# Characterization of Neuraminidase gene of Avian Influenza virus (H5N1) In Egypt from 2006-2009

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**Abstract:** Highly pathogenic avian influenza (AI) caused by the influenza A H5N1 virus, posses a significant threat to the poultry industry and human worldwide. Since 2006, the disease has become enzootic in poultry throughout Egypt and still circulates in the poultry population. The limitness of treatment options of avian influenza infection and the criticality of the antiviral drugs susceptibility highlighted the importance of the neuraminidase enzyme (NA) as a target for this study. The present study aimed to monitor genetic changes in the NA gene, specially the highly conserved active site to detect emerging possible strains of H5N1 that have antiviral resistant nature. Viral RNAs were isolated from thirty eight clinical samples, collected from infected different species of poultry at different governorates in Egypt during the period of 2006-2009. Real time RT-PCR was performed using specific primers for the matrix (M), Hemagglutinin (H5) and neuraminidase (N1) genes to confirm the viral subtype (H5N1). Sequencing analysis was used to monitor and detect the NA sequences in the isolated samples. Fifteen strains in the present study have mutations at the target of primers and probe without effect on the binding of the primer and probe in PCR reaction. The following mutations were recorded: Asp151His (NA active site mutation), Leu223Met (located between catalytic amino acid and framework one), Ser228Asp (located away from active site) and twenty amino acids deletion in the stalk motif of neuraminidase enzyme.
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**Key Words:** Avian Influenza, Neuraminidase, Mutations.

## 1-Introduction

Since its emergence, highly pathogenic avian influenza (HPAI) has attracted a considerable public and media attention ***(Horimoto and Kawaoka, 2001),*** due to the fatal cases in human which give rise to fear from the possible capacity for human-to-human transmission and global influenza pandemic ***(Earhart et al.,2009).*** By the end of 2014, there were a total of 695 confirmed human cases of the H5N1 virus infections, 403 of which were fatal from the beginning of AIV outbreak in 2003. Egypt shared by 203 confirmed human cases of the H5N1 virus infection, 72 have been fatal and by that Egypt ranking the first position in infected cases and second position in fatal cases number after Indonesia, as reported in World Health Organization. H5N1 virus is endemic in few countries including Indonesia, Egypt, China, Vietnam and maybe Bangladesh, and continues to cause outbreaks in poultry and sporadic human infections ***(WHO, 2015).*** Two major membrane glycoproteins; hemagglutinin (HA) and neuraminidase (NA), together play important roles in the interactions between the virus and the host cell surface receptors. HA is responsible for binding virus with the cell that is being infected (i.e., the virion entry is mediated by HA), while the viral shedding is facilitated by NA through cleavage of sialic acid linkage formed between the HA and sialic receptors on the surface of the host cell, so the newly formed viruses are released and able to spread to uninfected cells ***(Matrosovich et al., 2004).***

The influenza A virus NA exist as a mushroom-shape projection on the surface of the influenza virus. They have a highly conserved short cytoplasmic tail, which comprises a single polypeptide chain that is oriented in the opposite direction to the hemagglutinin antigen. The NA polypeptide is a single chain of six conserved polar amino acids, followed by hydrophilic, variable amino acids and a hydrophobic trans-membrane region that provides the anchor for the stalk and the head domains The structure of the head is a homotetramer consisting of four roughly spherical subunits each monomer of which is composed of six topologically identical of 4-stranded anti-parallel beta sheets arranged like the blades of a propeller ***(Varghese et al., 1992).***

Eight amino acids are referred as catalytic residues due to their direct contact to the substrate, or their criticality for function { Arginine (R-118), Aspartic (D-151), Arginine (R-152), Arginine (R-224), Glutamic (E-276), Arginine (R-292), Arginine (R-371), and Tyrosine (Y-406) }; (in N2 numbering), and others are referred as framework residues consequently to their implication in the stabilization of the active site structure {Glutamic(E-119), Arginine (R-156),Tryptophan (W-178), Serine (S-

179), Aspartic (D-198), Isoleucine (I-222), Glutamic (E-227), Glutamic (E-277), Asparagine (N-294), and Glutamic (E-425)} ***(Colman et al., 1983; Burmeister et al., 1992; Colman et al.,1993 and Gubareva, 2004).***

Neuraminidase was chosen as a suitable drug target because of its major role in the virus propagation ***(Moscona, 2005),*** and the strict conserved nature of its active site residues. Consequently, several NA inhibitors were raised against influenza A and B as oseltamivir ***(Li et al., 1998),*** and zanamivir ***(Von Itzstein et al., 1993),*** which are two currently licensed used inhibitors. These inhibitors are transition state analogues that prevent the hydrolysis of the connection existing between sialic acid, preferably N-acetylated and the adjacent carbohydrate molecule, of cellular glycoprotein ***(Bucher and Kilbourne, 1972).*** So, neuraminidase activity inhibition, reduces virus penetration through secretion, and the liberation of progeny virions budding out from cell surface. Neuraminidase inhibitors were developed using knowledge of the enzyme structure to inhibit virus replication in vitro and in vivo ***(Kim et al., 1997).***

The criticality of the antiviral drugs and the drug resistance developed by the virus highlighted the importance of a continuous monitoring of avian influenza (H5N1) neuraminidase enzyme (NA) sequences. In this context, the current study was targeted to detect possible mutations of NA gene for both diagnosis and treatment.

## 2. Methods

### Sampling

Clinical samples were collected from suspected infection of different species of poultry cases from different farms and backyards, from different governorates in Egypt during the period from 2006 to 2009. The samples were collected under the Governmental authority of National Lab. for Quality Control on Poultry Production (NLQP) and General Organization for Veterinary Service (GOVS) without need for permission from the owner.

### Viral RNA isolation and sub-typing

Viral RNA was isolated from each sample using QIAamp® Viral RNA Mini Kit (QIAGEN, Valencia, Calif., USA) according to the manufacturer procedures. The samples were tested by real time RT-PCR for AI using specific primers and probe for the Matrix (M) gene of all type A influenza viruses ***(Spackman, et al., 2002),*** then the positive samples are subtyped using specific primers and probe for H5 gene ***(Slomka, et al.,2007),*** and N1 gene ***(WHO, 2007).***

### NA amplification and sequencing

The positive thirty eight samples (H5N1) were inocuolated in 9 day old SPF ECE and the allantoic fluids were tested for HA activity in Virology unit in NLQP to amplification of overlapping fragments of neuraminidase enzyme (NA) by RT-PCR. Amplicon DNA sequencing was performed using BigDye Terminator V3.1 cycle sequencing kit (Foster city, USA) with the specific primers.

### The three dimensional structure analyses

The crystal structure of the complex of H5N1 NA with oseltamivir was obtained from the Protein Data Bank(PDB ID: 2hu0) ***(Russell et al. 2006),*** then the substitutions of the mutated amino acids were carried out with the local energy minimization and angle torsion correction by the Swiss PDB viewer (SPDBV) program version 4.1 and the binding of NA enzyme active site with Oseltamivir are viewed by AutoDock. Version (4.1) in Bioinformatics unit in Nile University.

### Phylogenetic tree

Phylogenic analysis was carried out on the full-length NA gene of the Egyptian strains that rooted with the original 2006 Egyptian strain using Molecular Evolutionary Genetics Analysis (MEGA 4.2 software). The phylogenetic relationships were estimated by neighbor joining method using 1000 bootstrap resampling

## 3. Results

The tested samples from the beginning of the outbreak 2006 till 2009 were positive for common gene (M), H5 subtype and N1 subtype.

Comparing NA sequence of H5N1 Egyptian strains with the primers and probe sequence targets, we found one mismatch at the forward primer (N1-For 474-502v2) target as in HQ908465 and GQ184289 in different positions, and one mismatch at the probe (N1 Probe 501-525v3) target as in GQ184259, GQ184260, GQ184262, GQ184265, GQ184278, GQ184279, GQ184287, HQ908466, HQ908464, HQ908470 and HQ908463. All of them have mismatches at the same position as adenine nucleotide base changed to thymine. Also GQ184266 has a mismatch but in different position. Other samples have two mismatches at the probe (N1 Probe 501-525v3) target as HQ908465 and HQ908459. No mismatches in the reverse primer (N1-Rev603-631v2) have been found, however all these mismatches did not affect qualitatively (positive or negative result) the binding efficiency of real time RT-PCR reaction for this test and all gave positive results.

The present study clearly showed that, in a total of 38 H5N1 studied neuraminidases; the only one that has mutation in the catalytic site is GQ184276,

where the catalytic residue Asp (D)151(N2 numbering) is replaced by His(H). In the same context, Leu223Met (N2 numbering) mutation has also been found in 4 of the sequenced H5N1-NAs; HQ908450, HQ908455, HQ908461 and GQ184282. This mutation located between Arg224 (N2 numbering) (one of the catalytic site residue of NA) and the Ile222 (N2 numbering) (one of the framework residue of NA). Another type of mutation, Ser228Asp (N1 numbering) was encountered in the present study in GQ184272 where amino acid residue serine in position 228 was replaced by asparagine.

The crystal structure of the complex of H5N1 NA with oseltamivir with the substitutions of the mutated amino acids was illustrated in the present work (Figures 1 – 5). All studied Egyptian H5N1-NAs had deletion in stalk region (a 20-amino acid deletion in the 49th to 68th in the stalk region). The phylogenetic tree of the sequenced isolates for neuraminidase enzyme gene (Figure 6) illustrated the high evolution rate of AI H5N1 to multiple clusters of neuraminidase at 2006 - 2009 in Egypt.

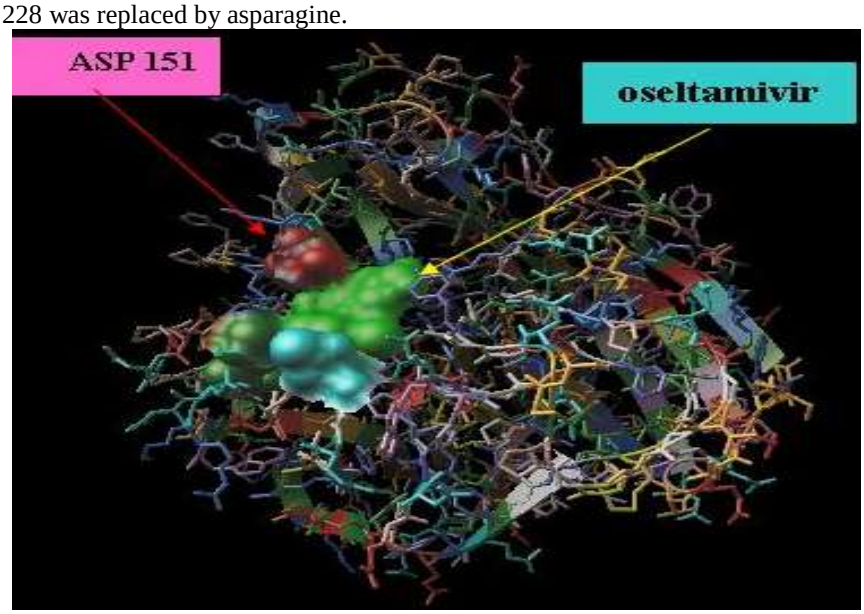


**Figure 1:- Show the affecting zone of mutation at amino acid 151 in N2 numbering (ASP 151 HIS)**.
The figure shows the site of amino acid ASP 151 which considered one of The catalytic amino acids of the neuraminidase enzyme, and it is appear as very close to oseltamivir site in more than one aspect.

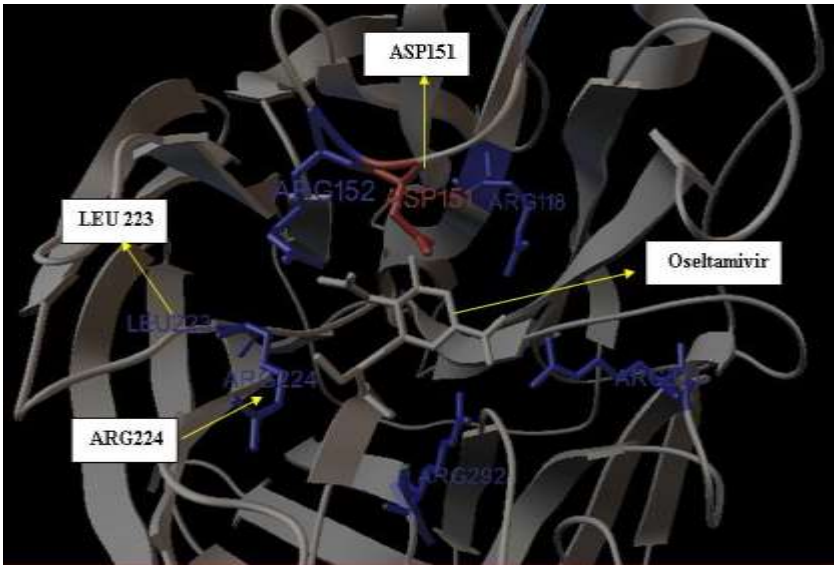


**Figure 2:- Show the importance of the site of the amino acid at 151 in N2 numbering (ASP 151).**
This Ribbon structure illustrates oseltamivir binding with the main amino acids in the active sites of the enzyme highlighte the importance of the Asp 151 on this structure, and Leu 223 in N2 numbering (Asp131, Leu 204) in N1 numbering.

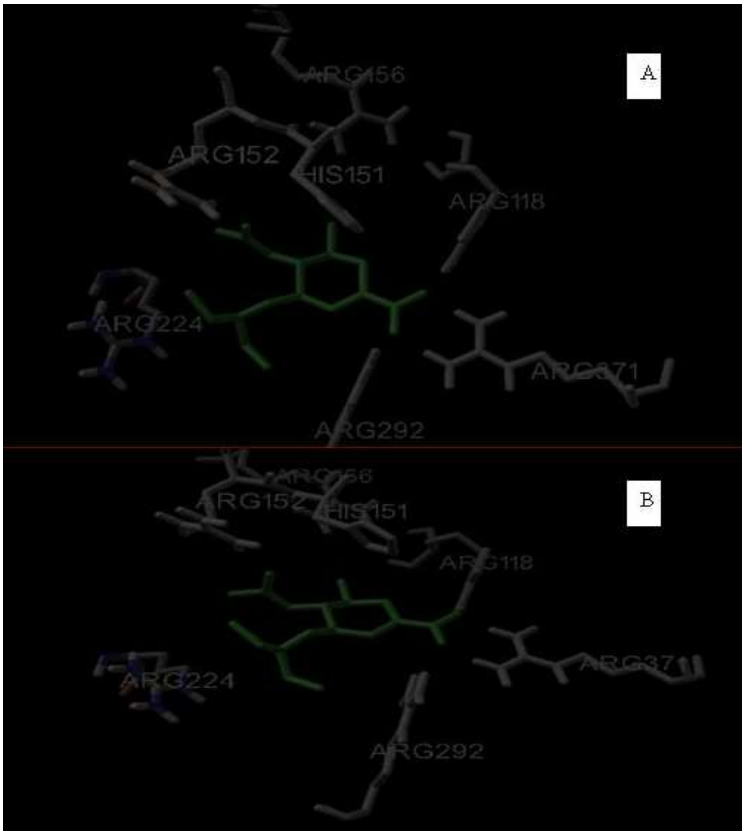


**Figure 3:- Show the mutation prediction of the amino acid at site 151 in N2 numbering (ASP 151 HIS) in neuraminidase enzyme of H5N1**.

This figure illustrates the prediction of mutation (Asp 151 His) in 2 different dimentions A, B to illustrate that imidazole side chain of the histidine is the part that directed toward the oseltamivir (green colour).This docking appeared the importance of the site 151 in N2 numbering as it is one of the catalytic site amino acids, and so it is directly contact to the oseltamivir.

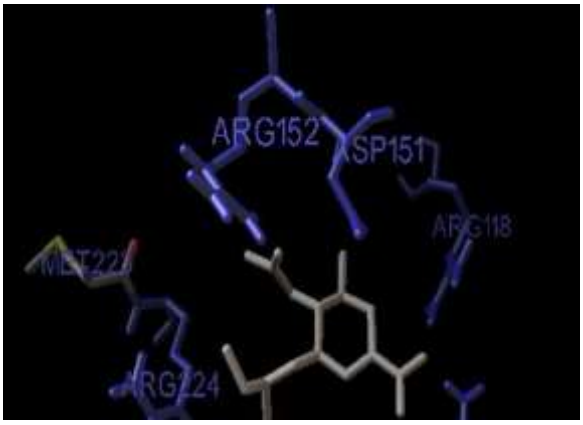


**Figure 4:- Show the mutation prediction of the amino acid at site 223 in N2 numbering (LEU 223 MET) in neuraminidase enzyme of H5N1.**

This figure illustrates the prediction of mutation (Leu 223 Met). Mutations were done in the protein structures, then local energy minimization was performed and local torsion angle correction was done using the software SPDBV. The output was visualized again using Autodock Tools, this docking appeared the importance of the site 223 in N2 numbering as it is beside directly Arg 224 which is one of the catalytic site amino acids, and is very close to oseltamivir.

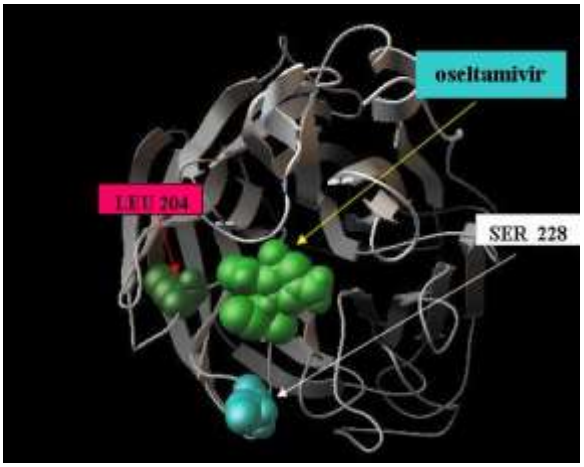


**Figure 5:- the affecting zone of mutation at amino acid 204 in N1 numbering (Leu 204 Met) and amino acid 228 in N1 numbering (Ser 228Asn).**

The figure shows in more than one aspect the sites of amino acids nearby an important amino acid in active site as (Ile 203) in the frame of the active site, (Arg 205) in the catalytic site of the NA enzyme, and Ser228.

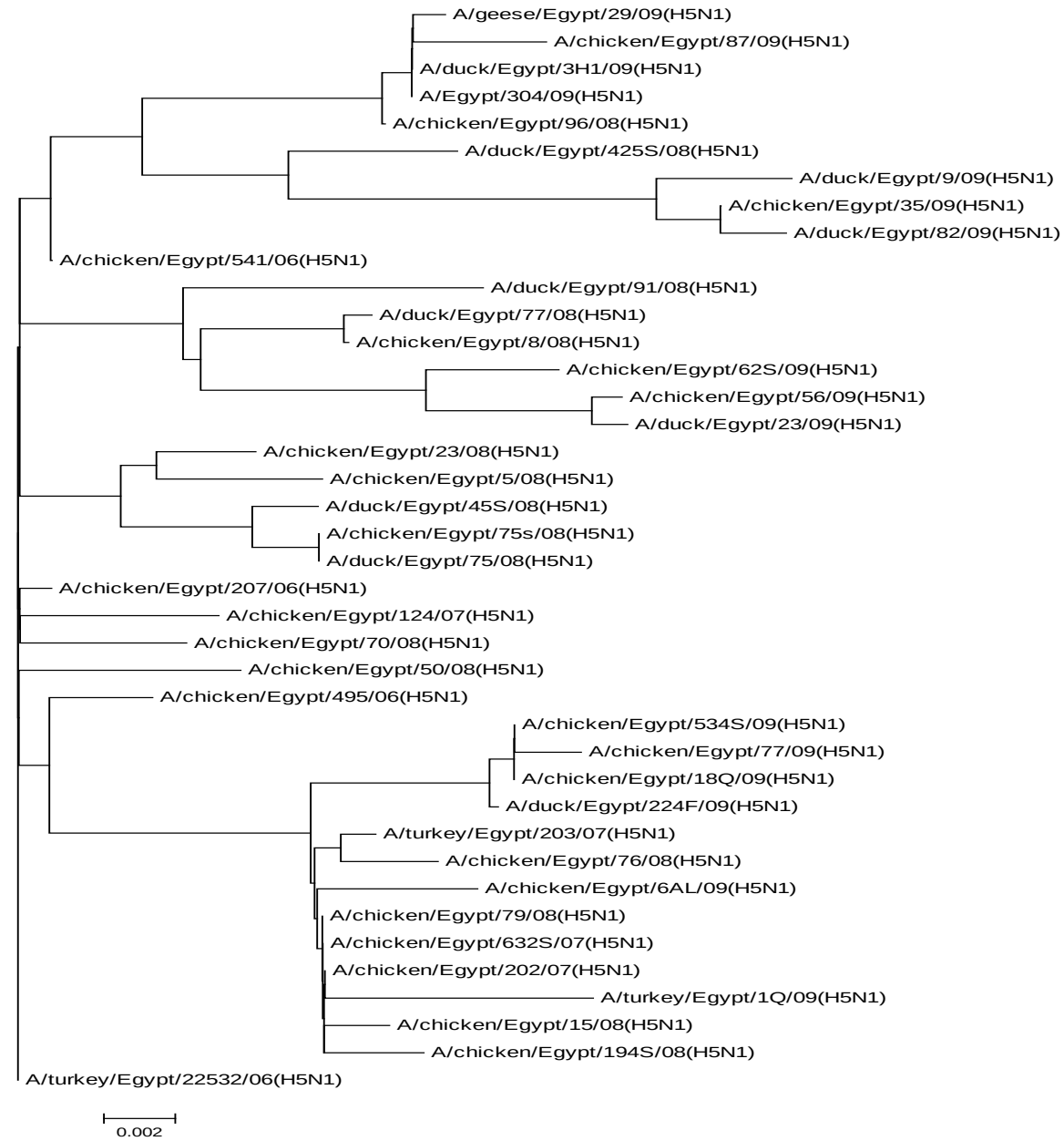


**Figure 6: The nucleotides sequences based phylogenetic tree of 38 sequenced isolates rooted with the original 2006 Egyptian strain.**

The figure shows the evolution of AI H5N1 to multiple clusters of neuraminidases between 2006 -2009 in Egypt.

## 4. Discussion

Our results confirmed the presence of H5N1 AI subtype in different poultry species, in different governorates and in different Farms and Backyards in Egypt from the beginning of the outbreak in 2006 till 2009 according to tested samples**.** The different mismatches in the primers and probes target sequences in NA gene, however they did not affect the binding efficiency of the real time RT-PCR reactions, give rise attention to the importance of continuous monitoring of the gene sequence to avoid false negative results due to additional mutations in these specific targets, as previously detected in H5 sub-typing real time RT-PCR ***(Arafa et al., 2010a).***

Sequence analysis of neuraminidases in the current study, revealed the occurrence of Asp151His mutation in GQ184276, where the catalytic amino acid residue aspartate in position 151 is replaced by

histidine. There was no oseltamivir-resistant report about this substitution. Although other natural variations (Gly/Val/Asn/Glu) at residue Asp151 have been identified in N1, N2, and influenza B NA in a large-scale influenza virus NA inhibitors susceptibility screening, suggesting that Asp151 may not be as conserved as previously thought ***(McKimm-Breschkin et al., 2003).*** Using reverse Genetics, seven charged, conserved NA residues were studied including Asp151 ***(Yen et al., 2010)*** that directly interact with the NA inhibitors with slight resistance to zanamivir and oseltamivir, since the drug sensitivity decreased 2.2- and 10.8- fold, respectively**.**

In the same line and by using site–directed mutagenesis, Asp151 was reported to have an important role in the catalysis but not as proton donor and Asp151Glu caused 10 fold reduction in the activity of the enzyme, and they attributed the change in mutant NA kinetic parameters could be due to the effect of the mutation on the chemistry of the reaction ***(Ghate and Air,1998).***Natural variations were identified at residue Asp151 in circulating influenza viruses without significant decrease in either the enzyme activity or the yields of the Asp151 variant viruses including Asp151Glu ***(McKimm- Breschkin et al., 2003).*** Furthermore the role of Asp151 was still unclear as residues Asp151Asn, Asp151Gly, and Asp151Val could not act as proton donors, and the lack of apparent critical role played by Asp151 in catalysis supporting the earlier findings ***(Ghate and Air, 1998).***

Using one of the bioinformatics tools illustrated the importance of the Asp151His mutation, where Asp151, as one of the catalytic site residue directly in contact with enzyme substrate explaining the important role of this amino acid mutation. Docking calculations for Asp151His mutation showed that the predicted three dimensional structure of the active site NA of H5N1 with this mutation revealed that imidazole side chain of Histidine in the active site of the neuraminidase, spatially oriented towards the amino group at position 4 of the oseltamivir inside the active site pocket of the neuraminidase (Figures 1, 2 & 3).

The present work recorded another type of mutation; Leu223Met, in 4 of the sequenced H5N1-NAs in different Governorates (Menia, Fayoum, Alexandria, Giza) also this mutation recorded and predominate in human strains H5N1 in Egypt as published in gene bank. This mutation located between Arg224 (one of the catalytic site residue of NA) and the Ile222 (one of the framework residue of NA). The predicted three dimensional structure of the active site of the NA of H5N1 with mutation Leu223Met revealed that the methionine side chain spatially protruded outwards the active site pocket of the neuraminidase, suggested that Met(M) could not be directly affect on bonding of NA with oseltamivir, but could be have indirect effect. As methionine was located between Arg224 and Ile222, where Arg224 was spatially oriented toward the pentyl ether group of oseltamivir (Figure 4). The hydrophobic faces of Ile222, together with Arg224 and Ala246 form a hydrophobic pocket to accommodate the glycerol side chain of sialic acid and zanamivir, while Glu276 forms a hydrogen bond with the O8 and O9 hydroxyls of the glycerol group ***(Varghese et al., 1992 and Stoll et al., 2003).***

The interaction of Arg224 and Glu276with oseltamivir is different in that the glycerol side chain is substituted by a pentyl ether group, Glu276 and Arg224 must form a salt bridge to accommodate the large hydrophobic pentyl ether group of oseltamivir ***(Smith et al., 2002 and Mihajlovic and Mitrasinovic, 2008).*** So any change in Leu223 could affect the bonding between Arg224 and Ile222 with oseltamivir.

The other type of mutation encountered in the present study was Ser228Asp also this mutation recorded in human strain H5N1 in Egypt (EF222322) which recorded for oseltamivir resistance as it contain N294S (N2 numbering) ***(Earhart et al.,2009),*** where amino acid residue Serine in position 228 was replaced by Asparagine. The predicted three dimensional structure of the NA of H5N1 with mutation Ser228Asp revealed that the affected zone of NA from this site of mutation was far away from the active site (Figure 5). In the same context, some differences were illustrated between NA of N9 and N1 of AI, as the lipophilic amino acid Ala248 in N9-NA was replaced by Ser 228(N1 numbering) in the H5N1-NA, so a new hydrogen bond is formed between Ser228-OH and O9-hydroxyl group of the glycerol side chain of the inhibitor as in Zanamivir ***(Wei et al., 2006).*** So these mutations worth further investigation by using biological analysis.

Notably, the 38 Egyptian H5N1-NAs had deletions in stalk region (a 20-amino acid deletion in the 49th to 68th in the stalk region) which could be have impact in the virulence of the virus as the Egyptian H5N1 NAs considered **A/chicken/ Hubei/327/2004(H5N1)** like group. These results are consistent with the observations of some researches that AI NA stalk region varies considerably among different viruses, even within the same subtypes ***(Zhou et al., 2009).***

The NA stalk-motif played a critical role in virulence and pathogenesis of H5N1 avian influenza virus and the special NA stalk-motif (a 20-amino acid deletion in the 49th to 68th in the stalk region) may be an important one among the reasons contributing to the emergence of H5N1 isolates with increased virulence since 2000. Published NA sequences of

1411 H5N1 influenza A viruses used for comparison were obtained from 1996 to 2007 from the Influenza Virus Resource ***(Zhou et al., 2009).*** These NA sequences were aligned and the stalk regions were compared. It was speculated that the deletion in the NA stalk may be associated with adaptation of influenza viruses to land-based poultry and increased virulence and pathogenesis in poultry and mammals ***(Matrosovich et al., 1999 and Li et al., 2004).*** There is a comparably strong rationale for the gradual increasing emergence of the special NA stalk motif in H5N1 influenza virus that deserves the great attention ***(Zhou et al., 2009).***

The phylogenetic tree of the sequenced isolates for Neuraminidase enzyme gene (Figure 6) illustrated the evolution of AI H5N1 to multiple clusters of neuraminidases between 2006 -2009 in Egypt, this marked genetic variations was also confirmed among the Egyptian and the international H5N1 strains ***(Arafa et al., 2010b).*** The Preliminary analysis did not show viruses clustering based on geographic location where the samples were taken. Additionally, isolates did not cluster based on the species of origin. It is obvious from the data of the present work that, H5N1 in Egypt is continuously mutating and that multiple heterogenic strains persist inside Egypt

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