Original Article

Northward movement of East Central South African genotype of Chikungunya virus causing an epidemic between 2006-2010 in India

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Abstract

Introduction: Re-emergence of chikungunya virus in South India after a gap of 32 years in 2006 affected over a million people in the Indian subcontinent. We kept a close vigil over the emerging trend of this virus between 2006-2010 with a view to establish the identity of the circulating genotype(s) and to determine the route of virus transmission in different parts of India.

Methodology: Nucleotide sequencing of the *E1* gene region from 36 strains of chikungunya virus from three states in northern India was performed for this present study. Forty-four previously reported *E1* sequences, retrieved from the global genome data base were used for making a phylogenetic tree.

Results: BLAST search revealed 99% homology of the northern Indian strains of the 2006-2010 outbreak with the Reunion Island isolates of 2006. Northern Indian strains of this study clustered with the East Central South African (ECSA) genotype.

Conclusions: Findings indicate that the currently circulating strain of chikungunya virus in northern India had its origin from the 2006 epidemic strain of South India that moved toward northern India via the western central India between 2006-2010 in a phased manner with dominance of the ECSA genotype and not the Asian genotype.

Key words: Chikungunya virus; *E1* gene; Sequence; Genotype; India

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Introduction

Chikungunya, a mosquito borne viral disease caused by chikungunya virus, belongs to the family Togaviridae, genus alphavirus, and is primarily transmitted by the *Aedes aegypti* and *Aedes albopictus* mosquitoes. Chikungunya virus (CHIKV) is serologically classified as a member of the Semliki forest antigenic complex [1]. Chikungunya is derived from a word in Makonde, one of the local languages in Tanzania, meaning "that which bends up", which is a reference to the stooped posture that patients develop as a result of the arthritic symptoms of the disease. CHIKV was first isolated from human serum of a febrile patient during a dengue epidemic that occurred in the Newala district, Tanzania, in 1953 [2]. CHIKV infection is notably characterized by fever, fatigue, headache, rash, nausea, vomiting, myalgia and debilitating arthralgia. This is followed by constitutional symptoms that include maculopapular rash on the trunk and limbs. Symptoms are generally self-limiting and can last from one to ten days, although arthralgia or arthritic symptoms may persist for many months afterward [3].

The genome of CHIKV consists of a linear, single-stranded, positive sense RNA of approximately 11.7 kb in length having four nonstructural proteins, capsid region and three structural proteins [4]. CHIKV has three distinct genotypes based on the structural *E1* gene: East Central South African (ECSA), West African and Asian genotypes [5]. The geographic range of the virus is Africa and Asia. The transmission of CHIKV in Asia appears to be maintained in an urban cycle with *Aedes aegypti* mosquito vectors, while in Africa transmission involves a sylvatic cycle with *Aedes furcifer* and *Aedes africanus* mosquitoes [6]. The natural cycle of the virus is human-mosquito-human; however, in Asia and in West Africa the epizootic cycle involves monkeys [7]. In South East Asia, the virus causes large outbreaks and virtually disappears

for long periods, at which time asymptomatic disease may be present [8].

India had its first documented CHIKV outbreak during 1963-1964 in Calcutta, in eastern India, followed by outbreak in southern India (Madras, Pondicherry and Vellore) in 1965 which affected 0.3 million people [9,10,11]. CHIKV circulation appeared to decline during 1965-1972; only sporadic cases were reported from southern India [12,13]. The last CHIKV outbreak in India was reported from Barsi, Maharashtra, in 1973 with a morbidity of 37.5% [14]. CHIKV had almost disappeared from India after 1975 and since then, no case was reported until the end of 2005 [15,16].

CHIKV reemerged after 32 years, in 2005 with unprecedented magnitude in Southeast Asia and the Pacific region. Massive outbreaks have been reported from many islands in the Indian Ocean such as Seychelles, Mayotte, Mauritius, Madagascar, and Reunion [17]. After the quiescence of about three decades, CHIKV reemerged in India causing a large outbreak affecting 213 districts of 15 states/union territories in 2005-2006. Major outbreaks have been reported from South India, with a few sporadic cases reported from Northern India [18]. CHIKV isolates causing epidemics in the 1960s and 1970s in India were of the Asian genotype while the strains of the current outbreaks since 2006 are of the ECSA genotype [19,20,21,22].

CHIKV has been declared a high-priority pathogen by the National Institutes of Health in the United States [23]. The morbidity and disability caused due to chikungunya is enormous. The national burden was 45.26 disability adjusted life years lost per million population as a result of the 2006 epidemic in India. The economic loss caused by the 2006 chikungunya epidemic in India was nearly 391 million INR. About 565.42 million Indian people are at risk of infection [24].

The National Vector Borne Disease Control Programme, India, reported 1.38 million cases in 2006; however, this number declined to 29000 cases in 2007, 95000 in 2008, 73000 in 2009, and 48000 cases in 2010 [18]. CHIKV first reemerged in southern India in the end of 2005. In northern India, confirmed cases of CHIKV infection were first reported in October 2006; thereafter, in 2010, CHIKV infection cases increased significantly [18,25]. The present study was designed to determine the circulating genotype(s) of CHIKV in three northern Indian states, Delhi, Haryana, and Uttar Pradesh (Figure 1).

Methodology

The present study was approved by the Institutional Ethical Committee, National Centre for Disease Control, Delhi, India. A total of 90 acute phase serum samples were collected from suspected clinical cases of CHIKV infection from Delhi, Haryana and Uttar Pradesh, three states of North India, during the post monsoon season (August to November) from 2006 to 2010. Seventeen serum samples were collected in 2006, eighteen in 2007, fifteen in 2008, nine in 2009, and thirty-one in 2010. A specialized disease investigation team from the National Centre for Disease Control frequently visited affected towns, villages and hospitals in different states and collected samples that fit the case definition of the patient having joint pain, rash and fever for less than five days of duration [6]. Informed consent from all patients was obtained before clinical samples were collected.

The standard procedure was followed for isolation of viral RNA from serum samples (140 µl) using a QIAamp Viral RNA Mini kit (Qiagen, Germany). The reverse transcription polymerase chain reaction (RT-PCR) was carried out in a 25µl reaction volume using the access quick one-step RT-PCR kit (Promega, USA) containing PCR master mix, AMV-RT, and respective forward and reverse primers [(E1-F: 5'- TACCCATTCATGTGGGGC-3') (E1-R: 5'- GCCTTTGTACACCACGATT-3')] [26] in a thermal cycler (Applied Biosystems 9700, USA). The thermal profile of the RT-PCR includes- RT step at 42°C for 30 minutes, followed by PCR step of initial denaturation at 95°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 54°C for 60 seconds, extension at 68°C for 90 seconds and final extension at 68°C for 7 minutes. The amplified fragments of 294 bp were visualized after electrophoresis on ethidium bromide stained 1.2% agarose gel.

The PCR products were purified using QIAquick PCR purification kit (Qiagen, Germany). Purified amplicons were subjected to automated nucleotide sequencing for both forward and reverse primers separately. Sequencing was carried out using Big dye terminator cycle sequencing ready reaction kit (Applied Biosystems, USA). Approximately 25 ng of purified PCR product was mixed with 3.2 pmol of respective primer (E1-F/ E1-R) and a reaction mixture containing AmpliTaq DNA polymerase and

Figure 1. Map of India. Regions included in the present study have been shaded.

four dyelabeled di-deoxy nucleotide terminators. The reaction mixture was placed onto a pre-heated thermal cycler (Applied Biosystems 9700, USA). Cycle sequencing parameters included 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The reaction mixture was purified by precipitation with 3M sodium acetate (pH 4.6) and 75 % isopropanol. The purified product was lyophilized, reconstituted in 10µl template suppression reagent, incubated at 95°C for 2 minutes and immediately chilled on ice. The chilled sample was mixed and briefly centrifuged before loading onto an ABI 3130*xl* automated capillary DNA sequencer (Applied Biosystems, USA). After obtaining sequencing data, sequence alignment was done by using Clustal W Multiple Alignment [27]. Thirty six *E1* gene sequences were determined in this study, submitted to GenBank and accession numbers acquired. A BLAST search was carried out to confirm the virus type. For comparison, we retrieved Indian and global chikungunya sequences belonging to diverse geographical locations from the National Center for Biotechnology Information (NCBI) database. The DNAstar software (DNASTAR Inc, USA) was used to examine the percent identity and diversity among the sequences. Phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis (MEGA) software *version 4* and phylogenetic tree was constructed using the neighbor-joining method with a bootstrap value of 1000 replicates [28,29].

Results

In the present study, out of 90 clinical samples, 36 samples were found positive for CHIKV infection by RT-PCR of *E1* gene region. Out of 36 samples, 25 were male and 11 were female of different age groups. Five, 24 and 7 samples belonged to ≥60 years, 18-59 years and <18 years respectively. The most common clinical symptoms were fever (100%), arthralgia (94%), myalgia (31%) and rashes (39%). Details of the CHIKV positive samples of the present study enlisted in Table 1.

E1 gene of 36 positive CHIKV were sequenced and subjected to BLAST search to determine the type

Table 1. Details of Chikungunya patients from Delhi, Haryana and Uttar Pradesh states of India.

PIDN.	Date of Collection	Age [a]	Sex	State	Symptoms	Acc. No.
NI/01	6/10/2006	34	M	Delhi	Fever, arthralgia	JN091130
NI/02	6/10/2006	25	M	Delhi	Fever, arthralgia, rashes	JN091131
NI /03	18/10/2006	37	F	Delhi	Fever, arthralgia, redness in eyes	JN091132
NI /04	18/10/2006	51	M	Delhi	Fever, arthralgia	JN091133
NI /05	17/11/2006	07	M	Delhi	Fever, arthralgia	JN091134
NI /06	17/11/2006	50	F	Delhi	Fever, arthralgia, myalgia	JN091135
NI /07	7/9/2007	04	M	Delhi	Fever, arthralgia, rashes	JN091136
NI /08	7/9/2007	23	F	Delhi	Fever	JN091137
NI /09	8/10/2007	39	M	Delhi	Fever, arthralgia, rashes	JN091138
NI /10	8/10/2007	45	M	Delhi	Fever, arthralgia, myalgia	JN091139
NI /11	12/9/2007	60	M	Haryana	Fever, arthralgia, myalgia, rashes	JN091140
NI /12	23/11/2007	33	F	Haryana	Fever, arthralgia, rashes	JN091141
NI /13	24/8/2007	28	M	Uttar Pradesh	Fever, arthralgia	JN091142
NI /14	19/10/2007	31	M	Uttar Pradesh	Fever, arthralgia, myalgia	JN091143
NI /15	13/11/2007	41	F	Uttar Pradesh	Fever, arthralgia	JN091144
NI /16	29/8/2008	56	M	Delhi	Fever, arthralgia, rashes	JN091145
NI /17	15/9/2008	32	M	Delhi	Fever, arthralgia, rashes	JN091146
NI /18	15/9/2008	42	M	Delhi	Fever, arthralgia, rashes	JN091147
NI /19	10/10/2008	12	F	Delhi	Fever, arthralgia, rashes, myalgia	JN091148
NI /20	24/9/2008	08	M	Haryana	Fever, arthralgia, myalgia	JN091149
NI /21	14/11/2008	45	M	Uttar Pradesh	Fever, arthralgia	JN091150
NI /22	14/9/2009	33	F	Delhi	Fever, arthralgia, rashes, myalgia	JN091151
NI /23	19/10/2009	70	F	Delhi	Fever, arthralgia, myalgia	JN091152
NI /24	29/9/2010	18	M	Delhi	Fever, arthralgia	JN091153
NI /25	4/10/2010	76	M	Delhi	Fever, arthralgia	JN091154
NI /26	4/10/2010	22	M	Delhi	Fever, arthralgia, myalgia	JN091155
NI /27	4/10/2010	44	M	Delhi	Fever, arthralgia, rashes	JN091156
NI /28	25/10/2010	21	F	Delhi	Fever, arthralgia, myalgia	JN091157
NI /29	28/10/2010	16	M	Delhi	Fever, arthralgia, rashes	JN091158
NI /30	28/10/2010	76	F	Delhi	Fever, arthralgia	JN091159
NI /31	12/11/2010	39	M	Delhi	Fever, arthralgia, rashes	JN091160
NI /32	22/11/2010	68	M	Delhi	Fever, arthralgia, redness in eyes	JN091161
NI /33	27/9/2010	43	M	Haryana	Fever	JN091162
NI /34	18/10/2010	30	F	Haryana	Fever, arthralgia, myalgia	JN091163
NI /35	21/9/2010	14	M	Uttar Pradesh	Fever, arthralgia	JN091164
NI /36	31/10/2010	09	M	Uttar Pradesh	Fever, arthralgia, rashes	JN091165

PIDN, Patient identification number; [a] in years; Acc. No., Accession numbers.

of circulating CHIKV, which confirmed the circulation of ECSA genotype. Accession numbers of *E1* gene region sequences of chikungunya virus isolated from three north Indian states are summarized in Table 1. The alignment of study sequences with respect to the S-27 African prototype revealed nucleotide substitution without insertion or deletion leads to synonymous mutation. The sequences of present study shared almost 99% nucleotide identity with referred Indian sequences of 2005-2006 belonging to Kerala, Tamil Nadu, Karnataka, Goa, Andhra Pradesh, Maharashtra, Gujarat and Rajasthan states and Indian Ocean island strains of Reunion, Seychelles and Mauritius. These studied sequences also revealed 99% sequence identity with the S-27 African prototype strain. The deduced amino acid sequences from the study indicated 100% identity among themselves and with the other Indian referred sequences.

A phylogenetic tree was constructed using study sequences and reference sequences (Table 2). The sequences segregated in to 3 genotypes: East Central South African, Asian and West African genotype (Figure 2). The phylogenetic analysis revealed that all studied CHIKV sequences from North India, referred Indian sequences along with 2006 Reunion Island grouped with ECSA genotype. The finding also indicates that a similar strain was circulating throughout India. Chikungunya sequence from S-27 African prototype, Tanzania, South Africa, Uganda and Republic of Congo also belonged to ECSA genotype. However, earlier chikungunya Indian isolates (1963-1973) along with a large number of isolates from Thailand, Philippines and Indonesia formed a separate cluster of Asian genotype. The strains from Senegal and Nigeria represented the West African genotype. O'nyong-nyong virus was used as an out group.

Discussion

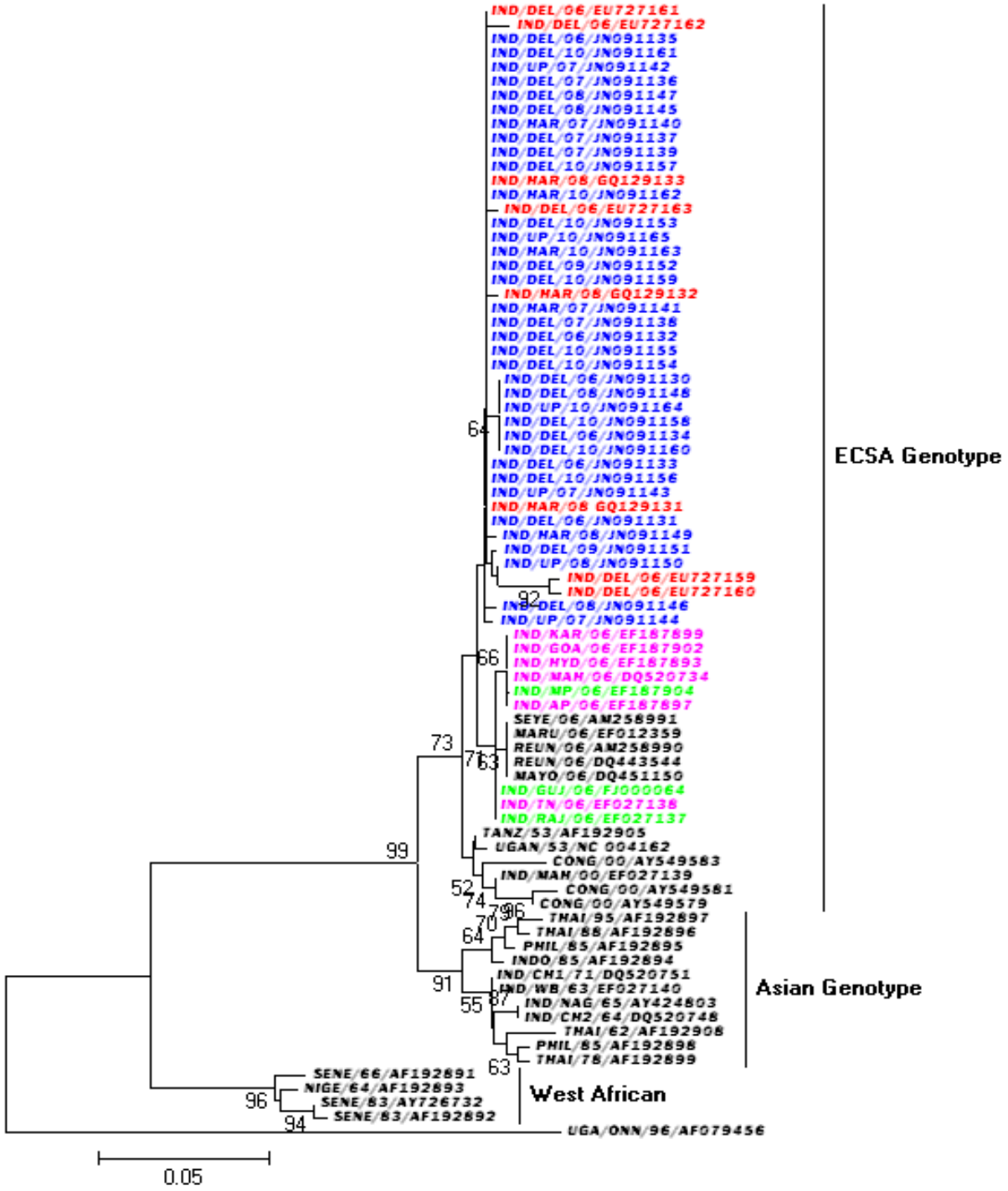
India is endemic for many arboviral infections which emerged in different parts of the world as a result of increased human and mosquito population, urbanization, global changes in human activities and ecology [21]. The actual incidence of the disease may possibly be higher; and may be underreported due to lack of accurate reporting. This may be because most symptoms of chikungunya fever mimic dengue fever and are therefore, misdiagnosed, especially in dengue endemic areas [30]. In 2005, a chikungunya epidemic started in several Indian Ocean islands, specifically Reunion, Mauritius, Madagascar and Seychelles, and subsequently CHIKV infection started in the southern part of India towards the last quarter of 2005 and is still ongoing. In October 2005, it was first reported from Andhra Pradesh and Karnataka [31]. However by early 2006, almost all parts of South India viz. Kerala, Tamil Nadu, Goa, Maharashtra along with Andaman Nicobar Island were gripped with CHIKV infection [20,21,32,33]. Subsequently, Central Western Indian states (Gujarat, Madhya Pradesh and Rajasthan) were affected [6,21]. By the end of 2006, sporadic cases of CHIKV infection along with massive dengue outbreak in Delhi were reported [22,34]. Few cases were also reported from Haryana and Uttar Pradesh [18, 35]. With the view to establish identity of circulating genotype(s) in North India, we kept looking at the emerging trend of CHIKV since 2006, which caused an outbreak of CHIKV during post monsoon period of 2010 in Delhi [25].

In the present study, molecular epidemiology of CHIKV circulating in North India was carried out using sequence comparison and phylogenetic analysis. CHIKV genome has three major structural genes along with four nonstructural genes. Study of literature and Genbank sequence survey revealed that the maximum number of sequences of geographically diverse chikungunya virus is available for *E1* gene. Therefore, to include all three representative genotypes, we have selected this genomic region for phylogenetic analysis. In conclusion, the phylogenetic analysis revealed that the southern, central, western and northern Indian chikungunya virus strains were very closely related to the strains of 2006 Reunion islands, all of them representing the ECSA genotype. This study confirms the presence of ECSA genotype in North India. The earlier CHIKV isolates of 1963-1973 belonged to Asian genotype [19]. The clinical manifestations of CHIKV during the outbreak in 1963-1973 are quite different from the current outbreak (2006-2010). From 1963-1973, the common symptoms were fever, rash, severe and crippling arthritis, lymphadenopathy, gingivitis and bleeding gums [11,36]. However, in 1963 in Calcutta, severe febrile illness sometimes associated with severe haemorrhagic manifestations and occasionally terminating in death was reported [37]. In the current outbreak fever, rashes, arthralgia and severe joint pain involving knees, ankles, wrists, hands and feets have been reported. However, rare manifestations includes meningoencephalitis, lymphadenopathy and bleeding of gums have occasionally been reported. Haemorrhagic manifestations were also relatively very uncommon.

Table 2. Chikungunya sequences used for phylogenetic analysis*

Sequence Number	Lab. ID/ isolate name	Year	State/Country	Genotype	Genbank accession number
1	DEL/968/06	2006	Delhi / India	ECSA	EU727161
2	DEL/1307/06	2006	Delhi / India	ECSA	EU727162
3	HAR-AIIMS/11/08	2008	Haryana / India	ECSA	GQ129133
4	DEL/8668/06	2006	Delhi / India	ECSA	EU727163
5	HAR-AIIMS/09/08	2008	Haryana / India	ECSA	GQ129132
6	HAR-AIIMS/06/08	2008	Haryana / India	ECSA	GQ129131
7	DEL/1795/06	2006	Delhi / India	ECSA	EU727159
8	DEL/758/06	2006	Delhi / India	ECSA	EU727160
9	HYR023	2006	Karnataka / India	ECSA	EF187899
10	GOA018	2006	Goa / India	ECSA	EF187902
11	HYD394	2006	Hyderabad / India	ECSA	EF187893
12	IND06MH1	2006	Maharashtra /India	ECSA	DQ520734
13	GWL008	2006	Madhya Pradesh / India	ECSA	EF187904
14	CHTR54	2006	Andhra Pradesh / India	ECSA	EF187897
15	05-209	2005	Seychelles	ECSA	AM258991
16	D570/06	2006	Mauritius	ECSA	EF012359
17	05-115	2005	Reunion	ECSA	AM258990
18	LR2006-OPY1	2006	Reunion	ECSA	DQ443544
19	MCF2006-OPY4	2006	Mayotte	ECSA	DQ451150
20	IND-GJ51	2006	Gujarat / India	ECSA	FJ000064
21	IND-06-TN1	2006	Tamil Nadu / India	ECSA	EF027138
22	IND-06-RJ1	2006	Rajasthan / India	ECSA	EF027137
23	TAN53	1953	Tanzania	ECSA	AF192905
24	S27AFRICA	1953	Tanzania	ECSA	NC_004162
25	CONGO01	2000	Congo	ECSA	AY549583
26	IND00 MMH4	2000	Maharashtra / India	ECSA	EF027139
27	CONGO02	2000	Congo	ECSA	AY549581
28	CONGO03	2000	Congo	ECSA	AY549579
29	THAI95	1995	Thailand	ASIAN	AF192897
30	THAI88	1988	Thailand	ASIAN	AF192896
31	PHILLIP85	1985	Philippines	ASIAN	AF192895
32	INDON85	1985	Indonesia	ASIAN	AF192894
33	IND71CH1	1971	Tamil Nadu / India	ASIAN	DQ520751
34	IND63WB1	1963	West Bengal / India	ASIAN	EF027140
35	Nagpur653496	1965	Madhya Pradesh / India	ASIAN	AY424503
36	IND64CH2	1964	Tamil Nadu / India	ASIAN	DQ520748
37	THAI62	1962	Thailand	ASIAN	AF192908
38	THAI75	1985	Thailand	ASIAN	AF192898
39	THAI78	1978	Thailand	ASIAN	AF192899
40	SENEG66	1966	Senegal	WEST AFRICAN	AF192891
41	NIGER64	1964	Nigeria	WEST AFRICAN	AF192893
42	SENEG83A	1983	Senegal	WEST AFRICAN	AY726732
43	SENEG83B	1983	Senegal	WEST AFRICAN	AF192892
44	O'NYONG-NYONG	1996	Uganda	OUT GROUP	AF079456

Figure 2. Phylogenetic tree of CHIKV based on partial nucleotide sequence of E1 gene (276 bp).



Each strain is denoted by the name of country of isolation followed by the name of the state (only in case of Indian strains) which, in turn, is followed by the last 2 digits of year of isolation and finally the accession number. Sequences derived from the study are shown in blue color, other north Indian sequences are depicted in red color, central western Indian sequences are shown in green color and South Indian sequences are denoted in pink color.

Since its reemergence in 2006, no deaths primarily due to CHIKV infection have been reported [6].

The main purpose of the present study was to find out the movement of CHIKV strain from southern part of India to northern states via central-western India. The study was limited to a specific and smaller region of *E1* gene of CHIKV. However, if complete *E1* gene is amplified, possibly, more information on mutational changes may be available. Emergence of newer strain of CHIKV with increased transmission potential may be responsible for long term persistence in the community. The findings of this preliminary study on the phylogeny of CHIKV infection in North India warrant a large scale investigation involving more samples, so as to have a thorough understanding of the evolutionary trends of the virus.

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