzakoya KK, Agarwal CS. Entamoepidemiological investigations on Chikungunya outbreak in the Lakshadweep islands, Indian Ocean. *Indian J Med Res* 2009; **129**: 442-5.

[5] Santhosh SR, Dash PK, Parida MM, Khan M, Rao PVL. Appearance of E1: A226V mutant Chikungunya virus in Coastal Karnataka, India during 2008 outbreak. *Virol J* 2009; **6**: 172.

[6] Kalantri SP, Joshi R, Riley LW. Chikungunya epidemic: An Indian perspective. *Natl Med J India* 2006; **19**: 315-22.

[7] Powers AM, Logue CH. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. *J Gen Virol* 2007; **88**: 2363-77.

[8] Naresh Kumar CVM, Saigopal DVR. Reemergence of Chikungunya virus in Indian Subcontinent. *Indian J Virol* 2010; **21**(1) doi: 10.1007/s13337-010- 0012-1.

[9] Yergolkar PN, Tandale BV, Arankalle VA, Sathe PS, Sudeep AB, Swati SG, et al. Chikungunya outbreaks caused by African genotype, India. *Emerging Infect Dis* 2006; **12**: 1580-3.

[10] Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, Vaney MC, et al. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLOS Medicine* 2006; **3**: e263.

[11] Naresh Kumar CVM, Johnson AMA, Sai Gopal DVR. Molecular characterization of chikungunya virus from Andhra Pradesh, India and phylogenetic relationship with Central African isolates. *Indian J Med Res* 2007; **126**: 534-40.

[12] Dash PK, Parida MM, Santhosh SR, Verma SK, Tripathi NK, Ambuj S, et al. East Central South African genotype as the causative agent in reemergence of Chikungunya outbreak in India. *Vector Borne Zoonotic Dis* 2007; **7**(4): 519-27.

[13] AbuBakar S, Sam IC, Wong PF, Mat Rahim N, Hooi PS, Roslan N. Reemergence of endemic Chikungunya, Malaysia. *Emerg Infect Dis* 2007; **13**(1)147-9.

[14] Sam IC, Chan YF, Chan SY, Loong SK, Chin HK, Hooi PS, et al. Chikungunya virus of Asian and Central/East African genotypes in Malaysia. *J Clin Virol* 2009; **46**(2): 180-3.

[15] Schwartz O, Albert ML. Biology and pathogenesis of chikungunya virus. *Nat Rev Microbiol* 2010; **8**(7): 491-500.

[16] Labadie K, Larcher T, Joubert C, Mannioui A, Delache B, Brochard P, et al. Chikungunya disease in non human primates involves long-term viral persistence in macrophages. *J Clin invest* 2010; **120**: 894-906.

[17] NVBDCP. *Epidemiological profile of chikungunya fever in the country.(prov.)*. NewDelhi: National Vector Borne Disease Control Programme; 2009.

[18] NVBDCP. *Status report on Dengue and Chikungunya as on31.12.09 (Dengue and chikungunya update)*. New Delhi: National Vector Borne Disease Control Programme; 2010.

[19] Ravi V. Re-emergence of Chikungunya virus in India. *Indian J Med Microbiol* 2006; **24**: 83-4.

[20] Naresh Kumar CVM, Mahathi N, Vishnu VR, Sai Gopal DVR. Association of ABO blood groups with Chikungunya virus. *Virol J* 2010; **7**: 140.

[21] Moro ML, Gagliotti C, Silvi G, Angelini R, Sambri V, Rezza G, et al. Chikungunya virus in North-Eastern Italy: A Seroprevalence study. *Am J Trop Med Hyg* 2010; **82**: 508-11.

[22] Lakshmipathy DT, Dhanasekaran D. Molecular epidemiology of Chikungunya virus in Vellore distrct,Tamilnadu,India in 2006. *East Afr J Public Health* 2008; **5**: 122-5.

[23] Bhatia R, Narain JP. Re-emerging chikungunya fever: some lessons from Asia. *Trop Med Int Health* 2009; **14**(8): 940-6.

[24] Saxena SK. Re-emergence of the knotty chikungunya virus:facts, fear or fiction. *Future Virol* 2007, **2**: 121-6.

[25] NVBDCP. *Facts about Chikungunya*. NewDelhi: National Vector Borne Disease Control Programme; 2006.

[26] Seyler T, Hutin Y, Ramachandran V, Ramakrishnan R, Manickam P, Murhekar M. Estimating the burden of disease and the economic cost attributable to Chikungunya, Andhra Pradesh, India 2005-2006. *Trans R Soc Trop Med Hyg* 2010; **104**: 133-8.

[27] Murty US, Dhanwada N, Arora N. Chikungunya outbreak in Kurnool district, Andhra Pradesh. *Internet J Health* 2007; **6**(2). [Online].Available from: http://www.ispub.com/ostia/index.php?xmlFilePath=journals/ijh/vol6n2/chickv.xml.[Accessed on 12 Feb 2009].

[28] Mohan A. Chikungunya fever strikes in Andhra Pradesh, *Natl Med J India* 2006; **19**: 240.

[29] Saxena SK, Singh M, Mishra N, Lakshmi V. Resurgence of Chikungunya virus in India: an emerging threat. *Euro Surveill* 2006; **11**(32): pii=3019.

[30] Lakshmi V, Neeraja M, subbalaxmi MVS, Parida MM, Dash PK, Santhosh SR, et al. Clinical features and Molecular diagnosis of Chikungunya fever from South India. *Clin Infec Dis* 2008; **46**: 1436-42.

[31] Mohan A. Chikungunya fever: clinical manifestations and management. *Indian J Med Res* 2006; **124**: 471-4.

[32] Theamboonlers A, Rianthavorn P, Praiananthavorn K, Wuttirattanakowit N, Poovorawan Y. Clinical and molecular characterization of Chikungunya virus in South Thailand. *Jpn J Infect Dis* 2009; **62**: 303-5.

[33] Sreekumar E, Issac A, Nair S, hariharan R, Janaki MB, Arathy DS, et al. Genetic characterization of 2006-2008 isolates of Chikungunya virus from Kerala, South India by whole genome sequence analysis. *Virus genes* 2010; **40**: 14-27.

[34] Mukhopadhyay S, Zhang W, Gabler S, Chipman PR, Strauss EG, Strauss JH, et al. Mapping the structure and function of the E1 and E2 glycoproteins in alphaviruses. *Structure* 2006; **14**: 63-73.