Phylogenetic Analysis of H1N1 Proteins for Understanding Its Allocation

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Abstract: *A serious Influenza pandemic infection has rapidly spread across the world since 2009 causing nearly 300,000 deaths globally within the first year of the pandemic. In 2014 and 2015, the swine flu pandemic hits again with increased rate of reported cases. H1N1, a swine influenza virus is a known causative agent of swine flu. This outbreak caused by subtype H1N1 in humans is due to transfer of swine influenza virus from pig to human. The entire Influenza A virus genome contained eight RNA segments such as Matrix protein, Hemagglutinin, Neuraminidase, Nucleocapsid protein, Polymerase PB1, Polymerase PA, Polymerase PB2 and Non-structural protein. In this study, phylogenetic analysis of protein sequences of human H1N1 viruses was carried out using MEGA 6 software to demonstrate the route map of its infection. Phylogenetic analysis of the sequences from Asian and other foreign countries, available at NCBI protein database were retrieved and analyzed. The result shows that many proteins of Indian entries were clustered within India while few with close Asian and foreign countries and very few remained non-clustered as evidenced in all sequences. The study helps understood the distribution and flow pattern of H1N1 virus across the world and Phylogenetic tools predicted the defined phyletic clusters available in that population.*

Keywords: *Swine flu, H1N1, Influenza, Phylogenetic analysis.*

Introduction

H1N1 influenza virus responsible for causing a global pandemic was originated from Mexico followed by USA. In 2009, a novel strain of H1N1 influenza virus emerged in California and rapidly spread throughout the world [14]. A recent study estimated that > 284,000 deaths occurred globally during the first 12 months of 2009 pandemic H1N1virus circulation [4]. Since last two years, there is gradual increase in the reported case of H1N1 infection and according to World Health Organization (WHO) report, influenza activity increased in several areas of the Southern Hemisphere which is dominated by the H1N1 pandemic strain of 2009 [1]. According to Union Health Ministry, India the number of affected people across the country has mounted to 25,190 with 1370 death till March, 2015. Due to the reoccurrence of H1N1 pandemic there is a need to study the evolution of different H1N1 proteins so as to make out spread of the disease.

Influenza A virus is a lipid-enveloped orthomyxovirus and a cause of human disease by strains that arise through seasonal variation and through pandemic infection resulting from viral adaptation that introduces new influenza viruses into the human population [2]. Influenza A virus strains are assigned an H and an N number based on these two proteins, the strain contains. There are 16 H and 9 N subtypes known in birds, but only H 1, 2 and 3, and

N 1 and 2 are commonly found in humans. Influenza A (H1N1) virus is a subtype of influenza virus A and the most common cause of influenza (flu) in humans [13]. The genetic material of Influenza virus contains eight segments of single stranded RNA. Out of its 8 segments of RNAs, 2 polymerase genes, PB2 and PA, were from the avian virus of North American lineage and were introduced into swine populations around 1998. The other polymerase gene, PB1, also evolved recently from a human seasonal influenza (H3N2) virus around the same year. Hemagglutinin (HA), nucleoprotein (NP) and nonstructural (NS) protein coding genes descended directly from the classic swine influenza A virus of North American lineage, which can be traced back to the 1918 virus. Originating from the Eurasian Swine virus, the remaining 2 genes, neuraminidase (NA) and matrix (M), were introduced from birds around 1979 [6]. The matrix (M1) protein of influenza A virus is a multifunctional protein that plays essential structural and functional roles in the virus life cycle [12]. HA (hemagglutinin) has been demonstrated to be particularly important for virus infection against the host, by mediating the attachment of the virus to the host cell surface and the entry of viral RNA into the host cell. Therefore, the properties of the HA protein in H1N1 virus are very worthwhile to be studied, which will provide a clue to better understand the infection mechanism of influenza viruses and monitor the interspecies transmission of influenza virus [9]. HA (hemagglutinin) and NA (neuraminidase) play roles in viral attachment and release from host cells, respectively [3]. The nonstructural gene (NS) of the influenza A virus has a crucial role in viral virulence and replication [16].

Sequence alignment is one of the important tools used in bioinformatics and computational Biology [15]. Besides, phylogenetic analysis is employed to determine the evolutionary relationships between organisms that further draw a hierarchical cladogram or phylogram (phylogenetic tree) to analyze the results. The aim of this study is to understand distribution and flow pattern of H1N1 virus across world and definitely phylogenetic tools predicted the defined phyletic clusters available in that population.

Materials and methods

Data collection

Protein sequence of eight segments of H1N1 virus namely: Matrix protein, Hemagglutinin, Neuraminidase, Nucleocapsid protein, Polymerase PB1, Polymerase PA, Polymerase PB2, Non-structural protein from Asian countries such as India, China, Japan and Korea were retrieved from NCBI Protein database. The study was restricted by retrieving random two sequences from each country for particular segment except India, where nine sequences were used in analyses in each group. This assisted the phylogenetic study of sequenced H1N1 virus across India in detail in accord with other countries and its relation to transmission in India from other countries. While to consider sequences of other continent except Asia, several countries like Australia, Canada, England, Germany, Russia and USA were considered in the study.

Coding of sequences

Total 27 protein sequences in fasta format retrieved from NCBI for each protein were decoded for their groups with respect to protein name, continent and country and according to series to which they belong. For example, first sequence of the matrix protein of china named as “MACHI1” where M stands for matrix, A for Asia, CHI for China and 1 for number. Such a decision helps in easy discrimination between sequences in phylogenetic tree. Details of each group are presented in Table 1.

Table 1. Assigned code details to H1N1 protein sequences along with NCBI Accession No.

				India		China	Country
5	4	3	2	1	2	1	Sl. No.
MAIND5 / AEM63460.1	MAIND4 / AEB71165.1	MAIND3 / AFO38712.1	MAIND2 / AGQ88547.1	MAIND1 / AGQ88635.1	MACHI2 / ACR67247.1	MACHI1 / ACR55007.1	**Matrix protein**
HAIND5 / AGQ88621.1	HAIND4 / AGQ88632.1	HAIND3 / AGQ88643.1	HAIND2 / AGQ88654.1	HAIND1 / AGQ88442.1	HACHI2 / ADU02152.1	HACHI1 / ACR54994.1	**Hemagglutinin protein**
NAIND5 / AGQ88535.1	NAIND4 / AGQ88568.1	NAIND3 / AGQ88634.1	NAIND2 / AGQ88656.1	NAIND1 / AGQ88444.1	NACHI2 / ADU02143.1	NACHI1 / ACR49238.1	**Neuraminidase protein**
NCAIND5 / AEM63458.1	NCAIND4 / AGQ88556.1	NCAIND3 / AGQ88633.1	NCAIND2 / AGQ88655.1	NCAIND1 / AGQ88443.1	NCACHI2 / ADU02141.1	NCACHI1 / ACR67245.1	**Nucleocapsid protein**
PB1AIND5 / ADV39697.1	PB1AIND4 / AFO38705.1	PB1AIND3 / AGQ88541.1	PB1AIND2 / AGQ88629.1	PB1AIND1 / AEB71172.1	PB1ACHI2 / AEC46380.1	PB1ACHI1 / ADW93941.1	**Polymerase PB1 protein**
PAAIND5 / AGQ88542.1	PAAIND4 / AGQ88553.1	PAAIND3 / AGQ88630.1	PAAIND2 / AGQ88652.1	PAAIND1 / AGQ88440.1	PAACHI2 / AEC46381.1	PAACHI1 / ADU02131.1	**Polymerase PA protein**
PB2AIND5 / AEM63443.1	PB2AIND4 / AFO38703.1	PB2AIND3 / AGQ88540.1	PB2AIND2 / AGQ88551.1	PB2AIND1 / AGQ88438.1	PB2ACHI2 / ADW93962.1	PB2ACHI1 / ACR49233.1	**Polymerase PB2 protein**
NSAIND5 / AGQ88637.1	NSAIND4 / AGQ88659.1	NSAIND3 / AGQ88447.1	NSAIND2 / ADD85879.1	NSAIND1 / ADD85897.1	NSACHI2 / ACR67259.1	NSACHI1 / ADU02134.1	**Non-structural protein**

	Korea		Japan					
2	1	2	1	9	8	7	6
MAKOR2 / ADG58979.1	MAKOR1 / AGE83974.1	MAJAP2 / BAM78371.1	MAJAP1 / BAM78411.1	MAIND9 / AEE68991.1	MAIND8 / AEM62793.1	MAIND7 / AEM62802.1	MAIND6 / AEM62838.1
HAKOR2 / AFF57168.1	HAKOR1 / AFF57188.1	HAJAP2 / ACT79133.1	HAJAP1 / ADC45736.1	HAIND9 / ADX31425.1	HAIND8 / AEN79399.1	HAIND7 / AHB72860.2	HAIND6 / AHB72862.2
NAKOR2 / AGE83994.1	NAKOR1 / AFF57189.1	NAJAP2 / BAJ05804.1	NAJAP1 / ACT79135.1	NAIND9 / AGL96807.1	NAIND8 / AGL96809.1	NAIND7 / AGL96820.1	NAIND6 / AGL96818.1
NCAKOR2 / AGE84003.1	NCAKOR1 / ACQ84452.1	NCAJAP2 / ADC45836.1	NCAJAP1 / ACT79134.1	NCAIND9 / ADH29485.1	NCAIND8 / ADH29486.1	NCAIND7 / ADX31428.1	NCAIND6 / ADX31429.1
PB1AKOR2 / AGE84000.1	PB1AKOR1 / ACR08503.1	PB1AJAP2 / BAM34407.1	PB1AJAP1 / BAJ05800.1	PB1AIND9 / ADH29493.1	PB1AIND8 / ADH29494.1	PB1AIND7 / ADH29495.1	PB1AIND6 / ADH29496.1
PAAKOR2 / AEA48988.1	PAAKOR1 / AGE84001.1	PAAJAP2 / ADC45823.1	PAAJAP1 / BAJ05801.1	PAAIND9 / AEM62826.1	PAAIND8 / ADX31431.1	PAAIND7 / AEM63445.1	PAAIND6 / AFO38707.1
PB2AKOR2 / ADG58818.1	PB2AKOR1 / AGE83999.1	PB2AJAP2 / ADC45765.1	PB2AJAP1 / BAJ05799.1	PB2AIND9 / ADH29498.1	PB2AIND8 / ADH29500.1	PB2AIND7 / ADH29501.1	PB2AIND6 / ADX31432.1
NSAKOR2 / ADV41682.1	NSAKOR1 / ACQ84456.1	NSAJAP2 / BAK23317.1	NSAJAP1 / BAK23309.1	NSAIND9 / AHH25567.1	NSAIND8 / AGQ88538.1	NSAIND7 / AGQ88549.1	NSAIND6 / AGQ88615.1

Russia		Germany		England		Canada		Australia
1	2	1	2	1	2	1	2	1
MORUS1 / ADA70332.1	MOGER2 / ACR10229.1	MOGER1 / ADL41176.1	MOENG2 / ADJ37585.1	MOENG1 / ADJ37623.1	MOCAN2 / ADN24461.1	MOCAN1 / ADN24469.1	MOAUS2 / ACD37496.1	MOAUS1 / ACS34665.1
HORUS1 / ACZ27810.1	HOGER2 / CAZ68645.1	HOGER1 / CAZ68643.1	HOENG2 / ADJ37855.1	HOENG1 / ADJ37854.1	HOCAN2 / ACU31147.1	HOCAN1 / ACU31151.1	HOAUS2 / ACS34667.1	HOAUS1 / ACM90865.1
NORUS1 / ADA79619.1	NOGER2 / AGA19237.1	NOGER1 / ACV42020.1	NOENG2 / ADJ37666.1	NOENG1 / ADJ37693.1	NOCAN2 / ACU31185.1	NOCAN1 / ACU31188.1	NOAUS2 / ABP49385.1	NOAUS1 / ACM90871.1
NCORUS1 / ACZ27814.1	NCOGER2 / AGA19236.1	NCOGER1 / ACV42019.1	NCOENG2 / ADJ37761.1	NCOENG1 / ADJ37778.1	NCOCAN2 / ACT68190.2	NCOCAN1 / ACQ89929.2	NCOAUS2 / ACX46230.1	NCOAUS1 / ADD22945.1
PB1ORUS1 / ACZ27818.1	PB1OGER2 / AGG86870.1	PB1OGER1 / ACV42016.1	PB1OENG2 / ADJ37994.1	PB1OENG1 / ADJ38009.1	PB1OCAN2 / ACU31247.1	PB1OCAN1 / ACQ73409.2	PB1OAUS2 / AEI29233.1	PB1OAUS1 / ADV74742.1
PAORUS1 / ACZ27817.1	PAOGER2 / AGA19304.1	PAOGER1 / ACV42017.1	PAOENG2 / ADJ37915.1	PAOENG1 / ADJ37932.1	PAOCAN2 / ACU31233.1	PAOCAN1 / ACU31236.1	PAOAUS2 / AEI29232.1	PAOAUS1 / ADV74743.1
PB2ORUS1 / ADA79604.1	PB2OGER2 / AGG86869.1	PB2OGER1 / ACV42015.1	PB2OENG2 / ADJ38065.1	PB2OENG1 / ADJ38086.1	PB2OCAN2 / ACU31140.1	PB2OCAN1 / ACQ82709.1	PB2OAUS2 / AEI29234.1	PB2OAUS1 / ADV74741.1
NSORUS1 / ACZ27815.1	NSOGER2 / ACV42006.1	NSOGER1 / ACV42023.1	NSOENG2 / AFU09750.1	NSOENG1 / AFU09860.1	NSOCAN2 / ADN25077.2	NSOCAN1 / ADN25081.2	NSOAUS2 / AEI29230.1	NSOAUS1 / ADV74749.1

	2	MORUS2 / AEQ33578.1	HORUS2 / ADA79597.1	NORUS2 / AEQ33560.1	NCORUS2 / AEQ33551.1	PB1ORUS2 / AHA38638.1	PAORUS2 / AHA38597.1	PB2ORUS2 / AHA38595.1	NSORUS2 / ADB81464.1
USA	1	MOUSA1 / AAT65434.1	HOUSA1 / ACP41105.1	NOUSA1 / ADZ05393.1	NCOUSA1 / ADZ05394.1	PB1OUSA1 / ACQ76357.1	PAOUSA1 / ACP41104.1	PB2OUSA1 / ACP41102.1	NSOUSA1 / ACD88521.1
	2	MOUSA2 / ADZ05391.1	HOUSA2 / ADZ05390.1	NOUSA2 / ACR18924.1	NCOUSA2 / ACY77664.1	PB1OUSA2 / ACQ76320.1	PAOUSA2 / ACY77602.1	PB2OUSA2 / ADN78220.1	NSOUSA2 / ACV93295.1

Multiple sequence alignment and phylogenetic analysis

All the eight protein groups containing 27 sequences in each group were subjected to ClustalW module of MEGA 6 (Molecular Evolutionary Genetics Analysis) software for performing multiple sequence alignment (MSA). The gap penalty of 10 was set for both pair wise as well as MSA where as gap extension penalty of 0.1 and 0.2 was set for pair wise and MSA respectively. BLOSUM was selected as protein weighted matrix for performing MSA. Then mega (.meg) file were exported from MSA results. The corresponding mega files were then subjected to phylogenetic tree construction using UPGMA method as all the strains are closely related to each other and there are strong sequence similarities among themselves.

Amino acids variation in protein cluster analysis

A customize PERL program was designed and used to obtain the mutation/variation (amino acid substitutions/insertions/deletions) from MSA result for each protein group.

Results and discussion

Three new Influenza A viruses (SARS virus in 2003, Influenza H5N1 ("Avian flu") in 2004 and Influenza virus A/H1N1 in 2009) coming out as a global pandemic in last few years. The H1N1 was first identified in Mexico and the pigs in North America play a vital role in interspecies transmission of this H1N1 virus [11]. With in short time duration this virus had been circulated in USA, Europe, Australia and Asia [6]. Our present study shows that the pandemic infection is due to the rapid spread of this virus across the globe through phylogenetic analysis.

The phylogenetic analysis of eight proteins of H1N1 virus isolated from across the globe revealed that the spreading of this virus was not a slow process of evolution rather than it was spreading very fast due to global travel. Phylograms of 8 proteins clearly indicated the pandemic situations associated with spread of H1N1. It was evidenced from the phylogram, that transmission of virus occurring very rapidly across the world as most of the sequences accounting its homology with geographically close and distant countries also. The multiple sequence alignment revealed that all these sequences from each group were genetically very close [13]. As our study, involved nine protein sequence entries of India compared to two from other countries, it had been hypothesized that all proteins isolated from India should be clustered only within India only, but few protein entries of India were clustered within India

while few clustered with close Asian countries and foreign countries and very few remain non-clustered.

Each H1N1 protein was found to have at least one evolutionary fully conserved region. Our analysis showed that in matrix protein sequences from particular one region in India were clustered within the other countries, i.e. Australia and USA. Very few mutations occurred in China, other different regions in India, Korea and Russia. Heamagglutinin and Neuraminidase are very important protein in H1N1 virus as they play important role in viral attachment and release from host cells, respectively. The phylogenetic analysis of 8 H1N1 proteins revealed that different clades obtained from phylogenetic tree were not confined to the region or countries from where they had isolated, rather proteins isolated from one country clustered well with the proteins isolated from other countries.

As there were 16 isolates of matrix proteins, obtained from different countries had identical amino acid sequence, they clustered in one clade (Fig. 1). Due to single amino acid substitution in MACHI1 (D94N), MAIND4 (A83V), MAIND6 (F62C), MAIND8 (A142G) and these sequences showed little divergence as shown in Fig. 1.

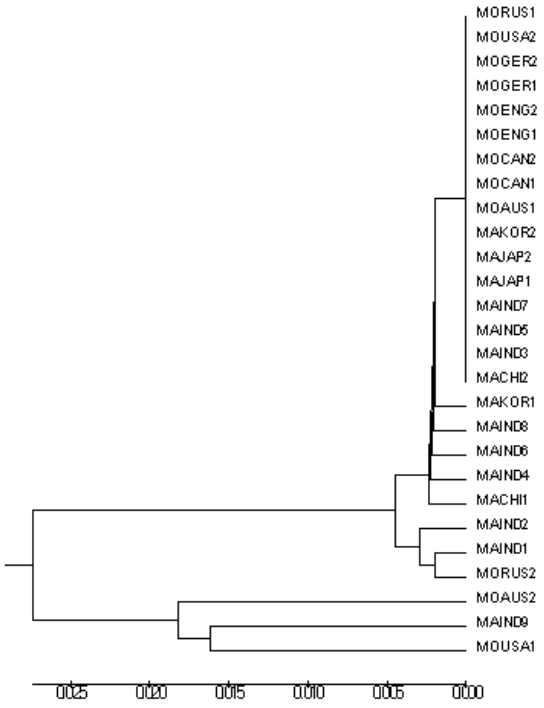

Fig. 1 Phylogram of matrix protein

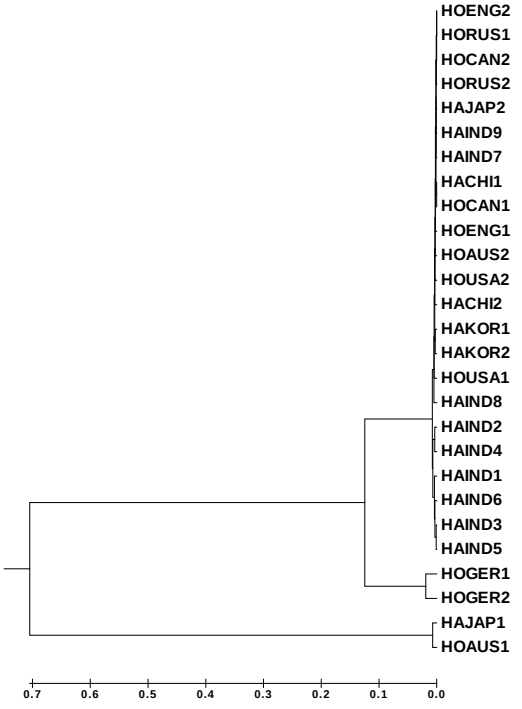

Fig. 2 Phylogram of heamagglutinin

Further, MAIND1, MAIND2 and MORUS2 were clustered in another clade while due to amino acid substitution at various positions in MAIND9, MOAUS2 and MOUSA1, they clustered in one clade. Heamagglutinin protein sequence entries from different regions in India were clustered in one clade i.e. HAIND2, HAIND4, HAIND1, HAIND6, HAIND3 and HAIND5 as shown in Fig. 2. As there was no mutation observed in HOENG2, HORUS1 and HOCAN2, they clustered in one clade (Fig. 2). Due to various positions of amino acid substitutions in HOGER1 & HOGER2 clustered in one clade and HAJAP1 & HOAUS1 clustered in another clade while due to single amino acid substitution in HORUS2 (S179N), HAJAP2 (T249K), HAIND9 (E391K), HAIND7 (I527V), HACHI1 (T220S), HOCAN1 (T220S), these sequences showed slight deviation. Further, HOENG1, HOAUS2, HOUSA2, HACHI2, HAKOR1, HAKOR2, HOUSA1, HAIND8 were clustered in another clade.

Neuraminidase cladogram (Fig. 3) showed NAKOR1 as a distinct clade as there was no mutation observed in its protein sequence. Further few divergences in sequences NOENG2, NOGER1, NOENG1, NAJAP2, NAJAP1, NACHI2, NOUSA1, NACHI1, NOCAN1, NOCAN2, and NOUSA2 were also observed (Fig. 3) and these sequences were clustered in one clade. Our analysis also showed that Neuraminidase sequences from several Indian isolates clustered with Russia, Korea & Germany. Due to amino acid substitution at various positions in NOAUS2, NOAUS1, NAIND7, NAIND8, NAIND6, NAIND9 were clustered in one clade. MSA result gave an idea about many mutations occurred in Heamagglutinin and Neuraminidase protein entries from different countries. Fig. 4 shows that little deviation in Nucleocapsid protein sequences from different countries were clustered in one clade except NCAJAP2 which diverged into another clade due to many amino acid substitutions at various positions.

In Polymerase PB1 phylogram (Fig. 5), PB1AIND6, PB1AIND2, PB1AIND3, PB1ORUS2 and PB1AIND1 were clustered in one clade due to more than one amino acids substitution in their sequences. Further, the sequences isolated from Japan, England, Germany, USA and Russia were clustered in another clade due to only single amino acid substitution in their sequences. In Polymerase PA phylogram, as there was no mutation observed in PAOGER1, PAORUS1, PAOCAN2, PAOAUS2 and PAAIND9, they clustered in one clade (Fig. 6). Due to amino acid substitutions at different positions in PAACHI2 and PAAJAP2, they clustered in another clade. Further, the sequences isolated from different regions were clustered in different clades due to a range of amino acid substitutions in their sequences. In Polymerase PB1 phylogram, India is well clustered with china and in polymerase PA, Japan clustered within China as shown in Figs. 5 and 6, respectively. Single amino acid substitution occurred in Asian countries and foreign countries also in both protein sequences of Polymerase PB1 and Polymerase PA. In Polymerase PB2, few protein sequence entries from India were clustered within India while few clustered within Asian countries.

In Polymerase PB2 cladogram (Fig. 7), PB2OENG1, PB2OENG2, PB2ORUS1 were clustered in one clade due to single amino acid substitution (K340N) at same position. As there was no mutation observed in the sequences of PB2OUSA1, PB2OUSA2, PB2GER1 and PB2CAN2, they clustered in another clade. Further, the sequences isolated from other regions, were clustered in different clades due to a range of amino acid substitutions in their sequences. In Phylogram of non-structural protein (Fig. 8), NSOUSA2, NSAJAP2, NSOUSA1 and NSAJAP1 were clustered in one clade due to various positions of amino acid substitutions in their sequences while NSAIND3, NSAIND4 and NSAIND9, clustered in another clade due to amino acid substitution at same positions (L90I, N205S) in their sequences. As there was no mutation observed in NSOENG1, NSOGER1, NSOAUS2, NSOAUS1, NSAKOR2 and NSACHI1, they clustered in one clade.

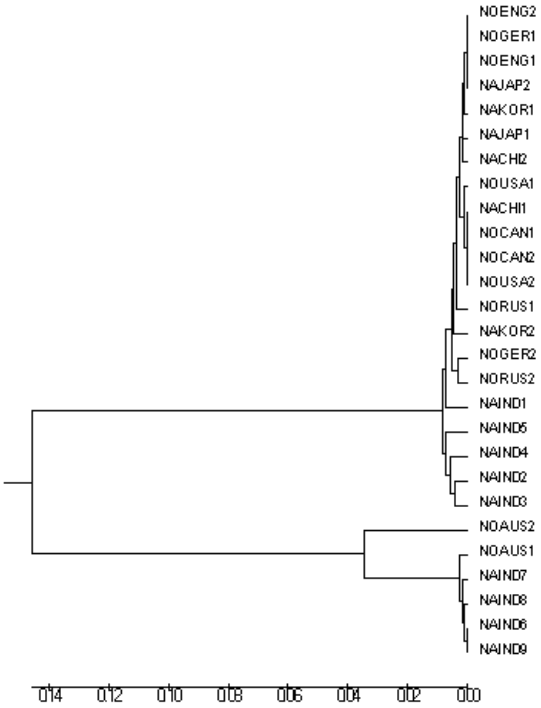

Fig. 3 Phylogram of neuraminidase

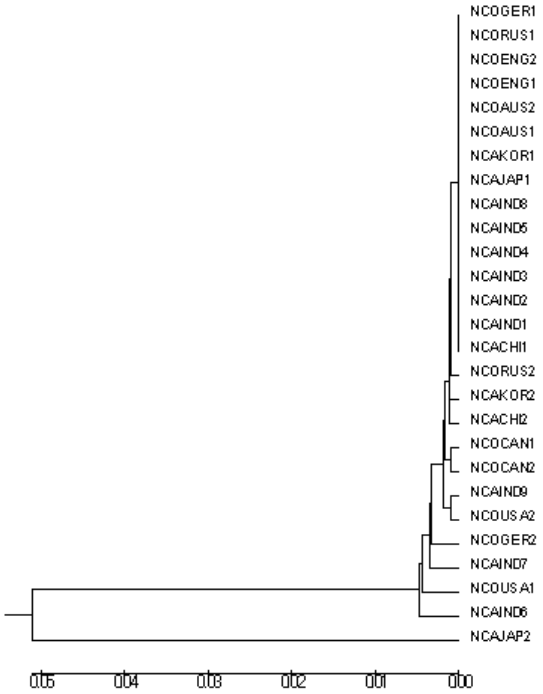

Fig. 4 Phylogram of neucleocapsid

Due to amino acid substitution at various positions observed in PB2AJAP2, it diverged into different clade. After that Non-structural protein entries from USA and Japan showed amino acid substitution at various positions and these sequences showed many divergences and they clustered in one clade as shown in Fig. 8. Due to single amino acid substitution obtained from MSA results, in the sequences isolated from Germany, Korea, China, some regions in India, Canada, England and Russia showed little divergence (Fig. 8).

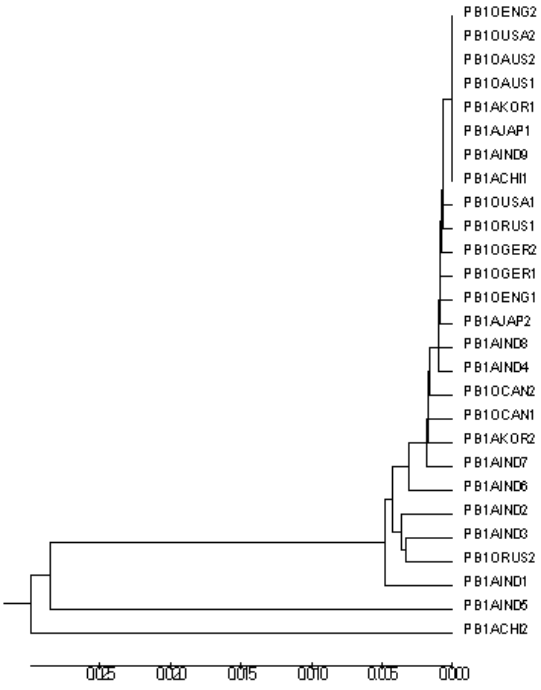

Fig. 5 Phylogram of Polymerase PB1

Fig. 6 Phylogram of PolymerasePA

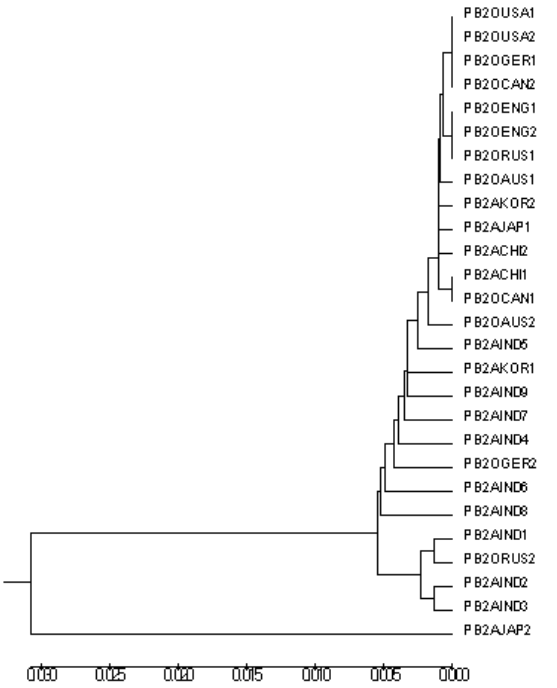

Fig. 7 Phylogram of Polymerase PB2

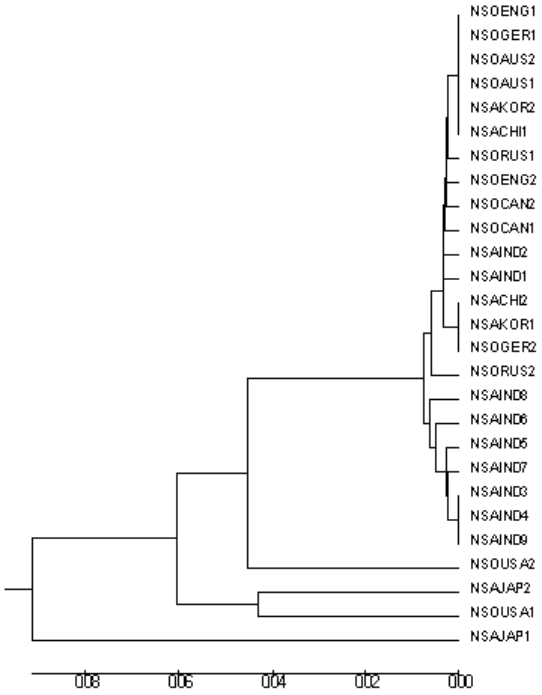

Fig. 8 Phylogram of non-structural protein

As we are entering the "era of pandemics" more threats like H1N1 will be a severe to mankind and role of phylogenetics always helps us to understand flow of severity and resistance pattern of the causative agents. The progressive adaptation of the new virus to the host could be the reason for the increase in severity. It is also evident that as the virus continues to spread there could be antigenic drifts and shifts that could increase the virulence, hence continuous monitoring of the various strains will aid to evolve appropriate vaccination strategies and prevention of outbreaks as well [7]. In our study also it has been evidenced that transmission of H1N1virus across globe has been obvious because of no barrier of continents as it was earlier. Further, out at different amino acid substitutions at different positions of Neuraminidase, the mutation H275Y was reporter to be associated with oseltamivir-resistant prepandemic seasonal subtype H1N1 viruses that emerged in 2007–08 [8] and the mutation at 248 position (N248D) was also reported as a major nonsynonymous mutations associated with 2009 pandamic influenza viruse [10]. Further, a mutation in Polymerase PA (N321K) also reported to associate with pandemic 2009 influenza virus [5].

Conclusion

In India, it has been evidenced that H1N1 virus entered and distributed more prominently from India followed by Asian countries further followed by foreign countries which represents that continental barrier (distance) and easy transport may be responsible for mix H1N1 population available in India and also available across the world. In decision it could be said that random sequencing of the bio-samples and its available data obtained from infected population of variant localities helped understand distribution and flow pattern of H1N1 virus across the world and phylogenetics tools predicted the defined phyletic clusters available in that population.

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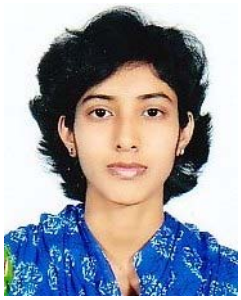

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