Evolution of nonstructural (NS1) gene of Egyptian high pathogenic H5N1 *Avian influenza viruses*

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ABSTRACT

In the present study, 24 H5N1 viruses were selected on the basis of the phylogenetic analysis of the HA gene sequences to study the genetic evolution of the NS1 gene of such viruses. The viruses were isolated from chickens and ducks in both live bird markets and backyard populations. NS1 gene was amplified, sequenced and the phylogenetic analysis of the obtained sequences revealed deletion of 5 amino acids at positions 80 to 84 in the NS1 protein along all Egyptian isolates under study. Ala149, which is a pathogeneicity marker for interferon antagonism was found in the studied indicating capability of these viruses to transmit between different avian species. PL motif ESEV was found in 6 isolates from chicken and ducks circulating in such population whereas 17 viruses have ESKV similar to those reported in human in 2006. Based on NS1 sequence analysis of the H5N1 Egyptian viruses, neither geographic nor species signature were reported. This study indicates independent evolution of NS1 gene in Egyptian viruses that is not related to HA gene grouping.

Key words: Avian influenza H5N1, NS1 gene, NS1 protein, Egyptian H5N1 strains, Pathogenicity markers on the NS1 protein, PL motif.

Abbreviations

NS: Nonstructural, RT-PCR: Reverse transcriptase polymerase chain reaction, PI3K: Phosphoinositide 3 kinase, IFN: Interferon, HPAI: Highly pathogenic avian influenza, CPSF30: Cleavage and polyadenylation specificity factor 30.

INTRODUCTION

Influenza A viruses are genetically differing according to their hosts and geographical origin, and they belong to orthomyxoviruses of negative sense segmented single-stranded RNA *(*Chen et al,2001).. The eight gene segments of influenza A virus encode 10 proteins: hemagglutinin (HA), neuraminidase (NA), matrix proteins M2 and M1, nonstructural (NS) proteins NS1 and NS2, the nucleocapsid, and the three polymerases, the PB1 (polymerase basic 1), PB2, and PA (polymerase acidic) proteins For some influenza viruses, the PB1 gene has recently been discovered to encode an additional PB1-F2 protein *(*Chen et al,2001). Influenza type A viruses are subtyped based upon the HA and NA antigens, which are surface proteins found on the viral envelope *(*Murti et al,1986) The NS gene is the segment number eight in the genome of avian influenza with RNA length of 890 nucleotides which translated into two different proteins, nuclear exporting protein (NEP) of 121 amino acids length and nonstructural protein-1 (NS1) of 230 amino acids *(*Dongzi et al,2007) . NS1 protein can be divided into two major groups, originally termed alleles A and B. A number of NS1 proteins from avian influenza viruses together with those of all human, swine and equine influenza viruses are described as allele A NS1 protein, whereas those of allele B are exclusively from avian viruses. The level of homology within each allele is 93–100%; however, between alleles it can be as little as 62% *(*Hale et al, 2008a) . but there are some exception like one previously reported virus of equine origin (A/equine/ Jilin/1/1989/H3N8), one virus strain of human origin (A/New York/107/2003 (H7N2) and finally one swine origin virus (A/Swine/Saskatchewan/18789/2002/H1N1) are related to allele B (CDC 2004 ; Guo et al,1992; Karasin et al,2004). NS1 is a multi-functional protein plays a role in virus replication, virulence, IFN antagonism of the virus. It is one of the pathogenicity marker proteins of avian influenza and it can trigger the infected cell to secrete the pro- and anti-inflammatory cytokines *(*Hale *et al,2008b)* . The protein-protein and protein–RNA interactions of NS1 including : temporal

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regulation of viral RNA synthesis, control of viral mRNA splicing, enhancement of viral mRNA translation, suppression of host immune/apoptotic responses, activation of phosphoinositide3-kinase (PI3K) and involvement in strain-dependent pathogenesis. *(*Dongzi et al,2007).

Influenza A viruses are classified into subtypes on the bases of the antigenic of the antigenic relationships in the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). There are at present 16 HA subtypes and 9 NA subtypes *(*Fouchier et al,2005) . Recently, two novel influenza A viruses were discovered in bats from Guatemala (H17N10) and Peru (H18N11) suggesting that these species may constitute another reservoir with even greater genetic diversity *(*Tong et al,2012 ; Tong *et al,2013)*

Genetic characterization of H5N1 strains involved 10 unique first order numbered clades for the HPAI viruses (H5N1) in the GS/GD-like lineage (clade 0-9) *(*WHO,2012) . In Egypt , the first highly pathogenic avian influenza H5N1 outbreak was recorded from February to December 2006 and all Egyptian strains were very closely related and belonging to subclade 2.2 of the H5N1 virus of Eurasian origin, the same one circulating in the Middle East region and introduced into Africa at the beginning of 2006 *(*Aly et al,2008) the Egyptian strains of avian influenza H5N1 classified into 2 groups named as 2.2.1 and 2.2.1.1 *(*WHO,2012) and the most recent study recorded a new cluster 2.2.1.2 which raised in Egypt since October 2014 (Arafa et al,2015)

The aim of this work is to study the evolution of NS1 gene of AIV highly pathogenic H5N1 in Egypt .

MATERIALS AND METHODS

Propagation of selected H5N1 virus isolates

Twenty four samples were selected based on grouping of H5 gene of the Egyptian viruses from 2006 to 2011 which have been isolated from chicken and duck farms and backyards by the National Laboratory of Veterinary Quality Control on Poultry Production (NLQP). (Table 1)

Table (1): Results of HA assay and real time PCR on the isolated viruses

	Isolate ID	HA Genebank Accession No.	Year	Species
1	**A/chicken/Egypt/06959-NLQP/2006**	EU372947	2006	chicken
2	**A/chicken/Egypt/07632S-NLQP/2007**	EU496395	2007	chicken
3	**A/chicken/Egypt/07175-NLQP/2007**	GQ184215		chicken
4	**A/duck/Egypt/07322S-NLQP/2007**	EU496392		duck
5	**A/chicken/Egypt/0859-NLQP/2008**	GQ184231	2008	chicken
6	**A/chicken/Egypt/0813-NLQP/2008**	GQ184220		chicken
7	**A/duck/Egypt/0867-NLQP/2008**	GU811711		duck
8	**A/chicken/Egypt/0870-NLQP/2008**	GQ184234		chicken
9	**A/duck/Egypt/0891/ NLQP /2008**	GQ184240		duck
10	**A/duck/Egypt/0923-NLQP/2009**	GU002675	2009	duck
11	**A/chicken/Egypt/0962S-NLQP/2009**	GU002688		chicken
12	**A/duck/Egypt/09224F-NLQP/2009**	GU002686		chicken
13	**A/chicken/Egypt/09534S-NLQP/2009**	GU002694		chicken
14	**A/duck/Egypt/093-NLQP/2009**	GU002699		duck
15	**A/chicken/Egypt/091317S- NLQP /2009**	HQ198254		chicken
16	**A/chicken/Egypt/0960-NLQP/2009**	GU002680		chicken
17	**A/chicken/Egypt/1034-NLQP /2010**	HQ198263	2010	chicken
18	**A/duck/Egypt/1053-NLQP /2010**	HQ198267		duck
19	**A/duck/Egypt/1025SF-NLQP /2010**	HQ198286		duck
20	**A/chicken/Egypt/1055-NLQP /2010**	HQ198268		chicken
21	**A/duck/Egypt/1017-NLQP /2010**	HQ198259		duck
22	**A/chicken/Egypt/1063-NLQP /2010**	HQ198269		chicken
23	**A/chicken/Egypt/115D-NLQP /2011**	JN807840	2011	chicken
24	**A/duck/Egypt/11193SF-NLQP /2011**	JN807861		duck

The propagation of viruses was done at 9-11 days old SPF-ECE through intra-allantoic inoculation (OIE,2009) . The haemagglutination (HA) test was applied on the harvested allantoic fluid and the viruses had HA titer more than 6log2 were selected for sequencing of NS1 gene

The detection and subtyping of H5 AIV was done by using Real-time RT-PCR. The viral RNAs were extracted using QiAamp viral RNA mini Kit (Qiagen, Germany) and identified the H5 subtype using Real-time RT-PCR as described by (Slomka et al, 2007)

RT-PCR for nonstructural gene (NS):

The NS gene was amplified from the extracted RNA and one step RT-PCR using Qiagen kit *(*Qiagen, Valencia, CA*)* and primers specific for the NS gene: NS-8FV2 and NS-8RV2, the sequences of the primers were described in Table 2 . The RT-PCR was carried out as follow: one cycle at 50 °C for 30 min, one cycle at 95 °C for 15 min and 40 cycles of 94 °C for 10 sec , 54 °C for 30 sec and 72 °C for 10 sec., a final extension at 72°C for 10 min. in thermocycler 2720 ABI (Applied Biosystems , USA) .

Table (2): Primers used in RT-PCR and Sequence of NS gene

Prime ID	Nucleotide Sequence	Reference
NS-8FV2	5'AAA AGC AGG GTG ACA AAG ACA TAA 3'	FLI
NS-8RV2	5'AGT AGA AAC AAG GGT GTT TTT TAT CA 3'	FLI
NS-R598	5'GAG TTA TCA TTC CAT TCA AGT CC 3'	SEPRL
NS-F327	5'GAM TGG TWC ATG CTC ATG CC 3'	SEPRL

The primer sequences were kindly supplied from FLI- Germany and SEPRL, USA.

The electrophoresis of PCR products were done on ethidium bromide stained agarose gel and the amplified products of expected correct size at 875 bp of NS gene was visualized by gel documentation system BDA digital – Image capture (Biometra, Germany). The PCR Products were purified from the gel using QIAquick gel extraction kit (Qiagen, Valencia, CA).

Sequencing for non-structural (NS1) gene

The sequencing of NS1 gene was carried out using Big dye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer, Foster city, CA), 4µl Big dye terminator v.3.1, 1µl of each primer specific for NS1 gene (Table S1). The cycling protocol for sequence reactions in thermal cycler 2720 ABI (Applied Biosystems , USA) was as follow: 1 cycle at 96˚ C for 1 min, 25 Cycles repeated at 96˚ C for 10 sec, 50˚ C for 5 sec and 60˚C for 2 min. the sequencing reactions were purified using spin column Centrisep kit (Applied Biosystems , USA) to remove the free dNTP bases from the sequence reactions, then loading in sequencer plate of ABI (Applied Biosystems 3130 genetic analyzer, USA). The 24 sequences of NS1 gene in this study were submitted to the gene bank under accession numbers: KC841879 to KC841902 (Table 3).

Phylogenetic analysis of NS1 gene:

The Bioedit (version 7.0.9) and DNAstar software Lasergene v7.1.0 (MD, WI, USA) were used for analysis of the nucleotides sequence of NS1 gene and amino acids of NS1 protein. The Phylogenetic analysis was carried out using Maximum Parsimony method - Bootstrap method (No. of Bootstrap Replications 100) with the aid of Mega5 software (MEGA version 5) (Tamura et al,2011).

Table 3: GenBank accession numbers of NS1 gene:

	Isolate ID	NS1 Genebank
1	A/chicken/Egypt/06959-NLQP/2006	KC841879
2	A/chicken/Egypt/07632S-NLQP/2007	KC841882
3	A/chicken/Egypt/07175-NLQP/2007	KC841881
4	A/duck/Egypt/07322S-NLQP/2007	KC841880
5	A/chicken/Egypt/0859-NLQP/2008	KC841884
6	A/chicken/Egypt/0813-NLQP/2008	KC841883
7	A/duck/Egypt/0867-NLQP/2008	KC841885
8	A/chicken/Egypt/0870-NLQP/2008	KC841886
9	A/duck/Egypt/0891/ NLQP /2008	KC841887
10	A/duck/Egypt/0923-NLQP/2009	KC841890
11	A/chicken/Egypt/0962S-NLQP/2009	KC841889
12	A/duck/Egypt/09224F-NLQP/2009	KC841893
13	A/chicken/Egypt/09534S-NLQP/2009	KC841892
14	A/duck/Egypt/093-NLQP/2009	KC841888
15	A/chicken/Egypt/091317S- NLQP /2009	KC841894
16	A/chicken/Egypt/0960-NLQP/2009	KC841891
17	A/chicken/Egypt/1034-NLQP /2010	KC841896
18	A/duck/Egypt/1053-NLQP /2010	KC841897
19	A/duck/Egypt/1025SF-NLQP /2010	KC841899
20	A/chicken/Egypt/1055-NLQP /2010	KC841898
21	A/duck/Egypt/1017-NLQP /2010	KC841895
22	A/chicken/Egypt/1063-NLQP /2010	KC841900
23	A/chicken/Egypt/115D-NLQP /2011	KC841901
24	A/duck/Egypt/11193SF-NLQP /2011	KC841902

RESULTS

Virus propagation and real time PCR results:

Harvested allantoic fluids were titerated using the HA test and the obtained titers were ranged from 7 to 9 log2. Table (4) The rRT-PCR for H5 gene detection was done and The Ct. Values of the tested viruses in rRT-PCR were ranged from 19.1 to 30.8 as shown in Table (4), fig.(1).

RT- PCR amplification of NS gene:

NS genes of the selected 24 isolates with specific size of 875 bp were obtained from the RT-PCR products using agarose gel 1.5% electrophoresis. **Fig.(2)**

Table 4: HA activity and real time PCR results after virus isolation

	Isolate ID	HA titer[1] (Log2)	H5 rRT-PCR (Ct value)[2]
1	A/chicken/Egypt/06959-NLQP/2006	8	23.14
2	A/chicken/Egypt/07632S-NLQP/2007	7	23.09
3	A/chicken/Egypt/07175-NLQP/2007	8	23.99
4	A/duck/Egypt/07322S-NLQP/2007	7	23.85
5	A/chicken/Egypt/0859-NLQP/2008	9	24.36
6	A/chicken/Egypt/0813-NLQP/2008	9	24.27
7	A/duck/Egypt/0867-NLQP/2008	8	23.43
8	A/chicken/Egypt/0870-NLQP/2008	9	24.83
9	A/duck/Egypt/0891/ NLQP /2008	7	27.68
10	A/duck/Egypt/0923-NLQP/2009	7	25.20
11	A/chicken/Egypt/0962S-NLQP/2009	8	24.36
12	A/duck/Egypt/09224F-NLQP/2009	7	22.97
13	A/chicken/Egypt/09534S-NLQP/2009	8	30.77
14	A/duck/Egypt/093-NLQP/2009	8	22.24
15	A/chicken/Egypt/091317S- NLQP /2009	9	23.56
16	A/chicken/Egypt/0960-NLQP/2009	7	24.35
17	A/chicken/Egypt/1034-NLQP /2010	8	23.40
18	A/duck/Egypt/1053-NLQP /2010	9	23.61
19	A/duck/Egypt/1025SF-NLQP /2010	8	23.45
20	A/chicken/Egypt/1055-NLQP /2010	9	20.59
21	A/duck/Egypt/1017-NLQP /2010	9	20.14
22	A/chicken/Egypt/1063-NLQP /2010	9	19.08
23	A/chicken/Egypt/115D-NLQP /2011	9	20.24
24	A/duck/Egypt/11193SF-NLQP /2011	9	20.08

[1]The HA titers more than 6log2 were selected for further analysis, [2]Ct. value: The point at which the fluorescence crosses the threshold in the real time PCR

Fig. 1: Real time PCR results for HA gene of 24 isolates of Egyptian high pathogenic H5N1 avian influenza viruses

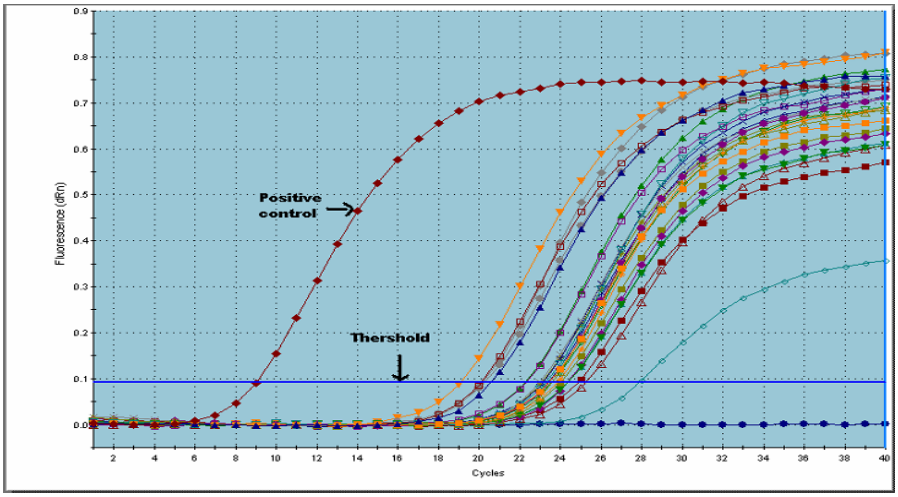

Fig.2: Conventional RT PCR results for NS gene of 24 isolates of Egyptian high pathogenic H5N1 avian influenza viruses

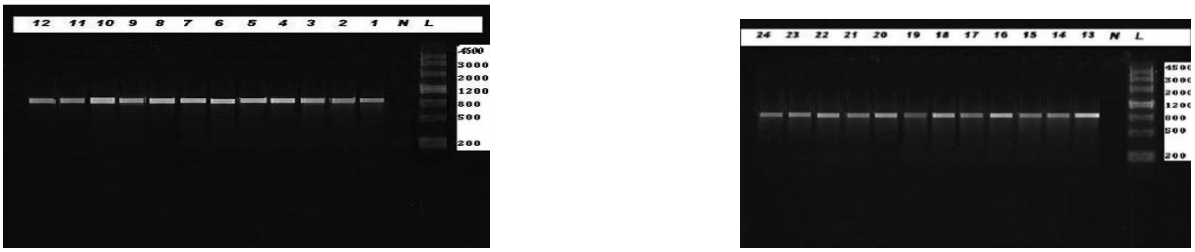

Table (5): Amino acids differences in NS1 protein among the examined Egyptian isolates

Isolate ID	Amino acids differences[1]	PL motif[2] (227-230)
A/chicken/Egypt/06 959-NLQP/2006	R44 , N48 , E55 , I64 ,E71 ,T127 ,Y138 ,V180 ,G183 ,I198 ,P216	ESKV
A/chicken/Egypt/07 632S-NLQP/2007	R44 , N48 , E55 , I64 ,E71 ,I127 ,Y138 ,V180 ,G183 ,I198 ,P216	ESKV
A/chicken/Egypt/07175-NLQP/2007	R44 , N48 , E55 , I64 ,E71 ,T127 ,Y138 ,V180 ,G183 ,I198 ,P216	ESKV
A/duck/Egypt/07322S-NLQP/2007	R44 , S48 , E55 , I64 ,E71 ,T127 ,Y138 ,V180, G183 ,I198 ,P216	ESKV
A/chicken/Egypt/0859-NLQP/2008	R44 , N48 , E55 , I64 ,E71 ,I127 ,Y138 ,V180 ,G183 ,I198 ,P216	ESKV
A/chicken/Egypt/0813-NLQP/2008	R44 , N48 , E55 , I64 ,E71 ,T127 ,Y138 ,I180 ,R184 ,I198 ,P216	ESKV
A/duck/Egypt/0867-NLQP/2008	R44 , N48 , E55 , I64 ,E71 ,T127 ,Y138 ,V180 ,G183 ,I198 ,P216	ESKV
A/chicken/Egypt/0870-NLQP/2008	R44 , N48 , E55 , I64 ,E71 ,T127 ,Y138 ,V180,G183 ,I198 ,P216	ESKV
A/duck/Egypt/0891/ NLQP /2008	R44 , N48 , E55 , I64 ,E71 ,T127 ,Y138 ,I180 ,G183 ,I198 ,P216	ESKV
A/duck/Egypt/0923-NLQP/2009	R44 , N48 , E55 , I64 ,E71 ,T127 ,C138 ,I1780 ,R183 ,I198 ,P216	ESKV
A/chicken/Egypt/0962S-NLQP/2009	R44 , N48 , E55 , I64 ,E71 ,T127 ,C138 ,I180 ,R183 ,I198 ,P216	ESKV
A/duck/Egypt/09224F-NLQP/2009	K44 , N48 , E55 , L64 ,E71 ,D96 ,I127 ,Y138 ,V180 ,G183 ,I198 ,P216	ESKV
A/chicken/Egypt/09534S-NLQP/2009	K44 , N48 , E55 , L64 ,E71 ,I127 ,Y138 ,V180 ,G183 ,I198 ,S216	ESKV
A/duck/Egypt/093-NLQP/2009	K44 , S48 , E55 , I64 ,E71 ,T127 ,Y138 ,V180 ,G183 ,V198 ,P216	ESEV
A/chicken/Egypt/091317S-NLQP /2009	K44 ,S48 ,K55 ,I64 ,K71 ,T127 ,Y138 ,V180 ,G183 ,V198 ,S216	ESEV
A/chicken/Egypt/0960-NLQP/2009	K44 , N48 , E55 , I64 ,E71 ,P127 ,N138 ,V180 ,G183 ,I198 ,P216	GSKV
A/chicken/Egypt/1034-NLQP /2010	K44 , S48 , K55 , I64 ,K71 ,I79 ,T127 ,Y138 ,V180 ,G183 ,V198 ,S216	ESEV
A/duck/Egypt/1053-NLQP /2010	K44 , S48 , K55 , I64 , K71 ,D75 ,T127 ,Y138 ,V180,G183 ,V198 ,S216	ESEV
A/duck/Egypt/1025SF-NLQP /2010	K44 , S48 , E55 , I64 ,E71 ,T127 ,Y138 ,V180 ,G183 ,V198 ,P216	ESEV
A/chicken/Egypt/1055-NLQP /2010	R44 , N48 , E55 , I64 ,E71 ,I127 ,Y138 ,V180 ,G183 ,I198 ,S216	ESKV
A/duck/Egypt/1017-NLQP /2010	K44 , N48 , E55 , L64 ,E71 ,I127 ,Y138 ,V180 ,G183 ,I198 ,S216	ESKV
A/chicken/Egypt/1063-NLQP /2010	K44 , N48 , E55 , L64 ,E71 ,I127 ,C138 ,V180 ,G183 ,I198 ,P216	ESKV
A/chicken/Egypt/115D-NLQP /2011	K44 , N48 , E55 , L64 ,E71 ,I127 ,Y138 ,V180 ,G183 ,I198 ,S216	ESKV
A/ duck /Egypt/11193SF-NLQP /2011	K44 , S48 , K55 , I64 , K71 ,T127 ,Y138 ,V180 ,G183 ,V198 ,S216	ESEV

[1]Amino acids differences between Egyptian isolates was calculated in comparison with A/Goose/Guangdong/1/96, [2]**PL motif** : PDZ ligand motif

Sequence analysis of NS 1 gene and protein:

The obtained nucleotides and amino acids sequences of the NS1 gene of the selected 24 HPAI H5N1 isolates were aligned and the similarity as well as the diversity % with A/Goose/Guangdong/1/96 (accession number: AF144307) the reference of H5N1 clades and A/bar-headed goose/Qinghai/5/2005 (access number: DQ095697) the reference of clade 2.2 H5N1. All Egyptian isolates demonstrate a deletion in 5 amino acids located at position

80-84 comparing with A/Goose/Guangdong/1/96, there are different PL motif and many differences in the amino acids of Egyptian isolates when compared with each other, Table 5.

Phylogenetic analysis of NS 1 gene and protein:

The NS1 gene of Egyptian isolates found to be belonging to clade 2.2. There are at least 2 evolutionary groups for NS1 gene independent to evolutionary characters of HA gene**, fig. (3).**

Fig. 3: Phylogenetic tree for the nucleotide sequence of the 24 Egyptian isolates in this study for NS1 gene in comparison to A/Goose/Guangdong/1/96 and other H5N1 world circulated strains.

Table (6): The characteristic genetic markers in the NS1 protein of Egyptian viruses

Position on NS1 protein[1]	Amino acids Markers of NS1 protein of Egyptian isolates
42	**S**
44	**R , K**
48	**N , S**
55	**E , K**
64	**I , L**
71	**E , K**
5 A.A (80-84)	**Deletion**
92 / 97	**E**
127	**T , L, P, I**
149	**A**
180	**I , V**
198	**I , V**
216	**P , S**
PL motif	**ESKV , ESEV & GSKV[2]**

[1]Amino acids position on NS1 protein of Egyptian strains aligned with A/Goose/Guangdong/1/96, [2]GSKV only in Egyptian isolate named A-chicken-Egypt-0960-NLQP/2009

DISCUSSION

The NS1 protein is a virulence factor for H5N1 avian influenza viruses and that multiple domains within this protein may be suitable targets for the development of antiviral drugs and attenuated vaccines. There are many amino acids residues on NS1 protein affect the virulence of AIV (Table 6). In the present study , the analysis of nucleotides and amino acids sequence of NS1 gene in 24 Egyptian AIV H5N1 revealed the presence of amino acid serine (S) at the position 42 that highly conserved in the human, swine, and equine influenza viruses, This amino acid plays a key role in the antiviral immune response as it antagonizes host cell interference and prevents the double-stranded RNA mediated activation of the NF-β pathway and the IRF-3 pathway (Jiao et al,2008). Also, conserved amino acid residues from 67 to79 which are the linker region between the two domains found critical for the functions of NS1 protein during influenza A virus infection (Bornholdt & Prasad, 2008). The two highly conserved leucine residues at amino acid positions 69 and 77 (L69, 77) has binding capabilities to double stranded RNA (dsRNA), CPSF30, and the p85β subunit of PI3K as The PI3K/Akt pathway is the cellular pathway mediates various anti-apoptotic responses (Franke et al,1997).

The Egyptian AIV H5N1 viruses have amino acid deletions at 80 to 84; this deletion is located in the linker region between the N-terminal RNA-binding domain and the C-terminal effector domain of the protein that explain the conformational changes observed in the RNA-binding domain of the H5N1 virus (Bornholdt & Prasad , 2006), and this deletion was also recorded in European, Middle Eastern and African H5N1 viruses from 2006 (Zohari et al,2008) . However, the two strains A/chicken/Egypt/Q1182/2010 and A/chicken/Egypt/Q1011/2010 had a longer NS1 protein (Kayali et al,2011).

The deduced NS1 amino acid sequences of viruses in one branch of the A allele revealed this 5-amino acid deletion (Neumann et al, 2010) , moreover, this deletion in addition to amino acid change at position 92 (D92E) is responsible on the cytokine imbalance that enhances the virulence of H5N1 viruses (Hyland et al,2006).

The amino acid at NS1-138, in combination with the NS1 PDM, affects HPAI H5N1 virulence. In fact, there was a study found that the large, polar tyrosine residue found in subgenotype Z.1 NS1 proteins NS1-138Y confers stronger interaction with p85than NS1-138F, but weaker PI3K/ Akt activation, some studies speculated that the large tyrosine residue causes a minor conformational change in PI3K that affects efficient activation, and that strong binding by NS1-138Y locks the

complex in this state and interferes with the kinase activity of PI3K*(Fan et al,2013).* In our work to study the evolution of NS1 of Egyptian viruses, we found that all viruses have NS1-138Y except three viruses: A/chicken/Egypt/0960-NLQP/2009 has NS1-138N and both A/chicken/Egypt/0962S-NLQP/2009 and A/chicken/Egypt/1063-NLQP /2010 have NS1-138C.

As well as, The NS1 protein of Egyptian AIV H5N1 viruses have amino acid residue Ala149 (A) that correlates with the ability of these viruses to antagonize interferon induction and critical for the pathogenicity of avian influenza virus in chickens, Previous work on the NS1 gene of GS/GD/1/96 revealed that it is important for this virus to be able to antagonize the host IFN- α/β response and to replicate with lethality in chickens because it has amino acid residue (A) 149 but for the virus A/goose/Guangdong/2/96 (GS/GD/2/96) encoding valine (V) 149 substitution is not capable of the same effect (Li et al,2006) .

Briefly, there are many host markers that can discriminate human influenza viruses from avian influenza viruses. NS1 protein has three host markers locate in regions of the molecule with known host cell binding functions (Finkelstein et al, 2007). In the Egyptian AIV H5N1, the N-terminal domain of NS1 has a deletion in the residue 81 I or M when compared to strain A/Guangdong/ 96. The second host marker is the P215T in SH3 recognition motif where the 212 PPLPP 216 motif is preserved in avian influenza viruses and is altered to 212 PPLTP 216 in human viruses, the 16 Egyptian H5N1 viruses out of 24 viruses in the study from 2006 – 2010 have 212 LPLPP 216 motif while there were eight viruses have motif 212 LPLPS 216 from 2009-2011. The third persistent host marker in NS1 is at residue R227E in the PDZ binding domain (PL motif) which is specifically short C-terminal peptide motifs of 4–5aa consists of residues 227–230 (Finkelstein et al, 2007). In the examined Egyptian isolates, the ESEV motif at the C–terminus of the NS1 protein was existed in six isolates from backyard chicken and duck populations that were belonging to group 2.2.1 within 2009–2011 similar to most of human H5N1 isolates. However the other 17 Egyptian isolates have ESKV motif like the previously reported Egyptian H5N1 isolates from human (A-Egypt-902782-2006 & A-Egypt-902786-2006) and only one isolate (A-chicken-Egypt-0960-NLQP(2009)) in the study has GSKV PL motif (Fig. 1). This finding may indicate a role of NS1 protein in the transmission of avian influenza H5N1 from chicken to human. PDZ domain containing cellular proteins play important roles in the transport, localization, and assembly of supramolecular signaling complexes, organizing cell polarity, receptors and downstream effectors *(*Harris & Lim, 2001). Furthermore, the interaction of NS1-ESEV (but not NS1-ESKV) with Dlg-1 stimulates the PI3K/Akt pathway, resulting in efficient virus replication. The significance of the NS1-ESKV motif is to attenuate HPAI H5N1 viruses and acts synergistically with the amino acid at position 138 of NS1*(Fan et al,2013).*

In conclusion, the present study reports the genetic evolution in the NS1 gene of avian influenza H5N1 viruses circulating among avian populations from 2006 to 2011. There were specific amino acids markers in NS1 protein which play an important role in virus pathogenicity and host specificity of HPAI H5N1 viruses in Egypt. There are at least two separate groups of Egyptian viruses were characterized in phylogenetic analysis. One group of viruses isolated during 2006 to 2010 and another group of recent viruses from 2009 to 2011. There is no geographical limitation for AIV H5N1 in Egypt based on NS1 gene as the viruses isolated from different governorates have the same molecular signature, also there is no genetic signature related to the breeding and poultry species. This work indicates independent evolution of NS1 gene in Egyptian viruses that is not related to HA gene evolution groups. Further studies are needed to study the effects of some mutations in NS1 amino acids of Egyptian isolates reported in this study to answer many raised questions related to host range and virulence determination of Egyptian H5N1 viruses.

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