

been considered as an important possible source for such barite vein<sup>18</sup>. The source of Ba and the oxidized fluids that led to precipitation of post-ore barite at Hutti needs further investigation.

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## Molecular characterization of chikungunya virus in febrile patients from central Kerala by RT-PCR assay

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**Chikungunya fever, caused by infection with mosquito-borne alphavirus is an acute nonfatal illness characterized by fever, rash, myalgia, arthralgia and sometimes inflammatory arthritis. During 2007, central Kerala experienced a chikungunya fever epidemic of unprecedented magnitude. There were unusual skin manifestations like peeling of scrotal skin, non-healing ulcer and soft tissue necrosis. cDNA copy of a portion of the viral genome was synthesized in a reverse transcription reaction in the presence of specific primers for the E1 region of the S27 African strain of chikungunya virus (CHIKV) followed by polymerase chain reaction (PCR) amplification. Among 23 patients sampled, nine cases were found CHIKV positive. In PCR confirmed cases, the knees and ankles were the joints commonly affected. Our results suggest that the viraemia could be detected in blood during the first seven days of illness only. Three mismatches observed in the DNA sequence indicate that the virus may be a deviant of the African prototype.**

**Keywords:** Blood samples E1 gene, chikungunya virus, polymerase chain reaction.

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CHIKUNGUNYA fever, an acute arthropod-borne (insect) viral illness reported in many parts of Kerala became epidemic in rubber-growing regions of central Kerala during 2006–07. The causative agent, chikungunya virus (CHIKV), is a single-stranded positive RNA virus and is transmitted from primates to humans generally by *Aedes aegypti*. In India, CHIKV was first reported in 1963 in Kolkata and subsequently the virus was isolated from mosquitoes and patients during the several well-documented outbreaks from 1963 to 1973 (refs 1, 2). The massive outbreak of this disease during 2006 affected more than 1,000,000 persons in Andhra Pradesh, Maharashtra and Karnataka, and this was caused by the African genotype of CHIKV<sup>3,4</sup>. A similar investigation (based on partial sequences of *E2* gene) in Tamil Nadu also confirmed the involvement of the African genotype of CHIKV<sup>2</sup>. But there was no report of the involvement of any specific strain of CHIKV in the epidemic that was prevalent in coastal areas of Kerala in 2006 and in central Kerala during 2007.

CHIKV infection causes a disease syndrome characterized by sudden onset of fatigue, nausea, rash, myalgia, and arthralgia and arthritis. Polyarthralgia is a typical clinical sign and is painful<sup>5</sup>. Symptoms are generally self-limiting and last 1–7 days. However, arthralgia may persist for months or even years. Its association with a fatal haemorrhagic condition was also reported in India<sup>6</sup>. Symptoms closely mimic dengue fever and may cause misdiagnosis. The 2007 outbreak in Kerala had many unusual manifestations like peeling off of scrotal skin, non-healing ulcers, skin and soft tissue necrosis, etc.

Viral isolation and culture by the gold standard method of detection is a time-consuming procedure and needs a specialized laboratory. The IgM antibody ELISA test takes more than a week to diagnose the infection and cannot be used to detect the same during the acute febrile phase. In contrast, reverse transcription polymerase chain reaction (RT-PCR) can detect the virus during early viraemia phase itself. The objective of this study was to confirm if the 2007 outbreak of suspected chikungunya fever in central Kerala was indeed due to CHIKV and to establish a rapid and simple method to detect the virus. We accomplished this objective using RT-PCR technique and the results are discussed here. Simultaneously, dengue PCR was also done to differentiate both dengue fever and chikungunya.

Patients who were presented within 7 days of fever, myalgia, arthralgia or arthritis were included while patients who had upper respiratory symptoms were excluded from the study. In addition, patients suffering from chronic illnesses were also excluded. A total of 23 blood samples (3 ml from each patient) were collected from suspected chikungunya patients from the District Hospital, Kottayam which included two negative controls and two dengue suspected patients. Blood samples were mixed with anticoagulant (EDTA) and then immediately

frozen in liquid nitrogen before transporting to Rubber Research Institute of India for RNA isolation and PCR amplification.

Total RNA from blood samples was isolated using Ambion Blood RNA purification kit. Briefly, blood samples were passed through columns, total RNA was eluted in 100 µl of elution buffer and stored at –80°C for further use. RNA was used for PCR with CHIKV and dengue virus gene-specific primers.

The oligonucleotide primers used for RT-PCR amplification of CHIKV were designed from a 205 bp region in the structural gene (*E1*) and were synthesized by MWG Biotech Pvt Ltd, Bangalore. The nucleotide sequence of the *E1* gene was retrieved from GenBank (accession no. AF369024). The primers were selected based on the criteria described previously by Parida *et al.*<sup>4</sup> and Notomi *et al.*<sup>7</sup>. The primer sequences were: forward 5'-ACGCA-ATTGAGCGAAGCA-3' and reverse 5'-CTGAAGACA-TTGGCC CCAC-3'. Two micrograms of total RNA was used for RT-PCR to obtain first strand cDNA and subsequently the *E1* gene fragment was amplified with specific primers<sup>8</sup>. The amplification was carried out in a 25 µl reaction mixture, which consisted of 2.5 µl buffer mix, 1 µl dNTPs (50 µM), 250 pmol each of forward and reverse primers, 1 µl reverse transcriptase enzyme and 2 µl total RNA by using a Promega Access Quick One-Step RT-PCR kit according to the manufacturers protocol (Promega, USA). The thermal profile of RT-PCR was 48°C for 45 min and 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 52°C for 45 s and 72°C for 1 min and a final extension cycle at 72°C for 10 min. PCR amplified products were analysed on agarose gel for detection of DNA bands using 500 bp ladder as a DNA size marker.

The PCR amplified CHIKV cDNA fragment (approximately 200 bp) was cloned into pCR4-TOPO vector system (Invitrogen) according to the manufacturer's instructions. The recombinant plasmids were used for colony PCR with *E1* gene specific primers as well as T3 and T7 sequencing primers to confirm the presence of the cloned insert. The plasmid with cloned insert was sequenced (Bangalore Genei, Bangalore). Nucleotide sequence was compared with GenBank database using BLASTN or BLASTX program. The partial nucleotide and deduced amino acid sequences of the cloned product were compared with the sequences of CHIKV isolates/strains reported from different geographical regions of Asia, Africa and Europe (Table 1).

Sensitivity was worked out for the group of subjects. In this study, specificity was not worked out as the test will always be negative in the case of a person without disease. The sensitivity was calculated as follows:  $[a/(a + c)] \times 100$ , where *a* is the total number of positives and *c* the total number of negatives.

A one-step, single-tube RT-PCR assay was established for the rapid detection of chikungunya virus targeting the

## RESEARCH COMMUNICATIONS

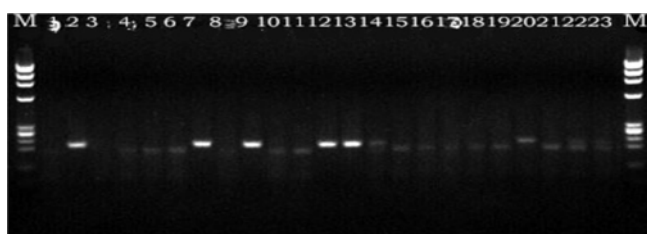
**Table 1.** Details of clinical specimens (blood samples) collected on 16 August 2007 (1–16 samples) and 20 August 2007 (17–23 samples) and processed for this experiment

Lane no.	Sample no.	Age	Gender	Locality/district	Day of collection (since fever first occurred)	PCR result	Remarks
M				DNA marker			
1	1	29	M	Killoor, Kottayam	8th day	Negative	A
2	2	41	F	Muttambalam, Kottayam	3rd day	Positive	B
3	3	40	F	Aymanam, Kottayam	2 weeks	Negative	B
4	4	52	F	Kollad, Kottayam	One month	Negative	B
5	5	42	F	Kanjiram, Kottayam	2 months	Negative	B
6	6	40	F	Kummanam, Kottayam	2 months	Negative	B
7	7	40	M	Muttambalam, Kottayam	2nd day	Positive	B
8	8	32	M	Kummanam, Kottayam	One month	Negative	B
9	9	45	F	Veloor, Kottayam	3rd day	Positive	B
10	10	38	F	Puthuppally, Kottayam	2 months old	Negative	B
11	11	25	M	Paruthikuzhiyil, Kottayam	3 weeks old	Negative	B
12	12	78	M	Puthuppally, Kottayam	2nd day	Positive	B
13	13	42	F	Karapuzha, Kottayam	2nd day	Positive	B
14	14	41	M	District Hospital, Kottayam	3rd day	Positive	B
15	15	41	M	District Hospital, Kottayam	Symptom free	Negative	C
16	16	55	M	District Hospital, Kottayam	Symptom free	Negative	C
17	17	28	M	Thiruvappu, Kottayam	8th day	Negative	A
18	18	53	F	Eerayilkadavul, Kottayam	8th day	Negative	B
19	19	24	F	Thiruvanchoor, Kottayam	3 weeks	Negative	B
20	20	48	F	Channanikadavu, Kottayam	4th day	Positive	B
21	21	44	M	Pallom, Kottayam	One month	Negative	B
22	22	55	F	District Hospital, Kottayam	6th day	Positive	B
23	23	44	M	Palathinkal, Kottayam	7th day	Positive	B
M				DNA marker			

A, Blood samples collected from patients suspected with chikungunya/dengue viral fever symptoms.

B, Blood samples collected from patients suspected with chikungunya viral fever symptoms.

C, Blood samples collected from patients without chikungunya/dengue (negative control) viral fever symptoms.



**Figure 1.** RT-PCR for detection of the chikungunya virus (CHIKV) *E1* gene in blood samples of suspected patients as observed by agarose gel analysis. Lanes: M: Molecular weight marker; 2, 7, 9, 12–14, 20, 22, 23: Presence of CHIKV *E1* gene amplicons and 1, 3–6, 8, 10, 11, 15–19, 21: Absence of CHIKV *E1* gene amplicons.

highly conserved regions of the *E1* gene based on multiple sequence alignments of all the circulating strains. Out of the 23 suspected chikungunya patients tested, nine patients were found positive (sample nos 2, 7, 9, 12–14, 20, 22 and 23) showing 205 bp bands (Figure 1). In addition, a 177 bp faint band was noticed in all samples. All these nine patients were sampled within one week from the onset of the first symptom. These patients also had unusual skin manifestations, skin peeling from scrotum and non-healing ulcers in the post-viraemia phase, which was not reported earlier. The two suspected dengue/

CHIKA patients did not show any PCR positive with either dengue or CHIKA viral infection. As expected, the two negative controls (sample nos 15 and 16) did not amplify, confirming the authenticity of the methodology and accuracy of the results obtained (Table 1). PCR was repeated at least twice in order to confirm the results. The PCR amplified DNA products (205 and 177 bp) were sequenced after cloning into a T/A cloning vector. The sequenced data of 205 bp when verified with the corresponding gene sequences available in the GenBank database by BLAST analysis matched with the *E1* protein of chikungunya virus (Figure 2). The other band (177 bp) showed homology to human 28S ribosomal RNA. This band was amplified due to partial homology at both priming sites. It had a mismatch of one base with S27 African strain (accession no. AF369024) and two bases with the Indian strains reported earlier (accession nos EU727162, EF555200, EF187893). DNA sequences of a few other accessions reported from Indian patients (accession nos EU119154, EF187882, EF187905, EF027137, EF027138, EF027139) also exhibited similar mismatch. Though a difference of up to four bases was noticed between the sequence reported from this study and earlier reported ones at nucleotide level (Figure 2), there was no change in the amino acid sequence. For phylogenetic analysis,

CLUSTAL 2.0.12 multiple sequence alignment

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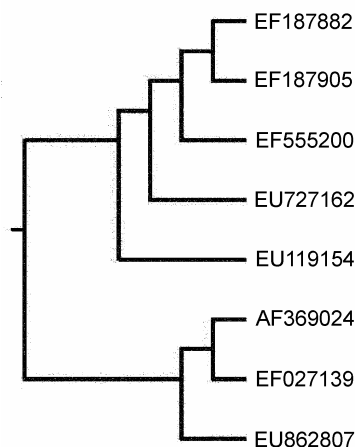
EF187882      TTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAAATTTGCATCAGCATA 60
EF187905      TTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAAATTTGCATCAGCATA 60
EU862807      TTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAAATTTGCATCAGCATA 60
EU119154      TTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAAATTTGCATCAGCATA 60
EU727162      TTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAAATTTGCATCAGCATA 60
EF555200      TTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAAATTTGCATCAGCATA 60
AF369024      TTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAAATTTGCATCAGCATA 60
EF027139      TTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAAATTTGCATCAGCATA 60
*****

EF187882      AGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAACAAACATC 120
EF187905      AGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAACAAACATC 120
EU862807      AGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATTAACATC 120
EU119154      AGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATTAACATC 120
EU727162      AGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATTAACATC 120
EF555200      AGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATTAACATC 120
AF369024      AGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATTAACATC 120
EF027139      AGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATTAACATC 120
*****

EF187882      ACTGTAAGTGCCTATGCAAAACGGCGACCATGCCGTACAGTTAAGGACGCCAAATTCATT 180
EF187905      ACTGTAAGTGCCTATGCAAAACGGCGACCATGCCGTACAGTTAAGGACGCCAAATTCATT 180
EU862807      ACTGTAAGTGCCTATGCAAAACGGCGACCATGCCGTACAGTTAAGGACGCCAAATTCATT 180
EU119154      ACTGTAAGTGCCTATGCAAAACGGCGACCATGCCGTACAGTTAAGGACGCCAAATTCATT 180
EU727162      ACTGTAAGTGCCTATGCAAAACGGCGACCATGCCGTACAGTTAAGGACGCCAAATTCATT 180
EF555200      ACTGTAAGTGCCTATGCAAAACGGCGACCATGCCGTACAGTTAAGGACGCCAAATTCATT 180
AF369024      ACTGTAAGTGCCTATGCAAAACGGCGACCATGCCGTACAGTTAAGGACGCCAAATTCATT 180
EF027139      ACTGTAAGTGCCTATGCAAAACGGCGACCATGCCGTACAGTTAAGGACGCCAAATTCATT 180
*****

EF187882      GTGGGGCCAATGTCTTCAG 199
EF187905      GTGGGGCCAATGTCTTCAG 199
EU862807      GTGGGGCCAATGTCTTCAG 199
EU119154      GTGGGGCCAATGTCTTCAG 199
EU727162      GTGGGGCCAATGTCTTCAG 199
EF555200      GTGGGGCCAATGTCTTCAG 199
AF369024      GTGGGGCCAATGTCTTCAG 199
EF027139      GTGGGGCCAATGTCTTCAG 199
*****
    
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**Figure 2.** Comparison of partial nucleic acid sequences of the chikungunya virus E1 protein gene between EU862807 and other sequences (EF187882, EF187905, EU119154, EU727162, EF555200, AF369024 and EF027139) by the CLUSTALW method. The asterisk denotes conserved sequence of CHIKV E1 gene.



**Figure 3.** Phylogenetic tree based on amino acid sequence, showing the phylogenetic relation between EU862807 and other chikungunya E1 protein sequences (EF187882, EF187905, EU119154, EU727162, EF555200, AF369024 and EF027139). Analysis was performed with the CLUSTALX (neighbour-joining method) using default parameters.

only 199 bases were considered instead of 205 bases as there was a mismatch in the 6th base of the primer annealing site. This analysis clearly indicates that this particular strain (obtained from Kottayam) is closely related to EU119154, EU727162 and EF555200 and a subgroup containing EF187882 and EF187905 altogether forming one group while AF369024 and EF027139 were clubbed in another group (Figure 3).

PCR detection of the chikungunya virus was possible only in the blood samples of the patients (nine) who were in the typical viraemia phase at the time of sample collection, but this was not possible in the blood samples collected one week after the onset of fever. This suggests that PCR-detectable viral particles were present in the blood only for a maximum period of one week (since commencing of fever). But, patients continued to suffer from post-viraemia complications such as arthritic pain, swelling in joints, etc. for much longer periods of months or beyond. In none of the cases investigated here, dengue virus could be detected, although two patients exhibited

clinical symptoms of dengue infection. This could be either because the patient did not have dengue infection or the blood samples did not contain PCR-detectable viral particles at the time of sample collection.

This study demonstrates that RT-PCR is a rapid, reliable and sensitive method to detect the presence of chikungunya virus in blood samples of patients in the first week of infection. The results also indicated that the viraemia stage of this particular virus does not last for more than a week. Therefore, blood sampling from suspected chikungunya patients has to be done within a week from the onset of fever for RT-PCR detection of CHIKV. The DNA sequencing analysis of the partial E1 protein gene amplified from the patients revealed that this particular virus was a deviant of the S27 prototype.

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