Changes in Hemagglutinin and Neuraminidase Genes of pH1N1 Influenza Virus Strains Collected from a North Indian Tertiary Care Hospital during 2015

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Keywords

Influenza virus · Epidemic · Haemagglutinin · Neuraminidase · Mutation

Abstract

Objective: The study was conducted to analyze changes in HA and NA genes of pandemic pH1N1 strains, collected from North Indian tertiary care hospital during 2015 and studied the nucleotide change since 2009. Differences in clinical features of cases positive and negative for influenza viruses were also studied. **Methods:** All samples referred for H1N1 testing, were tested for influenza viruses. Randomly selected 25 strains of pH1N1 were studied for nucleotide sequences of HA & NA gene. The nucleotide changes occurring since 2009 were studied by mutational and phylogenetic analysis. Clinical details of cases were recorded and analysed. **Results:** A total of 3319 cases of acute respiratory infections (ILI/SARI) were tested for influenza viruses during Jan to April 2015, of which 815 cases tested positive for pH1N1. Nucleotide variation of 2015 strains, from influenza A/California/07/2009 strain at HA1 and NA1 gene was 1.9% and 3.8% respectively. Both HA1 and NA1 coding sequence showed eight mutations. Four of HA1 (K180Q, S202T, S220T, and A273T) and NA1 (N200S, V241I, N248D, and N270K) mutations were observed in all pH1N1 study strains. **Conclusions:** Strains of pH1N1

isolated during year 2015 diverged from previously circulating strains. Their association with severity of illness needs to be further studied.

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Background

A novel influenza A (pH1N1) strain emerged in 2009, which caused a major pandemic worldwide. At the beginning of the year 2015, the pH1N1 flu epidemic re-emerged in India. Around 33,000 number of cases with death of more than 2,000 people was reported by mid-February 2015 [1]. States of Rajasthan, Gujarat, Telangana, Delhi, and Jammu and Kashmir were worst affected [2]; however, an unusual increase in number of cases was seen in 2015 across the whole of India including the state of Uttar Pradesh.

The changes in influenza virus antigens especially hemagglutinin (HA) and neuraminidase (NA) due to antigenic shifts/drifts are largely responsible for change in viral characters including pathogenicity [3]. It is important to characterize influenza strains on the basis of these antigens especially the active sites of HA1 domain of HA gene and NA domain of NA gene to determine the genetic changes on a regular basis. Hence, we studied the

changes in HA1 and NA genes of pH1N1 strains isolated during January to April, 2015, that is the flu epidemic period in India, and analyzed the changes that had occurred in HA1 and NA genes of pH1N1 strains since 2009. The clinical features of influenza positive and negative patients were analyzed to study the significant association of clinical features with influenza positivity.

Material and Methods

From January 25 to April 26, 2015, patients presenting with clinical diagnosis of influenza-like illness (ILI) and/or severe acute respiratory illness (SARI), referred to Virus diagnostic and research laboratory, Department of Microbiology, King George's Medical University, Lucknow, Uttar Pradesh, India, were tested for influenza viruses (pH1N1, influenza A H3N2, and influenza B). The center for disease control (CDC) guidelines for ILI and SARI case definitions were followed [4]. Nasal and throat swabs/nasopharyngeal aspirates were collected from each patient and processed for molecular diagnosis of human influenza viruses by real-time reverse transcriptase polymerase chain reaction (PCR) [5]. Informed consent was obtained from all the patients/their guardians (in case of children). Institutional ethical clearance was taken.

Clinical details of all the cases were collected on a predesigned questionnaire and compared for influenza pH1N1 positive and negative patients. Significant association was determined by chi-square test. All statistical analyses were done using SPSS 16.0 version software.

A total of 25 randomly selected pH1N1 strains were characterized for HA1 and NA genes. The 447 bp segment of HA1 gene (nucleotide position 434–905) and 1,159 bp segment of NA gene (nucleotide position 10–1,159) were amplified by one-step RT PCR assay as described earlier [6, 7]. Amplified products were confirmed by running on 2% agarose gel-electrophoresis and visualized on a UV trans-illuminator after staining with ethidium bromide solution. The PCR product seen as a single band was directly used for further purification process by Exo-Sap treatment (by Exonuclease I, 1 U/ μ L and Shrimp Alkaline Phosphatase, 20 U/ μ L; FastApTM, Fermentas) and ethanol precipitation. Purified products were sequenced using BigDye Terminator Cycle-Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on ABI 3130 genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Nucleotide sequences were edited and subjected to BLAST search for similar sequences in GenBank Database. To determine genetic changes in study strain, the nucleotide and amino acid sequences were compared to influenza A/California/07/2009 strain (2010 vaccine strain). Nucleotide and amino acid sequences were retrieved from the Influenza Research Database (www.fludb.org).

The amino acid sequences of study strains were deduced from nucleotide sequences in EXPASY translate tool. The percent nucleotide and amino acid identity was determined as pairwise p distances by comparing sequences in MEGA 5.05 software.

To study the changes from previous strains and other global data base, nucleotide and coding sequences of 2015 study strains were compared with published cognate sequences of cor-

responding genes from our previous published data [8]. Pair-wise p distances analysis of their HA1 and NA genes was carried out to decipher the divergence from previously circulating strains.

A phylogenetic analysis of 2012, 2013, and 2015 influenza pH1N1 strains was done. Phylogenetic tree was constructed using the maximum likelihood approach in PhyML program [9]. The HKY85+I+G4 nucleotide substitution model was utilized in this analysis and SH-like branch support was employed. The statistical inference of phylogenetic tree was measured on 500 bootstrap replicates.

Results

A total of 3,319 cases of acute respiratory infections (ILI/SARI) were referred to the study site during January 25–April 30, 2015, and tested for influenza viruses, of which 815 cases tested positive for influenza A (pH1N1), 6 for influenza A (H3N2), and 17 for influenza B. Complete clinical details were available in only 3,292 cases; hence 28 subjects were deleted from analysis. Patient's age, clinical symptoms (cough, coryza, chills, breathlessness, vomiting, headache, body ache), and duration of fever were analyzed as independent variables. Details are given in the Table 1. Cough, duration of illness <3 days, and age >45 years and <2 years, demonstrated a positive association with influenza positivity.

Comparative and Mutational Analysis of pH1N1 Strain of Year 2015, 2012, and 2013

Nucleotide variation of 2,015 strains from reference influenza A/California/07/2009 strain at the HA1 and NA gene was 1.9 and 3.8%, respectively, while divergence at protein level was 4.2 and 5.5%, respectively. Nucleotide divergence of 2,012 strains from reference strain at the HA1 and NA gene both was 1.3% while divergence at protein level for HA1 and NA protein was 3.0 and 1.7%, respectively. Nucleotide divergence of 2,013 strains from reference strain at the HA1 and NA gene was 1.7 and 1.6%, respectively, while divergence at protein level for HA1 and NA protein was 3.7 and 2.5%, respectively (Table 2).

Comparison of the HA1 coding sequence of 2015 pH1N1 strains with prototype strain showed 8 mutations. Four mutations (K180Q, S202T, S220T, and A273T) were observed in all pH1N1 2015 strains (Table 3). Figure 1 shows the phylogenetic analysis of HA1 gene; pH1N1 strains isolated during 2015 years diverged from clade 7 genotype of 2,009 pandemic H1N1 strains. Three of the mutations seen in 2,015 strains (D185N, D213N, and H290Q) were not reported before.

Table 1. Comparative analysis of different parameters (age group analysis, clinical presentations, and durations of fever) in patients positive for influenza A (H1N1)pdm09 virus and patients negative of respiratory viruses

Parameters	Patients with ARI (<i>n</i> = 3,292), <i>n</i> (%)	Influenza-negative cases (<i>n</i> = 2,478), <i>n</i> (%)	Influenza-positive cases (<i>n</i> = 814), <i>n</i> (%)	OR (95% CI)	<i>p</i> value
Age group, years					
0–2	224 (6.8)	183 (7.4)	41 (5.0)	0.665 (0.47–0.94)	0.021*
>2–5	255 (7.7)	189 (7.6)	66 (8.1)	1.069 (0.798–1.43)	0.65
>5–15	414 (12.5)	307 (12.3)	107 (13.1)	1.07 (0.845–1.35)	0.572
>15–45	1,805 (54.8)	1,382 (55.7)	423 (52.6)	0.86 (0.73–1.00)	0.058
>45	594 (18.0)	417 (16.8)	177 (21.7)	1.37 (1.13–1.67)	0.002*
Clinical presentations					
Cough	2,859 (86.8)	2,134 (86.1)	725 (89.1)	1.31 (1.025–1.682)	0.03*
Coryza	1,781 (54.1)	1,341 (54.1)	440 (54.1)	0.997 (0.851–1.169)	0.97
Chills	336 (10.2)	257 (10.4)	79 (9.7)	0.93 (0.71–1.21)	0.58
Breathlessness	733 (22.3)	543 (21.9)	190 (23.3)	1.085 (0.898–1.31)	0.39
Vomiting	347 (10.5)	260 (10.5)	87 (10.7)	1.21 (0.789–1.32)	0.87
Headache	656 (19.9)	499 (20.1)	157 (19.3)	0.948 (0.776–1.157)	0.59
Body ache	658 (20.8)	501 (20.2)	157 (19.3)	0.943 (0.772–1.151)	0.56
Duration of fever, days					
≤3	1,994 (60.6)	1,537 (62.0)	457 (56.1)	0.78 (0.67–0.92)	0.003*
>3 and ≤7	813 (24.7)	559 (22.6)	254 (31.2)	1.56 (1.31–1.86)	0.00
>7 and ≤15	347 (10.5)	269 (10.9)	78 (9.6)	0.87 (0.67–1.13)	0.30
>15	138 (4.2)	113 (4.6)	25 (3.1)	0.66 (0.43–1.03)	0.06

* $p \leq 0.05$ considered as significant.

Table 2. Divergence of HA1 and N1 nucleotide and/coding sequences of study strains isolated during 2012, 2013, and 2015 with reference strain (A/California/07/2009)

	2012	2013	2015
HA1 gene			
Nucleotide divergence, %	1.3	1.7	1.9
Amino acid divergence, %	3.0	3.7	4.2
N1 gene			
Nucleotide divergence, %	1.3	1.6	3.8
Amino acid divergence, %	1.7	2.5	5.5

Eight mutations were identified in NA amino acid sequences of 2,015 pH1N1 strains when compared to prototype strains (Table 4). All strains showed 4 mutations at different amino acid positions, that is, N200S, V241I, N248D, and N270K. In the phylogenetic analysis of NA gene, all 2015 pH1N1 strains got separated from 2,009 vaccine strains and made a single cluster in phylogenetic tree (Fig. 2).

The nucleotide sequences of pandemic HA1 gene and NA genes derived in the study were deposited into the GenBank database under accession numbers KR140136,

KR140138 – KR140141, KR140143, KR140144, KR140147 – KR140150, KT873519 – KT873527, KT873529 – KT873532, and KT873494 – KT873518.

Discussion

The present study demonstrated that pH1N1 study strains showed more changes in active sites of NA gene as compared to HA gene. Molecular analysis at gene level (especially HA1 and NA genes) identified few mutational changes which were different from the previously circulating strains of pH1N1 virus as described earlier [6, 10]. Mutational changes in the active site of HA1 protein showed 5 mutations, similar to previously circulating pH1N1 strains of Indian region along with 3 new mutations in 2015 strains at different positions (D185N, D213N, and H290Q) [11]. These mutations are also not reported in any other regions of India. There was higher nucleotide and amino acid variation in NA gene, in reference to HA gene when compared to reference influenza A/California/07/2009 strain. However, when the Lucknow strains from years 2012 and 2013 were compared, the nucleotide divergence rate was higher in HA gene, as reported in ear-

Table 3. Amino acid changes at various positions in HA1 coding sequences (from position 160aa to 300aa) of Lucknow strains

With Signal Peptide*, §	Without Signal Peptide	Amino acid mutations in particular positions			
		reference sequence A/Cal/07/2009	2012 study strains* (n = 20)	2013 study strains* (n = 14)	2015 study strains (n = 25)
180	163	K	Q (2/20); T (2/20); I (5/20)	Q (12/14); T (1/14)	Q (25/25)
185	168	D	No change	No change	N (1/25)
190	173	V	I (2/20)	I (1/14)	I (1/25)
200	183	S	No change	P (6/14)	No change
202	185	S	T (18/20)	T (14/14)	T (25/25)
203	186	A	No change	T (5/14)	No change
213	196	D	No change	No change	N (1/25)
214	197	A	T (9/20)	No change	No change
220	203	S	T (18/20)	T (14/14)	T (25/25)
251	234	V	No change	I (5/14)	No change
273	256	A	No change	T (14/14)	T (25/25)
279	262	G	E (2/20)	E (1/14)	No change
290	273	H	No change	No change	Q (1/17)

* Dangi et al. [6], 2014.

§ Initial 16–20 base pair cleaved, Suzuki et al. [11], 2002.

Table 4. Amino acid changes at various positions in N1 coding sequences (from position 180aa to 300aa) of Lucknow strains

Amino acid positions in N1 protein	Amino acid mutations in particular positions			
	reference sequence (A/Cal/07/2009)	2012 study strains* (n = 37)	2013 study strains (n = 33)	2015 study strains (n = 25)
200	N	D (12/37); S (10/37)	S (21/33)	S (25/25)
232	A	T (3/37)	T (6/33)	No change
234	V	I (6/37)	No change	No change
241	V	I (37/37)	I (33/33)	I (25/25)
248	N	D (37/37)	D (27/33)	D (25/25)
257	R	No change	K (6/33)	No change
260	K	No change	E (3/33)	No change
264	V	No change	No change	I (21/25)
270	N	No change	No change	K (25/25)
273	N	No change	K (6/33)	No change
285	S	No change	No change	Y (1/25)
287	E	No change	No change	K (1/25)
314	I	No change	No change	M (20/24)

* Dangi et al. [6], 2014.

Fig. 1. Phylogenetic tree of the pH1N1 hemagglutinin (HA1) sequences: Phylogenetic tree with HA1 gene from influenza A (pH1N1) viruses circulating in Northern India during year 2015, rooted by HA1 reference sequences of influenza A/

California/4/2009 (shown in circle). Representative strains of years 2012 and 2013 from Northern India are shown for comparison. The reliability of branches was evaluated on 500 bootstrap replicates.

(For figure 1 see next page.)

Fig. 2. Phylogenetic tree of the pH1N1 neuraminidase (NA) sequences: Phylogenetic tree with NA gene from influenza A (pH1N1) viruses circulating in Northern India during year 2015, rooted by NA sequences from A/California/07/2009 and A/India.

P131027/2013 (reference strains are shown in circle). Representative strains of years 2012 and 2013 from Northern India are shown for comparison. The reliability of branches was evaluated on 500 bootstrap replicates.

lier studies. From an evolutionary perspective, the HA gene has been accumulating mutations more rapidly than the NA and NP genes; however, the rate of amino acid substitution in HA gene is lower. The cumulative amino acid changes in HA gene can be explained as lack of proofreading of viral RNA polymerase activity [12, 13].

For a new epidemic to occur, the HA protein of influenza virus has to mutate enough to become antigenically distinct to a significant percentage [14, 15]. As shown by the earlier studies, the probability of a new epidemic will increase when the amino acid substitutions in the HA protein exceeds 10 [16, 17] or the num-

ber of amino acid substitutions in the epitope regions exceeds 4 [18]. One study done at Massachusetts Institute of Technology, USA, documented unidentified mutations (K166Q, D225, and T200A mutations in HA1 gene) in 2015 Indian outbreak strains and interpreted that these new acquired mutations are responsible for more virulence of Indian pH1N1 strain seen in year 2015 [19]. We did not find any of these mutations in our strains. According to Indian health officials, the pH1N1 strain has not changed much from the first strain that emerged in 2009 [20]. The phylogenetic analysis based on partial HA1 gene sequences of study strains revealed the presence of 2 mutations (S220T and S202T) in HA1 gene of all study strains which are specific to clade 7 genotype of pH1N1 [6, 10, 21–23].

Results of present study show that NA gene showed more divergence in coding sequences as compared to HA gene. In study strains, 3 mutations (N200S, V241I, and N248D) were similar to those reported in 2012 and 2013 pH1N1 strains reported by our group; while, 5 new mutations in NA protein were identified (V264I, N270K, S285Y, E287K, and I314M) causing diversion of 2015 pH1N1 strains from reference strains as observed in the phylogenetic tree (Fig. 2). There are 8 functional amino acids sites (R118, D151, R152, R224, E276, R292, R371, and Y406 – N2 numbering throughout) and 11 structural amino acid site (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, D293, and E425) that are conserved in almost all IAV subtypes and serve as target of drug zanamivir (Relenza) and oseltamivir (Tamiflu). There was no mutation seen in the mentioned active sites leading to minimal possibility of resistance to the drugs [24]. However, presence of new mutations in the coding region of HA1 and NA gene may have possibly contributed to the severity of outbreak.

At the beginning of 2009 pandemic, young adults were found to be more affected by pH1N1 infection and mortality was high in elders (age over 60 years) and children [25, 26]. Similar observation was depicted during 2015 flu outbreak where higher influenza positivity was seen in patients belonging to >45 years of age group ($p \leq 0.05$) but significantly low positivity was observed in children <2 years of age. Cough was significantly associated with affected groups; however, cough, fever, and sore throat are included as enrolment criteria as defined by CDC case definition. Higher influenza positivity was seen in patients presenting with fever <3 days duration.

India is a vast country with diverse climate and demonstrates 2 seasonal activities of influenza, one during monsoon months which is observed in most part of the

country with tropical climate and other in winter period which is observed in the temperate region of northern part of the country [27–29]. Previous studies on influenza seasonality in most parts of India and other parts of world demonstrated peak influenza activity in monsoon season and some showed positive association with humidity and rainfall [30, 31]. In contrast to this, 2015 flu outbreak demonstrated a major peak in pH1N1 outbreak in the winter months of 2015. The data collected in the present study cannot provide any possible explanation for this phenomena; however, this remains a query for inquisitive minds. No unusual increase in cases was seen in January to April 2016.

Conclusion

Strains of pH1N1 isolated during year the 2015 diverged from previously circulating strains and new mutations were seen. Their association with severity of illness need to be studied.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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