

First report of Chikungunya virus circulation during a dengue outbreak in Arunachal Pradesh, a Northeastern state of India

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Received 20 June 2016; received in revised form 5 January 2018; accepted 8 January 2018

Abstract. Dengue has become a major public health problem in Pasighat, Arunachal Pradesh since 2012 outbreak. This region is rich in *Aedes* mosquitoes, the common vector for Dengue and Chikungunya (CHIK), however, active surveillance of CHIK is lacking in this part of India. Therefore, this study has been undertaken to detect the presence of CHIKV infection during 2014 and 2015 dengue outbreak in Arunachal Pradesh. A total of 116 (52 during 2014 and 64 during 2015 outbreak) dengue negative serum samples were collected from General Hospital, Pasighat and were screened for the presence of CHIK IgM antibodies. All the samples were further processed for CHIKV RNA detection by RT-PCR using specific primers. Phylogenetic tree was constructed by Neighbor Joining method using Kimura-2 parameter model. A total of 8 samples were found to be positive for IgM antibodies. RT-PCR showed CHIKV RNA positivity in 2 samples. Both of these samples belonged to 2014 outbreak. Phylogenetic tree revealed that the CHIKV circulating during the outbreak belonged to Eastern, Central and Southern African genotype. Sequence analysis showed two uniform nucleotide substitutions. Circulation of CHIKV masked by dengue is reported from this study. Therefore, CHIKV diagnosis should be made as a routine test in areas where dengue is already endemic so as to prevent misdiagnosis of the disease and to check its accomplishment before commencing an outbreak.

INTRODUCTION

Chikungunya (CHIK) fever is caused by the Chikungunya virus (CHIKV) that belongs to the genus Alphavirus, family Togaviridae (Calisher and Karabatsos, 1988). The virus is transmitted by the *Aedes* mosquitoes specially *Ae. aegypti* and *Ae. albopictus*. The clinical spectrum of CHIKV is similar to Dengue, and often misdiagnosis occurs. The main symptoms include fever, headache, fatigue, nausea, vomiting, rash, myalgia, and severe polyarthralgia. The infection is usually self-limiting, but sometime polyarthralgia may persist for months to years (Dutta *et al.*, 2011; Lakshmi *et al.*, 2008). As the vectors for both dengue and CHIK are

same, in some cases, both the infections occur alongside and concurrent isolation of both viruses from blood of the same patients showing co-infection has been reported (Leroy *et al.*, 2009).

In recent years, regular dengue outbreaks have been occurring in Northeast region of India. The first reported dengue outbreak in Arunachal Pradesh was from Pasighat (2012) when more than 100 cases were reported. Again during 2014- 2015, two outbreaks of dengue strike Pasighat, Arunachal Pradesh. During 2014, only 27 dengue positive cases were detected but in 2015, it became more intense and infected more than 2,000 people of the area. During all these outbreaks, entomological studies

revealed high pre-valence of *Aedes* mosquitoes in the area. On the other hand, there were significant number of patients suffered from dengue like symptoms but were diagnosed as dengue negative. As there was high presence of the common mosquito vector, we undertook this study to determine the presence of CHIKV during the dengue outbreak in this state.

MATERIALS AND METHODS

Study area

During December 2014, a small scale dengue outbreak occurred in Pasighat (28.07°N 95.33°E), East Siang district, Arunachal Pradesh (Figure 1). Again in June-October, 2015, dengue virus activity was found to be circulated in Pasighat. Serum samples were collected from the dengue suspected cases who visited Health Training and Research Centre, General Hospital, Pasighat for diagnosis. After diagnosing dengue, the dengue negative serum samples were included in the study to observe any CHIKV infection during the Dengue outbreak.

Sample collection

After diagnosing dengue, the dengue negative serum samples were included in the study to detect if any active CHIKV circulation was

also occurring during the Dengue outbreak. Out of total 215 samples tested for dengue, 99 were found to be positive for Dengue by NS1 and/or IgM ELISA. The remaining 116 dengue negative cases (all were Dengue NS1 negative) were included in the present study. The Dengue diagnosis was performed by either NS1 or IgM ELISA based on the onset of symptoms. NS1 ELISA was done when the symptoms were for less than 5 days and IgM ELISA when symptoms were for more than 5 days. Clinical symptoms of the patients were recorded at the time of hospitalization. Ethics clearance was obtained from Institutional Ethics Committee of Regional Medical Research Centre, NE Region (ICMR), Dibrugarh. Informed consent was taken from the study participants at the time of sample collection after informing them about performing diagnostic test for Dengue and CHIK.

Serological and molecular diagnosis

All the samples were first screened serologically for presence of Dengue (Figure 2). Subsequently, the dengue negative samples were screened for the presence of CHIK IgM antibodies using CHIK specific IgM Antibody Capture-Enzyme Linked Immunosorbent Assay (MAC-ELISA) (National Institute of Virology, Pune) as per manufacturer's instruction. Following MAC-

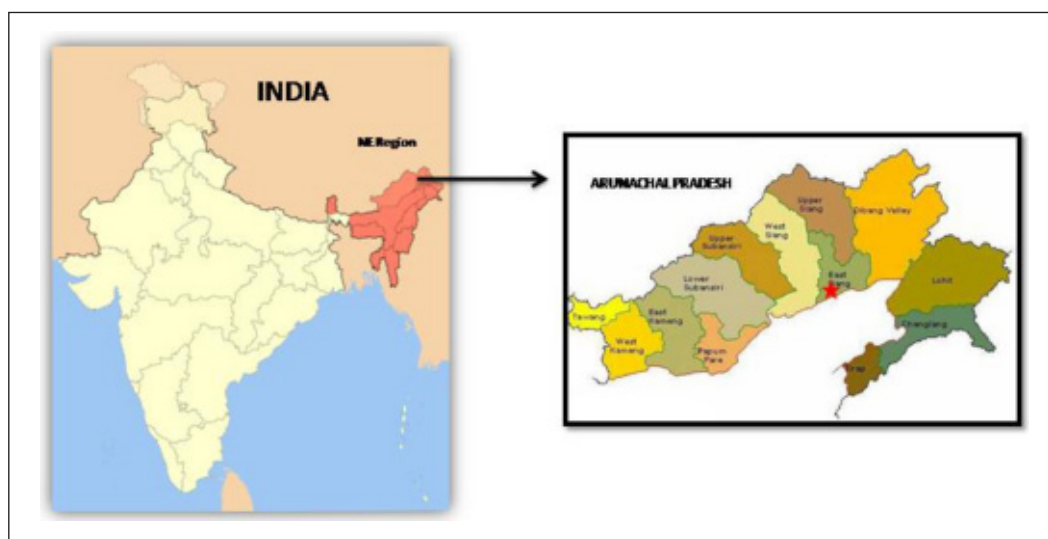


Figure 1. Map of Arunachal Pradesh showing the study area Pasighat (star). The map is not to scale.

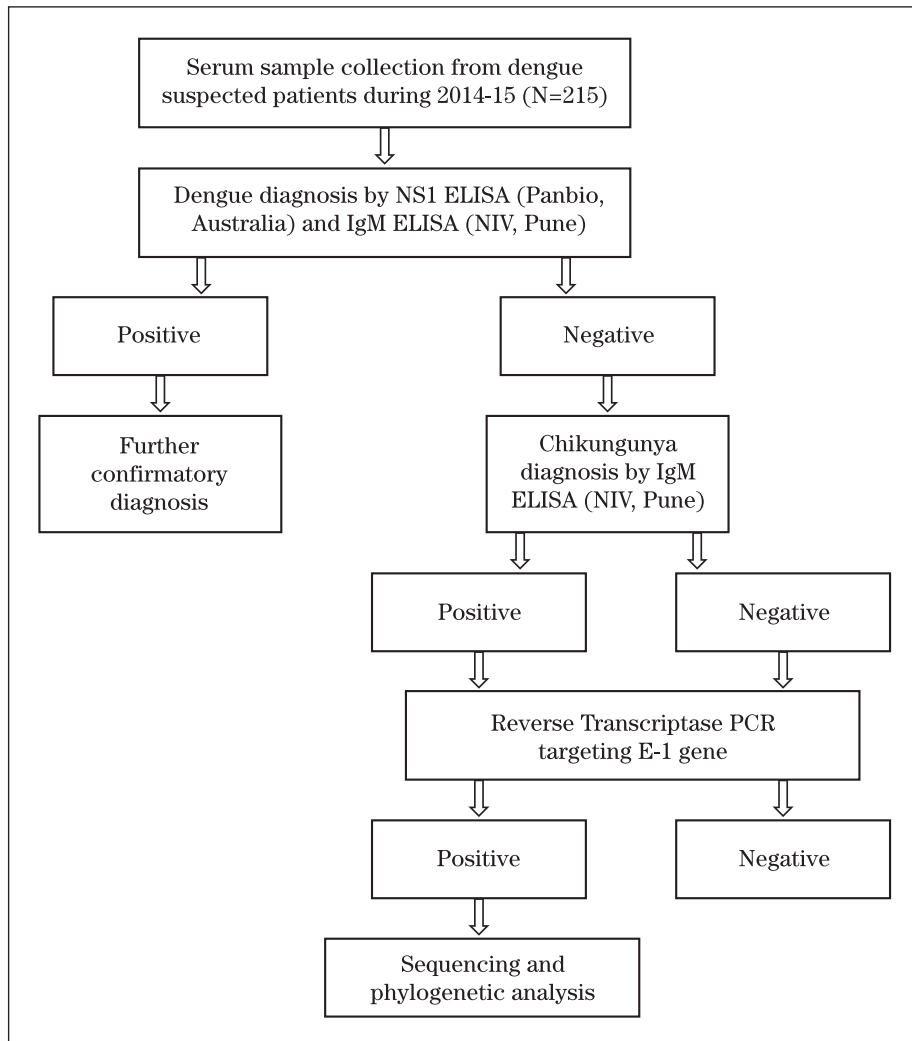


Figure 2. Flow chart of methodology adopted.

ELISA, RNA was extracted from each sample using QIAamp RNA mini kit (QIAGEN, Germany) and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was done using specific primers for CHIKV *Envelope-1 (E-1)* gene as per given protocol (Hasebe *et al.*, 2002) with modifications.

Sequencing and phylogenetic analysis

The resulted amplified product was outsourced to IDT, Malaysia for sequencing analysis using Sanger's method. Post sequencing analysis was done using BioEdit Sequence Alignment Editor Software (BioEdit, California, USA) and the sequences were submitted to GenBank (NCBI) to

obtain accession number. Translation was done by ExPasy translation tool, Bioinformatics Resource Portal (Swiss Institute of Bioinformatics). Phylogenetic tree was constructed by Neighbour Joining method using MEGA version 6 (Tamura *et al.*, 2013). Strains of all the three genotypes, namely East, Central and South African (ECSA), Asian and West African were taken as references.

RESULTS

Among the serum samples processed for CHIKV IgM antibody detection, 8 samples

Table 1. Clinical symptoms of the diagnosed samples

Clinical Symptoms	No. of Positive cases (% positive case), N=9	Total IgM ELISA/PCR negative cases (%), N=107
Fever	9 (100)	107 (100)
Headache	5 (55.6)	46 (43)
Redness in eyes	4 (44.4)	0 (0)
Myalgia	3 (33.3)	22 (20.6)
Arthralgia	2 (22.2)	0 (0)
Abdominal pain	2 (22.2)	10 (9.3)
Vomiting	2 (22.2)	7 (6.5)

were positive for IgM antibody. The year wise IgM positivity rate was 5.8% (3/52) in 2014 and 7.8% (5/64) in 2015. Following RT-PCR, amplification in two samples (band size 294bp) confirms their positivity for CHIKV RNA. Both RT-PCR positive samples were from the 2014 dengue outbreak and one out of these two was also IgM positive. No viral RNA could be amplified from samples collected in 2015 from Pasighat. Based on both IgM ELISA and RT-PCR, nine samples were found to be positive from Arunachal Pradesh, India. Most common symptom observed was fever (100%), followed by headache (55.5%) (Table 1).

Both the amplified samples were sequenced and after obtaining consensus sequence, submitted to NCBI. The accession numbers of the samples are KU216360 and KU216361. The BLAST search was done and the sequences of CHIKV from this region showed proximity to a strain isolated in Calcutta (Now Kolkata) [GenBank accession no.: KJ679577.1] with 99.0% homology. The E-1 gene region of the virus was found to be AG rich with an average percentage of 29.0% for A and 23.7% for G. The sequences of Arunachal Pradesh were compared with 28 other sequences retrieved from GenBank (NCBI) and the phylogenetic tree constructed revealed that the sequences belonged to ECSA genotype (Figure 3).

Comparison of the entire CHIKV partial E1 gene regions sequenced from Arunachal Pradesh during the study with the S27 African prototype revealed nucleotide substitutions (Figure 4). All the substitutions observed

were transition in nature of which two transitions (T→C at positions 10,512 and 10,539) were uniform in all CHIKV sequences of this region.

The translation results showed that this region is rich in amino acid Alanine with an average of 14.9% followed by equal proportion of Valine and Serine (8.5%). When the amino acid sequences of the virus from this region were compared with the prototype CHIKV, no point mutations were observed.

DISCUSSION

It is believed that CHIKV was originated in Africa and only later introduced to Asia (Lahariya & Pradhan, 2006). Severe epidemics of CHIKV infection occurred during 2005-2006, in which Réunion Island, France was affected with around 244,000 cases and further broadening to adjacent islands like Comoros, Mayotte, Madagascar, Mauritius and the Seychelles (Renault *et al.*, 2007). At the same duration i.e. in 2005, CHIKV reemerged in India with considerable outbreaks after a gap of 32 years (Joseph *et al.*, 2008). After this period, severe CHIKV outbreaks have been reported from different parts of India viz. Kolkata (Shah *et al.*, 1964) in 1963, Chennai, Pondicherry and Vellore in 1964, Vishakapatnam, Rajmundry, Kakinada and Nagpur in 1965 and Barsi in 1973 (Kannan *et al.*, 2009).

Northeast India is experiencing regular dengue outbreaks in recent years. In 2008 and 2010, CHIK was first reported from Assam

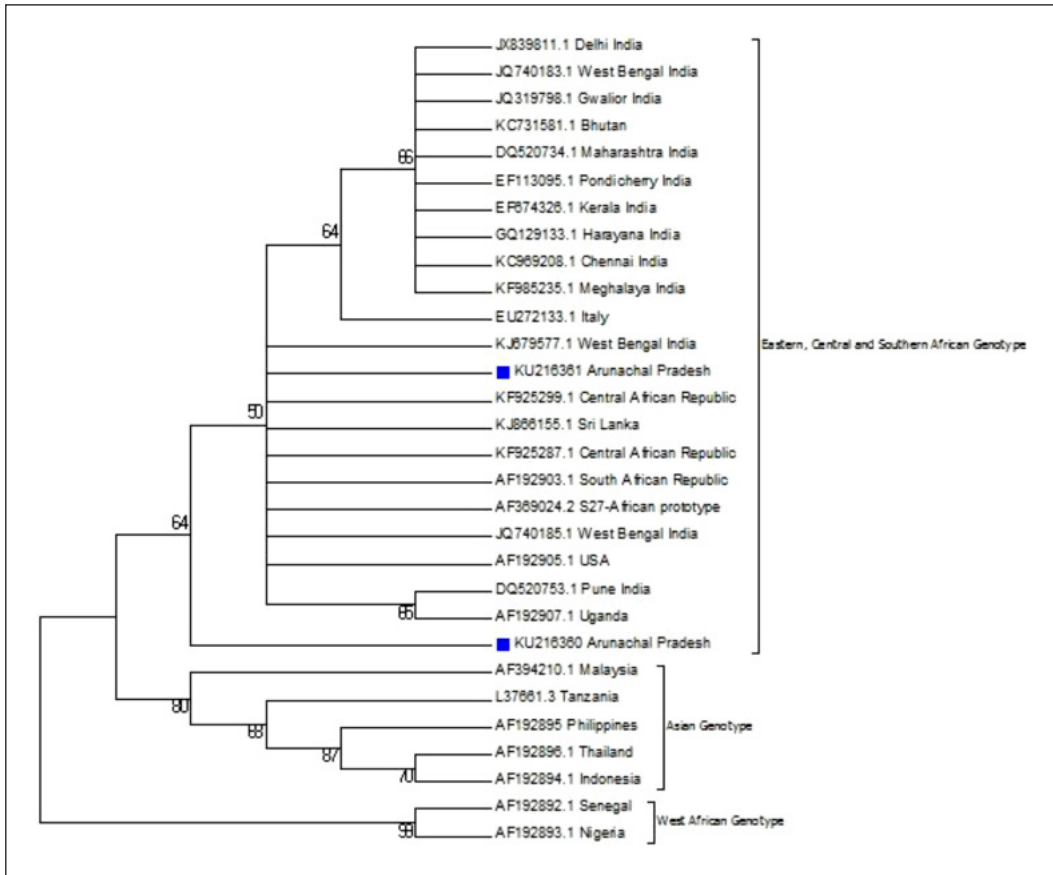


Figure 3. Molecular phylogenetic analyses using the Neighbour Joining method based on the Kimura 2-parameter model. Only bootstrap values of >50% are shown. The sequences from this study are marked by blue squares.

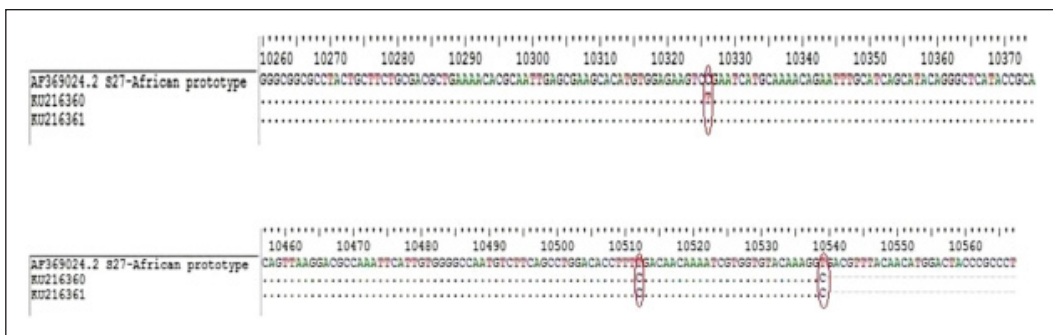


Figure 4. Nucleotide substitutions when compared with S-27 African prototype nucleic acid sequence.

and Meghalaya respectively from this region of India (Dutta *et al.*, 2011; Khan *et al.*, 2015). Both the studies showed the prevalence of IgM antibodies against CHIKV in the suspected patient, however the genotype of

the virus circulated during the period could not be established. The studies also revealed the presence of abundant *Aedes* vector mosquitoes in both of the states (Dutta *et al.*, 2011; Khan *et al.*, 2015).

During 2014-2015, a high number of people suffered due to the dengue outbreaks in Pasighat, Arunachal Pradesh. Here, in this study, we report the circulation of CHIKV alongside dengue during the outbreak. Total 215 samples were collected of which 99 (46.1%) were positive for dengue. This study area of Arunachal Pradesh was found to contain enormous breeding habitat for *Aedes* vector mosquitoes (Khan *et al.*, 2014). As the same mosquito vector species is responsible for transmitting both dengue and CHIK, the chances of circulating CHIKV along with dengue is high. This co-circulation of dengue and CHIK has already been reported by many investigators (Ratsitorahina *et al.*, 2008; Chahar *et al.*, 2009; Leroy *et al.*, 2009). Despite this, the diagnosis of CHIKV is not routinely practiced in this region.

Based on E1 gene sequences of CHIKV isolated in Africa, Asia and islands of the Indian Ocean and in India during outbreaks of 2005-2007, three distinct CHIKV phylogenetic groups West African, Asian and ECSA were reported (Powers *et al.*, 2000; Powers and Logue, 2007; Arankalle *et al.*, 2007). The phylogeny of the sequenced samples from Arunachal Pradesh revealed the presence of ECSA genotype of CHIKV in the region. Studies showed that initially Indian isolates of CHIKV belonged to Asian genotype; but after reemergence of CHIKV in 2005, the genotype that is primarily circulating in India is ECSA (Dash *et al.*, 2007). Apart from this present study, this genotype has also been reported for causing outbreaks in different states of India like Andhra Pradesh, Goa, Karnataka, Madhya Pradesh, Maharashtra etc. (Dash *et al.*, 2007; Kumar *et al.*, 2007). In 2010, this same ECSA genotype of CHIKV caused outbreak in Meghalaya (Khan *et al.*, 2015).

The partial E-1 gene sequences of the two samples from this region were compared with CHIK S-27 prototype sequence of Africa and it showed two uniform nucleotide substitutions in both of the sequences. However, no mutation was found in any of the samples.

The existence of CHIKV along with Dengue has been reported in this study. This raises concern of vector control as the common vector for both the diseases are abundant in the region. Also, Dengue negative samples should be routinely subjected for investigation of CHIK in this region as a public health measures to prevent future outbreak.

Acknowledgments. The authors would like to acknowledge Indian Council of Medical Research, New Delhi for funding the study. Authors also acknowledge State Surveillance Officer- Integrated Disease Surveillance Programme, Itanagar and District Medical Officer, Pasighat, Arunachal Pradesh along with all the health authorities of Pasighat, Arunachal Pradesh for their help and cooperation to carry out the study. In field and laboratory work the immense help and support of Mr. C.K. Sarma, Mr. R.C. Doloi, Mr. Hirok J. Borchetia, Mr. Khageswar Das and Mr. Dipanjal Bhuyan along with the entire staffs of Entomology are highly acknowledged.

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