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# Comparative full genome analysis revealed E1: A226V shift in 2007 Indian Chikungunya virus isolates

S.R. Santhosh*, P.K. Dash, M.M. Parida, M. Khan, M. Tiwari, P.V. Lakshmana Rao

*Division of Virology, Defence R&D Establishment (DRDE), Jhansi Road, Gwalior 474 002, MP, India*

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ABSTRACT

The resurgence of Chikungunya virus (CHIKV) in the form of unprecedented explosive epidemic after a gap of 32 years in India is a point of major public health concern. In 2007 again there was outbreak in Kerala, India, affecting more than 25,000 cases with many reported mortalities. To understand the molecular basis of this high virulence and its implication in large-scale epidemic, a detailed systematic serological, virological and molecular investigation was undertaken with the epidemic samples of Kerala-2007. The comparative analysis of full genome sequence of Chikungunya virus isolate of 2007 with 2006 revealed three unique substitutions in structural and non-structural genes of 2007 isolate [two in E1 region (V14A and A226V) and one in Nsp1 (M184T)]. Our finding further substantiates the association of A226V shift in E1 gene with evolutionary success possibly due to adaptation in the mosquito vector with progression of epidemic, as observed in Reunion Island. This A226V shift which was absent in all 2006 Indian isolates, is found to be present in the four 2007 isolates, analysed in this study. These unique molecular features of the 2007 isolates with the progression of the epidemic from 2005 to 2007 demonstrate their high evolutionary and epidemic potential and thereby suggesting possible implication in higher magnitude and virulence of this outbreak.



## 1. Introduction

Chikungunya (CHIK) is an acute mosquito-borne febrile arthritis caused by an alphavirus belonging to family *Togaviridae* (Strauss and Strauss, 1986; Porterfield, 1980). The disease is characterized by abrupt onset of high fever, arthralgia, myalgia, headache and rash (Johnston and Peters, 1996; Jupp and McIntosh, 1988). The typical clinical sign of the disease is the poly-arthralgia which is very painful and may persist for several months in some cases (Jupp and McIntosh, 1988). Chikungunya virus (CHIKV) has re-emerged as an important pathogen causing epidemics in several parts of the world.

Geographically, the virus is distributed in Africa, India and South-East Asia. The virus appears to have spread from Africa to other parts of the world and caused epidemics in the Asian tropics (Powers et al., 2000). Since the 1952 Tanzania outbreak, CHIKV has caused outbreaks in East Africa (Tanzania and Uganda), Austral Africa (Zimbabwe and South Africa), West Africa (Senegal and Nigeria), Central Africa (Central African Republic and Democratic Republic of the Congo) and Asia (Thailand, Cambodia, Vietnam, Laos, Myanmar, Malaysia, Philippines, Indonesia and India) (Johnston and Peters, 1996; Jupp and McIntosh, 1988; Pastorino et al., 2004). In both Africa and Asia, the re-emergence was unpredictable, with intervals of 7–20 years between consecutive epidemics. Since 2004, CHIKV has emerged in eastern Africa (Kenya), the islands of the south-western Indian Ocean. The virus spreads to Comoros in January 2005. Later, the virus has circulated in the other islands, i.e., Mayotte, Seychelles, Reĭunion and Mauritius. Starting in December 2005, the rainy season gave rise to a renewed epidemic circulation of the virus. The most affected island is Reunion (total population: 770,000), with an estimated 244,000 cases (16 April 2006). During this epidemic in Reunion severe clinical signs like neurological signs, fulminant hepatitis were documented (Schuffenecker et al., 2006). Several cases of encephalopathy and major algic syndrome have been associated with vertical transmission of the virus. An outbreak of CHIKV also occurred in northern Italy (Ravenna) during July–August 2007 affecting 205 cases (Rezza et al., 2007).

CHIKV is an enveloped, positive-strand RNA virus. The phylogenetic analyses based on E1 gene sequences grouped CHIKV isolated worldwide into three genotypes: Asian, East/Central/South African (ECSA) and west African (Powers et al., 2000; Schuffenecker et al., 2006; Powers and Logue, 2007). The complete nucleotide sequence for the African prototype strain, S27, Ross and West African Senegal strains was determined and the presence of an internal polyadenylation [I-poly(A)] site and repeated sequence elements within the

* Corresponding author. Tel.: +91 751 2233495; fax: +91 751 2341148.
*E-mail address:* santhuvet4u@rediffmail.com (S.R. Santhosh).

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3′ non-translated region (3′-NTR) was observed (Khan et al., 2002) in S-27 strain. Recently, several isolates from the recent epidemics of Reunion (2005–2006) and India (2006) have been sequenced (Schuffenecker et al., 2006; Arankalle et al., 2007).

In India, the first outbreak of CHIK was recorded in 1963 in Kolkata (Calcutta) (Neogi et al., 1995; Shah et al., 1964), followed by epidemics in Tamil Nadu, Andhra Pradesh and Maharashtra (Rao, 1966; Rodrigues et al., 1972; Yadav et al., 2003). The last outbreak was recorded in Barsi in 1973 before disappearing from Indian subcontinent (Padbidri and Gnaneswar, 1979; Burke et al., 1985; Pavri, 1986). The Chikungunya reemerged in several Indian states again in 2005. By the end of 2006, it spread to 15 other states including Kerala, before subsiding (Yergolkar et al., 2006; Dash et al., 2007). Suddenly during April–July 2007, an outbreak of febrile arthritis started in Kottayam and Pathanamthitta districts of south Kerala affecting more than 25,000 persons with suspected mortalities. The clinical presentations by the patients during this outbreak were found to be more severe compared to the classical cases reported from 2006 Indian outbreak. There was lot of media speculation on the identity and the cause of this outbreak. We report here the investigation of this unusual outbreak with special emphasis on the molecular characterization of etiology. We also tried to link the association of the more virulent strain in this outbreak through complete genome sequencing of two Indian isolates; one isolated from Kerala in 2007 and the other representative of 2006.

## 2. Materials and methods

### 2.1. Clinical samples

An outbreak of febrile illness with polyarthritis was reported in Kottayam, Kerala, India, during April–July 2007. A total of 57 blood samples from clinically suspected Chikungunya patients were collected from Kottayam district hospital during this period. In addition, 20 viremic blood samples were also collected from Kuruchi Hospital, Kottayam. Two sets of blood samples were collected with and without anticoagulant for virus isolation and serology, respectively. (Informed consent from all the patients and/or their parents (in minors) were obtained, before collection of clinical samples.) The samples were transported to the laboratory under cold condition and stored at −80 °C till further analysis.

### 2.2. Serosurveillance

All serum samples were tested for the presence of chikungunya specific IgM and IgG antibodies using an in-house dipstick ELISA system, following a previously described protocol (Dash et al., 2007).

### 2.3. RNA extraction, RT-PCR and sequencing

Methods for extraction, amplification and sequencing of viral RNA have been described previously (Dash et al., 2007). Briefly, viral RNA was isolated by using a QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions, followed by RT-PCR amplification of RNA was carried out using the Access quick one-step RT-PCR kit (Promega, USA). The 5′ and 3′ ends were amplified using 5′ and 3′ Full RACE kit (Takara, Japan) according to manufacturer's instructions. Amplified fragments were purified from the gel and both strands were sequenced using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA). The nucleotide sequence of the S27 strain (GenBank accession no. AF369024) (Khan et al., 2002) was used for primer designing. Supplementary Table S1 provides list of primers used for PCR/sequencing.

### 2.4. Virus isolation

Isolation of viruses from 10 representative RT-PCR positive samples was attempted in BHK-21 cells following the published protocol (Yamada et al., 2002). Briefly, delta tubes (Nunc, Denmark) containing preformed monolayers of BHK-21 cells were adsorbed with 0.2 ml of plasma samples (diluted 1:10). The inoculum was then replenished with 2 ml of maintenance medium after 2 h post-inoculum. Suitable cell controls were also kept along side. The cells were harvested on appearance of cytopathic effects or on 96 hpi, which ever is earlier. The identification of the virus isolates obtained from the clinical samples was carried out by RT-PCR.

### 2.5. Complete genome sequencing and phylogenetic analyses

The two CHIKV sequenced during the present study includes two isolates representative of 2006 and 2007 outbreaks (DRDE-06 and DRDE-07) in India isolated from Hyderabad, AP and Kottayam, Kerala, respectively. The partial E1 gene of three more CHIKV isolates from the current outbreak are also sequenced. The nucleotide sequences were aligned, edited and analysed using Seqscape v.3 software. The phylogenetic analysis was performed separately based on the available full-coding region and E1 gene (1044 nt) sequences of CHIK viruses using MEGA version 3.1 (Kumar et al., 2004). CLUSTALW version 1.83 (Thompson et al., 1994) was used to perform multiple nucleotide and amino acid sequence alignments. For the construction of phylogenetic trees, the neighbour-joining algorithm and the Kimura two-parameter distance model were utilized. The reliability of the analysis was evaluated by a bootstrap test with 10,000 replications.

## 3. Results

### 3.1. The epidemic

An outbreak of febrile arthritis started in Kottayam and Pathanamthitta districts of south Kerala during April–July 2007, affecting more than 25,000 persons. A total of 77 clinical samples were collected from Kottayam district hospital and Kuruchi hospital. The clinical history revealed that all the patients had suffered from fever with severe arthralgia. There is always abrupt onset of symptoms with a shorter fever (median duration 4 days). Most of the common symptoms include severe arthritis, headache, fatigue, myalgia and rash. Small joints of the hands and feet involving wrist, phalanx, ankle and knee joints are most commonly involved. The symptoms shown by the patients during this outbreak were found to be more severe in comparison to the classical cases of 2006 outbreak including hemorrhages, lymphadenitis and icterus. A local name called 'tomato-fever' has been given to the febrile patients, who developed a tomato type of swellings on the skin. The disease was reported to have occurred in Alappuzha district in December 2006, whereas in 2007 the cases in this district were less and majority of the cases reported this time were from Kottayam and Pathanamthitta. Most of the 2007 cases were found to be new cases affecting more than 25,000 persons with suspected mortalities.

### 3.2. Serology

All 77-serum samples were screened by the dipstick ELISA kit for the presence of IgM and IgG anti-Chikungunya antibodies. The results showed 16 cases (21%) as serologically positive and 61 cases (79%) as negative. Among these antibody positive cases, 8 (10%)

**Table 1**
Amino acid substitutions in isolates of ECSA genotype with respect to MH4-2000 (Yawat) strain

| Region | Polypeptide position | Protein position | MH4-00 (Yawat) | RU-05 (115) | RU-06 (21) | RU-06 (OPY1) | KA15-06 | AP3-06 | MH2-06 | DRDE-06 | DRDE-07 | Italy-07 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nsp1 | 128 | 128 | T | – | – | – | K | K | K | K | K | K |
| | 184 | 184 | M | – | – | – | – | – | – | – | T | – |
| | 230 | 230 | G | – | – | – | – | – | – | – | – | R |
| | 314 | 314 | M | – | – | – | – | – | – | L | L | – |
| | 326 | 326 | M | V | V | V | V | V | V | V | V | V |
| | 376 | 376 | T | – | – | – | M | M | M | M | M | M |
| | 488 | 488 | Q | R | R | R | R | R | R | R | R | R |
| Nsp2 | 589 | 54 | S | N | N | N | N | N | N | N | N | N |
| | 1328 | 793 | A | V | V | V | V | V | V | V | V | V |
| Nsp3 | 1550 | 217 | Y | H | H | H | H | H | H | H | H | H |
| | 1661 | 328 | P | Q | Q | Q | Q | Q | Q | Q | Q | Q |
| | 1670 | 337 | T | I | I | I | I | I | I | I | I | I |
| | 1691 | 358 | P | S | S | S | S | S | S | S | S | S |
| | 1709 | 376 | T | – | – | – | – | – | – | – | – | I |
| | 1768 | 435 | R | C | C | C | C | C | C | C | C | C |
| | 1771 | 438 | A | V | V | V | V | V | V | V | V | V |
| | 1782 | 449 | T | M | M | M | M | M | M | M | M | M |
| | 1794 | 461 | L | P | P | P | P | P | P | P | P | P |
| | 1804 | 471 | P | S | S | S | S | S | S | S | S | S |
| Nsp4 | 1938 | 75 | T | A | A | A | A | A | A | A | A | A |
| | 2117 | 254 | T | A | A | A | A | A | A | A | A | A |
| Capsid | 23 | 23 | P | – | – | – | S | S | S | S | S | S |
| | 27 | 27 | V | – | – | – | I | – | I | I | – | I |
| E2 | 487 | 162 | V | A | A | A | A | A | A | A | A | A |
| | 536 | 211 | I | T | T | T | T | T | T | T | T | T |
| | 637 | 312 | T | M | M | M | M | M | M | M | M | M |
| | 643 | 318 | M | V | V | V | V | V | V | V | V | V |
| | 700 | 375 | S | T | T | T | T | T | T | T | T | T |
| | 702 | 377 | V | I | I | I | I | I | I | I | I | I |
| | 711 | 386 | V | A | A | A | A | A | A | A | A | A |
| 6K | 756 | 8 | V | I | I | I | I | I | I | I | I | I |
| E1 | 813 | 14 | V | – | – | – | – | – | – | – | A | – |
| | 828 | 19 | I | V | V | V | V | V | V | V | V | V |
| | 1020 | 211 | K | N | – | – | – | – | – | – | – | – |
| | 1035 | 226 | A | – | V | V | – | – | – | – | V | V |
| | 1093 | 284 | D | E | E | E | E | E | E | E | E | E |
| | 1186 | 377 | T | A | A | A | A | A | A | A | A | A |

were found positive for IgM, 4 (5%) for IgG and 4 (5%) had both IgM and IgG antibodies.

### 3.3. RT-PCR

A total of 33 (43%) serum samples were found positive for the presence of CHIKV specific RNA, through demonstration of CHIKV E1 gene specific 500 bp amplicons on agarose gel.

### 3.4. Virus isolation

A total of 10 representative plasma samples were processed for virus isolation, which yielded 10 isolates. The isolates revealed discernable cytopathic effects characterized by appearance of foci of rounded, swollen, refractile and increasing granular cells at 24 hpi, followed by aggregation of enlarged and vacuolated cells. There was foamy degeneration and death of cells resulting in detachment of cell monolayer from the surface after 3–4th dpi. The mock infected BHK-21 cells remained healthy, with no changes in the cellular morphology.

### 3.5. Full genome sequence

We determined the full-genome sequences of two CHIKV isolates representative of 2006 and 2007 outbreaks of India in 25 overlapping fragments. The lengths of genomic RNA of both Indian CHIKV isolates are found to be 11,774 nt; with 5′ and 3′ NTR of 76 nt; and 495 nt.

### 3.6. Sequence comparisons

Description of CHIKV isolates from diverse geographical origin used in this study are given in the Supplementary Table S2. The nucleotide alignment of complete ORF of all 14 strains analysed in this study are provided in Supplementary Fig. S1. DRDE-06, DRDE-07 and all the Indian 2006 isolates were found very closely related (99.9% identity). The DRDE-06 and DRDE-07 share 99.8% identity with all the Reunion island isolates. The DRDE-07 share 99.8% identity with Italy-07 isolate. The DRDE-06, DRDE-07 isolates differed from the S27 isolate by 2.8% and Yawat-2000 isolate by 1.8% and 1.9%, respectively.

### 3.7. Sequence analysis

#### 3.7.1. Non-structural region

The DRDE-06, DRDE-07 and other 2006 Indian and Reunion isolates in this region exhibited 99.85 ± 0.06% identity at the amino acid level. The detailed amino acid substitutions in isolates belonging to RU-IND clade of ECSA genotype with respect to MH 4-2000 (Yawat) strain are given in Table 1. In comparison with the Yawat strain, 15 identical substitutions were present in DRDE-06, DRDE-07, India-06, Italy-07 and Reunion (RU) isolates: M326V, Q488R, S589N, A1328V, Y1550H, P1661Q, T1670I, P1691S, R1768C, A1771V, T1782M, L1794P, P1804S, T1938A and T2117A (Table 1). The two unique substitutions that were reported in all the Indian-06 isolates responsible for genetic divergence of Indian viruses were also present in both DRDE-06 and DRDE-07 and Italy-07 isolates. The DRDE-06 and DRDE-07 isolates

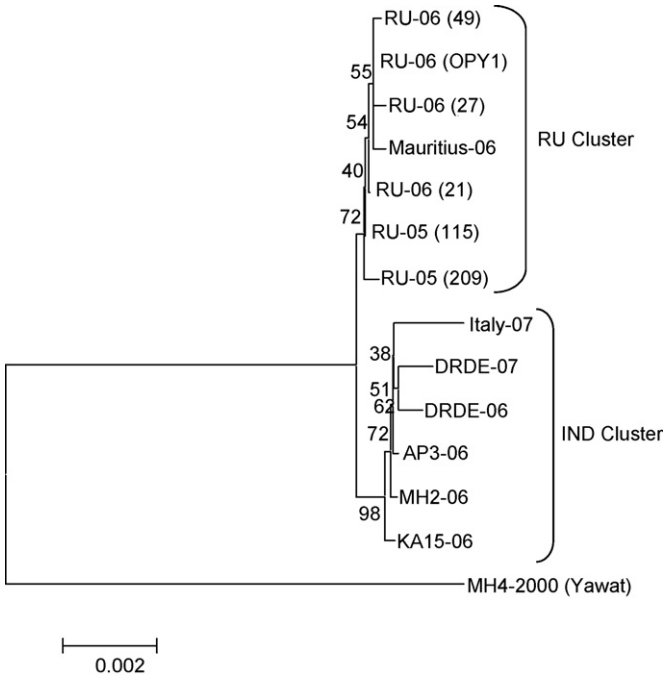


**Fig. 1.** Phylogenetic tree among Chikungunya viruses generated by neighbour-joining method based on the nucleotide sequence of Complete ORFof 14 isolates belonging to ECSA genotype. Numbers at nodes indicate bootstrap support (%). Each strain is abbreviated with virus strain ID and the details of the strain is as given in Supplementary Table S2.

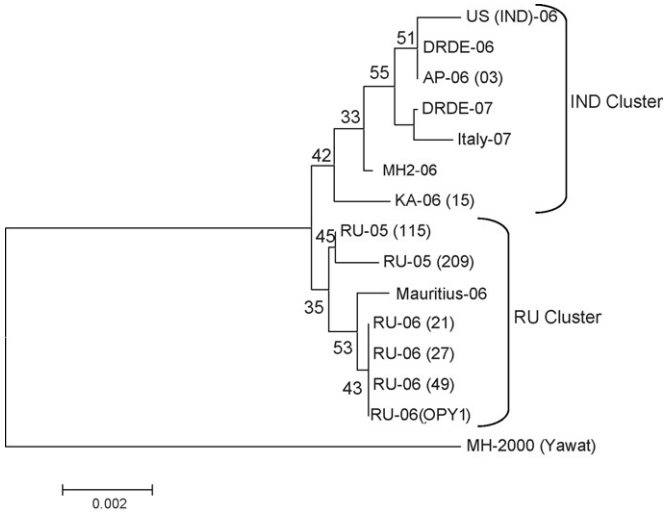


**Fig. 2.** Phylogenetic tree among Chikungunya viruses generated by neighbour-joining method based on the nucleotide sequence of Partial E1 gene of 15 isolates belonging to ECSA genotype. Numbers at nodes indicate bootstrap support (%). Each strain is abbreviated with virus strain ID and the details of the strain is as given in Supplementary Table S2.

exhibited one unique substitution (M314L) in Nsp1 region. The Kerala isolate DRDE-07 exhibited one additional unique substitution (M184T) in Nsp1 region. The Italy-07 isolate exhibited two unique substitutions (G230R and T1709I) in Nsp1 and Nsp3 regions, respectively.

#### 3.7.2. Structural region

Compared to Yawat strain, the DRDE, IND-06, Italy-07 and RU isolates shared 11 substitutions in the structural region: seven in E2 (V487A, I536T, T637M, M643V, S700T, V702I, V711A) one in 6K (V756I) and three in E1 protein (I828V, D1093E, T1186A). Between the two unique mutations reported in the capsid region of India-06 isolates the P23S is present in DRDE-06, DRDE-07 and Italy-07 isolates, whereas V27I is present only in the DRDE-06 and Italy-07 isolates. The most interesting part of the result is that DRDE-07 exhibited two unique substitutions (E1:V14A and A226V) in comparison to DRDE-06 and all the India-06 isolates in the E1 protein. The partial E1 region of the viral genome from three more isolates from the Kerala-2007 outbreak also revealed the same E1 A226V shift (data not shown).

### 3.8. Phylogenetic analyses

Fig. 1 depicts the phylogenetic tree based on full genome analysis. Two DRDE isolates (DRDE-06 and DRDE-07), Italy-07 isolate, three other Indian isolates from the 2005 to 2006 (KA15-06, MH2-06 and AP3-06) are clustered together into a close branch (IND subclade), whereas all Reunion isolates of 2005–2006, Mauritius-06 isolate were clustered together into RU subclade of ECSA genotype. Similar results were obtained when the structural and non-structural regions were analysed separately (data not shown). As E1 gene sequences were available for additional isolates and also because of its importance in phylogenetic analysis a separate phylogenetic tree was constructed (Fig. 2). It revealed that the DRDE isolates (DRDE-06 and DRDE-07), the Italy-07 isolate, US (IND)-06 isolate and three IND-06 isolates are grouped together into IND subclade of ECSA genotype. However, all the RU isolates including the Mauritius-06 were clustered into the RU subclade of ECSA genotype. Within the IND subclade, a state wise segregation can also be observed as the DRDE-06, an isolate from AP segregated along with another AP isolate (AP3-06) and a US isolate of Indian traveler from AP [US(IND)-06], whereas, the DRDE-07 and Italy-07 clustered together. Among RU subclade, there is a segregation of 2005 and 2006 isolates.

### 3.9. 5′ and 3′ NTRs

The 5′ NTR of both isolates were found to be of 76 nt and were completely conserved. The predicted secondary structures were also found to be similar. The 3′ NTR sequenced through sequence specific primer revealed 495 nt; and showed maximum divergence (10.1–17.4% between different genotypes). Within the 3′ NTR, deletion of a stretch of 10 nt (between positions 11377 and 11378) was observed in all the ECSA genotype in comparison to 1963 and 1973 Indian isolates of Asian genotype. A stretch of 19 'A' nucleotides, a possible I-poly(A) site at 3′ NTR was reported in S27 (Khan et al., 2002). Deletion of a stretch of 14 of the 19 'A' nucleotides was also reported in RU and 2006 Indian isolates (Schuffenecker et al., 2006; Arankalle et al., 2007). The present study also revealed same deletion of stretch of 14 of 19 'Á' nucleotides in both the DRDE isolates.

## 4. Discussion

Although the re-emergence of CHIKV in the form of epidemic in Indonesia was noted during the 2001–2003 after a gap of 20 years in Asia, the shift in genotype was not reported due to lack of sequence-based phylogenetic analysis (Laras et al., 2005). The clear shift in genotype from Asian to African was reported in India after a gap of 32 years during 2006 epidemic (Yergolkar et al., 2006; Dash et al., 2007). These same strains (ECSA genotype) were also attributed to the unprecedented epidemic in several Indian Ocean island nations including Reunion during 2005–2006 (Schuffenecker et al., 2006).

In India the disease spread to most part of the country including northern Kerala (Alappuzah district) by December 2006. The disease subsided for a brief period before emerging in southern Kerala (Kottayam and Pathanamthitta districts) in March 2007 and continued up to August 2007.

The present study revealed less seropositivity which may be attributed to the collection of samples at very early or acute stage of the illness. The genomic detection by RT-PCR results in good amount (43%) of CHIKV positivity. The success rate for virus isolation from the selected RT-PCR positive samples in BHK 21 cells was very high. This has been attributed to the very high viral load of the acute phase patients as reported earlier (Parola et al., 2006; Parida et al., 2007; Santhosh et al., 2007).

The four unique amino acid substitutions, two from non-structural (Nsp1: T128K and T376M) and two from structural proteins (Capsid: P23S and V27I) that were reported in all the Indian-2006 and 2007 isolates may be responsible for genetic divergence of Indian viruses as shown in phylogenetic analysis. We also confirm the presence of an opal stop codon at position 1857 in both DRDE-06 and DRDE-07 isolates, as reported between RU and 2006 Indian isolates. The deletion of 14 of the 19 'A' nucleotide in the I-poly (A) site in both DRDE-06 and DRDE-07 isolates is similar to RU and 2006 Indian isolates. However, the predicted secondary structure of the both 3′ and 5′ NTR did not reveal any significant changes.

During this outbreak, patients also reported non-classical symptoms including hemorrhage, lymphadenitis, ictures and liver involvement, etc. Few suspected fatalities were also reported, similar to the cases in Reunion 2005–2006 (Schuffenecker et al., 2006). These types of unusual chikungunya cases were not reported in India during 2006, leading to speculation about the involvement of a more virulent CHIKV in this 2007 epidemic of Kerala. Earlier during the Reunion outbreak, the E1-A226V shift was noticed from the isolates collected after September 2005, which led to increased rate of CHIKV transmission and also reports of more severe non-classical symptoms. However, this shift was not noticed among any of the Indian isolates reported so far. The sequencing of CHIKV strains from Kerala 2007 led to identification of this shift for the first time among Indian isolates. This shift may be attributed to the large-scale outbreak, in Kerala in 2007. The Kerala 2007 isolates also revealed many additional mutations across its genome (Nsp1: M184T in non-structural and E1:V14A, E1:A226V in the structural protein), which makes Kerala isolates unique in comparison to all other 2006 Indian isolates. However, the exact time point of this shift could not be ascertained, which can be taken up by including range of isolates from different time points.

Recently, the study on mosquitoes (*Aedes albopictus*) using both wild (E1: 226A) and mutant (E1: 226V) viruses revealed that the mutant strain is better adaptable and possess higher epidemic potential with increased transmissibility. The E1: A226V mutation in CHIKV results in increased fitness in *A. albopictus* mosquitoes with respect to midgut infectivity, dissemination to the salivary glands and transmission to vertebrate species (Konstantin et al., 2007). The higher prevalence of *A. albopictus* in Kerala, compared to *Aedes aegypti* in other parts of India may be one of the important factor for the unusual spread of the epidemic. It will also be important to increase surveillance in other southern states of India with higher prevalence of *A. albopictus* where there is a scope for evolution of mutant strains. The correlation of E1: A226V shift in India to the increase in outbreak size and rapid spread in Kerala further supports the Schuffenecker hypothesis (Schuffenecker et al., 2006). The rapid spread and larger outbreak may attribute to higher viral load in *A. albopictus.*

All the 2006 and 2007 Indian isolates share more than 99.8% sequence identity with Indian Ocean island isolates at full-genome level, implies circulation of the similar strains. However, exact origin of CHIK epidemic in India could not be resolved due to identification of an ECSA genotype strain from a mosquito captured in 2000 in India. The possibility of recombination between ECSA genotype with Asian/West African genotype was also earlier ruled out (Schuffenecker et al., 2006; Arankalle et al., 2007). A recent hypothesis suggests that Indian strains (IND cluster) and Reunion strains (RU cluster) were evolved independently from a yet to identified common ancestor belonging to ECSA genotype (Brisse and Iteman, 2007). The exact identity of this ancestor may be traced through isolation and sequencing of CHIKV from suspected samples from 2000 to 2006 in Africa and India. However, most probably, the origin of Kerala strains may be due to local evolution from earlier 2006 CHIKV strains, as documented in Reunion. The sharing of E1: A226V and the phylogenetic analysis in this study further proved the evolution of Italy isolate from the Kerala isolates (Rezza et al., 2007).

Overall, the comparison of full genome of two Indian CHIKV isolates from different clinical presentation revealed many mutations. An attempt to link all these unique molecular features associated with more virulence, higher evolutionary potential from the analysed isolates of unusual outbreak suggest possible clues for the unclassical and higher epidemic potential nature of the current outbreak. Further studies to find out the time of shift, and to characterize transmissibility and virulence of various isolates at molecular level is needed.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2008.02.004.

## References

Arankalle, V.A., Shrivastava, S., Cherian, S., Rashmi, S., Gunjikar, A.M., Walimbe, S.M., Jadhav, A.B., Sudeep, Mishra, A.C., 2007. Genetic divergence of Chikungunya viruses in India (1963–2006) with special reference to the 2005–2006 explosive epidemic. J. Gen. Virol. 88, 1967–1976.

Brisse, S., Iteman, I., 2007. Chikungunya outbreaks. N. Engl. J. Med. 356 (25), 2650–2651.

Burke, D.S., Nisalak, A., Nimmannitya, S., 1985. Disappearance of Chikungunya virus from Bangkok. Trans. R. Soc. Trop. Med. Hyg. 79, 419–420.

Dash, P.K., Parida, M.M., Santhosh, S.R., Verma, S.K., Tripathi, N.K., Ambuj, S.P., Gupta, N., Chaudhary, M., Pradeep Babu, J., Lakshmi, V., Mamidi, N., Subhalaxmi, M.V.S., Lakshmana Rao, P.V., Sekhar, K., 2007. East Central South African genotype as the causative agent in reemergence of Chikungunya outbreak in India. Vector Borne Zoonotic Dis. 7, 519–528.

Johnston, R.E., Peters, C.J., 1996. Alphaviruses associated primarily with fever and polyarthritis. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fields Virology. Lippincott-Raven Publishers, Philadelphia, pp. 843–898.

Jupp, P.G., McIntosh, B.M., 1988. Chikungunya disease. In: Monath, T.P. (Ed.), The Arboviruses: Epidemiology and Ecology. CRC Press, Boca Raton (Florida), pp. 137–157.

Khan, A.H., Morita, K., Parquet Md Mdel, C., Hasebe, F., Mathenge, E.G., Igarashi, A., 2002. Complete nucleotide sequence of Chikungunya virus and evidence for an internal polyadenylation site. J. Gen. Virol. 83, 3075–3084.

Konstantin, A., Tsetsarkin, D.L., Vanlandingham, C.E., McGee, S.H., 2007. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog. 3 (12), e201.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5, 150–163.

Laras, K., Sukri, N.C., Larasati, R.P., Bangs, M.J., Kosim, R., 2005. Tracking the re-emergence of epidemic chikungunya virus in Indonesia. Trans. R. Soc. Trop. Med. Hyg. 99, 128–141.

Neogi, D.K., Bhattacharya, N., Mukherjee, K.K., Chakraborty, M.S., Banerjee, P., Mitra, K., Lahiri, M., Chakravarti, S.K., 1995. Serosurvey of chikungunya antibody in Calcutta metropolis. J. Commun. Dis. 27, 19–22.

Padbidri, V.S., Gnaneswar, T.T., 1979. Epidemiological investigations of chikungunya epidemic at Barsi, Maharashtra state, India. J. Hyg. Epidemiol. Microbiol. Immunol. 23, 445–451.

Parida, M.M., Santhosh, S.R., Dash, P.K., Tripathi, N.K., Lakshmi, V., Mamadi, N., Shrivastav, A., Guptha, N., Saxena, P., Pradeep Babu, J., Lakshmana Rao, P.V., Morita, K., 2007. Rapid and real-time detection of chikungunya virus by reverse transcription loop mediated isothermal amplification assay. J. Clin. Microbiol. 45 (2), 351–357.

Parola, P., Lamballerie, X., Jourdan, J., Rovery, C., Vaillant, V., Minodier, P., Brouqui, P., Fluhault, A., Raoult, D., Charre, R.N., 2006. Novel Chikungunya virus variant in travellers returning from Indian ocean Islands. Emerg. Infect. Dis. 12 (10), 1493–1499.

Pastorino, B., Muyembe-Tamfum, J.J., Bessaud, M., Tock, F., Tolou, H., et al., 2004. Epidemic resurgence of Chikungunya virus in democratic Republic of the Congo: Identification of a new central African strain. J. Med. Virol. 74, 277–282.

Pavri, K.M., 1986. Disappearance of Chikungunya virus from India and South East Asia. Trans. R. Soc. Trop. Med. Hyg. 80, 491.

Porterfield, J.H., 1980. Antigenic characteristics and classification of the Togaviridae. In: Schlesinger, R. (Ed.), The Togaviruses. Academic Press, New York, pp. 13–46.

Powers, A.M., Logue, C., 2007. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. J. Gen. Virol. 88, 2363–2377.

Powers, A.M., Brault, A.C., Tesh, R.B., Weaver, S.C., 2000. Reemergence of chikungunya and o'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships. J. Gen. Virol. 81, 471–479.

Rao, T.R., 1966. Recent epidemics caused by Chikungunya virus in India, 1963–1965. Sci. Cult. 32, 215–220.

Rezza, G., Nicoletti, L., Angelini, R., Romi, R., Finarelli, A.C., Panning, PCordioli, M., Fortuna, C., Boros, S., Magurano, F., Silvi, G., Angelini, P., Dottori, M., Ciufolini, M.G., Majori, G.C., Cassone, A., 2007. Infection with chikungunya virus in Italy: an outbreak in temperate region. Lancet 370, 1840–1846.

Rodrigues, F.M., Patankar, M.R., Banerjee, K., Bhatt, P.N., Goverdhan, M.K., Pavri, K.M., Vittal, M., 1972. Etiology of the 1965 epidemic of febrile illness in Nagpur city, Maharashtra State, India. Bull. World Health Organ. 46, 173–179.

Santhosh, S.R., Parida, M.M., Dash, P.K., Pateriya, A., Pattnaik, B., Pradhan, H.K., Tripathi, N.K., Ambuj, S., Gupta, N., Saxena, P., Lakshmana Rao, P.V., 2007. Development and Evaluation of SYBR Green I based One Step Real time RT-PCR assay for detection and quantification of Chikungunya virus. J. Clin. Virol. 39, 188–193.

Schuffenecker, I., Iteman, I., Michault, A., Murri, S., Frangeul, L., Vaney, M., Lavenir, R., Pardigon, N., Reynes, J.M., Francois, P., Leon, B., Diancourt, L., Michel, S., Duquerroy, S., Guigan, G., Frankiel, M., Brehin, A., Cubito, N., Despres, P., Kunst, F., Rey, F.A., Zeller, H., Brisse, S., 2006. Genome croevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS Med. 3, e263.

Shah, K.V., Gibbs Jr., C.J., Banerjee, G., 1964. Virological investigation of the epidemic of haemorrhagic fever in Calcutta: isolation of three strains of chikungunya virus. Indian J. Med. Res. 52, 676–683.

Strauss, E.G., Strauss, J.H., 1986. Structure and replication of the alphavirus genome. In: Schlesinger, S., Schlesinger, M.J. (Eds.), The Togaviridae and Flaviviridae. Plenum Press, New York, pp. 35–90.

Thompson, J.D., Higgins, D.G., Gibson, T.J., Clustal, W., 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalities and weight matrix choice. Nucl. Acid Res. 22, 4673–4680.

Yadav, P., Shouche, Y.S., Munot, H.P., Mishra, A.C., Mourya, D.T., 2003. Genotyping of Chikungunya virus isolates from India during 1963–2000 by reverse transcription-polymerase chain reaction. Acta Virol. 47, 125–127.

Yamada, K., Takasaki, T., Nawa, M., Kurane, I., 2002. Virus isolation as one of the diagnostic methods for dengue virus infection. J. Clin. Virol. 24, 203–209.

Yergolkar, P.N., Tandale, B.V., Arankalle, V.A., Sathe, P.S., Sudeep, A., Gandhe, S.S., Gokhle, M.D., Jacob, G.P., Hundekar, S.L., Mishra, A.C., 2006. Chikungunya outbreaks caused by African genotype, India. Emerg. Infect. Dis. 12, 1580–1583.