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Molecular characterization of influenza A(H1N1)pdm09 viruses circulating at various geographical locations in India, 2017

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***Background & objectives*: Influenza virological surveillance is an essential tool for the early detection of novel genetic variants of epidemiologic and clinical significance. This study was aimed to genetically characterize A(H1N1)pdm09 virus circulating in 2017 and to compare it with the global data.**

***Methods*: The regional/State Viral Research and Diagnostic Laboratories (VRDLs) provided influenza diagnosis for referred clinical samples and shared influenza A(H1N1)pdm09 positives with the Indian Council of Medical Research-National Institute of Virology (ICMR-NIV), Pune, India, for hemagglutinin (*HA*) gene phylogenetic analysis. Sites at Manipal, Jaipur and Dibrugarh performed the sequencing and shared the sequence data for analysis. The antiviral susceptibility of influenza viruses was assessed for known molecular marker H275Y at the ICMR-NIV, Pune.**

***Results*: All the eight VRDLs had well-established influenza diagnostic facilities and showed increased activity of influenza A(H1N1)pdm09 during 2017. Phylogenetic analysis showed that the viruses from the different regions of the country were similar to A/Michigan/45/2015 strain which was the 2017-2018 recommended vaccine strain and were clustered with the globally circulating clade 6B.1 with signature mutations S84N, S162N and I216T. The clade 6B.1 showed further subgrouping with additional mutations S74R, S164T and I295V; however, there was no significant association between the presence of these mutations and severity of disease due to influenza. All the study viruses were sensitive to oseltamivir.**

***Interpretation & conclusions*: During the study period, all the study sites reported globally circulating A/Michigan/45/2015 vaccine strain of influenza A(H1N1)pdm09 viruses and remained sensitive to oseltamivir. Further genetic and antigenic characterization of influenza viruses is recommended to address public health concerns.**

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Pandemic influenza A(H1N1)2009 virus first appeared in India in May 2009 and thereafter continued to circulate with considerable morbidity and mortality in many parts of the country[1-5]. In India, multisite epidemiological and virological influenza surveillance established previously reported peak influenza activity to be associated with rainy season and a secondary peak in the winter months[2]. Hemagglutinin (*HA*) gene sequencing and whole-genome analysis of viruses from three interseasonal upsurges *i.e.,* 2012, 2015 and 2017 have been studied[6]. The outbreaks of the years 2012, 2015 and 2017 were more widespread across many States such as Rajasthan, Gujarat, Maharashtra, Madhya Pradesh and Telangana[7]. India has also experienced high mortality associated with influenza A(H1N1)pdm09 virus[8].

During the past 10 years, antiviral drugs have provided an important intervention for the treatment and prophylaxis of influenza virus infection. In India, antiviral drugs such as oseltamivir and zanamivir are licensed, and national guidance on their use in clinical management is followed[9]. The use and stockpiling of antiviral drugs are key components of the pandemic preparedness plans. Surveillance of antiviral susceptibility is, therefore, essential for public health. Initial studies from our group showed that the emergence of neuraminidase inhibitor-resistant viruses was rare[10,11], and H275Y mutation in neuraminidase (*NA*) gene was responsible for reducing susceptibility.

This study was undertaken to investigate the *HA* gene evolution and antiviral drug susceptibility of the 2017 influenza A(H1N1)pdm09 virus and compare it with the Indian, global data, including contemporary influenza vaccine components[5,12,13]. The study was undertaken at eight Viral Research and Diagnostic Laboratories (VRDLs) across the country established under the Department of Health Research/Indian Council of Medical Research (DHR/ICMR), Government of India, New Delhi, India.

Material & Methods

The samples of suspected influenza patients in category C as defined by the Government of India[9] were referred to the VRDLs for diagnosis of influenza. Influenza A(H1N1)pdm09-positive clinical samples were stored at −70°C for future use at the respective VRDLs. Complete *HA* gene sequencing was done by the VRDLs on randomly selected 10 positive A(H1N1) pdm09 clinical samples representative of severe and mild cases using the WHO sequencing protocol[12]. Of the eight VRDLs, those of Dibrugarh (365-1650 bp), Jaipur and Manipal carried out sequencing in their respective laboratories and submitted the *HA* sequences to the ICMR-NIV, Pune. The remaining laboratories submitted the clinical samples tothe ICMR-NIV, Pune, for gene sequencing. Viral RNA was extracted from clinical samples using the Mag Max 96 Kit (Ambion, CA, USA). One-step reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen Superscript III Platinum Kit, CA, USA) was used to amplify the entire *HA* gene (~1700 bp) in five overlapping fragments of A(H1N1)pdm09 using the WHO protocol[12].

PCR amplicons were subjected to DNA sequencing using Big Dye Terminator Kit Ver.3.1. (Austin, TX, USA). The expected amplicons for each fragment were visualized on two per cent agarose and purified using Charge switch magnetic beads PCR purification kit (Invitrogen, CA, USA). DNA sequencing was carried out using Big Dye terminator V 3.1 cycle sequencing ready reaction kit (ABI, CA, USA), and unincorporated labelled ddNTPs (dideoxynucleotide triphosphates) were purified using DyeEx 2.0 dye-terminated removal kit (Qiagen, Hilden, Germany). The sequencing was done on ABI 3730 DNA analyzer. The obtained sequence information was edited by Seqscape V2.5 software (Applied Biosystems, USA), and pairwise sequence alignment and phylogeny were performed with MEGA6 program[13].

The ICMR-NIV, Pune (Apex laboratory) collated all the data and performed quality checks on the raw sequence data received from the three laboratories. A phylogenetic tree was constructed based on the sequences of this study together with the sequences from India and neighbouring countries available from genebank data set and the 2017-2018 WHO recommended vaccine strains[14]. A neighbour-joining tree was generated using Tamura-Nei best-fit Model for influenza[12]. Sequence data were deposited in the gene bank, and accession numbers are listed in the Table.

The sequences were also analyzed using the free online tool BII Fluserver (*http://flusurver.bii.a-*

star.edu.sg/). Further, for antiviral susceptibility of the influenza A(H1N1)pdm09, the clinical samples and isolates were tested for the detection of H275Y mutation by allelic discrimination real-time RT-PCR using protocol shared by the National Institute of Health, Thailand[15] and isolates by phenotypic fluorescent assay described earlier[10,11]. Minor and major phenotypic outliers were identified based on IC_{50} cut-off values. The Dibrugarh VDRL tested their study clinical samples for H275Y mutation by allelic discrimination real-time RT-PCR.

Results & Discussion

In 2017, an increased activity of influenza A(H1N1) pdm09 was observed with substantial mortality from most parts of India[8]. In the present study, analysis of the *HA* gene-based, 2017 influenza A(H1N1)pdm09 viruses circulating in different regions of India was carried out. Phylogenetic analysis revealed that these were similar to the influenza A/Michigan/45/2015 and were clustered in the globally circulating clade 6B.1 with signature mutations S84N, S162N and I216T

Table. Details of isolate number, accession number, N-glycosylation sites and mutations in the *HA* gene of the 56 Indian isolates with respect to A/Michigan/45/2016

Strain name	Accession number	Glycosylation site	Amino acid change	Susceptibility to antiviral (oseltamivir)
A/India/Dib-1704/2017	MG271753	5 (162,276,287,481,540)	S74R, S164T, I295V	Sensitive
A/India/Dib-1572/2017	MG271752	5 (162,276,287,481,540)	S74R, S164T, I295V	Sensitive
A/India/Dib-1301/2017	MG279393	5 (162,276,287,481,540)	S74R, S164T, I295V	Sensitive
A/India/Dib-1328/2017	MG271752	5 (162,276,287,481,540)	S74R, S164T, I295V	Sensitive
A/India/Dib-1737/2017	MG271754	5 (162,276,287,481,540)	S74R, S164T, I295V	Sensitive
A/India/Dib-1172/2017	MF951088	5 (162,276,287,481,540)	S74R, I295V	Sensitive
A/India/Dib-1745/2017	MG271754	5 (162,276,287,481,540)	S74R, I295V	Sensitive
A/India/Guw-20/2017	MH229485	9	S74R, S164T, I295V	Sensitive
A/India/Guw-22/2017	MH229486	9	S74R, S164T, I295V	Sensitive
A/India/Guw-19/2017	MH229487	9	S74R, I295V	Sensitive
A/India/Guw-12/2017	MH229489	9	S74R, I295V	Sensitive
A/India/Guw-30/2017	MH229488	9	S74R, I295V	Sensitive
A/India/Jai-8/2017	MH333271	9	S74R, I295V	Sensitive
A/India/Jai-4/2017	MH333272	9	S74R, I295V	Sensitive
A/India/Jai-13/2017	MH333273	9	S74R, I295V	Sensitive
A/India/Luc-1842492/2017	MH211344	9	S74R, S164T, I295V	Sensitive
A/India/Luc-1842495/2017	MH211345	9	S74R, S164T, I295V	Sensitive
A/India/Luc-1842484/2017	MH211341	9	S74R, S164T, I295V	Sensitive
A/India/Luc-1842489/2017	MH211342	9	S74R, S164T, I295V	Sensitive
A/India/Luc-1842490/2017	MH211343	9	S74R, S164T, I295V	Sensitive
A/India/Ahm-1841326/2017	MH229460	9	S74R, S164T, I295V	Sensitive
A/India/Ahm-1841337/2017	MH229458	9	S74R, S164T, I295V	Sensitive
A/India/Ahm-1841328/2017	MH229459	9	S74R, S164T, I295V	Sensitive
A/India/Bhu-33170/2017	MH229454	9	S74R, S164T, I295V	Sensitive
A/India/Bhu-33201/2017	MH229456	9	S74R, S164T, I295V	Sensitive
A/India/Bhu-33206/2017	MH229457	9	S74R, S164T, I295V	Sensitive
A/India/Bhu-33174/2017	MH229455	9	S74R, S164T, I295V	Sensitive
A/India/Bhu-33144/2017	MH229453	9	S74R, I295V	Sensitive
A/India/Pun-1722287/2017	MF319590	9	S74R, I295V	Sensitive

Contd...

Strain name	Accession number	Glycosylation site	Amino acid change	Susceptibility to antiviral (oseltamivir)
A/India/Pun-1720775/2017	MF319572	9	S74R, I295V	Sensitive
A/India/Pun-1722256/2017	MF319588	8 (Loss of N-glycosylation)	S74R, I295V	Sensitive
A/India/Pun-1722376/2017	MF319587	9	S74R	Sensitive
A/India/Pun-1726441/2017	MG271886	9	S74R, I295V	Sensitive
A/India/Pun-1728697/2017	MG271900	9	S74R, S164T, I295V	Sensitive
A/India/Pun-1727283/2017	MG271898	9	S74R, S164T, I295V	Sensitive
A/India/Pun-1728161/2017	MG271899	9	S74R, I295V	Sensitive
A/India/Man-AF7821/2017	MG572210	9	S74R, S164T, I295V	Sensitive
A/India/Man-AF7638/2017	MG572213	9	S74R, S164T, I295V	Sensitive
A/India/Man-AF9834/2017	MG572216	9	S74R, S164T, I295V	Sensitive
A/India/Man-AF9709/2017	MG572215	9	S74R, S164T, I295V	Sensitive
A/India/Man-AF7736/2017	MG572209	9	S74R, S164T, I295V	Sensitive
A/India/Man-AF7881/2017	MG572211	9	S74R, S164T, I295V	Sensitive
A/India/Man-AF7809/2017	MG572214	9	S74R, I295V	Sensitive
A/India/Man-AF3154/2017	MG572212	8 (Loss of N-glycosylation)	S74R, I295V	Sensitive
A/India/Man-AF7729/2017	MG572208	8 (Loss of N-glycosylation)	S74R	Sensitive
A/India/Man-AG4823/2017	MH236898	9	S74R, S164T, I295V	Sensitive
A/India/Che-1721549/2017	MF319575	9	S74R, S164T, I295V	Sensitive
A/India/Che-1721030/2017	MF319568	9	S74R, I295V	Sensitive
A/India/Che-1720871/2017	MF319569	9	S74R, I295V	Sensitive
A/India/Che-1721657/2017	MF319573	9	S74R, I295V	Sensitive
A/India/Che-1720872/2017	MF319571	9	S74R, I295V	Sensitive
A/India/Che-1722045/2017	MF319566	9	S74R, I295V	Sensitive
A/India/Che-1722634/2017	MH794229	9	S74R, I295V	Sensitive
A/India/Che-1722637/2017	MH794228	9	S74R, I295V	Sensitive
A/India/Che-1722631/2017	MH794231	9	S74R, I295V	Sensitive
A/India/Che-1722633/2017	MH794230	9	S74R, I295V	Sensitive
A/India/Che-1722639/2017	MF319562	9	S74R, I295V	Sensitive
A/India/Che-1721552/2017	MF319574	9	S74R, I295V	Sensitive
A/India/Che-1722640/2017	MF319563	9	S74R	Sensitive

S, serine; R, arginine; T, threonine; I, isoleucine; V, valine

(Figure). Though the global frequencies of these mutations were more than 66 per cent, antigenically, the virus remained similar to the A/Michigan/45/2015 strain[16]. These mutations seem to be fixed after 2016. The clade 6B.1 showed further subgrouping with additional mutations S74R, S164T and I295V, and analysis of clinical data and outcome of the influenza disease showed no significant association between the presence of mutations and severity of disease.

The accession numbers, amino acid (aa) changes, glycosylation sites and virus susceptibility are summarized in the Table. All viruses were found to be sensitive to oseltamivir with H275. The IC_{50} values of all the study viruses showed normal inhibition (IC_{50} range 0.1 to 20 nMols) as compared to that of mutant virus (450-1200 nMols) in phenotypic assay.

Since the emergence of the pandemic influenza A/H1N1 virus in 2009, it has undergone considerable molecular evolution. However, the virus remained antigenically similar to the influenza A/California/07/2009 strain which was the recommended vaccine component from 2009 to 2016. The WHO updated the vaccine components for 2016-2017 northern hemisphere (NH) and 2017 southern

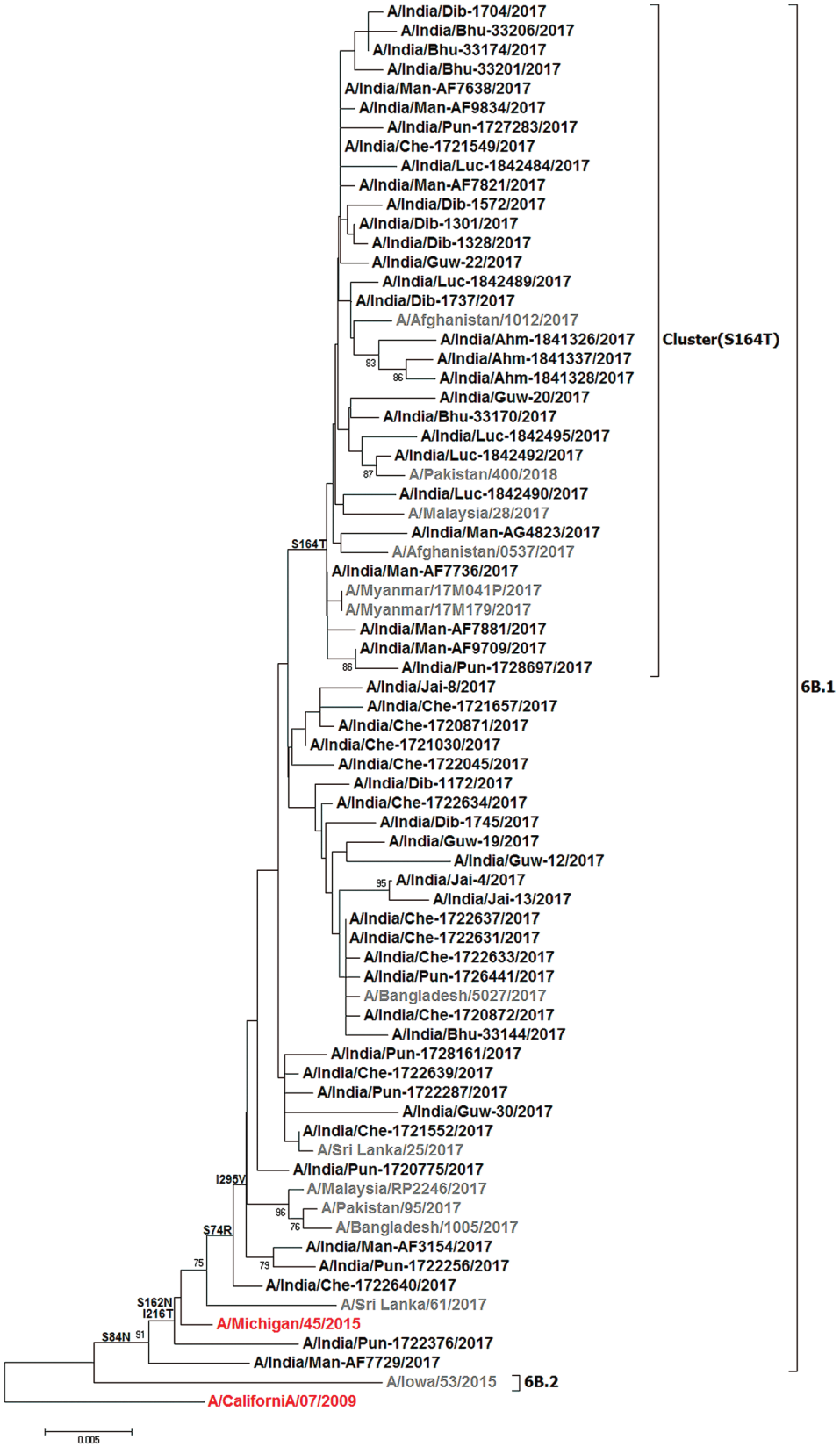

Figure. Phylogenetic analysis of the *HA* gene of A(H1N1)pdm09 2017 strains from India. The phylogenetic tree was constructed using the MEGA6 software with the neighbour-joining method. The Indian strains are represented in black and global representative strains in grey. The vaccine components are indicated in red; the scale bar indicates the nucleotide substitutions per site.

hemisphere (SH) from A/California/07/2009 virus to A/Michigan/45/2015 strain, and these viruses were detected globally since 2015-2016 representing Clade 6B.1[14]. During the study period, it was found that the influenza A(H1N1)pdm09 viruses circulating in several parts of India were similar to the globally circulating A/Michigan/45/2016-like viruses which were 2017 SH and 2017-2018 NH vaccine components. Further, these viruses on *HA* gene phylogeny showed evolution and subgrouping with S74R, S164T and I295V mutations. These mutations were found to be signature mutations of the 2017 strains. It is presumed that these are going to predominate in the future also[17]. The S164T was the most recent mutation seen in the 2017 strains, and analysis with the global strains showed that about 32 per cent of sequences had this mutation; however, analysis of clinical data and outcome of the influenza disease did not indicate any significant association between the presence of mutation and severity.

The laboratories from Pune, Chennai, Manipal and Dibrugarh had the capacity for virus isolation and isolated 2017 viruses. Thus, these influenza A(H1N1) pdm09 viruses antigenically remained the same as that of A/Michigan/45/2016 strain. The amino acid changes in *HA* genes S74R and S164T observed in the Indian strains and globally are believed to play a role at the viral oligomerization interfaces, antibody recognition sites and binding small ligands, while I295V also has a probable role in binding ligands (*http://flusurver.bii.a-star.edu.sg/*).

The previously reported influenza A/California/07/2009-like Indian viruses[12,13] possess eight glycosylation sites at positions 10, 11, 23, 87, 276, 287, 481 and 540 in *HA* gene. Notably, three virus isolates in this study from Manipal (2) and Pune (1) had eight glycosylation sites similar to the A/California/07/2009 strain. However, all the other Indian A/Michigan/45/2015-like viruses possessed an amino acid change at S162N that resulted in a gain of N-glycosylation sites at 162 positions in *HA* gene. Seven partial sequences from Dibrugarh showed five glycosylation sites at amino acid position 162, 276, 287, 481 and 540. Influenza A(H1N1)pdm09 antibodies were directed to each of the two-strain specific (Sa and Sb) and common antigenic sites (Ca and Cb) of the virus HA[18]. In the present study, A/Michigan-like A(H1N1)pdm09 study viruses showed altered amino acids in the antigenic sites.The sites S157L (singleseq from Jai-8), L161I (singleseq from Luc-1842490) and S164T (28seq) falls in Sa; R205K (singleseq from Pun-1720775) falls in Ca1; while S74R(50) fall in Cb. These antigenic sites could be important for host immune response against influenza A(H1N1)pdm09.

In conclusion, increased activity of influenza A(H1N1)pdm09 was observed in 2017, and A/Michigan/45 2016-like viruses were in circulation in different parts of India. The study also highlighted the capacity of the VRDL network to determine the antiviral susceptibility of circulating viruses which would be helpful in patient and contact management in addition to controlling the outbreaks of influenza. Continued surveillance throughout the country is required for the early detection of genetic changes in the virus and emergence of antiviral-resistant viruses, especially if this occurs in clusters.

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***Conflicts of Interest*:** None.

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