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Research paper

Spatio-temporal distribution analysis of circulating genotypes of dengue virus type 1 in western and southern states of India by a one-step real-time RT-PCR assay

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ABSTRACT

Dengue virus type 1 (DENV-1) Asian and American/African (AM/AF) genotypes were reported to be co-circulating in southern and western states of India based on envelope (E) gene sequencing of few representative samples. The objective of the present study was to develop a one-step real-time RT-PCR to discriminate between Asian and AM/AF genotypes of DENV-1 and investigate the spatio-temporal distribution of the DENV-1 genotypes in southern and western states of India. A one-step real-time RT-PCR to discriminate the Asian and AM/AF genotypes of DENV-1 was developed and validated using 40 samples (17 Asian and 23 AM/AF), for which the envelope (E) gene sequence data was available. DENV-2, DENV-3 and DENV-4 isolates, one each and DENV negative samples ($n = 17$) were also tested by the assay. Additional 296 samples positive for DENV-1 from selected Southern and Western states of India were genotyped using the real-time RT-PCR assay. Among the samples used for validation, the genotyping results were concordant with sequencing results for 39 samples. In the one discordant sample which was positive for AM/AF by sequencing, the genotyping assay tested positive for both Asian and AM/AF genotype. DENV-2, DENV-3 and DENV-4 isolates were not reactive in the assay. None of the DENV negative samples were positive (sensitivity 100% and specificity 98.2%). A total of 336 samples (40 samples with sequence data and 296 samples without sequence data) were used for spatio-temporal distribution analysis. The results revealed that the Asian genotype was the predominant genotype in Tamil Nadu and Kerala, the southern states. The AM/AF genotype was the predominant genotype in Maharashtra, a western state of India. In Nashik district of Maharashtra, Asian genotype was observed in 32.6% of DENV-1 samples during 2017 while the same decreased to 7.3% during 2018. In Pune district, Asian genotype was observed in 40.0% of DENV-1 samples during 2018 only. To conclude, a one step real-time RT-PCR has been developed for discriminating Asian and AM/AF genotypes of DENV-1. This assay can act as a complement to sequencing but not a substitute and can be utilized in resource limited settings for molecular surveillance of DENV-1. DENV-1 Asian genotype was the dominant genotype in South India while, AM/AF genotype was dominant in Western India.

1. Introduction

Dengue virus (DENV) has four antigenically distinct serotypes (DENV-1 to DENV-4) and each serotype has multiple genotypes. Genotypes within serotypes differ in their fitness and replication potential in mosquitoes and humans (Vu et al., 2010). Changes in the circulating serotypes of DENV and the genotypes within serotypes have been shown to be associated with the occurrence of major outbreaks/epidemics of DENV (Santiago et al., 2012; Tan et al., 2017; Villar et al., 2015; Saha et al., 2016; Cecilia et al., 2017; Patil et al., 2018). Despite the observation that homotypic DENV infection is not bound to occur in the same individual again, recurrent outbreaks due to a single serotype

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are observed in a same geographical region and has been attributed to changes in the circulating genotype/clade of that particular serotype (Teoh et al., 2013; Dupont-Rouzeyrol et al., 2014).

Conventional as well as real-time based reverse transcriptase (RT)-PCR assays are available for serotyping of DENV but not for genotyping (Patil et al., 2018; Santiago et al., 2013). Sequencing of E gene or C-PrM region or whole genome followed by phylogenetic analysis is generally performed to deduce the circulating genotypes of DENV (Kumar et al., 2010; Patil et al., 2011; Patil et al., 2012; Cecilia et al., 2017; Patil et al., 2018; Santiago et al., 2019). Since the sequencing of DENV RNA is a time-consuming procedure requiring higher viral load, high quality RNA and is not economical, only a few samples which do not represent the entire outbreak/epidemic from an outbreak are processed for genotyping. This poses a limitation in understanding the role of different genotypes within a serotype in disease severity. When multiple genotypes of DENV serotype are circulating, the spatial and temporal dynamics of different genotypes are not understood completely.

DENV-1 has five genotypes namely Asian (GI), American/African (AM/AF) (GV), South Pacific (GIV), Malaysia (GIII) and Thailand (GII) genotypes (Goncalvez et al., 2002). Among the five genotypes, only Asian, AM/AF and South Pacific genotypes were reported to be circulating worldwide while in India, only Asian and AM/AF were reported to be circulating. Only few sequences of Thailand and Malaysia genotypes are available in the GenBank (Villabona-Arenas and Zanotto, 2013; Cecilia et al., 2017; Patil et al., 2018). DENV-1 has been the dominant serotype during the 2012 epidemic of dengue in Tamil Nadu and still continues to be the dominant even during 2017–2018 (Cecilia et al., 2017; Rose et al., 2019). DENV-1 dominated 2014–2015 outbreak season in Karnataka, a southern state of India (Ahamed et al., 2019). In the western region of India, DENV-1 emerged as predominant serotype along with DENV-3 during 2017–2018 (Patil et al., 2018). DENV-1 AM/AF genotype was the only genotype circulating in India till 2012 while the emergence of DENV-1 Asian genotype was reported in the southern part of India during 2012. Asian genotype was the only observed DENV-1 genotype during 2012 and 2013 in Tamil Nadu and Kerala, southern states of India. In 2015, circulation of both the genotypes of DENV-1 was reported in the southern states (Cecilia et al., 2017; Ahamed et al., 2019; Pooja et al., 2017). DENV-1 Asian genotype was first detected in the western region of India during 2017 and was co-circulating with AM/AF genotype (Patil et al., 2011; Patil et al., 2018).

Based on the limited sequences available from these studies, it is not possible to find out the dominant circulating genotype of DENV-1 and hence, the molecular epidemiology of DENV representing a complete outbreak is not usually understood. Hence, in the present study, a one-step real-time RT-PCR for discrimination of two genotypes of DENV-1 circulating in India (Asian and AM/AF) was developed, validated with known isolates and samples and used for investigating spatio-temporal dynamics and dominance of Asian and AM/AF genotypes in Western and Southern states of India.

2. Samples and methods

2.1. Virus isolates and clinical samples

DENV-1 isolates/clinical samples with E gene sequence data obtained during different years from Western and Southern India available ($n = 40$) in the Dengue/Chikungunya Group, ICMR-National Institute of Virology were used for the valiadation of the one step real time RT-PCR assay. Among the 40 samples, 19 were sequenced during the current study. Virus isolates one each belonging to other serotypes (DENV-2, DENV-3 and DENV-4) and DENV negative clinical samples ($n = 17$) were also used for validating the assay. Additionally, leftover RNA from clinical samples received for serotyping of dengue viruses and positive for DENV-1 but without sequence data ($n = 296$) were also used in the present study. Details of the virus isolates/clinical samples positive for DENV-1 used with their geographical locations were given in Table 1. A map of India indicating sampled states has been provided (Fig. 1). Samples from the states of Karnataka, Kerala and Tamil Nadu were used for finding out the predominant genotype of DENV-1 circulating in the Southern region of India, while samples from the state of

Table 1
Geographical location and numbers of DENV-1 positive clinical samples/isolates tested in the assay and genotyping results.

State	District/City	Year	Number of samples/isolates tested	Numbers of positive samples		
				AM/AF	Asian	Dual
Andhra Pradesh	Anantapur	2015	4	4	–	–
Gujarat	Surat	2014	2	2	–	–
Karnataka	Bengaluru	2017	2	2	–	–
		2018	4	3	1	–
Kerala	Alappuzha	2010	1	1	–	–
	Alappuzha	2017	3	–	3	
	Ernakulam	2017	1	1	–	–
	Kottayam	2017	1	–	1	
	Alappuzha	2018	4	–	4	–
	Pathanamthitta	2018	1	–	1	–
Maharashtra	Pune	2012	1	1	–	–
		2013	6	6	–	–
		2014	1	1	–	–
		2017	31	31	–	–
		2018	25	14	10	1
	Nashik	2017	147	98	48	1
		2018	41	38	3	–
	Aurangabad	2018	1	1	–	–
	Kohlapur	2018	4	4	–	–
	Nagpur	2018	1	1	–	–
Odisha	Bhubaneshwar	2011	3	3	–	–
Tamil Nadu	Tirunelveli	2012	21	1	20	–
	Vellore	2015	2	1	1	–
		2016	2	2	–	–
		2017	5	4	1	–
	Coimbatore	2017	5	2	3	–
	Dindigul	2017	4	1	3	–
	Tiruppur	2017	14	4	9	1

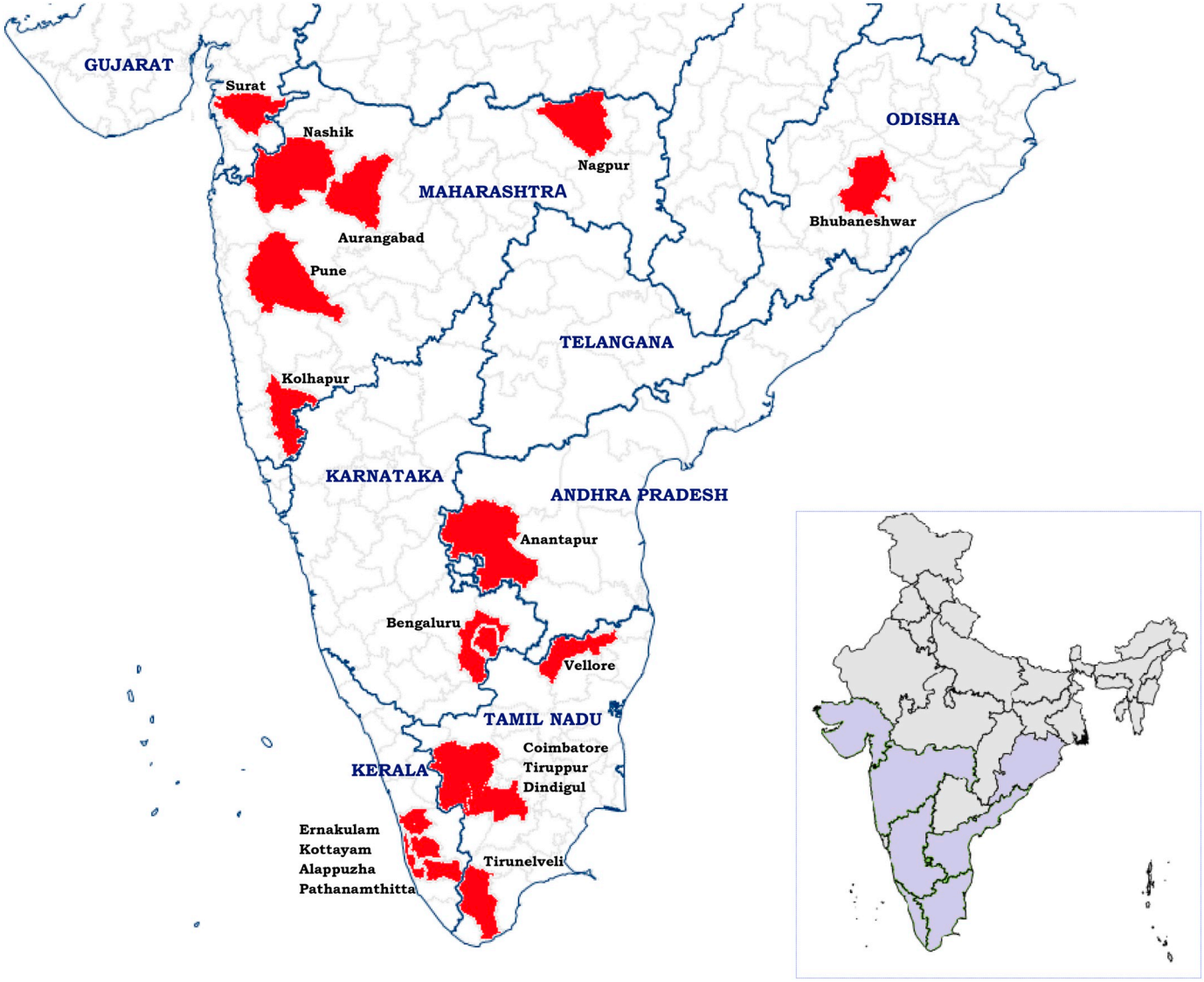


Fig. 1. Geographical location of the sampled states indicating the districts/city. Map not to the scale.

Maharashtra was used to study the DENV-1 genotypes circulating in the western part of India. The study was approved by the institutional ethics committee of ICMR-National Institute of Virology, Pune (NIV/IHEC/2015/D-7274, NIV/IEC/2018/D-8 and NIV/IEC/2018/D-34).

2.2. E gene sequencing and phylogenetic analysis

E gene sequencing of DENV-1 virus isolates and clinical samples positive for DENV-1 was performed as described earlier (Patil et al., 2011, 2018). MEGA7.0 was used for multiple sequence alignment and phylogenetic analyses (Kumar et al., 2016). The best-fit model for construction of phylogenetic tree was determined using model test option available in MEGA7.0. General time reversible (GTR) model was observed to be the best-fit model and used for generation of phylogenetic tree. Phylogenetic tree was constructed using DENV-1 E gene sequences available in GenBank from India ($n = 46$) and E gene sequences generated from the DENV-1 virus isolates/clinical samples positive for DENV-1 ($n = 19$) in the laboratory during the present study along with the global sequences from GenBank (65 DENV-1) representing all genotypes. Genotypes were defined as group of viruses within a serotype with a nucleotide divergence of < 6% (Rico-Hesse, 1990). A separate lineage is considered as a group of viruses within a genotype with a nucleotide divergence of < 3% (Alfonso et al., 2012). Nucleotide divergence within and between clusters was calculated using p-distance model implemented in MEGA7.0.

2.3. Designing of primers and probes for discrimination of DENV-1 genotypes

Whole genome sequence of DENV-1 for different genotypes available in the Virus Pathogen Database and Analysis Resource (ViPR) (Pickett et al., 2012) were downloaded, aligned using MAFT online version 7.0 (Katoh et al., 2017). The consensus sequence for DENV-1 serotype and each genotype were generated from aligned sequences. From the consensus sequence for each genotype, regions with signature sequences specific for each genotype flanked by regions common in all genotypes were identified and used for designing of primers and probes. A set of common primers amplifying the signature sequences specific to all the genotypes of DENV-1 were designed. Two probes specific for each genotype were designed. The designed probes were synthesized as dual-labeled probes.

Table 2
Position wise conservation of nucleotides in primers and probes among the analysed sequences of DENV-1.

Position	Primers				Probes[#]			
	D1F[a]		D1R[b]		Asian[c]		AMAF[d]	
	Nucleotide	Number of sequences conserved (%)[#]	Nucleotide	Number of sequences Conserved (%)	Nucleotide	Number of sequences conserved (%)[*]	Nucleotide	Number of sequences conserved (%)[*]
1	G	1633 (99.9)	G	1630 (99.7)	T	1242 (100)	A	212 (66.2)
2	A	1632 (99.9)	C	1632 (99.9)	T	1242 (100)	A	200 (62.5)
3	A	1630 (99.7)	T	1622 (99.3)	T	1241 (99.9)	T	320 (100)
4	C	1630 (99.7)	G	1632 (99.9)	C	1200 (96.6)	T	306 (95.6)
5	A	1633 (99.9)	T	1634 (100)	G	1234 (99.3)	T	319 (99.7)
6	G	1634 (100)	T	1633 (99.9)	A	1242 (100)	C	311 (97.1)
7	A	1634 (100)	A	1634 (100)	C	1242 (100)	A	227 (70.9)
8	T (Y)[*]	1580 (96.7)	T	1607 (98.3)	C	1237 (99.6)	A	319 (99.7)
9	G	1634 (100)	C (Y)[*]	1403 (85.9)	C	1242 (100)	C	311 (97.1)
10	C	1629 (99.6)	A	1634 (100)	A	1240 (99.8)	C	307 (95.9)
11	A (R)[*]	1549 (94.8)	A (R)[*]	1363 (83.4)	A	1240 (99.8)	C	320 (100)
12	C	1632 (99.9)	T	1632 (99.9)	G	1241 (99.9)	A	320 (100)
13	C	1632 (99.9)	C	1634 (100)	A	1242 (100)	A	320 (100)
14	A	1634 (100)	T	1634 (100)	T	1242 (100)	G	320 (100)
15	T	1634 (100)	C	1599 (97.8)	G	1242 (100)	A	320 (100)
16	G	1634 (100)	C	1631 (99.8)	A	1234 (99.3)	T	320 (100)
17	C	1613 (98.7)	C	1632 (99.9)	A	1166 (99.9)	G	320 (100)
18	A	1634 (100)	A	1615 (98.8)	A	1242 (100)	A	320 (100)
19	A	1634 (100)	T	1632 (99.9)	A	1231 (99.1)	G	320 (100)
20	G	1634 (100)	T	1634 (100)	A	1231 (99.1)	A	320 (100)
21	A	1633 (99.9)	C	1630 (99.7)	G	1242 (100)	A	320 (100)
22			T	1634 (100)	G	1242 (100)	A	320 (100)
23			G	1632 (99.9)	A	1238 (99.7)	G	320 (100)
24					G	1237 (99.6)	G	320 (100)
25					T	1231 (99.1)	A	312 (97.5)
26					A	1164 (93.7)	G	320 (100)
27					A	1238 (99.7)	T	320 (100)
28					C	1240 (99.8)	G	320 (100)
29							A	314 (98.1)
30							C	320 (100)

[#] The total number of DENV-1 sequences used for primer designing was 1634. The total number of sequences used for designing probe specific to Asian genotype was 1242. For AM/AF genotype, 320 sequences were used. The remaining 72 sequences among the 1634 DENV-1 sequences belonged to South Pacific genotype (data not shown).

[*] Degenerate bases in the parenthesis are present in the primers.

[a] Position in the whole genome 1917–1937 and in the E gene 983–1003.

[b] Position in the whole genome 1973–1995 and in the E gene 1039–1061.

[c] Position in the whole genome 1924–1951 and in the E gene 1010–1037.

[d] Position in the whole genome 1942–1971 and in the E gene 1008–1037.

2.4. Validation of the primes and probes in one-step real-time RT-PCR for DENV-1 genotyping

The primers and probes were tested in a one-step real-time RT-PCR using RNA extracted from the virus isolates ($n = 33$) and clinical samples ($n = 7$) for which genotype was known based on sequencing data and phylogenetic analyses ($n = 40$; Asian 17; AM/AF 23). Moreover, the specificity of the assay was tested against virus isolates one each belonging to other serotypes and samples ($n = 17$) negative for DENV.

2.5. One step real-time RT-PCR assay for discrimination of DENV-1 genotypes

Leftover RNA from clinical samples positive for DENV-1 but without sequence data ($n = 296$) were genotyped using the one step real-time RT-PCR assay. Briefly, 12.5 µl of 2× RT-PCR buffer and 0.5 µl of RT-PCR enzyme (Superscript III platinum one step qRT-PCR system, Invitrogen, CA, USA), 0.5 µM to 1.0 µM of common primers and probes specific for Asian and AM/AF genotypes of DENV-1 and 5 µl RNA in a total volume of 25 µl was used for real-time RT-PCR. The sequences of the primers and probes with their respective fluorescence dyes and quenchers were given in Table 2. The reaction was carried out in MX3005P real time PCR system. The reaction conditions were as follows: 50°C for 30 min, followed by 95°C for 2 min, and 40 cycles of amplification (15 s at 95°C and 1 min at 60°C). Respective fluorescence signals for different genotypes were detected and samples which gave a cycle threshold (Ct) value of ≤37 was considered positive and genotypes assigned accordingly.

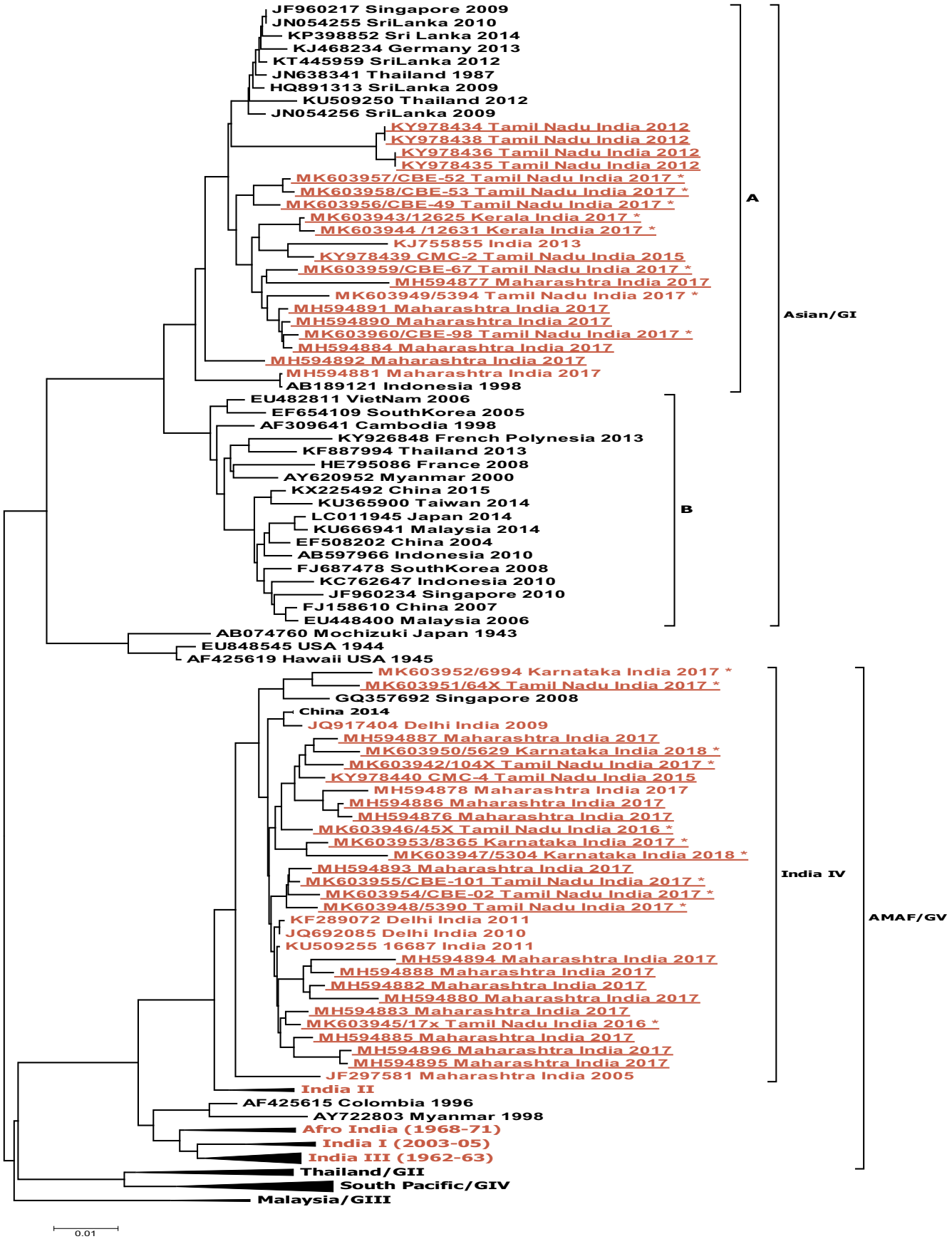
2.6. Statistical analysis

The genotyping results from 336 samples (40 DENV-1 isolates/clinical samples with E gene sequence data and 296 DENV-1 positive samples without sequence data) were included in the analysis for finding out the prevalence and spatio-temporal distribution of DENV-1 genotypes in the western and southern states of India. Percentage of DENV-1 genotypes were calculated from the total number of DENV-1 samples genotyped from that district or state.

3. Results

3.1. Phylogenetic analysis of DENV-1

Phylogenetic analysis of DENV-1 was performed using 65 sequences from Indian strains (19 sequenced in the current study) and 65 global sequences. The accession numbers of the viruses sequenced in the present study were provided in supplementary table 1. The analyses



(caption on next page)

Fig. 2. Phylogenetic tree of DENV-1 based on E gene sequences. All the sequences highlighted in Red are from India. Sequences that are underlined were available earlier and were genotyped by the one step real-time RT-PCR. Sequences with asterix and underlined were sequenced in the present study and genotyped by the one step real-time RT-PCR. Sequences with only asterix were sequenced in the present study, but not used for genotyping. Sixty five global sequences and 65 Indian sequences were used for generating the phylogenetic tree. The year ranges given in the Afro India, India I, India II and India III lineages within AM/AF genotype were given only for Indian isolates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

revealed that sequences from Indian strains clustered in two genotypes (Fig. 2). DENV-1 strains from the year 1962 to 2011 were found in AM/AF genotype while those collected during 2012–2017 were observed in both AM/AF and Asian genotypes. Within AM/AF genotype, Indian sequences clustered in five lineages: India I - India IV and Afro India. The inter lineage nucleotide divergence ranged from 3.0% to 5.7%. Strains collected after 2005 clustered within the India IV lineage.

Among the Asian genotype sequences, the strains clustered into two lineages A and B. The inter lineage nucleotide divergence between the two lineages was 3.0%. The intra lineage nucleotide divergence within lineage A was 2.0% and lineage B was 1.8%. All the Indian sequences from 2012 to 2018 clustered with lineage A sequences which consisted of sequences from Sri Lanka, Singapore and Thailand strains collected during 2009 and afterward (Fig. 2).

3.2. Primers and probes for discrimination of DENV-1 genotypes

Signature sequences specific to each genotype of DENV-1 flanked by consensus sequences common to both genotypes were identified in the E gene and found to be highly conserved (Fig. 3 and Table 2) and were used for the designing of primers and probes for genotyping of DENV-1. The designed primer and probe sequences were provided in the Table 3.

3.3. Validation results of the primer and probes in discriminating DENV-1 genotypes

The designed primer-probe set was tested in a one-step real-time RT-PCR assay using virus isolates or clinical samples for which the genotypes were known and E gene sequences were available: Asian genotype $n = 17$; AM/AF genotype $n = 23$. The genotyping results matched with the genotypes assigned on the basis of E gene phylogenetic analysis except for the one sample which was positive for both Asian and AM/AF genotype. The virus isolates belonging to other three serotypes as well as DENV negative samples by real time RT-PCR were not reactive in the assay (Sensitivity 100% and specificity 98.2%). The E gene sequence from the sample which was positive for both genotypes showed the presence of AM/AF by phylogenetic analysis (sequence accession number MH 594878) (Fig. 2). The probes were specific and not reactive with other genotype.

3.4. Prevalence and spatio-temporal distribution of different DENV-1 genotypes in Western India

In the Pune district of Maharashtra, a western state of India, AM/AF genotype was the only genotype detected in limited DENV-1 samples received during 2012–2015. All the 31 DENV-1 positive samples tested from 2017 belonged to AM/AF genotype. Out of the 25 samples tested in 2018, 10 samples were positive for Asian genotype (40.0%) and 14 (56.0%) for AM/AF genotype. One sample (4.0%) was positive for both the genotypes (Table 1).

In Nashik district, AM/AF genotype was detected in 98 (66.8%) samples while Asian genotype was detected in 48 (32.6%) samples out of the 147 DENV-1 positive samples tested during 2017. One sample (0.6%) was positive for both the genotype. Out of the 31 samples tested during 2018, three samples (7.3%) were positive for Asian genotype while AM/AF genotype was detected in 38 (92.7%) samples (Table 1).

DENV-1 samples from all the other districts of Maharashtra belonged to AM/AF genotype. Among the limited number of samples tested from the states of Gujarat (a western state) and Odisha (an eastern state), all were positive for AM/AF genotype (Table 1).

3.5. Prevalence of different DENV-1 genotypes in the southern states of India

In Tamil Nadu, among the DENV-1 positive samples tested from 2012, 90.5% belonged to Asian genotype while one each sample was positive for AM/AF and dual genotypes. Among the samples received during 2017 from different districts of Tamil Nadu, Asian genotype was the predominant genotype in all the districts except Vellore (Table 1).

Asian genotype was detected in higher numbers among the DENV-1 positive samples from Kerala during both 2017 and 2018 (Table 1).

Among the samples from the states of Karnataka and Andhra Pradesh, AF/AM genotype was detected in all samples except one which was positive for Asian genotype (Table 1).

4. Discussion

In the present study, a one-step real-time RT-PCR was developed to discriminate the Asian and AM/AF genotypes of DENV-1 and validated using isolates and clinical samples for which the sequences are available. To the best of our knowledge, this is first report to use a one-step real time RT-PCR to discriminate the Asian and AM/AF genotypes of DENV-1. The assay was observed to be very specific and sensitive in detecting Asian and AM/AF genotypes of DENV-1. Only one sample provided a discordant result. Though the assay detected the AM/AF genotype assigned on the basis of sequencing and phylogenetic analysis, the assay additionally detected Asian genotype in the discordant sample. Coinfection with multiple genotypes of DENV-1 has been reported in a dengue patient (Aaskov et al., 2007). It is possible that the traditional Sanger sequencing method may not be able to detect the minor population in the sample and next-generation sequencing (NGS) method is required to validate the presence of two genotypes in the sample. However, NGS method also requires high quality RNA and is not cost effective. The present assay is a cost effective and less time-consuming one as compared to the sequencing methods. The assay provides an opportunity to study the spatio-temporal distribution of

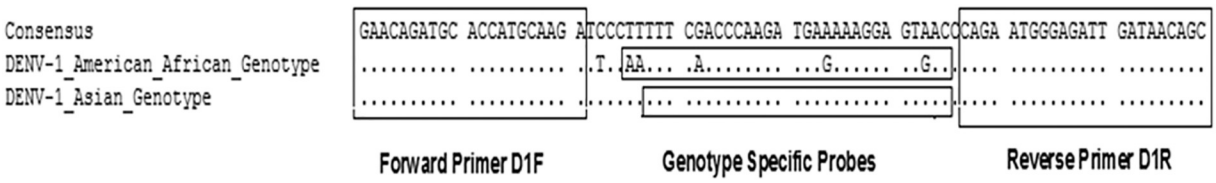


Fig. 3. Consensus sequence for DENV-1 serotype and each genotype used in primer-probe designing. A total number of 1634 DENV-1 sequences were used to generate the consensus sequence. Among the 1634, 1242 were from Asian and 320 from AM/AF genotypes. Rest of the sequences belonged to South Pacific genotype (data not shown).

Table 3
Primer and Probe sequences used for genotyping of DENV1 with their positions in the whole genome as well as the E gene.

Primers and probes name	Primers and probes nucleotide sequence	Position in the whole genome	Position in the E gene
Asian	TTTCGACCCAAGATGAAAAAGGAGTAAC	1924–51	1010–37
AM/AF	AATTTCAACCCAAGATGAGAAAGGAGTGAC	1942–71	1008–37
D1F	GAACAGAYGCRCCATGCAAGA	1917–37	983–1003
D1R	GCTGTTATYARTCTCCCATTCTG	1973–95	1039–61

[#]Probe for Asian genotype was labeled with 5′FAM and 3′ BHQ-1.
[#]Probe for AM/AF genotype was labeled with 5′HEX and 3′ BHQ-1.

DENV-1 genotype circulating in a region and can be readily used by resource limited public health laboratories to monitor the changes in the circulating genotypes over time and can serve as a component of early warning systems for dengue. If large number of DENV-1 positive samples were available from an outbreak, the assay can be utilized to initially find out the number of samples positive for each genotype and can guide in selection of samples for sequencing. The assay can also be utilized to find out whether the infecting DENV-1 genotype is Asian or AM/AF in a patient and the role of the these genotypes in their ability to cause severe disease may be investigated. If a particular genotype is proved to be associated with severe disease, then this assay can serve to predict severe disease. Moreover, the assay can be utilized in experimental coinfection studies to investigate the effect of one genotype on the replication of the other genotype. For samples with lower viral load, the success rate for sequencing is limited (Santiago et al., 2019) and can be overcome with the present genotyping assay. One of the limitation of the present assay was that the assay may not be useful in finding out inter genotypic recombinants for which usually nucleotide sequences are required to find out recombination.

The DENV-1 genotypic assay was further utilized to investigate the spatio-temporal distribution of circulating DENV-1 genotypes in India. Earlier studies based on limited sequences revealed that the Asian genotype displaced the AM/AF during the 2012 epidemic of dengue in Tamil Nadu (Cecilia et al., 2017). Higher percentage Asian genotype strains were observed during the 2014–2015 season of dengue in Karnataka (Ahamed et al., 2019). However, in the present study, among the DENV-1 positive samples from Tamil Nadu in 2012 tested, one was positive for AM/AF genotype suggesting that AM/AF genotype was not completely displaced by Asian genotype in 2012 and both the genotypes were circulating in Tamil Nadu since 2012 with the dominance of the Asian genotype even during 2017. Based on a single sequence, studies have reported the presence of the Asian genotype in Kerala between 2013 and 2015 (Cecilia et al., 2017; Pooja et al., 2017). In the present study, among the ten samples from 2017 to 2018 studied, 90% belonged to the Asian genotype suggesting its dominance in Kerala. Among the samples from Karnataka and Andhra Pradesh states, a dominance of AM/AF strains was observed, however, the sample size was limited to derive any conclusion.

During 2017, the study revealed that the both the Asian and AM/AF genotype were observed in the Nashik region while in the Pune district, only AM/AF was circulating. However, during 2018, the Asian genotype strains were observed in Pune also. Two year data from Nashik and Pune districts revealed that AM/AF is the dominant genotype suggesting that AM/AF circulating in western region of India might have more transmission potential than the circulating Asian genotype strains.

The study has revealed two contrasting observations. DENV-1 Asian genotype is dominant in the southern region of India, while DENV-1 AM/AF genotype is dominant in the western region of India. Data from different studies indicate that Asian genotype might have introduced from Singapore to Sri Lanka and South India followed by introduction in Western India. Introduction of DENV-1 Asian genotype might be one of the reasons for the larger number of DENV-1 cases in Pune and Nashik districts of Maharashtra during 2017 and 2018. The dominance of AM/AF genotype despite the introduction of the Asian genotype suggests that the Asian genotype virus might have acquired a phenotype with a lower transmissibility in mosquitoes compared to AM/AF genotype and this needs confirmation. Complete genome analyses coupled with transmission and replication studies of the circulating genotypes under *in-vitro* and *in-vivo* conditions might reveal the possible reasons for contrasting observations in Southern and Western regions of India.

The main limitation of the study is the sample size, which is not uniform across the years for different states. The assay cannot substitute sequencing since sequencing allows for phylogenetic analysis, which is powerful tool to investigate the transmission dynamics among sampled locations, the time of introduction of new lineages and the selection pressures impacting viral evolution. However, the assay can complement sequencing to understand the molecular epidemiology and spatio-temporal distribution of different genotypes in a particular region. Further follow up studies in the coming years investigating a larger number of samples across different regions are needed.

To conclude, a one step real-time RT-PCR has been developed for discriminating Asian and AM/AF DENV-1 genotypes and can be used as a complement to sequencing, but not a substitute in molecular surveillance of DENV-1 in public health laboratories and as an alternative to sequencing, in regions where the sequencing facilities are not available. Further, the study revealed the co-circulation of both the Asian and AM/AF genotypes of DENV-1 with a dominance of the Asian genotype in South India and AM/AF genotype in Western India.

Ethical approval

The study was approved by the institutional ethics committee of ICMR-National Institute of Virology, Pune (NIV/IHEC/2015/D-7274, NIV/IEC/2018/D-8 and NIV/IEC/2018/D-34).

Declaration of Competing Interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103989.

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