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ORIGINAL ARTICLE

Molecular characterization and evolutionary dynamics of influenza A(H1N1) strains isolated from 2015 to 2017 in North India

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ABSTRACT

Background and Objectives: The influenza A(H1N1) virus is known for large outbreaks, epidemics and pandemics worldwide owing to its genome plasticity which evolves constantly. In the year 2015 and then in 2017, India witnessed an upsurge in cases.
Materials and Methods: The study was carried out in this period (2015-2017) with samples from 5 states across north India. The hemagglutinin 1 (HA1) and non-structural 1 (NS1) gene segments of the viral genome were characterised by phylogenetic analysis, selection pressure analysis, prediction of potential glycosylation sites and phylodynamic analysis of the study strains.
Results: The study strains belonged to genogroup 6B. A total of 12 mutations were observed, half of which were located on the key receptor binding region of the HA1 protein. Established virulence markers D222G, S183P were observed in 2017 samples. Acquisition of an extra glycosylation site was observed in few strains from 2017 and 2016. Selection pressure analysis found the average dN/dS (v) ratio of 0.2106 and few codon sites in particular showed significant evidence of being under negative selection.
Conclusion: The genogroup 6B continues to be the dominant circulating strain in Indian subcontinent region however the presence of pathogenic mutations in the 2017 strains from north India underlines the importance of continued molecular surveillance.

Keywords: Influenza A virus; H1N1 subtype; Phylogeny; Heamagglutinins; Mutation; Genotype; India

INTRODUCTION

Influenza A viruses happen to be the most diverse of all the influenza viruses that exist in nature and have been a major public health threat in both developed and developing countries. Constant genomic evolution within influenza viruses, results in the emergence of new viral strains, with altered antigenicity, receptor preference, fusion properties and virulence (1). Antigenic shift helps the virus in evading the host defence, resulting in epidemics and pandemics even 100 years after the devastating Spanish flu pandemic of 1918. The Influenza A(H1N1) pandemic of 2009 had affected over 214 countries including the Indian subcontinent where more than 50000 cases were reported from India alone (2). The Influenza A(H1N1) pdm09 virus circulation declined thereafter at a low level in India till 2015, where it re-emerged

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with 42,000 cases and 2,990 deaths and in 2017 with 38000 cases and 2200 deaths (3). The present study is aimed at characterizing the genes of circulating Influenza A(H1N1) strains isolated during 2015 and 2017 outbreaks and the intervening period.

Hemagglutinin (HA1) proteins, the predominant viral antigens, are prone to mutations and remain under high selective pressure and involved in down regulating interferon production as well as enhancement of host cell apoptosis (4). Hence, the molecular characterisation of HA and NS1 genes will help in understanding virus evolution, mutations at amino acid levels and unfolding the emerging trends of virus circulation, during the period coinciding with the change in influenza vaccine strains from A/California/07/2009 to A/Michigan/45/2015.

MATERIALS AND METHODS

Sample processing and sequencing of HA and NS1 gene of Influenza A(H1N1). Respiratory samples (throat swab, nasopharyngeal aspirate or endotracheal aspirate) routinely received for Influenza A(H1N1) diagnosis from category C patients defined as those suffering from fever, cough, sore throat, nasal catarrh, or shortness of breath, as detailed in the guidelines of IDSP, GOI (5) were included during the period 2015 to 2017 were included in the study population. The study was approved by the institute ethics committee (NK/2550/MD/1923).

The samples were tested by CDC recommended qualitative real-time reverse-transcription PCR (RT-PCR). Samples positive for Influenza A(H1N1) were stored at -80°C till further processing. Viral RNA was extracted using QIAmp viral RNA mini kit (Qiagen, Germany) from Influenza A(H1N1) samples confirmed by RT-PCR. The first complementary DNA was synthesized using RevertAid kit (Fermentas). The protocol was optimised further by adding specific primers for the gene of interest (HA 1, NS1) to the master mix. The HA1 and NS1 domains from the respective HA and NS genes of Inf A(H1N1) strains were amplified by CDC recommended RT PCR protocol using Hot Start Hi-Fidelity Taq Polymerase (6). A 943 bp Region of the HA1 gene was amplified in two fragments consisted of the first segment from HA 1-461 bp followed by HA 351-943 bp, therefore included an overlapping region of 110 bp length. The NS1 gene was amplified in a single segment (NS24-482) in a 458 bp long region. Sanger sequencing of the amplified fragments were carried out using M13-forward and reverse primers on ABI 3130XI/Genetic Analyser. Consensus sequences of HA and NS gene from this study were deposited into GISAID database. The accession numbers of the sequences have been detailed in (Table 1).

Table 1. Clinical & demographic details of the study samples.

S.no	Sample ID	Accession no.	Sampling time	Age/Sex	Region	Clinical symptoms
1.	S1/PGI/2016	EPI_ISL_451075(A/India/PGIMER-1/2016)	22 March 2016	52/M	Chandigarh, India	Fever, cough , difficulty in breathing
2.	S3/PGI/2015	EPI_ISL_280130 (A/India/PGIMER-3/2015)	16 Mar 2015	46/F	Punjab, India	Fever, chest pain, shock
3.	S4/PGI/2015	EPI_ISL_420133 (A/INDIA/PGIMER-4/2015)	8 Apr 2015	78/F	Chandigarh, India	Fever, difficulty in breathing, shock
4.	S5/PGI/2016	EPI_ISL_312675 (A/INDIA/PGIMER-5/2016)	1 Apr 2016	34/M	Chandigarh, India	Fever, breathlessness, cough Fever,
5.	S6/PGI/2016	EPI_ISL_312676 (A/INDIA/PGIMER-6/2016)	11 Apr 2016	52/F	Chandigarh, India	chest pain, breathlessness
6.	S7/PGI/2016	EPI_ISL_312677 (A/INDIA/PGIMER-7/2016)	4 Apr 2016	65/M	Haryana, India	Fever, cough , difficulty in breathing
7.	S8/PGI/2016	EPI_ISL_312678 (A/INDIA/PGIMER-8/2016)	11 Apr 2016	27/F	Punjab, India	Fever, chest pain, breathlessness
8.	S9/PGI/2016	EPI_ISL_312719 (A/INDIA/PGIMER-9/2016)	21 Apr 2016	42/M	Punjab, India	Fever, chest pain, drowsiness
9.	S10/PGI/2016	EPI_ISL_312720 (A/INDIA/PGIMER-10/2016)	3 May 2016	72/M	Haryana, India	Fever, cough , difficulty in breathing
10.	S11/PGI/2016	EPI_ISL_312721 (A/INDIA/PGIMER-11/2016)	16 May 2016	58/M	Chandigarh, India	Fever, chest pain, breathlessness
11.	S12/PGI/2016	EPI_ISL_312722 (A/INDIA/PGIMER-12/2016)	19 May 2016	38/F	Chandigarh, India	Fever, cough , difficulty in breathing
12.	S13/PGI/2016	EPI_ISL_420896 (A/INDIA/PGIMER-13/2016)	15 Apr 2016	7/M	Chandigarh, India	Fever, chest pain, drowsiness
13.	S14/PGI/2017	EPI_ISL_397104 (A/India/PGIMER-14/2017)	7 Feb 2017	22/M	Chandigarh, India	Fever, cough , difficulty in breathing
14.	S15/PGI/2017	EPI_ISL_420893 (A/India/PGIMER-15/2017)	10 Feb 2017	66/F	Himachal Pradesh, India	Fever, chest pain, breathlessness
15.	S16/PGI/2017	EPI_ISL_420894 (A/India/PGIMER-16/2017)	6 March 2017	48/M	Punjab, India	Fever, cough , difficulty in breathing
16	S17/PGI/2017	EPI_ISL_420895 (A/India/PGIMER-17/2017)	15 March 2017	21/F	Chandigarh, India	Fever, cough , difficulty in breathing

Phylogenetic analysis and molecular characterisation. Homology of nucleotide and protein sequence search was carried out using the NCBI, NIH BLAST server at GenBank database. The H1N1 sequences for phylogenetic analysis database preparation were retrieved from NCBI. Sequences from all geographical regions across the globe, with special emphasis on Indian isolates and those submitted in the recent past were included. Phylogenetic analysis was performed using MEGA7: Molecular Evolutionary Genetics Analysis version 7.0, (Center for Evolutionary Medicine and Informatics, Arizona State University, USA) through the maximum likelihood method following 500-replicate bootstrapping method. Mean genetic distance within and between the clusters were determined by MEGA v7.0. Strains were analysed using WHO reference sequences of A/Michigan/45/2015(H1N1) and A/California/07/2009(H1N1) strains. Each mutation was analysed through flu-server emphasizing the demographic and epidemiological information related to frequency of the mutation, geographical distribution and its significance in viral pathogenesis.

Quaternary structure analysis of HA1 and NS1 proteins. 3D structure analysis was done on the model generated by the coordinates of (Protein database) PDB ID:1ruz and 3mr through pymol 2.0. The mutation sites were localised on the 3D crystallographic structure through PyMOL 2.0 (Schrodinger, LLC, New York, NY, USA) to improve spatial clarity and better appreciation of the structural domain effected by the mutation.

Adaptive evolution and molecular clock analysis. Selection pressure acting upon the codons of HA1 and NS1 genes of Influenza A(H1N1) virus was studied using HyPhy open-source software under the datamonkey web server (http://www.datamonkey.org/). The ratio of nonsynonymous (dN) to synonymous (dS) substitutions per site (dN/dS) was estimated using single likelihood ancestor counting (SLAC) approach. The best nucleotide substitution model for different data sets available in datamonkey server was adopted in the analysis. In order to assess and compare the evolutionary dynamics of Influenza A(H1N1) strains, global HA data sets were downloaded from GenBank and GISAID for H1N1 extending from 2009 to 2017. For reconstructing past population dynamics, the coalescent- based Gaussian Markov random field (GMRF) method with the time-aware smoothing parameter was used in BEAST v.1.8.1. Uncorrelated lognormal relaxed molecular clock, the SRD06 codon position model 65, was used for the analysis to allow partitioning for codon positions with the HKY85.G substitution model applied to these codon divisions. Convergence of runs was checked in Tracer v.1.6 to ensure adequate mixing. The GMRF plots and the maximum clade credibility trees were visualized in FigTree v1.4.2 (University of Edinburgh).

RESULTS

Sample details. The study included H1N1 positive samples collected over a three year period (2015-2017). A total of 16 positive samples from patients presenting between February 2015 to March 2017 were included (2015: 2 samples, 2016: 10 samples, 2017:4 samples) which fulfilled the inclusion criteria mentioned below.

All the cases included in the study presented with breathlessness, 2 cases presented with shock. Of the 16 cases included, one had a travel history to middle east 2 weeks prior to presentation. The cases were followed up for a period of 1 month, showed a mortality of 25%.

Inclusion criteria. Category C patients as per IDSP (Integrated disease surveillance program) guidelines.

Amino acid divergence. The percentage amino acid identity of the study strains was compared with the existing and previous H1N1 Influenza vaccine strains (A/California/07/2009 and A/Michigan/45/2015). The analysis sequences of study strains (n=18) comprising 9 study strains, 5 Indian reference strains and 4 global reference isolate. The study isolates were found to share a greater similarity to the present vaccine strain A/Michigan/45/2015.

The sequences of A/Michigan/45/2015 and A/California/07/2009 were aligned using MEGA7.0 and the mutation sites were identified as represented in (Table 2). The functional role of the mutations along with their epidemiological reference was assessed through the Flu server database. On comparison of the HA1 polypeptide chain of the isolates with reference to A/Michigan/45/2015 strains, non-synonymous amino acid mutations were observed in fusion protein (T4A, R45K), Vestigial esterase (E68K, S74R, N84S, D86N)

Table 2. Mutations in the HA1 and NS1 gene segments of InfA(H1N1)pdm09 isolates of the study with respect to A/Michigan/45/2015 and A/California/07/2009. Residues not identical to A/Michigan/45/2015 are represented in coloured fonts

	Amino acid positions showing variations (HA1)																	
	Fusion		Vestigial esterase						Receptor binding domain									
	4	45	68	74	83	84	86	97	129	162	163	183	185	203	216	222	223	256
A/Michigan/45/2015	T	R	E	S	P	N	D	N	N	N	Q	S	T	T	T	D	R	T
S1/2016/PGI	T	R	E	S	P	N	D	N	N	S	Q	S	T	T	I	D	Q	T
S3/2015/PGI	A	R	E	S	P	S	N	N	N	S	Q	S	T	T	I	D	Q	T
S4/2015/PGI	A	R	E	S	P	S	N	N	D	S	Q	S	T	T	I	D	Q	T
S5/2016/PGI	T	K	E	S	P	N	D	N	N	S	Q	S	T	T	I	D	Q	T
S6/2016/PGI	T	R	E	S	P	N	D	N	N	N	Q	S	T	T	T	D	Q	T
S7/2016/PGI	T	R	E	S	P	N	D	N	N	S	Q	S	T	T	I	D	Q	T
S8/2016/PGI	T	K	E	S	P	N	D	N	N	S	Q	S	T	T	I	D	Q	T
S9/2016/PGI	A	R	E	S	P	S	D	N	N	S	Q	S	T	T	I	D	Q	T
S10/2016/PGI	T	K	K	S	P	N	D	N	N	S	Q	S	T	T	I	D	Q	T
S11/2016/PGI	T	K	E	S	P	N	D	N	N	S	Q	S	T	T	I	D	Q	T
S12/2016/PGI	T	R	E	S	P	N	D	N	N	N	Q	S	T	T	T	D	Q	T
S13/2016/PGI	T	K	E	S	P	N	D	N	N	S	Q	S	T	T	I	D	Q	T
S14/2017/PGI	T	R	E	S	P	N	D	N	N	S	Q	S	T	T	T	D	Q	T
S15/2017/PGI	T	R	E	R	P	N	D	N	N	S	Q	S	T	T	T	G	Q	T
S16/2017/PGI	T	R	E	S	P	N	D	N	N	S	Q	S	T	T	T	D	Q	T
S17/2017/PGI	T	R	E	R	P	N	D	N	N	S	Q	P	T	T	T	D	Q	T
A/California/07/2009	A	R	E	S	S	S	D	D	N	S	K	S	S	S	I	D	Q	A

and the receptor binding regions (N129D, N162S, S183P, T216I, D222G, R223Q) (Table 2). Novel non-synonymous mutation E68K was located in the vestigial esterase region in one of the isolates from 2016. Present isolates on comparison to the A/California/07/2009 strains had 15 mutations in HA protein of which the mutated AA residues at positions 83, 97, 163, 185, 203 and 256 were found to be identical to the corresponding amino acids in A/Michigan/45/2015 reference strain. The mutational analysis of NS1 gene among the study sequences on comparison to the reference strain A/California/07/2009 showed a total of 6 non-synonymous mutations (E55K, T86A, L90I, I123V, E125D, K131E) in the NS1 protein. Among them the notable mutation I123V was present in 100% of the study strains. The T86A mutation has been reported only on 7 instances so far in the flu server, similar is the finding in the present study where only one strain had the mutation. It might play a role in viral oligomerization because of its position on the NS1 protein.

Modelling of mutation sites on 3D crystallographic structure of HA. The 3D structure of trimeric HA and NS1 protein was generated by the coordinates of PDB ID: 1ruz and 3mr from Research Collaboratory for Structural Bioinformatics (RCSB) protein data base (PDB) webserver, using PyMOL(TM) Molecular Graphics System, Version 1.8.2.0. The crystallographic structure generated was modelled to depict mutations at corresponding AA site by sphere representation of the mutated amino acid for appreciation of the structural domain involved mutation (Fig. 1).

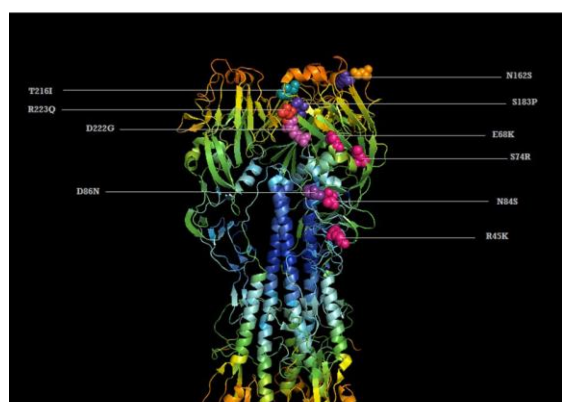

Fig. 1. Three-dimensional quaternary structure of HA 1 trimeric protein of Influenza A(H1N1)pdm09. Mutations observed in the study strains of (2015-2017) compared to A/Michigan/45/2015 have been represented by spheres

Prediction of potential glycosylation sites in the HA1 protein. The pattern of glycosylation sites was studied using N-glyc web server for the study strains (sequence length-310 AA). A/Michigan/45/2015 strain was found to show an extra glycosylation site at position 162 when compared to the glycosylation pattern of A/California/07/2009. Out of the strains studied (2015: 2, 2016: 10, 2017: 4), two each from 2016 and 2017 (S6/2016/PGI, S12/2016/PGI, S15/2017/PGI, S17/2017/PGI) showed the presence of extra glycosylation site at position 162, while remaining strains shared the glycosylation pattern similar to A/California/07/2009.

Phylogenetic analysis. Phylogenetic analysis of sixteen HA sequences (2015: 2, 2016: 10, 2017: 4), together with globally distributed Influenza A (H1N1) strains representing all genogroups from various geographical regions and timeline was performed, with a special emphasis on isolates from the northern part of the Indian subcontinent. In the phylogenetic tree, the isolates from 2015 to 2017 clustered with the strains of genogroup VI (Fig. 2) retaining the clade 6B specificity.

Evolutionary dynamics of circulating H1N1 strains. The mean nucleotide substitution rate for HA1 gene through Flu Skyline strict molecular clock was found to be: $3.41X10^{-3}$ [95% highest posterior density (HPD): $2.44X10^{-3}$-$4.47X10^{-3}$] for the global isolates and $3.38X10^{-3}$ [95% HPD interval: $2.23X10^{-3}$-$4.39X10^{-3}$] for the present Indian isolates.

Selection pressure analysis. Selection pressure studies were performed using datamonkey webserver HyPhy web package. Site specific selection pressure of HA1 gene was analysed by maximum likelihood procedure, using HKY85 substitution model as predicted by the webserver. P-value < 0.1 for SLAC indicated high statistical significance. Considering that the predominant clade in circulation was clade 6B the isolates included in the dataset belonged to clade 5, 6A, 6B and 6C in addition to the circulating strains of past 5 years (2012-2017). The average dN/dS (v) ratio for the HA1 was 0.210686, implying a non-synonymous substitution has about 21% as much chance as a synonymous mutation of being fixed. Among the study isolates, 4 negatively selected sites in HA protein were found at amino acid positions 2, 10, 48 and 132.

The isolates included in the dataset for selection pressure analysis of NS1 gene were the circulating strains obtained from the flu server, from the past few years (2012-2017). The average dN/dS (v) ratio for the NS1 was found to be 0.35377, implying a nonsynonymous substitution has about 35% as much chance as a synonymous mutation of being fixed. Among the study isolates, 3 negatively selected sites were observed at residues 29, 83 and 127 of NS gene.

DISCUSSION

The influenza A (H1N1) virus is well known for pandemic potentiality, owing to its constantly evolving segmented RNA genome helping the virus to escape the host immune mechanism which has led to its worldwide persistence even 100 years after the Spanish flu pandemic. During the post pandemic period since 2011 the cases declined and the circulation of Influenza A (H1N1) pdm09 persisted only at a low level in the population. However the occurrence of two large outbreaks in India (2015 and 2017) within a gap of two years was intriguing (7). The host population having been already exposed to the virus in 2015, a recurring outbreak in 2017 could possibly indicate the virus gaining virulence rather than immune naivety of the population.

The present study, thus investigated the circulating Influenza A (H1N1) strains from the two outbreaks (2015 and 2017) and of the intervening period of low disease activity (2016). Earlier studies had reported predominant co-circulation of clades 6 and 7 viruses with infrequent clade 5 strains of the Influenza A(H1N1) pdm09 (8). However since 2015, clade 6B appears to have replaced the other circulating clades in north, central and eastern India (2, 6, 7) and continues to be the dominant clade even in the 2017 outbreak.

Interestingly, the study period coincided with the change of influenza vaccine candidate by the WHO from A/California/07/2009 to A/Michigan/45/2015 in April 2017. All the study strains from northern India had a greater similarity to the A/Michigan/45/2015 (97.6-99.3%) than A/California/07/2009 strains (95.7- 97.8 %) justifying the change in vaccine strain in the Indian subcontinent as well. A total of 12 mutations were observed on mutational analysis of the strains in HA1 proteins. However mutations (T200A, D225N) predicted by *in silico* analysis from MIT, were not found which is in agreement with oth-

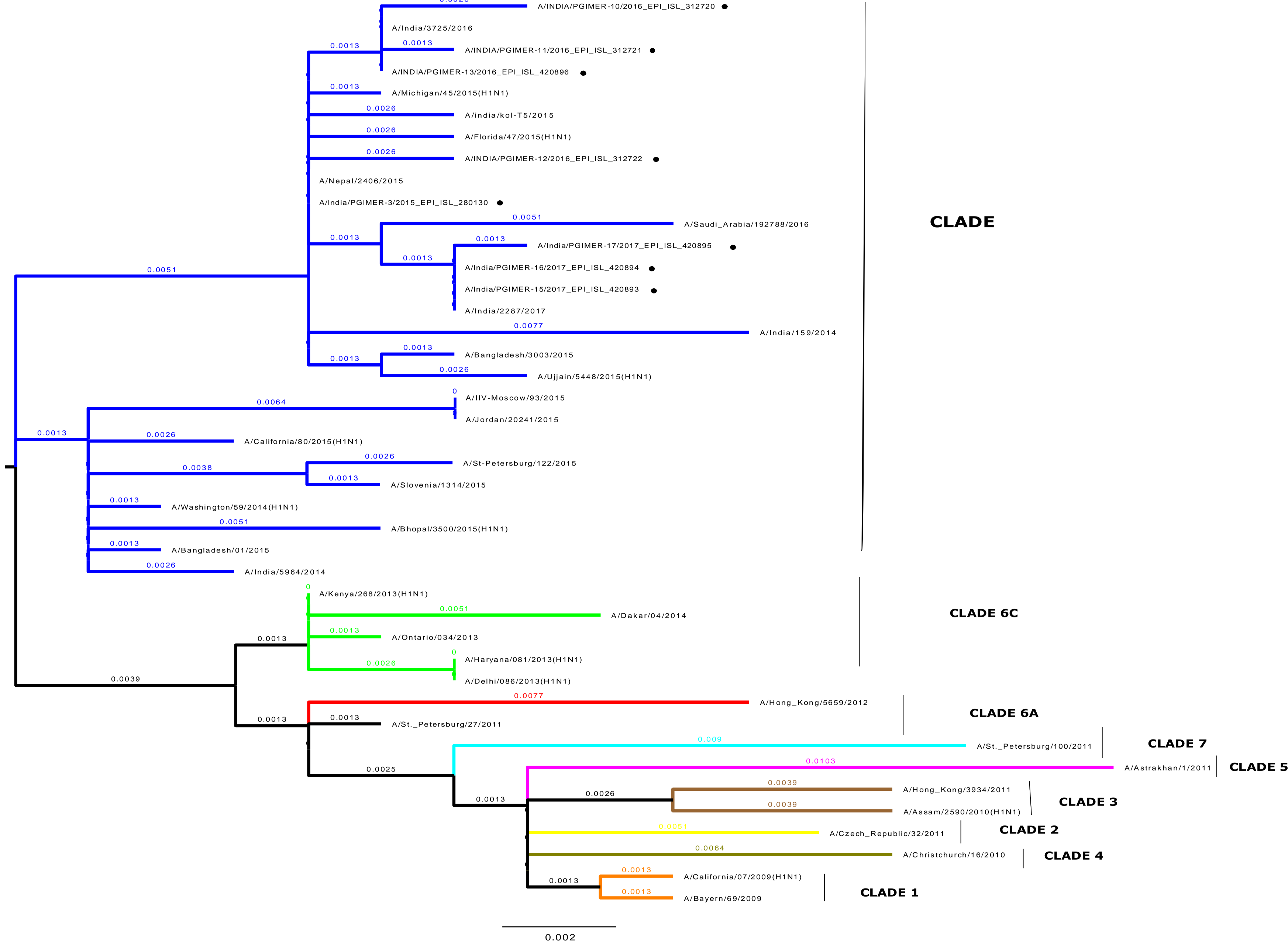

Fig. 2. Phylogenetic analysis of influenza A(H1N1)pdm09 representative study sequences obtained from 2015 to 2017 from India and representative global isolates using the maximum likelihood method

er studies from central and eastern India (9). Of the total 12 mutations reported, 6 (50%) clustered within the receptor binding site at the globular head region of the HA1 polypeptide molecule as has been depicted in (Fig. 2) (10). The Receptor binding domain plays a key role in the viral-host protein interaction and in governing the viral pathogenesis. The most significant mutation observed in the present study was at the residue 222(D222G) in a viral strain from 2017 (S15/2017/PGI). The D222G mutation is known to cause shift in the receptor preference of the virus from α 2, 6 to α 2,3 sialic acid receptors. The α 2,3-linked sialic acid receptors are found in avian hosts as well as in the lower respiratory tracts of human beings. Hence, shift in receptor preference might predispose increased avian transmission and lower respiratory tract involvement in humans, implying disease severity and increased mortality (11, 12). The acquisition of a highly pathogenic marker (D222G) in 2017 outbreak (even in an isolated case) appears to be relevant and necessitates the continuous monitoring of the mutation in subsequent years. A single strain (S17/2017/PGI) from 2017 outbreak had a significant change in the receptor binding domain, with proline at residue 183 instead of serine, a situation resembling the 1918 (H1N1) virus, which had a proline at 183 residue. The S183P mutation observed in S17/2017/PGI strain has previously been reported in the H1N1(2009) isolates from New Delhi but not with the 2015 strains (12). The significance of S183P mutation in HA protein needs to be elucidated. S74R mutation at residue 74 in the antigenic site Cb within the vestigial esterase domain near the base of the globular head was seen in two strains of 2017. N162S mutation was located in the Sa antigenic site, near the spike tip of HA1 transmembrane protein where serine replacing asparagine was present in 81% of the isolates (Table 2). indicating a possible drift at position 162 from serine to asparagine rendering incidentally an extra glycosylation site. Asparagine residue at this site facilitates a common post transcriptional modification in the form of N linked glycosylation. Glycosylation of a protein is predicted to occur when amino acid sequence of Asn-X-Ser/Thr-Y occurs, where X/Y may be any amino acid apart from proline which sterically hinders glycosylation. The attachment of glycans at the glycosylation site of HA, masks the regions vulnerable to antibody neutralization thereby helping the virus to evade the host immune system. The loss in glycosylation site exposes the surface to antibody mediated response promoting viral elimination. A novel mutation E68K was also observed in S10/2016/PGI strain of 2016, lying on the globular head, specifically in the vestigial esterase region of the HA1 protein with unknown significance. The mutation analysis of NS1 gene segment in comparison to A/California/07/2009 identified a total 6 mutations, of which 4 (E55K, L90I, I123V, K131E) were present in 100% of the samples (16/16) examined. The known polymorphism I123V based on which the NS1 strains are grouped was found in all the isolates (13). E125D mutation was present in 6 out of 16 (37%) of the study isolates. However on comparison of the NS1 sequences to the present reference strain (Table 2), they showed high similarity. A single mutation was observed in an isolate from 2017 (S15/2017/PGI), at residue 86, a rare mutation in the linker region of NS1 protein.

The HA1 domain containing antigenic sites are driven by the host's immune response and are under continuous selection pressure. Selection pressure analysis on HA1 protein of pandemic H1N1 viruses showed the average dN/dS (v) ratio for the HA was 0.2106. The four codon sites (2, 10, 48, 132) showed significant evidence of being under negative selection i.e an property to resist any change at the residue level as it might lead to proteins with reduced functionality. Past selection pressure studies had showed the HA gene of isolates to be under a stronger purifying selection both for Indian (dN/dS =0.373) as well as global isolates (dN/dS =0.345). The early pandemic period demonstrated a significantly higher dN/dS and strong purifying selection mostly driven by adaptation to the human host. This decrease in global dN/dS rates may be attributed to a relaxed selection in most genes including the HA gene following adaptation of Influenza A(H1N1) pdm09 strains to the human host.

The temporal phylogenies of global genomic sequences in the circulatory strains revealed significantly slower evolutionary rate in the HA than the global H1N1/2009 strains during the pandemic period [5.3×10^3 (95% HPD: 4.76-5.95×10^3)] (14). Thereby, indicating graded adaptation of the circulatory strains with the population exposed over the years similar to the observation with seasonal influenza viruses (15, 16).

Due to the diverse nature of influenza viruses along with their capacity to undergo genetic shifts and drifts over the years, there is a property for with

emergence of virulent strains with high transmissibility and virulence. Thus, it is the need of the hour to establish a mechanism for continuous surveillance of the circulating strains for active molecular mapping and virulence markers.

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REFERENCES

1. Nelson MI, Holmes EC. The evolution of epidemic influenza. *Nat Rev Genet* 2007; 8: 196-205.
2. Parida M, Dash PK, Kumar JS, Joshi G, Tandel K, Sharma S, et al. Emergence of influenza A (H1N1) pdm09 genogroup 6B and drug resistant virus, India, January to May 2015. *Euro Surveill* 2016; 21: 6-11.
3. Central Surveillance Unit, Integrated Disease Surveillance Programme, NCDC, Delhi. Seasonal Influenza (H1N1)–State/UT-wise, Year-wise number of cases and death from 2010 to 2017 (till 31st December 2017). 2018 Jun 5; Available from: https://ncdc.gov.in/index4.php?lang=1&level=0&linkid=119&lid=276
4. Caglioti C, Selleri M, Rozera G, Giombini E, Zaccaro P, Valli MB, et al. In-depth analysis of HA and NS1 genes in A(H1N1)pdm09 infected patients. *PLoS One* 2016; 11(5): e0155661.
5. Ministry of Health & Family Welfare Seasonal Influenza. Guidelines on categorization of Seasonal Influenza cases during screening for home isolation, testing, treatment and hospitalization (25.02.2019), 2019. https://health.py.gov.in/guidelines-categorization-seasonal-influenza-cases-during-screening-home-isolation-testing-treatment
6. WHO. Sequencing primer and protocol. 2009. https://cdn.who.int/media/docs/default-source/influenza/antiviral-wg-surveillance/genomeprimers_20090512.pdf?sfvrsn=d3b48809_8
7. Murhekar M, Mehendale S. The 2015 influenza A (H1N1) pdm09 outbreak in India. *Indian J Med Res* 2016; 143: 821-823.
8. Mir MA, Lal RB, Sullender W, Singh Y, Garten R, Krishnan A, et al. Genetic diversity of HA1 domain of hemagglutinin gene of pandemic influenza H1N1pdm09 viruses in New Delhi, India. *J Med Virol* 2012; 84: 386-393.
9. Tharakaraman K, Sasisekharan R. Influenza surveillance: 2014–2015 H1N1 "Swine"-derived influenza viruses from India. *Cell Host Microbe* 2015; 17: 279-282.
10. Caton AJ, Brownlee GG, Yewdell JW, Gerhard W. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* 1982; 31: 417-427.
11. Liu Y, Childs RA, Matrosovich T, Wharton S, Palma AS, Chai W, et al. Altered receptor specificity and cell tropism of D222G hemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus. *J Virol* 2010; 84: 12069-12074.
12. Barde PV, Sahu M, Shukla MK, Bharti PK, Sharma RK, Sahare LK, et al. The high frequency of non-aspartic acid residues at HA222 in influenza A(H1N1) 2009 pandemic viruses is associated with mortality during the upsurge of 2015: a molecular and epidemiological study from central India. *Epidemiol Infect* 2017; 145: 2656-2665.
13. Campanini G, Piralla A, Paolucci S, Rovida F, Percivalle E, Maga G, et al. Genetic divergence of influenza A NS1 gene in pandemic 2009 H1N1 isolates with respect to H1N1 and H3N2 isolates from previous seasonal epidemics. *Virol J* 2010; 7: 209.
14. Su YCF, Bahl J, Joseph U, Butt KM, Peck HA, Koay ESC, et al. Phylodynamics of H1N1/2009 influenza reveals the transition from host adaptation to immune-driven selection. *Nat Commun* 2015; 6: 7952.
15. Jones S, Nelson-Sathi S, Wang Y, Prasad R, Rayen S, Nandel V, et al. Evolutionary, genetic, structural characterization and its functional implications for the influenza A (H1N1) infection outbreak in India from 2009 to 2017. *Sci Rep* 2019; 9: 14690.
16. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* 2009; 459: 1122-1125.