Journal of General Virology (2008), **89**, 1945–1948 DOI 10.1099/vir.0.83628-0

Short Communication

A226V mutation in virus during the 2007 chikungunya outbreak in Kerala, India

N. Pradeep Kumar,[1] Rajan Joseph,[2] T. Kamaraj[2] and P. Jambulingam[1]

[1]Vector Control Research Centre (ICMR), Indira Nagar, Pondicherry, India

[2]Kerala State Institute of Virology and Infectious Disease, Alappuzha, Kerala, India

Correspondence
N. Pradeep Kumar
dr_n_pradeepkumar
@hotmail.com

Received 30 November 2007
Accepted 23 April 2008

Kerala State in India was gripped by a renewed and widespread outbreak of Chikungunya virus (CHIKV) infection during 2007. Here, we report the A226V mutation in the glycoprotein envelope 1 (E1) gene of the virus among isolates collected from the three worst-affected districts of the state during this outbreak. This mutation had already been suggested to be directly responsible for a significant increase in CHIKV infectivity in *Aedes albopictus*. The badly affected districts in Kerala State during 2007 have abundant rubber plantations, which supported prolific breeding of *Ae. albopictus* mosquitoes. The abundance of *Ae. albopictus* in the region and molecular evolution of CHIKV may be contributing factors for the renewed epidemic of chikungunya fever during 2007.

An outbreak of Chikungunya virus infection (CHIKV; 00.073.0.01.007; family *Togaviridae*; genus *Alphavirus* (ICTV, 2006)] occurred during 2006 in 15 states or union territories in India. The official number of suspected cases in the country was 1.39 million (NVBDCP, 2007). The southern state of Kerala (Fig. 1) contributed 70 731 cases during this epidemic; these cases were mostly from Alappuzha district (82.44 %), while sporadic infections were recorded from other districts. Our investigation in Alappuzha revealed that the virus involved in the outbreak was of the East, Central and South African (ECSA) genotype (Kumar *et al.*, 2007).

Since May 2007, a renewed outbreak of fever has occurred in the state, more widespread than the earlier one (NVBDCP, 2008). About 3.6 million fever cases were recorded during 2007 (11.3 % of the total population of the state), compared with about 1.8 million in 2006 and 1.2 million in 2005. These fevers were classified as either viral fever, which would have included chikungunya and dengue fever, or other fevers, such as malaria and leptospirosis (Arogyakeralam, 2008). The Department of Health and Family Welfare of the Government of Kerala reported 24 052 cases as suspected chikungunya fever during 2007, distributed across all 14 administrative districts of the state. The hilly and forested districts Kottayam and Pathanamthitta were the worst affected, respectively contributing 44.33 and 14.37 % of the total cases. These districts are situated immediately to the east of the coastal Alappuzha district (Fig. 1), which was badly affected during 2006. However, Alappuzha district contributed only 7.70 % of the total cases during 2007.

A supplementary table and two supplementary figures are available with the online version of this paper.

Kerala was the worst affected state in India during 2007, with 55.8 % of the suspected chikungunya fever cases in the country, compared with only 5.8 % cases during 2006 (NVBDCP, 2008). We investigated the genetic structure of the virus involved in the renewed outbreak of CHIKV in Kerala to obtain a better understanding of the epidemiology of the disease.

Blood samples were collected from 33 patients belonging to different age groups and both sexes (see Supplementary Table S1 in JGV Online). These patients were sampled from five hospitals: the Medical College Hospital, Alappuzha (1), Taluk Hospital, Cherthala (2), Taluk Hospital, Adoor (3), Taluk Hospital, Vaikom (4) and Santo Hospital, Achinakom (5) (Fig. 1). The scale of the fever outbreak during May–June 2007 was very great, creating a panic in the public. The flow of patients to the hospitals exceeded the inpatient capacity of the hospitals in the districts of Alappuzha, Kottayam and Pathanamthitta. Hence, after clinical diagnosis, they were admitted to hospital and were administered treatment. Immunological diagnosis of cases was not carried out, as these hospitals did not have the required laboratory facilities. Cases that were clinically diagnosed as suspected chikungunya fever and that were identified within 0–4 days of the onset of symptoms were included in the study. The fever history of all these cases included the classical symptoms of chikungunya fever (WHO/SEARO, 2008) such as fever, arthralgia and erythematous rashes. Standard ethical guidelines were followed during the collection of blood samples from patients.

Samples collected in 2 ml RNase-free Eppendorf vials were stored at 4 °C in ice-packs and transported to the Vector Control Research Centre (VCRC), Pondicherry, India,

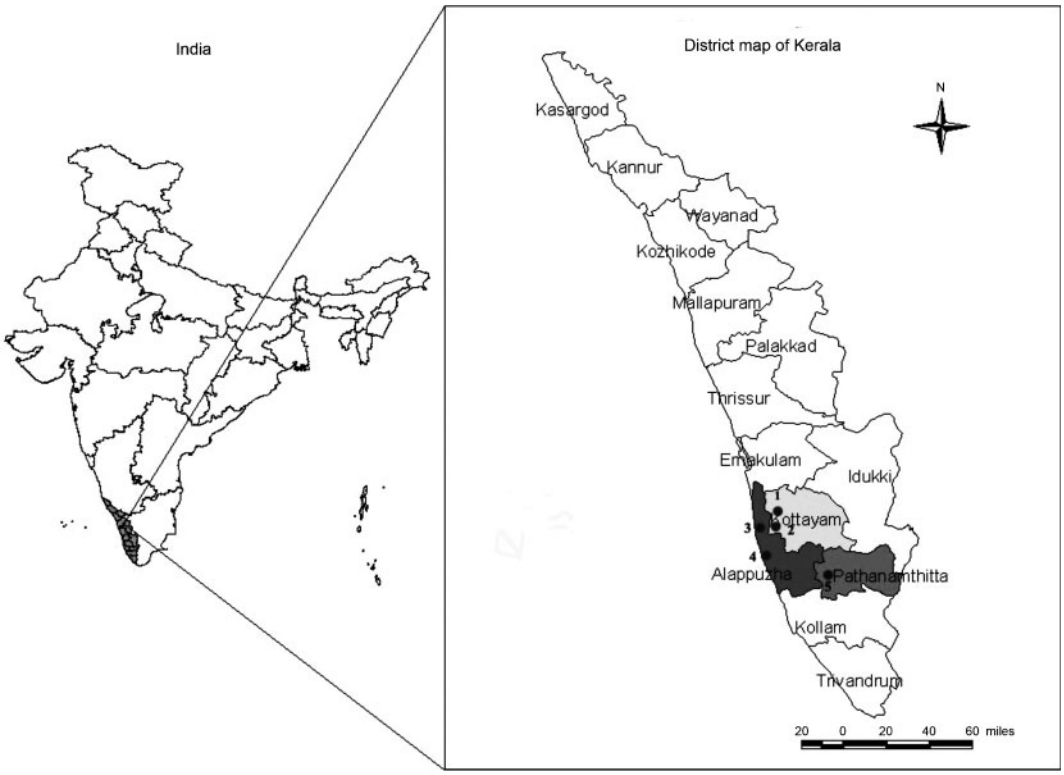

Fig. 1. Map of Kerala State, India, showing the study area and locations of hospitals (indicated by numbers 1–5) from where samples used in the study were collected.

where they were processed further. Serum from these blood samples was separated by centrifugation at 6000 r.p.m. for 15 min at 4 °C. Viral RNA was isolated using the QIAamp Viral RNA Mini kit (Qiagen) following the kit protocol. A negative control was also maintained. The diagnostic non-structural protein 1 (nsP1) gene (Hasebe *et al.*, 2002) was amplified by RT-PCR in order to identify CHIKV infection in the samples, using the Titan One Tube RT-PCR kit (Roche). Reverse transcription was carried out at 50 °C for 30 min, followed by 35 cycles of 50 s of denaturation at 94 °C, 60 s annealing at 54 °C and 90 s extension at 68 °C following a modified protocol of Hasebe *et al.* (2002), according to the manufacturer's instructions (Roche). Reaction products were separated on a 1.0 % agarose gel to resolve any amplified DNA fragments. Of the samples diagnosed positive for CHIKV infection, five viral RNA extractions from each of the three districts were processed further to amplify a large section (about 900 bp) of the phylogenetically informative glycoprotein envelope 1 (E1) gene (Schuffenecker *et al.*, 2006; Kumar *et al.*, 2007). The amplified E1 gene was sequenced and cDNA sequences were aligned using CLUSTAL W software. Phylogenetic analysis was performed using MEGA4 software (Tamura *et al.*, 2007) including E1 sequences of all three genotypes of CHIKV (from GenBank) and those of isolates from the 2006 outbreak in India. The neighbour-joining (NJ) method was followed using Kimura two-parameter (K2P) genetic distances (Kimura, 1980) to deduce the phylogenetic tree. The interior branch test was carried out using 1000 replications to examine the statistical significance of the branching pattern.

Of the 33 serum samples processed, 24 (seven, 12 and five, respectively, from Alappuzha, Pathanamthitta and Kottayam districts) were found to be positive for CHIKV infection (Supplementary Table S1). These yielded DNA fragments corresponding to 354 bp of the nsP1 gene, while no amplification products were observed in negative samples. The size of the amplified E1 DNA fragments of 15 isolates ranged from 891 to 957 bp. The region of E1 that was common to all these fragments (834 bp) corresponded to positions 10264–11097 of the genome of CHIKV strain LR2006_OPY1 (GenBank accession no. DQ443544).

The NJ phylogenetic tree constructed based on sequences of the E1 gene is given in Fig. 2. There was not much genetic variability (K2P genetic distance 0.001) among the 15 isolates obtained in 2007 from different districts of Kerala. The genetic distance between these isolates from Kerala and isolates from the island of Réunion (Schuffenecker *et al.*, 2006) was only 0.003, thus denoting them to be of the ECSA genotype, closely related to CHIKV involved in the Réunion outbreak. However, the Asian and West African (WA) genotypes of CHIKV were clearly

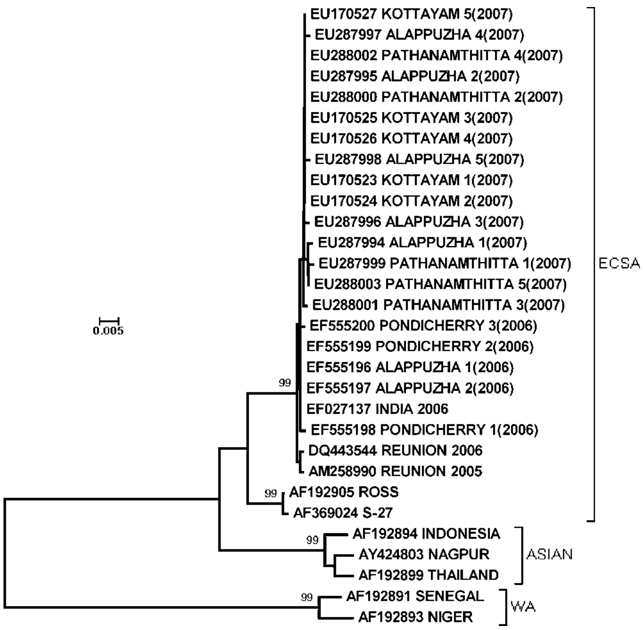

Fig. 2. Phylogenetic analysis of E1 gene sequences of different isolates of CHIKV. The tree was constructed using the neighbour-joining method. Bar, 0.005 substitutions per nucleotide position. GenBank accession numbers are shown for each sequence.

different from the Kerala isolates, with respective genetic distances of 0.058 and 0.171. Comparison of the sequences of the E1 gene showed that all 15 virus isolates from Kerala in 2007 had the non-synonymous mutation C/T at position 10670 of the E1 gene, resulting in a change from alanine to valine at position 226 of the deduced protein sequence (Fig. 3).

This is the first report of the A226V mutation in the E1 gene from CHIKV in India. Another non-synonymous mutation, D284E, recorded during the present investigation among 2007 isolates, was also observed among isolates from various regions of the country during 2006 (Kumar *et al.*, 2007; Arankalle *et al.*, 2007). Mutations A226V and D284E had been reported previously during the renewed circulation of CHIKV on Réunion in 2006, affecting about 30 % of the entire population of the island. During the initial outbreak on Réunion, both of these mutations were absent (Schuffenecker *et al.*, 2006). This study therefore indicates that the virus may be undergoing similar microevolutionary changes in Kerala.

The A226V mutation had been proposed to provide cholesterol-independence to a Semliki Forest virus population (Vashishtha *et al.*, 1998). Schuffenecker *et al.* (2006) proposed that this mutation might provide a selective advantage to the virus in mosquitoes, which are cholesterol auxotrophs. Recently, Vazeille *et al.* (2007) have reported a higher efficiency of replication and dissemination in the vector species *Aedes albopictus* for CHIKV with the A226V mutation, and Tsetsarkin *et al.* (2007) have proved that this mutation is directly responsible for CHIKV adaptation to *Ae. albopictus* mosquitoes.

Entomological investigations carried out during the 2006 outbreak of chikungunya in Kerala recorded high population densities of *Ae. albopictus* in affected areas as well as

```
                                                 221  *                                                         285
AF192905    ROSS                                 LQRPAAGTVH VPYSQAPSGF KYWLKERGAS LQHTAPFGCQ IATNPVRAMN CAVGNMPISI DIPDA
AF369024    S-27                                 .......... .......... .......... .......... .......... .......... .....
DQ443544    REUNION_2006                         .....V.... .......... .......... .......... ........V. .......... ...E.
AM258990    REUNION_2005                         .......... .......... .......... .......... ........V. .......... ...E.
EF027137    INDIA_2006                           .......... .......... .......... .......... ........V. .......... ...E.
EF555196    ALAPPUZHA_1 (2006)                   .......... .......... .......... .......... ........V. .......... ...E.
EF555197    ALAPPUZHA_2 (2006)                   .......... .......... .......... .......... ........V. .......... ...E.
EF555198    PONDICHERRY_1 (2006)                 .......... .......... .......... .......... ........V. .......... ...E.
EF555199    PONDICHERRY_2 (2006)                 .......... .......... .......... .......... ........V. .......... ...E.
EF555200    PONDICHERRY_3 (2006)                 .......... .......... .......... .......... ........V. .......... ...E.
EU170523    KOTTAYAM_1 (2007)                    .....V.... .......... .......... .......... ........V. .......... ...E.
EU170524    KOTTAYAM_2 (2007)                    .....V.... .......... .......... .......... ........V. .......... ...E.
EU170525    KOTTAYAM_3 (2007)                    .....V.... .......... .......... .......... ........V. .......... ...E.
EU170526    KOTTAYAM_4 (2007)                    .....V.... .......... .......... .......... ........V. .......... ...E.
EU170527    KOTTAYAM_5 (2007)                    .....V.... .......... .......... .......... ........V. .......... ...E.
EU287994    ALAPPUZHA_1 (2007)                   .....V.... .......... .......... .......... ........V. .......... ...E.
EU287995    ALAPPUZHA_2 (2007)                   .....V.... .......... .......... .......... ........V. .......... ...E.
EU287996    ALAPPUZHA_3 (2007)                   .....V.... .......... .......... .......... ........V. .......... ...E.
EU287997    ALAPPUZHA_4 (2007)                   .....V.... .......... .......... .......... ........A. .......... ...E.
EU287998    ALAPPUZHA_5 (2007)                   .....V.... .......... .......... .......... ........V. .......... ...E.
EU287999    PATHANAMTHITTA_1 (2007)              .....V.... .......... .......... .......... ........V. .......... ...E.
EU288000    PATHANAMTHITTA_2 (2007)              .....V.... .......... .......... .......... ........V. .......... ...E.
EU288001    PATHANAMTHITTA_3 (2007)              .....V.... .......... .......... .......... ........V. .......... ...E.
EU288002    PATHANAMTHITTA_4 (2007)              .....V.... .......... .......... .......... ........V. .......... ...E.
EU288003    PATHANAMTHITTA_5 (2007)              .....V.... .......... .......... .......... ........V. .......... ...E.
AF192891    SENEGAL                              .......... .......... .......... .......... ........V. .....I.... .....
AF192893    NIGER                                .......... .......... .......... .......... ........V. .....I.... .....
AY424803    NAGPUR                               ....S..... .......... .......... .......... .......... .......... .....
AF192899    THAILAND                             ....S..... .......... .......... .......... .......... .......... .....
AF192894    INDONESIA                            ....S..... .......... .......... .......... .......... .......... .....
```

Fig. 3. Alignment of amino acid sequences deduced from nucleotide sequences of the E1 gene of CHIKV isolates; amino acid positions 221–285 are shown. The position of the A226V mutation is indicated by an asterisk. Sequences are identified by their GenBank accession numbers, followed by the strain name or source of the virus and, in some cases, the year of isolation.

in areas not affected by the disease (WHO, 2006). The worst affected districts in Kerala during the 2007 outbreak, Kottayam and Pathanamthitta, are hilly and heavily forested, with vast rubber plantations [109 582 ha (the largest area in the state) in the former district and 61 016 ha in the latter]. *Ae. albopictus* breeds profusely in rainwater that collects in the hemispherical containers fitted to the trunks of rubber trees for latex collection in the region (National Informatics Centre, Government of India, 2008; NVBDCP, 2008). The role of this mosquito species in the transmission of dengue fever has already been documented (Sumodan, 2003; NVBDCP, 2008). Surveys of immature mosquitoes carried out by the VCRC during June 2007 in the districts of Alappuzha and Kottayam showed that *Ae. albopictus* constituted 85–92 % of the total mosquito larvae/pupae collected. Only 0–3.6 % of the total larvae/pupae belonged to *Aedes aegypti*. In another survey, carried out in Alappuzha, Kottayam and Pathanamthitta districts during July 2007, *Ae. albopictus* was recorded to make up 58–76 % of the larvae/pupae collected, while *Ae. aegypti* represented only 2–17 % (VCRC, unpublished reports). These reports show that *Ae. albopictus* was the predominant mosquito species in the region during the renewed chikungunya outbreak.

It is clear that there was an upsurge in fever cases in Kerala during 2007 compared with previous years (Arogyakeralam, 2008). During 2007 there was a peak of cases of fever at the beginning of the outbreak, in May–June 2007 (Supplementary Fig. S1). A similar trend was recorded in suspected cases of chikungunya fever (Supplementary Fig. S2). It may be presumed that most fever cases went undiagnosed owing to the lack of infrastructure in the state to handle such a large number of fever cases and were grouped as viral fever, which may likely include more chikungunya fever cases than were officially reported.

We conclude that the molecular evolutionary adaptation acquired by CHIKV as a result of the A226V mutation in the E1 gene and the high population density of *Ae. albopictus* in the region may have contributed to the widespread and renewed chikungunya fever epidemic in Kerala during 2007.

Acknowledgements

The investigators are grateful to Dr M. Kalyanasundaram, Officer-in-Charge, Vector Control Research Centre (VCRC), for his encouragement to take up the study. We also thank Mr N. Krishnamoorthy and Mr P. M. Ajithlal, VCRC, for technical assistance.

References

Arankalle, V. A., Shrivastava, S., Cherian, S., Gunjikar, R. S., Walimbe, A. M., Jadhav, S. M., Sudeep, A. B. & 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.

Arogyakeralam (2008). *Prevention & control of emerging fevers in Kerala*. State Disease Control & Monitoring Cell. http://www.arogyakeralam.gov.in/images/nwcg07/peid%20edited%20on%2004-02-08.ppt.pdf

Hasebe, F., Parquet, M. C., Pandey, B. D., Mathenge, E. G. M., Balasubramaniam, V., Saat, Z., Yusop, A., Sinniah, M. & other authors (2002). Combined detection and genotyping of Chikungunya virus by a specific reverse transcription-polymerase chain reaction. *J Med Virol* **67**, 370–374.

ICTV (2006). 00.073. *Togaviridae*. In *ICTVdB – The Universal Virus Database*, version 4. Edited by C. Büchen-Osmond. New York: Columbia University. http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.073.htm

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kumar, N. P., Madhu Mitha, M., Krishnamoorthy, N., Kamaraj, T., Joseph, R. & Jambulingam, P. (2007). Genotyping of virus involved in the 2006 Chikungunya outbreak in South India (Kerala and Puducherry). *Curr Sci* **93**, 1412–1416.

National Informatics Centre, Government of India (2008). *Health Bulletin. Dengue fever*. http://india.gov.in/citizen/health/archive_index.php

NVBDCP (2007). *Chikungunya fever situation in the country during 2006 (prov.)*. New Delhi: National Vector Borne Disease Control Programme. http://www.nvbdcp.gov.in/Chikun-cases.html

NVBDCP (2008). *Dengue/DHF*. New Delhi: National Vector Borne Disease Control Programme. http://www.nvbdcp.gov.in/Dengueupdate.html

Schuffenecker, I., Iteman, I., Michault, A., Murri, S., Frangeul, L., Vaney, M.-C., Lavenir, R., Paradigon, N., Reynes, J.-M. & other authors (2006). Genome microevolution of Chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med* **3**, e263.

Sumodan, P. K. (2003). Potential of rubber plantations as breeding source for *Aedes albopictus* in Kerala, India. *Dengue Bull* **27**, 197–198http://archives.hellis.org/documents/dbull/dbv27p197.pdf

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

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

Vashishtha, M., Phalen, T., Marquardt, M. T., Ryu, J. S., Ng, A. C. & Kielian, M. (1998). A single point mutation controls the cholesterol dependence of Semliki Forest virus entry and exit. *J Cell Biol* **140**, 91–99.

Vazeille, M., Moutailler, S., Coudrier, D., Rousseaux, C., Khun, H., Huerre, M., Thiria, J., Dehecq, J., Fontenille, D. & other authors (2007). Two chikungunya isolates from the outbreak of La Réunion (Indian Ocean) exhibit different patterns of infection in the mosquito, *Aedes albopictus*. *PLoS One* **2**, e1168.

WHO (2006). *Chikungunya in India*. Epidemic and Pandemic Alert and Response, 17 October 2006. Geneva: World Health Organization. http://www.who.int/csr/don/2006_10_17/en/index.html

WHO/SEARO (2008). *Chikungunya fever, a re-emerging disease in Asia*. New Delhi: WHO South-East Asia Regional Office. http://www.searo.who.int/en/Section10/Section2246.htm