Rapid detection and characterization of Chikungunya virus by RT-PCR in febrile patients from Kerala, India

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There has been a resurgence and prevalence of fever with symptoms of Chikungunya (CHIK) and increased death toll in Kerala, the southern-most state of India. The objective of this study was to develop a rapid detection method to determine the presence of CHIK virus in the serum samples collected from febrile patients in Kerala, India. Serum specimens were analyzed for CHIK viral RNA by RT-PCR using primers specific for nsP1 and E1 genes. Five out of twenty clinical samples were positive for CHIK virus. The partial sequences of the E1 and nsP1 genes of the strain, IndKL01 were highly similar to the Reunion strains and the recently isolated Indian strains. A novel substitution, A148V, was detected in the E1 gene of the isolate, IndKL02. The detection procedure used in this study was simple, sensitive and rapid (less than 4 hr). This result suggests that CHIK viruses similar to the Reunion strains, which had resulted in high morbidity and mortality rates, may have caused the recent Chikungunya outbreak in India. The effect of the variant, E1-A148V, in the virulence and the rate of transmission of the virus deserves further investigation.

Keywords: Chikungunya, E1, nsP1, Phylogenetic analysis, RT-PCR

Chikungunya (CHIK) virus is an alphavirus (family Togaviridae, genus Alphavirus) causing Chikungunya fever. It is serologically classified as a member of the Semliki Forest antigenic complex. It is a single stranded, positive, RNA–enveloped virus and is transmitted to human beings by various Aedes mosquito species. CHIK virus was first isolated from human serum during an epidemic in Tanzania in 1953. Since then, it has been widely reported in sub-Saharan Africa, tropical Asia including India and the Western Pacific. Recently, Chikungunya has been reported for the first time in Italy and Japan.

CHIK virus is generally considered self-limiting and has been reported as non-fatal in the past. But, recent reports indicated that one-third of the 770,000 people in the Indian Ocean Island of Réunion have been affected by Chikungunya with 237 deaths. The Chikungunya strain found on the Réunion Island has also undergone some mutation and microevolution and therefore better adapted to the mosquito vector.

Chikungunya virus was first reported in India in 1963 in Kolkata. Chikungunya re-emerged in India in December 2005 after a gap of 32 years. Official figures from the Government of India indicated, 1.39 million suspected Chikungunya cases from 152 districts across 10 states in India. The epidemic spread rapidly and affected many communities with an attack rate as high as 40-60%. It has affected a large section of the population of Southern India and some parts of North India.

CHIK virus is responsible for an acute disease of abrupt onset in man, characterized by high fever, arthralgia, myalgia, headache and rash. Haemorrhagic forms of the disease have been reported in a few cases in South–East Asia and India. Polyaarthralgia is the most prominent symptom and may persist for months or years. There have been reports of concurrent isolation of dengue and CHIK virus. The differential diagnosis of these infections is essential for clinical management and epidemiological studies.

Recently, there has been an outbreak of viral infections in Kerala, India. Most of these have been reported as Chikungunya infections based on the clinical symptoms. Although a few cases have been tested ELISA positive for CHIK virus, the virus itself
has not been isolated so far. Isolation of the virus is
the gold standard for the detection of CHIK virus.
This requires propagation of virus from the infected
samples. Although this method is sensitive, it is time
consuming. The aim of this study was to develop a
rapid and sensitive method to detect CHIK virus from
serum samples using RT-PCR so that effective
clinical management of the disease can be done
promptly.

Materials and Methods

Serum samples—A total of 20 clinical samples
from febrile patients were collected from three
different medical centers of Kerala, India. The serum
samples collected were from patients who were
suspected to have Chikungunya infection. Apart from
fever, the patients also exhibited other symptoms,
which included arthralgia, myalgia, headache,
reddishness of eyes and rashes, which are all clinical
symptoms, connected to Chikungunya. In these
samples, clinicians had ruled out other possible causes
of the infection.

Extraction of RNA—The genomic viral RNA was
extracted from 200 μl of patient serum samples by
using the GF-Viral Nucleic acid Extraction Kit
(Vivantis). The RNA was eluted from the spin
columns in a final volume of 30 μl of elution buffer
and was stored at -70 °C until use.

RT-PCR—Nucleic acid amplification was performed
using RNA extracted directly from the patient’s sera.
Two different primer pairs specific for the envelope
glycoprotein E1 (E1) and non-structural protein (nsP1)
genes of CHIK virus were used29. The RT mixture
contained 1mM dNTP, 30 units of RNase inhibitor
(GeNei), 50 pmol of antisense primer, 35 units of
reverse transcriptase (Vivantis) and 10 μl of RNA
sample in a final volume of 25 μl. cDNA synthesis
was carried out at 42°C for 1 hr followed by 95°C for 5
min. The PCR reaction mixture contained 2.5 μl
cDNA, 0.2 mM dNTP, 50 pmol of sense and antisense
primers, 1.25 units of Taq DNA Polymerase (Vivantis)
and 1.5 mM MgCl2 in a final volume of 25 μl. The
mixture was subjected to an initial denaturation at 94°C
for 2 min followed by 35 cycles of PCR (94°C for 30
sec, 54°C for 30 sec and 72°C for 30 sec, for each
cycle) and a 5 min final elongation step.

The amplified cDNA was sequenced using an
automated sequencer ABI310 at Chromous Biotech,
Bangalore. All amplicons were sequenced on both
strands.

Phylogenetic analysis—Phylogenetic analyses were
performed using the 295bp partial E1 sequences
obtained from the samples. A phylogenetic tree
comparing 27 Chikungunya strains isolated from
different endemic regions of the world was generated.
A sequence of O’nyong-nyong virus was included as
an outgroup. Nucleotide evolutionary distances were
calculated using the PHYLIP software package
(version 3.2)30. Phylogenetic tree was generated using
Neighbour Joining Method31, and the distance
analyses were done using the Kimura-2 parameter
formula. Bootstrap analysis was done with 1000
replicates to determine confidence values on the
clades within trees.

Results

RT-PCR detection—Among the 20 samples
collected from Kerala, five were tested positive.
These isolates were named IndKL01, IndKL02,
IndKL03, IndKL04 and IndKL05. Amplification was
observed in all the five samples using nsP1 as well as
E1 specific primers. The product sizes of cDNA
fragments obtained for the nsP1 and E1 genes were
355 base pairs and 295 base pairs respectively
(Fig. 1). Of the 20 samples only two were tested
positive for ELISA at the time of collection. The RT-
PCR detection method could identify 5 Chikungunya
positive samples, of which 3 were ELISA negative.

Molecular analysis of chik virus amplicons—Direct
sequencing of the nsP1 and E1 RT-PCR products of
all the five positive samples were carried out. The
partial nsP1 gene sequences did not show any
differences in the sequence among the 5 samples from
Kerala whereas one of these samples showed
variation in the E1 gene sequence. The partial nsP1
gene of IndKL01 (EU119155) when compared with
33 CHIK viral strains (data not shown) showed 96-
100% similarity with the recently reported Indian
strains. It also showed 99% sequence similarity with
that of strains reported from Reunion islands and less
than 95% similarity with other Asian CHIK viral
genotypes.

The partial nucleotide sequence of E1 gene of
IndKL01 (EU119154) and IndKL02 (EU131893)
were compared. IndKL02 showed a C to T
substitution at 443nt position. Comparison of the
amino acid sequences of IndKL01 and IndKL02
showed that alanine at position 148 in IndKL01 was
substituted with valine in IndKL02. When both these
strains were compared with other CHIK viral
sequences obtained from GenBank, it was seen that
A148V substitution was unique to IndKL02. In all the other reported Chikungunya strains, residue 148 was alanine (Fig. 2). IndKL01 exhibited 100% similarity with other recently reported Indian isolates while IndKL02 showed 99% similarity. The Reunion isolates exhibited 98% similarity with IndKL01 and IndKL02. The isolates from Central and East Africa showed more than 98% similarity with IndKL01 and IndKL02 whereas it showed only less than 95% similarity with other Asian genotypes and the Indian isolates reported in 1963 (EF192901) and 1973 (EF192902).

**Phylogenetic analysis of the chik viral amplicons specific for E1 gene**—In order to determine the phylogroup from which the isolates of the present study emerged, a 295-nt region within the E1 coding sequence (nt positions 253-547 i.e., codons 85–182) of isolates IndKL01 (EU119154) and IndKL02 (EU131893) were compared with the E1 sequences of 25 other CHIK viral strains. The 295bp partial E1 sequences were aligned using ClustalX. Phylogenetic analysis (Fig. 3) divided the CHIK virus isolates into three distinct genotypes, based primarily on geographical origins. The IndKL01 and IndKL02 isolates represent a homogenous clade within a broad group comprising isolates from East, Central and South Africa, Reunion Islands and the recent Indian isolates. Isolates of the present study were very closely related to the Indian strains isolated in 1963 and 1973 (AF192901 & AF192902), but were distantly related to the Asian isolates whereas, the West-African isolates were more divergent and formed a separate clade.

**Discussion**

CHIK virus is causing the largest fever outbreak in the recent times. The re-emergence of Chikungunya in India has been coupled with increased mortality and...
morbidity. Nearly 1.5 million cases were reported. The most likely explanation of this rapid penetration of the virus could be lack of herd immunity in the population, poor vector control systems and perhaps mutations in the virus. E1 sequences have strong phylogeographic structure. There are a few conserved and variable regions identified in CHIK virus, of which nsP1 belongs to the conserved region. Since nsP1 is conserved among the various Chikungunya strains, it is better to use a gene, which is less conserved for phylogenetic analysis. Conventionally, E1 gene has been the choice for phylogenetic analysis. The phylogenetic analysis of the partial E1 nucleotide sequences (253nt–547nt position) demonstrated that IndKL01 and IndKL02 fall into a single phylogroup comprising Indian isolates from recent outbreaks and those from Reunion Islands. These had a higher sequence similarity to East, Central and South African strains while the Indian strains isolated in 1963 and 1972 (GenBank Accession No. AF192901, AF192902) clustered together with the Asian strains forming a distinct clade. Phylogenetic analyses based on the partial E1 polyprotein sequence indicated that most of the CHIK viral strains causing Chikungunya outbreaks in the recent years have evolved from the African strains.

Analysis of the amplified nsP1 and E1 partial sequences showed 98-100% similarity with some recently isolated Indian strains whereas they showed less than 95% similarity to other Asian strains (data not shown). Gene nsP1 is better conserved than E1 among the Chikungunya strains and hence could be used for its diagnosis while, the E1 sequence could be used for the phylogenetic analysis of the isolates.

One of the major reasons attributed to the explosive epidemic in Reunion Islands was the A226V mutation, which offers cholesterol independence to the virus. It was proposed that the alanine to valine substitution at the 226aa position would have enhanced the virulence of the virus. When analyzing the amino acid sequence of strains in the present study, a novel substitution in IndKL02, A148V was detected. The residue 148 was alanine in all the other reported Chikungunya strains. The link between this mutation and the increased virulence and the rate of transmission of the virus deserves further investigation.

In contrast to the numerous species involved in maintenance of CHIK virus infection in Africa, it is opined that Aedes aegypti and Ae. albopictus are the only vector species known to transmit CHIK virus in Asia. These are urban and peridomestic, anthropophilic mosquitoes that maintain close associations with humans. It is, therefore, not surprising that outbreaks of CHIK virus infection are noted more frequently in Asia than in Africa. The epidemiological and clinical features of Chikungunya have a number of similarities to those of dengue virus. CHIK and dengue viruses are transmitted by the same mosquito species in Asia and are prevalent in the tropics and subtropics. There is a possibility of simultaneous outbreaks involving these two viruses. Hence it becomes crucial to differentiate Chikungunya from dengue virus infection for the effective management and treatment of the disease. Due to the lack of a simple and rapid diagnostic method for the identification of these viruses, assessing the epidemic potential and implementing appropriate control measures are often delayed. Conventional assays for CHIK virus detection include virus isolation by inoculating cell cultures and serological testing. Virus isolation is time consuming, expensive and...
requires subsequent diagnostic techniques to identify the virus. Serological testing involves antibody response, which does not usually develop until 4 to 5 days after the infection. However, RT-PCR amplification of the viral RNA provides a sensitive and fast detection of the CHIK viruses. In the present study, an RT-PCR technique was used, which did not require viral culture for RNA extraction. RNA isolated directly from 200 μl of the patient serum was found to be sufficient for the detection of the CHIK virus. The entire procedure took less than 4 hr and did not require any high-precision instruments other than a PCR machine. Among the 20 samples tested only two samples were positive for ELISA at the time of collection. The RT-PCR detection method could identify 5 Chikungunya positive samples, of which 3 were ELISA negative. The result indicated that the RT-PCR was sensitive enough to detect Chikungunya positive samples, which went undetected by ELISA. This method is simple, rapid and relatively less expensive and might be the best suited for the basic clinical settings in developing countries.

The magnitude and intensity of the current Chikungunya outbreak underlines the lack of knowledge and effective control of the CHIK virus. Despite infecting millions, study of the Chikungunya disease has been neglected. Vector control is the only way to limit this infection currently, due to the lack of efficient vaccine or antiviral therapy. From the present results it can be concluded that the recent outbreaks of Chikungunya in Kerala, India have been caused by CHIK viral strains similar to the virulent Reunion strains. The molecular data from the present study will serve as a powerful tool in the rapid detection and characterization of CHIK viral infections in developing countries.

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References


