Phylogenetic Analysis of the Neuraminidase Gene Reveals that the H5N1 Strains Prevalent in Chickens During 2006 Bird Flu Outbreaks in Two Regions of Maharashtra, India Are Genetically Different

Mohd Danishuddin and Asad U. Khan*

Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India.

*Corresponding author. E-mail: asad.k@rediffmail.com

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In February 2006, two outbreaks of highly pathogenic avian influenza A virus subtype H5N1 occurred in chickens in two neighboring districts (first in Nandurbar and second in Jalgaon) of Maharashtra, India, in a span of 12 days. In the present study, the neuraminidase (NA) gene of the two Indian H5N1 isolates was taken into consideration to find if the two strains are genetically similar. Phylogenetic analysis of the NA gene showed that the H5N1 strains isolated from the two outbreaks were not originated from the same source. The first Indian isolate (Nandubar/7972/06) was clustered closest to an isolate from chicken in Vietnam in 2004, whereas the second Indian isolate (Jalgaon/8824/06) showed resemblance to strains isolated from swan in Italy and Iran in 2006. Moreover, amino acid sequence analysis showed varying hot spots for substitutions between these two Indian isolates, and three substitutions were found at functional domain sites. Secondary structure changes due to these substitutions were also reported. This study reveals that the H5N1 strains isolated from chickens during 2006 bird flu outbreaks in two neighboring districts of Maharashtra, India are genetically different.

Key words: avian influenza virus, H5N1, neuraminidase, phylogeny, mutation

Introduction

Influenza viruses are members of the family Orthomyxoviridae (*1*) with a genome of single-stranded negative-sense RNA composed of 8 gene segments encoding at least 10 proteins (*2*). These viruses are classified into three major types A, B, C based on the antigenic differences in their nucleoprotein and matrix protein. The type A virus is pleomorphic and spherical (approximately 120 nm in diameter) and can be further classified into subtypes according to the antigenicity of two surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (*3*). To date, 16 HA subtypes and 9 NA subtypes of influenza A virus have been identified (*4*). The NA protein contains an N-proximal anchor and a C-terminal ectodomain. Its main function is to cleave terminal sialic acids from glycoconjugates to facilitate the release of new virus progeny, thereby causing infection from cell to cell (*1*).

The avian influenza virus (AIV) belongs to the type A virus. All the identified subtypes of influenza A virus have been reported to be present in wild aquatic bird populations such as wild ducks, shorebirds and gulls, indicating that wild aquatic birds are the natural reservoir of AIV (*5*, *6*). However, this avian virus usually does not have the ability to be pandemic in wild aquatic birds but once transmitted to poultry (such as chickens), it will show high mortality rate and cause great economic loss (*7*).

In 1997, avian influenza A virus subtype H5N1 acquired the ability to be transmitted from bird to human in Hong Kong and caused at least 6 deaths out of 18 infected persons (*8*, *9*). Since then, this highly pathogenic avian influenza virus has frequently emerged in poultry in various Asian, European and African countries, causing serious losses. It has not yet been reported that this subtype could spread from human to human but this ability can be acquired by mutations, which may cause either antigenic drift or shift to human influenza virus (*10–12*).

The first cases of H5N1 outbreaks in India were reported in chickens in two neighboring districts (first in Nandurbar and second in Jalgaon) of Maharash-

tra state in February 2006 within a span of 12 days (*13*). Previous phylogenetic analysis of the HA gene of both isolates suggested that the two outbreaks were possibly due to different populations of the virus introduced at two different times (*13*). In view of the above background, we conducted phylogenetic analysis of the NA gene of these two H5N1 strains with other H5N1 strains isolated from bird flu outbreaks. The main objective of this study was to validate whether the H5N1 strains from the two outbreaks in India are genetically similar or are reassorted leading to antigenic drift or shift.

Results and Discussion

Phylogenetic analysis

Phylogenetic analysis of the NA gene of the two Indian isolates and other H5N1 isolates from bird flu outbreaks occurred during 2001 to 2006 revealed that the two Indian isolates were heterogeneous and were clustered in two different groups (Figure 1). The first Indian isolate (Nandurbar/7972/06) was clustered in Group 2, a group comprised of isolates mainly from outbreaks occurred in Vietnam and Thailand during

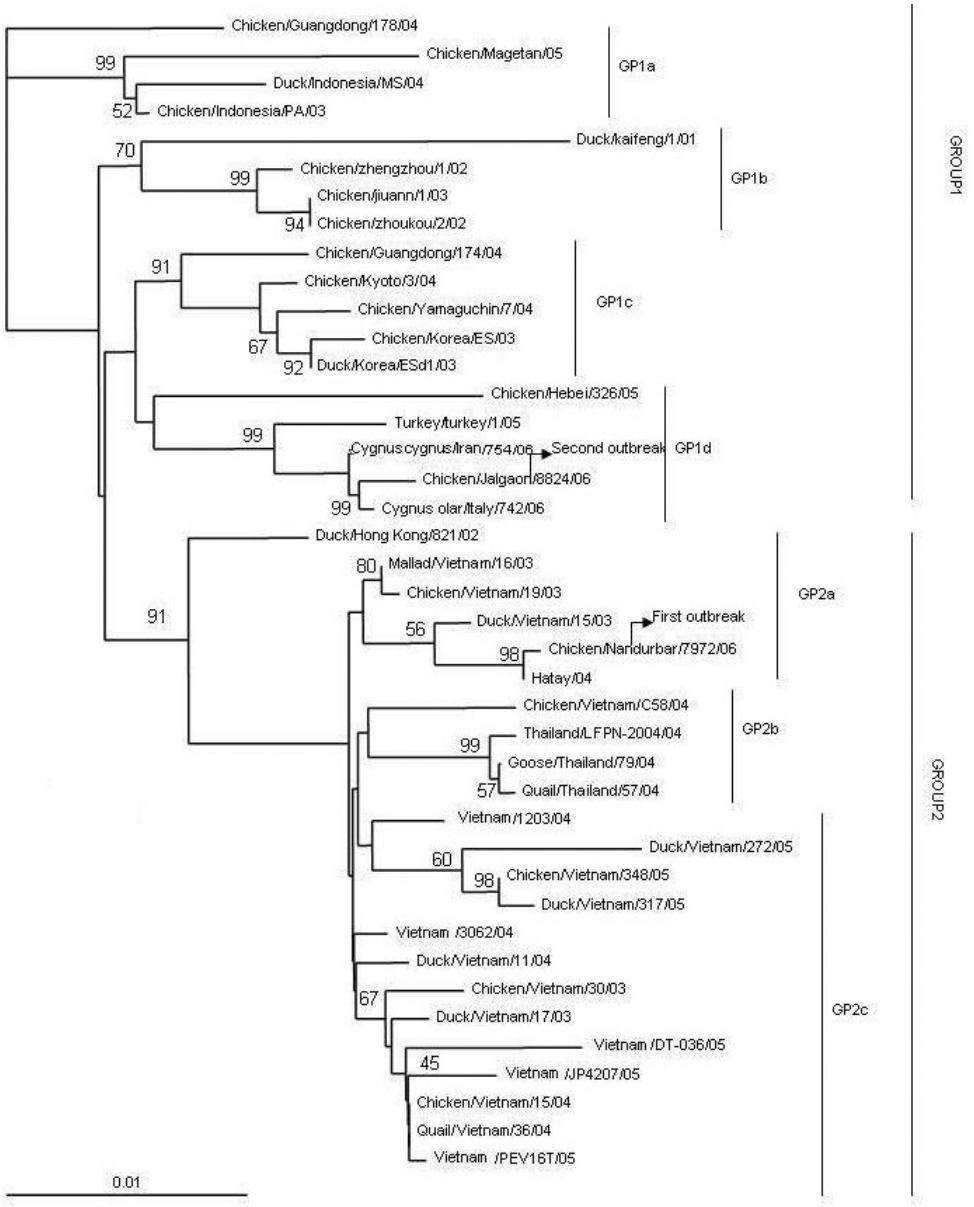

Figure 1 Phylogenetic tree for the NA gene of the two Indian isolates and other H5N1 isolates from bird flu outbreaks occurred during 2001 to 2006.

2003 to 2005. The second Indian isolate (Jalgaon/8824/06) was clustered in Group 1, the most heterogeneous group of isolates from outbreaks occurred in different countries (China, India, Indonesia, Iran, Italy, Japan, Korea, Turkey) during 2001 to 2006. Group 1 can be further divided into four subgroups (GP1a to GP1d) (Figure 1). The second Indian isolate is in subgroup GP1d and shows maximum homology (>99%) with isolates Cygnus olar/Italy/742/06 and Cygnus cygnus/Iran/754/06 from swan in Italy and Iran in 2006, indicating their close relationship. Group 2 can be further divided into three subgroups (GP2a to GP2c) (Figure 1). The first Indian isolate is in subgroup GP2a and shows maximum homology (>98%) with an isolate Hatay/04 (NCBI Accession No. AJ867075) from chicken in Vietnam in 2004. Thus, our findings suggest that the NA gene of H5N1 isolates from chickens during two outbreaks in Maharashtra does not belong to the same strain. The most probable reason is reassortment of the genome as has also been reported earlier (*14*).

Sequence comparison

Amino acid sequences of the NA gene of these two Indian isolates (Nandurbar/7972/06 and Jalgaon/8824/06) were compared and differences were found in fourteen amino acids (Table 1). Three substitutions were identified to be important as they were found on functional domains: substitution 64R→T (that is, amino acid substitution at position 64 from R in Nandurbar/7972/06 to T in Jalgaon/8824/06) occurred at protein kinase C (PKC) phosphorylation domain (positions 62–64; Domain SvR), substitution 91K→R at cell attachment site (positions 91–93; Domain RGD), and substitution 362E→G at N-myristoylation domain (positions 362–367; Domain GTdsSF). The PKC pathway is one of the significant signal transduction pathways that regulate various biological functions (*15*, *16*), especially in control of subcellular transport and trafficking (*17*). The N-myristoylation domain is found in viruses, fungi and higher eukaryotes and is formed as a consequence of post-translation modification catalyzed by N-myristoyltransferase enzyme. In this domain, myristic acid is covalently attached via an amide bond to the alpha-amino group of a glycine residue at N-terminus. This domain plays a vital role in membrane targeting and signal transduction (*18*). Myristoylation has the ability to influence the conformational stability of individual proteins. Moreover, it also interacts with membranes or the hydrobhobic domains of other proteins (*19*).

In addition, of these fourteen substitutions, six were observed to be non-synonymous. Among them, substitutions 17T→I, 233H→Y, 320S→P, 362E→G and 434G→S changed the amino acid property from hydrophilic to hydrophobic, whereas substitution 145V→E changed the amino acid property from hydrophobic to hydrophilic (Table 1). Such mutations

Table 1 Comparison of neuraminidase amino acid sequences of two Indian isolates

Position	AA*[1] in Nandurbar/7972/06	AA in Jalgaon/8824/06	Change in property	Change in secondary structure*[2]
17	T	I	Hydrophilic → Hydrophobic	E
39	H	Q	Hydrophilic	C
44	H	R	Hydrophilic	C
53	N	K	Hydrophilic	H → C
64#	R	T	Hydrophilic	E
75	N	S	Hydrophilic	C
91#	K	R	Hydrophilic	C
145	V	E	Hydrophobic → Hydrophilic	C
233	H	Y	Hydrophilic → Hydrophobic	H
247	V	I	Hydrophobic	E
320	S	P	Hydrophilic → Hydrophobic	C
332	R	K	Hydrophilic	E
362#	E	G	Hydrophilic → Hydrophobic	E
434	G	S	Hydrophilic → Hydrophobic	E → C

*[1]AA, amino acid; *[2]Secondary structure: E, extended strand; C, random coil; H, helix.

#Mutation at functional domain site.

have also been found in previous studies to alter the binding property of NA for the binding of antiviral drugs, like oseltamivir, making viral strains become resistant against these drugs (*20–22*). Other substitutions were found to be synonymous and did not change the chemical property at their respective sites.

Secondary structure changes

It has been reported that mutation not only alteres the polarity or hydrophobicity but also changes the propensity of each amino acid residue to stabilize the secondary structure (*23*). Changes in protein secondary structure due to substitutions were also reported in this study. When comparing the amino acid sequences, we found that substitution 53N→K changed secondary structure from helix to coil, whereas substitution 434G→S changed secondary structure from extended strand to coil (Table 1). In addition, amino acids at positions 139–140 were found to have extended strand in Jalgaon/8824/06 whereas have coil in Nandurbar/7972/06.

Conclusion

Our study concludes that the H5N1 outbreaks occurred in chickens in two districts of Maharashtra, India with the gap of 12 days in February 2006 were caused by strains from two different sources. The first isolate Nandurbar/7972/06 was clustered closest to an isolate from chicken in Vietnam in 2004. The second isolate Jalgaon/8824/06 showed resemblance to strains isolated from swan in Italy and Iran in 2006. Our study supports the suggestion in the previous study that these two Indian isolates were possibly due to different populations of the virus introduced at two different times (*13*).

Materials and Methods

Nucleotide sequences of the NA gene of the two Indian isolates and other H5N1 isolates were accessed from the NCBI database (http://www.ncbi.nlm.nih.gov). Sequences taken for analysis were from the H5N1 outbreaks occurred in different Asian and European countries during 2001 to 2006 (Figure 1). Protein sequences of the NA gene were also downloaded from NCBI with GenBank Accession No. ABG88886 for Nandurbar/7972/06 and ABJ98950 for Jalgaon/8824/06. Multiple sequence alignment of all the nucleotide sequences was carried out by ClustalX program with default parameters (*24*). Phylogenetic tree was built by neighbor-joining program with kimura 2 parameter available in Phylip 3.65 package (http://evolution.genetics.washington.edu/phylip.html). Phylogenies were determined by bootstrap analysis of 500 replicates with seqboot program in Phylip 3.65 package. The tree was drawn by TreeView 1.6 program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Secondary structure analysis was performed by GOR IV method (http://npsa-pbilibcp.fr). Different domains/motifs were searched at ExPASy server (http://expasy.org/tools/scanprosite). Amino acid sequences of both Indian isolates were compared by MAP MUTATION program (*25*) to analyze mutations among these isolates.

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Authors' contributions

MD collected the dataset, performed data analyses and drafted the manuscript. AUK designed the study, supervised the project and co-wrote the manuscript. Both authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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