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LETTER TO THE EDITOR

Genetic diversity of 2006–2009 Chikungunya virus outbreaks in Andhra Pradesh, India, reveals complete absence of E1:A226V mutation

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Chikungunya, an emerging arboviral infection of public health concern, was first reported from Makonde plateau, Tanzania, during 1952–1953 and since then has been held responsible for explosive epidemics in Africa, India and South East Asia, respectively (*1*). In Andhra Pradesh, Chikungunya virus (CHIKV) outbreaks due to Asian genotype were first recorded in Visakhapatnam, Rajahmundry and Kakinada in 1965. After a gap of 40 years, CHIKV re-emerged in the state of Andhra Pradesh in December 2005 and since then has been continuously (2007–2015) reported (*2*). Genetic analyses of CHIKV sequences from the state of Andhra Pradesh were limited and the virus has not been studied in detail. In the present study, we determined the complete E1 gene sequences from sera of 9 patients representing 2006–2009 CHIKV outbreaks. The phylogenetic origin, and microevolution of the CHIKV strains were examined in detail.

During 2006–2009, we confirmed CHIKV infections by virus isolation, RT-PCR and serological analysis, respectively (*3*). Nine representative CHIKV-positive serum samples pertaining to different time intervals and geographical locations from the above outbreaks (2006–2009) were further selected for genomic characterization of the virus. Primers for complete amplification of E1 gene of CHIKV (DVR-E1F: GGATCCAGCGCGTACGAACACGTA, genome position 9988–10005 and DVR-E1R: AAGCTTTTAGTGCC TGCTGAACGACAC, genome position 11293–11313) were designed based on strain 05-115 CHIKV (AM258990) from Reunion, using Primer 3 software. The sequence of CHIKV was determined directly from clinical samples without the risk of altering the genome by *in vitro* passaging. *Pfu* DNA polymerase (Fermentas, USA) was used to minimize polymerization errors. The RT-PCR products were cloned into pJET1.2/blunt cloning vector (Fermentas, USA). The resulting recombinant plasmids were transformed into *Escherichia coli,* strain DH5α cells. The transformants were screened by colony PCR and the potential recombinant clones with DNA insert of expected size were sequenced and submitted to the GenBank database. Using MEGA version 4.0, a phylogenetic tree was constructed (using complete E1 gene nucleotide sequences) taking *O'nyong-nyong virus* as the outgroup. Phylogenetic tree produced three major and distinctly branched groups corresponding to East Central South African (ECSA), Asian and West African genotypes, respectively (Fig. 1). Within ECSA genotype, we observed 2 subgroups. CHIKV isolates of Indian Ocean lineage (IOL) formed a subgroup while older ECSA isolates formed a separate subgroup. IOL subgroup consisted of CHIKV isolates from India, Kenya, Indian Ocean Islands (Mauritius, Reunion, Seychelles) and other Asian countries (China, Singapore,

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Abbreviations: CHIKV = Chikungunya virus; ECSA = East Central South African; IOL = Indian Ocean lineage

Malaysia, Srilanka, Bangladesh). Our present study strains grouped together within this IOL subgroup (Fig. 1). The most extensive series of CHIKV outbreaks during 2004–2011 were associated with CHIKV IOL subgroup, which first emerged in Kenya (2004) and subsequently spread to several Indian Ocean islands, India, Southeast Asia, China, Italy and France (*4*). CHIKV ECSA isolates from 1950's to 2000 clustered together and formed a separate subgroup (Older ECSA isolates). Within this subgroup Uganda-1982 and Yawat-2000 isolates clustered together while South Africa-1956, India-1986, Tanzania-1953 and Senegal-1963 isolates formed a separate cluster.

Second group consisted of isolates belonging to Asian genotype. Within the Asian genotype, older Indian isolates (India-1963 and India 1973) grouped together whereas later isolates from Indonesia (1983), Thailand (1995) and Philippines (1985) grouped together with recent endemic Asian isolates from Malaysia (2006) and Indonesia (2007), respectively.

The third group consisted of isolates of West African genotype. Evolutionary studies described earlier traced the CHIKV epidemics to at least 3 independent CHIKV lineages, which have emerged simultaneously from different parts of Africa and Asia: IOL, Democratic Republic of Congo lineage and endemic Asian lineage (*5*). The present CHIKV strains circulating in Andhra Pradesh, India, from 2006-09 belong to the IOL subgroup of ECSA genotype. The last reported CHIKV epidemic due to Asian genotype in India was in 1973 in Barsi. In India, the ECSA genotype has replaced the Asian genotype since the explosive epidemic of 2005, and has been isolated in the recurring outbreaks since then. Grouping of CHIKV strains from 2006–2009 (present study strains), under IOL subgroup within ECSA genotype further rules out the possible circulation of Asian genotype of CHIKV in Andhra Pradesh, India. It seems that Asian genotype has been completely displaced by ECSA genotype. The Asian genotype, however, has not completely disappeared, as Malaysia reported an outbreak in 2006 (*6*) and Taiwan reported the import of a strain of the Asian genotype from Indonesia during 2006-2009 (*7*). The nucleotide and deduced amino acid sequences of the present study strains were compared with 17 other CHIKV representative sequences corresponding to ECSA genotype using BIOEDIT software. Manually, we looked into the mutations present in our study strains, as to understand the evolutionary trends of CHIKV.

Out of 21 random substitutions observed in the E1 gene of present study strains, 18 were present in the E1 ectodomain of CHIKV: V813A, E839V, N949I, S977L, D992V, K1020N, D1021G, F1049S/L, G1057V, N1079D, D1101G, T1106A, A1138V, M1142T, E1152G, T1167M, S1180F. These non-conservative changes might affect the mobility (internal variation of confirmation of E1 & E2 proteins, which aroused due to mutations) and the strength of E1-E1 and E1-E2 interactions as was hypothesized earlier (*8*). Mutation E1:A226V provides a selective advantage to CHIKV in mosquitoes (9). Higher efficiency of replication and dissemination of CHIKV carrying A226V mutation in mosquitos *Aedes albopictus* was reported earlier. CHIKV A226V mutation was also proved to be directly responsible for CHIKV adaptation to *Aedes albopictus*. A226V mutation was completely absent in 2006 CHIKV isolates from different states of India. (*10, 11*). It was, however, observed in 2007–2009 CHIKV outbreaks in Kerala (*8, 12–14*) and 2008 CHIKV outbreaks in Karnataka (*15*). A226V mutation was completely absent in all 9 CHIKV strains sequenced in this study from 2006–2009 from the state of Andhra Pradesh, India. A recent study also showed absence of this mutation in 2009-10 CHIKV isolates from Andhra Pradesh and Tamil Nadu (*7*). CHIKV was detected in mosquitoes *Aedes aegypti* collected in Andhra Pradesh during 2006 Chikungunya outbreaks, while *Aedes albopictus* mosquitoes were implicated as the main vectors of CHIKV in Kerala and Karnataka. It has been reported that there are two sublineages of the ECSA genotype co-circulating in South India, the E1-226A along the East coast (Andhra Pradesh, Tamil Nadu), and the E1-A226V variant along the West coast (Kerala, Karnataka) since 2007 (*7*). Sublineages of the ECSA genotype circulating in India parallel the abundance of *Aedes albopictus* and *Aedes aegypti* mosquitoes, respectively (*7*). Our finding, i.e. circulation of CHIKV E1-226A from 2006–2009 in Andhra Pradesh (East Coast), further supports the above hypothesis of co-circulation of the ECSA sublineages in Southern India. E1-K211N mutation was observed in a lone CHIKV strain from our study (CHIK-THM-01). The E1-K211N mutation was reported to be under positive selection with a posterior probability of >75% (10). The E1-K211N mutation was observed in India (2006) and Srilanka (2008) in the background of both E1-226A and E1-A226V mutation (*10, 16*). It appears that E1-211 has a high propensity for mutation. E1-211 is located in the domain II of E1 glycoprotein (*17*) and in proximity to the ij loop. The ij loop interacts with the mosquito cell target membrane in contact with the fusion peptide (*9, 18*). A net change in polarity could possibly destabilize the intra-spike contacts within E1 or with the E2 protein in this region (*7*). Hence, it is an interesting research question for experimentation, whether E1-K211N mutation that is under significant positive selection is an adaptive mutation for increasing transmissibility and fitness in *Ae. aegypti* mosquitoes. In our CHIKV study strains, three mutations E1-4VA, E1-226A and E1-G248V fell within the T-cell epitopic region, which was earlier predicted to influence immunogenicity (8).

In conclusion, we have sequenced 9 complete E1 gene sequences of CHIKV strains circulating during 2006-09 in the state of Andhra Pradesh, India. Phylogenetic analysis revealed the circulating CHIKV strains to be of IOL subgroup of ECSA genotype. Circulation of CHIKV strains

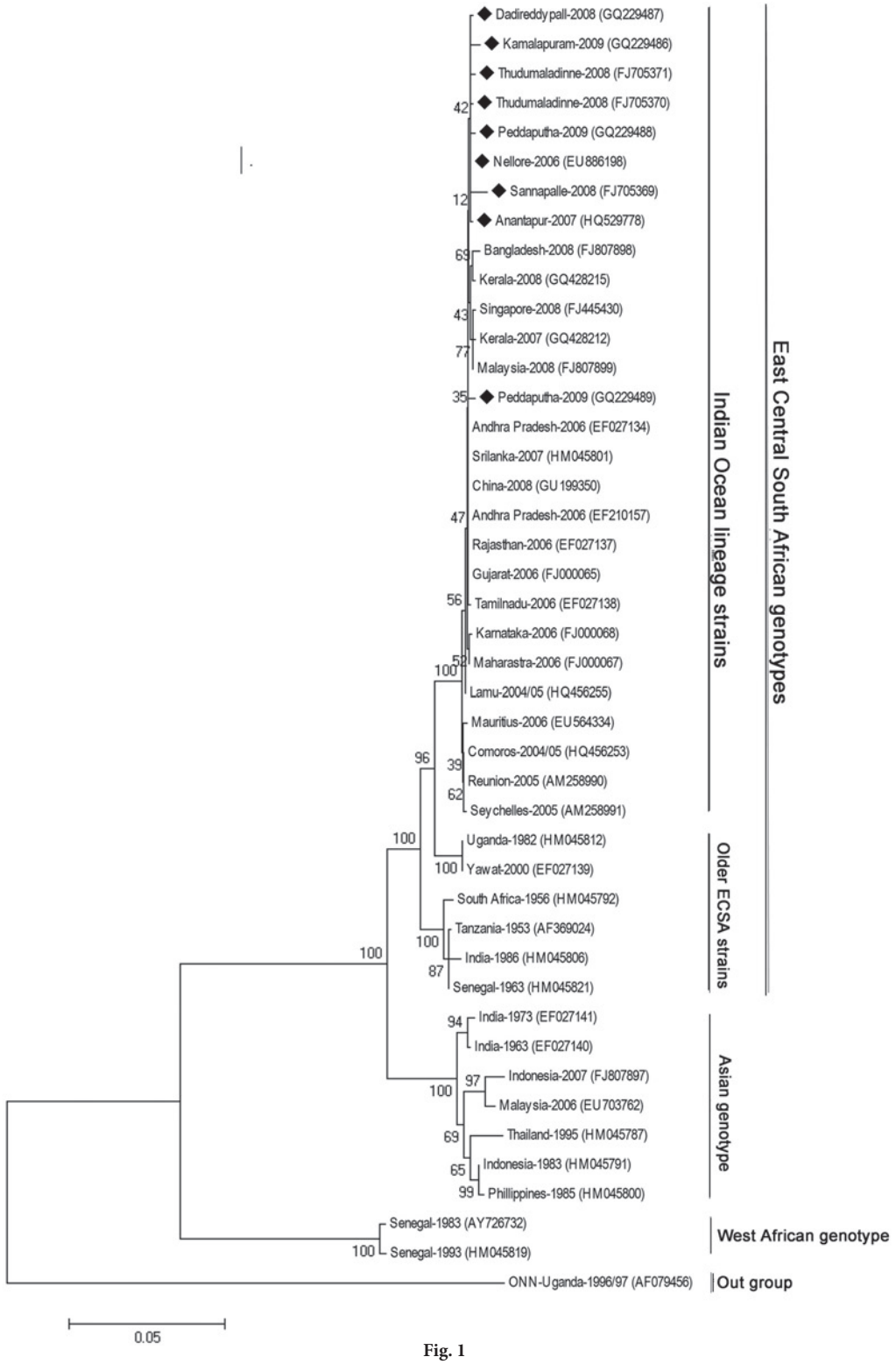

Fig. 1

Phylogenetic tree of complete E1 gene nucleotide sequences of the present study strains and those of other reported CHIKV sequences taking O'nyong-nyong virus (ONN) as an outgroup

The tree was constructed using MEGA version 4.0. The present study strains grouped within IOL subgroup of ECSA 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.

having E1-226A was observed in our study. Our study reveals circulation of CHIKV with novel genetic changes in Andhra Pradesh, India, during 2006–2009 disease outbreaks. The samples analyzed represent only a small cross section of the cases from massive CHIKV outbreaks in the state of Andhra Pradesh. However, the study points out genome microevolution of the viral strains, which might have affected the disease profile. Functional studies on these mutant viruses would help to understand the correlation of these genetic changes to CHIKV virulence and pathogenesis.

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