SHORT COMMUNICATION

Molecular analysis of influenza A H1N1pdm09 virus circulating in Madhya Pradesh, India in the year 2017

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Abstract Influenza A (H1N1) pdm09 emerged as a major public health problem in 2009. The virus has evolved since then and has acquired several mutations that are epidemiologically important. Viral hemagglutinin (HA) protein is crucial determinant for virus entry, antigenicity, and virulence. The information regarding amino acid substitutions in HA protein of viruses circulating in India during 2017 is lacking. We sequenced HA gene of Influenza A (H1N1) pdm09 detected in Central India and compared the amino acid sequences with present vaccine component and contemporary Influenza A (H1N1) pdm09 sequences. We observed 7 amino acid changes of which two (T508A and I510T) were novel to Central Indian strain. Further, the substitution of Aspartate by Glycine at 222, which is known to have clinical implications, was detected in sequences from western India. The phylogenetic analysis revealed that the circulating virus belonged to clade 6B.1. The appreciable acquired mutations by the virus are not important antigenically and the present vaccine provides effective protection. On the other hand, the amino acid substitutions may play important role in epidemiology; we suggest rigorous molecular monitoring and documentation for timely interventions.

Keywords Influenza A H1N1 pdm09 · Hemagglutinin protein · 2017 · Central India

Influenza A virus, owing to its ability to undergo antigenic shifts and drifts, is a serious health concern across the globe. Every year about 5 million cases and up to 650,000 fatalities are attributed to Influenza ([http://www.who.int/news-room/fact-sheets/detail/influenza-\(seasonal\)](http://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal))). In 2009, a new subtype of the Influenza A virus, designated as A (H1N1) pdm09 emerged, resulting in a pandemic; still causing local outbreaks and sporadic cases around the world ([http://www.who.int/news-room/fact-sheets/detail/influenza-\(seasonal\)](http://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal))). Since its inception in May 2009, till date, more than 117,000 laboratory confirmed cases and 8847 deaths are reported from India (<http://idsp.nic.in/index4.php?lang=1&level=0&linkid=453&lid=3906>).

The virus since its emergence has undergone several molecular changes that are clinically and epidemiologically important [1–4, 6, 14]. The changes in surface antigen hemagglutinin (HA) are known to affect severity, antigenicity, transmissibility of virus and are pith of vaccine design; therefore even a point mutation resulting in antigenic drift is avidly monitored across the world [5, 9]. The studies have shown that the viruses belonging to the clade 6B.1 with high polymorphism were circulating in India during 2015–2016 [1, 10]. However, no information is available on this aspect for the year 2017, to fulfill the lacuna, we sequenced HA gene of A(H1N1)pdm09 detected in 2017 in Central India and analyzed those sequences by comparing them with vaccine strain (A/Michigan/45/2015) and other sequences reported from India.

The samples of suspected Influenza patients are referred to virology laboratory of ICMR-NIRTH for diagnosis (<http://www.tngmssh.tn.gov.in/news/guidelines/h1n1.pdf>). After the diagnosis, part of the sample was stored at – 70 °C for future use. The Influenza A(H1N1)pdm09 positive samples showing high viral RNA load in diagnostic real time RT-PCR (qRT-PCR) were randomly

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selected for the HA gene sequencing. The sequencing and molecular analysis were done by following WHO protocol as described earlier [12] (http://www.who.int/influenza/resources/documents/molecular_diagnosis_influenza_virus_humans_update_201108.pdf). Resulting sequences were analyzed and submitted to GenBank. The sequences were analyzed for homology by comparing them with sequences available in GenBank (Table 1). Further in order to, understand the importance of mutations and their frequency, sequences were analyzed using free online tool BII Flusurver (<http://flusurver.bii.a-star.edu.sg/>). Clade of the submitted sequences was established by comparing 35 sequences of clade 6 and 7 downloaded from GenBank, using the software MEGA5.05.

During the study period of January to December 2017, a total of 887 throat swab samples were tested of which, 229 (25.8%) were found positive for A(H1N1)pdm09. Out of randomly picked six positive samples having high viral RNA; HA gene of two could be successfully amplified and the sequences of 1701 bases of these were submitted to GenBank (GenBank accession No.: MH160789 and MH160790). Sequence 1 (GenBank accession No.: MH160789), was from sample of a female patient of age 56 years, from West Madhya Pradesh. She had history of a lung tumor for which she was operated. Four weeks after operation, she had influenza like illness i.e. fever, cough, sore throat, breathlessness, chest pain, headache etc. and was admitted to hospital. She succumbed to infection on second day of hospitalization. The second sequence (GenBank accession No.: MH160790) was from patient of age 3.5 years girl from Central Madhya Pradesh. She was sick for 3 days with fever, sore throat, cough and breathlessness; she recovered from illness after 10 days of hospitalization and was discharged. When these sequences were compared with A/Michigan/45/2015, seven and three amino acid (a.a.) changes were recorded in MH160789 and MH160790 sequences respectively. We further compared these two sequences of 2017 with the sequences reported in 2015 from Central India and seven a.a changes were noted. The comparison of sequences with that reported from Kolkata, Eastern India and Pune, Dhule Western India in 2016–2017, revealed that sequences from Central India had variations. The sequence from Western India (accession No. MF319594) had five substitutions, whereas sequences (accession Nos. MF319590 and MF319587) had four changes and one sequence (accession No. MF319588) had two mutations when compared with Central India sequences. Interestingly sequences from western India, (accession Nos. MF319594 and MF319590) had substitution of Aspartate (D) by Glycine (G) at position 222; but this was not detected in sequences of 2017 from Central and Eastern India. Phylogenetic analysis revealed that the virus strain circulating in 2017 belonged to Clade 6B.1 (Fig. 1).

In comparison to A/Michigan/45/2015 we detected 7 changes at a.a. level, indicating that the virus has undergone changes since 2015. We observed a.a. change at position 73 from Alanine (A) to Valine (V). This change was not detected in Indian strains so far; globally too, this change was less frequent and was reported from Shiraz, Iran in 2015 (accession No.: KY930507.1) and Texas, USA in 2016 (accession No.: KX407851.1). Further, we detected Serine (S) 74 to Arginine (R) and Isoleucine (I) 295 to V mutations; these mutations are acquired by the virus recently, and are detected at high frequency (66%) globally. These are presumed to predominate in the future [15]. The S164 to Threonine (T) is the most recent mutation seen in 2017 strains and the analysis of global strains showed, about 32% sequences are having this mutation [15]. The change at a.a. 321 from Valine (V) to I observed in one strain (accession no.: MH160789) was also detected from Kolkata in 2017 (accession No.: MG905591). Previously, this mutation had been reported from Russia in 2016 and USA in 2016–2017, occurring at an estimated frequency of 0.52% (<http://flusurver.bii.a-star.edu.sg/>). Amino acid substitution of T508A and I510T are unique changes to the Central Indian sequence (accession No.: MH160789) and are not so far reported from India. However, these mutations have been reported in 2016 from Texas, USA. A73V, T508A, and I510T have low global occurrence with a frequency of 0.08, 0.12, and 0.04%, respectively (<http://flusurver.bii.a-star.edu.sg/>).

On comparison of sequences with sequences from Western India, we detected mutations having significant clinical implications. The substitution of Asparagine (N) by S at 162 was observed in the sequence (accession No. KT369728) of 2015 from Central India, but was missing from 2017 sequences of the region, however, was detected in Western Indian sequences from Pune (accession No.: MF319590) and Dhule (accession No.: MF319594). The substitution of NQS/T at 162–164 by SQS, results in a loss of a potential N-glycosylation site at position 162 which may affect antigenic properties of this strain [7, 11, 13]. Influenza A virus 2017 strains of Central India seems to have reverted back to NQT (162–164) gaining a glycosylation site when compared with 2015 Central Indian strains possessing SQS (162–164). Accumulation of glycosylation site at antigenic site by the Influenza virus helps it to escape from neutralizing antibodies. However, the substitution N162S was noted in strains from Western India (Pune accession. No.: MF319588 and Dhule accession. No.: MF319594). It will be worth to monitor the clinical-epidemiological role of this mutation in the near future.

Another important a.a. change of D222N/G affects virus tropism, helping virus to bind with α -2,3 receptors present mainly in the lower respiratory tract, thus enhancing the severity of disease and mortality [1, 8, 9] was detected in

Table 1 Showing changes at amino acid level in Influenza A H1N1pdm09 virus circulation in Madhya Pradesh when compared with Vaccine component, Strain Circulated in 2015 in Madhya Pradesh and Strains circulated in 2017 in other parts of India

Vaccine Component (2017–18)	Sequence ID	Accession No.	SPR	Mutations in HA1 and HA2															
Central Indian strain (2015) Indian Strains (Other than Central India: 2016–2017)	A/Michigan/45/2015(H1N1)	KY117023	T	A	N	V	D	R	A	S	N	F	P	N					
		AA H1 numbering	11	13	15	16	30	35	45	73	84	114	118	129					
	A/Barwani/6024/2015(H1N1)	KT369728	-	A	-	-	-	-	-	-	S	-	-	D					
	A/Kolkata/NICED-0001/2017(H1N1)	MG905591	-	-	-	-	-	-	-	R	-	-	-	-					
	A/Kolkata/NICED-0005/2017(H1N1)	MG905595	-	-	-	-	I	N	-	R	-	-	-	-					
	A/Kolkata/NICED-0007/2017(H1N1)	MG905597	-	-	-	S	-	-	-	R	-	-	-	-					
	A/India/P1722376/2017/	MF319587	I	-	T	-	-	-	-	-	-	-	-	-					
	A/India/P1722256/2017/	MF319588	-	-	-	-	-	-	-	-	-	-	-	-					
	A/India/P161674/2016/	MF319590	-	-	-	-	-	-	-	-	-	C	L	-					
	A/India/P161761/2016/	MF319594	-	-	-	-	-	-	K	-	-	-	-	-					
Central Indian Strains (2017)	A/Barwani/8117/2017(H1N1)	MH160789	-	-	-	-	-	-	-	V	R	-	-	-					
	A/Jabalpur/8504/2017(H1N1)	MH160790	-	-	-	-	-	-	-	-	R	-	-	-					
Vaccine Component (2017–18)																			
Central Indian strain (2015) Indian Strains (Other than Central India: 2016–2017)	A/Michigan/45/2015(H1N1)	KY117023	N	S	A	T	D	A	I	K	V	I	Y	E	R	T	I	V	C
		AA H1 numbering	162	164	215	216	222	285	295	308	321	372	489	491	497	508	510	520	545
	A/Barwani/6024/2015(H1N1)	KT369728	S	-	-	I	X	-	-	-	-	V	-	G	-	-	-	-	-
	A/Kolkata/NICED-0001/2017(H1N1)	MG905591	-	T	-	-	-	-	V	-	I	-	-	-	-	-	-	-	-
	A/Kolkata/NICED-0005/2017(H1N1)	MG905595	-	-	-	-	-	-	V	R	-	-	-	-	-	-	-	-	G
	A/Kolkata/NICED-0007/2017(H1N1)	MG905597	-	T	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-
	A/India/P1722376/2017/	MF319587	-	-	G	-	-	-	-	-	-	-	-	-	K	-	-	-	-
	A/India/P1722256/2017/	MF319588	S	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-
	A/India/P161674/2016/	MF319590	-	-	-	-	G	-	-	-	-	-	F	-	-	-	-	-	-
	A/India/P161761/2016/	MF319594	S	-	-	I	G	-	-	-	-	-	-	-	-	-	-	A	-
Central Indian Strains (2017)	A/Barwani/8117/2017(H1N1)	MH160789	-	T	-	-	-	-	V	-	I	-	-	-	-	A	T	-	-
	A/Jabalpur/8504/2017(H1N1)	MH160790	-	T	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-

Fig. 1 The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.13746011 is shown. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 35 nucleotide sequences. There were a total of 1701 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.05. The sequences from this study are marked with Solid Square (filled square) whereas the Vaccine component is marked with solid triangle (filled triangle)

sequences from Western India (accession Nos.: MF319590, MF319594). This change is estimated to occur at a frequency of 0.40% at global level. The other observed mutations in strains of Western India as listed in the Table 1 are hypothesized to play role in viral oligomerization, binding small ligands, and antibody recognition site (<http://flusurver.bii.a-star.edu.sg/>).

Although the mutations we detected are not known to affect the antigenicity of the virus [15]; the a.a. changes A73V, S74R, and S164T, are believed to play a role in viral oligomerization interfaces, antibody recognition sites and binding small ligands. I295V too has a probable role in binding ligands. The substitution of V321I is involved in viral oligomerization and antibody recognition sites (<http://flusurver.bii.a-star.edu.sg/>). The phenotypic effect of T508A and I510T are not known yet. Further, the mutations K308R and C545G reported from Kolkata, West Bengal, India (accession No.: MG905595.1), and V520A (accession No.: MF319594) were not present in sequences of Central India indicating spatial and temporal changes in the virus within the country.

Sequencing small number of strains is a limitation of our study; further limited availability of 2017 sequences from

India in public databases restrained us from a comprehensive analysis. Nonetheless our study shows that while A(H1N1)pdm09 has undergone changes and has acquired few mutations of high frequencies, the 2015 vaccine (A/Michigan/45/2015(H1N1)) remains effective as the changes are not at antigenic sites. On the other hand, the changes such as D222G and N162S that are known to have clinical importance were documented. Further the changes detected needs to be monitored for phenotypic characteristics.

We observed a.a. changes in HA that are known to have epidemiological importance; it is important to monitor and report molecular changes occurring in A(H1N1)pdm09 genome in real time at the local level for evolving timely intervention.

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Ethical statement This study was undertaken as part of virology research and diagnostic laboratory (VDRL) network project. This project has the approval of institutional ethics committee (No: NIRTH/IEC/1157/2017 dated 7th July 2017).

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