Molecular characterization of chikungunya virus isolates from two localized outbreaks during 2014-2019 in Kerala, India

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Abstract

After the 2005-2009 chikungunya epidemic, intermittent outbreaks were reported in many parts of India. The outbreaks were caused by either locally circulating strains or imported viruses. Virus transmission routes can be traced by complete genome sequencing studies. We investigated two outbreaks in 2014 and 2019 in Kerala, India. Chikungunya virus (CHIKV) was isolated from the samples, and whole genomes were sequenced for a 2014 isolate and a 2019 isolate. Phylogenetic analysis revealed that the isolates formed a separate group with a 2019 isolate from Pune, Maharashtra, and belonged to the East/Central/South African (ECSA) genotype, Indian subcontinent sublineage of the Indian Ocean Lineage (IOL). A novel mutation at amino acid position 76 of the E2 gene was observed in the group. The phylogenetic results suggest that the outbreaks might have been caused by a virus that had been circulating in India since 2014. A detailed study is needed to investigate the evolution of CHIKV in India.

Introduction

Chikungunya virus (CHIKV) is an enveloped, single-stranded positive-sense RNA virus that belongs to the family Togaviridae, genus Alphavirus. The CHIKV genome is about 12 kb in length and has two open reading frames (ORF): ORF 1 and ORF 2. ORF 1 encodes the non-structural proteins (nsP) nsP1, nsP2, nsP3, and nsP4, while ORF 2 encodes the five structural proteins E1, E2, E3, C (capsid), and 6K [1]. The genotype of CHIKV is determined by sequencing the E1 gene.

CHIKV has three distinct genotypes: West African (WA), East/Central/South African (ECSA), and Asian [2]. These genotypes represent the independent evolution of the virus in different geographical regions. Evolutionary studies have revealed that the WA genotype originated in Africa and subsequently moved into Asia, where it evolved into the Asian genotype. In 2004, there was a major outbreak in Kenya, and in 2005 the virus spread to Reunion via neighboring islands. Afterwards, the virus spread across several islands in the Indian Ocean. The Indian Ocean lineage (IOL) within the ECSA genotype was responsible for the Reunion outbreak [3].

In Asia, CHIKV was first isolated in Bangkok, Thailand, in 1958. In India, the first outbreak was recorded in Kolkata during 1963. It spread to the states of Tamil Nadu, Andhra Pradesh, and Maharashtra from 1964 to 1965 and disappeared in 1973. These outbreaks in India were caused by the Asian genotype [4]. The virus re-emerged in India in 2006 after a gap of 32 years and caused an explosive outbreak that affected 13 states. The states of Andhra Pradesh, Karnataka, Maharashtra, Madhya Pradesh, Tamil Nadu, Gujarat, and Kerala were the worst-affected states. ECSA was the predominant genotype of the virus in these outbreaks [5]. In 2007, the epidemic spread from India to Sri Lanka, Thailand, Malaysia, and Italy.

In Reunion, another outbreak occurred in the year 2009 that subsequently spread to Europe in 2010. During 2011 and 2012, the circulation of CHIKV was reported in many countries of Central and Western Africa, Oceania, South and Southeast Asia (SEA), Europe, and Western Indian Ocean islands. The Asian genotype continues to circulate in the regions of SEA and the Western Pacific [6]. In 2018, Pyke et al. reported a case of Asian genotype CHIKV imported from the Philippines into Australia in 2016 [7]. The Indian subcontinent sublineage within the IOL of the ECSA
genotype continues to circulate in India and surrounding countries [8].

Chikungunya outbreaks have been recorded in India every year since 2009 (IDSP: www.idsp.nic.in). Two CHIKV outbreaks occurred in two separate locations in the state of Kerala between 2014 and 2019. We carried out epidemiological and virological investigations during these outbreaks.

Molecular characterization of virus genomes is important for studying the molecular epidemiology of the virus. In this study, we observed the accumulation of many synonymous and non-synonymous mutations in both the structural and non-structural genes over a 5-year time period. Some of the mutations observed in the Kerala sequences are known to increase virus fitness [9]. In this communication, we report the results of molecular characterization of CHIKV strains responsible for the outbreaks in Kerala between 2014 and 2019.

**Materials and methods**

**Outbreaks and isolates**

The first outbreak occurred in 2014 in Mattancherry ward, under Cochin Municipal Corporation in the district of Ernakulam. The Indian Council of Medical Research (ICMR)-National Institute of Virology (NIV), Kerala unit, a state-level viral diagnostics and research laboratory, investigated the outbreak. A house-to-house survey was carried out in 11 colonies (n = 305 houses), and blood samples were collected from 197 individuals who had symptoms/signs suggestive of chikungunya at the time of the survey. All specimens were subjected to anti-CHIKV and anti-dengue virus (DENV) IgM ELISA using in-house IgM capture-ELISA kits. The samples (n = 52) collected within 7 days after onset of fever were tested for CHIKV RNA by real-time RT-PCR as described earlier [10]. RNA was extracted from 50 µl of the serum sample using a MagMax Viral RNA Isolation Kit (Ambion, USA) and used in real-time RT-PCR.

Samples that were positive by CHIKV real-time RT-PCR were processed for isolation of CHIKV.

The second outbreak occurred in the Rosapookkandam area of Kumily in the district of Idukki during 2019. Seven localities in the area were surveyed. In each locality, ten houses were visited and a total of 59 samples from suspected cases were collected. All of the samples were tested by anti-CHIKV and anti-DENV IgM ELISA. Three acute samples were processed for CHIKV real-time RT-PCR. Virus isolation was attempted from RT-PCR-positive samples.

**Virus isolation**

Virus isolation was performed in Vero cells. Briefly, 80-90% confluent monolayers of Vero cells in 6-well tissue culture plates were inoculated with 200 µl of 1:1-diluted human patient sera in serum-free DMEM medium. The plate was incubated at 37 °C for an hour with intermittent shaking for virus adsorption. After incubation, 2 ml of DMEM with 2% foetal bovine serum (FBS) was added to each well. The plate was further incubated at 37 °C with 5% CO₂. The plate was observed for 6 to 7 days for the appearance of 70-80% cytopathic effect (CPE). Samples that were negative in the first passage were subjected to two blind passages in Vero cells.

**Genome sequencing**

Primers for sequencing the entire length of the genome were synthesised as described earlier [11]. Four fragments of approximately 2–4 kb were amplified (Table 1). Culture supernatant from virus-infected cells was used as a source of viral RNA. The viral RNA was extracted from 140 µl of culture supernatant using a QIAmp Viral RNA Mini Kit as per the manufacturer’s protocol. The RNA was eluted in nuclease-free water with a volume of 50 µl. Complementary DNA (cDNA) was synthesized, and the fragments were amplified using Taq DNA polymerase (TaKaRa Bio, Japan). The amplified PCR products were gel purified using a NucleoSpin Gel and PCR Clean-Up Kit (MACHEREY-NAGEL, Duren, Germany).

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**Table 1** Details about primers for full-length sequencing of the chikungunya virus genome and PCR conditions

| Fragment | Primer name | Sequence | Product size | Annealing temperature |
|----------|-------------|----------|--------------|-----------------------|
| 1        | 1F          | 5'-GCCGGGCCCAAGAGACACGCTCATCTAGCT-3' | 2.3 kb | 64 °C |
|          | 1R          | 5'-GTGGGATACTTACCATGACCTCTATGTCT-3' |        |        |
| 2        | 2F          | 5'-GCAAGATATGCTCGACCGTACGGTTAGTATG-3' | 2.8 kb | 62 °C |
|          | 2R          | 5'-CTTACCGGCAGAATTCCGATTTCT-3' |        |        |
| 3        | 5065F       | 5'-TGTACAGGAGCGAGGACGAGAAGACAAGAC-3' | 3.0 kb | 50 °C |
|          | 8034R       | 5'-CCGCTTAAAGGCCCATTTTG-3' |        |        |
| 4        | 7910F       | 5'-TCGAAGTCACGACCGAAG-3' | 3.9 kb | 50 °C |
|          | 11770R      | 5'-TTTGCAATTTATGGTATTCA-3' |        |        |
Germany). Sequencing was outsourced to Thermo Fisher Scientific Ltd, India, which uses the ion-torrent next-generation sequencing platform.

**Phylogenetic analysis**

Phylogenetic analysis was performed using MEGA 7.0 software [12]. Full genome sequences (n = 32) available in the GenBank database representing all three genotypes were retrieved for analysis. The 32 sequences were from Kerala and other parts of India as well as neighbouring countries. The sequences obtained from GenBank were aligned using ClustalW in MEGA v 7.0.26 software. A phylogenetic tree was constructed using the maximum-likelihood method implemented in MEGA software. Genetic distances were calculated using the Tamura Nei model of nucleotide substitution. The robustness of the resulting tree was assessed with 1000 bootstrap replicates.

**Results**

Of the 52 samples from the 2014 outbreak tested by real-time RT-PCR, 14 were positive for CHIKV RNA. CHIKV was successfully isolated from 12 samples. In the 2019 outbreak, one of the three acute samples was positive for CHIKV by RT-PCR. The virus was isolated from the PCR-positive sample. One isolate from each outbreak was subjected to sequencing (GenBank accession nos. MW042254 and MW042255).

For analysis of mutations, all sequences were compared to that of the reference virus, the South African strain S27, and synonymous and non-synonymous mutations were identified. Phylogenetic analysis revealed that the Kerala isolates (2014 and 2019) belonged to the ECSA genotype, the IOL lineage, and the Indian subcontinental sublineage (Fig. 1).

With reference to the prototype virus, the 2014 Kerala isolate had 0.72% non-synonymous and 2.4% synonymous mutations, while the 2019 isolate had 0.62% non-synonymous and 2.63% synonymous mutations. The phylogenetic tree showed that both the 2014 and 2019 Kerala isolates clustered together with an isolate from Pune in 2019 and formed a separate group. This group has a common mutation, A76T in E2, which was absent in other viruses analyzed in this study (Supplementary Table S1). In the 2014 Kerala isolate, a novel amino acid shift was observed in the nsP1 protein (A10T). Five non-synonymous mutations were observed in the nsP2 gene, and one each in the nsP3 and nsP4 genes. Two novel amino acid mutations were observed in the E2 protein (Q158R and R395I), and one novel mutation was observed in the capsid protein (N79S) in the 2019 isolate.

In both the 2014 and 2019 isolates, the A226V mutation in the E1 protein was absent. However, the other mutations in the E1 protein were present (M269V, D284E, and K211E). The E2 protein mutations (V264A and I211T) were also present in both isolates (Supplementary Table S1).

**Discussion**

Full genome sequences of virus isolates are indispensable for molecular epidemiology studies. There is a dearth of full genome sequences of recent CHIKV isolates from Kerala. The sequence data from the present study will improve our understanding of CHIKV strains circulating in Kerala with regard to their genetic evolution in different parts of the country. During 2005, CHIKV resurfaced in India and affected many South Indian states. The epidemics continued until 2009. Since then, there have been many clusters of cases in many parts of India. We carried out investigations during two outbreaks that occurred in Kerala State, the first in 2014 and the second in 2019. We isolated the virus from patients affected during both outbreaks, and the whole genome of one representative virus strain each from these two outbreaks was sequenced.

Phylogenetic analysis revealed that the strains that caused these two outbreaks occurring five years apart were closely related. A novel mutation in the E2 gene (A76T) was present in strains isolated from both outbreaks. This mutation is in the region that codes for the top part of the spike in domain A of the E2 protein (amino acids 52-82), which is exposed on the surface of the virion and has been reported to be the point of contact for cellular receptors. This domain has been reported to be more stable and exposed in the mature conformation, which might be beneficial for host-cell binding [13].

The common mutation A226V, which confers the ability of the virus to adapt to Aedes albopictus, was not observed, but two other mutations in the E1 protein (M269V and D284E) were observed. The E1 protein mutations A226V, M269V, and D284E are considered to be a molecular signature of IOL [14]. The CHIKV outbreak occurred in Pune and Kerala at the same time during 2019. The Pune 2019 isolate is very similar to the Kerala 2019 isolate, and they share a common ancestor.

The New Delhi 2010 isolate, which was suspected to be the source of an outbreak in India and neighbouring countries, was not in the cluster formed by the recent isolates [15]. A previous phylogeography study traced the geographical ancestry of the IOL lineage to Kenya around 2004 [16]. A study by Chen et al. revealed that India was the source of a CHIKV strain that caused outbreaks in surrounding countries and that IOL strains have established persistent transmission in Southeast Asian countries, including Thailand [17]. Another Indian study also suggested that the IOL
Fig. 1 Phylogenetic tree highlighting the position of CHIKV Kerala isolates relative to other CHIKV sequences. Sequences were aligned using ClustalW (1000 bootstrap replicates), and phylogenetic inferences were obtained using the maximum-likelihood method in MEGA7. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The scale bar corresponds to 0.02 change per nucleotide.

lineage from India is associated with persistent global transmission [8]. However, the unique mutation in the E2 protein (A76T in the 2014 and 2019 Kerala isolates), which is absent in other strains except the Pune 2019 isolate, indicates that the same virus has been in circulation in Kerala from 2014 to 2019. The resurgence of CHIKV outbreaks in these regions may be attributed to indigenous evolution rather than importations.

To conclude, the present study improves our understanding of CHIKV strains circulating in Kerala, India, from 2014 to 2019 compared to CHIKV strains circulating in other parts of the world. The significance of the A76T mutation in the E2 protein requires further research.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00705-021-05186-9.

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