Genetic variations of the Hemagglutinin gene of Pandemic Influenza A (H1N1) viruses in Assam, India during 2016

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Abstract

Since its emergence in 2009, Influenza A/H1N1pdm09 virus has evolved continuously. Marked genetic variations have occurred in the HA1 domain of the hemagglutinin gene causing the emergence of new variants. The present study genetically characterized the hemagglutinin (HA) gene of Influenza A/H1N1pdm09 strains from Assam circulating in 2016 that caused a mild outbreak without any reported mortality. Sequence analysis of the HA gene of 20 positive Assam/H1N1pdm09 strains revealed 3 mutations (K180Q, S202T, S220T) at the antigenic sites along with several other reported mutations which are in close proximity to the antigenic sites and therefore might affect the viral antigenicity. Phylogenetically, the Assam/H1N1pdm09 strains clustered into genogroup 6B. These genetic variations highlight the importance of continuous surveillance and characterization of Influenza A/H1N1pdm09 virus activity to track the genetic makeup and diversification that may affect the behavior of the virus.

Keywords Influenza A virus · Swine flu H1N1 · Hemagglutinin · Genetic variation · Assam

The main driving force underlying the emergence of annual human Influenza epidemic and occasional pandemic is the continuous evolution of Influenza A virus (Webster et al. 1992). Among the many viral respiratory infections, the Influenza virus poses significant clinical and epidemiological importance in humans (Korsun et al. 2017) and remains a major public health problem in the developing as well as developed countries (Gatherer 2009). India was affected with around 50,000 cases with a case fatality rate of 6% during the 2009–2010 pandemic period (Parida et al. 2016). The virus continued to circulate at a low level in the population after the end of the 2010 pandemic period because of the immunity acquired among the population (Broor et al. 2012). From January to May 2015, over 39,000 persons in India were affected by a new epidemic of Influenza A/

H1N1pdm09, with more than 2500 deaths making it the largest epidemic since 2009 (Cousins 2015). This sudden re-emergence of Influenza A/H1N1pdm09 was a major public health concern, as it caused a higher number of hospitalizations with simultaneous widespread reporting from all parts of the country (Parida et al. 2016).

The hemagglutinin (HA) protein of the Influenza virus, being the principal surface antigen targeted by antibodies, is also attributed to the high frequency of mutational changes which contributes to the recurring epidemics (Gatherer 2009). During the first quarter of 2015, a sudden increase in Influenza A(H1N1)pdm09 activity was observed in India (Mishra et al. 2010). Reports from the Massachusetts Institute of Technology (MIT), USA, reported that the 2015 outbreak of A(H1N1)pdm09 virus in India might have occurred due to the mutation of potential amino acids at the receptor binding site (RBS) of the HA gene (Tharakaraman and Sasisekharan 2015). To understand and track the virus evolution and amino acid mutations, continuous surveillance of the Influenza A pandemic H1N1 is very much essential. This would help in monitoring the pathogenicity of the circulating strains and update for pandemic preparedness and response accordingly (Graham et al. 2011). The primary objective of the

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study is to monitor the antigenic and genetic changes occurring in Influenza A/H1N1pdm09 strains of Assam during 2016. The study was approved by the Institutional Human Ethics Committee.

During 2016, a total of 1407 nasopharyngeal/throat swab samples were collected from patients who presented with Influenza-like illness (ILI) as per WHO case definition for Influenza. Patients of all age and both sexes from outpatient and inpatient departments representing both rural and urban areas of Dibrugarh, Assam were included in the study. Written informed consent was obtained from all the patients or from their authorized representatives. The clinical samples were processed and subjected to RNA extraction using QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's instructions. Nucleic acid-based qRT-PCR was done for detection of Influenza virus followed by subtyping of the Influenza A virus positive samples for pandemic H1N1 by a modified WHO-CDC protocol with specific MGB probes (WHO 2017). Out of 119 Influenza A positive cases, 20 cases were found to be Influenza A/H1N1pdm09 type strains and are referred here as Assam/H1N1pdm09 strains which constituted the study group. Among the 20 Assam/H1N1pdm09 typed strains, 85% (17/20) were males and 15% (3/20) were females and the maximum positivity was high in the age group below five years. Circulation of Influenza A/H1N1pdm09 virus started in the month of March and lasted till mid of July with maximum positivity detected in the month of June. No reports of death associated with Influenza A/H1N1pdm09 were reported during the study period. The clinical features of confirmed cases revealed mild to moderate severity. Vomiting and diarrhea were present only in 2 of the 20 cases. Clinical and microbiological evidence of bacterial

co-infection were found in eight cases who were suffering from pneumonia due to *Streptococcus pneumoniae*.

Direct sequencing from clinical samples targeting the HA gene segment of 20 positive Assam/H1N1pdm09 strains were done in 2 segments using WHO recommended primers (WHO 2017). DNA sequencing was carried out using Big Dye terminator V 3.1 cycle sequencing ready reaction kit (ABI, Foster City, CA, USA) and 14 samples could be successfully sequenced. The sequences were analyzed in an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Massachusetts, USA). Raw sequence data were edited and consensus sequences were constructed using Bioedit version 7.0 (Hall 1999). The sequences from this study were deposited to GenBank under accession numbers MF564203–MF564216. Sequence analysis showed nucleotide and amino acid identity of 97.7–97.9% and 97.1–97.5%, respectively, with the vaccine strain (A/California/07/2009, GenBank id: NC_026433.1). Comparison of HA gene segment at protein level with respect to the vaccine strain revealed a total of 17 substitutions (Fig. 1) scattered throughout. Amino acids are numbered from the start codon of the HA segment (ATG: Methionine) throughout the study. Of these mutations, nine were observed at the receptor binding site (RBS₈₀₋₃₀₃) of the HA1 polypeptide. The 130 loop and 220 loop sequences were found to be highly conserved. Two mutations were observed at the 190 helix (S202T and T214A) in all the sequences examined. The T214A mutation which is localized in the RBS is reported to increase the binding affinity of the virus to α -2,6 glycan receptors which are predominately expressed in upper respiratory tract of human (Xu et al. 2012). This change was found to be conserved in all the pandemic strains of 2014–2015 from India analyzed so far (Mukherjee et al. 2016). In Assam/

H1N1/pdm Strains	13	62	100	101	114	180	202	214	220	286	300	338	391	465	468	516	537
A/California/07/2009	A	R	P	S	D	K	S	T	S	D	K	I	E	K	S	E	V
MF564203	T	K	S	N	N	Q	T	A	T	D	E	V	K	K	N	K	A
MF564204	T	K	S	N	N	Q	T	A	T	D	E	V	K	K	N	K	A
MF564205	T	K	S	N	N	Q	T	A	T	D	E	V	K	R	N	K	A
MF564206	T	K	S	N	N	Q	T	A	T	D	E	V	K	K	N	K	A
MF564207	T	K	S	N	N	Q	T	A	T	D	E	V	K	K	N	K	A
MF564208	T	K	S	N	N	Q	T	A	T	N	E	V	K	K	N	K	A
MF564209	T	R	S	N	N	Q	T	A	T	D	E	V	K	K	N	K	A
MF564210	T	K	S	N	N	Q	T	A	T	D	E	V	K	K	N	K	A
MF564211	T	K	S	N	N	Q	T	A	T	N	E	V	K	K	N	K	A
MF564212	T	K	S	N	N	Q	T	A	T	D	E	V	K	K	N	K	A
MF564213	T	K	S	N	N	Q	T	A	T	D	E	V	K	K	N	K	A
MF564214	-	-	-	-	-	Q	T	A	T	D	E	V	K	K	N	K	A
MF564215	-	-	-	-	-	Q	T	A	T	D	E	V	K	K	N	K	A
MF564216	-	-	-	-	-	Q	T	A	T	D	E	V	K	K	N	K	A

Fig. 1 Amino acid variable positions of HA in Assam/H1N1pdm09 strains in comparison to the vaccine strain. Antigenic sites are shaded with green color

H1N1pdm09 strains, three mutations, viz., K180Q, S202T and S220T were observed in the antigenic sites Sa, Sb and Ca1, respectively, which may have contributed towards the pathogenicity leading to epidemic during the study period. A previous study suggested that K180Q mutation which lies in the antigenic site Sa was observed in the strains that circulated after 2013 (Tharakaraman and Sasisekharan 2015). Mutation S220T was found in all the Assam/H1N1pdm09 strains which is located in the antigenic site Ca1. This substitution affected the infectivity and transmissibility of the virus in humans (Ramos et al. 2013). The D239G mutation in the Ca domain has been reported as one of the important manifestation for bringing out the clinical severity in Influenza-infected cases (Parida et al. 2016; Tharakaraman and Sasisekharan 2015). Earlier, this mutation was reported in two different strains from India (A/India/Delhi/VE442/2009 and A/India/6427/2014) (Mukherjee et al. 2016). However, this change was not observed in any of the Assam/H1N1pdm09 strains. This might be one of the reasons for less severity among the population during the study period. A novel mutation R62K, was observed in ten full-length amino acid sequences of Assam/H1N1pdm09 strains, and its potential biological role has not been defined. K465R mutation was observed in one strain and D286N was observed in two strains among the Assam/H1N1pdm09 strains. E391K mutation, which is globally on the rise after 2009 pandemic was observed in all the Assam/H1N1pdm09 strains. This change was first identified in New York in July 2009 and appeared thereafter globally (Maurer-Stroh et al. 2010). Based on tertiary structure, this mutation could alter salt bridge patterns and stability in a region of the HA oligomerization interface which is important for membrane fusion (Maurer-Stroh et al. 2010). Several other conserved mutations K300E, I338V, S468N, E516K and V537A in the HA2 polypeptide and P100S in the HA1 polypeptide were also observed in all the Assam/H1N1pdm09 strains. The identified mutations are labeled in the three-dimensional structure of HA and shown in supplementary figure 1.

A phylogenetic analysis of Assam/H1N1pdm09 strains (Fig. 2) was performed by comparing the nucleotide sequences retrieved from GenBank and the Global Initiative on Sharing Avian Influenza Data (GISAID) showed that

all the Assam/H1N1pdm09 strains formed a monophyletic clade with 6B genogroup. Sequence variations correlates with the phylogenetic analysis as the characteristic amino acid variations of this genogroup (D114N, K180Q, S202T, S220T, K300E) (Jimenez-Jorge et al. 2014; ECDC 2016) except D239G were found in all the Assam/H1N1pdm09 strains. It is reported that these characteristic substitutions are responsible for loss of neutralization by antibodies. Several reports on epidemiological data on the effectiveness of Influenza vaccine found that overall vaccine effectiveness after the emergence of clade 6B gradually decreased from 69% in 2014 to 47% in 2016 (Cheng and Subbarao 2018). This genogroup was first evolved from a Russian isolate (A/Moscow-Oblast/CRIE-08/2013) and has been circulating worldwide thereafter (Parida et al. 2016). In India, the first report on the circulation of this genogroup was from Madhya Pradesh in the year 2015 (Parida et al. 2016). However, this is the first report on the circulation of genogroup 6B in Assam during the season 2015–2016.

Results from identification of N-glycosylation sites on the HA gene revealed no alterations or emergence of new glycosylation sites in the Assam/H1N1pdm09 strains. Synonymous to non-synonymous amino acid substitution ratio (ds/dn) of the full-length sequences of Assam/H1N1pdm09 strains calculated using SNAP resulted a ds/dn ratio of more than 1 (5.4299) indicating low or no immune pressure (supplementary figure 2). The rate of ds/dn was almost constant at the HA1 polypeptide which constitutes the receptor binding as well as the antigenic sites of the gene. However, the high rate of synonymous substitutions at the HA2 polypeptide surpasses the overall substitution rate of the HA gene and resulted negative selection.

The present study revealed the circulation of Assam/H1N1pdm09 strains with marked genetic variations compared to the vaccine strain. These variations along with further adaptive changes may turn it into a more virulent strain which would escape antibody binding and viral neutralization. This might further increase the disease severity resulting in fatal outcomes. Therefore, the study warrants a continuous surveillance and monitoring of Influenza activity for disease prevention and formulating effective vaccination strategies.

Fig. 2 Phylogenetic tree of the HA gene of Assam/H1N1pdm09 strains along with globally distributed A/H1N1pdm09 strains representing different clades or genogroups. The trees were generated with the MEGA program (version 6.0) using maximum likelihood method based on the Hasegawa–Kishino–Yano model. Assam/H1N1pdm09 strains are indicated in red and vaccine strain in blue

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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