

# Phylogenetic Tree and Antiviral Resistance Analysis of Neuraminidase Gene of Influenza A Virus in H5N1 Strains Found in 2005-2007

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**Citation:** Saha RK, Mir H, Abdullah MD, et al. Phylogenetic Tree and Antiviral Resistance Analysis of Neuraminidase Gene of Influenza A Virus in H5N1 Strains Found in 2005-2007. *Transl Biomed.* 2015, 6:4.

## Abstract

This study brings the analysis of phylogenetic tree and amino acid sequences of Neuraminidase (NA) from the influenza A virus that can infect a wide variety of birds and mammals. We have analyzed strains of three different years (2005; 2006 & 2007) of H5N1 from different country to investigate the antiviral resistance patterns with respect to reported mutant positions of amino acids. We found that similar types of amino acids near the reported mutated positions of several H5N1 strains that may cause reduce sensitivity to Neuraminidase and its inhibitors. The analysis of phylogenetic tree reveals some diverse strains of H5N1 which can show antigenic drift.

**Keywords:** Phylogenetic tree; Sequence similarities; Antiviral resistance; DNA mutation

**Abbreviations:** FJ: Fujian; VN: Vietnam; GX: Guangxi; JX: Jiangxi; HN: Hunan; AKH: Astrakhan; YN: Yunnan; GY: Guiyang; XZ: Xinjiang; KH: Cambodia; EGP: Egypt; NG: Nigeria; BVR: Bavaria; QH: Qinghai; SE: Sweden; LAO: Laos; KW: Kuwait; CK: Chicken; DK: Duck; GS: Goose; SN: Swan; BHGS: Bar-Headed Goose; MDK: Muscovy Duck; MALL: Mallard; WDK: Wild Duck; SN: Swan; MSN: Mute Swan; CGO: Cygnus Olor; TDK: Tufted Duck; GDK: Goldeneye Duck; HGL: Herring Gull

**Received:** September 21, 2015; **Accepted:** December 04, 2015; **Published:** December 10, 2015

## Introduction

Neuraminidase; also referred to as sialidase; is an antigenic glycoprotein anchored in the surface envelope of the influenza virions; which hydrolytically cleaves the terminal sialic acid from the host cell receptors. Thus; it plays a crucial role in the release of viral progeny from the membranes of infected cells; prevents self-aggregation of virions and facilitates the movement of the infectious viral particles in the mucus of the respiratory epithelia [1,2]. Influenza neuraminidase has been established as a key drug target for the prophylaxis and treatment of influenza infections; predominantly for the following reasons: Firstly; the structure of the influenza neuraminidase active site is highly conserved between influenza A and B strains; making neuraminidase an attractive target for the development of broad-spectrum inhibitors [3]. Secondly; resistance to neuraminidase inhibitors develops less commonly than to other anti-influenza drugs. Nevertheless; the intensive application of neuraminidase inhibitors for influenza treatment results in a permanently increasing number

of drug-resistant strains [4]. Thirdly; in contrast to amantadines; neuraminidase inhibitors are mostly well tolerated in patients under therapy [5]. Finally; neuraminidase protein is a freely accessible target for antiviral molecules with an extracellular mode of action.

The anti-influenza drugs are usually classified according to their target in the viral life-cycle. Such antiviral molecules are particularly used as inhibitors of the following processes: attachment of the virus to host cell receptors; endocytosis and fusion of viral and cell membranes; replication and transcription of the viral genome; synthesis of viral proteins; assembly of the viral progeny and release of the new virions into the outside environment. The following paragraphs are focused on the description of basic classes of influenza virus inhibitors.

Avian influenza A consists of two major glycoproteins which are Hemagglutinin (HA) and Neuraminidase (NA) [6]. HA glycoproteins are more prone to attach to the cell surface sialic acid receptors. There is a difference between host surface receptors on the

target cell which is believed to be the possible restrictive factor of avian influenza. HA gene of avian cell binds to Sia2-3 Galactose-containing receptor which is different from human Sia2-6Galactose containing receptor [7]. Before functioning as a virus it needs post translational cleavage by host proteases [8]. HA followed by NA are important antigenic determinant from which neutralizing antibodies are directed. There are several subtypes of HA and NA. 18 different HA subtypes (H1 to H18) and 11 different NA subtypes (N1 to N11) are found [9]. There is a membrane protein named M2 protein which regulates the internal P<sup>H</sup> level of the virus. This membrane protein is responsible for uncoating the virus during early stages of viral replication [10]. Amantadine and rimantadine block this function. NA catalyze the cleavage of glycosidic linkages to sialic acid on the surface of the viral particle and host cell thus preventing the aggregation and facilitating the release of progeny viruses from the infected cell. Antiviral drugs like Oseltamivir and zanamivir (NA inhibitors) inhibits this important function are the key to the antiviral treatment.

Neuraminidase inhibitors (NAIs) are first-line agents for the treatment and prevention of influenza virus infections. Resistance to the NAIs can be both drug and virus type or subtype specific. The neuraminidase (NA) inhibitors (orally administered oseltamivir and inhaled zanamivir) are currently an important class of antiviral drugs available for the treatment of seasonal and pandemic influenza. Although administration of NA inhibitors may significantly reduce influenza virus transmission; it risks the emergence of drug-resistant variants [11,12]. Several mutations in seasonal or pandemic strains confer resistance to oseltamivir; which is currently the most widely used drug. These mutations are found mainly in the framework of the enzyme and they appear to destabilize drug binding to the target enzyme; thereby reducing viral susceptibility to the treatment [13-15]. Oseltamivir-resistant H5N1 viruses with the H274Y NA mutation have been isolated from three patients during drug treatment or prophylaxis [16,17] and those with the N294S NA mutation; from two patients in Egypt [18]. In addition; the highly pathogenic A/ Hanoi / 30408 / 2005(H5N1) influenza virus that was isolated from a patient treated with oseltamivir had a mixed oseltamivir-sensitive and oseltamivir-resistant population [19].

### Materials and methods: Sequence and data source

Data used in this study are obtained using nucleotide BLAST search from publicly available database of National Centre for Biotechnology Information (NCBI). Multiple sequence alignments; editing; assembly of strains were performed in windows platform with the Geneious program version 7.1.3 (trial). Numbers at nodes in the tree indicate Neighbor-Joining bootstraps value generated from 1,000 replicates.

## Result and Discussion

In 2005 we have selected total 169 strains of Neuraminidase (NA) strain of H5N1 (**Figure 1** and **Table 1**).

In 2006 we have selected total 148 strains of Neuraminidase (NA) strain of H5N1 (**Figure 2** and **Table 2**).

In 2007 we have selected total 158 strains of Neuraminidase (NA) strain of H5N1 (**Figure 3** and **Table 3**).

From three years we have got some strains which seems to diverse from our analysis. We did our literature search but we did not get any information about these diverse strains. So it seems to us that these strains are not responsible for antigenic shift. Neighbor joining method and bootstrap value shows that this diverse strain is showing antigenic drift which may transfer to the other avian in the same country or other different country as well through migratory process.

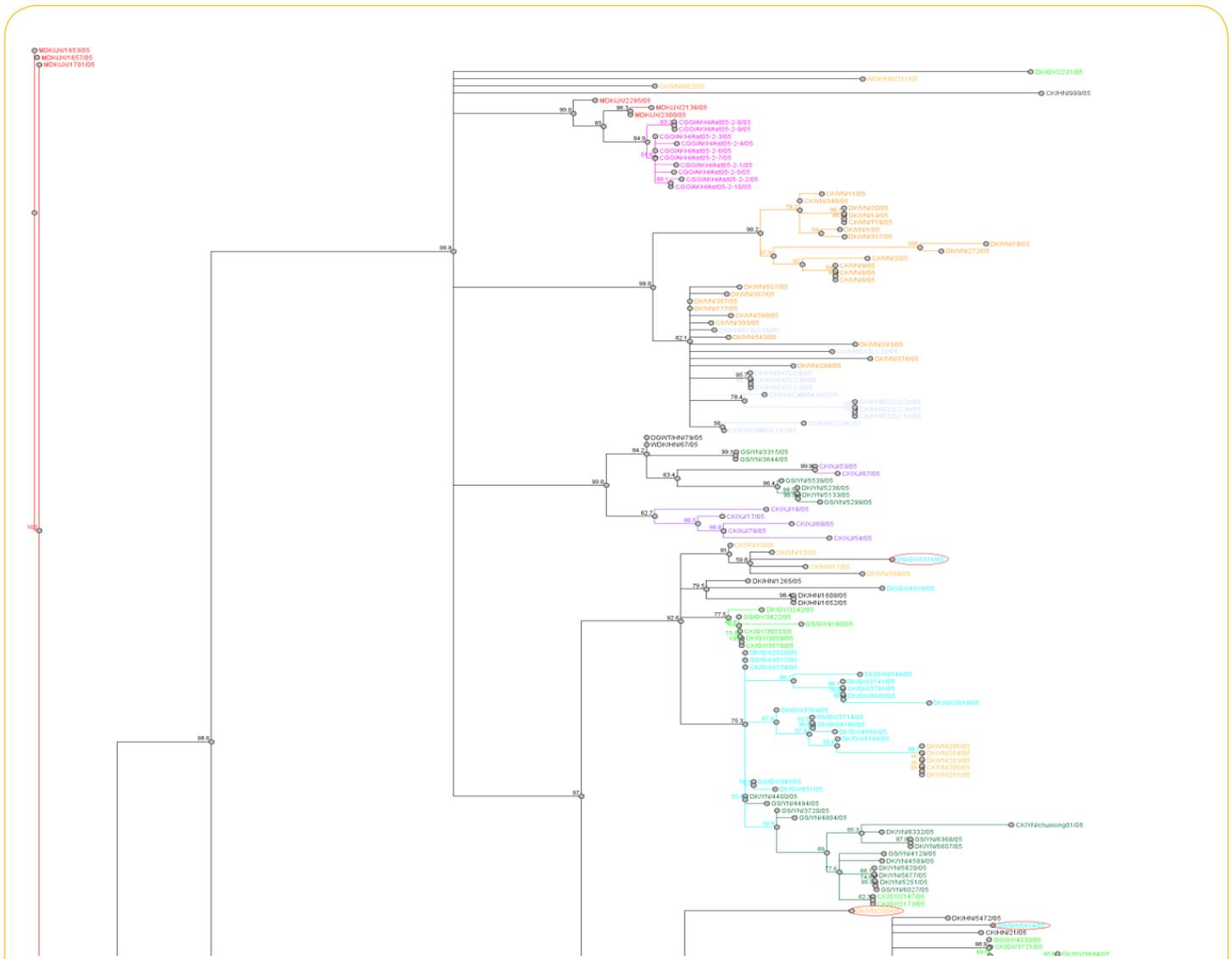
From the phylogenetic tree of 2005 we came to see that all VN like viruses formed a cluster. Among them GS/GX/3316/05 shows 59.8 bootstrap value with DK/VN/12/05. Number of bootstrap value is showing the similarities between these two strains. Strain GS/GX/3316/05 may be migrated or may be mutated which can show antigenic drift. GS/GX/5414/05 shows 65.7 bootstrap values with DK/HN/5472/05. Number of bootstrap value is showing the similarities between these two strains. Strain GS/GX/5414/05 may be migrated or may be mutated which can show antigenic drift.

DK/JX/80/05 and WDK/HN/021/05 viruses are closely related to each other as they both are detached from their region and showing the same bootstrap value 69.8. One VN virus (DK/VN/205/05) forming a clade with HB like viruses. Genetic character of this virus is different from other VN like viruses as this is detached from the cluster of VN like viruses showing the antigenic drift.

Phylogenetic tree analysis of 2006 shows that there are few diverse strains which may show genetic drift. One virus from Egypt (CK/EGP/RIMD6-1/06) form clade with the QG like viruses. HGL/SE/V1116/06 shows 94.6 bootstrap values with MSN/BVR/12/06. Number of bootstrap value is showing the similarities between these two strains. Strain HGL/SE/V1116/06 may be migrated or may be mutated which can show antigenic drift. Among them GS/GX/532/06 shows 50.8 bootstrap values with DK/HN/344/06. Number of bootstrap value is showing the similarities between these two strains. Strain GS/GX/532/06 may be migrated or may be mutated which can show antigenic drift. One HN like virus (DK/HN/29/06) forming a clade with Guangxi (GX) like viruses. One KH virus (DK/KH/D14AL/06) forming a clade. Genetic character of this virus is different from other KH like viruses as this is detached from the cluster of KH like viruses showing the antigenic drift.

Phylogenetic tree analysis of 2007 shows that there are few diverse strains which may show genetic drift. Among them DK/VN/NCVD-31/07 shows 92.4 bootstrap values with DK/LAO/PO127/07. Number of bootstrap value is showing the similarities between these two strains. Strain DK/VN/NCVD-31/07 may be migrated or may be mutated which can show antigenic drift. TWO QH virus (BHGS/QH/13/07 and BHGS/QH/F/07) forming a separate clade. Genetic character of this virus is different from other QH like viruses as these are detached from the cluster of QH like viruses showing the antigenic drift.

The molecular mechanisms that enable avian influenza viruses to cross the species barrier and transmit efficiently in humans are incompletely understood. Some experiments have been done to identify the transmission pattern and it shows poorer transmission from infected to susceptible animals [20-22].



**Figure 1** Phylogenetic tree of the year 2005.

**Table 1** List of diverse strains from our analysis of 2005 are given below.

Accession number	Strain name
EU930918	DK/VN/205/05
EF124227	GS/GX/5414/05
HM172200	DK/JX/80/05
EU329179	WDK/HH/021/05
EF124280	GS/GX/3316/05

Migration process can influence transmission of viruses. Migratory birds can carry pathogens from country to country thereby playing a role distributing influenza viruses. Avian influenza A consists of two major glycoproteins which are Hemagglutinin (HA) and Neuraminidase (NA) [23]. HA glycoproteins are more prone to attach to the cell surface sialic acid receptors. There is a difference between host surface receptors on the target cell which is believed to be the possible restrictive factor of avian influenza.

Human infections are periodic. In some cases these viruses are accompanied by high mortality. As a result they are the major

concern about the potential H5N1 as an endemic virus.

Although human infections are sporadic; they are accompanied by high mortality; raising major concerns about the potential of H5N1 as a pandemic virus [24]. Fortunately; H5N1 viruses have not yet naturally acquired the ability to stably transmit between humans [25,26]. One factor that limits transmission of avian viruses in humans is the receptor specificity of the hemagglutinin (HA) [27]. Avian viruses; like H5N1; preferentially bind to  $\alpha$  2; 3 sialosides (avian-type receptors); whereas human viruses prefer  $\alpha$  2; 6 sialosides (human-type receptors that are found in the human respiratory tract).

Before functioning as a virus it needs post translational cleavage by host proteases [28]. HA followed by NA are important antigenic determinant from which neutralizing antibodies are directed. There are several subtypes of HA and NA. 18 different HA subtypes (H1 to H18) and 11 different NA subtypes (N1 to N11) are found [29].

In humans; the SA  $\alpha$  2;6 Gal receptor is expressed mainly in the



**Figure 2** Phylogenetic tree of the year 2006.

upper airway; while the SA  $\alpha$  2;3 Gal receptor is expressed in alveoli and the terminal bronchiole [30]. A virus with good affinity to both SA  $\alpha$  2;3 Gal and SA  $\alpha$  2;6 Gal receptors may be a very dangerous one; which could both infect efficiently via its binding to SA $\alpha$ 2; 6Gal in the upper airway and cause severe infection in

the lung via its binding to SA  $\alpha$  2; 3Gal.

Data used in this study are obtained inside using nucleotide BLAST search from publicly available database of National Centre for Biotechnology Information (NCBI). Multiple sequence alignments;



**Figure 3** Phylogenetic tree of the year 2007.

editing; assembly of strains were performed in windows platform with the Geneious program version 7.1.3 (trial).

In this study we will analyze some avian neuraminidase (H5N1) of different years. Analysis includes building nucleotide sequence

and translating them into amino acid sequence. Then we will study amino acid positions with respect to some reported mutation to see the genetic pattern. After analyzing we will try to find out whether there are any similarities between avian and

**Table 2** List of diverse strains from our analysis of 2006 are given below.

Accession number	Strain name
HQ200451	DK/KH/D14AL/06
AB601142	CK/EGP/RIMD6-1/06
EU889100	HGL/SE/V1116/06
HM172177	DK/HN/29/06
EF124273	GS/GX/532/06

**Table 3** List of diverse strains from our analysis of 2007 are given below.

Accession number	Strain name
FJ602852	BHGS/QH/13/07
FJ461727	BHGS/QH/F/07
CY030455	DK/VN/NCVD-31/07

human or not. There are some reported avian H5N1 strains that affect human which are A/Goose/Hong Kong/739.2/2002 [31]; A/duck/Egypt/D1Br12/2007 [32]; A/Duck/Singapore/3/97 [33]; A/egret/Egypt/1162/2006 [34]. All of these strain show preferential binding to Sia  $\alpha$  (2; 6) Gal receptor that can infect a human. Few specific positions of amino acids are responsible for this binding. The NA of VN1203 in a PR8 background (designated N158D/N224K/Q226L or N158D/N224K/Q226L/T318I; respectively) preferentially bind to Sialic acid (Sia) and attach to human tracheal epithelia [35,36] (Tables 4 and 5).

In case of N158D (Asn-158-Asp) reported position; we found amino acid Threonine (Thr) in our software (Geneious). We found two mutations here; Threonine (Thr) to Serine (Ser) in one strain and from Threonine (Thr) to Alanine (Ala) in two strains. We found amino acid Asparagine (Asn) in two positions (142 and 147) which is located within twenty positions before 158. We found one mutation here from Asparagine (Asn) to Serine (Ser) in one strain. We have also found Asparagine (Asn) in one position (176) which is located within twenty positions after 158. We found no mutation here. We found T158S and T158A mutation which indicates that polarity is changed from polar to polar and from polar to non-polar as Threonine (Thr); Serine (Ser) is Polar and Alanine (Ala) is non-polar.

In case of N224K (Asn-224-Lys) reported position; we found amino acid Tryptophan (Trp) in our software (Geneious). We found amino acid Asparagine (Asn) in two positions (205 and 214) which is located within twenty positions before 224. We found two mutations here from Asparagine (Asn) to Serine (Ser) in seven strains and from Asparagine (Asn) to Aspartic acid (Asp) in one strain. We have also found Asparagine (Asn) in three positions (226; 227 and 240) which is located within twenty positions after 224. We found three mutations here from Asparagine (Asn) to Serine (Ser) in one strain; Asparagine (Asn) to Tyrosine (Try) in one strain and from Asparagine (Asn) to Serine (Ser) in one strain. We found N205S and N205D mutation which indicates that polarity is changed from polar to polar and from polar to negative as Threonine (Thr); Serine (Ser) is Polar and Aspartic acid (Asp) is negative. We also found N227Y mutation which indicates that polarity is changed from polar to polar as Threonine (Thr); Serine (Ser) is Polar and Tyrosine is polar.

In case of Q226L (Gln-226-Leu) reported position; we found amino acid Threonine (Thr) in our software (Geneious). We found one

**Table 4** Summary of some reported position which can be responsible for antigenic shift from avian to human. Reported neuraminidase amino acid position with mutation that bind  $\alpha$  2, 6 receptor can affect human.

Amino acid mutation position with reference	Short form	Amino acid in Geneious in reported position
Asn-158-Asp (Maines et al.) [34]	N158D	T
Asn-224-Lys (Chen et al.) [35]	N224K	W
Gln-226-Leu (Maines et al.) [34]	Q226L	N
Thr-318-Ile (Chen et al.) [35]	T318I	E

**Table 5** Correlation of Reported neuraminidase amino acid position with mutation with our experimental strain, and their mutation pattern (exact and around twenty positions): Original Amino Acid analysis.

Reported AA with mutation point	AA in Geneious in reported position	Exact AA which matches with the reported data with respect to specific mutation point (before 20 positions)	Exact AA which matches with the reported data with respect to specific mutation point (after 20 positions)
N158D	T158S (1) Polar to Polar T158A (2) Polar to Nonpolar	N142 N147S (1) Polar to Polar	N176
N224K	W224	N205S (7) Polar to Polar N205D (1) Polar to Negative N214	N226S (1) Polar to Polar N227Y (1) Polar to Polar N227S (1) Polar to Polar N240
Q226L	N226S (1) Polar to Polar		Q232P (1) Polar to Nonpolar
T318I	E318K (4) Electrically charged Negative to Positive		T239A (1) Polar to Nonpolar T239K (1) Polar to Positive

mutations here; Threonine (Thr) to Serine (Ser) in one strain. We found amino acid Glutamine (Gln) in one position (232) which is located within twenty positions after 226. In this position there is one mutation here from Glutamine (Gln) to Proline (Pro) in one strain. We found N226S and Q232P mutation which indicates that polarity is changed from polar to polar and from polar to non-polar as Threonine (Thr); Serine (Ser) is Polar and Proline (Pro) is non-polar.

In case of T318I (Thr-318-Ile) reported position; we found amino acid Glutamic acid (Glu) in our software (Geneious). We found one mutation here from Glutamic acid (Glu) to Lysine (Lys) in four strain. We found amino acid Threonine (Thr) in position (239) which is located within twenty positions after 318. We found two mutations here from Threonine (Thr) to Alanine (Ala) in one strain and from Threonine (Thr) to Lysine (Lys) in one strain. In T239K; T239A and E318K mutations which indicate that polarity is changed from polar to polar; polar to positive and negative to polar as Threonine (Thr)

**Table 6** Correlation of Reported neuraminidase amino acid position with mutation with our experimental strain, and their mutation pattern (exact and around twenty positions): Mutated Amino Acid analysis.

Reported AA with mutation point	AA in Geneious in reported position	Exact AA which matches with the reported data with respect to specific mutation point (before 20 positions)	Exact AA which matches with the reported data with respect to specific mutation point (after 20 positions)
N158D	T158S (1) Polar to Polar T158A (2) Polar to Nonpolar	D143 D152	
N224K	W224	K212	
Q226L	N226S (1) Polar to Polar	L211V (1) Nonpolar to Nonpolar	L229
T318I	E318K (4) Electrically charged Negative to Positive		I321T (1) Nonpolar to Polar I321M (3) Nonpolar to Nonpolar I321V (2) Nonpolar to Nonpolar I324

is Polar; Alanine (Ala) is non-polar; Lysine (Lys) is positive and Glutamic acid (Glu) is negative (**Table 6**).

In case of N158D (Asn-158-Asp) reported position; we found amino acid Threonine (Thr) in our software (Geneious). We found two mutations here; Threonine (Thr) to Serine (Ser) in one strain and from Threonine (Thr) to Alanine (Ala) in two strains. We found amino acid Asparagine (Asn) in two positions (143 and 152) which is located within twenty positions before 158. There is no mutation here. We found T158S and T158A mutation which indicates that polarity is changed from polar to polar and from polar to non-polar as Threonine (Thr); Serine (Ser) is polar and Alanine (Ala) is non-polar.

In case of N224K (Asn-224-Lys) reported position; we found amino acid Tryptophan (Trp) in our software (Geneious). We found amino acid Lysine (Lys) in one positions (212) which is located within twenty positions before 224. There is no mutation here.

In case of Q226L (Gln-226-Leu) reported position; we found amino acid Threonine (Thr) in our software (Geneious). We found one mutations here; Threonine (Thr) to Serine (Ser) in one strain. We found amino acid Leucine (Leu) in one position (211) which is located within twenty positions before 226. . In this position there is one mutation here from Leucine (Leu) to Valine (Val) in one strain. We found amino acid Leucine (Leu) in one position (229) which is located within twenty positions after 226. There is no mutation here. We found N226S and L211V mutation which indicates that polarity is changed from polar to polar and from non-polar to non-polar as Threonine (Thr); Serine (Ser) is Polar and Proline (Pro); Valine (Val) is non-polar.

In case of T318I (Thr-318-Ile) reported position; we found amino acid Glutamic acid (Glu) in our software (Geneious). We found one mutation here from Glutamic acid (Glu) to Lysine (Lys) in four strains.

We found amino acid Isoleucine (Ile) in two positions (321 and 324) which is located within twenty positions after 318. We found three mutations here from Isoleucine (Ile) to Threonine (Thr) in one strain; from Isoleucine (Ile) to Methionine (Met) in three strains and from Isoleucine (Ile) to Valine (Val) in two strains. In

I321T; I321M; I321V and E318K mutations which indicate that polarity is changed from non-polar to non-polar and non-polar to polar as Isoleucine (Ile); Methionine (Met) and Valine (Val) is nonpolar and Threonine (Thr) is polar (**Tables 7 and 8**).

In case of H274Y (His-274-Try) reported position; we found amino acid Valine (V) in our software (Geneious). We found one mutation here; Valine (V) to Isoleucine (I) in two strains. We found amino acid Histidine (H) in one positions (282) which are located after twenty positions 274. We found V 274 I (2) mutation in two strains which indicates that there is polarity changed from non-polar to non-polar as Valine (V) is non-polar and Isoleucine (I) is non-polar.

In case of H275Y (His-274-Try) reported position; we found amino acid Glutamic acid (E) in our software (Geneious). There are no mutations here. We found amino acid Histidine (H) in one positions (282) which are located after twenty positions 274. There are no mutations here.

In case of N294S (Asn-294-Ser) reported position; we found amino acid Glutamic acid (E) in our software (Geneious). We found one mutation here; Glutamic acid (E) to Glycine (G) in one strain. We found Asparagine (Asn) in one position (280) which is located within twenty positions before 294. In 280 positions there are four mutations here from Asparagine (N) to Aspartic acid (D) in one strain; from Asparagine (N) to Tyrosine (Y) in one strain; from Asparagine (N) to Threonine (T) in one strain and from Asparagine (N) to Lysine (K) in one strain. We found amino acid Asparagine (N) in three positions (302; 307 and 314) which are located after twenty positions 294. There are no mutation here. In N280D; N280Y; N280T and N280K mutations which are indicate that there is polarity changed from polar to electrically charged negative; to positive and to polar as Asparagine (N) is polar; Aspartic acid (D) is electrically charged negative; Tyrosine (Y) is polar; Threonine (T) is polar and Lysine (K) is electrically charged positive. In E294G mutation which is indicates that there is polarity change from electrically charged negative to nonpolar as Glutamic acid (E) is negative and Glycine (G) is nonpolar.

In case of N295S (Asn-295-Ser) reported position; we found

**Table 7** Summary of some reported position which can be responsible for N1 (H5N1) neuraminidase mutations causing reduced sensitivity to NAIs [37-39].

Amino acid mutation position with reference	Short form	Amino acid in Geneious in reported position	NAIs
His-274-Try (Le QM et al.) [36]	H274Y	V	Oseltamivir Peramivir
His-275-Try (Pizzorno et al.) [38]	H275Y	E	Oseltamivir Peramivir
Asn-294-Ser (Le QM et al.) [36]	N294S	E	Oseltamivir
Asn-295-Ser (Pizzorno et al.) [38]	N295S	I	Oseltamivir
Asp-198-Gly (Hurt et al.) [37]	D198G	I	Oseltamivir Zanamivir
Asp-199-Gly (Pizzorno et al.) [38]	D199G	G	Oseltamivir Zanamivir
Val -116- Ala (Nguyen et al.) [39]	V116A	V	Oseltamivir Zanamivir
His -252- Try (Nguyen et al.) [39]	H252Y	S	Oseltamivir
Ser -246- Asn (Nguyen et al.) [39]	S246N	V	Oseltamivir
Ile -222- Thr (Nguyen et al.) [39]	I222T	K	Oseltamivir

amino acid Isoleucine (I) in our software (Geneious). We found one mutation here; Isoleucine (I) to Valine (V) in seven strains. We found Asparagine (Asn) in one position (280) which is located within twenty positions before 295. In 280 positions there are four mutations here from Asparagine (N) to Aspartic acid (D) in one strain; from Asparagine (N) to Tyrosine (Y) in one strain; from Asparagine (N) to Threonine (T) in one strain and from Asparagine (N) to Lysine (K) in one strain. We found amino acid Asparagine (N) in three positions (302; 307 and 314) which are located after twenty positions 295. There are no mutation here. We found I295V mutation which indicates that there is polarity changed from non-polar to non-polar as Isoleucine (I) is non-polar and Valine (V) is non-polar. In N280D; N280Y; N280T and N280K mutations which are indicate that there is polarity changed from polar to electrically charged negative; to positive and to polar as Asparagine (N) is polar; Aspartic acid (D) is electrically charged negative; Tyrosine (Y) is polar; Threonine (T) is polar and Lysine (K) is electrically charged positive.

In case of D198G (Asp-198-Gly) reported position; we found amino acid Isoleucine (I) in our software (Geneious). There are no mutations here. We found amino acid Aspartic acid (D) in one positions (191) which are located before twenty positions 198. We found amino acid Aspartic acid (D) in one positions (204) which are located after twenty positions 198. There are no mutations here.

In case of D199G (Asp-199-Gly) reported position; we found amino acid Glycine (G) in our software (Geneious). There are no mutations here. We found amino acid Glycine (G) in one positions (191) which is located within twenty positions before 199. There are no mutations here. We found amino acid Aspartic acid (D) in two positions (204 and 219) which are located after twenty positions 199. There are no mutations here.

In case of V116A (Val-116-Ala) reported position; we found amino acid Valine (V) in our software (Geneious). There is one mutation here from Valine (V) to Isoleucine (I) in two strains. We found amino acid Valine (V) in two positions (99 and 114) which is located within twenty positions before 116 and one mutation here from Valine (V) to Isoleucine (I) in one strain. We found V116I mutation which is indicates that there is polarity changed from non-polar to non-polar as Valine (V) is non-polar and Isoleucine (I) is non-polar.

In case of H252Y (His-252-Try) reported position; we found amino acid Serine (S) in our software (Geneious). There are two mutations here from Serine (S) to Asparagine (N) in four strains and from Serine (S) to Glycine (G) in four strains. We found S252N mutation which is indicates that there is polarity changed from polar to polar as Serine (S) is polar and Asparagine (N) is polar. We found S252G mutation which is indicates that there is polarity changed from Polar to non-polar as Serine (S) is polar and Glycine (G) is non-polar.

In case of S246N (Ser-246-Asn) reported position; we found Valine (V) in our software (Geneious). There are two mutations here from Valine (V) to Leucine (L) in three strains and from Valine (V) to Isoleucine (I) in six strains. We found V246L and V246I mutation which is indicates that there is polarity changed from non-polar to non-polar as Valine (V) is non-polar; Leucine (L) is non-polar and Isoleucine (I) is non-polar. We found amino acid Serine (S) in two positions (234 and 242) which is located within twenty positions before 246. In 234 positions there is no mutation here. In 242 positions there is one mutation from Serine (S) to Phenylalanine (F) in one strain.

We found S242F mutation which is indicates that there is polarity changed from polar to non-polar as Serine (S) is polar and Phenylalanine (F) is non-polar. We found amino acid Serine (S)

**Table 8** Analysis of our study data with respect to reported mutation point causing reduced sensitivity to NAIs of avian H5N1. Correlation of Reported NAIs resistance specific avian amino acid position with our experimental strain, and their mutation pattern (exact and around twenty positions): Original Amino Acid analysis.

Reported AA with mutation point	AA in Geneious in reported position	Exact AA which matches with the reported data with respect to specific mutation point (before 20 positions)	Exact AA which matches with the reported data with respect to specific mutation point (after 20 positions)
H274Y	V274I (2) Nonpolar to Nonpolar		H282
H275Y	E275		H282
N294S	E294G Electrically charged Negative to Nonpolar	N280D (1) Polar to Electrically charged Negative N280Y (1) Polar to Polar N280T (1) Polar to Polar N280K (1) Polar to Electrically Charged Positive	N302 N307 N314
N295S	I295V (7) Nonpolar to Nonpolar	N280D (1) Nonpolar to Electrically charged Negative N280Y (1) Polar to Polar N280T (1) Polar to Polar N280K (1) Polar to Electrically Charged Positive	N302 N307 N314
D198G	I198	D191	D204
D199G	G199	D191	D204 D219
V116A	V116I (2) Nonpolar to Nonpolar	V99 I Nonpolar to Nonpolar V114	
H252Y	S252N (4) Polar to Polar S 252 G (4) Polar to Nonpolar		
S246N	V246L (3) V246I (6) Nonpolar to Nonpolar (L,I)	S 234 S 242 F (1) Polar to Nonpolar	S252G (4) Polar to Nonpolar S252N (4) Polar to Polar S257
I222T	K222Q (1) K222R (3) K222N (1) Electrically charged Positive to Polar (Q, N)and to Positive (R)	I216V(1) I217 I221V(1) Nonpolar to Nonpolar	I228V (1) I228T (2) I228L (1) Nonpolar to Nonpolar (V,L) and to Polar (T)

in two positions (252 and 257) which is located within twenty positions after 246. In 252 positions there are two mutations here from Serine (S) to Glycine (G) in four strains and from Serine (S) to Asparagine (N) in four strains. In 257 positions there is no mutation here. We found S252G mutation which indicates that

there is polarity changed from polar to non-polar as Serine (S) is polar and Glycine (G) is non-polar. We found S252N mutation which indicates that there is polarity changed from Polar to Polar as Serine (S) is polar and Asparagine (N) is polar.

In case of I222T (Ile-222-Thr) reported position; we found Lysine

**Table 9** Correlation of Reported NAIs resistance specific avian amino acid position with our experimental strain, and their mutation pattern (exact and around twenty positions): Mutated amino acid analysis.

Reported AA with mutation point	AA in Geneious in reported position	Exact AA which matches with the reported data with respect to specific mutation point (before 20 positions)	Exact AA which matches with the reported data with respect to specific mutation point (after 20 positions)
H274Y	V274I (2) Nonpolar to Nonpolar	Y258	Y281F (2) Polar to Nonpolar Y283 Y289N (1) Polar to Polar
H275Y	E275	Y258	Y281F (2) Polar to Nonpolar Y283 Y289N (1) Polar to Polar
N294S	E294G Electrically charged Negative to Nonpolar	S287	S306 S312
N295S	I295V(7) Nonpolar to Nonpolar	S287	S306 S312
D198G	I198	G192	G199 G202 G206A (1) Nonpolar to Nonpolar G206E (2) Nonpolar to Electrically Charged Negative G215
D199G	G199	G 192	G202 G206A (1) Nonpolar to Nonpolar G206E (2) Nonpolar to Electrically Charged Negative G215
V116A	V11I (2) Nonpolar to Nonpolar	A98	
H252Y	S252N (4) Polar to Polar S252G(4) Polar to Nonpolar		Y258
S246N	V246L (3) V246I (6) Nonpolar to Nonpolar (L,I)	N240	N253D (1) Polar to Electrically charged Negative N253H (1) Polar to Electrically charged Positive N253S (2) Polar to Polar
I222T	K222Q (1) K222R (3) K222N (1) Electrically charged Positive to Polar (Q,N)and to Positive (R)	T220 T218	T231

(K) in our software (Geneious). There are three mutations here from Lysine (K) to Glutamine (Q) in one strain; from Lysine (K) to Arginine (R) in three strains and from Lysine (K) to Asparagine (N) in one strain. We found K222Q; K222R and K222N mutation which indicates that there is changed electrically charged from

positive (K) to positive (R) and from positive (K) to polar (Q and N).

We found amino acid Isoleucine (I) in three positions (216; 217 and 221) which is located within twenty positions before 222. In

216 positions there is one mutation here from Isoleucine (I) to Valine (V) in one strain and in 221 positions there is one mutation here from Isoleucine (I) to Valine (V) in one strain. In 217 positions there is no mutation here. In I216V and I221V mutations which are indicate that there is polarity changed from non-polar to non-polar as Isoleucine (I) is non-polar and Valine (V) is non-polar.

We found amino acid Isoleucine (I) in one positions (228) which are located within twenty positions after 222. In 228 positions there are three mutations here from Isoleucine (I) to Valine (V) in one strain; from Isoleucine (I) to Threonine (T) in two strains and from Isoleucine (I) to Leucine (L) in one strain. In I228V; I228T and I228L mutations which are indicate that there is polarity changed from non-polar to non-polar and non-polar to polar as Isoleucine (I) is non-polar; Valine (V) is non-polar; Leucine (L) is non-polar and Threonine (T) is polar (**Table 9**).

In case of H274Y (His-274-Try) reported position; we found amino acid Valine (V) in our software (Geneious). We found one mutation here; Valine (V) to Isoleucine (I) in two strains. We found amino acid Tyrosine (Y) in one position (258) which is located before twenty positions 274. There are no mutations here. We found amino acid Tyrosine (Y) in three positions (281; 283 and 289) which are located after twenty positions after 274. There are two mutations here from Tyrosine (Y) to Phenylalanine (F) in two strains and from Tyrosine (Y) to Asparagine (N) in one strain. We found V274I mutation in two strains which indicates that there is polarity changed from non-polar to non-polar as Valine (V) is non-polar and Isoleucine (I) is non-polar. We found Y281F and Y289N mutations which indicate that there is polarity changed from polar to non-polar and to polar as Tyrosine (Y) is polar; Phenylalanine (F) is non-polar and Asparagine (N) is polar.

In case of H275Y (His-274-Try) reported position; we found amino acid Glutamic acid (E) in our software (Geneious). We found amino acid Tyrosine (Y) in one position (258) which is located before twenty positions 275. There are no mutations here. We found amino acid Tyrosine (Y) in three positions (281; 283 and 289) which are located after twenty positions after 275. There are two mutations here from Tyrosine (Y) to Phenylalanine (F) in two strains and from Tyrosine (Y) to Asparagine (N) in one strain. We found Y281F and Y289N mutations which indicate that there is polarity changed from polar to non-polar and to polar as Tyrosine (Y) is polar; Phenylalanine (F) is non-polar and Asparagine (N) is polar.

In case of N294S (Asn-294-Ser) reported position; we found amino acid Glutamic acid (E) in our software (Geneious). We found one mutation here; Glutamic acid (E) to Glycine (G) in one strain. We found amino acid Serine (S) in one position (287) which is located within twenty positions before 294. We found amino acid Serine (S) in two positions (306 and 312) which is located within twenty positions after 294. There are no mutations here. In E294G mutation which is indicates that there is polarity change from electrically charged negative to nonpolar as Glutamic acid (E) is negative and Glycine (G) is nonpolar.

In case of N295S (Asn-295-Ser) reported position; we found amino acid Isoleucine (I) in our software (Geneious). We found one mutation here; Isoleucine (I) to Valine (V) in seven strains. We

found amino acid Serine (S) in one position (287) which is located within twenty positions before 295. We found amino acid Serine (S) in two positions (306 and 312) which is located within twenty positions after 295. There are no mutations here. We found I295V mutation which indicates that there is polarity changed from non-polar to non-polar as Isoleucine (I) is non-polar and Valine (V) is non-polar.

In case of D198G (Asp-198-Gly) reported position; we found amino acid Isoleucine (I) in our software (Geneious). There are no mutations here. We found amino acid Glycine (G) in one position (192) which is located within twenty positions before 198. We found amino acid Glycine (G) in four positions (199; 202; 206 and 215) which is located within twenty positions after 198. We found two mutations here from Glycine (G) to Alanine (A) in one strain and from Glycine (G) to Glutamic acid (E) in two strains. In G206A and G206E mutations which indicates that there is polarity changed from nonpolar to nonpolar and nonpolar to electrically charge negative as Glycine (G) and Alanine (A) is nonpolar and Glutamic acid (E) is electrically charged negative.

In case of D199G (Asp-198-Gly) reported position; we found amino acid Isoleucine (I) in our software (Geneious). There are no mutations here. We found amino acid Glycine (G) in one position (192) which is located within twenty positions before 199. We found amino acid Glycine (G) in three positions (202; 206 and 215) which is located within twenty positions after 199. We found two mutations here from Glycine (G) to Alanine (A) in one strain and from Glycine (G) to Glutamic acid (E) in two strains. In G206A and G206E mutations which indicates that there is polarity changed from nonpolar to nonpolar and nonpolar to electrically charge negative as Glycine (G) and Alanine (A) is nonpolar and Glutamic acid (E) is electrically charged negative.

In case of V116A (Val-116-Ala) reported position; we found amino acid Valine (V) in our software (Geneious). There is one mutation here from Valine (V) to Isoleucine (I) in two strains. We found amino acid Alanine (A) in one position (98) which is located within twenty positions before 116. There are no mutations here. We found V116I mutation which is indicates that there is polarity changed from non-polar to non-polar as Valine (V) is non-polar and Isoleucine (I) is non-polar.

In case of H252Y (His-252-Try) reported position; we found amino acid Serine (S) in our software (Geneious). There are two mutations here from Serine (S) to Asparagine (N) in four strains and from Serine (S) to Glycine (G) in four strains. We found S252N mutation which is indicates that there is polarity changed from polar to polar as Serine (S) is polar and Asparagine (N) is polar. We found amino acid Tyrosine (Y) in one position (258) which is located within twenty positions after 252. There are no mutations here. We found S252G mutation which is indicates that there is polarity changed from Polar to non-polar as Serine (S) is polar and Glycine (G) is non-polar.

In case of S246N (Ser-246-Asn) reported position; we found Valine (V) in our software (Geneious). There are two mutations here from Valine (V) to Leucine (L) in three strains and from Valine (V) to Isoleucine (I) in six strains. We found amino acid Asparagine (N) in one position (240) which is located within twenty positions before

246. There is no mutation here. We found amino acid Asparagine (N) in one position (253) which is located within twenty positions after 246. There are three mutations here from Asparagine (N) to Aspartic acid (D) in one strain; from Asparagine (N) to Histidine (H) in one strain and from Asparagine (N) to Serine (S) in two strains. We found N253D; N253H and N253S mutation which indicates that there is polarity changed from polar to electrically charged negative; to positive and to polar as Asparagine (N) is polar; Aspartic acid (D) is electrically charged negative; Histidine (H) is electrically charged positive and Serine (S) is polar. We found V246L and V246I mutation which indicates that there is polarity changed from non-polar to non-polar as Valine (V) is non-polar; Leucine (L) is non-polar and Isoleucine (I) is non-polar.

In case of I222T (Ile-222-Thr) reported position; we found Lysine (K) in our software (Geneious). There are three mutations here from Lysine (K) to Glutamine (Q) in one strain; from Lysine (K) to Arginine (R) in three strains and from Lysine (K) to Asparagine (N) in one strain. We found amino acid Threonine (T) in two positions (220 and 218) which is located within twenty positions before 222. We found amino acid Threonine (T) in one position (231) which is located within twenty positions after 222. There are no mutations here. We found K222Q; K222R and K222N mutation which indicates that there is changed electrically charged from positive (K) to positive (R) and from positive (K) to polar (Q and N).

## Conclusion

Neuraminidase inhibitors (NAIs) are first-line agents for the treatment and prevention of influenza virus infections. Resistance to the NAIs can be both drug and virus type or subtype specific. The effects of NAI resistance NA mutations on the fitness and transmissibility of influenza viruses may vary depending on

several factors: location of the mutation (catalytic or framework residue); NA type / subtype; virus genetic background; existence of permissive secondary NA mutations; degree of NA functional loss; and an appropriate functional NA–HA balance. In addition; differences in the host's immune response and genetic background can contribute to such variation. No single measure can easily describe the extent of fitness of influenza viruses carrying drug resistance mutations.

The most frequently reported change conferring oseltamivir resistance in that viral context is the H274Y neuraminidase mutation (N1 numbering). Recent studies have shown that; in the presence of the appropriate permissive mutations; the H274Y variant can retain virulence and transmissibility in some viral backgrounds. Most oseltamivir-resistant influenza A virus infections can be managed with the use of inhaled or intravenous zanamivir; another NAI. New NAI compounds and non-neuraminidase agents as well as combination therapies are currently in clinical evaluation for the treatment for severe influenza infections.

At present; it is clear that mutation conferring resistance to the currently approved antiviral drugs is a growing problem; so it is very important to continue research in various areas that would allow better resolution of the problem: First; the knowledge of mutation's type that allows virus avoids the action of drugs; will allow an understanding as to how these mutations arise and how we must avoid them. Also; this knowledge; will allow a better design of new drugs.

## Conflict of Interest

None

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