Emergence of influenza A(H1N1)pdm09 genogroup 6B and drug resistant virus, India, January to May 2015

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To investigate the aetiology of the 2015 A(H1N1)pdm09 influenza outbreak in India, 1,083 nasopharyngeal swabs from suspect patients were screened for influenza A(H1N1)pdm09 in the state of Madhya Pradesh. Of 412 positive specimens, six were further characterised by phylogenetic analysis of haemagglutinin (HA) sequences revealing that they belonged to genogroup 6B. A new mutation (E164G) was observed in HA2 of two sequences. Neuraminidase genes in two of 12 isolates from fatal cases on prior oseltamivir treatment harboured the H275Y mutation.

An epidemic of influenza A(H1N1)pdm09, affecting over 39,000 persons and causing more than 2,500 deaths occurred in India in 2015 [1]. We show that genotype 6B strains forming two sub-lineages circulated during the outbreak. Comparison of the sequences of six outbreak strains recovered in this work, to other published genotype 6B sequences, also reveals a unique combination of previously-reported mutations in the haemagglutinin (HA) gene. Two of the six sequences additionally display a E164G mutation in HA2, which has not been reported to date, moreover a N129D mutation in HA1 is observed for two sequences derived from patients with severe disease. Among strains analysed from 12 fatal cases on prior oseltamivir treatment, two harbour the H275Y mutation in the neuraminidase (NA) gene, which confers resistance to this antiviral.

Description of the study

Sampling and testing for influenza A(H1N1) pdm09

A total of 1,083 acute phase nasopharyngeal swab specimens from patients suspected of influenza (as prior defined [2]), were referred by 13 district health authorities of Madhya Pradesh, India between 29 January and 7 May 2015. Upon specimen collection, the travel history, treatment status, and symptoms of the patients were recorded in addition to age, sex and place of residence. The samples were handled in a designated biosafety level (BSL) 3 laboratory and viral RNA was extracted using QIAamp viral RNA mini kit (Qiagen). The RNA samples were screened by World Health Organization (WHO)–Centers for Disease Control and Prevention (CDC) approved quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) for influenza A(H1N1)pdm09 [3].

Molecular analyses of the strains

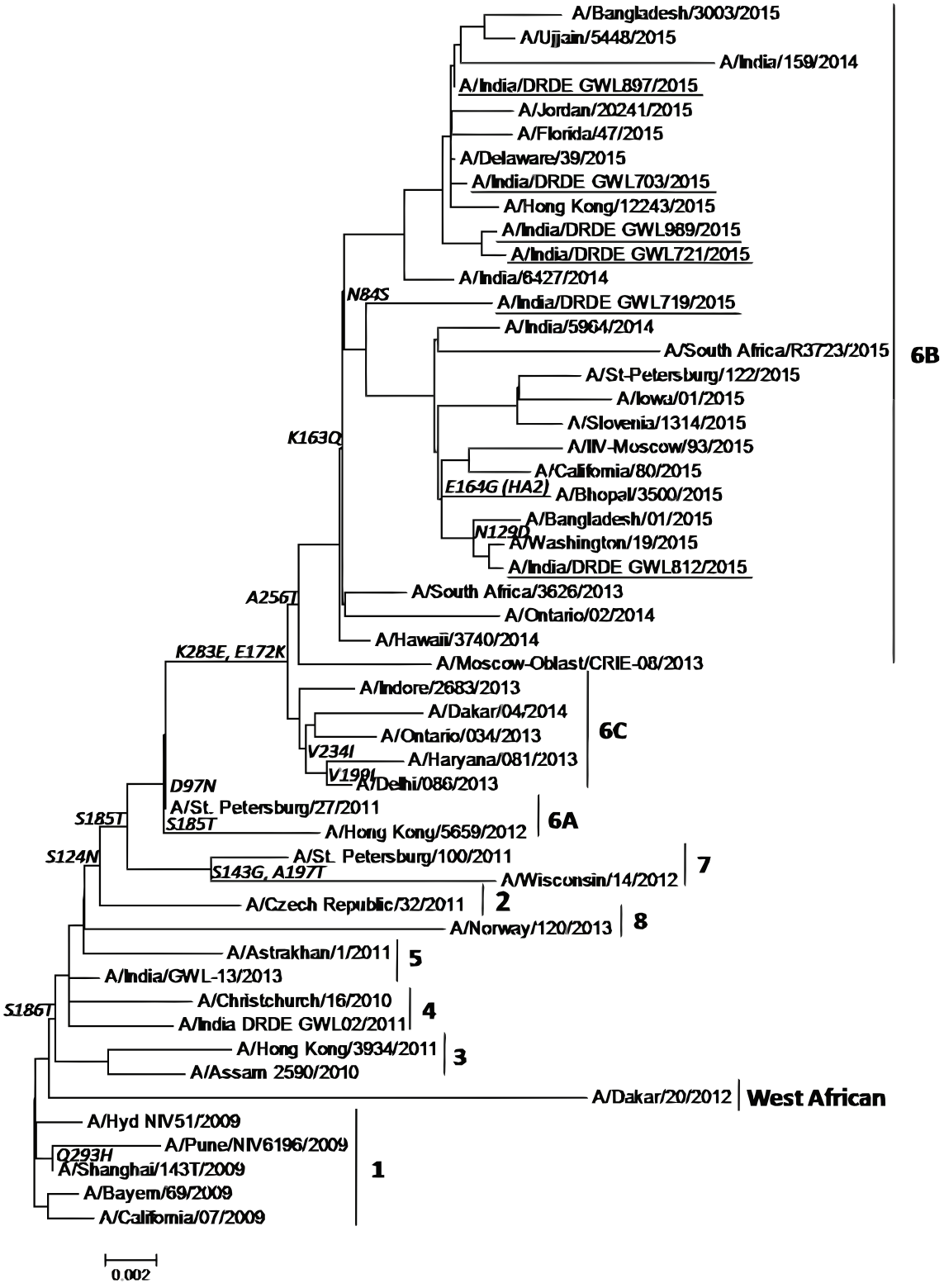
Six clinical samples testing positive for influenza A(H1N1)pdm09 by qRT-PCR were selected based on patients' disease severity category A (n=2; A/India/DRDE_GWL897/2015 and A/India/DRDE GWL721/2015), B (n=2; A/India/DRDE_GWL703/2015, A/India/DRDE_GWL989/2015), and C (n=2; A/India/DRDE GWL719/2015 and A/India/DRDE_GWL812/2015) as previously described [2], and used for direct nucleotide (nt) sequencing of the haemagglutinin (HA) gene. A phylogenetic analysis was performed by comparing with nt sequence of 45 globally diverse influenza A(H1N1)pdm09 viruses retrieved from GenBank (as further shown in the phylogenetic tree) and the Global Initiative on Sharing Avian Influenza Data (GISAID) (Table 1). The phylogenetic tree in this analysis was constructed with maximum likelihood and bootstrap analysis of 1,000 replicates using Mega 5.03 software [4]. Further the amino acid substitutions were marked at the major branches for better clarity.

Influenza A(H1N1)pdm09 HA amino-acid sequences were inferred from the genetic sequences obtained in this study, and the protein structures were modelled using Modeller software and compared to prototype A/California/07/2009 through Discovery studio client 4.1.

The qRT-PCR positive samples from 12 fatal cases, all on prior oseltamivir therapy, were also tested for a mutation (H275Y) conferring resistance to this antiviral by PCR–restriction fragment length polymorphism (RFLP)

Figure 1

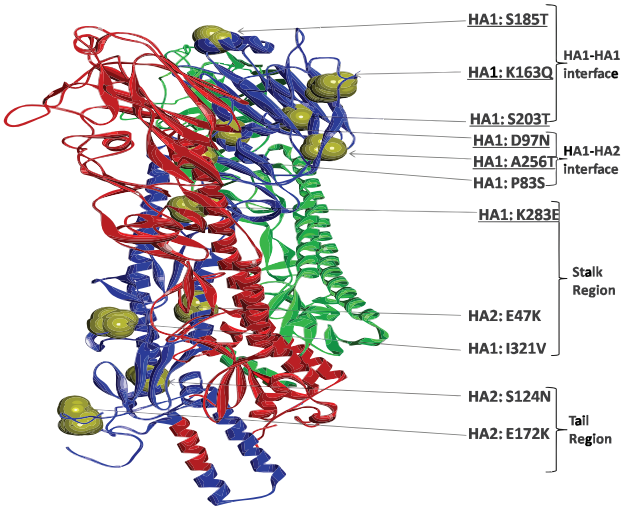
Phylogenetic analysis of influenza A(H1N1)pdm09 viral sequences derived from an outbreak in India, 2015



Amino acid substitutions are depicted on the major branches at the nodes. Samples recovered in this study are underlined. GenBank accession numbers of sequences in the tree are: A/Bhopal/3500/2015 (KT426698); A/California/80/2015 (KT836680); A/Delaware/39/2015 (KT836926); A/Delhi/086/2013 (KP317290); A/Florida/47/2015 (KT836928); A/Haryana/081/2013 (KP317285); A/Hawaii/3740/2014 (CY187658); A/India/DRDE_GWL703/2015 (KT867221); A/India/DRDE_GWL719/2015 (KT867219); A/India/DRDE_GWL721/2015 (KT867223); A/India/DRDE_GWL812/2015 (KT867224); A/India/DRDE_GWL897/2015 (KT867220); A/India/DRDE_GWL989/2015 (KT867222); A/India/GWL-13/2013 (KF683625); A/Indore/2683/2013 (KF886296); A/Iowa/01/2015 (KT836709); A/Moscow-Oblast/crie--08/2013 (KF013860); A/Ontario/02/2014 (KP864396); A/Ontario/034/2013 (KF886365); A/Ujjain/5448/2015 (KT369727); A/Washington/19/2015 (KT836815); A/Wisconsin/14/2012 (KC891394); A_Assam_2590_2010 (JN600357); A_Hyd_NIV51_2009 (GU292350); A_India_DRDE_GWL02_2011 (JQ319657); A_Pune_NIV6196_2009 (GU292352); A_Shanghai_143T_2009 (GQ411907).

Figure 2

Three-dimensional quaternary structure of trimeric haemaglutinin protein, identifying mutations compared to A/California/07/2009 in the proteins in this study, India, 2015



In this structure two monomers are coloured green and red. In the third monomer, residues are coloured blue and amino acid residues differing from A/California/07/2009 are denoted as yellow spheres. The mutations are listed to the right of the molecule, along with the four structural regions that contain the mutations. Mutations characterising genogroup 6B are underlined.

analysis of the NA gene [5]. Of these 12 cases, one (with corresponding sample: A/India/DRDE GWL719/2015) also belonged to the group of six patients, from whom the HA gene was sequenced. The PCR–RFLP positive samples were further confirmed through nt sequencing of the target sites of the NA gene.

Results of screening for influenza A(H1N1) pdm09

A total of 1,083 patients, including 525 males, were screened for influenza A(H1N1)pdm09 by qRT-PCR. The age range of these patients varied from 0 to 90 years-old, with age groups between 21 and 30 year-old (n=284) as well as between 31 and 40 year-old (n=179) representing 26% and 17% of the total respectively (Table 2). Of the 1,083 clinical samples tested, 412 (38%) were found positive for influenza A(H1N1)pdm09 virus. Similar to patients screened, most of those testing positive were from young age groups, with 21 to 30 years-old (n=104; 25%) representing the majority, followed by 31 to 40 year-olds (n=69; 17%). The positivity rate among the different age groups varied from 25 to 59% (Table 2). The female to male sex ratio of PCR positive patients was found to be 1.20:1.

The clinical features of PCR confirmed patients revealed presence of cough (n=378; 92%), fever >38°C (n=350; 85%), sore throat (n=331; 80%), shortness of breath (n=271; 66%) and catarrh (n=253; 61%).

Molecular characteristics of outbreak strains

HA sequences from six samples of influenza A(H1N1) pdm09-positive patients in this study were recovered and deposited in National Center for Biotechnology Information (NCBI)-GenBank under the accession numbers KT867219, KT867220, KT867221, KT867222, KT867223 and KT867224. The HA open reading frame was found to be 1,701 nt in length.

Phylogenetic analysis of the six sequences, together with geographically diverse global influenza A(H1N1) pdm09 viral sequences, including sequences recovered in India in previous years, revealed that the six sequences clustered with genogroup 6B sequences. Sequences from India in 2014 also belonged to this genogroup (e.g. A/India/159/2014, A/India/6427/2014 and A/India/5964/2014). Moreover, within this genogroup, two distinct lineages could be observed (Figure 1).

Four study sequences (A/India/DRDE GWL703/2015, A/India/DRDE GWL721/2015, A/India/DRDE GWL897/2015 and A/India/DRDE GWL989/2015), which were derived from patients with disease severity categorised as A and B, were found grouped into one lineage (lineage 1) of genogroup 6B. Lineage 1 additionally included some Indian sequences (A/India/159/2014 and A/India/6427/2014) from 2014. The two remainder study sequences (A/India/DRDE GWL719/2015 and A/India/DRDE GWL812/2015), both originating from category C patients, segregated into the other genogroup 6B lineage (lineage 2). A 2014 Indian sequence (A/India/5964/2014) also belonged to lineage 2. The two lineages differed by an amino acid substitution at position 84 in HA1, whereby lineage 1 sequences had an N and lineage 2 sequences an S.

No clear difference was observed between 2015 and 2014 Indian sequences included in the analysis, except that 2015 strains in lineage 2 (A/India/DRDE GWL719/2015 and A/India/DRDE GWL812/2015) encoded a N129D mutation in HA1 (HA1 numbering system).

The comparative analysis of inferred peptide-sequences confirmed that the 2015 Indian viruses harboured the signature amino acid substitutions of genogroup 6B (D97N, K163Q, S185T, S203T, A256T and K283E) [6,7].

In addition to the six substitutions defining genotype 6B, all HA-sequenced viruses in this study presented five mutations compared to prototype A/California/07/2009, namely, P83S, I321V in HA1, as well as E47K, S124N, and E172K in HA2 (Figure 2). Further to these total 11 mutations, N129D was found in HA1 sequences of two specimens (A/India/DRDE GWL719/2015 and A/India/DRDE GWL812/2015) from patients with severe disease (both category C including one fatal case). Also, E164G was found in HA2 of A/India/DRDE GWL721/2015 and A/India/DRDE GWL812/2015.

Table 1

Details of the A(H1N1)pdm09 sequences retrieved from the Global Initiative on Sharing Avian Influenza Data (GISAID)'s EpiFlu Database for complete haemagglutinin-gene-based phylogenetic analysis in this study

ID	S	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI624748	HA	Russian Federation	2015-Feb-26	A/St-Petersburg/122/2015	WHO National Influenza Centre Russian Federation	Crick Worldwide Influenza Centre	–
EPI630634	HA	Hong Kong (SAR)	2015-Jun-14	A/Hong Kong/12243/2015	Government Virus Unit	Crick Worldwide Influenza Centre	–
EPI630684	HA	South Africa	2015-Jun-29	A/South Africa/R3723/2015	Sandringham, National Institute for Communicable Diseases	Crick Worldwide Influenza Centre	–
EPI630652	HA	Slovenia	2015-Mar-05	A/Slovenia/1314/15	Laboratory for Virology, National Institute of Public Health	Crick Worldwide Influenza Centre	–
EPI624704	HA	Russian Federation	2015-Mar-10	A/IIV-Moscow/93/2015	Ivanovsky Research Institute of Virology RAMS	Crick Worldwide Influenza Centre	–
EPI589565	HA	Jordan	2015-Mar-22	A/Jordan/20241/2015	Laboratory Directorate	Crick Worldwide Influenza Centre	–
EPI253705	HA	Germany	2009-Jan-01	A/Bayern/69/2009	Robert-Koch-Institute	Robert-Koch-Institute	Biere, B; Schweiger, B
EPI278607	HA	New Zealand	2010-Jul-12	A/Christchurch/16/2010	Canterbury Health Services	WHO Collaborating Centre for Reference and Research on Influenza	Deng, Y-M; Iannello, P; Caldwell, N; Leang, S-K; Komadina, N
EPI319590	HA	Russian Federation	2011-Feb-28	A/Astrakhan/1/2011	WHO National Influenza Centre Russian Federation	National Institute for Medical Research	–
EPI319527	HA	Russian Federation	2011-Feb-14	A/St. Petersburg/27/2011	WHO National Influenza Centre Russian Federation	National Institute for Medical Research	–
EPI416411	HA	Norway	2013-Jan-02	A/Norway/120/2013	WHO National Influenza Centre	National Institute for Medical Research	–
EPI390473	HA	Hong Kong (SAR)	2012-May-21	A/Hong Kong/5659/2012	Government Virus Unit	National Institute for Medical Research	–
EPI326206	HA	Hong Kong (SAR)	2011-Mar-29	A/Hong Kong/3934/2011	Government Virus Unit	National Institute for Medical Research	–
EPI466626	HA	South Africa	2013-Jun-06	A/South Africa/3626/2013	Sandringham, National Institute for Communicable Diseases	National Institute for Medical Research	–
EPI539474	HA	Senegal	2014-Feb-05	A/Dakar/04/2014	Institut Pasteur de Dakar	National Institute for Medical Research	–
EPI417122	HA	Senegal	2012-Dec-09	A/Dakar/20/2012	Institut Pasteur de Dakar	National Institute for Medical Research	–
EPI319447	HA	Czech Republic	2011-Jan-18	A/Czech Republic/32/2011	National Institute of Public Health	National Institute for Medical Research	–
EPI320141	HA	Russian Federation	2011-Mar-14	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	–
EPI626148	HA	Bangladesh	2015-May-04	A/Bangladesh/3003/2015	Institute of Epidemiology Disease Control and Research (IEDCR) & Bangladesh National Influenza Centre (NIC)	Centers for Disease Control and Prevention	–
EPI626140	HA	Bangladesh	2015-May-10	A/Bangladesh/01/2015	Institute of Epidemiology Disease Control and Research (IEDCR) & Bangladesh National Influenza Centre (NIC)	Centers for Disease Control and Prevention	–
EPI176620	HA	United States	2009-Apr-09	A/California/07/2009	Naval Health Research Center	Centers for Disease Control and Prevention	–
EPI536832	HA	India	2014-May-24	A/India/5964/2014	National Institute of Virology	Centers for Disease Control and Prevention	–
EPI537951	HA	India	2014-Mar-06	A/India/6427/2014	National Institute of Virology	Centers for Disease Control and Prevention	–
EPI644248	HA	India	2014-Feb-05	A/India/159/2014 (H1N1)	National Centre for Disease Control	National Centre for Disease Control (NCDC)	–

S: segment.

We acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which this research is based. All submitters of data may be contacted directly via the GISAID website www.gisaid.org.

Table 2

Age distribution of persons with confirmed influenza A(H1N1)pdm09 positive samples, Madhya Pradesh, India, 29 January–7 May 2015 (n=412)

Age group in years	Positivity rates n/N (%)[a]
0–5	31/92 (34)
6–10	13/30 (43)
11–20	44/111 (40)
21–30	104/284 (37)
31–40	69/179 (38)
41–50	56/150 (37)
51–60	48/134 (36)
61–70	27/69 (39)
71–90	20/34 (59)

[a] Where in each age group, n is the number of positive samples and N the total number of samples screened and the percentage is the positivity rate.

A thorough *in silico* analysis revealed that all of the 11 mutations common to the 2015 Indian sequences studied here, have been reported in different strains of influenza A(H1N1)pdm09 virus isolated from various parts of the world in the past [8-10]. However, to date, no single strain was reported to possess all these 11 mutations together, except the Indian 2015 strains sequenced in this study. Moreover, the E164G mutation found in HA2 of A/India/DRDE GWL812 and A/India/DRDE GWL721 has not previously been reported.

Modelling reveals that mutations are found in the head, stalk and tail region of HA protein but the majority were found in the head region which covers the major antigenic binding region. The HA2 E172K mutation showed distinct structural changes in the tail region compared to the influenza A(H1N1)pdm09 virus prototype [11].

Two influenza A(H1N1)pdm09 strains from 12 fatal cases were found to possess H275Y oseltamivir resistance mutation.

Discussion

Influenza A viruses have been responsible for four influenza pandemics in last century viz., Spanish influenza (H1N1) in 1918, Asian influenza (H2N2) in 1957, Hong Kong influenza (H3N2) in 1968 and pandemic influenza (H1N1) in 2009, which was caused by influenza A(H1N1)pdm09. During the 2009 pandemic period (2009–2010), India was affected with around 50,000 cases and a case fatality of 6% [12]. After the end of the 2009 pandemic, the virus continued to circulate at low level in the population, and during the period from 2011 to 2014 the circulation of the virus declined [13]. From January to May 2015 however, over 39,000 persons in India were affected by a new epidemic of influenza A(H1N1)pdm09, with more than 2,500 deaths [1]. The outbreak spread across 22 of the 29 states in the country, making it the largest since 2009. This sudden re-emergence and wide spread simultaneous reporting of influenza A(H1N1)pdm09 along with higher number of hospitalisations and deaths was a major public health concern.

By further characterising the strains infecting patients positive for influenza A(H1N1)pdm09 through HA phylogeny, this study finds that sequences of genogroup 6B were circulating during the 2015 epidemic. The genogroup 6B was found to evolve from a Russian isolate (A/Moscow-Oblast/CRIE-08/2013) and is since then circulating in many parts of the world. However, this is the first report from India regarding circulation of genogroup 6B, coinciding with a large scale outbreak [1].

Researchers from Massachusetts Institute of Technology (MIT) have recently reported mutations D225N, and T200A in a 2014 Indian strain (A/India/6427/2014, which also clusters with genogroup 6B sequences in the phylogenetic tree Figure 1) making the virus more infectious [14]. Although we did not find these two mutations in our study, all the sequences that we characterised harboured five mutations (P83S, I321V in HA1, as well as E47K, S124N, and E172K in HA2), which although previously described, have not been reported in combination. Moreover, two isolates from patients with severe disease harboured a N129D mutation in HA1 and two isolates had a mutation in HA2, E164G, that has not been observed to date. These unique features of the viruses found here may have played a role in shaping the large scale epidemic with cases of severe disease. On the other hand, the 2015 epidemic in India may be attributed to lack of immunity among an immune-naïve population. It is also noteworthy that seasonal influenza vaccination is not very common in India.

Some limitations of the study include that the samples were only tested for influenza A(H1N1)pdm09 virus, whereby only 38% of samples tested were positive. Therefore, co-circulation of other influenza subtypes or types could not be ruled out. Moreover the sequence analysis was conducted with only few positive samples that did not cover other gene segments than the HA and NA genes.

The influenza A(H1N1)pdm09 virus represents a quadruple reassortment of two swine, one human, and one avian strain of influenza virus [15]. The largest proportion of genes comes from swine influenza viruses (30.6% from North American swine strains, 17.5% from Eurasian swine strains), followed by North American avian strains (34.4%) and human influenza strains (17.5%). It will be interesting to investigate the involvement of any gene reassortment in the 2015 outbreak in India through complete genome sequencing.

Two of 12 strains from fatal cases were found to harbour a mutation conferring resistance to oseltamivir. Learning more about the 2015 strains circulating

in India could help public health officials determine treatment options and inform on vaccines for the next influenza season, which is likely to include currently circulating strains [16].

Our findings show the importance of systematic molecular surveillance to provide insight into strains circulating during influenza epidemics.

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Conflict of interest

None declared.

Authors' contributions

Manmohan Parida: Design & supervision; Paban Kumar Dash: Sequence and Phylogeny; Jyoti S Kumar: RT-PCR; Gaurav Joshi: Sample processing, modeling & phylogeny; Kundan Tandel: Sample processing; Shashi Sharma: RT-PCR; Ambuj Srivastava: Sample processing; Ankita Agarwal: Sample processing; Amrita Saha: Sample processing; Shweta Saraswat: Sample processing; Divyanshi Karothia: Sample processing; Vatsala Malviya: Sample processing.

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