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Monitoring of dengue and chikungunya viruses in field-caught *Aedes aegypti* (Diptera: Culicidae) in Surat city, India

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A virological surveillance was carried out in various places of Surat Metropolitan Region (SMR) to monitor the chikungunya and dengue activity in *Aedes aegypti* populations. A total of 556 pools constituting 4918 *Ae. aegypti* mosquitoes were collected and processed in the laboratory for the detection of chikungunya virus (CHIKV) and dengue virus (DENV) using Immuno Fluorescence Assay (IFA) and reverse transcription polymerase chain reaction (RT-PCR). Thirteen pools were found positive for DENV and one pool was found positive for CHIKV. Molecular phylogeny results revealed that DENV-3 GIII, DENV-4 GI and CHIKV ECSA are circulating in Surat. Based on GIS information and climate conditions, the risk map for *Ae. aegypti* and DENV infections was predicted. Risk maps suggest that the central and western parts of Surat are at high risk of *Ae. aegypti* spread and associated DENV infection.

Keywords: Arboviruses, GIS mapping, phylogenetic analysis, risk map, surveillance.

DENGUE virus (DENV) and chikungunya virus (CHIKV) cause a major public health challenge in the tropical and subtropical areas of the world. The four antigenically related dengue serotypes (1–4) are responsible for dengue fever (DF) and dengue haemorrhagic fever (DHF)[1]. Recently, DENV-5, phylogenetically distinct from the other four types, was detected in human patient samples from Sarawak state of Malaysia (presence of DENV-5 was announced in the Third International Conference on Dengue and Dengue Haemorrhagic Fever, Bangkok). Yearly, over 100 million cases of dengue infections worldwide are estimated to occur and have become not only a huge health problem, but also a serious economical burden in those areas which are now endemic for dengue infections. CHIKV is endemic in Africa and Southeast Asia[2], and millions of cases have been reported from these areas since 2005. DENV and CHIKV are transmitted by the

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Aedes mosquitoes through an urban transmission cycle. Globalization, industrialization, rapid urbanization, water-storage practices and exponential increase in human and mosquito populations are responsible for the frequent epidemics and outbreaks covered by these viruses across many countries and the continents. Currently no commercial vaccines are available for these arboviral diseases and strict attention is needed to be given for the mosquito vector control programmes. Virological surveillance of dengue and chikungunya in vector mosquito populations will help in the prediction, prevention and control of impending outbreaks. It would act as a radar to trigger the healthcare system to undertake precautionary measures and quick response against disease outbreak.

Dengue is an endemic disease in India, and circulation of all the four dengue serotypes has been reported from urban and semi-urban areas of the country[3–13]. A change in the genotype of DENV-2 and a change in the lineage of DENV-1 have been recently reported from India[4,9,11,13]. DENV-3 genotype III has been reported to cause major outbreaks in various parts of India[4,7,14]. Reemergence of DENV-4 was reported in India and Brazil after three decades[5,12,15,16].

Three distinct CHIKV phylogenetic groups, viz. one containing all the isolates from West Africa, one containing the isolates from Asia, and one corresponding to Eastern, Central and Southern African (ECSA) isolates[17–22] were reported from India. The molecular information about DENV and CHIKV serotypes and genotypes in wild mosquitoes will help in understanding the phylogenetic similarity of these viruses and genotypes during future outbreaks. Therefore, detection of DENV and CHIKV viruses in mosquito populations constitutes an important element as part of an early warning system.

Surat, which is one of the important port cities in India, has a high incidence of CHIKV and DENV infection cases. In Surat, environmental and social factors such as high temperature and humidity during the year, population, water-storage habits, industrialization and trade centre, provide ideal conditions for the proliferation of *Aedes aegypti*. This situation necessitates constant monitoring of DENV and CHIKV in wild mosquito populations in Surat city on urgent basis. The present study describes the virological detection of DENV and CHIKV among the wild-caught *Ae. aegypti* mosquitoes from different parts of Surat city during the period 2009–2013. Detection and serotyping of DENV and CHIKV viruses in *Ae. aegypti* mosquitoes were carried out using immunofluorescence assay (IFA), reverse transcription polymerase chain reaction (RT-PCR) and sequencing. The surveillance data along with climatic data were used for the prediction of *Ae. aegypti* risk map.

We used mice for anti-CHIKV and anti-DENV antibody production. Similarly rabbit and hen were used for rearing the mosquitoes that are used as negative control. All animals were handled in strict accordance with good animal practice as defined by Institutional Animal Ethics Committee (IAEC). All animal work was approved by the IAEC and Institutional Biosafety Committee (IBSC).

Surat Metropolitan Region, Gujarat, India is divided into seven zones, viz. east zone (EZ), central zone (CZ), north zone (NZ), southeast zone (SEZ), southwest zone (SWZ), south zone (SZ) and west zone (WZ). Adult mosquitoes of *Ae. aegypti* were collected randomly during daytime from the households of Surat Metropolitan Region (see Supplementary Information, Table S1 online). Following collection, mosquitoes were anesthetized on ice, identified and pooled by gender, date and place of capture. Pools ranged from 1 to 10 individual mosquitoes. Head of individual mosquitoes was used for IFA and the carcasses of 10 mosquitoes were pooled.

The presence of viral antigen was determined by IFA. The head squashes were prepared on glass slides with dual impression. The slides were immersed in the blocking buffer (0.1% Tween 20 and 2% BSA in PBS) for 1 h at room temperature; the slides were then incubated with mouse anti DENV or CHIKV antibodies respectively, followed by FITC-conjugated goat anti-mouse IgG (Sigma Aldrich, Germany) for 1 h each at 37°C. These slides were then mounted with ProLong Gold anti-fade reagent (Promega, USA) with Evans Blue (Sigma Aldrich, Germany), and visualized under the fluorescent microscope. Mosquito pools containing head squash-positive (CHIKV/DENV) mosquito carcasses were used for RNA isolation and subsequently detection/isolation.

Chikungunya and dengue virus RNA was isolated (a) from CHIKV/DENV-positive pools of field-caught *Ae. aegypti* and (b) CHIKV and DENV infected C6/36 cell culture supernatants, using the QIAAmp Viral RNA mini kit (Qiagen, Germany), according to the manufacturer's recommended procedures.

Detection of DENV in *Ae. aegypti* was performed using RT-PCR, as described by Lanciotti *et al.*[23]. Detection of CHIKV was carried out using the procedure described by Arankalle *et al.*[20] (see Supplementary Information, Table S2 online).

For cDNA synthesis, the viral RNA was converted into cDNA using Goscript cDNA synthesis system. The reaction mixture contained 4 µl of 5× AMV reaction buffer, 3 µl $MgCl_2$ (25 mM), 1 µl of each dNTP (2.5 mM), 20 pmol reverse primer (D2 (ref. 23) and CHIKVR (ref. 20)), 1 µl AMV reverse transcriptase, 20 U RNAase inhibitor and 5 µl extracted RNA was used as a template in 20 µl of reaction volume. Reverse transcription was conducted at 42°C for 60 min, followed by denaturation at 70°C for 15 min.

The cDNA samples 5 µl along with 5 µl of 5× PCR buffer, 3 µl $MgCl_2$ (25 mM), 1 µl dNTP mix, 1 µl platinum *Taq* polymerase, primers (20 pmol each; D1 and D2 for DENV (ref. 23) and CHIKVF and CHIKVR for CHIKV (ref. 20)) were mixed and then incubated at 94°C for 30 sec, 55°C for 1 min, 72°C for 2 min for 35 cycles,

Table 1. Environmental variables used in the analysis

Layer	Description	No. of layers
Altitude	Altitude	1
	Monthly minimum temperature	12
	Monthly mean temperature	12
	Monthly maximum temperature	12
	Monthly precipitation	12
BIO1	Annual mean temperature	1
BIO2	Mean diurnal range (mean of monthly (max. temp. – min. temp.))	1
BIO3	Isothermality (BIO2/BIO7) (standard deviation * 100)	1
BIO4	Temperature seasonality (standard deviation *100)	1
BIO5	Maximum temperature of warmest month	1
BIO6	Minimum temperature of coldest month	1
BIO7	Temperature annual range (BIO5–BIO6)	1
BIO8	Mean temperature of wettest quarter	1
BIO9	Mean temperature of driest quarter	1
BIO10	Mean temperature of warmest quarter	1
BIO11	Mean temperature of coldest quarter	1
BIO12	Annual precipitation	1
BIO13	Precipitation of wettest month	1
BIO14	Precipitation of driest month	1
BIO15	Precipitation seasonality (coefficient of variation)	1
BIO16	Precipitation of wettest quarter	1
BIO17	Precipitation of driest quarter	1
BIO18	Precipitation of warmest quarter	1
BIO19	Precipitation of coldest quarter	1

Based on total 68 layers at 30 arc-sec (~ 1 km) resolution.

and the final extension was done at 72°C for 10 min. The amplification was conducted in a Veriti thermocycler (Life technology, USA). Negative controls consisted of uninfected male *Ae. aegypti* pools reared in the laboratory and the PCR negative control without RNA. The second round was initiated with 2 µl of sample from initial amplification reaction. The reaction mixture consisted of 1 µl platinum *Taq* polymerase (2.5 U), 5 µl of 10× buffer, 1.5 mM $MgCl_2$, 1 µl of dNTP mix, 20 pmol D1 primer, 25 pmol each of dengue virus type specific primers TS1, TS2, TS3 and TS4 (ref. 23) in a 50 µl reaction. The temperature profiles for amplification consisted of 30 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 2 min, and final extension at 72°C for 10 min.

C6/36 cells were grown in Mitsuhashi and Maramorosch (MM; Life Technology, USA) insect growth media supplemented with 10% fetal bovine serum (FBS; Gibco, USA) until cell monolayers were formed in 12-well plates. Pooled mosquitoes were homogenized on ice in 1 ml MM medium. Then 100 µl each of the homogenate was passed through 0.2 µm filters and inoculated into respective culture plates. The culture plates were then gently rotated and left to incubate for 2 h at ambient temperature for adsorption. MM medium containing 2% FBS was added and the culture tubes were incubated at 28°C for 5 days. After growth for 5 days at 28°C, the cell culture supernatant was collected, clarified by centrifugation and stored at –70°C until further use. The detection and serotypic characterization of dengue virus was performed by the previously described method[20,23].

The amplicons were subsequently gel-purified and sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Sequencing was performed in an ABI Prism model 3100 genetic analyser (Applied Biosystems, USA), and the sequences were edited, assembled and deposited in the GenBank database. All amplicons were sequenced on both strands.

DENV and CHIKV sequences were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/). Molecular phylogeny was performed using the freeware MEGA6 (ref. 24). Best-fit model for nucleotide substitution was selected from 24 models available in MEGA6 based on minimum Akaike Information Criterion (AIC) value[25]. Reliability of the phylogenetic tree was estimated using bootstrap values run for 1000 iterations. Accession numbers, isolate name and country information of virus sequences used in this study are given in the phylogenetic tree of the respective virus type. The maximum likelihood phylogenetic tree for DENV-3, DENV-4 and Chikungunya was constructed using K2+G, K2+G+I and TN93+G nucleotide substation models respectively.

DENV and CHIKV-positive *Ae. aegypti* collection sites in Surat were mapped using DIVA GIS software. Climatic and landscape variables used in the present study are listed in Table 1. The baseline (1950–2000) temperature and precipitation layers were obtained from WorldClim-Global Climate data repository (www.worldclim.org). Temperature and precipitation layers of the future (year 2020) were obtained from repository (www.ccafs-climate.org). The data were resampled at the

Table 2. DENV and CHIKV isolates obtained in this study

Isolate	Virus	Place of collection	Accession number
Surat-D3-PN	DENV-3	Patelnagar	KF543365
Surat-D3-GN	DENV-3	Gomatinagar	KF543366
Surat-D3-BC	DENV-3	Bombay Colony	KF543367
Surat-D3-HN	DENV-3	Hitendranagar	KF543368
Surat-D3-AG	DENV-3	Behind Shri Darshan Apartment, Althangam	KF543369
Surat-D3-JS	DENV-3	Jai Ambey Society	KF543370
Surat-D4-KKA	DENV-4	Sanskar park, Khanda Kuwa (Adajan)	KF543371
Surat-D4-KKP1	DENV-4	Khanda Kuwa, Palgam	KF543372
Surat-D4-KKP2	DENV-4	Khanda Kuwa, Palgam	KF543373
Surat-CHIKV-PRH	CHIKV	Pratham Row House	KF543374

native WorldClim 30 arcsec (approximately 1×1 km) resolution. Statistical modelling was conducted with DIVA GIS software with the present data and from the environmental variables. It is especially suited for mosquito surveillance since these records typically represent a reliable presence. In addition to modelling current species distribution, DIVA GIS has built-in capabilities (distribution modelling tool) to predict the range by using environmental variables.

Between 2008 and 2013, 556 pools constituting 4918 adult *Ae. aegypti* mosquitoes were collected from 106 collection sites from Surat Metropolitan Region (see Supplementary Information, Table S1 online). The individual mosquitoes were checked for the presence of DENV and CHIKV using IFA test. Thirty-three mosquitoes from 10 sites (two from NZ, two from SZ, three from SWZ and three from WZ sites respectively) were found positive for DENV-3, ten mosquitoes from two sites (WZ) for DENV-4, and four mosquitoes from one site (WZ) for CHIKV (see Supplementary Information, Table S1 online). Pools containing virus-positive samples were processed for virus-isolation and RNA isolation. Six isolates of DENV-3, three isolates of DENV-4 and one isolate of CHIKV were isolated in C6/36 cells (Table 2). The prM region of all isolates obtained in C6/36 cells was amplified and sequenced.

DENV-3 was detected in 10 mosquito pools and prM partial sequence was obtained from six mosquito pools. Nucleotide sequences of six DENV-3 isolates of the present study and 52 reference sequences from diverse geographical locations were used for the construction of a maximum likelihood phylogenetic tree (Figure 1). The maximum likelihood phylogenetic tree revealed that all the six DENV-3 isolates circulating in Surat belonging to genotype III.

Sequences of the *prM* gene from the three DENV-4 isolates were aligned and compared with 45 reference sequences in GenBank. Figure 2 shows the phylogenetic tree of the sequences using the maximum likelihood analysis and indicates that Surat DENV-4 isolates grouped with GI DENV-4 sequences.

CHIKV was detected in one mosquito pool and *ns1* gene partial sequence was amplified from this mosquito pool. A maximum likelihood phylogenetic tree was constructed using the partial ns1 region of 550 bp at nucleotide positions (1130–1680), numbered according to the CHIKV prototype S27. The isolate from Surat grouped together with recent Central/East African isolates from 2005 to 2008, sharing 98–99% nucleotide identity (Figure 3).

Based on 106 collections and 68 layers of altitude, rainfall and temperature, risk map for dengue in Surat Metropolitan Region was predicted. The prediction map suggests that the central and western parts of Surat Metropolitan Region have favourable conditions for dengue infection (Figure 4 *a*) and *Ae. aegypti* growth (Figure 4 *b*). We generated a prediction map based on climatic conditions of year 2020 to evaluate the possible expansion of areas currently populated by *Ae. aegypti*. The prediction map revealed that *Ae. aegypti* has the potential to spread to new areas (Balkash, Kunkani, Bhesan, Bhatpor, etc.) of Surat Metropolitan Region and that the central and western areas of the region will have the best conditions for *Ae. aegypti* propagation and survival (Figure 4 *c*).

Surat Metropolitan Region has an ideal environment for DENV transmission; it has a tropical climate, a population of over four million, industrialization, water-storage practices and the presence of the vector mosquito. The region reported more than 1000 cases from 2005 to 2010. The vertical transmission of DENV ensures the retention of virus across the mosquito generations and may serve to keep DENV in nature during inter-epidemic periods. It may also play an important role in contributing to and complementing the ongoing disease outbreak. Similarly, the continuous presence of an infected individual/s within the vector populations is the critical factor in periodic emergence of outbreaks. Therefore, Surat like many other rapidly developing cities in India, urgently needs a dengue surveillance system to monitor and control the mosquito-borne viral disease. We screened *Ae. aegypti* for the presence of CHIKV and DENV using IFA and RT-PCR methods. DENV-3 was more predominant (isolated from 10 mosquito pools) compared to DENV-4 (isolated from two mosquito pools). DENV-4 was only isolated from Khanda Kuwa area. In the present study we

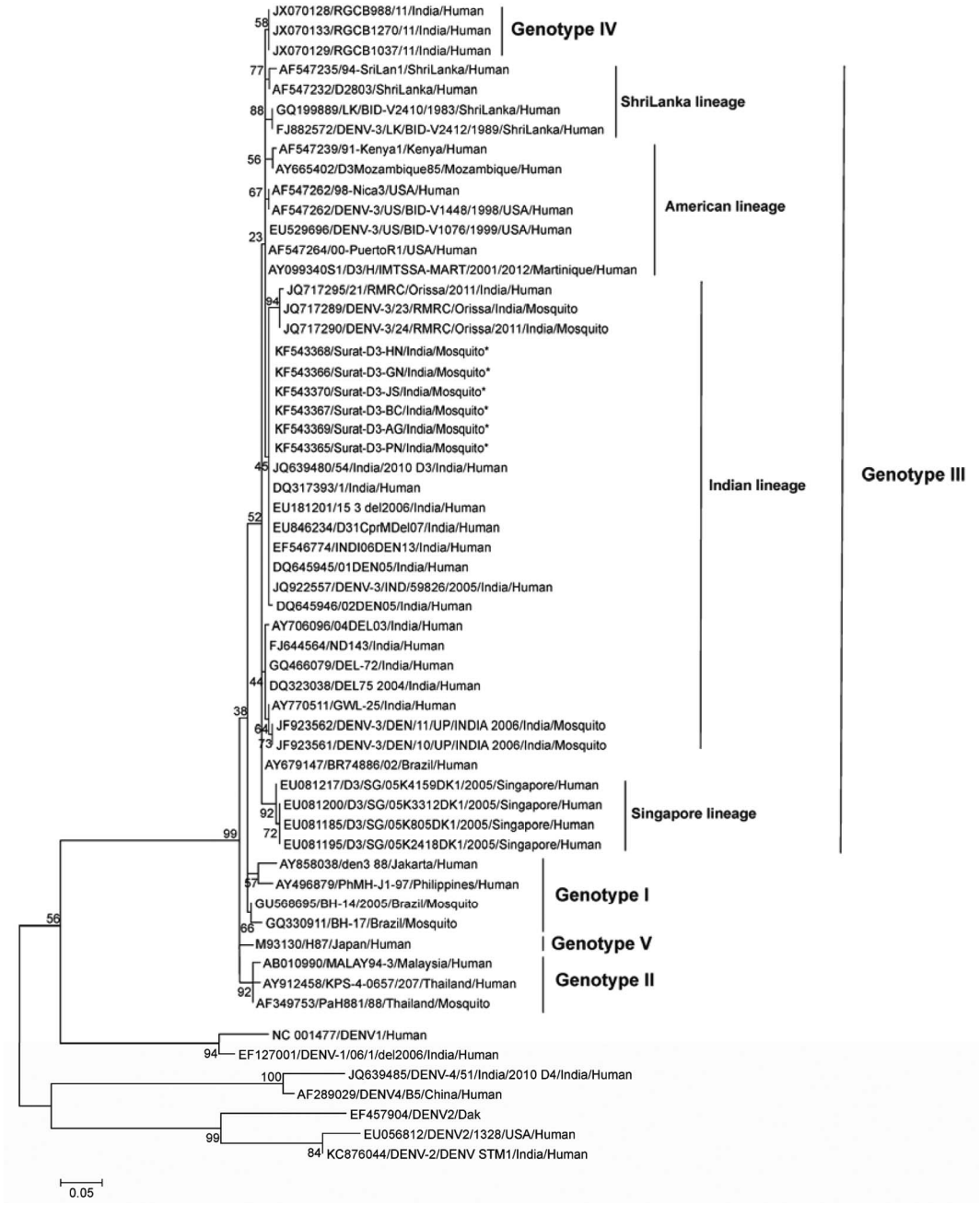

Figure 1. Phylogenetic relationship of DENV-3 isolates of Surat, India. Nucleotide sequences of six DENV-3 isolates of the present study and reference sequences from diverse geographical locations were aligned using MUSCLE software. Phylogenetic tree was constructed by the maximum likelihood method (1000 bootstrap replicates) using MEGA v.6 and K2+G model. DENV-1, DENV-2 and DENV-4 sequences were used as out group. *Denotes the isolates of the present study (accession number KF543365, KF543366, KF543367, KF543368, KF543369 and KF543370). Scale represents genetic distance.

could not detect DENV-1 and DENV-2 in mosquito pools. Chikungunya was detected and isolated from one mosquito pool. Maximum number of DENV and CHIKV-positive mosquito samples was recorded from CZ and WZ of Surat.

The molecular identification of DENV and CHIKV serotypes and genotypes in wild mosquitoes is useful in understanding the phylogenetic similarity of these viruses and genotypes during future outbreaks. RT-PCR and sequencing methods confirmed the circulation of DENV-3, DENV-4 and CHIKV in Surat. Earlier reports suggest that the DENV-3 genotype III (GIII) has a high potential for spread and adaptation; hence, it seems predominant in a large geographical area[4,7,14]. Previous studies from India have reported circulation of genotypes I and III of DENV-3 virus, with subtype III being the common one[4,7,14]. In

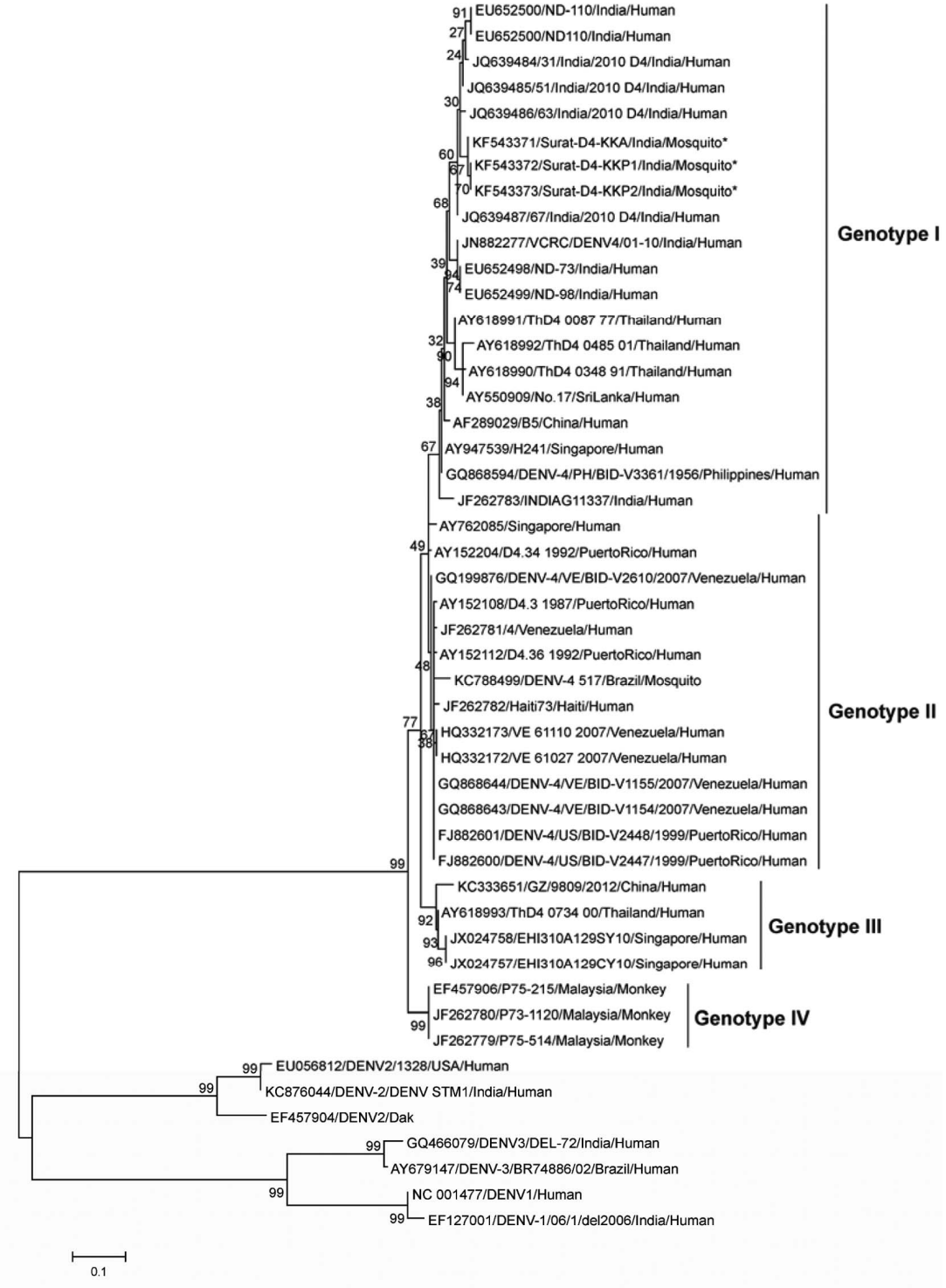

Figure 2. Phylogenetic relationship of DENV-4 isolates of Surat. Sequences DENV-4 isolated from Surat and reference sequences from GenBank were aligned using MUSCLE. Phylogenetic tree was constructed with the maximum likelihood method (1000 bootstrap replicates) with K2+G+I model using MEGA v.6. DENV-1, DENV-2 and DENV-3 sequences were used as out group. *Denotes the isolates of the present study (accession number KF543371, KF543372 and KF543373). Scale represents genetic distance.

this study, all six DENV-3 isolates belonged to GIII. DENV-3 isolated from mosquitoes showed maximum identity with DENV-3 isolated from human patient samples from the same geographic areas. These DENV-3 GIII strains have a close phylogenetic relationship to the previously reported GIII strains from India. This study reports that although DENV-3 GIII is still the predominant genotype in certain parts of India, continuous monitoring

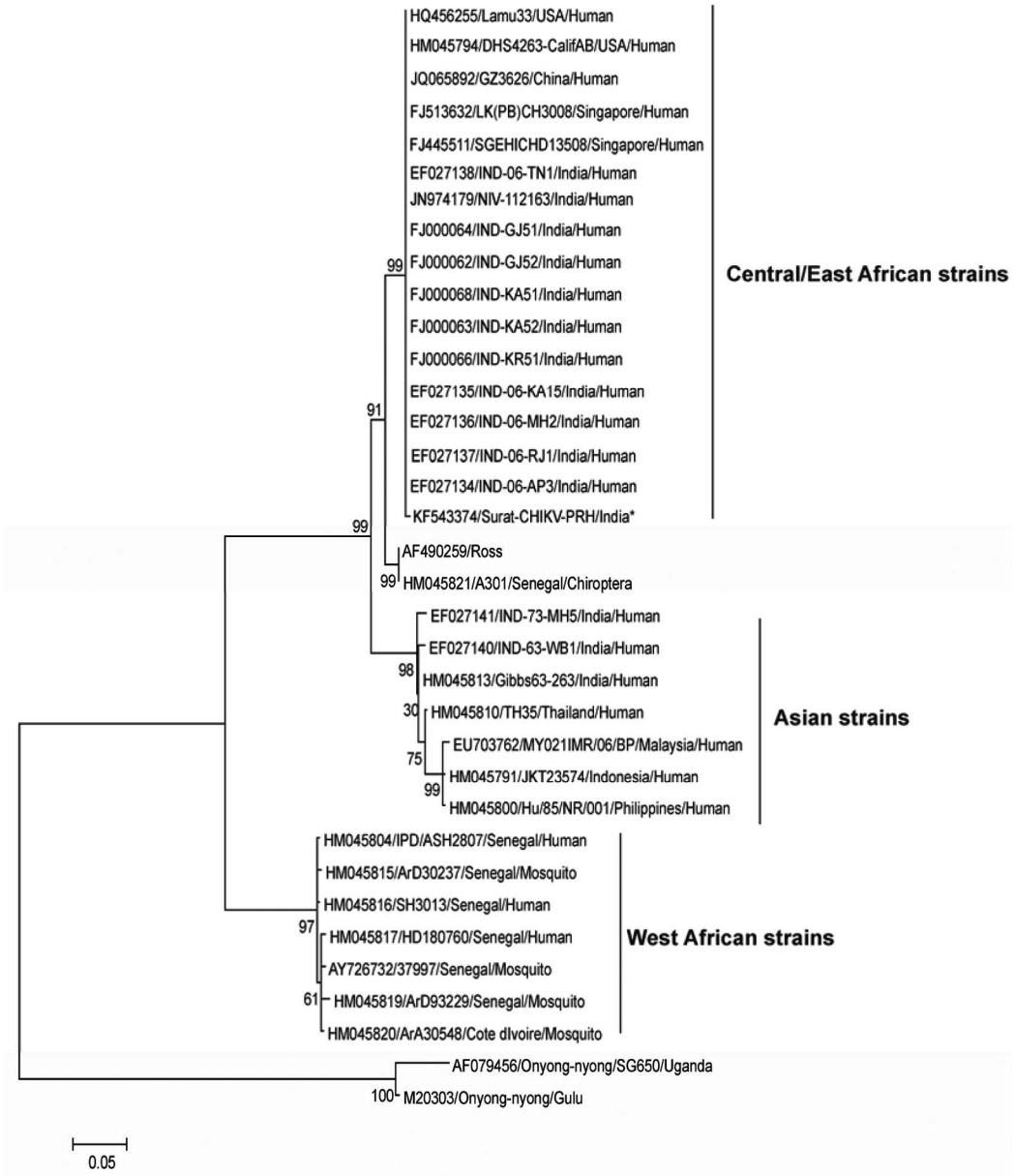

Figure 3. Phylogenetic relationships among CHIKV isolates from Surat. Nucleic acid sequences of *ns1* gene of CHIKV from different geographic locations and Onyong Onyong virus as out group were aligned using MUSCLE software. The maximum likelihood phylogenetic tree (1000 bootstrap replicates) was constructed using MEGA v.6 and TN93+G model. *Denotes the isolate of the present study (accession number KF543374). Scale represents genetic distance.

of circulating dengue virus strains is required to understand the emergence of newer strains and their effect on the epidemiology of dengue in India.

The DENV-4 serotype has been broadly classified into four genotypes (GI to GIV). GI is further divided into three lineages – lineage A of Indian and Sri Lankan isolates, lineage B of Thai isolates and lineage C of Far East isolates[12]. An earlier study showed that DENV-4 isolates from South India were closely related to a strain recovered from Japan[26]. In North India, two lineages of DENV-4 GI strains are circulating; one shows similarity to South Indian strains and the other is closer to the Thailand strain[16]. Earlier reports suggest that DENV-4 GI strains isolated from South India and North India were closely related to the India 1996 and Sri Lanka 1978 isolates[12,26]. All three of the Surat DENV-4 isolates grouped in DENV-4 GI were closely related with the Indian strains and the Sri Lanka 1978 isolate.

Several states in India experienced massive outbreaks of CHIKV during 2005–06, when CHIKV of ECSA lineage replaced the CHIKV Asian strain in various parts of India[20]. Recent outbreaks of CHIKV have documented

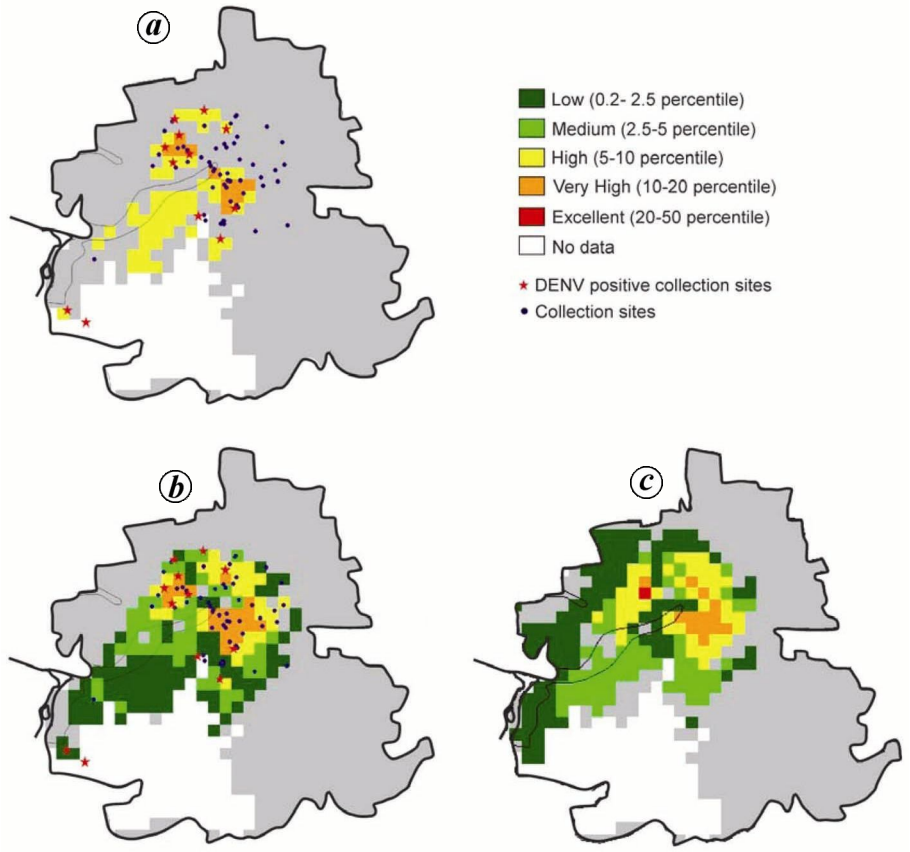

Figure 4. Areas predicted to be environmentally suitable for DENV transmission in Surat in 2013. Based on baseline data (1950–2000) temperature and precipitation layer (total 68 layers) prediction maps for (***a***) DENV and (***b***) *Aedes aegypti* were developed. Based on future data (2020) prediction map for *Ae. aegypti* was developed (***c***). Values from 0 to 0.2 (grey) indicate conditions not favourable for transmission, as opposed to green to dark red areas that delineate conditions increasingly favourable for transmission (values from 0.2 to 50 percentile).

Table 3. Reported human dengue cases in Surat during 2010–2012

	2010	2011	2012
Central zone	70	5	6
East zone	47	6	4
North zone	73	12	8
Southeast zone	95	14	48
Southwest zone	29	6	25
South zone	71	18	41
West zone	40	8	34
Total	425	69	166

the circulation of CHIKV ECSA lineage in different parts of India[22,27–29]. Phylogenetic analysis based on ns1 of Surat samples with the previously reported CHIKV strains revealed that Surat CHIKV isolates were of ECSA lineage.

In addition to the globalization, climate significantly influences the transmission of infectious diseases. Based on field observations and environmental parameters, dengue risk map for Surat Metropolitan Region was predicted. The spatial distribution of dengue risk shows that disease incidence is clustered in central and western parts of Surat and in these areas urgent monitoring of *Ae. aegypti* is recommended. The outskirts of Surat Metropolitan Region are currently predicted as not suitable because little or no data are available for these regions therefore this region requires continuous monitoring.

The climate change models have been used to predict and forecast a number of infectious diseases[30–32]. Similarly, climate change models have been used for prediction of risk maps for *Ae. aegypti*[33,34]. Based on future climatic conditions, *Ae. aegypti* risk map for the year 2020 was predicted. The future prediction suggests that *Ae. aegypti* will spread in newer areas of Surat Metropolitan Region in 2020. The central and western parts of Surat Metropolitan Region will become more suitable for *Ae. aegypti* reproduction and survival. The risk map developed in this study will help local health authorities in specific areas for focused interventions. Based on surveillance data, the Surat Municipal Corporation employed various approaches to control the *Ae. aegypti* populations. This initiative resulted in reduced DENV cases in Surat Metropolitan Region in the years 2011 and 2012 (Table 3).

In conclusion, DENV-3 (GIII), DENV-4 (GI) and CHIKV (ECSA) strains are circulating in Surat Metropolitan Region. Our model provides a climate-based prediction for the current and future distribution of *Ae. aegypti* in Surat. The risk map suggests that *Ae. aegypti* is spreading and will continue to spread to new areas of Surat. Data from RT-PCR-based surveillance of dengue coupled with disease mapping data could serve as a useful epidemiological tool that might provide early warnings of DF/DHF epidemics.

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