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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[1]. It is a single stranded, positive, RNA–enveloped virus and is transmitted to humanbeings by various *Aedes* mosquito species[2,3]. CHIK virus was first isolated from human serum during an epidemic in Tanzania in 1953[4]. Since then, it has been widely reported in sub-Saharan Africa, tropical Asia including India and the Western Pacific[5-12]. Recently, *Chikungunya* has been reported for the first time in Italy and Japan[13,14].

Chikungunya 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[15,16]. The *Chikungunya* strain found on the Réunion Island has also undergone some mutation and microevolution and therefore better adapted to the mosquito vector[17].

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CHIK 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[18,19]. The epidemic spread rapidly and affected many communities with an attack rate as high as 40-60%[20]. It has affected a large section of the population of Southern India and some parts of North India[21-23].

CHIK virus is responsible for an acute disease of abrupt onset in man, characterized by high fever, arthralgia, myalgia, headache and rash[24,25]. Haemorrhagic forms of the disease have been reported in a few cases in South–East Asia and India[26,27]. Poly-arthralgia is the most prominent symptom and may persist for months or years. There have been reports of concurrent isolation of dengue and CHIK virus[28]. 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 used[29]. The RT mixture contained 1m*M* 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 $MgCl_2$ 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 Method[31], 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

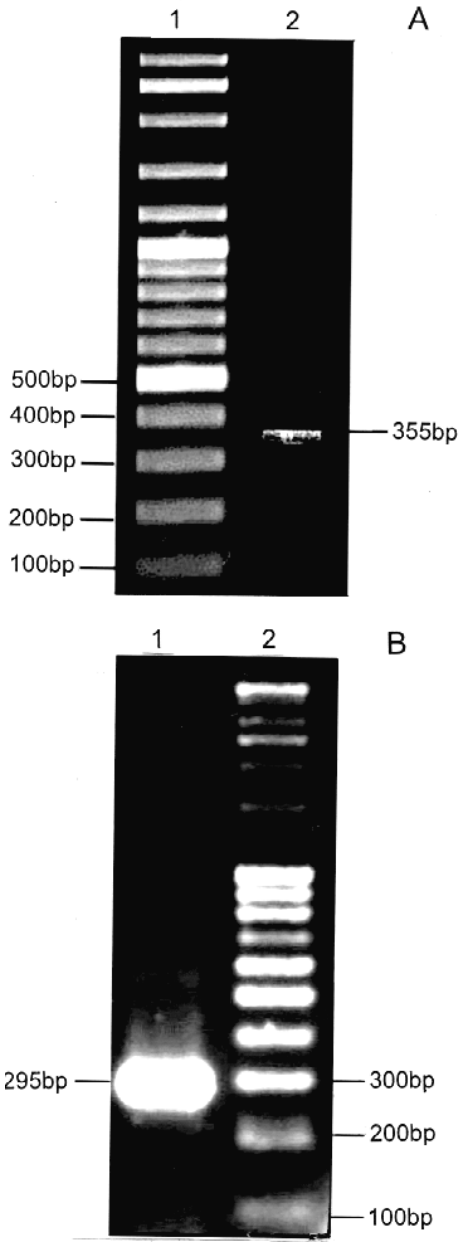

Fig. 1—Ethidium bromide-stained 1.5% agarose gel showing CHIK virus–specific amplification [A: Lane 1-100bp Ladder, Lane 2-Partial nsP1 amplicon (355 bp), B: Lane 1-Partial E1 amplicon (295 bp), Lane 2-100bp ladder]

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%

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AF192902    VLYQGNNVTVSAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192897    VLYQGNNVTVSAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192899    VLYQGNSVTVSAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192898    VLYQGNNVTVSAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192894    VLYQGNNVTVSAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192895    VLYQGNNVTVSAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
EF452494    VLYQGNNVTVSAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
L37661      VLYQGNNVTVSAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192900    VLYQGNNVTVSAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192901    VLYQGNNVTVYAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVNKG
AY549577    VLYQGNNITVTAYANGDHAVTVKDAKFIVGPMSSAWTPFDSKIVVYKG
AF192908    VLYQGNNVTVSAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192896    VLYQGNNVTVSAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192904    VLYQGNNITVTAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AM258991    VLYQGNNITVTAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
DQ443544    VLYQGNNITVTAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
DQ520736    VLYQGNNITVTAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192907    VLYQGNNITVTAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192906    VLYQGNNITVTAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
EF210157    VLYQGNNITVTAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
AF192893    VLYQGNNITVAAYANGDHAVTVKDAKFVVGPMSSAWTPFDNKIVVYKG
AF192892    VLYQGNNITVAAYANGDHAVTVKDAKFVAGPMSSAWTPFDNKIVVYKG
AF192891    VLYQGNNITVAAYANGDHAVTVKDAKFVVGPMSSAWTPFDNKIVVYKG
AF394210    VLYQGNNITVAAYANGDHAVTVKDAKFIVGPMSSAWTPFDN-------
AM397005    VLYQGNNITVAAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
IndKL01     VLYQGNNITVTAYANGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
IndKL02     VLYQGNNITVTAYVNGDHAVTVKDAKFIVGPMSSAWTPFDNKIVVYKG
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Fig. 2—Multiple sequence alignment of E1-protein sequence of various CHIK viral strains [Alignment of the deduced amino acid sequences derived from the partial nucleotide sequence of E1 gene of IndKL01 and Ind KL02 with other CHIK viral strains. IndKL02 has valine whereas all the other CHIK viral strains have alanine at positon 148]

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

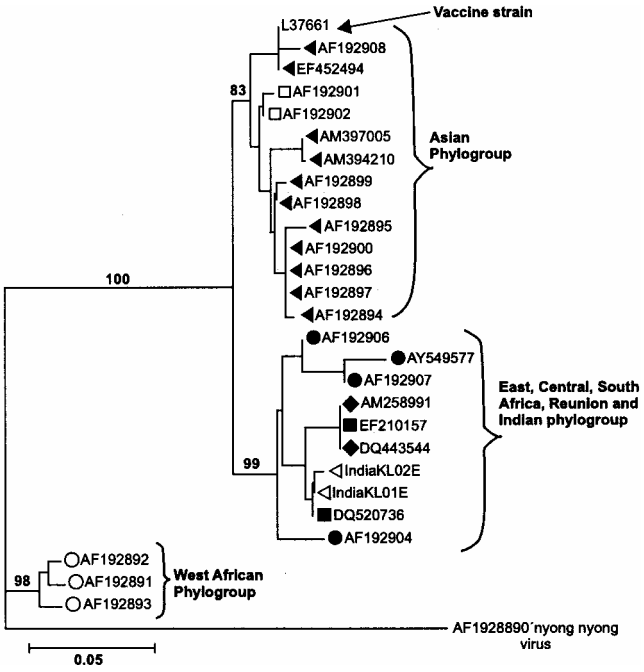

Fig. 3—Phylogenetic relationships among Chikungunya isolates based on partial E1 nucleotide sequences [The tree was constructed for a 295bp E1 partial sequence using PHYLIP. GenBank accession numbers and genotypes are shown. Bootstrap resampling values are indicated at major nodes. ◀Asia; ♦ Reunion; ● East Central and South Africa; □ Indian strains (1963 and1972); ■ Recent Indian strains; O West Africa]

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[11,32]. There are a few conserved and variable regions identified in CHIK virus, of which nsP1 belongs to the conserved region[32]. Since nsP1 is conserved among the various *Chikungunya* strains, it is better to use a gene, which is less conserved for phylogenetic analysis. Conventionally, E1gene has been the choice for phylogenetic analysis[17,22,23,29,32]. 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 1973 (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[32] 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[17]. 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[28,33,34]. 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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